**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.

**Key words**

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

Allergens are environmental proteins that interact with innate immune receptors, leading to Th2 polarization and consequently IgE production. 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. 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.

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. 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, 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. 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. In mice possessing a disrupted TGFβ1 gene or lacking the TGFβ1 receptor II (TGFβRII), severe inflammatory responses, tissue necrosis, and early death were observed, 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, 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. 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.

MATERIALS AND METHODS

Synthetic peptide

LPS-free TGFβ1-mim (pm26TGFβ1) 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 × 10⁶/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% CO₂. 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 59 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 3Rs22 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 (murRBL) 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 | TGF\(\beta\)1-mim modulates cytokine production and IgE-mediated basophil degranulation in vitro

We first tested the ability of the TGF\(\beta\)1-mim peptide to recognize TGF\(\beta\)RII on Jurkat cells, using ELISA. Recombinant TGF\(\beta\)1 was used as a positive control. Although not statistically significant, TGF\(\beta\)1-mim showed slightly elevated reactivity to TGF\(\beta\)RII than rTGF\(\beta\)1 (Figure 1A). In PMA-stimulated Jurkat cells, both mimetic and rTGF\(\beta\)1 significantly decreased the secretion of TNF-\(\alpha\) (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, 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-\(\alpha\)-stimulated A549 cells, both TGF\(\beta\)1-mim and rTGF\(\beta\)1 decreased IL-8 gene expression (Figure 1D), whereas only TGF\(\beta\)1-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 pre-treated with TGF\(\beta\)1-mim (Figure 1F), indicating an suppression of degranulation. This effect was also observable when pretreating with rTGF\(\beta\)1. Titration curves for every patient are presented in Figure E1. These results indicate that the TGF\(\beta\)1-mim was able to modulate the allergic inflammatory microenvironment in vitro.

3.2 | TGF\(\beta\)1-mim modulates antibody response in vivo

We next tested the ability of the TGF\(\beta\)1-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 \(\mu\)mol/L TGF\(\beta\)1-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, TGF\(\beta\)1-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 TGF\(\beta\)1-mim also showed significant higher levels of allergen-specific IgA (Figure 2F). Therefore, TGF\(\beta\)1-mim modulated the Phl p 5-specific antibody responses in mice.

3.3 | TGF\(\beta\)1-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-\(\gamma\), IL-2, IL-4, IL-5, and IL-13 was significantly lower, whereas secretion of IL-10 was higher in mice treated with TGF\(\beta\)1-mim than in untreated mice (Figure 3A). To determine the antigen-specific T cell polarization upon TGF\(\beta\)1-mim treatment, the number of IFN-\(\gamma\), 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-\(\gamma\) and IL-4 release upon allergen stimulation. In mice treated with TGF\(\beta\)1-mim, levels of IFN-\(\gamma\) 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, TGF\(\beta\)1-mim downregulated Th1 and Th2 cytokines, and upregulated IL-10.

3.4 | TGF\(\beta\)1-mim induces allergen-specific Treg cell production

To investigate the influence of TGF\(\beta\)1-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. The percentage of CD4\(^+\)CD25\(^+\)Foxp3\(^+\) Treg cells expressing CTLA4 in 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 TGF\(\beta\)1-mim had 36.2 ± 10% (Figure 4C-D). The percentage of CD4\(^+\)Foxp3\(^+\) Treg cells expressing GATA3 in 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 TGF\(\beta\)1-mim had 36.2 ± 10% (Figure 4C-D). The percentage of CD4\(^+\)Foxp3\(^+\) Treg cells expressing GATA3 in 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 TGF\(\beta\)1-mim had 36.2 ± 10% (Figure 4C-D). The percentage of CD4\(^+\)Foxp3\(^+\) Treg cells expressing GATA3 in 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 TGF\(\beta\)1-mim had 36.2 ± 10% (Figure 4C-D). The percentage of CD4\(^+\)Foxp3\(^+\) Treg cells expressing GATA3 in 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 TGF\(\beta\)1-mim had 36.2 ± 10% (Figure 4C-D). The percentage of CD4\(^+\)Foxp3\(^+\) Treg cells expressing GATA3 in 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 TGF\(\beta\)1-mim had 36.2 ± 10% (Figure 4C-D). The percentage of CD4\(^+\)Foxp3\(^+\) Treg cells expressing GATA3 in 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 TGF\(\beta\)1-mim had 36.2 ± 10% (Figure 4C-D). The percentage of CD4\(^+\)Foxp3\(^+\) Treg cells expressing GATA3 in 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 TGF\(\beta\)1-mim had 36.2 ± 10% (Figure 4C-D). The percentage of CD4\(^+\)Foxp3\(^+\) Treg cells expressing GATA3 in 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 TGF\(\beta\)1-mim had 36.2 ± 10% (Figure 4C-D). The percentage of CD4\(^+\)Foxp3\(^+\) Treg cells expressing GATA3 in 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 TGF\(\beta\)1-mim had 36.2 ± 10% (Figure 4C-D).
**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.
Th2 lymphocytes produce the cytokines IL-4, IL-5, and IL-13, and drive the class switching toward IgE in B cells. 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. 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 responses.
enhanced IgA production, consistent with other studies reporting
sensitized with sera from Phl p 5-sensitized mice. TGF
previously described in a mouse model of peritonitis
exacerbation of the allergic response. Firstly, in vivo treatment with
zation resulted in the modulation of various events related to the
inflammation. 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
deficiencies, suggesting further layers of complexity. In addition,
approaches aiming at the induction of Treg cells could modulate cyto-
tokine 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 in-
flammatory sites. 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, reinforcing the impor-
tance of this cytokine in the allergic response. We showed here that
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 could be explained by its suppressive effects on IL-8 secretion. Similarly, TGFβ1 has been shown to inhibit IL-8 production.

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 antibod-
ity production and suppressed degranulation in basophils pas-
vively 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. Furthermore, allergic disorders appear to be more common among patients with deficiency for IgA. 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 produc-
duction 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,
the suppression of these two cytokines strongly suggests that the
TGFβ1-mim treatment suppressed the allergen-induced inflamma-
tory 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-10
cells. In addition, Phl p 5-restimulated splenocytes from mice
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.

The induction of peripheral T cell tolerance and production of
Treg cells are key mechanisms in allergen-specific immunother-
TGFβRII in mice showed that
TGFβ signaling is required for the maintenance and survival of pe-
ipheral Foxp3-expressing T cells. In addition, treatment with
TGFβ1 induces Foxp3 expression. 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 re-
quired 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, main-
maintain immune tolerance, and prevent autoimmunity. Intrinsic
expression of GATA3 controls T cell polarization and maintains
Treg cell Foxp3 expression during inflammation in mice. 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. Since the balance between allergen-specific Treg cells and

**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.
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. 57 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. 57 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. 58, 67 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 high-throughput screening method based on stably transformed human cells was used to determine the immunotoxic effects of fluoranthene and other PAHs. Toxicol in Vitro. 2008;22(5):1301-1310.

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**SUPPORTING INFORMATION**

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

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