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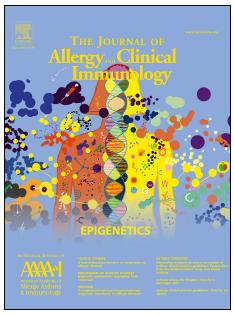

Immunologic Mechanisms of Short-course of *Lolium Perenne* Peptide Immunotherapy: A Randomized Double-Blind Placebo-Controlled Trial

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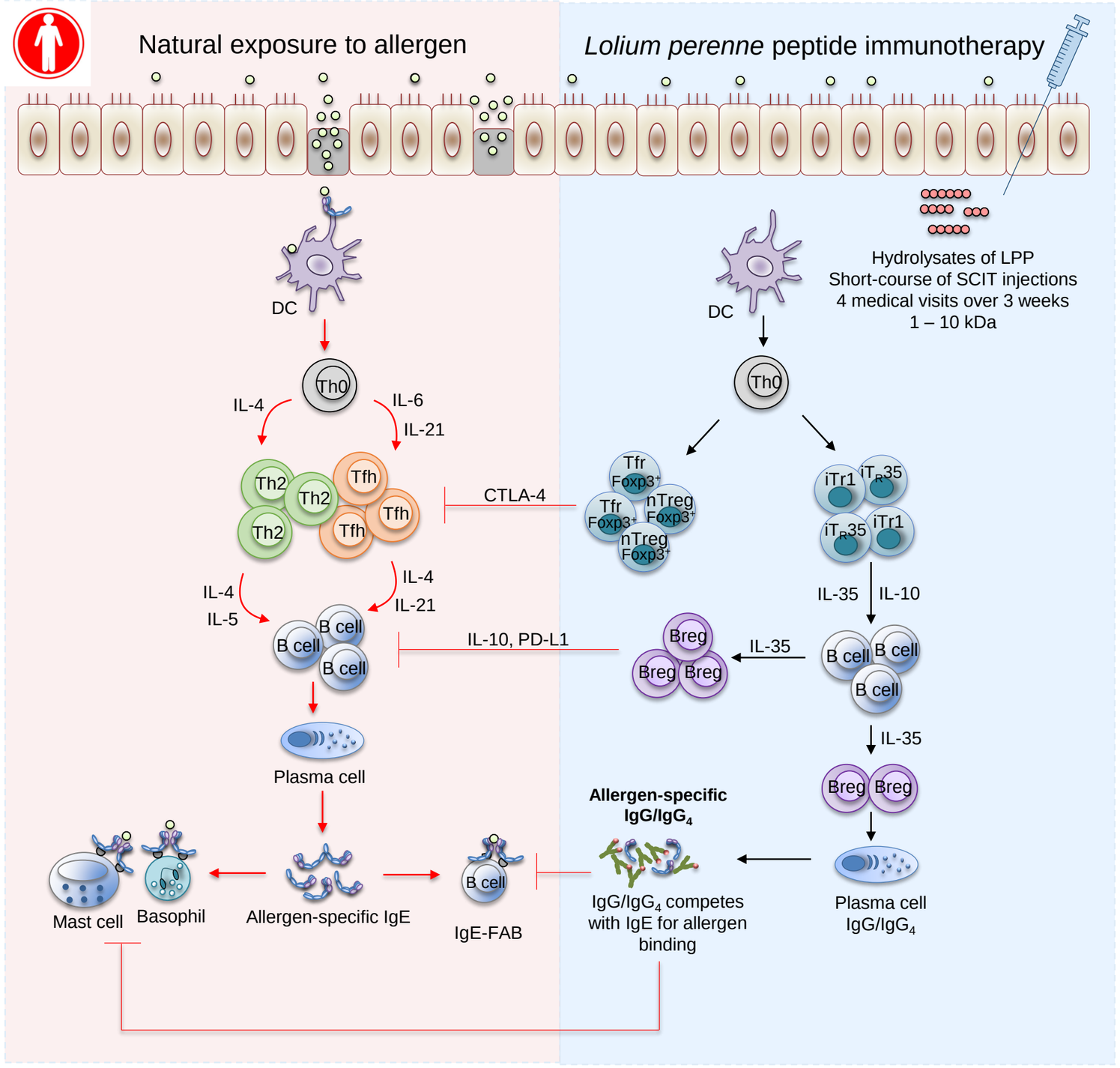
Short-course *Lolium perenne* peptide immunotherapy modulates T and B cell subsets



Abbreviations: LPP (*Lolium perenne* peptide), SCIT (subcutaneous), DC (dendritic cells), Th0 (naïve T cells), Th2 (T helper 2 cells), Tfh (T follicular helper cells), Tfr (T follicular regulatory cells), nTreg (natural T regulatory cells), iTr1 (inducible IL-10-producing T regulatory cells), iT_R35 (inducible IL-35-producing T regulatory 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).

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

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M. H. Shamji reports grants from Immune Tolerance Network, ASIT Biotech S.A., ALK, Regeneron, Merck and personal fees from ASIT Biotech S.A., ALK and Allergopharma. S. R. Durham reports grants from Biotech tools, during the conduct of the study; grants and personal fees from ALK, Denmark, personal fees from Anergis, Adiga, Biomay, Allergy Therapeutics, med Update GmbH and grants from Food Standards Agency, National Institute of Health Research. S. Pirotton has a patent EP 2 043 684 issued. J. Duchateau has patent numbers 631271 and 6709672 and publication numbers 20020102632 and 20150150956 issued. C. Bachert received research grants via Ghent University Hospital from ASIT biotech S.A. T. Legon reports personal fees from ASIT biotech S.A., and a patent EP 2 043 684 issued. N. Wathelet, S. Pirotton, N. Bovy, MA Bonny are employed by ASIT biotech S.A. S. Pirotton and T. Legon are shareholders of ASIT biotech S.A. R. Mosges received research grants via the Institute of Medical Statistics, Informatics and Epidemiology from ASIT biotech S.A. R. von Frenckell is consultants for ASIT biotech S.A. H. Sharif, I. Singh, L. Kouser, A. Karamani, R. V. Parkin, U. Kishore, A. Robb, M. Katotomichelakis, G. Holtappels, L. Derycke, F. Corazza declare that they have no relevant conflict of interest.

Total word count: 3456.

Key Messages

- Pre-seasonal 3-week short-course of adjuvant-free peptide hydrolysates of *Lolium perenne* (LPP) over four medical visits inhibited basophil response and attenuated Th2 pro-allergic responses.
- LPP immunotherapy induced peripheral FoxP3 regulatory T and T follicular regulatory cells, stimulated the induction of IL-35^+ T cells (iT_R35) which promoted production of IL-10 from $CD19^+$ B cells and Breg subsets.
- LPP immunotherapy was associated with the induction of grass pollen-specific neutralizing IgG_4 blocking antibodies which competes with IgE and suppress allergen-IgE binding to B cells.

Capsule Summary

Pre-seasonal short-course of hydrolysates of *Lolium perenne* peptides (LPP) immunotherapy is clinically effective and accompanied by modulation of T and B cell subsets.

Keywords

Allergy, peptide immunotherapy, T follicular helper cells, Tregs, Bregs.

Abbreviations

AIT, Allergen-specific immunotherapy; SAR, Seasonal allergic rhinitis; NAC, Non-atopic controls; LPP, *Lolium perenne* peptides; Breg, Regulatory B cells; iT_R35, IL-35 inducible regulatory T cells; Treg, Regulatory T cells; Tfh, T follicular helper cells; Tfr, T follicular regulatory cells.

Abstract:

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

Objective: 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 (PL; n=11) for 3 weeks over 4 visits. Grass pollen-induced basophil, T and B cell responses were evaluated before (V2), end of treatment (V6) and after the pollen season (V8).

Results: Combined symptom and rescue medication scores (CSMS) were lower during the peak (-35.1%, *P*=.03) and throughout pollen season (-53.7%, *P*=.03) in LPP- compared to PL-treated group. $CD63^+$ and $CD203c^{bright}CRTH2^+$basophils were decreased following LPP treatment at V6 (all, *P*<.0001) and V8 (all, *P*<.001), compared to V2. No change in PL-treated group was observed. Blunting of seasonal increases of grass pollen-specific IgE was observed in LPP- but not PL-treated group. LPP immunotherapy but not PL was associated with a reduction of IL-4^+ Th2 (V6, *P*=.02), IL-4^+ (V6, *P*=.001;V8, *P*=.0095) and IL-21^+ (V6, *P*=.0002) T follicular helper cells. Induction of $FoxP3^+$, follicular regulatory T and IL-10^+ Breg 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 immunological mechanisms of LPP immunotherapy are underscored by immune modulation in the T and B cell compartments which is necessary for its effect.

Abstract word count: 250

INTRODUCTION

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 symptoms-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 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 whilst 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 studies (clinicaltrials.gov NCT01308021)[10], and identified the optimal treatment schedule (4 x 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, *Lolium perenne* allergen can be used to treat allergic rhinitis induced by other grasses.[11] The advantages over the whole-protein allergens[12] are that linear peptides do not bind to IgE and cross-link FcεRI on the surface of mast cells and basophils, therefore, do not release mediators such as tryptase and histamine.

We have recently evaluated the efficacy of LPP treatment in a prospective, multicenter, randomized, double-blind, placebo-controlled (RDBPC) Phase III trial (ClinicTrials.gov no.

NCT02560948; EudraCT no. 2015-002105-11),[13] which was carried out in 57 different sites in Europe. 372 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 the 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, quality of life and a decrease in grass pollen conjunctival provocation test (CPT) scores.[13] The study was designed to demonstrate safety and efficacy of LPP and to investigate mechanistic endpoints using blood samples from LPP- and PL-treated groups collected from a single center site (Belgium).

This sub-study was specifically conducted to assess whether LPP immunotherapy would suppress early and late phase allergic responses. We wanted to identify the immunological 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 towards a Th1 response.[14]

We therefore hypothesized that short-course LPP immunotherapy leads to suppression of early allergic effector cell (basophils) response, deletion of pro-allergic Th2[15] and Tfh cells[16] which are known to promote IgE responses and induction of T regulatory 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 one clinical site in Belgium that 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 (V1), eligible participants (n=32) were randomized 2:1 to receive subcutaneous injections of LPP immunotherapy or placebo (PL) (Fig 1, *A*; Repository Fig E1; Table I). 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 mins later. Doses were increased progressively as follows: 2 × 5 µg for treatment at visit (V) 2 (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 as 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 the treatment), V6 (after the treatment) and V8 (after the pollen season). Daily combined symptom and rescue medication scores (CSMS) was collected from each participant during the peak (14 consecutive days within weeks 23–25) and the entire pollen season (weeks 22–30).

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 of *Phleum pratense* (Phlp) were added to heparinized whole blood and incubated at 37ºC in water bath for 15 mins. Cells were stained with cell surface antibodies (see Online

Repository). Red blood cells were lyzed with BD lysing solution (BD Biosciences, San Jose, CA) at room temperature in the dark for 10 mins and fixed using CellFix solution (BD Biosciences), prior to 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/without Phlp or CpG ODN 2006 (1 μg/mL; Invivogen, CA, USA) and CD40L (0.01 μg/mL; R&D Systems, Abingdon, UK) for up to 48 hours, respectively. To investigate the effect of IL-35 on the induction of 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, UK) in the presence or absence of rhIL-35 (100 ng/mL; Enzo Life Sciences, Exeter, UK) for 48 hours. Cells were washed using culture medium and stimulated with PMA (50 ng/mL; Sigma-Aldrich) and Ionomycin (1 μg/mL; Sigma-Aldrich) in the presence of monensin (20 μg/mL; BioLegend, London, UK) 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, UK). Cells were immunostained with cell surface and intracellular antibodies (see Online Repository) and acquired on BD FACSCanto™ II and BD LSRFortessa™ (BD Biosciences).

Serum allergen specific IgE and IgG_4

Specific IgE and IgG_4 to a grass pollen mixture (*anthoxanthum odoratum, lolium perenne, phleum pratense, secale cereale, holcus lanatus)* were measured in serum samples using ImmunoCAP system (Thermo Fisher Scientific, Pierce, UK) according to the manufacturer's instructions.

IgE-facilitated allergen binding assay

The allergenicity of LPP was tested by IgE-facilitated allergen binding to B cells as previously described.[18] Serum from allergic patients were pre-incubated with Phlp for 1 hour at 37ºC, followed by the addition of 1×10^5 EBV-transformed B cells (5 μL) and incubated for 1 hour at 4°C. Binding of allergen-IgE complexes to B cells were determined by polyclonal human anti-IgE PE-labelled antibody (Miltenyi Biotec) and acquired by 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 were enrolled in the Phase III trial[13] and attended the clinical site in Ghent, Belgium. The Phase III study was powered for the primary endpoint which was the reduction of 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 pre-planned and were included in the study protocol and a statistical analysis plan (SAP) was also predefined and finalized prior to performing biological analyses. For this study, sample size and power calculation was based on immunological parameters including grass pollen-specific IgG_4 and serum inhibitory antibody as measured by the IgE-FAB assay obtained from the Phase IIa[9] (clinicaltrials.gov NCT02156791) and Phase IIb[10] study (clinicaltrials.gov NCT01308021) (See Tables E1 and E2 in the Online Repository).

Statistical data analysis was performed using GraphPad Prism 7.02 (GraphPad Software Inc., San Diego, CA, USA). Non-parametric Mann-Whitney test was used to statistically compare between different groups of patients and non-parametric Wilcoxon paired signed-rank test was used to compare data within the same sample. Normally distributed data was analyzed

using parametric Welch's t-test. A *P* value of <.05 was considered to be statistically significant.

RESULTS

Reduction in symptom scores following LPP treatment

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

LPP immunotherapy but not placebo inhibits grass pollen-induced basophil responsiveness

The effect of LPP on FcεRI-mediated allergic inflammation, a surrogate endpoint of early type I-mediated hypersensitivity reaction was investigated by measuring basophil responsiveness. At V2, the proportion of $CD203c^{bright}CRTH2^{+}$ (Fig 1, *D* and *E*, and see Table E3 in the Online Repository) and $CD63^{+}CRTH2^{+}$ basophils (Fig 1, *F*, and see Table E4 in the Online Repository) 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 allergens in the LPP- (*P*<.05; compared to 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 following FcεRI cross-linking in LPP- and PL-treated groups. The proportion of $CD203c^{bright}CRTH2^{+}$ and $CD63^{+}CRTH2^{+}$ basophils following FcεRI cross-linking by anti-human IgE antibody was decreased at V6 and V8 compared to V2 in the LPP- but not in the PL-treated group (see Fig E3 and Table E5 in the Online Repository).

Blunting of seasonal increase in grass pollen-specific IgE in LPP but not placebo-treated groups

Specific IgE (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 induction of IgE following natural exposure during the pollen season) was assessed, sIgE induction in the PL-treated group was significantly higher compared to the LPP-treated group (*P*=.0004; Fig 2, *A*, right).

Attenuation of IL-4-producing Th2 cells, IL-4, IL-21 and dual IL-4, IL-21-producing Tfh cells following LPP immunotherapy but not in placebo

Following LPP treatment, there was a significant reduction of IL-4-producing Th2 ($CRTH2^+CD27^-$) cells at V6 (*P*=.02) but this was lost at V8 in LPP- but not PL-treated group. In contrast, Th1 cells ($CD4^+IFN\text{-}\gamma^+$) cells were significantly higher in LPP-treated group at V6 (*P*=.01) compared to PL, but this was lost at V8 (Table E6 in the Online Repository). Immune deviation from a Th2 to Th1 response has been demonstrated previously in conventional immunotherapy. However, there has been increasing evidence that a subset of T helper (Th) cells, called T follicular helper (Tfh) cells also play a crucial role in the pathology of allergic disease and IgE class-switching.[19,20] They are defined as $CD4^+$ cells that co-expressed CXCR5 and PD-1 and these $CD4^+CXCR5^+PD\text{-}1^+$ cells are henceforth referred to as Tfh cells (Fig 2, *B*). Tfh cells secrete IL-4 and IL-21 and has been shown to induce IgE production through STAT3 signalling.[21] IL-4-producing Tfh cells were significantly lower in LPP- compared to PL-treated group at V6 and V8 (*P*=.003 and *P*=.004, respectively; Fig 2, *C*). IL-21-producing Tfh 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 $IL\text{-}4^+IL\text{-}21^+$ Tfh cells were also

enumerated and this was significantly lower in LPP- compared to 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 the Online Repository). In contrast, IFN-γ-producing Tfh cells were significantly higher in LPP- compared to PL-treated group at V6 and V8 (*P*=.03 and *P*=.01, respectively; Fig 2, *F*).

Induction of FoxP3$^+$ Treg and Tfr cells following LPP immunotherapy but not placebo

The regulatory counterparts of T helper cells were investigated. LPP-treated group showed induction of FoxP3$^+$ Treg ($CD4^+CD25^+CD127^{low}FoxP3^+$; Fig 3, *A*) cells but not in PL-treated group (V6; *P*=.03), nonetheless the effect became non-significant at V8 (Fig 3, *B*). We further analyzed the functional counterparts of these Treg cells. Studies have shown GARP expression and SATB1 repression in Treg cells represent a suppressive subset of Treg cells.[22,23] GARP$^+$ Treg cells were significantly higher in LPP- 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 that was higher in LPP- compared to PL-treated group at both V6 (*P*=.002) and V8 (*P*=.01; Fig 3, *D*, and see Table E8 in the Online Repository).

A subset of Treg cells, called T follicular regulatory (Tfr; $CD4^+CXCR5^+PD\text{-}1^+FoxP3^+$) cells have been shown to regulate the interaction between B and Tfh cells. There was significantly higher Tfr cells in LPP- compared to PL-treated group at V6 and V8 (*P*=.004 and *P*=.004, respectively; Fig 3, *E* and *F*). Tfr cells have also been shown to exert their suppressive ability through the expression of CTLA-4.[24] CTLA-4$^+$ Tfr cells were significantly higher in LPP- 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 the Online Repository).

LPP immunotherapy but not placebo induced IL-35$^+$ and IL-10$^+$ Tregs that promoted B regulatory cells induction

The induction of IL-35- and IL-10-producing Treg cells upon stimulation with Phlp was investigated in PBMCs obtained from LPP- and PL-treated individuals at V2, V6 and V8. Inducible IL-35$^+$ Treg cells (iT$_R$35) were increased in LPP- at V6 (*P*=.01) compared to PL-treated group (Fig 4, *A* and *B*). Additionally, proportion of IL-10$^+$ Treg cells were significantly increased in LPP- at V6 (*P*=.0004) and V8 (*P*=.001) compared to PL-treated group (Fig 4, *C*, and see Table E10 in the Online Repository).

To assess the effect of IL-35 on the conversion of human B cells into Breg cells, PBMCs from grass pollen allergic individuals, 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*).

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- 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 as compared to PL-treated group (Fig 4, *G*, and see Table E11 in the Online Repository).

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 to 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 the PL-treated group. The ability of these antibodies to compete for IgE binding to B cells was decreased at V6 in the LPP- 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 the Online Repository).

Relationship between immune parameters and clinical effect

We assessed the relationship between combined symptom and rescue medication scores (CSMS), rescue medication scores (RMS) and rhinoconjunctivitis total symptom scores (RTSS) with inducible Treg cell subsets (iT_R35 and IL-10^+ Treg cells). There was a negative correlation observed between iT_R35 cells with 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 the Online Repository).

DISCUSSION

Here, we show in a RDBPC trial that a 3-week short-course of adjuvant-free hydrolysates of LPP over four medical visits reduce CSMS and RTSS. LPP immunotherapy inhibited allergen-induced basophil responsiveness and reactivity. Blunting of seasonal increases of grass pollen-specific IgE and attenuation of circulating IL-4^+ Th2, IL-4^+, IL-21^+ and dual $IL4^+IL\text{-}21^+$ Tfh cells was observed in LPP-treated patients. Circulating Treg and Tfr cells were induced following LPP treatment. Moreover, LPP immunotherapy stimulated the induction of iT_R35 cells which favoured *de novo* IL-10 production from $CD19^+$ B 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 surface of B cells. These findings are from a subset of participants in a larger Phase III clinical trial[13] in whom we were able to collect blood samples for mechanistic analysis. The design of the study included a mechanistic analysis in a 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 pre-planned and were included in the study protocol. In addition to this, the reported clinical data represents the studied cohort in the single center and therefore, it needs to be considered in the context of the whole study.

The immunological assays performed throughout this study involved stimulation of PBMCs with timothy grass pollen allergen (Phlp). Despite the patients undergoing LPP treatment, previous studies have shown the extensive cross-reactivity among members of the subfamily Pooideae.[25] Both Phlp and *Lolium perenne* belong to the subfamily Pooideae. Sequence analysis performed on both allergens showed that both Phlp and *Lolium perenne* shared an extensive homology. *Lolium perenne* isoallergens shared between 30-90% homolog sequences with Phlp 1, which contributes to their cross-reactivity.[11] In addition, Phlp 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 justify the use of timothy grass pollen allergen in *in vitro* assays.

In this study, allergen-induced basophil responsiveness was decreased as early as three 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 receptor cross-link and release allergic effector molecules.[17] We showed that the induction of IgE following LPP treatment during the grass pollen season may be due to the priming effect of the grass pollen season resulting in the 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 pollen season in LPP- was less than that in PL-treated group, suggesting that LPP treatment suppresses Th2 cell responses which is responsible for the production of IgE by B cells. It was also apparent from the levels of IgE at baseline that both the LPP- and PL-treated groups were moderate-to-severely allergic towards grass pollen allergen. Nevertheless, LPP-treated group showed significantly improved symptom scores during the pollen season compared to PL-treated group.

To address the factors that drive B cell responses, we investigated a subset of T cells known as Tfh cells.[27–29] Here, we demonstrated that IL-4- and IL-21-producing Tfh cells were lower in LPP- compared to PL-treated group, suggesting that IL-4- and IL-21-producing Tfh cells may be pathogenic in allergy. 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 have also been shown to induce IgE production by B cells through the activation of STAT3.[21,30] The

observed effect of LPP on IL-4- and IL-21-producing Tfh may 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 Tfh cells, including IFN-γ-producing Tfh cells. In this study, IFN-γ-producing Tfh cells were elevated in LPP-treated group. Similarly, high levels of IFN-γ^+ Th1 cells were observed in the same group, with a significant reduction of Th2 cells. This finding is consistent with the previous finding that reported immune deviation towards a Th1 response following conventional immunotherapy.[31]

Previous studies have shown transient induction of Treg cells following immunotherapy.[32] Treg cells that expressed FoxP3, 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, they are expressed in activated T cells.[33] Recent studies have shown that the 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 the Treg cells.[23,34] In addition, GARP has been shown to be highly expressed in Treg cells.[22] Together, the expression of FoxP3, GARP and repression of SATB1 within Treg subsets can be used to identify suppressive Treg cells. A Treg cell subset, Tfr cells, have been previously described as a subset of T cells that regulates B and Tfh cell interaction.[35] LPP induces Tfr and CTLA-4^+ Tfr cells which persists even after the grass pollen season. Previous studies have shown CTLA-4 to be crucial for Tfr cells to exert their suppressive functions,[24] and it is speculated that these functional Tfr cells may suppress cytokine production by Tfh cells, therefore disrupting the cytokine-mediated stimulation of B cells.[36] These observations on Tfh, Tfr and Treg cells suggest that these cells may act in a similar mechanism that mirrors the fate of Th2, Th1 and Treg cells following conventional immunotherapy.

Several studies have highlighted the role of IL-35 in the immune regulation autoimmune disease *in vivo*.[37] IL-35 induces the expansion of Bregs, Tregs and iT_R35 cells.[38] These regulatory cells promote immune regulation that can control Th2 inflammation. In our study, we have shown for the first time that a short-course LPP treatment induced iT_R35 cells. Moreover, previous studies have illustrated that IL-35 has the ability to induce IL-10^+ Breg cells by activating STAT1/STAT3.[37] It is likely that IL-35 promotes the induction of iT_R35 cells which in turn can differentiate B cells into IL-10^+ Bregs that produce allergen neutralizing IgG_4 antibodies during LPP treatment.

We have shown that LPP treatment enhanced IgG_4 production and prevented allergen-IgE complexes binding to B cells which subsequently inhibit Th2 cell activation. This observation is in agreement with the findings obtained using 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, the regulation of Tregs and Bregs leading to IgG_4 production may therefore 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 the 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. Immunological parameters were measured at baseline, at the end of the treatment (after 3 weeks) and end of the pollen season.

Previous studies on conventional AIT showed association of AIT with a reduction in the pro-inflammatory Th2 cell responses and an induction of T regulatory cells.[14] This was accompanied by the induction of blocking IgG_4 antibodies. In this study, we have shown that short-course LPP treatment results in the attenuation of the pro-allergic inflammatory T cells and induction of regulatory T and B 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 three years to achieve if given subcutaneously or sublingually. It is likely that a short-course immunotherapy treatment (4 physician visits over 3 weeks) may improve patient compliance which currently is 25% for SCIT and 12.5% for SLIT.[39]

To date, there is very limited studies that investigate the tolerance endpoint for short courses AIT. A recent phase IIb study was performed in cat allergic patients treated with short-course peptide immunotherapy using major cat allergen peptide, Fel d 1, referred to as Cat-PAD. The study showed persistent tolerance towards cat allergen for up to two 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 participants from the phase III study were exposed to cat and this may have resulted in the induction of IgG antibodies that may have been protective even in the placebo-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 two cycles of treatment off-season over a period of two years to induce $sIgG_4$ antibodies and blocking activity in serum of treated patients.[41] Intralymphatic immunotherapy indicated in allergic patients have also been shown to be clinically effective when administered as a short-course (three intralymphatic

allergen administrations within 8 weeks) and induced long-term tolerance following cessation of treatment.[42] These studies showed that short-course of 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 clinical as well as immunologic responses. In addition, previous studies have shown that a booster AIT injection prior to the pollen season following cessation of immunotherapy treatment resulted in a significant reduction in the CSMS of grass pollen allergic patients during the pollen season.[43] One could therefore give booster injection before the second pollen season to evaluate the persistence of 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 of rescue medications intake during the peak and the entire pollen season. The immunologic mechanisms of LPP immunotherapy are underscored by immune modulation in the T and B cell compartments.

491 **TABLE I**: Patient demographics

Characteristic	Placebo N=11	LPP N=21
Age (years), mean ± SD	33.27 ± 8.26	32.52 ± 11.19
Sex, n (%)		
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-specific IgE (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, n (%)		
Intermittent	1 (9.1)	0 (0.0)
Persistent	10 (90.9)	21 (100.0)
Asthmatic	1 (9.1)	3 (14.3)
Co-sensitizations (SPT > 3mm), n (%)		
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)

492

493 Data shown for the population with immunogenicity data. n= number of patients. N= total
494 number of patient per group. Abbreviations: IU, international units; kU_A, kilounits; kU_A,
495 allergen-specific kilounits; SD, standard deviation; SPT, skin prick test.

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FIGURE LEGENDS

Figure 1. Reduction of basophil activation following LPP. (A) Study design for patients with grass pollen related allergic rhinitis in the RDBPC Phase III trial. **(B)** Reduction in daily combined symptoms and medication scores (CSMS) in Belgium was -35% (*P*=.03) during peak period and -53.7% (*P*=.03) during the entire pollen season in the LPP (n=21) compared to PL-treated group (n=11). **(C)** Reduction of rhinoconjunctivitis total symptom scores (RTSS) in LPP-treated group in Belgium during 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 displayed surface activation markers CD63 and CD203c on $CRTH2^+$ basophils. Representative plots of $CD203c^{bright}CRTH2^+$ basophils of LPP- (n=21) or PL- (n=11) treated patients at V2, V6 and V8. **(E** and **F)** A dose dependent response of **(E)** $CD203c^{bright}CRTH2^+$ and **(F)** $CD63^+CRTH2^+$ basophils in LPP- and PL-treated groups at V2, V6 and V8. Green dotted lines represent peak pollen season. *denotes statistical significance for V2 vs. V6 while ω denotes statistical significance for V2 vs. V8. Data are shown as mean (±SEM). **P*<.05, ***P*<.01, ****P*<.001, Mann-Whitney test.

Figure 2. LPP inhibits pro-inflammatory Tfh cells. (A) Levels of grass pollen sIgE (kU_A/L) in serum samples of LPP- (n=21) and PL- (n=11) treated 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 treatment period (V6), and after grass pollen season (V8) and cultured for 6 days in the presence of Phlp. **(B)** $CD4^+$ cells that are $CXCR5^+PD\text{-}1^+$ were defined as Tfh cells. **(C** to **F)** Percentages of IL-4^+, IL-21^+, dual IL-4^+IL-21^+ and IFN-γ^+-producing Tfh cells were assessed within Tfh cells population by FACS. Data are shown as mean (±SEM). **P*<.05, ***P*<.01, ****P*<.001, Mann-Whitney test.

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

Figure 4. Induction of regulatory cells. (A) Representative plots analysis of $EBI3^+p35^+$ Treg cells. IL-35 producing Treg cells were assessed using FACS in LPP- (n=21) and PL- (n=11) treated group at V2, V6 and V8. **(B)** Percentage of inducible Treg (iT_R35) within $CD4^+CD25^+$ cells. **(C)** Proportion of IL-10-producing Treg cells within $CD4^+CD25^+$ cells. **(D to F)** IL-10^+CD19^+ Breg cells production was examined by FACS. **(D)** Representative plots of IL-10 induction in $CD19^+$ B cells by IL-35. **(E)** IL-35 induced IL-10^+ Breg cells production in grass pollen allergic patients in the presence of CpG. **(F)** Frequency of IL-10-producing cells was 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 were

increased in LPP-treated patients. Data are shown as mean (±SEM). **P*<.05, ***P*<.01, ****P*<.001, Mann-Whitney Test.

Figure 5. LPP enhances IgG_4 blocking activities. (A) The effect of LPP on the production of IgG_4 levels in serum samples of patients obtained from 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. The effect of LPP on IgE-facilitated allergen binding to B cells was determined in serum from allergic patients incubated with B cells. Data are shown as mean (±SEM). **P*<.05, ***P*<.01, Mann-Whitney test.

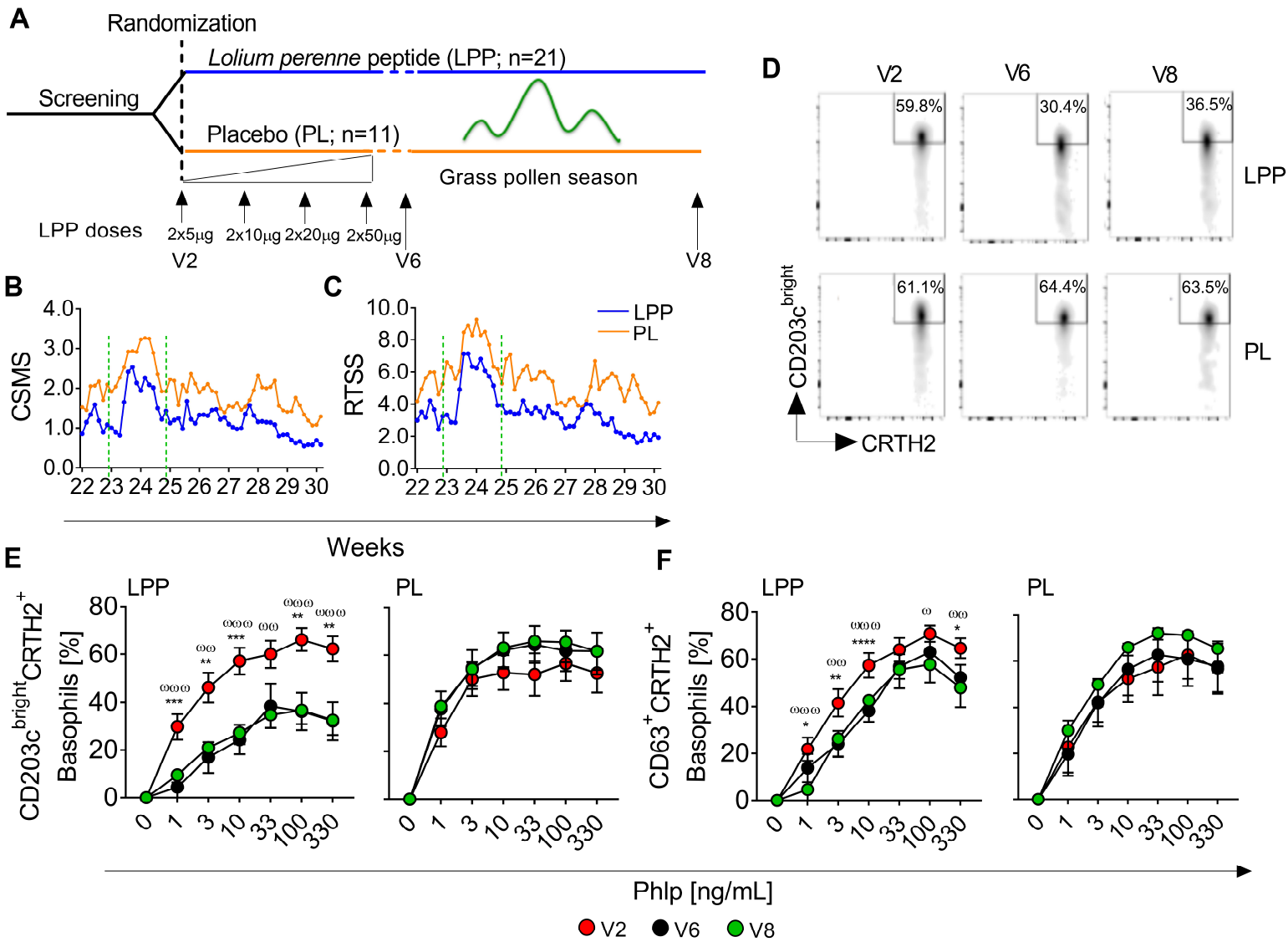
A
Randomization
Lolium perenne peptide (LPP; n=21)
Screening
Placebo (PL; n=11)
Grass pollen season
LPP doses
2x5µg
2x10µg
2x20µg
2x50µg
V2
V6
V8
B
CSMS
C
RTSS
LPP
PL
Weeks
D
V2
V6
V8
59.8%
30.4%
36.5%
61.1%
64.4%
63.5%
LPP
PL
CD203c bright
CRTH2
E
LPP
PL
CD203c bright CRTH2+ Basophils [%]
F
CD63+CRTH2+ Basophils [%]
Phlp [ng/mL]
V2
V6
V8

Figure 1.

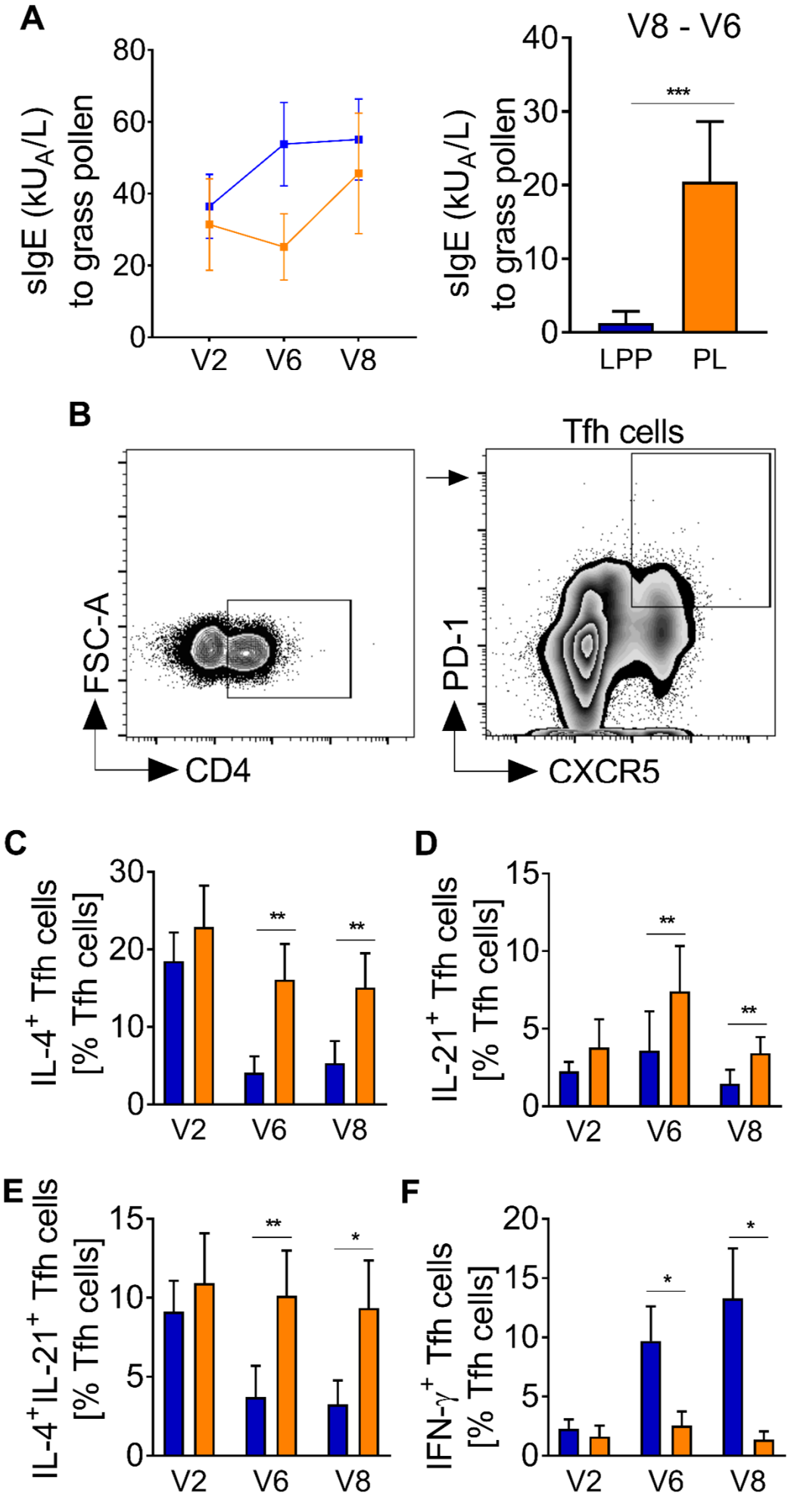
A
sIgE (kU$_A$/L) to grass pollen
80
60
40
20
0
V2
V6
V8
V8 - V6
***
40
30
20
10
0
LPP
PL
B
Tfh cells
FSC-A
CD4
PD-1
CXCR5
C
IL-4$^+$ Tfh cells [% Tfh cells]
30
20
10
0
**
**
V2
V6
V8
D
IL-21$^+$ Tfh cells [% Tfh cells]
15
10
5
0
**
**
V2
V6
V8
E
IL-4$^+$IL-21$^+$ Tfh cells [% Tfh cells]
15
10
5
0
**
*
V2
V6
V8
F
IFN-γ$^+$ Tfh cells [% Tfh cells]
20
15
10
5
0
*
*
V2
V6
V8
LPP
PL

Figure 2

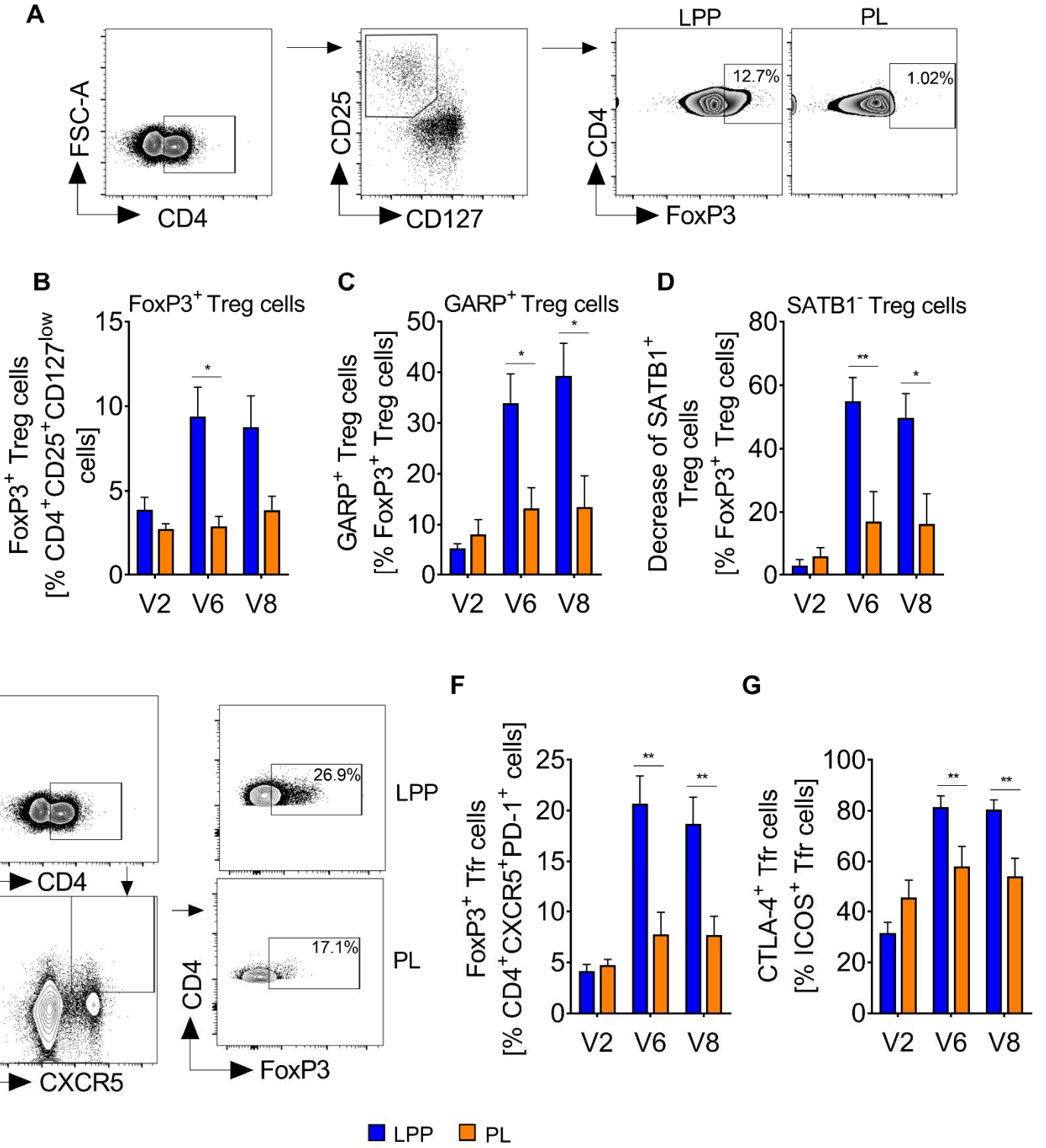
A
FSC-A
CD4
CD25
CD127
LPP
PL
12.7%
1.02%
CD4
FoxP3
B
FoxP3+ Treg cells
FoxP3+ Treg cells [% CD4+CD25+CD127low cells]
V2 V6 V8
C
GARP+ Treg cells
GARP+ Treg cells [% FoxP3+ Treg cells]
D
SATB1- Treg cells
Decrease of SATB1+ Treg cells [% FoxP3+ Treg cells]
E
FSC-A
CD4
PD-1
CXCR5
26.9%
17.1%
LPP
PL
CD4
FoxP3
F
FoxP3+ Tfr cells [% CD4+CXCR5+PD-1+ cells]
G
CTLA-4+ Tfr cells [% ICOS+ Tfr cells]
LPP
PL

Figure 3

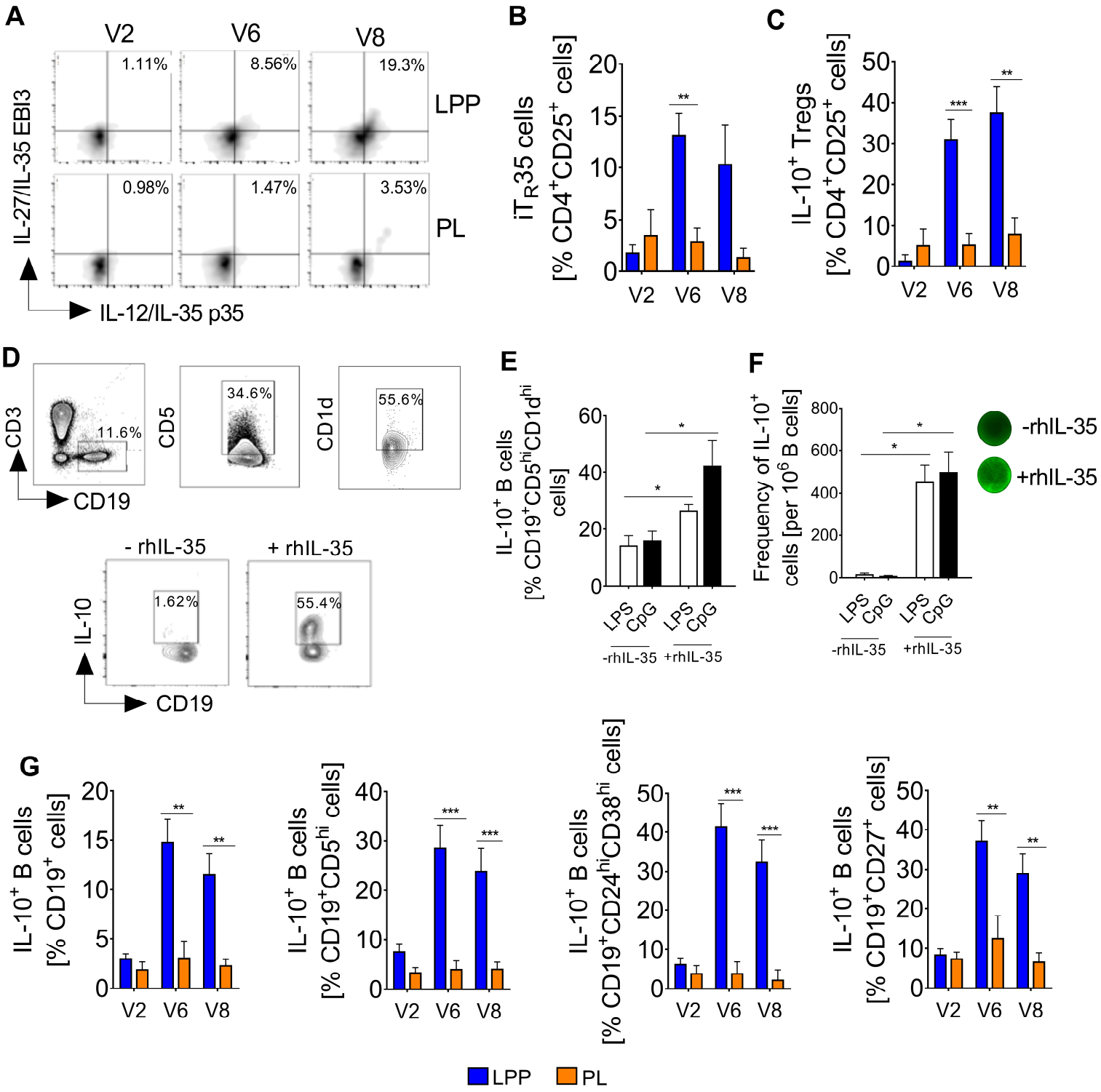
A
V2
V6
V8
1.11%
8.56%
19.3%
LPP
0.98%
1.47%
3.53%
PL
IL-27/IL-35 EBI3
IL-12/IL-35 p35
B
iTR35 cells [% CD4+CD25+ cells]
C
IL-10+ Tregs [% CD4+CD25+ cells]
D
CD3
11.6%
CD19
CD5
34.6%
CD1d
55.6%
- rhIL-35
+ rhIL-35
IL-10
1.62%
55.4%
CD19
E
IL-10+ B cells [% CD19+CD5hiCD1dhi cells]
LPS
CpG
-rhIL-35
+rhIL-35
F
Frequency of IL-10+ cells [per 10^6 B cells]
-rhIL-35
+rhIL-35
G
IL-10+ B cells [% CD19+ cells]
IL-10+ B cells [% CD19+CD5hi cells]
IL-10+ B cells [% CD19+CD24hiCD38hi cells]
IL-10+ B cells [% CD19+CD27+ cells]
V2
V6
V8
LPP
PL

Figure 4.

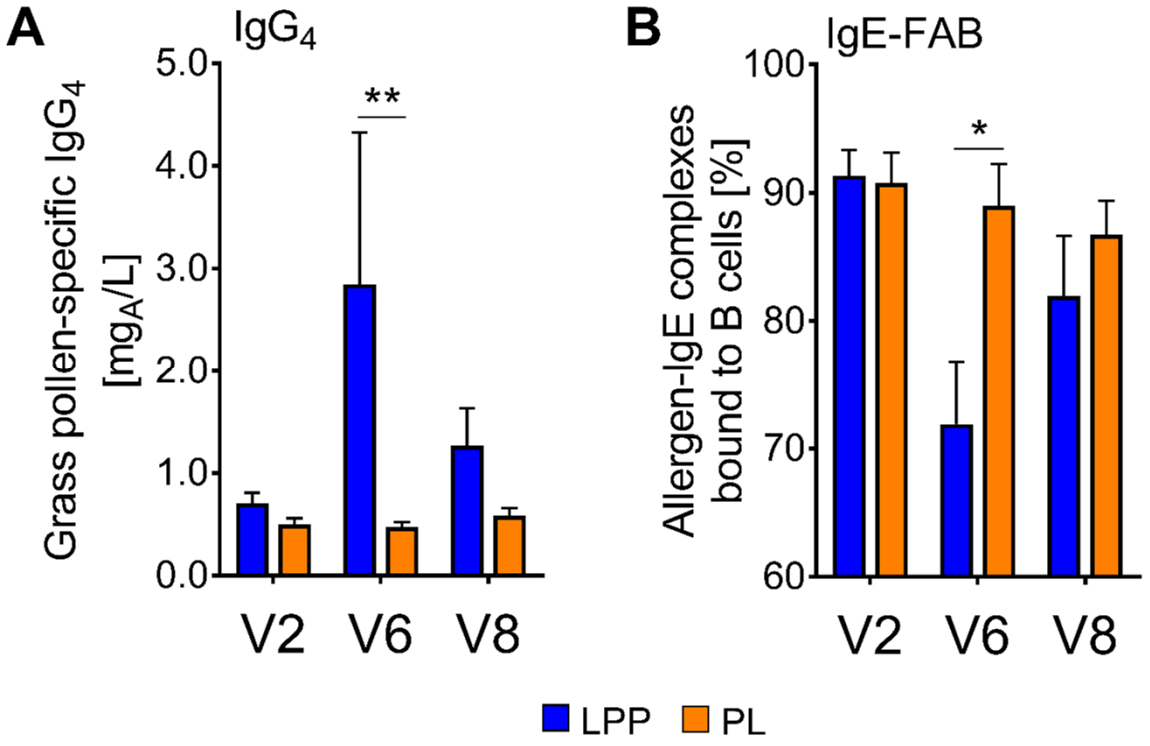
A
IgG4
Grass pollen-specific IgG4 [mgA/L]
5.0
4.0
3.0
2.0
1.0
0.0
**
V2
V6
V8
B
IgE-FAB
Allergen-IgE complexes bound to B cells [%]
100
90
80
70
60
*
V2
V6
V8
LPP
PL

Figure 5

ONLINE REPOSITORY

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

Hanisah Sharif, MSc[a*], Iesha Singh, MSc[a*], Lubna Kouser, PhD[a*], Ralph Mösges, MD[b], Marie-Alix Bonny[c], Angeliki Karamani, MSc[a], Rebecca V. Parkin, BSc[a], Nicolas Bovy, PhD[c], Uday Kishore, PhD[g], Abigail Robb, BSc[a], Michael Katotomichelakis, MD[d], Gabriele Holtappels, BSc[d], Lara Derycke, MD[d], Francis Corazza, PhD[e], Rémy von Frenckell[f], Nathalie Wathelet, PhD[c], Jean Duchateau, PhD[c], Thierry Legon, MBA[c], Sabine Pirotton, PhD[c], Stephen R. Durham, MD. FRCP[a], Claus Bachert, MD[d*], Mohamed H. Shamji, PhD. FAAAAI[a*]

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Acknowledgment: This research was supported by ASIT biotech S.A., Brussels, Belgium.

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 x 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 (Diaclone, 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

Hanisah Sharif, MSc[a*], Iesha Singh, MSc[a*], Lubna Kouser, PhD[a*], Ralph Mösges, MD[b], Marie-Alix Bonny[c], Angeliki Karamani, MSc[a], Rebecca V. Parkin, BSc[a], Nicolas Bovy, PhD[c], Uday Kishore, PhD[g], Abigail Robb, BSc[a], Michael Katotomichelakis, MD[d], Gabriele Holtappels, BSc[d], Lara Derycke, MD[d], Francis Corazza, PhD[e], Rémy von Frenckell[f], Nathalie Wathelet, PhD[c], Jean Duchateau, PhD[c], Thierry Legon, MBA[c], Sabine Pirotton, PhD[c], Stephen R. Durham, MD. FRCP[a], Claus Bachert, MD[d*], Mohamed H. Shamji, PhD. FAAAAI[a*]

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Acknowledgment: This research was supported by ASIT biotech S.A., Brussels, Belgium.

Table E1

Experimental readout	Patient group	Mean ± SD	Power rank	Calculated sample size (vs. PL)
$sIgG_4$ (mg_A/L)	PL	0.74 ± 0.60	0.9	
	LPP	2.82 ± 3.49	0.9	n = 2
Allergen-IgE complexes bound to B cells (%)	PL	85.30 ± 13	0.9	
	LPP	61.58 ± 25	0.9	n = 7

Sample size calculation based on previously published data of induction of $sIgG_4$ and formation of allergen-IgE complexes bound to B cells measured in 170 μg LPP- and placebo-treated patients.[1] Sample size calculations was performed using Statulator software (http://statulator.com/SampleSize/ss2M.html). PL, placebo-treated group; LPP, *Lolium perenne* peptide-treated group.

Table E2

Experimental readout	Visit	Mean ± SD	Power rank	Calculated sample size (vs V1)
$sIgG_4$ (mg_A/L)	V1	0.7 ± 0.7	0.9	
	V6	5.65 ± 13.56	0.9	n = 1
	V8	8.57 ± 18.36	0.9	n = 1
Allergen-IgE complexes bound to B cells (%)	V1	92.79 ± 11.11	0.9	
	V6	76.25 ± 19.03	0.9	n = 10
	V8	69.76 ± 19.51	0.9	n = 5

Sample size calculation based on previously published data of induction of $sIgG_4$ and formation of allergen-IgE complexes bound to B cells following LPP treatment measured at screening (V1), during (V6) and after (V8) treatment.[2] Sample size calculations was performed using Statulator software (http://statulator.com/SampleSize/ss2M.html).

Table E3

Concentration of Phlp	Visit	n (LPP)	LPP (mean ± SEM)	n (PL)	Placebo (mean ± SEM)	*P* value (LPP vs PL)	*P* value of LPP (V6 or V8 vs baseline)	*P* 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

Expression of $CD203c^{bright}CRTH2^{+}$ basophils in LPP and PL-treated group. **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 baseline) LPP/PL: Wilcoxon's Test.

Table E4

Concentration of Phlp	Visit	n (LPP)	LPP (mean ± SEM)	n (PL)	Placebo (mean ± SEM)	*P* value (LPP vs PL)	*P* value of LPP (V6 or V8 vs baseline)	*P* 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	0.007	> .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

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

Table E5

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

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

Table E6

T cell subset	Visit	n (LPP)	mean ± SEM (LPP)	n (PL)	mean ± SEM (PL)	*P* value (LPP vs PL)	*P* value of LPP (V6 or V8 vs baseline)	*P* 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

Proportion of IFN-γ^+ Th1 (CD4$^+$IFN-γ^+) cells following stimulation with different concentration of *Phleum 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 for normalized data. **P* value (V6 or V8 vs baseline) LPP/PL: Wilcoxon's Test.

Table E7

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

Proportion of T follicular helper (Tfh; CD4$^+$CXCR5$^+$PD-1$^+$) cells and its subsets following stimulation with different concentration of *Phleum 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 for normalized data. **P* value (V6 or V8 vs baseline) LPP/PL: Wilcoxon's Test.

Table E8

Treg cell subsets	Visit	n (LPP)	mean ± SEM (LPP)	n (PL)	mean ± SEM (PL)	*P* value (LPP vs PL)	*P* value of LPP (V6 or V8 vs baseline)	*P* 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

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): Mann-Whitney's Test. **P* value (V6 or V8 vs baseline) LPP/PL: Wilcoxon's Test.

Table E9

Tfr cell subsets	Visit	n (LPP)	mean ± SEM (LPP)	n (PL)	mean ± SEM (PL)	*P* value (LPP vs PL)	*P* value of LPP (V6 or V8 vs baseline)	*P* 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

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 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: Wilcoxon's Test.

Table E10

Inducible Treg cell subsets	Visit	n (LPP)	mean ± SEM (LPP)	n (PL)	mean ± SEM (PL)	*P* value (LPP vs PL)	*P* value of LPP (V6 or V8 vs baseline)	*P* value of PL (V6 or V8 vs baseline)
iT_R35	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

Proportion of inducible Treg ($CD4^+CD25^+$) cells following stimulation with grass pollen (*Phleum pratense*) 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 for normalized data. **P* value (V6 or V8 vs baseline) LPP/PL: Wilcoxon's Test.

Table E11

Breg cell subsets	Visit	n (LPP)	mean ± SEM (LPP)	n (PL)	mean ± SEM (PL)	*P* value (LPP vs PL)	*P* value of LPP (V6 or V8 vs baseline)	*P* value of PL (V6 or V8 vs baseline)
$CD19^{+}IL\text{-}10^{+}$	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
$IL\text{-}10^{+}CD19^{+}CD5^{+}$	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
$IL\text{-}10^{+}CD19^{+}CD27^{+}$	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
$IL\text{-}10^{+}CD19^{+}CD5^{+}$ $CD24^{hi}CD38^{hi}$	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

Proportion of IL-10 producing Breg cells following stimulation with CpG in LPP and 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 V8 vs baseline) LPP/PL: Wilcoxon's Test.

Table E12

Concentration of Lol p	Visit	n (LPP)	mean ± SEM (LPP)	n (PL)	mean ± SEM (PL)	*P* value (LPP vs PL)	*P* value of LPP (V6 or V8 vs baseline)	*P* 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

Induction of IgG-associated blocking antibodies in LPP and 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 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	*P* value	Spearman r	*P* value	Spearman r	*P* value	Spearman r	*P* value	Spearman r	*P* value	Spearman r	*P* value
Inducible Treg cell subsets												
iT_R35 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

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

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2. Mosges 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(6):1254-62.

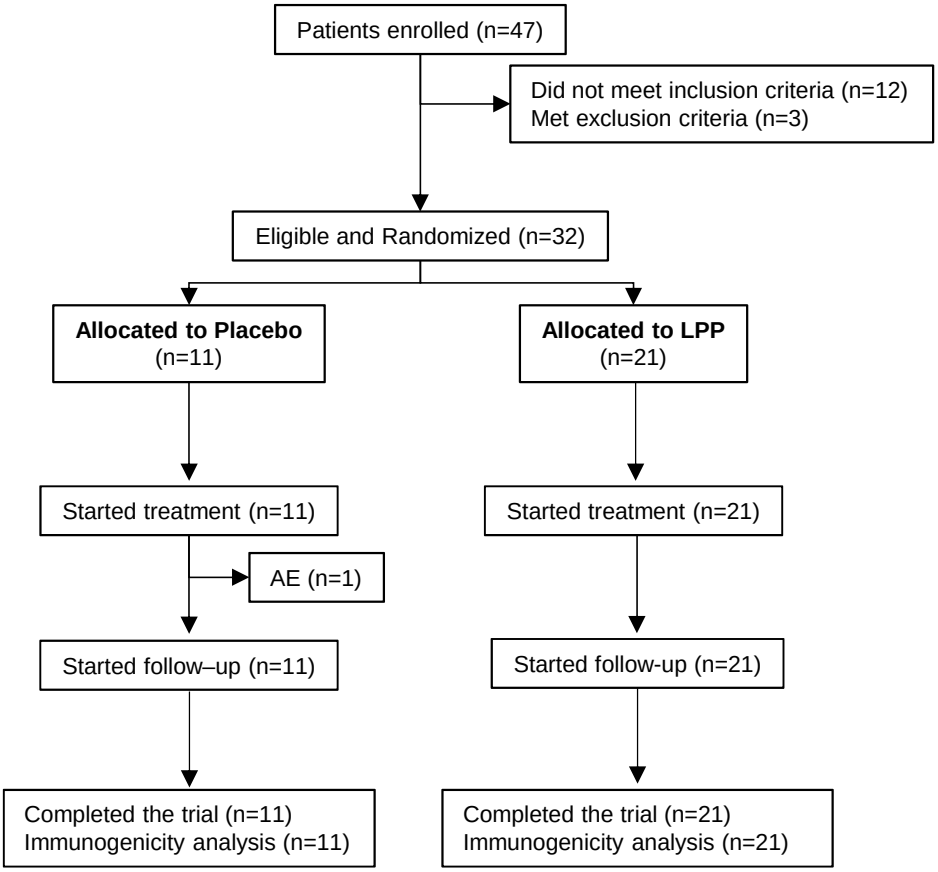
Patients enrolled (n=47)
Did not meet inclusion criteria (n=12)
Met exclusion criteria (n=3)
Eligible and Randomized (n=32)
Allocated to Placebo (n=11)
Allocated to LPP (n=21)
Started treatment (n=11)
Started treatment (n=21)
AE (n=1)
Started follow–up (n=11)
Started follow-up (n=21)
Completed the trial (n=11)
Immunogenicity analysis (n=11)
Completed the trial (n=21)
Immunogenicity analysis (n=21)

Figure E1

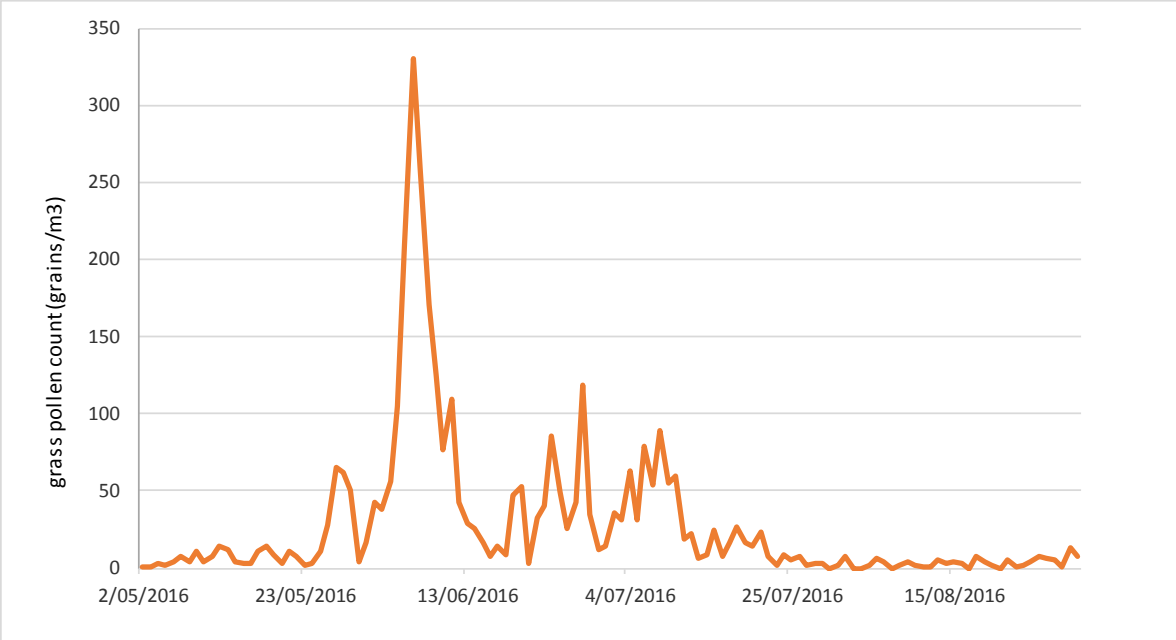
350
300
250
200
150
100
50
0
grass pollen count (grains/m3)
2/05/2016
23/05/2016
13/06/2016
4/07/2016
25/07/2016
15/08/2016

Figure E2

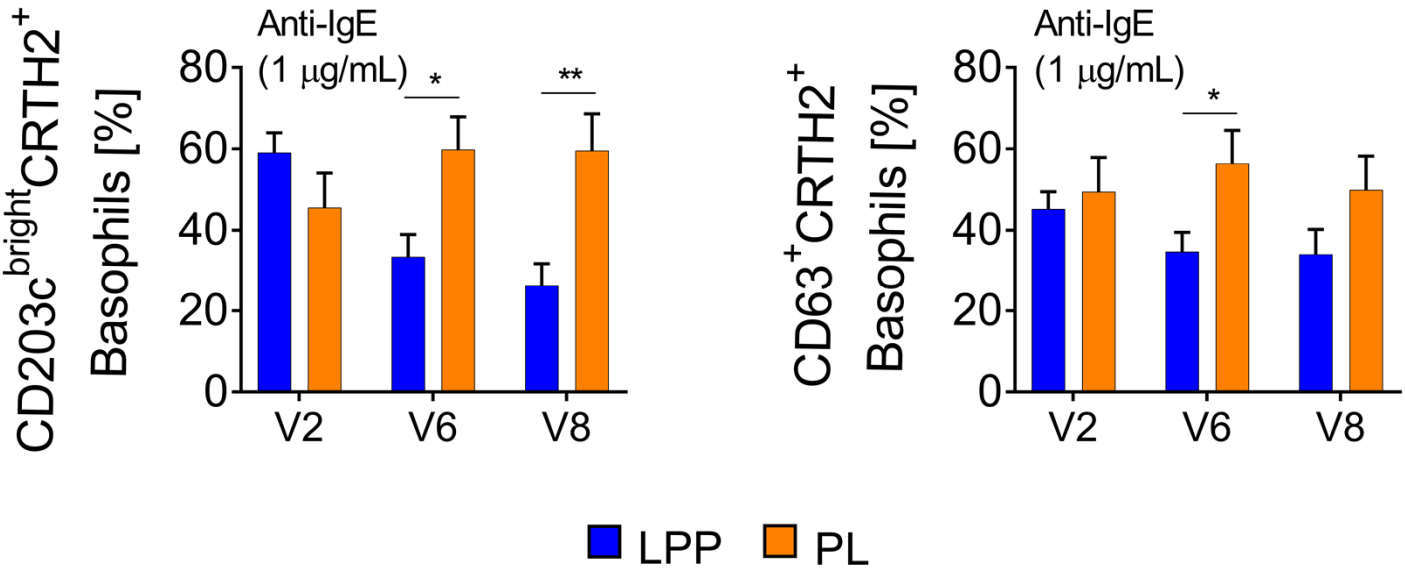
CD203c^bright CRTH2^+ Basophils [%]
Anti-IgE
(1 μg/mL)
80
60
40
20
0
*
**
V2
V6
V8
CD63^+ CRTH2^+ Basophils [%]
Anti-IgE
(1 μg/mL)
80
60
40
20
0
*
V2
V6
V8
LPP
PL

Figure E3