Vaccine 36 (2018) 2816–2824

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Allergic patients with and without allergen-specific immunotherapy mount protective immune responses to tick-borne encephalitis vaccination in absence of enhanced side effects or propagation of their Th2 bias

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ARTICLE INFO

Article history:
Received 3 November 2017
Received in revised form 9 March 2018
Accepted 28 March 2018
Available online 16 April 2018

Keywords:
Allergy
Specific immunotherapy
Th2 polarization
Immunomodulation
Vaccine efficacy
Vaccine reactogenicity

ABSTRACT

Background: Allergic diseases are caused by Th2-driven immune responses and their treatment with specific immunotherapy (SIT) leads to immunomodulation via IL10, TGF-ß and Th1/Tr1 shift. This phase IV, open-label clinical trial investigated whether allergies and SIT treatment influenced immune responses to routine vaccination.

Methods: We studied three groups: 49 allergic patients (allergic group), 21 allergic patients receiving maintenance doses of SIT (SIT group), and 49 non-allergic controls. All subjects received tick-borne encephalitis (TBE) booster vaccines and humoral and cellular immune responses were evaluated after one week, four weeks and six months.

Results: The levels and kinetics of neutralizing TBE-specific antibodies, reflecting protection against TBE, were not significantly different in the three groups. The allergic group showed Th2 polarization pre-booster as indicated by increased TBE-specific IgG1 and elevated mitogen-induced IL5 production. Alum-adjuvanted TBE vaccine led to Th2 biased immune responses in the controls, but to no further enhancement of Th2 polarization in the allergic and SIT group. Furthermore, in the SIT group cellular parameters reflected the induction of immunomodulation due to increased Tregs, elevated baseline IL10 and lack of TBE-specific IL5. Importantly, these cellular regulatory responses did not limit the ability to mount sufficient TBE-specific antibodies after the booster. All groups tolerated the vaccine well with no exacerbation of allergic symptoms.

Conclusion: TBE booster vaccinations were immunogenic and safe in both the allergic and SIT group and contributed to balanced immune responses. Our data indicate that all allergic patients, even when undergoing SIT, should be vaccinated without hesitation and at regular intervals according to standard recommendations.

ClinicalTrials.gov (NCT02511535).

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1. Introduction

Allergic diseases, which are caused by Th2-driven immune responses that lead to severe alterations of immune-homeostasis, are highly prevalent and afflict up to 30% of Western populations [1,2]. Two aspects are frequently discussed with regard to vaccinating individuals with allergies. Firstly, there is an ongoing debate about whether increased allergy prevalence can be linked to the

Abbreviations: TBE, tick-borne encephalitis; SIT, specific immunotherapy; NT, neutralization test; GMT, geometric mean titer; CI, confidence interval.

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https://doi.org/10.1016/j.vaccine.2018.03.076
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increased use of vaccines in developed countries [3]. However, existing solid data show no causal relationship between childhood vaccination and the development of allergies and asthma [4–6]. On the contrary, certain vaccines, such as measles-mumps-rubella, have been proved to have a protective effect against allergy development in early childhood [7]. The second issue of interest is whether vaccine efficacy might be altered in allergic individuals. Only limited data are available thus far and indicate no reduction of humoral vaccine responses [8,9]. However, specific immunotherapy (SIT), the only causative treatment for allergies, induces immunomodulation and mechanisms of tolerance via allergen-specific regulatory cells, suppressive cytokines IL10 and TGF-β, IgG4 production, repression of effector cells and Tr1/Th1 shift [10]. This condition further amplifies questions about whether vaccine efficacy might be impaired in allergic patients. The concern is supported by our recent finding that non-responder vaccinees who displayed increased IL10 levels, similar to allergic patients undergoing SIT, had impaired vaccine responses [11]. So far, the current SIT guidelines only consider safety aspects of vaccination during immunotherapy mainly based on theoretical considerations [12,13], but vaccine efficacy in allergic patients, with and without SIT, has not yet been sufficiently addressed.

This study therefore aimed to investigate whether vaccine responses were modified in allergic patients due to the generally Th2-polarized immunologic profile associated with a variety of sensitizations. In addition, specific immunotherapy during the maintenance phase represents long-term immunomodulation that could possibly limit vaccine responses. Given the hesitancy to vaccinate allergic patients in clinical practice, we monitored the side effects of vaccinations, together with their potential to exacerbate allergies. We chose to focus on routine booster vaccines for tick-borne encephalitis (TBE), because TBE virus is highly endemic in Austria and, due to past immunization campaigns, the vaccination coverage is high [14]. Three groups of subjects were recruited: allergic patients, allergic patients undergoing SIT, and non-allergic controls. All received a TBE booster and were then evaluated for vaccine-specific antibodies and cellular immune responses. This broader approach was chosen to identify potential differences in cellular responses in addition to antibody titers, which could represent cellular prediction markers for vaccine efficacy. Our goal was to determine whether allergic patients, especially those receiving SIT, could be vaccinated according to routine recommendations without limitations or whether vaccination schedules need to be adapted for this particular patient group.

2. Materials and methods

2.1. See supplementary material for further information

2.1.1. Study design and study subjects

Three parallel groups were investigated in this open-label, non-randomized, phase IV clinical trial: allergic patients receiving no or symptomatic treatment (n = 49), allergic patients receiving maintenance doses of subcutaneous SIT (n = 21) and non-allergic controls (n = 49). These are henceforth referred to as allergic group, SIT group and controls. All included participants had received a documented primary TBE vaccination and at least one booster. The subjects in the two allergic groups had been sensitized to one or several of the following allergen sources: perennial and seasonal inhalative allergens and insect venom allergens. Sensitization was confirmed by anamnestic clinical symptoms and positive specific IgE reactivity or positive skin-prick test. After providing written informed consent, the study subjects received a TBE booster vaccination. Venous blood was taken prior to and one week, four weeks and six months after vaccination and measured for TBE-specific antibodies. Peripheral blood mononuclear cells (PBMC) were prepared before and one week after booster to assess cellular responses. The occurrence, duration and intensity of any local and systemic reactions were reported by study subjects using a diary that covered the week after their vaccination. The study was approved by the local ethical committee (EK Nr.2083/2012), the national regulatory authorities and registered at ClinicalTrials.gov (NCT02511535).

2.1.2. Vaccines

Subjects received the TBE vaccine *FSME-IMMUN® 0.5 ml* (Lot: VNR1N02C), containing 2.4 μg inactivated TBE virus (strain Neudoerfl) and 0.35 mg $Al(OH)_3$ by intra-muscular application into the M. deltoideus (needle Sterican® (B|Braun) 0.50 × 25 mm BL/LB 25Gx1″). *FSME-IMMUN® 0.5 ml* (Pfizer Corporation Austria GmbH, Vienna, Austria) is licensed in Austria since 1996 and vaccines were stored at 2–8 °C until usage.

2.1.3. Preparation and storage of PBMC and serum

PBMC were prepared from heparinized blood by Ficoll Paque centrifugation, as previously described [11] and re-suspended in RPMI 1640 containing 50% FCS (both Biochrom, Berlin, Germany) and 10% dimethyl sulfoxide (Merck, Darmstadt, Germany) for storage in liquid nitrogen until evaluation. Serum was obtained from native venous blood and stored at −20 °C.

2.1.4. TBE-specific neutralization test titers

Anti-TBE virus antibodies were measured by neutralization test (NT) in sera from all sampling points. Neutralization assays were carried out in microtiter plates using baby hamster kidney cells (American Type Culture Collection BHK-21) as previously published [15,16] at the Department of Virology, Medical University of Vienna. Twofold serial dilutions of polyclonal sera were mixed with 25 PFU virus (starting dilution of serum in the mixture 1:10) and incubated for 1 h at 37 °C. Then BHK-21 cells were added and incubation was continued for 3 days. The presence of virus in the supernatant was determined by four-layer ELISA. The virus neutralization titer was defined as the reciprocal of the serum dilution that gave a 90% reduction in the absorbance readout in the assay compared to the control without Ab.

2.1.5. Immunoglobulin G subclasses

Serum concentrations of total IgG1, IgG2, IgG3, and IgG4 were determined before and four weeks after booster with the turbidimetric immunoassay Optilite IgG1-4 Kit (The Binding Site, Birmingham, UK) at the Institute of Immunology, Medical University of Vienna.

2.1.6. TBE-specific IgG subclasses and IgE

TBE-specific IgG1, IgG3, and IgG4 subclass antibodies and TBE-specific IgE antibodies were measured by in-house ELISA (see supplementary material).

2.1.7. TBE-specific in-vitro re-stimulation of PBMC

PBMC were re-stimulated with *TBE TICOVAC-like* antigen, superantigen *Staphylococcus* Enterotoxin B as positive control and medium alone for cytokine baselines (see supplementary material).

2.1.8. Quantification of cytokine production in supernatants

Cytokines IL2, IFNy, IL5, and IL10 were quantified in culture supernatants using Luminex 200 platform and Fluorokine MAP Human Cytokine Base-Kit A (R&D Systems, Minneapolis, Minnesota, USA) as previously described [11].

2.1.9. Flow-cytometric lymphocyte analyses

PBMC were surface-stained with fluorochrome-conjugated monoclonal antibodies and transcription factor FOXP3 was stained intra-cellularly for characterization of regulatory T cells (see supplementary material). Data were acquired on a FACS Canto II flow-cytometer by gating on cells with forward/side light scatter properties of lymphocytes and analyzed with FACS Diva 8.0 software (BD Biosciences, San Jose, California, USA).

2.1.10. Statistical evaluation

Sample size determination was based on the variance estimate of the log neutralization test (NT) titers from a previous study [17]. As primary endpoint the titer at day 28 was chosen and an effect size of $f = 0.31$, equivalent to a twofold mean titer difference between the allergic groups and the controls, was specified. Applying a significance level of 5% and 90% power resulted in a sample size of $n = 44$ per group. Since it turned out that allergic patients undergoing SIT were difficult to enroll, a post hoc power calculation was performed using the actual sample sizes and this gave an achieved power of 89.6%.

Antibody titers from NT were log-transformed and results are expressed as geometric mean titers with 95% confidence intervals. Normality was assessed by Kolmogorov-Smirnov tests and homogeneity of variance by Levene's tests. Comparisons of groups were done based on analysis of variance and linear contrasts within and between time points. The same procedure was applied for the other endpoints; however, relative counts of PBMC were arcsine-transformed to remove correlation of means and standard deviations, as well as to obtain homogeneity of variance. For all tests, p values below 0.05 were considered significant.

3. Results

3.1. Cohort description

The investigated groups were (1) 49 allergic patients, (2) 21 allergic patients receiving SIT at maintenance dose and (3) 49 non-allergic controls (Fig. 1A). The demographic parameters age, gender, and body mass index and the interval since last TBE booster were similar between the groups and the average duration of SIT treatment was 2.4 years (Fig. 1B). The majority of patients were allergic to more than one allergen with grass and tree pollens being the most frequent allergens and rhinitis and conjunctivitis as major allergic symptoms (Table 1A). Due to the fact that the immunomodulatory mechanisms induced by different types of SIT allergens (i. e. insect venom, seasonal/perennial inhalative allergens) are described to be similar [18,19], we allowed for recruitment of a higher percentage of insect venom allergics in the SIT group compared to the allergic group. Anti-histamines to be taken on demand had been prescribed to 19 of 49 subjects in the allergic group. The sensitization(s) and specificity/duration of immunotherapy for individual SIT patients are provided in Table 1B. Controls fulfilled general study eligibility criteria (Supp. Table I) and reported no anamnestic allergies.

3.2. Neutralization test titers

Neutralizing TBE-specific antibody titers (NT) were measured as a correlate of protection and titers >1:10 were considered protective [20]. Controls and both the allergic and the SIT group had protective geometric mean titers (GMT) before booster (110 in controls, 106 in the allergic group and 83 in SIT patients). Already one week after booster antibody titers significantly increased in all groups and reached peak levels after four weeks with GMT at 282 in controls, 318 in the allergic group and 250 in the SIT group (Fig. 2A, B). The titer difference between the allergic and SIT group after four weeks was not significant. Six months after vaccination, GMT declined in all three groups to equal levels, which were about twofold higher than pre-booster levels. No significant differences in fold increases after one week and four weeks were present between the three groups (Fig. 2B). When titers were analyzed according to gender, female controls and females in the SIT group showed a significantly higher fold increase than the males. This gender difference was not observed in the allergic group (Fig. 2C).

To address potentially altered TBE antibody avidity due to allergy/immunotherapy, the mean TBE IgG avidity indexes were determined according to published protocols [21] and we observed comparably high-avidity TBE IgG antibodies in all three groups (data not shown).

3.3. Total IgG subclasses

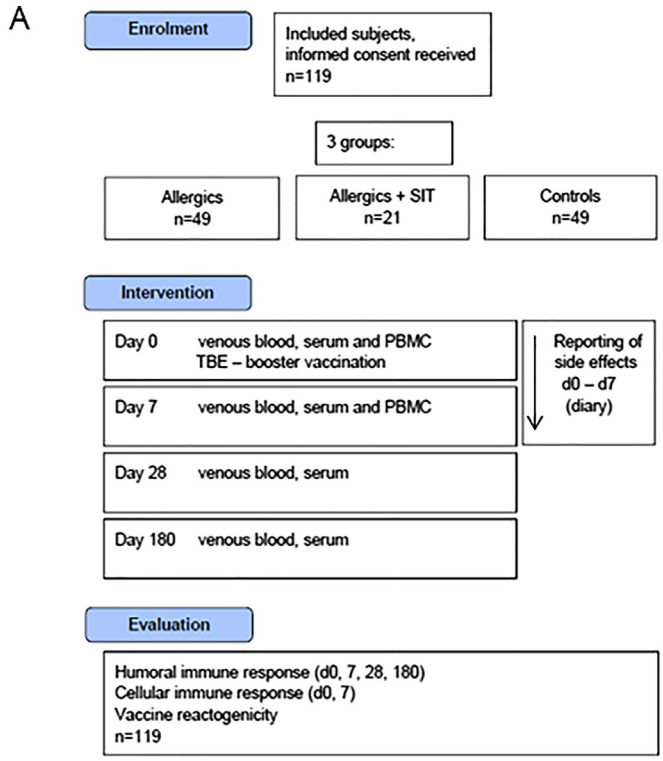
Allergic immune responses are associated with the production of allergen-specific IgE and IgG, especially IgG1 subclass [22,23], while allergen-specific IgG4 is induced during immunotherapy [10,24]. In our study population, concentrations of serum IgG1, 2, 3, and 4 were determined before and four weeks after booster and mean levels were within normal ranges. After vaccination, total IgG1 concentrations significantly increased in controls, but decreased in both the allergic and SIT group. In addition, total IgG2 ($p < 0.001$, data not shown) and IgG3 ($p < 0.05$) significantly decreased in these two groups, but remained unchanged in the controls. The changes in total IgG4 concentrations four weeks after booster did not differ between the three groups (Supp. Fig. 1).

3.4. TBE-specific IgG subclasses and IgE

We measured TBE-specific IgG1, IgG3, and IgG4 before and four weeks after booster to investigate whether the allergic phenotype influenced the quality of the elicited TBE-specific immune response in terms of IgG subclass. Before vaccination, TBE-specific IgG1 was significantly higher in the allergic group compared to the control and SIT group ($p < 0.05$). Four weeks after booster, IgG1 increased significantly in the controls ($p < 0.001$) but not in the allergic and SIT group. In contrast, TBE-specific IgG3 was at similar levels prior to booster and significantly increased four weeks after vaccination in all three groups (Fig. 2D). Also TBE–specific IgG4 levels were comparable in all groups before booster, but showed only marginal increases after vaccination (Supp. Fig. 2A). TBE-specific IgE was measured to investigate whether allergic patients produced Abs of IgE subclass to the vaccine antigen. Only few individuals in the allergic and SIT group (4 per group) produced low levels of TBE-specific IgE, while the majority did not. In controls, except for one vaccinee showing a higher IgE response, no TBE-specific IgE Abs were detectable (Supp. Fig. 2B).

3.5. Cytokine production upon in-vitro re-stimulation

In order to assess the cellular responses to TBE vaccine antigen, cytokine concentrations were measured in culture supernatants of re-stimulated PBMC. TBE-specific IFNy levels increased significantly after booster in controls and the allergic group, but the increase was markedly lower in the allergic patients (GM 37.8 to 54.2 pg/ml) than in controls (GM 38.5 to 77.3 pg/ml). A non-significant increase in IFNy was present in SIT patients (42.5 to 59.4 pg/ml, $p = 0.059$) (Fig. 3A). IL5 mediates activation and recruitment of eosinophils in allergic inflammation [2], and is thus regarded as a classic Th2 cytokine. Mitogenic stimulation with SEB induced significantly more IL5 in the allergic and SIT group than in controls before and after booster (Fig. 3B). TBE-specific IL5 levels increased significantly in the allergic group and in controls but



B

	allergics	allergics+SIT	controls
n	49	21	49
gender (m/f)	19/30	9/12	19/30
age (y)	30.1 (28.0-33.4)	37.2 (31.8-42.7)	30.5 (27.7-33.3)
BMI	22.6 (21.6-23.5)	23.7 (21.4-25.6)	23.3 (22.1-24.5)
interval (y) to last booster	6.5 (5.6-7.4)	5.4 (4.0-6.9)	6.6 (5.2-7.8)
SIT duration (y, mean & range)		2.4 (5mo – 4.8y)	
total IgE at d0 (kU/l, mean & range)	140.8 (66.8 – 214.9)	155.2 (3.5 – 306.9)	24.6 (12.4 – 36.8)

Fig. 1. *Study design and patient characteristics.* (A) Flow-diagram of study design and participant flow. (B) Demographic data of study subjects. Values are given as mean with 95% CI. Abbreviations: y, year; mo, month; f, female; m, male; BMI, body mass index.

not in SIT patients after vaccination (Fig. 3C). Production of the suppressive cytokine IL10 by regulatory cells is a hallmark of effective SIT [10]. We observed higher baseline IL10 in SIT patients than in controls. However, TBE-specific IL10 production was in parallel with IFNy: controls and the allergic group showed significant IL10 increases after booster, while the SIT group did not (Fig. 3D).

3.6. Quantification of lymphocyte sub-populations with flow cytometry

In order to investigate whether allergic phenotype and immunotherapy influenced the distributions of lymphocyte subsets we performed flow-cytometric analysis of PBMC.

This showed that CD3+ T cells and CD19+ B cells as percentage of total lymphocytes were within normal ranges. The numbers of B cells were similar in all three groups, whereas T cells were increased in the SIT group compared to controls. Analysis of subsets showed that CD4+ T-helper cells were significantly expanded in SIT patients (49.1% in SIT vs. 42.3% in allergics and 41.6% and controls), while CD8+ T cells did not differ between groups. Neither group showed significant shifts in subsets after the booster (Supp. Table II).

T-regulatory (Treg) cells (CD4+/CD25+/FOXP3+) as percentage of CD4 T cells were significantly higher in the SIT group than in controls (5.48% vs. 4.58%) before booster. Tregs marginally increased after vaccination in this group, while significantly decreasing in controls (Fig. 4A). Detailed analyses of naïve, resting Tregs (CD4+/CD45RA+/FOXP3^{low}) and highly suppressive CD4+/CD45RA-/FOXP3^{high} effector Tregs [25,26] revealed that before booster effector Tregs were significantly increased in the SIT group compared to controls (2.05% vs. 1.73%). After booster effector Tregs slightly decreased in SIT patients and, again, were significantly reduced in controls (Fig. 4B). Both Treg subsets in the allergic group did not significantly differ from the controls before and after vaccination.

Plasmablasts are precursors of antibody-secreting cells [27] and have been shown to increase one week after vaccination or infection [28]. We observed more plasmablasts (CD19+/CD27++/CD38^{high}) in the SIT, but not the allergic group compared to controls before booster (1.70% in SIT vs.1.12% in controls, $p = 0.09$). After vaccination plasmablasts expanded in all groups, but increased most prominently in the allergic group (1.33% to 2.22%, Fig. 4C).

Low-affinity IgE-receptor (FcεRII, CD23) is expressed on B cells and other hemato-poietic cells and up-regulated in the presence of IL4 and IgE [29,30]. We observed more FcεRII-expressing B cells (CD19+/CD23+) in the allergic group compared to control and SIT group before vaccination (60.1% vs. 53.9% and 53.1%, $p < 0.05$). Post-booster CD23+ B cells decreased in both the allergic and SIT group, but not in controls (Fig. 4D).

Table 1
Description of allergic patients.

		Allergic			Allergic + SIT	
(A) Sensitizations in allergic and SIT group						
n		49			21	
		x/n	%		x/n	%
Mono-sensitized		**8**	**16.3**		**5**	**23.8**
	Grass pollen	3	6.1	Wasp venom	3	14.3
	House dust mite	3	6.1	Bee venom	2	9.5
	Tree pollen	2	4.1			
Poly-sensitized		**41**	**83.7**		**16**	**76.2**
	2x	12	24.5	2x	4	19.0
	3x	4	8.2	3x	3	14.3
	4x	9	18.4	4x	4	19.0
	≥5x	16	32.7	≥5x	5	23.8
Most frequent allergens	Tree pollen	33	67.3	Tree pollen	11	52.4
	Grass pollen	32	65.3	Grass pollen	12	57.1
	House dust mite	25	51.0	House dust mite	9	42.9
	Weed pollen	20	40.8	Weed pollen	7	33.3
	Animal dander	19	38.8	Animal dander	3	14.3
	Rye pollen	12	24.5	Rye pollen	5	23.8
	Mold	7	14.3	Mold	2	9.5
	Insect venom	0	0	Insect venom	9	42.9

Patient	Gender	Age (y)	Allergens	SIT for	Duration of SIT (mo)
(B) Description of patients with specific immunotherapy					
1	f	20	b, t	b	21
2	f	21	a, h, t, we (f)	h, t	19
3	f	25	a, g, h, m, t, we	g, h, t	19
4	f	30	g, r, t, we	g, r, t	32
5	f	30	b	b	45
6	f	33	wa	wa	26
7	f	40	b	b	36
8	f	40	g, h, r, t	g, r, t	42
9	f	52	g, h, r, t, wa, we	g, h, wa	36
10	f	55	m, we	m	9
11	f	55	wa	wa	5
12	f	56	g, t, wa	wa	18
13	m	22	g, r, we	g, r, we	58
14	m	30	a, g, h, t, we	h	21
15	m	33	g, h	h	32
16	m	33	g, h, t	h, t	55
17	m	34	g, h	g, h	37
18	m	34	g, r, t (f)	g, r	8
19	m	36	g, h	g, h	30
20	m	45	t, wa	wa	32
21	m	58	wa	wa	34

(A) Frequency of mono- and poly-sensitizations, specificity of mono-sensitizations and ranking of the most frequent allergens are indicated as n/group and percentage.
(B) Gender, age, and sensitization to the respective allergen(s), as well as specificity and duration of SIT are provided for each patient.
Abbreviations for allergens:
insect venom: b, bee venom; wa, wasp venom.
inhalant perennial allergens: a, animal dander; h, house dust mite; m, mold; [f, food].
inhalant seasonal allergens: g, grass pollen; t, tree pollen; r, rye pollen; we, weed pollen.
f, female; m, male; mo, month; y, years.

3.7. Sample size and data evaluation

The sample size for the SIT group was less than half of that for the other groups. For comparison of antibody titers, this difference in sample size was tolerated according to statistical power calculations as outlined in Material & Methods. For comparison of the cellular results, we believe that the interpretation of our data is not limited by sample size difference, since there was a strong coherence of the results obtained with different outcomes.

3.8. Reactogenicity

The occurrence, duration and intensity of local and systemic side effects were reported by study subjects, who rated intensity as one (mild), two (intermediate) or three (strong). We found that the incidence of local and systemic reactions to the intramuscularly applied TBE vaccine was slightly lower in the allergic and slightly higher in the SIT group compared to controls. Gender-specific analysis showed higher reactogenicity in females in the allergic and control group, but no gender differences in the SIT group (Table 2). Intensity and duration of local and systemic side effects were augmented in SIT patients: local symptoms were slightly more intense and of longer duration in both genders, while systemic effects were only more intense and longer lasting in females. Local pain, redness and swelling at the injection site and headache, fatigue and muscle pain were the most frequent reactions (Supp. Fig. 3A, B). No exacerbation of allergic symptoms and no serious adverse events attributable to the TBE booster were reported in neither of the groups.

4. Discussion

In the present study we investigated whether allergic disease and allergen-specific immunotherapy influenced immune responses to a TBE booster vaccine. As our objective was to

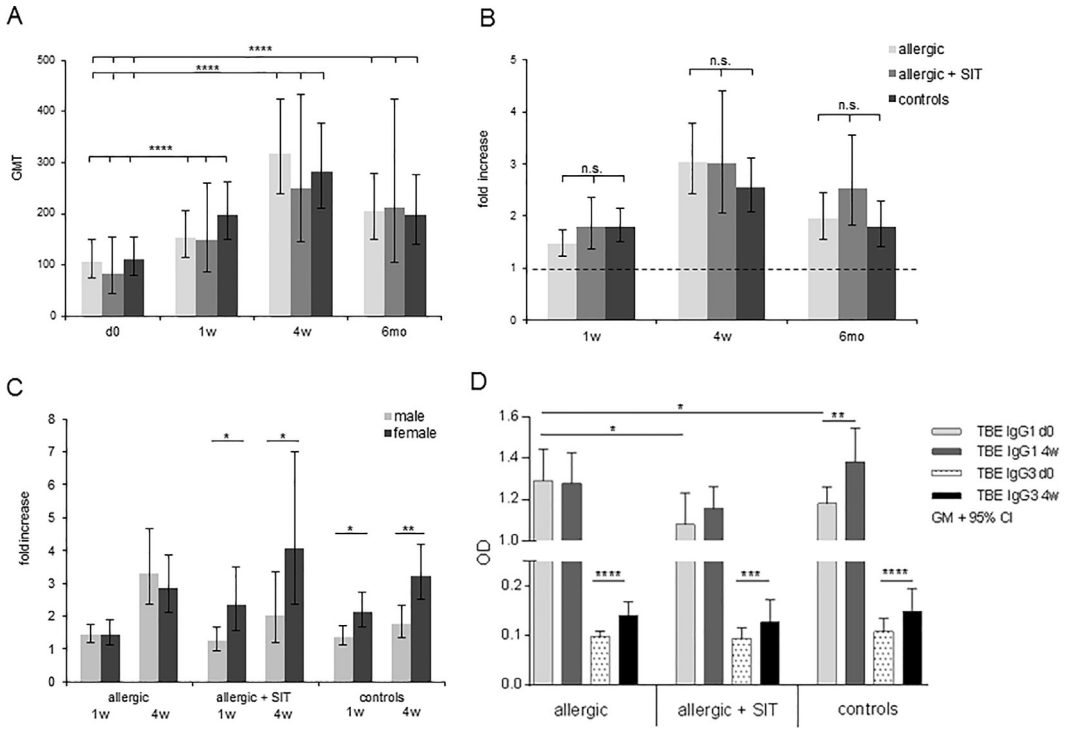


Fig. 2. *Geometric mean titers (GMT) of Neutralization Tests (NT) and TBE-specific IgG1 and IgG3 levels.* Anti-TBE virus antibody titers were measured by NT in sera obtained before (day 0) and 1 week, 4 weeks and 6 months after booster. (A) Titer kinetics of neutralizing TBE-specific antibodies, GMT with 95% CI. Titer increases from d0 to 1w, d0 to 4w and d0 to 6mo are in all groups significant with $p < 0.0001$. (B) Fold increase of GMT 1 week, 4 weeks and 6 months post-booster with 95% CI non-overlapping with 1, indicating a significant increase form baseline with $p < 0.05$. The differences between groups are at all time points not statistically significant. (C) Fold increase of GMT 1 week and 4 weeks post-booster in males and females. TBE-specific IgG1 and IgG3 were measured with in-house ELISA in sera obtained before (d0) and 4 weeks after booster. (D) TBE-specific IgG1 and IgG3, Geometric mean OD with 95% CI. ANOVA with linear contrasts; $^{****}p < 0.0001$; $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$.

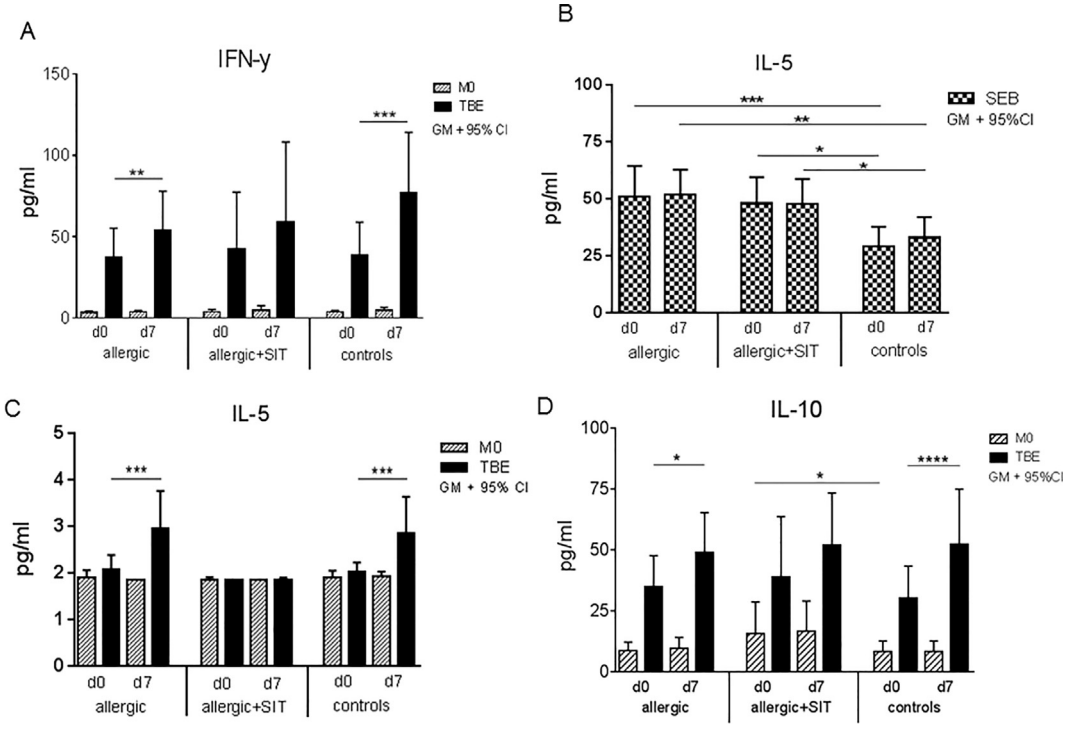


Fig. 3. *Cytokine production of re-stimulated PBMC.* IFNy, IL5, and IL10 concentrations in culture supernatants were measured with Luminex technology. Geometric mean (GM) concentrations (pg/ml) with 95% CI from PBMC obtained before (d0) and 1 week (d7) after booster are shown. (A, C, D) IFNy, IL5, and IL10 concentrations (pg/ml) from PBMC incubated with medium (hatched bars) and 0.4 μg/ml TBE antigen (full bars) for 48 h. (B) IL5 concentrations (pg/ml) in supernatants from PBMC re-stimulated with 1 μg/ml super-antigen *Staphylococcus* Enterotoxin B (SEB) for 48 h. ANOVA with linear contrasts; $^{****}p < 0.0001$; $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$.

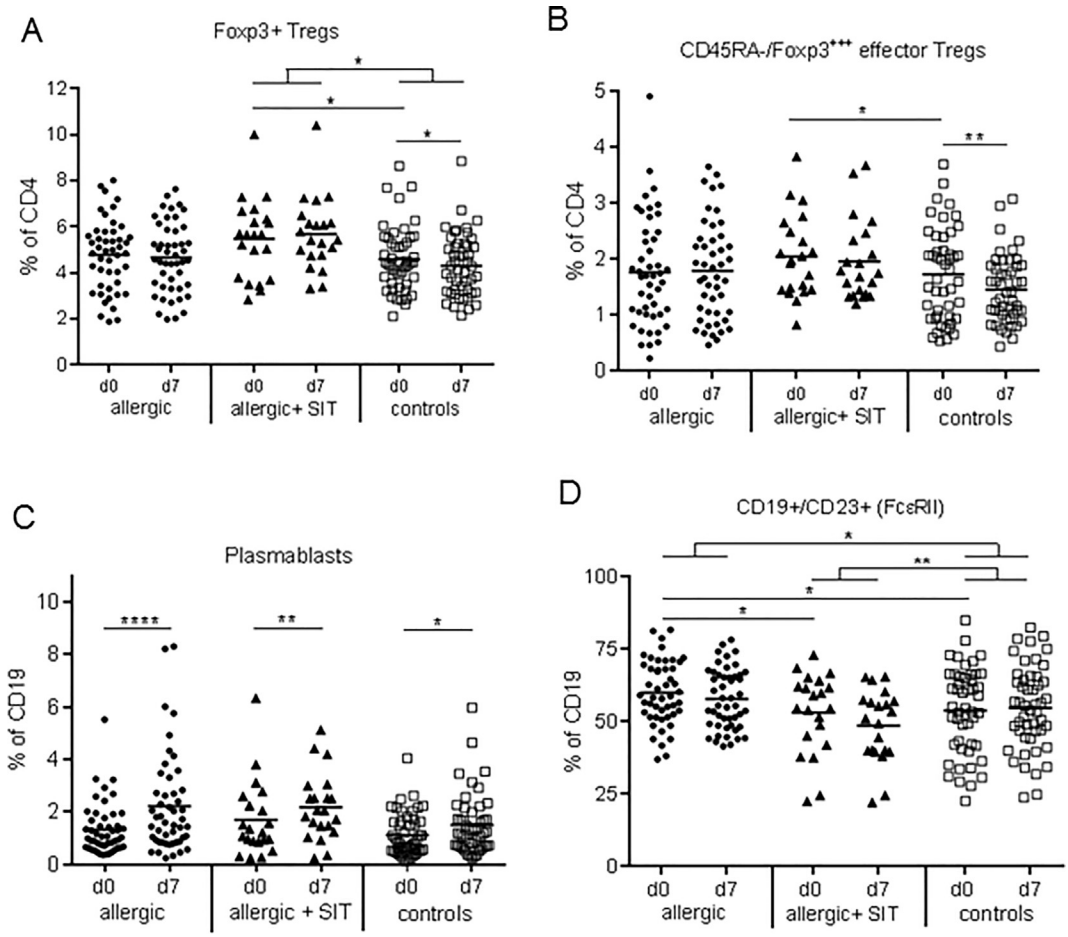


Fig. 4. *Quantification of lymphocyte subsets.* PBMC were stained with fluorochrome-labelled mAbs and analyzed on a BD FACS Canto II flow cytometer. (A, B) T regulatory cells (Tregs, CD4+/CD25+/FOXP3+) and effector Tregs (CD4+/CD45RA-/FOXP3^{high}) as percentage of total CD4+ T cells before (d0) and 1 week (d7) after booster. (C) Plasmablasts (CD19+/CD27++/CD38^{high}) as percentage of total CD19+ B cells before (d0) and 1 week (d7) after booster. (D) *FcεRII* (CD23) expression on B cells as percentage of total CD19+ B cells before (d0) and 1 week (d7) after booster. ANOVA with linear contrasts; $^{****}p < 0.0001$; $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$. Line is arithmetic mean.

Table 2
Incidence of local and systemic reactions to intra-muscularly applied TBE vaccine.

Reactogenicity	Allergic		Allergic + SIT		Controls	
Local	n	%	n	%	n	%
Total	23/49	46.9	12/21	57.1	27/49	55.1
Males	0/19	0.0	5/9	56.0	6/19	32.0
Females	23/30	77.0	7/12	58.0	21/30	70.0
Systemic						
Total	19/49	38.8	12/21	57.1	23/49	46.9
Males	0/19	0.0	4/9	44.0	5/19	26.0
Females	19/30	63.0	8/12	67.0	18/30	60.0

Frequency of local and systemic reactions as n/group and as percentage for entire group (total), males, and females are given.

investigate the general impact of a Th2-polarized immune system on vaccine responsiveness, we chose to include a heterogeneous collective of allergic sensitization profiles. A similar approach accounted for the selection of allergic patients undergoing SIT. TBE vaccine was chosen as a representative vaccine which has a high vaccination coverage rate and is accurately applied according to the national recommendations in Austria.

Our results showed that upon TBE booster vaccination, the levels and kinetics of neutralizing TBE-specific antibodies were not significantly different in the two allergic groups compared to non-allergic controls (Fig. 2A, B). This indicates that protection against the TBE virus was sufficiently induced in allergic individuals and that SIT as immune-modulating therapy had no significant effect on the protective humoral vaccine responses. The allergic group even showed a trend towards higher titers, confirming our earlier finding of increased TBE titers in an allergic subgroup of vaccinees tested for long term protection following TBE vaccination [9]. In the current trial we also evaluated titers by gender, revealing that in the control group females had a significantly higher fold increase than males. This is in line with other studies showing that vaccination leads to increased innate and early adaptive responses in females, which result in stronger vaccine-specific Th2 polarization and higher antibody levels [31,32]. A similar gender difference was observed in the SIT patients probably due to successful immunotherapy that re-directed immune responses towards those of healthy controls (Fig. 2C). In contrast, no gender differences were seen in the allergic group with high titer increases in both males and females, which might have been mediated by the existing intrinsic allergen-driven Th2 bias.

Measurement of antibody subclasses helps to identify the immunological bias of the respective humoral vaccine response. Before vaccination, TBE-specific IgG1 antibodies were significantly increased in the allergic group compared to the controls as well as to the SIT group. After vaccination, however, only controls showed significant increases in TBE-specific IgG1 (Fig. 2D). TBE vaccine is alum-adjuvanted and the increase of TBE-specific IgG1 – and also total IgG1 (Supp Fig. 1) – in controls likely represented the expected Th2-dominated immune responses elicited by alum in healthy subjects [33]. However, we did not observe a significant

increase of TBE-specific IgG1 after booster in either of the allergic groups even when they were vaccinated with alum-containing adjuvants, suggesting that vaccination contributes to normal, protective vaccine responsiveness rather than the further propagation of the existing allergen-driven Th2 responses. This is also supported by the fact that none of these vaccinees showed a substantial increase in vaccine-specific IgE antibodies. However, the levels of TBE-specific IgG3 significantly increased four weeks after TBE booster in all three groups (Fig. 2D), which is in line with the observed increases of NT titers. IgG3 is described to have strong virus-neutralizing capacity [34–37], and TBE-specific IgG3 subclass might therefore substantially contribute to the increased titers of neutralizing Abs especially in the allergic and SIT group.

We previously observed in certain vaccine non-responders that humoral and cellular immune responses are not always in correlation [11]. Thus we were interested to study whether allergic patients, especially those receiving SIT, showed impaired cellular responses to the vaccine antigen despite detectable normal antibody levels. Indeed, the allergic group produced less TBE-specific IFNy compared to controls. High IL5 production during mitogenic SEB stimulation, however, was indicative for the allergen-driven Th2 bias in these patients (Fig. 3A, B). In contrast to controls and the allergic group, the SIT group only had moderately increased TBE-specific IFNy and IL10 (Fig. 3A, D) and no TBE-specific IL5 production after vaccination (Fig. 3C). The latter, together with increased baseline IL10 (Fig. 3D), might be indicative of immune-modulatory effects by on-going immunotherapy.

With regard to lymphocyte distributions, we observed increased total and CD4+ T cells in SIT patients (Supp. Table II), which could be due to expanded allergen-specific CD4 memory T cells with an "anergic" phenotype induced by SIT, congruent with a recent study by Ryan et al. [38]. Induction of regulatory cells is the main mechanism of successful immunotherapy [10,39]. Accordingly, we observed increased CD4+/CD25+/FOXP3+ Tregs and also highly suppressive CD4+/CD45RA-/FOXP3^{high} effector Tregs in the SIT group prior to booster (Fig. 4A, B). These effector Tregs, which have previously been shown to dampen anti-tumor immunity [26], might have contributed to SIT-related immunosuppression/modulation in our study. Despite these increased Treg subsets, which were most likely allergen-specific, the established vaccine-specific antibody responses indicated that there was no general immuno-suppressive effect from the on-going immunotherapy in the SIT group. Controls showed significantly decreased percentages of total Tregs after the booster, as previously reported [11,40], and also a decreased effector Treg population. To our knowledge this is the first description of effector Tregs in the context of vaccination in allergic patients.

Circulating plasmablasts have been shown to increase one week after vaccination [28] and to correlate with titer increases after four weeks [41]. We observed more plasmablasts prior to vaccination only in the SIT group which could be a result of multiple allergen administrations during immunotherapy. After vaccination, the highest increase of plasmablasts was seen in the allergic group (Fig. 4C), which correlated with the stronger increase of NT antibody titers in this group. It is unlikely that this plasma blast expansion in the allergic group was only due to bystander activation of allergen-specific B memory cells as it has been demonstrated that plasmablasts are highly antigen-specific [42]. Thus, the increased number of plasmablasts post vaccination could rather have been induced by enhanced activity of T follicular helper cells, which have been shown to produce IL4 in addition to Th2 cells in allergic patients, thereby leading to enhanced B-cell maturation, plasma blast formation and antibody production [43,44].

Our observation of increased CD23+ B cells (FcεRII) in the allergic group compared to controls was in line with reports that IL4 and IgE lead to up-regulation of this low-affinity receptor for IgE [29]. Reduction of CD23+ B cells is associated with effective immunotherapy [45], which was confirmed here, showing that pre-booster CD23+ B cells in the SIT group were similar to controls. CD23 regulates IgE-production [46] and increases allergen presentation to T cells by binding IgE-allergen complexes to the B-cell surface [47]. Vaccination caused a reduction of CD23+ B cells in both allergic groups, but not in the controls (Fig. 4D). This shows that vaccination might have a beneficial effect in allergic individuals due to displacement of mostly naïve CD23+ B cells by vaccine-specific memory B cells.

Allergic reactions to vaccines are very rare in the general population [48] and the frequency of production residues in vaccines that cause allergic reactions is also very low [49]. Nevertheless, as hesitancy to vaccinate is particularly prominent in allergic patients, we monitored the reactogenicity profile upon vaccination in our study population. Overall, the incidence of side effects after TBE booster in both the allergic groups did not differ from controls and no exacerbations of allergic symptoms were reported. When we analyzed the gender-specific reactogenicity profile, which is generally documented to be higher in females [32], we found that women in both the allergic and control group reported more side effects than men, whereas in the SIT group males reported side effects with similar frequency as females (Table 2). Overall, the SIT group experienced higher intensity and longer duration of side effects; this might however be a matter of enhanced pain perception rather than increased inflammatory processes. Most importantly, neither group reported severe side effects.

In summary, our findings provide solid evidence that TBE booster vaccination in allergic patients, even those receiving SIT, can be performed efficiently and safely according to established routine vaccination schedules. The TBE vaccine, which was used as a model antigen in this study, is likely to be representative for other inactivated vaccine antigens in these patient groups. Whether primary vaccination would lead to similar outcomes is the subject of further investigation.

Funding

The Medical University of Vienna received funding from Pfizer Corporation Austria GmbH (at that time Baxter Innovations GmbH) for this work. The funders had no involvement in any aspect of this study or paper.

Conflicts of interest

The following potential conflicts of interest are declared but do not relate to this specific study. Tamar Kinaciyan provided consultancy for Shire and Leti; Karin Stiasny's institution, the Department of Virology, received research support from Pfizer for TBE epidemiology studies in Austria; Gerhard Zlabinger received lecture fees from Alexion Pharmaceuticals, Pfizer, UCB Pharma and MSD; Michael Kundi was a board member for Baxter and provided consultancy for Baxter, Pfizer and Sanofi. The other authors declare no conflicts of interest.

Author contributions

Ursula Wiedermann conceived the clinical trial. Claudia Seidl-Friedrich and Ines Zwazl facilitated the recruitment of allergic patients with Tamar Kinaciyan and Reinhart Jarisch. Michael Hofer and Claudia Seidl-Friedrich administered the study. Erika Garner-Spitzer obtained ethics approval, performed experiments and analyzed data. Karin Stiasny provided NT results and Gerhard Zlabinger measurement of total immunoglobulins. Michael Kundi

did statistical planning and final statistical analyses. Erika Garner-Spitzer and Ursula Wiedermann wrote the manuscript.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2018.03.076.

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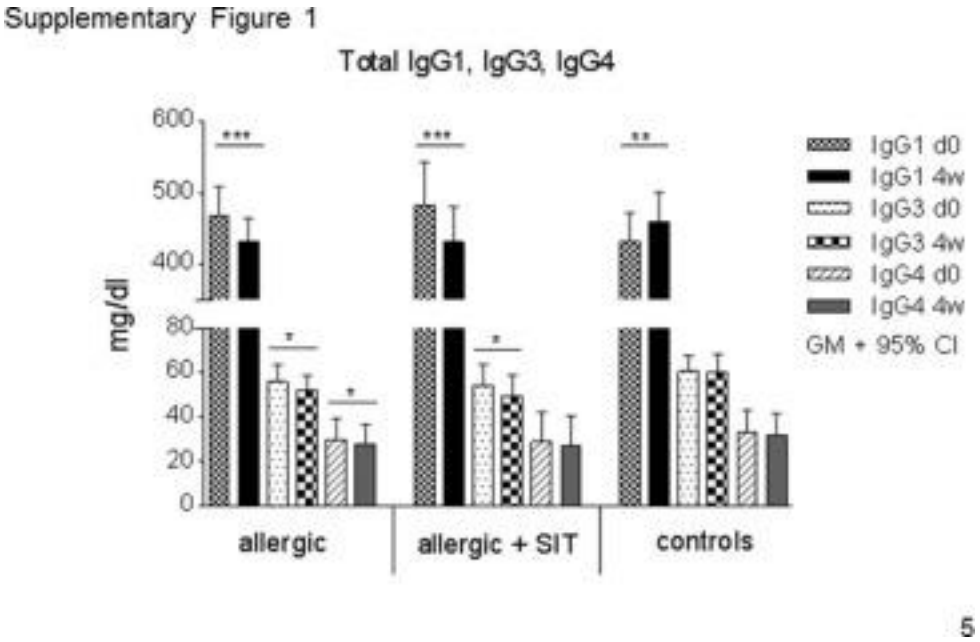


Supplementary Fig. 1.

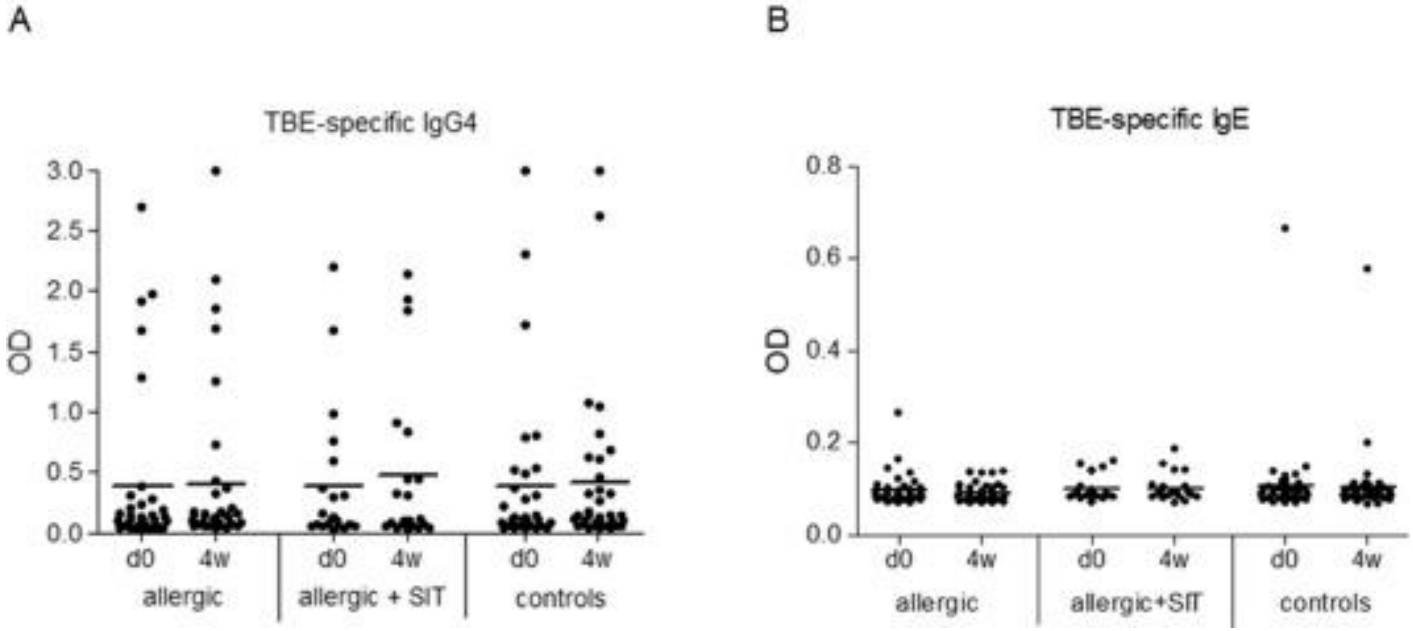
Erika Garner-Spitzer, Claudia Seidl-Friedrich, Ines Zwazl, Michael Hofer, Tamar Kinaciyan, Reinhart Jarisch, Karin Stiasny, Gerhard J. Zlabinger, Michael Kundi, Ursula Wiedermann

Allergic patients with and without allergen-specific immunotherapy mount protective immune responses to tick-borne encephalitis vaccination in absence of enhanced side effects or propagation of their Th2 bias

Vaccine, Volume 36, Issue 20, 2018, 2816–2824

http://dx.doi.org/10.1016/j.vaccine.2018.03.076

Supplementary Figure 2



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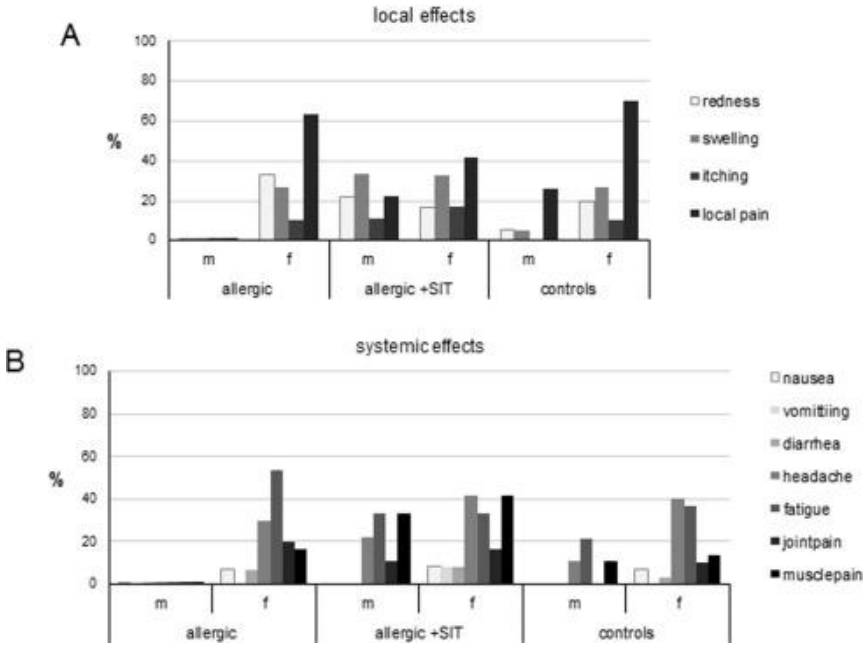
Supplementary Figure 3



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Appendix A

Supplement to Materials and Methods:

Study subjects

Allergic patients were screened at the Division of Immunology, Allergy and Infectious Diseases, AKH Vienna, the Floridsdorf Allergy Center in Vienna and the outpatient clinic of our institute between September 2013 and July 2015. All study subjects were of Caucasian ethnicity and were enrolled at our outpatient clinic.

Immunoglobulin G Subclasses

Test-specific (Optilite IgG1-4 Kit) normal ranges (95 percentiles in mg/dl) were 382.4 - 928.6 for IgG1, 241.8 - 700.3 for IgG2, 21.8 - 176.1 for IgG3 and 3.9 - 86.4 for IgG4.

TBE-specific IgG subclasses and IgE

Nunc-Maxisorp plates were coated with antigen *TBE TICOVAC-like* (2.4 µg inactivated TBE virus, strain Neudoerfl per 500µl solvent) (Pfizer Corporation Austria GmbH, Vienna, Austria) at 0.16 µg/well in coating buffer (0.1M carbonate-bicarbonate buffer, pH 9.6) overnight at 4°C. Sera obtained before and four weeks after booster were diluted at 1/100 for IgG1, at 1/50 for IgG3, and at 1/20 for IgG4 detection. Dilutions at 1/10 were used for detection of TBE-specific IgE. HRP-conjugated mouse anti-human IgG1 (clone HP6069), mouse anti-human IgG3 (clone HP6047), mouse anti-human IgG4 (clone HP6025, all Thermo Fisher Scientific, Waltham, Massachusetts, USA), and HRP-conjugated mouse anti-human IgE (clone E411 [5H2], Bio-Rad Laboratories Inc., Hercules, California, USA) at 1µg/ml and ABTS substrate were used to measure absorbance. Since standard preparations for TBE-specific IgG subclasses are not available, dilutions of control sera were used to achieve plate-to-plate comparability and results are indicated as optical density (OD).

TBE-specific in-vitro re-stimulation of PBMC

Frozen PBMC were re-established in culture medium RPMI 1640 supplemented with 10% human AB serum (Biochrom, Berlin, Germany) and 2mM L-glutamine, 50µM 2-mercaptoethanol and 0.1 mg/ml gentamycin (all Sigma Aldrich, St. Louis, Missouri, USA). Cells in duplicates were plated in 96-well round-bottom plates at $8x10^5$/well and cultured with *TBE TICOVAC-like* antigen (0.08 µg/well), super-antigen *Staphylococcus* Enterotoxin B (0.2 µg/well) and medium only for cytokine baselines (total culture volume 200µl). Cultures were maintained for 48 hours (37°C, 5% CO_2, 95% humidity); thereafter supernatants were pooled and stored at -20°C until analyses.

Flow-cytometric lymphocyte analyses

PBMC were surface-stained with the monoclonal antibodies anti-human CD19 FITC (clone HIB19), anti-human CD3 PE-Cy5 (clone HIT3a), anti-human CD4 FITC (clone RPA-T4), anti-human CD8 APC (clone RPA-T8), anti-human CD23 PerCP-Cy5.5 (clone EBVCS2), anti-human CD27 PE (clone L128), anti-human CD38 PerCP-Cy5.5 (clone HIT2) (all BD Biosciences, San Jose, California, USA). Regulatory T cells were characterized with anti-human CD3 PE-Cy5 (clone HIT3a), anti-human CD4 FITC (clone RPA-T4), anti-human CD25 PE (clone M-A251), anti-human CD45RA BV510 (clone HI100), (all BD Biosciences, San Jose, California, USA), and intra-cellular anti-human FOXP3-APC (clone PCH101) (eBioscience, now Thermo Fisher Scientific, Waltham, Massachusetts, USA). Foxp3/Transcription Factor Staining Buffer Set (eBioscience) was used for the fix/perm procedure and specific staining was verified with isotype controls.

Supplementary Figure Legends:

Figure S1: *Total IgG1, IgG3, and IgG4 concentrations*

Total IgG1, IgG3, and IgG4 were determined with turbidimetric immunoassay in sera obtained before (d0) and 4 weeks after booster.

Total IgG1, IgG3, and IgG4; Geometric mean (GM) concentrations (mg/dl) with 95% CI

ANOVA with linear contrasts; ***$p < 0.001$; **$p<0.01$; *$p<0.05$

Figure S2: *TBE-specific IgG4 and IgE levels*

TBE-specific IgG4 and IgE were measured with in-house ELISA in sera obtained before (d0) and 4 weeks after booster.

A) TBE-specific IgG4, and B) TBE-specific IgE; OD, line is arithmetic mean

ANOVA with linear contrasts; *$p<0.05$

Figure S3: *Incidence of local and systemic reactions to intra-muscularly applied TBE booster vaccination.*

A) Incidence of single local and B) single systemic side effects according to gender.

Supplementary Table I: *General study eligibility*

Inclusion criteria:

- adults of both sexes between 18 and 60 years of age
- completed primary TBE vaccination and at least one booster (received at least 2 years prior to enrolment)
- willingness to sign written informed consent form

Exclusion criteria:

- prior TBE infection
- pregnancy and breast feeding
- acute infection on day of inclusion (day 0), body temperature >37,9°C
- concomitant medications: systemic cortisone therapy, chemotherapy, immune-suppressive therapy 4 weeks prior to or during study
- administration of other vaccines 4 weeks before/after TBE vaccination
- planned surgery within 2 weeks before/after TBE vaccination
- start of de-sensitization and the first 4 weeks of allergen dose escalation
- any contraindication to administration of FSME-Immun® vaccine according to manufacturer's instructions
- history of malignant disease(s) within the last 5 years
- autoimmune diseases
- drug addictions
- plasma donors
- receipt of blood transfusions or immuno globulins within 3 month before study entry

Supplementary Table II: *B and T cells as % of total lymphocytes*

T cells, B cells	**CD3**		**CD19**	
as % of total ly	d0	1 week	d0	1 week
allergic	69.32 (66.86 -71.78)	68.67 (66.14 – 71.20)	7.63 (6.67 – 8.57)	7.22 (6.33 – 8.11)
allergic + SIT	73.68 (70.85 – 76.50)*	74.72 (72.37 – 77.07)*	7.01 (6.04 - 7.98)	6.34 (5.34 – 7.34)
controls	68.21 (65.47 – 70.96)	70.59 (68.28 – 72.90)	7.11 (6.34 – 7.89)	6.39 (5.78 – 7.01)
T-helper cells, CTL	**CD4**		**CD8**	
allergic	42.27 (39.91 – 44.63)	42.67 (40.37 – 44.97)	21.53 (19.77 – 23.29)	20.94 (19.25 – 22.63)
allergic + SIT	49.13 (45.47 – 52.42)**	50.46 (47.55 – 53.38)**	20.33 (17.04 – 23.37)	20.65 (17.54 – 23.75)
controls	41.57 (38.71 – 44.43)	42.97 (40.14 – 45.79)	20.56 (18.38 – 22.75)	21.16 (19.03 – 23.30)

PBMC were stained with fluorochrome-labelled mAbs and analyzed on a BD FACS Canto II flow cytometer.

Distributions of CD19+ B cells, CD3+ T cells, CD4+ helper T cells and CD8+ cytotoxic T cells are shown as mean percentages with 95% CI before (d0) and 1 week after booster.

ANOVA with linear contrasts;

**$p<0.01$; *$p<0.05$