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ORIGINAL ARTICLE

Experimental Models of Allergic Disease

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Subcutaneous immunotherapy suppresses Th2 inflammation and induces neutralizing antibodies, but sublingual immunotherapy suppresses airway hyperresponsiveness in grass pollen mouse models for allergic asthma

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Summary

Background: Both subcutaneous and sublingual allergen immunotherapy (SCIT and SLIT) have been shown to effectively suppress allergic manifestations upon allergen exposure, providing long-term relief from symptoms in allergic disorders including allergic asthma. Clinical studies directly comparing SCIT and SLIT report a different kinetics and magnitude of immunological changes induced during treatment. Comparative studies into the mechanisms underlying immune suppression in SCIT and SLIT are lacking.

Objective: We aimed to establish an experimental model for grass pollen (GP) SCIT and SLIT that would allow a head-to-head comparison of the two treatments.

Methods: BALB/c mice were sensitized with GP extract, followed by SCIT and SLIT treatments with various GP dosages. Subsequently, we challenged mice with GP and measured airway responsiveness (AHR), GP-specific immunoglobulins, ear swelling tests (EST), eosinophilic inflammation in bronchoalveolar lavage fluid (BALF), and T cell cytokine release after restimulation of lung cells (IL-5, IL-10, and IL-13).

Results: We find that SLIT treatment was able to suppress allergen-induced AHR, while allergic inflammation was not effectively suppressed even at the highest GP dose in this model. In contrast, SCIT treatment induced higher levels of GP-specific IgG1, while SLIT was superior in inducing a GP-specific IgG2a response, which was associated with increased Th1 activity in lung tissue after SLIT, but not SCIT treatment. Interestingly, SCIT was able to suppress Th2-type cytokine production in lung cell suspensions, while SLIT failed to do so.

Conclusions and clinical relevance: In conclusion, GP-SCIT suppresses Th2 inflammation and induced neutralizing antibodies, while GP-SLIT suppresses the clinically relevant lung function parameters in an asthma mouse model, indicating that the two application routes depend on partially divergent mechanisms of tolerance induction. Interestingly, these data mirror observations in clinical studies, underscoring the translational value of these mouse models.

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KEYWORDS
allergic asthma, grass pollen, mouse models, subcutaneous immunotherapy, sublingual immunotherapy, tolerance induction

1 | INTRODUCTION

To successfully treat allergic airway disease, international guidelines recommend allergen-specific immunotherapy (AIT).[1,2] Administration of allergen extracts via the subcutaneous (SCIT) and sublingual (SLIT) route has both been found to be effective therapies. For instance, SCIT treatment with grass pollen (GP) or house dust mite has shown clinical success in restoring long-term allergen-specific tolerance.[3,4] In a double-blinded, randomized placebo-controlled trial (RCT) in patients with allergic rhinoconjunctivitis, GP-SLIT treatment was also found to induce a significant and durable induction of neutralizing antibody responses as well as decreased symptom score up to 2 years after completion of a 3-year treatment period.[5] Moreover, a recent meta-analysis comparing SLIT tablets, SLIT drops, and SCIT injections for GP allergies reported comparable reduction in symptom scores and supplemental medication use for SLIT tablets and SCIT injections.[6]

Recent studies comparing SCIT and SLIT have reported differences in the kinetics and magnitude of immunological changes induced by these treatments.[7-12] For instance, Aasbjerg et al directly compared GP-SCIT vs GP-SLIT treatment in patients with allergic rhinitis and found both treatments to be effective compared to placebo controls.[8,13] SCIT treatment induced twofold to threefold greater induction of specific IgG4, while the effects on facilitated antigen presentation and basophil sensitivity induced by SCIT treatment were more pronounced in the first few months of treatment.[8] Moreover, SCIT, but not SLIT, treatment induced suppression of IL-5 production by $CD4^+$ T cells.[13] While an initial meta-analysis reported that SCIT was more effective in symptom control and reduction in medication,[14] a more recent study provided indirect evidence that SCIT and SLIT have a similar efficacy for the treatment of allergy.[6] Patients have been reported to show a preference for SLIT over SCIT.[15]

The mechanism of action for successful specific immunotherapy, irrespective of administration route, is thought to involve induction of neutralizing antibodies, an increased activity of regulatory T cells characterized by IL-10 production, and a gradual decline in specific IgE. The mechanisms underlying clinical efficacy in either SCIT or SLIT and the exact differences between the two treatments are not fully characterized. In clinical studies, immunological comparison of SCIT and SLIT is hampered by the limited amount of data from head-to-head comparisons and by the variability of the end-points used between studies.[9] Consequently, it remains unknown whether the differences in the immunological changes induced by SCIT and SLIT are relevant to clinical efficacy of either treatment.[8,9,16,17]

We have previously used experimental models of allergic airway disease to characterize critical immunological mechanisms underlying the mode of action of AIT.[18,19] Therefore, in this study, we aimed to establish an experimental model for GP-SCIT and GP-SLIT that allows direct comparison of the two treatments, to characterize the immunological changes and suppression of clinically relevant outcome parameters induced by either treatment as a platform to test further optimization of either form of AIT.

2 | METHODS

2.1 | Animals

BALB/cByJ mice (8-9 weeks old) were purchased from Charles River (L'Arbresle, France) and bred in individually ventilated cages and fed a hypoallergen GP-free diet (4 kcal/gr, 25% protein, 11% fat, 47% sugars, 5% fibres; AB Diets, Woerden, The Netherlands). Female 7-9-week-old progeny on the same diet were used for the experiments (8 mice/group). The Institutional Animal Care and Use Committee at the University of Groningen approved experiments.

2.2 | Allergic asthma and treatment protocols

All mice received two intraperitoneal injections of 5000 standardized quality (SQ) units (5kSQ = 8 μg allergen extract of GP (*Phleum pratense*, Phl p; ALK-Abelló, Hørsholm, Denmark) adsorbed to 1.6 mg Alum (Imject Alum, Pierce, USA) in 100 μL Phosphate-buffered Saline (PBS, Figures 1A,B, and 4A,B). SCIT was performed by three 100 μL injections or SLIT was performed by 40 5 μL sublingual administrations.[20] Inhalation challenges were administered as droplets of 25kSQ GP in 25 μL PBS after light isoflurane anaesthesia. After 2 days, airway responsiveness was determined, and serum samples, bronchoalveolar lavage fluid (BALF), and lung lobes were stored for further analyses (−80°C).

2.3 | Early-phase hypersensitivity: the EST

Similarly, as the skin prick test used in the clinic, we used ear swelling tests (ESTs), which were performed 1 week prior to AIT, to confirm a GP-specific response and 10 days thereafter to evaluate suppression of swelling (Figure 1A and 4A). Herein, 1kSQ GP in 10 μL PBS was injected intradermally in the right ear of anaesthetized mice, while as a control, 10 μL PBS was injected in the left ear.[21,22] After two hours, thickness was measured using a force micrometer at 0.5N (±0.15N, Mitutoyo, Japan). The net thickness (Δ, μm) was calculated by subtracting the thickness of the left from the right ear.

2.4 | Evaluation of Airway hyperresponsiveness

Airway responsiveness was assessed 48 hours after the last challenge by measuring airway resistance (R in $cmH_2O.s/mL$) and lung

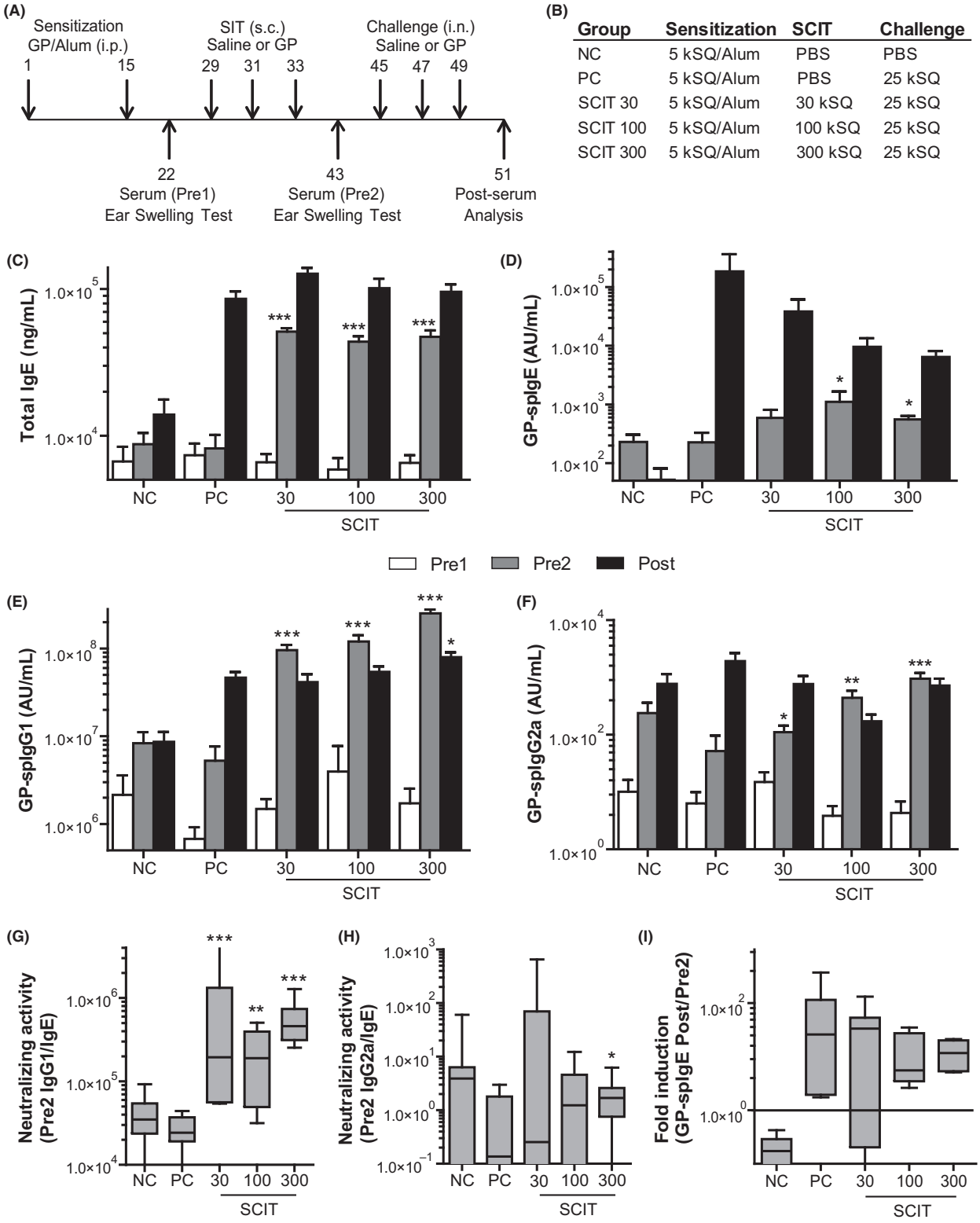

FIGURE 1 Overview and immunoglobulin response after GP-SCIT treatment. A, Outline of the SCIT protocol. B, Outline of the treatment groups. C, Serum total IgE (ng/mL) taken before SCIT (white bars, Pre1), before challenge (grey bars, Pre2), and after challenges (black bars, Post). D, Serum GP-spIgE (Arbitrary Units (AU)/mL, Pre2, and Post). E, Serum GP-spIgG1 (AU/mL, Pre1-2, and Post). F, Serum GP-spIgG2a (AU/mL, Pre1-2, and Post). G, Neutralizing activity plotted as ratio of GP-spIgG1/GP-spIgE levels in Pre2-sera. H, Neutralizing activity plotted as ratio of GP-spIgG2a/GP-spIgE levels in Pre2-sera. I, Fold induction of GP-spIgE after challenge (Post-sera/Pre2-sera). In Figure 1C-F, values are expressed as mean ± SEM (n = 8). In Figure 1G-I, values are expressed in box-and-whiskers plots (min-max). *$P < .05$, **$P < .01$, and ***$P < .005$ compared to PC at the same time-point. NC: negative control, PBS challenged; PC: positive control, GP challenged; 30, 100, 300: different doses of SCIT-treated mice (kSQ), GP challenged

compliance (C in mL/H_2O) in response to intravenous administration of increasing doses of methacholine (Sigma-Aldrich, MO) using a computer-controlled small animal ventilator (FlexiVent; SCIREQ, Quebec).[23]

2.5 | Evaluating inflammation in BALF

Directly after the AHR measurements, the lungs were lavaged and cytospin preparations were made according to the previous published protocols.[24]

2.6 | Analysis of T cell responses: restimulation of lung single cell suspensions

Lung single cell suspensions ($5x10^5$/well) were stimulated (*in triplo*) for 5 days in supplemented RPMI1640 with 0 μg or 30 μg GP/well. Supernatant was stored *in triplo* (−80°C), for ELISA measurements of IL-5, IL-10, IL-13, IFNγ, and TGFb1 according to the manufacturer's instructions (R&D Systems, Bio-Techne). The lower detection limits of the ELISAs were 3 pg/mL for IL-5, 10 pg/mL for IL-10, 15 pg/mL for IL-13, 30 pg/mL for IFNγ, and 30 pg/mL for TGFβ1.

2.7 | Measurement of GP-specific Immunoglobulins in serum

Blood was collected in MiniCollect Serum Tubes (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) via orbital puncture (pre-sera) and after the FlexiVent via the vena cava inferior (post-sera, Figure 1A,B, and 4A,B). Grass pollen-specific IgE (GP-spIgE), GP-spIgG1, and GP-spIgG2a levels were measured by ELISA as described previously in all collected sera samples.[25-27]

2.8 | Statistical analyses

All data were expressed as means ± SEMs. The Mann-Whitney *U* Test was used to analyse the results, and $P < .05$ was considered significant. Within the ELISA data, an AU value which was more than three times the interquartile (IQ) range higher than the upper Q or more than three times the IQ range lower than the lower Q was considered to be an extreme outlier and was removed for further analysis. Within the AHR measurements, a generalized estimated equation (GEE) analysis was used, using SPSS Statistics 20.0.0.2.[28] Nonparametric Spearman correlations were performed in Figure S4A-F.

See additional Methods description in the Data S1.

3 | RESULTS

3.1 | Serum immunoglobulin levels in GP-SCIT

To study the efficacy of GP-SCIT for suppression of asthmatic manifestations upon intranasal GP challenges, we started with a dose-finding experiment in which three doses GP extract (30, 100, or 300kSQ) were included for SCIT treatment (Figure 1A,B). To examine whether SCIT affected GP-specific serum immunoglobulin responses, we measured total IgE, GP-spIgE, GP-spIgG1, and GP-spIgG2a in serum taken at different time-points: after sensitization (white, Pre1), after SCIT treatment (grey, Pre2) and after the challenges (black, Post). Compared to the PBS-SCIT group, GP-SCIT injections resulted in significantly increased levels of total IgE (Figure 1C), that did not show a significant further increase after subsequent GP challenges. Furthermore, after GP-SCIT treatment the levels of GP-spIgE, GP-spIgG1 levels, as well as GP-spIgG2a were significantly increased as compared to PBS-SCIT-treated controls (PC, Figure 1D,E,F).

After the GP challenges, although not significant, we observed a slight dose-dependent decrease in serum levels of GP-spIgE, which implies an inverse correlation between GP dose in SCIT and spIgE in serum after challenges (Figure 1D). Interestingly, GP-spIgG1 levels after the GP challenges were significantly increased in the 300kSQ GP-SCIT treatment group (Figure 1E), compared to the sham-treated, GP-challenged asthma control group (PC).

As a measure of neutralizing capacity after GP-SCIT, the ratios of GP-spIgG1/GP-spIgE levels and GP-spIgG2a/GP-spIgE levels showed significant increases as compared to the PBS-SCIT-treated group (PC, Figure 1G,H). In contrast, when establishing fold inductions of GP-spIgE levels after GP challenges (GP-spIgE post/Pre2), we did not find significant reduction in the GP-SCIT-treated groups as compared to the positive controls (Figure 1I).

Overall, these results indicate GP-SCIT treatment induces a strong GP-spIgG1 and GP-spIgG2a response, while the use of three subcutaneous injections increased GP-spIgE serum levels after treatment and prevented further induction of spIgE after subsequent allergen challenges.

3.2 | Suppression of ear swelling and airway responsiveness after GP-SCIT

In the EST, we measured the net ear swelling (right ear minus left ear) two hours after intradermal GP injection at two time-points in our experimental protocol (Figure 1A). As expected, all experimental groups showed a net increase in ear swelling 1 week after the last GP/Alum injection (Figure S1A). After GP-SCIT, we observed a positive EST in all GP sensitized, placebo-treated control mice, similar to the EST after sensitization only (plotted together as Controls, Figure 2A). This EST showed a significant decrease in the 300kSQ GP-SCIT-treated group as compared to the sensitized controls (74.9 ± 12.3 vs 117.4 ± 10.4, Figure 2A). Similar results were found when plotting ratios of the EST value/average of the controls to allow intertreatment comparison (Figure S1C).

In addition, we measured methacholine-induced airway hyperresponsiveness in all experimental groups. We observed marked AHR in the PBS-SCIT, GP-challenged mice (PC) as compared to the PBS-SCIT, PBS challenged mice (NC; Figure 2C). Administering

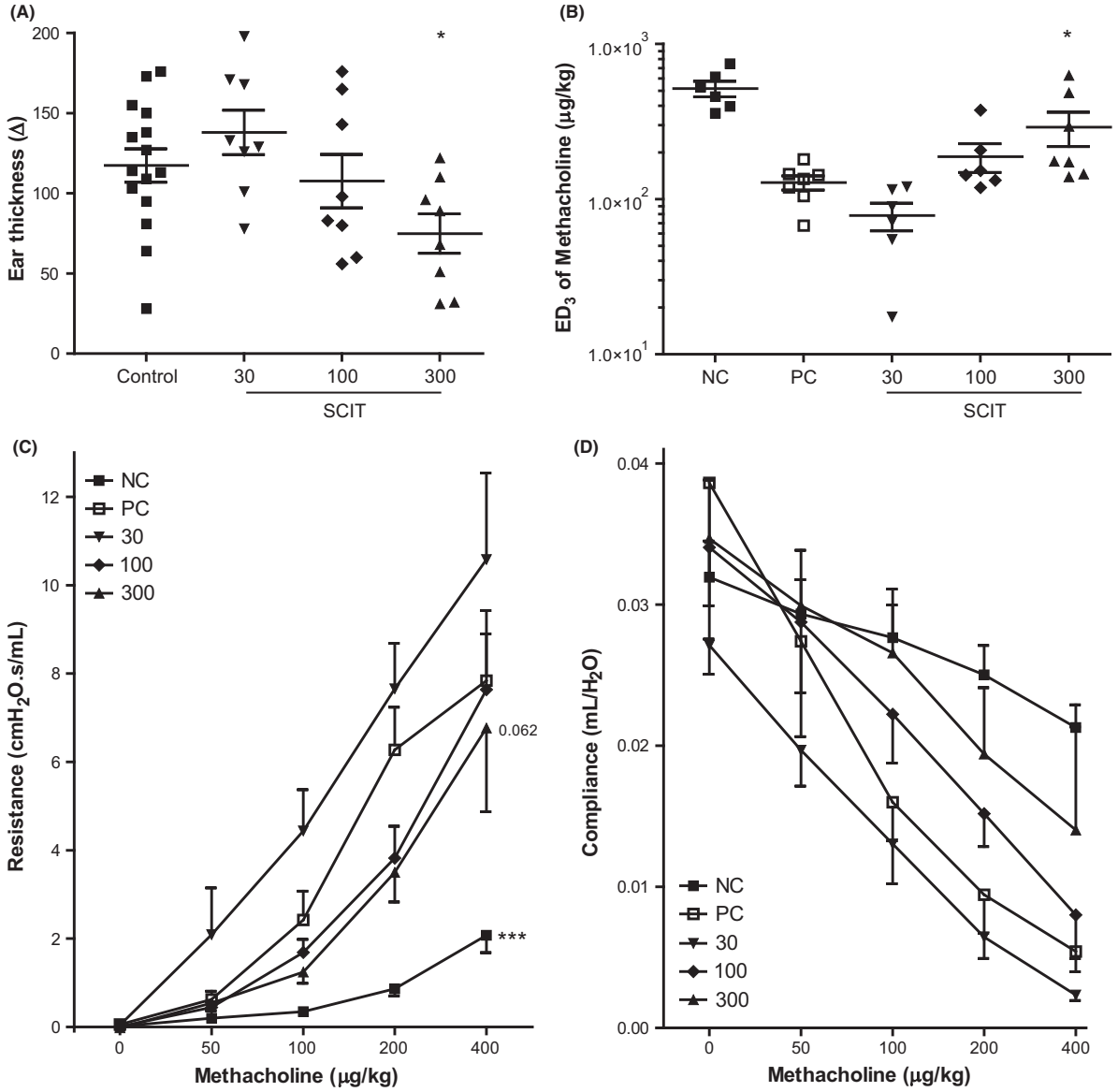

FIGURE 2 Clinical manifestations after GP-SCIT treatment. A, IgE-dependent allergic response plotted as net ear thickness (μm) two hours after GP injection (1kSQ) in the right ear and PBS in the left ear as a control, performed after SCIT. Placebo-SCIT-treated mice were plotted together as Controls (NC and PC). B, Effective dose (ED) of methacholine, when the airway resistance reaches 3 $cmH_2O.s/mL$ (ED_3). C, Airway hyperreactivity (AHR) was measured by FlexiVent and plotted as airway resistance (R in $cmH_2O.s/mL$) and as D, airway compliance (C in mL/cmH_2O). Absolute values are expressed as mean ± SEM (n = 8). *$P < .05$, **$P < .01$, and ***$P < .005$ compared to PC. NC, negative control, PBS challenged; PC, positive control, GP challenged; 30, 100, 300: different doses of SCIT (kSQ), GP challenged

the highest dose of GP-SCIT provided a trend to suppression of the dose-response curve of the airway resistance ($P = .062$, Figure 2C). In testing the effect of GP-SCIT on the suppression of this response, we found that the effective dose (ED) of methacholine necessary to increase AHR to an R of 3 $cmH_2O.s/mL$ (ED_3) was significantly increased in the 300kSQ GP-SCIT group as compared to the sham-treated control group (Figure 2B). Identical results can be found when testing for differences in ratio of ED_3/average of ED_3 of PC (Figure S1E). Remarkably, in examining the comparative stiffness of the lung, compliance values showed no significant differences between any of the experimental groups as compared to the PBS-SCIT-treated positive control group (Figure 2D).

Altogether, the decrease in EST, increase in ED_3 and trend towards a reduced airway resistance showed that the highest dose of GP administered in a SCIT protocol actively suppresses the asthmatic manifestations in this experimental immunotherapy protocol.

3.3 | SCIT suppresses of eosinophilic airway inflammation and cytokine levels

To evaluate the suppression of airway inflammation and Th2 cell activity after GP-SCIT, we measured inflammatory cell numbers in BALF and Th2 cytokines in restimulated lung cells. Within the GP-SCIT groups, we observed a dose-dependent decrease wherein the highest dose of SCIT resulted in a significant decrease in total cell count (Figure 3A). Next, we analysed numbers of mononuclear cells (M), eosinophils (E), and neutrophils (N) in BALF and single cell suspensions of lung tissue in the GP-SCIT groups and found a marked dose-dependent decrease in numbers of eosinophils in BALF as compared to the PBS-treated GP-challenged group (Figure 3B). The counts in lung single cell suspensions are comparable, in that the 100kSQ and 300kSQ groups showed significantly decreased numbers of eosinophils as compared to the PBS-SCIT, GP-challenged mice (Figure 3C). To clarify, both eosinophil counts were included as dot plots in Figure S2A,B. To allow for intertreatment comparison, the eosinophils found in BALF and lungs showed identical significance when plotted as ratio of eosinophil count divided by the average eosinophil count in the PBS-SCIT-treated positive controls (Figure 3D,E).

Next, we analysed cytokine release in the supernatant of GP-stimulated lung single cell suspensions and observed a significant suppression of IL-13 levels (Figure 4F), while the highest dose of SCIT showed a trend towards reduced IL-5 levels, but failed to reach significance (P = .052, Figure 4F). Interestingly, GP stimulation of lung cells from all SCIT groups failed to induce an enhanced IL-10 production. GP-SCIT did not alter the Th2 cytokine profile leading to enhanced Th1 cell IFNγ production.

In addition, we measured IL-5, IL-10, and TGFβ1 levels in lung tissue homogenates, which resulted in a significant IL-5 suppression after high dose GP-SCIT (Figure S3A). Similarly, as with the restimulated lung cells, SCIT failed to induce enhanced production of IL-10 and TGFβ1 (Figure S3C,E).

Overall, we observed a reduced eosinophilic airway inflammation in BALF and lung cells and decreased production of the prototypic Th2 cytokine IL-13 in response to GP stimulation in GP-SCIT-treated mice.

3.4 | Serum immunoglobulin responses induced by GP-SLIT

Next, we sought to evaluate the optimal dosage of GP for sublingual application in the experimental mouse model of allergic airway disease (Figure 4A,B). Serum was taken at five time-points for determination of total- and GP-specific immunoglobulin levels. In sera taken 3 weeks after starting GP-SLIT, we observed a marked increase in total IgE (Figure 4C) and GP-spIgE (Figure 4D). After GP challenges, PBS-SLIT control mice exhibit a strong GP-spIgE response. In contrast, GP-SLIT-treated groups showed a limited (30kSQ) or no (100/300kSQ) further induction of spIgE by the challenges after completion of the SLIT treatment protocol. In the 100 and 300kSQ SLIT groups, GP-spIgE serum levels were significantly reduced after the final GP challenges as compared to the sham-treated controls.

Additionally, significant increases of GP-spIgG1 were observed after 6 weeks of treatment onwards in the 100kSQ and 300kSQ GP-SLIT groups as compared to the sham-treated mice at the same time-point (Figure 4E). GP-spIgG1 levels did not further increase after GP challenges in GP-SLIT-treated groups, while challenges induced a marked increase in GP-spIgG1 responses in the PBS-treated control group. In contrast, GP-spIgG2a levels in the GP-SLIT-treated mice were significantly induced after GP challenges (Figure 4F).

Next, we calculated the neutralizing activity after GP-SLIT treatments in ratios of GP-spIgG1/GP-spIgE levels (not significant) and ratios of GP-spIgG2a/GP-spIgE levels, in which the latter showed significant increases as compared to the PBS-SCIT-treated group (Figure 4G,H). Furthermore, in contrast to the GP-SCIT treatments, the fold inductions of GP-spIgE levels after GP challenges (GP-spIgE post/Pre2 sera), showed a significant decrease as compared to the positive controls (Figure 4I).

These data indicate that GP-SLIT treatment induced increased specific immunoglobulin responses while providing a significant decrease in GP-spIgE by subsequent GP challenges.

3.5 | GP-SLIT reduces ear swelling and hyperresponsiveness

Next, we assessed whether the early-phase response to intradermal grass pollen challenge in the ear was suppressed in GP-SLIT-treated mice. Increasing doses of GP-SLIT resulted in a progressive reduction in the GP-induced ear swelling, reaching statistical significance only in the highest SLIT dose when compared to the PBS-treated control group (80 ± 15.9 vs 141.7 ± 12.7, Figure 5A). Similar results were found when plotting ratios of the EST value/average of the controls to allow intertreatment comparison (Figure S1B).

To measure the effect of GP-SLIT treatment on translational parameter of AHR to methacholine, we measured airway resistance (R) and compliance (C) in all experimental groups (Figure 5C,D). GP-challenged mice that had received PBS-SLIT treatment (PC) showed a marked increase in airway resistance as compared to the PBS challenged mice that had received PBS-SLIT treatment (NC; Figure 5C). Interestingly, we observed a strong reduction in the methacholine dose-dependent airway resistance in the 300kSQ GP-SLIT group as compared to the sham-treated positive controls. In addition, the ED_3 values (R of 3 $cmH_2O.s/mL$) of the GP-SLIT mice treated with the highest dose of GP (300kSQ) confirmed a significantly reduced sensitivity to methacholine (Figure 5B). Identical results were obtained when calculating the ratio of ED_3/average of ED_3 in the positive controls to allow efficient comparison between groups (Figure S1F). Finally, our data revealed a strong trend towards increased lung compliance for the highest SLIT group (P = .057, Figure 5D).

In all, the progressive decrease in ear swelling upon higher dosages of GP-SLIT, the significantly reduced airway resistance in response to methacholine challenges and the strong trend in increased compliance showed that the highest GP-SLIT dose (300kSQ) successfully suppresses the asthmatic manifestations in this experimental immunotherapy protocol.

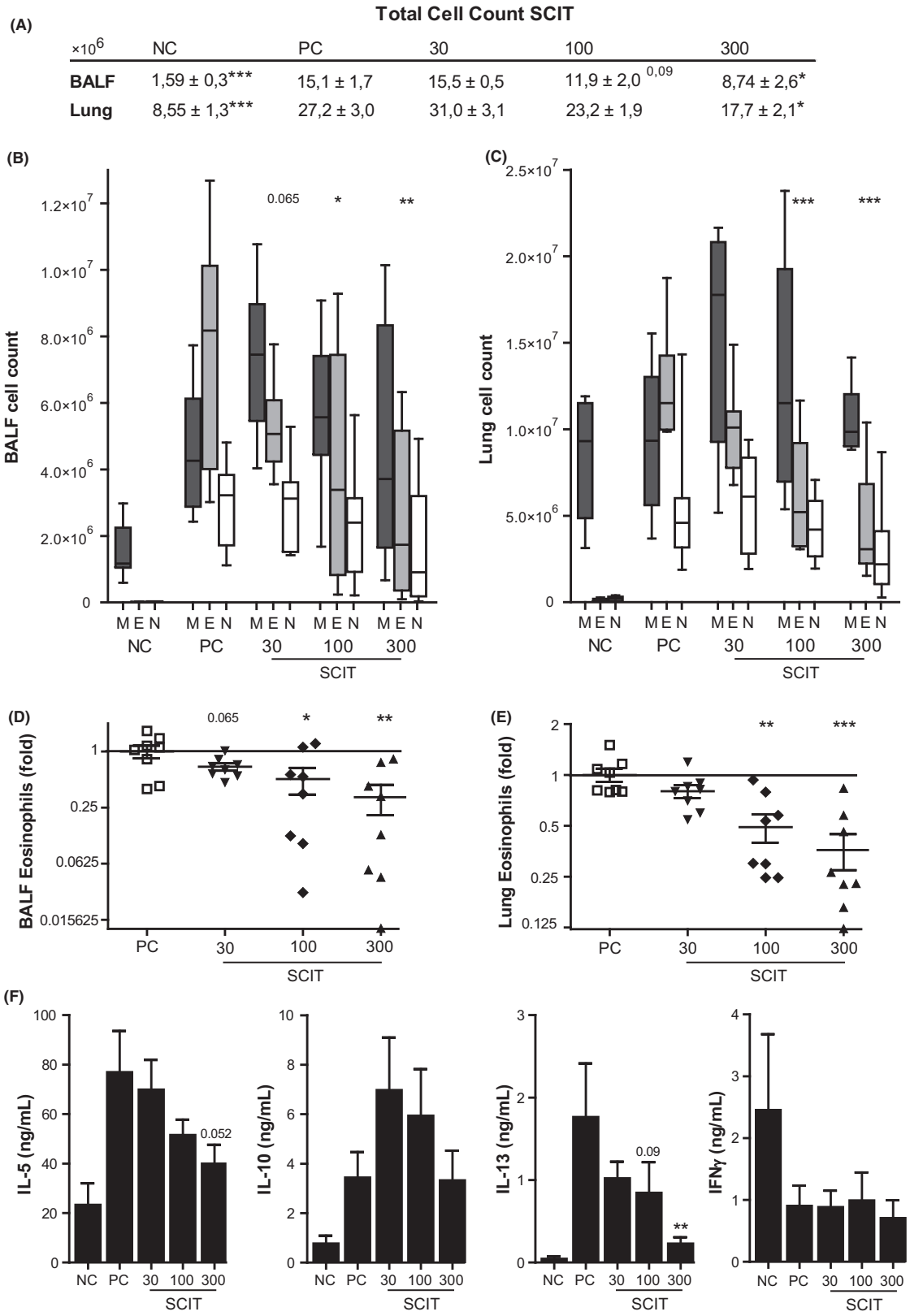

FIGURE 3 The eosinophilic and cytokine response after GP-SCIT treatment. A, Total cell counts in bronchoalveolar fluid (BALF) and lung single cell suspensions (Lung). B, Differential cytospin cell counts in BALF and in C, lung. M, mononuclear cells; E, eosinophils; N, neutrophils. Absolute numbers are plotted in box-and-whiskers plots (min-max). D, BALF eosinophils and E, lung eosinophils, both plotted as ratio of suppression (absolute eosinophils/average PC eosinophils; mean ± SEM). F: Net levels of IL-5, IL-10, IL-13, and IFNγ measured in restimulated lung single cell suspensions. Concentrations were calculated as the concentration after restimulation minus control (mean ± SEM, n = 8). *$P < .05$, **$P < .01$, and ***$P < .005$ compared to PC. NC: negative control, PBS challenged; PC: positive control, GP challenged; 30, 100, 300: different doses of SCIT (kSQ), GP challenged

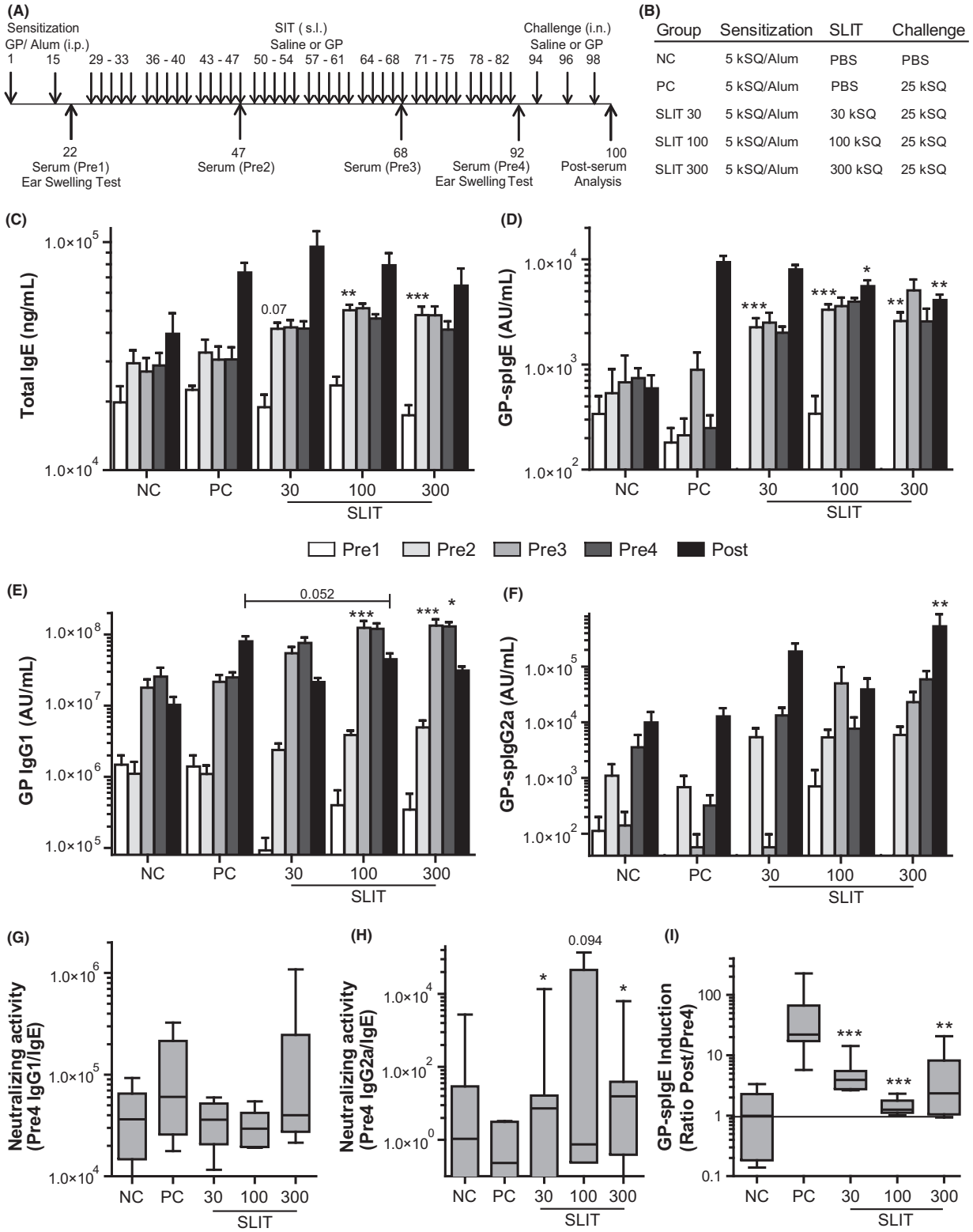

FIGURE 4 Overview and immunoglobulin response after GP-SLIT treatment. A, Outline of the SLIT protocol. B, Outline of the treatment groups. C, Serum levels of total IgE (ng/mL) taken before SLIT (white bars, Pre1), after 3 weeks of SLIT (light grey bars, Pre2), after 6 weeks of SLIT (middle grey bars, Pre3), before challenge (dark grey bars, Pre4), and after challenges (black bars, Post). D, Serum GP-spIgE (Arbitrary Units (AU)/mL, Pre1-4, and Post). E, Serum GP-spIgG1 (AU/mL, Pre1-4, and Post). F, Serum GP-spIgG2a (AU/mL, Pre1-4, and Post). G, Neutralizing activity plotted as ratio of GP-spIgG1/GP-spIgE levels in Pre4-sera. H, Neutralizing activity plotted as ratio of GP-spIgG2a/GP-spIgE levels in Pre4-sera. I, Fold induction of GP-spIgE after challenge (Post-sera/Pre4-sera). In Figure 4C-F, values are expressed as mean ± SEM (n = 8). In Figure 4G-I, values are expressed in box-and-whiskers plots (min-max). *$P < .05$, **$P < .01$, and ***$P < .005$ compared to positive control at the same time-point. NC: negative control, PBS challenged; PC: positive control, GP challenged; 30, 100, 300: different doses of SCIT-treated mice (kSQ), GP challenged

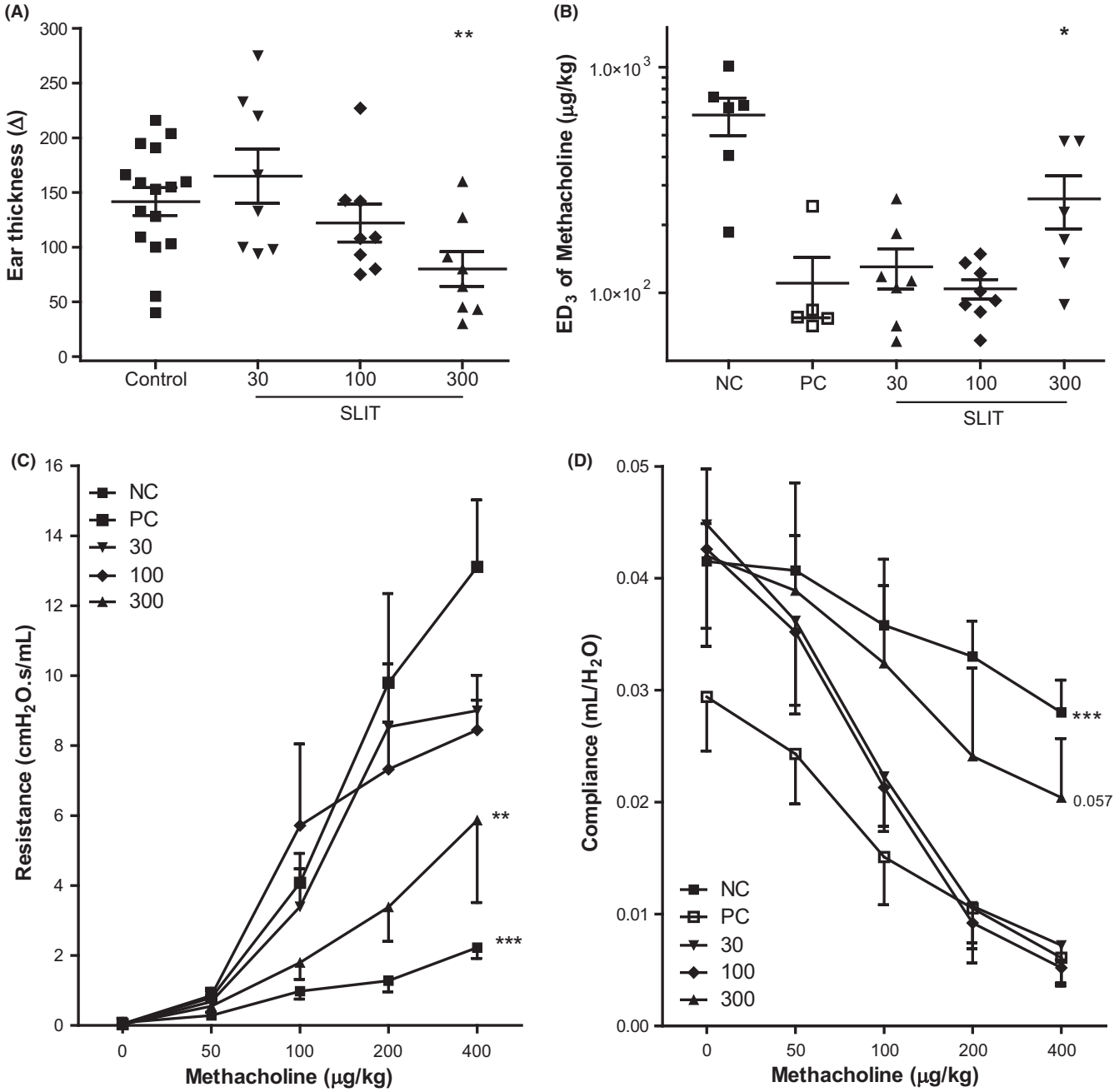

FIGURE 5 Clinical manifestations after GP-SLIT treatment. A, IgE-dependent allergic response plotted as net ear thickness (μm) 2 hours after GP injection (1kSQ) in the right ear and PBS in the left ear as a control, performed after SLIT. Placebo-SLIT-treated mice were plotted together as Controls (NC and PC). B, Effective dose (ED) of methacholine, when the airway resistance reaches 3 $cmH_2O.s/mL$ (ED_3). C: Airway hyperreactivity (AHR) was measured by FlexiVent and plotted as airway resistance (R in $cmH_2O.s/mL$) and as D: airway compliance (C in mL/cmH_2O). Absolute values are expressed as mean ± SEM (n = 8). *$P < .05$, **$P < .01$, and ***$P < .005$ compared to PC. NC: negative control, PBS challenged; PC: positive control, GP challenged; 30, 100, 300: different doses of SLIT-treated mice (kSQ), GP challenged

3.6 | Effects of GP-SLIT on eosinophilic inflammation and cytokine responses

To assess the effect of GP-SLIT on suppression of airway inflammation, we compared the eosinophilic airway inflammation and evaluated Th2 cytokines in restimulated lung cell homogenates of GP-SLIT-treated mice. We found that the numbers of eosinophilic granulocytes in BALF were comparable between GP-SLIT-treated mice and PBS-SLIT control-treated mice (Figure 6A,B). In contrast, differential cell counts in lung single cell suspensions revealed significantly decreased numbers of eosinophils in all GP-SLIT-treated groups as compared to the PBS-treated control group (Figure 6C and Figure S2C,D). Also when expressed as fold reduction in eosinophils compared to PBS-SLIT group, allowing straightforward comparisons between the groups, significantly, reductions in eosinophils in lung tissue, but not in BALF, were observed in all experimental groups (Figure 6D,E).

To evaluate the effect of GP-SLIT on Th2 cell driven inflammation, we measured cytokine levels in the supernatant of lung cells restimulated with GP ex vivo. In PBS-treated GP-challenged mice, we observed high levels of IL-5, IL-10, and IL-13, while IFNγ levels decreased compared to cultures from PBS challenged mice (Figure 6F). Remarkably, GP-SLIT treatment did not affect the cytokine production after GP stimulation of lung cells. However, we measured IL-5, IL-10, and TGFβ1 levels in lung tissue homogenates, which resulted in a trend towards IL-5 suppression after high dose GP-SLIT (Figure S3B). Comparable to our restimulated cell cytokine production and the SCIT model, SLIT failed to induce enhanced production of IL-10 and TGFβ1 (Figure S3D,F).

In conclusion, we observed reduced numbers of eosinophilic granulocytes in lung cell suspensions but not in BAL of all GP-SLIT-treated mice. Although none of the Th2 cytokines measured in supernatant of ex vivo restimulated lung cells were significantly reduced after GP-SLIT treatment, high dose GP-SLIT did result in a trend towards suppression of IL-5 in the lung tissue homogenates.

3.7 | Comparing GP-SCIT and GP-SLIT

Altogether, we can conclude that both administrative routes render suppression of certain phenotypes of the experimental mouse model of GP-driven allergic asthma. To allow quantitative evaluation of the relative efficacy of either administration route of GP-AIT on the various parameters of the allergic asthma mouse model, we provide an overview of all parameters measured in Table 1. To start, the immunoglobulin responses showed marked differences: the total and specific IgE responses measured after GP challenges were in the same range in SCIT as well as SLIT. The fold induction of IgE after GP challenges, however, was far more effectively suppressed by GP-SLIT (SCIT: 11,7-fold induction of IgE (vs 49,6) and SLIT 2,4 (vs 25,4), Table 1). The blocking immunoglobulins differ based on isotype: GP-spIgG1 is superior in the (faster) SCIT protocol, while the IgG2a levels are higher after SLIT treatment. Consequently, as expressed by the comparison of the median of the 300kSQ group of both administrative routes (Table 1), the neutralizing capacity by IgG1 ($78x10^6$ AU/mL) was much better in SCIT than in SLIT ($39x10^6$ AU/mL), while the opposite accounts for the IgG2a measurements (SCIT, 430 AU/mL vs. SLIT 220×10^3 AU/mL).

When comparing the airway resistance, GP-SLIT showed a significant reduction, while the highest dose of GP-SCIT merely provided a trend towards suppression of AHR (Figure 2C and 5C). This difference is further highlighted when calculating the fold reduction in resistance at 400 μg/kg MCh vs the average of the positive controls, results showed more than twice the reduction rate in SLIT as compared to SCIT treatments (0924 vs 2712, Table 1). Moreover, only SLIT treatment was able to provide a trend in increased compliance values ($P = .052$, Figure 5D), while SCIT was unable to improve compliance even at the highest dose of GP (Figure 2D).

In contrast, when comparing eosinophilic inflammation in BALF and Lung, marked responses can be detected in the SCIT protocol (suppression of 4386 vs PC in BALF), while in SLIT these findings were less profound (suppression of 2018 vs PC, Table 1). These findings are accompanied by an increased Th1 activity in lung tissue after SLIT ($P = .06$, Figure 6F), but not SCIT treatment. Interestingly, SCIT was able to suppress IL-13 production by lung cells, while SLIT failed to do so (Figure 3F). Although not equally strong, both administrative routes showed reduced levels of IL-5 in lung tissue homogenates.

Next, we asked whether the levels of GP-specific neutralizing antibodies after SCIT and SLIT correlated with a decreased AHR or immunological response after GP challenges. Here, we found a negative correlation between GP-spIgG1 (Post-SCIT) vs eosinophils in BALF and IL-5 in both administrative routes, whereas GP-spIgG1 correlated significantly with IL13 only after SLIT (Figure S4A-F).

4 | DISCUSSION

We aimed to establish an experimental mouse asthma model allowing direct comparison of GP-SCIT and SLIT to identify the opportunities for improvement of these treatment modalities. The results of this study clearly demonstrate that GP-SCIT and GP-SLIT can both suppress specific parameters of GP-induced allergic airway inflammation. However, there are marked differences in the efficacy of either treatment towards specific outcome parameters. We find that GP-SCIT treatment mainly modulated the allergen-specific adaptive immune response, with reduced Th2 inflammation and airway eosinophilia, and increased levels of spIgG1 and spIgG2a. In contrast, GP-SLIT treatment mainly affected lung function parameters, with significantly suppressed airway hyperresponsiveness and a trend towards improved lung compliance, while Th2 cytokines and eosinophil numbers in BALF were not markedly affected, while eosinophils in lung tissue were suppressed. Also, GP-SLIT treatment did not induce markedly increased levels of neutralizing antibodies, while spIgE levels were decreased. Of note, SCIT treatment did induce a trend towards suppression of AHR, indicating that with a larger group size this effect might have become statistically significant as well. Notwithstanding, the effect size of SLIT on AHR was stronger

FIGURE 6 The eosinophilic and cytokine response after GP-SLIT treatment. A, Total cell counts in bronchoalveolar fluid (BALF) and lung single cell suspensions (Lung). B, Differential cytospin cell counts in BALF and in C, lung. M, mononuclear cells; E, eosinophils; N, neutrophils. Absolute numbers are plotted in box-and-whiskers plots (min-max). D, BALF eosinophils and E, lung eosinophils, both plotted as ratio of suppression (absolute eosinophils/average PC eosinophils; mean ± SEM). F: Net levels of IL-5, IL-10, IL-13, and IFNγ measured in restimulated lung single cell suspensions. Concentrations were calculated as the concentration after restimulation minus control (mean ± SEM, n = 8). $*P < .05$, $**P < .01$, and $***P < .001$ compared to PC. NC, negative control, PBS challenged; PC, positive control, GP challenged; 30, 100, 300: different doses of SLIT (kSQ), GP challenged

(A)

Total Cell Count SLIT

$\times 10^6$	NC	PC	30	100	300
BALF	1,56 ± 0,1***	8,49 ± 0,8	10,3 ± 1,6	8,42 ± 1,3	10,2 ± 1,6
Lung	10,7 ± 0,7***	18,9 ± 1,3	14,7 ± 2,4	19,0 ± 1,8	16,7 ± 1,8*

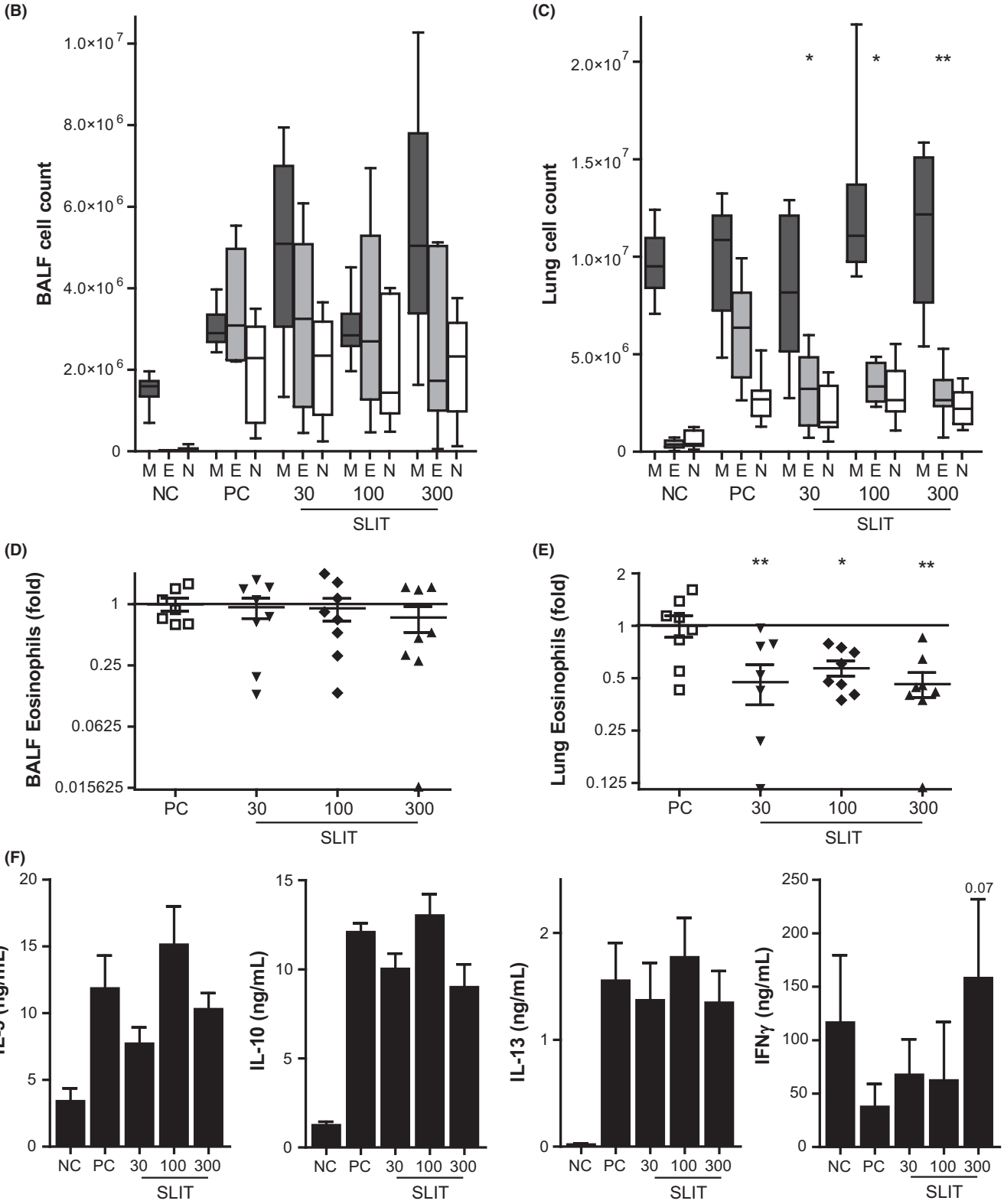

TABLE 1 Overview of parameters of inflammation after SCIT and SLIT using GP to allow for direct comparison of both administrative routes

Immunoglobulins	SCIT Median of 300kSQ	SLIT Median of 300kSQ
Post-total IgE	96 × 10^3	81 × 10^3
Post-GP-spIgE	4,3 × 10^3	4,0 × 10^3
Post-GP-spIgG1	78 × 10^6	39 × 10^6
Post-GP-spIgG2a	430	220 × 10^3
IgG1 neutralizing activity before challenge (GP-spIgG1/ GP-spIgE Pre)	0,46 × 10^6	40 × 10^3
IgG2a neutralizing activity before challenge (GP-spIgG2a/ GP-spIgE Pre)	2	16
Fold induction of IgE after challenge (vs Median PC)	11,7 (vs 49,6)	2,4 (vs 25,4)
AHR		
Fold induction of ED3	1.372	1.814
Fold reduction in resistance at 400 μg/kg MCh vs average of PC	0.924	2.712
EST		
Fold reduction in EST after 2 h	1.968	2.173
Inflammation		
Suppression of EO in BALF (ratio vs mean PC)	4.386	2.018
Suppression of EO in Lung (ratio vs mean PC)	4.063	2.327
Cytokines		
Cytokines in lung cells after GP stimulation ex vivo	IL-5, IL-13 suppression	IFNγ induction
Cytokines in lung tissue homogenates	Large IL-5 suppression	Small IL-5 suppression

than that of SCIT. For full suppression of AHR, a further increase in SCIT dose or a more chronic application of SCIT might be warranted. Conversely, the effect size of SCIT on suppression of Th2 cytokines and eosinophilic airway inflammation was stronger than that of SLIT, indicating that a higher allergen dose in SLIT, or a modified delivery method, might be warranted for full efficacy. Overall, these data indicate that the route of administration of a therapeutic GP vaccine in an experimental model for AIT plays a major role in determining the efficacy of the treatment towards suppressing specific parameters of the experimental asthma model.

In this initial study, we did not assess into great detail the biological mechanisms underlying the differences in the efficacy of SCIT vs SLIT mediated suppression of parameters of allergic airway inflammation in our experimental mouse model. The main differences are the route of allergen administration, the duration of treatment and the amount of allergen administered. Whereas SCIT relies on direct injection of high doses of allergen for a short duration, SLIT relies on sublingual application of lower amounts of allergen for a prolonged period. This will first of all affect the antigen-presenting cell population responsible for allergen presentation to the available memory T cell population in the draining lymph nodes. SLIT has been shown to mainly target oral CD11b+CD11c− cells, which have a strong tolerogenic capacity.[29] In contrast, SCIT injection mainly induces allergen presentation by mPDCA+ CD11c+ plasmacytoid dendritic cells (our unpublished observations). The difference in the antigen-presenting cell subset between the two administration routes might contribute to efficacy of immune modulation between the two treatment options. In addition, the dose of allergen in the SCIT treatment protocol is much higher per administration, which likely results in a higher number of naïve T cells activated (in a tolerogenic fashion) after the SCIT injections compared to the SLIT administration, where antigen presentation and therefore immune modulation will likely be restricted to antigen-experienced memory T cells. The mechanistic details underlying SCIT and SLIT are therefore an important subject for further research.

To our knowledge, this is the first study directly comparing SCIT and SLIT treatments in a mouse model for allergic airways disease using clinically relevant allergens. To date, experimental studies dissecting the mechanisms of allergen immunotherapy or attempting to enhance its efficacy have focused on a single route of application, precluding direct comparison of the efficacy of sublingual vs subcutaneous administration of allergen extracts for induction of neutralizing antibodies and suppression of allergic inflammation or lung function parameters. In agreement with our results, SCIT treatment with allergen extracts of birch pollen (BP) or recombinant phospholipase A2 (PLA2) induces strong neutralizing antibody responses, even resulting in protection from anaphylaxis upon subsequent allergen challenge.[30] Interestingly, in the BP-SCIT model, a higher number of allergen injections were needed to achieve suppression of AHR than to induce a neutralizing antibody response.[31] Experimental mouse models of HDM-SLIT treatment have rendered variable results regarding the efficiency of suppressing the different parameters of allergic airway disease, but generally show limited induction of neutralizing antibody responses, in line with our own results. For instance, in one study using *Dermatophagoides farinae* (Der f) extracts, SLIT treatment had a more pronounced effect on AHR than on Th2-driven, eosinophilic airway inflammation.[32] In contrast, a study using both *Dermatophagoides pteronyssinus* (Der p) and Der f extracts showed a stronger effect on suppression of eosinophilic airway inflammation, while AHR was only suppressed at higher doses of extracts used for SLIT treatment.[33] The latter study also reported limited effect on spIgG antibody titres, although spIgA was markedly increased. In mouse models of grass pollen SLIT treatment, induction of a neutralizing antibody response by GP-SLIT treatment is also limited,[20,34] although daily application of lower amounts of GP extract was able to induce a modest spIgG1 and IgG2a response in

comparison with less frequent application of higher doses of GP extract.[35] In BP driven mouse models of allergic airway disease, SLIT with BP extracts or rBet v1 dose-dependently suppressed airway eosinophilia as well as AHR, in the absence of induction of spIgG or any effect on spIgE responses.[36] Overall, the results in other models for AIT seem to confirm our observations with regard to the modulation of adaptive immune responses by SCIT, as evidenced by induction of neutralizing antibody responses and suppression of Th2 driven eosinophilia, whilst SLIT has more of an effect on lung function parameters, but not so much specific antibodies or Th2 cells. Moreover, the wide variety of allergen extracts used, the differences in treatment schemes, and the resulting variable outcomes of SCIT or SLIT treatment on the clinically relevant parameters of airway inflammation and lung function underscore the added value of our approach in combining the two application routes in a single mouse model using the identical allergen extract for efficient comparison on immunological and translational parameters.

In clinical studies on AIT, the number of studies that directly compare sublingual vs subcutaneous application of immunotherapy in the same patient population, and using allergen preparations is also very limited. In one study, allergic rhinitis patients sensitized to Phl p were randomized across three treatment groups: SCIT, SLIT, and sham control treatment and followed up for 15-24 months.[8,13] In this carefully conducted study with limited numbers of patients, SCIT was observed to induce a more rapid and robust change in spIgG4, IgE-blocking factor, and inhibition of facilitated antigen presentation and basophil activation test. SLIT treatment induced a more rapid and very strong increase in spIgE, while both treatments prevented seasonal induction of spIgE.[8] Of note, after 15 months SLIT and SCIT treatment resulted in similar levels of inhibition of facilitated antigen presentation and symptom score as measured by visual analogue scale. Subsequent analysis of GP-specific T cell responses up to 24 months into treatment revealed transient induction of IL-10 producing T cells and suppression of IL-5 producing T cells in both SLIT- and SCIT-treated patients, although the reduction in IL-5 producing T cells was significantly stronger in SCIT-treated patients compared to those that had received SLIT treatment.[13] SCIT treatment in this clinical study involved the use of Alum as an adjuvant, in contrast to the SLIT treatment which was free of adjuvantia, which might have potentiated the immunoglobulin responses induced by SCIT treatment.[37] Notwithstanding these limitations and the small patient numbers enrolled in this study, it is clear that SCIT treatment induced immunological changes with faster kinetics and stronger magnitude compared to SLIT treatment, which mirrors the findings in our experimental mouse model. In a very recent study, larger numbers of patients with severe seasonal allergic rhinitis were randomized in three 2-year treatment arms (SCIT, SLIT, or placebo) and followed up for nasal symptom score after allergen provocation up to 1 year after discontinuation of treatment.[38] Around 30 patients per treatment arm finished the study, and while SCIT-treated patients had significantly reduced nasal symptoms scores in year 1 and year 2 of treatment, SLIT treatment only achieved this in year 2 of treatment. After treatment discontinuation, SLIT was not effective in reducing nasal symptoms scores, underscoring the need for prolonged treatment periods or optimized allergen vaccines for SLIT application.[38] Interestingly, spIgG4 responses were stronger and showed faster kinetics in SCIT treatment compared to SLIT, while SLIT but not SCIT induced an increased spIgE titres, indicating that the ratio of spIgG over IgE was much higher in SCIT compared to SLIT, which was also reflected in the early allergic skin response upon allergen challenge. Our mouse model shows interesting parallels to this clinical study, where SCIT treatment induces a more rapid (after 1 week of treatment) and a more pronounced induction of IgG1 compared to SLIT (only after 6 weeks of treatment and with lower titres). We did not analyse long-term protection by either SCIT or SLIT treatment after discontinuation of treatment, however.

Recent insight into the relevance of allergen-specific IgG titres over specific IgE underscores the potential contribution of the neutralizing antibody response to the clinical benefit of AIT. A recent 3-centre study assessing the relation between serum levels of spIgE and IgG and symptom score in children with allergic rhinitis and asthma.[39] This well-designed study finds that the ratio of spIgG over IgE was a far better predictor of being symptomatic than spIgE titres per se, with asthma and rhinitis being associated with low IgG/IgE ratios. Interestingly, these authors identify a possible role for IL-10 producing Th cells in this protective response,[39] which is also considered to be a hallmark of successful allergen-specific immunotherapy.[40] In light hereof, SLIT treatment might be more efficacious if higher spIgG/IgE ratios are accomplished during treatment. In our experimental mouse model, the serum responses to the allergen challenges in sensitized and SCIT-, SLIT-, or control-treated mice were measured at day 6 after the allergen challenge, which is not optimal for the IgE responses. Nevertheless, we observed a clear induction of the (memory) IgE response mice that did not receive SCIT or SLIT treatment. Moreover, while we did calculate ratios of specific IgE over IgG1 as a measure of neutralizing capacity, we did not measure the affinity of the neutralizing IgG1 antibodies, which is important to the quality of the neutralizing antibody response.[41] Of note, the increased allergen-specific IgG1 levels in our mouse model protected against IgE-dependent allergic responses induced by allergen challenges, without any signs of IgG1-dependent anaphylaxis, which has been shown previously to occur in the mouse.[41,42] Therefore, we conclude that our experimental mouse model offers a promising platform for further optimization of GP-SLIT therapy to this end.

Our experimental study sought to achieve this by directly comparing SCIT and SLIT treatments in the model using a standardized protocol and on the basis of immunological and translational outcome parameters. We uniquely treat the mice with either SCIT or SLIT after parenteral immunization with the allergen, which means in the presence of an immunological memory population. This design also resulted in some limitations that we need to consider when interpreting our results. To allow a more accurate interpretation, we included interim serum bleeds in SLIT-treated mice, enabling the observation that induction of neutralizing antibody responses by SLIT treatment was delayed and reduced compared to SCIT treatment in our experimental model. In addition, measurement of allergen-induced ear swelling as a parameter of an early allergic response was quite variable within the groups,

precluding in-depth comparison of suppression of the early allergic response between SCIT and SLIT treatment in the experimental mouse model. Finally, we have previously shown that, while SCIT treatment in the mouse model induces a transient increase in FoxP3$^+$ T cells in circulation, the depletion of these cells at the time of allergen challenges has a rather modest effect on suppression of allergic phenotypes by SCIT, indicating that regulatory T cells have a relatively limited role in the mouse model.[43] While IL-10 is critical for suppression for allergic manifestations in allergen immunotherapy mouse models,[44] IL-10 production by T cells is not.[45] The lack of increased IL-10 and TGFβ responses after SCIT and SLIT treatment in the current study is in line with this. Nevertheless, our experimental mouse model recapitulates the differences seen in face-to-face comparisons between GP-SCIT and SLIT in clinical studies, underscoring its relevance as a platform for testing improvements for SLIT treatment for allergic disease.

The selection of the optimal application route for GP immunotherapy is not at all trivial, as shown in a recent study exploring the use of intradermal GP immunotherapy for allergic rhinitis, where treatment had no effect on primary outcome parameters (daily combined symptom-medication scores), while secondary end-points such as nasal and asthma symptoms were worsened, with fewer symptom-free days.[46] Although SCIT treatment might be able to achieve faster and more robust immunological changes, SLIT treatment has been proven to be an effective therapy. For instance, in a double-blinded RCT in patients with allergic rhinoconjunctivitis, GP-SLIT treatment was found to induce a significant and durable induction of neutralizing antibody responses as well as decreased symptom score up to 2 years after completion of a 3-year treatment period.[5] Moreover, a recent meta-analysis comparing SLIT tablets, SLIT drops, and SCIT injections for GP allergies reported comparable reduction in symptom scores and supplemental medication use for SLIT tablets and SCIT injections.[6] In addition, in a systematic review on adverse events reported with SCIT and SLIT treatment for GP allergic rhinitis, the authors note that, while data are not available for especially older treatment regimens, SLIT seemed to have a better overall safety profile when compared to SCIT.[47] A literature review of the aforementioned RCT of SLIT treatment for allergic rhinitis to an RCT of SCIT treatment for allergic rhinitis employing a very similar study design and the same GP allergen extract reports very similar effect sizes for both treatment regimens in suppressing nose and eye symptoms.[12] Nevertheless, the authors conclude that SCIT might be a more effective treatment than SLIT at the cost of having a greater risk for severe side-effects and the need for administration in a specialist clinic, while SLIT treatment has a greater risk for poor patient adherence.[12] Further improvements in SLIT efficacy in modulating immunological parameters such as the neutralizing antibody response might therefore increase its clinical efficacy, although it must be noted that no direct evidence for a causal relationship between levels of neutralizing antibodies and clinical efficacy of allergen immunotherapy is available to date. Our experimental mouse model can be used to test improvements of SLIT treatment using a validated SCIT treatment regime as a reference, making this model a valuable translational research tool for improvement of SLIT efficacy in the future.

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DISCLOSURE

The authors LH, UB, AHP, RG, LB, and JNGOE confirm that there is no conflict of interest to disclose. JB is an employee of ALK-Abelló. AjvO is GSK employee and shareholder. MCN reports consultancy fees paid by DC4U for scientific advice.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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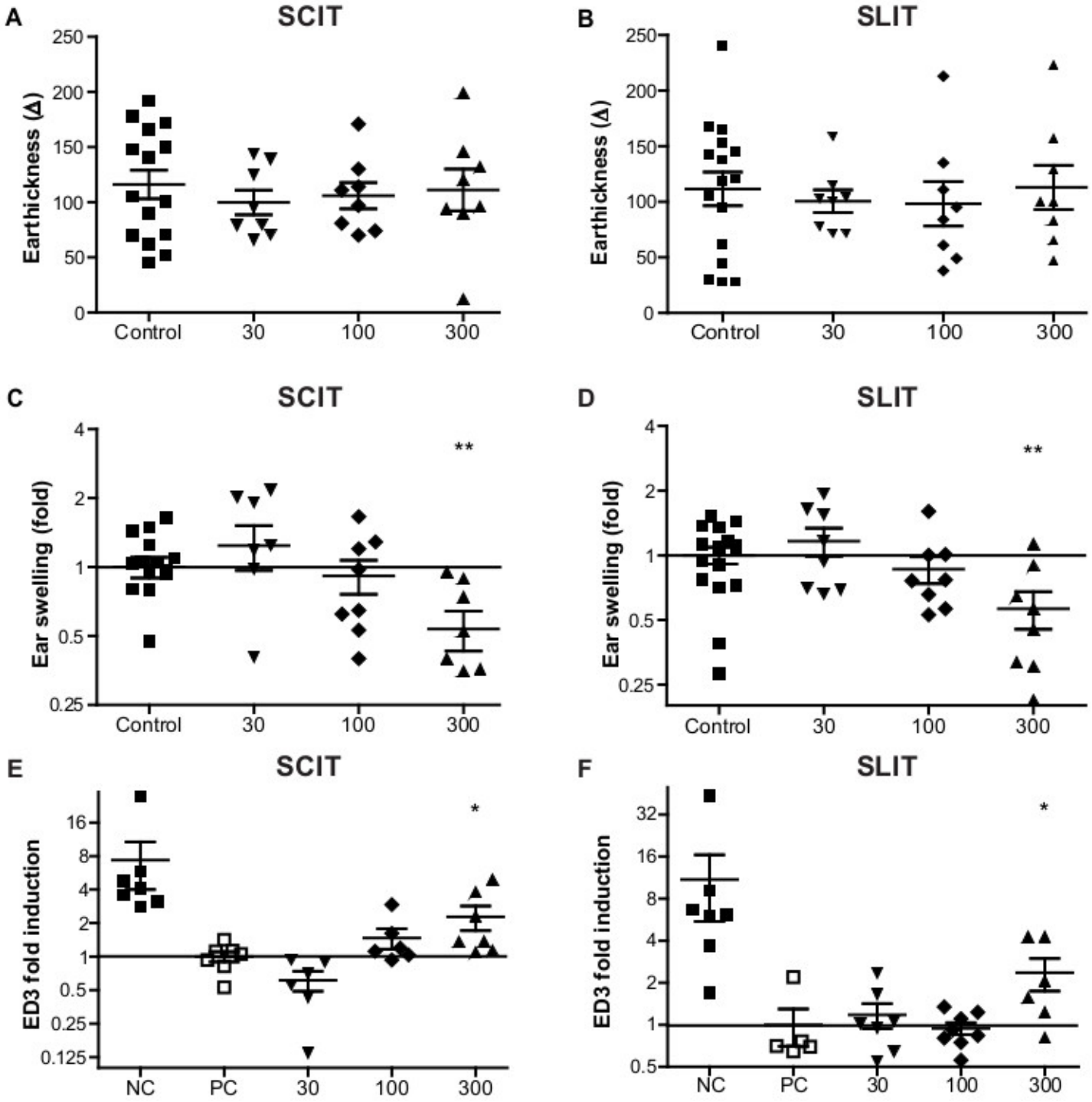
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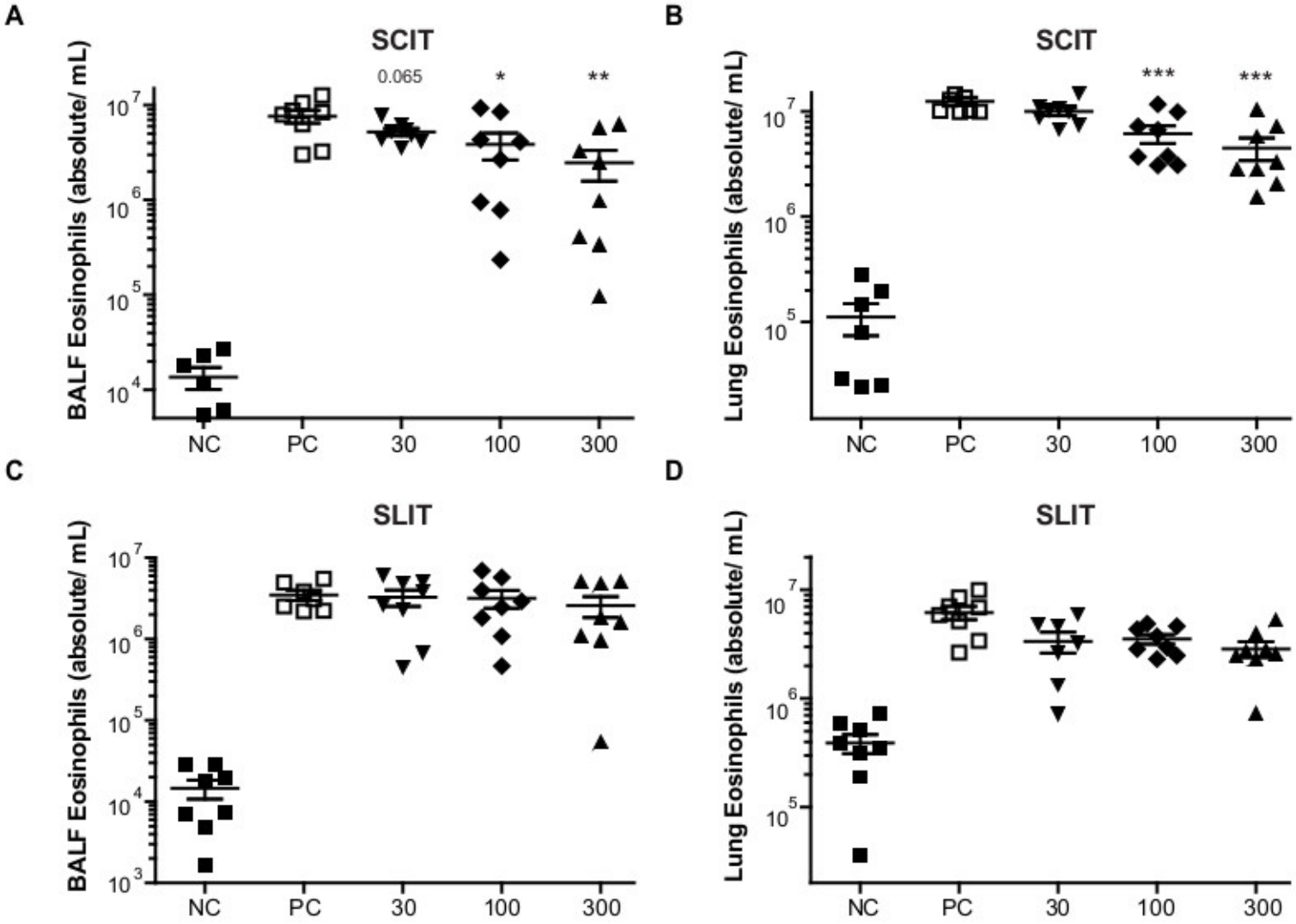
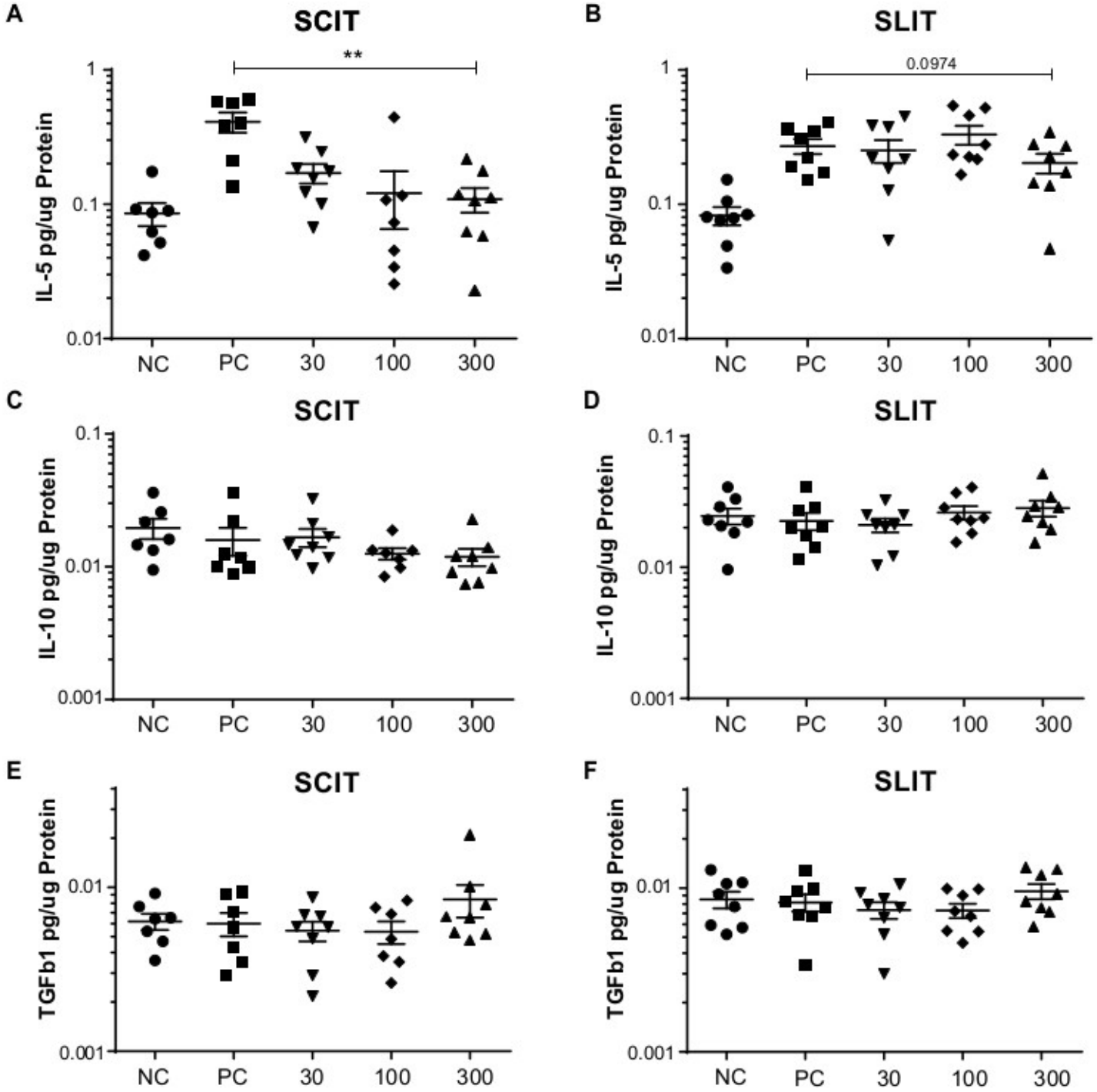
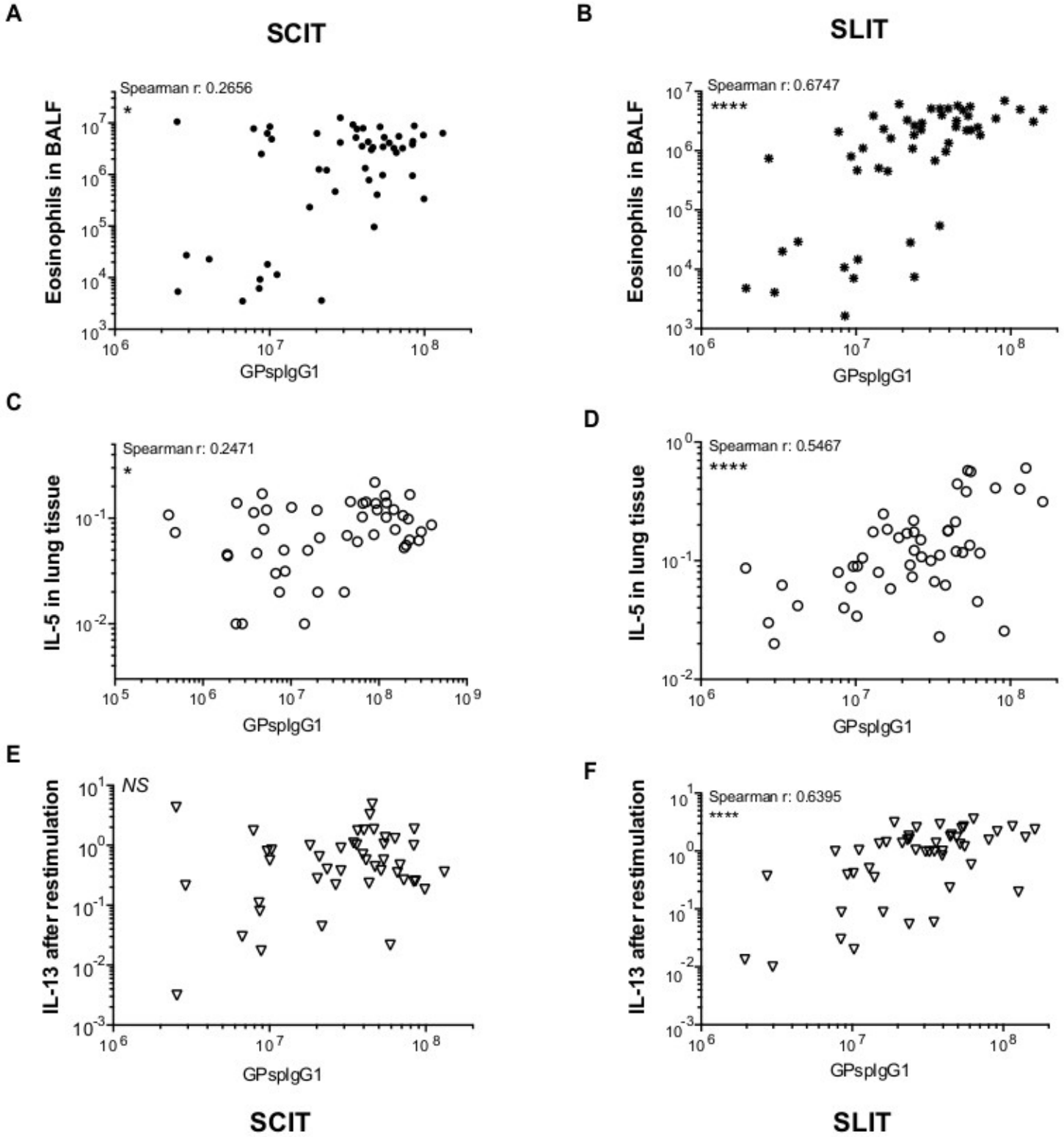
D
SLIT
Ear swelling (fold)
4
2
1
0.5
0.25
**
Control
30
100
300
E
SCIT
ED3 fold induction
16
8
4
2
1
0.5
0.25
0.125
*
NC
PC
30
100
300
F
SLIT
ED3 fold induction
32
16
8
4
2
1
0.5
*
NC
PC
30
100
300


A
SCIT
BALF Eosinophils (absolute/ mL)
0.065
*
**
NC
PC
30
100
300
B
SCIT
Lung Eosinophils (absolute/ mL)
***
***
NC
PC
30
100
300
C
SLIT
BALF Eosinophils (absolute/ mL)
NC
PC
30
100
300
D
SLIT
Lung Eosinophils (absolute/ mL)
NC
PC
30
100
300


A
SCIT
**
IL-5 pg/ug Protein
1
0.1
0.01
NC
PC
30
100
300
B
SLIT
0.0974
IL-5 pg/ug Protein
1
0.1
0.01
NC
PC
30
100
300
C
SCIT
IL-10 pg/ug Protein
0.1
0.01
0.001
NC
PC
30
100
300
D
SLIT
IL-10 pg/ug Protein
0.1
0.01
0.001
NC
PC
30
100
300
E
SCIT
TGFb1 pg/ug Protein
0.01
0.001
NC
PC
30
100
300
F
SLIT
TGFb1 pg/ug Protein
0.01
0.001
NC
PC
30
100
300


A
SCIT
Spearman r: 0.2656
*
Eosinophils in BALF
GPsplgG1
B
SLIT
Spearman r: 0.6747
****
Eosinophils in BALF
GPsplgG1
C
Spearman r: 0.2471
*
IL-5 in lung tissue
GPsplgG1
D
Spearman r: 0.5467
****
IL-5 in lung tissue
GPsplgG1
E
NS
IL-13 after restimulation
GPsplgG1
SCIT
F
Spearman r: 0.6395
****
IL-13 after restimulation
GPsplgG1
SLIT

Supplementary

Methods

Evaluating inflammation in BALF

Lungs were lavaged with 1mL PBS containing 5% Bovine Serum Albumin (BSA, Sigma Aldrich, Zwijndrecht, The Netherlands) and a cocktail of protease inhibitors (Complete mini tablet; Roche, Germany). Subsequently, four lavages were performed with 1mL non-supplemented PBS. After centrifugation (500xg, 4min), the cell-free supernatant of the first mL was stored as BALF (*in duplo*, -80°C). The cells from the first mL were added to the cells from the 4mL PBS lavages and counted using the Z2 coulter particle count and size analyzer (Beckman Coulter, Woerden, The Netherlands).

Cytospin preparations of the BALF and Lung cells were stained with Diff-Quick (Merz&Dade, Dudingen, Switzerland) and 300 cells per cytospin were evaluated and differentiated into mononuclear cells (M), neutrophils (N), and eosinophils (E) by standard morphology.

Airway Responsiveness

Mice were injected intraperitoneal with a mixture of 100mg/kg ketamine (Pfizer, NY) and 1mg/kg dormitor (Pfizer). After tracheotomization using a 20-gauge intravenous cannula (Becton Dickinson, The Netherlands) and canalization through the jugular vein, all mice were attached to a small animal ventilator; the FlexiVent (SCIREQ, Canada), and then ventilated (280 breaths/min) with a tidal volume of 10mL/kg, pressure limited at 300mmH_2O. Airway resistance and compliance was calculated from the pressure response to a 2-second pseudorandom pressure wave (COD>0.8). Moreover, responsiveness was expressed as the effective dose (ED) of methacholine required to induce a resistance of 3 cmH_2O.s/mL (ED_3).

GP-spIgE, GP-spIgG1, and GP-spIgG2a ELISA

Briefly, for GP-spIgE, NUNC MaxiSorp flat-bottom 96-well plates (Sigma, MO) were coated with 1µg/mL anti-mouse IgE (BD Pharmingen) in PBS (overnight, 4°C), washed five times (wash buffer; PBS 0.05% Tween-20), blocked using 3% skimmed milk powder (ELK, Campina, Amersfoort, The Netherlands) in ELISA buffer, and sera samples (diluted 1:8 in PBS 1%BSA) were incubated for 2hrs (room temperature). Then, the plates were incubated with 100µL 1:100 biotin labeled GP in PBS 1%BSA (homemade, see below) for 1.5hrs, washed five times and incubated for 1h with Streptavidin-Horseradish Peroxidase (1:200, R&D Sytems). Again plates were washed, following which SigmaFast^{TM} OPD substrate (Sigma-Aldrich) was added and incubated for 8min. The reaction was stopped by adding 75µL of 1.8M H_2SO_4. Optical density (OD) values were measured at 490nm and analyzed using a classic logit-log transformation model.

For GP-spIgG1 and GP-spIgG2a, plates were coated using 10µg/mL rough extract GP, blocked using 3%BSA in ELISA buffer, incubated with sera samples (1:300,000 for GP-spIgG1 and 1:100 for GP-spIgG2a), and labeled using biotinylated anti-mouse IgG1 or –IgG2a (1µg/mL, BD Pharmingen). Concentrations were calculated according to the standard curve (using reference serum) and the results are expressed as arbitrary unit (AU)/ mL.

Biotinylation of GP extract was performed using EZ-link Sulfo-NHS-LC-Biotin according to the manufacturers operating instructions (Thermo Scientific) and using a Slide-A-Lyzer cassette (3.5K MWCO, Thermo scientific) for purification by dialysis overnight.