Increased levels of IL-4 in CD8+ T cells in atopic asthma

Luminita A. Stanciu, MD, PhD, Janis Shute, PhD, Charuporn Promwong, MD, Stephen T. Holgate, MD, DSc, and Ratko Djukanović, MD, DM
Southampton, England

Background: In view of reports that CD8+ T cells may produce T_{H2}-type cytokines and our own finding that levels of intracellular IL-4 are higher in CD8+ than CD4+ T cells in healthy nonatopic subjects, we have hypothesized that the capacity of CD8+ T cells to produce IL-4 may be increased in atopic asthma, a disease characterized by high production of T_{H2} cytokines.
Methods: Levels of IL-4 and interferon-γ were measured by ELISA in cell lysates and in 20- and 48-hour cultures of concanavalin A-stimulated purified peripheral blood CD8+ T cells in seven patients with mild atopic asthma and seven healthy nonatopic subjects.
Results: Resting CD8+ T cells in patients with asthma contained significantly more IL-4 than those of healthy nonatopic subjects (median, 26 pg/10^6 cells; range, 17 to 84 pg/10^6 cells vs 16 pg/10^6 cells; 10 to 28 pg/10^6 cells), with no difference in intracellular interferon-γ levels. In the healthy control subjects, but not in the patients with asthma, levels of intracellular IL-4 correlated negatively with levels of interferon-γ in resting CD8+ T cells ($r_s = -0.9411$, $p = 0.005$). Stimulation with concanavalin A produced a consistent and significant increase in secretion of interferon-γ, but not IL-4, with no difference between the two groups of subjects.
Conclusion: The results of this study suggest that CD8+ T cells from patients with asthma may be an important source of the T_{H2}-type cytokine IL-4. This capacity appears to be acquired in vivo, possibly by conditioning by IL-4 produced in the inflamed airways. (J Allergy Clin Immunol 1997;100: 373-8.)

Key words: Atopic asthma, CD8+ T cells, IL-4, IFN-γ, Con A

It is now widely appreciated that purified CD8+ T cells possess the capacity to produce a wide range of cytokines and chemokines, which in some instances (interferon-γ [IFN-γ], granulocyte-macrophage colony-stimulating factor, macrophage inflammatory protein-1α, IL-16, and RANTES) exceeds that of CD4+ T cells.[1-3] Recently, distinct cytokine-secreting subsets of CD8+ T cells (Tc), similar to their CD4+ T_{H1}- and T_{H2}-type counterparts, have been identified and designated Tc1 cells, which secrete predominantly IL-2 and IFN-γ, and Tc2 cells, which secrete IL-4 and IL-5.[4-9] Activated/memory CD8+ T cells bearing CD45RO can be stimulated with phorbol myristate acetate and ionomycin to secrete appreciable levels of IL-4, which cannot be detected if the whole population of CD8+ T cells is cultured.[1] We have recently reported that when positively selected by immunomagnetic separation, a process that involves ligation of the CD8 molecule by monoclonal anti-CD8 antibodies, blood-derived CD8+ T cells from healthy nonatopic subjects contain higher amounts of IL-4 than CD4+ T cells from the same subjects.[10]

Abbreviations used
Con A: Concanavalin A
IFN-γ: Interferon-γ
mAb: Monoclonal antibody

There is abundant evidence that the T_{H2}-type cytokines IL-4 and IL-5 play a central role in the inflammatory response that characterizes asthma in both the atopic and nonatopic forms of the disease.[11, 12] The source of these cytokines has not been entirely elucidated. Although other cells, including mast cells and eosinophils, can be shown to transcribe messenger RNA for these two cytokines, the majority of cells expressing the cytokines are CD3+ T cells.[13-15] Studies of T-cell clones specific for allergens, such as Der p 1 from house dust mite, have ascribed most if not all of the allergen-induced T_{H2}-type response to CD4+ T cells in atopic diseases.[16, 17] In contrast, there has been less evidence for involvement of CD8+ T cells in human allergic responses. Recently, CD8+ T-cell lines derived from bronchoalveolar lavage in patients with atopic asthma have been shown to secrete significantly higher quantities of IL-5 relative to those from nonatopic control subjects.[18]

We have hypothesized that in atopic diseases, such as asthma, CD8+ T cells may be conditioned in vivo to produce increased amounts of IL-4. As with CD4+ T cells, the presence of IL-4 in vitro favors the differentiation of CD8+ T cells toward a T_{H2}-like immune response, resulting in production of IL-4 and IL-5, suppression of IFN-γ production, and assistance in IgE synthesis.[5, 6] To further investigate the potential of CD8+ T cells to engage in allergic inflammatory responses, we have analyzed peripheral blood CD8+ T

From the University Department of Medicine, University of Southampton.
Supported in part by the Medical Research Council, U.K. Charuporn Promwong was funded by a grant from the Government of Thailand.
Received for publication Jan. 21, 1997; revised Apr. 8, 1997; accepted for publication Apr. 10, 1997.
Reprint requests: Luminita Stanciu, MD, PhD, University Medicine, Level D, Centre Block, Southampton University General Hospital, Southampton SO16 6YD, UK.

0091-6749/97 $5.00 + 0 **1/1/83000**

cells from patients with atopic asthma and nonatopic control subjects for their intracellular content of IL-4 and their ability to produce this cytokine when stimulated ex vivo. We have also studied the capacity of these cells to produce IFN-γ, a cytokine that together with IL-4 appears to most clearly distinguish between T_{H1}/Tc1 and T_{H2}/Tc2 cells. In our study we have used highly purified $CD8^+$ T cells to exclude cell-cell interactions with $CD4^+$ T cells and non-T cells (B cells, natural killer cells, and monocytes) and to avoid production and/or consumption of IL-4 and/or IFN-γ by the same cells.

METHODS

Subjects

Heparinized blood was taken from seven patients with mild atopic asthma and seven nonatopic healthy subjects. All the patients with asthma fulfilled the American Thoracic Society criteria for asthma,[19] and in all instances the PC_{20} was less than 8 mg/ml. Their disease was mild and was controlled with inhaled albuterol only. They all had positive skin prick test responses (defined as a wheal 3 mm greater than that caused by physiologic saline control) to one or more of the following allergens: house dust mite, mixed grass pollens, mixed tree pollens, mixed feathers, cat fur, and dog hair (ALK, Denmark). Their total serum IgE levels were between 10 and 520 IU/ml (geometric mean, 121 IU/ml). None had been receiving oral corticosteroids within 6 weeks before the study. The healthy subjects had no history of allergic disease, and all had negative skin prick test responses and serum IgE levels less than 80 U/ml.

All the subjects gave their written consent, and the study was approved by the Southampton Joint University and Hospitals Ethics Committee.

Isolation of peripheral blood $CD8^+$ T cells

Highly enriched peripheral blood T cells were obtained as previously described.[10] In brief, this involved separation of mononuclear cells on Lymphoprep (Nycomed, Oslo, Norway); removal of monocytes by adherence to plastic at 37° C for 2 hours; and further removal of B cells, natural killer cells, and nonadherent monocytes by panning with anti-CD19, anti-CD16, anti-CD14, anti-CD11b, and anti-CD33 mouse monoclonal antibodies (mAbs). Thereafter, $CD8^+$ T cells were negatively selected by immunomagnetic cell sorting with anti-CD4 mAb.[10] The purity of $CD3^+CD8^+$ T cells was shown by flow cytometry to be greater than 90%, with mean contamination with $CD3^+CD4^+$ T cells being 2.8% and 3.3% in the control and asthmatic subjects, respectively.

Detection of intracellular cytokine in $CD8^+$ T cells

To determine the concentrations of IL-4 and IFN-γ contained within $CD8^+$ T cells, purified resting $CD8^+$ T cells were lysed in phosphate-buffered saline containing 2% Triton X-100 (1×10^6 cells/ml) and centrifuged at 400 *g* for 10 minutes at 20° C. The supernatants were stored at –20° C for measurement of IL-4 and IFN-γ at a later date.

Cell culture for cytokine production

We have opted to use concanavalin A (Con A) to stimulate $CD8^+$ T cells because this mitogen does not require the presence of accessory cells to activate T cells or induce their cytokine production.[20] Freshly isolated $CD8^+$ T cells (1×10^6) were cultured for 20 and 48 hours in 1 ml of RPMI-1640 medium (Gibco BRL, Life Technologies, Uxbridge, U.K.) supplemented with 10% fetal calf serum with or without 10 μ/ml Con A. Culture supernatants were kept at –20° C until measurement of cytokines was performed.

Measurement of cytokines

Cytokine levels in lysed cells and culture supernatants were analyzed by solid-phase ELISA. To detect IL-4, a mouse IgG_1 anti-human IL-4 mAb (donated by Dr. Christoph H. Heusser, Ciba-Geigy, Basle, Switzerland) and a commercially available polyclonal rabbit anti-human IL-4 antibody (Genzyme, West Malling, Kent, U.K.) were used. The ELISA for IFN-γ used a mouse IgG_1 anti-human IFN-γ mAb and a biotinylated mouse IgG_1 anti-human IFN-γ mAb (donated by Dr. Sefik Alkan, Ciba-Geigy, Basle). The sensitivities of the IL-4 and IFN-γ assays were 10 and 5 pg/ml, respectively.

Statistical analyses

Statistical analyses were performed by using the Mann-Whitney U and Wilcoxon tests for between- and within-group comparisons. Values of p less than 0.05 were accepted as being statistically significant. Correlations between cytokine production and IgE levels were sought by the Spearman rank correlation test.

RESULTS

Intracellular IL-4 and production in culture

IL-4 was detected in $CD8^+$ T-cell lysates in all the subjects with significantly ($p < 0.03$) higher levels in $CD8^+$ T cells from subjects with atopic asthma (median, 26 pg/10^6 cells; range, 17 to 84 pg/10^6 cells) when compared with control nonatopic, nonasthmatic subjects (median, 16 pg/10^6 cells; range, 10 to 28 pg/10^6 cells) (Fig. 1). IL-4 levels did not correlate with the percentage of contaminating $CD4^+$ T cells ($r_s = -0.32$).

In unstimulated culture, low amounts of IL-4 were secreted spontaneously by $CD8^+$ T cells (Fig. 2), and although measurable concentrations of IL-4 were detected in a greater proportion of patients with asthma than control subjects, this was not significantly different. In both groups of subjects stimulation with Con A failed to increase the production of IL-4 (Fig. 2).

Intracellular IFN-γ and production in culture

IFN-γ was detected in cell lysates of freshly isolated $CD8^+$ T cells in three of seven patients with asthma (median, 14.5 pg/10^6 cells; range, 0 to 26) and four of seven control subjects (median, 9 pg/10^6 cells; range, 0 to 62), with no significant difference between the two groups of subjects studied (Fig. 1).

In both groups of subjects, $CD8^+$ T cells responded to Con A stimulation by producing significantly greater amounts of IFN-γ when compared with that produced by cells in control, unstimulated cultures (Fig. 3). Although the median levels of IFN-γ detected after 20 hours of culture tended to be higher in the control subjects (478 pg/10^6 cells; range, 0 to 2462 pg/10^6 cells) than in the patients with asthma (184 pg/10^6 cells; range, 53 to 374 pg/10^6 cells), this was not statistically significant.

In the control subjects, but not in the patients with

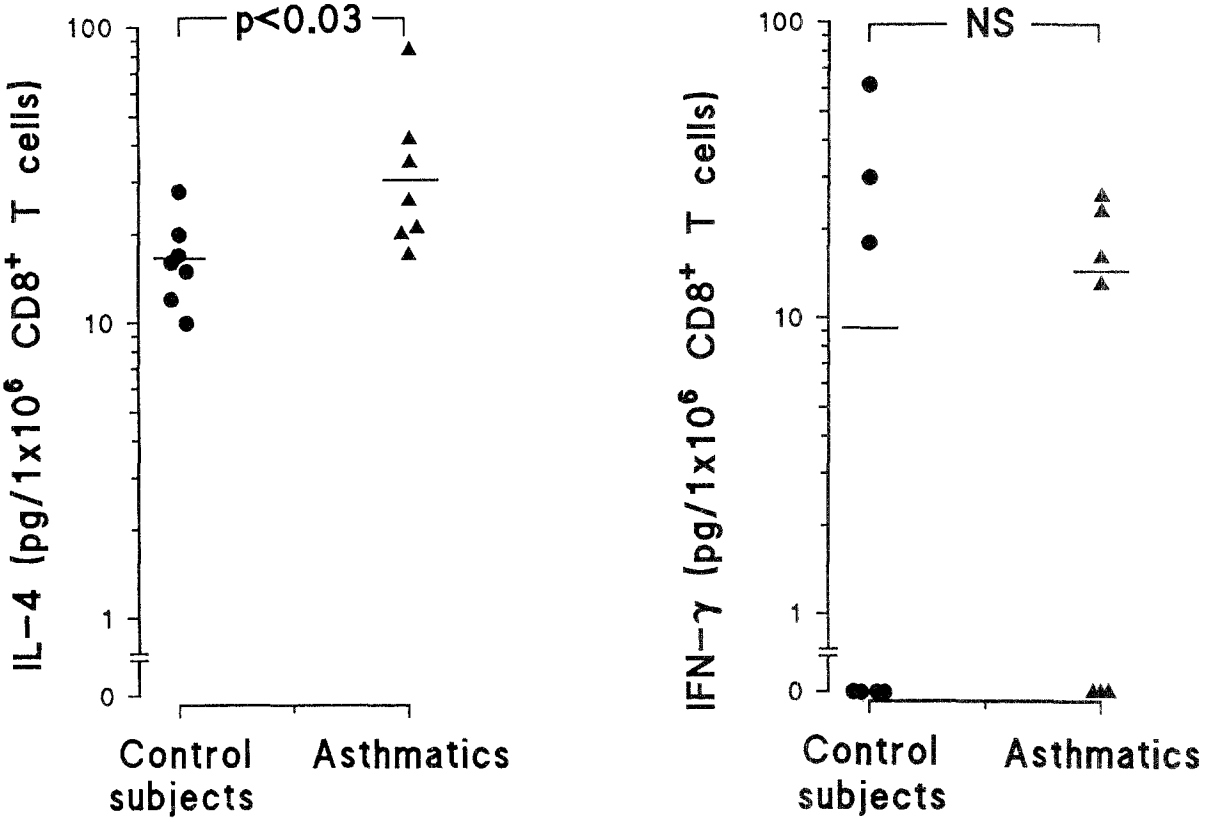

FIG. 1. Levels of intracellular cytokines in $CD8^+$ T cells from control and asthmatic subjects. Purified $CD8^+$ T cells were lysed with phosphate-buffered saline/Triton X-100 and IL-4 and IFN-γ measured by ELISA. *Horizontal bars* indicate medians. *NS,* Nonsignificant.

asthma, there was a strong negative correlation (r_s = −0.91, p = 0.01) between the intracellular concentrations of IL-4 and IFN-γ detected in freshly separated $CD8^+$ T cells. After 48 hours of culture with Con A, there was a strong (this time positive) correlation between the concentrations of IL-4 and IFN-γ in the culture supernatants (r_s = 0.97, $p < 0.001$).

In the patients with asthma there was no correlation between serum total IgE and either intracellular IL-4 levels in freshly isolated $CD8^+$ T cells or the amounts of IL-4 produced in culture.

DISCUSSION

We have shown that $CD8^+$ T cells isolated from peripheral blood of patients with mild atopic asthma contain higher levels of intracellular IL-4 than those from healthy nonatopic subjects. Together with the finding that additional ex vivo stimulation with Con A does not enhance IL-4 production, this suggests that in asthma $CD8^+$ T cells are conditioned in vivo to produce greater amounts of this T_{H2}-type cytokine.

Several studies have demonstrated increased production of T_{H2}-type cytokines by T cells in allergic disease both in blood and the airways.[11, 21-23] IL-4 and IL-5 play key roles in atopy by inducing IgE production and promoting the inflammatory effects of eosinophils.[24] Because of a previously held view that only $CD4^+$ T cells respond to soluble allergen in the context of an interaction between the T-cell receptor and major histocompatibility complex class II molecules on antigen-presenting cells, studies of T-cell cytokines involved in allergic responses have focused mainly on $CD4^+$ T cells.[16, 25, 26] However, it is now evident that $CD8^+$ T cells can also recognize and respond to soluble antigen, such as ovalbumin, in a class I major histocompatibility complex–restricted fashion.[27] Recent studies have shown that in health, subsets of $CD8^+$ T cells with a Tc2 and Tc1 cytokine profile co-exist, with Tc2 cells expressing surface markers, such as CD45RO and CDw60, and producing IL-4 and IL-5 and little or no IFN-γ.[1, 9]

Our recent study with intracellular staining and flow cytometry at the single-cell level has shown no difference between patients with asthma and nonatopic subjects in frequencies of blood T cells producing IL-2, IFN-γ, or IL-4 induced by stimulation with phorbol myristate acetate and ionomycin.[28] The proportions of $CD4^+CD45RO^+$ and $CD8^+CD45RO^+$ T cells producing IL-2, IFN-γ, IL-4, IL-5, and IL-10 in healthy subjects have also been found to be similar.[29] It is unclear to what extent the observations of cytokine production induced by such potent stimuli can be extrapolated to the in vivo setting. In keeping with these observations, we have found that stimulation of $CD8^+$ T cells ex vivo with Con A results in a loss of difference between cells from

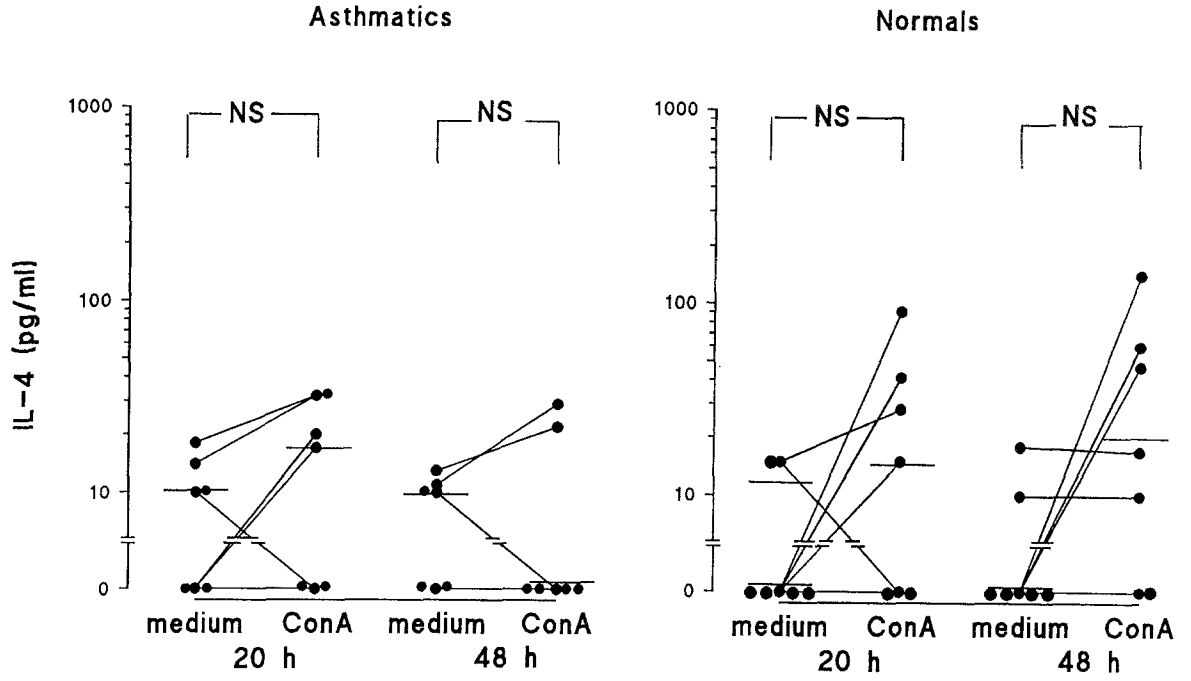

FIG. 2. Spontaneous and Con A-induced production of IL-4. Purified $CD8^+$ T cells were obtained from patients with atopic asthma and control subjects and cultured either in medium alone or medium stimulated with Con A. Supernatants were harvested after 20 and 48 hours and assayed by ELISA. *NS,* Nonsignificant.

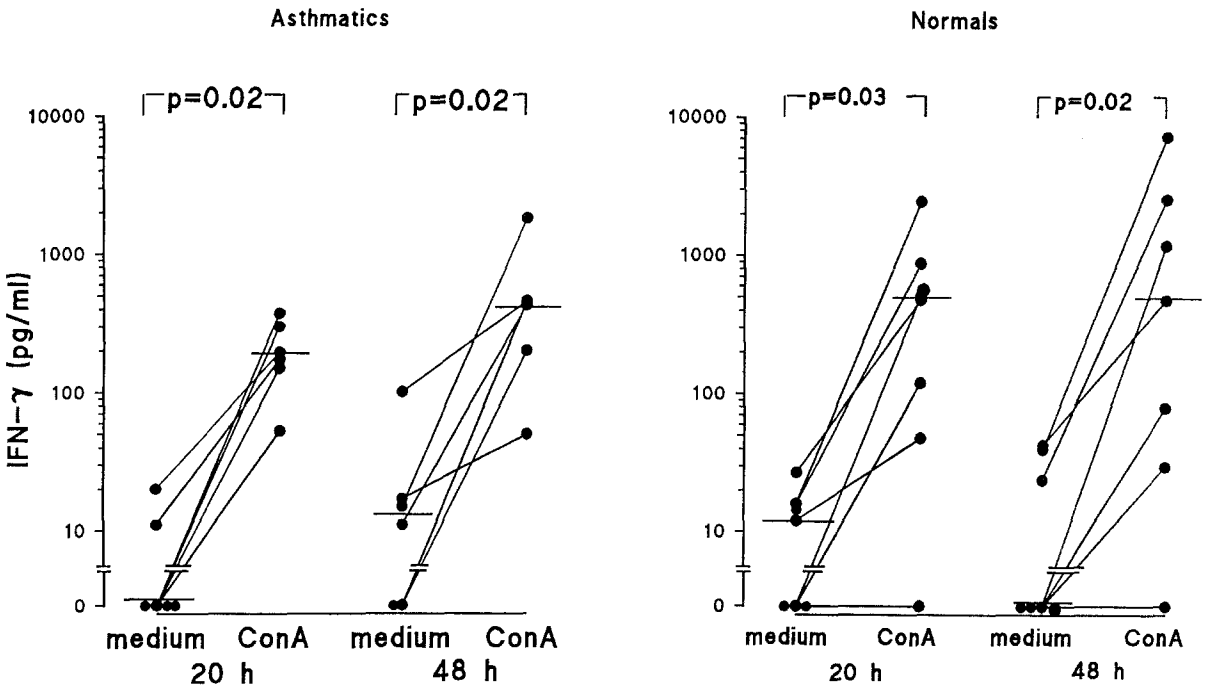

FIG. 3. Spontaneous and Con A–induced production of IFN-γ. Purified $CD8^+$ T cells were obtained from patients with atopic asthma and control subjects and cultured either in medium alone or medium stimulated with Con A. Supernatants were harvested after 20 and 48 hours and assayed by ELISA.

patients with asthma and control subjects with respect to both IL-4 and IFN-γ production. This would suggest that the increased amounts of IL-4 detected intracellularly in patients with asthma immediately after cell purification are the result of in vivo conditioning, possibly by IL-4 produced as part of the allergic response in the airways, and that other more appropriate stimuli present in vivo may be required for secretion of IL-4.

Thus in a study of ovalbumin-sensitized mice, infection with lymphocytic choriomeningitis virus resulted in a switch to IL-5 production by $CD8^+$ T cells and an induction of airway eosinophilia.[30] Other modes of activation of $CD8^+$ T cells to produce IL-4 may involve ligation of the surface CD8 molecule, which we have found to result in sustained production of IL-4 in culture.[10]

In experiments with $CD8^+$ T cells from control subjects, there was a negative correlation between the amounts of intracellular IFN-γ and IL-4. This is in keeping with the negative feedback between these two opposing cytokines, which has been observed with $CD4^+$ T cells.[31] After stimulation with Con A, this correlation became positive, suggesting similar activation of genes for these T_{H1}- and T_{H2}-type cytokines by this mitogen. For reasons that are unclear, no association was seen between IL-4 and IFN-γ in patients with asthma, suggesting that the IL-4/IFN-γ counterregulatory mechanisms operating in atopic disease may be different.

The relevance of our observation of increased IL-4 production by $CD8^+$ T cells in asthma remains to be elucidated. A balance between T_{H1}/Tc1 and T_{H2}/Tc2 cytokines is required for normal immune responses, with Tc1 $CD8^+$ T cells producing IFN-γ and exerting cytolytic activity in addition to having an important role in protecting against intracellular parasites and viruses.[32] Tc2 $CD8^+$ T cells have been isolated from patients with severe (lepromatous) forms of *Mycobacteria leprae* infection.[4] A similar increase in Tc2-type responses has not been demonstrated in allergic diseases. Increased IgE production in patients infected with the human immunodeficiency virus has been attributed to $CD8^+$ T cells, which produce IL-4, IL-5, and IL-6, express CD40L, and provide helper activity for IgE synthesis.[8, 33] Because this has also been associated with reduced production of IFN-γ, reduced cytolytic activity, and increased production of IL-10, it has been proposed as being responsible for defective defense against viral infections and intracellular parasites in these patients.[8] Whether and to what extent any imbalance between Tc1 and Tc2 $CD8^+$ T cells is relevant to both the increased susceptibility and severity of respiratory viral infections in patients with asthma[34, 35] are unknown. Similarly, the importance of the observations made in a recent study,[36] in which single-cell analysis by intracytoplasmic staining was used to show that $CD8^+$ T cells from patients with asthma contain increased amounts of IL-4 and are able to stimulate IgE synthesis, requires further study.

In conclusion, we have shown that $CD8^+$ T cells are potentially an important source of IL-4 in asthma. The mechanism(s) responsible for the increased production of IL-4 by these cells and the mode(s) of activation leading to the release of this cytokine remain to be elucidated.

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