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

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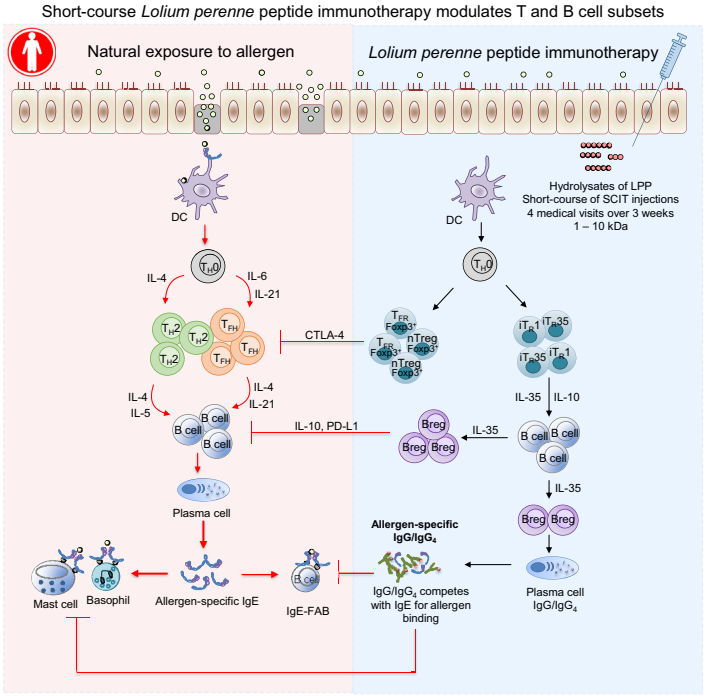
GRAPHICAL ABSTRACT



Abbreviations: LPP (*Lolium perenne* peptide), SCIT (subcutaneous), DC (dendritic cells), T_H0 (naïve T cells), T_H2 (T helper 2 cells), T_{FH} (follicular helper T cells), T_{FR} (follicular regulatory T cells), nTreg (natural regulatory T cells), iT_R1 (inducible IL-10-producing T regulatory cells), iT_R35 (inducible IL-35-producing regulatory T cells), PD-L1 (programmed death-ligand 1), CTLA-4 (cytotoxic T lymphocyte associated protein 4), IL (interleukin), Ig (immunoglobulin), IgE-FAB (IgE-facilitated allergen binding).

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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 (clinical.gov 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 IgG_4-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*

Abbreviations used

AIT: Allergen-specific immunotherapy
Breg: Regulatory B
CD40L: CD40 ligand
CRTH2: Chemoattractant receptor–homologous molecule expressed on T_H2 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_R35: IL-35–inducible regulatory T
LPP: *Lolium perenne* 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

Conventional allergen-specific immunotherapy (AIT) using purified whole aeroallergen extracts[1] or recombinant allergens[2] 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 NCT01111279).[8] We have performed safety, dose-escalation (clinicaltrials.gov NCT02156791),[9] and dose-finding (clinicaltrials.gov NCT01308021) studies[10] and identified the optimal treatment 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 allergens[12] 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 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

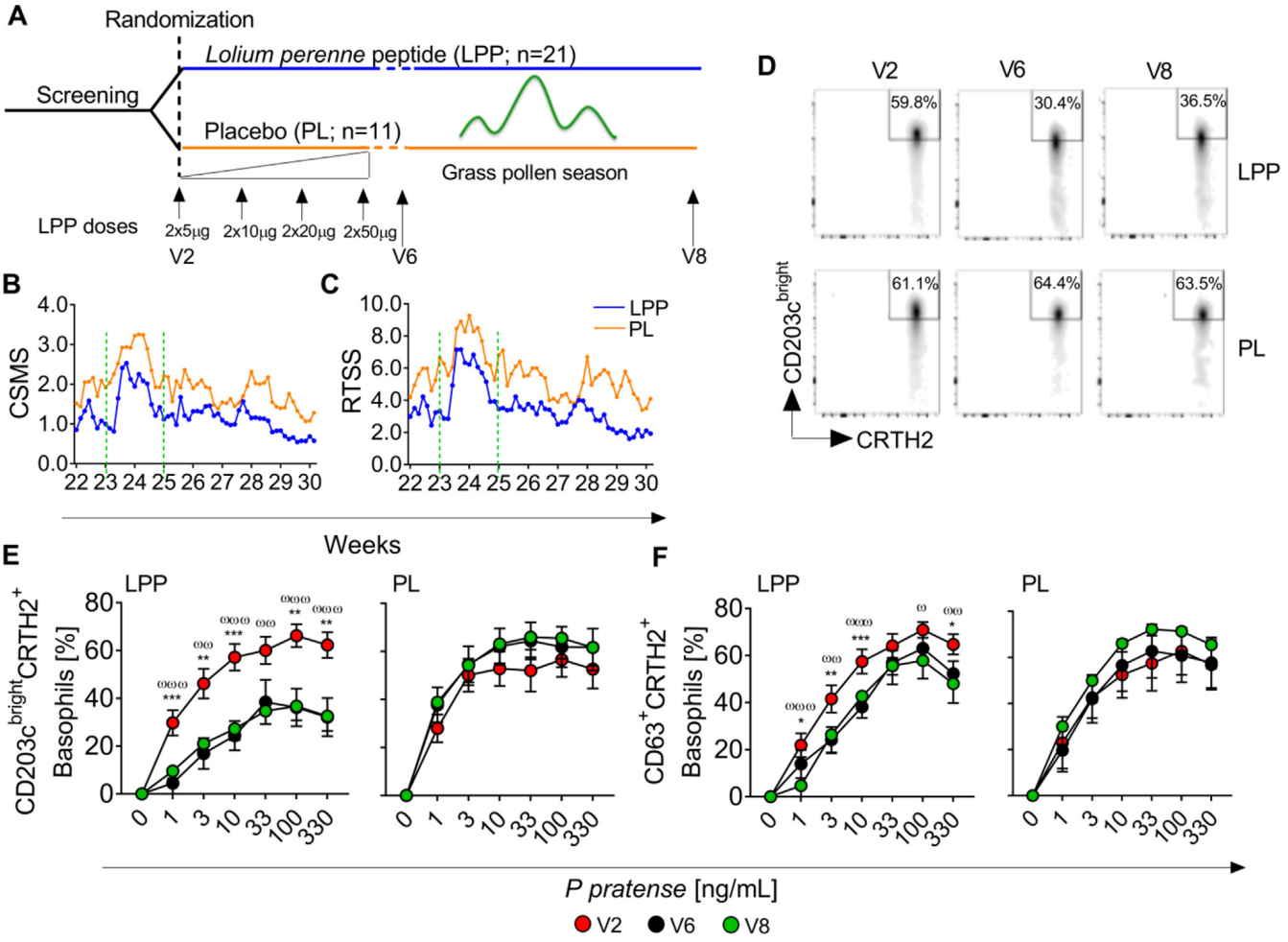


FIG 1. Reduction of basophil activation following LPP immunotherapy. **A,** Study design for patients with grass pollen–related allergic rhinitis in the RDBPC phase III trial. **B,** Reduction in daily CSMS in Belgium was −35.1% ($P = .03$) during the peak period and −53.7% ($P = .03$) during the entire 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. ωStatistical significance for V2 versus V8. Data are shown as means ± SEMs. * and ω$P < .05$, ** and ωω$P < .01$, and *** and ωωω$P < .001$, Mann-Whitney test.

(basophils) response, deletion of proallergic T_H2[15] 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 IgG_4 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 trial[13] 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 I; 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 × 5 µg for treatment at V2, 2 × 10 µg for treatment at V3, 2 × 20 µg for treatment at V4, and 2 × 50 µg for treatment at V5. A cumulative dose of 170 µg 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 ± SD	33.27 ± 8.26	32.52 ± 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 ± SD	23.19 ± 3.23	23.47 ± 3.59
Disease duration (y), mean ± SD	15.73 ± 9.95	18.19 ± 10.33
Grass pollen skin prick test (mm), mean ± SD	5.00 ± 1.79	6.05 ± 1.32
Grass pollen sIgE (kU$_A$/L), mean ± SD	20.76 ± 25.58	27.65 ± 31.89
Total IgE (IU/mL), mean ± SD	156.44 ± 211.28	219.83 ± 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 ≥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 *(Dermatophagoides farinae)*	1 (9.1)	3 (14.3)
House dust mite *(Dermatophagoides pteronyssinus)*	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 lyzed 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 μg/mL; InvivoGen, San Diego, Calif) and CD40 ligand (CD40L; 0.01 μg/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 μg/mL; R&D Systems) and CpG ODN 2006 (1 μg/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 μg/mL; Sigma-Aldrich) in the presence of monensin (20 μg/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 IgG$_4$

Specific IgE (sIgE) and sIgG$_4$ 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 1×10^5 EBV-transformed B cells (5 μL) 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 trial[13] 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–sIgG$_4$ 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.

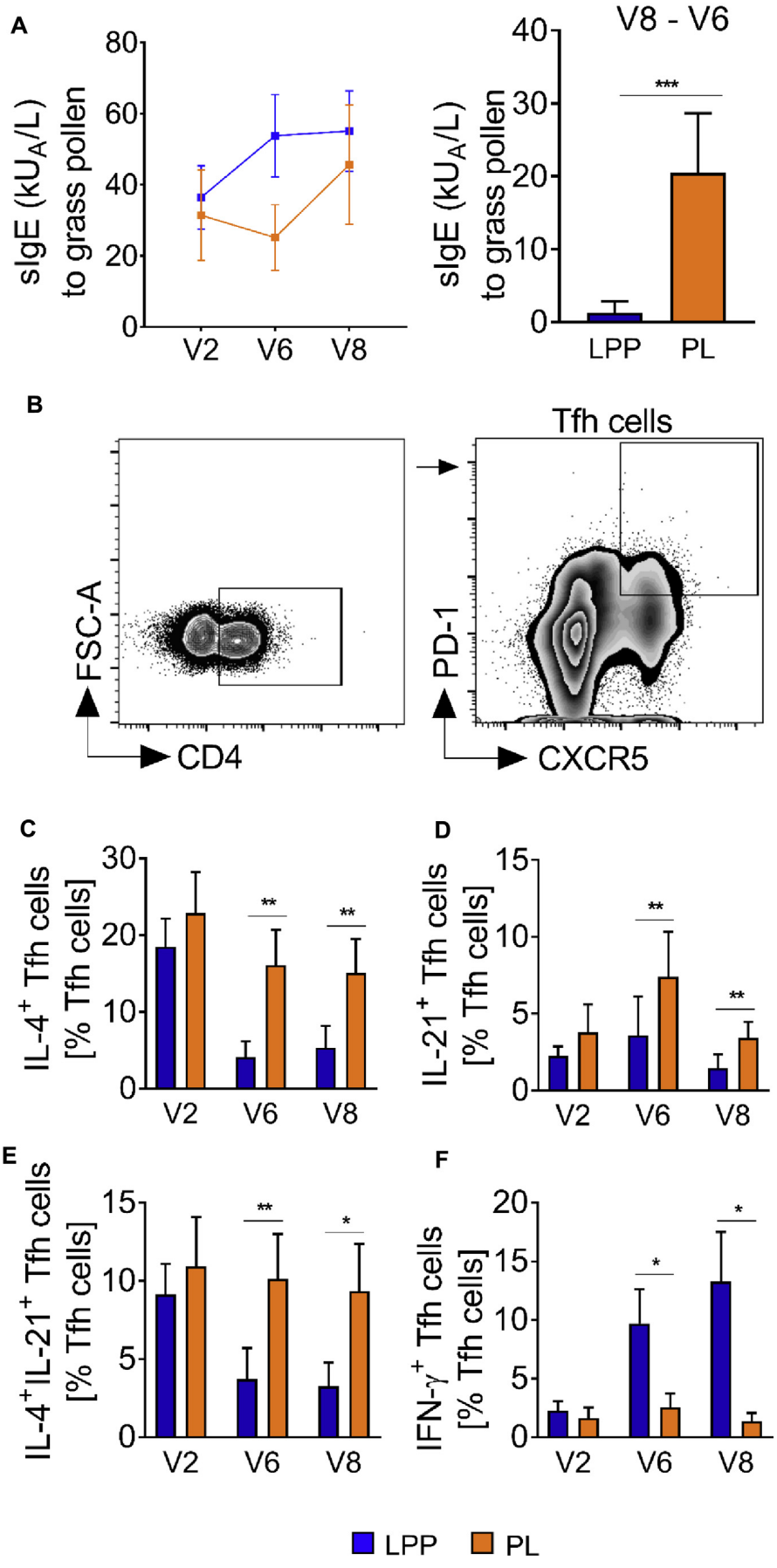
RESULTS

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


A
sIgE (kUA/L) to grass pollen
80
60
40
20
0
V2
V6
V8
V8 - V6
***
40
30
20
10
0
LPP
PL
B
Tfh cells
FSC-A
CD4
PD-1
CXCR5
C
IL-4+ Tfh cells [% Tfh cells]
30
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V2
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D
IL-21+ Tfh cells [% Tfh cells]
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IFN-γ+ Tfh cells [% Tfh cells]
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LPP
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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 μg/mL) on basophil activation after FcεRI cross-linking in LPP- and PL-treated groups. The proportions of $CD203c^{bright}CRTH2^+$ and $CD63^+CRTH2^+$ basophils following FcεRI 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 $(CD4^+IFN\text{-}\gamma^+)$ 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 $CD4^+CXCR5^+PD\text{-}1^+$ 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 $IL\text{-}4^+IL\text{-}21^+$ 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 $(T_{FR}$; $CD4^+CXCR5^+PD\text{-}1^+FoxP3^+)$ 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 $CTLA\text{-}4^+$ 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 $IL\text{-}35^+$ and $IL\text{-}10^+$ 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 $CXCR5^+PD\text{-}1^+$ were defined as T_{FH} cells. *FSC*, Forward scatter. **C-F,** Percentages of $IL\text{-}4^+$, $IL\text{-}21^+$, dual $IL\text{-}4^+IL\text{-}21^+$, and $IFN\text{-}\gamma^+$ T_{FH} cells were assessed within T_{FH} cell population by using FACS. Data are shown as means ± 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$_R$35) cells were increased in LPP-treated group at V6 ($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$^+$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 IgG$_4$ antibodies. We assessed whether such blocking antibodies were induced in LPP- and PL-treated groups. Levels of grass pollen–specific IgG$_4$ 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$_R$35 and IL-10$^+$ Treg cells). There was a negative correlation observed between iT$_R$35 cells and RTSS at V6 ($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$_H$2, IL-4$^+$, IL-21$^+$ and dual IL4$^+$IL-21$^+$ T$_{FH}$ cells was observed in LPP-treated patients. Circulating Treg and T$_{FR}$ cells were induced following LPP treatment. Moreover, LPP immunotherapy stimulated iT$_R$35 cell induction, which favored *de novo* IL-10 production from CD19$^+$ B cells and Breg cell subsets. This leads to the production of allergen-neutralizing IgG$_4$ 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 trial[13] 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$_H$2 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.

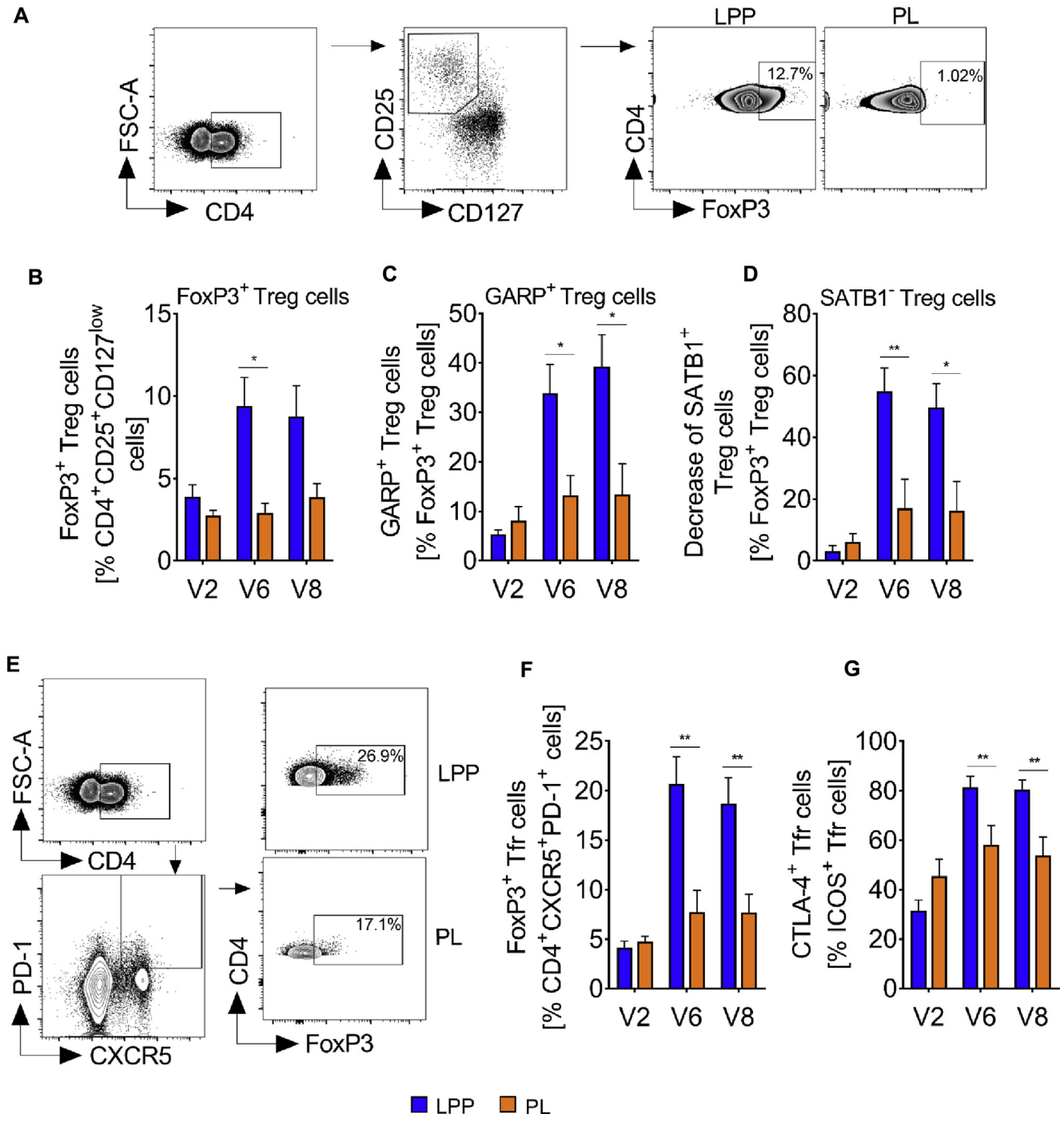

FIG 3. LPP induces expression of regulatory cells. **A,** Representative plots of Treg cells in LPP-treated (n = 21) and PL-treated (n = 11) groups. *FSC*, Forward scatter. **B,** Percentage of $FoxP3^+$ Treg ($CD4^+CD25^+CD127^{low}FoxP3^+$) cells within $CD4^+CD25^+CD127^{low}$ cells in LPP-treated (n = 21) and PL-treated (n = 11) groups were assessed by FACS. *Ex vivo* staining was performed on isolated PBMCs from whole blood collected before the pollen season (V2), after the treatment period (V6), and after the grass pollen season (V8). **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} ($CD4^+CXCR5^+PD\text{-}1^+FoxP3^+$) cells in LPP-treated (n = 21) and PL-treated (n = 11) groups. **F,** Percentage of T_{FR} ($CD4^+CXCR5^+PD\text{-}1^+FoxP3^+$) cells within $CD4^+CXCR5^+PD\text{-}1^+$ cells. **G,** Percentage of CTLA-4^+ T_{FR} ($CD4^+CXCR5^+PD\text{-}1^+FoxP3^+ICOS^+CTLA\text{-}4^+$) cells within $CD4^+CXCR5^+PD\text{-}1^+FoxP3^+ICOS^+$ cells. Data are shown as means ± SEMs. $^*P < .05$ and $^{**}P < .01$, Mann-Whitney test.

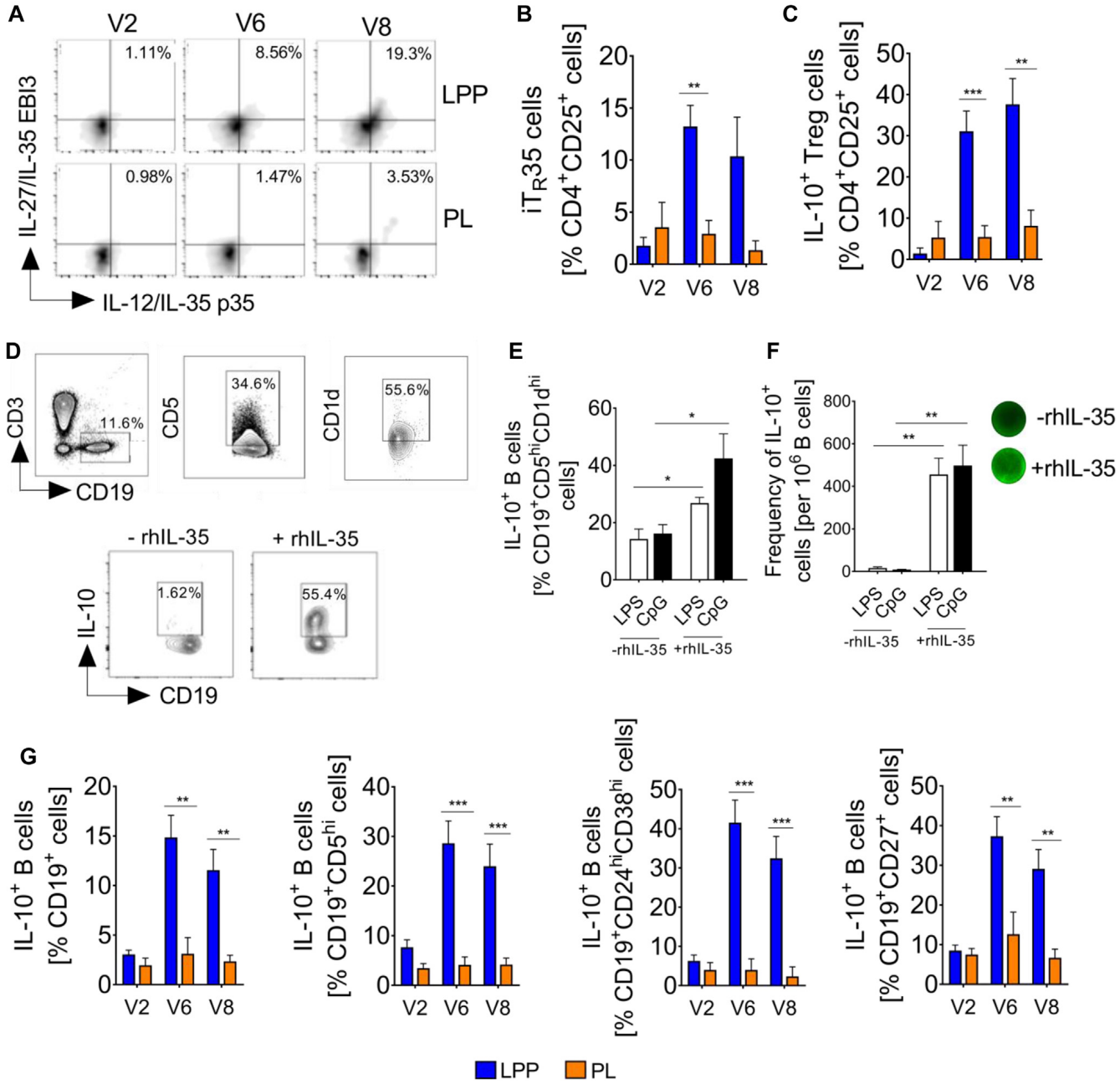

FIG 4. Induction of regulatory cells. **A,** Representative plots analysis of EBI3$^+$p35$^+$ Treg cells. IL-35–producing Treg cells were assessed by using FACS in LPP-treated (n = 21) and PL-treated (n = 11) groups at V2, V6, and V8. **B,** Percentage of iT$_R$35 cells within CD4$^+$CD25$^+$ cells. **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$^+$CD24^{hi}CD38^{hi}, and IL-10$^+$CD19$^+$CD27$^+$ Breg cells was increased in LPP-treated patients. Data are shown as means ± 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

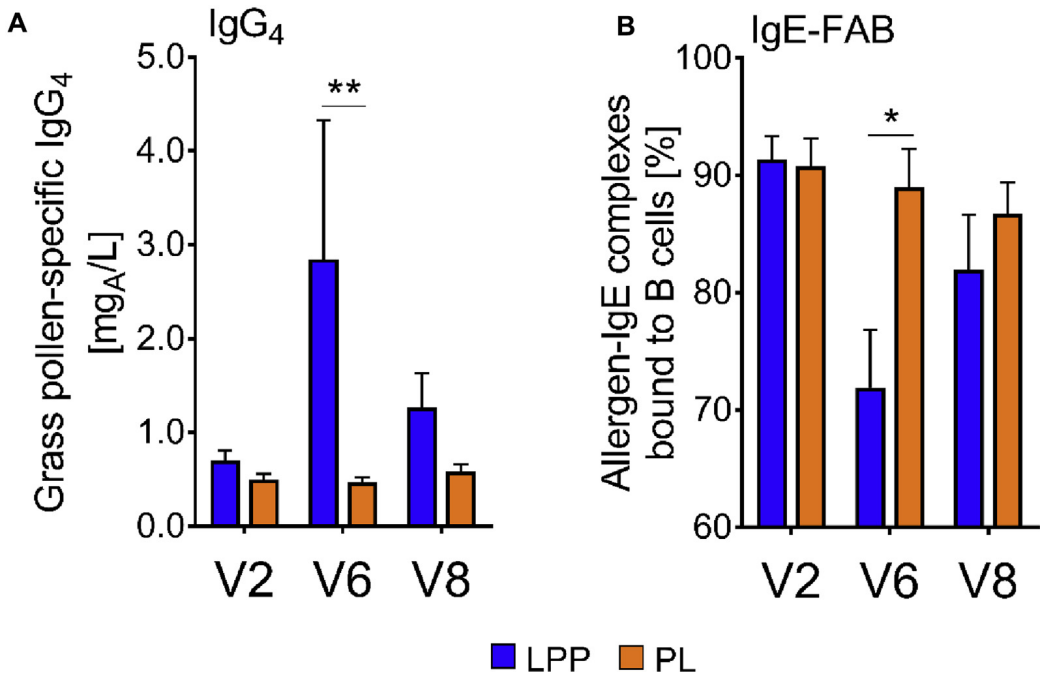

FIG 5. LPP enhances IgG_4 blocking activities. **A,** Effect of LPP immunotherapy on IgG_4 production in serum samples of patients obtained at V2, V6, and V8 were measured by ImmunoCAP. **B,** Induction of IgG-associated blocking antibodies that inhibit IgE-facilitated allergen-IgE binding to B cells. 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 ± 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 IgG_4 antibodies during LPP treatment.

We have shown that LPP treatment enhanced IgG_4 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 IgG_4 antibodies can compete with IgE to inhibit allergen-IgE complexes binding to CD23 (FcεRII) present on B cells, thus inhibiting facilitated antigen presentation to T cells.[18] Altogether, Treg and Breg cell regulation leading to IgG_4 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 IgG_4 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 IgG_4 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 IgG_4 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 IgG_4-blocking antibodies, which compete with IgE and suppress allergen-IgE binding to B cells.**

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