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Basic

Research Article

IL-10 signaling in dendritic cells is required for tolerance induction in a murine model of allergic airway inflammation

Anja Dolch[*1,2], *Stefanie Kunz*[*1], *Britta Dorn*[1,7], *Francesca Alessandrini*[3], *Werner Müller*[4], *Robert S. Jack*[5], *Stefan F. Martin*[1], *Axel Roers*[6] *and Thilo Jakob*[1,7]

[1] Allergy Research Group, Department of Dermatology, Medical Center, University of Freiburg, Freiburg, Germany
[2] Faculty of Biology, University of Freiburg, Freiburg, Germany
[3] Center of Allergy and Environment (ZAUM), Technische Universität München and Helmholtz Zentrum München, Munich, Germany
[4] Faculty of Life Sciences, University of Manchester, Manchester, UK
[5] Department of Immunology, Institute of Immunology and Transfusion Medicine, University Hospital of Greifswald, Greifswald, Germany
[6] Institute of Immunology, Medical Faculty Carl Gustav Carus, University of Technology Dresden, Dresden, Germany
[7] Department of Dermatology and Allergology, Experimental Dermatology and Allergy Research Group, University Medical Center Gießen-Marburg, Justus Liebig University Gießen, Gießen, Germany

Allergen specific tolerance induction efficiently ameliorates subsequent allergen induced inflammatory responses. The underlying regulatory mechanisms have been attributed mainly to interleukin (IL)-10 produced by diverse hematopoietic cells, while targets of IL-10 in allergen specific tolerance induction have not yet been well defined. Here, we investigate potential cellular targets of IL-10 in allergen specific tolerance induction using mice with a cell type specific inactivation of the IL-10 receptor gene. Allergic airway inflammation was effectively prevented by tolerance induction in mice with IL-10 receptor (IL-10R) deficiency in T or B cells. Similarly, IL-10R on monocytes/macrophages and/or neutrophils was not required for tolerance induction. In contrast, tolerance induction was impaired in mice that lack IL-10R on dendritic cells: those mice developed an allergic response characterized by a pronounced neutrophilic lung infiltration, which was not ameliorated by tolerogenic treatment. In conclusion, our results show that allergen specific tolerance can be effectively induced without a direct impact of IL-10 on cells of the adaptive immune system, and highlight dendritic cells, but not macrophages nor neutrophils, as the main target of IL-10 during tolerance induction.

Correspondence: Prof. Thilo Jakob
e-mail: thilo.jakob@derma.med.uni-giessen.de

*A.D. and S.K. contributed equally.

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Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Induction of immunological tolerance is the hallmark of an efficient allergen specific immunotherapy (AIT) and attributed mainly to regulatory mechanisms mediated by interleukin (IL)-10 [1]. IL-10 is a well-known anti-inflammatory cytokine binding with high affinity to the IL-10 receptor (IL-10R) that consists of two different chains: the specific IL-10Rα subunit and the ubiquitously expressed IL-10Rβ subunit [2]. Albeit at low level, IL-10Rα expression is detected on most immune cells and also on some non-hematopoietic cells explaining the pleiotropic effect of IL-10 on diverse cell types [3]. IL-10 modulates effector cells of the allergic immune response such as mast cells and granulocytes and decreases their secretion of pro-inflammatory mediators. It influences maturation and antigen presentation of dendritic cells, modifies phagocytosis and cytokine release of macrophages/monocytes, suppresses allergen-specific effector T cell subsets, promotes the induction of IL-10 secreting regulatory T cells, and alters B cell function and immunoglobulin class switching (reviewed in [3, 4]). Immunological tolerance induced by AIT is associated with these various biological effects of IL-10. A functional role of IL-10 has been suggested in AIT using a murine model of tolerance induction, in which the reduction of allergen induced airway inflammation achieved by subcutaneous OVA injections was demonstrated to be IL-10 dependent [5].

Using a similar approach, we recently analyzed the relevance and cellular sources of IL-10 during allergen specific tolerance induction in OVA induced allergic inflammation of the airways [6]. In IL-10 competent mice, tolerance induction was effective. In contrast, when the IL-10 was neutralized prior to tolerogenic treatment, the beneficial effects were completely abrogated. Using transcriptional reporter mice, we identified T cells, B cells, and to a lesser extent DC as sources of IL-10 during tolerance induction. Interestingly, in mice with T cell-, B cell-, B and T cell-, DC-, or macrophage/neutrophil-specific IL-10 deficiency tolerance induction was still effective. Failure of tolerance induction in IL-10$^{FL/FL}$Vav-Cre^{+} mice, which lack IL-10 from all hematopoietic sources, confirmed the role of IL-10 from hematopoietic cells and suggested a high redundancy of diverse cellular sources of IL-10 in tolerance induction [6].

Using the same mouse model of allergen specific tolerance induction in allergic airway inflammation, we now analyze the potential target cells of IL-10 during tolerance induction in mice with a cell-type specific deletion of the IL-10Rα subunit [7] and demonstrate that IL-10 signaling in DCs but not in T cells, B cells or macrophages, and neutrophils is essentially required for successful tolerance induction.

Results

IL-10 signaling in T cells is not required for successful tolerance induction

With the goal to identify IL-10 target cells in allergen specific tolerance induction, we first analyzed mice with a T cell-specific deficiency of the IL-10R generated by Cre-mediated recombination (IL-10R$^{FL/FL}$CD4-Cre^{+} mice). Specific deletion was verified by absent *IL-10R* gene detection in sorted T cells and functionally by a selective lack of STAT3 phosphorylation upon IL-10 stimulation (Supporting Information Fig. S1A and B). Sensitized and allergen challenged IL-10R$^{FL/FL}$CD4-Cre^{+} mice developed an allergic airway inflammation that was comparable to their Cre^{-} littermates and characterized by an eosinophil dominated cellular influx into the bronchoalveolar space (Fig. 1A and B) and goblet cell hyperplasia in lung sections (Fig. 1E). It was accompanied by high serum titers of allergen-specific IgE (Fig. 1C left panel) and a pronounced Th2 cytokine response of lung draining LN cells upon restimulation (Fig. 1D). Tolerance induction by s.c. allergen treatment effectively reduced the allergic airway inflammation in IL-10R$^{FL/FL}$CD4-Cre^{+} mice and their Cre^{-} littermates, as documented by a drop in total BAL cell counts (Fig. 1A), reduction of BAL eosinophil numbers (Fig. 1B), reduced allergen specific IgE, increased IgG1 in serum (Fig. 1C), and dampened Th2 cytokine responses of lung draining LN cells in vitro (Fig. 1D). Along the same line, lung sections displayed reduced cellular infiltration and mucin staining upon tolerance induction in both IL-10R$^{FL/FL}$CD4-Cre^{+} mice and their Cre^{-} littermate controls (Fig. 1E). In conclusion, these results showed that a direct effect of IL-10 on T cells was not required for effective allergen specific tolerance induction in mice.

IL-10 signaling in B cells is dispensable for effective tolerance induction

We next addressed B cells as possible targets of IL-10 in tolerance induction using mice with a B cell specific IL-10R deficiency (IL-10R$^{FL/FL}$CD19-Cre^{+}). Specific deletion was verified by absent *IL-10R* gene detection in sorted B cells and functionally by a selective lack of STAT3 phosphorylation upon IL-10 stimulation (Supporting Information Fig. S2A and B). Allergic airway inflammation (Fig. 2A, B, and E), serum IgE and IgG1 responses (Fig. 2C), and Th2 cytokine response of lung draining LN cells (Fig. 2D) were similar in IL-10R$^{FL/FL}$CD19-Cre^{+} and Cre^{-} littermate controls. Again tolerance induction effectively dampened the allergic

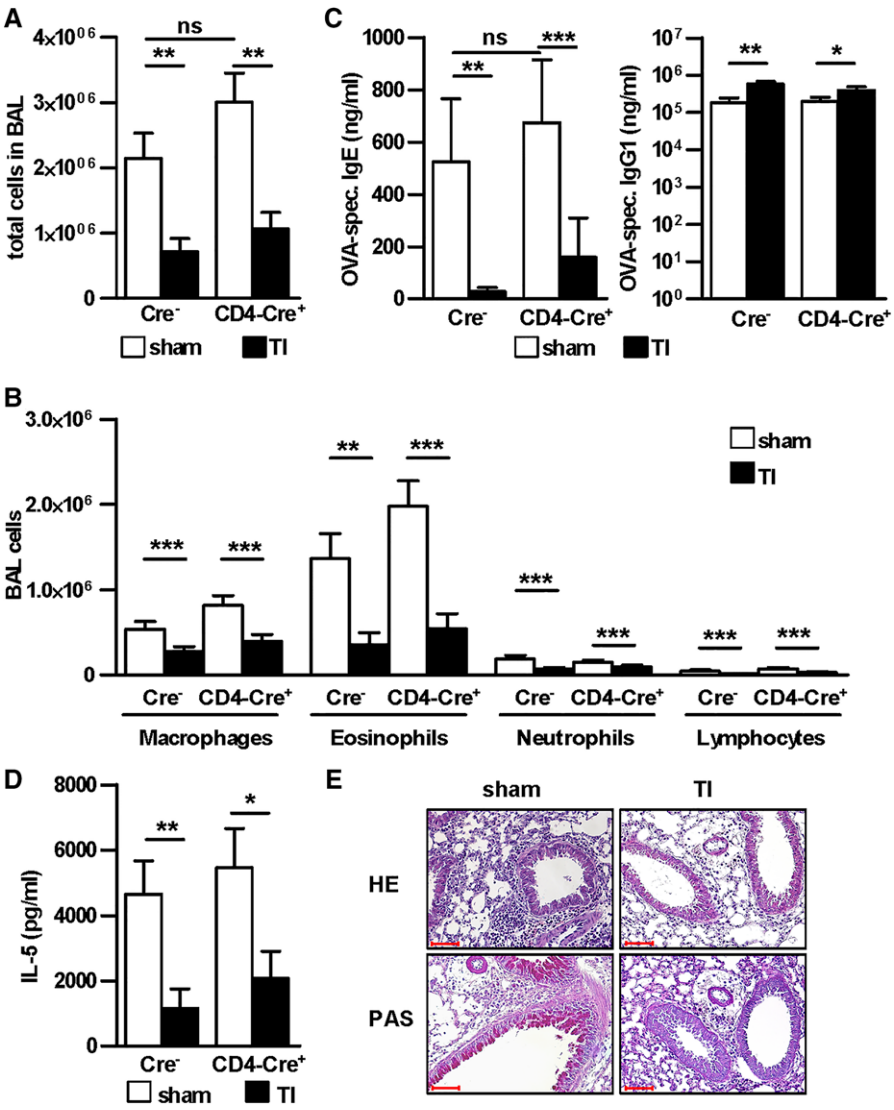

Figure 1. Tolerance induction in mice with a T cell-specific IL-10Rα deficiency. Sensitized T cell specific IL-10Rα deficient mice (IL-10R$^{FL/FL}$CD4-Cre^{+}) and Cre^{-} littermate controls (Cre^{-}) were sham treated (sham) or tolerized (TI) by s.c. injections, allergen challenged by aerosol treatment, and analyzed 24 h after the last challenge as described in *Materials and methods*. (A) Total cell numbers in BAL fluid. (B) Immune cells in BAL fluid were counted on Diff-Quick stained cytospins. (C) Serum levels of allergen-specific IgE and IgG1 were measured by ELISA. (D) IL-5 release by allergen-stimulated lung draining LN cells was measured by ELISA. (E) Representative H&E and PAS-stained lung sections of IL-10R$^{FL/FL}$CD4-Cre^{+} mice (20×, scale bar = 100 μm). Images shown are representative of two independent experiments. (A–D) Data are shown as mean + SEM (n = 4–6 mice/group per experiment) and are pooled from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ Mann–Whitney U test

airway inflammation in both IL-10R$^{FL/FL}$CD19-Cre^{+} and control mice (Fig. 2A–E). This data suggested that IL-10 sensing by B cells was not required for effective tolerance induction in allergic airway inflammation.

IL-10 signaling in monocytes, macrophages, and neutrophils is dispensable for tolerance induction

Looking at cells of the innate immune system as potential targets of IL-10 in tolerance induction, we next focused on macrophages and neutrophils. Tolerance induction in IL-10R$^{FL/FL}$LysM-Cre^{+} mice, which lack IL-10R expression in monocytes, macrophages, and neutrophils [7], was as effective as in the Cre^{-} littermate controls (Fig. 3): allergic airway inflammation (Fig. 3A, B, and E), serum Ig response (Fig. 3C) were comparable at baseline, and reduced equally in tolerized animals of both genotypes (Fig. 3A–C and E). Th2 cytokine release by allergen stimulated LN cells (Fig. 3D) is comparable at baseline and slightly, but not significantly, reduced in tolerized animal of both genotypes, this reduction is less pronounced in the Cre^{+} group. This indicated that allergen specific tolerance can be achieved independent from IL-10R signaling in monocytes/macrophages and neutrophils.

IL-10R deficiency in dendritic cells results in a neutrophil-rich allergic airway inflammation

The effect of IL-10 on DCs during tolerance induction was addressed by crossing IL-10R$^{FL/FL}$ mice to CD11c-Cre^{+} mice [8]. This strain expresses the Cre recombinase primarily in DCs. Accordingly, no IL-10R was detectable in sorted DCs from IL-10R$^{FL/FL}$CD11c-Cre^{+} mice (Supporting Information Fig. S3A) and stimulation of splenocytes with IL-10 in vitro failed to induce STAT3 phosphorylation selectively in DCs of IL-10R$^{FL/FL}$CD11c-Cre^{+} mice, while no obvious difference

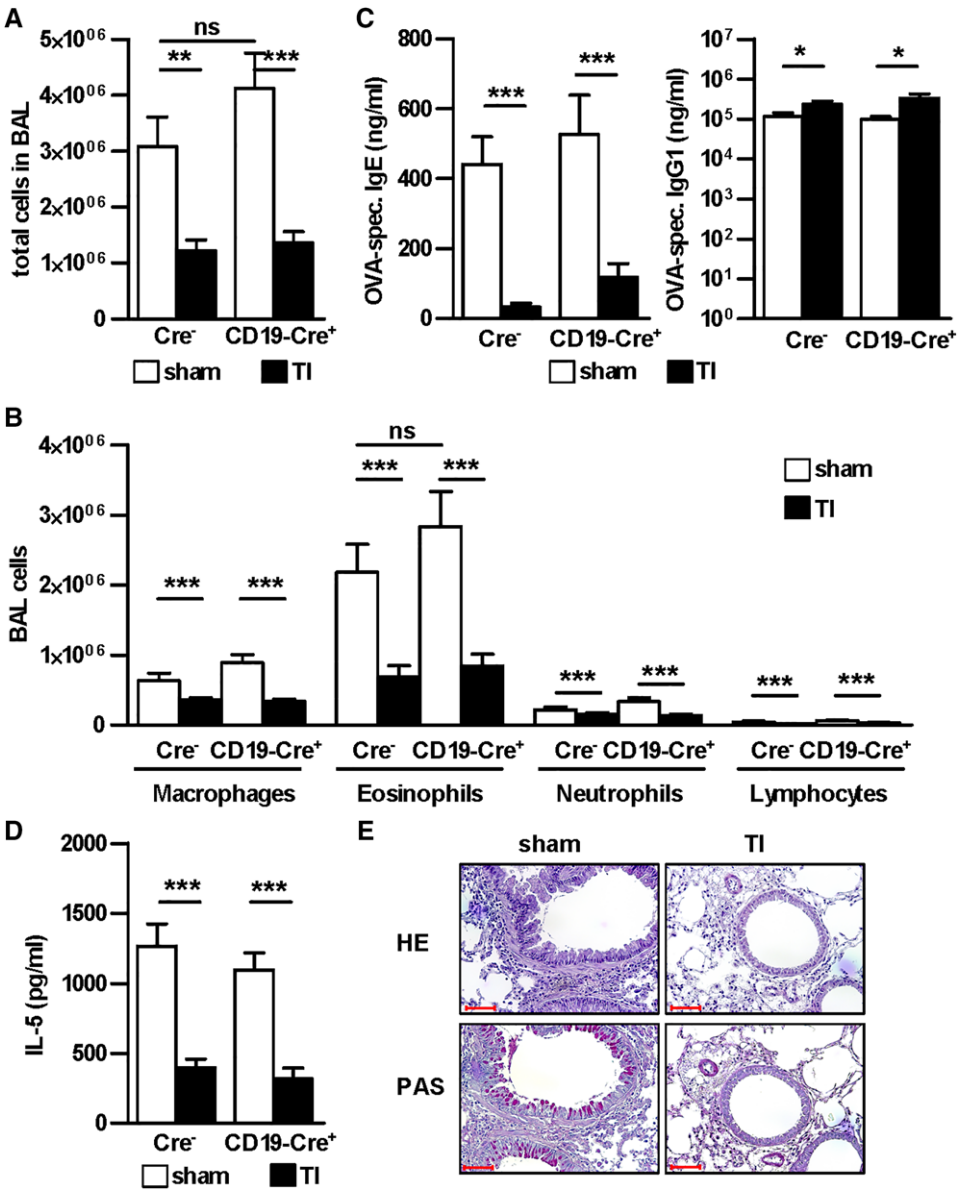

Figure 2. Tolerance induction in mice with a B cell specific IL-10Rα deficiency. Sensitized B cell specific IL-10Rα deficient mice (IL-10R$^{FL/FL}$CD19-Cre^{+}) and littermate controls (Cre^{-}) were sham treated (sham) or tolerized (TI) by s.c. injections, allergen challenged by aerosol treatment, and analyzed 24 h after the last challenge as described in *Materials and methods*. (A) Total cell numbers in BAL fluid. (B) Immune cells in BAL fluid were counted on Diff-Quick stained cytospins. (C) Serum levels of allergen-specific IgE and IgG1 were measured by ELISA. (D) IL-5 release by allergen-stimulated lung draining LN cells was measured by ELISA. (E) Representative H&E and PAS-stained lung sections of IL-10R$^{FL/FL}$CD19-Cre^{+} mice (20×, scale bar = 100 μm). Images shown are representative of two independent experiments. (A–D) Data are shown as mean + SEM (n = 4–6 mice/group per experiment) and are pooled from four independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ Mann–Whitney U test

in STAT3 phosphorylation was detectable in T cells, B cells, neutrophils, or macrophages when compared to Cre^{-} littermates (Supporting Information Fig. S3B). Sensitized IL-10R$^{FL/FL}$CD11c-Cre^{+} mice and Cre^{-} littermates displayed a rather similar allergic airway inflammation upon allergen challenge. While the total BAL cell counts were almost identical (Fig. 4A) and the cellular composition dominated by eosinophils, IL-10R$^{FL/FL}$CD11c-Cre^{+} mice displayed by trend increased numbers of neutrophils and less eosinophils in comparison to Cre^{-} littermates (Fig. 4B). This was also reflected by increased neutrophil and decreased eosinophil counts in lung sections from IL-10R$^{FL/FL}$CD11c-Cre^{+} mice (Fig. 4F and G). An allergen independent lung infiltration in naïve mice was not detected (Fig. 4E left panels). Serum levels of allergen-specific IgG1 and IgE did not differ between both genotypes (Fig. 4C).

Tolerance induction fails in the absence of IL-10R signaling in dendritic cells

Tolerance induction by s.c. allergen injections efficiently dampened allergic lung inflammation in Cre^{-} control mice as indicated by a reduced total cell influx to the bronchoalveolar space, but it only marginally and insignificantly reduced the cellular influx in IL-10R$^{FL/FL}$CD11c-Cre^{+} mice (Fig. 4A). No alteration in the neutrophil influx as well as influx of macrophages and lymphocytes was observed in IL-10R$^{FL/FL}$CD11c-Cre^{+} mice upon tolerance induction (Fig. 4B). In contrast, the level of eosinophils within the BAL of those mice was diminished (Fig. 4B). In both groups allergen treatment for tolerance induction increased specific IgG1 and not significantly reduced allergen specific IgE serum levels (Fig. 4C). The Th2 cytokine response of lung draining LN cells

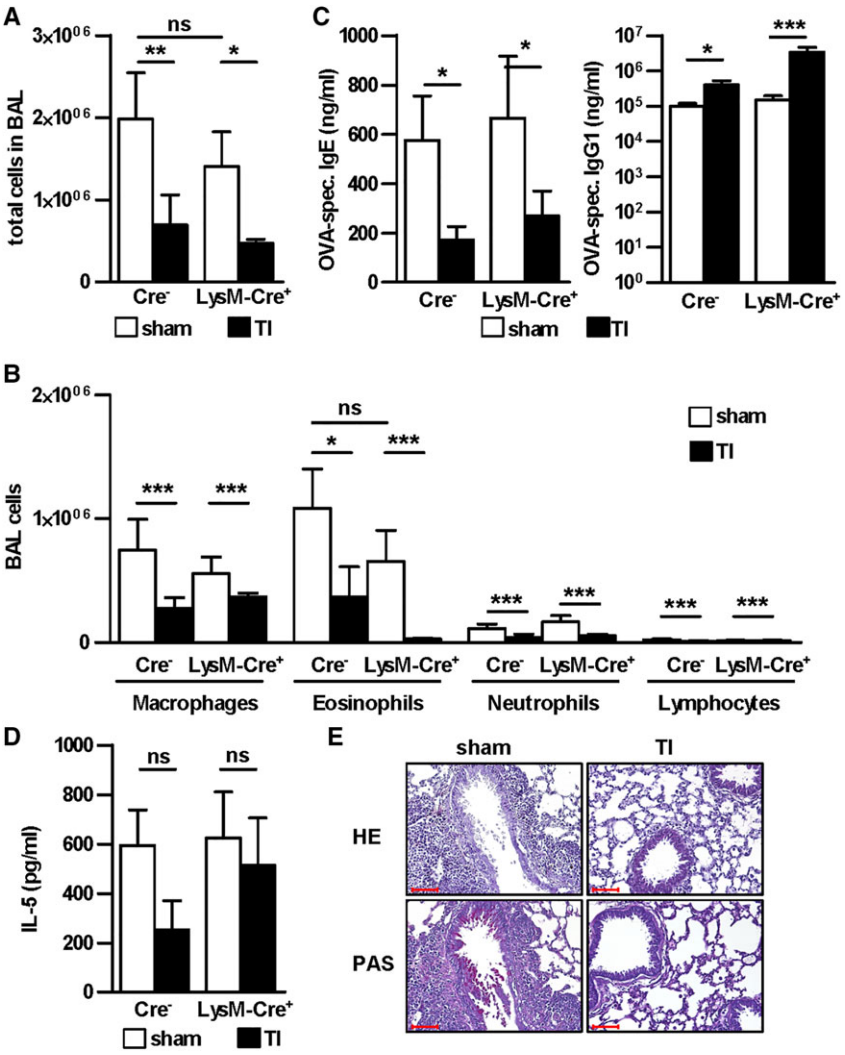

Figure 3. Tolerance induction in mice with IL-10Rα-deficiency in monocytes/macrophages and neutrophils. Sensitized macrophage/monocyte and neutrophil specific IL-10Rα deficient mice (IL-10R$^{FL/FL}$LysM-Cre^{+}) and littermate controls (Cre^{-}) were sham treated (sham) or tolerized (TI) by s.c. injections, allergen challenged by aerosol treatment, and analyzed 24 h after the last challenge as described in *Materials and methods*. (A) Total cell number in BAL fluid. (B) Immune cells in BAL fluid were counted on Diff-Quick stained cytospins. (C) Serum levels of allergen-specific IgE and IgG1 were measured by ELISA. (D) IL-5 release by allergen-stimulated lung draining LN cells was measured by ELISA. (E) Representative H&E and PAS-stained lung sections of IL-10R$^{FL/FL}$LysM-Cre^{+} mice (20×, scale bar = 100 μm). Images shown are representative of two independent experiments. (A–D) Data are shown as mean +SEM (n = 5–6 mice/group per experiment) and are pooled from two independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ Mann–Whitney U test

upon in vitro allergen stimulation was decreased in tolerized control mice, while tolerization had an only minor and insignificant effect on the IL-5 release of cells from IL-10R$^{FL/FL}$CD11c-Cre^{+} mice (Fig. 4D). In contrast to Cre^{-} littermate controls, tolerance induction in IL-10R$^{FL/FL}$ CD11c-Cre^{+} mice did not reduce the inflammatory cell infiltrate around blood vessels nor the goblet cell hyperplasia as assessed by semi-quantitative scoring of lung sections (Fig. 4E and H). Finally, the number of eosinophils and neutrophils in lung sections was clearly reduced in tolerized Cre^{-}, while tolerization in IL-10R$^{FL/FL}$CD11c-Cre^{+} failed to reduce eosinophil numbers and only slightly reduced neutrophil numbers (Fig. 4F and G).

These results from IL-10R$^{FL/FL}$CD11c-Cre^{+} corresponded well with data obtained in mice with IL-10R deficiency in all hematopoietic cells due to cre expression under the control of the vav promoter (IL-10R$^{FL/FL}$Vav-Cre^{+}). The functional lack of IL-10R in different leukocyte populations was documented by failure to induce STAT3 phosphorylation upon in vitro IL-10 stimulation (Supporting Information Fig. S4) [9]. In IL-10R$^{FL/FL}$Vav-Cre^{+} mice tolerance induction failed to ameliorate the allergic airway inflammation as documented by a prominent cellular influx to lung and bronchoalveolar space (Fig. 5A and E). This was again dominated by neutrophils and macrophages (Fig. 5B). Tolerogenic treatment failed to reduce the Th2 cytokine release of lung draining LN cells upon in vitro allergen stimulation (Fig. 5D). Again, serologic parameters did not differ from Cre^{-} littermate control mice (Fig. 5C). The similarity between the data obtained in IL-10R$^{FL/FL}$CD11c-Cre^{+} mice and in mice with an IL-10R deficiency in all hematopoietic cells may indicate that DCs are the main cellular target of IL-10 during allergen specific tolerance induction in mice.

Discussion

IL-10 plays a substantial role during the development of tolerance in allergic patients that undergo AIT [1]. In the current study, we addressed potential target cells of IL-10 in a mouse model of allergen specific tolerance induction. IL-10 dependency of the experimental system was confirmed previously, as tolerance induction failed in mice in which IL-10 signaling had been neutralized with an IL-10R blocking antibody [5, 6]. Failure in mice with an IL-10R-deficient hematopoietic system suggested that cells of the hematopoietic lineage were the primary targets of IL-10.

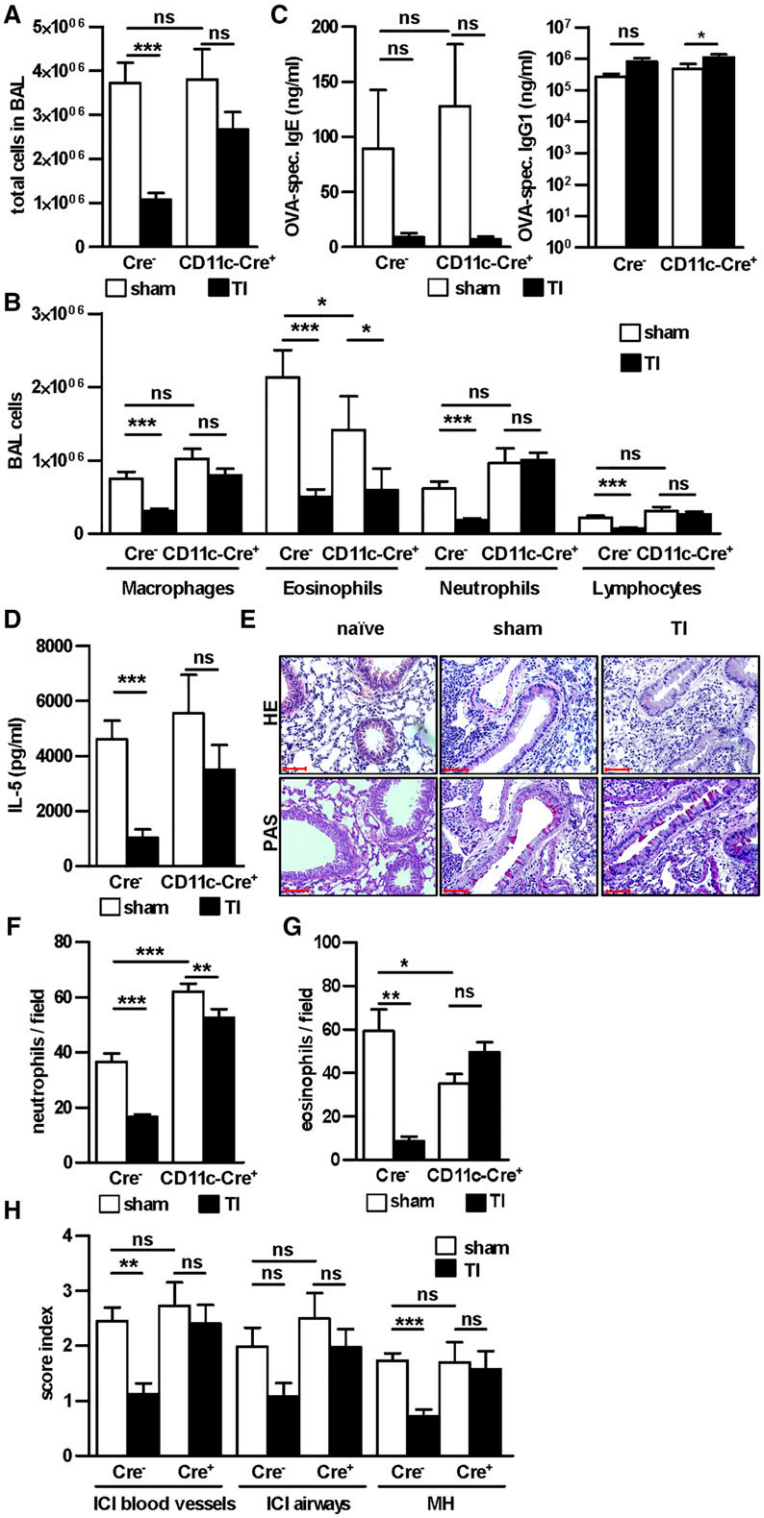

Figure 4. Allergic airway inflammation and tolerance induction in the absence of IL-10R signaling in DCs. Sensitized DC specific IL-10Rα deficient mice (IL-10R$^{FL/FL}$CD11c-Cre^{+}) and littermate controls (Cre^{-}) were sham treated (sham) or tolerized (TI) by s.c. injections, allergen challenged by aerosol treatment, and analysed 24 h after the last allergen challenge as described in *Materials and methods*. (A) Total cell numbers in BAL fluid. (B) Immune cells in BAL fluid were counted on Diff-Quick stained cytospins. (C) Serum levels of allergen-specific IgE and IgG1 were measured by ELISA. (D) IL-5 release by allergen-stimulated lung draining LN cells was measured by ELISA. (E) Representative H&E and PAS-stained lung sections of IL-10R$^{FL/FL}$CD11c-Cre^{+} mice (20×, scale bar = 100 μm). Images shown are representative of two independent experiments. (F) Quantification of neutrophils in lung sections. (G) Quantification of eosinophils in lung sections. (H) Evaluation of lung histology; ICI, inflammatory cell infiltrate; MH, mucus hypersecretion. (A–D, F–H) Data are shown as mean + SEM (n = 4–6 mice/group per experiment) and are pooled from (A–D) four or (F and H) two independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ Mann–Whitney U test

The use of cell-type specific IL-10Rα deficient mice allowed us to exclude a crucial, direct effect of IL-10 on cells of the adaptive immune system as well as on monocytes/macrophages and/or neutrophils in this model. In contrast, loss of IL-10 signaling in DCs resulted in an allergic lung inflammation with a pronounced neutrophilic infiltration that was resistant to tolerance induction. Our data suggests that IL-10 acts on DCs and that IL-10 signaling in DCs is essential for efficient tolerance induction in this murine model of allergic airway inflammation.

For allergen specific tolerance induction, we initially assumed a direct effect of IL-10 on T cells since human studies had suggested that AIT induces anergy in T cells primarily through IL-10

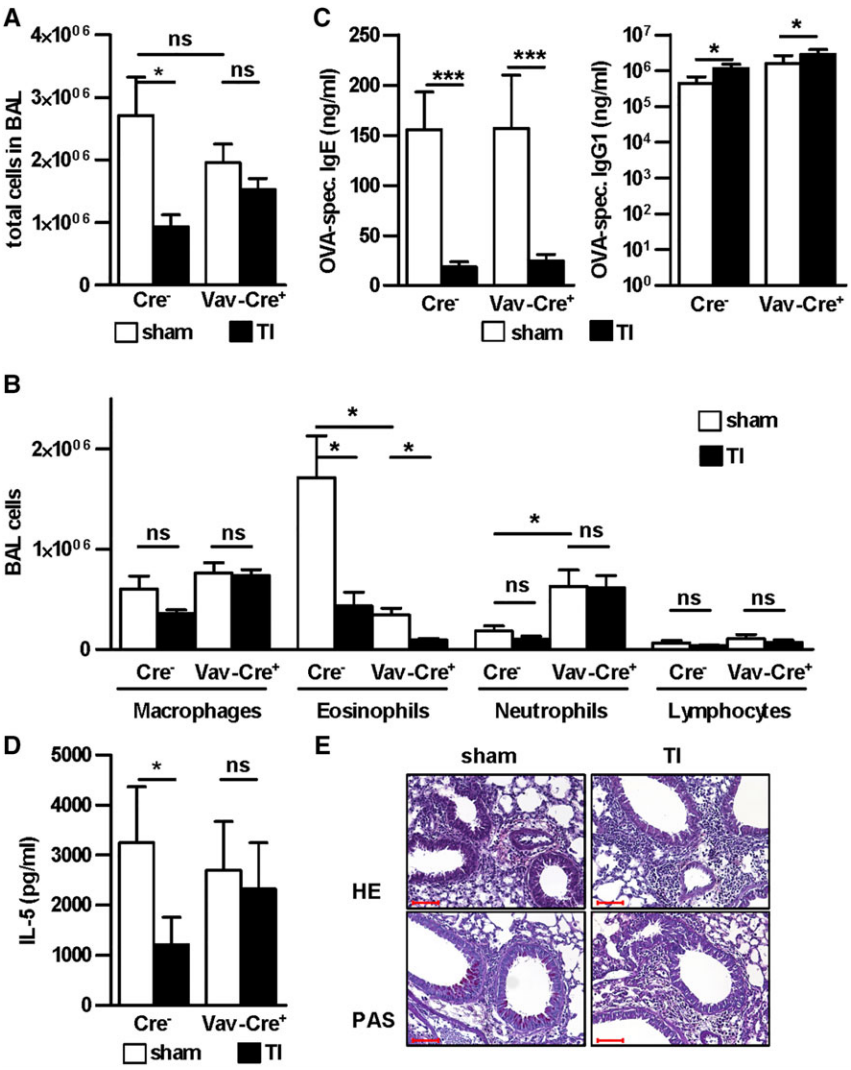

Figure 5. Allergic airway inflammation and tolerance induction in the absence of IL-10R signaling in hematopoietic cells. Sensitized mice with an IL-10Rα deficiency in all hematopoietic cells (IL-10R$^{FL/FL}$Vav-Cre^{+}) and littermate controls (Cre^{-}) were sham treated (sham) or tolerized (TI) by s.c. injections, allergen challenged by aerosol treatment and analyzed 24 h after the last challenge as described in *Materials and methods*. (A) Total cell numbers in BAL fluid. (B) Immune cells in BAL fluid were counted on Diff-Quick stained cytospins. (C) Serum levels of allergen-specific IgE and IgG1 were measured by ELISA. (D) IL-5 release by allergen-stimulated lung draining LN cells was measured by ELISA. (E) Representative H&E and PAS-stained lung sections of IL-10R$^{FL/FL}$Vav-Cre^{+} mice (20×, scale bar = 100 μm). Images shown are representative of two independent experiments. (A–D) Data are shown as mean + SEM (n = 5–6 mice/group per experiment) and are pooled from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ Mann–Whitney U test

signaling [10, 11]. In addition, T cells responding to IL-10 in an autocrine manner have been attributed to the regulation of lung inflammation in a viral infection model [12] and regulatory T cells have been reported to require IL-10 signaling for suppression of Th17 cell-mediated inflammation [13]. Against our initial hypothesis, we observed allergen-specific tolerance induction to be independent of IL-10 signaling in T cells (Fig. 1). Tolerization of both IL-10R$^{FL/FL}$CD4-Cre^{+} and control mice resulted in a decreased Th2 cytokine response excluding a direct inhibition of T cells by IL-10 in this model. Instead modifications of T cell function due to tolerance induction were more likely to be a consequence of indirect effects of IL-10. In line with this, regulation of Th1 and Th2 cytokine release in mice has been reported to be unaffected by a deficient IL-10R signaling in Foxp3^{+} Treg [13], while the influence of IL-10 on antigen presenting cells has been shown to result in a strong inhibition of T cell cytokine production and proliferation [14, 15]. IL-10 has been shown to suppress costimulatory molecules on DCs and thereby modifies T cell responses [16, 17]. Furthermore, DCs stimulated with IL-10 were demonstrated to support the differentiation of IL-10 producing T cells. This indirect effect of IL-10 on T cells has been suggested to maintain an anti-inflammatory status [2, 18]. Quite recently, Coomes et al. suggested a direct regulatory effect of IL-10 on T cells in allergic airway inflammation [19]. Lack of IL-10 signaling in T cells in IL-10R$^{FL/FL}$CD4-Cre^{+} mice resulted in enhanced house dust mite induced allergic airway inflammation [19], which is in contrast to our findings in which the baseline allergen induced lung inflammation in IL-10R$^{FL/FL}$CD4-Cre^{+} mice was comparable to Cre^{-} littermate controls. Differences in allergen composition (OVA vs. HDM extract), allergen dose, or route of application may account for the differences observed. In particular, endogenous triggers of the innate immune response such as the MD2 homologue Der p 2 may act as auto adjuvant in the HDM extract model, while this is not the case when using the model allergen OVA. In addition, Coomes et al. used a short-term model with a rather mild allergic inflammation, while our model was designed to obtain a severe allergic lung inflammation in order to get a clear phenotype in case of tolerance induction. So differences in model design may also account for the diverging findings. In conclusion, with the given experimental model we could not confirm the in vivo

relevance of IL-10 effects on T cells in tolerance induction but rather demonstrated that TI is also effective in the absence of IL-10R signaling in T cells. Of course, this may be different in the human setting, however, we lack the experimental systems to functionally analyze this in the human in vivo system.

We next addressed the effect of IL-10 on B cells and demonstrated that IL-10 signaling in B cells was dispensable for the establishment of allergen specific tolerance in the analyzed model (Fig. 2). Tolerized mice displayed increased allergen specific IgG_1 and reduced IgE serum titer. This was detectable in all analyzed IL-10R deficient mice including IL-10R$^{FL/FL}$Vav-Cre^{+} mice and was in line with a previous report, demonstrating that the success of allergen immunotherapy in this model was independent of immunoglobulins [20]. In humans it has been reported that IL-10 can inhibit IgE and enhance IgG4 synthesis by IL-4 stimulated PBMC in vitro [21]. As mice do not bear IgG4 isotype and we do not see a reduction of specific IgE nor a change in specific IgG1, we assume that the secretion of specific IgE and IgG1 is independent of direct IL-10 signaling on B cells in our model.

Monocytes/macrophages are regarded as main target cells of IL-10 [7, 22–26], which is in line with the high expression level of IL-10Rα found in macrophages (Supporting Information Fig. S5). Extensive effects of IL-10 on macrophages have been previously described [3] and allergic airway inflammation in mice has been previously reported to be suppressed/regulated by macrophages [27, 28]. In addition, lung-resident tissue macrophages have been suggested to promote airway tolerance by generating Treg [29]. In contrast to this, our data obtained in mice with an IL-10R deficiency in monocytes/macrophages and neutrophils did not suggest that IL-10 signaling in macrophages was necessary for allergen specific tolerance induction (Fig. 3). Differences between previous studies [27–29] and our observations may be related to the fact that cre expressing mice analyzed for this study exhibit the specific IL-10R deficiency during their entire life. This may have led to compensatory mechanisms that may hide the actual contribution of a specific target cell. The use of mice with an inducible cre/lox-system could be helpful to address this issue.

Finally, DCs were addressed as central link between innate and adaptive immunity that initiate either a protective immune response or exert regulatory function [30]. IL-10 has been reported to modify DCs and inhibit their inflammatory cytokine release, maturation [2], and antigen presentation ability [31]. In vitro treatment of DCs with IL-10 has been demonstrated to result in the development of a tolerogenic phenotype and regulatory capacity [32]. This appeared to be in line with our in vivo observation, that IL-10R signaling in DCs was required to achieve tolerance. Similarly, IL-10 signaling in skin DCs has been shown to prevent an exaggerated T cell response during contact hypersensitivity reactions [33] and DCs were one cell type that sensed IL-10 in MCMV infection [34] and DCs were regulated by IL-10 during an anti-*Leishmania* major immune response [35].

Interestingly, mice with IL-10R deficiency in CD11c^{+} DCs displayed a prominent neutrophil infiltration into the lung and bronchoalveolar space that could not be reduced by tolerance induction. IL-10 has been described to inhibit neutrophil recruitment during sepsis [36], in a rat model of arthritis [37] and in cases of plasmacytosis-associated overexpression [38]. In addition, migration of in vitro cultured neutrophils could be inhibited by IL-10, however, only at high concentrations [38]. Interestingly, we did not observe a pronounced neutrophilic inflammation in mice with IL-10R deficiency in neutrophils but instead in DCs, suggesting indirect effects of IL-10 on neutrophil recruitment. Neutrophil-dominated cell influx has been associated with severe and steroid resistant asthma in humans [39] that may be driven by IL-17A/F production [40]. IL-17 and IL-17F in turn has been demonstrated to induce lung structural cells to secrete proinflammatory cytokines and chemokines resulting in neutrophil infiltration [41]. In a mouse model of allergic lung inflammation, IL-17 deficient mice developed a reduced Th2 response with reduced lung inflammation [42]. In addition, mice lacking IL-17R exhibited a declined neutrophil recruitment [43]. Consistent with these observations, high mobility group box 1 protein activated DCs induced neutrophilic inflammation in a murine model of neutrophilic asthma probably via IL-23 mediated Th17 differentiation [44]. Finally, IL-10 signaling in DCs was reported to decrease IL-23p19 mRNA expression [45]. Together these data may indicate a possible indirect effect of IL-10 on neutrophil recruitment via suppressed IL-23 production by DCs resulting in decreased Th17 differentiation and IL-17 secretion. Lack of IL-10 signaling in DCs may thus result in an augmented IL-17 mediated neutrophilic influx. However, preliminary analysis of IL-17 A and F in BAL fluid and supernatants of allergen stimulated lung draining LN cells in our experimental setting did not reveal increased IL-17 levels in IL-10R$^{FL/FL}$CD11c-Cre^{+} mice (data not shown). Currently, we can only speculate about other parameters that may be involved, such as increased production or altered kinetics of neutrophil chemoattractants (e.g., IL-8, IL-18, or CXCL). Further analyses need to be performed to dissect the exact mechanism that leads to neutrophil rich airway inflammation in IL-10R$^{FL/FL}$CD11c-Cre^{+} mice.

In conclusion, our data showed that in a murine model of s.c. tolerance induction in allergic airway inflammation direct targeting of T cells, B cells, and monocytes/macrophages or neutrophils by IL-10 was dispensable to achieve allergen specific tolerance. In contrast, we observed that the loss of IL-10 signaling in DCs resulted in a neutrophil dominated allergic airway inflammation and that tolerance induction failed to abrogate the allergic phenotype. Our data highlight a crucial role of DCs targeted by IL-10 in the establishment of allergen specific tolerance.

Material and methods

Animals

All mice were bred at the Center for Experimental Models and Transgenic Services (CEMT-FR), Medical Center, University of Freiburg and housed under specific-pathogen-free conditions. C57BL/6NCrl mice and the following strains were used in age- and sex-matched groups: breeding pairs of IL-10R$^{FL/FL}$CD4-Cre^{+},

IL-10R$^{FL/FL}$CD19-Cre^{+}, and IL-10R$^{FL/FL}$LysM-Cre^{+} mice [7]. IL-10R$^{FL/FL}$CD11c-Cre^{+} mice were obtained by crossing IL-10R$^{FL/FL}$ to CD11c-Cre^{+} mice [8], provided by B. Reizis, Columbia University Medical Center, NY and IL-10R$^{FL/FL}$Vav-Cre^{+} mice by crossing IL-10R$^{FL/FL}$ mice to Vav-Cre^{+} mice [9] provided by D. Kioussis, Department of Molecular Immunology, National Institute for Medical Research, London, UK. All of the experimental procedures were in accordance with institutional, state, and federal guidelines on animal welfare. The animal experiments were approved by the Regierungspräsidium Freiburg and supervised by the animal protection representatives of the Medical Center, University of Freiburg.

Analysis of cell type specific IL-10R deficiency

Specific deletion of the *IL-10R* gene in FACS-sorted spleen cell populations (FACSAria, BD Biosciences, Heidelberg, Germany) was confirmed by PCR. The following primers result in a PCR product in case of an intact IL-10R gene with a length of 373 bp: forward primer 5'-AGC AGC TGA GGC TTT CTG TG-3' and reverse primer 5'-TCA AGG CGC CTA TTG ACA CT-3'.

Functional IL-10R expression at single cell level was analyzed by using a STAT3 phosphorylation assay. In brief, splenocytes of naïve mice from the various mouse strains were stimulated in vitro with 100 ng/mL IL-10 (Invivogen, Toulouse, France). Dead cells were marked with a fixable viability dye (eBiosciences, San Diego, USA) before all cells were fixed in 4% formaldehyde (ChemSolute), followed by permeabilization with 90% methanol (Sigma–Aldrich, Taufkirchen, Germany). Cells were stained with antibodies against surface lineage markers and pSTAT3 (clone pY705; BD Bioscience), and analyzed by flow cytometry.

Flow cytometry

Extracellular staining was performed according to standard protocols using the following antibodies: CD4-FITC (clone RM4-5; BD Bioscience), B220-PE (RA3-6B2; eBioscience), CD11c-FITC (HL3; BD Bioscience) or CD11c-PECy7 (N418; eBioscience), F4/80-PerCP-Cy5.5 (BM8; BioLegend, San Diego, USA), Ly6G-FITC or Ly6G-PECy7 (1A8; BioLegend), CD45-APC (30-F11; BD Bioscience) or CD45-Brilliant Violet 421™ (30-F11; BioLegend), and CD210-Biotin (1B1.3a; BioLegend) with streptavidin-APC (BD Bioscience) as second step reagent. Dead cells were detected with a fixable viability dye (eFluor® 506; eBiosciences). Antibody binding was detected using a BD FACSCanto flow cytometer (BD Bioscience) and evaluated with FlowJo software Version 9 (TreeStar Inc.). The IL-10Rα expression of different immune cells from lung, lymph nodes, and spleen of naïve mice and the full gating strategy for the pStat3 and the IL-10R staining are shown in Supporting Information Figs. S6 and S7. Panel setup, flow cytometry settings and analysis of the data was performed according to the guidelines for the use of flow cytometry and cell sorting in immunological studies [46].

Induction of allergic airway inflammation, tolerance induction, and bronchoalveolar lavage (BAL)

Sensitization to OVA, tolerance induction, allergen challenge and BAL were performed as described recently [6]. In brief, mice were sensitized to OVA by three, weekly intraperitoneal injections of 10 μg OVA (Grade V, Sigma–Aldrich) adsorbed to 2 mg alum, a suspension of aluminium hydroxide and magnesium hydroxide (Imject alum, Pierce, Rockford, USA). Tolerance induction (TI) was conducted 14 days after last sensitization by subcutaneous injections of OVA (1 mg in PBS) on three alternate days. Two weeks later mice were challenged by 1% OVA aerosol for 20 min three times 72 h apart. Animals were sacrificed and analyzed 24 h after the last challenge.

Cytokine production of lung draining LN cells and OVA-specific IgE and IgG1 detection

Single-cell suspensions of mediastinal lymph nodes were cultured in the absence or presence of OVA (10 μg/mL) as described recently [6]. In brief, IL-5 content in supernatants was analyzed by ELISA according to the manufacturer´s instructions using matched antibody pairs purchased from BioLegend. OVA-specific IgE and IgG1 concentrations were measured in serum samples by ELISA as described previously [6].

Histology

Two micrometer sections of formalin-fixed, paraffin-embedded left lung lobes obtained after BAL were stained with H&E and periodic-acid Schiff (PAS) according to standard protocols as described previously [6]. Sections were evaluated regarding the extent of inflammatory cell infiltrates (ICI) around blood vessels and airways and of mucus hypersecretion (PAS^{+} goblet cells) using a scoring from 0 (none) to 4 (severe) as previously described [47].

Neutrophil specific immunohistochemistry was done after antigen retrieval with proteinase K and blocking of endogenous peroxidase activity with H_2O_2. Neutrophil specific staining was obtained by incubation with the NIMP-R14 antibody [48] followed by a biotinylated goat-anti-rat-IgG secondary antibody (Southern Biotech, Birmingham). Counterstaining was achieved with hematoxylin. Neutrophils were counted on ten visual fields per animal using AxioVision software (Carl Zeiss Microscopy, Jena, Germany). Eosinophil-specific IHC was done after antigen retrieval with pepsin (Abcam, Cambridge, UK) and blocking of endogenous peroxidase activity with dual endogenous block (Agilent Technologies, Santa Clara, CA). Eosinophils were detected using an anti-mouse major basic protein monoclonal antibody (MBP, clone MT-14.7.3; kindly provided by J.J. Lee and Nancy Lee, Mayo Clinic, Arizona, USA) followed by biotinylated rabbit anti-rat IgG secondary antibody (Vector Labs, Burlingame, CA). Counterstaining was achieved with a 0.1% methyl green solution. Eosinophils

were counted on up to 17 fields with 10× magnification using AxioScan.Z1 generated pictures and Zeiss Zen blue Software.

Statistical analysis

Statistical analysis was performed using Mann–Whitney *U* test for nonparametric data (GraphPad Prism, version 5.01). Differences were considered significant when *p*-values were <0.05. Data are presented as mean ± SEM unless otherwise indicated.

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Abbreviations: **AIT**: allergen specific immunotherapy · **alum**: suspension of aluminium hydroxide and magnesium hydroxide · **BAL**: bronchoalveolar lavage · **Cre**: cre recombinase · **ICI**: inflammatory cell infiltrate · **PAS**: Periodic acid-Schiff staining · **TI**: tolerance induction

Full correspondence: Prof. Thilo Jakob, MD, Justus Liebig University Gießen, University Medical Center Gießen-Marburg, Department of Dermatology and Allergology, Gaffkystr. 14, 35385 Gießen
fax: +49-641-985-43209
e-mail: thilo.jakob@derma.med.uni-giessen.de

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