AN INVESTIGATION OF THE IMPACT OF SUBLINGUAL IMMUNOTHERAPY IN EXPERIMENTAL MODELS OF FOOD ALLERGY AND ANAPHYLAXIS

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TITLE: An investigation of the impact of sublingual immunotherapy in experimental models of food allergy and anaphylaxis

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Lay Abstract

Food allergy is a potentially life-threatening disease which is primarily mediated by IgE antibodies. Strict allergen avoidance and use of rescue epinephrine upon accidental allergen exposure remain the standard of care. Oral immunotherapy, where individuals ingest small amounts of allergen, is currently the experimental treatment of reference to induce clinical tolerance; however, it is accompanied by a significant rate of adverse reactions. In contrast, sublingual immunotherapy (SLIT), which is less efficacious, upholds a superior safety profile. The primary objective of this thesis was to investigate the impact of SLIT in inducing clinical and immunological changes in murine models of food allergy. We demonstrated that when administered prophylactically, SLIT prevents mice from undergoing anaphylaxis. When administered to sensitized mice in a pre-allergic state, SLIT was protective against severe clinical reactivity after challenge. In conclusion, the work presented here establishes a useful platform to investigate the mechanisms underlying SLIT-mediated protection.

Abstract

Food allergy is a potentially life-threatening disease affecting up to 10% of individuals in Western countries. Clinical reactivity to food allergens is primarily mediated by immunoglobulin (Ig) E, with symptoms ranging from mild urticaria to anaphylaxis. Currently, food allergy remains a disease without a cure. Oral immunotherapy (OIT), which involves consuming small amounts of allergen, remains an experimental treatment in Canada, although has been approved by the Food and Drug Administration (FDA) in the United States for treatment of peanut allergy. While efficacious to induce desensitization, OIT is accompanied by a significant rate of adverse effects. Sublingual immunotherapy (SLIT) is a novel route of treatment for food allergy, where small amounts of allergen are placed under the tongue and held for 2-3 minutes. In contrast to OIT, SLIT offers not only treatment efficacy but also promises an excellent safety profile.

The first objective of this thesis was to first develop a SLIT regimen in murine models of food allergy where sensitization is carried out either epicutaneously or intragastrically. Secondly, we investigated the efficacy of SLIT in modulating the clinical and humoral responses in prophylactic and semi-therapeutic settings. In the prophylactic setting, where SLIT was administered prior to sensitizing allergen exposures, SLIT-treated mice were completely protected from allergic sensitization including absent production of serum ovalbumin-specific IgE. In the semi-therapeutic setting, where SLIT was administered to mice primed to develop food allergy, it produced a partial protection against food-induced clinical reactivity. This was associated with lower levels of IgE production in comparison to non-treated, allergic mice. Together, this work provides both an optimized SLIT

protocol, as well as evidence on the efficacy of SLIT in the treatment of food allergy in murine models. These findings will aid future work investigating the cellular and molecular mechanisms underlying SLIT-induced protection.

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Abbreviations

α-Gal	Galactose-α-1,3-galactose
ANOVA	Analysis of Variance
BC	B Cell
Breg	B Regulatory Cell
CHILD	Canadian Health Infant Longitudinal Development
CT	Cholera Toxin
DBPC	Double-Blinded, Placebo-Controlled
ELISA	Enzyme-Linked Immunosorbent Assay
EPIT	Epicutaneous immunotherapy
FDA	U.S. Food and Drug Administration
GI	Gastrointestinal
Ig	Immunoglobulin
i.g.	Intragastric
IFN	Interferon
MBC	Memory B Cells
ODN	Oligodeoxynucleotides
OFC	Oral Food Challenge
OIT	Oral Immunotherapy
OVA	Ovalbumin
PBS	Phosphate Buffered Saline
PC	Plasma Cell

PN	Peanut
s.c.	Subcutaneous
SCIT	Subcutaneous Immunotherapy
SEM	Standard Error of Mean
SLIT	Sublingual Immunotherapy
SPINK5	Serine Peptidase Inhibitor Kazal Type 5
SPT	Skin Prick Test
TC	T cells
Tfh	T Follicular Helper
Treg	Regulatory T Cell

Chapter 1: Background

1.1 Food Allergy

Food allergy is characterized by a pathogenic immune response against innocuous food antigens. Currently, challenge-diagnosed food allergy affects up to 10% of individuals in Western countries [1–4]. In Canada, the self-reported prevalence of food allergy is estimated at 6.67%, with peanut (PN) allergy affecting approximately 1.5% of individuals [5]. However, another study found that physician-reported food allergy occurs in 2.5% of Canadians, significantly lower than the self-reported prevalence [6]. Reactivity against food allergens ranges in severity as patients may experience reactions such as urticaria (skin rash), abdominal cramps, diarrhea, or coughing/trouble breathing, that are often restricted to a localized site [7]. In severe cases, patients may experience a systemic response known as anaphylaxis. This response affects multiple organs and can be life-threatening if not treated immediately with rescue medication, such as epinephrine [7].

Some allergies, such as those to milk and egg are often outgrown. [1]. For example, egg allergy is commonly acquired during the first year of life [8], and one retrospective study of 881 patients found that the median age of outgrowing egg allergy was 9 years old [9]. Similarly, milk allergy also presents during the first year of life, with the median age for outgrowing being 10 years of age [8,10]. In contrast, allergies to tree nuts, PN, fish or shellfish are generally lifelong with less than 20% of patients outgrowing their allergy by 20 years of age [10]. Despite the persistence of these allergies, no curative treatments exist, and the current standard of care is

strict allergen avoidance. However, cases of accidental exposures occur with an incidence rate of 14.3% annually, especially on cultural holidays, such as Easter and Halloween, when treats are common [11,12]. Food allergies further present a significant impact on the quality of life (QoL) of allergic individuals and their families [13–16]. QoL questionnaires administered to allergic individuals consistently report a worse QoL in comparison to individuals type 1 diabetes mellitus or rheumatological diseases [13–16]. Additionally, family members and or caregivers of the allergic individual report a similar impairment in QoL [13]. This is largely attributed to the possibility of undergoing anaphylaxis upon accidental exposures. In combination with the need to maintain allergen-free safe environments, this often increases anxiety amongst the allergic patients and their families [13–16]. Overall, the economic cost for food allergy was estimated at USD24.8 billion annually in the United States, indicating a substantial burden for both the healthcare system and the families of allergic individuals [17].

1.2 Mechanisms of food-induced reactivity

Allergic reactions to innocuous food allergens are a type 1 hypersensitivity reaction that is primarily mediated by IgE [7]. Typically associated with TH2 immunity, allergen-specific IgE has a short half-life of 2 days in circulation in humans [18]. However, evidence in mice has shown that IgE can remain bound to its high-affinity receptor (FcεR1) on the surface of mast cells for up to 67.3 days (CI 46.5-88.1 days) [19]. Upon a secondary allergen exposure, food allergens can cross-link multiple FcεR1-bound IgE antibodies to induce the degranulation of mast cells

and basophils thus leading to the release of preformed granules as well as rapidly synthesized mediators, such as PAF [7]. These granules contain vasoactive mediators, such as histamine, which are responsible for the signs and symptoms of an allergic reaction [20,21]. Histamine for example, has been shown to bind receptors on endothelial cells to cause vasodilation and a decrease in blood pressure, whereas PAF seems to be responsible for the magnitude and duration of the anaphylactic response [20,21]. During the degranulation phase, other mediators such as heparin, serotonin, proteases, tryptases, chymases, prostaglandins and leukotrienes contribute to the anaphylactic reaction [20–22]. Together, they mediate a wide array of physiological effects including airway constriction, increased mucous production, itching, and diarrhea, amongst others [20–22].

Following the immediate allergic response, patients may experience a secondary reaction known as a late phase response [22,23]. This response is primarily mediated by T helper 2 (TH2) cells through the secretion of interleukins (IL) such as IL-4, IL-5, and IL-13. In combination with mediators released during the acute phase of the allergic reaction, TH2 cells can recruit various immune cells such as eosinophils and basophils, in turn causing a secondary rise in histamine after the initial allergic reaction [22,23]. The secondary late-phase response is typically not lethal.

1.3 Skin as a potential site of allergic sensitization

Traditionally, the gut was considered the primary route for allergic sensitization because the gastrointestinal (GI) tract is the main site of interaction with food

allergens. However, individuals typically present to the hospital upon their first known allergen exposure, which suggests a prior allergic sensitization event that is clinically silent [8,24]. Numerous factors have been identified as contributors to this silent allergic sensitization. It has been shown that an altered gut microbiome during the early years of life is associated with food sensitization [25]. The Canadian Health Infant Longitudinal Development (CHILD) study found that a low microbiota richness in combination with elevated enterobacteriacaea/bacteroidaceae ratio by 3 months of age is associated with early food sensitization [25]. Therefore, these data identify a critical period of immunological development where environmental factors may facilitate allergic sensitization. However, the immune system remains largely tolerant in early life, thus providing an opportunity for interventions to prevent allergic sensitization. One study assigned 640 infants with severe eczema and/or egg allergy to either consume or avoid ingestion of PN until 60 months of age [26]. Amongst this at-risk population, the prevalence of PN allergy at 60 months was 13.7% in the avoidance group and only 1.9% in the consumption groups for those with an initial negative skin-prick test. For individuals with an initial positive egg allergy skin-prick test, the avoidance group had a prevalence of 35.3% and the consumption group of 10.6% [26]. The study demonstrates that early oral introduction of PN dramatically decreases the frequency of food allergy amongst high-risk populations.

Allergen exposures can occur at any one of the physical barriers within the body. It has become increasingly clear that the skin is another key barrier through

which individuals may become sensitized against food allergens. The skin behaves differently than the GI tract, as the outmost epidermal layer serves as a barrier against the entry of any antigens. Although barrier disruption through injury may occur, the rapid release of inflammatory cytokines by structural cells and activation of resident immune cells prevents the onset of any infection. In 2003, a study examined a cohort of children with a history of PN allergy, confirmed by a double-blind PN challenge [27]. Analysis of cord blood showed no PN-specific IgE, indicating a lack of prenatal sensitization; however, a significant relationship was found between the onset of PN allergy and the use of PN oil for treatment of inflamed skin [27]. Approximately 91% of individuals, in comparison to 53% of atopic controls and 59% of normal controls, had been exposed to skin creams containing PN oils [27]. Therefore, it was proposed that exposure to PN proteins on inflamed skin could be an avenue through which allergic sensitization occurs. As the PN protein is largely innocuous, it is likely that alarmins released as a result of inflamed skin are responsible for this phenomenon.

Additional studies have found associations between the filaggrin gene and missense mutations in the serine peptidase inhibitor Kazal type 5 (SPINK5) gene and the risk for IgE-mediated food allergy [28,29]. Both genes are responsible for maintaining the integrity of the epithelial barrier. Therefore, it was hypothesized that a disrupted epithelial barrier could lead to further complications such as atopic dermatitis and allergic sensitization [28,29]. Genotyping of allergic and non-allergic individuals revealed that a loss-of-function in the filaggrin gene and missense

mutations in the SPINK5 gene are significantly associated with a risk of IgE-mediated food allergy [28,29]. In addition to genetic mutations, diseases such as atopic dermatitis are also associated with an increased risk of PN allergy. Ha *et al* found a dose-response relationship between the levels of PN-protein in household dust and PN-skin prick test results amongst patients with atopic dermatitis, with a higher PN-protein concentration attributing to an increase in severity of the skin prick test [30]. Together, these studies provide evidence to illustrate sensitization to food allergens through a disrupted epithelial barrier.

1.4 Persistence of allergy and the IgE recall response

It was traditionally thought that long-lived IgE^+ plasma cells (PC) were the reservoir of allergic humoral memory. PCs are terminally differentiated B cells (BC) that reside primarily in the bone marrow and are responsible for the production of antibodies. As seen in the anti-viral IgG response, the antibody half-life ranges from 50 years for varicella-zoster virus, to more than 200 years for measles and mumps [31]. Additionally, in a murine model of house dust mite allergy, repeated chronic allergen exposure for 15 months demonstrated the emergence of an IgE^+ PC population, capable of inducing anaphylaxis [32]. However, this notion has been inconsistent with observations in both human and murine studies of food allergy. Multiple studies have concluded that IgE titres often wean with allergen avoidance. For example, patients allergic to pollen demonstrate an increase in serum IgE during pollen season, which wean off with the conclusion of the pollen season [33]. For individuals allergic to a common fish parasite, *Anisakis* spp., strict allergen-

avoidance for at least 6-10 months resulted in a drastic decrease of both total and allergen-specific IgE titres [34]. When examining the entirety of the patient-specific follow-up periods, a median of 76% decrease in allergen-specific IgE titres was noted [34]. However, upon consumption of the fish allergen, IgE titres and clinical symptoms re-emerged [34]. This finding is consistent with a recent report of the decline in galactose-α-1,3-galactose (α-Gal)-specific IgE titres in patients who avoid tick bites [35]. α-Gal is an oligosaccharide of non-primate mammals, that is targeted by α-Gal-IgE within individuals allergic to red meat [35,36]. Interestingly, subjects with serum IgE antibodies against α-Gal often have a history of tick bites [36]. Therefore, an association was established between IgE sensitization against α-Gal, and a history of tick bites. Together these observations suggest that despite the decline in IgE antibodies with allergen avoidance, the capacity to regenerate IgE antibodies and subsequent clinical reactivity are long-lived. In a murine model of PN allergy, our lab demonstrated that allergen-specific IgE titres and IgE^+ PCs contract and are undetectable 3-6 months post-allergic sensitization, although, the capacity to regenerate IgE antibodies persisted for over 15 months post-allergic sensitization [19]. These findings suggest a role for memory (M) BCs in reconstituting the IgE^+ PC population and the persistence of IgE-mediated food allergy.

Upon antigen exposure, MBCs undergo rapid proliferation and differentiation into PCs [37]. In human food allergy, IgE^+ MBCs were thought to be the reservoir of IgE-secreting cells. However, it has been demonstrated that IgE^+ MBCs in humans are extremely rare, and perhaps even absent [38,39]. These findings

were derived from human peripheral blood mononuclear cells (PBMCs), so they do not exclude the possibility of tissue resident IgE^+ MBCs. Interestingly, current literature points to a subset of non-IgE^+ MBCs which contribute to long-lived IgE immunity. For context, IgE antibodies can derive from two distinct pathways: 1) a direct pathway, where a naïve IgM^+ BC undergoes class switch recombination directly to IgE, or 2) a sequential pathway, where BCs first switch to an intermediate subclass prior to class switching to IgE [40]. Analysis of IGH repertoires in human PBMCs identified clonal lineages of IgE BCs derived predominantly from IgG and partially from IgA-expressing BCs [40]. These findings are consistent in murine models of allergy [41–44]. These studies suggest that IgE^+ PCs primarily originate from a memory population of $IgG1^+$ BCs. In this regard, our lab demonstrated that $IgG1^+$ MBCs persist even after the decline of serum IgE and loss of clinical reactivity [19]. Furthermore, non-sensitizing allergen exposure up to 15 months post-sensitization generated IgE antibodies in serum [19]. In addition, in a helminth model of TH2 immunity, $IgG1^+$ MBCs and IL-4^+ $CD4^+$ memory T cells isolated from mice infected with *Nippostrongylus brasiliensis* (N.b.) could induce a N.b.-specific IgE response upon re-infection when adoptively transferred to $Rag1^{-/-}$ recipient mice [42]. Since the recipient mice lack the ability to generate lymphocytes, it can then be concluded that the IgE response was generated from the transferred $IgG1^+$ MBCs. Collectively, these studies spotlight long-lived $IgG1^+$ MBCs as the reservoir of the IgE allergic response in humans and mice.

Activation of $IgG1^+$ MBCs can often occur through accidental allergen exposures, which in turn, drive the emergence of IgE-secreting cells and thus the maintenance of food-specific IgE titres. This secondary response, the allergic recall response, is strictly dependent on T cells (TCs) [45,46]. Without the presence of $CD4^+$ TCs, the production of IgE antibodies and subsequent clinical reactivity does not occur [45,46]. It is important to note that naïve $CD4^+$ TCs, rather than antigen experienced $CD4^+$ TCs, are sufficient to propagate the recall response [45]. In vitro models suggest an avenue through how $CD4^+$ TCs help during the secondary allergic response. Here, PN allergic mice were first treated with anti-CD4 antibody or vehicle *in vivo* to deplete $CD4^+$ TCs [19]. Next, splenocytes were harvested, labelled with CFSE, and stimulated *ex vivo* with PN in the presence of blocking antibodies against IL-4, CD40 ligand (CD40L) and/or an isotype control [19]. In the context of MBCs, proliferation upon allergen stimulation was observed in a $CD4^+$ TC-dependent, IL-4- and CD40L-independent manner [19]. In contrast, proliferation of plasmablasts was impaired by the lack of both $CD4^+$ TCs and IL-4 [19]. Therefore, this data suggests the importance of $CD4^+$ TCs in the differentiation of MBCs into plasmablasts, dependent on IL-4 secretion.

IL-4 is a critical signalling molecule to initiate IgE class switch recombination [47,48]. IL-4 interacts with one of two heterodimeric receptors, type I and type II. The type I receptor incorporates the IL-4Rα chain with the common γ chain, whereas the type II receptor combines the IL-4Rα chain with the IL-13Rα1 chain [49,50]. With respect to the source of IL-4, studies utilizing genetically

engineered IL-4 reporter mice, found that TH2 cells within the lymph nodes produced both IL-4 and IL-13, whereas T follicular helper cells (Tfh) primarily produced IL-4 in response to parasitic helminth infection [51]. The type II receptor has a secondary ligand, known as IL-13. In murine models of asthma, neutralization of IL-13 attenuated the asthma phenotype (airway hyperresponsiveness, eosinophil recruitment, and mucus overproduction) [52]. Similarly in atopic dermatitis, Bitton *et al.* demonstrated that dermatitis symptoms and expression of TNF-α (inflammatory cytokine) are dependent on IL-13 signaling via the type II receptor [53].

Since both IL-4 and IL-13 share the signaling pathway through the IL-4Rα chain, antibody-mediated blockade of this receptor prevents the effects of these cytokines. The efficacy of dupilumab, an anti-IL-4Rα monoclonal antibody, has been studied in humans with atopic diseases such as atopic dermatitis and uncontrolled asthma [54,55]. For individuals with moderate-to-severe asthma, receiving subcutaneous (s.c.) dupilumab every 2 weeks for 52 weeks led to a 47.7% lower rate of severe asthma exacerbations in comparison to placebo [54]. Additionally, patients exhibited an increase in the forced expiratory volume in 1 second, indicating improvement in lung function and asthma control [54]. For individuals with moderate-to-severe atopic dermatitis, dupilumab was evaluated as a monotherapy and in combination with topical glucocorticoids [55]. With 12-weeks of dupilumab monotherapy, 85% of patients exhibited a 50% reduction in the Eczema Area and Severity Index (EASI) score in comparison to 35% of those in the placebo group [55]. Reduction in the pruritus scores, itching, of 55.7% in the

dupilumab group in comparison to 15.1% in the placebo group [55]. Combination therapy found patients using 50% less of topical glucocorticoids and a decrease in the frequency of nasopharyngitis and headache when compared to patients without dupilumab [55]. In both studies, patients receiving dupilumab demonstrated a significant reduction in disease severity and improved symptom scores. Further studies have shown that antibody-mediated blockade of IL-4 signalling through the IL-4Rα chain or α-IL4 antibodies abolishes the food allergic recall response in human PBMCS and the allergic response towards helminth infections in mice [47,56]. Recent work from our lab has demonstrated that blockade of IL-4/IL-13 signaling with anti-IL-4Rα prevented the generation of IgE antibodies in a human *in vitro* culture system, and single-cell RNA-sequencing analysis demonstrated a skewed T cell polarization, away from the TH2 phenotype [56]. Furthermore, treatment with anti-IL-4Rα abolished the generation of IgE in allergic mice and prevented anaphylaxis upon a systemic allergen challenge [56]. Importantly, anti-IL-4Rα treated allergic mice remained unresponsive to a PN challenge 6 weeks after discontinuation of anti-IL-4Rα treatment [56]. Therefore, anti-IL-4Rα may potentially induce extended protection and, perhaps some form of immunological reprogramming. These findings suggest a vital role for anti-IL-4Rα treatment in the future of allergen immunotherapy.

1.5 Current state of allergen immunotherapy

Currently, no curative treatments exist for food allergy and, thus, the standard of care remains strict allergen avoidance [57]. Epinephrine and

antihistamines relieve allergic symptoms only after a reaction has occurred. Various allergen immunotherapy strategies have been extensively investigated towards establishing an efficacious treatment or even a cure for food allergy. The four principal approaches include subcutaneous immunotherapy (SCIT), epicutaneous immunotherapy (EPIT), oral immunotherapy (OIT), and sublingual immunotherapy (SLIT). Although these therapies differ in the route of allergen administration, they typically involve a similar approach to treatment [58]. An oral food challenge (OFC) determines the initial starting dose of allergen. After 1-2 weeks, patients begin small incremental dose escalations for weeks to months, until a predetermined maintenance dose is reached. Maintenance doses can range from low, such as 3-7 mg of allergen per day in SLIT, to high, such as 300-4,000 mg of allergen per day in OIT [58]. As reference, one PN typically contains around 300 mg of allergen protein. This maintenance dose can be extended indefinitely. Depending on the allergen immunotherapy protocol, patients undergo a second OFC after a predetermined period of the maintenance phase. A pass, defined as the ability to tolerate an increased amount of allergen in comparison to baseline measurements would indicate allergen desensitization. Serum measurements from blood collected during this second challenge often indicate a decrease in allergen-specific IgE, a decrease in basophil activation levels, and an increase in allergen-specific IgG4. Broadly, these changes suggest a cellular shift away from the allergic phenotype [58].

1.6 SCIT

SCIT involves the injection of allergen under the skin of the arm (subcutaneously) and has been shown to be efficacious in the treatment of seasonal allergic rhinitis [59,60]. A Cochrane meta-analyses on the efficacy of SCIT for allergic rhinitis demonstrated a standardized mean difference of -0.73 for symptom scores, favouring SCIT over placebo [59]. This was accompanied by a reduction in medication score (use of medication for relief of allergic symptoms) of -0.57, favouring SCIT over placebo [59]. Double-blind, placebo-controlled (DBPC) clinical trials of SCIT for ragweed and grass pollen demonstrated an approximate 30-40% reduction in both symptom and medication score during 3-4 years of immunotherapy [61–63]. This improvement was maintained for up to 3 years after discontinuation of immunotherapy; however, allergen reactivity did return thereafter [59,61–63]. A recent phase 2a, multicenter, DBPC parallel group study of adults with grass pollen-induced seasonal allergic rhinitis examined the effects of dupilumab in combination with SCIT [64]. The study found that 61.5% of patients in the SCIT + dupilumab group reached maintenance dose, versus 46.2% of patients who received SCIT alone [64]. Additionally, only 7.7% of SCIT + dupilumab-treated patients required rescue medication (epinephrine) as compared to 19.2% of patients receiving SCIT alone [64]. Withdrawal rate was also reported to be significantly lower in the combinational therapy (7.7%) as compared to SCIT alone (30.8%) [64]. However, this study found no significant differences in total nasal symptom scores between both treatments [64]. Although dupilumab treatment did not achieve a better

reduction in symptom scores, it did increase patient adherence, which is critical in the treatment of food allergy.

SCIT can result in adverse systemic effects, and thus, often confines allergen administration to a clinical setting with medical supervision, and access to rescue medication, such as epinephrine [59,60]. The frequency of adverse events is likely the reason for the discontinuation of clinical trials involving the treatment of food allergy with SCIT. One study reported a 67-100% decrease in symptoms (nausea, vomiting, urticaria, rhinitis, mucosal pruritus, asthma, and hypotension) for PN-allergic patients receiving PN-SCIT compared to baseline measurements; however, this was accompanied by a 13.3% rate of systemic reactions [65]. A second study examining the effects of SCIT for PN allergy reported a 23% systemic reaction rate during the escalation phase and 39% during the maintenance phase of immunotherapy, often treated with epinephrine [66]. Ultimately, these findings discouraged the use of SCIT for the treatment of food allergy.

1.7 EPIT

EPIT utilized a novel route of allergen delivery, through an epidermal patch containing the allergen [58,67]. These patches are usually placed on the back or upper arm and changed at 24-48-hour intervals over the course of treatment [58,67]. This treatment was initially explored in a DBPC trial for the treatment of grass pollen allergy [68]. Patients received treatment before and during the pollen season, with the primary outcome being nasal provocation tests [68]. Patients receiving the epidermal patch demonstrated an improvement in the nasal provocation tests [68]. A pilot study

examining the safety and efficacy of cow's milk EPIT was reported in 2010 [69]. The study followed a 3-month, DBPC trial in 18 children (mean age of 3.82 years), with treatment consisting of three 48-hour epidermal patch application on the upper back [69]. The cumulative tolerated dose was evaluated by OFC conducted in a clinical setting [69]. Adverse reactions to the patch were localized to the area of application, consisting of local eczema or erythema [69]. Although being well tolerated, the EPIT treatment failed to significantly improve the cumulative tolerated dose in the active group in comparison to placebo [69]. A 2016 randomized DBPC study examined the safety and efficacy of PN-EPIT [70]. Participants were treated with either 100 or 250 μg PN, or placebo and examined for treatment success after 52 weeks [70]. The primary outcome was defined as passing a 5,044 mg protein OFC or achieving a 10-fold or greater increase in dose consumption from baseline [70]. At 52 weeks, 12% of placebo-treated participants, 46% of 100 μg PN participants, and 48% of 250 μg PN participants achieved treatment success [70]. Approximately 14.4% of placebo doses and 79.8% of EPIT doses resulted in predominantly local patch-site reactions [70]. One phase 2b DBPC, dose-ranging trial assigned patients to receive a patch containing placebo, 50, 100 or 250 μg of PN protein [71]. The study was conducted for 12 months, with a DBPC food challenge to evaluate changes in eliciting doses [71]. At 12 months, a statistical significance was seen only between the 250 μg group (50% of patients) and placebo (25% of patients) [71]. When stratified for age, a statistical difference was found for the 6- to 11-year-old cohort at 250 μg, but no differences were found between the adolescents/adults receiving the same dose [71].

A recent phase 3, randomized, DBPC trial examined the efficacy of a patch containing 250 μg of PN protein *versus* placebo [72]. The study had a high treatment adherence (98.5%) but found the success rate, defined as percentage of patients meeting a defined eliciting dose to PN OFC, of the treatment group to be only 35.3% in comparison to 13.6% with placebo [72]. The pre-specified lower bounds of the confidence interval were not met, and thus, the authors could not report a positive trial result [72]. These studies demonstrated an initial increase, followed by a decrease in PN-IgE, increases in PN-IgG4, and reduction in basophil activation and PN-specific TH2 cytokines [58,67,69–72]. Although safe, EPIT remains contentious regarding efficacy, but the high degree of safety holds merit for further exploration in treatment of food allergy.

1.8 OIT

OIT is currently the leading experimental therapy for the treatment of food allergy, with peanut OIT (Palforzia) having been approved by the FDA in the United States of America. It involves ingesting small, tolerable amounts of food allergens that are gradually increased to a predetermined maintenance dose that aims for approximately 300-4,000 mg of allergen ingestion per day [58]. This maintenance dose can then be continued for an indefinite amount of time. Primary outcome of clinical trials is generally desensitization, defined as the successful completion of an OFC after the maintenance phase. Individuals who can successfully tolerate the oral food challenge are deemed desensitized and those who pass an OFC after an arbitrary period of treatment cessation, have achieved

"sustained unresponsiveness" [58,73]. Although applicable to a variety of food allergens, OIT has predominantly been examined in the treatment of egg, milk and PN allergy [58,73].

A randomized trial of egg OIT in 40 children (5-11 years) reported a 55% desensitization rate to an OFC of 5 g of egg white powder at 10 months, increasing to 75% for a 10 g egg white powder oral food challenge at 22 months [74]. Of those that were desensitized, only 27.5% of patients exhibited sustained unresponsiveness 6-8 weeks after treatment cessation [74]. Long-term treatment of patients for 4 years with egg OIT resulted in 20/40 (50%) participants achieving sustained unresponsiveness 4-6 weeks after treatment cessation [75]. Therefore, there seemed to be an association between treatment duration, and success in achieving sustained unresponsiveness, though sustained unresponsiveness still remains elusive.

Similar results were observed for cow's milk OIT. In a 2004 study, 21 cow's milk allergic children (6-10 years old) were placed on OIT or placebo [76]. Here, approximately 72% of participants achieved desensitization over the treatment course of 6 months [76]. Pajno *et al*. report a similar finding of 67% desensitization rate following 18 weeks of cow's milk OIT treatment [77]. When the treatment duration was extended to 1 year, a desensitization rate of 90% was observed in the allergic patients, although the study population included only 2–3-year-old children [78]. One study sought to examine the addition of omalizumab, an anti-IgE biologic, with cow's milk OIT [79]. This DBPC trial first randomized patients to omalizumab or placebo [79]. OIT was initiated after 4 months of omalizumab/placebo, dose

escalation for 22-40 weeks, followed by a maintenance phase. At 28 months, all patients underwent an OFC where 88.9% of omalizumab-treated subjects and 71.4% of placebo-treated subjects passed the OFC [79]. Next, OIT was discontinued for 2 months, with a rechallenge at month 32 to assess sustained unresponsiveness [79]. Here, 48.1% of patients in the omalizumab group and 35.7% of patients in the placebo group achieved sustained unresponsiveness (P = 0.18) [79]. Despite treatment with omalizumab, cow's milk OIT yields similar findings to other allergen immunotherapies in terms of the difficulties in achieving sustained unresponsiveness. Addition of omalizumab during treatment reduced the proportion of symptom-evoking doses by 14% [79]. Overall, this study demonstrates certain advantages of using a combination approach to allergen immunotherapy.

One of the first studies to explore PN OIT was reported in 2009 [73,80]. Subjects first had a one-day escalation phase from 0.1 mg of PN protein, doubling every 30 minutes to a final dose of 50 mg [80]. Next, there was a buildup phase where doses were increased by 25 mg every 2 weeks until 300 mg was reached [80]. The subjects then maintained 300 mg of PN protein daily for up to 36 months, after which they underwent an OFC where 300, 600, 1,200, and 1,800 mg of PN protein were given every 30 minutes for a total of 3.9 g PN-protein [80]. Approximately 93% of patients successfully ingested 3.9 g of PN protein during the OFC [80]. A decrease in the skin prick test (SPT) and activation of basophils at 6 months, and a decrease in PN-specific IgE at 12-18 months were reported [80]. On the other hand, IgG4 antibodies significantly increased [80]. Further analysis of PBMCs stimulated with

PN protein revealed an increase in the secretion of IL-10, IL-5, IFN-γ, and TNF-α at 6-12 months [80]. IL-4 and IL-17 were undetectable from baseline to after PN stimulation [80]. Overall, these data suggest suppression of TH2 immunity, and perhaps an induction of a regulatory response indicated by the secretion of IL-10 and IFN-γ. A shorter randomized, placebo-controlled clinical trial of PN-OIT for 48 weeks reported an 84% desensitization rate for a 5 g PN-protein OFC [81]. Similarly, the PN-OIT group demonstrated a decrease in skin prick test, IL-5, IL-13, and an increase in PN-specific IgG4 [81]. Interestingly, the study further reports an increase in the ratio of $FoxP3^{hi}$:$FoxP3^{intermediate}$ ratio $CD4^{+}CD25^{+}$ TCs (Treg cells) at the time of OFC in the PN-OIT treated patients [81]. As FoxP3 is a transcription factor associated with Treg cells, this finding suggests immune modulation provided by OIT. However, this immune modulation is temporary, as clinical reactivity returns upon treatment cessation. One clinical trial treated patients with PN-OIT for up to 5 years with a maximum dose of 4,000 mg/day of PN-protein [82]. Approximately 61.5% (24/39 subjects) of patients successfully completed the protocol [82]. These subjects had a smaller SPT, lower levels of PN-specific IgE and lower ratios of PN-specific IgE:total-IgE in comparison with subjects who were unsuccessful in completing the study protocol [82]. Of the 24 patients, 12 (50%) achieved sustained unresponsiveness after discontinuing treatment for 1 month [82]. Levels of PN-specific IgE, and IgG4 were no longer different between the subjects at the end of the treatment cessation period [82]. In a recent randomized DBPC phase 2 study, participants were built up to a 4,000 mg

of PN protein per day maintenance phase lasting until week 104 [83]. Next, patients were asked either to discontinue treatment (PN-0), ingest 300 mg of PN protein daily for 52 weeks (PN-300), or receive oat flour (placebo) [83]. OFC were conducted at weeks 104, 117, 130, 143, and 156 [83]. At the week 104 OFC, the OIT-treated groups had an approximate 84% desensitization rate, compared to 4% in the placebo group [83]. For the PN-0 group, the rate of sustained unresponsiveness was 54% at week 117, 20% at week 130, 15% at week 143, and 13% at week 156 [83]. The PN-300 group followed a similar trend with 54% of patients passing the OFC at week 117, 43% at week 130, 37% at week 143 and 37% at week 156 [83]. Merely 13 weeks after discontinuation of treatment, the protection gained from OIT is lost in 65% of individuals. This loss of protection does seem to be slowed down by daily consumption of a lower dose of PN protein, as at week 156, 24% more patients consuming a lower dose of PN were protected in comparison to those who discontinued treatment. Both these studies demonstrate that although OIT is efficacious in desensitizing individuals, discontinuation, or reduction in consumption of PN-protein can dramatically compromise the achieved level of protection. Therefore, patients are required to continue daily consumption of PN-protein, likely indefinitely, to maintain protection.

A major limitation of OIT lies in its safety-to-efficacy ratio. Although effective at achieving desensitization, OIT has a high rate of adverse reactions, in comparison to other forms of allergen immunotherapy, often requiring rescue medications, such as antihistamines or even epinephrine [58,73,84]. A recent systematic

review and meta-analysis examined randomized control trials comparing the safety of OIT for PN allergy [84]. Studies were evaluated for anaphylaxis occurrence, adverse events, epinephrine use, and quality-of-life [84]. Close examination of 12 trials highlights an increase in risk of anaphylaxis (RR=3.12), an increase in anaphylaxis frequency (incidence rate ratio=2.72), and an increase in epinephrine use (RR=2.21) during OIT in comparison to no OIT [84]. Additionally, OIT increased the risk of adverse events (RR=1.92), and non-anaphylactic reactions (RR=1.79), such as vomiting (RR=1.79), angioedema (2.25), upper tract respiratory reactions (RR=1.36), and lower tract respiratory reactions (RR=1.55) [84]. Some studies report up to 83% of doses resulting in a form of allergic reaction and a patient withdrawal rate up to 46% due to adverse reactions [73,83,85]. Additional factors, such as concurrent illness, sub-optimally controlled asthma, timing of dose administration after food ingestion, exercise and during menses can contribute to exacerbation of allergic symptoms during OIT [73,86,87]. Thus, patient-specific dosage adjustments may be required to prevent OIT-mediated allergic symptoms. An additional approach found that pre-treatment with omalizumab allowed all 13 patients to tolerate the 11 desensitization doses given on the first day, requiring minimal to no rescue treatment [88]. In a more recent study, patients underwent a rapid 1-day desensitization of up to 250 mg PN protein after either receiving omalizumab or placebo for 12 weeks [89]. Omalizumab was then discontinued, and patients were kept on 2 g of PN protein daily [89]. Omalizumab-treated patients tolerated a median PN dose of 250 mg on the initial desensitization day versus 22.5 mg for placebo-treated

subjects [89]. Additionally, 79% of omalizumab-treated patients and only 12% of placebo-treated patients passed the 4 g oral food challenge 12 weeks after cessation of omalizumab [89]. Pre-treatment with omalizumab was critical in shortening the desensitization period, allowing patients to tolerate an increased initial dose. Although the reaction rates were not significantly different between the two groups, this was likely due to omalizumab-treated subjects receiving much higher doses of PN protein [89]. If applied to a typical, longer OIT protocol, significant improvements in safety could be demonstrated. Recent studies are consistent with these findings where treatment with omalizumab during OIT conferred a rapid dose escalation and desensitization [90–92]. These changes are marked by a reduction in serum levels of allergen-specific IgE antibodies and an increase in allergen-specific IgG4 antibodies [90–92]. In one study, decreasing the dosage of omalizumab treatment increased the frequency of OIT-mediated adverse events, leaving only 48% of participants successful in the continuation of the maintenance dose [90]. In another study, discontinuation of omalizumab during the maintenance phase left only 54% of participants continuing with OIT [91]. The remainder discontinued therapy because of adverse events.

For patients with PN allergy, the evidence tends to favour allergen-avoidance in comparison to OIT, as adverse events are quite common. The significant increase in the risk of anaphylaxis and non-anaphylactic reactions may decrease patient adherence to treatment and increase in patient withdrawal. Combination of OIT with an adjunctive treatment, such as omalizumab, holds

potential in reducing the frequency of adverse events. Therefore, further trials exploring alternative treatment regimens and allergen delivery systems are required to optimized patient safety.

1.9 SLIT

SLIT involves the daily administration of allergen extract or tablets placed underneath the tongue and held for approximately two-three minutes [58,73]. The amount of allergen is then increased to achieve a maintenance dose of approximately 3-7 mg per day, which can be continued indefinitely [58,73]. SLIT has demonstrated efficacy in reducing symptom scores (*e.g.*, allergic rhinitis) and medication use for the treatment of seasonal allergic rhinitis due to aeroallergens such as tree, grass, and ragweed pollen [93,94]. In the case of birch pollinosis, SLIT reduced both the symptom and medication scores by more than 50% over the 3-year course of treatment [95]. Comparable results were found in patients with allergic rhinitis, with or without intermittent asthma, receiving either SLIT or placebo for 3 years [96]. Treatment with SLIT resulted in an average of 50% reduction in symptom scores and significantly reduced bronchial hyperreactivity [96]. In grass pollen allergy, a longitudinal DBPC study randomized subjects to receive either a grass allergen tablet or placebo daily [97]. Treatment began 16 weeks prior to the start of grass pollen season and continued throughout the season [97]. After the first season of treatment, patients demonstrated a 30% reduction in rhino-conjunctivitis symptom score, and a 38% reduction in rhino-conjunctivitis medication score in comparison to placebo [97]. Despite 44% of patients having moderate and 56% severe

grass pollen allergy, determined by daily symptom scores during the grass pollen season, the treatment was well tolerated with a withdrawal rate of less than 4% of participants [97]. Additionally, no serious local side effects or systemic adverse events were reported, as most pertained to mild reactions, including swelling of the tongue or an itchy throat [97]. After 2 consecutive grass pollen seasons, the reduction in rhino-conjunctivitis symptom and medication scores were maintained in SLIT-treated in comparison to placebo [98]. Furthermore, patients receiving SLIT had a 33% improvement in their quality of life, determined using Juniper's Rhino-conjunctivitis Quality of Life Questionnaire [98]. Treatment was well tolerated with less than 1% of participants withdrawing due to adverse events [98]. Allergen-specific IgE increased initially, but continued to decrease over time, consistent with the findings from other allergen immunotherapies [98]. Interestingly, the levels of allergen-specific IgG4 linearly increased over the course of the treatment, resulting in a 23-fold increase over the span of 2 years [98]. These findings remain consistent with those of OIT, revealing the importance of IgG4 as a marker of desensitization for allergen immunotherapies.

Although not yet approved for the treatment of food allergy, SLIT is a novel allergen delivery approach, capable of causing desensitization in various clinical trials. One of the first published cases was reported in 2003 for a patient allergic to kiwi fruit [99]. This patient suffered severe anaphylaxis upon the consumption of kiwi, including 3 episodes of allergic shock accompanied by a loss of consciousness and subsequent hospitalization [99]. The patient was severely allergic, as even small traces

of kiwi left on a knife used to prepare a desert at a restaurant elicited anaphylaxis [99]. The patient underwent a modified-SLIT protocol, where he/she received diluted kiwi extract 3 times a day, increasing the amount of allergen with subsequent doses [99,100]. The patient increased their tolerance to kiwi from 10^{-5} mg to 1 mg of kiwi pulp prior to reaching a 1 cm^3 cube of fresh or frozen kiwi maintained for 5 years [99,100]. Immunoglobulin analysis demonstrated an increase in the protective IgG4 antibodies, and a decrease in kiwi-specific IgE antibodies after treatment [99,100]. Interestingly, despite cessation of therapy for 4 months, the patient was able to tolerate a 1cm^3 cube of fresh kiwi without any adverse reactions upon resuming immunotherapy [100]. This case encouraged the exploration of SLIT for the treatment of food allergy.

One of the first randomized DBPC trials to examine the efficacy and tolerance of SLIT in food allergy was applied to hazelnut allergy [101]. Patients were randomly assigned to receive either hazelnut immunotherapy or placebo, and efficacy was determined by a DBPC food challenge after 8-12 weeks of treatment [101]. Approximately 95% of patients in the active group (receiving SLIT) were able to reach the maximal dose of 11.56 g from a baseline tolerance of ~2.29 g over the course of 4 days [101]. Additionally, approximately 50% of patients receiving active treatment were able to reach the highest dose of the study, 20 g, within the treatment period [101]. Despite administering doses in the range of grams, only 0.2% of administered doses resulted in mild reactions, such as itchiness or swelling of the

throat [101]. Immunoglobulin and cytokine analysis from blood samples revealed an increase in IgG4 and IL-10 after immunotherapy, only in the active group [101].

Similar results were obtained from the treatment of peach allergy with SLIT. With 6 months of peach SLIT, the active group had a 3-9-fold increase in the amount of peach tolerated, and 5.3 times decrease in SPT with no serious adverse events reported [102]. Immunoglobulin analysis demonstrated an increase in Pru p 3, the major peach allergen, specific IgG4 [102]. Interestingly, one murine study examined the effect of peach-SLIT in combination with a CpG motif-containing oligodeoxyribonucleotide (ODN-CpG) as an adjuvant to induce a Th1/Treg specific response [103,104]. Mice were intranasally sensitized with Pru p 3 and lipopolysaccharide (LPS) [103,104]. Mice then underwent 8 weeks of SLIT + ODN-CpG treatment and then challenged at 1- or 3-weeks following treatment cessation [103,104]. Treated mice were protected from a drop in core body temperature and demonstrated a decrease in Prup3-specific IgE and IgG1 antibody levels following treatment [103,104]. Additionally, an increase in Treg cells and intracellular levels of IL-10/IFN-γ cytokines within $CD4^+$ splenocytes was noted [103,104]. This protection was maintained for 3 weeks after stopping treatment [103,104]. Therefore, combining the SLIT treatment with an adjuvant, such as ODN-CpG, to induce a shift away from the TH2 phenotype may be a beneficial route for future treatments.

In an early non-placebo-controlled study of children with cow's milk allergy, 6 months of milk SLIT increased the threshold of milk tolerated from 39 to 143 mL [105]. In another study, children with cow's milk allergy were subjected to

either SLIT alone (7 mg daily) or SLIT followed by OIT at 2 different doses (1000 mg or 2000 mg daily) [106]. At 12 and 60 weeks of maintenance, the children were challenged with 8 g of milk protein [106]. The study revealed that SLIT followed by OIT was far more effective in comparison to SLIT alone (60-80% *versus* 10% in passing of the 8 g milk protein challenge) [106]. However, 40% of children across both groups regained reactivity as early as 1 week upon treatment cessation [106]. A decrease in the SPT results and an increase in cow's milk-specific IgG4 levels were observed in all groups [106]. Systemic reactions were more common in the OIT groups with antihistamine usage in up to ~11% of doses in comparison to ~1.5% of SLIT [106]. Although not as efficacious, SLIT maintains the high degree of safety in food allergy treatment, as seen in the treatment of aeroallergens. A limitation of this study pertains to the dosage protocol of SLIT. The OIT groups escalated to doses in the range of grams, whereas the SLIT group was continued on 7 mg for the period of 60 weeks. The challenge was administered at a dose only 4-8 times larger for the OIT groups and at a dose ~1142 times larger for SLIT. The volume of dosage that can be applied under the tongue further poses an obstacle, as exceeding this threshold would most likely result in ingestion of allergen. Therefore, SLIT may benefit from an extended dose escalation, to help decrease the fold difference between the maintenance dose and the OFC dose. Together, these reasons may be why the SLIT group had a lower success rate in comparison to OIT.

SLIT has also been examined for both efficacy and safety in the treatment of PN allergy. In one randomized controlled trial, PN-allergic patients were

administered either PN-SLIT or placebo for 44 weeks [107]. A 5 g OFC was conducted after 44 weeks, and subjects successfully consuming 5 g or 10-fold more PN powder from baseline were considered responders [107]. 14/20 (70%) of SLIT-treated patients and 3/20 (15%) of placebo-treated patients successfully passed the oral food challenge [107]. The median tolerated dose increased from 3.5 to 496 mg in the SLIT-treated group [107]. Furthermore, this tolerated dose increased to 996 mg after an additional 24 weeks of SLIT [107]. The 10,855 doses were well tolerated with 95.2% of them being symptom-free, and the rest being limited to oropharyngeal itching or tingling [107]. This degree of safety was upheld after 3 years of treatment where 98% of administered doses were well tolerated without any severe reaction or use of epinephrine [108]. However, an OFC at an increased dose of 10 g of PN powder at the 3-year mark yielded only 4 participants who successfully tolerated this dose [108]. Furthermore, these 4 patients exhibited sustained unresponsiveness upon passing a secondary 10 g OFC 8 weeks after treatment cessation [108]. Since the participants were unable to tolerate the high dose, this study would probably have benefited from a secondary dose escalation where the likelihood of participants tolerating a higher dose during the OFC would potentially increase. In another DBPC study subjects underwent a 6-month dose escalation, followed by 6 months of maintenance with PN-SLIT [109]. The treated group was able to successfully ingest 20 times more PN protein than the placebo group (median dose: 1710 mg versus 85 mg) [109]. A decrease in SPT and basophil activation after PN-stimulation was observed in the treated group [109]. As seen with other immunotherapies, PN-specific

IgE levels initially increased during the escalation phase prior to decreasing over the remaining months [109]. In contrast, PN-specific IgG4 levels increased over the course of treatment [109]. IL-5 levels were decreased after treatment; however, no statistically significant changes were reported for IL-13, IL-10, Treg cells, or IFN-γ measured from the supernatants of PN-stimulated PBMCs [109]. Continuation of PN-SLIT at 2 mg/day for 3-5 years resulted in 86.5% of participants successfully consuming >750 mg and 62% of participants successfully consuming >1750 mg of PN-protein during the OFC [110]. Furthermore 32.4% (12/37) of participants passed a 5,000 mg OFC, a dose 2,500 times larger than that of maintenance [110]. Of the 12 who passed the higher-dosed challenge, 10 subjects (27% of treated patients) achieved sustained unresponsiveness after discontinuing treatment for 2-4 weeks [110]. Over the course of treatment, the median PN-specific IgE levels decreased from 83.9 kU/L (range, 7.7-1636 kU/L) to 20.0 kU/L (range, 1.6-1051.8 kU/L), and to 28.0 kU/L (range, 10.3-219) for those who achieved sustained unresponsiveness [110]. The median PN-specific IgG4 increased from 0.3 mg/L (range, 0-13.1 mg/L) to 10.9 mg/L (range, 0-231.0 mg/L) at the end of the study [110]. The IgG4:IgGE ratio increased from ~1.45 to ~356, demonstrating induction of protective antibodies [110]. Doses were well tolerated with no epinephrine use [110]. Side effects were reported for 4.8% of doses, with the most common symptom being oropharyngeal itching [110]. Extending PN-SLIT for a long-term protocol does seem to increase the threshold of tolerated allergen. However, these studies could benefit from examining how well patients are able to tolerate additional dose escalation after the

maintenance period, to help increase the threshold of tolerated allergen. In conclusion, published literature demonstrates the capabilities of SLIT in desensitizing patients, while maintaining a high degree of safety.

A limitation to SLIT is that, in comparison to OIT, it is not as efficacious at achieving desensitization. In a double-blinded study, PN-allergic children were randomized to receive either SLIT with placebo OIT, or OIT with placebo SLIT [111]. Maintenance doses was 3.7 mg/day for SLIT and 2000 mg/day for OIT [111]. OFCs were conducted at 6 and 12 months of maintenance [111]. Subjects in both groups demonstrated >10-fold increase in the tolerated dose at 12 months [111]. OIT achieved a 141-fold increase in the allergen tolerance threshold, while SLIT only achieved a 22-fold increase [111]. A decrease in IgE levels and an increase in IgG4 were seen in both groups, although to a greater extent in OIT [111]. Therefore, current studies demonstrate that OIT exceeds SLIT in terms of efficacy; however, it is accompanied by more systemic side effects [106,111]. Indeed, systemic reactions occurred in 42.8% of doses for PN-OIT (requiring epinephrine, antihistamines, or β-agonists; and treatment withdrawals) and in only 9% of the doses for SLIT [111]. 3/11 participants in the OIT group discontinued the treatment due to side effects, *versus* 1 who received SLIT [111]. For the 10 participants who initially received SLIT and were then switched to OIT after 12 months, 2 discontinued the treatment due to side effects [111]. It is evident that although SLIT may not be as efficacious as OIT, it does deliver the allergen with a high degree of clinical safety and patient adherence. In conclusion, SLIT presents a novel approach for the delivery of food

allergens capable of inducing desensitization, while upholding a high degree of safety. Additional studies using differing dosing protocols and comparisons to other immunotherapies will be beneficial in determining the applicability of SLIT to the general food allergic population.

1.10 Thesis Objective

A number of immunotherapy approaches are being investigated for the treatment of food allergy, although OIT remains the gold standard. Despite demonstrating high efficacy, it is accompanied by severe systemic side effects that discourage patients from continuing treatment [58,73,84]. The failure to achieve sustained unresponsiveness in most patients following treatment discontinuation suggests a potential role for combinational therapies to induce lasting protection [58,73]. The end goal of this investigation was to evaluate the impact of SLIT + anti-IL-4Rα combination therapy in two murine models of food allergy. However, there were several steps and optimizations required prior to reaching this goal. Specifically, we first established a comprehensive understanding on the capabilities of SLIT in inducing clinical and cellular changes in our murine models of food allergy. In contrast to other models of murine food allergy, the models used here are those of severe food-induced anaphylaxis, and thus, evaluate the capabilities of SLIT under extreme conditions [112]. Additionally, our models allow us to examine the effect of SLIT treatment on the generation of IgE. In the epicutaneous model (Figure 1A), only allergen-specific IgG1 antibodies emerge after the tape-stripping protocol, while allergen-specific IgE antibodies are produced only after the non-

sensitizing s.c. allergen exposures. In the intragastric model (Figure 1B), IgE production is only measurable after the non-sensitizing intragastric allergen re-exposures. For these studies, we utilized chicken egg protein ovalbumin (OVA) as our model antigen, which we have demonstrated produces immune and physiological responses analogous to PN; also, selecting OVA as our model antigen allows us to eventually use novel allergen-specific cell purification methods for future experiments not covered within this thesis. Lastly, we conducted this experimentation in 3 phases: Phase 1) Prophylactic effects of SLIT alone, Phase 2) Semi-therapeutic/Therapeutic effects of SLIT alone, and Phase 3) Investigation of SLIT + anti-IL-4Rα as a combinational therapy.

This MSc thesis sought to first optimize SLIT protocols in two models of food allergy and, secondly, to investigate the efficacy of SLIT in modulating clinical and humoral responses in a prophylactic and semi-therapeutic setting.

Chapter 2: Methods

2.1 Animals

Six to eight weeks old female C57BL/6 mice were purchased from Charles River Laboratory (Ottawa, Ontario). Mice were housed in a pathogen-free environment and maintained on a 12-hour light/dark cycle. All experiments outlined were approved by the Animal Research Ethics Board of McMaster University. A total of 295 mice were used for the experiments reported in this document.

2.2 Murine models of allergic sensitization

Epicutaneous Sensitization

Mice were subjected to a previously reported protocol, which is outlined in Figure 1A [41,113,114]. Backs of mice were first trimmed with electric clippers, followed by shaving with a mechanical razor to remove any remaining hair (Figure 2A). Water could be used to help ease the shaving process and prevent irritation of the skin. If redness was seen during this process, shaving was stopped and moved onto another area on the back, as the development of scabs would hinder the tape stripping process. Next, using a piece of tape, wrapped around the fingers, the outermost skin layer was gently peeled off (Figure 2B). The back became slightly red, with no hair on the shaven area (Figure 2C). Next, 20 µL of chicken egg protein, ovalbumin (OVA) (Albumin from chicken egg white, A5378, Millipore Sigma, St Louis, USA) at 10 mg/mL was directly applied onto the shaven back (Figure 2D). This process was repeated Monday-Friday for 10 consecutive days.

Two weeks after the final tape stripping session, mice received 3 s.c. administrations of 100 µg OVA in 0.5 mLs of PBS, Monday, Wednesday, and Friday. Serum was collected prior to the s.c. re-exposures, and one day prior to the systemic allergic challenge. One day prior the systemic challenge, mice received 500 µg of anti-CD16/32 (Bio X Cell, BE0307, Clone 2.4G2) intraperitoneally as previously published [115].

Gut Sensitization

Mice were subjected to a modified gastric sensitization protocol, outlined in Figure 1B [21,116]. Held in the supine position, mice received an initial priming intragastric gavage of 1 mg OVA and 5 µg cholera toxin (CT, Biological Laboratories) in a final volume of 0.5 mL PBS. After this initial "priming gavage" mice were rested for 4 weeks. Next, mice received non-sensitizing gavages of 1 mg OVA alone once a week for 3 weeks. Two weeks following the last oral gavage, mice underwent a systemic allergen challenge. Serum was collected and stored prior to the OVA alone gavages and one day prior to the systemic allergen challenge.

2.3 SLIT and Oral Tolerance

Mice were held while maintaining a high scruff where both the ears fall in between the thumb and the index finger (Figure 3A). Upon inspection in the supine position, the mouth was open, with little room available for the head movement (Figure 3B). Next, 5 µL of OVA (20 µg, 100 µg or 1,000 µg) was administered directly underneath the tongue, behind the lower front teeth using a pipette (Figure

3C). Mice were held in the supine position, parallel to the floor, for 20 seconds, and then released back into the cage (Figure 3D). SLIT was done at the same time every Monday to Friday.

For the oral tolerance comparator group utilized in Phase 1 of experimentation, mice received 10 intragastric gavages of 1 mg OVA twice a week for 5 weeks.

2.4 Systemic Allergen Challenge

The challenge was conducted with 5 mg of OVA in 500 µL of PBS injected intraperitoneally into sensitized mice. Mice were then observed over the course of 40 minutes for clinical signs of anaphylaxis. Core body temperatures were recorded at 10-minute intervals using a rectal probe digital thermometer (VWR). Clinical symptoms were graded on a scale from 1-5 (1 = in-ear scratching, 2 = reduced motion, 3 = motionless, 4 = no response to whisker stimulus, 5 = prodding or seizure) as outlined in Table 1. Hemoconcentration was recorded by bleeding mice at 40 minutes post systemic challenge using a heparin coated microhematocrit capillary tube and measured using the Hemastat microhematocrit centrifuge (EKF Diagnostics PLC, San Antonio, USA). All mice received 1 mL of warmed PBS intraperitoneally and placed on a heating pad for 4-5 hours. Mice were then examined for recovery post-challenge and either left on the heating pad overnight or taken off and returned to the housing rack.

2.5 Serum Collection

Mice were first anesthetized with isoflurane and then peripheral blood was collected by bleeding from the retroorbital plexus using non-heparinized capillary tubes. For Phase 1 of experimentation, mice were bled: 1) prior to the allergen re-exposures, and 2) one day prior to the systemic allergen challenge (Figure 4A, Figure 7A). Mice in Phase 2 of experimentation were bled: 1) after the tape stripping protocol, or priming gavage, 2) After the cessation of the SLIT protocol, and 3) one day prior to the systemic challenge (Figure 10A, Figure 16A). Peripheral blood was spun at 4°C at 13,000 rpm (397 rcf) for 7 minutes, and serum was pipetted out into a separate Eppendorf tube. Serum was stored at -20°C.

2.6 Splenocyte Collection and Culture

Three days post-allergen challenge, mice were anesthetized, and spleens were harvested. Spleens were crushed through a 40-μm nylon strainers with Hanks buffer into a Petri dish within a biosafety cabinet. Samples were then spun at 1,240 rpm (361 rcf, 4°C, break on) for 10 minutes and shaken with ACK lysis buffer for 90 seconds to lyse red blood cells. ACK reaction was slowed down with 20-30 mL of Hanks and samples were spun at 1,240 rpm (361 rcf), 4°C, break on.

Splenocytes for culture were resuspended with sterile filtered RPMI (cRPMI) containing 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, and 55 μmol/L 2-mercaptoethanol (Gibco, Grand Island, USA). Samples underwent cell counts and were diluted to a final concentration of 8,000,000 live cells/mL (in cRPMI) and plated onto flat bottom 96-well plates at 100 μL/well (800,000

cells/well) in triplicates for both stimulated and unstimulated groups. Wells then received either 100 μL of cRPMI or 100 μL of OVA at 0.5 mg/mL. Samples were placed in the incubator for 5 days. On day 5, supernatants were collected from samples (with pooled triplicates) and stored at -80°C until further analysis.

2.7 ELISAs

OVA-specific IgG1

First, 96-well NUNC Maxi-Sorp plates (Thermo Fisher Scientific, Waltham, USA) were coated with 4 μg/mL OVA in carbonate-bicarbonate buffer (made in-house at 50 nM, Millipore Sigma, St Louis, USA) at 100 μL/well and incubated overnight at 4°C. Next morning, plates were aspirated, and blocked with 1% BSA/PBS at 100 μL/well for 2 h at room temperature. During the incubation, samples were prepared by dilution to 1/20, 1/200, 1/2000, 1/20000. Plates were washed 3 times and incubated with the prepared samples overnight at 4°C. On the third day, the plates were washed 3 times, and then coated with biotinylated goat anti-mouse IgG1 (Southern Biotech, Birmingham, USA) at 50 μL/well and incubated for 2 h at room temperature. After washing 3 times, plates were incubated with alkaline-phosphatase streptavidin (Sigma) for 1 h at room temperature. The assay was developed with *p*-nitrophenyl phosphate tablets (Sigma) in a solution of 4-parts water to 1-part 5x diethanolamine buffer (Sigma) and stopped with 2 M NaOH. Absorbance was read at 405 nm via Multiskan FC photometer (ThermoFisher Scientific).

OVA-specific IgE

First, 96-well NUNC Maxi-Sorp plates (Thermo Fisher Scientific) were coated with 2 μg/mL rat anti-mouse IgE (Clone: R35-72, BD Pharmigen, San Jose, USA) at 50 μL/well. Next, standards were prepared by diluting dig-oxygenated OVA (ANP technologies, Newark, USA, kit 90-1023-1KT) 8 times from 62.5 ng/mL to 0 ng/mL, by a factor of 2 during each dilution. Standards were plated in duplicates at 50 μL/well and the plates were incubated at 4°C overnight. Coated plates were washed 3 times and blocked with 5% skim milk powder in PBS for 1 h in a 37°C warm room. After washing 3 times, samples (diluted at 1:2 and 1:8 in 1% skim milk) were transferred to the plate and stored at 4°C overnight. The next day, plates were washed 5 times and incubated with 50 μL/well dig-oxygenated OVA (300 ng/mL, diluted in 1% skim milk) (excluding the standard columns) for 90 minutes at room temperature. Plates were washed 5 times and anti-digoxigenin-POD (poly) Fab fragments (Roche, Basel, Switzerland, 5 μL in 0.3% BSA/PBS) was added to the plate at 50 μL/well and incubated for 1 h at room temperature, covered from light. Plate was washed 5 times and the colour reaction was developed using TMB liquid substrate (3,3',5,5'-Tetramethylbenzidine, Sigma) and stopped with 2M H_2SO_4. Absorbance was read at 450 nm.

2.8 Luminex

Frozen supernatants from splenocyte cultures were tested in the MILLIPLEX Immunology Multiplex Assays (MCYTOMAG-70K kit, Millipore Sigma, St Louis, USA) and analyzed through the MAGPIX XMAP Technology

system (Luminex, Austin, USA). Samples were evaluated for the secretion of IFN-γ, IL-3, IL-4, IL-5, IL-9, IL-10, IL-12 (p70), IL-13, and IL-17. Cytokines for statistical analysis were included if they fell within the detectable range (3.2-10000 pg/mL).

2.9 Statistics

All data were analyzed using GraphPad Prism (v.6.0) and displayed as mean ± SEM. A one or two-way analysis of variance (ANOVA) with a Turkey's or Bonferroni's *post hoc* test were used for data analysis. Differences were considered significant when the p-value was less than 0.05 (*, °).

Chapter 3: Results

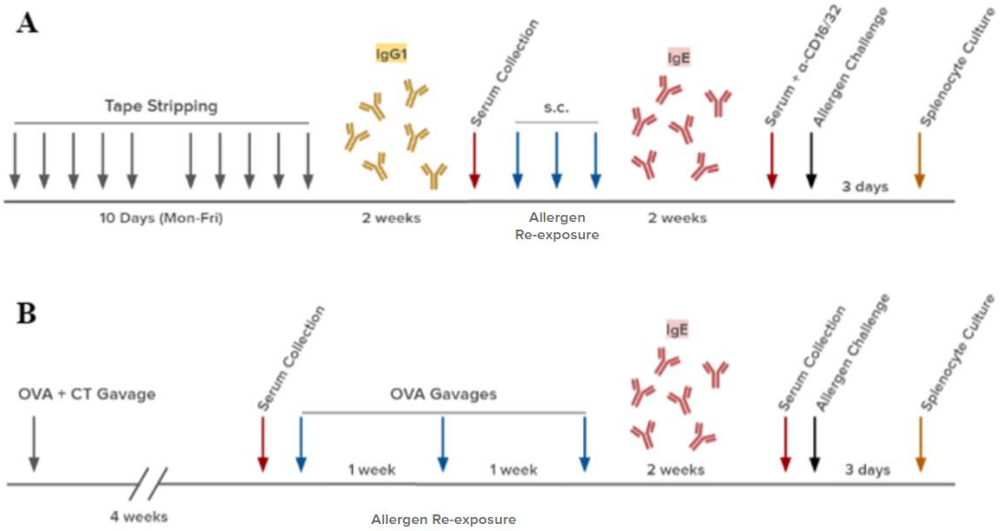


Figure 1. Schematics of models of allergic sensitization. **A**, epicutaneous sensitization with IgG1 generation occurring after tape stripping, and IgE being generated upon subcutaneous re-exposures. **B**, intragastric sensitization with mice producing IgE only after the OVA-alone intragastric exposures. S.c., subcutaneous; CT, cholera toxin; OVA, ovalbumin.

Figure 2. Visual representation of epicutaneous sensitization adapted from Jiménez-Saiz *et al* [41]. **A**, representation of back after shaving. **B**, tape stripping

technique. **C**, visual representation of the shaven back prior to OVA administration. **D**, OVA administration on the shaven back.

Figure 3. Visual representation of the SLIT administration protocol. A, maintain a high scruff when picking up the mouse, so that the ears are encompassed between the thumb and index fingers. **B**, ensure that the mouth is open and the tongue steady. **C**, administer 5 µL of OVA underneath the tongue, behind the front teeth. **D**, hold the mouse in the supine position for 20 seconds prior to release.

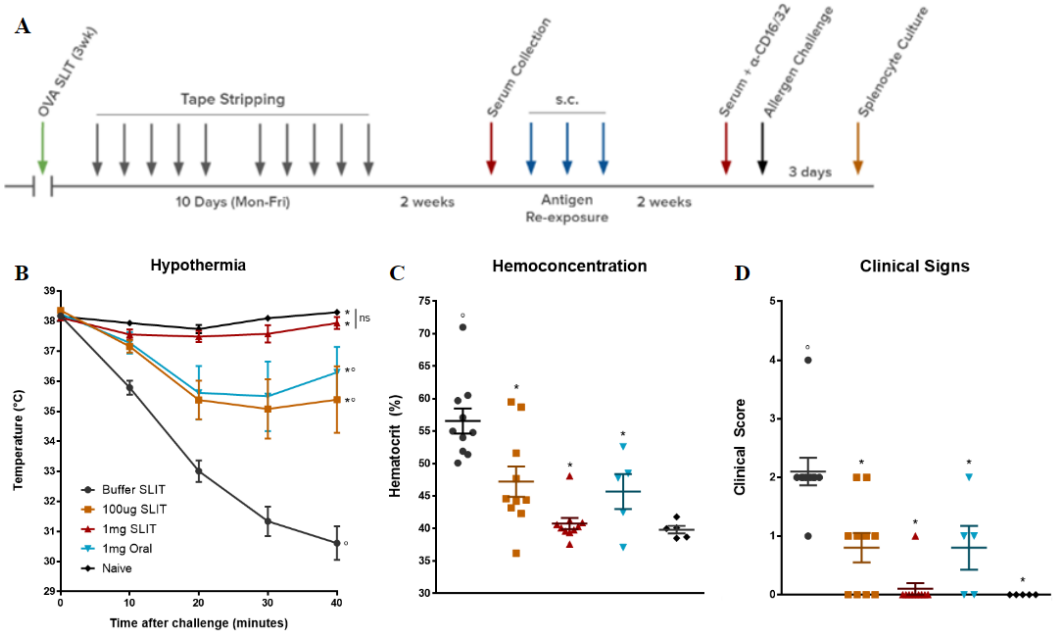


Figure 4. Prophylactic SLIT protects mice against epicutaneous allergic sensitization and allergen-induced clinical reactivity. **A**, schematic for the prophylactic SLIT prior to epicutaneous sensitization. **B**, core body temperature during systemic allergen challenge. **C**, hemoconcentration at 40 minutes post challenge. **D**, clinical scores during systemic challenge (scoring described in Table 1). Data are representative of 1 experiment with 5-10 mice per group, plotted as mean ± SEM in comparison to the buffer SLIT group (*) or naïve group (°). *, ° $P<0.05$. Buffer, PBS SLIT; oral, oral tolerance model, s.c., subcutaneous, OVA, ovalbumin.

Evaluation of prophylactic OVA-SLIT in establishing protection against epicutaneous sensitization

Our first aim was to examine the impact of OVA-SLIT in a prophylactic setting. This served two purposes: 1) to examine whether the sublingual route can be used to induce tolerance, thus protecting the mice from any subsequent allergic sensitization, and 2) to validate the SLIT technique and dosage protocol to be used in subsequent experiments. C57BL/6 mice were first administered three weeks of OVA-SLIT, Monday to Friday, at either 1,000 µg (high dose) or 100 µg (low dose)

(Figure 4A). Next, mice received 10 days of tape stripping, Monday to Friday, as shown in Figure 2. Serum was collected two weeks after the final tape strip, followed by 3 non-sensitizing, subcutaneous OVA exposures administered Monday, Wednesday, and Friday. Additionally, a group of mice underwent 10 intragastric gavages, twice a week for 5 weeks, to serve as a model of oral tolerance. This allowed us to compare the effects of sublingual exposure to a more conventional exposure to induce tolerance. Mice were rested for two weeks, prior to the final serum collection. At that point, mice were treated with an anti-CD16/32 monoclonal antibody to prevent the induction of IgG1-mediated anaphylaxis. One day after the serum collection, mice were challenged intraperitoneally with 5 mg of OVA and monitored for clinical signs of anaphylaxis (core temperature, hemoconcentration and clinical scores).

As expected, the PBS buffer-treated allergic mice had a significant drop in core body temperature over the course of 40 minutes, indicative of an IgE-mediated systemic shock (Figure 4B). One mouse in the buffer group was a non-responder, an atypical event in this model (Appendix 1A). The 100 μg SLIT-treated group exhibited a variable, yet intermediate level of protection (Figure 4B). Notably, these mice showed some drop in core body temperature up to the 20-minute mark, at which it plateaued at the 30-minute mark and begun to recover at the 40-minute mark (Figure 4B, Appendix 1A). Examination of individual mice showed that 6/10 mice had returned to baseline temperature at the 40-minute mark, 2/10 had an intermediate drop in temperature, and 2/10 were still fully responsive (Appendix

1A). In contrast, the 1 mg SLIT-treated group exhibited full protection from a drop in core body temperature at all time points and was not significantly different than the naïve group (Figure 4B). One mouse from this group had an intermediate drop in core body temperature for the first 30 minutes but proceeded to recover thereafter (Appendix 1B). Interestingly, the oral tolerance group exhibited an intermediate level of protection, similar to that of the 100 μg SLIT-treated group (Figure 4B). Individual temperatures showed 2/5 mice exhibiting full protection and 3/5 mice an intermediate level of protection (Appendix 1C). The naïve mice exhibited no drop in core body temperature (Figure 4B).

When undergoing anaphylaxis, mice also exhibit an increase in hemoconcentration due to vascular leakage. Buffer-treated mice exhibited the largest increase in hemoconcentration in comparison to naïve mice (Figure 4C). Consistent with the drop in core body temperature, the 100 μg SLIT-treated group exhibited a significantly less increase in hemoconcentration in comparison to the positive control (Figure 4C). The variability seen in the data reciprocate the temperature graphs, with the highest increase in hemoconcentration corresponding to the mice exhibiting the largest drop in core body temperature. Next, the 1 mg SLIT-treated group exhibited hemoconcentration, similar to that of the naïve group (Figure 4C). The orally exposed mice exhibited a variable but statistically significant lesser increase in hemoconcentration in comparison to buffer-treated mice (Figure 4C).

A third measure of reactivity was observation of clinical responses (Table 1). We utilize a 5-point based grading scheme, ranging from 0: no clinical signs, to 5: prodding or seizure. The buffer-treated mice exhibited clinical scores upwards of 4, indicating no whisker responses (Figure 4D). Next, the 100 μg SLIT-treated group demonstrated significantly better clinical scores in comparison to the positive control (Figure 4D). Within this group, 2/10 mice exhibited reduced motion, 4/10 mice exhibited in-ear scratching and 4/10 mice exhibited no symptoms, thus demonstrating a variable, intermediate level of protection (Figure 4D). Mice in the 1 mg SLIT-treated group were fully protected except for one mouse that exhibited in-ear scratching; 9/10 mice exhibited no symptoms (Figure 4D). This group was not statistically different than the naïve group. The orally exposed mice showed a variable response with 1/5 mice demonstrating reduced motion, 2/5 with in-ear scratching and 2/5 exhibiting no symptoms (Figure 4D). The naïve group experienced no clinical signs upon allergen challenge (Figure 4D).

In conclusion, administering three weeks of SLIT prophylactically was sufficient to protect mice from severe anaphylaxis upon challenge. Furthermore, the 1 mg dosage provided near full protection in comparison to the 100 μg dosage of OVA-SLIT.

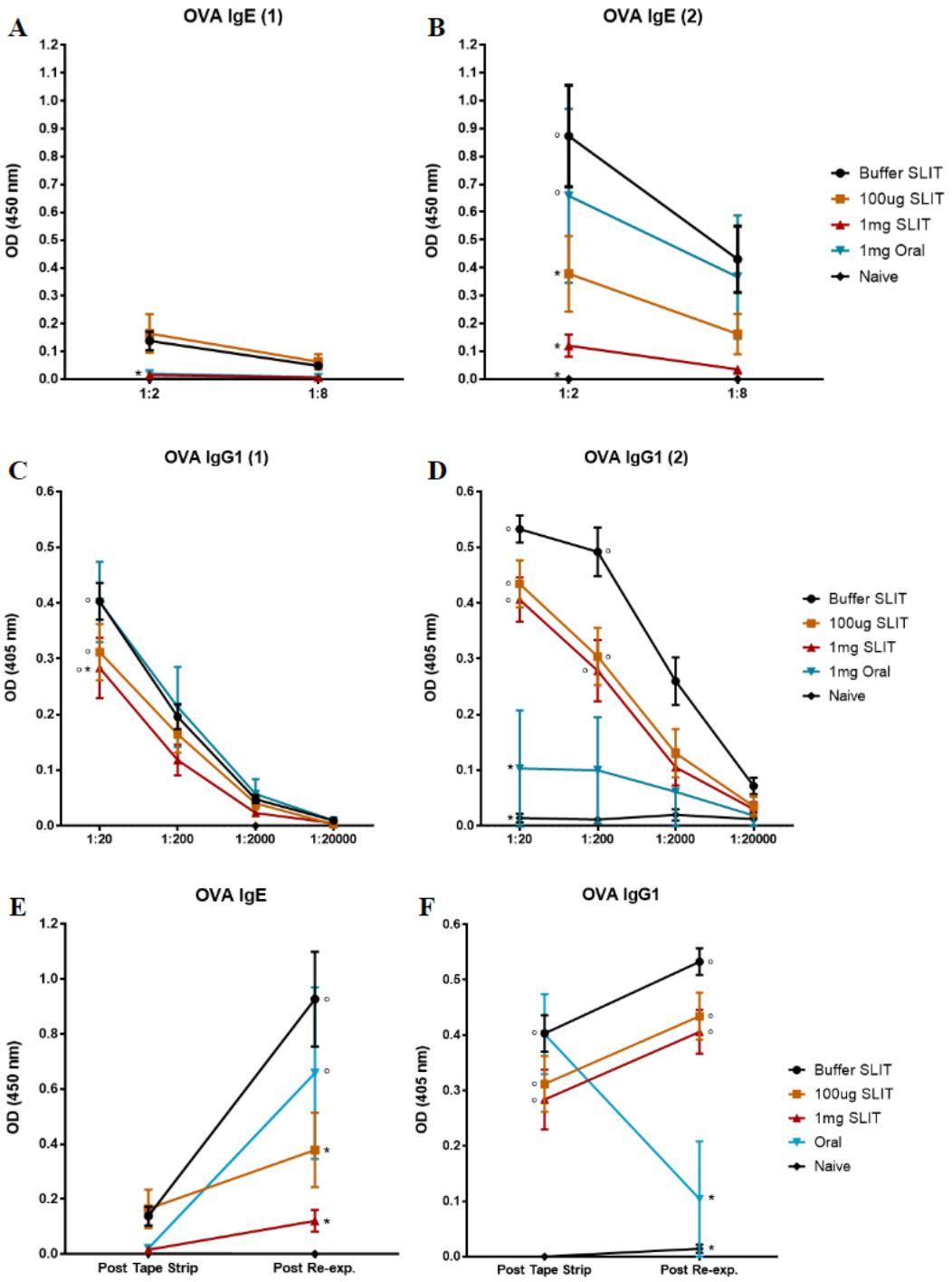


Figure 5. Prophylactic SLIT reduces the emergence of OVA-specific IgE after epicutaneous sensitization. **A-B**, serum OVA-specific IgE at two dilutions measured prior to subcutaneous allergen re-exposures, (**A**) and one day prior to challenge (**B**). **C-D**, serum OVA-specific IgG1 at four dilutions measured prior to subcutaneous allergen re-exposures (**C**) and one day prior to challenge (**D**). **E-F**,

comparison of OVA-specific IgE and IgG1 titres across the allergen subcutaneous re-exposures. Data are representative of 1 experiment with 5-10 mice per group, plotted as mean ± SEM in comparison to the buffer SLIT group (*) or naïve group (°). *, ° P<0.05. Buffer, PBS SLIT; Oral, oral tolerance model; Re-exp, allergen re-exposures. Buffer, PBS SLIT; oral, oral tolerance model; OD, optical density.

Serum was collected from mice at two timepoints (Figure 4A) and analysed for the levels of OVA-specific IgE and IgG1 using a sandwich-ELISA protocol. Post tape stripping samples demonstrated low levels of OVA-IgE amongst all groups, increasing upon subsequent allergen re-exposures (Figure 5A-B). As expected, the buffer-treated group showed the largest increase in OVA-IgE levels following allergen re-exposures (Figure 5E). The 100 μg SLIT-treated group showed a significant reduction in the level of OVA-IgE production post allergen re-exposures in comparison to the positive control (Figure 5E). Next, the 1 mg SLIT-treated group showed only a slight increase in titres of OVA-IgE antibodies at the secondary time point (Figure 5E), but these levels were not significantly different from those of naïve mice. The orally exposed mice demonstrated an increase in OVA-IgE antibodies, not significant from that of the positive control (Figure 5E). However, it is important to note the high degree of variability observed in this analysis, consistent with the variability seen within the hemoconcentration and clinical signs (Figure 4C, D). Additionally, it is plausible that these findings are the result of the protocol itself, 1mg intragastric OVA gavage twice a week for five weeks. Optimizing the frequency of the dose intervals may ameliorate the OVA-IgE production seen within this group. Together, these data suggest that this

protocol for oral tolerance was not sufficient in establishing complete protection against epicutaneous sensitization.

Analysis of OVA-IgG1 demonstrated a similar level of induction amongst all groups following tape stripping (Figure 5C-D). At the 1:20 dilution, the 1 mg SLIT-treated mice exhibited a statistically significantly lower level of OVA-IgG1 in comparison to the positive control. These results are in accordance with the epicutaneous model of sensitization, where only IgG1 antibodies are observed prior to subcutaneous allergen exposures [41,113,114]. Upon allergen subcutaneous exposures, the buffer-treated, 100 μg SLIT-treated, and 1 mg SLIT-treated mice all showed a similar increase in the levels of OVA-specific IgG1 antibodies (Figure 5F). Interestingly, the oral tolerance model had a significant decrease in levels of OVA-IgG1 following the subcutaneous allergen re-exposures (Figure 5F). It is important to note that the levels of OVA-IgG1 for this group was highly variable, resulting in no statistical difference from the levels of the naïve group (Figure 5F). We believe that the small number of mice allocated to this group may explain the large degree of variability seen within the OVA-IgG1 analysis. With more mice allocated to the group, we expect them to produce levels of OVA-IgG1 antibodies similar to that of the buffer group. Together, these data indicate that prophylactic treatment with SLIT in this model reduces the production of OVA-specific IgE.

Three days following the allergen challenge, we harvested the spleens of mice to investigate the impact of SLIT on the T cell compartment. Harvested splenocytes were cultured *in vitro* with OVA for five days. Supernatants collected

on the fifth day were examined for various cytokines through the Luminex cytokine detection assay. Unfortunately, the high degree of variability observed prevents definite conclusions from this analysis. Nevertheless, frozen replicates of the samples have been stored, and will be repeated to achieve a less variable result.

We first investigated the impact of prophylactic SLIT on the TH2-associated cytokines, namely, IL-4, IL-5, and IL-13. An induction of IL-4 with OVA was seen within both SLIT-treated groups, the oral tolerance group, and the positive control (Appendix 3A). A similar trend was demonstrated for IL-5 and IL-13 upon stimulation with OVA (Appendix 3B-C). Therefore, this suggests an induction of a TH2 response in the SLIT-treated mice, despite the observed significant protection from clinical reactivity upon challenge. Next, we sought to examine any changes in the regulatory cytokine, IL-10 and the Th1 cytokine, IFN-γ. No differences were observed for the induction of IFN-γ (Appendix 3D) although the naïve group does seem to have some induction of IFN-γ (Appendix 3D). However, IL-10 was significantly upregulated in both the SLIT-treated groups with OVA stimulation (Appendix 3E). IL-3, IL-9, IL-12 (p70), and IL-17 were also examined during this analysis; however, these cytokines remained unchanged upon OVA stimulation within all groups (Appendix 3F-I).

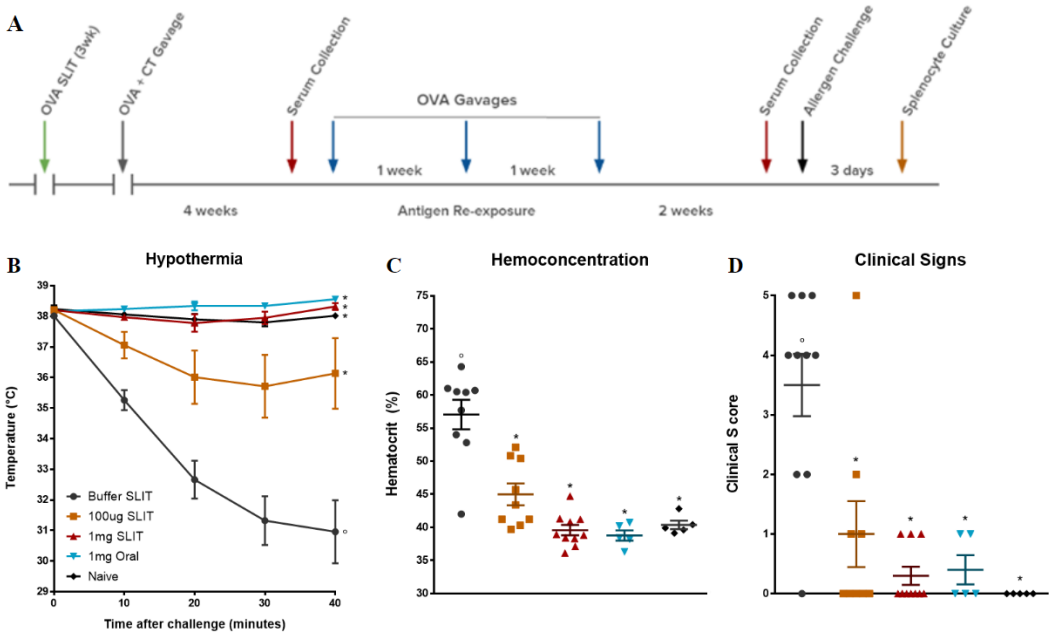


Figure 6. Prophylactic SLIT protects mice against intragastric sensitization and food-induced clinical reactivity. **A**, schematic for prophylactic SLIT prior to intragastric sensitization. **B**, core body temperature during challenge. **C**, hemoconcentration at 40 minutes post challenge. **D**, clinical scores during challenge (as described in Table 1). Data are representative of 1 experiment with 5-10 mice per group, plotted as mean ± SEM in comparison to the buffer SLIT group (*) or naïve group (°). *, ° P<0.05. OVA, ovalbumin; CT, cholera toxin; Buffer, PBS SLIT; oral, oral tolerance model.

Evaluation of prophylactic OVA-SLIT in establishing protection against intragastric sensitization

Next, we evaluated the impact of prophylactic OVA-SLIT on subsequent intragastric allergic sensitization. C57BL/6 mice were first administered three weeks of OVA-SLIT, Monday to Friday, at either 1,000 (high dose) or 100 μg (low dose) (Figure 6A). Next, the mice received a single priming gavage of OVA + CT, a known TH2 adjuvant [117]. The mice were rested for four weeks, followed by serum collection prior to allergen exposures. A non-sensitizing, OVA alone intragastric

gavage was administered once a week for three weeks. Serum was collected two weeks after the final gavage, followed by an allergen challenge with 5 mg of OVA the next day.

Like the epicutaneous model, the buffer-treated mice exhibited a significant drop in core body temperature upon challenge (Figure 6B). One non-responder (1/10 mice) in the buffer group resulted in large error bars (Figure 6B, Appendix 2A-C). The low dose 100 μg SLIT-treated group exhibited a partial level of protection (Figure 6B). These mice had an intermediate drop in core body temperature, plateauing at 20-30 minutes, and then showing a trend towards recovery (Figure 6B). Within this group, 2/10 mice were fully reactive, 2/10 mice were partially protected while demonstrating a trend towards recovery after 30 minutes, and 6/10 mice had no clinical response (Appendix 2A). The high dose 1 mg SLIT-treated group also responded similarly to the epicutaneous model (Figure 6B). Here, 9/10 mice were fully protected from clinical reactivity, and one mouse had a partial response, but recovered back to baseline after 20 minutes (Figure 6B, Appendix 2B). Interestingly, the orally exposed group was, in this model, fully protected from clinical reactivity, with all mice exhibiting no drop in core body temperature, similar to the naïve group (Figure 6B, Appendix 2C).

Analysis of hemoconcentration data demonstrated a statistically significant reduction in the SLIT-treated, and orally exposed groups (Figure 6C). The 100 μg SLIT-treated group showed variability, as seen in the drop in core body temperature

(Figure 6C). The 1 mg SLIT-treated group, and the orally exposed group showed low variability and results not different than those of naïve mice (Figure 6C).

With respect to clinical signs, we observed more severe reactions in the positive control group in comparison to the epicutaneous model (Figure 6D). Within the buffer group, 3/10 mice achieved the highest clinical score indicating seizure or prodding, 4/10 mice had no response to whisker stimulation, and 2/10 mice had reduced motion (Figure 6D). The scores were significantly reduced in the 100 μg SLIT-treated mice, with 1/10 mice undergoing seizure, 1/10 mice having reduced motion, 2/10 mice with in-ear scratching and 6/10 exhibiting no clinical symptoms (Figure 6D). The 1 mg SLIT-treated group had a less variable response with only 3/10 mice reacting with in-ear scratching, and 7/10 mice with no clinical response (Figure 6D). The orally exposed group was similar with 2/5 mice exhibiting in-ear scratching and 3/5 undergoing no clinical symptoms (Figure 6D).

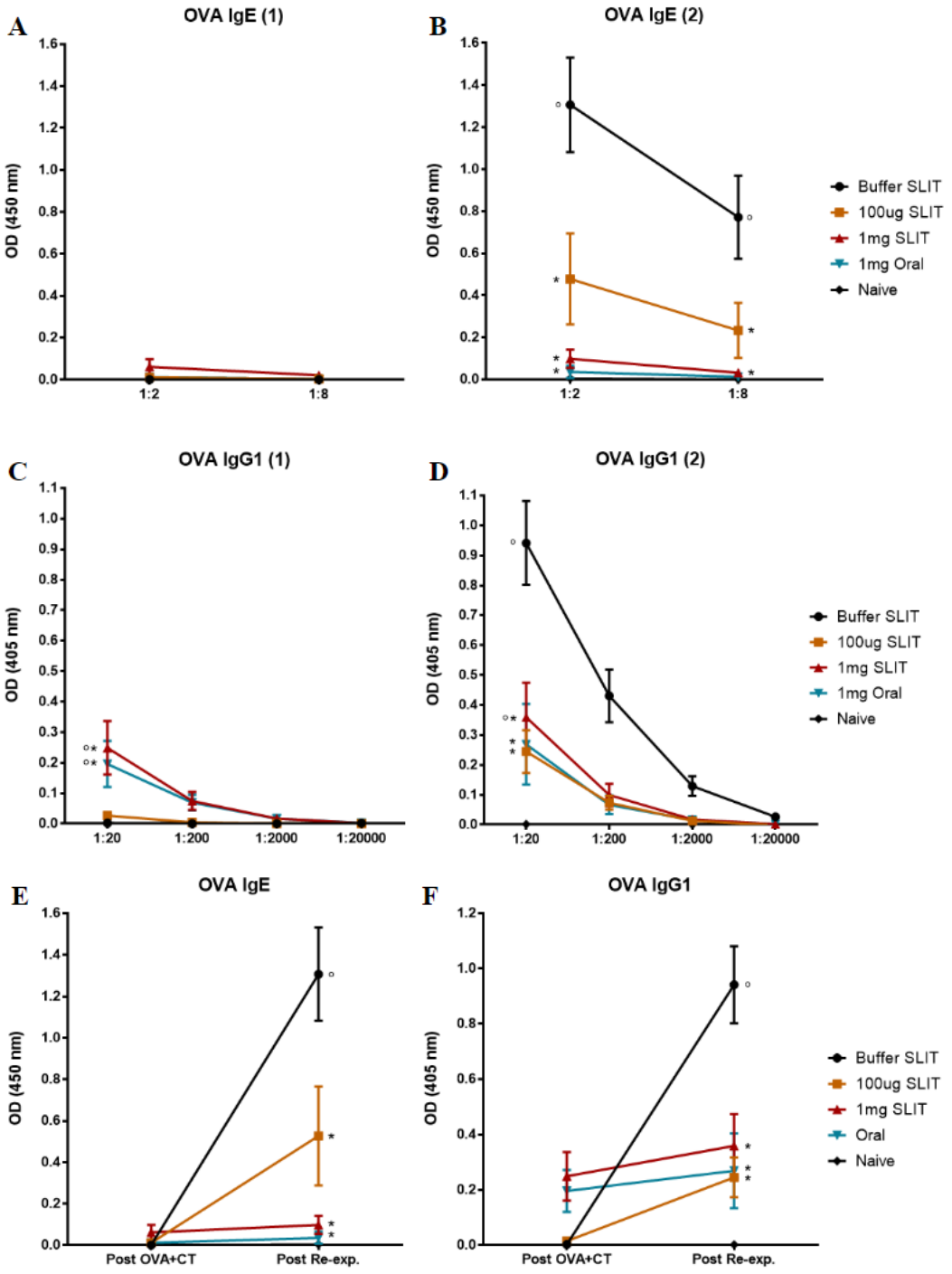


Figure 7. Prophylactic SLIT reduces the emergence of OVA-specific IgE antibodies after intragastric sensitization. **A-B**, serum OVA-specific IgE antibodies at two dilutions measured prior to subcutaneous allergen re-exposures (**A**) and one day prior to challenge (**B**). **C-D**, serum OVA-specific IgG1 antibodies at four dilutions measured prior to intragastric allergen re-exposures, (**C**) and one day prior

to challenge (**D**). **E-F**, comparison of OVA-specific IgE and IgG1 antibody titres across the allergen intragastric exposures. Data are representative of 1 experiment with 5-10 mice per group, plotted as mean ± SEM in comparison to the buffer SLIT group (*) or naïve group (°). *, ° $P<0.05$. OD, optical density; OVA, ovalbumin; CT, cholera toxin; Buffer, PBS SLIT; Oral, oral tolerance model; Re-exp, allergen re-exposures.

As expected, analysis of OVA-specific IgE levels in serum revealed no IgE production following the initial intragastric priming gavage (Figure 7A). IgE production was observed only at the second serum collection, *i.e.*, after the non-sensitizing OVA alone intragastric exposures (Figure 7B). The positive control group demonstrated a substantial increase in OVA-IgE production following allergen re-exposures (Figure 7E). The 100 μg SLIT-treated group demonstrated an intermediate, yet significant production of OVA-IgE in comparison to the positive control (Figure 7E). Consistent with no drop in core body temperature, both the 1 mg SLIT-treated group and the orally-exposed group had no increase in IgE production across both serum collection timepoints (Figure 7E). The levels of IgE in these groups were not statistically different from naïve mice (Figure 7E).

Previous data from our lab showed that following a single priming gavage, mice do not generate any measurable antibody response, as demonstrated by the buffer group and 100 μg SLIT-treated group (Figure 7C). Interestingly, both the 1 mg SLIT-treated, and oral tolerance group show a small, but significant level of OVA-IgG1 production following the priming gavage (Figure 7C). Upon the allergen re-exposures, the buffer- treated group had a large increase in OVA-IgG1 production (Figure 7D, F). The 100 μg SLIT-treated group had an increase in OVA-

IgG1 levels, although statistically lower than the levels of the buffer control group (Figure 7D, F). In the 1 mg SLIT-treated and oral tolerance group, there was little to no change in the levels of OVA-IgG1 (Figure 7D, F). The lack of change in the production of OVA-IgG1 was unique and may be a feature of establishing tolerance in the gut sensitization model.

Three days after the systemic allergen challenge, we harvested spleens to set up splenocyte cultures culture as mentioned previously. Since both the epicutaneous and priming model were analyzed at the same time, the following data also contain a high degree of variability, preventing us from drawing any definite conclusions. However, the trends observed in this set of data may provide some insight on markers of interest for future experimentation. Nonetheless, replicates have been stored and the analysis will be repeated to achieve a less variable response.

First, with respect to TH2 associated cytokines, we observed an induction of IL-4 by both the buffer and 100 μg SLIT-treated group upon OVA stimulation (Appendix 4A). However, upon stimulation with OVA, both the 1 mg SLIT-treated, and oral tolerance group showed no change in IL-4 secretion (Appendix 4A). As IL-4 is required for IgE class switching, the lack of it explains why both groups had no IgE production prior to challenge, and subsequent clinical reactivity. IL-5 seems to be induced in both the buffer-treated and 100 μg SLIT-treated groups (Appendix 4B). The 1 mg SLIT-treated, and oral tolerance group lacked an IL-5 response (Appendix 4B). Induction of IL-13 was seen to an extent in the buffer group, but

all other groups showed no response (Appendix 4C). In sum, we see a trend suggesting an inhibition of TH2 cytokines in the groups that exhibited low clinical reactivity upon challenge. No firm conclusions can be drawn with respect to IFN-γ and IL-10 due to the high variability. No changes were observed with OVA stimulation in IL-3, IL-12 (p70), and IL-9 (Appendix 4F-H). IL-17 was significantly induced in the buffer group and to a small degree in the oral tolerance group, but not in any other group (Appendix 4I).

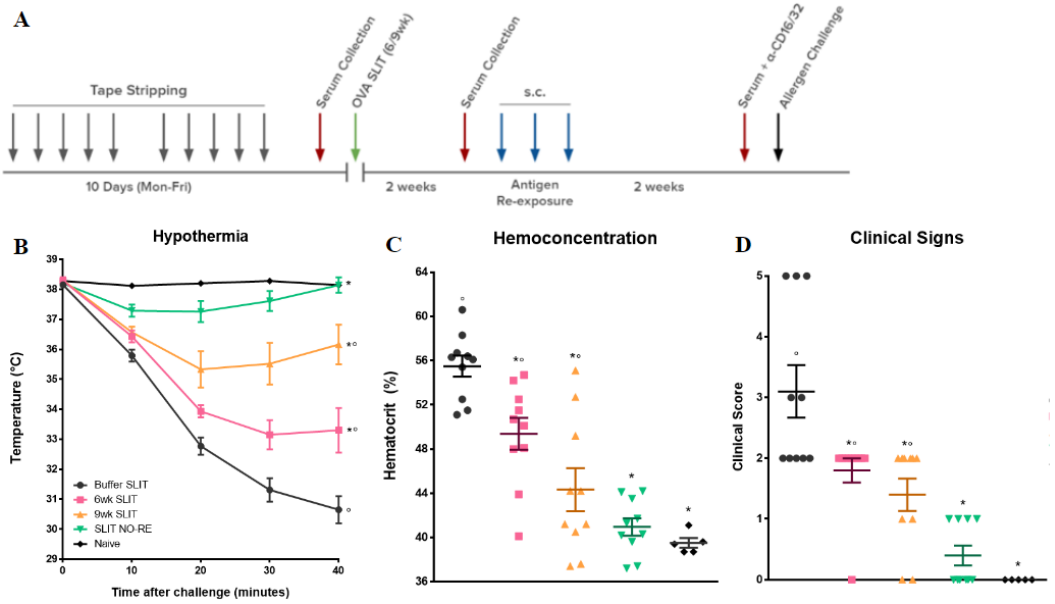


Figure 8. Semi-therapeutic SLIT (6/9 wk) partially protects epicutaneous sensitized mice from food-induced clinical reactivity. **A**, schematic for semi-therapeutic SLIT administration following tape-stripping. **B**, core body temperature during allergen challenge. **C**, hemoconcentration at 40 minutes post challenge. **D**, clinical scores during challenge (as described in Table 1). Data are representative of 1 experiment with 5-10 mice per group, plotted as mean ± SEM in comparison to the buffer SLIT group (*) or naïve group (°). *, ° $P<0.05$. SLIT no-re, SLIT treated mice that received no allergen re-exposures. OVA, ovalbumin; s.c., subcutaneous; Buffer, PBS SLIT; NO-RE, no allergen re-exposures.

Evaluation of semi-therapeutic OVA-SLIT in establishing protection against epicutaneous sensitization

The second phase of experimentation evaluated the efficacy of SLIT as a semi-therapeutic treatment. Here, C57BL/6 mice underwent the 10-day tape stripping protocol prior to receiving OVA-SLIT treatment (Figure 8A). Noted earlier, this model offers a unique feature in that the production of OVA-specific IgE only occurs after the 3 non-sensitizing subcutaneous OVA exposures. Therefore, we can examine whether administering OVA-SLIT prevented the transition from IgG1 to IgE. We denote the treatment as being semi-therapeutic, since the mice are sensitized as indicated by the presence of OVA-specific IgG1 and the capacity to generate an IgE response upon subsequent non-sensitizing exposures. We chose 1 mg of OVA to be the optimal dosage for SLIT, as it demonstrated the greatest level of protection in the prophylactic model. Additionally, we hypothesized that 3 weeks could be too short and, thus, extended it arbitrarily to 6 and 9 weeks. The 6-week SLIT-treated group was adjusted to start 3 weeks after the 9-week group. We also added a group that received 9 weeks of OVA-SLIT, but no subcutaneous OVA re-exposures (SLIT no-re), to examine if SLIT itself could induce OVA-IgE. Mice were rested for 2 weeks following SLIT, prior to receiving the OVA subcutaneous exposures (Figure 8A). An allergen challenge was conducted 2 weeks following the last subcutaneous exposure (Figure 8A).

The buffer group exhibited a significant drop in core body temperature, while the naïve mice showed no clinical responses (Figure 8B). Administering 6 weeks of OVA-SLIT resulted in partial protection (Figure 8B). Within this group, 5/10 mice had a partial drop in core body temperature prior to an increase towards recovery at the 30-minute mark (Appendix 5A). The response was variable with some mice recovering, others maintaining a partial level of protection and the rest reacting no differently than that of buffer control (Appendix 5A). Extending OVA-SLIT to 9 weeks statistically improved the level of protection (Figure 8B). Despite the degree of variability, the mean core body temperature of the 9-week OVA-SLIT group was ~36°C, in comparison to ~33°C for 6-weeks of OVA-SLIT and ~30°C for buffer control (Figure 8B, Appendix 5B). Closer analysis showed 4/10 mice with no change at the 40-minute mark in core body temperature in comparison to baseline, 5/10 mice with a partial response, and 1 mouse that initially had a 4°C drop in core body temperature but recovered back to baseline after 20-minutes (Appendix 5B). The SLIT no re-exposure group showed a small drop in core body temperature which recovered back to baseline at 40-minutes (Figure 8B). Although the temperature measurements were not statistically different than those of the naïve group (Figure 8B), there was some variability (Appendix 5C). This indicates that SLIT by itself can induce a slight degree of reactivity.

Examination of hemoconcentration data demonstrated a partial increase in comparison to the buffer group when treated with either 6 or 9 weeks of OVA-SLIT (Figure 8C). Despite variability, 9 weeks of OVA-SLIT had most mice clustered

towards a hemoconcentration similar to that of the naïve group (Figure 8C). Both the 9-week OVA-SLIT treated and SLIT no re-exposure group had a similar level of hemoconcentration to that of naïve mice (Figure 8C).

Clinical scores followed a similar trend as seen with the other outcomes. The buffer group had the highest score, with 3/10 mice having seizures/prodding, 2/10 mice motionless, and 5/10 mice exhibiting reduced motion (Figure 8D). Clinical scores were significantly better in all SLIT-treated groups (Figure 8D). In the 6-week SLIT-treated group, 9/10 mice exhibited only reduced motion and 1/10 mice had no response (Figure 8D). For the 9-week SLIT-treated group, 6/10 mice had reduced motion, 2/10 mice displayed in-ear scratching, and 2/10 mice had no clinical response (Figure 8D). Within the SLIT no-re-exposure group, 4/10 mice experienced in-ear scratching, and no clinical signs were observed in the naïve group (Figure 8D). Collectively, these findings indicate that 9 weeks of OVA-SLIT provides a superior protection compared to 6 weeks.

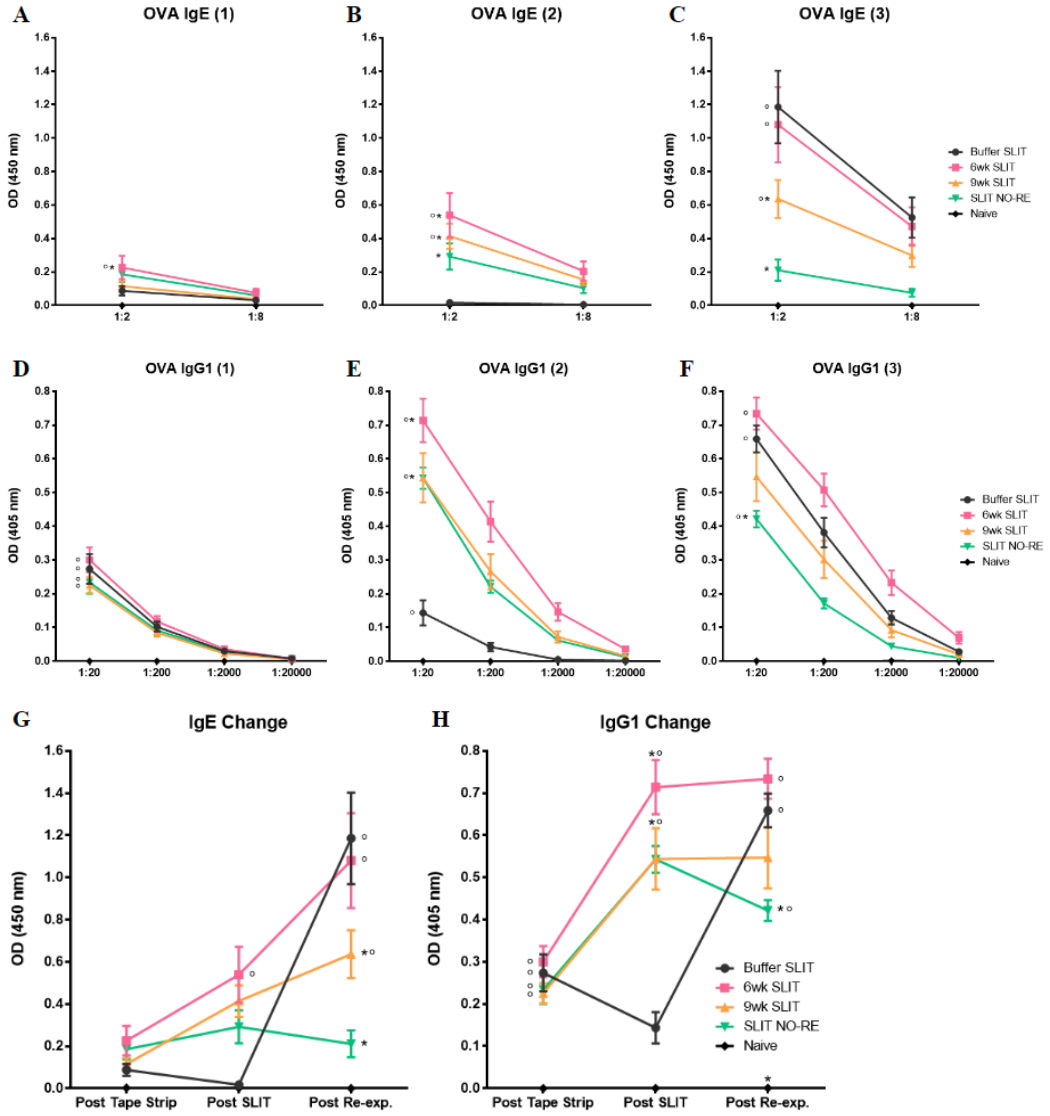


Figure 9. Semi-therapeutic SLIT partially prevents the emergence of OVA-specific IgE antibodies after epicutaneous sensitization. **A-C**, serum OVA-specific IgE antibodies at two dilutions measured after the tape stripping (**A**), after the cessation of SLIT (**B**), and one day prior to challenge (**C**). **D-F**, serum OVA-specific IgG1 antibodies at four dilutions measured after tape stripping (**D**), after the cessation of SLIT (**E**), and one day prior to challenge (**F**). **G-H**, comparison of OVA-specific IgE and IgG1 antibody titres across the measured timepoints. Data are representative of 1 experiment with 5-10 mice per group, plotted as mean ± SEM in comparison to the buffer SLIT group (*) or naïve group (°). *, ° $P<0.05$. OD, optical density; Buffer, PBS SLIT; NO-RE, no allergen re-exposures, Re-exp, allergen re-exposures.

Serum was collected at 3 time points: 1) after the 10 days of tape stripping, 2) after the 6-9 weeks of OVA-SLIT, and 3) one day prior to allergen challenge (Figure 8A). Following tape stripping, a small amount of OVA-IgE production is only seen in the 6-week SLIT-treated group, although at extremely low levels (Figure 9A). Post SLIT treatment, we saw low levels of OVA-IgE antibodies in all SLIT-treated groups (Figure 9B). Upon subcutaneous exposures, the buffer, 6- and 9-week SLIT-treated groups all showed IgE production (Figure 9C). Low levels of OVA-IgE are noted for the SLIT no re-exposures group, consistent with the slight reactivity seen during the allergen challenge (Figure 8D). Examining the change in IgE levels over the three serum collection points, the 6-week SLIT group produced levels of OVA-IgE no different than the buffer group (Figure 9G). The 9-week SLIT group demonstrated an intermediate level of OVA-IgE, significantly lower than both the buffer and 6-week SLIT groups (Figure 9G). The SLIT no re-exposures treated group maintained low levels of OVA-IgE across the serum collections (Figure 9G).

As expected, all tape-stripped mice (excluding naïve mice), had an OVA-IgG1 response (Figure 9D). Treatment with OVA-SLIT resulted in an increase in the levels of OVA-IgG1 in comparison to the buffer group (Figure 9E, H). Upon the administration of subcutaneous OVA re-exposures, all groups, excluding naïve mice, produced OVA-IgG1 (Figure 9E, H). Within the SLIT-treated groups, administration of OVA subcutaneous re-exposures did not increase the levels of OVA-IgG1, but rather the levels were sustained from the post-SLIT serum

collection (Figure 9H). The data indicate that the levels of OVA-specific IgG1 for both the 6- and 9-weeks of SLIT treatment were not significantly different from that of the buffer control.

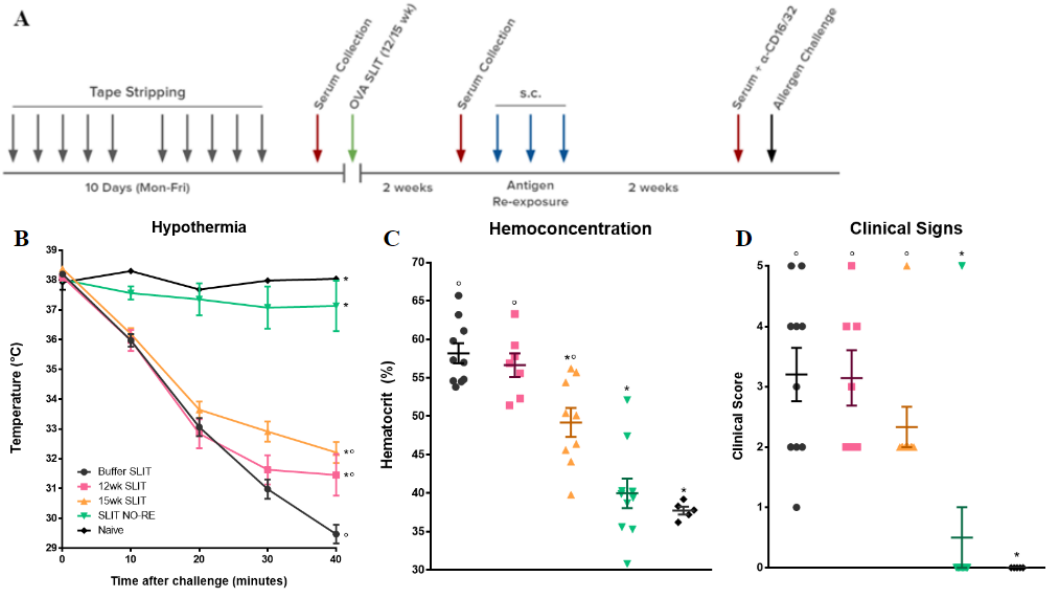


Figure 10. Impact of semi-therapeutic SLIT (12/15 wk) on food-induced clinical reactivity in epicutaneously sensitized mice. **A**, schematic for semi-therapeutic SLIT administration following tape-stripping. **B**, core body temperature during systemic allergen challenge. **C**, hemoconcentration at 40 minutes post systemic challenge. **D**, clinical scores during systemic challenge (as described in Table 1). Data are representative of 1 experiment with 5-10 mice per group, plotted as mean ± SEM in comparison to the buffer SLIT group (*) or naïve group (°). *, ° P<0.05. OVA, ovalbumin; s.c., subcutaneous; Buffer, PBS SLIT; NO-RE, no allergen re-exposures.

Evaluation of extended semi-therapeutic OVA-SLIT in establishing protection following epicutaneous sensitization

Following the previous set of experiments, we sought to further improve the level of protection offered by semi-therapeutic OVA-SLIT. Our results established that 9 weeks of OVA-SLIT provided a higher degree of protection in comparison to 6 weeks. Therefore, we hypothesized that extending the duration of SLIT to 12-15 weeks would provide better protection. This set of experimentation followed the same procedures as mentioned previously, except for a longer duration of OVA SLIT (Figure 10A).

All mice in the buffer group had a drastic drop in core body temperature reaching an average of ~29.5°C (8.5-degree change) at 40-minutes in comparison to the negative control (Figure 10B). Surprisingly, both SLIT-treated groups exhibited a significant drop in core body temperature, although slightly but significantly less than the buffer group (Figure 10B). The drop in core body temperatures between the two SLIT-treated groups was not significant (Figure 10B). Unlike previous data, we did not observe recovery in the SLIT-treated groups, and most mice became clinically reactive (Appendix 6A, B). The SLIT no re-exposures group had one mouse that fully responded, and one mouse that had a partial drop in core body temperature (Appendix 6C). The variability amongst the SLIT no re-exposures group was noticeably less in comparison to the previous set of experimentation.

Buffer and the 12-week SLIT group had similar levels of hemoconcentration (Figure 10C). The 15-week SLIT group had a significantly lower hemoconcentration, although a large degree of variability was seen (Figure 10C). The SLIT no re-exposures group demonstrated variable responses but, as a group, the response was not significantly different than that of the naïve group (Figure 10C).

The buffer, 12-week SLIT, and 15-week SLIT all had statistically similar clinical scores (Figure 10D). Within the buffer group, 2/10 mice had seizures/prodding, 3/10 had no whisker response, 1/10 was motionless, 2/10 were reduced, and 1 had in-ear scratching (Figure 10D). Within the 12-week SLIT group, 1/10 mice underwent seizure/prodding in, 2/10 had no whisker response, 1/10 was motionless, and 6/10 had reduced motion (Figure 10D). Within the 15-week SLIT group, only 1/10 experienced seizure/prodding, and 9/10 had reduced motion (Figure 10D). One mouse in the SLIT no re-exposures group had a seizure, but the remainder had no clinical response (Figure 10D). When examining the clinical scores, 15-weeks of OVA-SLIT appeared to better protect the mice against clinical reactivity, demonstrated by the large proportion of mice with lower clinical scores in comparison to 12-weeks of SLIT (Figure 10D). We expected OVA-SLIT to improve the level of protection following an extended duration, but this was not the case. Clearly, the drop in core body temperature and clinical scores indicate that the 12- and 15-weeks of OVA-SLIT was not protective.

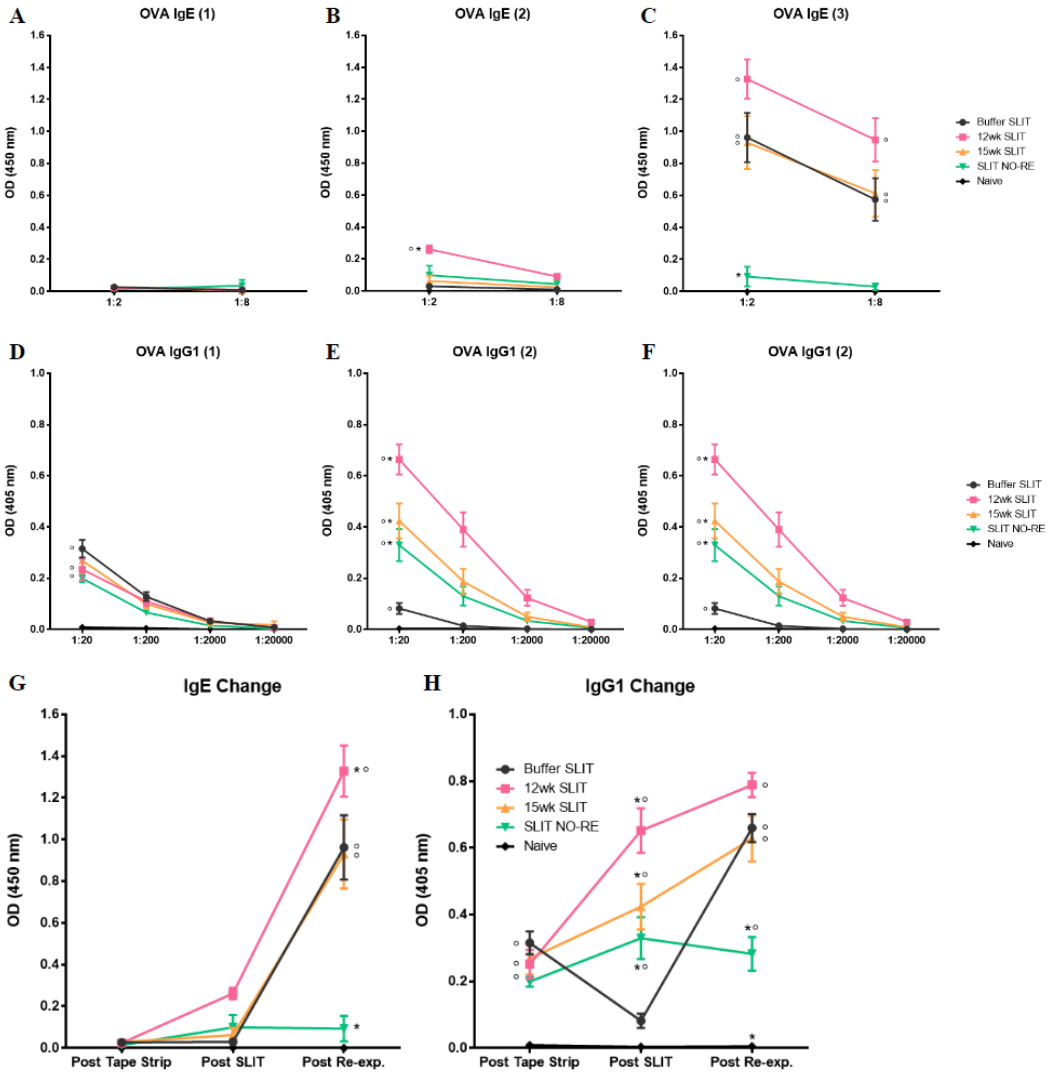


Figure 11. Impact of semi-therapeutic SLIT on the emergence of OVA-specific IgE and IgG1 after epicutaneous sensitization. **A-C**, serum OVA-specific IgE antibodies at two dilutions measured after the tape stripping (**A**), after the cessation of SLIT (**B**), and one day prior to challenge (**C**). **D-F**, serum OVA-specific IgG1 antibodies at four dilutions measured after tape stripping (**D**), after the cessation of SLIT (**E**), and one day prior to challenge (**F**). **G-H**, comparison of OVA-specific IgE and IgG1 antibody titres across the measured timepoints. Data are representative of 1 experiment with 5-10 mice per group, plotted as mean ± SEM in comparison to the buffer SLIT group (*) or naïve group (°). *, ° P<0.05. OD, optical density; Buffer, PBS SLIT; NO-RE, no allergen re-exposures, Re-exp, allergen re-exposures.

Analysis of the immunoglobulins demonstrated no increase in OVA-IgE in all groups at the post-tape stripping serum collection point, as expected (Figure 11A). Following the administration of OVA-SLIT, IgE only significantly increased in the 12-week OVA-SLIT group (Figure 11B). Interestingly, we did not see any induction of OVA-IgE in the 15-week OVA-SLIT-treated group (Figure 11B). The levels of OVA-IgE at this timepoint were extremely low, so lack of significance could be due to variability between groups. Buffer, 12-week, and 15-week SLIT-treated groups all produced OVA-IgE upon subcutaneous allergen re-exposures (Figure 11C). The levels of OVA-IgE were not significantly different amongst the three groups (Figure 11C, G). Over the course of the three bleeding points, the 12-week SLIT group had a significantly higher amount of OVA-IgE in comparison to the positive control (Figure 11G). Although not significant in comparison to the naïve group, we detected low levels of OVA-IgE in the SLIT no re-exposures group, possibly explaining the variable number of intermediate responses observed during the allergen challenge (Figure 11G).

All groups, except naïve mice, generated OVA-IgG1 upon the completion of tape stripping (Figure 11D). After the administration of OVA-SLIT, all SLIT-treated groups showed an increase in OVA-IgG1 (Figure 11E). The largest change in OVA-IgG1 production across these two bleeding points was seen in the 12-week OVA-SLIT-treated group (Figure 11E, H). Following the allergen re-exposures, OVA-IgG1 production was detected amongst all groups, although to a significantly lower extent in the SLIT no re-exposures group (Figure 11F). The SLIT no re-

exposures treated group maintained a similar level of OVA-IgG1 across all three bleeding points (Figure 11H). Overall, semi-therapeutic OVA-SLIT induced an OVA-IgG1 response, although no differences were observed in the production of OVA-IgG1 at the final bleed point (Figure 11H).

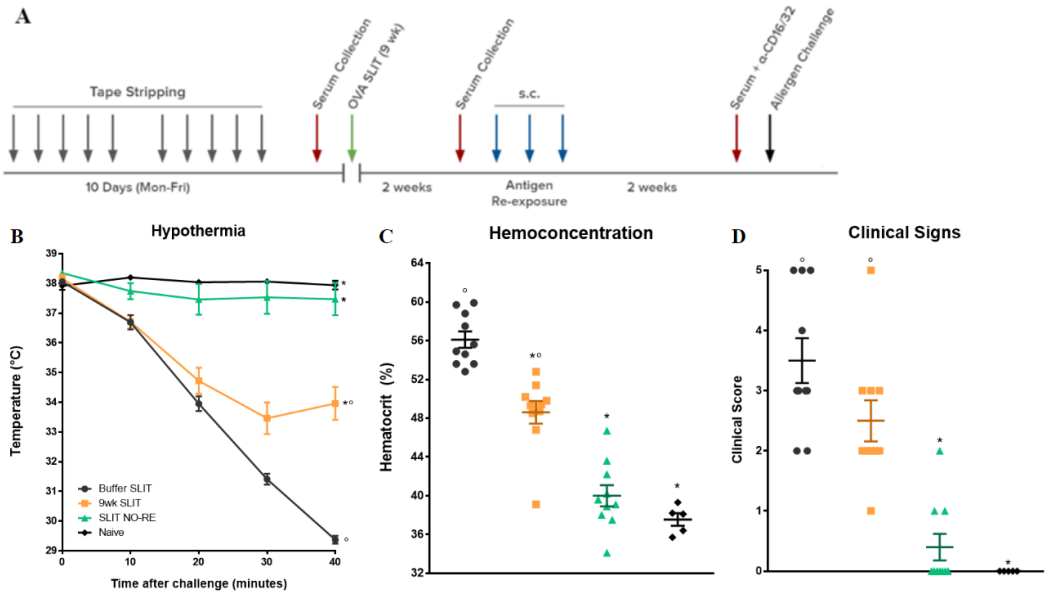


Figure 12. Experimental repeat of the 9-week semi-therapeutic SLIT protocol confirms protection from food-induced clinical reactivity in epicutaneously sensitized mice. A, schematic for semi-therapeutic SLIT administration following tape-stripping. B, core body temperature during systemic allergen challenge. C, hemoconcentration at 40 minutes post challenge. D, clinical scores during challenge (as described in Table 1). Data are representative of 1 experiment with 5-10 mice per group, plotted as mean ± SEM in comparison to the buffer SLIT group (*) or naïve group (°). *, ° P<0.05. OVA, ovalbumin; s.c., subcutaneous; Buffer, PBS SLIT; NO-RE, no allergen re-exposures.

Confirmation of the protection following epicutaneous sensitization and subsequent clinical reactivity by 9-weeks of semi-therapeutic OVA-SLIT

It was intriguing to find that 12 and 15 weeks of OVA SLIT conferred less protection than 9-weeks. Therefore, we conducted a repeat of the 9-week OVA SLIT group to confirm our initial findings (Figure 12A). This experiment followed the exact protocol as mentioned in Figure 10A, with the exclusion of the 6-week OVA-SLIT group.

Upon challenge, we saw very similar results in the drop in core body temperature, hemoconcentration, clinical scores, change in OVA-IgE and IgG1 in comparison to the initial experiment (Figure 8-9, 12-13, Appendix 7). Pooled results consistently demonstrated that 6 and 9 weeks of SLIT treatment provided partial protection against food-mediated clinical reactivity (Appendix 10).

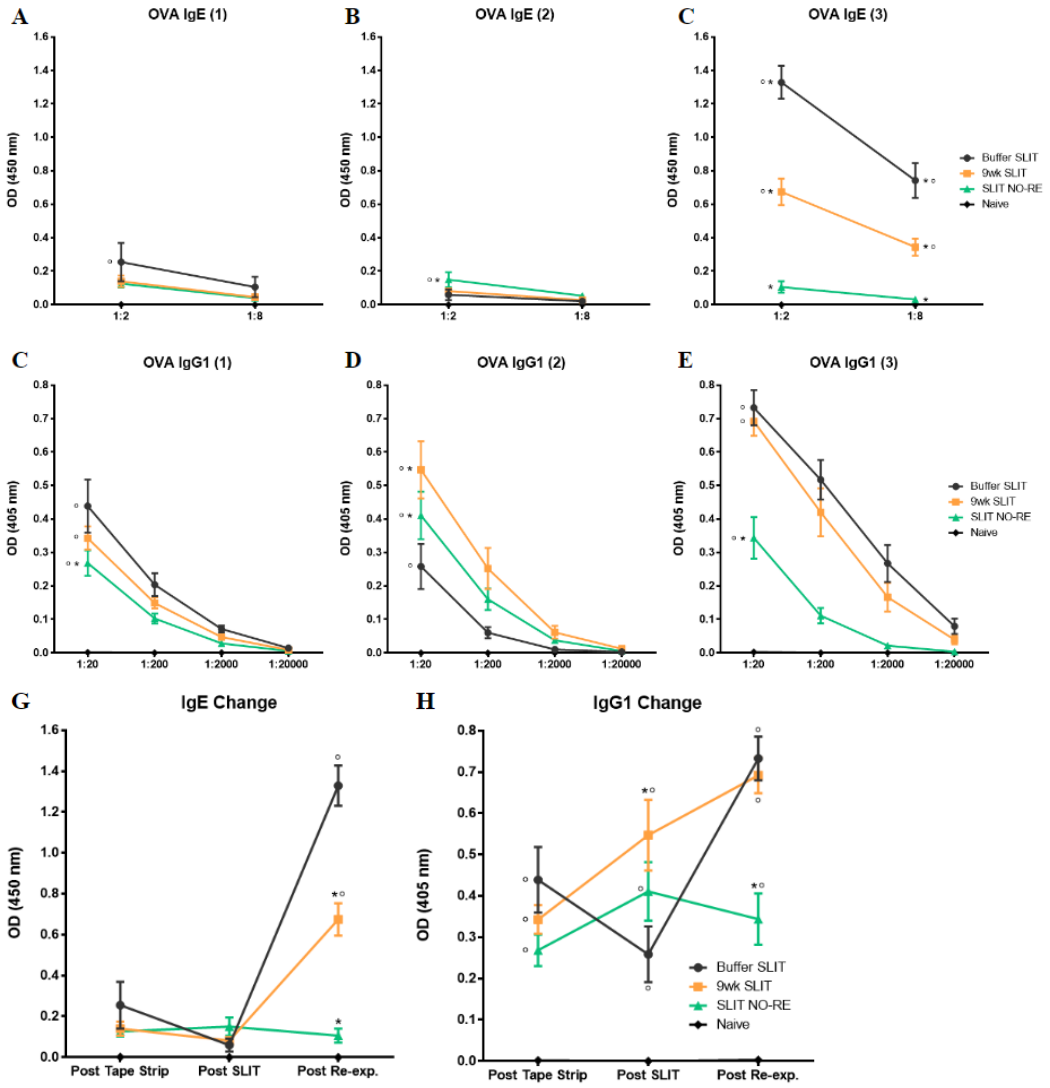


Figure 13. Impact of 9-week SLIT treatment on the emergence of OVA-specific IgE in epicutaneously sensitized mice. A-C, serum OVA-specific IgE at two dilutions measured after the tape stripping (A), after the cessation of SLIT (B), and one day prior to challenge (C). D-F, serum OVA-specific IgG1 antibodies at four dilutions measured after tape stripping (A), after the cessation of SLIT (B), and one day prior to challenge (C). G-H, comparison of OVA-specific IgE and IgG1 antibody titres across the measured timepoints. Data are representative of 1 experiment with 5-10 mice per group, plotted as mean ± SEM in comparison to the buffer SLIT group (*) or naïve group (°). *, ° P<0.05. OD, optical density; Buffer, PBS SLIT; NO-RE, no allergen re-exposures, Re-exp, allergen re-exposures.

Analysis of the immunoglobulin response was consistent with the initial set of experimentation. As expected, little to no OVA-IgE is detected in all groups following tape stripping (Figure 13A). Interestingly, a small amount of OVA-IgE was only detected in the SLIT no re-exposures group following the conclusion of SLIT treatment (Figure 13B). Data pooled with the initial experiment were consistent, with all SLIT-treated groups exhibiting production of OVA-IgE following SLIT treatment, and one day prior to challenge (Appendix 10D). However, the OVA-IgE one day prior to challenge in the 9 week SLIT-treated group was significantly lower than those in the buffer and the 6 week SLIT-treated group (Appendix 10D). Serum levels of OVA-IgG1 remained consistent with the initial findings amongst all groups (Figures 9H, 13H, Appendix 10E). Overall, the results of this experiment confirm our initial findings indicating that 9 weeks of semi-therapeutic OVA SLIT was partially protective against food-mediated clinical reactivity.

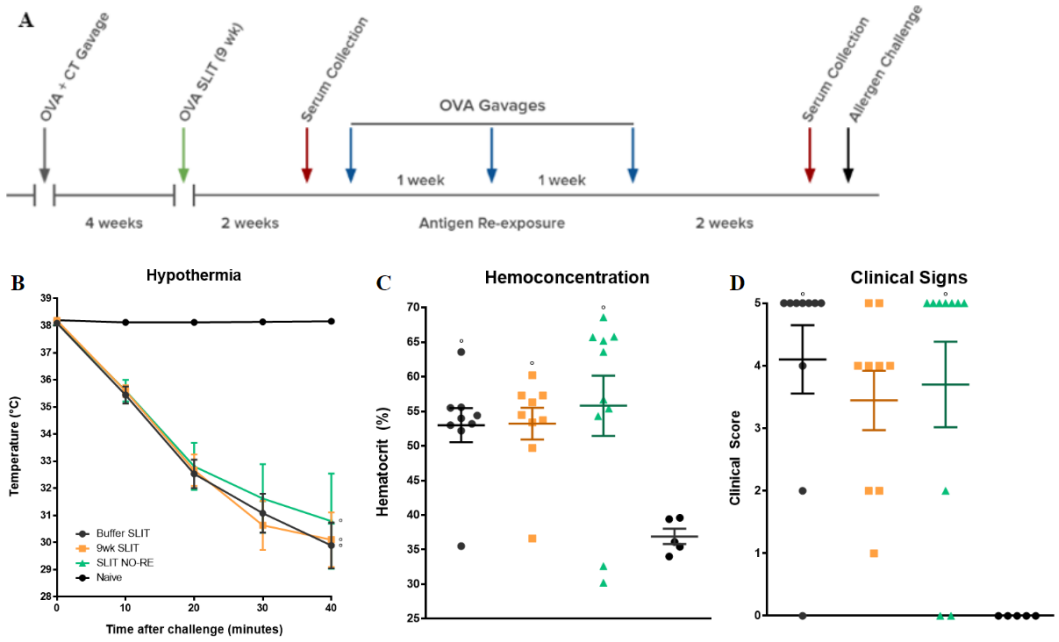


Figure 14. Semi-therapeutic SLIT treatment for 9 weeks following intragastric sensitization was not protective against food-induced clinical reactivity. **A,** schematic for semi-therapeutic SLIT administered following the initial priming intragastric gavage. **B**, core body temperature during allergen challenge. **C**, hemoconcentration at 40 minutes post challenge. **D**, clinical scores during challenge (as described in Table 1). Data are representative of 1 experiment with 5-10 mice per group, plotted as mean ± SEM in comparison to the buffer SLIT group (*) or naïve group (°). *, ° P<0.05. OVA, ovalbumin; CT, cholera toxin; Buffer, PBS SLIT; NO-RE, no allergen re-exposures.

Evaluation of semi-therapeutic OVA-SLIT in establishing protection following intragastric sensitization

The second part of phase 2 entails evaluating the efficacy of semi-therapeutic SLIT in a gut model of allergic sensitization. Here, C57BL/6 mice first received a single intragastric priming gavage of OVA + CT (Figure 14A). Mice were rested for four weeks prior to the administration of OVA-SLIT. As mentioned earlier, the mice do not generate a detectable OVA-specific antibody response following the priming oral gavage; however, IgE and IgG1 are both detected upon

the subsequent OVA-alone intragastric exposures. Like the epicutaneous model, the priming model allows us to examine whether OVA-SLIT is capable in preventing the generation of IgE in primed mice. The prophylactic gut model indicated 1 mg of OVA as the optimal protective dose for SLIT treatment, and thus, all SLIT-treated mice received this dose. Additionally, the duration of SLIT was increased to 9-weeks, as we hypothesized that a longer treatment could be required for establishing protection. Two weeks after the final administration of SLIT, mice received a non-sensitizing OVA-alone intragastric gavage, once a week, for a total of three weeks (Figure 14A). An allergen challenge was conducted two weeks following the final OVA gavage. During our initial experimentation, we had two groups, 6 and 9 weeks, for the duration of SLIT; however, this experiment failed due to variable responses in our positive control. Therefore, we used a second set of mice that had completed the priming gavage and were initially scheduled as a repeat. Due to the number of mice available at that time, we decided it was best to only include the 9-week SLIT group.

We found that treating mice with 9 weeks of OVA-SLIT was not protective in this setting (Figure 14B). The buffer group had a significant drop in core body temperature, with 1 mouse being a non-responder (Figure 14B, Appendix 8A). Within the SLIT-treated group, 8/9 mice responded to a similar degree as the positive control, and only 1 mouse had no drop in core body temperature (Figure 14B, Appendix 8A). The SLIT no re-exposures group had 8/10 mice respond to the same degree as the positive control and only 2/10 mice showed no drop in core

body temperature (Figure 14B, Appendix 8B). Consistent with the drop in core body temperatures, we observed that all groups, excluding naïve mice, had a similar increase in hemoconcentration and, thus, no different from each other (Figure 14C). Overall, 9 weeks of 1 mg OVA-SLIT did not prevent anaphylactic reactions.

Despite all groups demonstrating clinical scores not statistically significant from each other, we did observe variance in the SLIT-treated mice (Figure 14D). Within the buffer group, 7/10 mice had seizures/prodding, 1/10 had no whisker response, 1/10 had reduced motion, and 1/10 was a non-responder (Figure 14D). In the OVA-SLIT group, 2/9 mice had seizures/prodding, 4/9 mice had no whisker response, 2/9 were reduced and 1/9 had in-ear scratching (Figure 14D). In comparison to the positive control, a lower proportion of mice reached the highest clinical score, and thus, possibly indicating some protection conferred from SLIT. Within the SLIT no re-exposures group, 7/10 mice had seizures/prodding, 1/10 had reduced motion and 2/10 had no clinical symptoms. Together, these data indicate that 1 mg of SLIT following intragastric sensitization was not protective, suggesting that further optimization of dosage and duration was required.

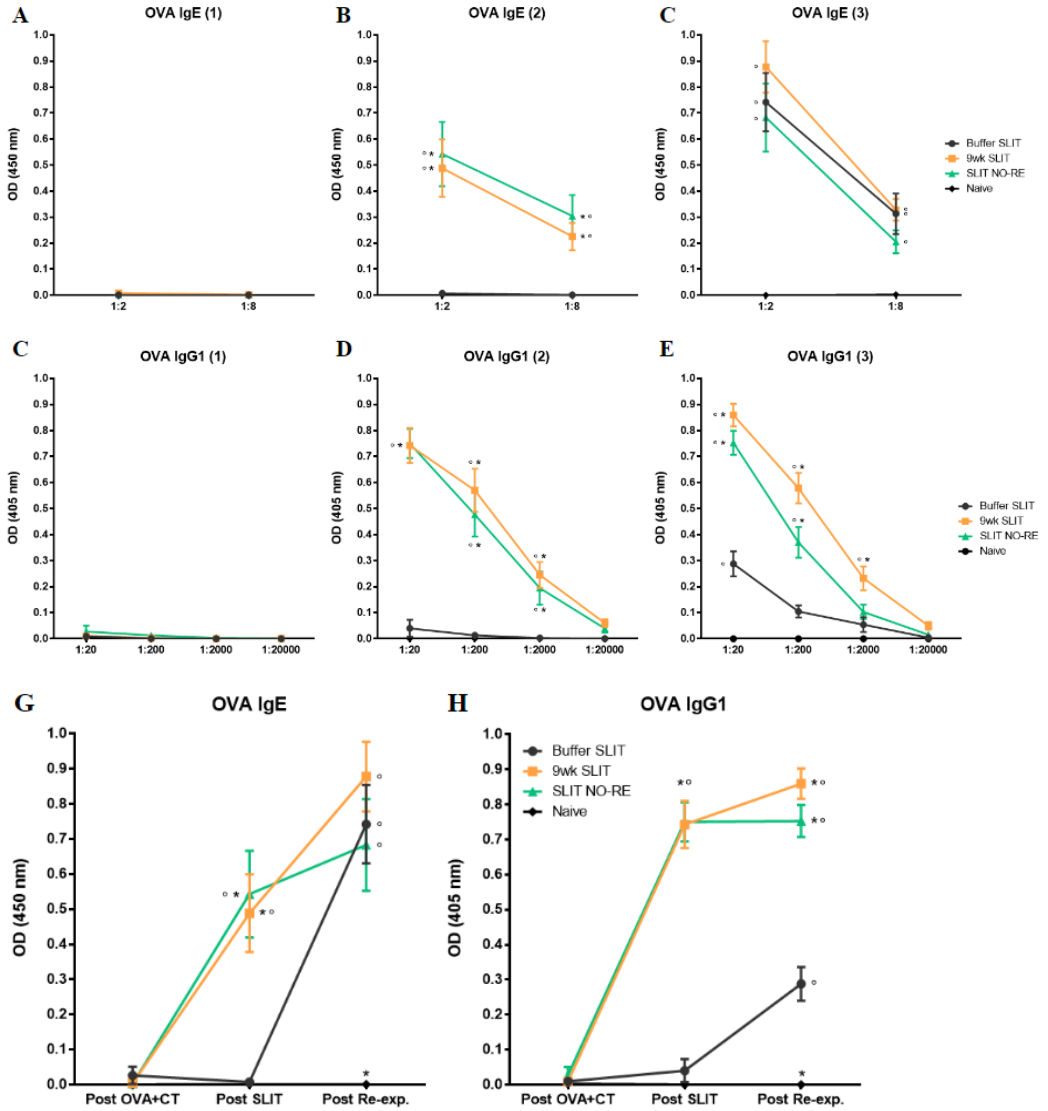


Figure 15. Semi-therapeutic SLIT does not prevent the emergence of OVA-specific IgE and IgG1 after intragastric sensitization. **A-C**, serum OVA-specific IgE at two dilutions measured after the priming gavage (**A**), after the cessation of SLIT (**B**), and one day prior to systemic challenge (**C**). **D-F**, serum OVA-specific IgG1 at four dilutions measured after the priming gavage (**D**), after the cessation of SLIT (**E**), and one day prior to challenge (**F**). **G-H**, comparison of OVA-specific IgE and IgG1 levels across the measured timepoints. Data are representative of 1 experiment with 5-10 mice per group, plotted as mean ± SEM in comparison to the buffer SLIT group (*) or naïve group (°). *, ° $P<0.05$. OD, optical density; Buffer, PBS SLIT; NO-RE, no allergen re-exposures; Re-exp, allergen re-exposures.

Serum was collected at three different timepoints: 1) four weeks after the initial priming gavage, 2) after the conclusion of SLIT treatment, and 3) one day prior to the systemic allergen challenge. As expected, no OVA-IgE was detected amongst all groups following the priming gavage (Figure 15A). OVA-IgE levels within the SLIT-treated groups were detected prior to the OVA-alone gavages (Figure 15B). The amount of OVA-IgE detected was not significantly different between the two SLIT groups. Upon the non-sensitizing OVA gavages, the buffer, OVA-SLIT and SLIT no re-exposures groups all had detectable levels of OVA-IgE (Figure 15C). Across the serum collection timepoints, treatment with SLIT resulted in the production of OVA-IgE, at amounts similar to the buffer group prior to challenge (Figure 15G). Similar levels of OVA-IgE between the SLIT-treated and buffer groups is consistent with the clinical findings.

No OVA-IgG1 was detected following the priming gavage within all groups (Figure 15D). Both SLIT-treated groups had a significant increase in OVA-IgG1 production following SLIT treatment, in comparison to the buffer group (Figure 15E, H). Following the OVA gavages, all groups, excluding naïve mice, showed detectable levels of OVA-IgG1 (Figure 15F, H). Interestingly, both SLIT-treated groups showed significantly more OVA-IgG1 antibodies in comparison to the buffer group, unlike what was observed in the semi-therapeutic SLIT, skin sensitized mice (Figure 15F, H). It is uncertain whether this change is meaningful or is the result of variance seen between these models. We do not suspect that the

difference in OVA-specific IgG1 levels had any impact on the clinical results of the systemic allergen challenge.

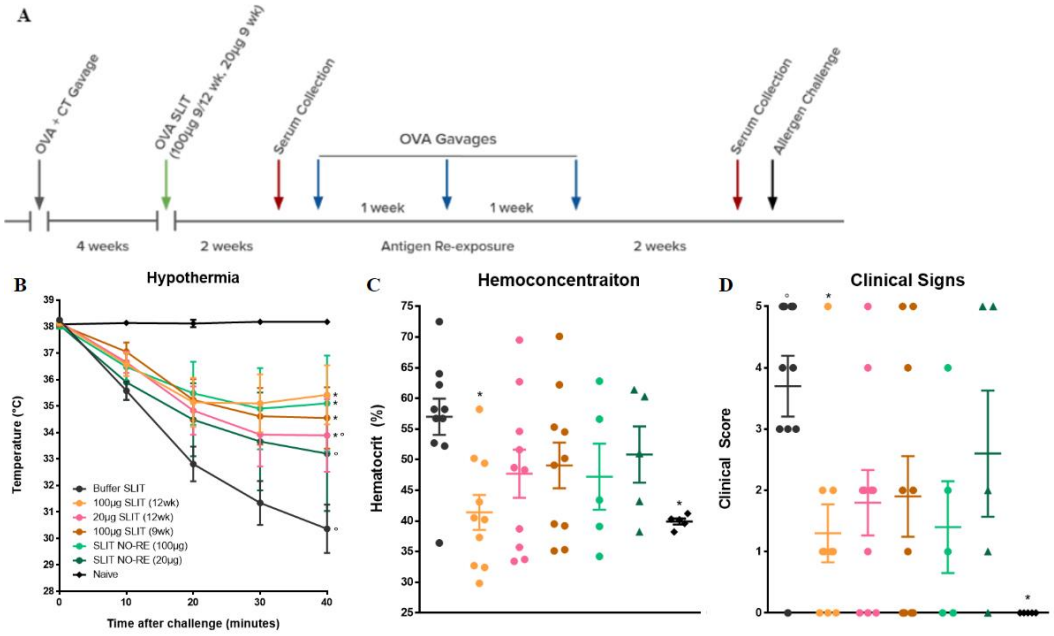


Figure 16. Lower dosed semi-therapeutic SLIT partially protects intragastric sensitized mice form food-induced clinical reactivity. **A**, schematic for semi-therapeutic SLIT administration following priming gavage. **B**, core body temperature during allergen challenge. **C**, hemoconcentration at 40 minutes post challenge. **D**, clinical scores during challenge (as described in Table 1). Data are representative of 1 experiment with 5-10 mice per group, plotted as mean ± SEM in comparison to the buffer SLIT group (*) or naïve group (°). *, ° P<0.05. OVA, ovalbumin; CT, cholera toxin; NO-RE, no allergen re-exposures.

Lowering the dose of OVA-SLIT confers partial protection against food-induced clinical reactivity within intragastric sensitized mice

The previous set of data demonstrated that treatment of primed mice with 9 weeks of 1 mg OVA-SLIT was not protective against food-induced clinical reactivity. Therefore, we sought to examine treatment with lower doses of SLIT, specifically 100 and 20 µg of OVA. Additionally, we hypothesized that increasing

the duration of SLIT with a lowered dosage could be required to achieve protection. With that in mind, we assessed the 100 μg dose for 9 and 12 weeks, and the 20 μg dose for 9 weeks. Additionally, we included two separate groups for the SLIT no re-exposures group, one for the 100 μg dose, and one for the 20 μg dose, both 12 weeks. Mice followed a similar protocol as the previous experiment, outlined above (Figure 16A).

The buffer control group had a drastic drop in core body temperature, with 9/10 mice dropping to a mean temperature of ~30.4°C (Figure 16B). One mouse was a non-responder and showed no drop in core body temperature (Figure 16B, Appendix 9A). Treatment with the 9-week 100 μg OVA-SLIT protocol was partially protective as the mean temperature drop was 34.6°C at 40 minutes (Figure 16B). Here, 3/10 mice had a severe response, 3/10 mice had an intermediate drop in core body temperature, and 4/10 mice had no drop in core body temperature (Appendix 9A). Extending this SLIT treatment to 12-weeks provided similar protection (Figure 16B). Here, 3/10 mice had a severe response no different than buffer, 2/10 mice had a partial response, recovering after 20 minutes, 1/10 had an initial drop in temperature, but proceeded to recover back to baseline at 40 minutes, and 4/10 mice had no drop in core body temperature (Appendix 9B). Decreasing the dose of SLIT to 20 μg for 12 weeks was still protective, demonstrating an intermediate drop in core body temperature, reaching ~33.9°C at 40 minutes (Figure 16B). Within the 20 μg SLIT group, 2/10 mice had a severe drop in core body temperature, 4/10 had an intermediate drop, and 4/10 had no drop in core body

temperature (Appendix 9C). The 12-week 20 μg SLIT group performed similarly to the 9-week 100 μg SLIT treated group. The 100 μg SLIT no re-exposures group did have a statistically significant drop in core body temperature lower than the buffer group, reaching ~35.1°C at 40 minutes (Figure 16B). Here, 2/5 mice had a complete response, 1/5 had a partial response, recovering towards baseline after 20 minutes, and 2/5 had no drop in core body temperature (Appendix 9A). The 20 μg SLIT no re-exposures group demonstrated a similar level of protection from a drop in core body temperature, although this was not statistically significant in comparison to the buffer group due to a large standard error (Figure 16B). Here, 2/5 mice had a complete response, 1/5 had an intermediate drop, and 2/5 had no drop in core body temperature (Appendix 9E).

Measurement of hemoconcentration yielded large variability amongst the groups. The buffer group had the highest level of hemoconcentration, which was not statistically significant from among all groups, excluding naïve and the 12-week 100 μg SLIT-treated group (Figure 16C). All SLIT-treated groups had a high degree of variability in their hemoconcentration, consistent with the variability seen within the drop in core body temperatures. The 12-week 100 μg SLIT group had many mice clustering towards the lower end of hemoconcentration, suggesting an optimal dose protocol (Figure 16C).

Clinical signs were consistent with findings in core temperature and hemoconcentration. Within the buffer group, 4/10 mice had seizures/prodding, 2/10 had no whisker response, 3/10 were motionless and 1 was a non-responder (Figure

16D). The 12-week 100 μg SLIT-treated mice were the only group with a significantly lower clinical score in comparison to the buffer control (Figure 16D). Within the 12-week 100 μg SLIT-treated group, 1/10 mice had a seizure/prodding, 2/10 mice had reduced motion, 4/10 mice had in-ear scratching, and 3/10 mice had no clinical symptoms (Figure 16D). In comparison to the buffer, we observed an increase in the number of mice clustering in the 1-2 clinical score range, suggesting protection by the SLIT treatment. The 9-week 100 μg SLIT-treated group had 1/10 mice experience seizure/prodding, 1/10 had no whisker response, 4/10 had reduced motion, 1/10 had in-ear scratching and 3/10 had no symptoms (Figure 16D). Similarly, the 12-week 20 μg SLIT-treated group had 2/10 mice experience seizure/prodding, 1/10 had no whisker response, 2/10 had reduced motion, 1/10 had in-ear scratching, and 4/10 had no symptoms (Figure 16D). For the 100 μg SLIT no re-exposures had 1/5 mice with no whisker response, 1/5 had reduced motion, 1/10 with in-ear scratching, and 2/5 with no symptoms (Figure 16D). For the 20 μg SLIT no re-exposures group, 2/5 mice had seizures/prodding, 1/5 had reduced motion, 1/5 with in-ear scratching and 1 had no clinical symptoms (Figure 16D). Therefore, SLIT by itself does induce a slight degree of clinical reactivity. Clearly, the best protection, although partial, was achieved with the 12-week 100 μg SLIT group protocol.

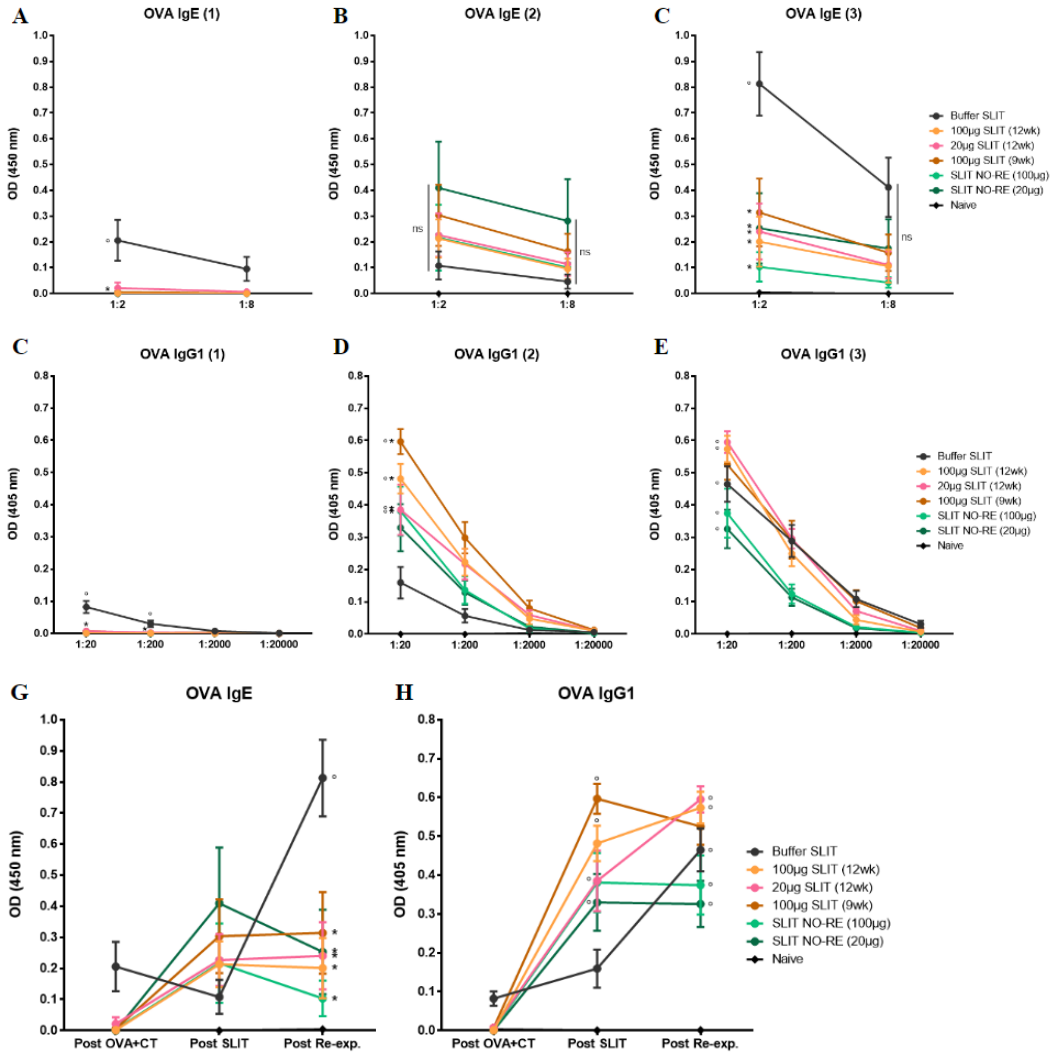


Figure 17. Semi-therapeutic SLIT after intragastric sensitization partially prevents the emergence of OVA-specific IgE. **A-C**, serum OVA-specific IgE at two dilutions measured after the intragastric priming gavage (**A**), after the cessation of SLIT (**B**), and one day prior to challenge (**C**). **D-F**, serum OVA-specific IgG1 at four dilutions measured after the intragastric priming gavage (**D**), after the cessation of SLIT (**E**), and one day prior to challenge (**F**). **G-H**, comparison of OVA-specific IgE and IgG1 levels across the measured timepoints. Data are representative of 1 experiment with 5-10 mice per group, plotted as mean ± SEM in comparison to the buffer SLIT group (*) or naïve group (°). *, ° P<0.05. OD, optical density; Buffer, PBS SLIT; NO-RE, no allergen re-exposures; Re-exp, allergen re-exposures.

Antibody analysis of serum collected at three different timepoints demonstrated a decrease in the levels of OVA-IgE within the SLIT-treated mice

(Figure 17A-C). Four weeks following the initial intragastric priming gavage, a low level of OVA-IgE was detected in the buffer-treated mice (Figure 17A). This level remained consistent at the second serum collection time point, where all groups exhibited similar levels of OVA-IgE (Figure 17B, G). Prior to the allergen challenge, all groups had detectable levels of OVA-IgE; however, all SLIT-treated groups had a statistically significant lower level of OVA-IgE (Figure 17C). Over the course of treatment, the initial generation of OVA-IgE within the SLIT-treated groups was sustained upon the OVA-alone intragastric allergen exposures (Figure 17G). The decrease in OVA-IgE is consistent with the protection seen during the allergen challenge.

OVA-IgG1 was detected in the buffer group following the initial priming gavage (Figure 17D). All other groups had no detectable OVA-IgG1 at this timepoint (Figure 17D). Upon the administration of SLIT, all SLIT-treated groups showed an increase in the production of OVA-IgG1 (Figure 17E, H). The levels of OVA-IgG1 produced by all SLIT-treated groups, excluding the 20 μg SLIT no re-exposures group, were significantly higher than the buffer group (Figure 17E). Following allergen re-exposures, all groups, excluding naïve mice, produced similar amounts of OVA-IgG1 (Figure 17F). SLIT-treated groups maintained the levels of OVA-IgG1 produced across the second and third serum collection points, as seen consistently in our previous SLIT experiments.

Chapter 4: Discussion

A plethora of studies have investigated the efficacy and safety of OIT for the treatment of food allergies. Clearly, OIT can successfully desensitize most patients. However, it is associated with a significant number of adverse events, greater in fact compared to allergen avoidance. Therefore, the decision to engage in OIT requires an informed joint evaluation of risks and benefits by patients, or their guardians, and health care professionals. SLIT is an alternative route of immunotherapy that has demonstrated similar efficacy to OIT but a significantly better safety profile. Most of the available evidence regarding SLIT has been generated in the context of aeroallergies. The primary goal of this MSc thesis was to first optimize and then determine the impact of SLIT in two murine models of food-induced anaphylaxis, a model of epicutaneous sensitization and a model of intragastric sensitization. In addition, SLIT was administered in two distinct settings, prophylactic and semi therapeutic. The terminology “semi therapeutic” was chosen to indicate that SLIT was administered to either primed mice (intragastric model) or mice that had generated an allergen-specific IgG1 response (epicutaneous model) but prior to the development in both instances of an allergen-specific IgE response. The allergen used in these studies was OVA. The reason for choosing this allergen is that future studies, beyond the scope of this thesis, will investigate in depth the impact of SLIT on allergen-specific B cells. These are very rare cells and to circumvent this limitation, our laboratory has developed a tetramer technology to harvest enriched populations of these cells. At this point, only fully

validated tetramers for OVA are available. It is important to note that, in these models, OVA yields similar outcomes as PN.

Prophylactic OVA-SLIT prevents epicutaneous and intragastric allergic sensitization, and subsequent food-mediated anaphylaxis.

In Phase 1 of the research plan, we optimized the sublingual delivery of OVA as an approach to potentially establishing tolerance in two murine models of food allergy and anaphylaxis (Figure 1A-B). We found that prophylactic treatment with 3 weeks of OVA SLIT at 1 mg completely prevented the generation of OVA-specific IgE in both models. As clinical and physiological outcomes in these models are largely dependent on IgE, SLIT-treated mice were, expectedly, fully protected from anaphylaxis (Figures 4 and 7). A limitation of the allergen-specific immunoglobulin analysis is that a serum collection timepoint immediately after the conclusion of SLIT was not included. This timepoint would have allowed a direct examination of the immunoglobulin response elicited by SLIT *versus* allergic sensitization. For comparison, we included a group of mice that were subjected to a conventional oral tolerance protocol [118–120]. Interestingly, we observed that these mice were completely protected from anaphylaxis in the intragastric sensitization model, but only partially in the epicutaneous sensitization model. The OVA-specific IgE data was concordant with the clinical readouts. We hypothesize that this divergence could be due to the distinct routes of treatment and sensitization. The draining sites in epicutaneous and intragastric sensitization are the inguinal and the mesenteric lymph nodes respectively. Thus, it could be argued that a pre-

existing tolerant response, induced by the oral gavages, in the mesenteric lymph nodes was sufficient to fully prevent the development of allergic sensitization in the same lymph nodes. In contrast, it could have been not sufficient to fully prevent a competing allergic response established in the inguinal lymph nodes, ultimately resulting in partial protection.

A limitation of these experiments is that we did not have an opportunity to examine the length of protection conferred by prophylactic SLIT in these models of food allergy. In other words, whether the mice would remain protected against allergic sensitization at 3- or 6-months following prophylactic SLIT treatment remains unknown. However, these experiments are currently being pursued by other members of the lab that are continuing this project.

To examine the mechanisms underlying SLIT-induced tolerance, we conducted a cytokine analysis of supernatants from OVA-stimulated splenocytes from SLIT-treated mice. Unfortunately, the high variability of the data prevents any firm conclusions. Within the epicutaneous model we observed an apparent induction of a TH2 response in SLIT-treated mice (Appendix 3A-C). These data were in sharp contrast with the clinical results, where the 1 mg SLIT-treated mice had essentially no reactivity upon allergen challenge (Figure 4). However, we also observed a trend towards an induction of IL-10, a regulatory cytokine (Appendix 3E). In contrast, we observed an absence of a TH2 response in the SLIT-treated, intragastric sensitized mice, and an induction of IL-10 (Appendix 4A-C, E). Together, these data may suggest that the SLIT-treated mice in the epicutaneous

model are undergoing two different responses, while the mice in the intragastric model lean towards one type of response. At this point, the mechanisms underlying clinical tolerance in SLIT-treated mice remain to be elucidated. Upcoming work from Lundsgaard *et al*. has examined the capacity and effects of SLIT with Ara h 6, a major PN allergen, in preventing allergic sensitization [121]. Similar to the protocol described here, mice received 3 weeks of SLIT prior to intraperitoneal sensitization [121]. Not only were the mice protected from Ara h 6 sensitization in a dose-dependent manner, but tissue analysis revealed an expansion of Treg cells within the sublingual lymph nodes and blood [121]. In combination with the trends in IL-10 induction observed in our prophylactic settings of SLIT, we hypothesize that Treg cells and IL-10 may play a critical role in the induction of tolerance. The source and specific mechanism behind this production of IL-10 warrants further investigation in future phases of this project.

Semi-therapeutic OVA-SLIT provides protection against food-mediated anaphylaxis in both the epicutaneous and intragastric models of allergic sensitization

In the second phase of experimentation, we sought to examine the semi-therapeutic effects of SLIT in the two models of food allergy and anaphylaxis. In the epicutaneous model, SLIT was administered immediately after the tape stripping *i.e.*, at the time mice had already generated an OVA-specific IgG1 response, but before the allergen re-exposures. Therefore, this experimental design was asking whether SLIT would prevent the transition from an IgG1 to an IgE

response. In the intragastric model, SLIT was administered 4 weeks following the priming gavage, but before the allergen re-exposures. Thus, the experiment was asking whether SLIT would prevent the activation of allergic memory.

Semi-therapeutic OVA-SLIT in the epicutaneous sensitization model

We found that 9 weeks of treatment with 1 mg OVA-SLIT provided significant, better than 50%, but partial protection, as indicated by the changes in core body temperature, hemoconcentration and clinical score in comparison to the buffer group. The change in the serum levels of OVA-specific IgE was concordant with the clinical outcome. As the impact of SLIT for 6 weeks was comparatively inferior, we argued that extending SLIT beyond 9 weeks could yield an additional benefit. However, treatment for 12-15 weeks did not improve protection and, in fact, resulted in worsening of all the outcome measurements. The protocol of SLIT administration that we used may explain this unexpected outcome. Typically, human trials of food immunotherapies begin with an extremely low dose, at the microgram level, followed by a dose escalation over a period of weeks to months [58]. The use of dose escalation protocols is intended to minimize adverse events in the early stages of treatment. In our studies, we used a single high dose of allergen from the beginning and throughout the entire treatment. It is, then, possible that maintaining that high dose of allergen beyond 9 weeks reinforced the pathogenic TH2 response.

Induction of allergen-specific IgG has been suggested as a possible mechanism through which allergen immunotherapy mediates tolerance, notably

IgG4 in humans [58]. In mice, it has been shown that IgG antibodies may induce tolerance to foods through their effects on the inhibitory FcγRIIb receptor on mast cells and basophils [122,123]. It is possible that the ratios of OVA-IgE to OVA-IgG1 antibodies varied depending on the duration of SLIT. Unfortunately, ELISAs for both the 6-9 week SLIT treatment and the 12-15 week SLIT treatment were ran at different times and, therefore, we are unable at this time to make a direct comparison of OVA-specific IgE:IgG1 ratios amongst the various groups. This hypothesis can be evaluated by repeating the OVA-specific IgE and IgG1 ELISAs with the samples stored from both experiments. Additionally, it is plausible that other antibody isotypes, such as IgG2a, IgG2b, and IgG2c, which we did not measure at this point, could have contributed to the partial protection observed. In fact, recent and ongoing work by a PhD candidate in the lab has shown an upregulation of OVA-specific IgG2b and IgG2c in this setting.

Semi-therapeutic OVA-SLIT in the intragastric sensitization model

We found that treatment of mice with 9 weeks of SLIT at a dose of 1 mg OVA was not protective against food-mediated anaphylaxis and, expectedly, serum levels of OVA-specific IgE were similar to the buffer group (positive control) amongst all SLIT-treated mice (Figure 15). Importantly, the SLIT-treated mice that received no allergen re-exposures also underwent anaphylaxis and produced OVA-IgE. These findings were in sharp contrast to those in the epicutaneous model, where this group did not show any significant response. These findings suggest the possibility that the SLIT-treated mice were swallowing a portion of the sublingually

administered OVA, which is not totally unexpected. As mentioned previously, the route of sensitization and treatment are anatomically separated in the skin model; however, both routes may be shared in the model of gastrointestinal sensitization, assuming that a portion of the allergen is swallowed during SLIT-treatment. We now know that the administration of a single gavage of OVA plus CT (priming) establishes long-lived allergic memory. Consequently, it is plausible that OVA swallowed during SLIT treatment activated a memory response. With this in mind, we hypothesized that lowering the dose of OVA would minimize the amount of OVA reaching the gastrointestinal tract. Indeed, we found that lowering the dose of OVA-SLIT from 1 mg to, particularly, 100 μg for 12 weeks achieved significant but partial protection from anaphylaxis and a drastic decrease in the levels of serum OVA-specific IgE. SLIT alone, *i.e.*, with no allergen re-exposures, induced only a very slight increase in OVA-specific IgE. These data strengthen the hypothesis that small amounts of OVA ingested during SLIT activated allergic memory in the gut. Although we were unable to achieve complete protection through a single-dose treatment approach, these data emphasize the importance of optimizing both the dosage and duration of treatment.

Summary of data findings and future directions

Currently, food allergy remains a disease without a cure. Despite immunotherapies, such as OIT, demonstrating high efficacy in achieving clinical desensitization, OIT is accompanied by a high rate of adverse effects, and protection that is lost upon discontinuation of treatment in most patients [58]. The goal

of this thesis project was to optimize and evaluate the use of SLIT in two distinct murine models of food allergy and anaphylaxis. In the first phase of experimentation, we optimized a prophylactic SLIT treatment regime that prevented both epicutaneous and intragastric sensitization. In the second phase, we evaluated OVA-SLIT in semi-therapeutic models. Here, we found that the dosage of OVA and the duration of treatment were unique to each model and were key in optimizing this therapy. However, we were unable to achieve complete protection in these set of experiments. This finding is not entirely surprising, as lack of complete protection from reactivity is commonly seen in food allergen immunotherapies in humans [58,101,102,107,108]. Typically, patients who received SLIT-treatment demonstrate an increase in the maximum tolerated dose of allergen in comparison to controls; however, these doses are often accompanied by non-severe allergic reactions, such as an irritated throat. Therefore, the partial level of protection seen within our SLIT-models may be characteristic of allergen immunotherapy. Nonetheless, further evaluation of dosage and treatment duration may confer greater protection.

Our data show that SLIT, given as a single constant dose, provides a partial level of protection in a semi-therapeutic setting. It would be valuable to optimize a dose-escalation SLIT protocol in these murine models that mimics human protocols of OIT. Mice could be started on a small dose that is escalated every week until a maximum dose of 1 mg is reached for maintenance. A starting dose of 100 μg would be appropriate as this dose demonstrated minimal reactivity within our semi-

therapeutic SLIT models of food allergy. The rate of dose escalation would require a degree of optimizations, as increasing the dosage too quickly may cause more harm than benefit. Upon reaching the 1 mg maintenance dose, the mice could continue it for at least 6 weeks, as demonstrated by our semi-therapeutic SLIT data. If successful, the semi-therapeutic SLIT-treated mice would exhibit full protection from clinical reactivity upon the systemic allergen challenge. With an established model, we may then proceed to explore the fundamental mechanisms through which this tolerance is established.

An additional consideration is the incorporation of serum collection at multiple time points, which would allow for the analysis of immunoglobulin kinetics. These mice may be bled every 2 weeks throughout the treatment protocol. Additionally, examining OVA-specific IgG subtypes (IgG2a, IgG2b), IgA, and IgM would provide additional information on how SLIT is impacting humoral immunity. We must also consider the analysis of the B and T cell compartments within the spleens, cervical lymph nodes, inguinal lymph nodes (in the skin model) and mesenteric lymph nodes (in the gut model). If SLIT is inducing lasting tolerance in these models, we hypothesize that there would be a shift away from the allergic TH2 phenotype towards a regulatory phenotype. This can be addressed through flow cytometric analysis of the various tissues following the systemic allergen challenge. Key cells would include TH2 cells, Treg cells, and a complete analysis of all BC isotypes. It may also be of interest to examine a relatively new and unexplored subset of BCs known as IL-10^{+} B regulatory (Breg) cells that play

a role in the suppression of pro-inflammatory responses [124]. Although relatively unexplored, these cells appear to play crucial roles in the suppression of clinical symptoms via IL-10 and induction of $Foxp3^+$ Tregs [124,125]. In addition to flow cytometry, Luminex analysis of cytokines from OVA/media stimulated splenocyte cultures of SLIT-treated, allergic, and naïve mice can further assist in the elucidation of how SLIT establishes its protective effects. We would expect to see a decrease in the production of TH2 cytokines (IL-4, -5, -13) and an induction of the regulatory cytokine, IL-10. Together, these set of experimentations could cohesively establish a mechanism by which SLIT exhibits its protective effects and the impact it has on both the T/B cell compartments and humoral immunity.

Lastly, it may be important to examine the role of SLIT in a combinational therapy for the treatment of food allergy within these models. It is evident from current literature that food allergen immunotherapies do confer a high degree of protection but is still accompanied by allergic reactions [58]. Additionally, the established protection is transient and is often lost upon treatment cessation. Therefore, an alternative form of treatment is required to achieve long-term tolerance, even after treatment cessation.

One strategy would be to combine a biologic, capable of inducing cellular changes within the allergic memory cell compartment, with an allergen immunotherapy to help shift the TH2 response towards a regulatory response. This concept has been previously explored in the context of both peach and cow's milk allergy where treatment with SLIT in combination with an adjuvant (*e.g.*, ODN-

CpG, IL-2/anti-IL-2Ab complex antibodies, or Omp16) conferred better, lasting protection in comparison to SLIT alone [103,126,127]. Within our models of food allergy, anti–IL-4Rα is the ideal candidate for the biologic component of treatment. As demonstrated previously by our lab, treatment with anti–IL-4Rα not only abolished IgE generation and clinical reactivity, but this protection was maintained for up to 6 weeks following treatment discontinuation [56]. We would then hope that treatment with anti–IL-4Rα in combination with allergen stimulation could drive the TH2 allergic response towards one of a regulatory phenotype. For administration of allergen, the sublingual route or oral route could both be effective for this combinational therapy. However, it is important to note that within both human and murine studies of food immunotherapy, SLIT, in comparison to OIT, exhibits a higher degree of safety [58]. In turn, this would likely promote a higher treatment adherence amongst patients, and thus, provide an effective treatment with a superior safety profile.

The first part of my MSc thesis optimized the SLIT protocol within the epicutaneous and intragastric models of food allergy. Here we demonstrated that prophylactic treatment with 3 weeks of 1 mg OVA SLIT was protective against allergic sensitization and subsequent clinical reactivity. The second component focused on the evaluation of SLIT as a semi-therapeutic treatment. Here, we demonstrated that SLIT, when administered for after tape stripping (9 weeks at 1 mg OVA) or the priming gavage (12 weeks at 100 μg), was only capable of achieving partial protection from food-induced clinical reactivity. The work

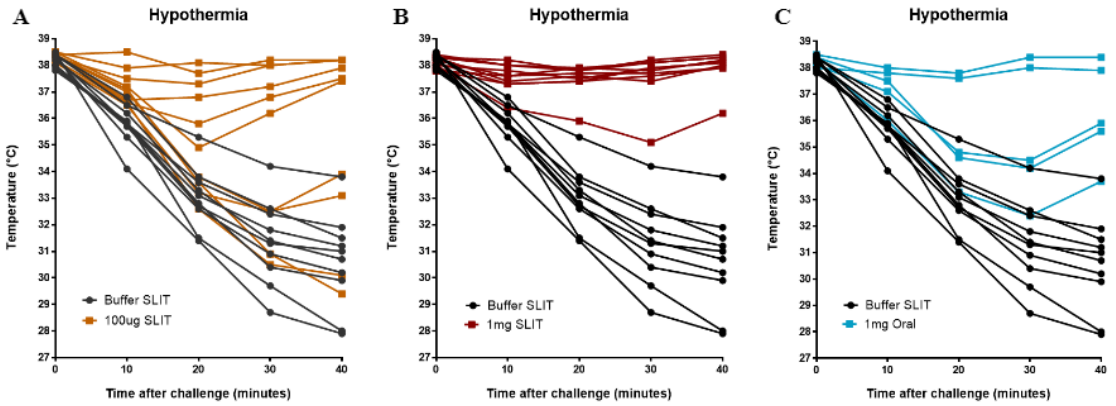
presented here provides both an optimized SLIT protocol and evidence for the efficacy of SLIT in the treatment of food allergy. Future work should aim to explore alternative SLIT dosing protocols to achieve complete protection against clinical reactivity when used both as a semi-therapeutic and therapeutic treatment.

Tables

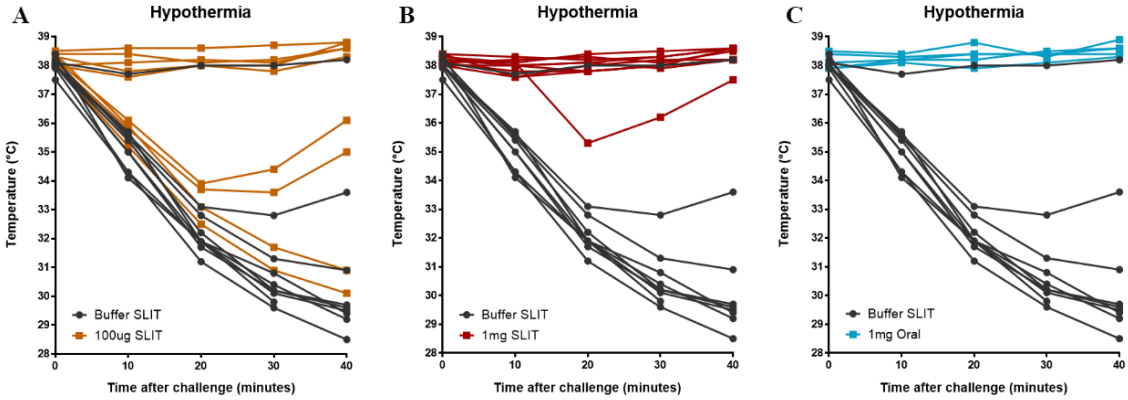
Table 1. Clinical grading scheme for evaluating mice undergoing a systemic allergic challenge

Score	**Symptom**
1	In ear scratching with hind foot
2	Reduced Motion
3	Motionless
4	No whisker response
5	Prodding and/or seizure

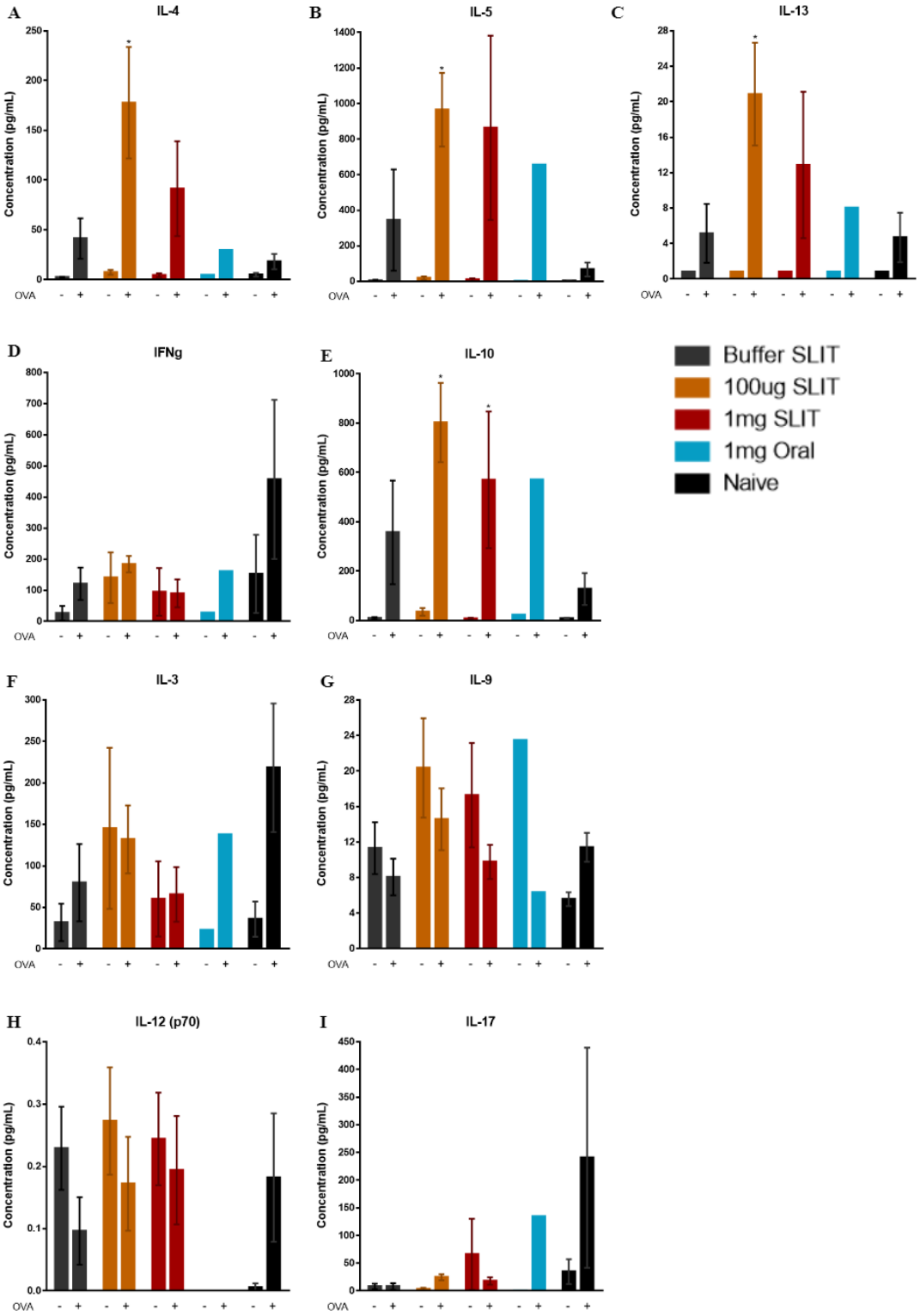
Appendices



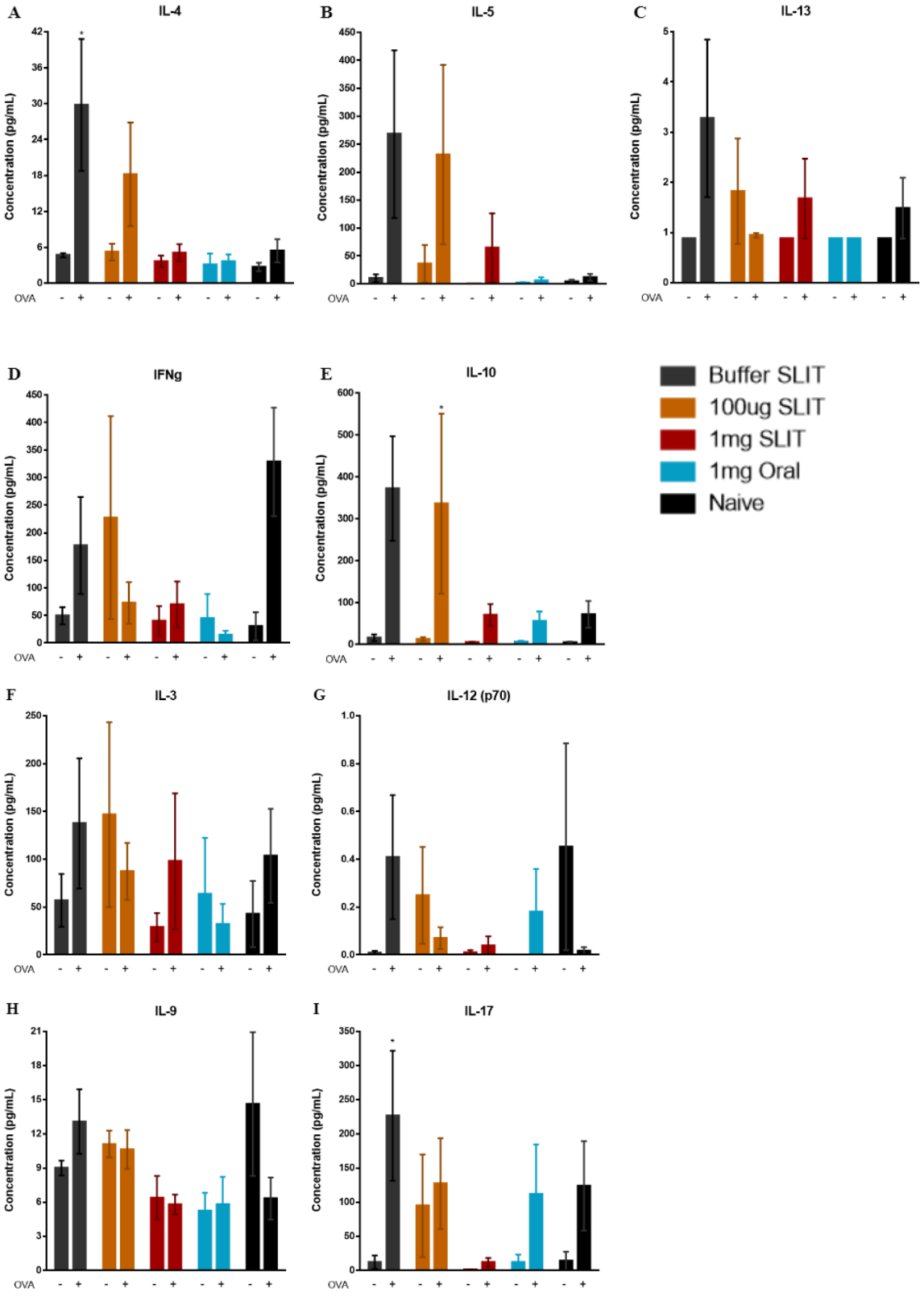
Appendix 1. Group-sorted drop in core body temperature graphs for Phase 1 OVA-SLIT treated, skin sensitized mice.



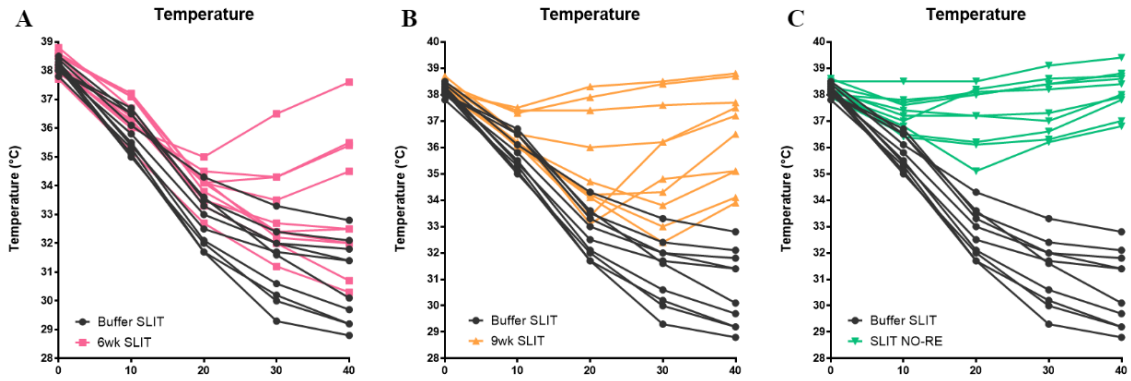
Appendix 2. Group-sorted drop in core body temperature for Phase 1, OVA-SLIT treated, gut sensitized mice.


A
IL-4
B
IL-5
C
IL-13
D
IFNg
E
IL-10
F
IL-3
G
IL-9
H
IL-12 (p70)
I
IL-17
Concentration (pg/mL)
OVA
Buffer SLIT
100ug SLIT
1mg SLIT
1mg Oral
Naive

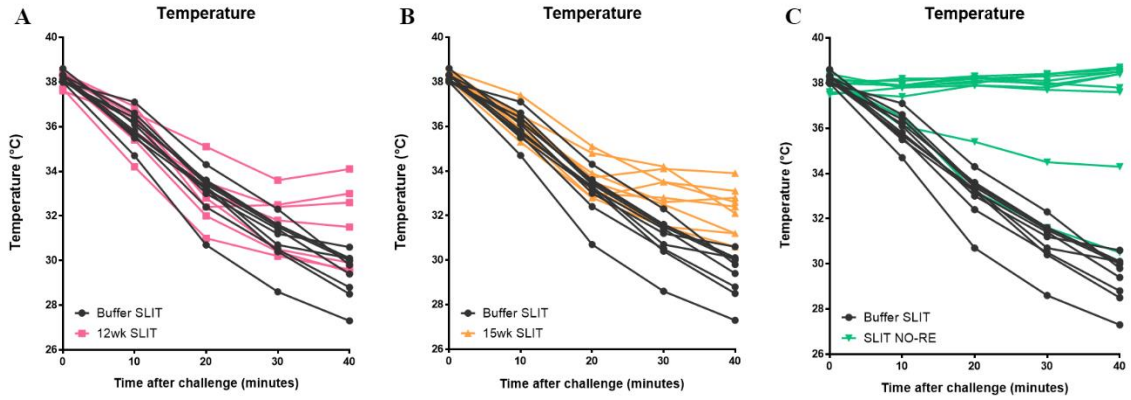
Appendix 3. Cytokine analysis of cultured splenocytes from Phase 1, skin-sensitized mice. The first bar of each group indicates media-stimulated splenocytes, and the second bar represents stimulation with OVA. Due to sample constraints in the Luminex assay, only one sample from the oral tolerance group was analyzed. **A-C**, analysis of TH2 associated cytokines. **D-E**, analysis of regulatory cytokines IFN-γ and IL-10. **F-I**, analysis of IL-3, IL-9, IL-12 (p70), and IL-17. Data are representative of 1 experiment with 5-10 mice per group, plotted as mean ± SEM relative to the media control per group. *P<0.05.


A
IL-4
B
IL-5
C
IL-13
D
IFNg
E
IL-10
F
IL-3
G
IL-12 (p70)
H
IL-9
I
IL-17
Concentration (pg/mL)
OVA
- + - + - + - + - +
Buffer SLIT
100ug SLIT
1mg SLIT
1mg Oral
Naive

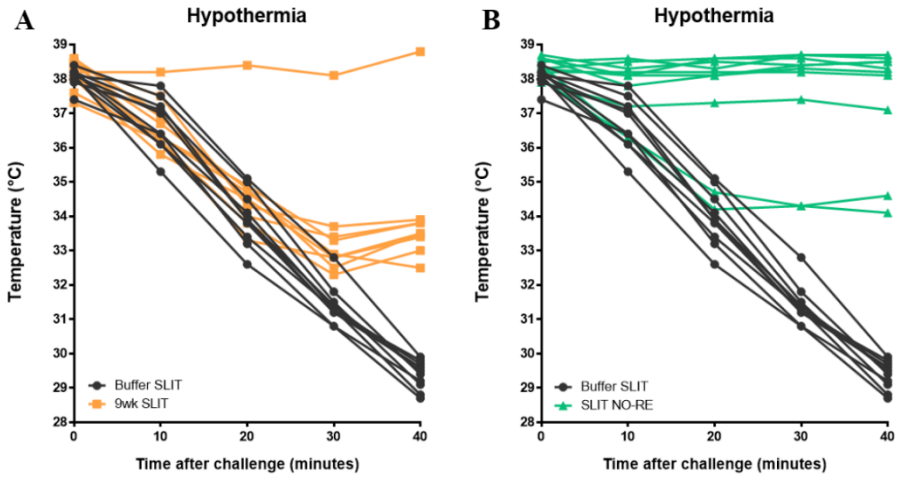
Appendix 4. Late-phase cytokine analysis of cultured splenocytes from Phase 1, intragastric-sensitized mice. The first bar of each group indicates media-stimulated splenocytes, and the second bar represents stimulation with OVA. **A-C**, analysis of TH2 associated cytokines. **D-E**, analysis of regulatory cytokines IFN-γ and IL-10. **F-I**, analysis of IL-3, IL-9, IL-12 (p70), and IL-17. Statistical significances are shown relative to the media control per group. Data are representative of 1 experiment with 5-10 mice per group, plotted as mean ± SEM relative to the media control per group. *P<0.05.



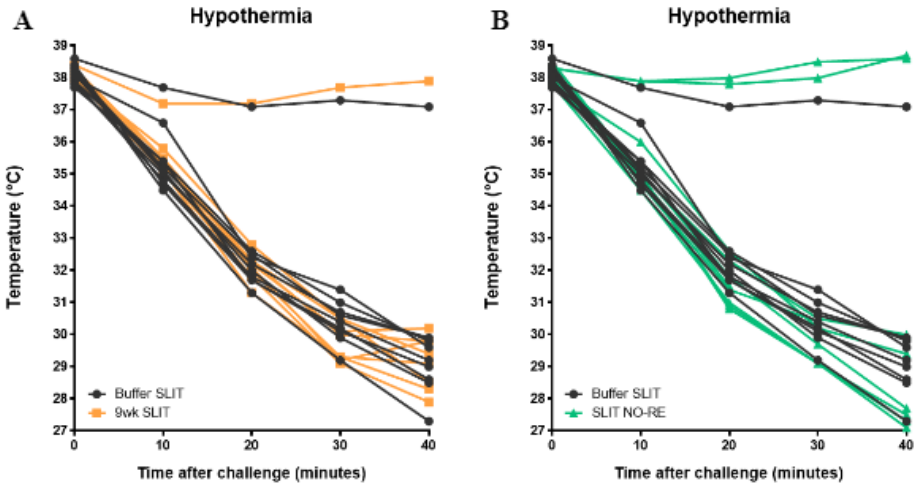
Appendix 5. Group-sorted drop in core body temperature for Phase 2, 6-9 week, OVA-SLIT treated, skin sensitized mice.



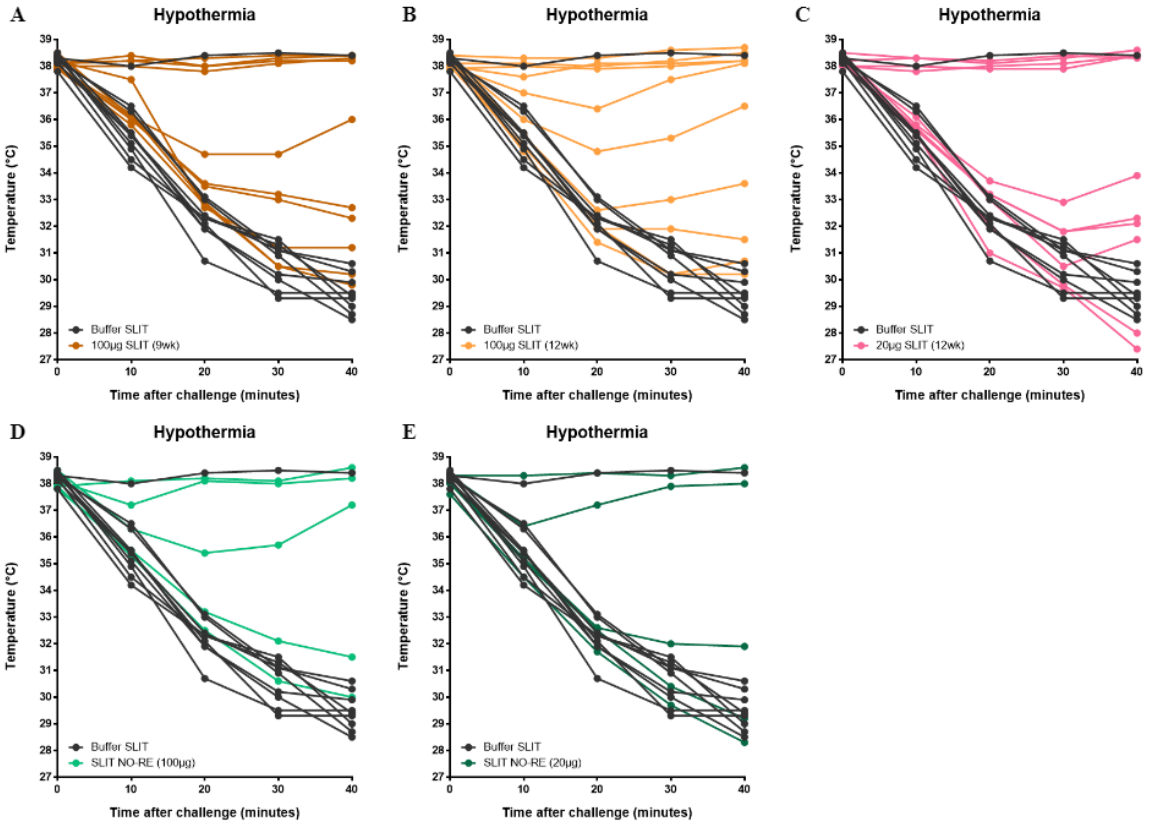
Appendix 6. Group-sorted drop in core body temperature for Phase 2, 12-15 week, OVA-SLIT treated, skin sensitized mice.



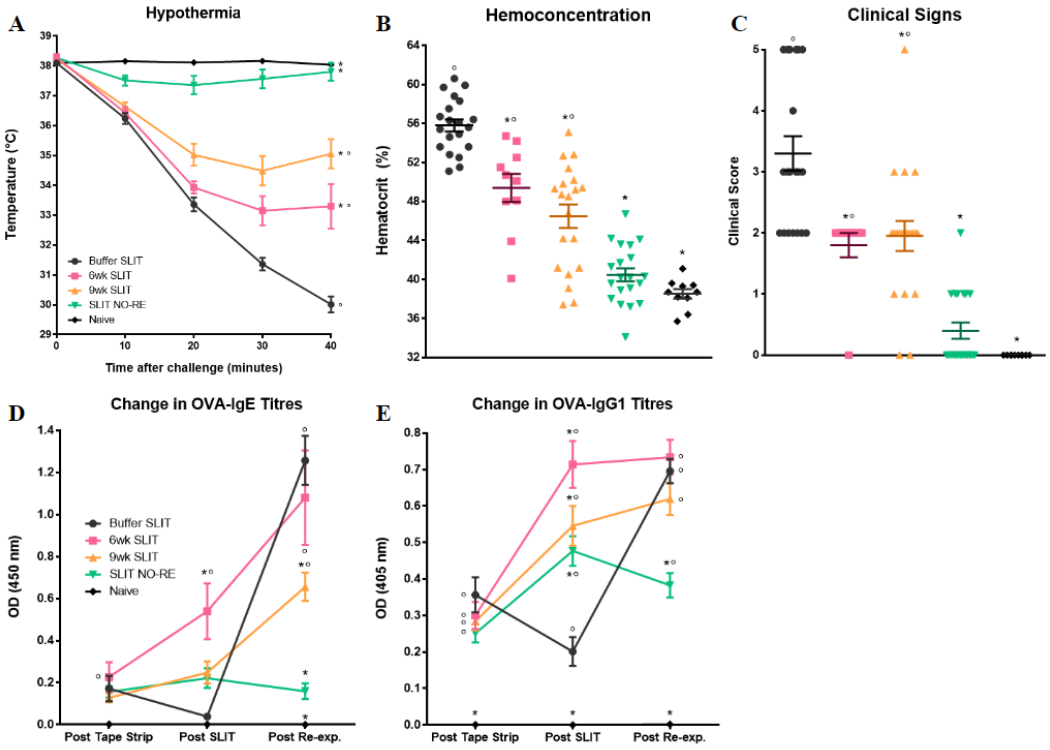
Appendix 7. Group-sorted drop in core body temperatures for Phase 2, 9-week OVA-SLIT treated, skin sensitized mice.



Appendix 8. Group-sorted drop in core body temperature for Phase 2, 9-week OVA-SLIT treated, gut sensitized mice.



Appendix 9. Group-sorted drop in core body temperature for Phase 2, OVA-SLIT treated, gut sensitized mice.



Appendix 10. Pooled results of semi-therapeutic SLIT (6/9 wk) demonstrating partial protection against food-induced clinical reactivity in epictuaneous sensitized mice. **A**, Drop in core body temperature during systemic allergen challenge. **B**, Hemoconcentration at 40 minutes post challenge. **C**, Clinical score of reactivity during challenge (as described in Table 1). **D**, Change in serum OVA-specific IgE levels measured after tape stripping, after cessation of SLIT, and one day prior to systemic allergen challenge. **E**, Change in serum OVA-specific IgG1 levels measured after tape stripping, after cessation of SLIT, and one day prior to systemic allergen challenge. Data are represented of 2 identical experiments (n = 20 mice for Buffer, 9wk SLIT, and SLIT no-re groups; n = 10 mice for 6wk SLIT, and naive groups). Statistical significances are shown relative to the buffer SLIT group (*) or naïve group (°). *, ° P<0.05. Buffer, PBS SLIT; NO-RE, no allergen re-exposures; OD, optical density; Re-exp, allergen re-exposures.

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