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Prophylactic Peanut Allergen Ara h 6 Sublingual Immunotherapy Drives Expansion of FoxP3$^+$Helios$^-$ Regulatory T Cells in the Absence of Allergen-Specific IgA

Jeppe Madura Larsen[1,2] | Emma Alberte Lundsgaard[1] | Niels Banhos Danneskiold-Samsøe[3] | Si Brask Sonne[3] | Neil Marcus Rigby[4] | Alan Robert Mackie[4] | Karsten Kristiansen[3] | Katrine Lindholm Bøgh[1]

[1]National Food Institute, Technical University of Denmark, Kgs. Lyngby, Denmark | [2]Department of Technology, University College Copenhagen, Copenhagen, Denmark | [3]Laboratory of Integrative Biomedicine, Department of Biology, University of Copenhagen, Copenhagen, Denmark | [4]School of Food Science & Nutrition, University of Leeds, Leeds, UK

Correspondence: Jeppe Madura Larsen (jmla@kp.dk)

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ABSTRACT

Insights into the underlying immunological mechanisms of prophylactic sublingual immunotherapy (SLIT) may support the development of new strategies for improved prevention and treatment of food allergy. Here, we investigated the humoral, regulatory and sublingual tissue immune response to prophylactic SLIT administration of a single purified peanut allergen in Brown Norway (BN) rats. BN rats received daily sublingual administration of peanut allergen Ara h 6 for three weeks. Suppression of sensitisation was evaluated by subsequent intraperitoneal administration of Ara h 6. Ara h 6-specific IgE, IgA, IgG1 and IgG2a-c levels were measured in serum. The frequency of regulatory T (Treg) cells was analysed using flow cytometry. The sublingual tissue response to Ara h 6 was analysed by transcriptional profiling using mRNA-sequencing. Ara h 6 SLIT protected rats from subsequent sensitisation without inducing a detectable humoral immune response (Ara h 6-specific IgE, IgA, IgG1 and IgG2a-c) in serum. SLIT furthermore promoted the relative expansion of induced Helios$^-$ Treg cells within the conventional CD4$^+$CD25$^+$FoxP3$^+$ Treg population in sublingual draining lymph nodes and blood. In conclusion, prophylactic Ara h 6 SLIT drives the relative expansion of induced Helios$^-$ Treg cells in the absence of Ara h 6-specific IgA highlighting a potential novel IgA-independent Treg-related immune response at the sublingual mucosal site.

1 | Introduction

Food allergy is a growing health concern affecting up to 8% of children and 4% of adults [1, 2]. Individuals and relatives living with food allergies experience a lower quality of life due to the perpetual risk of potential severe allergic reactions upon accidental exposure to the offending food [3]. Allergen-specific immunotherapy (AIT) is emerging as an avenue for the treatment of food allergy leading to desensitisation with higher thresholds for reaction to the culprit food [4, 5]. Food allergy AIT involves the administration of a low dose of food (whole food or isolated food allergens), typically via the oral route (oral immunotherapy, OIT). The treatment dose is gradually increased until a maintenance level is reached, at which point the dose is maintained for up to several years. The first AIT drug for food allergy was approved in 2020 [6]. This drug

Jeppe Madura Larsen and Emma Alberte Lundsgaard share the first authorship.

is a standardised preparation of peanut powder approved for OIT of peanut allergy. Although OIT for food allergy shows therapeutic efficacy in terms of desensitisation, treatment is associated with a high frequency of adverse reactions, including gastrointestinal and systemic allergic reactions [7]. Thus, there is a need to develop more effective and safe therapies for food allergies. Sublingual immunotherapy (SLIT) is well-established and has been used in the treatment of respiratory allergies for decades [8]. However, SLIT remains less explored in food allergy. A study directly comparing OIT and SLIT for the treatment of peanut allergy reported that SLIT showed a lower level of desensitisation but also a lower frequency of adverse reactions compared to OIT [9]. These findings are in line with the general notion that SLIT shows lower efficacy but increased safety due to the lower frequency of severe adverse reactions compared to OIT [10]. Thus, there is a need to develop new strategies to improve the efficacy of SLIT. Interestingly, recent studies indicate that prophylactic SLIT for respiratory allergies can modify disease trajectories by preventing the development of allergic sensitisation and asthma [11, 12]. These findings are in agreement with studies reporting that early introduction of peanut prevents the development of peanut allergy in high-risk infants [13]. More insights into underlying mechanisms of prophylactic administration of allergens may advance our understanding and ability to develop new immunotherapeutic strategies for improved efficacy. In the present study, we investigated the underlying humoral and regulatory immune responses of prophylactic SLIT using a single purified peanut allergen (Ara h 6) in a Brown Norway (BN) rat model of peanut allergy prophylaxis. Furthermore, the in vivo sublingual (SL) tissue response to the Ara h 6 allergen was investigated using genome-wide transcriptional profiling.

2 | Methods

2.1 | Peanut Protein Extract and Ara h 6 Preparation

Peanut protein extract (PPE) was prepared from whole raw defatted peanut flour and Ara h 6 was purified from PPE, as previously described [14].

2.2 | Brown Norway Rats

BN rats were from the in-house breeding colony at the National Food Institute, DTU. The rats were of >F10 generation kept on an allergen-free rice-based diet free from proteins with potential cross-reactivity to peanut allergens (e.g., legumes), as previously described [14, 15]. Ethical approval was provided by the Danish Animal Experiments Inspectorate (authorisation no. 2020-15-0201-00500-C1). Experiments were overseen by the National Food Institute's in-house Animal Welfare Committee for animal care and use.

2.3 | Experimental Design

BN rats ($n = 8$/group; 4/sex; age 9–11 weeks) were sublingually administered (SLIT) with 0 (two groups), 1, 10 or 100 (two groups) μg of Ara h 6 in 20 μL PBS once daily for 3 weeks (Day 0–20; see Figure 1A). Rats were restrained by the neck in a vertical position to suppress swallowing reflexes and allow access to the sublingual cavity, in which the SLIT solution was administered and allowed for absorption for 30 s before enabling the rats to swallow. One week after completed SLIT regimen, four groups of rats (0, 1, 10 and 100 μg of Ara h 6 groups; $n = 8$/group) were intraperitoneally (IP) immunised with 50 μg of Ara h 6 in 0.5 mL PBS once a week for 4 weeks (Day 28, 35, 42 and 49) to assess protection from sensitisation. Blood samples were collected by SL vein puncture on Day 0, 21, 28, 35, 42 and 49 (only rats with IP-immunisations following SLIT). An ear-swelling test (EST) using intradermal injections of 3 μg Ara h 6 or 10 μg PPE was performed on Day 54, as previously described [16]. On Day 56, rats received an oral gavage of 100 mg PPE in 1 mL PBS to assess immediate reactions and protein uptake in the gut. After 10 min, rats were sacrificed, and tissues were collected for further analysis. On Day 21, two SLIT-only subgroups of rats (0 or 100 μg of Ara h 6 SLIT; $n = 8$/group), which did not receive any IP immunisations or SL vein puncture blood sampling, were sacrificed for flow cytometry and SL tissue transcriptional analysis. Please refer to the Supporting Information for detailed information on procedures, sample collection and processing.

2.4 | ELISAs

Serum antibody titers were analysed by ELISA. Briefly, Ara h 6-specific IgA, IgG1, IgG2a, IgG2b and IgG2c levels were determined by indirect ELISA using horse radish peroxidase (HRP)-coupled anti-Ig antibodies. Ara h 6-specific IgE levels were determined by IgE-capture ELISA and digoxigenin (DIG)-coupled Ara h 6, as well as anti-DIG HRP-coupled antibodies for detection. Serum samples were 2-fold serially diluted starting at a 1:8 (v/v) dilution. For each plate, positive and negative control sera were included to evaluate and ensure consistent assay performance. Please refer to the Supporting Information for details on the ELISA assays.

2.5 | Serum and Intestinal Protein Uptake

Total protein from small intestine epithelium (EPI), lamina propria (LP) and Peyer's patches (PP) was extracted as described in the Supporting Information. Protein uptake following oral challenge with PPE was measured as Ara h 3 protein content in serum and intestinal tissue extracts using an Ara h 3 ELISA kit according to the manufacturer's recommendations (EPC-AH3-5, Indoor Biotechnologies, Charlottesville, VA, US). Ara h 3 was chosen as a marker for intestinal protein uptake as it is unaffected by potential immune responses developed against Ara h 6 and thus antibodies that may interfere with its detection using ELISA.

2.6 | Flow Cytometry

The relative frequencies of $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) and $CD4^+CD25^+FoxP3^+Helios^-$ induced Tregs (iTregs) were determined in SL draining lymph nodes

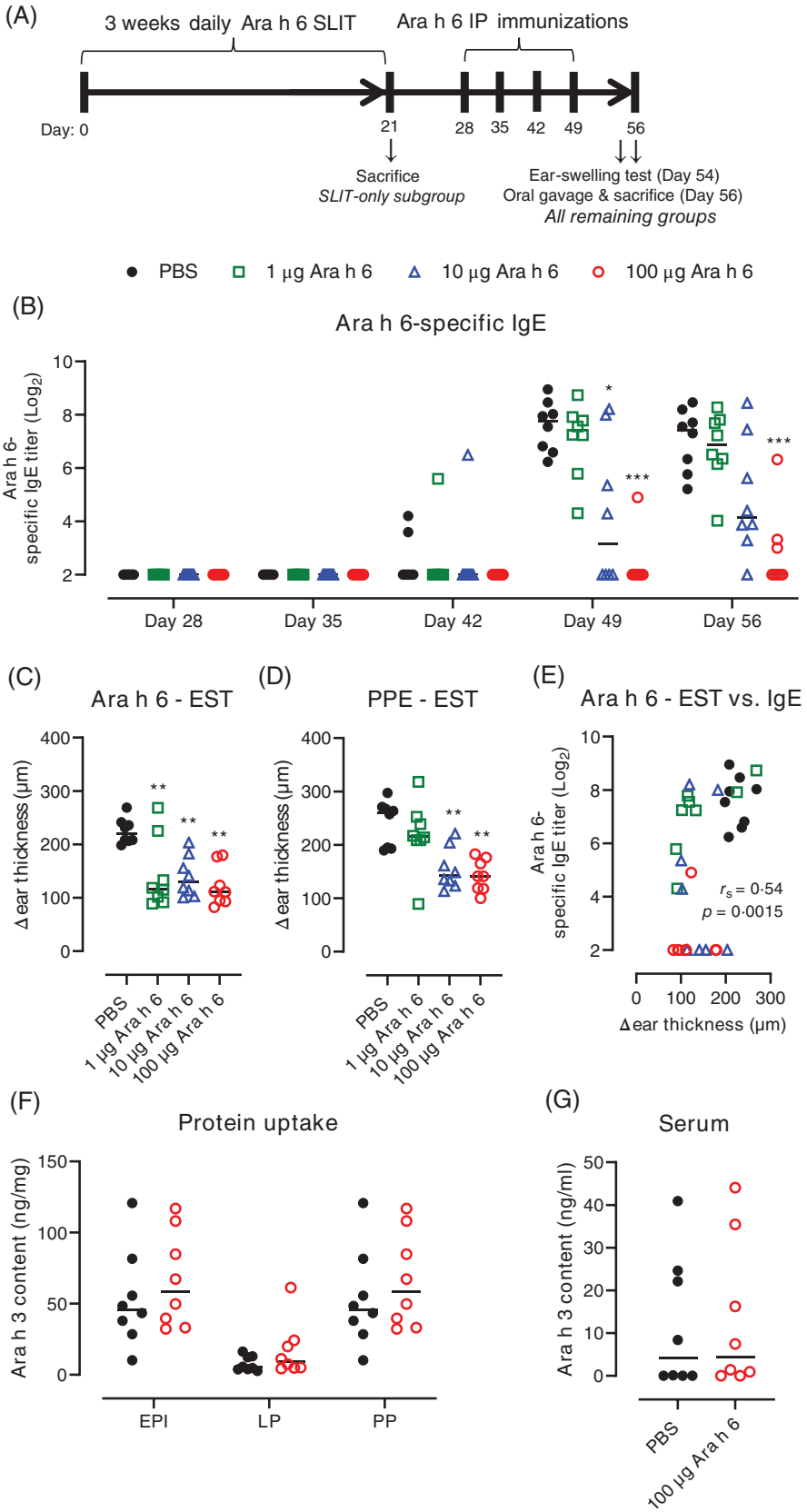

FIGURE 1 | Sensitisation, clinical reactivity and protein uptake. Animal experimental design (A). Ara h 6-specific IgE levels in serum (B). Ear swelling test (EST) response to intradermal injections of Ara h 6 (C) or peanut protein extract (PPE) on Day 54 (D). Correlation between EST response to Ara h 6 and Ara h 6-specific IgE levels (E). Protein uptake measured as Ara h 3 levels in small intestinal compartments (F) and serum (G) following oral gavage of PPE and sacrifice on Day 56. $n = 8$/group. EPI, epithelium; IP, intraperitoneal; LP, lamina propria; PP, Peyer's patches; SLIT, sublingual immunotherapy. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

(SL-LN), blood, mesenteric lymph nodes (mLN), PP, and small intestine tissue with PPs removed (SI) by flow cytometry on Day 21 (SLIT only) or Day 59 (SLIT and subsequent IP immunisations). Briefly, single cell preparations were surface stained by anti-CD45/APC-eF780 (clone OX-1, Invitrogen, Waltham, MA, US), anti-CD3/PerCp-eF710 (clone eBioG4.18, Invitrogen), anti-B220/PE (clone HIS24, Invitrogen), anti-CD4/PE-Cy7 (clone OX-35, BD Bioscience, San Jose, CA, US) and anti-CD25/BV421 (OX-39, BD Bioscience) antibodies. Cells were permeabilised and stained intracellularly by anti-FoxP3/FITC (clone FJK-16 s, Invitrogen) and anti-Helios/AF647 (clone 22F6, BD Bioscience) antibodies. Samples were analysed using a BD Fortessa flow cytometer. Please refer to the Supporting Information for details on tissue processing and staining procedures. The gating strategy and representative samples from each tissue are shown in the Supporting Information (Figure S1).

2.7 | Gene Expression Profiling of Sublingual Tissue

The SL tissue comprised the mucosa and submucosa covering the muscle on the floor of the SL cavity. The tissue was snap-frozen in liquid nitrogen and stored at −80°C until analysis. Total RNA was isolated from the tissue, and bulk RNA-seq was performed using a 3′-end sequencing methodology (BRB-seq), as previously described [17]. RNA-seq libraries were sequenced on an Illumina NextSeq500 system (San Diego, CA, US) using 26 cycles for read 1 and 56 cycles for read 2. Subsequent sample demultiplexing, genome reference alignment (mRatBN7.2) and counting of unique molecular identifiers (UMI) were performed using STARsolo [18]. UMI read counts were downsampled to 200 000 reads per sample for samples above 200 000 reads. Following downsampling the median library size was 199 996 (IQR, 199954–200 014) and 164 762 (IRQ, 122819–199979) for Day 21 and Day 56 samples, respectively. Differentially expressed genes (DEGs) were deduced from UMIs and controlled for gender effects, using the R package edgeR. Significance levels were adjusted for multiple testing by False Discovery Rate (FDR at 5%). Please refer to the Supporting Information for details on the preparation of the sequencing library and analysis of gene expression data.

2.8 | Statistics

Gene expression data were analysed as described in the paragraph above. Remaining data analysis and visualisation were performed in GraphPad Prism version 9.3 (San Diego, CA, US). Differences in antibody titers and EST results were analysed using the Kruskal–Wallis test comparing each group to the PBS control group. Correction for multiple comparisons was performed by controlling the FDR using the method of Benjamini, Krieger and Yekutieli. Dose–response of Ara h 6 versus IgE levels or EST response, and correlation between IgE level and the EST response were examined by Spearman's rank-order correlation test. Flow cytometry results were analysed by Welch's *t*-test and Pearson's correlation test. The levels of statistically significant differences are indicated in figures using asterisk(s) ($*p<0.05$, $**p<0.01$, $***p<0.001$, and $****p<0.0001$).

3 | Results

3.1 | Prophylactic SLIT Suppresses Sensitisation, IgE Levels and Clinical Reactivity to Ara h 6 in a Dose-Dependent Manner

BN rats received prophylactic SLIT administrations containing 1, 10 or 100 μg of purified Ara h 6 peanut allergen in PBS, or PBS alone, daily for 3 weeks (Day 0–20). Subsequently, the rats received four IP injections with Ara h 6 at one-week intervals (Day 28, 35, 42 and 49) to assess the protective effect of the prophylactic SLIT (Figure 1A). All control animals receiving PBS SLIT became sensitised to Ara h 6 after 3 IP immunisations (Figure 1B, Day 49). Ara h 6 SLIT suppressed sensitisation to Ara h 6 and reduced Ara h 6-specific IgE levels in a dose-dependent manner (Figure 1B; Ara h 6 SLIT dose versus Ara h 6-specific IgE: $r_s=-0.73$, $p<0.0001$ (Day 49), $r_s=-0.70$, $p<0.0001$ (Day 56)). Ara h 6 SLIT likewise suppressed the development of clinical reactivity to intradermal injections with Ara h 6 (Figure 1C) and PPE (Figure 1D) in a dose-dependent manner (Ara h 6 SLIT dose versus Ara h 6 EST response: $r_s=-0.59$, $p=0.0003$; Ara h 6 SLIT dose versus PPE EST response: $r_s=-0.68$, $p<0.0001$). Furthermore, the clinical EST response to Ara h 6 correlated with levels of Ara h 6-specific IgE at Day 56 (Figure 1E). No differences in the intestinal protein uptake were observed from the measurements of Ara h 3 content in the small intestine EPI, LP, PP or in serum following oral challenge with PPE (Figure 1F,G). Collectively, prophylactic SLIT using purified Ara h 6 protected rats from subsequent sensitisation and clinical allergy induced by IP immunisation with Ara h 6.

3.2 | Prophylactic SLIT Does Not Induce a Detectable Ara h-6-Specific IgA, IgG1 or IgG2a-c Immune Response

Ara h 6 SLIT-mediated protection from sensitisation may occur due to the development of Ig-related immune responses other than IgE, for example the development of mucosa-associated IgA responses [19]. To examine this, Ara h 6-specific IgA, IgG1 and IgG2a-c levels were analysed in serum after SLIT administration (Day 28) and following the subsequent Ara h 6 IP immunisations (Day 56), respectively. No Ara h 6-specific IgA, IgG1 or IgG2a-c were detected following the Ara h 6 SLIT administration (Figure 2A–E), indicating that the SLIT did not induce a specific Ig-related immune response. On the contrary, the subsequent Ara h 6 IP immunisations induced Ara h 6-specific IgA, IgG1 and IgG2a-c (Figure 2A–E), demonstrating the development and detection of Ig-related immune responses. Ara h 6 SLIT in animals subsequently receiving IP immunisations was associated with decreased Ara h 6-specific IgA, IgG1 and IgG2a-c levels, indicating that SLIT mediated a general suppression of Ara h 6-induced humoral immune responses. Total IgA levels in serum and faecal samples remained unaffected by Ara h 6 SLIT administration (Figure 3A,B), whereas Ara h 6-specific IgA was mainly detectable in faecal samples following IP immunisation regimen (Figure 3C). These findings indicate that Ara h 6 SLIT had no or limited effect on the overall IgA-related immune response

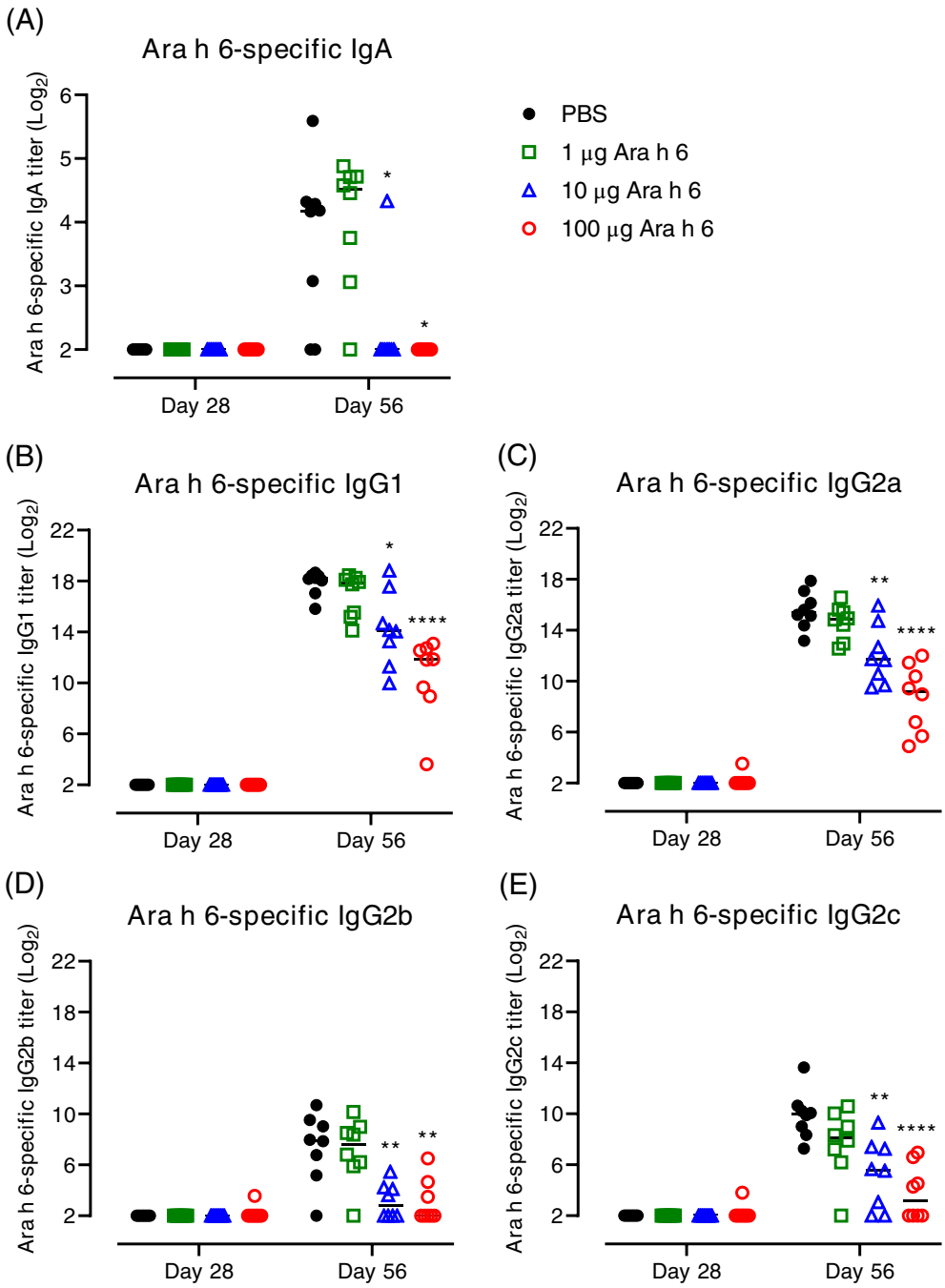

FIGURE 2 | Humoral immune responses. Ara h 6-specific IgA (A), IgG1 (B), IgG2a (C), IgG2b (D), and IgG2c (E) in serum following prophylactic Ara h 6 SLIT (Day 28) and subsequent intraperitoneal immunisations (Day 56). $n = 8$/group. *$p < 0.05$, **$p < 0.01$, and ****$p < 0.0001$.

and support that effective prophylactic SLIT does not necessarily drive mucosa-associated IgA responses.

3.3 | Prophylactic SLIT Drives the Relative Expansion of Helios-Negative Regulatory T Cells

Tregs play a central role in the maintenance of oral tolerance [20]. Therefore, the Treg compartment was analysed by flow cytometry of cells obtained from SL-LN, blood, mLN, PP and SI following SLIT (Day 21), as well as after subsequent Ara h 6 IP immunisations and oral gavage of PPE (Day 56). Ara h 6 SLIT was found to promote a relative expansion of induced Helios$^-$ iTregs within the conventional CD4$^+$CD25$^+$FoxP3$^+$ Treg population in the SL-LN on Day 21 (Figure 4A). Furthermore, SLIT promoted a systemic relative expansion of iTregs in the blood (Figure 4A), which correlated with the frequencies of iTregs in the SL-LN ($r_p = 0.63$, $p = 0.0088$). No relative iTreg expansion was detected in the intestinal mLN, PP or SI compartments on Day 21 (Figure 4A). The SLIT-mediated relative expansion of iTregs in SL-LN and blood was not associated with an overall change in the relative frequencies of conventional CD25$^+$FoxP3$^+$ Tregs within the CD4$^+$ helper T cell population (Figure S2). On Day 56, after the oral gavage with PPE, the Ara h 6 SLIT-induced relative expansion of iTregs was still indicated in the blood (Figure 4B). Interestingly, increased frequencies of

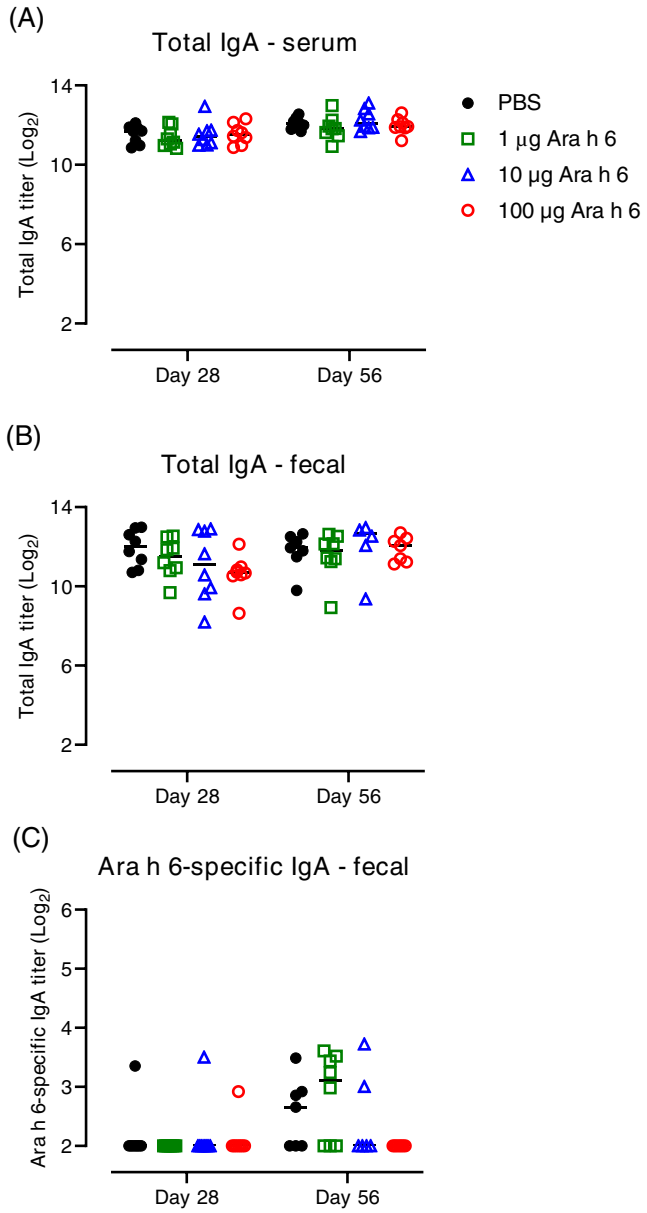

FIGURE 3 | IgA immune responses. Total IgA in serum (A) and faecal samples (B), and Ara h 6-specific IgA in faecal samples (C) following prophylactic Ara h 6 SLIT (Day 28) and subsequent intraperitoneal immunisations (Day 56). $n = 8$/group.

iTregs within the conventional $CD4^+CD25^+FoxP3^+$ Treg population were now also observed in the SI tissue (Figure 4B).

3.4 | Prophylactic SLIT Induces no Major Changes in Gene Expression Within the Sublingual Mucosa

The response of SL mucosal tissue to allergens during SLIT remains unknown. Here, we examined the transcriptional response of the SL tissue immediately following Ara h 6 SLIT (Day 21) and after subsequent IP immunisations (Day 56) using mRNA-seq. Even though Ara h 6 SLIT affected the frequencies of iTregs within the conventional Treg population, no significant differentially expressed genes (DEGs) could be identified within the SL mucosa after correction for multiple comparisons. We conclude that Ara h 6 SLIT does not result in major transcriptional changes in the SL mucosa. Please refer to the Supporting Information for mRNA-seq. results summarising the genes expressed in the SL tissue samples and the accompanying statistics when comparing PBS and Ara h 6 groups at Day 21 and 56, respectively.

4 | Discussion

More insights into the underlying mechanisms of SLIT may support the development of novel or improved strategies for prevention and treatment of allergic diseases. Here, we investigated the humoral, regulatory and SL tissue immune responses to prophylactic SLIT administration of a single peanut allergen Ara h 6 in BN rats. Prophylactic SLIT administration of Ara h 6 prevented sensitisation and suppressed the development of Ara h 6-specific IgE in response to IP immunisations with Ara h 6. Interestingly, prophylactic SLIT was not found to induce a detectable humoral immune response (Ara h 6-specific IgA, IgG1 or IgG2a-c). These findings suggest that mechanisms other than the development of IgE blocking antibodies play a role in suppressing the allergic response to Ara h 6 IP immunisations. IP immunisations with Ara h 6 were found to induce Ara h 6-specific IgA, IgG1 or IgG2a-c with lower Ig titre levels in rats having received Ara h 6 SLIT. This suppression of specific Ig levels may be explained by clonal deletion of Ara h 6-specific B cells during the SLIT regiment and/or the induction of immune regulatory mechanisms suppressing Ig production. The absence of detectable humoral immune responses after prophylactic SLIT is contrary to observations during therapeutic SLIT, where specific antibodies of different isotypes are detected [21]. This is likely a consequence of sensitisation being associated with the development of other Ig isotypes in addition to IgE (i.e., in therapeutic SLIT, allergen-specific antibodies of several isotypes are present before treatment initiation). This suggests that different immune regulatory mechanisms could be in play for prophylactic and therapeutic SLIT, or that Ig-unrelated mechanisms play a pivotal role in SLIT treatment efficacy. Indeed, studies have shown various effects of therapeutic SLIT on IgE levels, including suppression, upregulation or no effect even when therapeutic efficacy has been reached [21]. This variation in Ig levels may occur due to different treatment regiments, doses and/or duration of the clinical studies [21].

Treg cells play a central role in the maintenance of tolerance [20]. We investigated the induction of conventional $CD4^+CD25^+FoxP3^+$ Tregs and induced $CD4^+CD25^+FoxP3^+Helios^-$ Tregs after the prophylactic SLIT regiment (Day 21) as well as following the IP immunisations (Day 56). Prophylactic SLIT had little effect on the frequency of conventional $CD25^+FoxP3^+$ Tregs within the $CD4^+$ helper T cell population, which is in line with a previous study of prophylactic SLIT using house dust mite in mice [22]. However, prophylactic SLIT was found to drive a relative expansion of $Helios^-$ Tregs within the conventional $CD4^+CD25^+FoxP3^+$ Treg population in SL-LN and blood, but not small intestine compartments. These findings are in line with recent studies reporting a fixed population size of conventional $CD25^+FoxP3^+$ Tregs, in which the balance between thymus-derived and induced Tregs is maintained by different signals (CD28, ICOS and/or MHC-II) in response to environmental exposures [23]. Accordingly, we

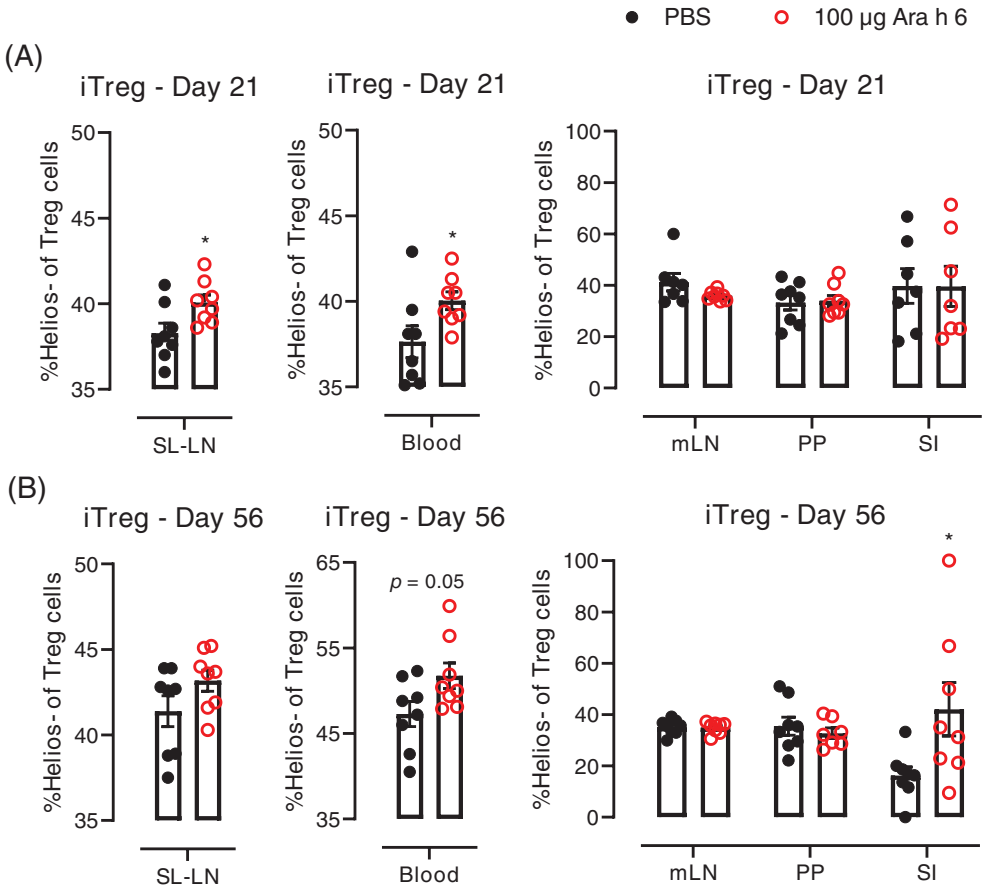

FIGURE 4 | Regulatory T cell responses. The frequency of Helios$^-$ induced regulatory T cells (iTreg) in sublingual draining lymph nodes (SL-LN), blood and small intestine compartments following the Ara h 6 SLIT regiment (Day 21) (A) and the subsequent intraperitoneal immunisation regiment (Day 56) (B). $n = 8$/group. mLN, mesenteric Lymph Nodes; PP, Peyer's patches; SI, small intestine. $n = 8$/group. *$p < 0.05$.

speculate that Ara h 6-specific Treg cells are induced and expanded within the conventional Treg cell population, but this needs confirmation by flow cytometry using MHC tetramer staining specific for Ara h 6 epitopes in future studies. It is possible that the relative expansion of iTregs may be due to SLIT-induced changes in innate mechanisms regulating Treg homeostasis independent of antigen specificity. Nevertheless, our findings are in line with observations in clinical trials in humans, indicating that peanut OIT induces Helios$^-$ Treg cells [24]. Following the IP immunisation regiment and PPE oral gavage administration the induced Helios$^-$ iTreg cell frequency was higher within the conventional CD4$^+$CD25$^+$FoxP3$^+$ Treg populations in the small intestine of rats having received prophylactic Ara h 6 SLIT compared to the PBS control group. These findings may suggest that the SLIT-induced Treg cells home to the small intestine to prevent an allergic reaction in response to oral administration of peanut. However, it cannot be ruled out that the observation may be driven by the response to Ara h 6 IP immunisations given to all groups sacrificed on Day 56. Interestingly, Treg cells have traditionally been linked to IgA-related immune responses, particularly in control of the gut microbiota [25]. However, in the present study, the relative expansion of Helios$^-$ iTregs was not accompanied by the production of allergen-specific IgA in response to Ara h 6 via the SL mucosa. This finding highlights that distinct Treg-related immune responses may be at play at different mucosal sites, which may again be affected by the nature or origin of the specific antigen (e.g., pathogens or foods).

The role of allergen-specific IgA in the natural response to food and in protection from food allergy remains largely elusive due to inconsistent observations in the relationship between IgA levels and allergy in humans [26]. Studies of feeding experiments in mice (ad libitum or oral gavage) reported the induction of allergen-specific IgA, however the levels were generally lower compared to mice sensitised to the food, and the role of this food-induced IgA in protection from food allergy remains unknown [27, 28]. Contrastingly, allergen-specific IgA is known to be affected by allergen-specific immunotherapy in humans and seems to play a protective role similar to that of allergen-specific IgG4 [29], possibly via blocking mast cell and basophil function [30]. It has been proposed that the induction of allergen-specific IgA may be site- and timing-specific [26]. Our findings support this notion, as we observed no induction of allergen-specific IgA via the SL mucosa in contrast to the reports of allergen-specific IgA induction during oral feeding (ad libitum or oral gavage) [27, 28]. However, a comparative study of site-specific IgA induction and function in response to food remains to be conducted. Interestingly, SLIT (10 and 100 µg Ara h 6) seems to suppress allergen-specific IgA levels to a greater degree than allergen-specific IgE levels indicating that SLIT potently suppresses the possibility of developing an IgA response in a specific manner. It is unknown if this occurs due to deletion or suppression of B cells recognising specific "IgA-prone" epitopes. Nevertheless, interestingly studies indicate that IgA and IgE epitopes are different in allergy [29] supporting this notion.

The SL tissue response to allergen during SLIT remains largely elusive. Here, we analysed the genome-wide gene expression profile in SL tissue by mRNA-sequencing following the SLIT regiment (Day 21) and the subsequent IP immunisation regimen (Day 56), respectively. We were unable to detect a significant SLIT-induced change in SL mucosal gene expression, as no specific DEGs were found after correcting for multiple testing. It should be noted that increasing sequencing depth may allow the detection and characterisation of more subtle changes in gene expression within the SL tissue in future studies. We propose that future studies compare the effect of SLIT administrations of low and high allergenic proteins on the SL tissue response.

The present study exhibits some limitations, particularly as it is observational in nature. The exact causative mechanism protecting SLIT-treated rats from subsequent sensitisation was not identified. Adoptive transfer experiments could be used to determine if the regulatory T cell population is the main effector cells mediating tolerance. Interestingly, adoptive transfer experiments have demonstrated phenotypic and functional differences in regulatory T cells induced by allergen-specific immunotherapy via different routes [31], highlighting the need to further dissect tolerogenic mechanisms at different sites in the body. Despite this limitation, the absence of allergen-specific IgE, IgA, IgG1 and IgG2a-c following SLIT strongly indicates that the tolerogenic mechanism is not mediated by the development of blocking antibodies, although we cannot rule out undetectable antibody levels or a role for allergen-specific IgM or IgD. It may be a further limitation that we used purified Ara h 6 for prophylactic SLIT due to the absence of other immune stimulatory allergens and natural adjuvants found in peanut [32, 33] that could potentially drive specific antibody responses. Thus, our findings cannot be generalised to the context of exposure to complex foods.

Author Contributions

Conceptualisation: K.L.B. and J.M.L. Funding acquisition: K.L.B. Investigation and data curation: E.A.L., J.M.L., N.B.D.-S. and S.B.S. Methodology: K.L.B., J.M.L., E.A.L. and K.K. Visualisation: J.M.L. and E.A.L. Formal Analysis: J.M.L. and E.A.L. Resources: N.M.R. and A.M. Supervision: K.L.B., J.M.L. and K.K. writing – original draft: J.M.L. and E.A.L. writing – review and editing: E.A.L and K.L.B. All authors made substantial intellectual contributions to the study, reviewed the manuscript critically and approved the final version of the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data is available upon reasonable request made to the corresponding author.

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Supporting Information

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