

Enhanced Generation of Helper T Type 1 and 2 Chemokines in Allergen-induced Asthma

Linying Liu, Nizar N. Jarjour, William W. Busse, and Elizabeth A. B. Kelly

Allergy and Immunology Section, and Pulmonary and Critical Care Section, Department of Medicine, University of Wisconsin, Madison, Wisconsin

Allergen-induced asthma is characterized by airway eosinophilia and recruitment of helper T (Th) Type 2 lymphocytes. We hypothesized that lymphocyte-associated chemokines contribute to allergen-induced airway inflammation. Sixteen subjects with asthma were phenotyped according to their response to inhaled antigen as single- or dual-phase responders, and then underwent bronchoscopy and segmental allergen bronchoprovocation. Bronchoalveolar lavage fluids were obtained before and 48 hours after segmental challenge with allergen to determine the cellular response and patterns of Th1 and Th2 chemokines and cytokines. Airway cells, cytokines, and lymphocyte-associated chemokines increased after segmental challenge. Th2 chemokines (thymus and activation-regulated chemokine, macrophage-derived chemokine) correlated with airway eosinophils and concentrations of interleukin-5 and -13. In contrast, airway lymphocytes correlated with both Th2 and Th1 (monokine-induced by IFN- γ , IFN- γ -inducible protein-10) chemokines. Notably, when subjects were analyzed according to the presence of a late-phase response, concentrations of both types of lymphocyte-associated chemokines were significantly greater in subjects with a dual-response phenotype. Our findings suggest that both Th2 and Th1 chemokines may be involved in allergen-induced airway inflammation. However, asthma subjects with a dual-responder phenotype have greater generation of chemokines that may lead to enhanced airway inflammation and obstruction after allergen exposure.

Keywords: bronchoalveolar lavage; chemokines; eosinophils; late-phase response; helper T Type 1/helper T Type 2 cells

Allergic airway inflammation in asthma involves eosinophils, lymphocytes, cytokines, and chemokines, which contribute to airway obstruction and hyperresponsiveness. Multiple molecules are generated in response to allergen challenge and direct trafficking of inflammatory cells, including activated T cells, into the airway (1). Chemokines can contribute to the extravasation of leukocytes by inducing expression of integrin and adhesion molecules (2). These actions suggest that chemokines and their receptors are important in the generation and regulation of allergic airway inflammation in asthma.

In vitro studies have demonstrated that human lymphocytes highly polarized toward a helper T Type 1 (Th1) or helper T Type 2 (Th2) phenotype selectively express distinct sets of

chemokine receptors (3–5). CC chemokine receptor (CCR) 5 and CXC receptor (CXCR) 3 are expressed primarily on Th1 cells. In contrast, CCR3, CCR4, CCR8, and CXCR4 are expressed primarily on Th2 cells. Although expression of these receptors may not be mutually exclusive, it is clear that the majority of polarized Th1 cells express CXCR3 and the majority of Th2 cells express CCR4 (6). Both the Th2 chemokine receptor, CCR4, and the Th1 chemokine receptor, CXCR3, are upregulated in allergic airway disease (1, 7–10). CCR4 recognizes thymus and activation-regulated chemokine (TARC, CCL-17) and macrophage-derived chemokine (MDC, CCL-22) and its expression is elevated in bronchial biopsies, serum, and induced sputum in patients with allergic asthma (8, 11–13) and is further increased after airway antigen challenge (14). However, the expression of CXCR3 ligands monokine-induced by IFN- γ (MIG, CXCL9), IFN- γ -inducible protein-10 (IP-10, CXCL10) and IFN-inducible T cell α chemoattractant (I-TAC, CXCL-11) has not been well characterized during allergic inflammation in asthma.

Airway allergen challenge is associated with eosinophilic inflammation and airway obstruction and is useful as a model to study mechanisms of airway inflammation in asthma. Two approaches have been used to evaluate the effect of antigen on allergic inflammation in the lower airway: aerosol inhalation challenge and localized segmental bronchoprovocation with antigen (SBP-Ag). SBP-Ag provokes a marked localized airway inflammatory response that facilitates analysis of recruited cells, mediator generation, and *ex vivo* cell function. Localized antigen challenge, however, does not induce measurable changes in lung function. In contrast, after inhaled antigen challenge, the immediate (within minutes) drop in lung function (early-phase response) and the later fall in forced expiratory volume in 1 second (FEV₁) between 3 and 8 hours (late-phase response, LPR) can be evaluated. The early response most likely represents the release of mast cell mediators, whereas the later response coincides with an initial influx of inflammatory cells (15). In this study, we combined both approaches to take advantage of their individual strengths. Inhalation challenge was used first to “phenotype” the subjects as single responders (early) or dual responders (early and late). SBP-Ag was then performed to define the characteristics of selective features of airway inflammation. Specifically, SBP-Ag was used to determine the pattern of Th1 and Th2 chemokines in bronchoalveolar lavage (BAL) fluid and to ascertain the relationship between these factors and airway cellular recruitment. Finally, the airway inflammatory response to SBP-Ag was compared between the single and dual responders on the basis of the previously defined pulmonary physiologic reaction to inhaled antigen. (Airway physiology and cellular data from 10 of the 16 subjects have been previously reported as part of a study to evaluate the expression of chemokine receptors on blood and airway eosinophils [7].)

METHODS

Subjects

Sixteen subjects with allergic asthma were studied (Table 1). Each subject underwent a medical history, physical examination, allergen

(Received in original form December 5, 2003; accepted in final form March 3, 2004)
Supported by an institutional Specialized Center of Research grant (NIH HL56396) and by a University of Wisconsin General Clinical Research Center grant (NIH M01RR03186).

Correspondence and requests for reprints should be addressed to Elizabeth A. (Becky) Kelly, Ph.D., Section of Pulmonary and Critical Care Medicine, 600 Highland Avenue, CSC K4/928, University of Wisconsin School of Medicine, Madison, WI 53792-9988. E-mail: eak@medicine.wisc.edu

This article has an online supplement, which is accessible from this issue's table of contents online at www.atsjournals.org

Am J Respir Crit Care Med Vol 169, pp 1118–1124, 2004
Originally Published in Press as DOI: 10.1164/rccm.200312-1659OC on March 4, 2004
Internet address: www.atsjournals.org

TABLE 1. SUBJECT CHARACTERISTICS

Subject	Age (yr)	Sex	Antigen	Ag-PD ₂₀ (C _{BU})*	PC ₂₀ (mg/ml)	FEV ₁ (% Predicted)	% Fall in FEV ₁	
							Early Phase	Late Phase
1	48	M	RW	795.0	6.9	110	34	6
2	24	F	RW	54.9	12.6	97	35	8
3	26	M	CAT	61.7	0.5	102	46	14
4	29	F	HDM	23.2	3.9	88	32	10
5	20	M	CAT	61.9	3.3	95	31	3
6	26	M	CAT	263.6	4.8	98	29	5
7	32	F	CAT	223.9	17.9	88	31	0
8	22	M	RW	81.2	9.0	90	37	0
9	19	M	HDM	56.0	8.6	100	57	43
10	20	M	HDM	325.8	0.3	84	25	20
11	20	F	CAT	4.7	0.4	89	34	28
12	19	F	HDM	9.3	0.2	84	23	15
13	30	F	HDM	10.7	4.8	95	38	25
14	24	F	CAT	1.7	1.5	90	29	18
15	19	F	CAT	51.9	1.9	112	38	15
16	30	M	CAT	17.8	5.4	92	39	36

Definition of abbreviations: Ag-PD₂₀ = PD of antigen producing a 20% fall in FEV₁; CAT = cat dander; HDM = house dust mite
PC₂₀ = provocative concentration of methacholine producing a 20% fall in FEV₁; RW = ragweed.

* In cumulative breath units.

skin prick test, and pulmonary function testing including spirometry to determine FEV₁, reversibility of FEV₁ to β -agonist, and airway hyper-responsiveness to methacholine at the screening visit. Exclusion criteria are included in the online supplement. Informed consent was obtained from each subject before participation. The study was approved by the University of Wisconsin-Madison (Madison, WI) Center for Health Sciences Human Subjects Committee.

Determination of Early- and Late-phase Response to Inhaled Allergen Challenge

At least 4 weeks before bronchoscopy, a graded inhaled antigen challenge was performed to determine Ag-PD₂₀ (the dose that caused a 20% fall in FEV₁), and to document the presence of early- and late-phase responses. Detailed methods are provided in the online supplement. Briefly, after determining the response to diluent (normal saline), five breaths of antigen were inhaled and spirometry was repeated 10 minutes later. Consecutively higher concentrations of antigen were given until the FEV₁ fell by 20% from baseline. The maximum immediate (within 15–30 minutes) drop in FEV₁ was determined and then subjects were monitored every 15 minutes until their FEV₁ returned to within 10% of their baseline. Thereafter, they were monitored at 1-hour intervals for up to 8 hours to determine the LPR (a sustained 15% fall in FEV₁ lasting at least 15 minutes and occurring 3 to 8 hours after challenge).

Segmental Bronchoprovocation and BAL

Bronchoscopy and SBP-Ag were performed as previously described (16) and details are provided in the online supplement. Bronchoscopy and BAL were performed in two different bronchopulmonary segments at baseline and 48 hours after SBP-Ag. BAL fluid (BALF) from the two antigen-challenged segments was pooled for analysis of fluid and cells.

Chemokine and Cytokine Measurements

As previously described (17), a sandwich ELISA was used to measure chemokines in neat (IP-10 and TARC) or 5 $\times$ -concentrated BALF (MIG, I-TAC, and MDC). Coating and biotinylated antibodies for MIG were purchased from BD Biosciences Pharmingen (San Diego, CA). I-TAC, TARC, and MDC coating and biotinylated antibodies were purchased from R&D Systems (Minneapolis, MN). The sensitivity for MIG, I-TAC, TARC, and MDC was less than 3 pg/ml. IP-10 was measured with a commercial ELISA kit (R&D Systems) with a sensitivity of less than 2 pg/ml. The levels of interleukin (IL)-5 and IL-13 in 5 $\times$ BALF and of IFN- γ in 20 $\times$ BALF were also measured by ELISA, with a sensitivity of less than 3 pg/ml.

Statistical Analysis

Data are presented as medians with interquartiles of 25 and 75% or as means $\pm$ SEM (for normally distributed data). The Wilcoxon signed rank test (or a paired *t*-test for normally distributed data) was used to compare data obtained at baseline and 48 hours after SBP-Ag. The Mann-Whitney rank sum test was used to compare data between groups (single versus dual responders). The Spearman rank-order correlation coefficient (*r*_s) was used to assess bivariate associations. A *p* value of less than 0.05 was considered significant. Statistical analyses were performed with the SigmaStat software package (Jandel Scientific Software, San Rafael, CA).

RESULTS

Subject Characteristics

(See Table 1.) All subjects met American Thoracic Society criteria for the definition of asthma. Twelve of the 16 subjects with asthma had a provocative concentration of methacholine producing a 20% fall in FEV₁ (PC₂₀) of less than 8 mg/ml. Of the other four subjects with asthma, one (Subject 8) had episodic asthma symptoms and 14% β_2 -agonist reversibility. Subjects 2, 7, and 9 had had physician-diagnosed asthma for more than 10 years, were taking inhaled albuterol as needed, and had previous exercise- or virus-induced asthma symptoms that required treatment with inhaled corticosteroid. All subjects had an immediate (15–30 minutes) fall in FEV₁ exceeding 20% and eight subjects had a second fall (LPR) in FEV₁ of 15% or more 3–8 hours later.

BAL Cells, Cytokines, and Chemokines after SBP-Ag

Forty-eight hours after SBP-Ag, concentrations of total protein, total numbers of cells, and all leukocyte populations including macrophages, neutrophils, lymphocytes, and eosinophils were significantly increased in BALF when compared with baseline values (Table 2). The concentrations of IL-5 and IL-13 in BALF were increased after SBP-Ag. IFN- γ also had a small, but significant, increase after SBP-Ag (Table 2). Compared with baseline values, BALF concentrations of Th1-type chemokines IP-10 and MIG (and, to a lesser extent, I-TAC), as well as Th2-type chemokines TARC and MDC (Table 3), were significantly increased after SBP-Ag.

TABLE 2. BRONCHOALVEOLAR LAVAGE CELLULAR AND PROTEIN PROFILE BEFORE AND 48 HOURS AFTER SEGMENTAL BRONCHOPROVOCATION WITH ANTIGEN

	Baseline (Pre-Ag Challenge)	48 Hours Post-Ag Challenge	p Value (Baseline versus 48 Hours)
Total BAL cells, $\times 10^4$ /ml BALF	12.0 (8.7, 12.8)	56.0 (25.5, 90.7)	< 0.001
Macrophages, $\times 10^4$ /ml BALF	10.3 (7.9, 11.5)	19.6 (13.6, 27.8)	< 0.001
Neutrophils, $\times 10^4$ /ml BALF	0.1 (0.1, 0.2)	1.7 (1.0, 3.2)	< 0.001
Lymphocytes, $\times 10^4$ /ml BALF	0.7 (0.5, 1.3)	3.3 (2.2, 7.9)	< 0.001
Eosinophils, $\times 10^4$ /ml BALF	0.0 (0.0, 0.1)	28.4 (5.9, 52.7)	< 0.001
Total protein, mg/ml BALF	63.2 (54.0, 94.2)	228.6 (125.5, 658.6)	< 0.001
IL-5, pg/ml $1 \times$ BALF	0.0 (0.0, 0.0)	22.7 (1.0, 84.0)	< 0.001
IL-13, pg/ml $1 \times$ BALF	0.1 (0.0, 0.5)	2.2 (0.7, 4.8)	< 0.001
IFN- γ , pg/ml $1 \times$ BALF	0.0 (0.0, 1.2)	0.6 (0.0, 2.6)	0.027

Definition of abbreviations: Ag = antigen; BAL = bronchoalveolar lavage; BALF = bronchoalveolar lavage fluid; IL = interleukin. Data are depicted as median (25 and 75% quartiles); n = 16.

Relationship of Th1 and Th2 Chemokines to BAL Eosinophils and Lymphocytes

Forty-eight hours after SBP-Ag, strong correlations were found between absolute numbers of airway eosinophils and concentrations of Th2-type chemokines TARC ($r_s = 0.74$, $p < 0.001$; Figure 1A) and MDC ($r_s = 0.67$, $p = 0.004$; Figure 1B). TARC and MDC also correlated with the percentage of eosinophils in the BALF (TARC: $r_s = 0.72$, $p = 0.001$; MDC: $r_s = 0.58$, $p = 0.012$). In contrast, the presence of BAL lymphocytes was associated not only with Th2-type chemokines (Figures 1C and 1D), but also with Th1-type chemokines IP-10 and MIG (Figures 1E and 1F) ($r_s > 0.6$, $p < 0.005$ for all chemokines). No significant relationships were noted between BAL lymphocytes, eosinophils, and chemokine concentrations before SBP-Ag.

Relationship between Th2 Chemokines and Th2 Cytokines

Forty-eight hours after SBP-Ag, the BALF concentrations of Th2-type chemokines (TARC and MDC), but not Th1-type chemokines, had striking correlations with IL-5 (Figure 2A: $r_s = 0.90$, $p < 0.001$; and Figure 2B: $r_s = 0.76$, $p < 0.001$) and IL-13 (Figure 2C: $r_s = 0.82$, $p < 0.001$; and Figure 2D: $r_s = 0.67$, $p = 0.004$). These correlations were not observed before SBP-Ag.

Relationship of Lymphocyte-associated Chemokines and the Pattern of Airway Response to Allergen Inhalation

To further assess the potential clinical relevance of lymphocyte-associated chemokine generation, we determined BALF chemokine concentrations 48 hours after SBP-Ag in subjects with different patterns of airway response (single versus dual responders) to whole lung inhalation of antigen.

Although IP-10, MIG, TARC, and MDC were augmented in BALF obtained from both groups 48 hours after SBP-Ag, the increase in both Th1- and Th2-type chemokines was remarkably greater in asthma subjects with a dual-responder phenotype (Figure 3). The concentrations of I-TAC were not significantly different between the two groups. After SBP-Ag, the dual-responder group also had increased BAL lymphocytes ($7.9 [4.2, 12.1] \times 10^4$ /ml BALF versus $2.2 [1.7, 2.7] \times 10^4$ /ml BALF, $p < 0.001$), a trend toward increased BAL eosinophils ($48.0 [20.6, 111.5] \times 10^4$ /ml BALF versus $7.4 [4.7, 32.3] \times 10^4$ /ml BALF, $p = 0.065$), and significantly increased concentrations of IL-5 ($56.2 [22.7, 339.9]$ pg/ml BALF versus $2.9 [0.0, 31.8]$ pg/ml BALF, $p < 0.05$). Subjects with the single- or dual-responder phenotype had similar concentrations of BAL cells, cytokines, and chemokines before SBP-Ag.

DISCUSSION

Using a model of allergen-induced asthma, we have demonstrated that lymphocyte-associated chemokines are associated with eosinophilic airway inflammation. Forty-eight hours after SBP-Ag, the Th2-type chemokines TARC and MDC had a highly significant correlation with the numbers of BAL eosinophils and concentrations of the Th2-associated cytokines IL-5 and IL-13. In contrast, airway lymphocytes correlated not only with Th2-type chemokines, but with the Th1-associated chemokines IP-10 and MIG as well. The potential clinical relevance of these chemokines was demonstrated by the fact that subjects with allergic asthma who had been phenotyped as dual responders on the basis of presentation of an LPR to inhaled antigen challenge had more intense airway inflammation and greater

TABLE 3. CONCENTRATIONS OF LYMPHOCYTE-ASSOCIATED CHEMOKINES IN BRONCHOALVEOLAR LAVAGE FLUID BEFORE AND 48 HOURS AFTER SEGMENTAL BRONCHOPROVOCATION WITH ANTIGEN

	Baseline (Pre-Ag Challenge)	48 Hours Post-Ag Challenge	p Value (Baseline versus 48 Hours)
IP-10, pg/ml $1 \times$ BALF	21.5 (14.5, 38.0)	123.5 (42.5, 154.5)	< 0.001
MIG, pg/ml $1 \times$ BALF	11.0 (3.0, 19.0)	53.0 (17.5, 93.0)	< 0.001
I-TAC, pg/ml $1 \times$ BALF	1.0 (0.0, 4.8)	5.0 (1.5, 13.0)	0.048
TARC, pg/ml $1 \times$ BALF	3.0 (1.5, 4.0)	131.0 (41.5, 567.0)	< 0.001
MDC, pg/ml $1 \times$ BALF	7.0 (0.0, 15.5)	118.0 (50.5, 1,152.5)	< 0.001

Definition of abbreviations: Ag = antigen; BALF = bronchoalveolar lavage fluid; IP-10 = IFN- γ -inducible protein-10; I-TAC = IFN-inducible T cell α chemoattractant; MDC = macrophage-derived chemokine; MIG = monokine-induced by IFN- γ ; TARC = thymus and activation-regulated chemokine.

Data are depicted as median (25 and 75% quartiles); n = 16.

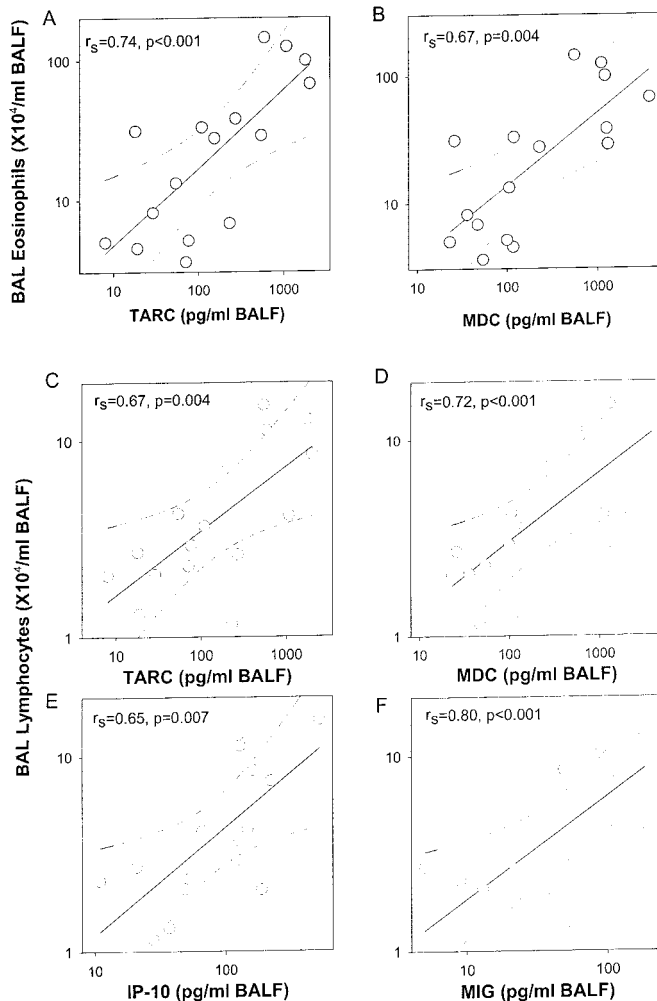


Figure 1. Relationship of helper T cell Type 1 and Type 2 (Th1 and Th2) chemokines to bronchoalveolar lavage (BAL) eosinophils and lymphocytes, 48 hours after segmental bronchoprovocation with antigen (SBP-Ag). BAL eosinophils were significantly correlated with thymus and activation-regulated chemokine (TARC) (A, $r_s = 0.74$, $p < 0.001$) and macrophage-derived chemokine (MDC) (B, $r_s = 0.67$, $p = 0.004$). BAL lymphocytes were significantly correlated with TARC (C, $r_s = 0.67$, $p = 0.004$), MDC (D, $r_s = 0.72$, $p < 0.001$), and the Th1 chemokines IFN- γ -inducible protein (IP)-10 (E, $r_s = 0.65$, $p = 0.007$) and monokine-induced by IFN- γ (MIG) (F, $r_s = 0.80$, $p < 0.001$).

production of Th2- and Th1-type chemokines after localized antigen challenge. Th2-type chemokines may contribute to the persistence of eosinophilic airway inflammation through lymphocyte recruitment and their production of Th2-associated cytokines. These data suggest that both Th2- and Th1-type chemokines are potentially involved in antigen-induced allergic airway inflammation and obstruction.

Although lymphocytes, in particular Th2-type cells, are considered to be an essential component of antigen-induced airway inflammation, little is known about how these cells are recruited to the airway. From *in vitro* studies, TARC and MDC are known to selectively recruit CCR4-expressing Th2 lymphocytes (3, 4). *In vivo* evidence that TARC plays a crucial role in antigen-induced airway eosinophilia was provided in a murine model as the administration of a specific antibody against TARC attenuated airway eosinophilia and decreased Th2-type cytokines (18). Our observations that BALF levels of TARC and MDC were

significantly correlated with IL-5 and IL-13, as well as with numbers of airway eosinophils, further support a role for these chemokines in antigen-induced eosinophilic airway inflammation. It was, however, somewhat surprising that these chemokines were present in the airway as late as 48 hours after antigen challenge. In general, chemokines, including eotaxin (15, 19), regulated upon activation, normal T cell expressed and secreted (15, 20, 21), macrophage inflammatory protein-1 α , and chemoattractant protein-1 (21), have been reported to increase within hours of antigen challenge and then return to baseline values by 24 hours (21). A possible explanation for the later presence of Th2-associated chemokines is that they contribute to the persistence of eosinophilic airway inflammation and, by inference, to the chronicity of airway inflammation in asthma. Interestingly, tumor necrosis factor- α , in combination with either Th2 cytokine, IL-4 or IL-13, enhances the expression of TARC in airway smooth muscle (22), epithelial cells (23), and macrophages (24). Thus, it is possible that a positive feedback loop exists whereby Th2-type cytokines induce expression of Th2-associated chemokines that in turn recruit Th2-type lymphocytes. The perpetuation of this Th2 milieu could then contribute to the persistence of eosinophilic airway inflammation and may enhance the inflammatory response to a subsequent antigen exposure.

Th1-associated chemokines IP-10 and MIG are produced by a variety of cell types and could play either a positive or negative role in the regulation of airway inflammation. There is compelling evidence in animal models that Th1 cytokines, and by implication Th1-associated chemokines, downregulate allergic inflammation. For example, IFN- γ , produced by Th1-type cells, inhibits the polarization of Th2 cells and/or antagonizes IL-4-mediated immune responses. In addition, IFN- γ is a potent inducer of IP-10 and MIG (25), and these chemokines attract IFN- γ -producing Th1-type lymphocytes. Surprisingly, although we demonstrated significant increases in IP-10 and MIG in BALF 48 hours after SBP-Ag, the levels of IFN- γ were low or undetectable. It is possible that Th1-associated chemokines are present at 48 hours to recruit a subsequent wave of Th1-type lymphocytes that may then downregulate the ongoing Th2-type response. Evaluation of BALF days to weeks after antigen challenge is needed to clarify this issue. In addition to attracting Th1-type lymphocytes, IP-10, MIG, and I-TAC may also directly antagonize allergen-induced eosinophilic inflammation by blocking the binding of eotaxin (an eosinophil-specific chemokine) to its receptor, CCR3 (26).

Despite the evidence that Th1-associated chemokines have the potential to downregulate allergic inflammation, there is also evidence that these same factors can promote airway eosinophilia. For example, overexpression of IP-10 in transgenic mice increased airway hyperresponsiveness, eosinophilia, and IL-4 in an IFN- γ -independent fashion (27). In addition, it has been demonstrated by us (7), and others (25), that eosinophils express CXCR3 and that the corresponding ligands, MIG and IP-10, can induce eosinophil chemotaxis and degranulation (25). Additional studies in animal models as well as humans are necessary to more clearly define the sources, inducers, and functional role of these chemokines in allergic inflammation.

Our observations corroborate a report by Bochner and colleagues (14) that demonstrated increased BALF concentrations of TARC, MDC, and IP-10, but not of I-309, in subjects with allergic asthma 20 hours after SBP-Ag. In their study, the Th2-associated chemokine response was confirmed to be antigen specific as levels remained at or below the level of detection in fluid from a corresponding saline-challenged segment. In contrast to their study, our data show that, when BAL is performed 48 hours after an SBP challenge with physiologic doses of allergen, TARC and MDC are strongly correlated with BAL eosinophilia

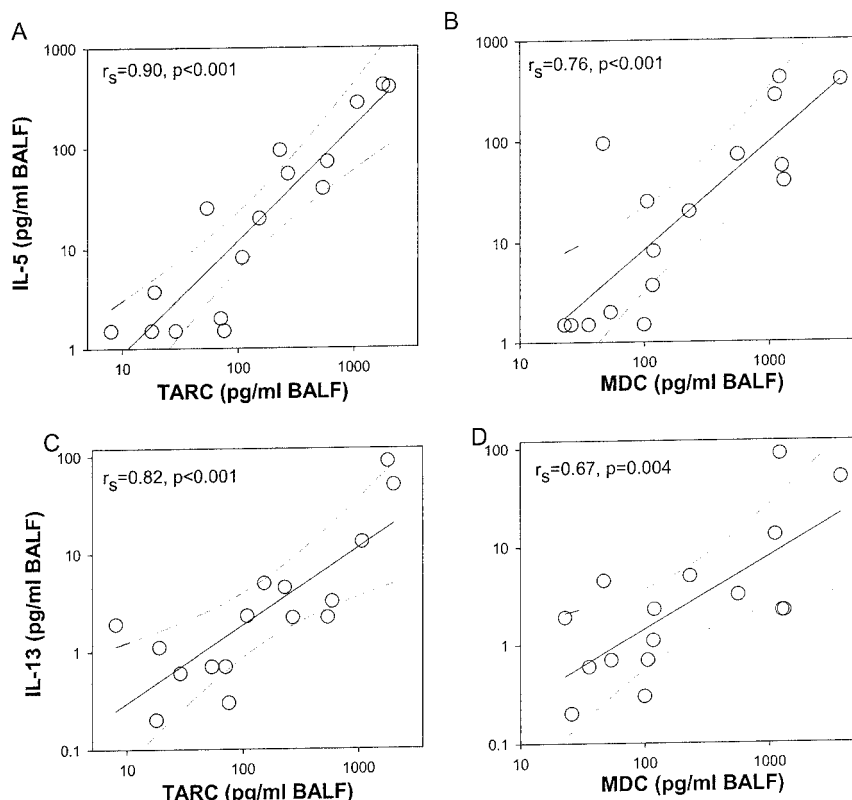


Figure 2. Relationship of Th2-type chemokines to IL-5 and IL-13, 48 hours after SBP-Ag. BAL fluid (BALF) concentrations of IL-5 were significantly correlated with TARC (A, $r_s = 0.90$, $p < 0.001$) and MDC (B, $r_s = 0.76$, $p < 0.001$). IL-13 was also significantly correlated with TARC (C, $r_s = 0.82$, $p < 0.001$) and MDC (D, $r_s = 0.67$, $p = 0.004$).

and Th2-associated cytokines (IL-5 and IL-13). The lack of correlation in the study by Bochner and coworkers may reflect the timing of the BAL (20 hours) or the dose of antigen given (all subjects received 500 units of either ragweed or house dust mite).

Our data, demonstrating that subjects with a dual-phase phenotype have higher concentrations of Th2- and Th1-associated chemokines after SBP-Ag, are in accord with previous reports (15, 28–30) that subjects with a documented LPR have greater eosinophilic airway inflammation and increased numbers of IL-5⁺ T cells in the bone marrow (30) between 24 and 48 hours after inhaled antigen challenge. The relationship between airflow obstruction seen between 3 and 8 hours after antigen challenge and eosinophilic airway inflammation present 48 hours later is not clearly understood. It has been implied, however, that the inflammatory events occurring at 48 hours reflect, to some extent, the initial airway inflammation associated with the antigen-induced LPR. Thus, it is tempting to speculate that the Th2-associated chemokines in the airway at 48 hours are also involved in, or are reflective of, the late asthmatic response. However, to prove a cause-and-effect relationship, it will be necessary to measure the LPR in the presence of inhibitors of these chemokines. Another possibility, and perhaps a more likely scenario, is that the immune response that occurs during the LPR initiates a cascade of events resulting in a more intense eosinophilic airway inflammation seen between 24 and 48 hours after antigen challenge. Thus, the magnitude of the later inflammatory response could reflect the intensity of the LPR and may be reflective and possibly dependent on the events associated with this response. Indeed, we found that subjects, who had previous evidence for greater airway obstruction at 3–8 hours postallergen challenge, had more airway inflammation (eosinophils, lymphocytes, Th2 cytokines, and lymphocyte-associated chemokines) 48 hours after SBP-Ag.

We recognize that there are certain limitations to our human studies. First, it is not feasible to perform detailed kinetic studies using bronchoscopy and BAL after SBP-Ag. Consequently, our data were limited to a single time point (48 hours) after antigen challenge. To more fully understand and define the roles of Th1- and Th2-associated chemokines in allergic inflammation, it would be of interest to study these patterns during the initiation (3–8 hours), peak (12 to 48 hours), persistence (7–10 days), and resolution (14–21 days) of the antigen-induced eosinophilic airway inflammation. Second, the use of segmental challenge did not allow us to determine simultaneous changes in pulmonary function in response to the locally administered antigen. Physiologic changes induced by SBP-Ag are generally limited because the antigen is deposited only in two bronchopulmonary segments. The previous characterization of the pulmonary function responses of these subjects to whole lung inhalation of antigen gives us a historical physiologic pattern by which to evaluate local airway inflammatory responses. Moreover, we have previously demonstrated that the cellular and protein changes after SBP-Ag are qualitatively similar to those induced by inhaled antigen, a method by which LPR can be measured (31). Thus, we feel that this combined approach provides a unique method to assess airway inflammatory responses in relationship to previously defined physiology. Third, our bronchial lavage study may not fully reflect the presence of chemokines in the bronchial mucosa. However, a report showing that TARC and MDC were strongly expressed in the airway epithelium 24 hours after local allergen challenge (10) suggests that lymphocyte-associated chemokines, particularly Th2-associated chemokines, are enhanced throughout the airway after exposure to antigen and thus supports our findings. Fourth, it should be emphasized that although the majority of *in vitro* polarized Th2 lymphocytes are CCR4⁺/CXCR3[−] whereas Th1 lymphocytes are CXCR3⁺/CCR4[−], there is likely to be a more diverse expression profile on both polarized and

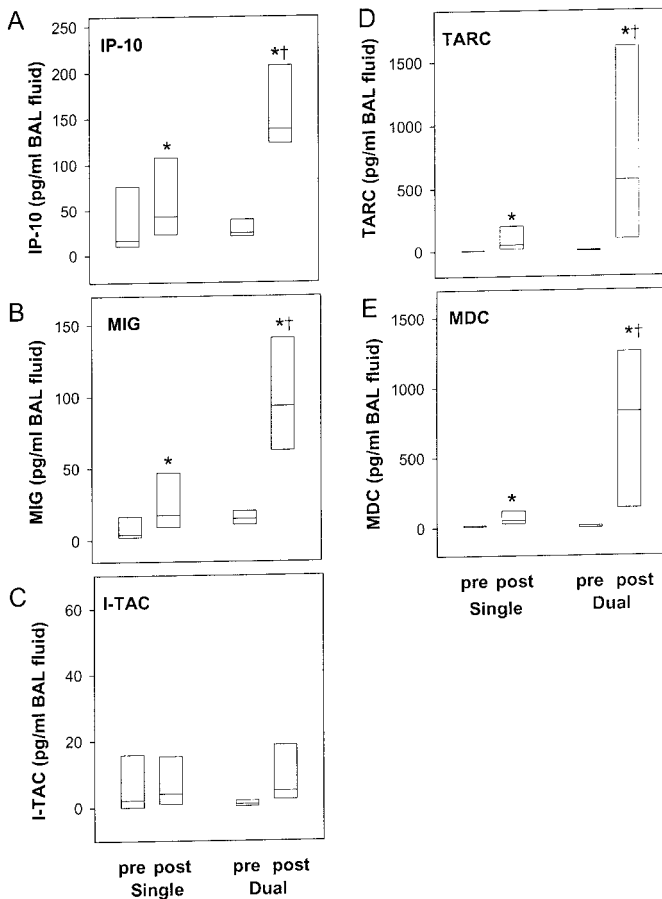


Figure 3. Comparison of chemokine concentrations in asthma subjects with a single- or dual-responder phenotype. IP-10 (A) and TARC (D) were measured in $1 \times$ BALF, and MIG (B), I-TAC (C), and MDC (E) were measured in $5 \times$ BALF, and data are expressed as the calculated amount of each chemokine in $1 \times$ BALF. Boxes represent medians within quartiles of 25 and 75%. * $p < 0.05$, within-group comparisons (preantigen versus postantigen); † $p < 0.05$, between-group comparisons (postantigen challenge values of single-responder [$n = 8$] versus dual-responder [$n = 8$] subjects).

nonpolarized cells *in vivo* (6). Indeed, studies of these receptors in human blood revealed a complex pattern that included a high percentage of CCR4⁺/CXCR3⁺ T cells, particularly within populations of Th0 and nonpolarized T cells (6). Clearly, single-cell analysis of BAL (and mucosal) T cells for expression of chemokine receptors and Th1/Th2 cytokines is necessary to fully understand and appreciate the contribution of lymphocyte-associated chemokines to allergen-driven recruitment Th1- and Th2-like lymphocytes. Finally, we recognize the possible contribution of lipopolysaccharides (LPS) to our findings. Although the Ag extract used for SBP contains only trace amounts of LPS (as determined by the manufacturer), it is possible that LPS is introduced into the airway when the bronchoscope is passed through the nose. The fact that there were minimal changes in numbers of neutrophils after antigen challenge (medians with 25th and 75th percentiles increased from 0.1 [0.1, 0.2] to 1.7 [1.0, 3.2] $\times 10^4$ cells/ml BALF) suggests that contamination by LPS was minimal. However, we cannot totally exclude the possibility that even trace amounts of LPS had an effect on chemokine generation.

In conclusion, our studies demonstrate that both Th2- and Th1-type chemokines are involved in antigen-induced airway inflammation. Asthma subjects with the dual-responder pheno-

type developed a more intense airway inflammation that was associated with enhanced levels of Th1- and Th2-associated chemokines. Our observations also suggest that TARC and MDC are involved in the eosinophilic airway inflammation through their recruitment of lymphocytes producing Th2 cytokines. The presence of these chemokines 48 hours after airway antigen challenge also suggests that they contribute to the persistence of allergic airway inflammation in asthma.

Conflict of Interest Statement: L.L. has no declared conflict of interest; N.N.J. has no declared conflict of interest; W.W.B. has no declared conflict of interest; E.A.B.K. has no declared conflict of interest.

Acknowledgment: The authors acknowledge research nurses Ann Dodge, B.S.N., Mary Jo Jackson, B.S.N., and Andrea Tweedie, B.S.N., for patient recruitment, screening, and assistance with bronchoscopies; Keith Meyer, M.D., for assistance with bronchoscopies; Sarah Panzer, B.S., and Rebecca Lawniczak, B.S., for BAL processing and help with the ELISA; and Michael Evans, M.A., for assistance with data analysis.

References

1. D'Ambrosio D, Mariani M, Panina-Bordignon P, Sinigaglia F. Chemokines and their receptors guiding T lymphocyte recruitment in lung inflammation. *Am J Respir Crit Care Med* 2001;164:1266–1275.
2. Thelen M. Dancing to the tune of chemokines. *Nat Immunol* 2001;2:129–134.
3. Bonecchi R, Bianchi G, Bordignon PP, D'Ambrosio D, Lang R, Borsatti A, Sozzani S, Allavena P, Gray PA, Mantovani A, et al. Differential expression of chemokine receptors and chemotactic responsiveness of Type 1 T helper cells (Th1s) and Th2s. *J Exp Med* 1998;187:129–134.
4. Sallusto F, Lenig D, Mackay CR, Lanzavecchia A. Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. *J Exp Med* 1998;187:875–883.
5. Imai T, Nagira M, Takagi S, Kakizaki M, Nishimura M, Wang J, Gray PW, Matsushima K, Yoshie O. Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. *Int Immunol* 1999;11:81–88.
6. Kim CH, Rott L, Kunkel EJ, Genovese MC, Andrew DP, Wu L, Butcher EC. Rules of chemokine receptor association with T cell polarization *in vivo*. *J Clin Invest* 2001;108:1331–1339.
7. Liu LY, Jarjour NN, Busse WW, Kelly EA. Chemokine receptor expression on human eosinophils from peripheral blood and bronchoalveolar lavage fluid following segmental antigen challenge. *J Allergy Clin Immunol* 2003;112:556–562.
8. Panina-Bordignon P, Papi A, Mariani M, Di Lucia P, Casoni G, Bellettato C, Buonsanti C, Miotto D, Mapp C, Villa A, et al. The C-C chemokine receptors CCR4 and CCR8 identify airway T cells of allergen-challenged atopic asthmatics. *J Clin Invest* 2001;107:1357–1364.
9. Lloyd CM, Delaney T, Nguyen T, Tian J, Martinez A, Coyle AJ, Gutierrez-Ramos JC. CC chemokine receptor (CCR)3/eotaxin is followed by CCR4/monocyte-derived chemokine in mediating pulmonary T helper lymphocyte type 2 recruitment after serial antigen challenge *in vivo*. *J Exp Med* 2000;191:265–274.
10. Nouri-Aria KT, Wilson D, Francis JN, Jopling LA, Jacobson MR, Hodge MR, Andrew DP, Till SJ, Varga EM, Williams TJ, et al. CCR4 in human allergen-induced late responses in the skin and lung. *Eur J Immunol* 2002;32:1933–1938.
11. Sekiya T, Yamada H, Yamaguchi M, Yamamoto K, Ishii A, Yoshie O, Sano Y, Morita A, Matsushima K, Hirai K. Increased levels of a TH2-type CC chemokine thymus and activation-regulated chemokine (TARC) in serum and induced sputum of asthmatics. *Allergy* 2002; 57:173–177.
12. Lezcano-Meza D, Negrete-Garcia MC, Dante-Escobedo M, Teran LM. The monocyte-derived chemokine is released in the bronchoalveolar lavage fluid of steady-state asthmatics. *Allergy* 2003;58:1125–1130.
13. Leung TF, Wong CK, Lam CW, Li AM, Ip WK, Wong GW, Fok TF. Plasma TARC concentration may be a useful marker for asthmatic exacerbation in children. *Eur Respir J* 2003;21:616–620.
14. Bochner BS, Hudson SA, Xiao HQ, Liu MC. Release of both CCR4-active and CXCR3-active chemokines during human allergic pulmonary late-phase reactions. *J Allergy Clin Immunol* 2003;112:930–934.
15. Gauvreau GM, Watson RM, O'Byrne PM. Kinetics of allergen-induced airway eosinophilic cytokine production and airway inflammation. *Am J Respir Crit Care Med* 1999;160:640–647.
16. Liu LY, Sedgwick JB, Bates ME, Vrtis RF, Gern JE, Kita H, Jarjour

- NN, Busse WW, Kelly EAB. Decreased expression of membrane IL-5R α on human eosinophils: I. Loss of membrane IL-5R α on eosinophils and increased soluble IL-5R α in the airway after antigen challenge. *J Immunol* 2002;169:6452–6458.
17. Kelly EA, Rodriguez RR, Busse WW, Jarjour NN. The effect of segmental bronchoprovocation with allergen on airway lymphocyte function. *Am J Respir Crit Care Med* 1997;156:1421–1428.
18. Kawasaki S, Takizawa H, Yoneyama H, Nakayama T, Fujisawa R, Izumizaki M, Imai T, Yoshie O, Homma I, Yamamoto K, *et al*. Intervention of thymus and activation-regulated chemokine attenuates the development of allergic airway inflammation and hyperresponsiveness in mice. *J Immunol* 2001;166:2055–2062.
19. Lilly CM, Nakamura H, Belostotsky OI, Haley KJ, Garcia-Zepeda EA, Luster AD, Israel E. Eotaxin expression after segmental allergen challenge in subjects with atopic asthma. *Am J Respir Crit Care Med* 2001;163:1669–1675.
20. Teran LM, Noso N, Carroll M, Davies DE, Holgate S, Schröder JM. Eosinophil recruitment following allergen challenge is associated with the release of the chemokine RANTES into asthmatic airways. *J Immunol* 1996;157:1806–1812.
21. Holgate ST, Bodey KS, Janezic A, Frew AJ, Kaplan AP, Teran LM. Release of RANTES, MIP-1 α , and MCP-1 into asthmatic airways following endobronchial allergen challenge. *Am J Respir Crit Care Med* 1997;156:1377–1383.
22. Faffe DS, Whitehead T, Moore PE, Baraldo S, Flynt L, Bourgeois K, Panettieri RA, Shore SA. IL-13 and IL-4 promote TARC release in human airway smooth muscle cells: role of IL-4 receptor genotype. *Am J Physiol Lung Cell Mol Physiol* 2003;285:L907–L914.
23. Sekiya T, Miyamasu M, Imanishi M, Yamada H, Nakajima T, Yamaguchi M, Fujisawa T, Pawankar R, Sano Y, Ohta K, *et al*. Inducible expression of a Th2-type CC chemokine thymus- and activation-regulated chemokine by human bronchial epithelial cells. *J Immunol* 2000;165:2205–2213.
24. Greaves DR, Hakkinen T, Lucas AD, Liddiard K, Jones E, Quinn CM, Senaratne J, Green FR, Tyson K, Boyle J, *et al*. Linked chromosome 16q13 chemokines, macrophage-derived chemokine, fractalkine, and thymus- and activation-regulated chemokine, are expressed in human atherosclerotic lesions. *Arterioscler Thromb Vasc Biol* 2001;21:923–929.
25. Jinquan T, Jing C, Jacobi HH, Reimert CM, Millner A, Quan S, Hansen JB, Dissing S, Malling HJ, Skov PS, *et al*. CXCR3 expression and activation of eosinophils: role of IFN- γ -inducible protein-10 and monokine induced by IFN- γ . *J Immunol* 2000;165:1548–1556.
26. Loetscher P, Pellegrino A, Gong JH, Mattioli I, Loetscher M, Bardi G, Baggiolini M, Clark-Lewis I. The ligands of CXC chemokine receptor 3, I-TAC, Mig, and IP10, are natural antagonists for CCR3. *J Biol Chem* 2001;276:2986–2991.
27. Medoff BD, Sauty A, Tager AM, MacLean JA, Smith RN, Mathew A, Dufour JH, Luster AD. IFN- γ -inducible protein 10 (CXCL10) contributes to airway hyperreactivity and airway inflammation in a mouse model of asthma. *J Immunol* 2002;168:5278–5286.
28. Shaver J, O'Connor J, Pollice M, Cho SK, Kane G, Fish J, Peters S. Pulmonary inflammation after segmental ragweed challenge in allergic asthmatic and nonasthmatic subjects. *Am J Respir Crit Care Med* 1995;152:1189–1197.
29. Aalbers R, Kauffman HF, Vrugt B, Smith M, Koeter GH, Timens W, de Monchy JG. Bronchial lavage and bronchoalveolar lavage in allergen-induced single early and dual asthmatic responders. *Am Rev Respir Dis* 1993;147:76–81.
30. Wood LJ, Sehmi R, Dorman S, Hamid Q, Tulic MK, Watson RM, Foley R, Wasi P, Denburg JA, Gauvreau G, *et al*. Allergen-induced increases in bone marrow T lymphocytes and interleukin-5 expression in subjects with asthma. *Am J Respir Crit Care Med* 2002;166:883–889.
31. Calhoun WJ, Jarjour NN, Gleich GJ, Stevens CA, Busse WW. Increased airway inflammation with segmental versus aerosol antigen challenge. *Am Rev Respir Dis* 1993;147:1465–1471.