

Tc9 cells, a new subset of CD8⁺ T cells, support Th2-mediated airway inflammation

Alexander Visekruna¹, Josephine Ritter¹, Tatjana Scholz¹, Lucia Campos¹, Anna Guralnik¹, Lucia Poncette¹, Hartmann Raifer¹, Stefanie Hagner², Holger Garn², Valerie Staudt³, Tobias Bopp³, Sebastian Reuter⁴, Christian Taube⁵, Karin Loser⁶ and Magdalena Huber¹

¹ Institute for Medical Microbiology and Hygiene, University of Marburg, Marburg, Germany

² Institute for Laboratory Medicine and Pathobiochemistry, University of Marburg, Marburg, Germany

³ Institute for Immunology, University Medical Center of the Johannes Gutenberg-University Mainz, Mainz, Germany

⁴ III-Medical Clinic, Department of Pulmonology, University Medical Center of the Johannes Gutenberg-University Mainz, Mainz, Germany

⁵ Department of Pulmonology, C3-P, Leiden University Medical Center, Leiden, The Netherlands

⁶ Department of Dermatology and Interdisciplinary Center of Clinical Research (IZKF), University of Münster, Münster, Germany

Similar to T-helper (Th) cells, CD8⁺ T cells also differentiate into distinct subpopulations. However, the existence of IL-9-producing CD8⁺ T (Tc9) cells has not been elucidated so far. We show that murine CD8⁺ T cells activated in the presence of IL-4 plus TGF- β develop into transient IL-9 producers characterized by specific IFN- γ and IL-10 expression patterns as well as by low cytotoxic function along with diminished expression of the CTL-associated transcription factors T-bet and Eomesodermin. Similarly to the CD4⁺ counterpart, Tc9 cells required for their differentiation STAT6 and IRF4. Tc9 cells deficient for these master regulators displayed increased levels of Foxp3 that in turn suppressed IL-9 production. In an allergic airway disease model, Tc9 cells promoted the onset of airway inflammation, mediated by subpathogenic numbers of Th2 cells. This support was specific for Tc9 cells because CTLs failed to exert this function. We detected increased Tc9 frequency in the periphery in mice and humans with atopic dermatitis, a Th2-associated skin disease that often precedes asthma. Thus, our data point to the existence of Tc9 cells and to their supportive function in Th2-dependent airway inflammation, suggesting that these cells might be a therapeutic target in allergic disorders.

Keywords: Allergic airway inflammation · Atopic dermatitis · CD8⁺ T cells · IL-9 · Tc9 cells



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Correspondence: Dr. Magdalena Huber
e-mail: magdalena.huber@staff.uni-marburg.de

Introduction

IL-9 is a pleiotropic cytokine produced by different cell types such as T cells, innate lymphoid cells, eosinophils, and mast cells [1, 2]. IL-9 secretion by T cells was originally linked to Th2-mediated (where Th is T-helper) diseases such as allergic airway inflammation [3, 4] and parasite infection [5, 6]. However, in vitro IL-9 is differently regulated from other classical Th2-cytokines such as IL-4, IL-5, and IL-13. IL-9 is induced by the combination of the Th2-skewing cytokine IL-4 with TGF- β [7] and, under these conditions Th2-cytokines are strongly inhibited [8, 9]. Therefore, besides Th1, Th2, Th17, and Treg cells, IL-9-producing cells have been established as an additional Th-cell subset, termed Th9 cells. The differentiation of Th9 cells is governed by the transcription factors IRF4, PU.1, and STAT6 [10, 11]. IRF4, also important for Th2-, Th17-, and Tfh-fate decisions [12–15] regulates IL-9 production directly by binding to the *Il9* promoter [10]. Likewise, PU.1 enhances IL-9 production, at least partly by binding to the *Il9* promoter [11]. In contrast, STAT6, activated by IL-4, contributes to IL-9 production indirectly. It represses the expression of two transcription factors, Treg-specific Foxp3 and Th1-associated T-bet that inhibit IL-9 production [8, 9, 16].

Similarly to Th cells, CD8⁺ T cells differentiate into at least four effector subsets with different phenotype: CTLs, Tc2, Tc17, or CD8⁺ Treg cells [17]. The best characterized CD8⁺ T-cell subpopulation, CTLs kill infected or tumorigenic cells by releasing cytotoxic molecules such as Perforin 1 and Granzyme B (GzmB) and by production of proinflammatory cytokines, IFN- γ and TNF- α . Eomesodermin (Eomes) and T-bet, two T-box transcription factors, are involved in the determination of their effector differentiation [17, 18].

Asthma is a chronic airway disorder characterized by bronchial inflammation, airway obstruction, and airway hyperresponsiveness. The most common form, allergic asthma, is associated with classical Th2-cytokine mediated inflammation [19]. However, Th9 cells have also been shown to contribute to allergic airway inflammation via secreted IL-9 [10, 11, 20]. Consistently, in lungs of asthmatic patients IL-9 expression is higher than in healthy controls [21, 22]. Moreover, mononuclear cells of atopic infants produce more IL-9 than those of healthy controls [23], suggesting that the presence of early IL-9 may promote subsequent allergic asthma induction. In addition to CD4⁺ cells, CD8⁺ T cells most likely are involved in asthma pathology [24, 25]. However, the exact role of different CD8⁺ T-cell subsets in the onset of the disease is unclear. CD8⁺ T cells contribute also to the development of atopic dermatitis (AD), a common chronic inflammatory skin disease that often precedes asthma in humans [26, 27]. AD is characterized, for example, by Th2- and Th9-associated immune abnormalities such as increased serum IgE levels, sensitization to allergens, and elevated Th2-cytokine, as well as IL-9 levels [28].

Here we investigated the ability of CD8⁺ T cells to produce IL-9, their cytokine expression patterns, molecular requirements for IL-9 production, the impact of these cells on the onset of allergic airway disease (AAD) and their presence in AD, a skin disease strongly associated with asthma and allergic sensitization.

Results

IL-9-producing CD8⁺ T cells display diminished cytotoxicity

To elucidate whether CD8⁺ T cells are able to acquire an IL-9-producing phenotype, we chose culture conditions applied for induction of Th9 cells. As expected, in the absence of skewing cytokines, CD8⁺ T cells developed CTL-effector phenotype with high expression of the cytotoxic molecule GzmB and the cytokine IFN- γ (Fig. 1A and B). The presence of IL-4 did not strongly influence GzmB and IFN- γ production. In contrast, addition of TGF- β inhibited expression of GzmB but not of IFN- γ . Similar to CD4⁺ T cells, the combination of IL-4 and TGF- β -induced IL-9 production also in CD8⁺ T cells and concomitantly suppressed GzmB (Fig. 1B).

To establish the transcriptional program of cultured CD8⁺ T cells, we performed quantitative RT-PCR analysis for IL-9 and molecules associated with Tc2 and CTL differentiation. Similar to Th9 cells, IL-9-producing Tc9 cells expressed the cytokines IL-5 and IL-13 at diminished levels as compared with Tc2 cells cultured in the presence of IL-4 alone (Fig. 1C). Consistent with impaired GzmB production, the expression of the CTL hallmark molecules T-bet (*Tbx21*), Eomes and Perforin 1 (*Prf1*) was strongly reduced in Tc9 cells, suggesting impaired CTL development and probably diminished cytotoxicity. To evaluate this assumption, we performed cytotoxic assays with CTL, Tc2, or Tc9 obtained after polyclonal stimulation under respective conditions of OVA_{257–264} peptide-specific TCR transgenic OT-I cells. Similar to WT CD8⁺ T cells, IL-9 production of OT-I cells was also associated with decreased expression of GzmB (Supporting Information Fig. 1). The cytotoxicity of Tc2 cells was similar to that of CTLs in accordance with their unaltered levels of GzmB and previously published data [29]. Consistently with the impaired expression of GzmB and *Prf1*, the cytotoxicity of Tc9 OT-I cells was greatly diminished as compared with CTLs (Fig. 1D). For *Runx3*^{−/−} CD8⁺ T cells has been reported that they display reduced cytotoxicity because of impaired proliferation [30]. To evaluate whether this phenomenon also applies to Tc9 cells, we assessed the proliferation of CD8⁺ T cells cultured under indicated conditions by CFSE dilution. The percentages of proliferating cells did not significantly differ between CTLs and Tc9 cells (Fig. 1E) suggesting that low cytotoxicity exerted by Tc9 cells is not caused by proliferative defects.

Thus, CD8⁺ T cells stimulated under TGF- β and IL-4 conditions produce IL-9 and display impaired expression of molecules specific for CTL development. This is reflected in their diminished cytotoxicity as compared with CTL or Tc2 cells.

Tc9 cells are characterized by specific cytokine expression patterns

To characterize the patterns of IL-9 and IFN- γ production by Tc9 cells we analyzed these cells kinetically in comparison with CTLs

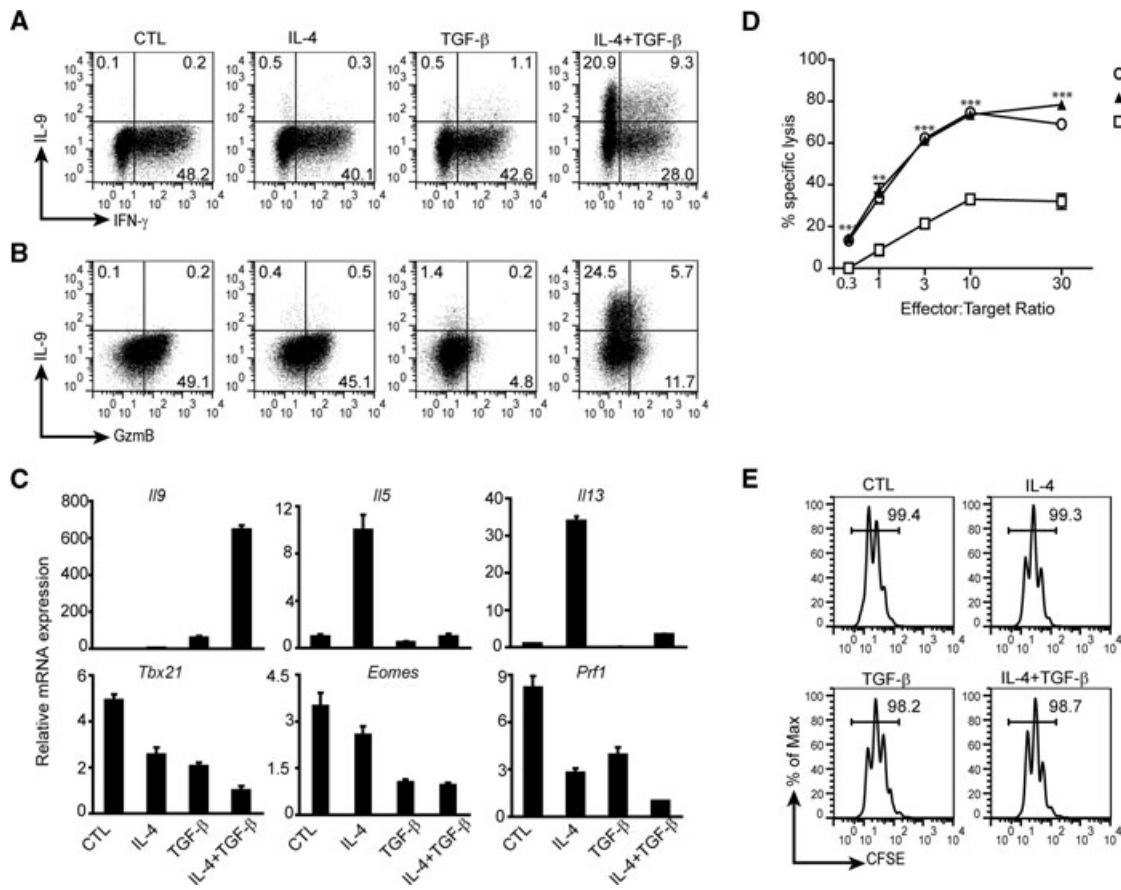


Figure 1. Characterization of Tc9 cells. (A, B) Flow cytometry of CD8⁺ T cells differentiated in the absence (CTL) or presence of the indicated cytokines, restimulated with PMA and ionomycin in the presence of brefeldin A on day 2, stained intracellularly for IL-9 and (A) IFN-γ or (B) GzmB. Numbers indicate the percentage of positive cells. (C) Quantitative real-time RT-PCR for the indicated genes in CD8⁺ T cells stimulated as in (A). Data are shown as mean ± SD of PCR duplicates. (D) Antigen-specific cytotoxicity of OT-I-CTL, -Tc2, and -Tc9 cells was determined by specific lysis of EL-4 cells. Data are shown as mean ± SD of triplicate determinations. ***p* < 0.005, ****p* < 0.0005, unpaired two-tailed *t*-test. (E) Flow cytometry of CD8⁺ T cells stained with CFSE and differentiated as in (A). Numbers above the lines indicate the percentage of cells that had proliferated. (A–E) Data shown are representative of three independent experiments.

at the mRNA and protein level. As expected, CTLs did not express IL-9 at any analyzed time points of culture and their ability to produce IFN-γ after restimulation increased gradually reaching the highest percentages at day 6 (Fig. 2A). At mRNA level, non-restimulated CTLs maintained high IFN-γ expression with peak at day 4 and decline at day 6 (Fig. 2B). Similarly, Tc9 cells expressed IFN-γ during the whole culture period however considerably less than CTLs at later time points (Fig. 2A and B), revealing that Tc9 and CTL cells differ in IFN-γ expression levels. In contrast, the IL-9 expression by Tc9 cells was transient with maximum at day 2 and rapid decline at day 3. Already at day 4, IL-9 was almost undetectable in restimulated cells and also at mRNA level (Fig. 2A and B). Consistently, in culture supernatants the IL-9 amounts increased till day 2 and then remained at the constant level (Fig. 2C). As Th9 cells have been shown to express IL-10 [8, 9, 11, 31], we examined Tc9 cells for IL-10 production. Similar to Th9 cells, Tc9 cells expressed increasing IL-10 mRNA levels reaching maximum at day 6 (Fig. 2B). In accordance with mRNA data, in culture supernatants from Tc9 cells we found ascending

IL-10 amounts with the highest value at day 6 (Fig. 2C). In contrast, CTLs failed to express IL-10 during the analyzed time period (Fig. 2B and C). Thus, like Th9 cells, also Tc9 cells produce IL-9 and IL-10 with different kinetics. Furthermore, IFN-γ production by Tc9 cells although maintained during the whole culture period was substantially lower as compared with CTLs, revealing distinct expression patterns.

STAT6 and IRF4 are important for Tc9 development

To elucidate whether STAT6 is involved in regulating Tc9 development, we cultured *Stat6*^{-/-} CD8⁺ T cells in the presence or absence of IL-4 and TGF-β alone, or in combination. Similar to Th9 cells, the IL-9 production in CD8⁺ T cells was dependent on STAT6, because *Stat6*^{-/-} CD8⁺ T cells cultured under Tc9 conditions displayed greatly diminished production of IL-9, both at the protein and mRNA level as compared with WT cells (Fig. 3A and B). As for Th2 cells, STAT6 deficiency also affected the

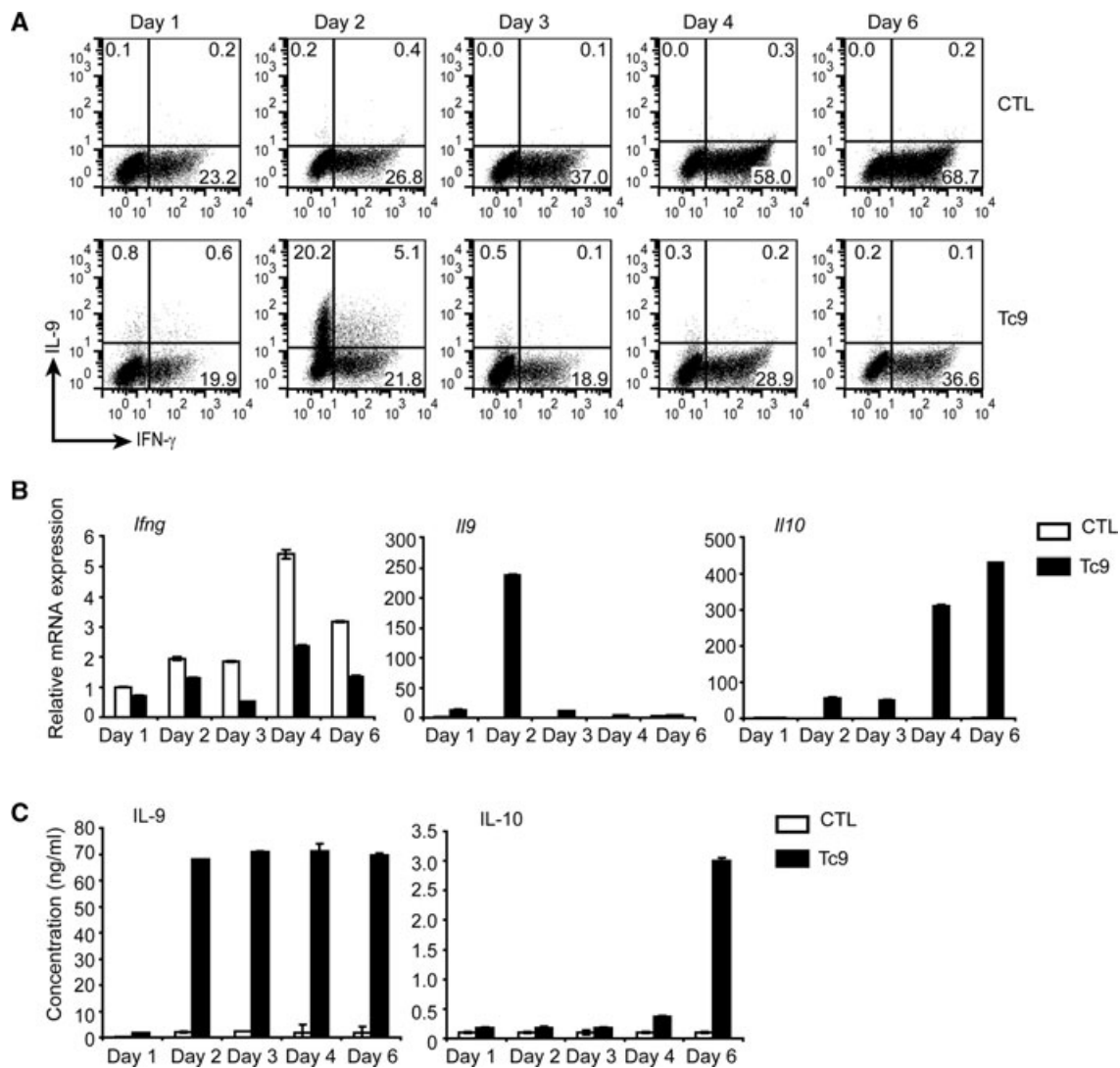


Figure 2. Expression patterns of IFN- γ , IL-9, and IL-10 by CTL and Tc9 cells. CD8⁺ T cells were differentiated in the absence (CTL) or presence of TGF- β and IL-4 (Tc9). (A) Flow cytometry of cells restimulated on the indicated days and stained intracellular for IFN- γ and IL-9. Numbers indicate the percentage of positive cells. (B) Quantitative real-time RT-PCR for IFN- γ , IL-9, and IL-10 in CD8⁺ T cells cultured under CTL and Tc9 conditions and collected at the indicated days. For all genes the expression level in CTLs on day 1 was set to 1. Data are shown as mean $\pm$ SD of PCR duplicates. (C) Culture supernatants were collected at the indicated days, and the amounts of IL-9 and IL-10 were determined by ELISA. Data represent mean $\pm$ SD of ELISA duplicates. (A–C) Data shown are representative of three independent experiments.

expression of Tc2 cytokines. Moreover, lack of STAT6 led to increased numbers of IFN- γ -producing cells under Tc2 and Tc9 conditions, suggesting that STAT6 downregulates IFN- γ , possibly by suppression of T-bet [16].

To analyze whether IRF4 influences Tc9 differentiation, we cultured WT and *Irf4*^{-/-} CD8⁺ T cells under CTL, Tc2, TGF- β , and Tc9 conditions. The result shows that, as for CD4⁺ T cells, IRF4 is also essential for IL-9 production by CD8⁺ T cells, both at the protein and mRNA level (Fig. 3C and D). Similar to *Stat6*^{-/-} CD8⁺ T cells, *Irf4*^{-/-} CD8⁺ T cells produced more IFN- γ under Tc2 and Tc9 conditions and failed to express Tc2 cytokines.

In summary, these data demonstrate that IL-9 production in CD8⁺ T cells is regulated by similar transcription factors as in CD4⁺ T cells, namely by STAT6 and IRF4.

Foxp3 suppresses IL-9 production in Tc9 cells

To evaluate whether in CD8⁺ T cells, IL-9 production is regulated by Foxp3, we analyzed its expression in WT, *Irf4*^{-/-} and *Stat6*^{-/-} CD8⁺ T cells cultured under TGF- β or TGF- β + IL-4 conditions. As compared with WT cells, we found increased expression of Foxp3 in *Irf4*^{-/-} CD8⁺ T cells cultured with TGF- β (Fig. 4A). The addition of IL-4 caused significant suppression of Foxp3 in *Irf4*^{-/-} CD8⁺ T cells. However, the percentages of Foxp3-positive cells were still significantly higher as compared with WT cells cultured with TGF- β and IL-4 (Fig. 4B). Thus, *Irf4*^{-/-} CD8⁺ T cells exert increased Foxp3 expression under Tc9 conditions.

As described previously for Th9 cells, the expression of Foxp3 during culture with TGF- β plus IL-4 was higher in *Stat6*^{-/-} CD8⁺

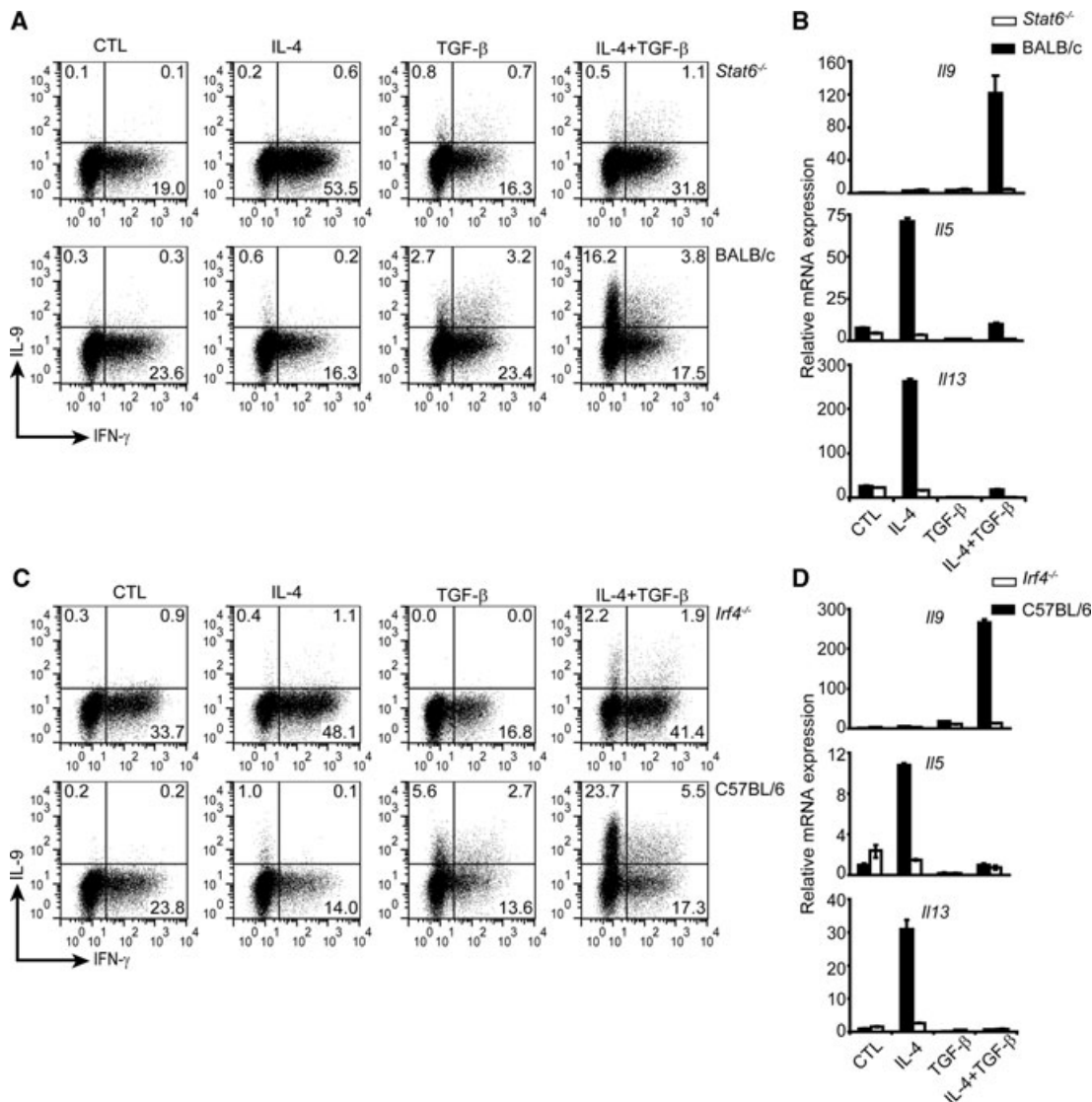


Figure 3. Molecular requirements for Tc9 cell differentiation. (A, C) Flow cytometry of CD8⁺ T cells from WT, *Stat6*^{-/-}, or *Irf4*^{-/-} mice differentiated in the absence (CTL) or presence of the indicated cytokines, restimulated on day 2, and stained for intracellular IL-9 and IFN- γ . (B, D) Quantitative real-time RT-PCR for the indicated genes in CD8⁺ T cells from WT, *Stat6*^{-/-}, or *Irf4*^{-/-} mice stimulated as in (A). For all genes the expression level in WT CTLs was set to 1. (B, D) Data are shown as mean $\pm$ SD of PCR duplicates. (A–D) Data shown are representative of three independent experiments.

T cells than in WT cells, due to impaired Foxp3 suppression by IL-4 (Fig. 4C). Thus, although mediated by different mechanisms, the expression of Foxp3 was markedly increased in Tc9 cells deficient for STAT6 as well as for IRF4. As both, *Stat6*^{-/-} and *Irf4*^{-/-} CD8⁺ T cells display greatly impaired IL-9 production and concomitantly increased Foxp3, we used retroviral infection to evaluate whether enhanced Foxp3 expression influences the IL-9 production. In CD8⁺ T cells infected with a virus expressing GFP and Foxp3 bicistronically, more than 70% of transduced GFP⁺ cells were also positive for Foxp3 resulting in considerably higher MFI for Foxp3 as compared with cells transduced with control-GFP retrovirus (Supporting Information Fig. 2A and B) thus, confirming forced expression of Foxp3. Compared with infection with the control retrovirus, transduction with Foxp3-encoding virus substantially

decreased the percentage of IL-9-producing CD8⁺ T cells, while IFN- γ and TNF- α were suppressed to a lower extent (Fig. 4D and Supporting Information Fig. 2C). Thus, enforced expression of Foxp3 inhibits IL-9 production in CD8⁺ T cells and this result suggests that increased Foxp3 levels contribute to the massive impairment of IL-9 production in both *Stat6*^{-/-} and *Irf4*^{-/-} cells.

Tc9 cells promote airway inflammation

As IL-9-producing T cells were originally linked to allergic airway inflammation and CD8⁺ T cells together with Th2 cells are suggested to play an important role in this disorder [32], we speculated that Tc9 cells may serve as additional source of IL-9, thus cooperating with Th2 cells to enhance AAD. To assess this

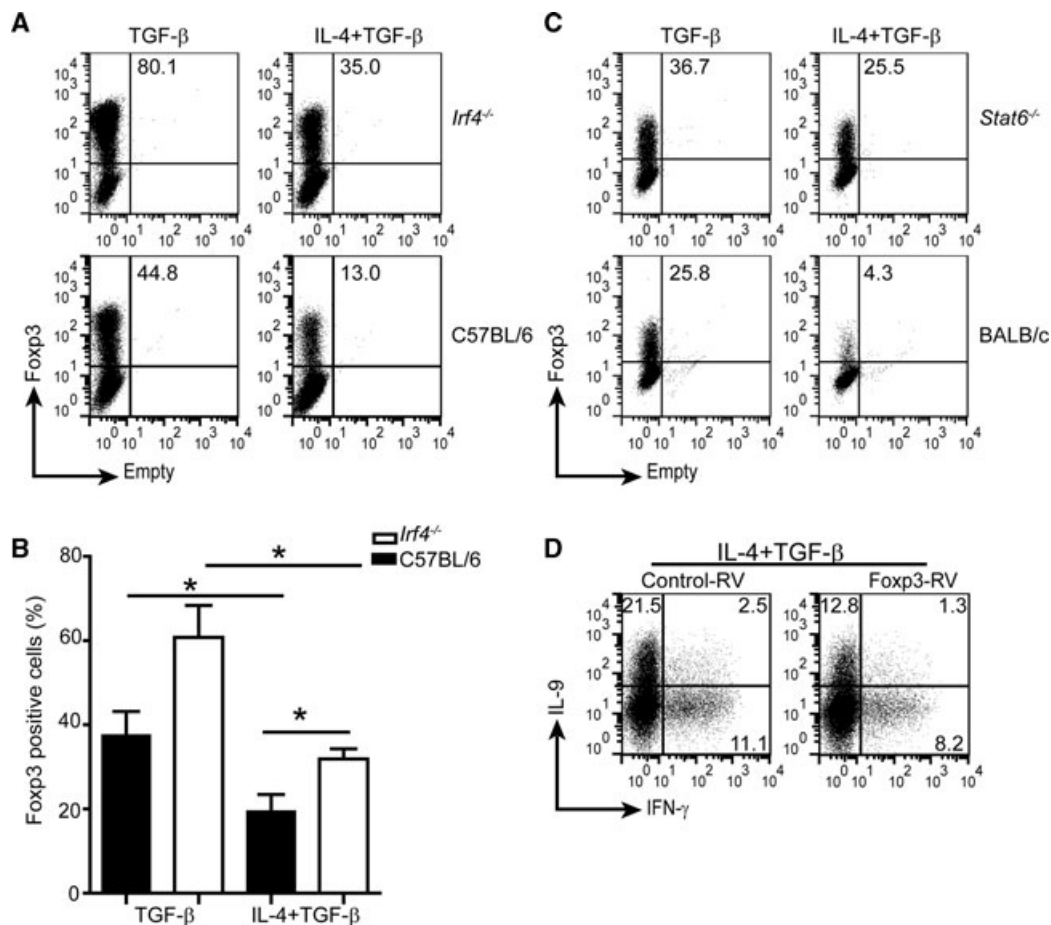


Figure 4. The function of Foxp3 during Tc9 differentiation. (A–C) Flow cytometry of CD8⁺ T cells from WT (C57BL/6, BALB/c), *Stat6*^{-/-}, or *Irf4*^{-/-} mice differentiated in the presence of the indicated cytokines and stained for Foxp3 at day 2 of culture. (B) The percentage of Foxp3-positive WT and *Irf4*^{-/-} cells cultured under indicated conditions are shown as mean ± SEM, n = 5. (D) WT CD8⁺ T cells were transduced with retrovirus expressing GFP (Control-RV) or retrovirus expressing Foxp3 and GFP (Foxp3-RV), cultured as indicated, restimulated at day 2, and stained intracellularly for IFN-γ and IL-9. Analyses were performed on a GFP⁺ gate. (A, C, D) Numbers indicate the percentage of positive cells. (A–D) Data shown are representative of three independent experiments. *p < 0.05, unpaired two-tailed t-test.

assumption, we first transferred titrated numbers of Th2 cells and evaluated AAD severity (data not shown). As source of Th2 cells we used OVA_{323–339} peptide-specific TCR transgenic OT-II cells polarized in vitro under Th2 conditions. Because our aim was to analyze a contribution of Tc9 cells to AAD development, we then applied subpathogenic numbers of Th2 cells. Transferred Tc9 cells were obtained from OT-I cells polarized in vitro under Tc9 conditions (Supporting Information Fig. 3A). We then injected either subpathogenic numbers of Th2 cells failing to cause airway inflammation (Fig. 5A–I) or Tc9 cells, alone or in combination, into *Rag2*^{-/-} mice. Subsequently, the recipient mice were exposed to the nebulized allergen OVA for 6 days. The transfer of Tc9 cells alone did not evoke symptoms of AAD (Fig. 5A–I). This result is consistent with previously published data demonstrating that IL-4-producing CD4⁺ T cells are central to the development of allergic airway inflammation [32]. Interestingly, the transfer of Th2 and Tc9 cells together caused severe symptoms such as increased eosinophil, neutrophil, and T-cell numbers in the BALF

(Fig. 5A–C), increased numbers of mucus-producing goblet cell numbers (periodic acid-Schiff (PAS)-positive cells) (Fig. 5H and I), and an increased inflammation score (Fig. 5F and G). Although we also notified increased airway reactivity to metacholine, the differences were not statistically significant, probably because of the limited size of experimental groups (Supporting Information Fig. 3). Furthermore, co-transfer of Th2 and Tc9 cells led to increased numbers of both CD4⁺ and CD8⁺ T cells in the BALF as compared with single transfers, suggesting that Th2 and Tc9 cells deliver reciprocal help for infiltration (Fig. 5D and E). Phenotype analysis of transferred CD8⁺ T cells from the lung revealed IL-13 and IFN-γ-positive cells, but no IL-9 and IL-4 (Supporting Information Fig. 4A and B). Also in the spleens and draining lymph nodes of substituted mice we could not detect IL-9 in CD8⁺ T cells, indicating that Tc9 cells had lost their marker cytokine expression. In contrast, Th2 cells upregulated IL-13, suggesting their enhanced pathogenic phenotype in vivo (Supporting Information Fig. 4C and D). Collectively, our results show that Tc9

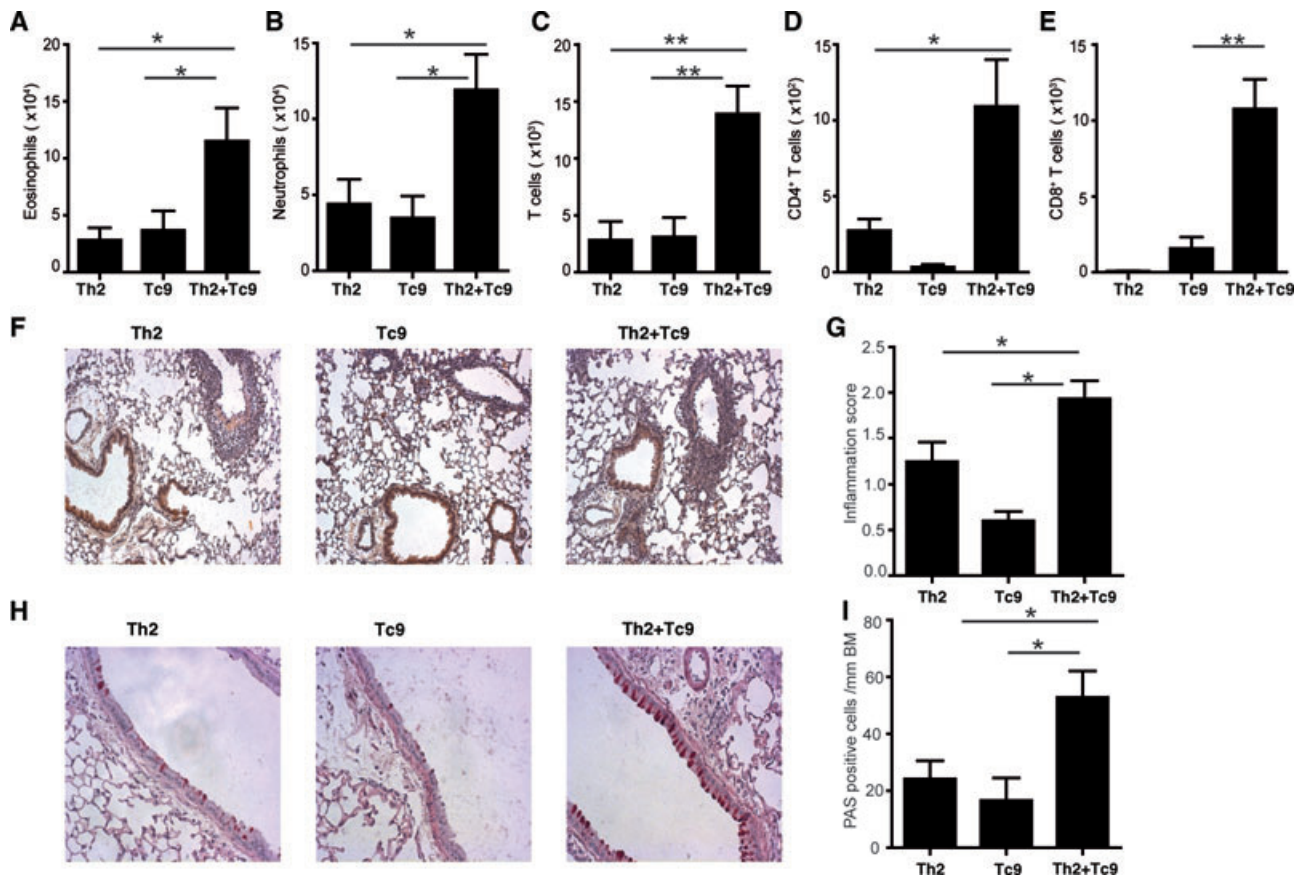


Figure 5. Tc9 cells support Th2-mediated airway inflammation. OT-I and OT-II cells were polarized in vitro under Tc9 or Th2 conditions, respectively. *Rag2*^{−/−} mice were substituted with subpathogenic numbers of Th2 cells alone or in combination with Tc9 cells and then challenged with nebulized OVA. (A–E) The numbers of (A) eosinophils, (B) neutrophils, and (C–E) T cells in the BALF are shown. Data are shown as mean + SEM of *n* = 10 pooled from three independent experiments. (F–I) Tissue inflammation was evaluated (F, G) with H&E staining, (F) original magnification 100 $\times$, and (H, I) with PAS staining for goblet cells, (H) original magnification 200 $\times$. (G) Slides were scored for peribronchial and perivascular inflammation with semiquantitative score from 0 to 4. (I) The numbers of mucus-positive cells were scored per millimeter basement membrane. (G, I) Data are shown as mean + SEM of *n* = 6 pooled from two independent experiments. **p* < 0.05, ***p* < 0.005, unpaired two-tailed *t*-test.

cells cooperated with Th2 cells for airway inflammation. During this process, Th2 cells maintained their cytokine pattern, whereas Tc9 cells probably switched into IFN- γ and IL-13 producers.

Tc9 cells but not CTLs support Th2-mediated airway inflammation

To evaluate whether the support for Th2-mediated AAD is specific for Tc9 cells we transferred CTL or Tc9 cells in combination with subpathogenic numbers of Th2 cells into *Rag2*^{−/−} mice. The transfer of Tc9 cells in combination with Th2 cells caused a significantly higher number of leukocytes, especially eosinophils, in the BALF as compared with that observed with CTL and Th2 co-transfer (Fig. 6A), suggesting that Tc9 but not CTLs possess properties to support Th2-mediated AAD. Accordingly, histological analysis of the lungs revealed significantly higher inflammation score (Fig. 6D) and increased presence of PAS-positive cells (Fig. 6E) in mice substituted with Tc9 cells in combination with Th2 cells.

Likewise, we found significantly higher numbers of both CD4⁺ and CD8⁺ T cells in the BALF of mice substituted with Tc9 and Th2 cells (Fig. 6B). These results indicate that Tc9 but not CTL cells support Th2-mediated airway inflammation and that Th2 and Tc9 cells but not CTLs deliver reciprocal help for airway infiltration. The comparison of the phenotype of transferred CD8⁺ T cells from the lungs of *Rag2*^{−/−} mice after challenge revealed that Tc9 cells were positive for IFN- γ and to a lower extent for IL-13, as previously. Interestingly, markedly higher percent of CTLs was positive for IFN- γ than of Tc9 cells (Fig. 6C) mirroring the cytokine expression patterns by Tc9 and CTLs during later time points of in vitro culture. Namely also in vitro, when IL-9 production was already extinguished, Tc9 cells produced considerably less IFN- γ than CTLs (Fig. 2A). Altogether, these data suggest that Tc9 but not CTLs provide support for Th2-mediated onset of airway inflammation. During this process transferred Tc9 and CTLs display IFN- γ expression pattern similar to that exerted during in vitro culture, reconfirming their distinct characteristics.

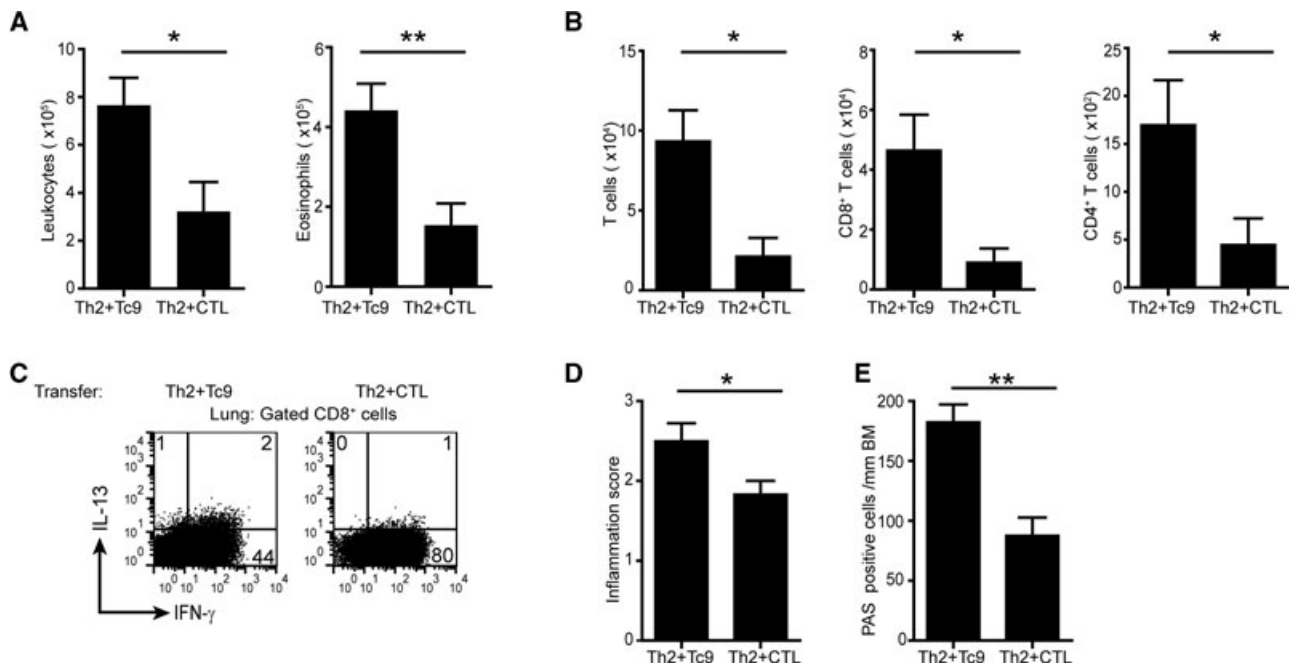


Figure 6. Tc9 but not CTL cells support Th2-mediated airway inflammation. OT-I and OT-II cells were polarized in vitro under CTLs, Tc9, or Th2 conditions, respectively. *Rag2*^{-/-} mice were substituted with subpathogenic numbers of Th2 cells in combination with CTLs or Tc9 cells and then challenged with nebulized OVA on six consecutive days. Twenty-four hours after the last airway challenge the mice were analyzed. (A, B) The numbers of (A) leukocytes, eosinophils, and (B) T cells in the BALF are shown as mean + SEM of *n* = 8 pooled from two independent experiments. (C) Flow cytometry of lymphocytes isolated from lungs, restimulated with PMA and ionomycin in the presence of brefeldin A, stained for surface CD8, and intracellular for IFN- γ and IL-13. Data are representative for individually analyzed mice (*n* = 8). (D, E) Tissue inflammation was evaluated (D) with H&E staining and (E) with PAS staining for goblet cells and shown as mean + SEM of *n* = 8 pooled from two independent experiments. (D) Slides were scored as described for Figure 5G. (E) The numbers of mucus-positive cells were scored per millimeter basement membrane. **p* < 0.05, ***p* < 0.005, unpaired two-tailed *t*-test.

Tc9 cells are increased in atopic mice and humans

Our data demonstrate supportive role of Tc9 cells for the onset of AAD mediated by subpathogenic numbers of Th2 cells. Since (i) the presence of early IL-9 has been suggested to promote asthma induction [23] and (ii) AD, a Th2-mediated disease, often precedes asthma, we analyzed whether Tc9 cells are detectable in AD and thus may influence the subsequent onset of allergic airway inflammation. Interestingly, we observed increased numbers of IL-9-producing CD8⁺ T cells (Fig. 7A and Supporting Information Fig. 5) and elevated IL-9 mRNA levels as well as elevated IL-9 secretion in CD8⁺ T cells purified from lymph nodes draining cutaneous lesions of OVA-treated mice (Fig. 7B). To assess whether Tc9 cells might be directly involved in tissue destruction, we analyzed lesional skin biopsies from OVA- and control-mice at different time points after induction of AD. Interestingly, we detected Tc9 cells only in lymph nodes, but not in cutaneous lesions (Fig. 7C), suggesting that Tc9 cells might contribute to the activation of effector T cells in regional lymph nodes. Alternatively, they might migrate to the inflammatory skin and thereby change their phenotype. Next, we analyzed human individuals with AD compared with healthy donors for the presence of Tc9 cells. As depicted in Figure 7D, we observed twofold increased numbers of CD8⁺IL-9⁺ T cells in peripheral blood from patients with chronic AD (Fig 7D and Supporting Information Fig. 5). Notably, similar to the obser-

vations made in the mouse model, human Tc9 cells were not detectable in cutaneous lesions from patients with AD (Fig. 7E). Together, these data indicate that Tc9 cells are increased in the periphery of mice as well as in humans with AD and might suggest influence of Tc9 cells on the subsequent onset of Th2-associated airway disease.

Discussion

In the current study, we found that, similarly to CD4⁺ T cells, the cytokine IL-4 along with TGF- β determines the differentiation of CD8⁺ T cells into an IL-9-secreting effector population. Tc9 cells differentiate along a developmental program that is very similar to the one described for Th9 cells, which includes low expression of IL-5 and IL-13 as well as reduced expression of the hallmark effector molecules that are central to CTL phenotype and function. We and others have previously described a similar low cytotoxic phenotype for Tc17 cells [33, 34]. These IL-17-producing CD8⁺ T cells differentiate likewise in the presence of TGF- β but in combination with IL-6 or IL-21 instead of IL-4. Apparently, under TGF- β -rich conditions and in the presence of proinflammatory cytokines, “non-classical” CD8⁺ T cells arise whose biological function is probably associated with cytokine production and support of CD4⁺-mediated inflammatory response rather than

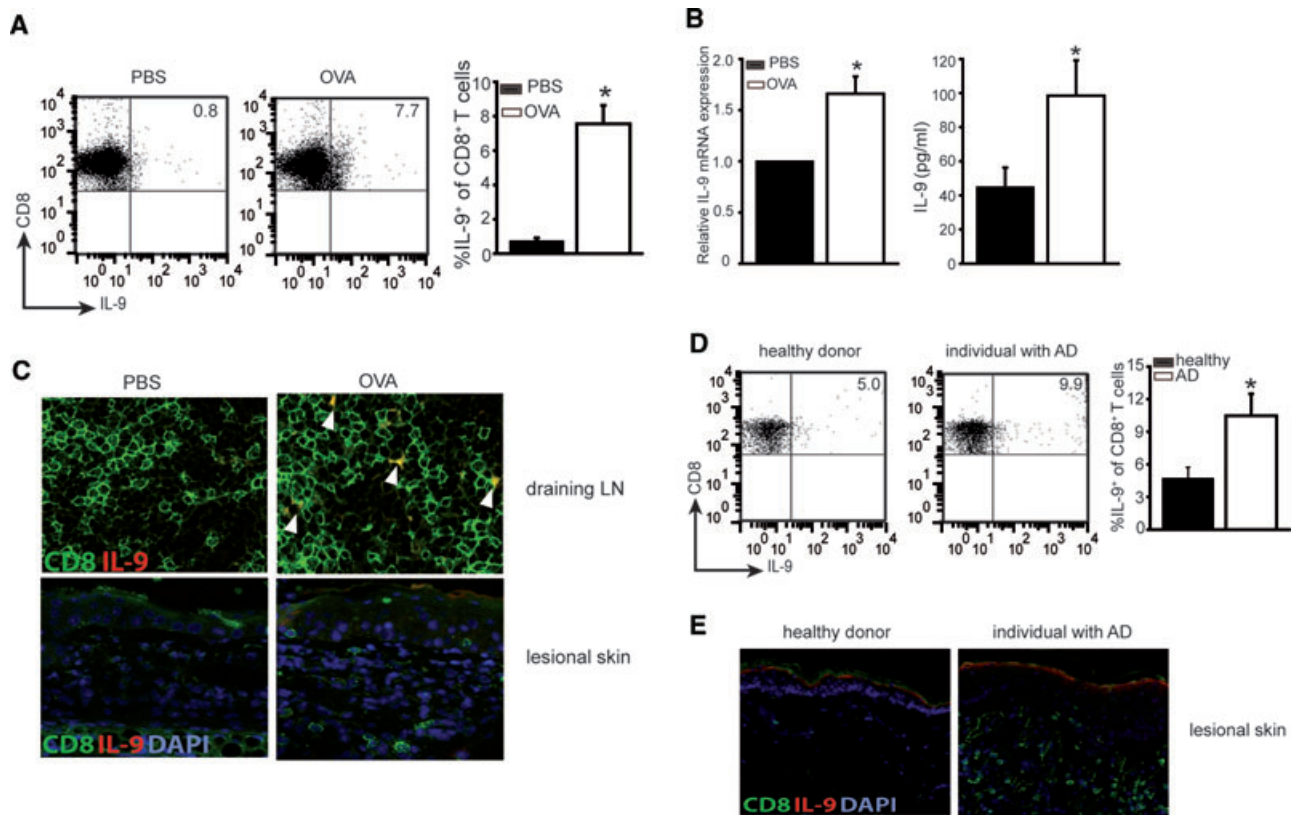


Figure 7. Increased numbers of Tc9 cells in atopic dermatitis. (A) IL-9 staining in CD8⁺ T cells from draining lymph nodes, 6 weeks after induction of AD. One representative dot plot (left) as well as statistical evaluation (mean + SEM, $n = 5$) are shown. (B) IL-9 mRNA and protein expression in purified CD8⁺ T cells from lymph nodes as above, quantified by real-time PCR or ELISA. (C) Immunofluorescence staining of lesional skin and draining lymph nodes 6 weeks after induction of AD, original magnification 400 $\times$ (lymph node) or 200 $\times$ (skin). (D) Flow cytometry of peripheral blood stained with anti-CD8 and anti-IL-9 antibodies. One representative dot plot (left) as well as statistical evaluation (mean + SEM, $n = 5$) are shown. (E) Immunofluorescence staining (lesional skin) in individuals with chronic AD. One representative image is shown, original magnification 400 $\times$. (A–E) Data shown are representative of three independent experiments. * $p < 0.05$, unpaired two-tailed t -test.

with cytotoxicity. Here we show that Tc9 cells display specific cytokine expression patterns. In contrast to Th9 cells, Tc9 cells expressed IFN- γ , however at markedly lower level as compared with those of CTLs during later time points of in vitro culture. Similarly to Th9 cells, IL-9 production by Tc9 cells is characterized by short retention, namely after peak at day 2, already at day 4 the IL-9 expression was extinguished both at mRNA and protein level. The IL-10 expression by Tc9 cells increased with time reaching the highest values at day 6 of culture, resembling the Th9 expression pattern. Thus, Tc9 cells express IL-9 and IL-10 with similar characteristics as compared with Th9 cells, however in contrast to Th9 cells, Tc9 cells produce IFN- γ . Mechanistically, the generation of Tc9 cells, similarly to Th9 cells, is regulated by the transcription factors STAT6 and IRF4 [8, 10, 16]. Both, *Stat6*^{-/-} and *Irf4*^{-/-} CD8⁺ T cells produced IL-9 at greatly diminished levels. Conversely, the expression of the CTL cytokine IFN- γ was upregulated in both CD8⁺ T-cell types cultured under Tc9 conditions, suggesting the involvement of these transcription factors in the reciprocal relationship between Tc9 and CTL development. Furthermore, the expression of Foxp3 was upregulated in *Stat6*^{-/-} and *Irf4*^{-/-} CD8⁺ T cells, and overexpression of Foxp3 in Tc9

cells suppressed IL-9 production. This result is consistent with published data demonstrating that also in Th9 cells forced expression of Foxp3 suppresses IL-9 production [16]. Altogether, these data indicate common molecular patterns governing IL-9 production in CD4⁺ and CD8⁺ T cells and suggest that additional indirect mechanisms such as the level of Foxp3 contribute to the regulation of IL-9 production by IRF4.

Functionally, in contrast to CTLs, Tc9 cells supported Th2-mediated AAD. This conclusion is drawn from our experiments performed in an adoptive transfer model into *Rag2*^{-/-} mice. The transfer of Tc9 cells alone did not induce onset of AAD, demonstrating that Tc9 cells by their own were not pathogenic. However, in combination with subpathogenic numbers of Th2 cells, which failed to evoke disease symptoms, Tc9 cells elicited increased eosinophil numbers in the BALF, increased numbers of mucus-producing cells in the lung and elevated lung inflammatory score. The capability to promote Th2-mediated airway inflammation was associated with Tc9 properties because CTLs failed to fulfill this function. Thus, Tc9 cells but not CTLs cooperated with Th2 cells for airway infiltration and for induction of AAD. The phenotype analysis of transferred cells from the lung revealed loss of IL-9

production by Tc9 cells in favor of IFN- γ and to small extent IL-13. This result is in agreement with our data obtained from in vitro experiments demonstrating transient IL-9, while continues IFN- γ production by Tc9 cells. Thus, resembling the phenotype of Tc9 cells from in vitro culture, CD8⁺ T cells obtained from *Rag2*^{-/-} mice substituted with Tc9 cells retained different phenotype as compared with CTLs, because they produced considerably less IFN- γ . When we analyzed spleens and lung lymph nodes of substituted *Rag2*^{-/-} mice with Tc9 and Th2 cells we failed to detect IL-9⁺CD8⁺ T cells, demonstrating that also in the periphery the transferred Tc9 cells changed the phenotype. Transient IL-9 production has been also described for innate lymphocytes [35] and Th9 cells [31]. Thus, similar to other lymphocytes, also Tc9 cells are characterized by short IL-9 retention. Consistent with the transient kinetic of IL-9 secretion, we could not detect IL-9-producing CD8⁺ T cells in lungs of mice during OVA/alum-induced AAD (data not shown). Nevertheless, our data obtained from transfer experiments into *Rag2*^{-/-} mice suggest a supportive function of Tc9 cells for the initiation of Th2-mediated airway inflammation at a stage when the amounts of Th2 cells are not sufficient to induce disease pathology by themselves. Therefore, we speculated that Tc9 cells might reside in diseases, which precede airway inflammation and which pathology is associated with the presence of CD8⁺ T cells and Th2 cells. Accordingly, we found Tc9 cells in the lymph nodes but not in the skin of mice and in peripheral blood of patients with AD, a disease that is Th2-associated and often precedes asthma in humans [27]. Therefore, we suppose that peripheral Tc9 cells arising during AD might contribute to the subsequent onset of asthma by promoting the pathogenic function of Th2 cells. Probably, in the periphery Tc9 cells use IL-9 and/or other so far unknown Tc9-associated cytokines or surface molecules to activate Th2 cells directly or/and via innate immune cells to enhance allergic inflammation. An activating function of IL-9 on T cells, antigen-presenting cells, and mast cells has already been described [1]. However, the pleiotropic activities of IL-9 open the possibility that Tc9 cells support Th2-mediated inflammation at different levels, which deserve further elucidation. As Tc9 cells isolated from the lung expressed IL-13, one could speculate that Tc9 cells directly via IL-13 support Th2-pathogenicity. However, it is rather unlikely, because the signaling via IL13R α 1 has been suggested to antagonize Th2 development in vivo [36].

Concerning the supportive function of Tc9 cells for AAD in the mouse model and their presence in the periphery in mice and humans with AD, closer characterization of their function may be important for better understanding of the mechanisms of allergic asthma and thus may reveal novel therapy strategies.

Materials and methods

Mice

WT BALB/c and C57BL/6 mice were from The Jackson Laboratory. *Rag2*^{-/-}, *Irf4*^{-/-}, and OVA-specific, TCR-transgenic OT-I and

OT-II mice, all on the C57BL/6 background, were bred at the animal facility of the Biomedical Research Center, University Marburg. *Stat6*^{-/-} mice on the BALB/c background were kindly provided by Dr. Vöhringer (University of Erlangen, Germany).

Human blood samples and ethics statement

After written informed consent was obtained, PBMCs were isolated from heparinized blood of healthy donors and patients with AD according to standard methods. Cells were stained using anti-CD8 (OKT8) and anti-IL-9 (MH9A4) antibodies from Biolegend. Isotype-matched controls were included in each staining. Subsequently, PBMCs were analyzed on a FACSCalibur™ using the CELLQuest™ software (BD, Germany). All experiments were carried out according to the declaration of Helsinki and were approved by the ethical committee of the University of Münster, Medical School (2008–180-f-S).

Murine CD4⁺ and CD8⁺ T-cell purification, in vitro stimulation, and staining

CD4⁺ and CD8⁺ T cells were isolated by magnetic cell sorting (MACS, Miltenyi, Germany) from spleens and lymph nodes of 8–12 week old mice and were primed with plate-bound 5 μ g/mL anti-CD3 (145–2C11) and 3 μ g/mL anti-CD28 mAb (37.51) in the presence of recombinant human IL-2 (50 U/mL; Novartis) and 5 μ g/mL anti-IFN- γ (XMG1.2) (CTL conditions). Some cultures also received 2 ng/mL rhTGF- β 1 (R&D Systems), 50 ng/mL recombinant murine IL-4 (Peprotech), or combinations of these stimuli (Tc9 conditions). For Th2 transfers, purified CD4⁺ OT-II cells were primed with plate-bound anti-CD3 (5 μ g/mL), soluble anti-CD28 (1 μ g/mL) and rhIL-2 (50 U/mL), anti-IFN- γ (5 μ g/mL) and 50 ng/mL rmIL-4 for 3 days, then the cells were split and cultured for further 3 days in the presence of rhIL-2 and rmIL-4 in the absence of anti-CD3. On day 6, the cells were harvested, washed, counted, and a total of 7×10^4 cells was injected into *Rag2*^{-/-} mice. For Tc9 and CTL transfers purified CD8⁺ OT-I cells were stimulated as described above for CTL and Tc9 conditions. On day 2 the cells were harvested, washed, counted, and a total of 10^6 was injected into *Rag2*^{-/-} mice.

Intracellular staining and ELISA

After indicated time points of in vitro culture or after isolation from lung, cells were restimulated with 50 ng/mL PMA and 750 ng/mL ionomycin in the presence of 5 μ g/mL brefeldin A (all from Sigma) for 4 h. The cells were intracellularly stained with anti-IL-9 (RM9A4, Biolegend), anti-IFN- γ (XMG1.2), anti-TNF- α (MP6-XT22), anti-IL-13 (eBio13A), or anti-GranzymeB (16G6) antibodies (eBiosciences). For Foxp3 detection, the Foxp3 staining kit (anti-Foxp3; FJK-16s; eBioscience) was used. Cells were analyzed with a FACSCalibur and the CellQuest Pro software. IL-10 levels in culture supernatants were measured with the ELISA kit provided by BD Biosciences. IL-9 was detected by mAb 229.4

(1 $\mu\text{g/mL}$) and biotinylated mAb C12 (1 $\mu\text{g/mL}$). ELISAs were evaluated according to reference standard curves by using known amounts of the specific cytokine.

Quantitative real-time PCR

RNA preparation and complementary DNA synthesis were performed as described previously [33]. Gene expression was examined with an ABI Prism 7700 Sequence Detection System (Applied Biosystems) using the SYBR green I qPCRTM Core Kit (Eurogentec) for detection of *Il5*, *Il9*, *Il10*, *Il13*, *Eomes*, *Prf1*, *Tbx21*, and *Hprt1* (hypoxanthine-guanine phosphoribosyl transferase). Levels of mRNA for each gene were normalized to *Hprt1* expression using the $\Delta\Delta\text{Ct}$ method, with the lowest experimental value set to 1. Primer sequences are provided in Supporting Information Table 1.

Cytotoxicity assay

EL-4 cells were labeled with 2.5 or 10 μM CFSE. Those labeled with 2.5 μM CFSE were pulsed with 10 nM of the peptide SIINFEKL and used as targets. Those labeled with 10 μM CFSE were used as controls. OT-I CD8⁺ T cells were primed as described above for 72 h. Control and pulsed EL4 target cells were mixed at a 1:1 ratio and were incubated in triplicates with skewed OT-I CD8⁺ T cells at indicated effector:target ratios. After 6 h, the cells were analyzed by flow cytometry. Percent specific lysis was calculated as $(1 - \% \text{ targets} / \% \text{ control cells}) \times 100$.

Retroviral transduction

The retroviral vector pMSCV containing Foxp3-IRES-GFP, the empty control vector containing IRES-GFP, and transduction were described previously [37]. After transduction, the cells were cultured under the conditions indicated in the experiments. On day 2 after infection, the cells were restimulated and then analyzed for GFP, IL-9, IFN- γ , and TNF- α expression.

Asthma adoptive transfer model

Rag2^{-/-} mice received PBS, 7×10^4 Th2 cells derived from purified CD4⁺ T cells from OT-II mice alone or in combination with 10^6 CTL or Tc9 cells derived from purified CD8⁺ T cells from OT-I mice on day 0 by i.p. injection. Mice were then challenged via the airways with nebulized OVA (1% in saline) with ultrasonic nebulizer (NE-U17; Omron, Hoofdrop, the Netherlands) for 20 min daily from day 1 to 6. On day 7, BALFs and lung tissue were collected.

Bronchoalveolar lavage

Cells were isolated by lavage of the lungs via a tracheal tube with PBS (1 mL). Numbers of BAL cells were counted with trypan

blue dye exclusion. Differential cell counts were made from cyto-centrifuged preparations, fixed and stained with the Microscopy hemacolor set (Merck, Darmstadt, Germany). The percentage and absolute numbers of each cell type were calculated.

Histology

Lungs were fixed by inflation (1 mL) and immersion in 10% formalin and embedded in paraffin. Tissue sections were stained with H&E and PAS. Slides were examined in a blinded fashion by two experienced observers by microscopy (BX40, Olympus, Germany), and peribronchial and perivascular inflammation was graded by a semiquantitative score (no inflammation = 0, severe inflammation = 4). For each slide, five randomly chosen areas were scored. As for PAS stained slides, the number of goblet cells was analyzed by imaging software (Analysis, Soft Imaging Systems, Stuttgart, Germany). The number of mucus-containing cells per millimeter of basement membrane was determined.

Lung cell isolation

Lung cells were isolated as described [32] using collagenase digestion. Mononuclear cells were isolated by collection of the interphase fraction between 30 and 70% Percoll.

OVA-induced AD

BALB/c mice were sensitized with OVA (100 μg) or PBS by applying the protein to a sterile patch and placing the patch onto the shaved and tape-stripped back skin of the mice for 1 week. Two weeks as well as 4 and 6 weeks after removal, an identical patch was reapplied to the same skin area, finally resulting in a total of four 1-week patch exposures. At the end of the fourth sensitization, mice were sacrificed and analyzed by flow cytometry or immunofluorescence staining using anti-CD8 (53–6.7) and anti-IL-9 (RM9A4) antibodies (Biolegend).

Statistical analysis

For determination of differences between two cell populations unpaired two-tailed Student's *t*-test was used. Calculations were performed using GraphPad Prism software (GraphPad Software, Inc, La Jolla, CA).

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Abbreviations: AD: atopic dermatitis · AAD: allergic airway disease · PAS: periodic acid-Schiff

Full correspondence: Dr. Magdalena Huber, Institute for Medical Microbiology and Hygiene, University of Marburg, Hans-Meerwein Straße 2, Marburg 35043, Germany
Fax: +49-6421-28-66420
e-mail: magdalena.huber@staff.uni-marburg.de

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