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ORIGINAL ARTICLE

Allergen-Specific Immunotherapy and Biologics

TGFβ1 mimetic peptide modulates immune response to grass pollen allergens in mice

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Abstract

Background: Transforming growth factor β1 (TGFβ1) is a cytokine that exerts immunosuppressive functions, as reflected by its ability to induce regulatory T (Treg) cell differentiation and inhibit Th1 and Th2 responses. Hence, peptides that mimic the active core domain of TGFβ1 may be promising candidates for modulation of the allergic response. This study aimed to investigate a synthetic TGFβ1 mimetic peptide (TGFβ1-mim) for its ability to modulate the immune response during allergic sensitization to grass pollen allergens.

Methods: The in vitro action of TGFβ1-mim was evaluated in human lung epithelial cells, Jurkat cells, and rat basophilic leukemia cells. The in vivo action was evaluated in a murine model of Phl p 5 allergic sensitization. Additionally, the Th2 modulatory response was evaluated in IL-4 reporter mice.

Results: In vitro, TGFβ1-mim downregulated TNF-α production, IL-8 gene expression, and cytokine secretion, upregulated IL-10 secretion, and inhibited Phl p 5-induced basophil degranulation. During Phl p 5 sensitization in mice, TGFβ1-mim downregulated IL-2, IL-4, IL-5, IL-13, and IFN-γ, upregulated IL-10, and induced Treg cell production. Furthermore, mice treated with TGFβ1-mim had lower levels of IgE, IgG1, IgG2a and higher levels of IgA antibodies than control mice. In a reporter mouse, the mimetic inhibited Th2 polarization.

Conclusion: The TGFβ1-mim efficiently modulated various important events that exacerbate the allergic microenvironment, including the production of main cytokines that promote Th1 and Th2 differentiation, and the induction of allergen-specific regulatory T cells, highlighting its potential use in therapeutic approaches to modulate the immune response toward environmental allergens.

KEYWORDS

allergic sensitization, grass pollen allergy, immune regulation, immunotherapy, TGFβ1

Abbreviations: 4get, IL-4/GFP-enhanced transcript; Phl p, *Phleum pratense*; RBL cells, Rat basophilic leukemia cells; TGFβ1, Transforming growth factor β1; TGFβ1-mim, TGFβ1 mimetic peptide; TGFβRII, TGFβ receptor II; Treg, Regulatory T cell.

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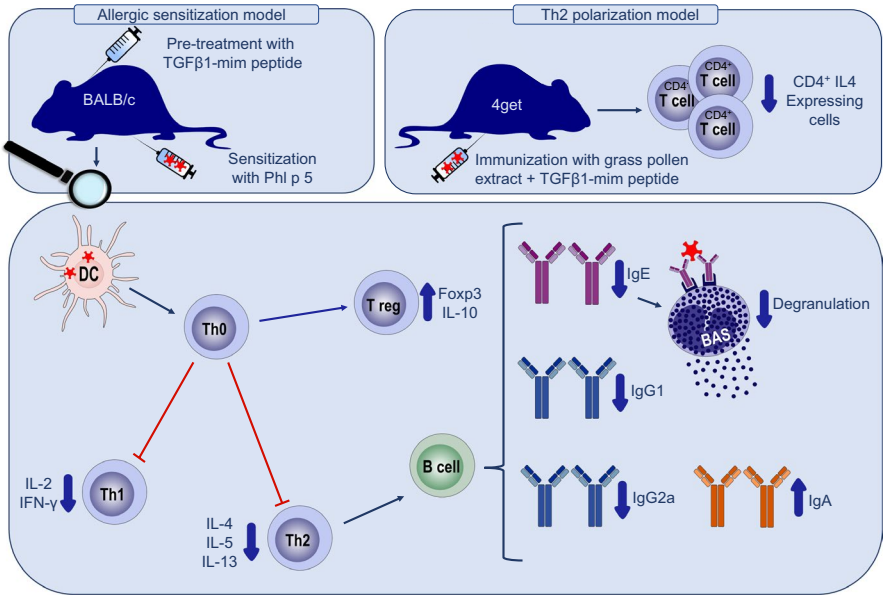

GRAPHICAL ABSTRACT

TGFβ1-mim downregulated major cytokines involved in Th1 and Th2 responses in mice sensitized to Phl p 5. Treatment of mice with the mimetic during sensitization to Phl p 5 induced Treg production, suppressed specific IgE and IgG1, IgE-mediated basophil degranulation, induced IgA antibodies and IL-10 secretion. In an IL-4 reporter model, TGFβ1-mim suppressed Th2 polarization induced by grass pollen extract.

1 | INTRODUCTION

Allergens are environmental proteins that interact with innate immune receptors, leading to Th2 polarization and consequently IgE production.[1] Grasses are among the most potent sources of allergens. They produce large amounts of pollen, are widely distributed, and are potent respiratory allergens. Although it is estimated that only 8% of grass pollen-allergic patients have been officially diagnosed, it is believed that around 20% of the population of the United States, Europe, and Brazil are affected by grass pollen allergy.[2,3] Hence, grass pollen allergy is a global problem, implying a need for novel therapeutic approaches. Almost all timothy grass (*Phleum pratense*) allergic patients displaying strong clinical reactions have high IgE antibody responses toward Phl p 5.[4-6]

Allergic sensitization is initiated when an allergen interacts with innate immune receptors, leading to Th2 polarization and IgE production. Upon re-exposure, allergen cross-links IgE bound to the high-affinity FcεRI receptor on mast cells leading to inflammatory reactions characterized by the secretion of chemical mediators, synthesis of leukotrienes, prostaglandins, and cytokines (eg, IL-4, IL-5, and IL-13), and by recruitment of other effector cells, such as Th17, Th9, basophils, and eosinophils.[7-9] Th1 cells also contribute to the effector phase and chronicity in allergic diseases.

Regulatory T (Treg) cells are essential for immune tolerance against autoimmune and inflammatory diseases, allergies, and various other events related to the breakdown of immune homeostasis. Treg cells are a heterogeneous population characterized by the constitutive expression of the transcription factor Foxp3 and represent the dominant subset specific for common environmental allergens in healthy individuals.[10-12] Transforming growth factor β1 (TGFβ1) is a key cytokine involved in the induction of Treg cells and in the regulation of effector T cells, B cells, and epithelial cells.[13] In mice possessing a disrupted TGFβ1 gene or lacking the TGFβ receptor II (TGFβRII), severe inflammatory responses, tissue necrosis, and early death were observed,[14,15] confirming the broad immune regulatory functions of TGFβ1. Hence, the modulation of allergen-specific $CD4^+$ effector T cells by TGFβ1 could potentially induce immune tolerance and suppress allergic inflammation.

Considering that the availability, specificity, and biological activity of TGFβ1 are normally controlled by numerous interactions with membrane-bound proteins,[16] short peptides that mimic the binding domain of TGFβ1 and are able to efficiently activate the TGFβRII on target cells might represent novel therapeutic tools for treating allergies. We have previously selected by phage display a TGFβ1 mimetic peptide (pm26TGFβ1/TGFβ1-mim) that was mapped into the TGFβ1:TGFβRII activation domain and shown to contain amino acid residues that are crucial for high-affinity binding and stabilization. Furthermore, in the same study we also showed that the TGFβ1-mim displayed potent anti-inflammatory activity.[17] Here, we investigated the ability of the TGFβ1-mim to modulate the allergic/inflammatory response in vitro and in mice sensitized with Phl p 5 or timothy grass pollen extract. Our findings showed that the TGFβ1-mim peptide efficiently modulated crucial events involved in allergen-specific immune responses.

2 | MATERIALS AND METHODS

2.1 | Synthetic peptide

LPS-free TGFβ1-mim (pm26TGFβ1)[17] was chemically synthesized by Bachem (Weil am Rhein, Germany). A detailed description is

provided in the Appendix S1. Unconjugated human TGFβ1 recombinant protein produced in CHO cells (eBioscience, Austria) was included as positive control in the in vitro experiments.

2.2 | IL-8 expression analysis

To investigate whether TGFβ1-mim affected IL-8 expression, an A549 immortalized human lung epithelial cell line (A549 - ATCC, LGC Promochem, Wesel, Germany) stably transfected with a luciferase reporter gene placed under the control of the IL-8 promoter was used according to the original protocol.[18] A detailed description is provided in the Appendix S1.

2.3 | IL-10 and TNF-α measurements

Levels of secreted IL-10 and TNF-α in the supernatant of Jurkat cells and IL-8 in the supernatant of A549 cells were measured by ELISA. Cells at a density of 4×10^5/mL were pretreated with 1 μmol/L TGFβ1-mim or recombinant TGFβ1 (rTGFβ1) for 1 hour and stimulated with 40 ng/mL phorbol 12-myristate 13-acetate (PMA) (Jurkat cells; Sigma-Aldrich) plus 500 ng/mL ionomycin (Sigma-Aldrich), or 20 ng/mL TNF-α (A549 cells; GenScript) for 24 hours at 37°C and 5% CO_2. Supernatant was collected for cytokine measurements, which were performed according to the manufacturer's protocol (eBioscience).

2.4 | Mice sensitization model

Female BALB/c mice (6-10 weeks old) were obtained from Charles River Laboratories and maintained in the animal facility of the University of Salzburg. To induce allergen-specific IgE response, five mice were immunized intradermally (i.d.) with 25 μg of recombinant Phl p 5[19] diluted in PBS without any added adjuvant. Five mice were pretreated subcutaneously with 100 μL of TGFβ1-mim diluted in PBS at 1 μmol/L, followed by i.d. injection with 25 μg of Phl p 5 diluted in PBS. Three mice constituted the naïve group. Immunization was performed on days 0, 14, 28, and 42, and mice were sacrificed on day 44. To investigate the induction of Phl p 5-specific IgE responses, blood samples were drawn from the saphenous vein on days 14, 28, and 42.

2.4.1 | In vivo Th2 polarization model

Bicistronic IL-4 reporter mice in the BALB/c background (8-13 weeks old) were obtained from The Jackson Laboratory. Because our preliminary data showed that rPhl p 5 alone did not induce IL-4 production, we investigated the Th2 polarizing capacity of grass pollen extract alone or in combination with the mimetic. Five mice received i.d. injection in the right lateral abdominal region either with 25 μg rPhl p 5 (n = 5), 10 μg grass pollen extract (n = 5), or 10 μg grass pollen extract in combination with TGFβ1-mim at 1 μmol/L diluted in PBS without added adjuvant. Five mice constituted the naïve group. Mice were sacrificed on the 5th day after allergen injection. IL-4 expressing cells in skin-draining inguinal lymph nodes were analyzed by flow cytometry. Following the principles of Russell and Burch's 3Rs[20] for animal experiments, we analyzed the effects of the mimetic peptide on Phl p 5 responses without including recombinant TGFβ1. All animal experiments were performed according to national guidelines approved by the Austrian Federal Ministry of Science, Research and Economy (normal mice: BMWF-66.012/0017-WF/V/3b/2017; 4get mice: BMWF-66.012/0041-WF/V/3b/2017).

2.5 | Rat basophil leukemia (RBL) cell mediator release assay

The capacity of TGFβ1-mim to inhibit Phl p 5-induced degranulation in murine RBL-2H3 (muRBL) cells (ATCC® CRL2256™) was performed as described elsewhere.[21] Humanized RBL-2H3 (huRBL) cells transfected with the cDNA encoding the human FcεRI were tested with nine sera from grass pollen-allergic patients, as described elsewhere.[22] Experiments using anonymized human serum samples were approved by the local Ethics Committee of the Medical University and General Hospital of Vienna (no. EK1263/2014), and informed written consent was obtained from all study participants. A detailed description is provided in the Appendix S1.

2.6 | Phl p 5-specific antibody detection by ELISA

The levels of Phl p 5-specific IgE, IgG1, IgG2a, and IgA antibodies were measured by ELISA in sera from all groups of mice. A detailed description is provided in the Appendix S1.

2.7 | Cytokine-producing cells detection by ELISPOT

Production of IFN-γ, IL-4, and IL-10 from splenocytes in response to Phl p 5 stimulation was assessed by ELISPOT. A detailed description is provided in the Appendix S1.

2.8 | Flow cytometry analysis

Flow cytometry analyses of proliferating mice splenocytes upon Phl p 5 stimulation for 5 days were performed as described in the Appendix S1. In this study, we investigated the percentage of allergen-specific $CD4^+Foxp3^+$ Treg cells expressing CD25, GATA3, CTLA4, or the cellular proliferation marker KI67, important factors associated to Treg cell function.

2.9 | Statistical analysis

Statistical analyses were conducted using GraphPad Prism 5 software (GraphPad software). Results are presented as mean with SD of each group. Comparisons between groups were performed using one-way ANOVA for all experiments, except for the human RBL assays where the paired Student's *t* test was employed. *P* values < .05 (*P < .05; **P < .01; ***P < .001) were considered significant.

3 | RESULTS

3.1 | TGFβ1-mim modulates cytokine production and IgE-mediated basophil degranulation in vitro

We first tested the ability of the TGFβ1-mim peptide to recognize TGFβRII on Jurkat cells, using ELISA. Recombinant TGFβ1 was used as a positive control. Although not statistically significant, TGFβ1-mim showed slightly elevated reactivity to TGFβRII than rTGFβ1 (Figure 1A). In PMA-stimulated Jurkat cells, both mimetic and rTGFβ1 significantly decreased the secretion of TNF-α (Figure 1B) and increased the secretion of IL-10 (Figure 1C). Since the lung epithelium plays an important role as a first line of defense toward external compounds,[18] we sought to investigate the production of IL-8 using a human lung epithelial (A549) cell line possessing a luciferase reporter gene under the control of the IL-8 promoter. In TNF-α-stimulated A549 cells, both TGFβ1-mim and rTGFβ1 decreased IL-8 gene expression (Figure 1D), whereas only TGFβ1-mim significantly decreased IL-8 secretion (Figure 1E). To investigate whether the mimetic modulates IgE-mediated basophil degranulation in an already established allergic microenvironment, we performed mediator release assays using RBL cells expressing the humanized FcεRI receptor. We found that the amount of Phl p 5 needed to induce half-maximal release in basophils, which were passively sensitized with sera from grass pollen-allergic patients, was significantly higher when cells were pretreated with TGFβ1-mim (Figure 1F), indicating an suppression of degranulation. This effect was also observable when pretreating with rTGFβ1. Titration curves for every patient are presented in Figure E1. These results indicate that the TGFβ1-mim was able to modulate the allergic inflammatory microenvironment in vitro.

3.2 | TGFβ1-mim modulates antibody response in vivo

We next tested the ability of the TGFβ1-mim to modulate the antibody response during Phl p 5 sensitization. Phl p 5 (Figure 2A) significantly induced allergen-specific IgE response, as observed from the capacity of mice sera to provoke degranulation in RBL cells (Figure E2 and Figure 2B). RBL cells sensitized with sera of mice treated with 1 μmol/L TGFβ1-mim had significantly lower levels of degranulation than the group injected with Phl p 5 alone. ELISA analysis showed that in serum from Phl p 5-sensitized mice, TGFβ1-mim significantly suppressed the levels of allergen-specific IgE (Figure 2C) and IgG1 (Figure 2D). No significant differences were observed for allergen-specific IgG2a antibodies (Figure 2E). Mice treated with TGFβ1-mim also showed significant higher levels of allergen-specific IgA (Figure 2F). Therefore, TGFβ1-mim modulated the Phl p 5-specific antibody responses in mice.

3.3 | TGFβ1-mim modulates cytokine production in vivo

To investigate the cytokine profile in the supernatants of restimulated splenocytes, we used a multiplex cytokine analysis kit. The secretion of IFN-γ, IL-2, IL-4, IL-5, and IL-13 was significantly lower, whereas secretion of IL-10 was higher in mice treated with TGFβ1-mim than in untreated mice (Figure 3A). To determine the antigen-specific T cell polarization upon TGFβ1-mim treatment, the number of IFN-γ, IL-4, and IL-10 producing splenocytes in all groups was assessed by ELISPOT. Sensitization with Phl p 5 resulted in high induction of IFN-γ and IL-4 release upon allergen stimulation. In mice treated with TGFβ1-mim, levels of IFN-γ and IL-4 secreted by Phl p 5-restimulated splenocytes were significantly lower, while levels of IL-10 were significantly higher than in untreated mice (Figure 3B). Therefore, TGFβ1-mim downregulated Th1 and Th2 cytokines, and upregulated IL-10.

3.4 | TGFβ1-mim induces allergen-specific Treg cell production

To investigate the influence of TGFβ1-mim on Phl p 5-specific Treg induction during allergic sensitization, we analyzed splenocytes from BALB/c mice using flow cytometry. We sought to investigate $CD4^+Foxp3^+$ cells expressing CD25, GATA3, CTLA4, or KI67, since these factors are required for Treg cell survival and function.[23,24] The percentage of $CD4^+CD25^+Foxp3^+$ proliferating Treg cells found in naïve and Phl p 5-sensitized mice was 5.81 ± 0.4 and 8.5 ± 2.5, respectively, whereas 13.5 ± 1.1 was found in mice treated with TGFβ1-mim (Figure 4A-B). Naïve and Phl p 5-sensitized mice had comparable levels of $CD4^+Foxp3^+$ Treg cells expressing GATA3 (20.2 ± 4.7% and 22.4 ± 4%, respectively), whereas mice treated with TGFβ1-mim had 36.2 ± 10% (Figure 4C-D). The percentage of $CD4^+Foxp3^+$ Treg cells expressing CTLA4 in naïve and Phl p 5-sensitized mice was 9.0 ± 1.9% and 13.1 ± 3.5%, respectively, whereas for mice treated with TGFβ1-mim this value was 17.5 ± 4.0% (Figure 4E-F). Although not significantly different when compared to Phl p 5-sensitized mice (15.7 ± 6.4%), the percentage of $CD4^+Foxp3^+$ Treg cells expressing the KI67 proliferation marker was enriched in the group of mice treated with TGFβ1-mim (24.6 ± 2.8) (Figure 4G-H). These results demonstrate that TGFβ1-mim promotes Treg cell production during Phl p 5 sensitization.

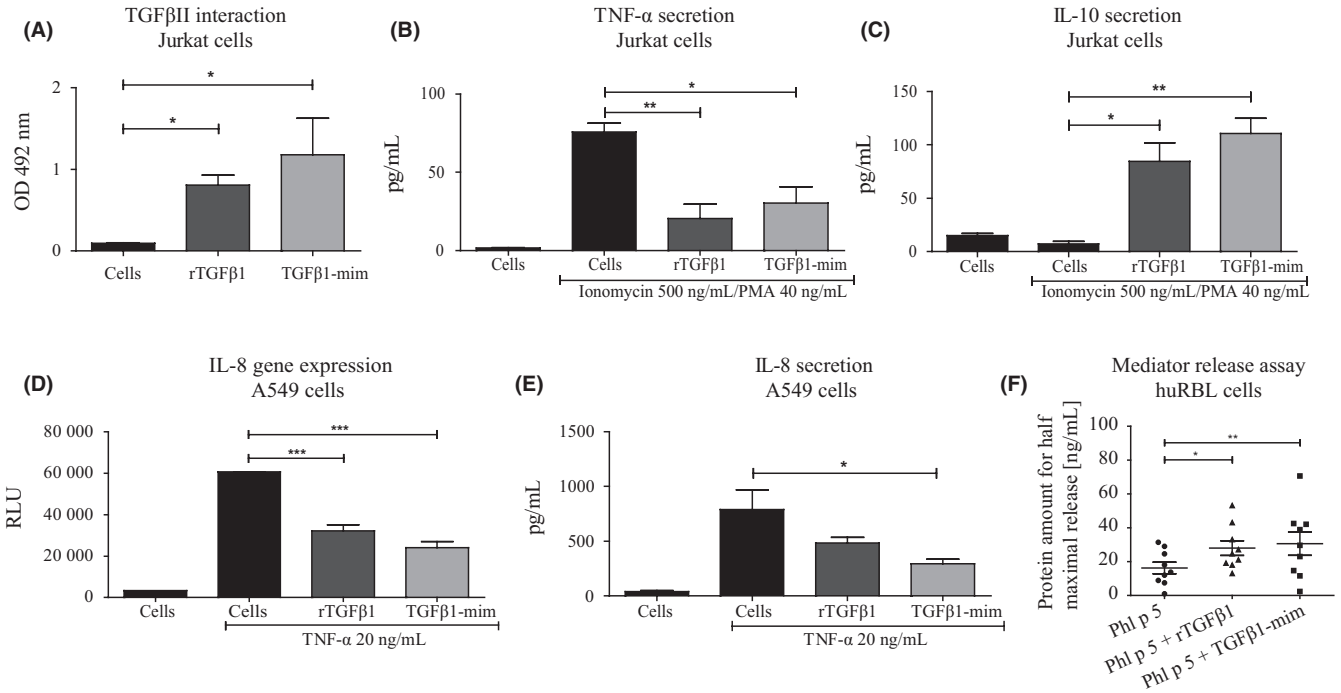

FIGURE 1 TGFβ1-mim modulates immune responses in vitro. A, ELISA assay to assess binding of rTGFβ1 and TGFβ1-mim to TGFβII on Jurkat cells. B, Secretion of TNF-α and IL-10 (C) in Ionomycin/PMA-stimulated Jurkat cells. D, Luciferase reporter assay showing IL-8 gene expression in TNF-α-stimulated A549 cells. E, IL-8 secretion in TNF-α-stimulated A549 cells. F, Mediator release assay in huRBL cells expressing the human FcεRI. The ability of TGFβ1-mim to suppress IgE-mediated degranulation in sensitized basophils was confirmed in nine grass pollen-sensitized patients. *P < .05, **P < .01, ***P < .001

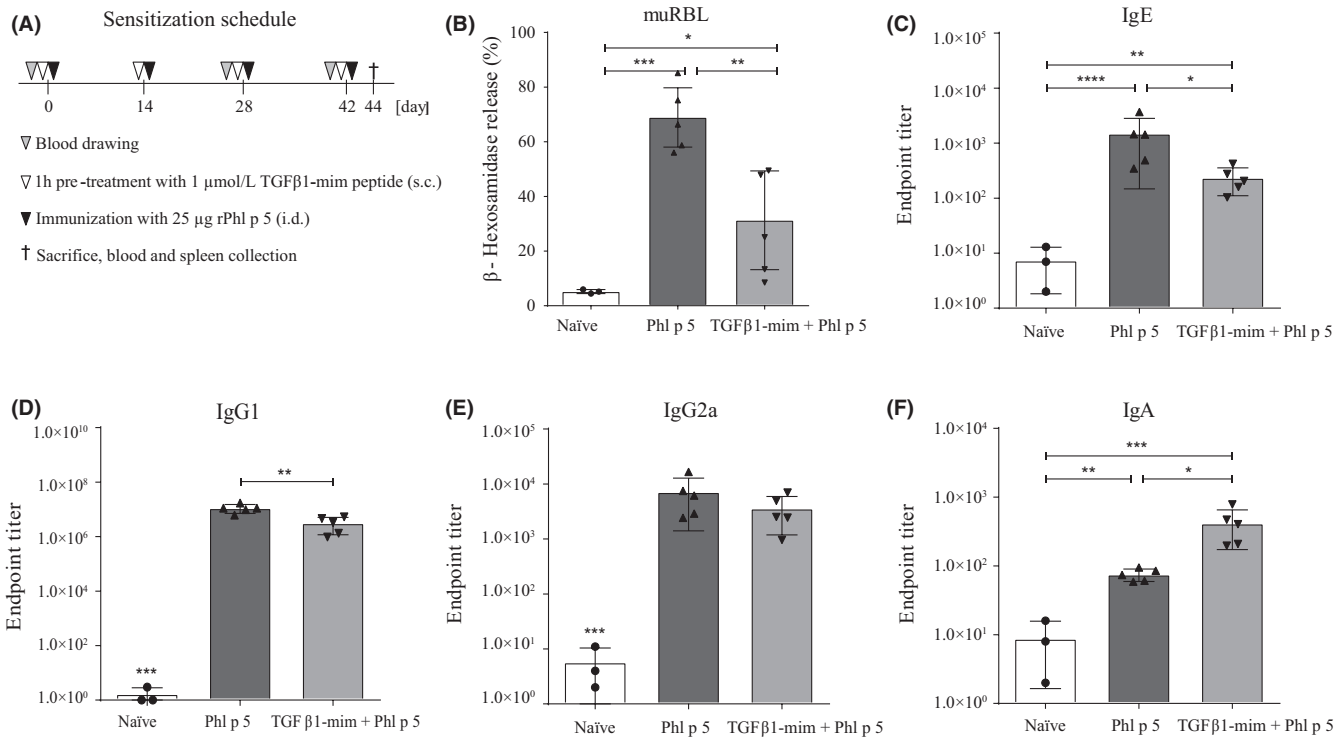

FIGURE 2 TGFβ1-mim peptide modulates antibody response in vivo. A, Scheme of animal sensitization. B, Immunization with the recombinant Phl p 5 significantly induced IgE-mediated basophil degranulation, whereas it was substantially decreased in mice that received the mimetic. TGFβ1-mim peptide was able to suppress Phl p 5-specific IgE (C), IgG1 (D) and IgG2a (E) and to induce IgA (F) antibody production in mice sera. *P < .05, **P < .01, ***P < .001. s.c., subcutaneous; i.d., intradermal

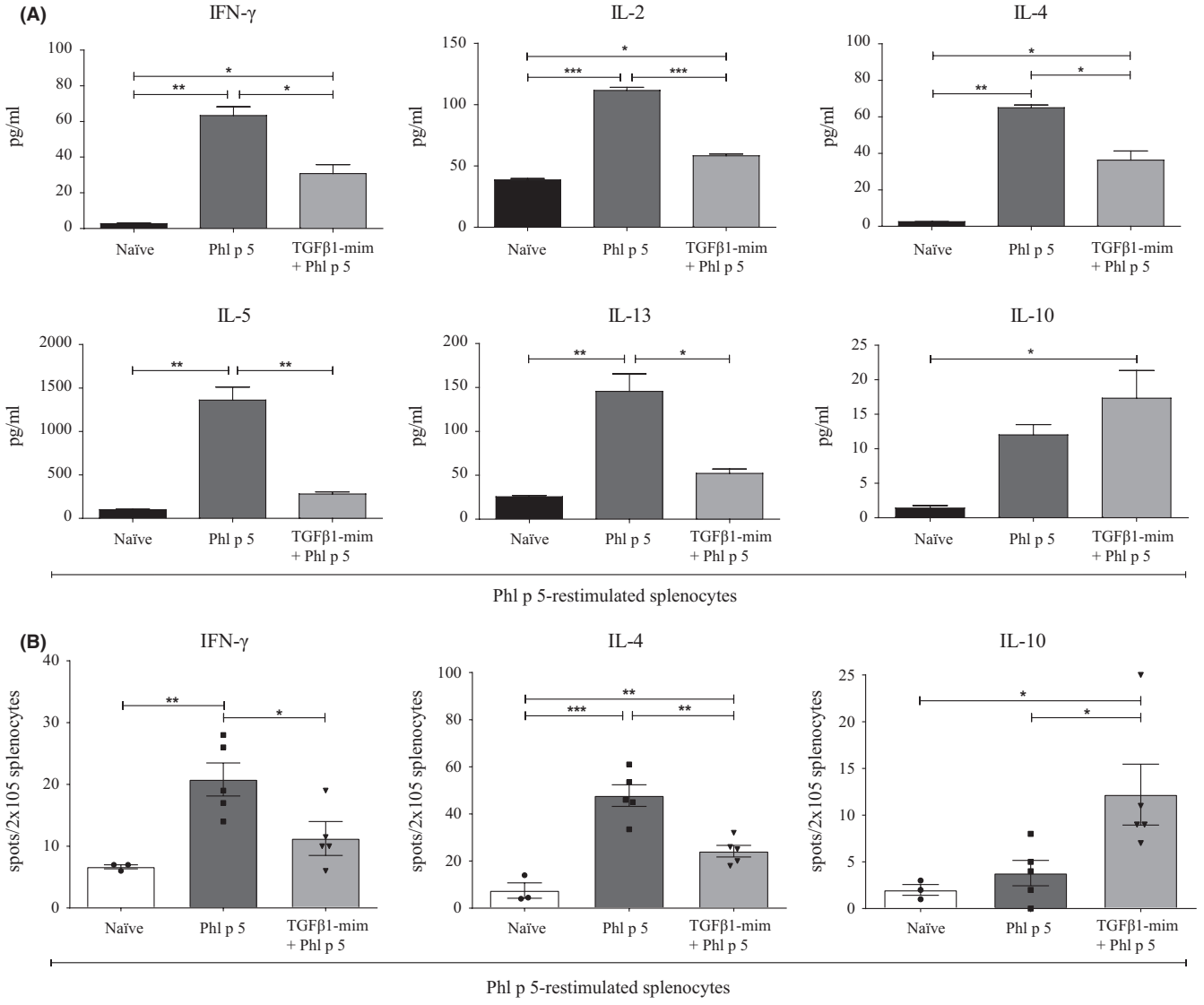

FIGURE 3 TGFβ1-mim peptide modulates Th1 and Th2 responses in vivo. A, Supernatant from Phl p 5-restimulated splenocytes assessed by multiplex bead-based flow cytometry had significant reduction in the levels of secreted IFN-γ, IL-2, IL-4, IL5 and IL-13, and induction of IL-10 in mice that received the TGFβ1-mim peptide. Multiplex flow cytometry results were obtained on pooled samples. B, Phl p 5-restimulated splenocytes assessed by ELISPOT rendered significant reduction in levels of secreted IFN-γ and IL-4, and induction of IL-10 in mice that received the TGFβ1-mim peptide. **P* < .05, ***P* < .01, ****P* < .001

3.5 | TGFβ1-mim suppresses Th2 polarization in vivo

Th2 lymphocytes produce the cytokines IL-4, IL-5, and IL-13, and drive the class switching toward IgE in B cells.[25] To investigate whether the TGFβ1-mim modulated Th2 polarization in the presence of grass pollen extract or rPhl p 5, we used an IL-4 reporter mouse. The 4get mice express eGFP as part of a bicistronic IL-4-IRES-GFP mRNA, allowing the identification of IL-4 expressing cells *in vivo*.[26,27] Flow cytometry analysis of $eGFP^{+}CD4^{+}$ lymphocytes isolated from skin-draining inguinal lymph node cells of 4get mice 5 days after allergen injection (Figure 5A) showed that TGFβ1-mim administered alongside the grass pollen extract suppressed Th2 polarization (Figure 5B-C). Phl p 5 injected alone did not induce Th2 polarization. In summary, the TGFβ1-mim administered in combination with grass pollen extract was able to inhibit Th2 polarization in vivo.

4 | DISCUSSION

For decades the Th1/Th2 balance has been the predominant concept for a healthy immune system. Accordingly, allergy has been proposed to result from a shift in the Th1/Th2 balance in favor of a Th2-biased response. In this respect, activated Th2 lymphocytes produce IL-4, IL-5, and IL-13, subsequently inducing B cells to produce allergen-specific IgE antibodies. More recently, there has been increasing evidence that other T helper cell subsets such as Th9, Th17, and Th22 participate in the progression of certain forms of T cell-mediated allergic

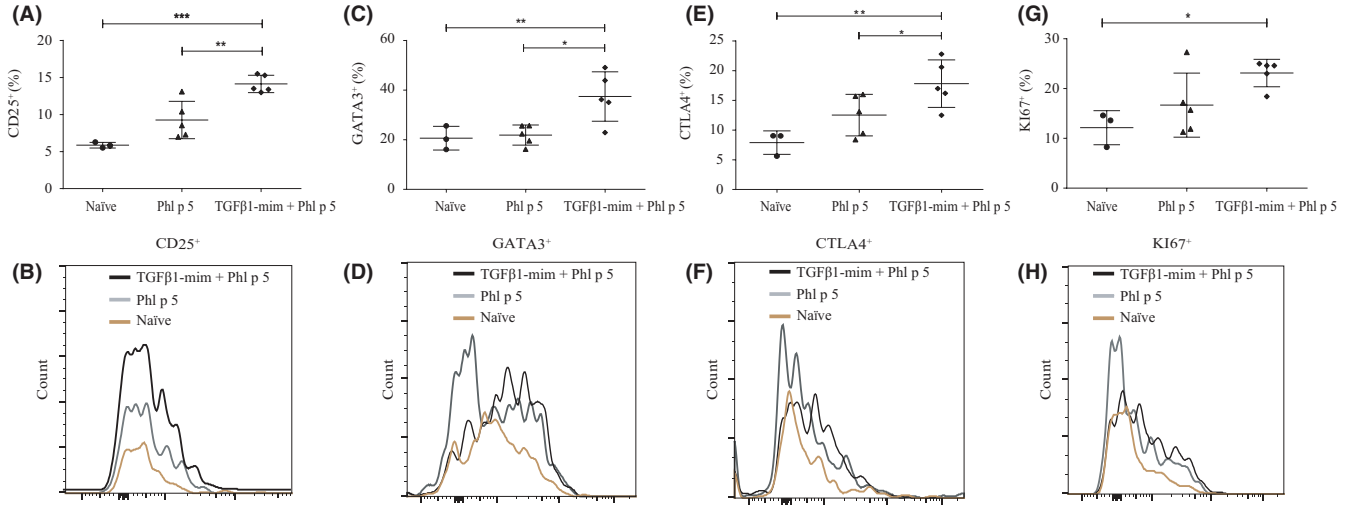

FIGURE 4 TGFβ1-mim peptide induces Treg cell in vivo. Frequency of CD4 Foxp3 T cells expressing CD25 (A-B), GATA3 (C-D), CTLA4 (E-F) and KI67 (G-H) in splenocytes was enriched in mice that received the TGFβ1-mim peptide. *P < .05, **P < .01, ***P < .001

disorders, suggesting further layers of complexity.[28-30] In addition, extensive data have reported the regulatory effect of antigen-specific Treg cells in the suppression of allergic responses.[31-34] Therefore, approaches aiming at the induction of Treg cells could modulate cytokine production, T cell polarization, and antibody profiles, all shown to be key events in inflammation and allergy. Here, we investigated the immunoregulatory action of a TGFβ1 mimetic peptide in vitro and in mice sensitized either with Phl p 5 or grass pollen extract.

IL-8 is a chemoattractant cytokine produced by a variety of tissue and blood cells that predominantly attract neutrophils to inflammatory sites.[35,36] It has been shown that activated neutrophils accumulate at the site of allergic provocation and have the ability to prime $CD4^+$ T cells,[37] a crucial event for the initiation of allergic inflammation. Grass pollen extract can induce neutrophil immune responses through the secretion of IL-8,[38,39] reinforcing the importance of this cytokine in the allergic response. We showed here that in TNF-α-stimulated lung epithelial cells, TGFβ1-mim suppressed IL-8 gene expression and cytokine secretion. Based on these results, we suggest that the reduction of neutrophil migration by TGFβ1-mim previously described in a mouse model of peritonitis[17] could be explained by its suppressive effects on IL-8 secretion. Similarly, TGFβ1 has been shown to inhibit IL-8 production.[40,41]

In our murine in vivo model, sensitization was induced by Phl p 5. The pretreatment with TGFβ1-mim followed by Phl p 5 sensitization resulted in the modulation of various events related to the exacerbation of the allergic response. Firstly, in vivo treatment with TGFβ1-mim significantly inhibited antigen-specific IgE and IgG1 antibody production and suppressed degranulation in basophils passively sensitized with sera from Phl p 5-sensitized mice. TGFβ1-mim enhanced IgA production, consistent with other studies reporting TGFβ1 as an IgA-specific class-switching factor.[42-44] Furthermore, allergic disorders appear to be more common among patients with deficiency for IgA.[45,46] We also observed that sera from mice treated with TGFβ1-mim had enhanced IgE-allergen blocking capacity (data not shown), which will be further investigated in future studies.

TGFβ1-mim efficiently modulated IFN-γ and IL-4 cytokine production in Phl p-5-restimulated splenocytes. Since IFN-γ and IL-4 are marker cytokines for Th1 and Th2 cells, respectively, and play major roles in the development and regulation of immune responses,[47] the suppression of these two cytokines strongly suggests that the TGFβ1-mim treatment suppressed the allergen-induced inflammatory response. In parallel, the TGFβ1-mim treatment significantly increased the number of IL-10-secreting cells. IL-10 downregulates the development of cell-mediated immune responses. It has been previously shown in a murine asthma model that the suppression of allergic airway disease correlates with an increase of $IL\text{-}10^+CD4^+$ cells.[48,49] In addition, Phl p-5-restimulated splenocytes from mice treated with TGFβ1-mim showed reduced levels of secreted IFN-γ, IL-2, IL-4, IL-5, and IL-13 cytokines, which act in concert to determine T helper polarization.[10]

The induction of peripheral T cell tolerance and production of Treg cells are key mechanisms in allergen-specific immunotherapy.[34,50] T cell-specific deletion of TGFβRII in mice showed that TGFβ signaling is required for the maintenance and survival of peripheral Foxp3-expressing Treg cells.[51] In addition, treatment with TGFβ1 induces Foxp3 expression.[17,52,53] Here, we found that mice administration of TGFβ1-mim during Phl p 5 sensitization induced $CD4^+Foxp3^+$ Treg cells expressing CD25, GATA3, CTLA4, and the proliferation-associated nuclear antigen KI67, important factors required for Treg cell survival and function. Treg cells characterized by the expression of CD4, CD25, and Foxp3 are a subpopulation of $CD4^+$ T cells specialized to modulate the immune system, maintain immune tolerance, and prevent autoimmunity.[34,54,55] Intrinsic expression of GATA3 controls Treg cell polarization and maintains Treg cell Foxp3 expression during inflammation in mice.[56] CTLA-4 is an inhibitory receptor crucial for the suppressive function of Treg cells in vivo. A specific deficiency of CTLA-4 in Treg cells results in spontaneous development of systemic lymphoproliferation, lethal T cell-mediated autoimmune disease, and hyperproduction of IgE in mice.[57] Since the balance between allergen-specific Treg cells and

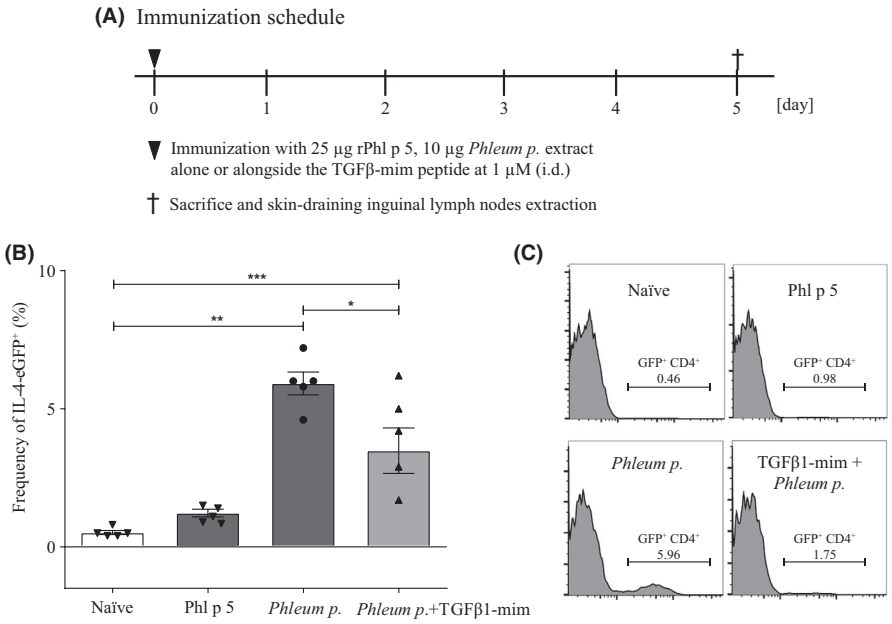

FIGURE 5 TGFβ1-mim peptide inhibits Th2 polarization in IL-4 reporter mouse model. (A) 4get mice were sacrificed 5 days after allergen injection to analyze the frequency of CD4+ EGFP+ expressing skindraining inguinal lymph node cells. (B-C) TGFβ1-mim administered alongside the grass pollen extract was able to downregulate Th2 polarization. Phl p 5 by itself was not able to induce a significant Th2 polarization. *P < .05, **P < .01, ***P < .001. i.d., intradermal

Th2 seems to play a decisive role in the development of the immune response to allergens,[58] we hypothesize that the production of Treg cell induced by the TGFβ1-mim in Phl p 5-sensitized mice suppressed Th2 polarization. It should be pointed out that besides the herein described Treg phenotypes, there are additional Treg-specific markers recently described allowing for a more thoroughly characterization of Treg subsets known to be associated with TGFβ and the induction of allergen tolerance. These include the transcription factor Helios, which is expressed in a large subset of Foxp3$^+$ Tregs, the activation-dependent surface marker TIGIT, and the leukocyte differentiation antigen CD226.[34,59,60] The capacity of TGFβ1-mim to induce Treg cells will be further explored in future studies.

In order to investigate the modulatory capacity of the TGFβ1-mim on Th2 polarization in vivo, we used the 4get mouse model, which possesses a bicistronic mRNA linking a readily identifiable reporter (eGFP) to the IL-4 gene expression.[26,27,61,62] In this model, recombinant Phl p 5 alone was not able to induce expression of eGFP in CD4$^+$ T cells. In a recent study, we could show that birch pollen extracts promoted Th2 polarization in the 4get mouse model whereas purified recombinant Bet v 1 did not. These findings led us to hypothesize that the context in which an allergen is presented to the immune system plays a crucial role in the outcome of the allergen-specific immune response.[27] In line with these observations, here we showed that grass pollen extract induced Th2 polarization in the 4get mice but not rPhl p 5, thus reinforcing the idea that the context (adjuvants) provided by the pollen is crucial in the initiation of the Th2 polarization.

TGF-β1 has been implicated in the pathogenesis of human fibrosis.[63] Additionally, several cytokines including IFN-γ, IL-4, and IL-13 have been shown to participate in the progression of fibrosis.[64-66] A crucial role of toll-like receptor 4 (TLR4) in fibrosis has also been suggested.[67] Although the implication of the TGFβ1-mim in the induction of fibrosis was not directly addressed in this study, we found that under in vivo inflammatory conditions induced by allergen sensitization, the mimetic was able to efficiently downregulate the production of IFN-γ, IL-4, and IL-13 (Figure 3). We also observed that the mimetic was able to downregulate the LPS-induced TLR4 expression in a human TL4 reporter cell line (data not shown). In this respect, overexpression of TLR4 after LPS challenge in mice or mouse lung fibroblasts was shown to significantly contribute to the induction of pulmonary fibrosis.[68,69] Taken together, we speculate that the ability to inhibit crucial players (IFN-γ, IL-4, and IL-13 cytokines; TLR4 expression) involved in the induction of fibrosis rather supports the idea of a beneficial regulatory property for the mimetic peptide. Nevertheless, the possible role of the mimetic in fibrosis needs to be fully investigated before clinical applications in humans.

In summary, we have shown a regulatory role of a TGFβ1 mimetic peptide in the modulation of the inflammatory allergic response. TGFβ1-mim regulated Th1 and Th2 responses *via* the modulation of cytokines and antibodies, induced Treg cell differentiation, and inhibited basophil degranulation. We propose that the application of TGFβ1 mimetic peptide prior to allergen vaccination might enhance the efficacy of conventional AIT protocols by modulating the allergic inflammation and increasing tolerance to the sensitizing allergen. Future studies should explore the immunomodulatory potential of the TGFβ1-mim within other models of allergic sensitization (eg, food and house dust mite) as well as different routes of application (eg, mucosal and transdermal).

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CONFLICT OF INTEREST

F. Ferreira is a member of Scientific Advisory Boards (HAL Allergy, NL; SIAF, Davos, CH; AllergenOnline, USA). The remaining authors declare that they have no relevant conflicts of interest.

AUTHOR CONTRIBUTIONS

GRA and LA performed immunoassays, cell culture, in vivo experiments and analysis. YM performed in vivo experiments. ERV performed RBL experiments. SH performed IgE-allergen blocking capacity analyses based on the IgE-facilitated allergen binding (FAB) assay. MH analyzed IL-8 expression on A549 cells. GRA, LA, AD, LRG, and FF wrote the manuscript. FF supervised all aspects of the study. All authors read and approved the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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TGFβ1 mimetic peptide modulates immune response to grass pollen allergens in mice

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Methods

TGFβ1-mim peptide synthesis and detection on the surface of Jurkat cells

The TGFβ1-mim peptide originally composed of seven amino acids was constructed with fourteen residues and contained two cysteine residues to induce disulfide bond formation and folding. Synthetic sequence: AC<u>ESPLKRQ</u>CGGGS (original peptide is underlined).

The capacity of the TGFβ1-mim peptide to recognize the TGFβ1 receptor on Jurkat cells (ATCC, LGC Promochem, Wesel, Germany) was evaluated by ELISA. Maxisorp microtiter 96-well plates (Nunc, Roskilde, Denmark) were coated with $1 \times l0^6$ cells diluted in PBS, and incubated overnight at 4°C. Cells were blocked with 3% PBS-BSA for 1 hour at 37°C, washed once with PBS and incubated with either 1 µg/ml of the TGFβ1-mim peptide or 5ng/ml of recombinant TGFβ1 (eBioscience, Vienna, Austria) for one hour at 37°C. After washing, the plate was incubated for 1 h at 37°C with anti-human TGFβ (eBioscience, Vienna, Austria). Plate was washed and incubated with HRP-labeled anti-human IgG (Southern Biotech, Birmingham, USA) antibody for 1 h at 37°C. After washing, TMB substrate solution (Sigma, Vienna, Austria) was added to the plate, the reaction stopped by 2N H_2SO_4 solution and read in a microplate reader (Tecan reader infinite m200).

IL-8 expression analysis

A549 cells used in this experiment were derived from a lung epithelial carcinoma and produce a range of cytokines that are typical for epithelial cells upon suitable stimulation. The experiment was conducted according to the original protocol with modifications [1]. These cells are cultured in RPMI 1640 without l-glutamine, supplemented with l-glutamine, penicillin/streptomycin, and 10% FCS. All cell culture reagents were obtained from PAA Laboratories (Pasching, Austria). A total of 4×10^5/ml were cultured in the presence of G418 (0.5 mg/ml) overnight at 37°C and 5% CO_2. Cells were pre-treated with 1µM TGFβ1-mim peptide or recombinant TGFβ1 for 1h, followed by addition of 20ng/ml TNF-α (GenScript, Piscataway, USA), or left untreated for 24 h at 37°C and 5% CO_2. Supernatant was removed and 50 µl lysis buffer (100 mM potassium phosphate pH 7.8, 0.1% Triton, 1 mM DTT) was added to the cells and incubated for 10 min at 37°C and 5% CO_2. Lysed cells were transferred to white 96-well microtiter plates and the luciferase signal was detected using a microplate reader (Tecan reader infinite m200)

immediately after lysis by using a freshly prepared D-luciferin substrate (Synchem, Felsberg, Germay).

RBL cell mediator release assay

For mediator release assays, RBL-2H3 transfected with the alpha chain of the mouse or human IgE receptor were passively sensitized with IgE antibodies from Phlp 5-sensitized mice or grass pollen allergic patients, respectively, according as previously described [2] with modifications. a Briefly, a total of 4×10^4 cells/well diluted in RPMI 1640 (supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 U penicillin and streptomycin/ml, 4 mM l-glutamine, 2 mM sodium pyruvate, 10 mM HEPES and 100 µM of 2-mercaptoethanol) were plated into 96-well tissue culture plates (Becton-Dickinson, Franklin Lakes, NJ, USA) and sensitized with individual sera from the different grass pollen allergic patients diluted at 1:20 by overnight incubation at 37°C and 7.5% CO_2. Subsequently, cells were washed twice with Tyrode's buffer (19 g/L Tyrode´s salt, 1 g/L $NaHCO_3$, pH 7.2) supplemented with 0.1% BSA (Sigma-Aldrich, St. Louis, MO, USA) and incubated with the TGFβ1-mim peptide diluted at 1µM for 24 hours at 37°C and 7.5% CO_2. For allergen stimulation, cross-linking of FcεRI-bound IgE was induced by addition of Phl p 5 diluted in Tyrode's buffer in 1:10 dilutions starting at 1µM/ml for 1 hour at 37°C and 7.5% CO_2. For maximal β-hexosaminidase release, 10 µl 10% Triton X-100 was added to the wells containing cells only. Thereafter, 50 µl of supernatants was transferred to a 96-well plate (Greiner) and incubated with 50 µl 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide (4-MUG) (Sigma, Deisenhofen, Germany) in citrate buffer (0.1 M, pH 4.5) for 1 hour at conditions indicated above. The reaction was stopped by adding 100 µl glycine buffer (0.2 M glycine and 0.2 M NaCl, pH 10.7) and fluorescence was measured at 465–360 nm using a microplate reader (Tecan reader infinite m200). For muRBL assay, a total of 4×10^4 cells/well diluted in RPMI 1640 (supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 U penicillin and streptomycin/ml, 4 mM l-glutamine, 2 mM sodium pyruvate, 10 mM HEPES and 100 µM of 2-mercaptoethanol) were plated into 96-well tissue culture plates (Becton-Dickinson, Franklin Lakes, NJ, USA) and sensitized with individual sera from the different immunization groups or naïve mice at a final dilution of 1:20 by overnight incubation at 37°C and 7.5% CO_2. Subsequently, cells were washed twice with Tyrode's buffer (19 g/L Tyrode´s salt, 1 g/L $NaHCO_3$, pH 7.2) supplemented with 0.1% BSA (Sigma-Aldrich, St. Louis, MO, USA). For stimulation, cross-linking of FcεRI-bound IgE was induced by addition of Phl p 5 diluted at

1ng/ml in Tyrode's buffer for 1 hour at 37°C and 7.5% CO_2. For maximal β-hexosaminidase release, 10 µl 10% Triton X-100 was added to the wells containing cells only. Thereafter, 50 µl of supernatants was transferred to a 96-well plate (Greiner) and incubated with 50 µl 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide (4-MUG) (Sigma, Deisenhofen, Germany) in citrate buffer (0.1 M, pH 4.5) for 1 hour at conditions indicated above. The reaction was stopped by adding 100 µl glycine buffer (0.2 M glycine and 0.2 M NaCl, pH 10.7) and fluorescence was measured at 465–360 nm using a microplate reader (Tecan reader infinite m200). The specific release was calculated in relation to the percentage of total β-hexosaminidase content that was obtained by lysing the cells with Triton X-100 (100%).

Phl p 5-specific IgE, IgG1, IgG2a and IgA antibodies detection by ELISA

For Phl p 5-specific antibody detection, maxisorp ELISA plates (Nunc, Thermo Fisher, Waltham, USA) were coated with Phl p 5 dilute in PBS at 2 µg/ml overnight at 4°C. After blocking and washing, plates were incubated with sera titrated using a starting dilution of 1:200, 1:50 or 1:3 for IgG1, IgG2a or IgA, respectively, following 1:2 dilution series for 2h at RT. Bound IgG1 was detected by an AP-conjugated rat anti-mouse IgG1 antibody and mouse IgG2a was detected using an AP-conjugated rat anti-mouse IgG2a antibody (all Southern Biotech, Birmingham, USA). Bound IgA was detected by an HRP-conjugated goat anti-mouse IgA antibody (Bio-Rad, Puchheim, Germany). After incubation for 1h at 37°C and 1h at 4°C, colorimetric detection with 10 mM 4-nitrophenyl phosphate (Sigma-Aldrich, St. Louis, MO, USA) was performed.
In order to enhance IgE detection, total IgG was captured from sera by using protein G sepharose beads (GE Healthcare), following the manufactures' protocol. After IgG removal, sera titrated out starting from 1:20 were added to the plates previously coated with Phl p 5. Bound IgE was detected by an AP-conjugated rat anti-mouse IgE antibody (Southern Biotech, Birmingham, USA). After incubation for 1h at 37°C and 1h at 4°C, IgE detection was performed by 10 mM 4-nitrophenyl phosphate or TMB (Sigma, Wien, Austria). As a cutoff value the LOD (limit of detection) was used, which is calculated as three times the mean of the background. ELISA results are presented as endpoint titer, which was determined using the limit of quantification (10 times the standard deviation) adapted from a protocol described elsewhere [3]. All measurements were performed in duplicates.

Isolation of splenocytes and lymphocytes

Spleens were aseptically removed immediately after mice sacrifice, disrupted and sedimented for 5 min in 1 ml minimal essential culture medium (MEM). Lysis of erythrocytes was performed by adding 5 ml ammonium chloride potassium (ACK) lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA in ddH_2O, pH 7.2) for 5 min at RT. White blood cells were collected by centrifugation at 300 × g for 5 minutes at RT. Cells were resuspended in proliferation medium (MEM, 1% (v/v) heat-inactivated fetal calf serum, 2mM L-glutamine, 100U penicillin, and streptomycin/ml, 20mM Hepes, 1mM sodium pyruvate, 2 µM of 2-mercaptoethanol, and 1x non-essential amino acids) and used in the subsequent experiments. Skin-draining inguinal lymph nodes of 4get mice were collected in PBS. Lymph node surrounding fat tissue was removed and lymphocytes obtained using a tissue grinder.

Splenocyte stimulation

Splenocytes diluted at a density of 2×10^5 in proliferation medium were added to a 96 well plate (BD Falcon) with Phl p 5 (20 µg/ml). The plates were incubated for 5 days at 37ºC, 5% CO_2, and 95% relative humidity, and then submitted to flow cytometry analysis. To quantify cytokine levels in the supernatant of splenocytes after Phl p 5 stimulation, a multiplex bead-based flow cytometry analysis was performed using the mouse Th1/Th2/Th17/Th22 13plex FlowCytomix Multiplex kit (eBioscience) according to the manufacturer's protocol. Measurements were performed on a Luminex MAGPIX instrument.

Flow cytometry analysis

After five days of stimulation, proliferating splenocytes were stained with anti-mouse CD25 (PE-conjugated), anti-mouse CD4 (V500-conjugated), and anti-mouse CTL4 (APC-conjugated) (all eBioscience, Vienna, Austria) at 4ºC for 30 minutes in the dark. For intracellular staining, a fixation/permeabilization buffer set was used according to the manufacturer's protocol (eBioscience, Vienna, Austria). Cells were washed with dPBS and incubated with fixation/permeabilization solution for 30 minutes at 4ºC in the dark. Cells were centrifuged at 1200 rpm for 5 min, washed twice with permeabilization solution and incubated with anti-mouse foxp3 (BV421-conjugated) and anti-mouse Ki-67 (PE/Cy7-conjugated) (Both BioLegend, San Diego, USA) for 30 minutes at 4ºC in the dark. Cells were washed twice with permeabilization solution and ressuspended in FACS buffer (1X PBS, 3mM EDTA). Fixable Viability Dye eFluor™ 780 (eBioscience) was used for live/dead discrimination. Doublet discrimination was

performed by gating FSC width versus FSC height, and SSC height versus SSC area. Only living CD4+ splenocytes were used for further analysis. The gating strategy for Treg characterization is shown in Figure E3. Extracted lymphocytes from 4get mice were incubated with APC-conjugated anti-mouse CD4 antibody for 30 minutes at 4ºC in the dark (eBioscience, Vienna, Austria). Measurements were performed on a FACS Canto II flow cytometer (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) and data analysis was performed using BD FACSDiva software (BD 78 Biosciences).

ELISPOT

For IFN-γ, IL-4 and IL-10 cytokines detection in splenocytes, 96 multiscreen filtration plates (Millipore) were activated with 70% ethanol for 10 minutes, washed 3 times with PBS, and coated with 2 µg/ml with anti-mouse IFN-γ (BioLegend), anti-mouse IL-4 (eBioscience), or anti-mouse IL-10 (BioLegend) overnight at 4°C in humidified chamber. Plates were washed 3 times with PBS and incubated for 2 hours with blocking solution (Proliferation medium supplemented with 5% fetal bovine serum) in humidified chamber at RT. Splenocytes were diluted at a density of 2×10^5 in proliferation medium, added to each plate in the presence of Phl p 5 diluted at 25 µg/ml, and incubated for 48 h at 37°C and 5% CO_2. After 3 washes with PBS supplemented with 0.1% Tween-20, IFN-γ, IL-4 and IL-10 (all BioLegend) detection biotin anti-mouse antibodies were added to the plates and held for 2h at RT in humidified chamber. After 3 washes, HRP-conjugated streptavidin was added to the plates and incubated for 1h at RT in humidified chamber. After 4 washes, TMB substrate (SeraCare Life Sciences) was added to each plate for 5 min prior to stopping by washing with ddH_2O. Immunospots were counted using the color deconvolution plugin in ImageJ software.

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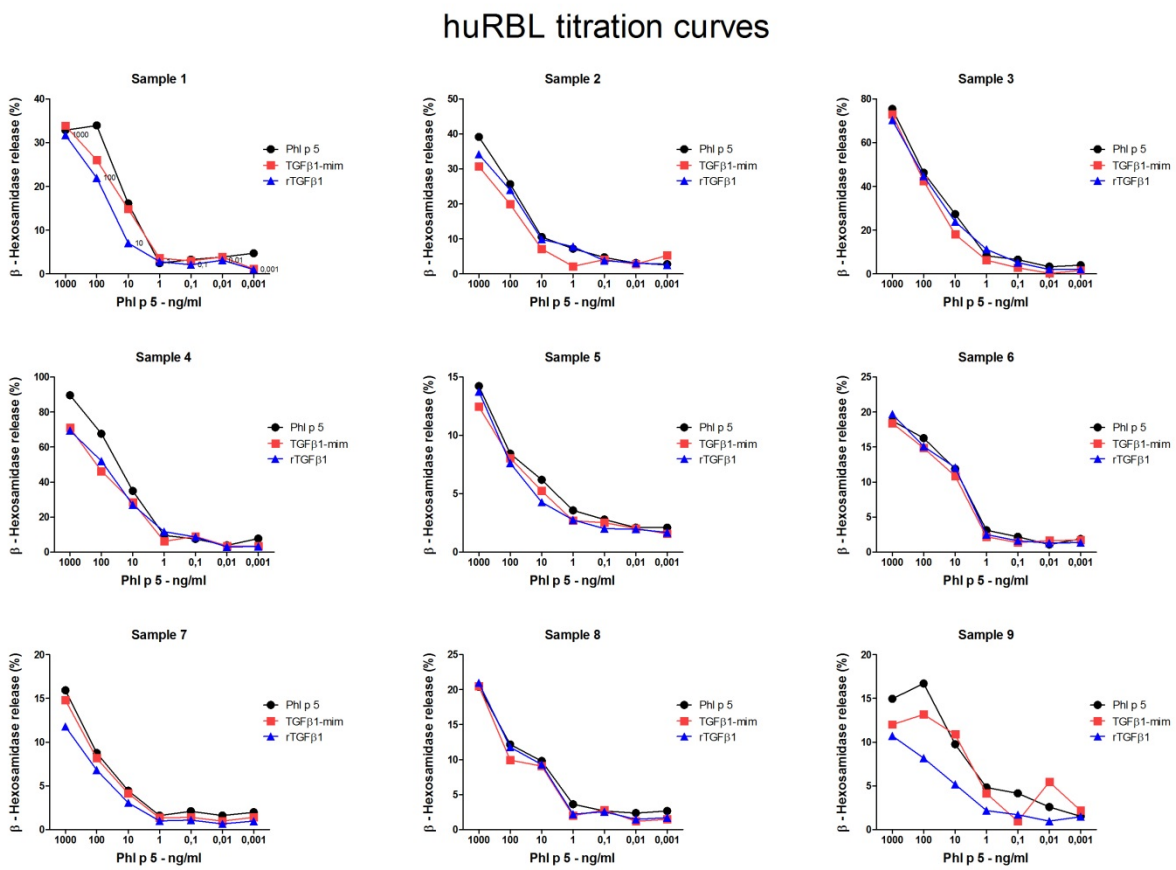

Figure E1. Titration curves of huRBL-2H3 cells encoding the human FcεRI receptor. Cells were sensitized overnight with sera from 9 different grass pollen allergic patients, treated for 24 h either with TGFβ1-mim peptide or recombinant TGFβ1, and then challenged for 1 h with the indicated concentrations of recombinant Phl p 5.

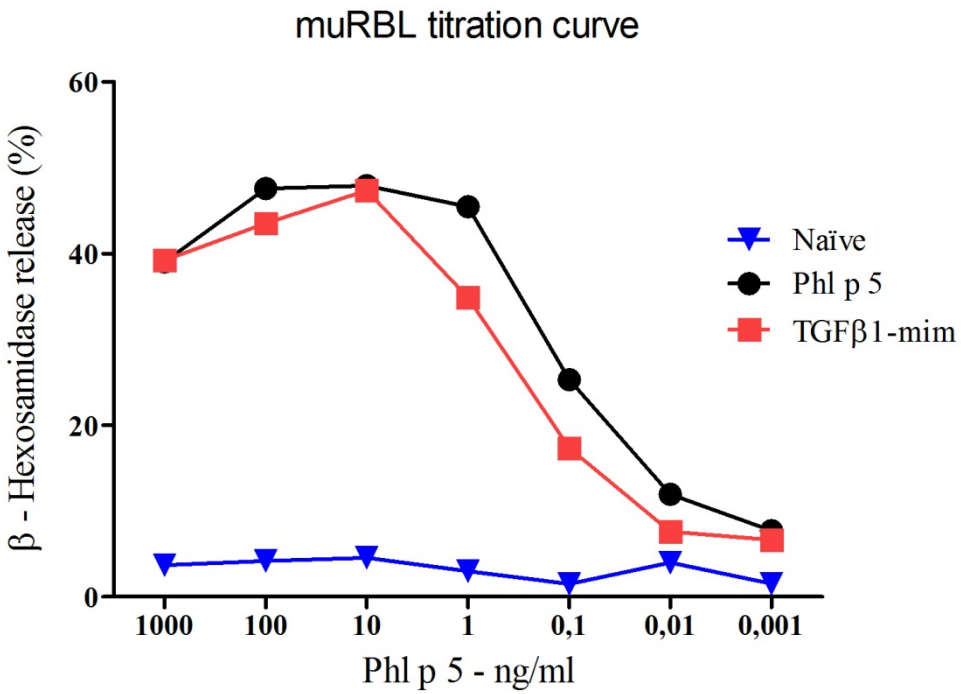

Figure E2. Titration curves of muRBL-2H3. Cells were sensitized overnight with pooled sera from the different immunization groups or naïve mice, and then challenged for 1 h with the indicated concentrations of recombinant Phl p 5. The optimal Phl p 5 concentration (1ng/ml) was used for maximal release assay with individual serum samples.

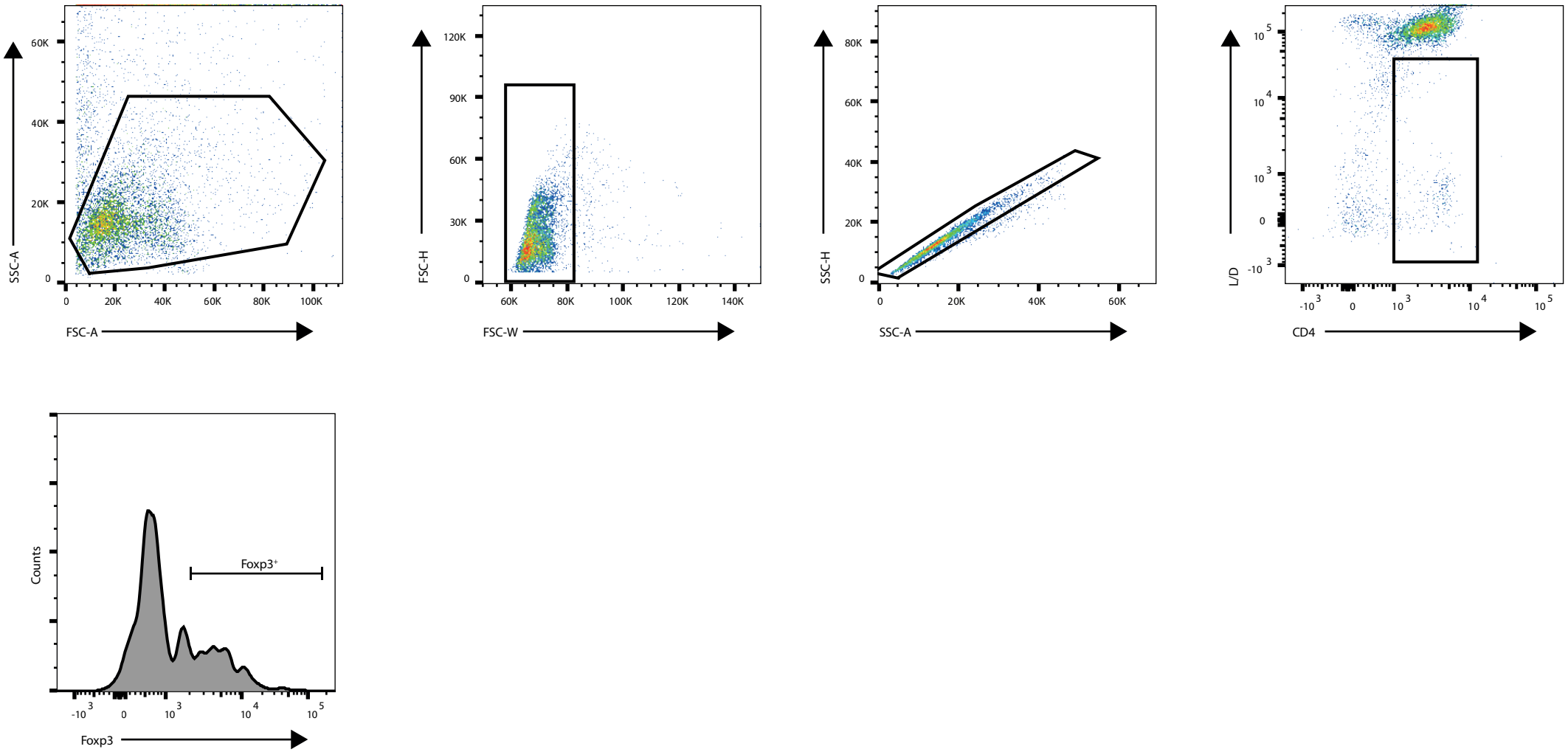

Figure E3. Gating strategy for identification of Treg cells. Splenocytes were gated based on scatter light (FSC, SSC) characteristics, followed by doublet discrimination (FSC width versus FSC height, and SSC height versus SSC area). Living CD4+ cells were gated (dot blot) and analyzed towards Foxp3 expression (histogram).