

Tc2 cells respond to soluble antigen in the respiratory tract and induce lung eosinophilia and bronchial hyperresponsiveness

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The key role of T helper 2 (Th2) cells in asthma is well established. In contrast, the function of CD8⁺ T cells producing a distinct cytokine profile similar to Th2 cells is largely unknown. To analyze a potential role of CD8⁺ T cell subsets, allergen-specific, *in vitro*-differentiated T cytotoxic (Tc1 or Tc2) cells from T cell receptor transgenic OT-I mice were adoptively transferred into naive C57BL/6 mice. Subsequent allergen challenge of mice injected with Tc1 cells (producing IFN- γ but no IL-4) resulted in a neutrophilic airway inflammation without induction of bronchial hyperresponsiveness (BHR). In contrast, the inflammatory response of recipients of adoptively transferred Tc2 cells (producing high levels of IL-4 but little IFN- γ) was characterized by significantly increased numbers of eosinophils and induction of BHR to methacholine. The response of antigen-specific CD8⁺ T cells to soluble antigen was also observed in an *in vitro* system. A low concentration of antigen was shown to favor the generation of Tc2 cells, whereas high antigen load resulted in the differentiation of Tc1 cells. Thus, allergen-specific Tc2 cells respond to inhaled soluble antigen, produce an inflammatory response qualitatively similar to Th2 cells and therefore may exacerbate the Th2-driven airway inflammation in asthma.

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1 Introduction

Increasing evidence indicates that cytokines secreted by selected T cell populations play a key role in the pathogenesis of asthma. T helper cell subsets can be subdivided into T helper type 1 (Th1) or T helper type 2 (Th2) cells based on the cytokine profiles they produce. Th1 cells produce IFN- γ and IL-2 but no IL-4 or IL-5 and are mainly involved in immune responses to intracellular microorganisms that may lead to tissue damage. In contrast, Th2 cells produce IL-4, IL-5, IL-9 and IL-13 but no IFN- γ or IL-2 and participate in responses to helminthic infections and in allergic diseases such as asthma [1]. Evidence for a key role of Th2 cells in the pathogenesis of asthma is mainly based on results from clinical studies demonstrating the predominant activation of Th2-like T cells that produce IL-4, IL-5 and IL-13 in blood, bronchoalveolar lavage (BAL) and bronchial mucosal biopsy specimens [2–8], suggesting that Th2 cytokines account directly or indirectly for a majority of

the characteristic features of asthma including eosinophilic airway inflammation, elevated levels of IgE, mucus hypersecretion and bronchial hyperresponsiveness (BHR).

Multiple experimental models in several species have been used by various investigators to model different aspects of the underlying inflammatory response in asthma [9–11]. Among these, murine models using adoptive transfer of defined T cell subsets from transgenic mice, which express T cell receptors recognizing well-defined peptides from known antigens, have become increasingly popular. These models were designed to study different subsets of cells in more detail and were used to define the role of Th2 cells in asthma. In such models, adoptive transfer of antigen-specific Th2 cells followed by exposure to allergens causes airway eosinophilia, mucus hypersecretion and BHR [12–14]. In addition, transgenic mice that over-express Th2 cytokines in the airway epithelium exhibit typical inflammatory features of asthma, indicating that the activation of Th2 cells is sufficient for induction of the characteristic inflammation and chronic pathological changes associated with asthma [15, 16].

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Abbreviations: BAL: Bronchoalveolar lavage BHR: Bronchial hyperresponsiveness Tc: T cytotoxic

There is increasing evidence suggesting that CD8⁺ T cells differentiate into subsets analogous to Th1 and Th2 cells with characteristic cytokine secretion patterns. T cytotoxic type 1 (Tc1) cells secrete IFN- γ , and Tc2 cells secrete IL-4, IL-5, IL-6 and IL-10 [17, 18]. The polarization of CD8⁺ T cell subsets has been studied in both humans and in murine models. CD8⁺ T cells have the potential to secrete high levels of IL-4 when primed in the presence of IL-4 [19, 20] and produce Th1 cytokines when stimulated by IL-12 [21]. CD8⁺ T cell polarization into Tc1 and Tc2 is not restricted to *in vitro* conditions but was also observed in numerous infectious disease states in mice [21, 22] and humans [23, 24]. CD8⁺ T cells displaying type 2 cytokine profiles have also been found in the lungs of asthmatic patients [24]. However, the roles of Tc1 and Tc2 cells in asthma is not well defined. One hypothesis is that Tc1 cells control allergic sensitization by suppressing Th2 cells and enhancing Th1-type immunity [25]. However, Tc2 cells might contribute to the pathology by amplifying the underlying Th2 inflammatory response. Generation of transgenic mice has made it possible to study the functions of Tc1 and Tc2 cells in more detail. The present study clearly demonstrates that like Th2 cells, transfer of antigen-specific Tc2 cells into naive mice followed by antigen challenge leads to the development of airway eosinophilia and BHR. The data indicate that CD8⁺ T cells respond to soluble antigen in the airways via the MHC class I pathway and may therefore contribute to the inflammatory response in asthma.

2 Results

2.1 *In vitro* generation of allergen-specific Th1, Th2, Tc1 and Tc2 cells

The methods for generating allergen-specific Th1 and Th2 cells *in vitro* and the study of their roles in airway inflammation are well established in the literature. However, to compare the effects of these cells with a potential functional role of distinct CD8⁺ T cell subsets, *in vitro*-polarized antigen-specific Th1 and Th2 as well as Tc1 and Tc2 cells were generated from T cell receptor-transgenic mice. For that purpose, CD4⁺ T cells purified from spleens of T cell receptor-transgenic OT-II mice, which recognize a specific immunogenic peptide sequence of ovalbumin (OVA^{323–339}), were cultured *in vitro* under polarizing condition to produce antigen-specific Th1 or Th2 cells. Following stimulation, these *in vitro*-differentiated Th1 and Th2 cells produced characteristic cytokine patterns, with high numbers of IFN- γ and no IL-4-producing cells in the Th1 population but high levels of IL-4 and no IFN- γ production by Th2 cells, as measured by intracellular cytokine staining and FACS analysis (Fig. 1A). To produce functionally distinct CD8⁺ T cell

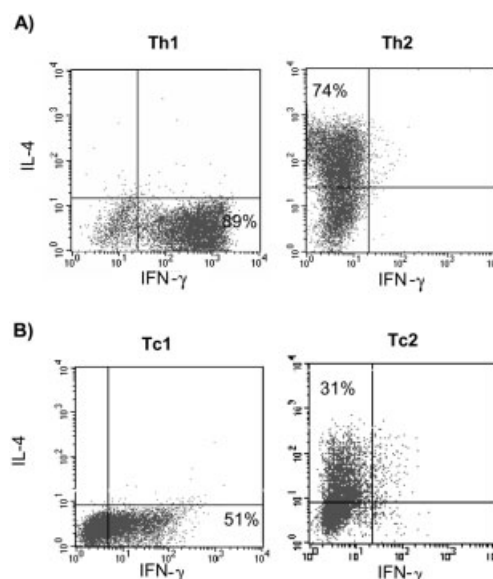


Fig. 1. FACS dot plots of intracellular cytokine staining of Tc1, Th1, Tc2 and Th2 cells. CD4⁺ (A) or CD8⁺ (B) T cells were isolated from spleens and lymph nodes of transgenic mice (OT-II and OT-I, respectively) using magnetic cell sorting. Cells were then cultured in type 1 (for Tc1/Th1)- or type 2 (for Tc2/Th2)-polarizing conditions. At the end of the differentiation period, 3×10^6 cells were washed and restimulated with PMA and ionomycin in the presence of brefeldin A for 5 h. Cells were double stained with anti-IFN- γ -FITC and IL-4-PE. Control samples were stained with isotype-matched antibodies.

subsets, CD8⁺ OVA^{257–264}-specific T cells were isolated from spleens and lymph nodes of T cell receptor-transgenic OT-I mice and polarized *in vitro* into Tc1 or Tc2 cells. For optimal polarization of Tc1 cells, the CD8⁺ T cells were cultured for 1 week in the presence of recombinant IL-12 and anti-IL-4 antibody, whereas for the differentiation into Tc2 cells, cells were stimulated with PMA and anti-CD3 and anti-CD28 antibodies in the presence of IL-4 and anti-IL-12 for 4 days. Similar to the cytokine profiles of Th1 and Th2 cells, re-stimulation of these differentiated CD8⁺ T cell subsets revealed characteristic cytokine patterns, with high levels of IL-4 and little IFN- γ production by Tc2 cells but high numbers of IFN- γ - and no IL-4-producing cells within the Tc1 cell population (Fig. 1B).

2.2 Airway inflammation induced by adoptive transfer of Th1 or Th2 cells

To study the functional roles of these *in vitro*-polarized CD4⁺ and CD8⁺ T cell subsets in airway inflammation, a murine T cell transfer model was established. In a first set of experiments, the effects of allergen-specific Th1 and

Th2 cells were analyzed. As shown in Fig. 2A, the transfer of 5×10^6 Th2 cells (optimal cell concentration as determined by pilot experiments, data not shown) into naive C57BL/6 mice followed by exposure of these mice to aerosolized OVA for 20 min on 3 consecutive days induced significant BHR to inhaled methacholine. Moreover, characteristic eosinophilic inflammation with significantly increased numbers of eosinophils and lymphocytes was found in BAL fluid of these mice, confirming results from multiple published studies using similar types of adoptive transfer models. In contrast to the eosinophilic inflammation induced by Th2 cells, the adoptive transfer of Th1 cells induced a characteristic airway neutrophilia and no induction of BHR, as published previously by our group [26]. Moreover, analysis of cytokines in BAL fluid revealed the expected profiles, with high levels of IL-4 and no IFN- γ in the Th2 cell transfer model and high levels of IFN- γ but no IL-4 following the transfer of Th1 cells [26].

2.3 Tc1 and Tc2 cells induce inflammation comparable to Th1 and Th2 cells

Having established a model of Th1 and Th2 cell-induced airway inflammation, the functional effects of allergen-specific Tc1 and Tc2 cells were analyzed in the same

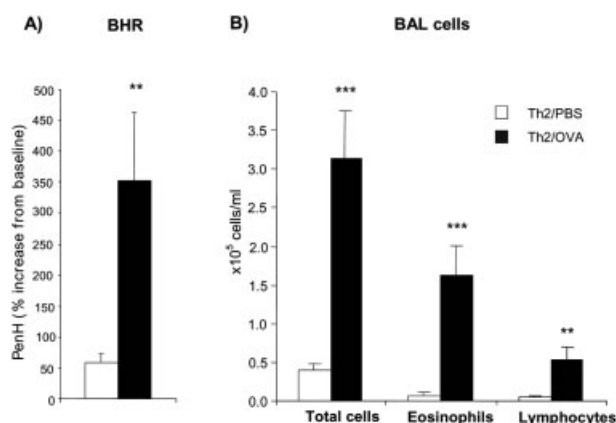


Fig. 2. Development of BHR and airway inflammation after the transfer of antigen-specific Th2 cells. Naive C57BL/6 mice were injected with 5×10^6 Th2 cells and exposed 1 day later to antigenic challenge on 3 consecutive days (Th2/OVA). Control animals received PBS challenge alone (Th2/PBS). (A) Bronchial hyperreactivity to inhaled methacholine was measured as an increase in Penh value 24 h after the last antigen exposure. (B) Mice were then killed for BAL collection, and total and differential (lymphocyte and eosinophil) cell counts were performed. Data are presented as the mean $\pm$ SEM of eight mice per group (* $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$).

model system. Tc2 cells (1.0×10^6 , 2.5×10^6 or 5×10^6) or Tc1 cells (5×10^6) were injected i.v. into naive C57BL/6 mice, followed by three daily aerosol challenges with 5% OVA for 20 min. Exposure to antigen induced a significant increase in total leukocyte numbers present in BAL fluid 24 h after the last challenge in both the Tc1 and Tc2 transfer models (Fig. 3A). Differential cell counts showed significant and dose-dependent increases in the number of lymphocytes in the Tc2 model. Transfer of Tc1 cells gave results comparable to those with Tc2 cells at the same cell concentration (Fig. 3B). However, whereas Tc1 cell transfer induced a massive increase in the number of neutrophils but not eosinophils, a characteristic eosinophilic inflammation was found following the injection of Tc2 cells (Fig. 3C, D). Moreover, cytokine levels in BAL fluid showed an increase in IFN- γ in mice that received Tc1 cells and antigen challenge (from 4 to 2,900 pg/ml), while the type 2 cytokine IL-4 was significantly elevated (from <3 to 261 pg/ml) in mice treated with Tc2 cells (Table 1). Even though mice that

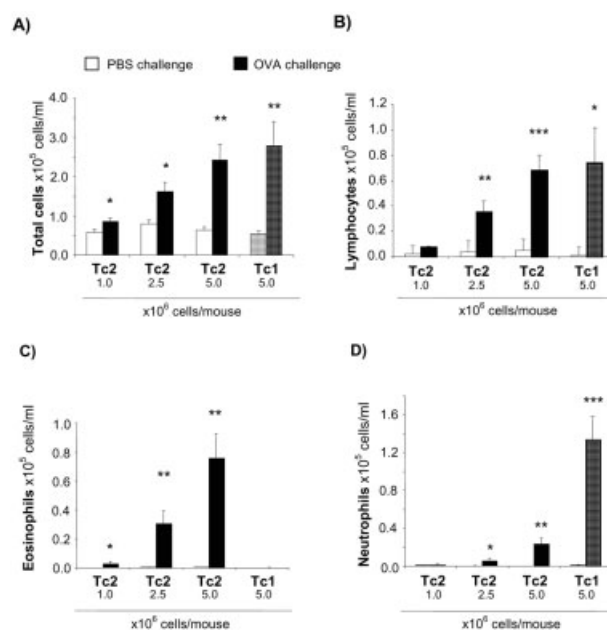


Fig. 3. Total and differential cell counts of BAL from the Tc1 and Tc2 transfer models. Naive C57BL/6 mice were injected i.v. with 1.0×10^6 , 2.5×10^6 or 5.0×10^6 highly polarized OVA-specific Tc2 or 5.0×10^6 Tc1 cells. Mice were challenged 1 day later with aerosolized OVA on 3 consecutive days. Control animals received Tc2 or Tc1 cells and were challenged with PBS alone. BAL was collected 24 h after the last challenge and analyzed for total (A) cell count. Cytospins were stained using Diff-Quik and differential cell count performed. Lymphocyte (B), eosinophil (C) and neutrophil (D) numbers are expressed per 1 ml BAL. Data are presented as the mean $\pm$ SEM of eight mice per group (* $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$).

Table 1. Cytokines in BAL fluid from PBS- or OVA-challenged recipients of Tc1 or Tc2 cells^{a)}

	Tc1		Tc2	
	PBS	OVA	PBS	OVA
IFN- γ	3.8 $\pm$ 2.6	2,907 $\pm$ 896	ND	342 $\pm$ 78.5
IL-4	ND	ND	ND	261 $\pm$ 0.2

^{a)} 5.0×10^6 OVA-specific Tc1 or Tc2 cells were injected i.v. into naive C57BL/6 mice. Values represent pg/ml $\pm$ SEM ($n=8$ mice per group; ND: not detected).

received Tc2 cells also had elevated levels of IFN- γ in BAL, the increase was tenfold lower than in mice receiving Tc1 cells. Overall, the airway inflammation induced by Tc1 or Tc2 cells was comparable to that obtained in the Th1 and Th2 transfer models, respectively.

To further analyze the Tc2-induced inflammation, histological sections of the airways were examined in order to

determine whether antigenic stimulation results in augmented accumulation of transgenic (V α 2⁺) CD8⁺ T cells in the lung. As shown in Fig. 4, the exposure of Tc2-recipient C57BL/6 mice to antigen resulted in the infiltration of antigen-specific V α 2⁺ CD8⁺ T cells into the lung, an effect not seen in the PBS-challenged control group, indicating that the migration of CD8⁺ T cells was induced by the local antigen challenge.

2.4 Tc2 cells induce BHR

To determine the effects of Tc1 and Tc2 cells on lung function, we studied their effects on BHR. Tc1 or Tc2 cells (5×10^6) were transferred into naive recipient mice that were then challenged with aerosolized OVA. Control animals were challenged with PBS alone. BHR to inhaled methacholine was measured as Penh value 24 h after the last challenge. The antigen challenge of mice given Tc2 cells resulted in significant methacholine-induced airway obstruction, whereas no change was observed after the transfer of Tc1 cells (Fig. 5).

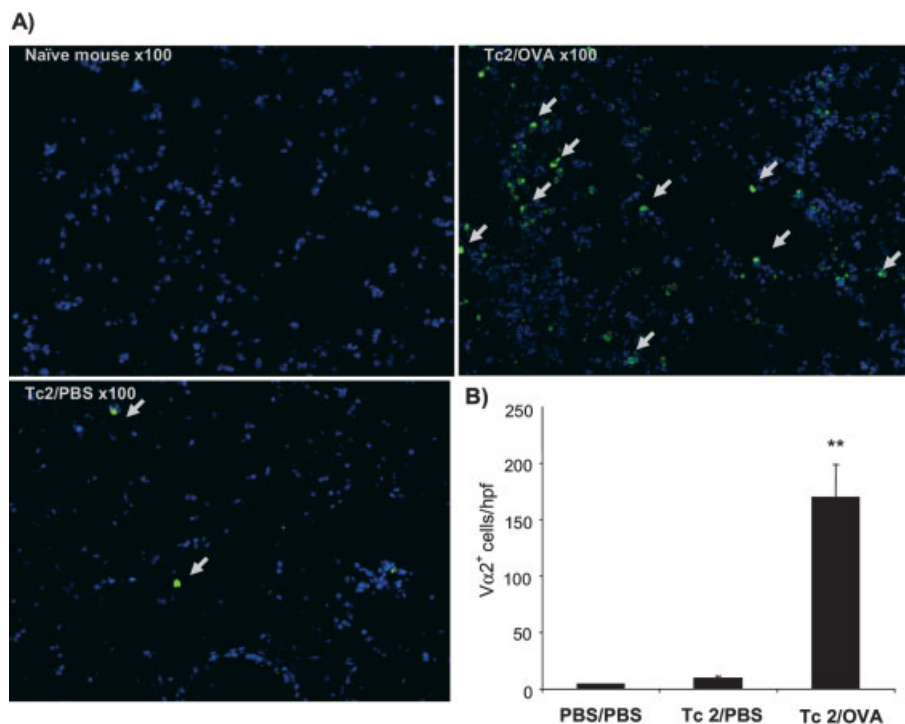


Fig. 4. Immunofluorescence staining of lung sections from the Tc2 transfer model. Animals were injected i.v. with 5×10^6 Tc2 cells. Mice were exposed 1 day later to antigenic challenge on 3 consecutive days. Control animals received PBS and were challenged with PBS alone. Lungs were collected 24 h after the last exposure. Sections were stained for the presence of transgenic V α 2⁺ cells using tyramide amplification system. Sections were counterstained with the nuclear stain DAPI. (A) Representative pictures of lung sections stained with anti-V α 2⁺ antibodies from animals that were injected and challenged with PBS (naive mouse) or injected with Tc2 cells and challenged with PBS (Tc2/PBS) or OVA (Tc2/OVA). (B) Total numbers of V α 2⁺ cells obtained per ten high-power fields (hpf) in histological sections from each lung. Data are shown as the mean $\pm$ SEM of eight mice per group (** $p < 0.005$).

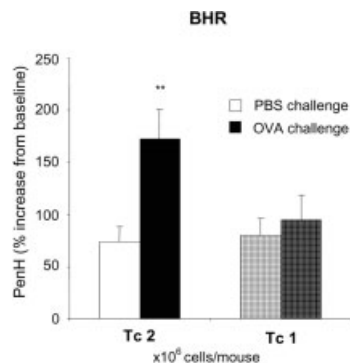


Fig. 5. Tc2-induced BHR. Animals were given 5×10^6 Tc1 or Tc2 cells and challenged with aerosolized OVA on 3 consecutive days. Bronchial hyperreactivity to inhaled methacholine was measured as an increase in Penh value 24 h after the last antigen exposure. Data are shown as the mean $\pm$ SEM of eight mice per group (** $p < 0.005$).

2.5 Antigen-specific CD8⁺ T cells respond to soluble antigen *in vitro*

The data obtained so far clearly demonstrate that fully polarized antigen-specific CD8⁺ T cells respond to soluble antigens and induce an inflammatory response comparable to the responses observed with Th1 or Th2 cells. However, these results were obtained with artificially polarized CD8⁺ T cell subsets and therefore do not indicate whether naive CD8⁺ T cells would differentiate into Tc1 or Tc2 cells in response to soluble antigen under more physiological conditions. In this respect, it is well established that differentiation into Th1 or Th2 cells depends on the concentration of antigen [27]: low antigen concentrations favor the generation of Th2 cells, whereas high antigen concentrations favor the generation of Th1 cells. Naive T cell receptor-transgenic CD8⁺ T cells were therefore cultured with increasing doses of OVA (0.1, 1, 10, 100 or 1,000 $\mu\text{g/ml}$) and analyzed for proliferation and their capacity to produce IFN- γ or IL-4 following re-stimulation with PMA and ionomycin in the presence of monensin. Cells proliferated in response to OVA in a dose-dependent manner (Fig. 6C). Moreover, OVA was able to trigger generation of cytokine-producing cells, and the cytokine profile depended on the amount of antigen used. Similar to the differentiation of Th1 versus Th2 cells, higher doses of OVA (≥ 100 $\mu\text{g/ml}$) induced Tc1-like cells producing IFN- γ (Fig. 6A), while lower doses of antigen (≤ 10 $\mu\text{g/ml}$) triggered IL-4 production (Fig. 6B). Thus, direct exposure to soluble antigens can induce the differentiation of CD8⁺ T cells into Tc1 or Tc2 cells, suggesting that these cells may amplify antigen-driven inflammatory responses in the lung.

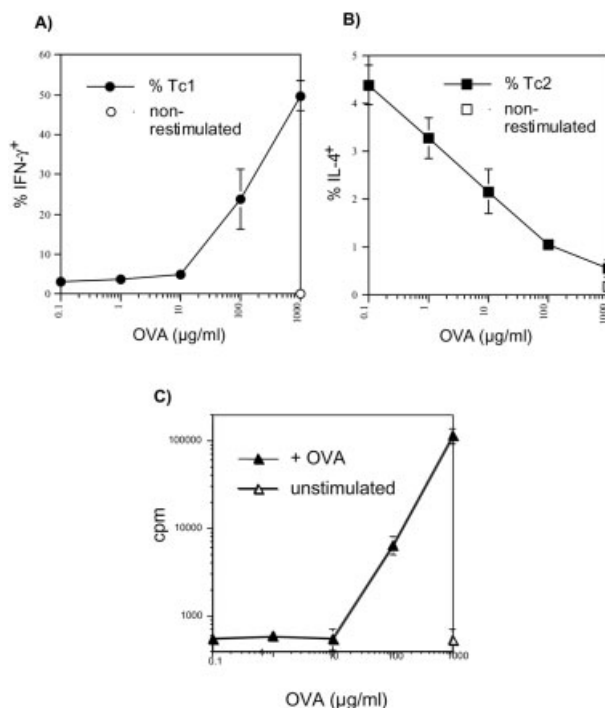


Fig. 6. Antigen dose regulates the differentiation of CD8⁺ OT-I T cells stimulated with whole OVA into Tc1 and Tc2 effectors. Lymph node and spleen cells (1:1) from OT-I mice were stimulated with the indicated doses of soluble OVA in 0.2 ml cultures. (A, B) Intracellular cytokines were measured after 4 days of culture and 5 h restimulation as in Fig. 1. (C) Proliferation was assessed after 48 h by addition of 0.5 μCi [^3H]thymidine for an additional 16 h before harvesting and liquid scintillation counting (Topcount NXT, Packard Bioscience). Results are the means $\pm$ SD of triplicate cultures and are representative of three independent experiments.

3 Discussion

CD8⁺ T cells can differentiate into Tc1 or Tc2 cells producing distinct cytokine patterns similar to Th1 or Th2 cells. However, the roles of these CD8⁺ T cells subsets in inflammatory diseases such as asthma are not well understood. The results in this study clearly demonstrate that Tc1 and Tc2 cells respond to aerosolized allergens and induce a neutrophilic airway inflammation without BHR or an eosinophilic inflammatory response with BHR, respectively. Thus, the functional roles of these CD8⁺ T cell subsets were found to be very similar to Th1 or Th2 cells, suggesting that these cells might amplify the mainly T helper cell-driven inflammatory responses in the lungs found in diseases such as asthma.

Although the responses induced by Tc1 or Tc2 cells were qualitatively very similar to the effects observed with Th1 or Th2 cells, the overall activity of the CD8⁺ T cell subsets

appears to be less potent. Both neutrophilic and eosinophilic inflammatory responses were quantitatively less pronounced compared to the inflammation induced by Th1 or Th2 cells. Similarly, the BHR induced by allergen-specific Tc2 cells was slightly lower than the response induced by similar numbers of Th2 cells. This lower potency of both Tc1 and Tc2 cells is most likely due to the lower level of polarization and cytokine production achieved by CD8⁺ cells compared to CD4⁺ subsets in our experiments. Moreover, the polarization of Tc2 cells was not as complete as with Th2 cells and there was still a substantial, although dramatically reduced, amount of IFN- γ produced by these cells, which may have subsequently interfered with the Tc2 cell-driven inflammatory response.

However, despite the reduced potency of Tc1 or Tc2 compared to Th1 or Th2 cells, the results of this study clearly demonstrate that these CD8⁺ T cell subsets respond to soluble antigens and therefore may contribute to inflammatory responses in the airways induced by inhaled antigens. Classically, MHC class I molecules present self- or pathogen-derived antigens that are synthesized within the cell, whereas exogenous antigens acquired *via* endocytic uptake are loaded onto MHC class II molecules for presentation to CD4⁺ T cells. Therefore, antigen-specific Tc1 or Tc2 cells should not directly respond to aerosolized antigens *in vivo* or to soluble antigens *in vitro*. However, it is becoming increasingly clear that some dendritic cells are specialized in processing exogenous antigens for presentation to CD8⁺ T cells. Processing of exogenous antigens in the MHC class I pathway, a process known as cross-priming, was first demonstrated in splenic APC [28], and recent studies indicate that the CD8⁺ population of dendritic cells is responsible for cross-priming of cytotoxic T cells *in vivo* [29]. This cross-priming effect is most likely also responsible for the response of CD8⁺ T cell transgenic splenocytes to soluble OVA in our *in vitro* experiments.

Similar to the well-described effect that differentiation into Th1 or Th2 cells depends on the concentration of allergen used, CD8⁺ T cells also differentiate into a predominantly Tc2 phenotype in response to a low allergen concentration, whereas a high antigen load drives the generation of Tc1 cells. However, the frequency of IL-4-producing CD8⁺ T cells generated in response to low antigen concentrations (~4%) was substantially lower than that obtained when cells were stimulated with anti-CD3, anti-CD28 and PMA (~30%) for *in vivo* transfer. This altered protocol was used to obtain optimal polarization of the cells, since previous work from our laboratory demonstrated that murine cell differentiation is regulated by the balance of protein kinase C (PKC)

and calcium signaling within T cells, and stimulation of PKC or inhibition of calcineurin results in type 2 effectors [30]. Nevertheless, the results obtained from culturing CD8⁺ T cells with cognate antigen indicate that CD8⁺ T cells not only respond to soluble antigen but also serve to demonstrate more physiological conditions in which development of a Tc2 phenotype may occur.

The role of CD8⁺ T cells in animal models of allergic inflammation is controversial. In some studies, CD8⁺ T cells have been demonstrated to have a suppressive role in the development of allergic inflammation and BHR [25, 31–33]; in others, the opposite has been shown. For example, mice depleted of CD8⁺ T cells prior to sensitization were unable to develop airway hyperresponsiveness, eosinophil infiltration of the lung parenchyma or IL-5 production in the local lymph nodes of the airway. Transfer of CD8⁺ T cells from naive animals during sensitization fully restored the ability to develop airway hyperresponsiveness, and this was accompanied by IL-5 production and eosinophil accumulation in the lung [34]. This opposing effect of CD8⁺ T cells was also shown by Holmes et al. [35]. This study demonstrated that the time of CD8⁺ T cell depletion is crucial for IgE production. While depletion of CD8⁺ T cells after immunization enhanced allergic sensitization, removal of CD8⁺ T cells prior to immunization inhibited the primary IgE response.

Since the cytokine gene expression profile produced by Tc2 cells was found to be almost completely overlapping with that of Th2 cells [36], it is not surprising that Tc2 cells are capable of inducing a comparable airway inflammation as observed with Th2 cells and thus may amplify the inflammatory response in diseases such as asthma. It has indeed been demonstrated that in certain experimental conditions, such as presence of viral infection, CD8⁺ T cells triggered in the presence of type 2 cytokines can differentiate towards Tc2. IL-5-producing CD8⁺ T cells, when challenged *via* the airways with cognate peptide, were able to induce a significant eosinophilia [37]. In addition, it has been reported that infection with influenza A virus leads to an increase in airway responsiveness and allergen-specific IgE production in mice exposed to aerosolized antigen [38]. Moreover, data from human studies point out a positive correlation between respiratory tract infections and the development of allergic disease [39, 40]. This suggests that CD8⁺ T cell activation can promote asthma either directly or indirectly. There are a number of respiratory viruses that can exacerbate the symptoms of asthma in humans, the most common of which are rhinovirus (RV) and respiratory syncytial virus (RSV). Rhinovirus is common in all age groups, whereas RSV is more important in infants and young children [41]. The mechanisms by which viral infections induce disease exacerbation are not well

understood but probably include the biased production of specific cytokines. IL-4 produced during allergic responses may switch a normally type 1-biased virus-specific T cell response towards type 2. Indeed, it has been shown that exposure of PBMC from asthmatics to rhinovirus induces a different cytokine pattern, with increased levels of IL-4 and decreased levels of IL-12 and IFN- γ , as compared with the exposure of cells from normal individuals to the same virus [42].

In conclusion, our results indicate that production of a cytokine pattern that is compatible with the allergic phenotype by CD8⁺ T cells might further amplify the underlying inflammatory response in diseases such as asthma and contribute to disease exacerbation. In this respect, it is important to note that CD8⁺ T cells secreting type 2 cytokines have indeed been reported in lung biopsies and BAL fluid of asthmatic patients [24, 43], suggesting that Tc2 cells may contribute to the pathology of asthma. However, the allergen specificity of these cells as well as their responsiveness to an allergen challenge has not been demonstrated. Further studies are therefore needed to elucidate the role of the MHC class I pathway of allergen presentation in both the etiology and immunopathology of allergic disease in man.

4 Materials and methods

4.1 Animals

C57BL/6 mice (5–8 weeks old) were obtained from Harlan (Oxon, GB). OVA peptide-specific, class I-restricted V α 2/V β 5.2 T cell receptor transgenic mice (OT-I) [28, 44] were a kind gift from Dr. M. Merkenschlager (Royal Postgraduate Medical School, Hammersmith Hospital, GB). OVA peptide-specific, MHC class II-restricted T cell receptor transgenic mice (OT-II) [45] were obtained from Ifa Credo, France. All inter-group and inter-experiment groups were age-, sex- and weight-matched. All experimental protocols complied with the Home Office 1986 Animals Scientific Procedures Act and were approved by the Novartis Horsham Research Centre Animal Welfare Committee.

4.2 Generation of OVA-specific T cell subsets

OVA-specific T cell receptor-transgenic CD4⁺ and CD8⁺ T cells were isolated from spleens and lymph nodes (cervical, inguinal, axillary and mesenteric) of OT-II and OT-I mice, respectively, using magnetic cell sorting (MACSTM). CD4⁺ T cells were cultured in complete medium with OVA^{323–339} (1 μ g/ml) and irradiated (4,000 rad) splenocytes (1:2 ratio) at 1×10^6 cells/ml. For Th1 development, recombinant murine IL-12 (BD Bioscience, Oxford, GB; 10 ng/ml) and neutralizing anti-IL-4 antibody (BD Bioscience; 5 μ g/ml) were added. For

Th2 development, recombinant murine IL-4 (BD Bioscience; 10 ng/ml) and anti-IL-12 (BD Bioscience; 5 μ g/ml) were used. IL-2 (BD Bioscience) was added every 3 days from day 3 onwards at 20 U/ml. Cells were cultured for three rounds of antigenic stimulation (3 weeks) under polarizing conditions.

For the Tc1 phenotype, CD8⁺ T cells were mixed with irradiated (4,000 rad) C57BL/6 splenocytes at a 1:2 ratio and suspended in complete medium (1×10^6 cells/ml) with OVA^{257–264} (1 μ g/ml), recombinant murine IL-12 (10 ng/ml) and neutralizing anti-IL-4 antibody (5 μ g/ml). Cells were then cultured for 1 week. IL-2 (20 U/ml) was added on day 3. For Tc2 differentiation, CD8⁺ T cells were suspended in complete medium at 1×10^6 cells/ml in the presence of PMA (Sigma-Aldrich, Poole, GB; 10 ng/ml), anti-CD28 (BD Bioscience; 1 μ g/ml), IL-4 (10 ng/ml) and blocking anti-IL-12 (5 μ g/ml). Cells were then cultured in flasks pre-coated with anti-CD3 (BD Bioscience; 4 μ g/ml) for 4 days. To determine cytokine profiles, cells were restimulated with PMA (10 ng/ml) and ionomycin (400 ng/ml) in the presence of 5 μ g/ml brefeldin A (Sigma-Aldrich) at 1×10^6 cells/ml for 5 h at 37°C.

Cells were then washed with PBS, placed in FACS tubes at 5×10^5 cells/tube and stained for intracellular IFN- γ , IL-2, IL-4 and IL-5 (antibodies from BD Pharmingen, Oxford, GB), using the Fix&Perm kit (Caltag Laboratories, Burlingame, CA) according to manufacturer's instructions. Samples were analyzed using a FACSCaliburTM (BD Bioscience) flow cytometer. Tc1 and Th1 cells produced high levels of IFN- γ but little IL-4 (<2%), whereas Th2 and Tc2 cells produced high levels of IL-4 but little IFN- γ (<5%). The viability of both Tc1 and Tc2 cells was >95%. These cells were subsequently resuspended in PBS for immediate adoptive transfer into mice.

4.3 T cell transfer model

Recipient C57BL/6 mice were given 1.0×10^6 , 2.5×10^6 or 5.0×10^6 Th1, Th2, Tc1 or Tc2 cells i.v. in 50 μ l PBS. Mice were exposed 24 h later to an aerosol of OVA (5%) for 20 min either once or on 3 consecutive days. Control groups received cells but were challenged with aerosolized PBS. Mice were killed 24 h after the last antigen exposure.

4.4 Determination of BHR

BHR was assessed 24 h after the last antigen challenge by methacholine-induced airflow obstruction using a whole-body plethysmograph (Buxco Europe Ltd., Petersfield, GB), as previously described [46]. Unanesthetized mice were placed into a plethysmograph and allowed to acclimate. Baseline averages of breathing frequency, tidal volume, aspiratory and expiratory times and airway resistance of all breaths over a 1-min period were recorded. Following

baseline measurements, animals were exposed for 1 min to an aerosol generated from PBS. Immediately following the aerosolization period, the animal's pulmonary function was measured over a 2-min period. Animals were then exposed to aerosol generated from a 0.1 M acetyl- β -methylcholine-bromide (methacholine; Sigma-Aldrich) solution for 1 min followed by a 4-min post-exposure recording. BHR is expressed as enhanced pause (Penh), a calculated value that correlates with measurements of airway resistance, impedance and intrapleural pressure in the same mouse: $\text{Penh} = (T_e/T_r - 1) \times (P_{ef}/P_{if})$, where T_e is the expiration time, T_r is the relaxation time, P_{ef} is the peak expiratory flow and P_{if} is the peak aspiratory flow $\times 0.67$ coefficient). The relaxation time is the time it takes for the box pressure to change from a maximum to a user-defined percentage of the maximum. Here, T_r measurement begins at the maximum box pressure and ends at 40%. Values are expressed as the percentage shift from baseline, which was measured by comparing the maximum Penh value of mice before (*i.e.* baseline) and after methacholine challenge.

4.5 Assessment of inflammation

At the specified time point, animals were anaesthetized with 60 mg/kg *i.p.*-injected pentobarbitone sodium. The trachea was cannulated and BAL collected with 3×0.4 ml PBS. Total cell counts were determined and cytopspins prepared (Shandon Scientific, Cheshire, GB). Cells were stained with Diff-Quik (Baxter Dade AG, Duding, Switzerland) and a differential count performed on 200 cells.

4.6 Proliferation assay

Lymph nodes and spleen cells (1:1) from OT-I mice were cultured for 48 h in 96-well round-bottom plates at 5×10^4 cells/per well in 0.2 ml medium. Increasing doses of OVA (0.1, 1, 10, 100, 1,000 g/ml) were added to the cultures for stimulation. Proliferation was assessed after 48 h of OVA stimulation by addition of 0.5 μCi [^3H]thymidine for further 16 h before harvesting and liquid scintillation counting (Topcount NXT, Packard Bioscience).

4.7 *In vivo* measurement of cytokine production

Cytokine levels (IL-4 and IFN- γ) in the BAL of recipient mice were determined by ELISA (R&D Systems, Oxon, GB) 24 h after a last OVA challenge.

4.8 Immunohistochemistry

Lungs were collected 24 h post-challenge, inflated with optimum cutting temperature (OCT) compound, snap-frozen in liquid nitrogen and stored at -80°C for histological staining.

Tissue sections were stained for the presence of $\text{Va}2^+$ cells using anti- $\text{Va}2$ antibody (BD PharMingen) and the tyramide signal amplification system (Perkin Elmer Life Sciences, Inc., Hounslow, GB) according to the manufacturer's instructions.

4.9 Statistical analysis

Results are representative of at least three independent experiments. Data were analyzed by standard statistical packages for one way analysis of variance (ANOVA) followed by Student's *t*-test for unpaired values. A value of $p < 0.05$ was considered significant. Results are expressed as means $\pm$ SEM.

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