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Nitrated food proteins induce a regulatory immune response associated with allergy prevention after oral exposure in a Balb/c mouse food allergy model

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Abstract

Background: Food allergy is associated with a high personal health and economic burden. For immunomodulation toward tolerance, food compounds could be chemically modified, for example, by posttranslational protein nitration, which also occurs via diet-derived nitrating agents in the gastrointestinal tract.

Objective: We sought to analyze the effect of pretreatment with nitrated food proteins on the immune response in a mouse food allergy model and on human monocyte-derived dendritic cells (moDCs) and PBMCs.

Methods: The model allergen ovalbumin (OVA) was nitrated in different nitration degrees, and the secondary structures of proteins were determined by circular dichroism (CD). Allergy-preventive treatment with OVA, nitrated OVA (nOVA), and maximally nitrated OVA (nOVAmax) were performed before mice were immunized with or without gastric acid-suppression medication. Antibody levels, regulatory T-cell (Treg) numbers, and cytokine levels were evaluated. Human moDCs or PBMCs were incubated with proteins and evaluated for expression of surface markers, cytokine production, and proliferation of Th2 as well as Tregs.

Results: In contrast to OVA and nOVA, the conformation of nOVAmax was substantially changed. nOVAmax pretreated mice had decreased IgE as well as IgG1 and IgG2a levels and Treg numbers were significantly elevated, while cytokine levels remained at baseline level. nOVAmax induced a regulatory DC phenotype evidenced by a decrease of the activation marker CD86 and an increase in IL-10 production and was associated with a higher proliferation of memory Tregs.

Abbreviations: BSA, bovine serum albumin; DCs, dendritic cells; h, hour; IFN, interferon; IL, interleukin; LEAP, learning early about peanut; min, minute; NIAID, National Institute of Allergy and Infectious Diseases; NOx, nitrogen oxides; NT, nitrotyrosine; OVA, ovalbumin; PPI, proton pump inhibitor; RT, room temperature; TBST, tris-buffered saline Tween-20; Th, T helper cells; TNM, tetranitromethane; Treg, regulatory T cells.

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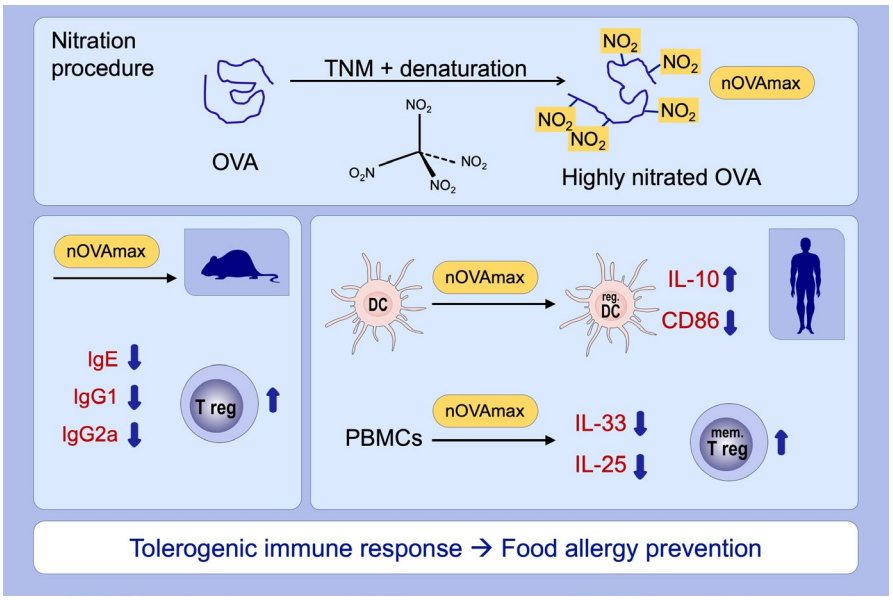
Conclusion: Oral pretreatment with highly nitrated proteins induces a tolerogenic immune response in the food allergy model and in human immune cells.

KEYWORDS
allergy prevention, food allergy, immune modulation, protein nitration, regulatory T cells



GRAPHICAL ABSTRACT
Oral pre-exposure to highly nitrated food proteins is associated with prevention of food allergy development and immune tolerance as evidenced by suppressed allergen-specific IgE levels and enhanced Tregs. A regulatory moDC phenotype indicated by low expression of costimulatory molecules and higher secretion of IL-10 was observed after pre-incubation with highly nitrated proteins. Secondary protein structure of highly nitrated allergens is substantially altered potentially being associated with the capacity to induce a tolerogenic immune response. OVA: ovalbumin; PBMCs, peripheral blood mononuclear cells; TNM, tetranitromethane

1 | INTRODUCTION

With the current absence of a commonly available causative treatment option, management of food allergy is primarily based on avoidance of ingestion and prompt medical treatment in the event of an inadvertent ingestion associated with allergic reactions. Thus, prevention of allergy development is an important aspect in management of disease. Early peanut introduction in high-risk infants represents the most convincing measure to support food allergy prevention. In the Learning Early About Peanut (LEAP) trial, infants with a high risk for developing peanut allergy were randomized to either consume or avoid peanut up to age 5 years.[1] Those who were sensitized to peanut and randomized to consumption had a 3-fold lower rate of peanut allergy development compared to the avoidance group. For the other food proteins, the potential of early introduction remains less certain due to the limited number of studies and controversial findings.[2]

As an alternative approach, modifications of dietary proteins may be used to beneficially modify the immune response.[3] Food preparation for example was shown to substantially influence protein structure and allergenicity.[4] Moreover, posttranslational protein modifications impact on protein allergenicity.

For nitration of proteins, which is the addition of a nitro group to the aromatic ring of amino acid residues, an increased allergenicity was reported.[5] Pollutants, such as nitrogen oxides (NOx) and ozone,[6] were defined to chemically modify airborne proteins by nitration,[5] affecting protein conformation and T-cell as well as B-cell epitopes.[7] In the case of Bet v 1, the major birch pollen allergen, nitration takes place with various degrees on individual tyrosine residues providing a mixture of nitration variants.[8] Besides that, Bet v 1-nitration shows effects on antigen processing and presentation via HLA-DR [9] as well as enhancement of oligomer formation, thereby postponing endolysosomal degradation. Moreover, a reduced production of pro-inflammatory cytokines in dendritic cells was reported, leading to less Th1 cells and potentially favoring a Th2 response.[10] Nitration happens as a posttranslational phenomenon in the context of environmental pollution but also during inflammation [11] and aging processes in the human body.[12,13] It has also been suggested that diet-derived nitrating agents, such as nitrate and nitrite, might promote nitration in the gastrointestinal tract.[14] The

acidic environment of the stomach causes the protonation of nitrite as the initial step for the formation of a different nitrating species, which might interact with proteins or allergens in the stomach.[15] While the presence of nitrated tyrosine residues has not been broadly understood,[14] food per se might already contain nitrated proteins.[16] Thus, exogenous and endogenous nitrotyrosine (3-NT) formation could influence food allergy. Our group has evaluated the impact of protein nitration on food allergy development. The nitrated form of ovalbumin (OVA), a major hen's egg white allergen, induced enhanced sensitization only after intraperitoneal injections. Oral administration, however, was associated with inhibited sensitization, potentially due to the accelerated gastric degradation of nitrated OVA.[17] In contrast, when IgE was already preexisting, nitrated food proteins triggered an enhanced allergic response.[18]

Based on this preceding work, we investigated the effect of pretreatment with nitrated food proteins in a mouse food allergy model in the present study. Moreover, the capacity of nitrated proteins to efficiently modulate a type 2 immune response was assessed by evaluating the response of human monocyte-derived dendritic cells (moDCs) and PBMCs after exposure to the nitrated and non-nitrated food allergens.

2 | MATERIALS AND METHODS

2.1 | Animals

Female BALB/cAnNCrl mice (15-20 g, provided with health report certificate) were purchased from Charles River Laboratories and housed under conventional conditions in the institutional mouse facility. Mice were divided into 10 groups (n = 8 mice per group) and had access to food (egg and cow's milk free diet, Ssniff) and water ad libitum. After 2 weeks of acclimation, the experimental procedures were performed according to the European Union guidelines of animal care and with permission of the animal ethics committee of the Medical University of Vienna and the Austrian Federal Ministry of Science and Research (permission number: BMWF-66.009/0229-WF/V/3b/2017).

2.2 | Nitration of OVA with two nitration degrees

Protein nitration was performed using the nitrating agent tetranitromethane (TNM) [Sigma]) as described[18] with slight modifications. OVA proteins (1 mg/mL; Sigma) dissolved in Na_2HPO_4 buffer (10 mmol/L, pH 7.4) were mixed with 0.5 mol/L TNM in methanol (Merck; molar ratio TNM: tyrosine = 10:1). The reaction was stopped by washing with Na_2HPO_4 buffer using an Amicon Ultra-15 centrifugal filter device (Merck Millipore) with a 10-kDa cutoff membrane for 8 minutes at 4000 rpm (nOVA). For the preparation of the OVA samples with the highest nitration degree (nOVAmax), OVA proteins were cooked for 60 minutes and then TNM and methanol were added. Resulting protein concentrations were determined using Pierce BCA Protein Assay Kit (Thermo Scientific) with an OVA standard curve. To determine the number of nitrated tyrosine residues per molecule, 3-NT (Sigma) was dissolved in 0.05 mol/L NaOH serving as a standard curve ranging from 6.125 to 200 μmol/L. Protein samples were diluted 1:2 in 0.1 mol/L NaOH. Absorbance was measured at 428-650 nm (TECAN, infinite M200 PRO), and number of 3-NT per molecule was calculated.

2.3 | Circular dichroism analysis

The secondary structure of OVA, nOVA, and nOVAmax was analyzed by UV Circular Dichroism (CD) spectroscopy as previously described.[19] Samples were analyzed using a J-715 spectropolarimeter (Jaco) at 0.250 mg/mL in Na_2HPO_4 buffer (10 mmol/L, pH 7.4) in a 1 mm path length quartz cuvette (Hellma) equilibrated at 20°C. The mean residue ellipticity was calculated at the respective wavelengths and analyzed using DichroWeb program.[20]

2.4 | Determination of LPS concentrations in OVA, nOVA, and nOVAmax

To detect LPS contaminations in OVA, nOVA, and nOVAmax proteins, an EndoLISA (Hyglos GmbH) was performed according to the manufacturer's instructions. The biological activity of LPS was measured using a cell-based assay as described previously.[21] Briefly, HEK-293 cells were transiently transfected with plasmids containing the LPS receptor complex and a NF-κB-dependent reporter gene (Luciferase) for 1 day. Cells were stimulated with a LPS standard and the three proteins (LPS concentration of nOVAmax was adjusted). After 24 hours, reporter gene expression was measured.

2.5 | Mouse immunization protocols

The oral preventive therapy (OVA, nOVA, and nOVAmax; 3 groups for each allergen preparation; 200 μg of protein per gavage) was performed daily for 14 days. One mouse group was kept naïve. After preventive therapy, final read-out experiments were performed for 1 group of each pretreatment (Figure S1). Blood was collected by cardiac puncture. Spleens, mesenteric lymph nodes (MLN), and intestines were harvested for further evaluations.

For sensitization, 1 group of each pretreatment (with OVA, nOVA, and nOVAmax) was gastric acid suppressed with 116 μg of the proton pump inhibitor (PPI) omeprazole (OMEP) dissolved in 0.9% sodium chloride on 3 consecutive days. On day 2 and 3, mice received 200 μg OVA orally mixed with the sucralfate (Gerot Lannach Pharma Company). For control, 1 group of each pretreatment received 200 μg OVA orally on days 2 and 3 (Figure S1).

Mice were immunized every second week for 4 times. Peripheral blood was collected from the facial vein before the experiment start and after the 14 days of prophylactic therapy. After the fourth immunization cycle, all mice were subjected to the final read-out experiments as described above. Immunized mice were challenged orally with 2 mg in 100 μL of OVA in distilled water, and the body temperature was measured before and 15, 30, and 45 minutes after challenge.

2.6 | Splenocyte stimulation experiments and cytokine measurements

After removal of spleens, splenocytes were further processed under sterile conditions as described.[22] For stimulation, 4×10^5 cells/well were incubated with 5 μg/mL of OVA, nOVA, nOVAmax, ConA, and medium, respectively, for 72 hours at 37°C. Supernatants were screened for IL-4, IL-5, IL-10, IL-13, and IFN-γ levels by multiplex technology following the manufacturer's instructions (Procartaplex, ThermoFisher). The samples were measured and analyzed using a Bio-Plex 200 (Bio-Rad).

2.7 | Flow cytometry analysis of murine $CD4^+$ and regulatory T cells

Splenocytes were stained for regulatory T cells (Tregs) using a mouse regulatory T-cell staining kit (eBioscience) according to the manufacturer's protocol. Staining procedure is described in Data S1. CD4+CD25+FOXP3+ cells were measured on a FACS Canto II (BD), and the results were analyzed by BD FACSDiva™ software (BD).

2.8 | Detection of OVA-specific antibodies in sera and intestinal lavages

Blood was collected from facial vein or cardiac puncture after killing. Small intestines were removed and flushed with 2 mL PBS and protease inhibitor (Complete Mini, Roche). Serum samples were screened for OVA-specific IgE, IgG1, and IgG2a and lavages for total and OVA-specific IgA by enzyme-linked immunosorbent assay (ELISA) as previously described.[23] To analyze the biological activity of OVA-specific IgE antibodies, a rat-basophil leukemia cell assay (RBL assay) was done as described elsewhere.[17]

2.9 | Isolation of human peripheral blood mononuclear cells (PBMCs) and generation of monocyte-derived dendritic cells (moDCs)

Human PBMCs were isolated from blood donations of healthy, anonymous donors (kindly provided by the blood bank Salzburg, Austria) using density gradient centrifugation.[23] 0.5×10^6 cells/mL PBMCs were seeded in IMDM (Sigma) supplemented with 5% FCS (PAA), 100 U/mL penicillin (Sigma), and 100 μg/mL streptomycin (Sigma). After 7 days of stimulation with 10 μg/mL of OVA, nOVA, nOVAmax (LPS concentration adjusted), or the respective amount of LPS, cells, and supernatants were harvested for flow cytometry and ELISA, respectively.

For the isolation of monocytes, PBMCs were allowed to adhere for 70 minutes. After removal of nonadherent cells, monocytes were differentiated to moDCs in the presence of 50 ng/mL GM-CSF and 50 ng/mL IL-4 (Novartis) for 6 days. There upon 1×10^5 moDCs/mL was seeded and either left untreated or stimulated with OVA, nOVA, nOVAmax (LPS concentration not adjusted), and LPS for indicated times.

2.10 | Flow cytometry analysis of human moDCs and T cells

Human moDCs were stained as indicated in Table S1, and surface marker expression was measured using a FACS Canto II. Analysis of human T-cell subsets within the PBMC population was based on a modified version of OMIP-030 by Wingender et al[24] using Cytoflex and CytExpert software. Cells were stained as indicated in Table S1. Gating of moDCs and T cells was performed as described in Figures S2, S3. Subsequent cell analysis was performed with FlowJo (version 10).

2.11 | Detection of cytokines in cell culture supernatants by ELISA

Cytokines secreted by moDCs or PBMCs were analyzed using Sandwich-ELISA for IL-10, IL-25, and IL-33 (Peprotech) according to the manufacturer's instructions.

2.12 | Statistics

All samples were analyzed in groups as defined in the immunization protocol. Data were statistically compared using GraphPad Prism version 5.00 for Windows (GraphPad Software, www.graphpad.com). All results were checked for normal distribution by Kolmogorov-Smirnov test. Treg levels in splenocytes, RBL assay, body core temperature measurement, IgE, IgG1, IgG2a, as well as total and specific IgA levels were analyzed with ANOVA and Tukey's post-test or with Kruskal-Wallis nonparametric test with Dunn's multiple correction. Surface marker expression and cytokines measurements from human moDCs as well as human Treg levels and Th2 levels were analyzed with ANOVA combined with the Tukey's post-test. P values of <.05 were considered as statistically significant.

3 | RESULTS

3.1 | OVA heat pretreatment is associated with enhanced protein nitration

The nitrated OVA samples, nOVA and nOVAmax, were prepared as described above, and the protein concentration was measured by BCA assay. The final concentration of nOVA samples was 11.4 mg/mL, and the nitration degree was 17.15%. After the pretreatment by heat, the final protein concentration of nOVAmax was 10.1 mg/mL and the nitration degree was determined to be 83.7% (Table 1).

3.2 | Nitration effects on secondary structure of OVA

Circular dichroism analysis (Figure S4) of OVA, nOVA, and nOVAmax revealed that treatment associated with maximal nitration influences secondary protein structure. The CD spectra indicated conformational changes in nOVAmax, with a decrease of α-helical

TABLE 1 Characterization of OVA, nOVA, and nOVAmax samples by protein concentration, degrees of tyrosine nitration, and LPS content

Proteins	Protein conc. (g/L)	Nitrotyrosine per molecule	Degree of nitration	LPS conc. (EU/mL)	LPS conc. in 10 µg protein (ng)
OVA	10	n/a	n/a	5	1
nOVA	11 402	1.7156	17.15%	5	1
nOVAmax	10 112	8.3708	83.7%	1.5	0.3

structures while unordered domains increased compared to OVA and nOVA (Table 2).

3.3 | Elevated levels of regulatory T cells are found after OVA sensitization only after nOVAmax pretreatment

T-cell characterization by flow cytometric analysis revealed comparable numbers of Tregs immediately after the 14 days of prophylactic treatment irrespective of the applied food protein preparation. Treg levels were similar to those observed in the naïve animals. After sensitization by oral OVA feeding under concomitant gastric acid suppression or after oral exposure to OVA alone, we observed significantly elevated signals for regulatory T cells, however, only in mice pretreated with nOVAmax samples (Figure 1).

3.4 | Pretreatment with nOVAmax suppresses systemic antibody response after immunizations, while OVA-specific intestinal IgA titers are elevated

Evaluations of sera collected on the day of final read-out experiments indicated significant differences regarding OVA-specific antibody induction capacity by immunizations after prophylactic therapy. Measuring OVA-specific IgE titers as surrogate for allergic sensitization indicated that 14 days of oral OVA, nOVA, or nOVAmax feeding did not induce elevated OVA-specific IgE titers. When we screened sera after subsequent sensitizations, significantly elevated titers were found in groups receiving nOVA pretreatment and in OVA pretreated group receiving subsequent OVA feeding. For nOVAmax pretreated animals, IgE levels remained at baseline comparable to titers measured in naïve animals (Figure 2A). These data were confirmed by RBL assay. While sera of nOVAmax pretreated mice triggered only baseline mediator release, significantly higher mediator release was detected after RBL cell incubation with sera of groups pretreated with nOVA, irrespective of the subsequent sensitization protocol (Figure 2B). Within these groups, animals with high mediator release revealed a drop of body temperature 15 minutes after oral challenges as indicated by core body temperature measurements (Figure 2C). However, apart from high IgE responders, the chosen prophylactic treatment approaches impeded the development of a clinically manifest food adverse response upon oral challenge, as no drop of the core body temperature beyond 0.5°C was observed 15 minutes after oral challenges (Figure 2C).

Moreover, groups were additionally compared for IgG1 and IgG2a levels by ELISA measurement. In line with IgE data, we observed suppression of OVA-specific IgG1 (Figure 3A) and IgG2a (Figure 3B) formation in groups after nOVAmax pretreatment and subsequent oral OVA sensitization under gastric acid suppression and to a lesser extent after continuous oral feeding of OVA. The 14 days pretreatment did not induce OVA-specific serum IgG elevation, irrespective of three different pretreatment strategies (Figure 3A,B).

When the mucosal immune response was evaluated by measurement of total and OVA-specific IgA titers in intestinal lavages, we observed a significant elevation of total IgA levels, however only in groups receiving nOVA and nOVAmax followed by OVA feeding (Figure 3C). Significantly elevated OVA-specific IgA levels were found after pretreatment compared to naïve animals, regardless of the chosen prophylactic treatment approach. Significant antibody suppression was again observed in mice after preventive therapy with nOVAmax, however, only in the group with subsequent OVA sensitization (Figure 3D).

TABLE 2 Circular dichroism

Secondary structure	α-helix	β-sheet	turn	Unordered
OVA	0.49	0.23	0.07	0.21
nOVA	0.48	0.25	0.09	0.19
nOVAmax	0.13	0.27	0.25	0.32

Note: Protein secondary structure was estimated by the program CDSSTR, and the model protein set 4 was used.

3.5 | Prophylactic therapy induces suppression of cytokine release

When supernatants of stimulated splenocytes were screened for release of Th2, Th1, and Treg cytokines (IL-4, IL-5, IL-13, IFN-γ, and IL-10) by multiplex technology, surprisingly, we observed a suppressed cytokine production as compared to the naïve mice. There was neither variation with regard to the three prophylactic treatment strategies, nor comparing before to after immunizations (data not shown).

3.6 | nOVAmax induces a regulatory DC and PBMCs phenotype

Since moDCs are responsive to LPS concentrations even lower than 1 ng/mL,[23] we determined the LPS content in OVA, nOVA, and nOVAmax by EndoLISA (Table 1). Next, we analyzed whether nitration

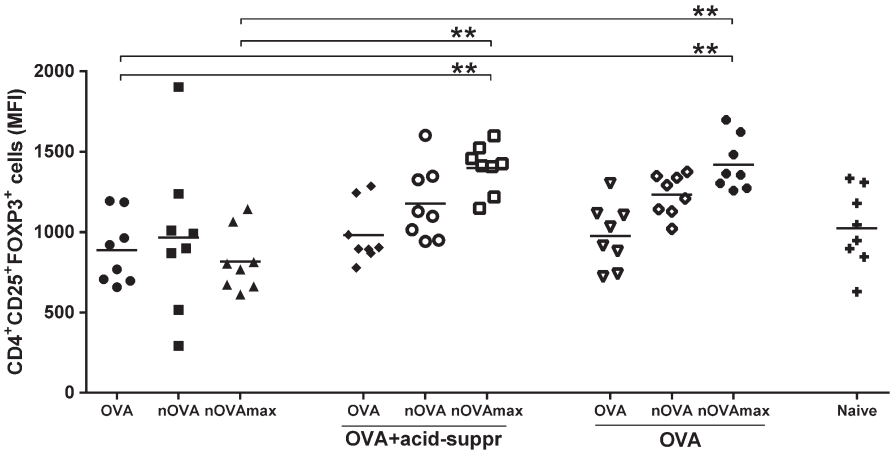


FIGURE 1 Analysis of regulatory T cells by flow cytometry. Treg cells were isolated from spleens, and the mean fluorescence intensity (MFI) was measured for the surface markers CD4, CD25, and after intracellular staining of FOXP3 by flow cytometry. 1×10^5 events (cells) were measured per sample, and the results were analyzed by BD FACSDiva™ software. (**$P < .01$)

of OVA had an impact on LPS signaling induced by the low amounts of LPS present in the OVA preparations. As shown in Figure S5, NF-κB activation was not affected by nitration.

To investigate the potential effect of OVA, nOVA, and nOVAmax on surface marker expression and cytokine production, moDCs were pre-incubated with 10 μg/mL of the respective protein for 4 hours and stimulated with 2 ng/mL LPS for another 24 hours. As shown in Figure 4A, nOVAmax downregulated the expression of CD86, while the release of IL-10 was significantly upregulated, indicating that nOVA max might induce a tolerogenic DC phenotype.

In addition, PBMCs were isolated and incubated with all 3 types of proteins for 7 days. Interestingly, the allergy-related cytokines IL-25 and IL-33 were suppressed by nOVAmax in comparison to nOVA and OVA (Figure 4B), whereas the proliferation of memory T regulatory cells was significantly increased with nOVAmax. The proliferation of Th2 cells was unaffected and stayed at baseline levels (Figure 4C).

4 | DISCUSSION

In the present study, we investigated the preventive effect of oral pretreatment with nitrated food proteins (with different nitration degrees) in our mouse food allergy model of oral immunizations under concomitant gastric acid suppression.[25] Furthermore, we analyzed the impact of exposure to nitrated food proteins on the response of human moDCs and on T cells.

Pre-exposure to food proteins was found to be associated with prevention of food allergy development irrespective of the protein used as evidenced by core body temperature measurements upon oral challenge. Previously, our group developed an oral immunization protocol in BALB/c mice with IgE-mediated food adverse response by oral feeding under concomitant anti-acid medication.[25] Using this immunization protocol, the lack of a clinical response toward food allergens was repeatedly shown to be associated with prevention of food allergies.[26] Exposure to high dose of food allergen, such as β-lactoglobulin, OVA, or peanut via the oral route, was found to be associated with tolerance and prevents food allergy.[27-29] Here, we confirmed the preventive effect of applying food proteins via the oral route in rather low allergen dosages as evidenced by low levels of mediator release from RBL cells.

Regarding OVA-specific antibodies, we could confirm the correlation between the degree of protein nitration and antibody productions. Our previous findings indicated that oral immunization of nitrated protein under anti-acid treatment did not induce IgG and IgE formation in mice.[18] In the present project, after pretreatment with nOVAmax, all antibody levels were comparable to those measured in naïve mice. In contrast, pretreatment with nitrated OVA resulted in elevated antibody titers after sensitizations. Moreover, the epitope diversity of the food-specific IgE response might be relevant. A greater number of sequential linear epitopes is associated with higher severity of allergenic reactions and presence of milk allergy.[30] As suggested previously, nitration might efficiently change epitope structure,[10] especially due to changes of secondary protein structure as observed for nOVAmax.

In the intestinal tract, elevated titers of OVA-specific IgA were measured after pretreatment but levels decreased again after OVA immunizations. IgA is the main mucosal immunoglobulin and contributes to mucosal homeostasis by limiting the antigen adhesion and colonization.[31] Thus, higher levels of OVA-specific IgA after pretreatment might contribute to allergy-preventive effects.

Moreover, we observed a significant induction of Tregs in mice receiving nOVAmax in comparison with the other groups supporting the evidence of food allergy prevention. We observed a similar impact of nOVAmax on human moDCs. Specific subtypes of DCs exhibit regulatory functions stimulating differentiation of naïve T cells into regulatory cells.[32] Regulatory DCs are characterized by low surface expression of costimulatory molecules such as CD86 and enhanced IL-10 expression.[33,34] Accordingly, upon treatment with nitrated proteins, moDCs revealed less CD86 surface expression and more IL-10 production. Moreover, IL-25 and IL-33 are important in allergic diseases and defined as type 2 cytokines.[35,36] Our data from human PBMCs indicated that stimulation with all types of proteins had no effect on Th2 cells proliferation. Interestingly, only nOVAmax induced suppression of type 2 cytokines and significantly increased the level of memory Treg.

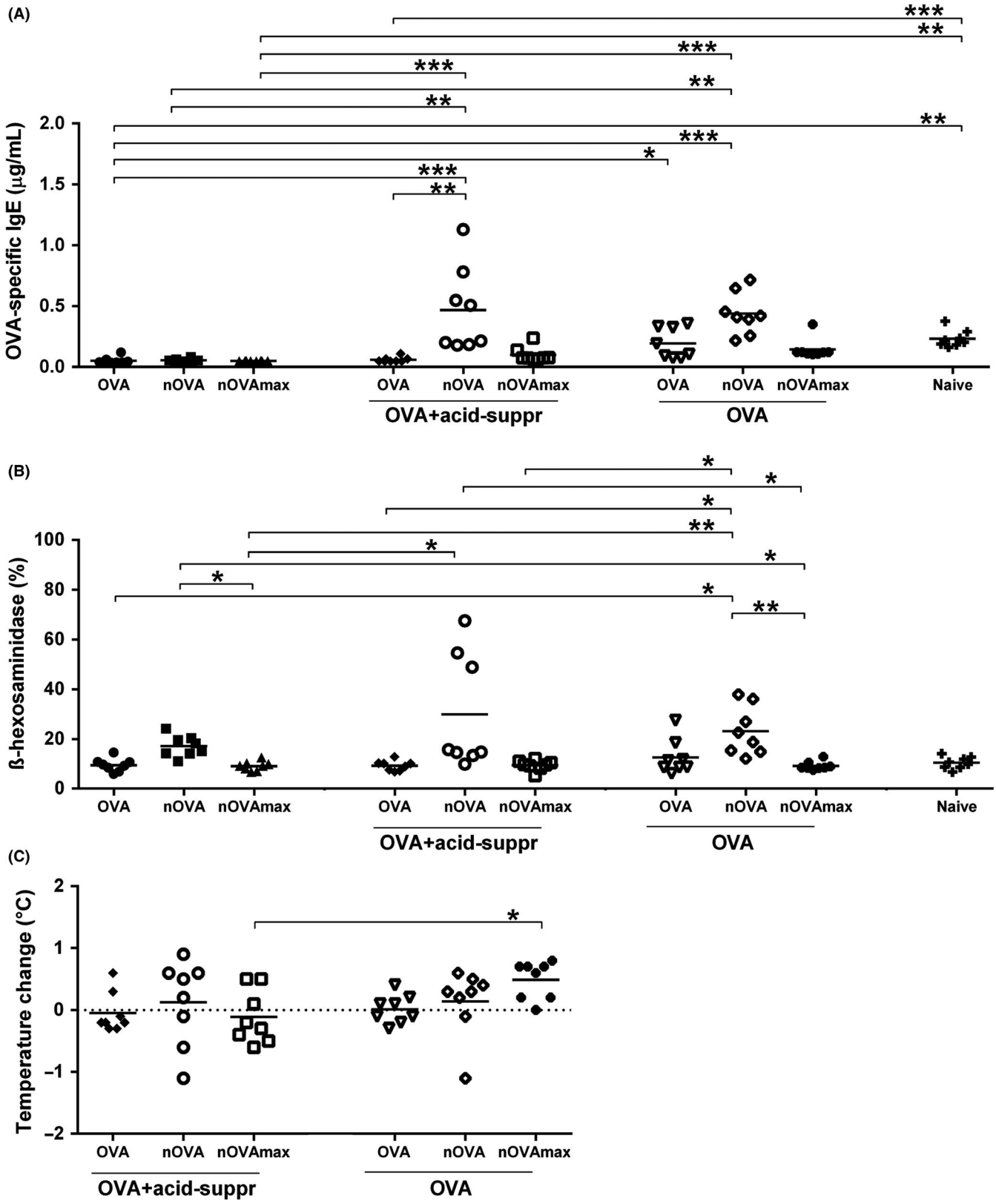


FIGURE 2 Evaluation of OVA-specific IgE titers in serum, biological functionality of IgE antibodies, and clinical reactivity. A, On day of final read-out experiments, sera were collected by cardiac puncture and ELISA was performed to determine the IgE antibody levels. B, The release of β-hexosaminidase from passively sensitized RBL cells was measured. C, Core body temperature of immunized mice orally challenged with OVA was measured, and the change of body temperature was calculated from before to 15 min after provocation. (*$P < .05$, **$P < .01$, ***$P < .001$)

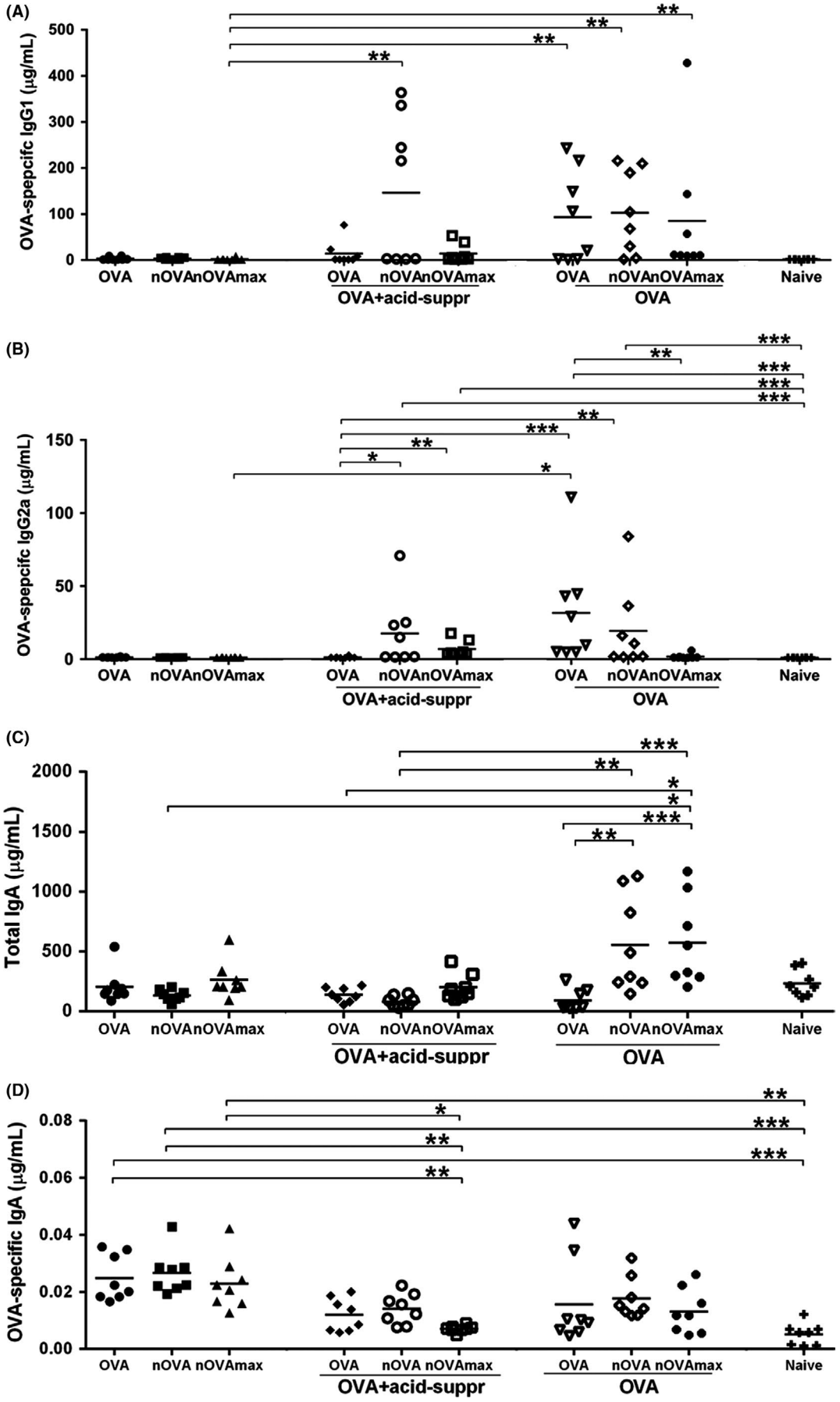

(A)
OVA-spepcifc IgG1 (µg/mL)
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FIGURE 3 Evaluation of OVA-specific IgG1 and IgG2a titers in mouse sera and total and OVA-specific IgA titers in intestinal lavages. Blood was collected by cardiac puncture and (A) OVA-specific serum IgG1 and (B) IgG2a were evaluated by ELISA. Intestinal lavages were collected and evaluated for (C) total and (D) OVA-specific IgA by ELISA. (*$P < .05$, **$P < .01$, ***$P < .001$)

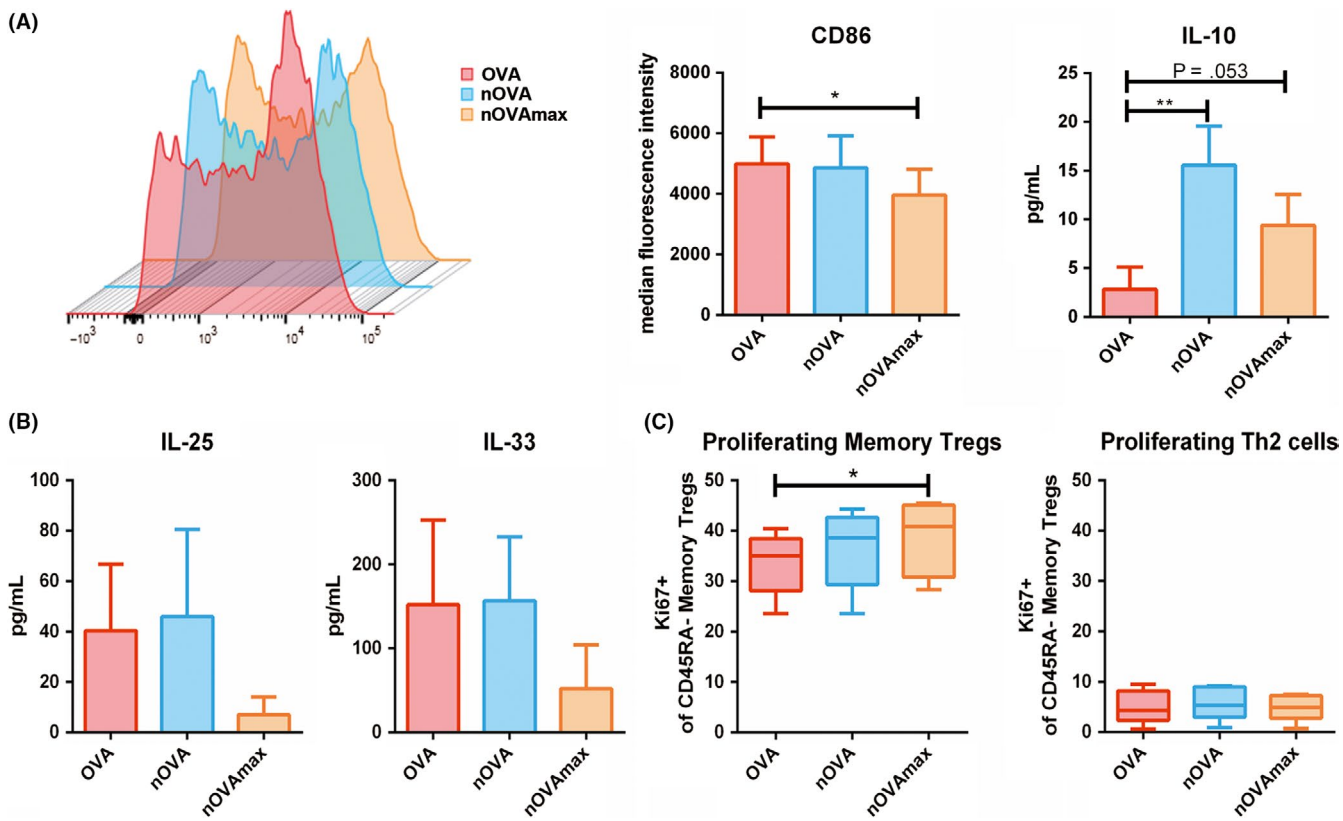

FIGURE 4 Treatment of human moDCs and T cells with nitrated OVA promotes a more regulatory phenotype. Human moDCs were pre-incubated with 10 μg/mL OVA, nOVA, and nOVAmax for 4 h. Subsequently, 2 ng/mL of LPS was added. After 24 h, CD86 surface marker expression (flow cytometry) and IL-10 cytokine secretion (ELISA) was analyzed. Human PBMCs were incubated for 7 days with 10 μg/mL OVA, nOVA, and nOVAmax. LPS concentration was adjusted according to Figure S5. Cytokine secretion and surface marker expression were analyzed. A, Histogram overlay of CD86 expression on moDCs of one representative donor (left panel) and median fluorescence intensity of CD86 as well as IL-10 secretion of at least 6 independent donors (right panel) are shown. Data represent means + SEM of. B, IL-25 and IL-33 cytokine secretion of human PBMCs was measured. Data represent means + SEM of 5 independent donors. C, Proliferating memory Tregs and Th2 cells out of PBMCs were analyzed. The figures show medians represented by a box whisker plot of 5 independent donors. (*$P < .05$, **$P < .01$)

It is well established that posttranslational protein modifications are associated with epitope changes.[9] Nitrated tyrosine residues potentially modify the steric properties thereby also the interaction with neighboring amino acids, leading to a reduced antibody response. Circular dichroism experiments indicated that nOVAmax had a substantially compromised secondary protein structure in comparison to nOVA and OVA. The relevant effect of structural integrity of allergens has been indicated in a study comparing adverse reactions against native and denatured allergens.[37] The antibody binding capacity was reduced when all these proteins (BLG, ALA, and β-casein) were denatured, due to the loss of conformational epitopes. By oral administration of these proteins, the sensitizing ability of the three allergens correlated with their susceptibility to digestion.[37] In line, we have demonstrated previously that nitrated OVA proteins are more susceptible to gastric digestion.[17] This mechanism might also play a role in our experiment. As we observed a protein-specific reduction of the allergic response, nitrated allergens might be specifically effective as a safe allergy-preventive approach for food allergy. It is therefore tempting to speculate that due to denaturation and loss of secondary protein structure, extensive nitration is associated with tolerance induction in food allergy.

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CONFLICTS OF INTEREST

OAS, DA, and UE are inventors of patent application No. EP18201031.4 of the Medical University of Vienna and the University of Salzburg. None of the other authors report any conflicts of interest related to this work.

AUTHOR CONTRIBUTIONS

SN, KM, and HD performed the animal experiments. BR and HHJ performed the experiments with human moDCs and PBMCs. SN, BR, and HHJ analyzed the data and SN drafted the manuscript. HHJ and DA planned the cell culture study with human cells. KC and SOA contributed to optimize the methodology. RD and WE contributed during final read-out experiments. HHJ and UE designed the study and revised and corrected the manuscript. UE and DA obtained funding for the study. All authors reviewed and approved the final version of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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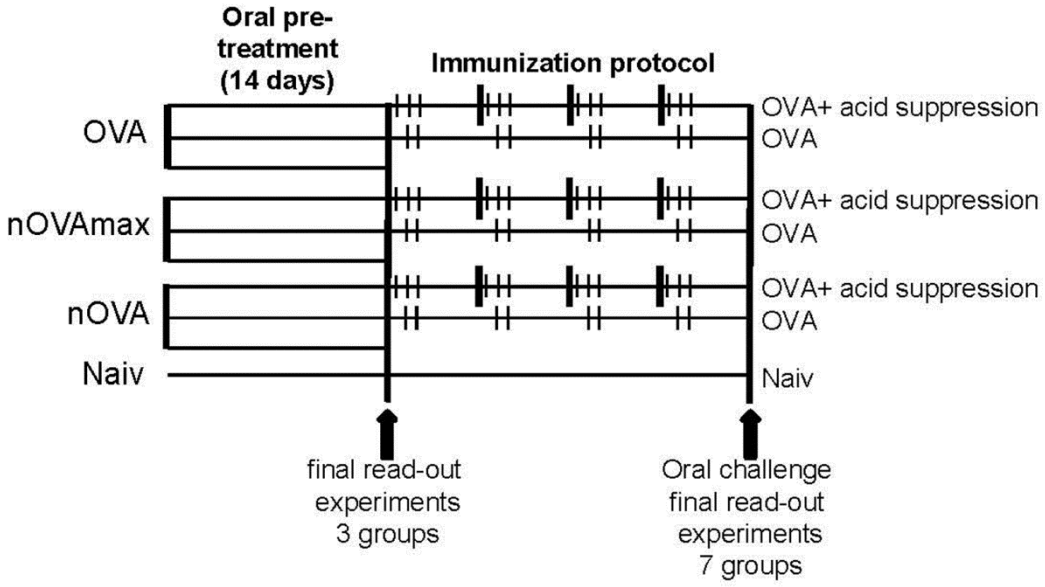
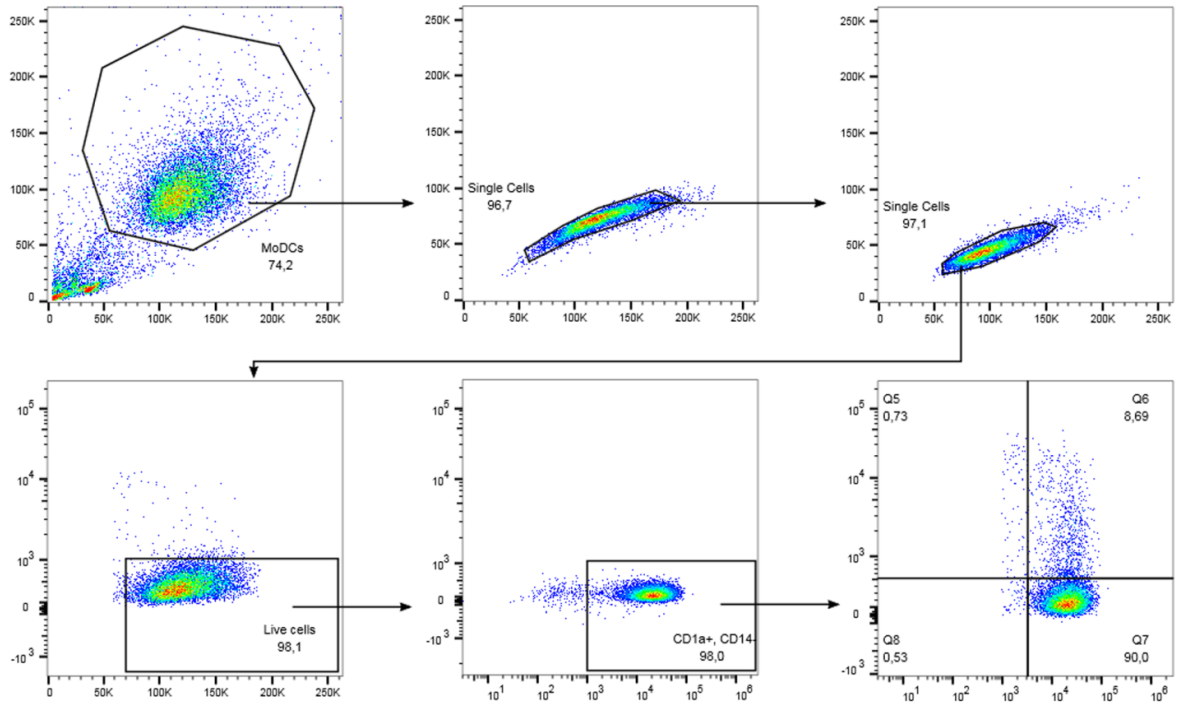
Supplementary figure legends

Suppl. Fig. 1:



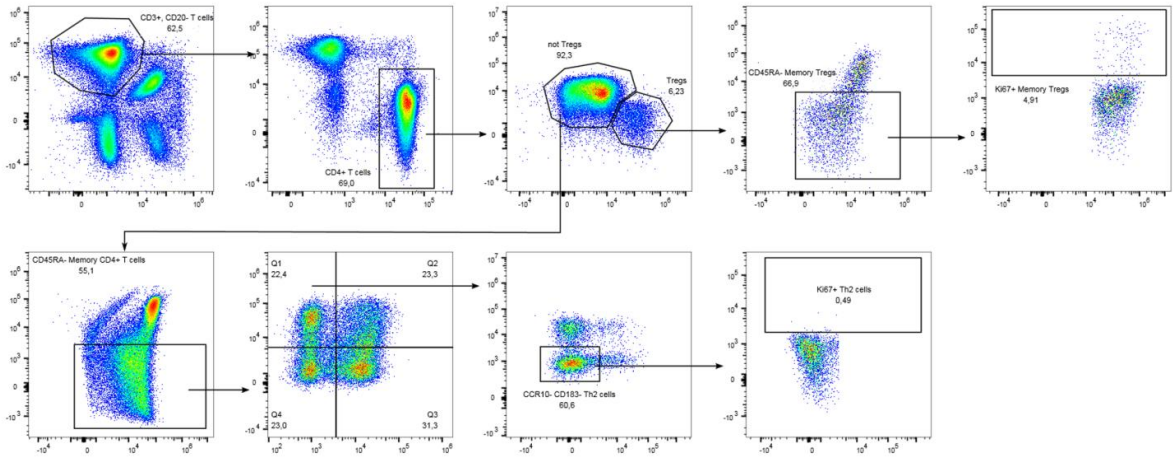
Suppl Fig 1. Schematic diagram of oral preventive treatment, immunization protocol and oral challenges in mice. Oral allergy preventive treatment (antigens: OVA, nOVA, nOVAmax; 3 groups of 8 Balb/c mice each; 200 µg of protein per gavage) was performed daily for 2 weeks. From each pre-treatment, one group was sacrificed and final readout experiments were performed. For subsequent food allergy induction, mice were sensitized every second week for 4 times on 3 consecutive days. 3 groups of mice received 200µg OVA orally under concomitant gastric acid suppression with the proton pump inhibitor (PPI) omeprazole and the sucralfate. For control purpose, mice were fed with 200µg OVA alone. Afterwards all mice were sacrificed and final read-out experiments were performed.

Suppl. Fig. 2:



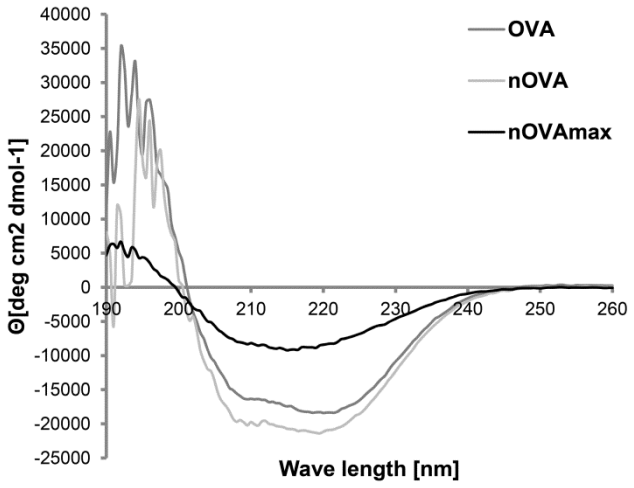
Suppl Fig 2. Gating strategy of moDCs. Doublets were excluded from the moDC population. Afterwards only CD1a+, CD3-, CD19-, CD14- and living cells were analyzed for the expression of CD86.

Suppl. Fig. 3:



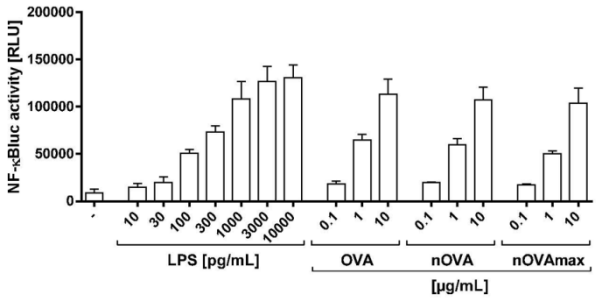
Suppl Fig 3. Gating strategy of T cell subsets. After doublet exclusion, $CD3^{+}/CD20^{-}$ T cells were gated for the $CD8^{-}/CD4^{+}$ subpopulation. $CD25^{high}/CD127^{low}/CD45RA^{-}$ Memory Tregs were analyzed for proliferation ($Ki67^{+}$). $CD25^{low}/CD45RA^{-}$ cells were gated for $CD194^{+}/CD196^{-}$ Th2 cells and further checked for proliferation ($Ki67^{+}$).

Suppl. Fig. 4:



Suppl Fig 4. Maximally nitrated OVA revealed an altered secondary structure. For nOVAmax (black) an alternation of the secondary structure was observed as indicated by changed spectra, which were not revealed for OVA (gray) and nOVA (light gray). Results are expressed as the mean residue ellipticity Θ (y-axis) and the plotted against the respective wavelength (x-axis).

Suppl. Fig. 5:



Suppl Fig 5. Activation of NF-κB reporter upon LPS, OVA, nOVA or nOVAmax stimulation.

HEK-293 cells were transiently transfected with plasmids encoding a NF-κB-dependent Luciferase and the full LPS receptor complex. After 1 day, cells were stimulated with different concentrations of LPS standard or proteins for further 24 hours. The LPS concentration of nOVAmax was adjusted before use. The activity of NF-κB-dependent Luciferase was measured in 4 independent experiments. Data represent means + SD.

Supplementary information

Staining procedure for flow cytometry analysis of murine $CD4^+$ and regulatory T cells

Splenocytes were stained for Tregs using a mouse regulatory T cell staining kit (eBioscience). Per sample, $1x10^6$ cells were counted and washed with flow cytometry buffer. Surface staining was performed by adding 50 µl master mix (anti-CD4 FITC, anti-CD25 APC in FACS buffer). Isotype controls were treated equally using the appropriate isotype controls (rat IgG2a, 1:400 and rat IgG1, 1:333 in flow cytometry buffer). Cells were incubated at RT in the dark for 30 min, centrifuged and fixed with fixation buffer (7.5% formaldehyde 1:2 in flow cytometry buffer) at 4 °C for 30min. Afterwards, cells were washed with Perm buffer and intracellular staining was performed using anti-FOXP3-PE (1:40 in Perm buffer). All samples were incubated for 30 min at 4 °C and washed with FACS buffer. CD4+CD25+FOXP3+ cells were measured on a FACS Canto II (BD) and the results were analyzed by BD FACSDiva™ software (BD).

Suppl. Table 1. Staining procedure for flow cytometry analysis of human moDCs and T cells

	Target (Color, company)
Human moDCs	CD3 (eFluor506, eBioscience)
	Fixable Viability Dye (eFlour506, eBioscience)
	CD19 (eFlour506, eBioscience),
	CD14 (PerCP-Cy5.5, BD)
	CD1a (BV421, Biolegend)

	CD86 (PE, eBioscience)
Human T cells	CD3 (PerCP/Cy5.5, BioLegend),
	CD20 (APC/Cy7, BioLegend)
	CD8 (PE/Cy5, BioLegend)
	CD4 (BV510, BioLegend)
	CD25 (PE, BioLegend)
	CD127 (AF700, BioLegend)
	CD197 (PE/Dazzle 594, BioLegend)
	CD45RA (BV605, BioLegend)
	CD196 (BB515, BD Horizon)
	CD194 (PE/Cy7, BioLegend)
	CCR10 (APC, BD Pharmingen)
	Ki67 (BV650, BD Horizon)

Human moDCs were stained with the indicated antibodies for 30 minutes at 4°C in the dark. After washing, the cells were fixed with 4% Paraformaldehyde for flow cytometric analysis. Human T cells within PBMCs were stained with antibodies as described above.