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A pilot study towards the immunological effects of omalizumab treatment used to facilitate oral immunotherapy in peanut-allergic adolescents

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Abstract

Anti-IgE treatments, such as omalizumab, have shown promising effects in allergy treatment. Our previous work has shown that individualized omalizumab treatment (OT) allows a safe initiation and rapid up-dosing of peanut oral immunotherapy (OIT) in peanut-allergic adolescents. However, the broader immunological effects of this OT are incompletely understood. In this pilot study, we longitudinally followed the total B- and T-cell immunity during OT, using flow cytometry, ELISpot and ELISA. Peripheral blood mononuclear cells (PBMCs) and plasma were collected from participants (n = 17) at several timepoints during treatment, before starting OT (baseline), prior to starting OIT during OT (start OIT) and at maintenance dose OIT prior to OT reduction (maintenance). OT did not affect the total B-cell compartment over treatment time, but our results suggest an association between the OT dosage scheme and the B-cell compartment. Further, in vitro polyclonal T-cell activation at the different timepoints suggests a cytokine skewing towards the Th1 phenotype at the expense of Th2- and Th9-related cytokines during treatment. No differences in the frequencies or phenotype of regulatory T cells (Tregs) over treatment time were observed. Finally, plasma chemokine levels were stable over treatment time, but suggest elevated gut homing immune responses in treatment successes during the treatment as compared to treatment failures. The novel and explorative results of this pilot study help to improve our understanding on the immunological effects of OT used to facilitate OIT and provide guidance for future immunological investigation in large clinical trials.

1 | BACKGROUND

The current incidence of food allergies among children is estimated to be 8%.[1] Peanut allergy is among the most prevalent allergies in older children in western Europe and is rarely outgrown with age.[2]

Moreover, peanut allergy causes about 19% of the anaphylactic reaction in Swedish children.[3] Hence, peanut allergy is a major health concern and effective and safe treatments are warranted.

Oral immunotherapy (OIT) has shown promising short-term efficacy in the treatment of food allergies, including peanut

allergy.[4,5] Nevertheless, side effects often occur during OIT, which are sometimes severe[6] and there are as of today no long-term follow-up studies of peanut OIT. Recently, it was discovered that peanut OIT induces blocking antibodies, but that it does not change the functional characteristics of peanut-specific IgE.[7]

Previous research indicated positive effects of combining peanut OIT with an anti-IgE treatment, such as omalizumab.[8-10] Omalizumab lowers the sensitivity of mast cells and basophils, by capturing circulatory free IgE as well as downregulating the expression of the FcεRI on the basophils and mast cells and subsequently prevents the binding of IgE to the high-affinity FcεRI on mast cells and basophils.[11] Our previous work has shown that individualized omalizumab treatment (OT) allows a safe initiation and rapid up-dosing of peanut OIT in peanut-allergic adolescents.[12-14] Within this small explorative one-armed phase-2 study, described in detail elsewhere,[12,14] 11 patients passed the final open peanut challenge and were considered treatment successes (TS), while 6 patients were unable to stop OT treatment during OIT and were classified as treatment failures (TF).

Despite these promising results obtained with omalizumab-facilitated OIT, broader effects of omalizumab treatment on the immune system remain largely unexplored. Previously, a limited number of studies reported declined B cell[15,16] and inflammatory Th2 cell frequencies[17,18] following OT, but effects on regulatory T (Tregs) and B (Bregs) cells, important for tolerance induction,[19-23] remain currently unknown.

In order to enhance our understanding of the immunological effects of OT, we aimed to longitudinally follow the total B- and T-cell immune responses during OT facilitated peanut OIT in peanut-allergic adolescents. We investigated a broad range of B-cell subsets, including naïve and memory B cells, Foxp3 expressing B cells, the antibody-secreting plasmablasts, as well as IgE and IgG4 antibody-secreting cells (ASCs). Moreover, T helper cell responses were followed during treatment on the basis of IL5, IL13 (Th2), IL9 (Th9) and IFNγ (Th1) secretion, as well as the frequency of Tregs, along with investigation of their phenotype and inhibitory capacity using the expression of CD45RA, Helios and CTLA4. In addition, plasma chemokine concentrations were used to monitor the immune profiles over treatment time. Finally, associations between these immune parameters with treatment success and omalizumab dosage schemes were investigated.

This explorative study enhances our understanding on the immunological effects of omalizumab treatment when used in a combined setting with OIT and provides guidance for large(r) clinical trials investigating future allergy treatments.

2 | METHODS

2.1 | Study population

This study is based on a previously described one-armed phase 2 study of omalizumab-facilitated peanut OIT in peanut-allergic adolescents (age 12-19 years old, n = 23), of which the study design is described in detail in two previous publications.[12,14] Figure 1 provides a schematic study flow chart.

In brief, 23 peanut-allergic adolescents with documented anaphylactic reactions to peanut and who all were positive in peanut-specific basophils activation tests (CD-sens, as described elsewhere[24]) were included in the study. All participants received an initial 8-week dose of omalizumab (based on body weight and IgE), after which the CD-sens level was checked again. If CD-sens was not suppressed to 0 after 8 weeks of treatment, the omalizumab treatment was continued in the same or increased dose until CD-sens levels became suppressed. Subsequently, as CD-sens suppression to peanut was reached, an open peanut challenge was performed, after which the peanut OIT, under protection of omalizumab, was started with 280 mg of peanut. Peanut dosages were increased every 2nd week, until a maintenance dose of 2800 mg of peanut was reached. CD-sens analysis was performed 8 weeks into the maintenance phase, and if still suppressed, the omalizumab dose was decreased by 50%. If CD-sens started to increase, the omalizumab dose was maintained for >8 weeks followed by repeated CD-sens analysis. This process was repeated until OT was completely stopped and peanut OIT alone was continued for an additional 12 weeks, after which an open peanut challenge was performed again.

After the treatment period, 4 years after the study start, 11 patients were considered treatment successes (TS), passing the final peanut challenge, whereas six patients were classified as treatment failures (TF), not being able to discontinue OT during OIT. Moreover, 6 patients dropped out during the conduct of the study. Detailed characteristics on peanut-specific IgE/IgG4-ab levels, basophil activation levels (CD-sens) and adverse events (AEs) of the TS and TF, as well as the dropout, have been described previously.[12,14]

Peripheral blood samples were taken at different timepoints during the conduct of the study, before starting the OT (Baseline), at the peanut challenge during OT and prior to starting the peanut OIT (start OIT), and at the maintenance dose prior to OT reduction (OT + OIT: maintenance). All longitudinally available material was used in this explorative study.

The study was approved by the ethics committee in Stockholm: 2013/827-31/3 and the Swedish Drug Agency: 5.1-2013-46183 and registered at EudraCT: 2012-005625-78, ClinicalTrails.gov; NCT02402231. All patients and (if <18 years old) their caregivers gave their informed consent.
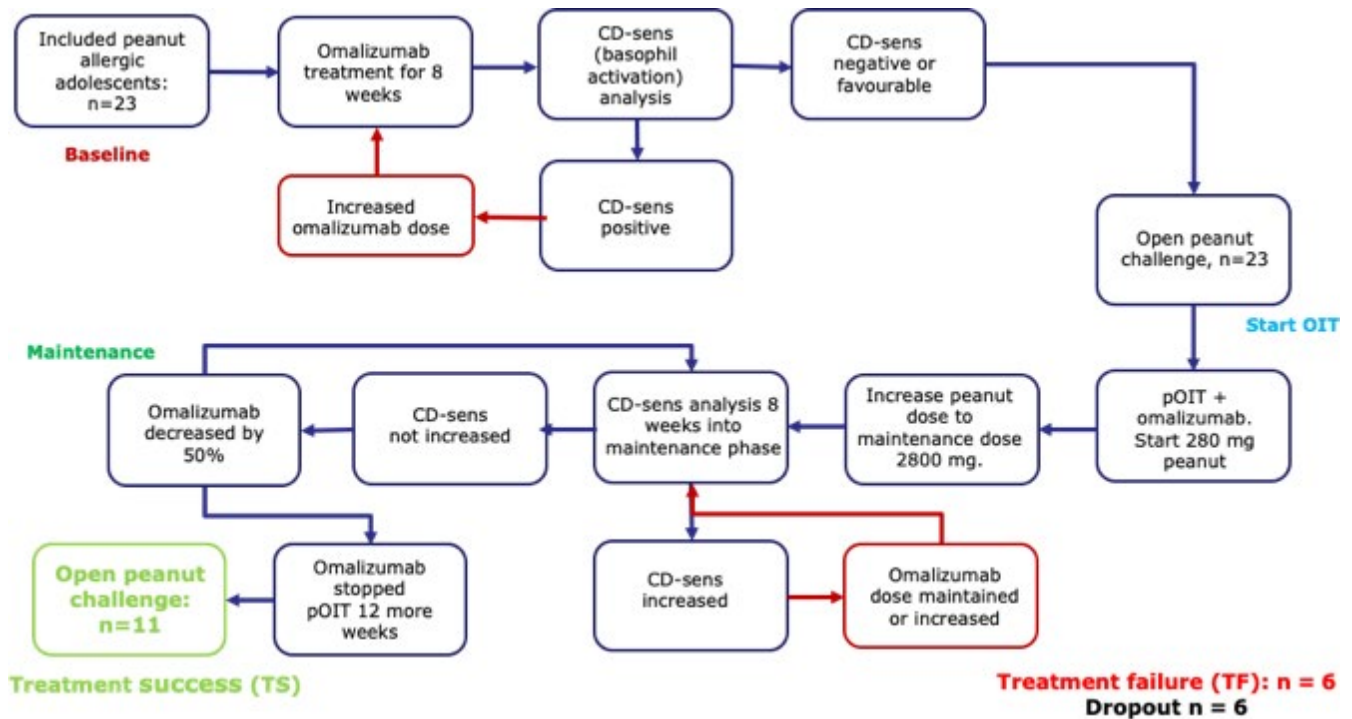
FIGURE 1 Schematic flow chart of the study. The different phases of the study, including the checkpoints and sampling timepoints, are indicated. Baseline: before starting the OT, Start OIT: at the peanut challenge during OT and prior to starting the peanut OIT and Maintenance: at the maintenance dose prior to OT reduction. The classification of TS is defined as passing the open peanut challenge 12 weeks post-omalizumab treatment.

2.2 | Sample collection, preparation and processing

Peripheral blood samples were collected in tubes containing heparin (BD Biosciences PharMingen) at the different timepoints. Plasma samples were collected and stored at −80°C until further use.

Peripheral blood mononuclear cells (PBMCs) were isolated with gradient separation using Ficoll-Hypaque (GE Healthcare Biosciences AB, cat-nr: 17-5446-02). The cells were washed using RPMI-1640 (Cytiva, HyClone laboratories, cat-nr: 11541811) and supplemented with 20 mmol/L HEPES (Cytiva, cat-nr: 16777-032) and thereafter frozen in freezing media containing 40% RPMI-1640, 50% FCS and 10% dimethyl sulphoxide (DMSO) (Sigma-Aldrich, cat-nr: 67-68-5) and stored in liquid nitrogen until further use.

Frozen PBMCs were thawed and washed with RPMI-1640 supplemented with 20 mmol/L HEPES. The cells were counted, and viability was assessed with Trypan Blue staining. Thereafter, the cells were resuspended in a concentration of 10^6 cells/mL in cell culture medium, consisting of RPMI-1640 supplemented with 20 mmol/L HEPES, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mmol/L L-glutamate (2 mmol/L) (all Cytiva, cat-nr's: HYCLSV30079.01 and SH3033603), and 10% heat-inactivated FCS and rested overnight at 37°C and 5% CO_2 in a flat-bottomed 6-well plate (Costar, cat-nr: 07-200-83).

2.3 | In vitro stimulation of the PBMCs

To analyse the T helper cell-specific cytokine production, 500 000 live PBMCs were stimulated for 48 hours (at 37°C and 5% CO_2) with Dynabeads™ Human T-activator CD3:CD28 (Thermo Fisher Scientific, cat-nr: 11161D) at a 2:1 cell:bead ratio.

In order to analyse the IgE and IgG4-ab secreting B cells, 500 000 live PBMCs were stimulated for 5 days with, respectively, a combination of CD40L (1 µg/mL) and IL4 (10 ng/mL) or R848 (1 µg/mL) and IL2 (10 ng/mL) (all from Mabtech AB, Nacka, Sweden, cat-nr's: 3810-2H, 3854-2A).

Cell culture supernatants were collected from all stimulations and stored at −20°C for up till a month until further use. All stimulations were performed in a flat-bottomed 48-well plate (Costar, cat-nr: 10065370).

2.4 | IgE and IgG4 ELISpot analysis

The ELISpots were performed according to the manufacturer's instructions (all reagents from Mabtech, cat-nr's: 3810-2H, 3854-2A). Briefly, the multiscreen filter plates were activated using 70% ethanol, washed with sterile water and subsequently coated overnight with 15 µg/mL of either IgE or IgG4 coating antibody in sterile PBS. The plates were stored overnight at 4–8 degrees.

After washing with sterile PBS, the plates were blocked for at least 30 minutes at room temperature with culture medium as described above. After stimulation of the PBMCs for 5 days as described above, the cells were counted and plated at 300 000 or 250 000 live cells per well for the IgE and IgG4 assays, respectively. The plates were incubated for 24 hours at 37°C and 5% CO_2. The next day, plates were washed with a washing buffer of PBS and 0.5% FCS and incubated with either 1 µg/mL of IgE- or IgG4-specific biotinylated detection antibody for 2 hours. Subsequently, plates were washed and incubated with a 500x dilution of Streptavidin-HRP antibody (IgE) or a 1000x dilution of Streptavidin-ALP (IgG4) for 1 hour at room temperature. Finally, after washing, the IgE plates were incubated with TMB substrate and the IgG4 plates with NBT substrate until distinct spots emerged.

After development of the spots, the plates were dried and spots were analysed with the ImmunoSpot reader (CTL Europe GmbH). The values are expressed as antibody-secreting cells (ASCs) per 1M PBMCs.

2.4.1 | Flow cytometry analysis

After incubation, the cells were washed with PBS and stained with live/dead aqua (Invitrogen, cat-nr: L34957) in PBS. After surface staining, cells were either washed and fixed with 4% PFA before analysis or treated with the intranuclear staining fixation kit (BioLegend, cat-nr: 424401) according to the manufacturers' instructions. A blocking step with 10% human serum was performed both before surface and intracellular staining. The flow cytometry antibodies used for the different panels are described in Table 1. All surface antibodies were stained in FACS wash buffer containing PBS,

0.1% BSA (Roche diagnostics GMBH) and 2mM EDTA (Invitrogen, Grand Island, NY, cat-nr: 15575020). The intracellular staining was performed in the intranuclear staining perm wash buffer, according to the manufacturer's instructions (BioLegend). Fluorescence-minus-one (FMO) and isotype controls were used for gating. Example gating strategies are provided in S. Figures 1 and 2.

The data were acquired with a Facs Verse in combination with the Facs Suite software (BD Biosciences). The analysis was performed with FlowJo Software (TreeStar).

2.5 | Supernatant and plasma analysis

IFNγ, IL5, IL13 (all from Mabtech, cat-nr's: 3420-1A-6, 3490-1A-6, 3471-1A-6) and IL9 (R&D systems, cat-nr: DY209-05) concentrations (pg/mL) in the cell culture supernatant derived from the CD3:CD28 beads stimulations were measured with a sandwich ELISA according to the manufacturers' instructions. The optical density of the plates was measured with a micro-plate reader, either at 405 or 450 nm (Molecular Devices Corp) and analysed using SoftMax Pro 5.2 rev C (Molecular Devices Corp).

The Ara h 2 IgE-ab concentrations (kU_A/L) in the cell culture supernatants after IL4 and CD40L stimulation were measured with ImmunoCAP® (Thermo Fisher Scientific) according to the manufacturers' instructions.

The plasma chemokine concentrations (pg/mL) were measured using a magnetic-based BioPlex Pro™ Human Chemokine Assay (Bio-Rad Laboratories, customized) according to the manufacturer's instructions. The plates were measured with the BioPlex® Magpix™ (Luminex) and results analysed with Bioplex Manager v6.1.

TABLE 1 Flow cytometry antibodies used for the different panels

Panel	Marker	Fluorochrome	Clone	Manufacturer	Cat-nr	Stain
B-cell panel	CD3	FITC	UCHT1	BD	561806	Surface
	CD19	APC-Cy7	HIB19	BioLegend	363010	Surface
	CD5	PerCp Cy5.5	L17F12	BioLegend	364006	Surface
	CD27	BV421	O323	BioLegend	302824	Surface
	CD38	APC	HIT2	BioLegend	356606	Surface
	IgD	Pe-Cy7	IA6-2	BioLegend	348210	Surface
	FOXP3	PE	259D/C7	BD	560046	Intracellular
Treg panel	CD4	APC-H7	RPA-T4	BD	560251	Surface
	CD127	PerCP Cy5.5	HIL-7R-M21	BD	560551	Surface
	CD25	BV421	BC96	BioLegend	302630	Surface
	CD45RA	FITC	HI100	BioLegend	983002	Surface
	FOXP3	PE	259D/C7	BD	560046	Intracellular
	Helios	APC	22F6	BD	563801	Intracellular
	CTLA-4	PE Cy7	L3D10	BioLegend	369614	Intracellular

2.6 | Statistical analysis

Due to the limited number of participants in the study, all data were analysed using non-parametric statistical tests. Different numbers of participants were included in each analysis, based on PBMC availability, which is clearly indicated in the figure legends. Due to the limited number of participants, and the explorative nature of the presented study, statistical tests were not corrected for multiple testing.

The three different timepoints of the treatment were compared with the repeated-measures ANOVA, followed by the uncorrected Dunn's test. The Mann-Whitney test was used to compare the TS and TF or the groups receiving the normal dosage or the increased dosage of omalizumab. A *P*-value <.05 was considered significant. Significances are indicated with *P < .05, **P < .01 and ***P < .001. For these analysis, GraphPad Prism V7 was used.

The principal components analysis (PCA) was performed using R studio 1.3.5033 (RStudio Inc). All data were log-transformed before performing the PCA analysis. The data were reduced into two principle components, of which the amount of variance in the data that is explained by the principle component is mentioned as a percentage on the axes.

The heatmaps were created using Microsoft Excel for Mac and GraphPad Prism V7. The group-specific median values for the different immune parameters were normalized to modified z-scores using the median and median average deviation (MAD) of the total group of values (all timepoints combined). The modified z-scores were displayed on a colour scale, ranging from blue (below median value in the total group) to red (above median value of the total group). The colour intensity indicates the strength of deviation from the total group median.

3 | RESULTS

3.1 | The B-cell compartment is not altered by OT, but associated with the OT dosage scheme

Omalizumab is known to capture free circulatory IgE, but is also suggested to affect the B-cell compartment in a broader perspective.[15,16] Therefore, we prioritized to study the effects of OT on the B-cell compartment. In addition, we investigated whether the B-cell compartment was associated with the initial dosage scheme of OT or treatment outcome.

As shown in the PCA plot in Figure 2A (Table S1), the total B-cell compartment was stable over treatment time. Interestingly, a slight difference in the B-cell compartment

between the group receiving only the initial normal dosage of omalizumab (referred to as normal dosage) and the group receiving prolonged and increased omalizumab dosages after 8 weeks (referred to as increased dosage) is visible (Figure 2B). In general, the normal dosage group showed trends towards lower frequencies in the majority of the B-cell subsets investigated, especially on the earlier treatment timepoints (Figure 2D).

Although no major differences were found between the TS and TF in the B-cell compartment (Figure 2C), some interesting trends were observed (Figure 2E). At the start of OIT, TF possessed higher frequencies of plasmablasts as compared to the TS. Moreover, trends towards higher IgE ASC were observed at all three timepoints of treatment, becoming most pronounced at the maintenance timepoint. In addition, a trend towards a higher IgG4/IgE ASC ratio was observed in the TS at the maintenance timepoint (Figure 2E).

3.2 | The T helper cell cytokine profile is skewed towards IFNγ over treatment time

Next, we investigated whether the omalizumab treatment was associated with alterations in T-cell polarization, by investigating the concentrations of IL5, IL13, IFNγ and IL9 in the cell culture supernatant after stimulation of PBMCs for 48 hours with a polyclonal T-cell activator. Trends towards decreasing concentrations of IL5, IL13 and IL9 (Figure 3A-C) over treatment time were observed, whereas the concentration of IFNγ was significantly enhanced at the maintenance timepoint (Figure 3D). Interestingly, these findings resulted in significantly decreased ratios of IL5, IL13 and IL9 to IFNγ towards the end of treatment (Figure 3E-G).

3.3 | The Treg compartment did not change during OT

Since regulatory T cells (Tregs) are suggested to be crucial for tolerance induction, we investigated the total Treg frequency and phenotype during the omalizumab-facilitated treatment. Although some participants showed clear fluctuations over treatment time, no significant longitudinal difference in the frequency of FoxP3$^+$CD25$^+$CD127^{low} CD4$^+$ T cells (hereafter called Tregs) was observed (Figure 4A). Also, the frequency of Tregs expressing Helios (Figure 4B) or CD45RA (Figure 4C) did not change over treatment time, nor did the expression level (MFI) of CTLA4 in the Tregs (Figure 4D).

Despite the low number of TF tested, we noted higher frequencies of Helios-expressing Tregs cells at baseline in the TFs, which disappeared at the later timepoints (S. Figure S3).
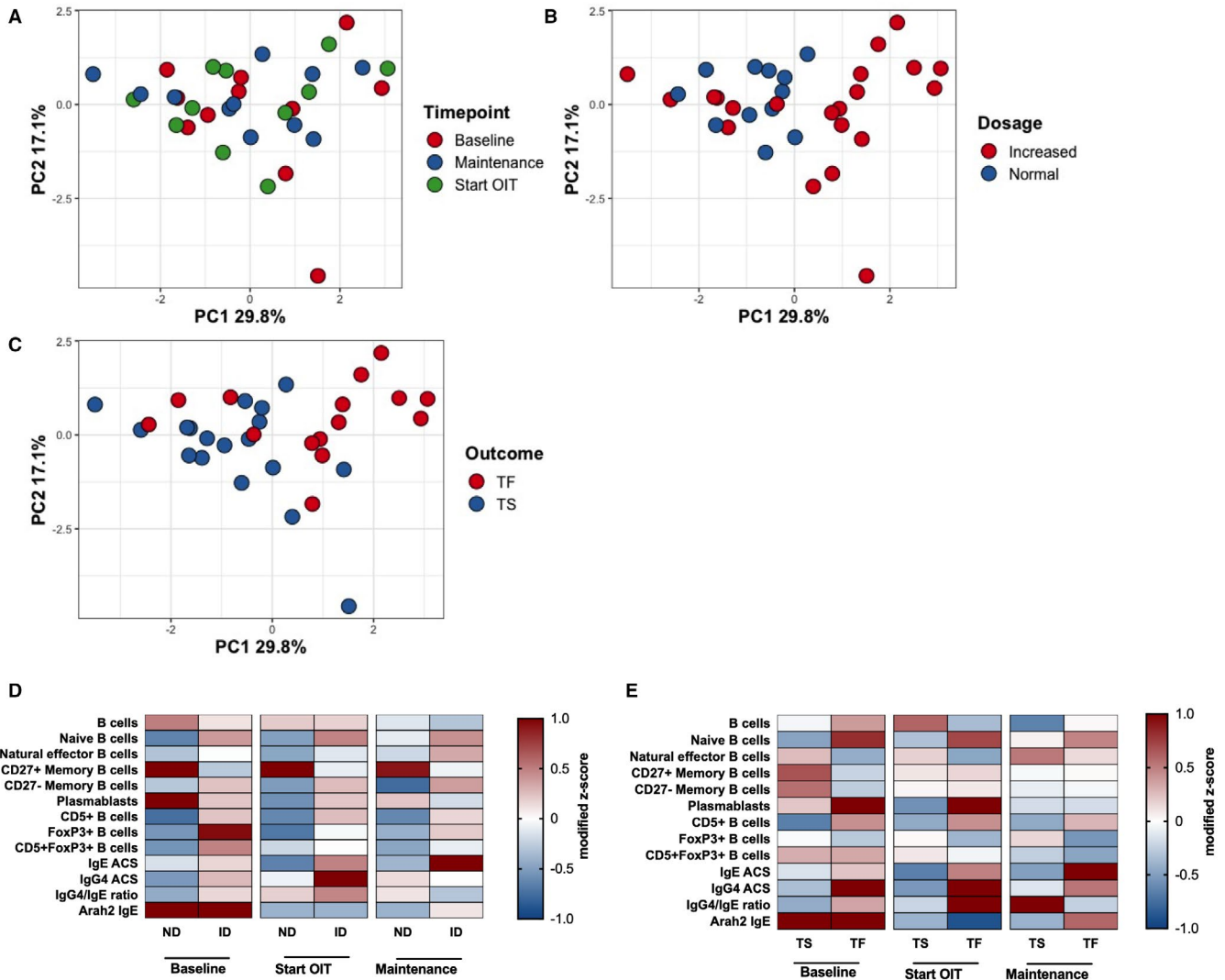
FIGURE 2 The B-cell compartment during OT treatment. Principal component analysis (PCA) comparing the B-cell compartment between the three different treatment timepoints (red: baseline, green: start OIT, blue: maintenance) (A), between the participants receiving the normal dosage (blue) or the prolonged and increased dosage of omalizumab treatment (OT) (red) (B), and between treatment successes (TS) (blue) and failures (TF) (red) (C). All three timepoints are included in the latter two analyses. Log-transformed B-cell parameters; total B cells, naïve B cells, natural effector B cells, CD27 + memory B cells, plasma cells, FoxP3 expressing B cells, CD5 expressing B cells, IgE and IgG4 production are used in the analysis. Heatmaps comparing the individual B-cell parameters at the different timepoints between the participants receiving the normal dosage (ND) (n = 4) or the prolonged and increased dosage (ID) (n = 8) of OT (D) and between the TS (n = 7) and TF (n = 5) (E). The modified z-scores were indicated on a scale from −1 (blue), below the median of the total group, to 1 (red) above the median of the total group.

3.4 | Stable plasma chemokine levels during OT

Finally, plasma chemokine levels were measured and analysed over omalizumab treatment time, as well as separately analysed per function group, gut homing, Th1 and Th2 chemokines.[25] No major differences in plasma chemokine levels during treatment were observed (Figure 5A). Moreover, no major differences were observed between the groups receiving the normal and increased omalizumab dosage as well as the TF and TS (plots not shown). However, some slight fluctuations were observed for separate chemokines. The group receiving only the normal dosage possessed higher concentrations of homing-associated chemokines at the maintenance phase. Moreover, the normal dosage group showed a significant higher concentration of BCA-1 (CXCL13) at the start of OIT (Figure 5B).

In addition, the TS possessed significantly higher concentrations of the gut homing-associated chemokines MIP3a (CCL20), and TECK (CCL25), as well as trends towards a higher concentration of the macrophage attractant chemokine MCP-1 (CCL2) at the maintenance timepoint. Moreover, the TF showed trends towards lower concentrations of the Th2-related chemokines, which became significant for BCA-1 (CXCL13) and MDC (CCL22) at the start of the OIT (all Figure 5C).
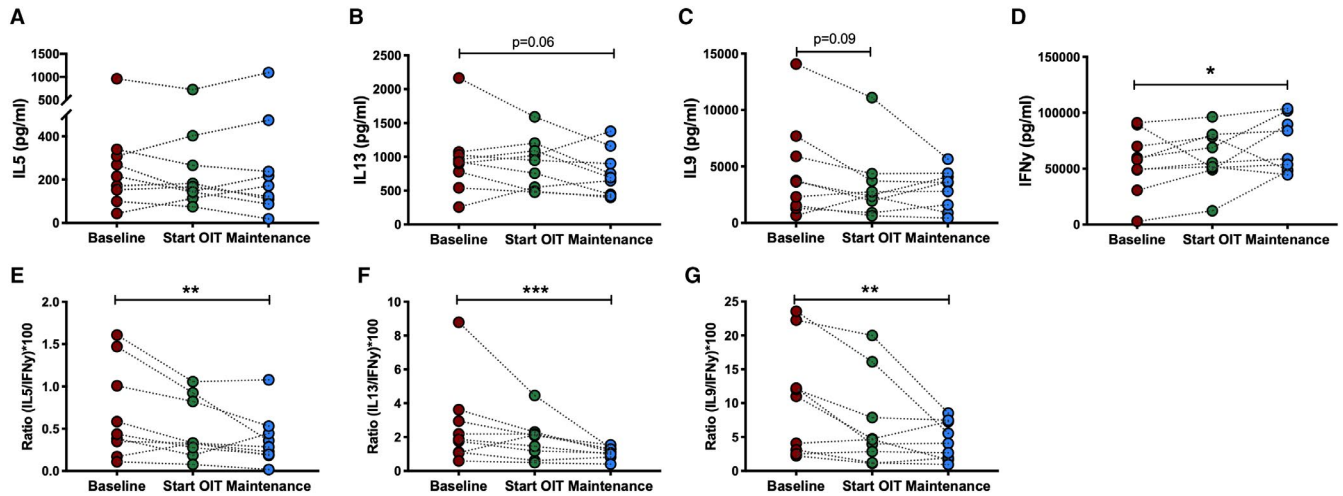
FIGURE 3 T helper cell responses over treatment time. Concentrations (pg/mL) of IL-5 (A), IL-13 (B), IL-9 (C) and IFNγ (D) in the cell culture supernatant of PBMCs that were stimulated for 48 h with a polyclonal CD3:CD28 bead-based T-cell activator at the three different timepoints of treatment (baseline—red, start OIT—green and maintenance—blue) (n = 9, both containing TF (3) and TS (6)). These secretion values of IL-5, IL-13 and IL-9 are expression as a ratio to the IFNγ secretion in (E, F and G), respectively. All timepoints were compared with the baseline values, and significances are indicated as $*P < .05$, $**P < .01$, $***P < .01$

FIGURE 4 Tregs cells during combined OT and OIT treatment. The frequencies of FoxP3$^+$CD25$^+$CD127^{low} cells among CD4$^+$ T cells (A), Helios$^+$ cells among FoxP3$^+$CD25$^+$CD127^{low} cells (B) and CD45RA$^+$ cells among FoxP3$^+$CD25$^+$CD127^{low} cells (C) and the mean fluorescent intensity (MFI) of CTLA4 among FoxP3$^+$CD25$^+$CD127^{low} cells (D) at the three different treatment timepoints (baseline—red, start OIT—green and maintenance—blue) (n = 9 at all timepoints, both containing TF (n = 3) and TS (n = 6)). All timepoints were compared with the baseline values
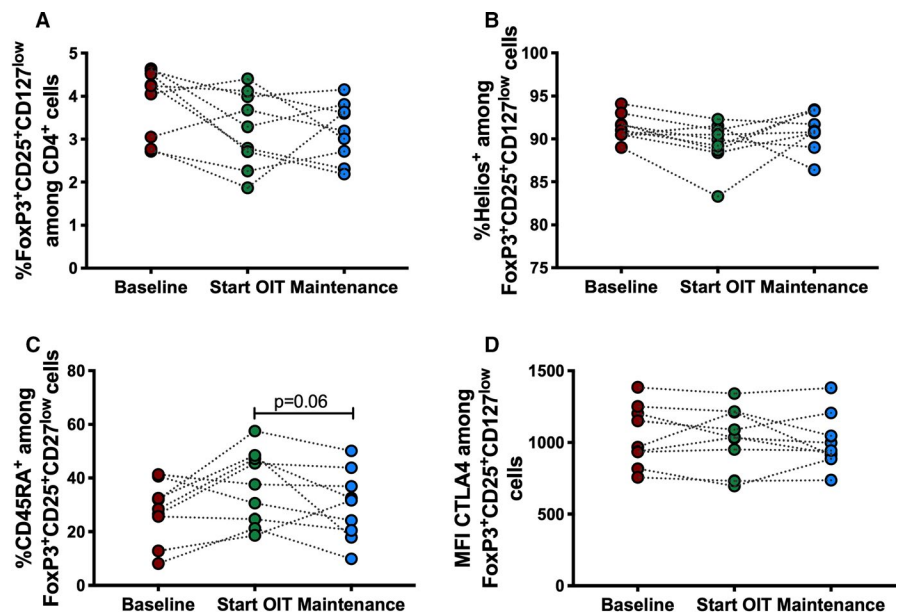
4 | DISCUSSION

In order to improve our understanding of potential immunological effects of omalizumab, we investigated the B- and T-cell responses during OT in peanut-allergic adolescents, where individualized OT dosing, monitored by CD-sense,[24] was used to facilitate peanut OIT. Within this pilot study, we show that the general B-cell compartment is not altered by OT. Nevertheless, we suggest an association between the B-cell compartment and the initial OT dosage scheme needed for suppressive treatment. Importantly, we report a skewing of part of the T-cell compartment towards a Th1 phenotype

over treatment time. In addition, we show that Treg frequencies and phenotype did not change during treatment. Finally, we observed stable plasma chemokine profiles, but suggest a potential difference in the gut homing immune responses between TS and TF during the combined OT and OIT phase.

Current knowledge on effects of OT on cells of the immune system is limited. Omalizumab is known to capture free circulatory IgE with high affinity and consequently prevents the binding of IgE to the FcεRI on mast cells and basophils.[11,26] Next to prevention of binding, omalizumab also reduces the expression of the FcεRI on these cells, as well as on peripheral dendritic cells.[17,26,27] Previously described
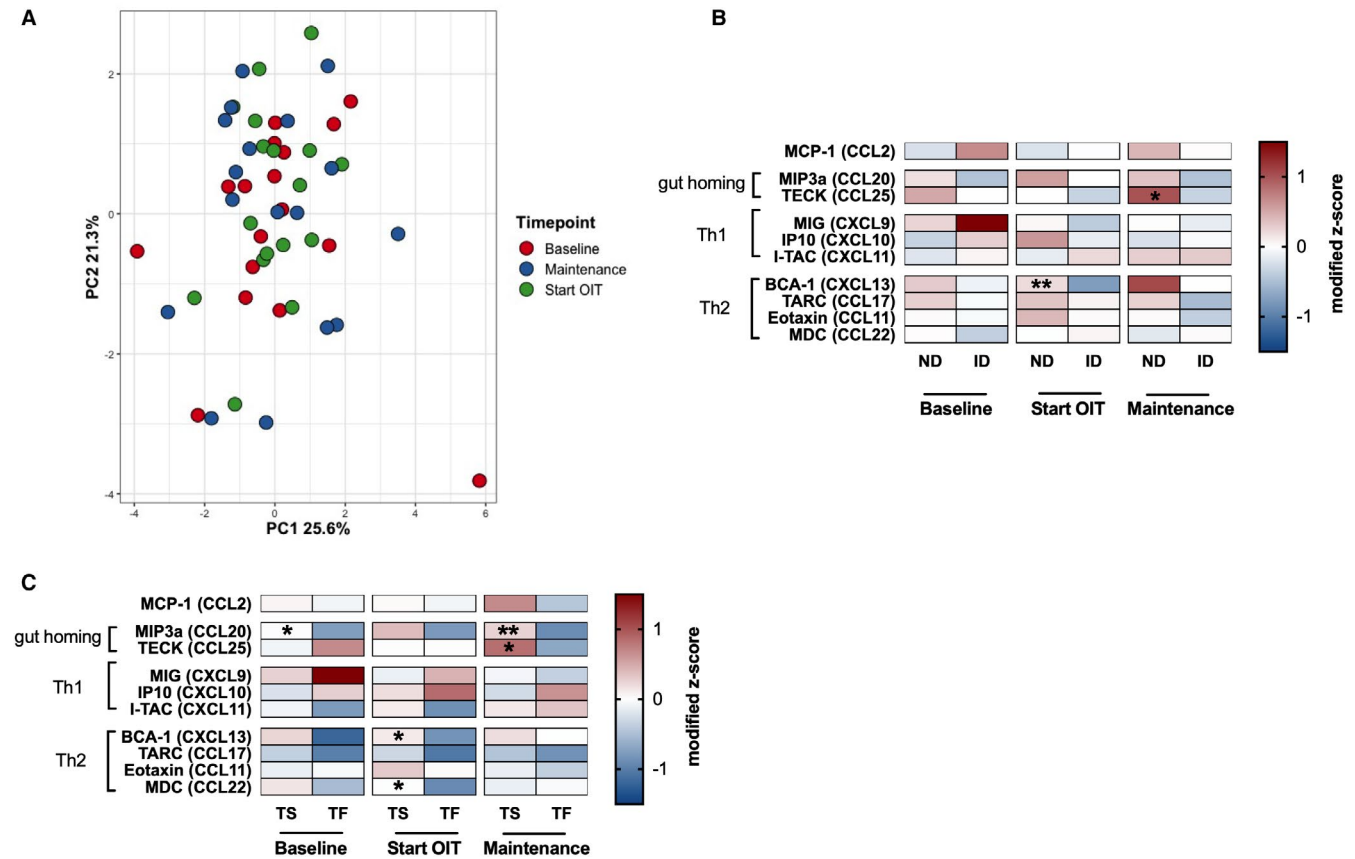
FIGURE 5 Plasma chemokine concentrations over treatment time. PCA comparing the plasma chemokine concentrations between the three different treatment timepoints (baseline—red, start OIT—green and maintenance—blue) (A). Heats maps comparing the plasma chemokine concentrations at the different treatment timepoints between participants receiving the normal dosage (ND) (n = 7) and the increased dosage (ID) (n = 9) of OT (B) and between the TS (n = 12) and TF (n = 5) (C) The chemokines were roughly classified as gut homing, Th1- and Th2-related chemokines based on current knowledge. The modified z-scores were indicated on a scale from −1 (blue), below the median of the total group, to 1 (red) above the median of the total group. Significant differences between the groups are indicated with as *P < .05 and **P < .01

associations between OT and cells of the adaptive immune system are more controversial. While some studies show effects of OT on B-cell numbers and phenotype,[15,16] others do not.[17] We here show no major effect of OT on the total B-cell compartment, using a broad range of parameters. Of interest, no difference in the number of IgE ASC was observed over treatment time, suggesting that omalizumab does not decrease the number of IgE-positive B cells nor the capacity of B cells to become IgE positive by switching from IgG1.

We are the first to suggest an association between the B-cell compartment and the dosage scheme of OT given. Clear trends towards lower frequencies and numbers of cells in the majority of the B-cell subsets were observed in the participants that were given only the initial round of 8 weeks OT. This trend was both visible at baseline and visible at the start of OIT and possibly suggests a more active B-cell compartment in participants in need of an elevated or prolonged dosage scheme.

No major differences were observed in the B-cell compartment between TS and TF. Nevertheless, several interesting trends were observed, where TF possessed higher frequencies of plasmablasts, naïve B cells, CD5 expressing B cells, as well as higher numbers of IgE and IgG4 ASCs. These results might suggest a slightly more active antibody-secreting B-cell compartment in TF. Importantly, a trend towards an association between a higher IgG4/IgE ASC ratio and TS was observed at the maintenance phase, which agrees with previous findings, indicating important roles for IgG4 relative to IgE during allergen tolerance induction.[28]

Influences of OT on the T-cell compartment are described by several studies,[17,18] whereas others report no influence.[29] A study by Maggi et al[17] reported decreased frequencies of CD154+ T cells in patients treated with omalizumab, whereas no differences in cytokine expression were observed. Others suggested anti-inflammatory effect of OT, since fewer IL2+ and IL13+ lymphocytes were observed in omalizumab-treated patients.[18] We here show a clear shift in the overall T-cell responses from IL5, IL13 and IL9 towards enhanced IFNγ secretion during the treatment, suggesting a change in the balance between Th2/Th9 and Th1 T-cell responses. This shift in balance away from the IgE stimulating Th2 and Th9 phenotypes[30-32]

towards the Th1 phenotype suggests a less allergy-prone T-cell phenotype.[33] A preliminary comparison of these T-cell responses did not point towards differences between TF and TS (data not shown).

Tregs are perceived as important players in the tolerance induction to food allergens,[19] but whether OT effects Tregs is currently incompletely understood.[29] We here observed stable frequencies and phenotype of Tregs over treatment time. Interestingly, despite the low numbers of TF, we observed higher frequencies of Helios-expressing Tregs in TF at baseline, a finding worth further investigation in future clinical trials and possibly indicating a higher frequency of recently thymic-derived Tregs with high suppressive capacity in the TF before the start of treatment.[34] Moreover, investigating the specificity of these Tregs is of interest in future trails.

Finally, besides in general stable chemokine profiles during treatment, we suggest potential differences in the underlying gut homing immune responses in the TS and TF during combined OT and OIT. This finding might indicate a deviating mucosal immune response between TF and TS which possibly results in less efficient peanut-specific immune responses during OIT in TF. This association warrants further investigation in future clinical trials.

We acknowledge that the low number of participants and lack of placebo group, already discussed elsewhere,[14] are weaknesses of our study, and consequently, our study should be considered as a pilot study. Also, our study is limited to the analysis of immune responses in peripheral blood only, which might not completely mirror the responses that are ongoing in the mucosal tissues, such as the gut.

Despite our aim to study general immune effects observed during OT, our study is limited by the absence of peanut-specific immune responses, specifically when it comes to the understanding of TF and TS. Therefore, it would also be essential in future studies to investigate peanut-specific immune responses during combined OT and OIT in clinical trials. Our previous results suggest a stronger peanut-specific baseline allergic response in the TF, based on peanut-specific IgE antibodies and basophil activation levels. Moreover, our previous results indicated an association between the treatment-induced peanut-specific IgG4 response, involved in immune tolerance induction due to allergen blocking,[20,35] and treatment success.[14] Consequently, it would be relevant to study these responses in relation to peanut-specific regulatory B cells (Bregs), previously observed to be important for IgG4 production and tolerance induction.[20-23] Moreover, others observed that during resolution of peanut allergy, the peanut-specific T cells shifted from a Th2 or Th9 phenotype towards the Th1 phenotype,[36-39] whereas also enhanced peanut-specific Treg activity during OIT was observed.[19,40] It is of importance to study these peanut-specific immune responses under the influence of OT in future trials.

In conclusion, we suggest that OT, when used to facilitate peanut OIT, does not impact the total B-cell compartment and nor does the B-cell compartment influence treatment success. We do, however, suggest an association between the phenotype of the B-cell compartment and the initial omalizumab dosage scheme needed. Importantly, we provide indications that the treatment associated with a phenotype switches towards Th1 in part of the total T-cell compartment. These explorative results improve our understanding on the immune responses underlying OT facilitated OIT, knowledge that can be used to design future clinical trials on OT facilitated allergy treatment.

5 | ETHICS APPROVAL

The study was approved by the ethics committee in Stockholm: 2013/827-31/3 and the Swedish Drug Agency: 5.1-2013-46183 and registered at EudraCT: 2012-005625-78, ClinicalTrails.gov: NCT02402231. Written informed consent was obtained from all patients or their caregivers (of <18 years old) before inclusion in the study.

CONFLICT OF INTERESTS
The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS
AN, CN and JB conceptualized and performed the clinical trial, and CCQ was involved in the material collection. MvdH, ESE, AN and CN conceptualized the immunological investigation. MvdH performed all laboratory experiments and together with AN, CN and ESE analysed the data. All authors wrote and critically revised the manuscript.

DATA AVAILABILITY STATEMENT
Data is available on request.

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SUPPORTING INFORMATION

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