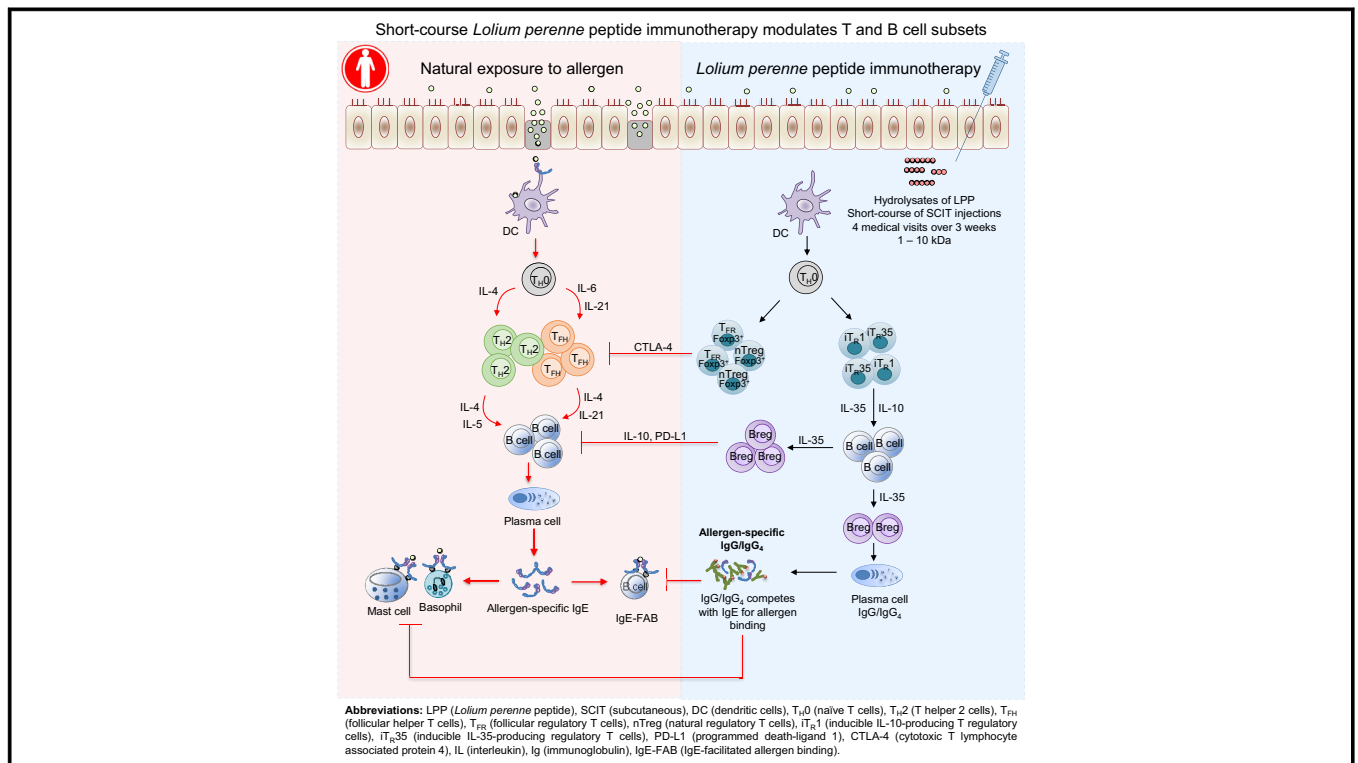


Immunologic mechanisms of a short-course of *Lolium perenne* peptide immunotherapy: A randomized, double-blind, placebo-controlled trial



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GRAPHICAL ABSTRACT



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Background: A 3-week short-course of adjuvant-free hydrolysates of *Lolium perenne* peptide (LPP) immunotherapy for rhinoconjunctivitis with or without asthma over 4 physician visits is safe, well tolerated, and effective.

Objective: We sought to investigate immunologic mechanisms of LPP immunotherapy in a subset of patients who participated in a phase III, multicenter, randomized, double-blind, placebo-controlled trial ([clinicaltrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT02560948) NCT02560948).

Methods: Participants were randomized to receive LPP (n = 21) or placebo (n = 11) for 3 weeks over 4 visits. Grass pollen-induced basophil, T-cell, and B-cell responses were evaluated before treatment (visit [V] 2), at the end of treatment (V6), and after the pollen season (V8).

Results: Combined symptom and rescue medication scores (CSMS) were lower during the peak pollen season (−35.1%, $P = .03$) and throughout the pollen season (−53.7%, $P = .03$) in the LPP-treated group compared with those in the placebo-treated group. Proportions of CD63⁺ and CD203c^{bright}CRTH2⁺ basophils were decreased following LPP treatment at V6 (10 ng/mL, $P < .0001$) and V8 (10 ng/mL, $P < .001$) compared to V2. No change in the placebo-treated group was observed. Blunting of seasonal increases in levels of grass pollen-specific IgE was observed in LPP-treated but not placebo-treated group. LPP immunotherapy, but not placebo, was associated with a reduction in proportions of IL-4⁺ T_H2 (V6, $P = .02$, IL-4⁺ (V6, $P = .003$; V8, $P = .004$), and IL-21⁺ (V6, $P = .003$; V8, $P = .002$) follicular helper T cells. Induction of FoxP3⁺, follicular regulatory T, and IL-10⁺ regulatory B cells were observed at V6 (all $P < .05$) and V8 (all $P < .05$) in LPP-treated group. Induction of regulatory B cells was associated with allergen-neutralizing IgG₄-blocking antibodies.

Conclusion: For the first time, we demonstrate that the immunologic mechanisms of LPP immunotherapy are underscored by immune modulation in the T- and B-cell compartments, which is necessary for its effect. (J Allergy Clin Immunol 2019;144:738-49.)

Key words: Allergy, peptide immunotherapy, follicular helper T cells, regulatory T cells, regulatory B cells

Conventional allergen-specific immunotherapy (AIT) using purified whole aeroallergen extracts¹ or recombinant allergens² for respiratory allergies is indicated in those patients who do not respond to conventional symptom-relieving medications, such as antihistamines and nasal corticosteroids. AIT is a disease-modifying therapy that requires long-term administration lasting up to 3 years to demonstrate a desirable clinically meaningful and persistent effect.³⁻⁵ The associated risks of adverse effects, including anaphylaxis, and poor patient compliance warrant the development of novel short-course therapeutic strategies for AIT to improve efficiency while reducing side effects and improving adherence. It is important to note that the prevalence of respiratory allergic disease is increasing and denotes a significant health problem and disease burden in both developed and developing countries.^{6,7}

We have characterized purified peptidic fragments of rye grass (*Lolium perenne* peptides [LPP]) suitable for short-course subcutaneous administration ([clinicaltrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT01111279) NCT01111279).⁸ We have performed safety, dose-escalation ([clinicaltrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT02156791) NCT02156791),⁹ and dose-finding ([clinicaltrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT01308021) NCT01308021) studies¹⁰ and identified the optimal treatment

Abbreviations used

AIT:	Allergen-specific immunotherapy
Breg:	Regulatory B
CD40L:	CD40 ligand
CRTH2:	Chemoattractant receptor-homologous molecule expressed on T _H 2 lymphocytes
CSMS:	Combined symptom and rescue medication score
CTLA-4:	Cytotoxic T lymphocyte-associated antigen 4
FoxP3:	Forkhead box P3
GARP:	Glycoprotein A repetitions predominant
iT _R 35:	IL-35-inducible regulatory T
LPP:	<i>Lolium perenne</i> peptide
PD-1:	Programmed cell death protein 1
PL:	Placebo
RDBPC:	Randomized, double-blind, placebo-controlled
RTSS:	Rhinoconjunctivitis total symptom score
sIgE:	Specific IgE
STAT:	Signal transducer and activator of transcription
SATB1:	Special AT-rich sequence-binding protein-1
T _{FH} :	Follicular helper T
T _{FR} :	Follicular regulatory T
Treg:	Regulatory T
V:	Visit

schedule (4 × 2 injections over 3 weeks) to elicit a clinical effect. Due to the extensive cross-reactivity of allergenic components of grass pollen from different species, *L perenne* allergen can be used to treat allergic rhinitis induced by other grasses.¹¹ The advantages over whole-protein allergens¹² are that linear peptides do not bind to IgE and cross-link FcεRI on the surfaces of mast cells and basophils and therefore do not release mediators, such as tryptase and histamine.

Recently, we have evaluated the efficacy of LPP treatment in a prospective, multicenter, randomized, double-blind, placebo-controlled (RDBPC) phase III trial ([ClinicTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT02560948) NCT02560948; EudraCT 2015-002105-11),¹³ which was carried out in 57 different sites in Europe. Three hundred seventy-two adults were treated with LPP and 182 were treated with placebo (PL) based on the medical history of moderate-to-severe seasonal allergic rhinoconjunctivitis. A short-course of grass allergen peptide immunotherapy over 3 weeks showed a significant reduction in daily combined symptom and rescue medication scores (CSMS) during the peak pollen season and over the entire season. The study provided useful safety data, improvement in symptoms and quality of life, and a decrease in grass pollen conjunctival provocation test (CPT) scores.¹³ The study was designed to demonstrate the safety and efficacy of LPP and to investigate mechanistic end points by using blood samples from the LPP- and PL-treated groups collected from a single-center site (Belgium).

This substudy was specifically conducted to assess whether LPP immunotherapy would suppress early- and late-phase allergic responses. We wanted to identify the immunologic mechanisms of short-course and fast-acting LPP immunotherapy, as compared to long-term conventional immunotherapy. It has been shown that conventional immunotherapy results in the production of blocking antibodies, induction of regulatory cells, and immune deviation toward a T_H1 response.¹⁴

Therefore we hypothesized that short-course LPP immunotherapy leads to suppression of the early allergic effector cell

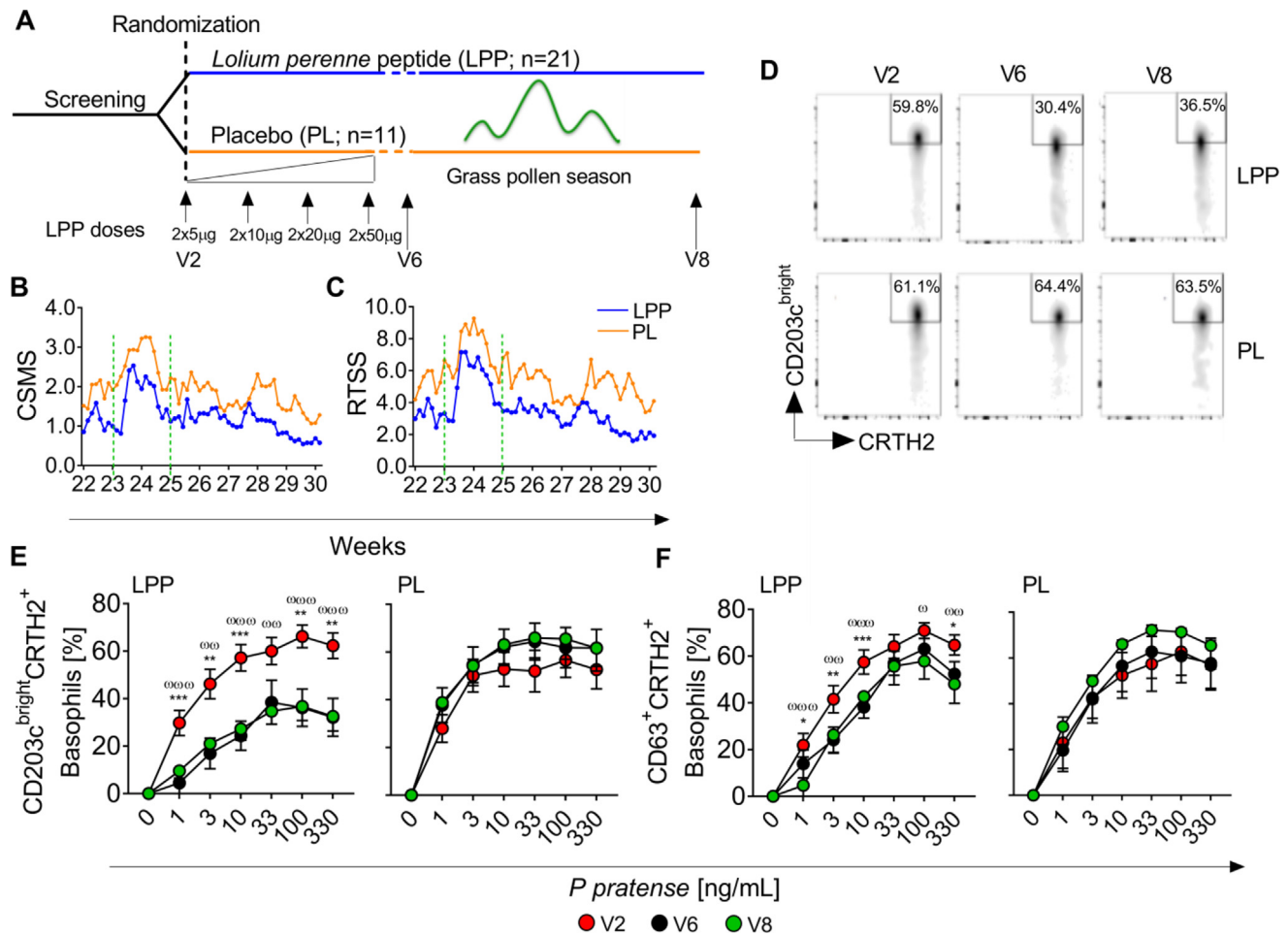


FIG 1. Reduction of basophil activation following LPP immunotherapy. **A**, Study design for patients with grass pollen-related allergic rhinitis in the RDBPC phase III trial. **B**, Reduction in daily CSMS in Belgium was -35.1% ($P = .03$) during the peak period and -53.7% ($P = .03$) during the entire pollen season in LPP-treated group ($n = 21$) compared to PL-treated group ($n = 11$). **C**, Reduction in RTSS in LPP-treated group in Belgium during the peak period was -27.4% ($P = .04$) and -56.9% ($P = .01$) during the entire pollen season. **D**, Grass pollen-induced basophil reactivity in LPP- and PL-treated groups showed surface activation markers CD63 and CD203c on CRTH2⁺ basophils. Representative plots of CD203c^{bright}CRTH2⁺ basophils of LPP-treated ($n = 21$) or PL-treated ($n = 11$) patients at V2, V6, and V8 are shown. **E** and **F**, Dose-dependent response of CD203c^{bright}CRTH2⁺ (Fig 1, **E**) and CD63⁺CRTH2⁺ (Fig 1, **F**) basophils in LPP- and PL-treated groups at V2, V6, and V8. Green dotted lines represent the peak pollen season. *Statistical significance for V2 versus V6. ω Statistical significance for V2 versus V8. Data are shown as means $\pm$ SEMs. * and $\omega P < .05$, ** and $\omega\omega P < .01$, and *** and $\omega\omega\omega P < .001$, Mann-Whitney test.

(basophils) response, deletion of proallergic TH2¹⁵ and follicular helper T (T_{FH}) cells,¹⁶ which are known to promote IgE responses, and induction of regulatory T (Treg) cells. We further hypothesized that allergen-neutralizing IgG₄ antibodies that can inhibit allergen-induced basophil responsiveness and CD23-mediated IgE-facilitated allergen presentation are also induced by B cells in LPP- but not PL-treated group.

METHODS

Study design

We assessed the immunologic effect of LPP immunotherapy in a subset of patients from 1 clinical site in Belgium who participated in a prospective, multicenter, RDBPC phase III trial¹³ evaluating the efficacy of LPP in patients

with grass pollen-induced allergic rhinitis with or without asthma. After screening (visit [V] 1), eligible participants ($n = 32$) were randomized 2:1 to receive subcutaneous injections of LPP immunotherapy or PL (Fig 1, **A**; Table 1; and see Fig E1 in this article's Online Repository at www.jacionline.org). Double blinding was maintained for all patients and clinical and laboratory staff throughout the entire duration of the study.

At each treatment visit, the patient received a first injection in one arm, followed by a second injection in the opposite arm 30 minutes later. Doses were increased progressively as follows: $2 \times 5 \mu\text{g}$ for treatment at V2, $2 \times 10 \mu\text{g}$ for treatment at V3, $2 \times 20 \mu\text{g}$ for treatment at V4, and $2 \times 50 \mu\text{g}$ for treatment at V5. A cumulative dose of $170 \mu\text{g}$ of LPP was reached, which appeared to be optimal in a previous dose-finding phase II study.¹⁰ All participants who attended the immunogenicity clinical study site were subjected to blood sampling at V2 (baseline [before treatment]), V6 (after treatment), and V8 (after the pollen season). Daily CSMS were collected from each participant

TABLE I. Patients' demographics

Characteristic	PL-treated group (n = 11)	LPP-treated group (n = 21)
Age (y), mean $\pm$ SD	33.27 $\pm$ 8.26	32.52 $\pm$ 11.19
Sex, no. (%)		
Male	5 (45.50)	8 (38.10)
Female	6 (54.50)	13 (61.90)
Body mass index (kg/m ²), mean $\pm$ SD	23.19 $\pm$ 3.23	23.47 $\pm$ 3.59
Disease duration (y), mean $\pm$ SD	15.73 $\pm$ 9.95	18.19 $\pm$ 10.33
Grass pollen skin prick test (mm), mean $\pm$ SD	5.00 $\pm$ 1.79	6.05 $\pm$ 1.32
Grass pollen sIgE (kU _A /L), mean $\pm$ SD	20.76 $\pm$ 25.58	27.65 $\pm$ 31.89
Total IgE (IU/mL), mean $\pm$ SD	156.44 $\pm$ 211.28	219.83 $\pm$ 173.08
Frequency of allergic rhinitis, no. (%)		
Intermittent	1 (9.1)	0 (0.0)
Persistent	10 (90.9)	21 (100.0)
Asthmatic, no. (%)	1 (9.1)	3 (14.3)
Cosensitization (SPT $\geq$ 3 mm), no. (%)		
None (other than grass)	0 (0.0)	0 (0.0)
Birch	2 (18.2)	8 (38.1)
Cat epithelia	4 (36.4)	2 (9.5)
Dog epithelia	1 (9.1)	3 (14.3)
House dust mite (<i>Dermatophagoides farinae</i>)	1 (9.1)	3 (14.3)
House dust mite (<i>Dermatophagoides pteronyssinus</i>)	2 (18.2)	7 (33.3)

Data are shown for the population with immunogenicity data. IU, International units; kU_A, allergen-specific kilounits.

during the peak (14 consecutive days within weeks 23–25) and the entire (weeks 22–30) pollen season.

Allergen-induced basophil responses

Ex vivo allergen-induced basophil responsiveness was measured by the expression of CD63 and CD203c markers, as previously described.¹⁷ Briefly, 1, 3, 10, 33, 100, and 330 ng/mL *Phleum pratense* were added to heparinized whole blood and incubated at 37°C in a water bath for 15 minutes. Cells were stained with cell-surface antibodies (see the [Methods](#) section in this article's Online Repository at www.jacionline.org). Red blood cells were lysed with BD lysing solution (BD Biosciences, San Jose, Calif) at room temperature in the dark for 10 minutes and fixed with CellFix solution (BD Biosciences) before acquisition on BD FACSCanto II (BD Biosciences).

In vitro T- and B-cell stimulation

For *in vitro* T- and B-cell culture experiments, PBMCs were cultured for up to 6 days with or without *P pratense* or CpG ODN 2006 (1 μ g/mL; InvivoGen, San Diego, Calif) and CD40 ligand (CD40L; 0.01 μ g/mL; R&D Systems, Abingdon, United Kingdom) for up to 48 hours, respectively. To investigate the effect of IL-35 on the induction of regulatory B (Breg) cells, PBMCs were cultured with CD40L (0.01 μ g/mL; R&D Systems) and CpG ODN 2006 (1 μ g/mL; InvivoGen) or LPS (100 ng/mL; Sigma-Aldrich, Dorset, United Kingdom) in the presence or absence of rhIL-35 (100 ng/mL; Enzo Life Sciences, Exeter, United Kingdom) for 48 hours. Cells were washed with culture medium and stimulated with phorbol 12-myristate 13-acetate (50 ng/mL; Sigma-Aldrich) and ionomycin (1 μ g/mL; Sigma-Aldrich) in the presence of monensin (20 μ g/mL; BioLegend, London, United Kingdom) or Brefeldin A (1:10; BD Biosciences) for 5 hours prior to staining. For B-cell culture, cells were blocked with Fc blocking agent (Miltenyi Biotec, Woking, United Kingdom). Cells were immunostained with cell-surface and intracellular antibodies (see the [Methods](#) section in this article's Online Repository) and acquired on BD FACSCanto II and BD LSRFortessa (BD Biosciences).

Serum allergen-specific IgE and IgG₄

Specific IgE (sIgE) and sIgG₄ levels to a grass pollen mixture (*Anthoxanthum odoratum*, *L perenne*, *P pratense*, *Secale cereale*, and *Holcus lanatus*) were measured in serum samples by using the ImmunoCAP system (Thermo Fisher Scientific, Pierce, United Kingdom), according to the manufacturer's instructions.

IgE-facilitated allergen-binding assay

The allergenicity of LPP was tested using IgE-facilitated allergen binding (IgE-FAB) to B cells, as previously described.¹⁸ Sera from allergic patients were preincubated with *P pratense* for 1 hour at 37°C, followed by the addition of 1 $\times$ 10⁵ EBV-transformed B cells (5 μ L) and incubation for 1 hour at 4°C. Binding of allergen-IgE complexes to B cells was determined by using polyclonal human anti-IgE phycoerythrin-labeled antibody (Miltenyi Biotec) and acquired on BD FACSCanto II (BD Biosciences).

Statistical analysis

This study was predominantly a mechanistic study to evaluate the immunologic mechanisms of short-course LPP or PL treatment in a subset of patients who enrolled in the phase III trial¹³ and attended the clinical site in Ghent, Belgium. The phase III study was powered for the primary end point, which was a reduction in CSMS over the pollen peak period.¹³ This study was not a *post hoc* selection of the site and neither of the analyses. The analyses were preplanned and included in the study protocol, and a statistical analysis plan was also predefined and finalized before performing biological analyses. For this study, sample size and power calculation were based on immunologic parameters, including grass pollen-sIgG₄ and serum inhibitory antibody levels, as measured by the IgE-FAB assay obtained from the phase IIa (clinicaltrials.gov NCT02156791)⁹ and phase IIb (clinicaltrials.gov NCT01308021)¹⁰ studies (see [Tables E1 and E2](#) in this article's Online Repository at www.jacionline.org).

Statistical data analysis was performed with GraphPad Prism 7.02 software (GraphPad Software, San Diego, Calif). Nonparametric Mann-Whitney test was used to statistically compare different groups of patients, and nonparametric Wilcoxon paired signed-rank test was used to compare data within the same sample. Normally distributed data were analyzed by using the parametric Welch *t* test. A *P* value of less than .05 was considered statistically significant.

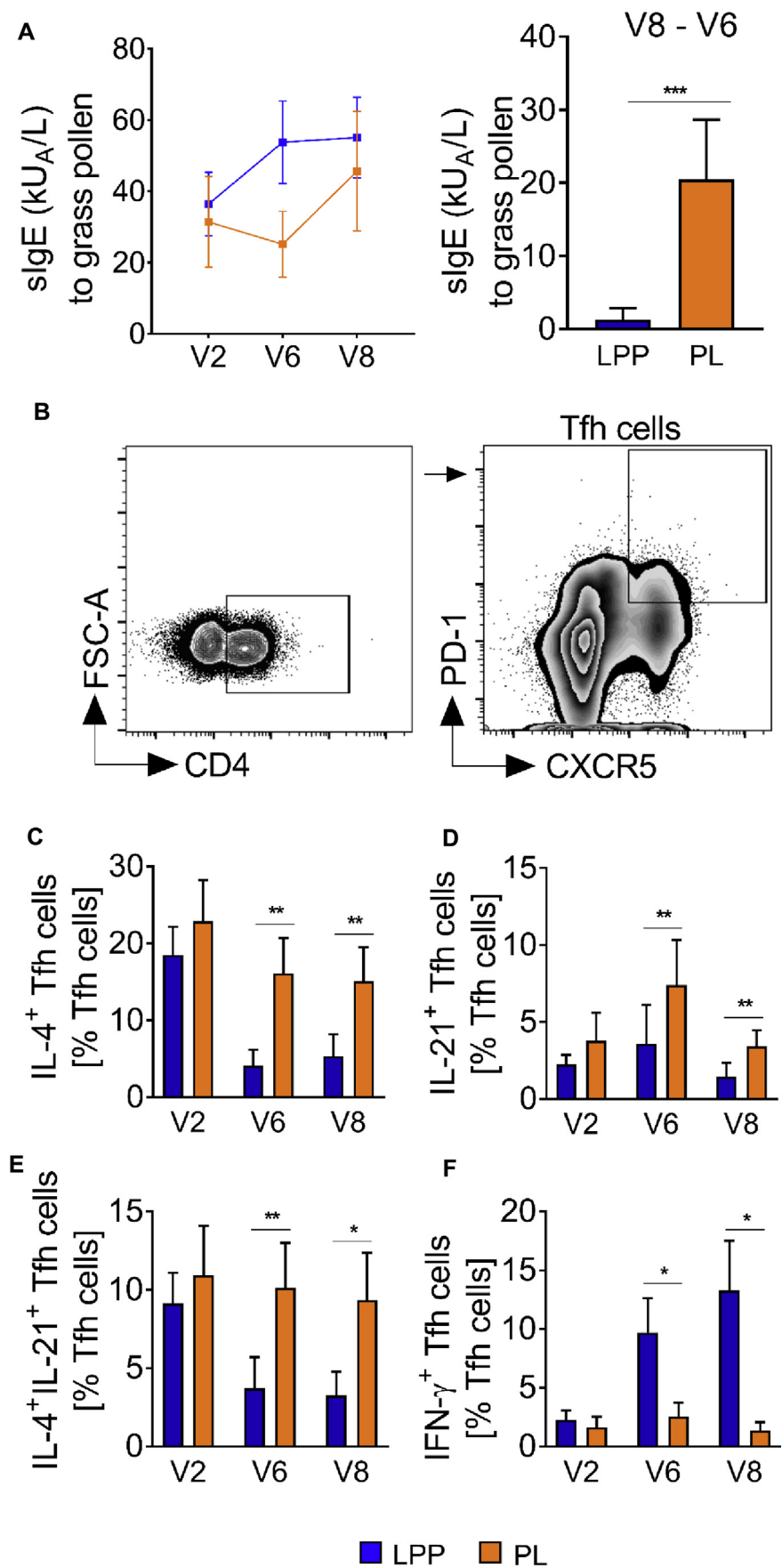
RESULTS

Reduction in symptom scores following LPP treatment

The clinical results of this study have been reported previously.¹³ Briefly, mean CSMS were significantly reduced by 15.5% during the peak pollen season and by 17.9% over the entire season in LPP- but not PL-treated subjects.¹³ In this study mean CSMS and mean rhinoconjunctivitis total symptom scores (RTSS) were also reduced during the peak (*P* = .03 and *P* = .04, respectively) and throughout the entire pollen season (*P* = .03 and *P* = .01; [Fig 1, B and C](#)). The pollen count for Belgium in 2016 is represented in [Fig E2](#) in this article's Online Repository at www.jacionline.org.

LPP immunotherapy, but not PL, inhibits grass pollen-induced basophil responsiveness

The effect of LPP immunotherapy on FcεRI-mediated allergic inflammation, a surrogate end point of the early type I-mediated hypersensitivity reaction, was investigated by measuring basophil responsiveness. At V2, proportions of CD203c^{bright} chemoattractant receptor-homologous molecule expressed on



T_H2 lymphocytes (CRTH2)⁺ (Fig 1, D and E, and see Table E3 in this article's Online Repository at www.jacionline.org) and CD63⁺CRTH2⁺ basophils (Fig 1, F, and see Table E4 in this article's Online Repository at www.jacionline.org) were increased in a dose-dependent manner in both LPP- and PL-treated groups. Interestingly, at V6 and V8, allergen-induced basophil responsiveness was reduced at 1, 3, 10, 33, 100, and 330 ng/mL of grass pollen allergen in LPP-treated group ($P < .05$ compared with V2) but not in the PL-treated group (Fig 1, D and E).

We also investigated the effect of anti-human IgE antibody (1 μ g/mL) on basophil activation after Fc ϵ RI cross-linking in LPP- and PL-treated groups. The proportions of CD203c^{bright}CRTH2⁺ and CD63⁺CRTH2⁺ basophils following Fc ϵ RI cross-linking by anti-human IgE antibody was decreased at V6 and V8 compared with V2 in LPP- but not PL-treated group (see Fig E3 and Table E5 in this article's Online Repository at www.jacionline.org).

Blunting of seasonal increase in grass pollen sIgE levels in LPP- but not PL-treated groups

sIgE to grass pollen mixture was measured in sera of study participants. There was an induction of grass pollen sIgE in LPP- but not PL-treated patients (Fig 2, A, left). However, when the difference in sIgE induction between V6 and V8 (corresponding to the IgE induction following natural exposure during the pollen season) was assessed, sIgE induction in the PL-treated group was significantly higher compared to that in the LPP-treated group ($P = .0004$; Fig 2, A, right).

Attenuation of IL-4-producing T_H2 cells and IL-4-, IL-21-, and dual IL-4 and IL-21-producing T_{FH} cells following LPP immunotherapy but not in PL

Following LPP treatment, there was a significant reduction of IL-4-producing T_H2 (CRTH2⁺CD27⁻) cells at V6 ($P = .02$), but this was lost at V8 in LPP-treated group but not PL-treated group. In contrast, T_H1 (CD4⁺IFN- γ ⁺) cells were significantly higher in LPP-treated group at V6 ($P = .006$) compared with those in PL-treated group, but this was lost at V8 (see Table E6 in this article's Online Repository at www.jacionline.org). Immune deviation from a T_H2 to a T_H1 response has been demonstrated previously in conventional immunotherapy. However, there has been increasing evidence that a subset of T_H cells, called follicular helper T (T_{FH}) cells, also plays a crucial role in the pathology of allergic disease and IgE class-switching.^{19,20} They are defined as CD4⁺ cells that coexpressed CXCR5 and programmed cell death protein 1 (PD-1), and these CD4⁺CXCR5⁺PD-1⁺ cells are henceforth referred to as T_{FH} cells (Fig 2, B). T_{FH} cells secrete IL-4 and IL-21 and have been shown to induce IgE production through signal transducer and activator of transcription (STAT) 3 signaling.²¹ Proportions of IL-4-producing T_{FH} cells were significantly lower in

LPP-treated group compared to those in PL-treated group at V6 and V8 ($P = .003$ and $P = .004$, respectively; Fig 2, C). IL-21-producing T_{FH} cells were significantly lower in LPP- compared to PL-treated group at V6 and V8 ($P = .003$ and $P = .002$, respectively; Fig 2, D). Dual IL-4⁺IL-21⁺ T_{FH} cells were also enumerated, and were significantly lower in LPP-treated group compared to those in PL-treated group at V6 ($P = .004$) and remained low in LPP-treated group at V8 ($P = .01$; Fig 2, E, and see Table E7 in this article's Online Repository at www.jacionline.org). In contrast, IFN- γ -producing T_{FH} cells were significantly higher in LPP-treated group compared to those in PL-treated group at V6 and V8 ($P = .03$ and $P = .01$, respectively; Fig 2, F).

Induction of forkhead box P3-positive Treg and follicular regulatory T cells following LPP immunotherapy but not PL

The regulatory counterparts of T_H cells were investigated. LPP-treated group showed induction of forkhead box P3 (FoxP3)⁺ Treg (CD4⁺CD25⁺CD127^{low}FoxP3⁺; Fig 3, A) cells but not in PL-treated group (V6; $P = .04$); nonetheless, the effect became nonsignificant at V8 (Fig 3, B). We further analyzed the functional counterparts of these Treg cells. Studies have shown glycoprotein A repetitions predominant (GARP) expression and special AT-rich sequence-binding protein-1 (SATB1) repression in Treg cells represent a suppressive subset of Treg cells.^{22,23} GARP⁺ Treg cells were significantly higher in LPP-treated group compared to PL-treated group at V6, and they remained elevated at V8 ($P = .03$ and $P = .01$, respectively; Fig 3, C). This is consistent with the repression of SATB1 within Treg cells, which was greater in LPP-treated group compared to PL-treated group at both V6 ($P = .002$) and V8 ($P = .01$; Fig 3, D, and see Table E8 in this article's Online Repository at www.jacionline.org).

A subset of Treg cells called follicular regulatory T (T_{FR}; CD4⁺CXCR5⁺PD-1⁺FoxP3⁺) cells has been shown to regulate the interaction between B and T_{FH} cells. There were significantly higher T_{FR} cells in LPP-treated group compared to PL-treated group at V6 and V8 ($P = .004$ and $P = .004$, respectively; Fig 3, E and F). T_{FR} cells have also been shown to exert their suppressive ability through the expression of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4).²⁴ Proportions of CTLA-4⁺ T_{FR} cells were significantly higher in LPP-treated group compared to PL-treated group at V6 ($P = .001$) and they remained elevated at V8 ($P = .002$; Fig 3, G, and see Table E9 in this article's Online Repository at www.jacionline.org).

LPP immunotherapy, but not PL, induced IL-35⁺ and IL-10⁺ Treg cells, which promoted Breg cell induction

Induction of IL-35- and IL-10-producing Treg cells upon stimulation with *P pratense* was investigated in PBMCs obtained

FIG 2. LPP inhibits proinflammatory T_{FH} cells. **A**, Levels of grass pollen sIgE (kU_A/L) in serum samples of LPP-treated (n = 21) and PL-treated (n = 11) groups were measured by ImmunoCAP. Difference in sIgE production in both groups was also measured between V8 and V6. PBMCs were isolated from whole blood collected before (V2) and after (V6) the treatment period and after the grass pollen season (V8) and cultured for 6 days in the presence of *P pratense*. **B**, CD4⁺ cells that are CXCR5⁺PD-1⁺ were defined as T_{FH} cells. FSC, Forward scatter. **C-F**, Percentages of IL-4⁺, IL-21⁺, dual IL-4⁺IL-21⁺, and IFN- γ ⁺ T_{FH} cells were assessed within T_{FH} cell population by using FACS. Data are shown as means $\pm$ SEMs. * $P < .05$, ** $P < .01$, and *** $P < .001$, Mann-Whitney test.

from LPP- and PL-treated subjects at V2, V6, and V8. Inducible IL-35⁺ regulatory T (iT_R35) cells were increased in LPP-treated group at V6 ($P = .01$) compared to PL-treated group (Fig 4, A and B). Additionally, proportions of IL-10⁺ Treg cells were significantly increased in LPP-treated group at V6 ($P = .0004$) and V8 ($P = .001$) compared to those in PL-treated group (Fig 4, C, and see Table E10 in this article's Online Repository at www.jacionline.org).

To assess the effect of IL-35 on the conversion of human B cells into Breg cells, PBMCs from patients with grass pollen allergy, independent of the study, were stimulated with LPS or CpG and CD40L in the presence or absence of IL-35. CD19⁺IL-10⁺ B cells were increased when stimulated with CpG in the presence of IL-35 (Fig 4, D). IL-35 significantly increased the proportion of IL-10⁺CD19⁺CD5^{hi}CD1d^{hi} B cells when stimulated with CpG and LPS ($P = .02$ and $P = .03$, respectively), which was decreased in the absence of IL-35 (Fig 4, E).

The frequency of IL-10⁺ cells was measured using FluoroSpot assay in the presence or absence of IL-35. The frequency of IL-10⁺ cells was significantly increased when stimulated with CpG ($P = .002$) and LPS ($P = .002$) in the presence of IL-35 (Fig 4, F). In addition, production of IL-10⁺ Breg cells was assessed in LPP- and PL-treated patients. PBMCs stimulated with CpG and CD40L resulted in an increase in IL-10-producing Breg cell subsets in LPP-treated group compared to PL-treated group. LPP-treated group showed significantly higher production of IL-10⁺CD19⁺ (V6, $P = .002$; V8, $P = .004$), IL-10⁺CD19⁺CD5^{hi} (V6, $P = .0007$; V8, $P = .0008$), IL-10⁺CD19⁺CD5^{hi}CD24^{hi}CD38^{hi} (V6, $P = .0004$; V8, $P = .001$), and IL-10⁺CD19⁺CD27⁺ (V6, $P = .004$; V8, $P = .002$) Breg cell subsets at V6 and V8 compared to PL-treated group (Fig 4, G, and see Table E11 in this article's Online Repository at www.jacionline.org).

Induction of allergen-specific neutralizing/blocking antibodies following LPP treatment

Conventional allergen immunotherapy has been shown to be induced by grass pollen-specific IgG₄ antibodies. We assessed whether such blocking antibodies were induced in LPP- and PL-treated groups. Levels of grass pollen-specific IgG₄ were increased at V6 compared with V2 ($P = .002$; Fig 5, A) and persisted until the end of the pollen season (V8) in LPP-treated group, whereas no change was observed in PL-treated group. The ability of these antibodies to compete for IgE binding to B cells was decreased at V6 in LPP-treated group compared to PL-treated group; however, no difference was observed at V2 and V8 ($P = .02$ at V6; Fig 5, B, and see Table E12 in this article's Online Repository at www.jacionline.org).

Relationship between immune parameters and clinical effect

We assessed the relationship between CSMS, rescue medication scores (RMS), and RTSS with inducible Treg cell subsets (iT_R35 and IL-10⁺ Treg cells). There was a negative correlation observed between iT_R35 cells and RTSS at V6 ($r = -0.60$, $P = .01$), IL-10⁺ Treg cells and CSMS at V6 ($r = -0.52$, $P = .02$) and V8 ($r = -0.45$, $P = .04$), and IL-10⁺ Treg cells and RMS at V8 ($r = -0.46$, $P = .0499$; see Table E13 in this article's Online Repository at www.jacionline.org).

DISCUSSION

Here we show in a RDBPC trial that a 3-week short-course of adjuvant-free hydrolysates of LPPs over 4 medical visits reduce CSMS and RTSS. LPP immunotherapy inhibited allergen-induced basophil responsiveness and reactivity. Blunting of seasonal increases in grass pollen sIgE levels and attenuation of circulating IL-4⁺ T_H2, IL-4⁺, IL-21⁺ and dual IL-4⁺IL-21⁺ T_{FR} cells was observed in LPP-treated patients. Circulating Treg and T_{FR} cells were induced following LPP treatment. Moreover, LPP immunotherapy stimulated iT_R35 cell induction, which favored *de novo* IL-10 production from CD19⁺ B cells and Breg cell subsets. This leads to the production of allergen-neutralizing IgG₄ antibodies that can compete with IgE and prevent allergen-IgE binding to CD23 on the surfaces of B cells. These findings are from a subset of participants in a larger phase III clinical trial¹³ from whom we collected blood samples for mechanistic analysis. The design of the study included a mechanistic analysis in the subset of participants who attended the clinical site in Ghent, Belgium. This was not a *post hoc* selection of the site and neither of the analyses. The mechanistic analyses were preplanned and included in the study protocol. In addition to this, the reported clinical data represent the studied cohort in the single center, and therefore the data need to be considered in the context of the whole study.

The immunologic assays performed throughout this study involved stimulation of PBMCs with *P pratense*. Despite the patients undergoing LPP treatment, previous studies have shown the extensive cross-reactivity among members of the subfamily Pooideae.²⁵ Both *P pratense* (Phl p) and *L perenne* belong to the subfamily Pooideae. Sequence analysis performed on both allergens showed that both *P pratense* and *L perenne* shared an extensive homology. *L perenne* isoallergens shared between 30% and 90% homolog sequences with Phl p 1, which contributes to their cross-reactivity.¹¹ In addition, Phl p 1 fusion protein has been shown to block reactivity of other grass pollen species.¹¹ This demonstrates the cross-reactivity between grass pollen allergens and therefore justifies the use of *P pratense* in *in vitro* assays.

In this study allergen-induced basophil responsiveness was decreased as early as 3 weeks and persisted throughout the grass pollen season. This is a faster response compared to conventional immunotherapy, which takes 6 to 12 months to achieve a similar decrease in basophil activation. CD63⁺ and CD203c^{bright} were used as activation markers. Basophils are activated when IgE receptors cross-link and release allergic effector molecules.¹⁷ We showed that the induction of IgE following LPP treatment during the grass pollen season might be due to the priming effect of the grass pollen season, resulting in IgE production by B cells. This increase has been observed previously as an effect of immunotherapy treatment.²⁶ Despite this increase, the magnitude of IgE production after the pollen season in LPP-treated group was less than that in PL-treated group, suggesting that LPP treatment suppresses T_H2 cell responses, which are responsible for production of IgE by B cells. It was also apparent from IgE levels at baseline that both LPP- and PL-treated groups were moderately to severely allergic to grass pollen allergen. Nevertheless, LPP-treated group showed significantly improved symptom scores during the pollen season compared to PL-treated group.

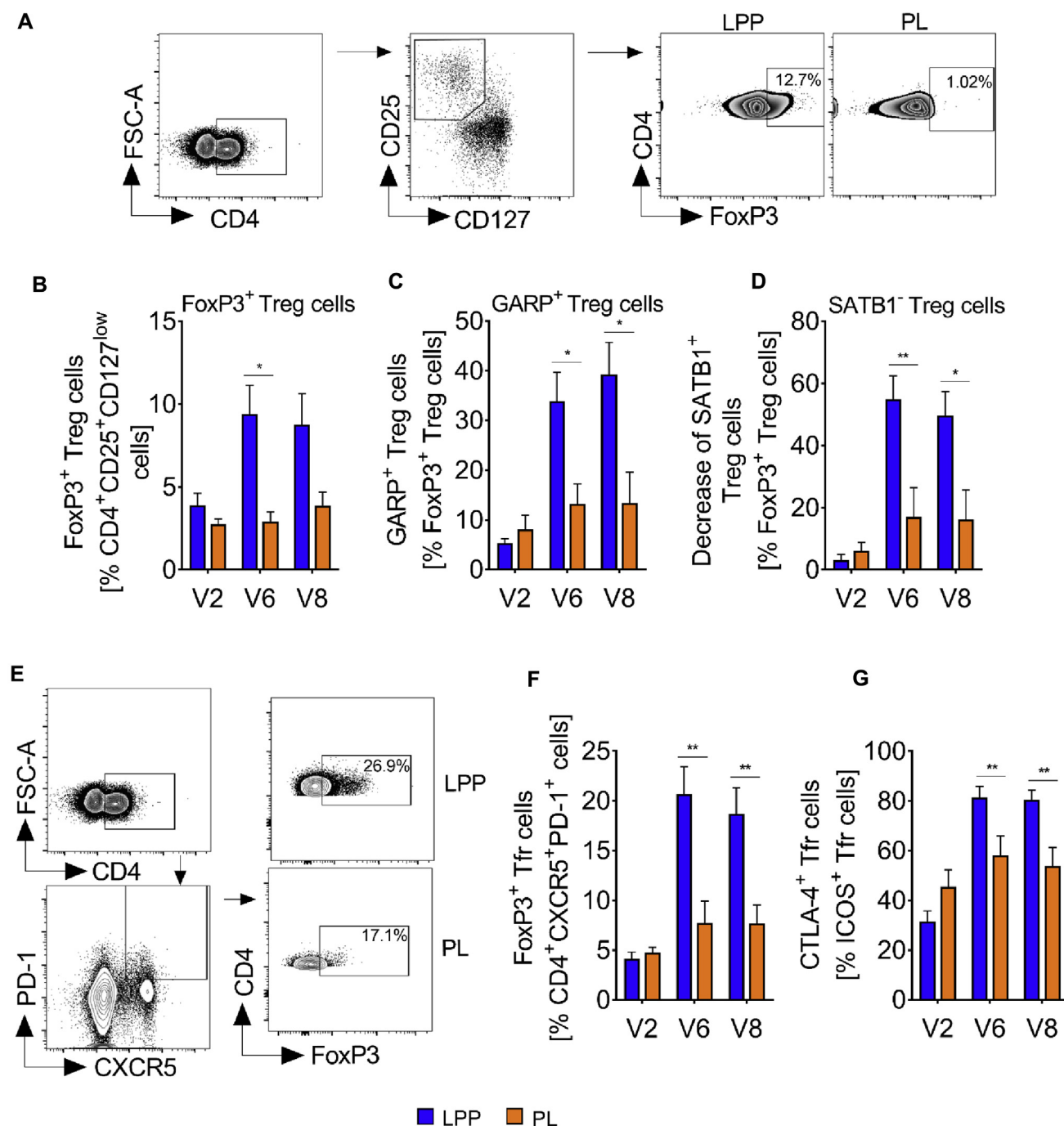


FIG 3. LPP induces expression of regulatory cells. **A**, Representative plots of Treg cells in LPP-treated ($n = 21$) and PL-treated ($n = 11$) groups. **B**, Percentage of FoxP3⁺ Treg (CD4⁺CD25⁺CD127^{low}FoxP3⁺) cells within CD4⁺CD25⁺CD127^{low} cells in LPP-treated ($n = 21$) and PL-treated ($n = 11$) groups were assessed by FACS. **C**, Percentage of GARP⁺ Treg (CD4⁺CD25⁺CD127^{low}FoxP3⁺GARP⁺) cells within CD4⁺CD25⁺CD127^{low}FoxP3⁺ cells. **D**, Percentage of SATB1⁻ Treg (CD4⁺CD25⁺CD127^{low}FoxP3⁺SATB1⁻) cells within CD4⁺CD25⁺CD127^{low}FoxP3⁺ cells. **E**, Representative plots of T_{FR} (CD4⁺CXCR5⁺PD-1⁺FoxP3⁺) cells in LPP-treated ($n = 21$) and PL-treated ($n = 11$) groups. **F**, Percentage of T_{FR} (CD4⁺CXCR5⁺PD-1⁺FoxP3⁺) cells within CD4⁺CXCR5⁺PD-1⁺ cells. **G**, Percentage of CTLA-4⁺ T_{FR} (CD4⁺CXCR5⁺PD-1⁺FoxP3⁺ICOS⁺CTLA-4⁺) cells within CD4⁺CXCR5⁺PD-1⁺FoxP3⁺ICOS⁺ cells. Data are shown as means $\pm$ SEMs. * $P < .05$ and ** $P < .01$, Mann-Whitney test.

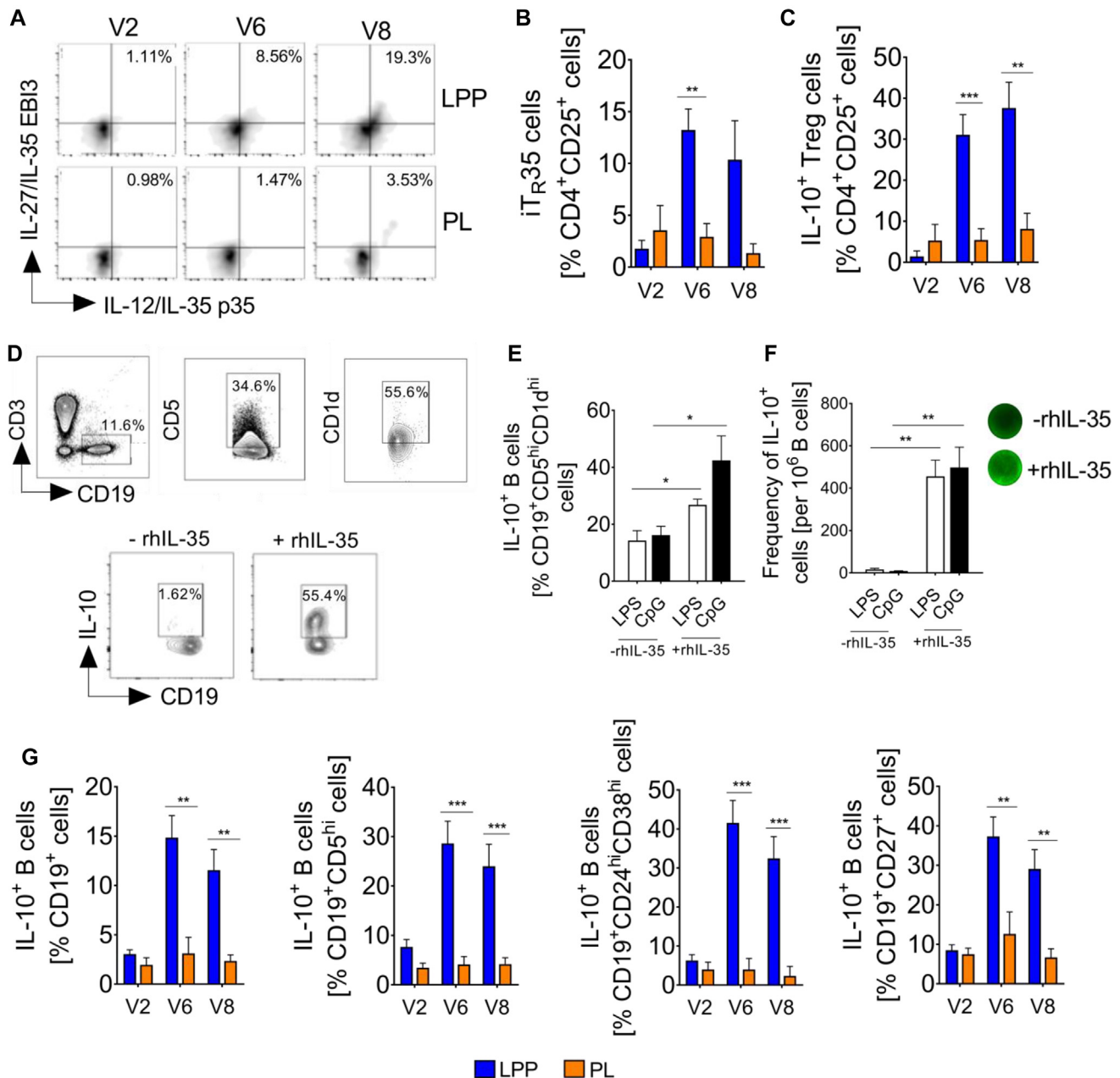


FIG 4. Induction of regulatory cells. **A**, Representative plots analysis of EB13⁺p35⁺ Treg cells. IL-35-producing Treg cells were assessed by using FACS in LPP-treated ($n = 21$) and PL-treated ($n = 11$) groups at V2, V6, and V8. **B**, Percentage of iT_R35 cells within CD4⁺CD25⁺ cells. **C**, Proportion of IL-10-producing Treg cells within CD4⁺CD25⁺ cells. **D-F**, IL-10⁺CD19⁺ Breg cells production was examined by FACS. Fig 4, **D**, Representative plots of IL-10 induction in CD19⁺ B cells by IL-35. Fig 4, **E**, IL-35-induced IL-10⁺ Breg cell production in patients with grass pollen allergy in the presence of CpG. Fig 4, **F**, Frequency of IL-10-producing cells measured by FluoroSpot. **G**, Production of IL-10⁺CD19⁺, IL-10⁺CD19⁺CD5^{hi}, IL-10⁺CD19⁺CD5^{hi}CD24^{hi}CD38^{hi}, and IL-10⁺CD19⁺CD27⁺ Breg cells was increased in LPP-treated patients. Data are shown as means $\pm$ SEMs. * $P < .05$, ** $P < .01$, and *** $P < .001$, Mann-Whitney test.

To address the factors that drive B-cell responses, we investigated a subset of T cells known as T_{FH} cells.²⁷⁻²⁹ Here we demonstrated that IL-4- and IL-21-producing T_{FH} cells were lower in LPP-treated group compared to those in PL-treated group, suggesting that IL-4- and IL-21-producing

T_{FH} cells can be pathogenic in allergic patients. It is well established that IL-4 induces IgE production, the key player in allergic hypersensitivity, and the synergistic effect between IL-4 and IL-21 has also been shown to induce IgE production by B cells through STAT3 activation.^{21,30} The observed effect

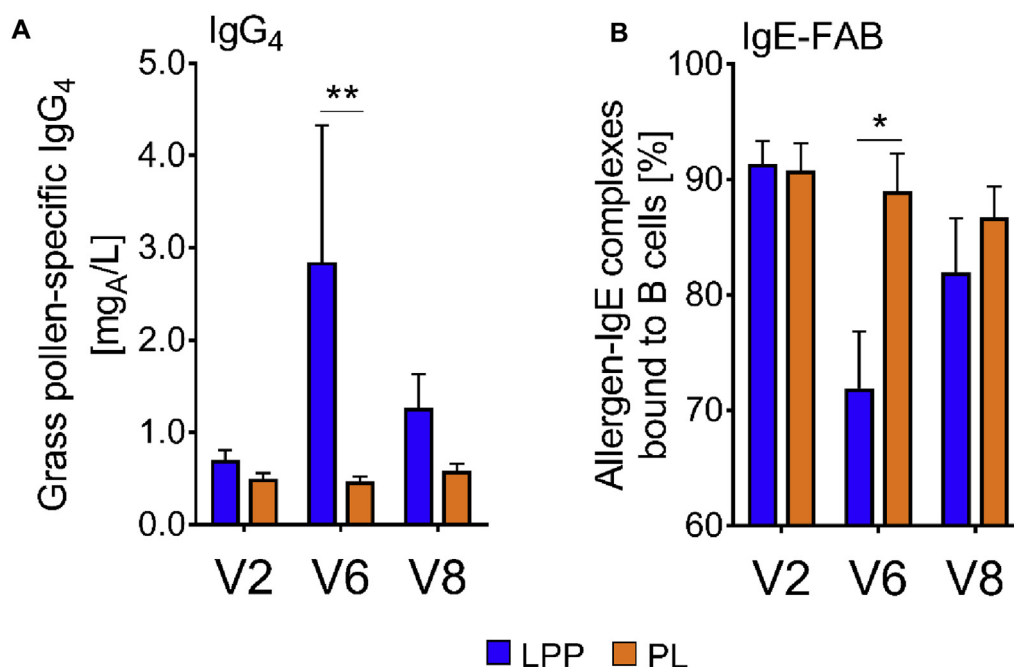


FIG 5. LPP enhances IgG₄ blocking activities. **A**, Effect of LPP immunotherapy on IgG₄ production in serum samples of patients obtained at V2, V6, and V8 were measured by ImmunoCAP. **B**, Induction of IgG-associated blocking antibodies that inhibit IgE-facilitated allergen-IgE binding to B cells. Effect of LPP on IgE-facilitated allergen binding to B cells was determined in sera from allergic patients incubated with B cells. Data are shown as means $\pm$ SEMs. * $P < .05$ and ** $P < .01$, Mann-Whitney test.

of LPP on IL-4- and IL-21-producing T_{FH} cells might play a role in the blunting of IgE production, consequently suppressing the symptoms in LPP-treated group. Previous studies have explored the different subsets of T_{FH} cells, including IFN- γ -producing T_{FH} cells. In this study, IFN- γ -producing T_{FH} cells were increased in LPP-treated group. Similarly, high levels of IFN- γ ⁺ T_{H1} cells were observed in the same group, with a significant reduction in numbers of T_{H2} cells. This finding is consistent with the previous finding that reported immune deviation toward a T_{H1} response following conventional immunotherapy.³¹

Previous studies have shown transient induction of Treg cells following immunotherapy.³² Treg cells that expressed FoxP3 and GARP and repressed SATB1 were induced following LPP treatment and remained high after the grass pollen season. It is well established that FoxP3 serves as a marker for Treg cells; nevertheless, FoxP3 is expressed in activated T cells.³³ Recent studies have shown that expression of GARP and repression of SATB1 is crucial in the suppressive function of Treg cells.^{22,23} SATB1 has been shown to be negatively regulated by FoxP3 expression in Treg cells, thus determining the fate of Treg cells.^{23,34} In addition, GARP has been shown to be highly expressed in Treg cells.²² Together, the expression of FoxP3 and GARP and repression of SATB1 within Treg cell subsets can be used to identify suppressive Treg cells.

A Treg cell subset, T_{FR} cells, has been previously described as a subset of T cells that regulates B-cell and T_{FH} cell interaction.³⁵ LPP induces T_{FR} and CTLA-4⁺ T_{FR} cells, which persist even after the grass pollen season. Previous studies have shown CTLA-4 to be crucial for T_{FR} cells to exert their

suppressive functions,²⁴ and it is speculated that these functional T_{FR} cells might suppress cytokine production by T_{FH} cells, therefore disrupting the cytokine-mediated stimulation of B cells.³⁶ These observations on T_{FH}, T_{FR}, and Treg cells suggest that these cells might act in a similar mechanism that mirrors the fate of T_{H2}, T_{H1}, and Treg cells following conventional immunotherapy.

Several studies have highlighted the role of IL-35 in the immune regulation of autoimmune disease *in vivo*.³⁷ IL-35 induces the expansion of Breg, Treg, and iT_R35 cells.³⁸ These regulatory cells promote immune regulation that can control T_{H2} inflammation. In our study, for the first time, we have shown that a short-course of LPP treatment induced iT_R35 cells. Moreover, previous studies have shown that IL-35 has the ability to induce IL-10⁺ Breg cells by activating STAT1/STAT3.³⁷ It is likely that IL-35 promotes iT_R35 cell induction, which in turn can differentiate B cells into IL-10⁺ Breg cells that produce allergen-neutralizing IgG₄ antibodies during LPP treatment.

We have shown that LPP treatment enhanced IgG₄ production and prevented allergen-IgE complex binding to B cells, which subsequently inhibited T_{H2} cell activation. This observation is in agreement with the findings obtained by using the IgE-FAB assay, illustrating that IgG₄ antibodies can compete with IgE to inhibit allergen-IgE complexes binding to CD23 (Fc ϵ R2) present on B cells, thus inhibiting facilitated antigen presentation to T cells.¹⁸ Altogether, Treg and Breg cell regulation leading to IgG₄ production might provide an alternative mechanism to induce tolerance in LPP-treated patients.

In this study LPP immunotherapy was associated with a reduction in seasonal symptoms and use of rescue medications, which was related to suppression of allergen-induced basophil responsiveness, induction of IgG-associated blocking antibodies, and immune modulation of T and B cells in peripheral blood. Immunologic parameters were measured at baseline, the end of treatment (after 3 weeks), and the end of the pollen season.

Previous studies on conventional AIT showed association of AIT with a reduction in proinflammatory T_H2 cell responses and an induction of Treg cells.¹⁴ This was accompanied by induction of blocking IgG₄ antibodies. In this study, we have shown that short-course LPP treatment results in the attenuation of proallergic inflammatory T cells and induction of Treg and Breg cell subsets and blocking IgG₄ antibodies. These results showed that the rapid mechanism of immunomodulation observed during treatment is somewhat similar to that in conventional immunotherapy, which takes 3 years to achieve if given subcutaneously or sublingually. It is likely that a short-course immunotherapy treatment (4 physician visits over 3 weeks) might improve patient compliance, which currently is 25% for subcutaneous immunotherapy and 12.5% for sublingual immunotherapy.³⁹

To date, there are very limited studies that investigate the tolerance end point for short courses AIT. A recent phase IIb study was performed in patients with cat allergy treated with short-course peptide immunotherapy using the major cat allergen peptide Fel d 1, which is referred to as Cat-PAD. The study showed persistent tolerance to cat allergen for up to 2 years after the treatment.⁴⁰ However, the phase III study resulted in a strong placebo effect, and it was not significant when compared to the treated group. It is important to note that participants from the phase III study were exposed to cat, and this might have resulted in the induction of IgG antibodies that could have been protective, even in the PL-treated group. However, the clinical and immunologic findings of this study are yet to be published. In another short-term immunotherapy study that involves administration of allergoids adjuvanted by monophosphoryl lipid (MPL), it was shown that it takes 2 cycles of treatment off season over a period of 2 years to induce specific IgG₄ antibodies and blocking activity in sera of treated patients.⁴¹ Intralymphatic immunotherapy indicated in allergic patients has also been shown to be clinically effective when administered as a short-course (3 intralymphatic allergen administrations within 8 weeks) and induced long-term tolerance after cessation of treatment.⁴² These studies showed that short-course immunotherapy treatment could potentially induce long-term tolerance in treated patients. It would be interesting to follow the study participants after cessation of treatment and evaluate both clinical and immunologic responses. In addition, previous studies have shown that a booster AIT injection before the pollen season after cessation of immunotherapy treatment resulted in a significant reduction in CSMS in patients with grass pollen allergy during the pollen season.⁴³ Therefore, one could provide a booster injection before the second pollen season to evaluate the persistence of the clinical and immunologic effect.

In summary, for the first time, we showed that a 3-week short-course of LPP immunotherapy reduces seasonal symptoms and the need for rescue medications intake during the peak and the entire pollen season. The immunologic mechanisms of LPP immunotherapy are underscored by immune modulation in T- and B-cell compartments.

Key messages

- Preseasonal 3-week short course of adjuvant-free peptide hydrolysates of *L perenne* (LPP) over 4 medical visits inhibited basophil response and attenuated T_H2 proallergic responses.
- LPP immunotherapy-induced peripheral FoxP3 Treg and T_{FR} cells, stimulated iT_R35 cell induction, which promoted production of IL-10 from CD19⁺ B cells and Breg cell subsets.
- LPP immunotherapy was associated with induction of grass pollen-specific neutralizing IgG₄-blocking antibodies, which compete with IgE and suppress allergen-IgE binding to B cells.

REFERENCES

1. Bousquet J, Lockey R, Malling HJ. Allergen immunotherapy: therapeutic vaccines for allergic diseases. A WHO position paper. *J Allergy Clin Immunol* 1998;102:558-62.
2. Valenta R, Niespodziana K, Focke-Tejkl M, Marth K, Huber H, Neubauer A, et al. Recombinant allergens: what does the future hold? *J Allergy Clin Immunol* 2011;127:860-4.
3. Durham SR, Walker SM, Varga EM, Jacobson MR, O'Brien F, Noble W, et al. Long-term clinical efficacy of grass-pollen immunotherapy. *N Engl J Med* 1999;341:468-75.
4. James LK, Shamji MH, Walker SM, Wilson DR, Wachholz PA, Francis JN, et al. Long-term tolerance after allergen immunotherapy is accompanied by selective persistence of blocking antibodies. *J Allergy Clin Immunol* 2011;127:509-16, e1-5.
5. Scadding GW, Calderon MA, Shamji MH, Eifan AO, Penagos M, Dumitru F, et al. Effect of 2 years of treatment with sublingual grass pollen immunotherapy on nasal response to allergen challenge at 3 years among patients with moderate to severe seasonal allergic rhinitis: the GRASS randomized clinical trial. *JAMA* 2017;317:615-25.
6. Bauchau V, Durham SR. Prevalence and rate of diagnosis of allergic rhinitis in Europe. *Eur Respir J* 2004;24:758-64.
7. Katelaris CH, Lee BW, Potter PC, Maspero JF, Cingi C, Lopatin A, et al. Prevalence and diversity of allergic rhinitis in regions of the world beyond Europe and North America. *Clin Exp Allergy* 2012;42:186-207.
8. Shamji MH, Ceuppens J, Bachert C, Hellings P, Placier G, Thirion G, et al. Lolium perenne peptides for treatment of grass pollen allergy: a randomized, double-blind, placebo-controlled clinical trial. *J Allergy Clin Immunol* 2018;141:448-51.
9. Mösges R, Koch AF, Raskopf E, Singh J, Shah-Hosseini K, Astvatsatourov A, et al. Lolium perenne peptide immunotherapy is well tolerated and elicits a protective B-cell response in seasonal allergic rhinitis patients. *Allergy* 2018;73:1254-62.
10. Mösges R, Kasche EM, Raskopf E, Singh J, Sohlich L, Astvatsatourov A, et al. A randomized, double-blind, placebo-controlled, dose-finding trial with Lolium perenne peptide immunotherapy. *Allergy* 2018;73:896-904.
11. Laffer S, Valenta R, Vrtala S, Susani M, van Ree R, Kraft D, et al. Complementary DNA cloning of the major allergen phl p I from timothy grass (*Phleum pratense*); recombinant phl p I inhibits IgE binding to group I allergens from eight different grass species. *J Allergy Clin Immunol* 1994;94:689-98.
12. Larché M. Mechanisms of peptide immunotherapy in allergic airways disease. *Ann Am Thorac Soc* 2014;11(suppl 5):S292-6.
13. Mösges R, Bachert C, Panzner P, Calderon MA, Haazen L, Piroton S, et al. Short-course of grass allergen peptides immunotherapy over three weeks reduces seasonal symptoms in allergic rhinoconjunctivitis with/without asthma: a randomized, multicenter, double-blind, placebo-controlled trial. *Allergy* 2018;73:1842-50.
14. Shamji MH, Durham SR. Mechanisms of allergen immunotherapy for inhaled allergens and predictive biomarkers. *J Allergy Clin Immunol* 2017;140:1485-98.
15. Wambre E, DeLong JH, James EA, LaFond RE, Robinson D, Kwok WW. Differentiation stage determines pathologic and protective allergen-specific CD4⁺ T-cell outcomes during specific immunotherapy. *J Allergy Clin Immunol* 2012;129:544-51, e1-7.
16. Schulten V, Tripple V, Seumois G, Qian Y, Scheuermann RH, Fu Z, et al. Allergen-specific immunotherapy modulates the balance of circulating Tfh and Tfr cells. *J Allergy Clin Immunol* 2018;141:775-7.e6.

17. Shamji MH, Layhadi JA, Scadding GW, Cheung DK, Calderon MA, Turka LA, et al. Basophil expression of diamine oxidase: a novel biomarker of allergen immunotherapy response. *J Allergy Clin Immunol* 2015;135:913-21.e9.
18. Shamji MH, Wilcock LK, Wachholz PA, Dearman RJ, Kimber I, Wurtzen PA, et al. The IgE-facilitated allergen binding (FAB) assay: validation of a novel flow-cytometric based method for the detection of inhibitory antibody responses. *J Immunol Methods* 2006;317:71-9.
19. Varricchi G, Harker J, Borriello F, Marone G, Durham SR, Shamji MH. T follicular helper (Tfh) cells in normal immune responses and in allergic disorders. *Allergy* 2016;71:1086-94.
20. Noble A, Zhao J. Follicular helper T cells are responsible for IgE responses to Der p 1 following house dust mite sensitization in mice. *Clin Exp Allergy* 2016;46:1075-82.
21. Avery DT, Ma CS, Bryant VL, Santner-Nanan B, Nanan R, Wong M, et al. STAT3 is required for IL-21 induced secretion of IgE from human naive B cells. *Blood* 2008;112:1784-93.
22. Wang R, Kozhaya L, Mercer F, Khaitan A, Fujii H, Unutmaz D. Expression of GARP selectively identifies activated human FOXP3+ regulatory T cells. *Proc Natl Acad Sci U S A* 2009;106:13439-44.
23. Beyer M, Thabet Y, Muller RU, Sadlon T, Classen S, Lahl K, et al. Repression of the genome organizer SATB1 in regulatory T cells is required for suppressive function and inhibition of effector differentiation. *Nat Immunol* 2011;12:898-907.
24. Sage PT, Paterson AM, Lovitch SB, Sharpe AH. The coinhibitory receptor CTLA-4 controls B cell responses by modulating T follicular helper, T follicular regulatory, and T regulatory cells. *Immunity* 2014;41:1026-39.
25. Weber RW. Patterns of pollen cross-allergenicity. *J Allergy Clin Immunol* 2003;112:229-39.
26. Shamji MH, Ljørring C, Francis JN, Calderon MA, Larché M, Kimber I, et al. Functional rather than immunoreactive levels of IgG4 correlate closely with clinical response to grass pollen immunotherapy. *Allergy* 2012;67:217-26.
27. Crotty S. Follicular helper CD4 T cells (TFH). *Annu Rev Immunol* 2011;29:621-63.
28. Bentebibel SE, Lopez S, Obermoser G, Schmitt N, Mueller C, Harrod C, et al. Induction of ICOS+CXCR3+CXCR5+ TH cells correlates with antibody responses to influenza vaccination. *Sci Transl Med* 2013;5:176ra32.
29. He J, Tsai LM, Leong YA, Hu X, Ma CS, Chevalier N, et al. Circulating precursor CCR7(lo)PD-1(hi) CXCR5(+) CD4(+) T cells indicate Tfh cell activity and promote antibody responses upon antigen reexposure. *Immunity* 2013;39:770-81.
30. Avery DT, Bryant VL, Ma CS, de Waal Malefyt R, Tangye SG. IL-21-induced isotype switching to IgG and IgA by human naive B cells is differentially regulated by IL-4. *J Immunol* 2008;181:1767-79.
31. Durham SR, Ying S, Varney VA, Jacobson MR, Sudderick RM, Mackay IS, et al. Grass pollen immunotherapy inhibits allergen-induced infiltration of CD4+ T lymphocytes and eosinophils in the nasal mucosa and increases the number of cells expressing messenger RNA for interferon-gamma. *J Allergy Clin Immunol* 1996;97:1356-65.
32. Matsuoka T, Shamji MH, Durham SR. Allergen immunotherapy and tolerance. *Allergol Int* 2013;62:403-13.
33. Gavin MA, Torgerson TR, Houston E, DeRoos P, Ho WY, Stray-Pedersen A, et al. Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. *Proc Natl Acad Sci U S A* 2006;103:6659-64.
34. Kitagawa Y, Ohkura N, Kidani Y, Vandenbon A, Hirota K, Kawakami R, et al. Erratum: Guidance of regulatory T cell development by Satb1-dependent super-enhancer establishment. *Nat Immunol* 2017;18:474.
35. Linterman MA, Pierson W, Lee SK, Kallies A, Kawamoto S, Rayner TF, et al. Foxp3(+) follicular regulatory T cells control the germinal center response. *Nat Med* 2011;17:975-82.
36. Sage PT, Alvarez D, Godec J, von Andrian UH, Sharpe AH. Circulating T follicular regulatory and helper cells have memory-like properties. *J Clin Invest* 2014;124:5191-204.
37. Wang RX, Yu CR, Dambuzza IM, Mahdi RM, Dolinska MB, Sergeev YV, et al. Interleukin-35 induces regulatory B cells that suppress autoimmune disease. *Nat Med* 2014;20:633-41.
38. Shamji MH, Layhadi JA, Achkova D, Kouser L, Perera-Webb A, Couto-Francisco NC, et al. Role of Interleukin-35 in sublingual allergy immunotherapy. *J Allergy Clin Immunol* 2019;143:1131-42.e4.
39. Kiel MA, Röder E, Gerth Van Wijk R, Al MJ, Hop WCJ, Rutten-Van Mölken MPMH. Real-life compliance and persistence among users of subcutaneous and sublingual allergen immunotherapy. *J Allergy Clin Immunol* 2013;132:353-60.e2.
40. Couroux P, Patel D, Armstrong K, Larché M, Hafner RP. Fel d 1-derived synthetic peptide immuno-regulatory epitopes show a long-term treatment effect in cat allergic subjects. *Clin Exp Allergy* 2015;45:974-81.
41. Rosewich M, Schulze J, Eickmeier O, Telles T, Rose MA, Schubert R, et al. Tolerance induction after specific immunotherapy with pollen allergoids adjuvanted by monophosphoryl lipid A in children. *Clin Exp Immunol* 2010;160:403-10.
42. Senti G, Prinz Vavricka BM, Erdmann I, Diaz MI, Markus R, McCormack SJ, et al. Intralymphatic allergen administration renders specific immunotherapy faster and safer: a randomized controlled trial. *Proc Natl Acad Sci U S A* 2008;105:17908-12.
43. Pfaar O, Lang S, Pieper-Fürst U, Astvatsatourov A, Gerich F, Klimek L, et al. Ultra-short-course booster is effective in recurrent grass pollen-induced allergic rhinoconjunctivitis. *Allergy* 2018;73:187-95.

1 ONLINE REPOSITORY

2 **Title: Immunologic mechanisms of short-course of *Lolium Perenne* Peptide**
3 **Immunotherapy: A Randomized Double-Blind Placebo-Controlled Trial**

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ONLINE REPOSITORY METHODS

Exclusion criteria

Patients were selected on the basis of having a medical history of moderate-to-severe seasonal allergic rhinitis for at least two years, a positive skin prick test (wheal diameter of ≥ 3 mm) to grass pollen mixture and histamine and specific IgE (>0.70 kU_A/L) to timothy grass pollen (*Phleum pratense*). Patients were excluded from the study if they had previous history of allergen immunotherapy within the last 5 years, anaphylaxis, perennial rhinitis, poorly controlled or uncontrolled asthma, or other significant medical illnesses. Women of childbearing potential who were not taking contraceptive precaution, pregnant or lactating were also excluded.

Allergen-induced basophil responses

Cells used to measure *ex vivo* allergen-induced basophil responsiveness were stained with anti-human CD3 PE-Cy7 (BD Biosciences, San Jose, CA), CD303 APC (Miltenyi Biotec, Woking, UK), CD294 (CRTH2) PE (Miltenyi Biotec, Woking, UK), CD203c PerCP-Cy5.5 (Biolegend, London, UK) and CD63 FITC (Biolegend, London, UK).

In vitro T and B cell stimulation

Peripheral blood mononuclear cells (PBMCs) were isolated from approximately 200 mL of heparinized whole blood using density gradient centrifugation without brakes using Ficoll[®]-Paque PLUS (GE Healthcare Bio-sciences AB, Uppsala, Sweden). For *in vitro* T and B cell culture experiments, cells were immunostained with the following fluorescent-labelled antibodies as per manufacturer's protocol (all from BD Biosciences unless stated): CD4

BUV395, CD25 BV650, CD185 (CXCR5) BB515, CD279 (PD-1) BUV737 for T cells or CD5 APC, CD27 BB515, CD1d BV421, CD19 PerCP-Cy5.5, CD3 APC-H7, CD24 BV510, CD38 PE-Cy7 for B cells. The cells were fixed for 20 mins with Cytofix/Perm buffer (BD Biosciences, San Jose, CA) and washed with Perm/wash buffer (BD Biosciences, San Jose, CA). Intracellular staining were performed using IL-4 PE-CF594, IL-21 Alexa Fluor 647, IL-10 BV786, IFN- γ BV605, IL-12/IL-35 p35 PE (R&D Systems, Abingdon, UK) and IL-27/IL-35 EBI3 APC (R&D Systems, Abingdon, UK) for T cells, while B cells were immunostained with IL-10 PE. The cells were then washed and re-suspended in cell stain buffer prior to acquisition on BD FACSCanto™ II and on BD LSRFortessa™ (BD Biosciences, San Jose, CA).

Ex vivo staining of T cells by flow cytometry

PBMCs were resuspended in PBS and 1×10^6 cells per tube were fixed with Transcription Factor Phospho Fix/Perm Buffer (BD Biosciences, San Jose, CA) for 50 mins and treated with Perm Buffer III (BD Biosciences, San Jose, CA) prior to cell surface and intracellular transcription factor staining according to manufacturer's instruction. The following antibodies were used (all from BD Biosciences unless stated): anti-human CD4 APC-Cy7, CD185 (CXCR5) BV421, CD279 (PD-1) PE, CD278 (ICOS) PerCP-Cy5.5, CD25 BV510, CD152 (CTLA-4) PE-Cy7, CD127 BB515, GARP PE, SATB1 Alexa Fluor 647, FoxP3 Alexa Fluor 647, FoxP3 BV421 and analyzed on BD FACS Canto II (BD Biosciences, San Jose, CA).

FluoroSpot assay

96-well polyvinylidene difluoride (PVDF) plate (Diacclone, Besançon, France) was pre-treated with ethanol and blocked for 30 mins with 10% FCS at room temperature. PBMCs were seeded at a density of 500,000 cells per well in the presence of rhIL-35 with LPS (1 µg/mL; Sigma-Aldrich) and CpG ODN 2006 (1 µg/mL; Invivogen) in triplicates for 72 hours. Plates were washed and anti-IL-4 (Cy3) and anti-IL-10 (FITC) antibodies were added and incubated for 1 hour. Fluorescent enhancer (1:10) was added and incubated for 15 mins. Fluorescent spots were read and quantified under a UV light using iSpot reader (Oxford BioSystems, Abingdon, UK).

FIGURE LEGENDS

Figure E1. Study Design. Flowchart illustration of patient recruitment, randomization and treatment. No patient drop-outs took place throughout the treatment period. AE: Adverse Events.

Figure E2. Grass Pollen Count in Belgium. Reported grass pollen count in Belgium between May and August in 2016. Peak pollen season was between week 23 and week 25.

Figure E3. LPP inhibits anti-IgE-mediated basophil activation. Heparinized whole blood was incubated with 1 µg/mL of anti-human IgE antibody for 15 mins prior to staining and acquisition by FACS. Proportion of CD203c^{bright}CRTH2⁺ and CD63⁺CRTH2⁺ basophils in LPP (n=21)- and PL (n=11)-treated groups at V2, V6 and V8 were assessed. Data are shown as mean (±SEM). **P*<.05, ***P*<.01, Mann-Whitney test.

ONLINE REPOSITORY

Title: Immunologic mechanisms of short-course of *Lolium Perenne* Peptide Immunotherapy: A Randomized Double-Blind Placebo-Controlled Trial

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33 **Table E1**

Experimental readout	Patient group	Mean $\pm$ SD	Power rank	Calculated sample size (vs. PL)
sIgG ₄ (mg _A /L)	PL	0.74 $\pm$ 0.60	0.9	n = 2
	LPP	2.82 $\pm$ 3.49	0.9	
Allergen-IgE complexes bound to B cells (%)	PL	85.30 $\pm$ 13	0.9	n = 7
	LPP	61.58 $\pm$ 25	0.9	

34

35 Sample size calculation based on previously published data of induction of sIgG₄ and formation of allergen-IgE complexes bound to B cells
 36 measured in 170 μ g LPP- and placebo-treated patients.¹ Sample size calculations was performed using Statulator software
 37 (<http://statulator.com/SampleSize/ss2M.html>). PL, placebo-treated group; LPP, *Lolium perenne* peptide-treated group.

38 **Table E2**

Experimental readout	Visit	Mean $\pm$ SD	Power rank	Calculated sample size (vs V1)
sIgG ₄ (mg _A /L)	V1	0.7 $\pm$ 0.7	0.9	
	V6	5.65 $\pm$ 13.56	0.9	n = 1
	V8	8.57 $\pm$ 18.36	0.9	n = 1
Allergen-IgE complexes bound to B cells (%)	V1	92.79 $\pm$ 11.11	0.9	
	V6	76.25 $\pm$ 19.03	0.9	n = 10
	V8	69.76 $\pm$ 19.51	0.9	n = 5

39

40 Sample size calculation based on previously published data of induction of sIgG₄ and formation of allergen-IgE complexes bound to B cells
 41 following LPP treatment measured at screening (V1), during (V6) and after (V8) treatment.² Sample size calculations was performed using
 42 Statulator software (<http://statulator.com/SampleSize/ss2M.html>).

43 **Table E3**

Concentration of Phlp	Visit	n (LPP)	LPP (mean ± SEM)	n (PL)	Placebo (mean ± SEM)	<i>P</i> value (LPP vs PL)	<i>P</i> value of LPP (V6 or V8 vs baseline)	<i>P</i> value of PL (V6 or V8 vs baseline)
0 ng/mL	V2	21	0.00 ± 0.00	11	0.00 ± 0.00	>.99		
	V6	21	0.00 ± 0.00	11	0.00 ± 0.00	>.99		
	V8	21	0.00 ± 0.00	11	0.00 ± 0.00	>.99		
1 ng/mL	V2	21	29.73 ± 5.32	11	27.96 ± 5.77	.78		
	V6	21	4.50 ± 6.11	11	37.73 ± 7.29	.0009	<.0001	.41
	V8	21	9.54 ± 3.01	11	38.82 ± 8.32	.002	<.0001	.18
3 ng/mL	V2	21	46.16 ± 6.18	11	50.10 ± 6.84	>.99		
	V6	21	16.88 ± 6.46	11	54.66 ± 7.50	.002	.006	.97
	V8	21	21.00 ± 4.05	11	54.21 ± 8.25	.001	.001	.83
10 ng/mL	V2	21	57.19 ± 5.57	11	52.82 ± 7.17	.33		
	V6	21	24.35 ± 6.13	11	62.02 ± 7.589	.0005	<.0001	.21
	V8	21	27.26 ± 4.75	11	63.07 ± 8.03	.0006	<.0001	.41
33 ng/mL	V2	21	60.05 ± 5.75	11	52.04 ± 8.76	.37		
	V6	21	38.46 ± 9.30	11	64.42 ± 7.69	.042	.06	.12
	V8	21	34.71 ± 5.46	11	65.92 ± 8.37	.002	.002	.05
100 ng/mL	V2	21	66.19 ± 4.78	11	56.57 ± 7.20	.11		
	V6	21	36.15 ± 7.88	11	61.85 ± 8.44	.042	.002	.52
	V8	21	36.72 ± 5.96	11	65.52 ± 8.56	.008	<.0001	.28
330 ng/mL	V2	21	62.27 ± 5.39	11	52.62 ± 8.06	.21		
	V6	21	32.12 ± 7.98	11	61.73 ± 7.77	.034	.002	.18
	V8	21	32.49 ± 6.10	11	61.64 ± 9.59	.034	<.0001	.24

44

45 Expression of CD203c^{bright}CRTH2⁺ basophils in LPP and PL-treated group. **P* value (LPP vs PL): Mann-Whitney's Test for non-normalized
 46 data and Welch's t test for normalized data. **P* value (V6 or V8 vs baseline) LPP/PL: Wilcoxon's Test.

47 **Table E4**

Concentration of Phlp	Visit	n (LPP)	LPP (mean ± SEM)	n (PL)	Placebo (mean ± SEM)	<i>P</i> value (LPP vs PL)	<i>P</i> value of LPP (V6 or V8 vs baseline)	<i>P</i> value of PL (V6 or V8 vs baseline)
0 ng/mL	V2	21	0.00 ± 0.00	11	0.00 ± 0.00	>.99		
	V6	21	0.00 ± 0.00	11	0.00 ± 0.00	>.99		
	V8	21	0.00 ± 0.00	11	0.00 ± 0.00	>.99		
1 ng/mL	V2	21	21.89 ± 5.04	11	22.99 ± 11.24	.81		
	V6	21	13.89 ± 5.95	11	19.60 ± 9.15	.13	.01	.76
	V8	21	4.71 ± 5.99	11	30.01 ± 7.84	.01	.0002	.41
3 ng/mL	V2	21	41.55 ± 5.85	11	42.59 ± 8.96	.94		
	V6	21	24.18 ± 5.43	11	42.06 ± 10.41	.13	.003	.83
	V8	21	26.30 ± 7.83	11	49.95 ± 9.46	.07	.007	>.99
10 ng/mL	V2	21	57.44 ± 5.17	11	52.46 ± 9.99	.88		
	V6	21	38.19 ± 4.75	11	56.54 ± 11.21	.08	<.0001	.24
	V8	21	42.70 ± 5.38	11	66.01 ± 9.03	.02	.0002	.58
33 ng/mL	V2	21	64.10 ± 5.09	11	57.45 ± 11.95	.51		
	V6	21	56.92 ± 5.16	11	62.75 ± 11.59	.19	.16	.08
	V8	21	55.64 ± 7.92	11	72.25 ± 8.29	.006	.08	.07
100 ng/mL	V2	21	70.87 ± 3.37	11	62.75 ± 10.32	.81		
	V6	21	63.03 ± 4.49	11	60.83 ± 11.57	.37	.10	.76
	V8	21	57.83 ± 7.66	11	71.40 ± 8.92	.02	.01	.41
330 ng/mL	V2	21	64.72 ± 4.30	11	56.86 ± 10.84	.78		
	V6	21	52.27 ± 5.35	11	57.74 ± 10.95	.27	.02	>.99
	V8	21	48.05 ± 8.31	11	65.49 ± 10.22	.04	.003	.70

48

49 Expression of CD63⁺CRTH2⁺ basophils in LPP and PL-treated group. **P* value (LPP vs PL): Mann-Whitney's Test for non-normalized data and

50 Welch's t test for normalized data. **P* value (V6 or V8 vs baseline) LPP/PL: Wilcoxon's Test.

51 **Table E5**

Marker expression	Visit	n (LPP)	LPP (mean $\pm$ SEM)	n (PL)	Placebo (mean $\pm$ SEM)	<i>P</i> value (LPP vs PL)	<i>P</i> value of LPP (V6 or V8 vs baseline)	<i>P</i> value of PL (V6 or V8 vs baseline)
CD63 ⁺	V2	20	45.21 $\pm$ 4.28	11	49.53 $\pm$ 8.40	.40		
	V6	21	34.77 $\pm$ 4.75	11	56.41 $\pm$ 8.15	.01	.05	.83
	V8	21	34.11 $\pm$ 6.09	11	49.97 $\pm$ 8.28	.12	.06	.37
CD107a ⁺	V2	20	16.45 $\pm$ 3.57	11	26.39 $\pm$ 5.54	.12		
	V6	21	13.11 $\pm$ 2.45	11	23.88 $\pm$ 5.41	.07	.67	.58
	V8	21	14.22 $\pm$ 3.89	11	22.83 $\pm$ 5.62	.17	.47	.41
CD203c ^{bright}	V2	20	59.10 $\pm$ 4.84	11	45.58 $\pm$ 8.49	.17		
	V6	21	33.41 $\pm$ 5.58	11	59.82 $\pm$ 8.06	.01	.002	.25
	V8	21	26.34 $\pm$ 5.45	11	59.56 $\pm$ 9.05	.006	<.0001	.41

52

53 Anti-human IgE effect on CRTH2⁺ basophils in LPP and PL-treated group. **P* value (LPP vs PL): Mann-Whitney's Test for non-normalized

54 data and Welch's t test for normalized data. **P* value (V6 or V8 vs baseline) LPP/PL: Wilcoxon's Test.

55 **Table E6**

T cell subset	Visit	n (LPP)	mean ± SEM (LPP)	n (PL)	mean ± SEM (PL)	<i>P</i> value (LPP vs PL)	<i>P</i> value of LPP (V6 or V8 vs baseline)	<i>P</i> value of PL (V6 or V8 vs baseline)
IL-4 ⁺ Th2 cells	V2	21	8.33 ± 3.03	11	19.91 ± 5.99	.10		
	V6	21	6.13 ± 3.21	11	18.95 ± 4.46	.02	.36	.90
	V8	21	15.22 ± 4.85	11	16.12 ± 4.70	.56	.03	.46
Th1 cells	V2	21	2.41 ± 0.79	11	3.70 ± 1.14	.36		
	V6	21	6.93 ± 1.34	11	2.58 ± 0.55	.006	<.0001	.41
	V8	21	5.98 ± 1.63	11	4.25 ± 1.42	.43	.01	>.99

56

57 Proportion of IFN- γ ⁺ Th1 (CD4⁺IFN- γ ⁺) cells following stimulation with different concentration of *Phleum pratense* (Phlp) allergen in LPP and
58 PL-treated groups. **P* value (LPP vs PL): Mann-Whitney's Test for non-normalized data and Welch's t test for normalized data. **P* value (V6 or
59 V8 vs baseline) LPP/PL: Wilcoxon's Test.

60 **Table E7**

Tfh cell subsets	Visit	n (LPP)	mean $\pm$ SEM (LPP)	n (PL)	mean $\pm$ SEM (PL)	<i>P</i> value (LPP vs PL)	<i>P</i> value of LPP (V6 or V8 vs baseline)	<i>P</i> value of PL (V6 or V8 vs baseline)
Tfh cells	V2	21	3.80 $\pm$ 0.61	11	4.17 $\pm$ 0.55	.60		
	V6	21	4.81 $\pm$ 0.59	11	5.02 $\pm$ 0.43	.66	.03	.12
	V8	21	4.41 $\pm$ 0.79	11	4.31 $\pm$ 0.84	.94	.60	.97
IL-4 ⁺ Tfh cells	V2	21	18.51 $\pm$ 3.69	11	22.88 $\pm$ 5.35	.47		
	V6	21	4.12 $\pm$ 2.11	11	16.12 $\pm$ 4.597	.003	.002	.52
	V8	21	5.34 $\pm$ 2.84	11	15.10 $\pm$ 4.41	.004	.008	.15
IL-21 ⁺ Tfh cells	V2	21	2.26 $\pm$ 0.59	11	3.79 $\pm$ 1.81	.97		
	V6	21	3.58 $\pm$ 2.54	11	7.41 $\pm$ 2.92	.003	.12	.46
	V8	21	1.45 $\pm$ 0.90	11	3.42 $\pm$ 1.04	.002	.12	.92
IL-4 ⁺ IL-21 ⁺ Tfh cells	V2	21	9.12 $\pm$ 1.95	11	10.93 $\pm$ 3.17	.61		
	V6	21	3.72 $\pm$ 1.97	11	10.14 $\pm$ 2.86	.004	.03	.64
	V8	21	3.26 $\pm$ 1.51	11	9.35 $\pm$ 3.01	.01	.02	.79
IFN- γ ⁺ Tfh cells	V2	21	2.28 $\pm$ 0.80	11	1.63 $\pm$ 0.91	.60		
	V6	21	9.69 $\pm$ 2.92	11	2.56 $\pm$ 1.18	.03	.003	.15
	V8	21	13.30 $\pm$ 4.21	11	1.35 $\pm$ 0.70	.01	.001	>.99

61

62 Proportion of T follicular helper (Tfh; CD4⁺CXCR5⁺PD-1⁺) cells and its subsets following stimulation with different concentration of *Phleum*
63 *pratense* (Phlp) allergen in LPP and PL-treated groups. **P* value (LPP vs PL): Mann-Whitney's Test for non-normalized data and Welch's t test
64 for normalized data. **P* value (V6 or V8 vs baseline) LPP/PL: Wilcoxon's Test.

65

66 **Table E8**

Treg cell subsets	Visit	n (LPP)	mean ± SEM (LPP)	n (PL)	mean ± SEM (PL)	<i>P</i> value (LPP vs PL)	<i>P</i> value of LPP (V6 or V8 vs baseline)	<i>P</i> value of PL (V6 or V8 vs baseline)
Treg cells	V2	21	3.88 ± 0.74	11	2.74 ± 0.32	.79		
	V6	21	9.41 ± 1.71	11	2.91 ± 0.58	.04	.0002	.99
	V8	21	8.77 ± 1.87	11	3.87 ± 0.82	.37	.0002	.41
GARP ⁺ Treg cells	V2	21	5.27 ± 0.93	11	7.99 ± 2.97	.99		
	V6	21	33.95 ± 5.74	11	13.15 ± 4.09	.03	.0001	.57
	V8	21	39.26 ± 6.48	11	13.43 ± 6.14	.01	<.0001	.64
SATB1 ⁺ Treg cells	V2	21	3.12 ± 1.93	11	6.01 ± 2.79	.46		
	V6	21	54.99 ± 7.42	11	17.05 ± 9.41	.002	<.0001	.94
	V8	21	49.72 ± 7.66	11	16.25 ± 9.49	.01	.0001	.94

67

68 Proportion of T regulatory (Treg; CD4⁺CD25⁺CD127^{low}FoxP3⁺) cells and its subsets in LPP and PL-treated groups. **P* value (LPP vs PL):
69 Mann-Whitney's Test. **P* value (V6 or V8 vs baseline) LPP/PL: Wilcoxon's Test.

70 **Table E9**

Tfr cell subsets	Visit	n (LPP)	mean ± SEM (LPP)	n (PL)	mean ± SEM (PL)	<i>P</i> value (LPP vs PL)	<i>P</i> value of LPP (V6 or V8 vs baseline)	<i>P</i> value of PL (V6 or V8 vs baseline)
Tfr cells	V2	21	4.18 ± 0.64	11	4.72 ± 0.59	.37		
	V6	21	20.69 ± 2.72	11	7.78 ± 2.17	.004	<.0001	.41
	V8	21	18.70 ± 2.62	11	7.69 ± 1.86	.004	<.0001	.41
CTLA-4 ⁺ ICOS ⁺ Tfr cells	V2	21	31.50 ± 4.28	11	45.50 ± 6.88	.15		
	V6	21	81.48 ± 4.39	11	58.10 ± 7.84	.001	<.0001	.08
	V8	21	80.48 ± 3.75	11	53.89 ± 7.40	.002	<.0001	>.99

71

72 Proportion of T follicular regulatory (Tfr; CD4⁺CXCR5⁺PD-1⁺FoxP3⁺) cells and its subsets in LPP and PL-treated groups. **P* value (LPP vs
73 PL): Mann-Whitney's Test for non-normalized data and Welch's t test for normalized data. **P* value (V6 or V8 vs baseline) LPP/PL:
74 Wilcoxon's Test.

75 **Table E10**

Inducible Treg cell subsets	Visit	n (LPP)	mean ± SEM (LPP)	n (PL)	mean ± SEM (PL)	<i>P</i> value (LPP vs PL)	<i>P</i> value of LPP (V6 or V8 vs baseline)	<i>P</i> value of PL (V6 or V8 vs baseline)
iT _R 35	V2	21	1.78 ± 0.81	10	3.23 ± 2.17	.34		
	V6	21	13.23 ± 2.03	10	2.92 ± 1.28	.01	<.0001	.92
	V8	21	10.35 ± 3.77	10	1.35 ± 0.90	.31	.08	.42
IL-10 ⁺ iTreg cells	V2	21	1.40 ± 1.35	10	5.79 ± 4.32	.26		
	V6	21	31.06 ± 4.93	11	5.43 ± 2.72	.0004	<.0001	.85
	V8	21	37.63 ± 6.26	11	8.14 ± 3.80	.001	<.0001	.61

76

77 Proportion of inducible Treg (CD4⁺CD25⁺) cells following stimulation with grass pollen (*Phleum pratense*) allergen in LPP and PL-treated
78 groups. **P* value (LPP vs PL): Mann-Whitney's Test for non-normalized data and Welch's t test for normalized data. **P* value (V6 or V8 vs
79 baseline) LPP/PL: Wilcoxon's Test.

80 **Table E11**

Breg cell subsets	Visit	n (LPP)	mean ± SEM (LPP)	n (PL)	mean ± SEM (PL)	<i>P</i> value (LPP vs PL)	<i>P</i> value of LPP (V6 or V8 vs baseline)	<i>P</i> value of PL (V6 or V8 vs baseline)
CD19 ⁺ IL-10 ⁺	V2	21	3.05 ± 0.44	11	1.95 ± 0.73	.06		
	V6	20	14.85 ± 2.23	9	3.11 ± 1.64	.002	<.0001	.91
	V8	20	11.55 ± 2.09	10	2.34 ± 0.62	.004	.0005	.38
IL-10 ⁺ CD19 ⁺ CD5 ⁺	V2	21	7.65 ± 1.51	11	3.44 ± 0.94	.06		
	V6	20	28.61 ± 4.51	9	4.11 ± 1.61	.0007	<.0001	.65
	V8	20	23.99 ± 4.46	10	4.19 ± 1.29	.0008	<.0001	.32
IL-10 ⁺ CD19 ⁺ CD27 ⁺	V2	21	8.79 ± 1.43	11	7.50 ± 1.54	.73		
	V6	20	37.30 ± 4.95	9	12.63 ± 5.56	.004	<.0001	.91
	V8	20	29.08 ± 4.90	10	6.70 ± 2.18	.002	.0003	.70
IL-10 ⁺ CD19 ⁺ CD5 ⁺ CD24 ^{hi} CD38 ^{hi}	V2	21	6.32 ± 1.47	11	3.99 ± 1.87	.47		
	V6	20	41.55 ± 5.77	9	3.99 ± 2.83	.0004	<.0001	.82
	V8	21	32.45 ± 5.59	10	2.34 ± 2.45	.001	.0001	.91

81

82 Proportion of IL-10 producing Breg cells following stimulation with CpG in LPP and PL-treated groups. **P* value (LPP vs PL): Mann-83 Whitney's Test for non-normalized data and Welch's t test for normalized data. **P* value (V6 or V8 vs baseline) LPP/PL: Wilcoxon's Test.

84 **Table E12**

Concentration of Lol p	Visit	n (LPP)	mean ± SEM (LPP)	n (PL)	mean ± SEM (PL)	<i>P</i> value (LPP vs PL)	<i>P</i> value of LPP (V6 or V8 vs baseline)	<i>P</i> value of PL (V6 or V8 vs baseline)
0.3 µg/mL	V2	21	91.35 ± 2.00	11	90.76 ± 2.40	.91		
	V6	21	71.90 ± 4.92	11	88.97 ± 3.28	.02	<.0001	.41
	V8	21	81.95 ± 4.72	11	86.74 ± 2.63	.70	.01	.04

85

86 Induction of IgG-associated blocking antibodies in LPP and PL-treated groups. **P* value (LPP vs PL): Mann-Whitney's Test for non-normalized
87 data and Welch's t test for normalized data. **P* value (V6 or V8 vs baseline) LPP/PL: Wilcoxon's Test. Lol p, *Lolium perenne*.

88 **Table E13**

	CSMS				RMS				RTSS			
	V6		V8		V6		V8		V6		V8	
	Spearman r	<i>P</i> value	Spearman r	<i>P</i> value	Spearman r	<i>P</i> value	Spearman r	<i>P</i> value	Spearman r	<i>P</i> value	Spearman r	<i>P</i> value
<i>Inducible Treg cell subsets</i>												
iT _R 35 cells	-0.36	.12	-0.14	.55	0.16	.52	0.17	.50	0-.60	.01	-0.31	.22
IL-10 ⁺ Treg cells	-0.52	.02	-0.45	.04	-0.33	.16	-0.46	.0499	-0.27	.27	-0.14	.59

89

90 Correlation statistics of clinical response and inducible T regulatory cell subsets in LPP- and PL-treated groups at V6 and V8.

91 **REFERENCES**

- 92 1. Mösger R, Kasche EM, Raskopf E, Singh J, Sohlich L, Astvatsatourov A, et al. A randomized, double-blind, placebo-controlled, dose-
93 finding trial with lolium perenne peptide immunotherapy. Allergy. 2018 Apr;73(4):896-904.
- 94 2. Mosges R, Koch AF, Raskopf E, Singh J, Shah-Hosseini K, Astvatsatourov A, et al. Lolium perenne peptide immunotherapy is well
95 tolerated and elicits a protective B-cell response in seasonal allergic rhinitis patients. Allergy. 2018;73(6):1254-62.