

CD8⁺ T cells regulate immune responses in a murine model of allergen-induced sensitization and airway inflammation

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The role of CD8⁺ T cells in the development of allergic airway disease is controversial. On the one hand, CD8⁺ T cells are known to inhibit the development of airway hyperreactivity (AHR) in murine models of asthma. In humans, IL-10-producing CD8⁺ T cells were shown to act as regulatory cells, inhibiting both proliferation and cytokine secretion of T cells. On the other hand, CD8⁺ T cells can promote IL-5-mediated eosinophilic airway inflammation and the development of AHR in animal models. To examine this, we investigated the role of CD8⁺ T cells during the induction of allergen-induced AHR and demonstrated a protective effect of CD8⁺ T cells. Depletion of CD8⁺ T cells prior to the immunization led to increased Th2 responses and increased allergic airway disease. However, after development of AHR, CD8⁺ T cells that infiltrated the lungs secreted high levels of IL-4, IL-5 and IL-10, but little IFN- γ , whereas CD8⁺ T cells in the peribronchial lymph nodes or spleen produced high levels of IFN- γ , but little or no Th2 cytokines. These data demonstrate protective effects of CD8⁺ T cells against the induction of immune responses and show a functional diversity of CD8⁺ T cells in different compartments of sensitized mice.

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1 Introduction

T lymphocytes have been described as one of the main effector cells in allergic airway diseases, such as bronchial asthma [1–3]. The numbers of T cells in bronchoalveolar lavage (BAL) fluids and bronchial biopsies of asthmatic patients are significantly increased and correlate with the severity of disease [4, 5]. These T cells secrete cytokines predominantly of the Th2 type, such as IL-4, IL-13, IL-5 and GM-CSF [6, 7].

Whereas the pivotal role of CD4⁺ T helper cells in the development of allergic diseases has been demonstrated in several experimental models, the exact role of CD8⁺ T cells in the induction and regulation of allergen-mediated inflammatory responses remains unclear. Ini-

tially, IFN- γ -producing CD8⁺ T cells were defined as suppressor T cells that counteract the initiation of allergic reactions [8, 9]. However, IFN- γ may actually contribute to the severity of allergic diseases [3, 10]. Th1 cells, when adoptively transferred together with Th2 cells, exacerbate pulmonary inflammation rather than counterbalancing the effects of Th2 cells [11].

This suggests that mechanisms involving other cell types, e.g. IL-10- or TGF- β -secreting regulatory T cells [12] might be more effective in relieving airway inflammation in asthma. The transfer of activated CD8⁺ T cells from sensitized animals reduces the production of allergen-specific IgE and the development of *in vitro* airway hyperreactivity (AHR) [9, 13]. Similar, protective effects of heat-killed *Listeria*, as an adjuvant suppressing the development of airway inflammation and AHR following allergen sensitization and airway challenge, are dependent on CD8⁺ cells [14]. More-recent findings demonstrate the existence of IL-10-producing CD8⁺ regulatory T cells in humans, inhibiting the proliferation of T cells and thus down-modulating immune responses [15].

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Abbreviations: **AHR:** Airway hyperreactivity **AR:** Airway reactivity **BAL:** Bronchoalveolar lavage **MNC:** Mononuclear cell **PBLN:** Peribronchial lymph node

In contrast, other authors demonstrate a functional role of CD8⁺ T cells that rather enhance allergen-mediated immune responses. Depletion of CD8⁺ T cells prior to allergen sensitization via the airways prevented the development of eosinophilic airway infiltration and *in vitro* AHR by a reduction of IL-5 production by peribronchial lymph node (PBLN) cells [16]. The capacity of CD8⁺ T cells to produce IL-5, the main eosinophil maturation and activation factor, has been confirmed by others [17]. The transfer of CD8⁺ T cells from RSV-infected mice into naive recipients induced the development of airway inflammation and *in vivo* AHR after subsequent inhalative allergen sensitization [18].

These seemingly controversial findings about the role of CD8⁺ T cells in the context of allergen-mediated airway disease may be explained by a functional diversity of CD8⁺ T cells. Indeed, CD8⁺ T cells may be divided into two subpopulations according to their secreted cytokine profiles [19, 20]. Whereas the majority of CD8⁺ T cells secrete Th1 type cytokines (T_C1 cells), a smaller fraction of Th2-like CD8⁺ T cells (T_C2 cells) produce IL-4, IL-5 and IL-10, but not IFN- γ or IL-2 [21–24].

In addition, CD8⁺ T cells can be separated into subpopulations according to their homing capacities [25]. CD8⁺ T cells primed with IL-15 appear to home primarily to LN and Peyer's patches and are stated to represent a memory phenotype. In contrast, CD8⁺ T cells primed with IL-2 demonstrate a predominant cytotoxic phenotype and primarily home to sites of inflammation.

To further delineate the role of CD8⁺ T cells in allergen-mediated immune reactions, we utilized a murine model of systemic sensitization and repeated airway allergen challenge. We investigated the role of CD8⁺ T cells at different sites and studied the effects of *in vivo* CD8⁺ T cell depletion at different time points. We show here that CD8⁺ T cells are functionally involved in the regulation of systemic sensitization and airway inflammation and have distinct roles depending on the time and site of action. The results of this study may help to further clarify the role of CD8⁺ T cells in allergic diseases.

2 Results

2.1 CD8⁺ T cells infiltrate the airways after systemic sensitization and subsequent airway challenge

CD4⁺ and CD8⁺ T cells were analyzed in the spleen, PBLN and lungs by flow cytometry. As shown in Fig. 1A, systemic sensitization followed by airway challenge significantly increased total numbers of mononuclear cells

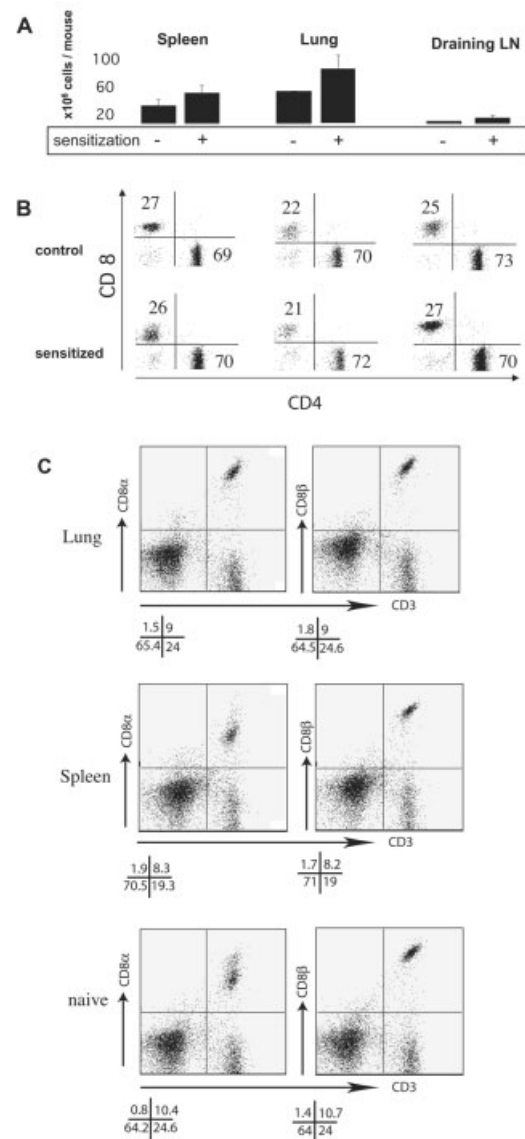


Fig. 1. CD4⁺ and CD8⁺ T cells increase proportionally after immunization. BALB/c mice (6–8 mice per group; representative values are shown) were systemically sensitized and intranasally challenged with OVA or PBS. (A) One day after the last airway challenge, the spleen, lungs and draining LN were collected and total cell numbers were counted. Cells from lung tissues were acquired by enzymatic digestion of tissue slices. Data are expressed as cell numbers per organ. (B) Subsequently, proportions of CD4⁺ and CD8⁺ T cells were analyzed by FACS staining. Results are shown as percentages of cells gated on CD3⁺ cells. (C) Data were confirmed by staining with T cell specific anti-CD8 β mAb and anti-CD3; cells were isolated from the lungs and spleen of sensitized and challenged mice, or from the spleen of naive mice.

(MNC) in the spleen ($43.7 \pm 11.9 \times 10^6$ vs. 25.3 ± 9.1 cells/mouse), in lungs ($86.2 \pm 22.4 \times 10^6$ vs. 46.60 ± 0.45 cells/organ) and in PBLN ($7.9 \pm 3.8 \times 10^6$ vs. 2.0 ± 1.4 cells/

organ), compared with unsensitized controls. The relative proportion of CD4⁺ vs. CD8⁺ T cells remained unchanged (Fig. 1B). Infiltration of CD8⁺ T cells was confirmed by staining with anti-CD8 β mAb in the lungs and spleens of mice, and no difference was found between the surface expression of CD8 α or CD8 β , indicating that infiltrating CD8 α ⁺ cells were T cells and not DC (Fig. 1C). There was no increase in CD3⁺CD4⁺CD8⁺ T cells during sensitization or challenge (data not shown),

indicating that there was no loss of CD8 expression of CD8⁺ T cells.

Infiltration of CD4⁺ and CD8⁺ T cells as well as granulocytes was analyzed (Fig. 2). Twelve hours after a single airway allergen challenge, CD4⁺ and CD8⁺ T cells were barely present in lung tissues (Fig. 2A). However, CD4⁺ as well as CD8⁺ T cells gradually infiltrated the lung 36 h and 60 h after intranasal allergen challenge (Fig. 2B, C). Staining for granulocytes (using Ly6G) showed no differences in cellular infiltration at any time point (positive control).

These data show that intranasal allergen challenge leads to the infiltration not only of CD4⁺, but to the same degree of CD8⁺ T cells into the lung tissues and PBLN of sensitized mice.

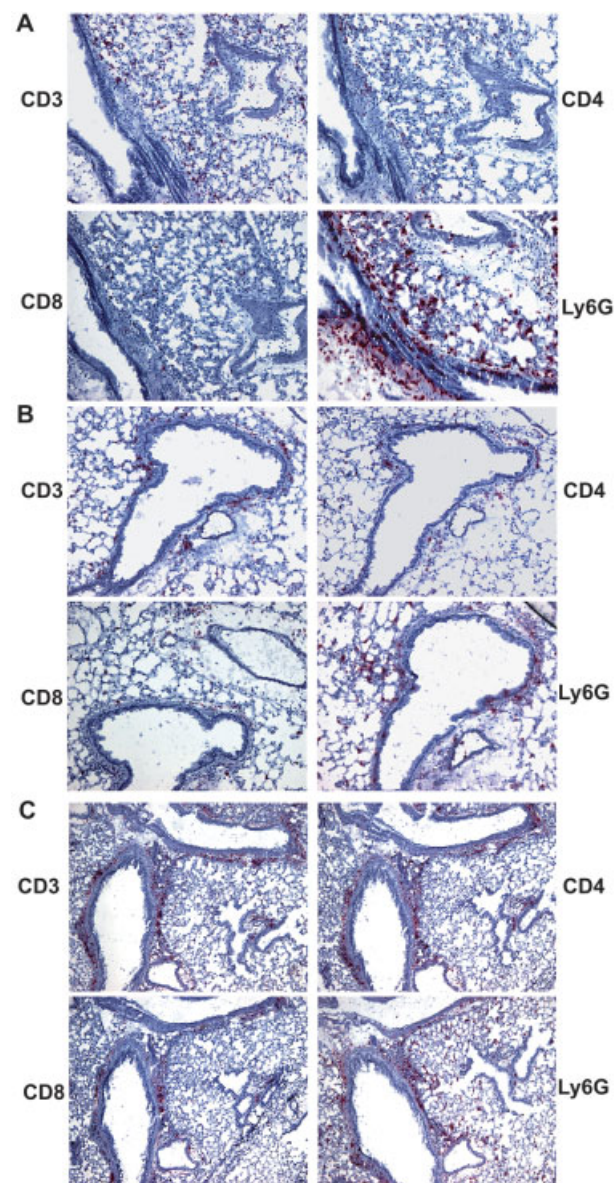


Fig. 2. Kinetics of T cell infiltration into lung tissues after allergen challenge. Mice were sensitized with OVA and lungs were removed (A) 12 h, (B) 36 h or (C) 60 h after a single intranasal challenge with OVA. Sections were stained with anti-CD3, anti-CD4, anti-CD8 or anti-Ly6G mAb.

2.2 CD8⁺ T cells of sensitized, challenged mice display distinct functional properties depending on their localization

CD8⁺ T cells from lungs and spleen of sensitized or naive animals were positively selected by MACS-purification (purity of CD8⁺ T cells was >95%; data not shown). Cells were re-stimulated and cytokines were measured by ELISA. As shown in Fig. 3a, CD8⁺ T cells derived from lungs of sensitized and challenged mice produced enhanced levels of IL-4 (205.3 ± 18.6 pg/ml vs. levels below the detection limit), IL-5 (1866.4 ± 205.6 pg/ml vs. levels below detection limit) and IL-10 (286.16 ± 47.60 pg/ml vs. 6.60 ± 4.60 pg/ml), compared with lung CD8⁺ T cells from naive mice. Moreover, production of these cytokines in sensitized animals was significantly higher by CD8⁺ T cells derived from lungs compared with spleen CD8⁺ T cells (in the latter case, IL-10 was 24.0 ± 8.3 pg/ml; IL-4 and IL-5 were below the detection limit). In contrast, CD8⁺ T cells from the spleen secreted significantly higher amounts of IFN- γ (5067.6 ± 101.7 pg/ml in sensitized mice; 4479.0 ± 271.1 pg/ml in naive mice), than CD8⁺ T cells from lungs (421.9 ± 19.7 pg/ml in sensitized mice; 232.7 ± 44.9 pg/ml in naive mice), regardless of sensitization status.

We asked whether CD8⁺ T cells from lungs or spleens proliferated in response to antigen (OVA) and which cytokines those proliferating CD8⁺ T cells secreted. As shown in Fig. 3b, the percentage of proliferating CD8⁺ T cells was highest in cell fractions obtained from the lungs, whereas a smaller number of CD8⁺ T cells from the spleen proliferated in response to antigen. Naive CD8⁺ T cells showed no signs of proliferation. Proliferating CD8⁺ T cells from the lungs produced IL-4 and IL-10, but little or no IFN- γ . In contrast, proliferating CD8⁺ T cells from

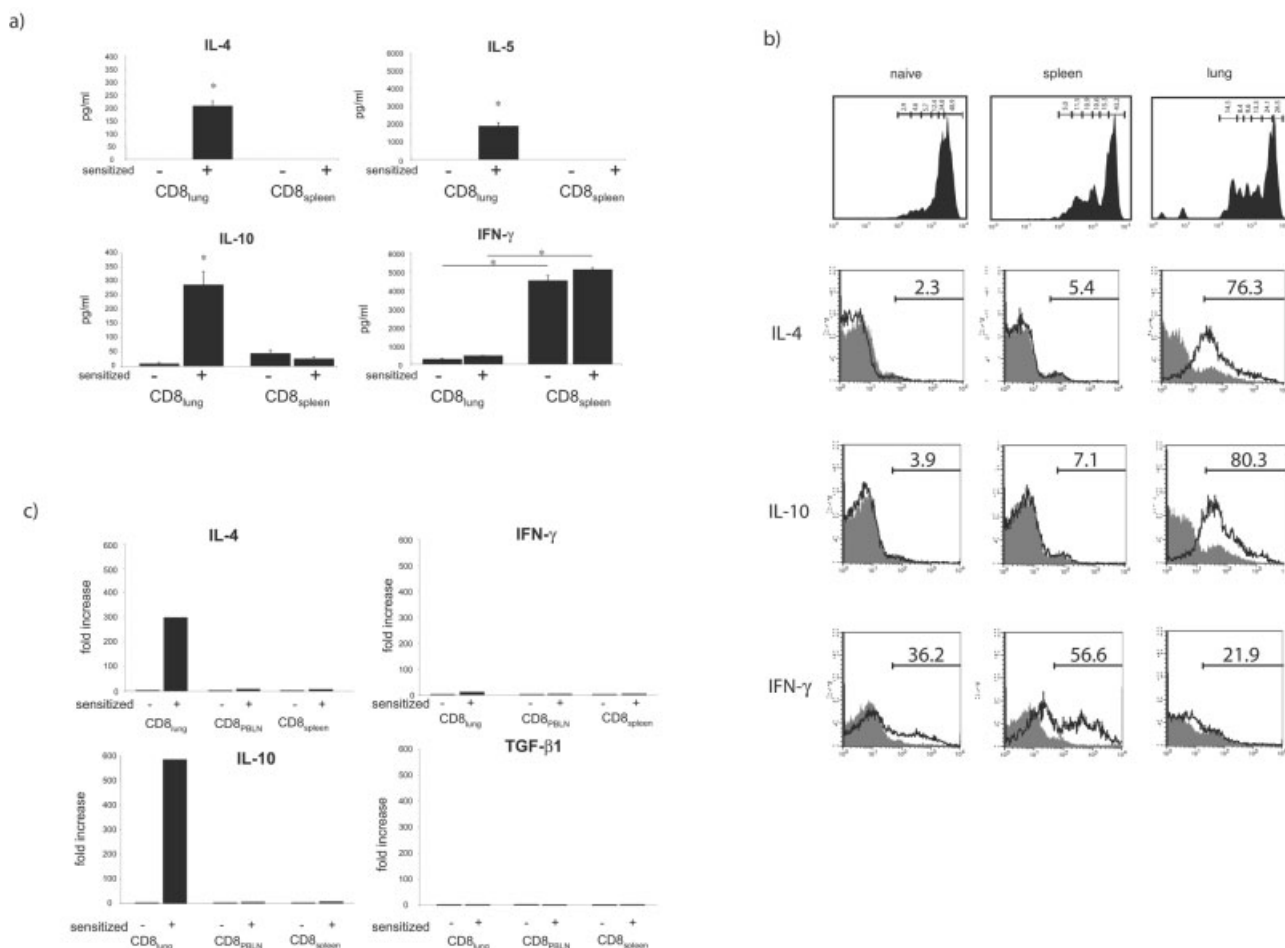


Fig. 3. Cytokine profiles of spleen and lung CD8⁺ T cells. Mice were systemically sensitized and intranasally challenged with OVA or PBS. CD8⁺ T cells were positively selected by MACS from lungs and the spleen 2 days after the last airway challenge. (a) CD8⁺ T cells were restimulated *in vitro* with antigen. Levels of cytokines in cell culture supernatants were measured by ELISA, and expressed as mean $\pm$ SD. (b) Cells were labeled with CFSE and restimulated *in vitro* with antigen for 3 days. Intracellular cytokines of proliferating cells was measured by FACS. (c) Levels of cytokine mRNA were determined by real-time PCR; values are expressed as relative quantification ($2^{-\text{average } \Delta \Delta C_T}$) of cytokine mRNA expression. One representative of three independent experiments is shown.

the spleen of sensitized mice showed little or no production of IL-4 or IL-10, but a large number of cells produced IFN- γ .

These data show that CD8⁺ T cells from lungs of sensitized and challenged mice were capable of producing high amounts of IL-4, IL-5 and IL-10, whereas IFN- γ production was low. In contrast, CD8⁺ T cells from the spleen produced no significant levels of IL-4, IL-5 and IL-10, but high levels of IFN- γ . In addition, the data indicate that antigen-specific CD8⁺ T cells were the main producers of the described cytokines.

To confirm these results, we compared mRNA expression of IL-4, IL-10, IFN- γ and TGF- β 1 by CD8⁺ T cells

from lungs, PBLN and spleen using quantitative real-time PCR (Fig. 3c). Upon sensitization and airway allergen challenge, CD8⁺ T cells from the lungs showed high expression of IL-4 and IL-10 (279.6-fold and 585.3-fold increases above baseline). In contrast, CD8⁺ T cells from PBLN or from the spleen of sensitized and challenged mice showed only minimal amounts of mRNA expression for these cytokines (IL-4 in PBLN increased 6.0-fold, and in spleen 4.9-fold above baseline; IL-10 in PBLN increased 2.2-fold, and in spleen 4.1-fold above baseline). Expression of IFN- γ as well as TGF- β 1 was not significantly altered from baseline in all samples.

These findings confirm the high capacity of CD8⁺ T cells derived from the lungs of sensitized and challenged mice

to express IL-4 and especially IL-10, but not IFN- γ (or TGF- β 1).

To analyze the functional properties of CD8⁺ T cells from different organs further, we compared the cytolytic capacity of CD8⁺ T cells from different organs. Two modes of cell lysis induced by CD8⁺ T cells have been described, namely the Fas–Fas-L and perforin pathways [26]. Thus, we compared CD8⁺ T cells from the spleen and from the lungs of immunized and challenged mice for surface expression of CD178 (Fas-L) and for intracellular levels of perforin by FACS. As shown in Fig. 4a, CD8⁺ T cells from the spleen expressed significantly higher levels of CD178 than did CD8⁺ T cells from the lungs of the same mice. Also, intracellular levels of perforin were markedly higher in splenic CD8⁺ T cells when

compared with CD8⁺ T cells from the lung (Fig. 4b); both of these markers thus indicated a decreased cytolytic potential of CD8⁺ T cells in the lungs.

In summary, these results indicate that CD8⁺ T cells found in the lung tissue of immunized and challenged mice express a different cytokine profile and show a reduced cytolytic capacity when compared with CD8⁺ T cells found in the spleen.

2.3 *In vivo* depletion of CD8⁺ T cells prior to sensitization increases the circulating levels of serum antibodies

To further investigate the role of CD8⁺ T cells in allergen-mediated sensitization, animals were depleted of these cells by systemic application of anti-CD8 mAb either prior to sensitization and airway allergen challenge (Fig. 5, Protocol A), or after completion of sensitization, but before airway allergen challenge (Fig. 5, Protocol B). This treatment resulted in effective depletion of CD8⁺ cells in spleen and PBLN, as determined by FACS analysis (see Supplemental Fig. 1 in Supporting Information at <http://www.wiley-vch.de/contents/jc-2040/2004/24623-s.pdf>).

As shown in Fig. 6, the depletion of CD8⁺ T cells prior to sensitization (protocol A) resulted in significant increases

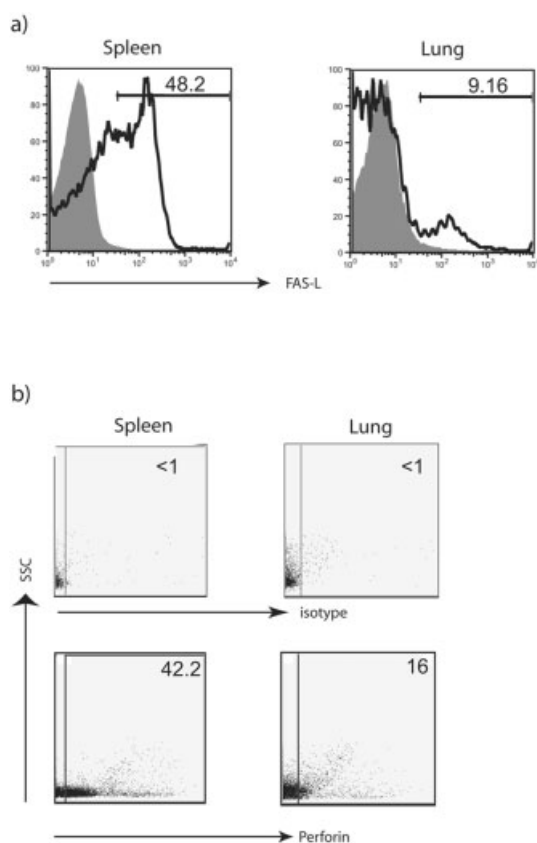


Fig. 4. Cytolytic capacities of spleen and lung CD8⁺ T cells. Mice were systemically sensitized and intranasally challenged with OVA or PBS. CD8⁺ T cells were positively selected by MACS from lungs and spleen. (a) CD8⁺ T cells (grey background, naive; overlay, immunized) were stained for surface expression of Fas-L (CD178) and analyzed by FACS. (b) CD8⁺ T cells were stained for intracellular levels of perforin and analyzed by FACS (top panel, isotype control; bottom panel, anti-perforin mAb). Both analyses were performed after gating on CD8 β ⁺ cells.

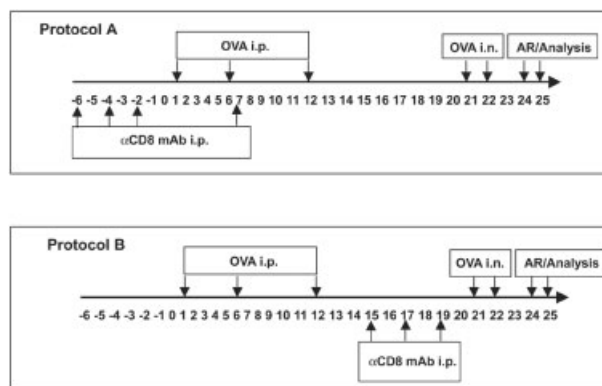


Fig. 5. Protocols for sensitization and CD8 depletion. Protocol A: BALB/c mice were injected with anti-CD8 (α CD8) mAb (or isotype control) i.p. on days –6, –4, –2 and 7. Subsequently, mice were sensitized with OVA/alum on days 1, 6 and 12, and intranasally (i.n.) challenged with OVA on days 21 and 22; two days after the last airway challenge (day 24), *in vivo* lung function was measured, and mice were killed for further analysis on day 25. Protocol B: BALB/c mice were sensitized, challenged and analyzed as described in protocol A; after sensitization and prior to airway challenges, mice were injected i.p. with anti-CD8 mAb on days 15, 17 and 19.

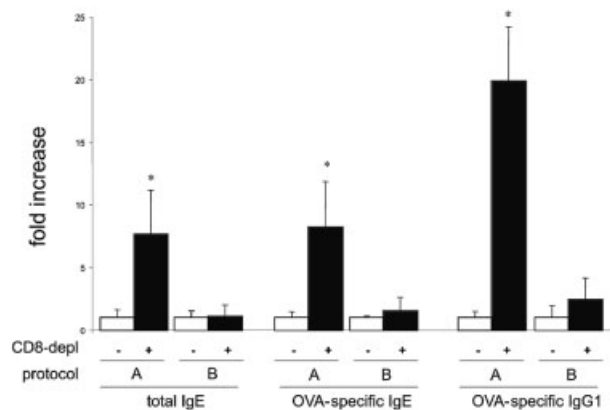


Fig. 6. Total and OVA-specific Ig serum levels in CD8-depleted mice. Serum levels of total IgE, OVA-specific IgE and OVA-specific IgG1 were measured by ELISA. Expressed is the mean $\pm$ SD fold-increase of Ig serum levels after sensitization in normal animals (white bars) and CD8-depleted animals (black bars) according to protocol A or protocol B and compared with non-sensitized mice. Three mice per group were tested and one representative of three independent experiments is shown.

in levels of total IgE and OVA-specific IgE and IgG1. Similar results were obtained when CD8-depleted and sensitized mice were analyzed prior to airway allergen challenge (data not shown).

In contrast, CD8-depletion of sensitized mice prior to intranasal challenges with OVA (protocol B) did not significantly alter the levels of total IgE and OVA-specific IgE or IgG1, as shown in Fig. 6.

2.4 *In vivo* depletion of CD8⁺ T cells prior to sensitization and airway allergen challenges exaggerates airway disease

The depletion of CD8⁺ T cells prior to sensitization (Fig. 5, Protocol A) resulted in significantly increased total numbers of leukocytes in BAL fluids upon airway allergen challenges, when compared with sensitized and challenged non-depleted animals (Fig. 7A, left panel). Likewise, staining and manual differentiation of BAL cells showed an average of a 9-fold increase in eosinophil numbers in the CD8-depleted group when compared with controls (Fig. 7A, right panel).

The depletion of CD8⁺ T cells in sensitized mice prior to intranasal allergen challenges (Fig. 5, protocol B) resulted in no significant changes in total numbers of leukocytes or eosinophils (Fig. 7A).

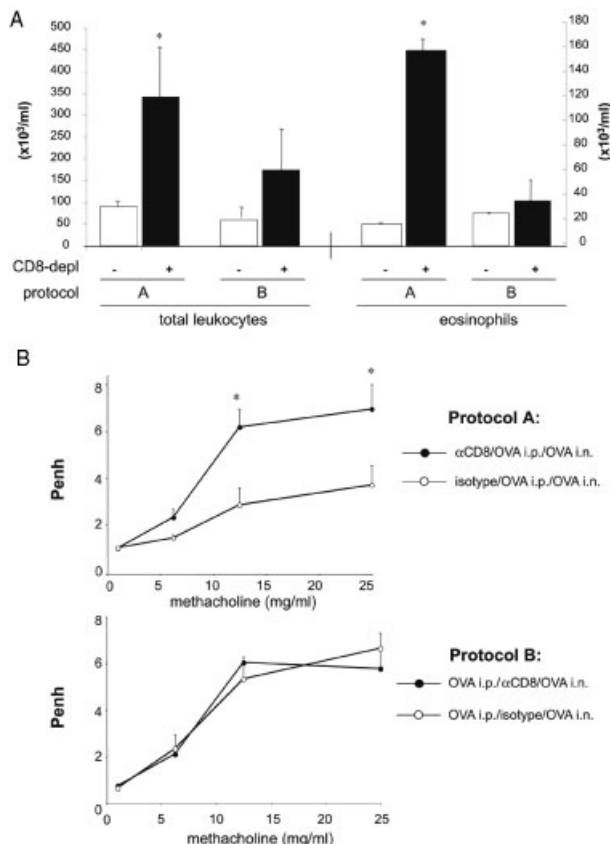


Fig. 7. Antigen-induced airway responses in CD8-depleted BALB/c mice. Differential cell counts in BAL fluids of sensitized normal animals (white bars) and CD8-depleted animals (black bars) according to protocol A or protocol B were determined 48 h after the last airway allergen challenge. (A) Representative numbers of total leukocytes and eosinophils are expressed as mean $\pm$ SD from three independent experiments, each containing four mice per group. (B) *In vivo* AR to increasing concentrations of inhaled methacholine was measured by barometric plethysmography 24 h after the last allergen airway challenge. Expressed are representative mean $\pm$ SEM values of enhanced pause (Penh) from three independent experiments, each containing four mice per group.

To further analyze the role of CD8⁺ T cells in allergic airway inflammation, the *in vivo* airway reactivity (AR) was assessed. As shown in Fig. 7B, the development of AHR was increased after early depletion of CD8⁺ T cells (Fig. 5, protocol A). In contrast, the depletion of CD8⁺ T cells after the completion of sensitization, but prior to the intranasal allergen challenges (Fig. 5, protocol B), resulted in no significant changes in the development of AHR. These results indicate that CD8⁺ T cells appear to play a protective role in the early stages of allergic sensitization.

3 Discussion

The role of CD8⁺ T cells in the development of allergen-induced AHR is controversial. To examine this issue and to resolve some of the discrepant previous data, we examined the role of CD8⁺ T cells both early and late during the development of allergen-induced AHR. We found that CD8⁺ T cells play an important inhibitory role early on during the sensitization phase of this response, but their role later during the challenge phase of AHR is limited.

Analyzing the functional capacities of the CD8⁺ T cells found in the lung tissues of sensitized and challenged animals and comparing them with CD8⁺ T cells derived from the spleen of the same animals, we found striking differences in the cytokine profiles and functional properties of these two cell subpopulations. Lung CD8⁺ T cells from sensitized and challenged mice that proliferated in response to antigen (*i.e.* were antigen-specific) showed a high capacity to produce Th2 cytokines (IL-4 and IL-5), especially high amounts of IL-10, and only little IFN- γ production. CD8⁺ T cells from the lung showed a reduced expression of cytolytic proteins, as determined by FACS. In contrast, antigen-specific CD8⁺ T cells derived from spleen of the same animals demonstrated rather a Th1-dominated cytokine profile with no IL-10, IL-4 or IL-5, but high IFN- γ production and a strong cytolytic potential. TGF- β 1 was not transcribed in either of the CD8⁺ T cell subpopulations, as demonstrated by quantitative PCR.

The vast increase of IL-10-producing CD8⁺ T cells in the lungs following sensitization and airway challenge may reflect various immunological functions, as the role of IL-10 in the development of AHR and airway inflammation is still controversial. IL-10 may play an important regulatory role late during ongoing immune responses by down-modulating Th2-driven inflammation [27–30]. Accordingly, the administration of IL-10 inhibited the development of AHR, and the transfer of regulatory T cells that predominantly produce IL-10 prevented the development of AHR, even after completion of allergen sensitization [31].

Our findings, that antigen-specific CD8⁺ T cell subpopulations exhibit divergent cytokine profiles in different compartments of the same animals, were not observed in CD4⁺ T cells (data not shown) and may be explained in at least two ways. First, a specific subpopulation of CD8⁺ T cells, characterized by predominant production of Th2 cytokines IL-4 and IL-5 as well as IL-10 but also characterized by low IFN- γ production and low cytolytic potential, may have differentiated in the periphery upon allergen sensitization. The homing properties of this subpop-

ulation may be specifically altered, thus leading to preferential infiltration to sites of allergen-induced airway inflammation. This hypothesis is supported by the recent findings of Weninger et al., who were able to demonstrate distinct migratory behaviors of CD8⁺ T cells depending on their functional phenotype [25]. Second, the distinct cytokine profiles of CD8⁺ T cells derived from lungs and spleen may be a result of local polarization of CD8⁺ T cells in the tissues after homing. Th2 polarization within sensitized and challenged lungs is greatly enhanced compared with that in naive lungs or that in the naive or sensitized spleen, thus possibly favoring Th2-skewing of CD8⁺ T cells. This is supported by *in vitro* data showing that CD8⁺ T cells are capable of producing Th2 cytokines after specific stimulation [20, 32].

The concept that CD8⁺ cells from extralymphoid organs may show markedly different properties than those in lymphoid organs is not by itself novel [33], but the current study is significant for delineating cytokine profiles within an exemplary Th2 disease.

To analyze the functional relevance of CD8⁺ T cells, we depleted mice of CD8⁺ T cells at two different time points of the immunization protocol. We chose an early time point of depletion, prior to the start of systemic sensitization (protocol A), and a later time point, after completion of systemic immunization but prior to the induction of airway inflammation (protocol B).

Depletion of CD8⁺ T cells before systemic sensitization resulted in significantly increased serum levels of IgE (both total and allergen-specific), indicating a protective role of CD8⁺ T cells in the early stages of allergic sensitization. Accordingly, the synthesis of Th2 type cytokines was increased in the spleen, lungs and PBLN of mice depleted of CD8⁺ T cells prior to sensitization, whereas IFN- γ production was reduced (data not shown). Consequently, mice showed significantly higher numbers of total leukocytes and eosinophils in the BAL fluid and an increased *in vivo* AR when CD8⁺ T cells were depleted prior to systemic sensitization and airway challenge. Thus, the regulatory potential of CD8⁺ T cells seems to be crucial at early time points of the immunization protocol, *i.e.* at time points when CD4⁺ T cells are expanding. During these early stages of immunization, CD8⁺ T cells may play a crucial role in regulating CD4⁺ T cell proliferation and function by antagonizing Th2 development. This view is supported by previous observations that assign to CD8⁺ T cells a rather suppressive function in allergic sensitization [15, 34].

Antigen-specific CD8⁺ T cells from the lungs of sensitized and challenged mice produced significant amounts of Th2 cytokines, but the depletion of CD8⁺ T cells prior

to allergen airway challenges (Fig. 5, protocol B) did not alter the subsequent allergic airway disease. This may well be explained by the hypothesis that CD4⁺ T cells are the major effector cells in the lungs, whereas CD8⁺ T cells may play a subordinate role in the local production of cytokines. Thus, the depletion of CD8⁺ T cells after the establishment of a Th2-driven immune response and after the expansion of allergen-specific CD4⁺ T cells did not significantly modify the overall secretion of cytokines by CD4⁺ T cells in the lungs (data not shown). Our data using protocol B support observations from other groups demonstrating no significant alterations in subsequent lung inflammation if CD8⁺ cell depletion was at or close to the time of antigen challenge [35–37]; these studies do not detract from the novelty of the current results using protocol A.

In summary, our results indicate a time-dependent role of CD8⁺ T cells. Thus, CD8⁺ T cells appear to protect the organism from systemic sensitization at an early stage, most likely by an increased production of IFN- γ . Once systemic sensitization is established, CD8⁺ T cells may play a bystander or pro-inflammatory role in the development of allergic airway disease upon airway challenge with allergen. This information helps to differentiate functional subpopulations of CD8⁺ T cells and to further delineate the specific role of CD8⁺ T cells in allergen-mediated systemic immune responses and local airway responses.

4 Materials and methods

4.1 Animals

Pathogen-free, female BALB/c mice (BgVV, Berlin, Germany), 6–8 weeks of age, that were maintained under institutional approved guidelines were used in all experiments.

4.2 Sensitization procedure

Mice were sensitized by i.p. injection of 20 μ g chromatographically purified OVA (Worthington Biochemical Corp) in 2 mg of aluminum hydroxide (alum) on days 1, 6 and 12. Intranasal treatment was with OVA (50 μ g in 50 μ l PBS) on days 21 and 22. Control animals received equal volumes of PBS at the same time points.

4.3 *In vivo* CD8⁺ T cell depletion

CD8⁺ T cells were depleted *in vivo* by i.p. injection (200 μ g each) of monoclonal anti-CD8 antibody (clone 53-6.72) either on days –6, –4 and –2 (protocol A), or on days 16, 18 and 20 (protocol B). Isotype-control animals received i.p.

injections of 200 μ g polyclonal rat IgG (Sigma Chemical Co., St. Louis, MO, USA) at the same time points.

4.4 Determination of serum antibody titers by ELISA

Total and antigen-specific IgE and antigen-specific IgG1 were analyzed by ELISA as previously described [16].

4.5 BAL

Mice were killed, and the trachea was cannulated. Lungs were rinsed twice with 1 ml PBS. BAL cells were stained and differentially counted (Diff Quick, Dade Behring AG, Dürdingen, Switzerland).

4.6 Histology

Histology of lungs was performed as described earlier [16]. The right ventricles were cannulated and the lungs were rinsed with 10 ml PBS to remove intravascular blood. Alkaline-phosphatase / anti-alkaline-phosphatase (APAAP) staining was performed according to standard protocols [38]. Briefly, cryostat sections were fixed in acetone for 10 min. Each section was incubated for 30 min with one of the following monoclonal antibodies: anti-CD3, anti-CD4, anti-CD8 or anti-Ly6G (used to stain neutrophil granulocytes). After incubation of the primary antibody, the sections were incubated with rabbit anti-mouse-immunoglobulin and rat APAAP complexes. The samples were developed using new-fuchsin as a chromogen. In addition, the sections were counterstained with hematoxylin. Isotype-matched immunoglobulins were used as controls.

4.7 Cell preparation

The spleen, draining LN, PBLN and lungs were removed and placed in PBS. The lungs were digested in collagenase (2105 U/ml) and DNase (2000 U/ml) for 1 h at 37°C, as previously described [39]. Single-cell suspensions were prepared by density gradient centrifugation (Lympholyte-M, Cedarlane Laboratories, Hornby, Canada).

4.8 Purification of T cells

CD8⁺ T cells were positively selected from lung and spleen MNC of naive (non-sensitized) and sensitized mice by magnetic cell sorting (Miltenyi Biotec, Bergisch-Gladbach, Germany). The selected fraction contained >95% CD8⁺ T cells, the depleted fraction contained less than 1% CD8⁺ T cells as determined by FACS analysis.

APC were isolated by negative selection of spleen MNC using anti-CD90 (anti-Thy-1.2) -conjugated magnetic beads (Miltenyi Biotec), depleting the fraction of $\geq 96\%$ of T cells.

The remaining fraction contained $\geq 3\%$ T cells as determined by FACS analysis. The cells were incubated with 50 $\mu\text{g/ml}$ mitomycin C (Sigma Chemical Co.) for 20 min at 37°C to abolish proliferation *in vitro*.

4.9 CFSE labeling

CD8⁺ T cells were purified to $>95\%$ with MACS. Labeling of CD8⁺ T cells with CFSE (Molecular Probes, Inc., Eugene, OR, USA) was done as described earlier [40]. In brief, cells were washed and resuspended at a concentration of $10^7/\text{ml}$ in PBS. CFSE was added at a final concentration of 5 μM and incubated for 15 min at 37°C. Cells were washed twice and 5×10^5 cells/well (in 96-well plates) were restimulated *in vitro* with OVA (300 $\mu\text{g/ml}$) for 3 days.

4.10 *In vitro* culture of cells

MNC (1×10^6 cells/well) or purified CD8⁺ T cells (2×10^5 cells/well plus APC at 4×10^5 cells/well) were restimulated with OVA (50 $\mu\text{g/ml}$). Supernatants were harvested after 96 h for the detection of cytokines.

4.11 Detection of cytokines by ELISA

Levels of cytokines were measured by ELISA as described previously [16]. The detection limits were 0.02 ng/ml for IL-4, 0.03 ng/ml for IL-5 and 0.08 ng/ml for IFN- γ .

4.12 FACS analysis

Surface molecules of cells were analyzed by FACS as follows. After blocking of Fc receptors with excess anti-Fc (HB197), cells were incubated with fluorescently coupled antibodies against CD4 (FITC-coupled), CD8 α (FITC or PE), CD8 β (FITC or PE), CD3 (PE-Cy5) (BD Biosciences Pharmingen, San Diego, CA, USA) and CD178 (PE) (eBioscience, San Diego, CA, USA). Flow cytometric measurement of cytokine production in T cells was done according to a standard protocol, with some modifications [41]. Briefly, CD8⁺ T cells were purified from the spleen and lungs of immunized mice. For some experiments, the CD8⁺ T cells were stained with CFSE and restimulated with 300 $\mu\text{g/ml}$ OVA *in vitro* for 3 days. Fixation and permeabilization was done according to cytofix-cytoperm plus with the GolgiPlug protocol (PharMingen). Staining for cytoplasmic cytokines was done by adding PE-anti-IL-10, PE-anti-IL4 or PE-anti-IFN- γ (PharMingen) and staining for cytoplasmic perforin was done by adding FITC-anti-perforin (clone CB5.4: Kamiya Biochemical, Seattle, WA, USA).

4.13 Isolation of mRNA

Total RNA was prepared from fresh samples of lung PBLN and spleen by using TRIzol (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions. Briefly, single-cell suspensions were prepared as described

above and resuspended in 1 ml of TRIzol reagent followed by chloroform-extraction and ethanol-precipitation. Air-dried RNA pellets were dissolved in diethylpyrocarbonate-treated water.

4.14 Taqman 5' nuclease fluorogenic quantitative PCR assay

Quantitation of IL-4, IL-10, IFN- γ and TGF- $\beta 1$ mRNA levels was performed using pre-qualified real-time PCR primers and probes (Biosource International) and TaqMan Universal PCR MasterMix (PE Applied Biosystems) according to the manufacturer's protocols and as described elsewhere [42]. Briefly, 50 ng mRNA was reverse transcribed using random hexamers (TaqMan Reverse Transcription Reagents, PE Applied Biosystems) and then PCR-amplified in the presence of gene-specific primers and a fluorescently labeled probe in a 7900 Sequence Detection System (PE Applied Biosystems). A TaqMan 18S ribosomal RNA internal control was used to ensure that the levels and quality of applied RNA samples showed no significant differences. The relative quantification and calculation of the mRNA levels was performed using the comparative $\Delta\Delta C_T$ method. All amplifications were conducted in triplicates.

4.15 Measurement of AR

Measurement of AR to increasing concentrations of inhaled methacholine (MCh) was performed by whole-body plethysmography (model PLY 3211, Buxco Electronics, NY, USA), as described previously [43]. AR was expressed as enhanced pause values for each concentration of MCh.

4.16 Statistics

Values were compared by the Mann-Whitney-U-Test; the level of significance (*) was set to $p \geq 0.05$.

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