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Short-course sublingual immunotherapy by mucoadhesive patch and tolerogenic particle enhanced allergen presentation

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To the Editor,

Allergen immunotherapy (AIT) is the only currently available medical intervention that can limit the natural course of allergic rhinitis and lead to long-term disease modification [1]. Although the subcutaneous route of immunization has previously been considered a reference for AIT, it is now well established that sublingual immunotherapy (SLIT) is an effective and safer alternative and has recently been explored in food allergy with promising results [2]. The excellent safety profile of SLIT can be explained by the fact that oral tissues constitute an immune-privileged gateway containing few proinflammatory cells and exhibiting an allergen-tolerogenic phenotype prone to orient immune responses toward a Th1/Treg-cell profile [3].

As a consequence, the default response initiated by oral APCs to allergens is immune tolerance, allowing for the administration of intact whole allergens without systemic inflammatory responses, unlike when allergens are administered via intravenous, intracutaneous or intradermal routes. However, issues still remain regarding SLIT because the requirement for daily administrations over 3-5 years leads to poor patient adherence with more than 70% abandoning treatment within the first year of treatment [4]. There is therefore a large unmet need for developing more efficacious next-generation SLIT with simplified short-course treatment regimens but with an equally safe profile. An explanation for suboptimal SLIT efficacy could be both the short retention time of the therapeutic agent and suboptimal presentation to APCs when formulated in drops or tablets at the oral mucosa. Previous studies in murine allergy models have indeed shown that allergen contact time and dose correlates with efficacy [5]. We hypothesized that extended oromucosal exposure of allergen in combination with enhanced allergen presentation in the context of tolerogenic adjuvants would address limitations in antigen exposure and potentiate efficacy in a short-course SLIT protocol. To test our hypothesis, we designed an innovative delivery system consisting of the major egg allergen ovalbumin (OVA; Gald1) formulated with *L. lactis* tolerogenic bacterial particles (TBP) [6] on mucoadhesive nanofiber-based patches [7]. In the formulation OVA was *ad mixed* with TBPs i.e. there was no coupling step involved (see methods in online supporting information). To evaluate the approach in a mucoadhesive patch-compatible translational model, we used pigs, which have an oral mucosa and immune system closely resembling humans. Also similar to humans and unlike laboratory murine models, pigs have non-keratinized oral surfaces and are not inbred [8]. A therapeutic protocol was adapted from Rupa *et al.* [9] applying a short-course administration schedule (**Fig. 1A**). Five litters

of piglets (n=42) were randomized for gender to either sensitisation intraperitoneally with OVA (piglets from 3 litters, n=31) or no sensitisation, as negative controls (piglets from 2 litters, n=8) and for use in the passive cutaneous anaphylaxis (PCA) tests (piglets from 1 litter, n=3). Sensitised piglets were randomised to sublingual treatment, 5 times using a dose-ascending administration schedule with mucoadhesive patches carrying OVA allergen alone (Group 1, OVA, n=12) or OVA *ad mixed* with TBP (Group 2, OVA+TBP, n=11). The positive control group (Group 3) consisted of OVA-sensitised piglets treated with patches containing PBS (n=8). The negative control group (Group 4, n=8) consisted of piglets that received no sensitization and no treatment. Randomisation was such that the gender distribution was even in all groups and the average value of the skin reaction size after sensitisation but before treatment (BT) was similar in all groups (see online supporting information). Briefly, desensitisation by mucoadhesive nanofiber patches carrying OVA or OVA+TBP was performed weekly using a short-course schedule with dose-ascending schedule for OVA: dose 1 contained 10 µg OVA, dose 2 contained 30 µg, and dose 3 contained 100 µg, followed by two maintenance doses with 100 µg. All patches used in the OVA+TBP treatment group contained 100 µg TBP. All patch administrations were well tolerated without local irritation at the application site. Immunologic assessment before (BT) and after desensitisation treatment (AT) was by intradermal test for immediate allergic reactions to OVA (**Fig. 1B**), and as adequate anti-pig IgE reagents were not available, passive cutaneous anaphylaxis (PCA) tests in naïve pigs were used to confirm IgE involvement (**Fig. 1C**).

The short-course mucoadhesive treatment was highly efficacious in the OVA+TBP group (Group 2) with 5 of the 11 pigs (45%) completely desensitized after treatment (AT). The 45% efficacy in Group 2 is considerably higher compared to 8% in the Group 1 with allergen alone (1 out of 12 pigs desensitized; **Fig. 1B**). These results indicated a strong synergistic effect of the TBPs. Interestingly, this resembles the synergistic effect observed in a recent randomized placebo-controlled peanut allergy oral immunotherapy clinical study with live probiotic *Lactobacillus rhamnosus*, see Sampath et al and references therein[2]. To investigate the mechanism of the enhanced mucoadhesive-induced desensitisation, fluorochrome-labelled TBPs and OVA were used in a parallel experiment to study their uptake by the sublingual mucosa and draining lymph nodes after applying the patches for 3 hours. Piglets were sacrificed and cross-sections of mucosal tissue and draining lymph nodes were analysed by confocal microscopy. The results demonstrated rapid and massive diffusion of OVA into submucosa (**Fig. 2**).

Uptake of TBPs was also evident but to a lower extent than OVA (**Fig. 2B-C**). TBP fluorescence was localised in the cytoplasm of cells present in the submucosa and OVA accumulated in vesicles that tended to be concentrated around the nuclei. Co-localisation of TBP and OVA was also observed, although separate localisation dominated (**Fig. 2D-E**). Interestingly, fluorescent TBPs and OVA were found in draining lymph nodes as soon as 3 hours after sublingual application (**Fig. 2F, G**). Further investigation is needed to identify the nature of the cells that contain OVA and/or TBPs, but based on previous mechanistic studies we believe that two tolerogenic APC subsets, Langerhans cells present in the epithelium and macrophage-like cells located along the *lamina propria*, are the most likely candidates [5] as these have also have been identified in pig oral mucosa [8]. Based on the observations, we hypothesise that allergen presentation by combining extended mucoadhesive exposure with TBPs synergistically enhanced the desensitisation effect of the immunotherapy.

The mechanism we propose is that prolonged mucoadhesive patch-based presentation of TBP in combination with OVA facilitates the phagocytic uptake of antigen by submucosal APCs and condition the local cytokine milieu towards better desensitisation compared to OVA presented alone. In support of this hypothesis we have in a human food allergic patient derived ex vivo system observed that TBP in combination with allergen, synergistically inhibits proliferation of allergic Th2 cells and expand IL-10 producing T regulatory cells (see online supporting information). Such an effect would translate into more efficient trafficking to the draining lymph nodes and ultimately higher systemic efficacy in the OVA+TBP treated group (Group 2). Further studies into the mode of action and the induction of tolerance are required to corroborate these assumptions. Interestingly, the incomplete responsiveness observed even in the high-efficacy OVA+TBP treated group (Group 2) has also been observed in human SLIT clinical studies [10], indicating that SLIT may lead to desensitisation or tolerance in only a subgroup of patients. As such the heterogenous response observed in our outbred pig model might represent a better model for human SLIT responses compared to the inbred mouse strains typically used and it will be interesting to study this further in a follow up study. Finally, our efficacious short-course SLIT protocol was achieved with maintenance doses in the 100 µg range. This is a much lower dose than in clinical oral immunotherapy and SLIT food allergy studies that use maintenance doses in the mg range [2], raising the possibility of further improving the efficacy of the enhanced mucoadhesive therapy. Interestingly, a recent AIT biomarker study with a grass tablet-based

product indicated that clinical benefit is exerted by two mechanisms: first an allergen desensitisation which occurs early and leads to clinical benefit for at least two years, while the second one is based on induction of regulatory networks which, although initiated early on intervention, is only consolidated with a sustained effect after 3 years treatment [11]. Thus, the dose used in our short-course study on one hand might have been sub-threshold for reaching a full desensitizing effect in all pigs, but on the other hand the combination with tolerogenic TBPs could have accelerated the regulatory response leading to a short-course treatment effect. At any rate, future dose finding studies are warranted to further optimise our approach. Finally, it's worth noting in this context that although the sublingual route is well documented as an efficacious route to induce tolerance in AIT, there is evidence that other sites in the oral cavity, e.g. the vestibular and buccal regions might be even more tolerogenic and worth pursuing with our approach.

In conclusion, these encouraging data suggest that a short-course SLIT treatment protocol is feasible by enhanced and prolonged allergen presentation using a combination of a mucoadhesive patch and proper stimulation of a privileged tolerogenic immune compartment by tolerogenic probiotic bacterial particles. Future studies need to explore the potential of this approach for inducing long-term disease-modifying tolerance in allergy and potentially its relevance in other immune disorders where T regulatory cells are involved in the natural resolution of inflammation.

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Conflicts of interest

PS and KL are shareholders of Allero Therapeutics.

Ethical approval

Ethical approval was obtained prior to the start of animal experiments by the Ethics Committee of the Ministry of Agriculture, Czech Republic.

Data availability

The data that supports the findings of this study are available in the supplementary material of this article

Author contributions:

Conception and design of study: P.Sørensen, K.Leenhouts, J.Masek, J. Turanek.

Acquisition of data: J.Masek, P.Turánek-Knotigová, J.Kotouček, F. Hubatka, P. Kulich, D.Lubasova.

Analysis and/or interpretation of data: P.Sørensen, P.Turánek-Knotigová, K.Leenhouts, J.Masek, M.Raska, J.Turanek.

Drafting the manuscript: P.Sørensen, J.Masek, M.Raska, K.Leenhouts, J.Turanek

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Figures and legends

Fig. 1A

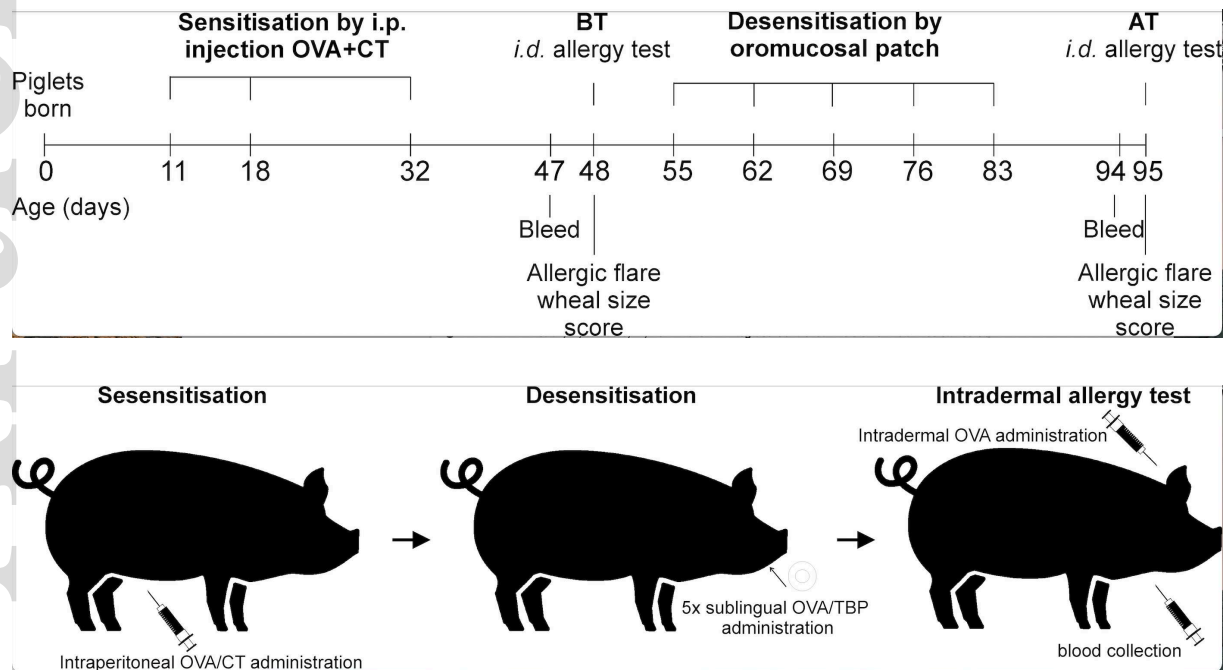


Fig. 1B

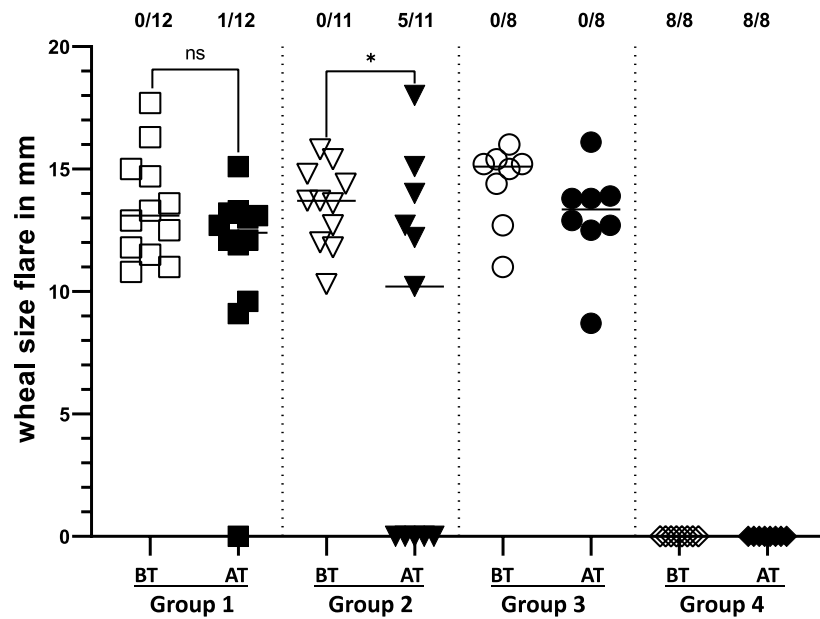


Fig. 1C

BT

AT

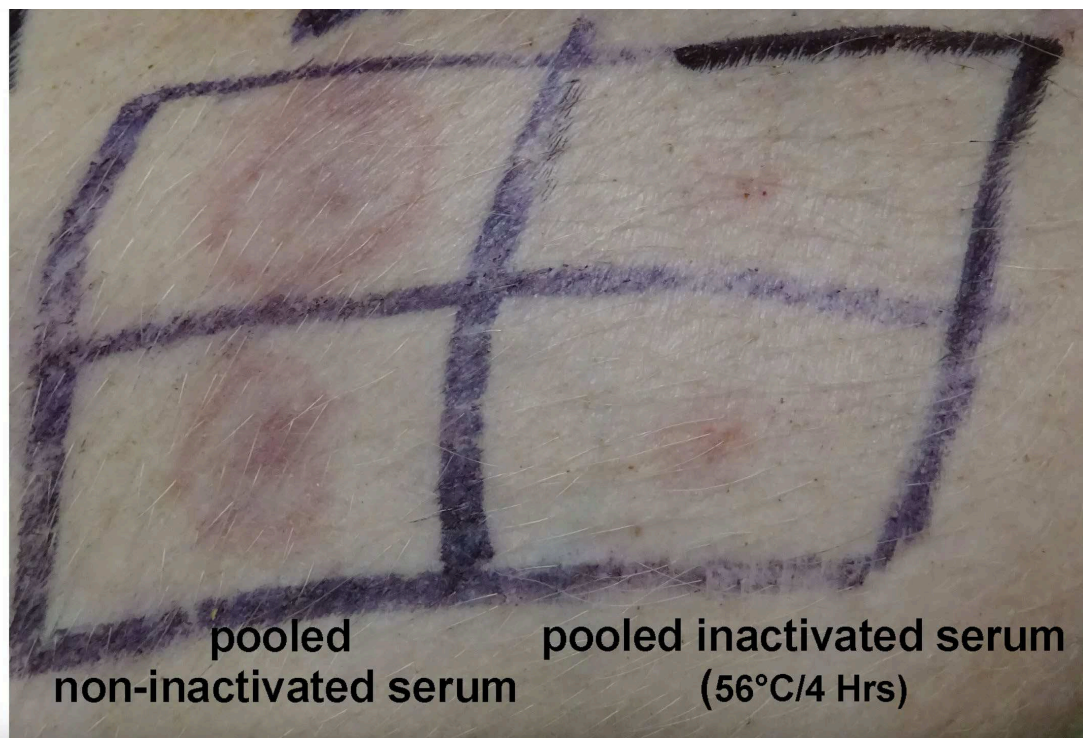


Fig. 2

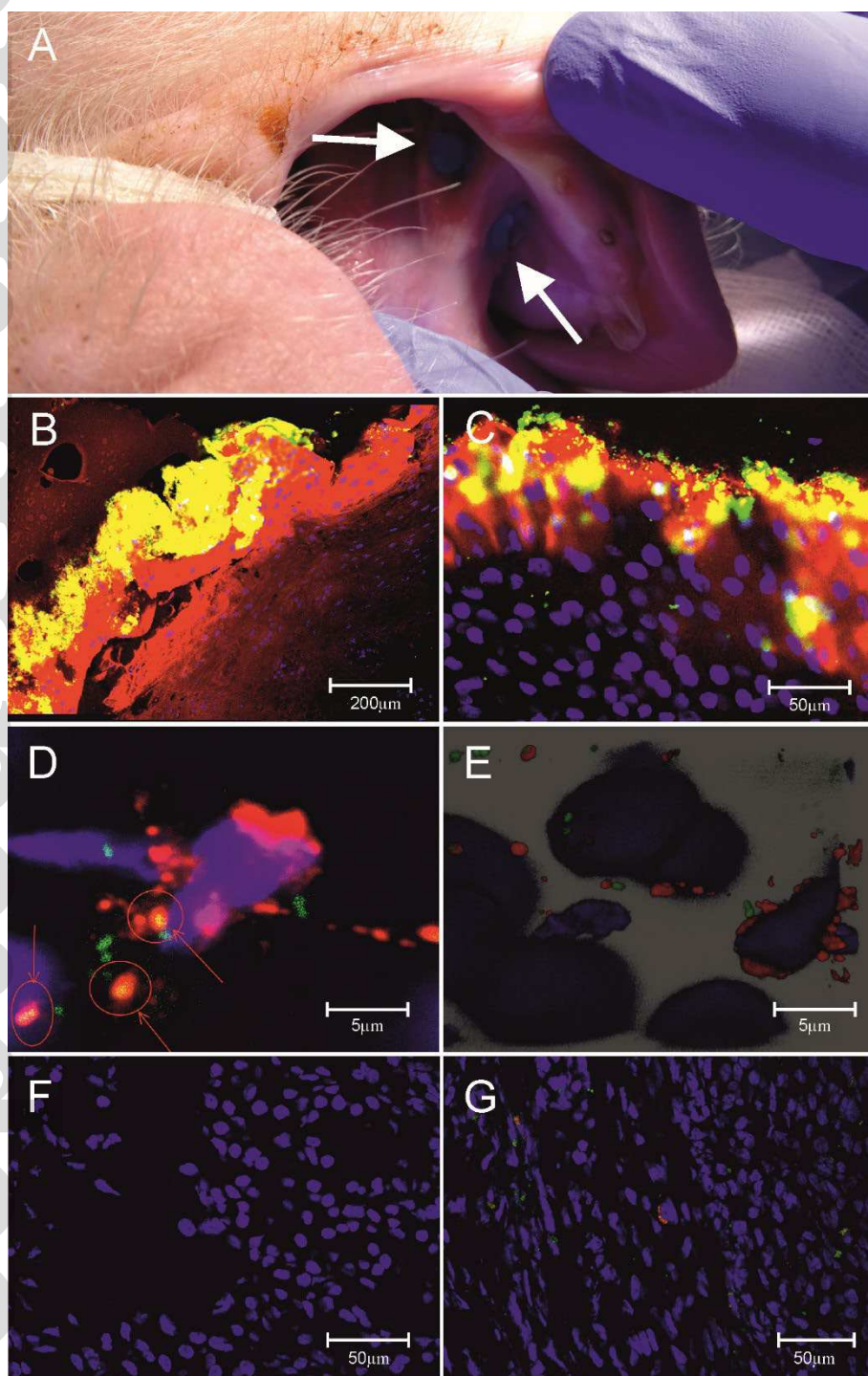


Figure legends

Fig. 1. Therapeutic food-allergic porcine model for testing short-course SLIT treatment.

A. The study results included a total of 39 outbred piglets from 5 different litters (See online supporting information) that were randomly divided into 3 litters that were sensitised (n=31) with 3 intraperitoneal administrations of OVA+CT, starting at day 11 after birth (based on a protocol adapted from Rupa *et al* [9]), and 2 litters (n=8) that were left non-sensitised for use as negative controls (NC, Group 4). Of available 31 sensitised piglets, 3 groups were given desensitisation patches: Group 1 with OVA-only patches (n=12), Group 2 with OVA+TBP patches (n=11), and Group 3 with PBS-infused patches as controls for spontaneous tolerance during the study (n=8). The desensitisation started at day 55 with sublingual treatment 5 times with weekly intervals using a dose-ascending administration schedule. Per administration two mucoadhesive patches were placed sublingually per piglets (to each piglet (see online supporting information). Blood was collected at day 47 and 94 for PCA testing in naïve pigs at the end of the study. Intradermal (*i.d.*) tests for immediate allergic reactions to OVA was done on all piglets in Groups 1-4 before treatment (BT) on day 48 and after treatment (AT) on day 95.

B. Results of intradermal (*i.d.*) tests for immediate allergic reactions to OVA expressed as flare wheal size in mm at the injection site. Group 1: desensitized with mucoadhesive patches containing OVA only. Group 2: desensitized with mucoadhesive patches containing OVA+TBP. Group 3: desensitized with mucoadhesive patches containing PBS (positive control). Group 4: not sensitized for OVA and not desensitized (negative control). Individual wheal size flares are shown before mucoadhesive patch treatment (BT) on day 48 and after treatment (AT) on day 95. The horizontal bars: group means. On the top of the Figure: number of completely desensitized animals in the group/number of animals per group. ns: no significant difference before and after treatment. Asterisk: significant difference before and after treatment ($P<0.05$). GraphPad Prism 9 software was used applying a Wilcoxon matched-pairs signed rank test.

C. Representative positive (left) allergic skin reactions of a naïve pig by passive cutaneous anaphylaxis (PCA) tests. In the shown PCA test, pooled sera were used from the animals in Group 2 obtained before desensitisation treatment at day 47 (BT) and after treatment at day 94 (AT) for those piglets that were still positive in the intradermal allergy test on day 95. Serum was incubated in a water bath (56 °C, 4 hr) to inactivate potential OVA-specific IgE. Exactly 100 µl of non-inactivated or inactivated pooled serum were administrated intradermally into naïve piglets for the PCA test. After 24 hr, 15 mg OVA in PBS (total volume 1 ml) was injected to a dorsal ear vein. The cutaneous hypersensitivity reaction develops at the site of serum sample administration within 15 min after OVA injection in case active OVA-specific IgE is present in the serum. The pooled non-inactivated serum is shown on the left-hand side of the Figure and the inactivated matching serum on the right-hand side. The absence of a wheal-shaped flare at the injection site of the heat-inactivated serum confirms the skin reaction is IgE mediated.

OVA: ovalbumin; CT: cholera toxin; TBP: tolerogenic bacterial particle; BT: before treatment; AT: after treatment.

Fig. 2. Confocal microscopy analysis demonstrating uptake of OVA and TBP by the submucosa and draining lymph nodes. A) Position of mucoadhesive patches on pig sublingual mucosa (arrows). B) Histological cross-section of sublingual mucosa with mucoadhesive patch located on the surface. Mucoadhesive patch contained FITC-labelled TBPs (green-yellow pseudocolour) and Alexa Fluor 647-labeled OVA (red pseudocolour). Nuclei of cells are labelled by Hoechst (blue). C) Enlarged section from B. D) Localisation of TBP and OVA in cytoplasm of cell (detail). Co-localised TBP and OVA (orange) as well as non-colocalised OVA (red signal) and TBP (green signal). E) Intracellular localisation of TBPs (green) and OVA (red). Note localisation of both TBPs and OVA close to nucleus. F) Confocal microscopy: histological cross-section of lymph node extracted from control piglet (no sublingual application of fluorescent TBP). G) Confocal microscopy: histological cross-section of lymph node extracted from piglet treated by sublingual application of mucoadhesive patch with fluorescent TBP. Lymph nodes extracted 3 h after application of mucoadhesive patch, which is based on our previous experience for most optimal delivery schedule for nanoparticles (7). Note several fluorescent TBP in lymph node tissue (green) and cells containing fluorescence labelled OVA (red).

FITC: Fluorescein isothiocyanate; TBP: tolerogenic bacterial particle; OVA: ovalbumin.

SUPPORTING INFORMATION

TBPs (Tolerogenic Bacterial Particles)

Preparation of TBPs derived from *Lactococcus lactis* has been described before (reference 6 in the manuscript). TBPs were diluted to the desired concentration with PBS and ultrasound was applied for 1 min at room temperature to obtain a homogenous suspension (Bandelin Sonorex 200, Germany). Fluorescently labelled TBPs were used for tracking experiments. Fluorescein isothiocyanate isomer I (FITC), F7250 (Sigma Aldrich, USA) was used for labelling of TBPs. Briefly, 74 μ L of TBP stock solution (54 mg/mL) was added to 1 mL of 0.05 M Borate buffer, pH 9.0. The sample was centrifuged at 10,000 g and 4°C for 5 min and the pellet was resuspended in 74 mL of Borate buffer (concentration $\approx 10^9$ TBP/mL). 296 μ L of FITC in DMSO (5 mg/mL) was added to the TBP suspension, immediately vortexed and then incubated in the dark under continuous mild stirring for 30 min at room temperature. Subsequently, TBPs were washed 3x with PBS. Finally, the pellet was resuspended in 200 μ L PBS yielding a 20 mg/mL TBP working solution.

Ovalbumin (OVA)

Chicken OVA (Sigma A5503-1G) was weighted and dissolved to desired concentration with PBS to be mixed with TBP solution. OVA and TBP were mixed together to get the desired final concentration indicated in the Animal experiment section. Fluorescently labelled OVA (Alexa Fluor 647 conjugate, ThermoFisher Scientific, USA) was used for tracking experiments.

Nanofiber-based mucoadhesive patches

Nanofiber-based mucoadhesive patches was prepared according to reference 7 in the manuscript. In brief, solvent casting method was used for preparation of mucoadhesive patches. Carbopol 974P and HPMC K4M plasticised with glycerol were used as mucoadhesive polymers. Ethylcellulose was used for the formation of the backing layer. Nanofiber-based non-woven tissue material (thickness of 15 μ m) made of silk fibroin and manufacturing processes of the final nanofiber-based mucoadhesive patch were selected according to [2].

Loading of mucoadhesive patch with OVA and TBP

Pre-formed nanofiber-based mucoadhesive patches (0.28 cm²) were loaded with a solution of OVA or OVA+TBP mixture before application to the sublingual mucosa of the experimental animals. The suspension of TBP in PBS was sonicated prior to the mixing with the OVA solution. Then, appropriate amounts of TBP and OVA were mixed to obtain solutions with 10 mg TBP/mL and 1, 3 or 10 mg OVA/mL. 5 μ L of the OVA, OVA+TBP, or PBS preparations were applied onto the nanofibrous reservoir layer of the premanufactured mucoadhesive patches followed by immediate sublingual administration. Two mucoadhesive patches were administered to each piglet in the Desensitisation experiment, one on right and one on left part of sublingual mucosa, thus the total volume of 10 μ L of preparation was applied to each animal (see Table in Desensitisation by specific oromucosal immunotherapy section).

Tracking of TBPs and OVA following sublingual administration

Nanofiber-based mucoadhesive patches loaded with fluorescently labelled OVA or OVA-TBP were attached to sublingual mucosa in anesthetized experimental pigs. After 3 hours, animals were sacrificed and the samples of sublingual tissues with attached mucoadhesive patches were surgically removed. Draining lymph nodes were extracted 3 h after application. Samples of tissues were immediately frozen using n-heptane and stored at -80°C until use. Cryocut (Leica, Germany) was used for cutting of the samples to obtain 15 µm tissue slices. Tissue sections were fixed using acetone and stained using Sytox Blue (ThermoFisher Scientific, Prague), nuclei acid staining dye.

Confocal microscopy (Leica SP8) was used for imaging and for tracking of FITC-labelled TBP and Alexa Fluor 647-labelled OVA following *in vivo* sublingual administration. Excitation wavelength of 488 nm was used for FITC-TBPs and 647 nm for Alexa Fluor 647-OVA. Emission spectra measured were 500-550 nm and 655-700 for the labelled TBP and OVA, respectively. To distinguish between autofluorescence of tissues and cells and FITC-labelled TBPs, spectral analysis of particular areas was done using white-light laser.

Experimental Animals

The animals used in the experiment were newborn outbred piglets (pigs were hybrids, mother: BU (Czech white high-grade pig) x Landrace; father: BU (Czech white high-grade pig) x Pietrain). The piglets were weaned at 21 days, housed with free access to food and water. The sow's diet did not contain any egg protein. Animal use was approved by the local Animal Care Committee at Veterinary Research Institute in Brno, under guidelines of the Central Commission for Animal Welfare. The piglets were not castrated.

A total of 46 piglets were obtained from 5 different litters of which 35 were treated with OVA+CT to induce allergy for OVA. Two piglets did not develop allergy for OVA and were excluded from the study. Of the remaining 33 sensitized piglets, two did not survive the study procedures. Hence, a total of 31 OVA sensitized piglets were part of the experiment and the piglets were randomly divided in Groups 1-3 as described under Characterization of particular litters entering the study. Another 8 piglets were not sensitized and used in Group 4 as a negative control for the intradermal skin tests. An additional 3 piglets were kept naive for use in the PCA testing.

Characterization of particular litters entering the study

Litter no. 1: 12 piglets

All piglets from litter 1 were sensitized 3 times. 1 piglet responded poorly to OVA at *i.d.* test BT (day 48) and was excluded from the experiment. The remaining 11 piglets were divided into 3 groups such that the gender distribution was even in all groups and the average value of the skin reaction size was similar in all groups.

Litter no. 2: 12 piglets

All piglets from litter 2 were sensitized 3 times and at *i.d.* test BT (day 48) it was found that 1 piglet did not respond and did not develop a skin reaction to OVA, thus being excluded from the experiment. The remaining 11 piglets were divided into 3 groups such that the gender distribution was even in all groups and the average value of the skin reaction size was similar in all groups.

Litter no. 3: 11 piglets

All piglets from litter 2 were sensitized 3 times. All piglets responded well to OVA in the *i.d.* test BT at day 48 and were included in the experiment. The piglets were divided into 3 groups such that the gender representation was even in all groups and the average size of skin reaction was similar in all groups.

Litter no. 4: 7 piglets

The piglets from litter 4 were not sensitized and served as a negative control. A group of negative controls was formed from 4 piglets; the remaining 3 piglets were used for PCA test. The OVA *i.d.* test BT (day 48) of all 7 piglets was negative.

Litter no. 5: 4 piglets

The piglets from litter 5 were not sensitized and were used as a negative control. The OVA *i.d.* test BT (day 48) of all 4 piglets was negative.

In conclusion, the sensitisation to OVA was highly effective with only two piglets that demonstrated a poor response to OVA in the *i.d.* test at day 48 (2 out of 35 sensitized piglets, being 5.7% non-responders). Two piglets died between days 50 and 58 (one from litter No. 2 in group 2 and one from litter No. 3 in group 3, these animals were not included in the results. Therefore, 31 piglets of the 33 in the active groups completed the experiment (93.9%). Overall, 42 piglets of the 46 newborn piglets could be included in the experiment (91.3%).

The use of the animals is summarized in Table 1 below.

Table 1

Randomisation of final experimental groups after intradermal allergy test BT (day 48)

Group	Treatment	Sensitised groups			Non-sensitised groups	
		Litter No. 1	Litter No. 2	Litter No. 3	Litter No. 4	Litter No. 5
1	Sensitisation followed by Desensitisation with OVA	4 piglets	4 piglets	4 piglets		
2	Sensitisation followed by Desensitisation with OVA + TBP	4 piglets	3 piglets	4 piglets		
3	Positive control Sensitisation followed by Desensitisation with PBS	3 piglets	3 piglets	2 piglets		
A	Passive transfer of cutaneous anaphylaxis				3 piglets	

B	Negative control				4 piglets	
C	Negative control					4 piglets

An additional consideration for the randomization was to obtain a similar distribution of the allergic reaction in the sensitized pigs among the different experimental groups before the start of the treatment (BT). The randomization resulted in experimental Groups 1-3 with similar average allergic flare wheal sizes as shown in Table 2 below.

Table 2

Experimental group		BT Intradermal allergy test (day 48) Average wheal size per group (mm)
Group 1	OVA	13.4 ± 2.2
Group 2	OVA+TBP	13.6 ± 1.6
Group 3	PBS (Positive Control)	14.1 ± 1.8
Group 4	(Negative Control)	0

OVA sensitisation of piglets

OVA sensitization was induced in piglets from litter No. 1 - 3 (see Table above) by three repeated intraperitoneal (*i.p.*) administration of 200 µg OVA (Sigma) plus 50 µg detoxified Cholera toxin (CT, Sigma) in total volume of 1 mL per piglet, starting at day 11 after birth. The interval between the first and second sensitisation was 7 days and between the second and third sensitisation was 14 days. Blood was collected by jugular venipuncture performed at day 94 (15 mL of blood/piglet). This approach has been previously shown to be effective by Rupa *et al.* (reference 9 in the manuscript). The reason for doing the sensitisation as early as possible in life of the animals is mainly because the animals are easier to handle in that life-phase.

Intradermal (*i.d.*) allergy test

Hypersensitivity to OVA was evaluated by an allergy skin test performed in Groups 1 - 4 on day 48 and 95 by *i.d.* administration of 100 µg OVA in 100 µL of PBS behind the right ear. The hypersensitivity reaction was evaluated 15 min later by measurement of the diameter by slide calliper

and recorded as wheal size flare in mm. As the allergen negative control 100 μ L PBS was applied *i.d.* behind the left ear.

Passive cutaneous anaphylaxis

For passive cutaneous anaphylaxis tests (PCA) the individual sera were used of animals of Group 1-3) as well as for pooled sera for each group with animals that were positive in the *i.d.* allergy test BT and AT, day 48 and 95, respectively. Only pooled sera of Group 4 (negative controls) were used in the PCA experiments. Serum aliquots were incubated in a water bath (56 °C, 4 hr) to inactivate OVA-specific IgE. Exactly 100 μ L of inactivated or non-inactivated serum was administrated intradermally into naive piglets. After 24 hr, 15 mg OVA in PBS (total volume 1 ml) was injected to a dorsal ear vein. The cutaneous hypersensitivity reaction develops at the site of serum sample administration within 15 min after OVA injection in case active OVA-specific IgE is present in the serum.

Desensitisation by specific oromucosal immunotherapy

The pigs from sensitized Groups 1-3 were desensitized by specific oromucosal immunotherapy by nanofiber patch administration of OVA, OVA+TBP or PBS, respectively, performed under anesthesia (mixture of Ketamine 50 mg, Xylazine 50 mg, Tiletamine 50 mg, Zolazepam 50 mg/mL; 1 mL of anesthesia mixture per 15 kg administered by *i.m.*). Enhanced SLIT was delivered by applying two mucoadhesive patches per piglet to the sublingual region. The desensitisation was performed 5 times according to a dose ascending schedule summarized in **Fig. 1** and in Table 3 below. The dose range was modelled based on the assumption that the enhanced presentation by combining TBPs and oral patch would allow for relative lower and safer dosing compared to a sublingual drop-based approach where most of the allergen is washed away almost immediately by saliva.

Table 3

Dosing and time schedule for enhanced SLIT desensitisation.

Group 1 (OVA)				
Day of administration	OVA (μ g) Total amount per treatment day	TBP (μ g) Total amount per treatment day	Volume applied per patch (μ L)	Number of patches per animal
55	10	0	5	2
62	30	0	5	2
69	100	0	5	2
76	100	0	5	2
83	100	0	5	2
Group 2 (OVA+TBP)				
Day of administration	OVA (μ g) Total amount per treatment day	TBP (μ g) Total amount per treatment day	Volume applied per patch (μ L)	Number of patches per animal
55	10	100	5	2
62	30	100	5	2

69	100	100	5	2
76	100	100	5	2
83	100	100	5	2
Group 3 (positive control)				
Day of administration	OVA (µg) Total amount per treatment day	TBP (µg) Total amount per treatment day	Volume applied per patch (µL)	Number of patches per animal
55	0	0	5	2
62	0	0	5	2
69	0	0	5	2
76	0	0	5	2
83	0	0	5	2

Ethical approval

Ethical approval was obtained prior to the start of animal experiments by the Ethics Committee of the Ministry of Agriculture, Czech Republic. The number of approval: MZe 2032

TBP in combination with allergen synergistically modulate both T effector and T regulatory cells from food allergic patients

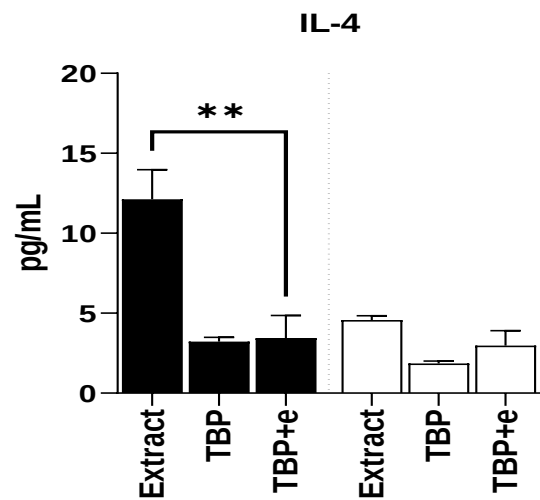
Functionality of TBPs in combination with allergen was tested in a human system. PBMCs from 5 healthy donors and 5 walnut allergic donors (diagnosis of walnut allergy confirmed by skin prick test and or specific-IgE levels > 3,5 kU) and not having previously received specific immunotherapy) were isolated and stimulated with walnut extract with and without TBPs and proliferation was determined by 5,6-carboxyfluorescein diacetate N-succinimidyl ester (CFSE) dilution assay analyzing the expression of the CFSEdim cells. The specific proliferation of different lymphocyte subpopulations was evaluated using autologous PBMCs (150.000 cells/well) stimulated with TBPs at 2×10^5 particles per well with or without walnut extract at 25µg/mL PMA and culture media was used as positive and negative control, respectively. Cells was incubated for 7 days at 37°C and 5% CO₂. After this, cells was stained with different specific fluorochrome-conjugated mAbs according to the cell subpopulation phenotypes defined as CD3+CD4+CRTH2+CCR4+ (Th2) and CD4+CD25+FOXP3+IL-10+ as IL-10 producing Treg cells (IL-10+Treg). For cell staining of the latter two populations, human Foxp3 buffer was used (BDTM PharMingen).

The cytokine pattern was evaluated by determining intracellular cytokines using BD Cytofix/CytoPerm Fixation/Permeabilization solution kit (BD Biosciences) was added during the last 6 hours of stimulation and staining with specific fluorochrome-conjugated mAbs, anti-IL-4-PE-Cy7A and anti-IL-10-PE (BD Pharmingen). After this, cells were sorted by flow cytometry (FACS Canto).

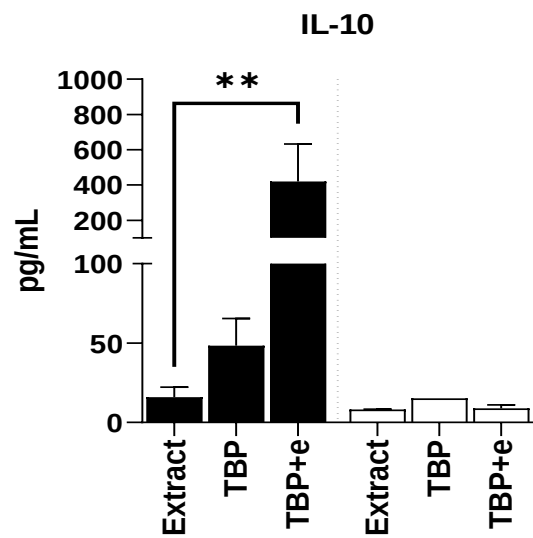
Below representative data demonstrate TBP based inhibition of Th2 proliferation and cytokine production (Fig 3 A) and synergistically increased proliferation of IL-10 producing T regulatory cells (Fig 3 B and C) when TBP was mixed with allergen extract.

Fig. 3:

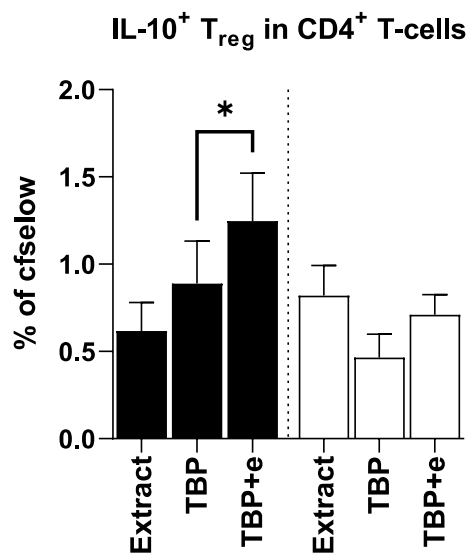
A



B



C



Black bars = walnut allergy patient PBMCs

Open bars = healthy subject PBMCs

Extract (e) = walnut extract

Asterisks = significant difference