Inflammatory Process of CD8+CD28− T Cells in Induced Sputum From Asthmatic Patients

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Previously unreported CD8+CD28− and CD8+CD28+ T-cell subsets occur in healthy individuals and expand in patients suffering from autoimmune disease. Here we studied, for the first time, the expression of CD8+CD28+, CD8+CD28−, and CD8+CD56+ subpopulations in induced sputum from asthmatics. Using sputum samples, purified CD8+ T cells were stained for surface antigen CD28, CD56, FITC-conjugated anti-perforin, and anti-IFN-γ. Cytotoxic activity was evaluated in a chromium releasing test. Induced sputum CD8+CD28− T cells were found to be more expanded and expressed low levels of IFN-γ in severe asthmatics than mild asthma and age-matched healthy controls. The predominance of CD8+CD28− T cells can be in part explained by the expansion of CD8+CD56+. CD8+CD28− T cells from severe asthmatics produced high intracytoplasmic perforin and exerted a potent cytotoxic activity. Considering their phenotyping and functional properties, the CD8+CD28− T-cell subset may constitute an intermediate phenotype in the process of CD8+ T-cell differentiation of effector-type cells in severe asthmatics. Functional studies showed that CD8+CD28− T cells had cytotoxic function.

INTRODUCTION

Asthma is one of the most common chronic diseases, characterized by inflammation of the airways, with infiltration of lymphocytes, eosinophils, macrophages, and mast cells [1]. A critical role in the pathophysiology of asthma has been attributed to the T helper 2 lymphocytes (T_H2) that promote IgE production and eosinophils activation by release of cytokines, such as interleukin (IL)-4, IL-5, IL-10, and IL-13. However, the exact mechanisms that cause this Th2 polarization remain to be elucidated [2, 3]. CD4+ T cells and their proinflammatory cytokines play a role in the allergic inflammatory process [4], but the role of CD8+ cells were often overlooked. Severe asthma involves different pathogenic pathways than mild form. Pathogenic pathways in severe asthma are considered to differ from those discussed during mild forms of asthma as the inflammatory process resists to steroids, and no usual anti-asthma treatment is efficient. We postulated that CD8 cells, particularly cytotoxic T cells, are involved in severe asthma. Human CD8+ T cells comprise cells that are in different states of differentiation and under the control of complex homeostatic process. The CD8 T-cell subset can be classified according to CD28 surface marker expression as either CD28+ or CD28−, with different biological properties. The CD8+CD28+ is predominant in healthy individuals, whereas CD8+CD28− cells increase during inflammation [5] and autoimmune disease model [6, 7]. Human CD8+ T lymphocytes are largely homogenous at birth and essentially all express the CD28 receptor, but healthy adults accumulate CD8+CD28− T cells. Moreover, CD8+CD28− T cells are increased in numbers and maintained for many years by viral carrier status, viral latency, or other chronic immunostimulative conditions [8, 9]. The origin of CD28− cells has been controversial, but recent data show that they derive from their CD28+ counterparts [10]. CD8+CD28− T cells are efficient producers of a variety of cytokines [11, 12]; they express perforin and exert potent cytotoxic activity [9, 12, 13, 14]. Links between T_H2 cells in asthma and NKT cells, a population of NK cells that express conventional T-cell receptor particularly CD8 , were reported [15, 16]. These data indicate new levels of complexity of interactions between CD8, CD28, CD56 markers and the activation of T_H2 cells during an asthmatic response.

Therefore, in this study we asked whether there are phenotypic differences in the induced sputum CD8 cell types, including CD56 cells in asthmatic patients.

The aim of this study was to quantify the CD8+CD28−, CD8+CD56+ cells in mild and severe asthmatics in comparison with a healthy control group, and to specify the level of their cytotoxic activity.

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TABLE 1. Subject's characteristics. Values are expressed as mean (SEM). (†) denotes significant differences ($P < .05$) compared to the same subset of mild asthmatic patients and healthy controls. Successful sputum induction was achieved in all patients.

	Healthy controls	Severe asthmatics	Mild asthmatics
Number	10	10	12
Age	38.2 (5.7)	53.8 (2.6)	54.6 (4.9)
FEV$_1$ (% predicted)	95.7 (4.8)	58.7 (6.2)	87.3 (2.5)
Total cell count $\times$ (10^6/g sputum)	1.8 (0.4)	2.9 (0.8)	3.1 (0.5)
Macrophages (%)	52.6 (4.2)	45.6 (3.8)	50.9 (4.5)
Neutrophils (%)	32 (4.8)	38.4 (5.6)	29.7 (5.3)
Eosinophils (%)	0.4 (0–2.5)	4.8 (3–60)†	0.9 (0–40)
Lymphocytes (%)	1.2 (0.9)	3.2 (0.6)†	1.9 (0.5)
Epithelial cells	1.6 (0.9)	2.2 (0.8)	2.8 (0.6)
Squamous-cell contamination (%)	3.8 (1.2)	4.8 (1.6)	6.2 (1.7)
Viability (%)	66.8 (6.7)	69.2 (4.9)	68.7 (4.2)

PATIENTS AND METHODS

Patients

Induced sputum samples were collected from 15 successive patients with asthma (8 patients in severe asthma) (Table 1). All were outpatients in steady state, regularly followed by an asthma specialist. The severity of the disease was classified according to Gina recommendations [17]. The samples were obtained on the fixed day of the visit. A precise history of the patient was previously obtained with functional respiratory tests. The following cases were excluded: acute exacerbation of asthma, concomitant respiratory infection, other pulmonary diseases, and smokers.

Ten induced sputum samples from healthy subjects (females, with a mean age of mean age 32.4 years; range 25–42 years), who had normal pulmonary radiographs and showed no clinical signs of respiratory diseases, acted as controls. Informed consent was obtained from all the patients. The study was approved by our Ethics Committee.

Sputum induction

Sputum induction and processing were realized following the method recently reported [18]. Briefly, after the inhalation of salbutamol ($2 \times 200\,\mu$g), subjects were asked to inhale sterile, pyrogen-free, hypertonic saline in increasing concentrations for a duration of 10 minutes. The hypertonic saline was nebulized via an ultrasonic nebulizer. Subjects were encouraged to cough throughout the procedure. Most patients were able to expectorate an adequate sample (7 mL and more) within the first 10 minutes.

Sputum processing

In order to reduce salivary contamination, plugs were selected and transferred into an Eppendorf tube. A freshly prepared 10% solution of dithiothreitol (1 mL) (DTT) was added. The tube was vortex mixed and the sputum was incubated for 5 minutes at room temperature, filtered

through 52 μm nylon gauze to remove debris and mucus, and subsequently centrifuged at 450 xg for 10 minutes. The cell pellet was resuspended in phosphate-buffered saline (PBS) in a volume equal to the sputum plus DTT solution volume. Total cell counting was carried out in a hemocytometer and the cell concentration was adjusted to 10^5 cells/mL.

In order to enrich CD8$^+$ T cells, CD4$^+$ T cells were depleted from induced sputum cells using magnetic beads (Dynal AS, Norway). Induced sputum samples were treated with beads directly conjugated to a monoclonal antibody (mAb) against CD4 (according to the manufacturers's recommendations) and then exposed to magnetic field. The unbound cells consistently contained less than 2% CD4$^+$ cells compared to 30%–40% CD4$^+$ cells in the pretreated population.

Monoclonal antibodies

Specific staining of the respective cell surface molecules was performed using FITC-conjugated anti-CD8, phycoerythrin-conjugated anti-CD28, and peridinin-chlorophyll-protein-conjugated anti-CD56. Anti-CD56 Cy-Chrome (Cy) (BD Biosciences, Franklin Lakes, NJ) was also used. For intracellular detection of perforin or IFN-γ, cells were stained for their surface antigens, CD28 and CD8, with FITC-conjugated anti-perforin, and for IFN-γ or control immunoglobulin, respectively. Monoclonal antibodies were provided from Becton Dickinson (San Diego, Calif).

Immunostaining and flow cytometry

Surface staining was performed using FITC-conjugated anti-CD8, phycoerythrin-conjugated anti-CD28, and peridinin-chlorophyll-protein-conjugated anti-CD8 or Cy-Chrome (Cy) anti-CD56 monoclonal antibodies, respectively (Becton Dickinson). Cells were simultaneously stained for 30 minutes at 4°C and, after washing with phosphate-buffered saline, fixed with 4% paraformaldehyde in phosphate-buffered saline for 60 minutes. For intracellular detection of perforin or

TABLE 2. CD28 and CD56 expression on CD8$^+$ T lymphocytes obtained from healthy controls and patients suffering from mild and severe asthma. Values are expressed as mean ± SD. (†) denotes significant differences ($P < .01$) compared to the same subset of healthy controls. (††) means significantly different than severe asthmatics.

	Healthy controls	Severe asthma	Mild asthma
CD8$^+$CD28$^+$	72.7% ± 8.5%	30.4% ± 5.4%†	44.5% ± 9.2%
CD8$^+$CD28$^-$	24.4% ± 10.4%	58.8% ± 9.3%†	46.6% ± 8.5%†††
CD8$^+$CD56$^+$	2.01% ± 1.04%	9.9% ± 5.4%†	3.09% ± 0.11%

IFN-γ, cells were stimulated with 25 ng/mL phorbol 12-myristate 13-acetate and 1 μg/mL ionomycin in the presence of 10 μg/mL brefeldin A for 4 hours (Sigma, St Louis, Mo). Cells were then stained for their surface antigens CD28 and CD8 and, after permeabilization, with FITC-conjugated anti-perforin, anti-IFN-γ, or control immunoglobulin, respectively. Fixed cells were analyzed on a FACSCalibur flow cytometry (Becton Dickinson). Data were analyzed using WinMDI software.

Cytotoxicity assay

The cytotoxic activity of CD8 T-cell subsets was evaluated in an anti-CD3-redirected cytotoxicity assay [19]. Briefly 5×10^5 Fc-receptor-bearing P815 target cells were labeled with 50 μCi of Na ^{51}CrO$_4$ for 2 hours at 37°C. Cells were then washed three times and incubated for 30 minutes at 4°C in the presence or the absence of 2 μg of anti-CD3 mAb. Gated and sorted CD28$^-$ cells were incubated for 4 hours at 37°C with 5×10^3 P815 target cells at E:T ratios ranging between 5:1 and 25:1. Supernatants were then collected and counted. Specific cytotoxicity was calculated as follows: cpm of experimental release − cpm of spontaneous release / cpm of maximum release − cpm of spontaneous release × 100. The SE of the mean percentage lysis never exceeded 7%.

Statistical analysis

The Mann-Whitney test, the two-sided Pearson test, and regression analysis by ANOVA were performed using the SPSS program, version 10.0 (Chicago, Ill). Bonferroni adjustment was performed in case of multiple testing. Significance was defined as $P < .05$.

RESULTS

Prevalence of CD8$^+$CD28$^-$ T cells in severe asthmatic patients

The clinical characteristics and sputum cell counts were as shown in Table 1. There were significant differences in the total cell count of the sputum cells between patients with mild asthma and patients with severe asthma when compared to healthy controls. Significant differences were observed in the percentages of eosinophils and lymphocytes between severe asthmatics and mild asthmatics ($P < .001$).

The presence of CD8$^+$ T lymphocytes was evaluated in sputum samples from asthmatics and healthy
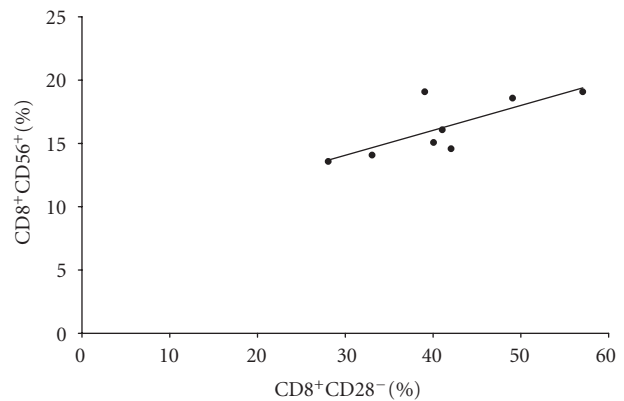

FIGURE 1. Relationship between CD8$^+$CD28$^-$ and CD8$^+$CD56$^+$ cells in induced sputum cells of severe asthmatics. Significant positive correlation ($r = 0.748$, $P = .0034$) was observed between CD8$^+$CD28$^-$ cells and CD8$^+$CD56$^+$ cells by Pearson's method.

individuals after staining with a CD28 mAbs, which revealed two subsets of CD8$^+$ T lymphocytes: CD8$^+$CD28$^+$ and CD8$^+$CD28$^-$ subsets, as shown in Table 2. In healthy individuals, the CD8$^+$CD28$^+$ subset prevailed over the CD8$^+$CD28$^-$ subset. In severe asthmatics, we observed a significant increase of CD8$^+$CD28$^-$ cells, compared to healthy subjects and mild asthmatics. This increase of CD8$^+$CD28$^-$ cells in severe asthma is paralleled by a decrease of the CD8$^+$CD28$^+$ subset. In mild asthma, CD8$^+$CD28$^-$ and CD8$^+$CD28$^+$ subsets population were expressed at similar levels.

The proportions of CD8$^+$CD56$^+$ cells in the induced sputum cells of patients with severe asthma were higher compared to mild asthma and healthy controls (Table 2). In terms of the relationship between CD8$^+$CD28$^-$ and CD8$^+$CD56$^+$ subsets, analyzed by Pearson's method, CD8$^+$CD28$^-$ cells were correlated significantly with CD8$^+$CD56$^+$ cells ($r = 0.748$, $P = .0034$) (Figure 1).

Perforin and interferon-γ production by CD8$^+$CD28$^-$ T cells

For functional characterization of CD8$^+$CD28$^-$ sputum T cells, the frequency of perforin and IFN-γ positive cells were studied. In healthy controls, perforin production was more frequent in CD8$^+$CD28$^-$ T cells (36.6% ± 8.3%) than in CD8$^+$CD28$^+$ T-cell counterparts
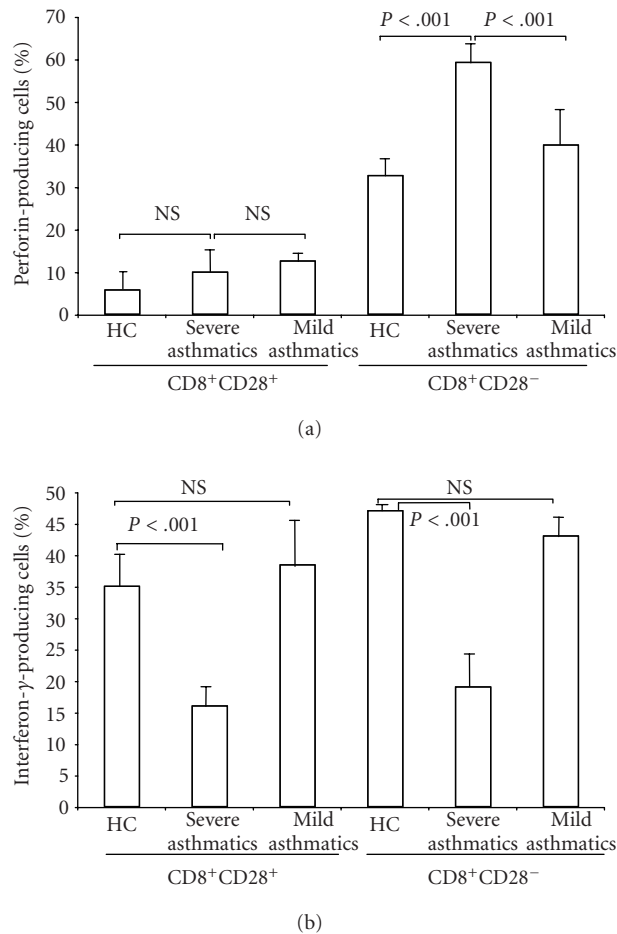
(a)

(b)

FIGURE 2. Intracellular production of perforin in CD8⁺CD28⁻ and CD8⁺CD28⁺ cells. Induced sputum cells of asthmatics and healthy controls (HC) were stimulated with phorbol 12-myristate 13-acetate and ionomycin in the presence of brefeldin A. Cells were stained with fluorescence-marked monoclonal antibodies (mAbs) directed against CD8, CD28, and perforin and counted by flow cytometry. The Mann-Whitney test was used to determine the statistical difference between perforin-CD28⁺ and perforin-CD28⁻ T cells in induced sputum from severe, mild asthmatics, and healthy controls.

FIGURE 3. CD8⁺CD28⁻ T lymphocytes perforin expression from a patient with severe asthma. Cells were permeabilized and stained for intracellular perforin. The histogram shows the intracellular protein after staining with anti-perforin-PE (——), and after staining with isotype-matched PE-labeled mouse IgG mabs, used as the negative control (------). Results are the mean of at least two independent experiments.

Cytotoxic activity

CD8⁺CD28⁺ and CD8⁺CD28⁻ T cells from 5 severe asthmatics, 4 mild asthmatics, and 4 healthy controls were investigated for their cytotoxic activity against the mouse cell line P815 as a nonspecific target in a CD3-restricted cytotoxicity assay (Figure 4).

CD8⁺CD28⁻ T cells from severe asthmatics exhibited efficient cytotoxic responses (30% ± 4%, 20% ± 6%, and 17% ± 5%) at different E:T ratios (25:1, 10:1, and 5:1), whereas CD8⁺CD28⁺ T cells lysed target cells at lower levels (12% ± 6%, 10% ± 2%, and 5% ± 3%) at the same E:T ratios ($P < .001$). In healthy controls and in mild asthmatics, CD8⁺CD28⁻ T cells, cytotoxicity was low (healthy controls: 17% ± 9%, 12% ± 5%, 8% ± 2%; mild asthmatics: 15% ± 4%, 8% ± 5%, and 6% ± 4%) when compared to severe asthmatics CD8⁺CD28⁻ T cells ($P < .001$), at all E:T ratios (25:1, 10:1, and 5:1). In healthy controls and mild asthma, CD8⁺CD28⁺ T cells were unable to efficiently lyse target cells (healthy controls: 3.5%; mild asthmatics: 4% at 25:1 ratio).

DISCUSSION

Our data clearly show the presence of two CD8⁺ T-cell subsets in induced sputum from both healthy controls and asthmatic patients, and particularly the enlargement of the CD8⁺CD28⁻ T-cell subset in severe asthma; they expressed low IFN-γ production. Intracytoplasmic perforin is highly increased in CD8⁺CD28⁻ T cells from severe asthmatics. This increased perforin expression is

(5.76% ± 2.6%) (Figure 2a). Perforin production was more important in CD8⁺CD28⁻ T cells of severe asthmatics (59.2.5% ± 8.5%) (Figure 3) compared to mild asthmatics (39.8 ± 12.3%) and healthy controls. CD8⁺CD28⁺ T cells from mild, severe asthmatics and healthy controls expressed similar perforin levels.

In severe asthmatics, the frequency of CD8⁺CD28⁻ T cells producing IFN-γ is lower than what is observed in healthy controls and mild asthmatics ($P < .001$) (Figure 2b). Mild asthmatics and healthy control CD8⁺CD28⁻ T cells and CD8⁺CD28⁺ T cells expressed similar IFN-γ levels. However, severe asthmatics expressed a significantly decreased percentage of cells positive for intracytoplasmic IFN-γ.
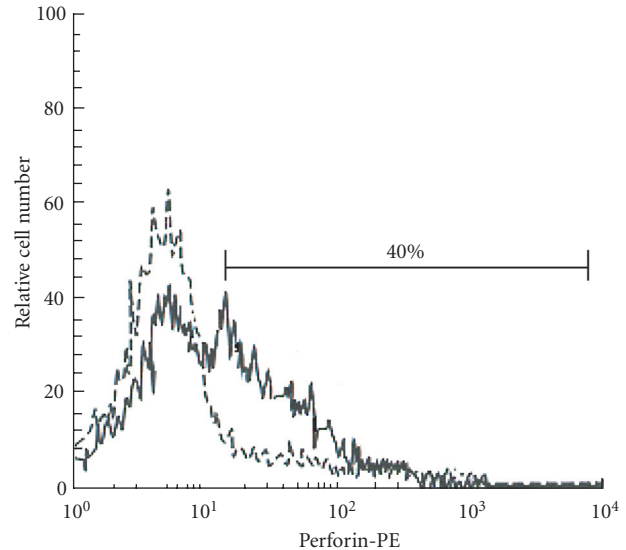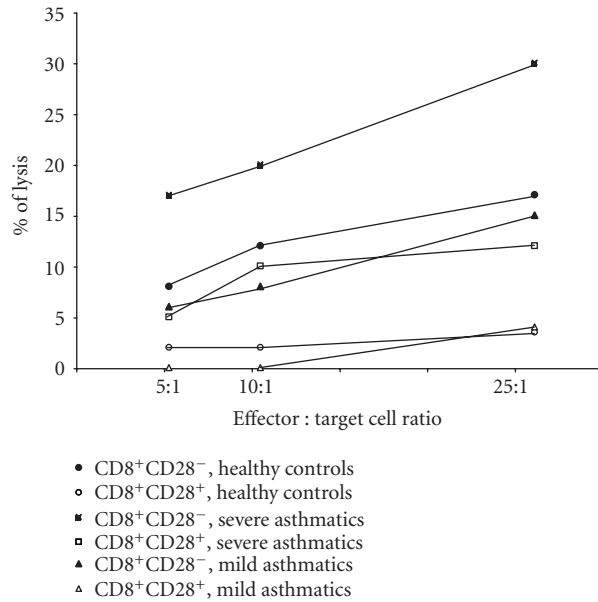
FIGURE 4. Cytotoxic activity of CD8$^+$ T-cell subpopulations. Purified CD8$^+$ T-cell subsets were directly analyzed for cytotoxicity against P815 target cells in the presence of anti-CD3 monoclonal antibody in a 4-hour ^{51}Cr release assay, in 4 healthy controls, 5 mild and 5 severe asthmatics. Results are the mean of independent experiments.

associated with high CD8$^+$CD28$^-$ cytotoxic activity in severe asthmatics. According to these results, we can speculate that effector cytotoxic function of CD8$^+$ T cells reside in the subpopulation lacking CD28 expression. Previous studies have shown that most healthy elderly humans harbor clonal CD8$^+$ T-cell expansions in their peripheral blood [20]. Chamberlain et al [21] reported that loss of CD28 expression marks functional differentiation to cytotoxic memory cells [22]. Some studies have also shown that polyclonal CD8$^+$CD28$^-$ T cells found in the peripheral blood have significantly shorter telomeres than CD8$^+$CD28$^+$ population, suggesting replicative senescence [23], with more resistance to apoptosis [24] and increased expression of BCL2 [25]. Patients with acute exacerbation of asthma were characterized by increased expression of BCL2 proto-oncogene in induced sputum lymphocytes from asthmatics [26].

Reports indicated that circulating CD8$^+$CD28$^-$ T cells are increased in various infectious diseases [27], in patients with autoimmune diseases [6], and in animal autoimmune model [7]. It has been reported that inflammation during an asthma exacerbation is more reminiscent of an antiviral response than an eosinophil-predominant response to allergen. This implies an independent role of airway T cells in mediating asthma flares and in determining glucocorticoid efficacy in the treatment of this disease [28].

An imbalance of T-cell subsets in asthma with a predominance of T$_H$2 type cells has been characterized [1]. Production of IFN-γ has been reported to be reduced in

patients with asthma [29], particularly in CD8$^+$ T cells [30]. However, antagonist results were reported [1], where IFN-γ-producing T cells are more abundant in the airways of asthmatics, and the proinflammatory activities of IFN-γ may play an important role in the pathogenesis of childhood asthma and may suggest that asthma is not simply a T$_H$2-driven response. Our result reported a decreased IFN-γ in severe asthma-producing CD8$^+$CD28$^-$ T cells. This suggests a real imbalance of T$_H$1/T$_H$2 cytokine-producing cells in asthma, which linked asthma to a chronic T-cell-mediated bronchial inflammation.

Because little is known about the physiologic role and the putative cytotoxic functions in asthma, we studied perforin production in CD8$^+$CD28$^-$ T cells. Perforin production was found increased in CD8$^+$CD28$^-$ T cells in severe asthmatics. Increased perforin expression has been reported by other authors in peripheral blood of asthmatics [31] and in patients with COPD [32]. Increased perforin expression has been reported in several other chronic inflammatory disorders with autoimmune phenomena such as multiple sclerosis [33, 34], Takayasu arteritis [35], or autoimmune thyroid disease [36], which is localized to CD4$^+$, CD8$^+$, CD16$^+\gamma\delta$ T cells or NK cells. Recently we have reported increased $\gamma\delta$ T-cell expression in induced sputum asthmatics, which mediated a potent natural killer cytotoxic activity [18]. CD8$^+$CD28$^-$ T cells have been reported to be effector cells, producing perforin, granzyme B, tumor necrosis factor-α, and IFN-γ [14]. CD28$^-$ T cells derive from their CD28$^+$ T-cell counterparts [37, 38]. In our asthmatic patients, CD28$^-$ T cells produced high levels of perforin-mediated cytotoxicity, inversely correlated to IFN-γ production. We assumed that the functional differentiation of CD8$^+$ T cells in severe asthmatics into mature effector cells, with the disappearance of CD28 makes them able to adhere to human microvascular endothelial cells as reported by Fiorentuni et al [27]. CD8$^+$CD28$^-$ T cells contain clonally expanded cytotoxic T cells for unknown antigen specificity [7] and act as suppressor cells on antigen-presenting cells, inhibiting their ability to elicit T helper cell activation and proliferation [39]. The role of CD8$^+$CD28$^-$ T cells in asthmatics is still unclear. Some recent publications have shed light on some of the key processes controlling CD8$^+$ cells, T$_H$2 cytokines, and NKT cells [15]. CD8$^+$CD56$^+$ cells was the predominant subtype in severe asthmatics. A significant correlation was found between CD8$^+$CD28$^-$ cells and CD8$^+$CD56$^+$ cells in severe asthmatics. NKT cells are heterogeneous T-cell populations that are characterized by the coexpression of TCRs and various NK receptors, including CD16, CD56, CD161, CD94, CD158a, and CD158b [40]. More investigations were needed to define NKT cells in asthmatics. Umetsu et al [16] bear on the driving roles of NKT cells and T cells in asthma. In asthmatic patients, occurrence of CD8$^+$CD28$^-$ T cells seemed to be independent of prior viral infection, as our patients were in steady state. A recent study highlights the relationship between the functional activities of lymphocytes and their migration properties. Cells migrating

to lymph nodes lack inflammatory and cytotoxic functions, whereas cells migrating to peripheral tissues are endowed with various effector functions [41]. Inflammation is present in the lungs of asthmatics despite treatment, but the inflammation in severe asthma may be distinct from the inflammation seen in mild asthma. These findings are not without limitations, including overlap among the groups and the unclear relationship to type and severity of disease [42]. The mechanisms associated with the development of severe asthma are poorly understood, but likely heterogenous. It was hypothesized that severe asthma could be divided pathologically into two inflammatory groups based on the presence or absence of eosinophils and that the inflammatory subtype would be associated with distinct structural, physiologic, and clinical characteristics [43].

The immunologic mechanisms reported are specific with the development of severe asthma. Severe asthmatics were characterized by a large expression of CD8$^+$CD56$^+$ cells and CD8$^+$CD28$^-$ cells. Functional studies showed that CD8$^+$CD28$^-$ T cells had a cytotoxic function.

However, more work is required to describe the relationship between: (i) CD8$^+$CD28$^-$ T cells and CD8$^+$CD56$^+$ cells, (ii) CD8$^+$CD28$^-$ T cells and NKT cells.

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