

Short communication

Altered intracellular expression of the chemokines MIP-1 α , MIP-1 β and IL-8 by peripheral blood CD4⁺ and CD8⁺ T cells in mild allergic asthma

Background: The ability of chemokines to regulate Th1 and Th2 responses suggests a role in the pathogenesis of atopic disorders such as allergic asthma where Th2 response dominance has been observed. Although the impact of allergic asthma on local chemokine production in the lung has been the subject of investigation, little is known about the influence of disease progression on peripheral chemokine production. We now report use of whole blood culture and flow cytometry to assess the influence of mild allergic asthma on peripheral T-cell chemokine expression.

Methods: Study participants included patients with mild allergic asthma ($n = 7$) and nonasthmatic controls ($n = 7$). Following *in vitro* stimulation of peripheral venous blood with phorbol 12-myristate acetate (PMA) and ionomycin, flow cytometry was used to estimate the percentage of CD4⁺ and CD8⁺ T cells producing a number of chemokines, including macrophage inflammatory proteins MIP-1 α and MIP-1 β , RANTES (regulated on activation, T-cell expressed and secreted), monocyte chemoattractant protein-1 (MCP)-1, and interleukin (IL)-8, or the cytokines interferon (IFN)- γ and IL-4. Serum levels of MIP-1 α , MIP-1 β , RANTES, MCP-1, IL-8, IFN- γ and IL-4 were also assessed by quantitative ELISA.

Results: Intracellular expression of MIP-1 β by CD4⁺ and CD8⁺ T cells from allergic asthmatics was significantly reduced in comparison to that observed for nonasthmatics (median = 2.29% (1.75–3.50) vs 4.57% (3.38–6.64), $P = 0.05$; 14.20% (13.18–17.88) vs 44.10% (30.38–48.70), $P = 0.01$). Similarly, intracellular expression of MIP-1 α by CD8⁺ T cells from allergic asthmatics was also significantly lower (3.67% (1.17–5.42) vs 17.10% (4.97–20.43), $P = 0.05$). Conversely, IL-8 expression by both CD4⁺ and CD8⁺ T cells from allergic asthmatics demonstrated significant enhancement (9.93% (7.77–11.28) vs 4.14% (3.61–7.11), $P = 0.05$; 8.40% (6.97–10.04) vs 4.98% (3.37–6.08), $P = 0.05$). Examination of intracellular IFN- γ and IL-4 revealed no significant difference in the expression of either cytokine by CD4⁺ T-cells from allergic asthmatics and nonasthmatics. In contrast, expression of IFN- γ was significantly reduced in CD8⁺ T-cells from allergic asthmatics (24.60% (21.08–32.50) vs 48.40% (41.50–55.28), $P = 0.01$).

Conclusions: The occurrence in mild allergic asthma of peripheral T-cell chemokine expression suggestive of a diminished Th1 response, coinciding with marginal change in cytokine profiles indicative of a Th2 response bias, confirms the importance of chemokine involvement in the etiology of allergic asthma. The ability to use whole blood culture to estimate chemokine expression in T cell subsets may ultimately provide a practical means to evaluate disease status and to monitor early intervention therapies which target chemokines.

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Key words: asthma; atopy; chemokines; IL-8; MCP-1; MIP-1 α ; MIP-1 β ; RANTES; T cells.

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Accepted for publication 10 September 2002

Evidence for chemokine contribution to the pathogenesis of atopic disorders is increasing (1). In particular, focus on imbalanced T-helper type 1 (Th1) and T-helper type 2 (Th2) cytokine responses during allergic asthma is

currently being expanded to encompass a parallel role for polarized chemokine responses (2).

The influence of Th2 cells in establishing early allergen sensitization and supporting subsequent inflammatory

processes has been confirmed by numerous studies (3, 4). Bias towards a Th2 cytokine profile during allergic asthma is evident from the participation of interleukin-4 (IL-4) and IL-5 in local eosinophil infiltration and activation (5) and the role of IL-10 and IL-13 in airway hyperreactivity and allergic inflammation (6). Although intensified Th2 responses have been attributed primarily to CD4⁺ T-cells (7), exacerbation of both early- and late-phase responses has also been shown to result from CD8⁺ T-cell depletion in animal models of allergic asthma (8, 9). However, in contrast to CD4⁺ T cells, the influence of CD8⁺ T cells derives primarily from the negative regulatory effect of IFN- γ secretion on subsequent IL-4 and IL-5 production (10).

Chemokine involvement in allergic asthma has been observed as increased levels of RANTES (regulated on activation, normal T-cell expressed and secreted), macrophage inflammatory protein-1 α (MIP-1 α), macrophage chemotactic protein-1 (MCP-1), MCP-3, eotaxin and IL-8 in bronchoalveolar lavage fluid and biopsy specimens (11–16). In keeping with the initial characterization of chemokines as factors responsible for leukocyte recruitment (17), this chemokine production can be correlated with levels of inflammatory cell infiltration (11–13). Observation of a temporal association between the presence of chemokines and subsequent development of biased cytokine responses (18–21) suggests a similar role for chemokines in the pathogenesis of allergic asthma (2, 22).

Definition of functional T-cell subsets characterized by Th1 and Th2 cytokine patterns has relied heavily on measurement of intracellular cytokine expression by flow cytometry (23). The ability to evaluate cytokine patterns at the single-cell level has allowed simultaneous assessment of the phenotype and frequency of responding cells in response to a wide range of stimuli (24). Adaptation of this approach to whole blood culture now allows detection of intracellular cytokines in the physiologically relevant presence of an autologous cellular and serological environment (25). In an effort to understand the impact of atopy on peripheral T-cell chemokine production, we have extended this approach to include assessment of intracellular chemokine expression. In particular, we now report use of an *in vitro* whole blood system to assess the influence of mild allergic asthma on intracellular production of MIP-1 α , MIP-1 β , MCP-1, RANTES and IL-8 by peripheral blood CD4⁺ and CD8⁺ T-cells.

Material and methods

Subjects

Symptomatic allergic asthmatic ($n = 7$) and nonasthmatic ($n = 7$) study participants were recruited from among outpatients attending the Allergy Unit, Department of Dermatology, University Hospital of Zürich and staff employed within the University Hospital of

Table 1. Study participant characteristics

Parameter	Allergic asthmatics	Nonasthmatics
Age (years)	22 (21–29)	37 (32–42)
Gender (female/male)	3/4	4/3
Severity	2.0 (1.0–2.0)	0.0
FEV ₁ (% predicted value)	85.04 (71.89–98.63)	ND
FVC (% predicted value)	104.19 (88.71–115.78)	ND
Total IgE (kU/l)	366.00 (96.45–1457.50)	20.40 (8.89–51.40)
Sx1 allergen panel-specific IgE (kU/l)	35.00 (10.72–98.05)	0.35 (0.35–0.35)
Fx5 allergen panel-specific IgE (kU/l)	0.35 (0.35–2.72)	0.35 (0.35–0.35)
ECP (μ g/l)	16.90 (12.12–27.50)	12.20 (8.64–13.60)
Eosinophils (cells/ μ l)	200 (180–420)	80 (70–110)
CD4 ⁺ T cells (cells/ μ l)	1021 (803–1110)	934 (702–1152)
CD8 ⁺ T cells (cells/ μ l)	582 (562–618)	447 (303–613)

Values are given as medians (interquartile range).

ECP, eosinophil cationic protein; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; ND, not done.

Zürich. Baseline characteristics of the study population are outlined (Table 1).

Clinical evaluation

Diagnosis of allergic asthma was based on guidelines established by the EAACI (26) and was scored for severity (27). Airway dynamics, as determined by forced expiratory volume in one second (FEV₁) and forced vital capacity (FVC), were assessed by spirometry (28) using published reference values (29). The atopic status of all study participants was additionally confirmed by measurement of total serum IgE levels and allergen specific IgE levels against panels of common inhalative (Sx1 panel: timothy and rye grass pollen, birch tree pollen, mugwort weed pollen, *Cladosporium herbarum*, house dust mite, cat and dog dander) and food (Fx5 panel: egg white, milk, fish, wheat, peanut, soya bean) allergens by UniCAP (Pharmacia Diagnostics, Dübendorf, Switzerland) (30,31). The possible occurrence of eosinophil inflammation was assessed by estimation of serum eosinophil cationic protein (ECP) concentrations by UniCAP (Pharmacia & Upjohn Diagnostics) (32). Allergic asthmatic participants were being treated with inhaled corticosteroids and none were undergoing systemic corticosteroid, antihistamine or leukotriene-antagonist therapies (33).

Intracellular chemokine levels

Detection of intracellular chemokine and cytokine production was performed on heparinized venous blood. Whole blood was diluted 1 : 2 in RPMI-1640 (Invitrogen Life Technologies, Basel, Switzerland) containing 100 ng/ml of phorbol 12-myristate acetate (PMA) (Sigma-Aldrich, Buchs, Switzerland), 2 μ g/ml of ionomycin (Sigma) and 20 μ g/ml Brefeldin-A (Sigma), followed by incubation for 5 h at 37°C in the presence of 5% CO₂. Stimulated whole blood was distributed in 50 μ l aliquots into 12 $\times$ 75 mm test tubes (Falcon, Allschwill, Switzerland) containing 10 μ l antihuman CD4-PC5 (Coulter, Nyon, Switzerland) or 10 μ l antihuman CD8-PC5 (Coulter), followed by vortexing and incubation in the absence of light at room temperature for 15 min. Leukocyte fixation was undertaken by addition of 100 μ l of IntraPrep Reagent-1 (Immuno-tech, Nyon, Switzerland), followed by vortexing, continued incubation for 15 min and removal of excess antibody by one

wash cycle in 4 ml phosphate-buffered saline (PBS). Red blood cell lysis and leukocyte permeabilization were undertaken by addition of 100 μ l of IntraPrep Reagent-2 (Immunotech) and incubation in the absence of light at room temperature for 5 min. Intracellular chemokines were then stained by addition of 2 μ l aliquots of the appropriate antichemokine, anticytokine or isotype control antibodies, followed by vortexing and further incubation for 15 min. Antibodies used included FITC-conjugated antihuman MIP-1 α or antihuman IL-8 and PE-conjugated antihuman MIP-1 β , antihuman RANTES, antihuman MCP-1, antihuman IFN- γ and antihuman IL-4 (BD Pharmingen, Allschwill, Switzerland). Excess antibody and red blood cell debris were then removed by one wash in 4 ml PBS. The remaining cell pellet was fixed in 500 μ l of PBS containing 1% paraformaldehyde and stored overnight at 4°C. Three-color flow cytometric data was collected using an EPICS XL/MCL flow cytometer (Coulter). Dead cells, monocytes and polymorphonuclear cells were initially excluded by forward- and side-scatter gating. Acquisition was then gated on CD4 $^{+}$ or CD8 $^{+}$ T cells and histogram plots set up to determine the percentage of these cells expressing chemokine or cytokine. Cell activation was controlled by assessment of intracellular CD69 levels, with all CD4 $^{+}$ or CD8 $^{+}$ T cells exhibiting expression levels = 96%.

Serum chemokine levels

Quantitative sandwich enzyme-linked immunosorbant assay (ELISA) kits were used to measure retrospectively serum levels of MIP-1 α , MIP-1 β , MCP-1, RANTES, IL-8, IFN- γ and IL-4 (R & D Systems) in duplicate according to the manufacturer's instructions. As described by the manufacturer, lower detection limits for the assays are 10 pg/ml for MIP-1 α , 11 pg/ml for MIP-1 β , 5 pg/ml for MCP-1, 8 pg/ml for RANTES, 10 pg/ml for IL-8, 8 pg/ml for IFN- γ and 10 pg/ml for IL-4.

Statistical analysis

Results are expressed as median values with interquartile ranges. The significance of differences between study parameters was estimated using the nonparametric Mann-Whitney *U*-test for unpaired datasets and a *P*-value of ≤ 0.05 was regarded as significant. All evaluations were implemented using the STATVIEW 5.0 (SAS Institute, Cary, North Carolina, USA) software package.

Results

Intracellular chemokine and cytokine expression

Following *in vitro* stimulation of whole blood with PMA and ionomycin, the percentage of CD4 $^{+}$ T cells expressing intracellular MIP-1 β was significantly lower ($P = 0.05$) in cultures derived from allergic asthmatics (2.29% (1.75–3.50)) in comparison to cultures derived from nonasthmatics (4.57% (3.38–6.64)) (Fig. 1A). In contrast, the percentage of CD4 $^{+}$ T cells expressing IL-8 was significantly higher ($P = 0.05$) for allergic asthmatics (9.93% (7.77–11.28)) than for nonasthmatics (4.14% (3.61–7.11)) (Fig. 1A). An analogous trend was observed for CD8 $^{+}$ T cells, with intracellular MIP-1 β expression being significantly lower ($P = 0.01$) in cultures derived from allergic asthmatics (14.20% (13.18–17.88)) in comparison to cultures derived from nonasthmatics (44.10%

(30.38–48.70)) (Fig. 1B). Again, the percentage of CD8 $^{+}$ T cells expressing IL-8 was also significantly higher ($P = 0.01$) for allergic asthmatics (8.40% (6.97–10.04)) than for nonasthmatics (4.98 (3.37–6.08)) (Fig. 1B). An additional significant decrease ($P = 0.05$) in the percentage of CD8 $^{+}$ T cells expressing MIP-1 α was observed for allergic asthmatics (3.67% (1.17–5.42)) in comparison to nonasthmatics (17.10% (4.97–20.43)) (Fig. 1B).

No significant difference was observed between the percentage of CD4 $^{+}$ T cell expressing MIP-1 α , MCP-1 or RANTES for allergic asthmatics (0.85% (0.50–1.72), 0.19% (0.06–0.33) and 0.11% (0.00–0.54), respectively) and nonasthmatics (1.09% (0.77–1.28), 0.17% (0.09–0.42) and 0.15% (0.07–0.49), respectively) (Fig. 1A). Similarly, no significant difference was observed between the percentage of CD8 $^{+}$ T cell expressing MCP-1 or RANTES for allergic asthmatics (0.34% (0.28–0.52) and 0.19% (0.12–0.26), respectively) and nonasthmatics (0.39% (0.07–0.48) and 0.39% (0.21–0.96), respectively) (Fig. 1B).

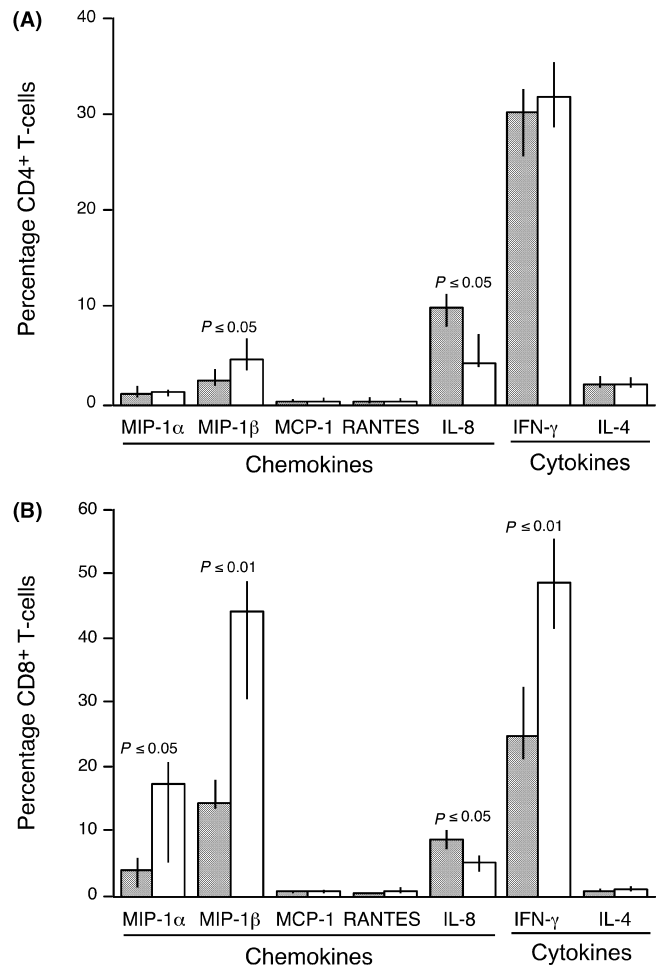


Figure 1. Intracellular chemokine and cytokine expression in CD4 $^{+}$ and CD8 $^{+}$ T-cells from allergic asthmatics (■) and nonasthmatics (□) following *in vitro* stimulation of whole blood with PMA and ionomycin.

Table 2. Chemokine and cytokine levels in serum from allergic asthmatics and nonasthmatics

Chemokine/cytokine	Allergic asthmatics	Nonasthmatics	Significance
MIP-1 α (pg/ml)	7.93 (6.56–20.25) ¹	29.54 (7.47–42.23)	NS
MIP-1 β (pg/ml)	2.41 (2.41–3.70)	3.18 (1.81–3.48)	NS
MCP-1 (pg/ml)	119.05 (59.90–242.29)	53.07 (29.16–243.00)	NS
RANTES (ng/ml)	18.49 (2.36–18.83)	14.14 (0.54–21.50)	NS
IL-8 (pg/ml)	55.02 (15.68–403.41)	38.12 (30.84–131.97)	NS
IFN- γ (pg/ml)	10.60 (2.38–21.62)	3.00 (0.72–13.53)	NS
IL-4 (pg/ml)	0.00 (0.00–0.62)	0.00 (0.00–0.30)	NS

¹median (interquartile range) NS = not significant.

Using identical *in vitro* culture conditions, no significant difference was observed in the percentage of CD4⁺ T cells expressing either intracellular IFN- γ or intracellular IL-4 for allergic asthmatics (30.10% (25.65–32.50) and 1.90% (1.50–2.76), respectively) or nonasthmatics (31.70% (28.65–35.30) and 1.80% (1.44–2.68), respectively) (Fig. 1A). A lack of significant difference in intracellular IL-4 expression was also observed for CD8⁺ T cells from allergic asthmatics (0.60% (0.41–0.86)) and nonasthmatics (0.90% (0.47–1.12)) (Fig. 1B). In contrast, the percentage of CD8⁺ T cells expressing IFN- γ was significantly lower ($P = 0.01$) in cultures derived from allergic asthmatics (24.60 (21.08–32.50)) in comparison to cultures derived from nonasthmatics (48.40 (41.50–55.28)) (Fig. 1B).

Serum chemokine and cytokine levels

Assessment of serum MIP-1 α , MIP-1 β , MCP-1, RANTES, IL-8, IFN- γ and IL-4 levels by quantitative ELISA revealed no significant differences between allergic asthmatics and nonasthmatics (Table 2).

Discussion

Chemokine contribution to the establishment of Th1 and Th2 response polarization (2) suggests a role for these chemotactic cytokines in the pathogenesis of atopic disorders demonstrating a skewed Th2 response (1). Recent focus on the lung as a source of elevated chemokine levels during allergic asthma has allowed the relationship between inflammatory cell infiltrate and chemokine production to be intensively investigated (11–16). In order to assess the impact of mild allergic asthma on chemokine production in the periphery, we have now examined intracellular production of the MIP-1 α , MIP-1 β , MCP-1, RANTES and IL-8 by T-cells following *in vitro* activation of whole blood in the presence of PMA and ionomycin. It is anticipated that use of peripheral blood to monitor asthma-related changes, while unable to directly reflect any lung-based pathophysiology, may prove a practical adjunct. We now report the occurrence of peripheral blood CD4⁺ T-cell

chemokine production characterized by enhanced IL-8 and reduced MIP-1 β expression. Similarly, we also report the occurrence of peripheral blood CD8⁺ T-cell chemokine production characterized by enhanced IL-8 and reduced MIP-1 α and MIP-1 β expression.

A role for chemokines in establishing the nature of cytokine responses has now been confirmed. Naive T cells stimulated *in vitro* in the presence of MIP-1 α demonstrate an enhanced propensity to produce Th1 cytokines such as interferon- γ (IFN- γ) (18). Conversely, incubation in the presence of MCP-1 results in increased production of the Th2 cytokine IL-4 (18). The complete inability of MCP-1-deficient mice to mount Th2 responses has also recently been reported (19). Similarly, following *in vitro* (20) and *in vivo* (21) challenge with mycobacterium-derived antigens, known to elicit Th1 cytokine profiles, predominant expression of MIP-1 α , MIP-1 β and RANTES has been reported. *In vivo* challenge with metazoan parasite-derived antigens, known to elicit Th2 cytokine profiles, is associated with skewed expression of chemokines such as eotaxin, MCP-2, MCP-3 and liver and activation-regulated chemokine (LARC) (21).

The participation of chemokines in preferential recruitment of Th2 cells is also evident. Monocyte-derived chemokine (MDC), TARC, eotaxin and I-309 all act *in vitro* to specifically attract Th2 cells (34–36). Increased expression of MDC (37) and TARC (38) during allergic asthma has since been confirmed in animal models and administration of blocking antibodies shown to effectively diminish Th2 cytokine production (38), cell infiltration and degree of airway hyperresponsiveness (37, 38). In humans, enhanced production of MDC and TARC following allergic challenge has also been demonstrated and the majority of T cells infiltrating the airway mucosa of allergic asthmatics shown to express the CCR4 receptor specific for MDC and TARC (39). The bidirectional nature of this interaction is apparent from the observation that, following commitment to a Th2 response, increased expression of the cytokines IL-4 and IL-13 can further enhance production of chemokines such as eotaxin (40, 41) and MCP-1 (42).

The importance of MIP-1 α in allergic asthma has been inferred from observation of enhanced MIP-1 α levels within the lungs of allergic asthmatics, where it is responsible for recruitment of a wide range of cell types, including basophils, eosinophils, macrophage and lymphocytes (43). In addition, the presence of MIP-1 α has been shown to be influential in establishing Th1 responses (18) and MIP-1 α is a predominant chemokine in the Th1 response itself (21). Given evidence that allergic asthma can be characterized by a failure to develop effective Th1 responses, our observation of diminished numbers of peripheral blood CD4⁺ T cells expressing MIP-1 α during mild allergic asthma most likely reflects polarization away from Th1 responses at the chemokine level. Nevertheless, this observed trend towards diminishing peripheral production of MIP-1 α

during mild allergic asthma contrasts with evidence for increasing MIP-1 α levels localized within the lung.

A similar role for MIP-1 β in influencing establishment of Th1 responses has been reported (20). However, the role of MIP-1 β during allergic asthma has not previously been investigated and reports are currently limited to related observation of MIP-1 β accumulation in lungs during chronic bronchitis (44) and acute lung injury (45). Our observation of diminished MIP-1 β production by both CD4⁺ and CD8⁺ T cells suggests the relevance of this chemokine to disease development and is likely to further reflect polarization away from Th1 responses at the chemokine level. In contrast to observation of MIP-1 α and MIP-1 β changes at the single cell level, no fluctuation in serum levels of these chemokines was evident for allergic asthmatics and nonasthmatics.

The important contribution made by IL-8 to the inflammatory features of diseases characterized by neutrophil recruitment is well recognized (46). During allergic asthma, increased levels of IL-8 have been detected in the lung (15,16) and a functional role for IL-8 assigned to local recruitment of both neutrophils (47) and, more recently, eosinophils (48,49). It is likely that our observation of elevated numbers of peripheral blood CD4⁺ and CD8⁺ T cells expressing IL-8 during mild allergic asthma reflects this chronic inflammatory component of allergic asthma. However, this increased production of IL-8 by peripheral T cells must be contrasted with the observed simultaneous diminishment in numbers of T-cells expressing MIP-1 α or MIP-1 β , suggesting the independent control of these inflammatory chemokines. Indeed, in contrast to MIP-1 α and MIP-1 β , the pronounced ability of Th2 cytokines to down-regulate IL-8 has previously been observed in an *in vitro* model of lung inflammation (50).

In contrast to the observed changes in peripheral CD4⁺ T-cell chemokine production, expression of the cytokines IFN- γ and IL-4 was similar for both allergic asthmatics and nonasthmatics enrolled in this study. Continued maintenance of normal IFN- γ and IL-4 expression would appear to reflect both the mild nature of allergic asthma under investigation and a subsequent lack of shift towards Th2 response predominance. However, this lack of polarization at the cytokine level contrasts noticeably with the significant drop in production of MIP-1 α by CD4⁺ T cells observed in the same

allergic asthmatics. In view of association between MIP-1 α and Th1 response establishment, this drop in MIP-1 α expression provides evidence at the chemokine level that a shift towards Th2-dominated responses is indeed underway. Furthermore, the early appearance of this chemokine shift suggests a temporal association with eventual establishment of the Th2 cytokine profile characterizing more advance stages of allergic asthma. Occurrence of a Th2 response shift at the chemokine level during mild allergic asthma was also evident from our observation of diminished production of both MIP-1 α and MIP-1 β by CD8⁺ T cells. However, in contrast to that observed for CD4⁺ T cells, the presence of a Th2 response shift was already discernible as a drop in the percentage of CD8⁺ T cells producing IFN- γ . This later observation confirms previous reports describing diminished IFN- γ production by CD8⁺ T-cells during allergic asthma (51–54). In contrast, recent reports have also described an enhancement of IFN- γ production by CD8⁺ T-cells during allergic asthma (55,56) which most likely reflects differences in methodology and disease staging (55).

In summary, using an *in vitro* whole blood culture system and flow cytometry, we now highlight an association between mild allergic asthma and reduced peripheral T-cell production of chemokines typically associated with development of Th1 responses. In particular, reduced percentages of CD4⁺ T-cells expressing MIP-1 α , coinciding with reduced percentages of CD8⁺ T-cells expressing both MIP-1 α and MIP-1 β , were observed. In contrast, diminished production of cytokines associated with Th1 responses was only evident as a reduced percentage of CD8⁺ T cells expressing IFN- γ . Taken together, these results suggest the utility of assessing peripheral T-cell chemokine production as an early indicator of polarization away from Th1 response dominance. Assessment of intracellular chemokine expression by peripheral T cells following *in vitro* whole blood culture may ultimately provide a practical means to evaluate early intervention therapies for allergic asthma which target chemokines (57).

Acknowledgments

The authors would like to thank P. Juraschek and her nursing team.

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