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Research Article

T cell derived IL-10 is dispensable for tolerance induction in a murine model of allergic airway inflammation

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Regulatory mechanisms initiated by allergen-specific immunotherapy are mainly attributed to T cell derived IL-10. However, it has not been shown that T cell derived IL-10 is required for successful tolerance induction (TI). Here, we analyze cellular sources and the functional relevance of cell type specific IL-10 during TI in a murine model of allergic airway inflammation. While TI was effective in IL-10 competent mice, neutralizing IL-10 prior to tolerogenic treatment completely abrogated the beneficial effects. Cellular sources of IL-10 during TI were identified by using transcriptional reporter mice as T cells, B cells, and to a lesser extent DCs. Interestingly, TI was still effective in mice with T cell, B cell, B and T cell, or DC-specific IL-10 deficiency. In contrast, TI was not possible in mice lacking IL-10 in all hematopoetic cells, while it was effective in bone marrow (BM) chimera that lacked IL-10 only in nonhematopoetic cells. Taken together, allergen-specific tolerance depends on IL-10 from hematopoetic sources. The beneficial effects of allergen-specific immunotherapy cannot solely be attributed to IL-10 from T cells, B cells, or even DCs, suggesting a high degree of cellular redundancy in IL-10-mediated tolerance.

Keywords: Allergy · IL-10 · Immunotherapy · T cells · Tolerance

Additional supporting information may be found in the online version of this article at the publisher's web-site

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Introduction

Adequate immune tolerance to harmless antigens is essential for immune homeostasis. Lack of tolerance and the ensuing inflammatory response to harmless environmental antigens is the hallmark of immediate type allergies such as allergic asthma [1]. In allergic individuals, tolerance can at least partly be reestablished through repeated administration of the relevant allergen, a procedure designated allergen-specific immunotherapy (AIT) [2]. AIT is well established for the treatment of immediate type allergies and the underlying mechanisms have been extensively studied (reviewed in [3]). Many of the observed immune alterations during this process of tolerance induction (TI) have been attributed to IL-10 [4], a cytokine well known for its anti-inflammatory and immune regulatory capacity [5]. The relevance of IL-10 in AIT is supported by several studies that show an induction of IL-10-producing cells upon exposure to high allergen doses, an effect that is especially seen in different Treg subsets [6–10]. Additionally, increased numbers of IL-10-producing B cells and monocytes in the peripheral blood of patients receiving bee venom AIT [6, 11], as well as increased IL-10 signals co-localizing to mucosal macrophages in patients receiving pollen AIT [8] have been reported.

Functional evidence for a role of IL-10 in AIT comes from a murine model of high dose TI, in which subcutaneous injections of ovalbumin (OVA) ameliorated the allergen-induced airway inflammation in an IL-10-dependent manner [12]. More recent studies in mice suggest that T cells are essential for peripheral TI in the context of AIT, however, results vary in terms of the respective T-cell subtype [13–15] and the experimental model [16]. The formal proof that T cell derived IL-10 is required for an effective AIT is still lacking.

Aside from T cells, other cellular sources of IL-10 have been implied in TI. IL-10-producing regulatory B cells have been described in allergy [17], autoimmune diseases, helminth infection [18], and pregnancy [19]. DC-derived IL-10 has been implied in long-lasting allergen-specific tolerance [20]; IL-10 from myeloid DCs, macrophages, and nonhematopoetic sinusoidal endothelial cells appeared to contribute to tolerance in the liver [21] and mast cell-derived IL-10 has been suggested to drive local tolerance in chronic bladder infection [22]. In conclusion, IL-10 can be produced by a wide range of cell types from hematopoetic as well as nonhematopoetic origin [5], many of which have been suggested to be involved in generating or maintaining tolerance in different experimental systems.

In the present study, we utilize IL-10 transcriptional reporter mice to monitor the cellular origin of IL-10 during TI in a mouse model of allergic airway inflammation. In addition, we analyze the functional relevance of cell type specific IL-10 during TI using cell type specific IL-10-deficient mice. We confirm that IL-10 is essential for successful TI but challenge the concept of T cell derived IL-10 as the critical cellular source: although allergen-specific TI depends on IL-10 from hematopoetic sources, T cell specific IL-10 deficiency does neither prevent TI, nor does B cell or DC-specific IL-10 deficiency.

Results

Allergen-specific TI is IL-10 dependent

As published previously for BALB/c mice [23], subcutaneous high-dose allergen injection efficiently induced tolerance to subsequent allergen aerosol challenge also in C57BL/6 WT mice (for protocol see Supporting Information Fig. 1). Treatment with IL-10-receptor blocking antibody once before the first subcutaneous allergen application abrogated the beneficial effects of TI as shown by a prominent lung inflammation, increased allergen-specific IgE, and enhanced Th2 cytokine responses of draining LN cells upon allergen challenge (Supporting Information Fig. 2A–D). Similarly, TI did not reduce allergen-induced inflammatory infiltration of the bronchoalveolar space in IL-10 knockout mice (Supporting Information Fig. 2E). In conclusion, allergen-specific TI was highly effective in C57BL/6 mice and the beneficial effect depends on IL-10.

IL-10 expression during TI measurably increases in T and B cells

Use of IL-10 transcriptional reporter mice (Vert-X) [24] enabled us to address cellular sources, kinetics, and localization of *in vivo* IL-10 transcription during TI. Vert-X mice were sensitized and subjected to s.c. immunotherapy. Green fluorescent protein (GFP) expression that served as IL-10 transcriptional reporter was analyzed by flow cytometry.

IL-10 reporter expression was quantified in all viable cells of different organs at different time points after initiation of immunotherapy. S.c. allergen injections increased the percentage of cells with IL-10 transcription as well as the IL-10 expression level in skin draining axillary LNs and lung draining mediastinal LNs, which was most evident 132 h after initiation of therapy, that is, 36 h after final injection (Fig. 1A). No significant induction of IL-10 signal was observed in spleen, peripheral blood, and bone marrow (BM) as compared to the sham injected control (Fig. 1A).

GFP expression of defined cell types during TI was analyzed at the time point of maximal GFP expression. Compared to sham injected controls, TI induced increased IL-10 signals in T and B cells of skin draining (Fig. 1 B and C) and mediastinal LNs (Supporting Information Fig. 4). Within the T cell compartment, the highest percentages and expression level (MFI) of the IL-10 signal was observed in $CD4^+CD25^+$ T cells, which was increased even further upon TI. Similarly, TI also increased IL-10 signals in $CD4^+CD25^-$ and $CD8^+$ T cells as well as in $CD19^+$ B cells, however at a lower expression level (Fig. 1B and C). Finally, comparable signals for IL-10 transcription were also detected in DCs ($CD11b^+CD11c^{high}$ cells; Fig. 1B and C), which however, were not significantly influenced by TI. GFP expression of NK cells, neutrophils, macrophages, and mast cells was at the detection limit at baseline and unaltered upon TI (data not shown).

IL-10 reporter expression was also analyzed during the allergen aerosol challenge in tolerized mice and sham injected

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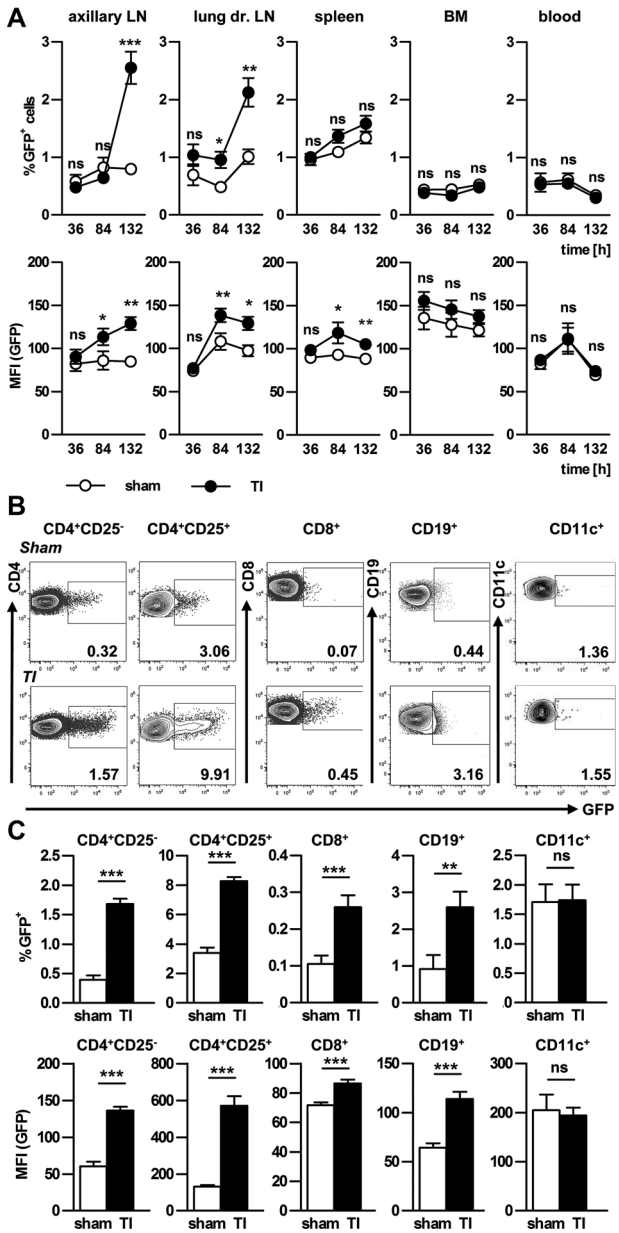

Figure 1. IL-10 reporter expression (GFP) in different organs and cell types upon initiation of tolerance. Sensitized transcriptional IL-10 reporter mice (Vert-X) were either sham treated (sham) or tolerized (TI) by subcutaneous allergen injections as described in Section "Materials and methods". (A) LNs, spleen, BM, and blood were analyzed at different time points upon the first subcutaneous allergen injection by flow cytometry for GFP expression in all viable cells of the respective organs. (B and C) Different cell populations of axillary LNs were analyzed 132 h after initiation of therapy, that is, 36 h after final injection by flow cytometry for reporter expression (GFP). (B) Representative dot plots upon sham treatment (upper panel) and TI (lower panel). (C) Percent GFP+ cells per indicated cell type (upper panel) and mean fluorescence intensity (lower panel). (A and C) Data are shown as mean ± SEM (n = 3–6 mice/group and experiment) and are pooled from four independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns: not significant Mann–Whitney U-test. (B) Plots are representative of four independent experiments.

controls comparing baseline GFP expression in the lung prior to first aerosol treatment (Supporting Information Fig. 5A) as well as GFP expression of bronchoalveolar lavage (BAL) cells after aerosol inhalation (Supporting Information Fig. 5C). However, no significantly different GFP expression was observed between tolerized and nontolerized mice during the elicitation phase of allergic airway inflammation (Supporting Information Fig. 5) as baseline GFP expression did not differ and increased comparably in lung T and B cells of both groups after allergen challenge. However, tolerized mice displayed a slightly increased percentage of $CD8^+$ T cells within the BAL cellular infiltrate (Supporting Information Fig. 5B).

In conclusion, induction of tolerance by s.c. allergen injections was accompanied by a significant increase in percentage and expression level of IL-10 transcribing T and B cells in skin draining and mediastinal LNs during the treatment phase.

IL-10 deficiency of the adaptive immune system does not prevent effective TI

The prominent IL-10 expression in T cells during TI prompted us to analyze the functional role of T cell derived IL-10 during this process using mice with a T cell specific deficiency of IL-10 generated by Cre-mediated recombination (IL-$10^{FL/FL}$CD4-Cre^+) [25]. T cell specific deletion of the IL-10 gene was confirmed by Southern blot (Supporting Information Fig. 6A). Upon sensitization and challenge, IL-$10^{FL/FL}$CD4-Cre^+ mice developed an allergic lung inflammation that was comparable to that of their Cre^- littermate controls, with the exception of a more prominent neutrophil and less prominent eosinophil infiltration in the BAL (Fig. 2A). Also allergen-specific IgE and IgG1 responses in serum (Fig. 2B) and Th2 cytokine responses of draining LN cells upon restimulation were comparable (Fig. 2C). Interestingly, induction of tolerance was equally effective in IL-$10^{FL/FL}$CD4-Cre^+ as documented by a reduced BAL cell infiltrate (Fig. 2A) and mucin staining in lung sections (Fig. 2D), reduced allergen-specific IgE, increased allergen-specific IgG1 (Fig. 2B), and a dampened Th2 cytokine response of draining LN cells upon allergen stimulation in vitro (Fig. 2C). These data suggested that T cell derived IL-10 is dispensable for successful allergen-specific TI in the present experimental setting.

The observed induction of IL-10 transcription in B cells during TI and previous reports on B cell derived IL-10 in the regulation of adaptive immune responses [26] prompted us to analyze the functional role of B cell derived IL-10 in our model. For this, we used mice with a B cell specific IL-10 deficiency (IL-$10^{FL/FL}$CD19-Cre^+) and confirmed the specific deletion by Southern blot (Supporting Information Fig. 6B). Upon sensitization and challenge IL-$10^{FL/FL}$CD19-Cre^+ mice developed an allergic lung inflammation that did not differ obviously from that of their Cre^- littermate controls (Fig. 2E). Also allergen-specific IgG1 responses (Fig. 2F) and Th2 cytokine responses of draining LN cells were comparable (Fig. 2G). In contrast, the allergen specific IgE response was increased in the absence of B cell derived IL-10. Again,

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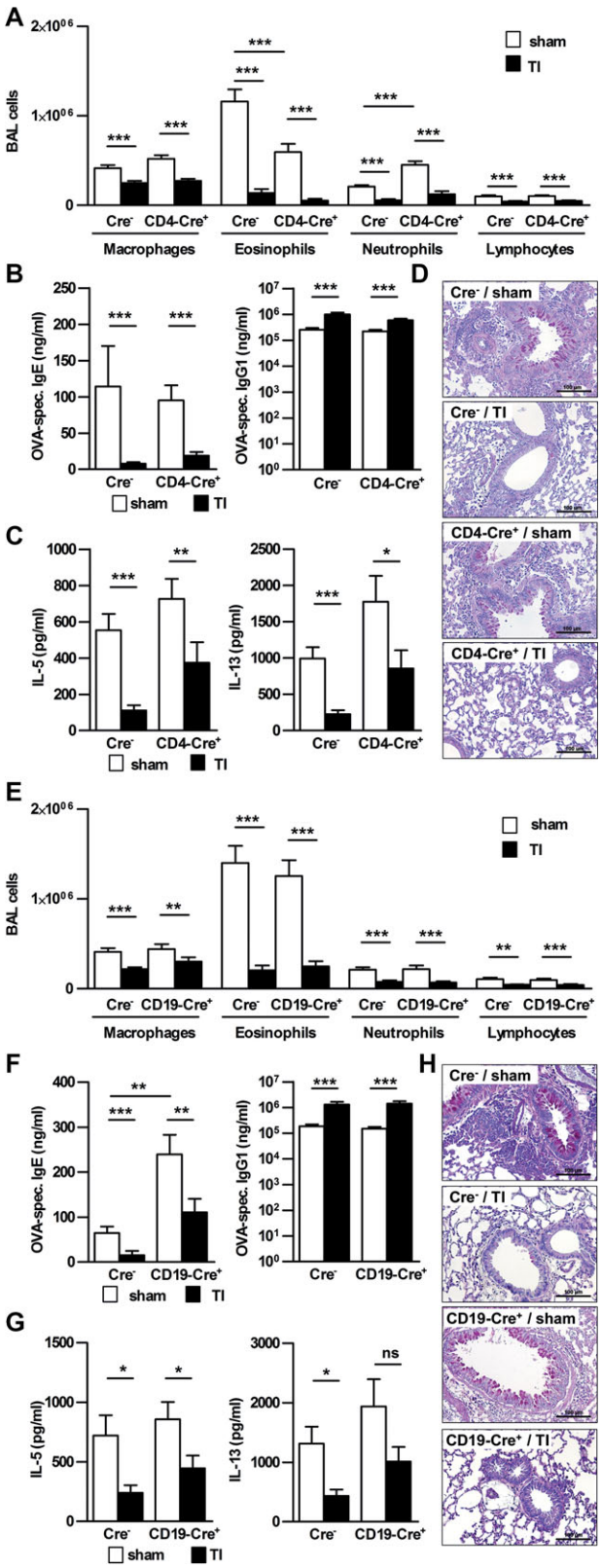

induction of tolerance was equally effective in IL-10$^{FL/FL}$CD19-Cre^{+} as documented by a reduced BAL cell infiltrate (Fig. 2E), reduced allergen-specific IgE, increased allergen-specific IgG1 (Fig. 2F), and a dampened Th2 cytokine response of draining LN cells upon allergen restimulation (Fig. 2G). This data suggested that also B cell derived IL-10 is dispensable for successful allergen-specific TI in mice.

Since it was conceivable that IL-10 deficiency of one cell type could be compensated by the IL-10 production of the other, we next generated mice with an IL-10 deficiency in both T and B cells (IL-10$^{FL/FL}$CD4-Cre^{+}CD19-Cre^{+}), in which the deletion of the IL-10 gene in both cell types was verified by Southern blot (Supporting Information Fig. 6C). However, even in the absence of B and T cell derived IL-10 induction of tolerance finally led to a drop in BAL cell numbers (Supporting Information Fig. 7A), an increase in allergen-specific IgG1 (Supporting Information Fig. 7B) and a declined Th2 response of local LN cells upon restimulation (Supporting Information Fig. 7C), while allergen-specific IgE was not reduced by TI (Supporting Information Fig. 7B). Thus, despite the lack of both T cell as well as B cell derived IL-10, induction of tolerance was still possible in regard to the majority of parameters analyzed. In conclusion, these data suggest that IL-10 derived from cells of the adaptive immune system is no requirement for successful allergen-specific TI in mice.

Absence of DC-derived IL-10 does not prevent effective TI

The role of IL-10 derived from cells of the innate immune system was investigated by using DC-specific IL-10 deficient mice (IL-10$^{FL/FL}$CD11c-Cre^{+}). Deletion of the IL-10 gene in DCs was confirmed by Southern blot. As reported previously [27], the expression of Cre recombinase in CD11c-Cre^{+} mice is not restricted to CD11c^{+} DC populations, but may also affect significant portions of other leukocyte populations such as B cells. In line with this, we observed the deleted as well as the undeleted IL-10 allele in B cells (Supporting Information Fig. 6D). In IL-10$^{FL/FL}$CD11c-Cre^{+} mice, allergen-induced airway inflammation, specific IgE and IgG1 responses, as well as cytokine responses of draining LN cells were comparable to that observed in Cre^{-} littermate controls (Fig. 3A–C). Induction

Figure 2. TI in mice with T or B cell specific IL-10 deficiency. (A–H) Sensitized (A–D) T cell specific (IL-10$^{FL/FL}$CD4-Cre^{+}) or (E–H) B cell specific IL-10 deficient mice (IL-10$^{FL/FL}$CD19-Cre^{+}) and corresponding control litter mates (IL-10$^{FL/FL}$Cre–) were sham treated (sham) or tolerized (TI) by subcutaneous injections, allergen challenged by aerosol treatment and analyzed 24 h later as described in the Section "Materials and methods". (A and E) Immune cells in BAL fluid were counted on Diff-Quick stained cytospins. (B and F) Serum levels of allergen-specific IgE and IgG1 were measured by ELISA. (C and G) IL-5 and IL-13 release by allergen-stimulated LN cells was measured by ELISA. (D and H) Representative periodic-acid Schiff stained lung sections. Images shown are representative of two independent experiments. (A–C and E–G) Data are shown as mean + SEM ($n = 3$–10 mice/group and experiment) and are pooled from (A–C) nine or (E–G) six independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; Mann–Whitney U-test.

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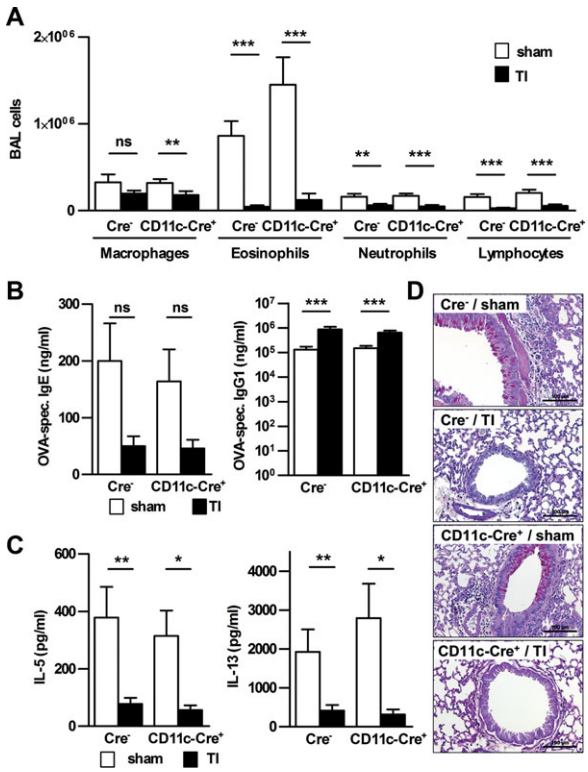

Figure 3. TI in mice with DC-specific IL-10 deficiency. Sensitized DC-specific IL-10 deficient mice (IL-10$^{FL/FL}$CD11c-Cre^{+}) and control mice (IL-10$^{FL/FL}$Cre^{-}) were sham treated (sham) or tolerized (TI) by subcutaneous injections, allergen challenged by aerosol treatment and analyzed 24 h later as described in Section "Materials and methods". (A) Immune cells in BAL fluid were counted on Diff-Quick stained cytospins. (B) Serum levels of allergen-specific IgE and IgG1 were measured by ELISA. (C) IL-5 and IL-13 release by allergen-stimulated LN cells was measured by ELISA. (D) Representative periodic-acid Schiff stained lung sections. Images shown are representative of two independent experiments. (A–C) Data are shown as mean + SEM ($n = 6$–7 mice/group and experiment) and are pooled from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns: not significant; Mann–Whitney *U*-test.

of tolerance in IL-10$^{FL/FL}$CD11c-Cre^{+} mice was as effective as in Cre^{-} littermate controls, as demonstrated by a reduction of BAL cell infiltrate (Fig. 3A), drop in total (Supporting Information Fig. 8) and allergen-specific IgE, and increase in sIgG1 (Fig. 3B) as well as reduction of Th2 cytokine responses in draining LNs (Fig. 3C). In conclusion, successful TI was possible also in the absence of DC-derived IL-10. Similar results were obtained for mice with a macrophage/neutrophil (IL-10$^{FL/FL}$LysM-Cre^{+} [28]) or mast cell specific deletion of the IL-10 gene (IL-10$^{FL/FL}$Mcpt5-Cre^{+} [29]; Supporting Information Fig. 9).

IL-10 from hematopoetic, but not nonhematopoetic, cells is required for TI

IL-10 production has also been attributed to nonhematopoetic cells such as keratinocytes and epithelial cells [5]. The influence of IL-10 derived from nonhematopoetic cells was analyzed using BM chimeras, in which transfer of WT donor cells into IL-10$^{-/-}$ recipients (B6→IL-10ko) allowed the generation of mice that lacked IL-10 in nonhematopoetic cells. Transfer of WT BM into WT recipients (B6→B6) served as controls. Despite the lack of IL-10 from nonhematopoetic sources, the allergen-induced airway inflammation and immune responses did not differ remarkably from control mice (Fig. 4). TI was effective in both control mice (B6→B6) and mice with a deficiency of IL-10 in nonhematopoetic cells (B6→IL-10ko) as indicated by reduced cellular influx into the bronchoalveolar space (Fig. 4A), diminished allergen-specific IgE (Fig. 4B), and reduced LN cytokine response upon in vitro restimulation (Fig. 4C).

Finally, we analyzed whether IL-10 production of cells of hematopoetic origin contributes to successful TI using IL-10$^{FL/FL}$Vav-Cre^{+} mice, in which all hematopoetic cells are deficient in IL-10 [30]. At baseline, IL-10$^{FL/FL}$Vav-Cre^{+} mice did not reveal any significant differences in T cell (CD4^{+}, CD8^{+}, and CD4^{+}CD25^{+}), B cell, or DC numbers in blood, axillary lymph nodes (LNs), spleen and lung tissue compared to IL-10$^{wt/FL}$Vav-Cre^{+} control mice (Supporting Information Fig. 10). While the magnitude of the allergen-induced airway inflammation in IL-10$^{FL/FL}$Vav-Cre^{+} mice was comparable to Cre^{-} control mice (Fig. 4D), the inflammation was dominated by infiltration of neutrophils (Fig. 4E). In contrast to Cre^{-} controls, induction of tolerance was not effective in IL-10$^{FL/FL}$Vav-Cre^{+} mice, as documented by a persisting airway inflammation dominated by neutrophils (Fig. 4D and E). Correspondingly, TI in IL-10$^{FL/FL}$Vav-Cre^{+} mice failed to reduce the Th2 cytokine production of draining LN cells upon allergen restimulation (Fig. 4G), while the amount of OVA-specific IgE was reduced upon TI in both groups (Fig. 4F) suggesting that humoral and cellular responses were regulated by independent pathways. In conclusion, the majority of parameters analyzed suggests that IL-10 from hematopoietic cells is required for successful TI in allergen-induced airway inflammation.

Discussion

In the present study, we used a murine model of high dose tolerance in allergen induced airway inflammation to investigate the functional role and cellular source of IL-10 during TI. Our data confirm the previously reported functional involvement of IL-10 in TI, however, challenge the concept of T cells as the crucial source of IL-10 responsible for immune tolerance.

IL-10 reporter mice (Vert-X mice) [24] allowed us to monitor *in vivo* IL-10 transcription during different phases of allergen-specific TI. IL-10 protein detection *ex vivo* is challenging [31], but IL-10 transcription has been demonstrated to be closely linked to IL-10 protein production in these mice [24]. Still the reporter mice only provide a surrogate signal for *in vivo* protein production. Induction of tolerance obtained by repeated subcutaneous injections of OVA was accompanied by significantly increased IL-10 transcription in skin draining and mediastinal LN cells but not in peripheral blood, BM, or spleen (Fig. 1). The prominent signal in mediastinal LN cells is most likely due to sensitization

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route via the peritoneum, which in mice predominantly drains to the mediastinal LNs [32]. At baseline, the strongest signal for IL-10 transcription was detected in $CD4^+CD25^+$ T cells, which is in line with their regulatory function. Signals for IL-10 were also detectable in $CD4^+CD25^-$ T cells, $CD8^+$ T cells, B cells, and DCs, albeit to a lesser extent. Initiation of tolerance increased IL-10 expression in both $CD25^+$ and $CD25^-CD4^+$ cells, as well as in $CD8^+$ T cells and $CD19^+$ B cells but not in $CD11c^+$ DCs. When analyzing the elicitation phase of allergic airway inflammation in lung and BAL no prominent differences between tolerized and sham-treated mice were detected (Supporting Information Fig. 5). The observations that IL-10 transcription was selectively induced primarily in T and B cells during the tolerization process (Fig. 1) and that blocking IL-10 prior to TI effectively abrogated the beneficial effects (Supporting Information Fig. 2), both seemed to support the concept that T cell or B cell derived IL-10 or both may play a functional role in establishing tolerance. This concept was in line with previous studies that describe the induction of Tregs and IL-10-producing $CD4^+$ T cells following immunotherapy [14], [15], [33] and with adoptive transfer experiments, in which allergen-specific $CD4^+CD25^+$ T cells [14, 34] or $CD4^+$ T cells engineered to produce IL-10 [35] were demonstrated to suppress allergic airway inflammation. Interestingly, successful suppression of allergic airway inflammation was also possible by transfer of allergen-specific $CD4^+CD25^+$ T cells from IL-10-deficient donor mice, suggesting that $CD4^+CD25^+$ regulatory cells can suppress the allergen-induced inflammation *in vivo* but that IL-10 production by Tregs themselves is not required [36]. More recently it has been suggested that the effectiveness of immunotherapy most probably relies on the induction of IL-10 in $Foxp3^-$ T cells by $Foxp3^+$ Tregs from thymic origin [13]. These studies seemed to suggest a close link between the effectiveness of allergen-specific immunotherapy and T cell derived IL-10, however, the functional evidence provided in these studies was somewhat circumstantial.

To address this issue in more detail, we therefore made use of cell type specific IL-10-deficient mice generated by Cre/loxP-mediated deletion. T cell specific IL-10 deficient mice have been reported to display increased T cell mediated immune responses in a murine contact hypersensitivity model, while responses to innate stimuli such as LPS or the skin irritant croton oil appeared to be normal [25]. Subsequent studies demonstrated that selective ablation of IL-10 in T cells resulted in an improved viral clearance in viral infection models [37, 38] and a reduced disease control in Leishmania infection [39]. The selective lack of IL-10 in T cells was associated with spontaneous development of colitis [25], similar to that observed in IL-$10^{-/-}$ mice. Similarly, selective ablation

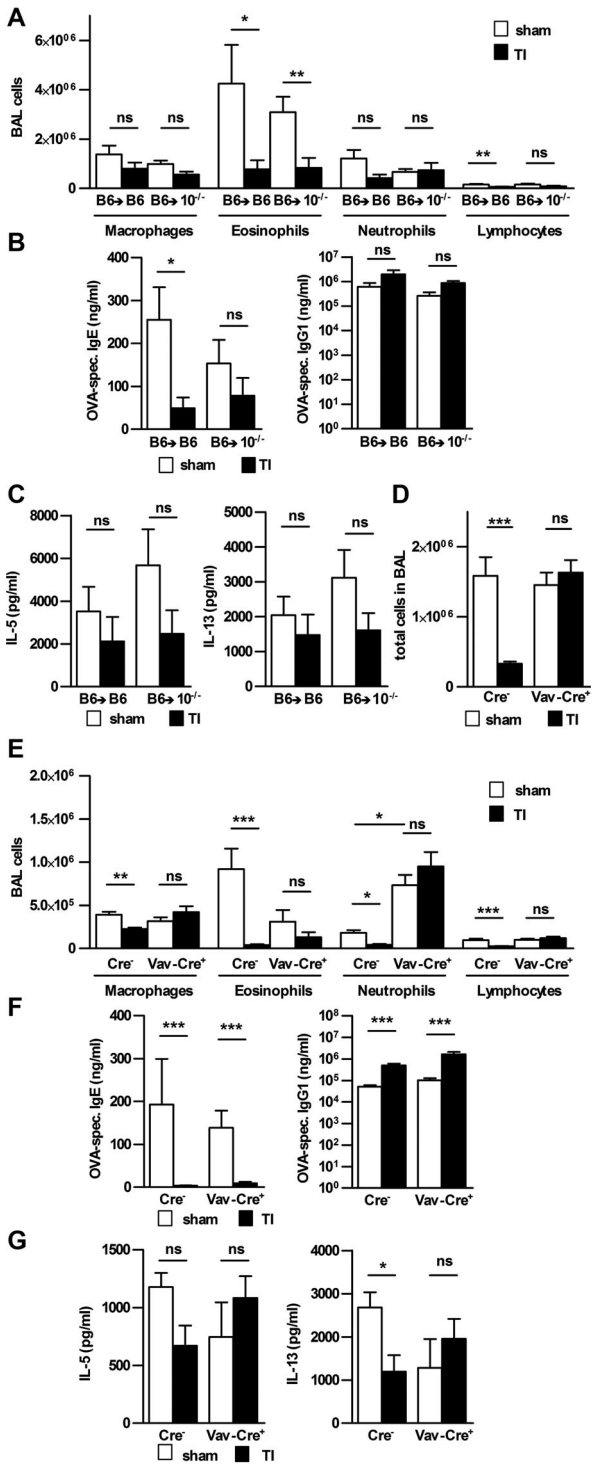

Figure 4. TI in mice with IL-10 deficiency in either nonhematopoetic or hematopoetic cells. (A–C) IL-10ko mice were irradiated and substituted with C57BL/6 WT bone marrow (B6→$10^{-/-}$; IL-10 deficiency in nonhematopoetic cells) as described in Section "Materials and methods". In parallel, substituted WT mice (B6→B6) served as control. (D–G) Mice with an IL-10-deficient hematopoetic system (IL-$10^{FL/FL}$Vav-Cre^+) and control mice (IL-$10^{FL/FL}Cre^-$). (A–G) All mice were sensitized, sham treated (sham), or tolerized (TI) by subcutaneous injections, allergen challenged by aerosol treatment and analyzed 24 h later as described in Section "Materials and methods". (A and E) Immune cells in BAL fluid were counted on Diff-Quick-stained cytospins. (D) Enumeration of total cells in BAL fluid. (B and F) Serum levels of allergen-specific IgE and IgG1 were measured by ELISA. (C G) IL-5 and IL-13 release by allergen-stimulated LN cells were measured by ELISA. (A–G) Data are shown as mean +SEM ($n = 4$–6 mice/group and experiment) and are pooled from (A–C) two or (D-G) four independent experiments. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns: not significant; Mann–Whitney *U*-test.

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of IL-10 in Foxp3$^+$ Tregs was reported to lead to spontaneous colitis and to an increased inflammatory response in a model of allergen-induced inflammation of the lung [40].

Based on these reports and the IL-10 expression pattern analyzed in IL-10 reporter mice, we expected to see an augmented allergen-induced airway inflammation in IL-10$^{FL/FL}$CD4-Cre$^+$ mice. To our surprise, the overall response to allergen challenge was comparable to that obtained in Cre$^-$ littermate controls (Fig. 2). The only exception was a more prominent neutrophil and less prominent eosinophil infiltration in the BAL of IL-10$^{FL/FL}$CD4-Cre$^+$ mice, suggesting a direct or indirect role of T cell derived IL-10 in the recruitment or survival of granulocytes (Fig. 2). In this line the inhibition of neutrophil migration due to plasmacytosis-associated overexpression of IL-10 points to a general effect of IL-10 on the recruitment of neutrophils [41]. However, selective deficiency in T cells (IL-10$^{FL/FL}$CD4-Cre$^+$) did not abrogate the beneficial effect of the tolerizing treatment (Fig. 2), suggesting that at least in the presently used model T cell derived IL-10 was dispensable for allergen-specific TI.

B cell derived IL-10 has also been implied in immune regulation in a number of different disease models [26]. A nonredundant role of B cell derived IL-10 has been reported in murine cytomegalovirus infection [24], in *Salmonella* infection [42], and in acute graft versus host disease [43]. However, using mice with a B cell specific IL-10 deficiency (IL-10$^{FL/FL}$CD19-Cre$^+$), we did not obtain evidence for a functional involvement of B cell derived IL-10 on allergen specific TI. Similar to results observed in IL-10$^{FL/FL}$CD4-Cre$^+$ mice, B cell IL-10 deficiency did not alter the degree of allergic airway inflammation nor the protective effect of TI (Fig. 2). We only observed increased allergen specific IgE in IL-10$^{FL/FL}$CD19-Cre$^+$ in line with the known function of IL-10 on Ig production and class switching [5, 44]. Since neither T cell specific nor B cell specific IL-10 deletion had a significant impact on TI, we hypothesized that lack of IL-10 production by T cells could be compensated by B cells and vice versa. This however, could not be confirmed when analyzing mice with an IL-10 deficiency in both populations (IL-10$^{FL/FL}$CD4-Cre$^+$CD19-Cre$^+$; Supporting Information Fig. 7). Successful TI despite the lack of IL-10 from cells of the adaptive immune system suggested that IL-10 from other sources may be involved in TI. Nonredundant functions of IL-10 from myeloid cells have been reported in different disease models [38, 45–47]. However, in our model IL-10 deficiency in DCs (IL-10$^{FL/FL}$CD11c-Cre$^+$; Fig. 3) had no functional impact on allergen-specific TI. Other myeloid cells such as neutrophils, macrophages, or mast cells are additional known sources of IL-10. But according to the lack of a significant upregulation of IL-10 transcription during TI in these populations, preliminary data from mice with an IL-10 deficiency of those cell types did not point to a functional involvement of either macrophages/neutrophils or mast cells alone in the development of allergen specific tolerance (Supporting Information Fig. 9).

Successful TI in mice with IL-10 deficiency in nonhematopoetic cells (Fig. 4A-C), but failure of TI in case of IL-10 deficiency in the hematopoetic system (IL-10$^{FL/FL}$Vav-Cre$^+$; Fig. 4D-G) confirmed that the functionally relevant IL-10 is generated by hematopoetic cells. This combined with the observation that IL-10 deficiency of single-cell populations (T cells, B cells, DCs, and presumably macrophages/neutrophils and mast cells) or a combination of cell populations (T cells + B cells) was not sufficient to have a major impact on the efficiency of TI, suggests either a functional redundancy of IL-10 from diverse hematopoetic sources or that IL-10 from different cellular source have to act together in an additive fashion to achieve the full biological activity required for successful allergen-specific TI. Along these lines, redundant functions of IL-10 from different cell sources have been discussed in endotoxemia [24], and additive functions of T cell and macrophage-derived IL-10 have been suggested in viral chronicity [38].

The mice studied were generated by Cre/loxP-mediated recombination that leads to a loss of cell type specific IL-10 throughout the ontogeny. This may lead to compensatory mechanisms that may not be adequately recognized and thus not taken into account when interpreting the data. Inducible Cre/loxP-mediated recombination, which allows temporal control of gene expression, may be necessary to dissect the full spectrum of cell type specific IL-10 functions during allergen-specific TI.

In conclusion, our data obtained in a murine model of high-dose tolerance confirmed the need of IL-10 for a successful induction of allergen-specific tolerance. In agreement with previous studies, IL-10 expression during immunotherapy was predominantly observed in T cells and to a lesser extent in B cells and DCs. Functional relevance for TI was confirmed in mice that lack IL-10 in hematopoetic cells. Cell type specific IL-10 deficient mice, however, revealed that neither T cell nor B cell or DC-derived IL-10 was exclusively required for successful TI and suggested a functional redundancy of IL-10 from different hematopoetic sources in this model. Studies that address the cellular target of IL-10 during the process of TI may provide additional insight on the mechanisms of IL-10-mediated immune regulation during allergen-specific immunotherapy.

Materials 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 WT and the following strains on C57BL/6N (B6) background were used in age- and sex-matched groups for experiments: Vert-X (B6(Cg)-*Il10*$^{tm1.1Karp}$) [24], IL-10$^{FL/FL}$CD4-Cre$^+$ [25], IL-10$^{FL/FL}$CD19-Cre$^+$ [24], IL-10$^{FL/FL}$CD4-Cre$^+$CD19-Cre$^+$ (generated by crossing IL-10$^{FL/FL}$CD4-Cre$^+$ x IL-10$^{FL/FL}$CD19-Cre$^+$), IL-10$^{FL/FL}$CD11c-Cre$^+$ [27], IL-10$^{FL/FL}$LysM-Cre$^+$ [28], IL-10$^{FL/FL}$Mcpt5-Cre$^+$ [29], IL-10$^{FL/FL}$Vav-Cre$^+$ [30], IL-10$^{FL/FL}$Cre$^-$ littermates, IL-10$^{-/-}$ [48], and B6.SJL-Ptprc^a Pepc^b/BoyJ (B6.CD45.1).

For the construction of BM chimeras, B6.CD45.2 IL-10$^{-/-}$ or WT recipient mice were sublethally irradiated twice with 6 Gy

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at an interval of 4 h. Subsequently, 5×10^6 BM cells from B6.CD45.1 WT mice were transferred via the tail vein. To allow complete chimerism and reconstitution (controlled by flow cytometric detection of CD54.1$^+$ cells) at least 12 weeks were waited until the experiment was started [49].

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.

Southern blot

Specific deletion of the IL-10 gene in defined cell populations (sorted with the help of immunostaining on a FACSAria, BD Biosciences, Heidelberg, Germany) was confirmed by Southern blot analysis as described previously [25].

Induction of allergic airway inflammation, TI, and BAL

Mice were sensitized to OVA by repetitive intraperitoneal injections of 10 μg OVA (Grade V, Sigma-Aldrich, Taufkirchen, Germany) adsorbed to 2 mg aluminiumhydroxide (Imject alum, Pierce, Rockford, USA) in PBS on days 0, 7, and 14. TI was conducted by three subcutaneous injections (neck) of OVA (1 mg in PBS) on three alternate days beginning at least 14 days after sensitization. Control animals received PBS injections instead. Mice were challenged 7–14 days after the last injection by 1% OVA aerosol for 20 min three times 72 h apart. Animals were sacrificed and analyzed 24 h after the last aerosol challenge (Supporting Information Fig. 1). BAL was performed as recently described [50]. The functional role of IL-10 during TI was assessed by a single intraperitoneal injection of an IL-10-receptor neutralizing antibody (clone 1B1.3a, 500 μg) 2–6 h prior to TI. Rat IgG (Jackson ImmunoResearch Europe Ltd., Newmarket, UK) served as control.

Flow cytometry

Antibody staining was performed according to standard protocols for extracellular staining. Antibody binding was detected by using BD Canto (BD Biosciences) and analyzed with FlowJo software, version 9 (TreeStar, Inc.). 4',6-diamidino-2-phenylindole (DAPI) positive, dead cells were excluded from further analysis. Antibodies against CD3 (clone 145-2C11), CD4 (RM4-5), CD25 (PC61), CD8 (53-6.7), CD19 (ID3), CD11b (M1/70), CD11c (N418), CD138 (281-2), NK1.1 (PK136), CD117 (2B8), FceRIα (MAR-1), SiglecF (E50-2440), GR-1 (RB6-8C5), CD5 (53-7.3), and CD1d (1B1) were purchased from BD Biosciences or eBioscience (San Diego, USA). For the analysis of GFP expression corresponding antibody stained cells of WT mice without reporter activity served as reference (Supporting Information Fig. 8).

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

Single-cell suspensions of mediastinal LNs were cultured in the absence or presence of OVA (10 μg/mL) as recently described [51]. Supernatants were taken after 5 days of incubation and cytokine content was analyzed by ELISA using matched antibody pairs purchased from BD Biosciences (IL-5) and AbD Serotec, Kidlington, UK (IL-13). Assays were performed according to the manufacturer´s instructions. OVA-specific IgE and IgG1 concentrations were measured in serum samples by ELISA as described previously [50].

Histology

Periodic-acid Schiff staining of lung tissue was performed as described previously [50].

Statistical analysis

Mann–Whitney *U*-test was used for unpaired, nonparametric data (GraphPad Prism version 5.01, GraphPad Software, Inc., La Jolla, USA). Differences were considered significant when p values were <0.05.

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Abbreviations: **AIT**: allergen-specific immunotherapy · **BAL**: bronchoalveolar lavage · **TI**: tolerance induction

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Supporting Information
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T cell derived IL-10 is dispensable for tolerance induction in a murine model of allergic airway inflammation

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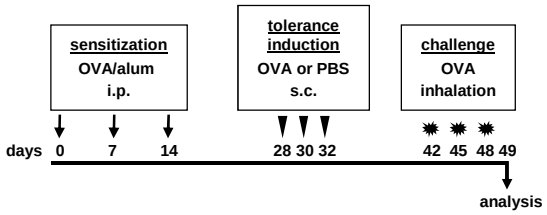
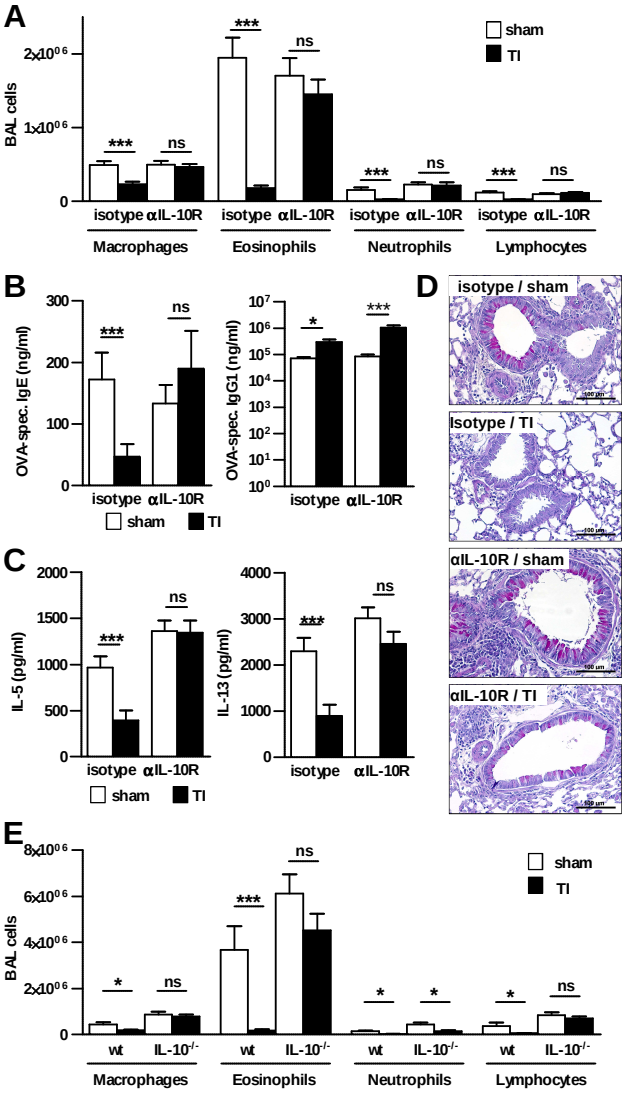
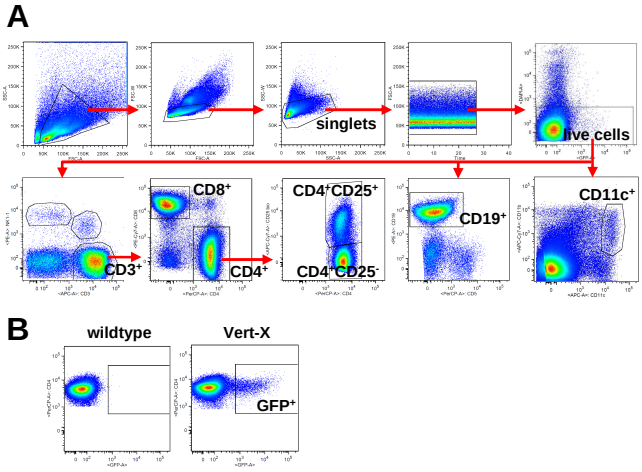


Supporting information Figure 1:
Standard protocol for sensitization, tolerance induction and allergen challenge.
Mice were sensitized to ovalbumin (OVA) by repetitive intraperitoneal injections of OVA adsorbed to aluminiumhydroxide (alum) on days 0, 7 and 14. Tolerance induction was conducted by three subcutaneous injections of OVA on three alternate days beginning at least 14 days after sensitization. Control animals received PBS injections instead. Mice were challenged 7 to 10 days after the last injection by OVA aerosol three times 72 hours apart. Animals were sacrificed and analyzed 24 hours after the last aerosol challenge.

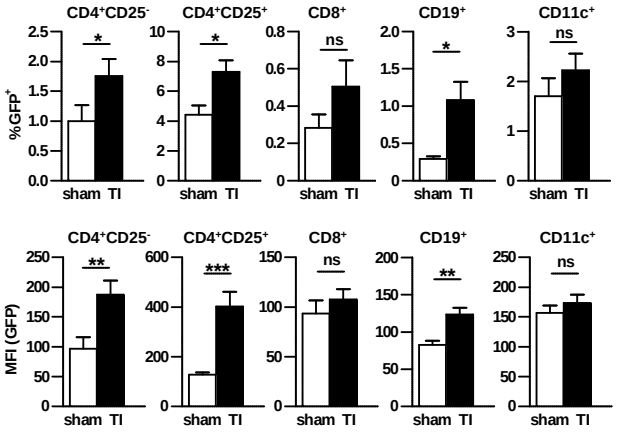


Supporting information Figure 2:
IL-10 dependent beneficial effect of allergen specific TI in C57BL/6 wild type mice.

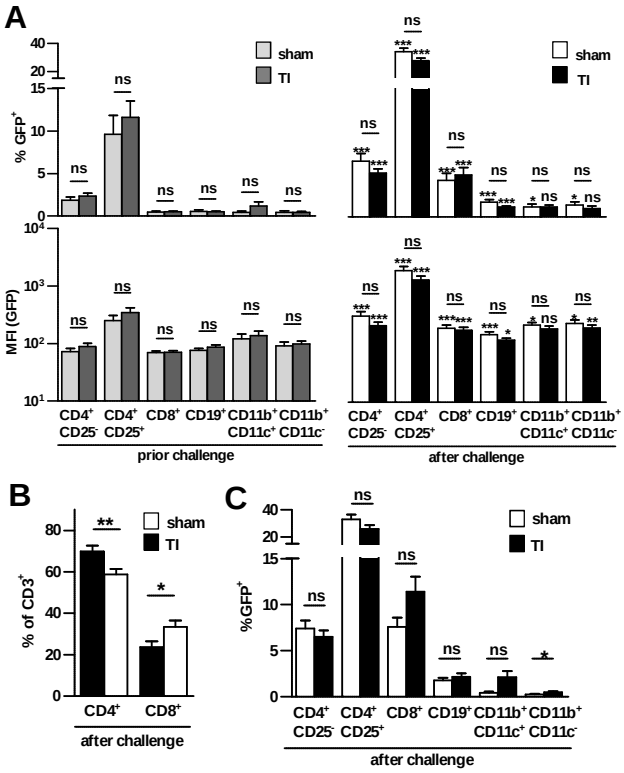
(A-D) Sensitized C57BL/6 wildtype mice treated with IL-10R blocking or isotype control antibody and (E) IL-10 knock-out mice (IL-10$^{-/-}$) were either sham treated (sham) or tolerized (TI) by s.c. allergen injections and analysed 24 hours after final allergen challenge as described in the *Materials and methods*. (A,E) Immune cells in BAL fluid were counted on Diff-Quick stained cytospins. (B) Serum levels of allergen-specific IgE and IgG1 were measured by ELISA. (C) IL-5 and IL-13 release by allergen-stimulated LN cells was measured by ELISA. (D) PAS-stained lung sections. Images shown are representative of 2 independent experiments. (A-E) Data are shown as mean+SEM (n=3-8 mice/group and experiment) and are pooled from (A-C) 7 or (E) 2 independent experiments. * $p<0.05$, *** $p<0.001$, ns: not significant; Mann- Whitney U test.



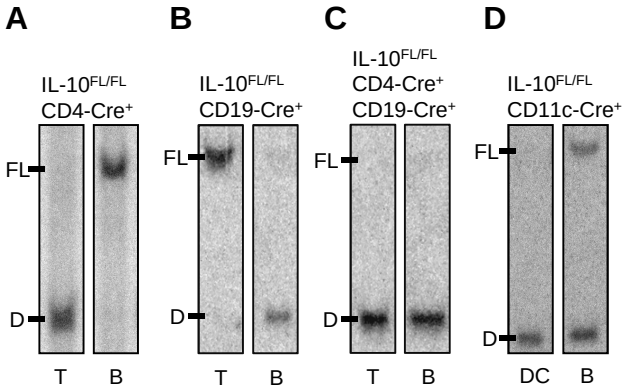
Supporting information Figure 3:
Flow cytometry gating strategy for determination of GFP+ immune cells.
(A) Definition of distinct cell populations. (B) Analysis of GFP expression by comparing wildtype and IL-10 reporter mice (Vert-X).



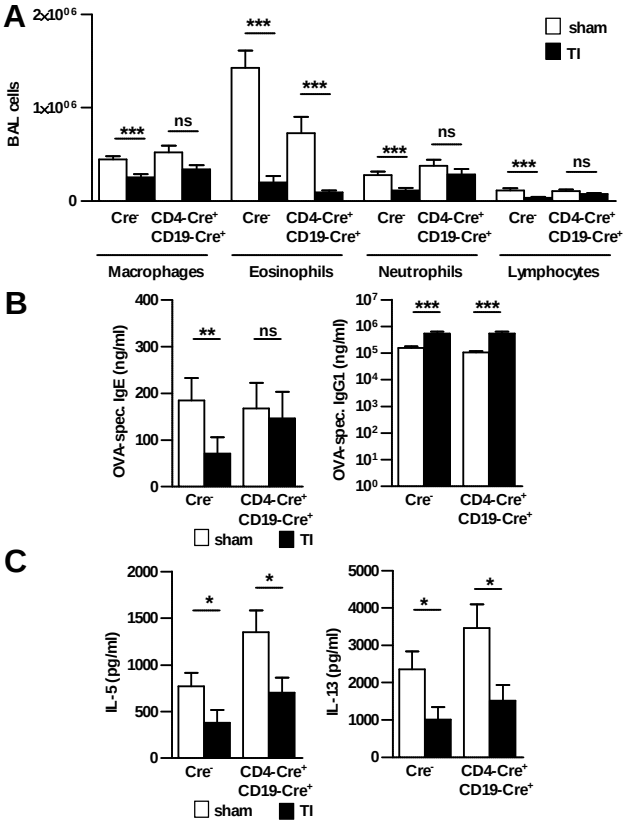
Supporting information Figure 4:
IL-10 reporter expression (GFP) in different cell types of mediastinal LNs upon initiation of tolerance.
Sensitized transcriptional IL-10 reporter mice (Vert-X) were either sham treated (sham) or tolerized (TI) by s.c. allergen injections as described in the *Materials and methods*. Different cell populations of mediastinal LNs were analysed 132 hours after initiation of therapy, i.e. 36 hours after final injection by flow cytometry for reporter expression (GFP). Percent GFP+ cells per indicated cell type (upper panel) and mean fluorescence intensity (lower panel). Data are shown as mean +SEM (n=3-6 mice/group and experiment) and are pooled from 4 independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns: not significant Mann-Whitney U test.



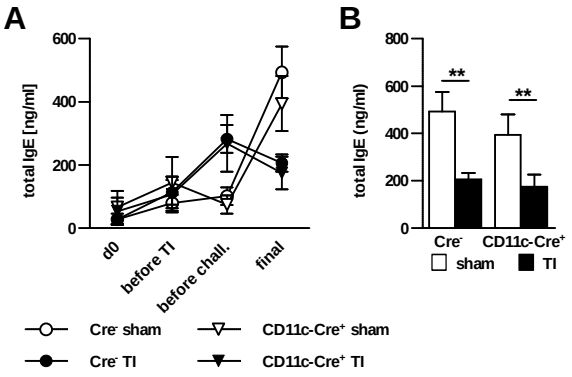
Supporting information Figure 5:
IL-10 expression levels in immune cells of lung tissue and BAL during the elicitation phase of allergic airway inflammation.
Sensitized transcriptional IL-10 reporter mice (Vert-X) were sham treated (sham) or tolerized (TI) and allergen challenged by aerosol treatment as described in the *Materials and methods.* (A) Percentage of GFP expression (upper panel) and mean fluorescence intensity (MFI, lower panel) of immune cells in lung tissue before and 24 hours after the last allergen challenge. Significance levels for the comparison of tolerized and sham treated mice are displayed above a bracket. Significance levels for the comparison before and after allergen challenge are only displayed in the right panel above each column. (B) T cell composition within BAL cells after allergen challenge. (C) GFP expression of different BAL cell populations after allergen challenge. Data are shown as mean+SEM (n=16-24 mice/group) and are pooled from 4 independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns: not significant Mann-Whitney U test.



Supporting information Figure 6:
Cell type specific deletion of the IL-10 gene in IL-10$^{FL/FL}$CD4-Cre^{+}, IL-10$^{FL/FL}$CD19-Cre^{+}, IL-10$^{FL/FL}$CD4-Cre^{+}CD19-Cre^{+} and IL-10$^{FL/FL}$CD11c-Cre^{+} mice.
Southern blot analysis of DNA extracted from different FACS-sorted spleen cell populations after PstI digest. (A-C) CD3^{+} T cells and CD19^{+} B cells from (A) IL-10$^{FL/FL}$CD4-Cre^{+}, (B) IL-10$^{FL/FL}$CD19-Cre^{+} and (C) IL-10$^{FL/FL}$CD4-Cre^{+}CD19-Cre^{+} mice. (D) CD11c^{high}MHCII^{+} DCs and CD19^{+} B cells from IL-10$^{FL/FL}$CD11c-Cre^{+} mice. Data are representative of three animals.
D: deleted allele, FL: loxP-flanked allele, T: T cells, B: B cells.

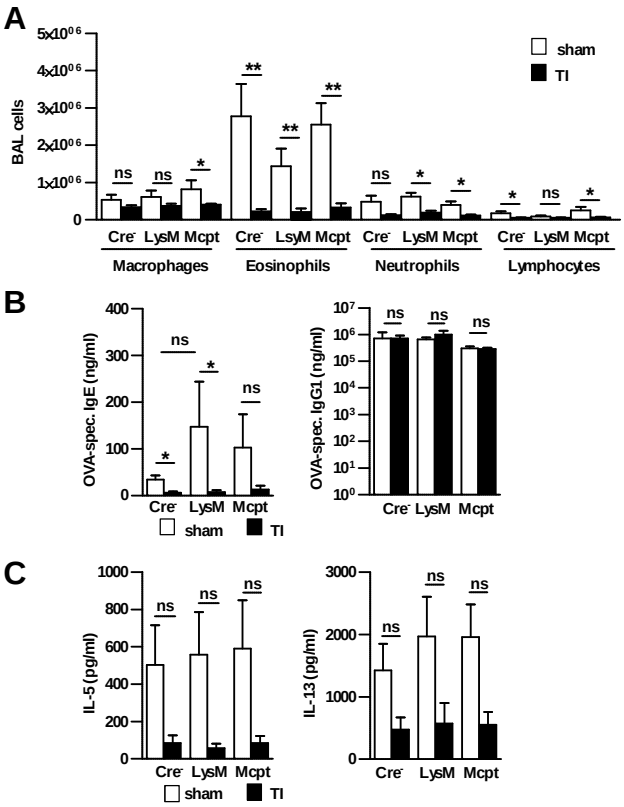


Supporting information Figure 7:
Lack of T and B cell derived IL-10 does not prevent tolerance induction.
Sensitized T and B cell specific IL-10 deficient mice (IL-10$^{FL/FL}$CD4-Cre^{+}CD19-Cre^{+}) and control mice (IL-10$^{FL/FL}$Cre^{-}) were sham treated (sham) or tolerized (TI) by s.c. injections, allergen challenged by aerosol treatment and analysed 24 hours later as described in the *Materials and methods*. (A) Immune cells in BAL fluid were counted on Diff-Quick stained cytospins.(B) Serum levels of allergen-specific IgE and IgG1 were measured by ELISA. (C) IL-5 and IL-13 release by allergen-stimulated LN cells was measured by ELISA. Data are shown as mean +SEM (n=3-10 mice/group and experiment) and are pooled from from (A) 5 or (B,C) 4 independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$; ns: not significant, Mann-Whitney U test.

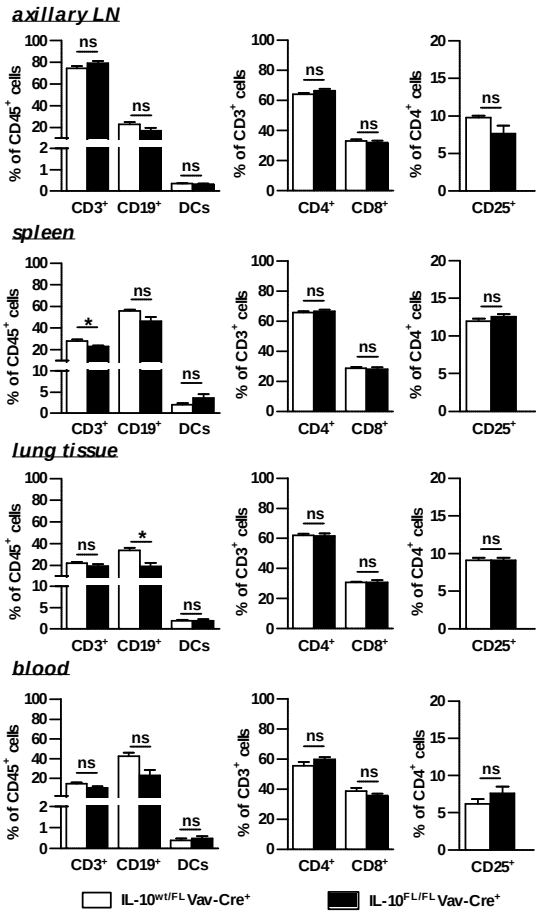


Supporting information Figure 8:
Serum level of total IgE in mice with DC specific IL-10 deficiency

Sensitized DC specific IL-10 deficient mice (IL-10$^{FL/FL}$CD11c-Cre^{+}) and control mice (IL-10$^{FL/FL}$Cre^{-}) were sham treated (sham) or tolerized (TI) by s.c. injections, allergen challenged by aerosol treatment and analysed as described in the *Materials and methods*. (A) Kinetic of total IgE levels and (B) total IgE 24 h after challenge (final) was determined by ELISA. Data are shown as mean+SEM (n=6-7 mice/group and experiment) and are pooled from 3 independent experiments. ** $p<0.01$, Mann-Whitney U test.



Supporting information Figure 9:
Lack of macrophage/neutrophil or mast cell derived IL-10 does not prevent tolerance induction.
Sensitized macrophage/neutrophil (IL-10$^{FL/FL}$LysM-Cre^{+}) or mast cell (IL-10$^{FL/FL}$Mcpt5-Cre^{+}) specific IL-10 deficient mice and control mice (IL-10$^{FL/FL}$Cre^{-}) were sham treated (sham) or tolerized (TI) by s.c. injections, allergen challenged by aerosol treatment and analysed 24 hours later as described in the *Materials and methods*. (A) Immune cells in BAL fluid were counted on Diff-Quick stained cytospins. (B) Serum levels of allergen-specific IgE and IgG1 were measured by ELISA. (C) IL-5 and IL-13 release by allergen-stimulated LN cells was measured by ELISA. Data (n=5-6 mice/group) of 1 experiment are shown as mean+SD. LysM: IL-10$^{FL/FL}$LysM-Cre^{+} mice, Mcpt: IL-10$^{FL/FL}$Mcpt5-Cre^{+}mice, * $p<0.05$, ** $p<0.01$, ns: not significant, Mann-Whitney U test.



Supporting information Figure 10:
Composition of significant immune cell populations in IL-10$^{FL/FL}$Vav-Cre^{+} mice and litter mate controls.

Axillary lymph nodes (LN), spleen, lung tissue and blood of naïve, 5 week old IL-10$^{FL/FL}$Vav-Cre^{+} mice and IL-10$^{wt/FL}$Vav-Cre^{+} were analyzed by flow cytometry for total T cells (CD3^{+}), B cells (CD19^{+}) and dendritic cells (DCs, CD11c^{+}MHCII^{+}) (left panel), as well as for CD4^{+} and CD8^{+} T cells (middel panel) and CD4^{+}CD25^{+} T cells (right panel). Data are shown as mean+SEM (n=2-3 mice/group and experiment) and are pooled from from 2 independent experiments. ** $p<0.01$, Mann-Whitney U test.