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Effect of malondialdehyde on the ovalbumin structure and its interactions with T84 epithelial cells

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*Running title: *Effects of MDA on the ovalbunin interactions with T84 epithelial cells*

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ABSTRACT

Background: Protein oxidation can occur as a consequence of lipid peroxidation during food processing. The aim of this work was to explore the effect of malondialdehyde (MDA) modification of ovalbumin (OVA) on its interaction with T84 intestinal cells.

Methods: Molecular dynamics simulation was employed for the prediction of MDA modification in the OVA, while introduced structural changes were evaluated by measurement of carbonyl group content, fluorescence spectra, MS/MS analysis, and IgE reactivity. Effects of MDA modified OVA on T84 epithelial cells were analyzed by gene expression for pro-inflammatory cytokines and protein secretion.

Results: Out of 9 predicted, five modified Lys residues were confirmed by MS/MS analysis: ^{51}TQINKVVR^{58}, ^{85}DILNQITKPNDVYSFSLASR^{104}, ^{111}YPILPEYLQCVKELYR^{126}, ^{187}AFKDEDTQAMPFR^{199}, ^{277}KIKVYLPR^{284}, and ^{278}IKVYLPR^{284}. The introduced MDA modifications influenced profile of IgE reactivity to OVA. Treatment of T84 epithelial cells with OVA and OVA modified with 1 mM MDA, induced up-regulation of pro-inflammatory cytokines (IL-1β, IL-25, IL-33, TSLP and TNFα), while OVA modification with 10 mM MDA induced down regulation of the cytokine expression profile, except for IL-1β. OVA and OVA modified with 1 mM MDA induced secretion of epithelial cells specific cytokine IL-33.

Conclusions: This finding indicated that OVA and its MDA modified form have the potential to trigger the innate immunity by inducing up-regulation and secretion of pro-allergenic IL-33 in T84 intestinal epithelial cells.

General significance: Interactions of ovalbumin and its MDA modified form with intestinal epithelial cells can induce a specific immunological priming necessary for the downstream activation of innate immunity.

Abbreviations: DNPH, 2,4-dinitrophenylhydrazine; DC, dendritic cell; DHP, dihydropyridine; IgE, immunoglobulin E; IgG, immunoglobulin G; MDA, malondialdehyde; MD, molecular dynamics; OVA, ovalbumin; TSLP, thymic stromal lymphopoietin; TNFα, tumor necrosis factor α.

Keywords: epithelial cells, interleukin-33, malondialdehyde, molecular dynamics, ovalbumin

1. Introduction

Food allergy is a growing public health concern in the Western society that affects 3-8% of children and 1-3% of the adult population [1,2]. Hen eggs are generally regarded as one of nature's perfect foods which contain a unique and well-balanced mix of essential nutrients however, they are among the top eight food allergen sources that induce a wide range of clinical manifestations of type I hypersensitivity reaction which are particularly prevalent in the pediatric population [3]. The molecular basis for hen egg allergy is attributed to seven registered allergens from *Gallus domesticus* (www.allergen.org), with the following allergens as predominant: ovomucoid (Gal d 1), ovalbumin (Gal d 2), ovotransferrin (Gal d 3) and lysozyme (Gal d 4) [4,5]. Among this protein panel, ovalbumin is the most abundant, representing 54% of the egg white proteins.

Ovalbumin (Gal d 2, OVA) is a polypeptide of 385 amino acid residues [6]. OVA shares sequence identity with protease inhibitors of the serpin family, including antitrypsin [7], but it lacks protease inhibitory activity. It is a phosphoglycoprotein having carbohydrate (3% by weight) and phosphate moieties attached to the polypeptide. The protein conformation is stabilized by one disulfide bond (Cys74-Cys121), and contains four free thiol (SH) groups (Cys11, Cys30, Cys367, and Cys382), which sometimes causes dimerization, while the amino terminus of the protein is acetylated [8]. The post-translational modifications of OVA increase the sequence-derived molecular mass of 42750 Da to 44000-45000 Da [9]. Five immunodominant IgE-binding epitopes were detected in OVA: AA38-49, AA95-102, AA191-200, AA243-248, and AA251-260 [10]. In a recent study seven IgE-reactive peptides were detected in the OVA after digestion with human and simulated gastroduodenal fluids: AA125-134, AA141-154, AA159-172, AA164-176, AA188-198, AA326-336, and AA370-385. In the group of egg allergic persons, 80% were positive to the peptide AA370-385 [11].

Food processing can have an impact on the IgG- and IgE-binding properties of food proteins and consequently on their ability to elicit allergic reactions. However, much less information is available on the impact of food processing on the potential of a protein to induce the immunological priming, necessary for the induction of IgE antibody responses and the acquisition of allergenic sensitization.

During the food processing, generation of lipid peroxidation end-products can cause protein deteriorations, including changes in the allergenic properties [12]. Malondialdehyde (MDA), very often the most abundant product of lipid peroxidation, can cause protein damage by reacting with lysine amino group, cysteine sulfhydryl group, arginine guanidine group and histidine imidazole moiety [13]. Effects of aldehydes are important because of their propensity to form Schiff bases with amino groups, and in particular the bifunctional MDA can modify and cross-link protein via Schiff base formation [14]. MDA reacts with lysine generating *N*-(2-propenal)lysine (*N*-propenal-Lys), and also forms fluorescent products such as the dihydropyridine (DHP)-type adducts [15], which are often found in proteins after lipid peroxidation.

Epithelial cells play a crucial role in the process of sensing insults from the environment and regulate both innate and adaptive immunity through the production of functional molecules and via physical interactions with the cells of the immune system [16]. Activation of epithelial cells results in immediate host defense responses by producing cytokines, growth factors, chemokines, and danger signals which recruit and activate other mucosal innate immune cells [17,18]. Recent evidences that allergens can interact with the components of the innate immune system in ways that promote Th2 response, provide a new pathway for investigation of allergenic sensitization. This should open novel concepts in allergen immunotherapy [19]. IL-33, a member of the IL-1-related cytokines, is considered to be a pro-allergenic molecule (an endogenous danger signal - alarmin) that is especially involved in Th2-type immune response [20], while thymic stromal lymphopoietin (TSLP), a member of IL-2 family of cytokines, is able to modulate dendritic cells (DCs) to induce the production of Th2-attracting chemokines [18].

The aim of this study was to explore 1) structural and immunological features of MDA modified ovalbumin, and 2) its effects on T84 intestinal epithelial cells in terms of up-regulation of genes for pro-inflammatory cytokines, and production of pro-allergenic IL-33 and TSLP.

2. Material and methods

2.1 Molecular modeling of OVA

To predict dynamics of OVA amino acid residues exposure to the solvent and hence their susceptibility to MDA modifications, molecular dynamics simulation was employed. Ten nanoseconds of unconstrained molecular dynamics (MD) simulations were performed using OVA structure deposited in Protein Data Bank, PDB ID: 1OVA [21]. *N*-Acetylglucosamine bound to Asn298 was retained and phosphate groups bound to Ser87 and Ser350 were removed. Protein was neutralized with counterions and embedded in the sphere of explicit water molecules (TIP3P). Input files were prepared in CHARMM-GUI [22,23], the CHARMM36 force field was used. The system was equilibrated in two cycles of minimization and heating, yielding sphere of ~ 96 Å radius. MD simulations were performed in NAMD 2.9 [24], on Linux-based HPC facility. MD trajectories were analyzed in VegaZZ 3.1 [25]. pK_a values of protonable side-chains were estimated using PropKa 3.0 [26].

2.2 Purification of OVA

Before modification with MDA, OVA (A5253, Sigma – Aldrich, St. Luis, USA) was fractionated by gel filtration on a Sephadex G100 column (Pharmacia, Uppsala, Sweden, 80 mL) equilibrated with 20 mM sodium phosphate, 150 mM NaCl buffer pH 7.4. The fractions containing OVA were pooled and resolved on Fe^{3+}-immobilized-metal affinity chromatography (IMAC, IDA-Sepharose 6B matrix, Clontech Laboratories, Inc. , Mountain View, USA) [27]. Purity of OVA (95%) was confirmed by SDS-PAGE analysis (12% polyacrylamide gel), which was performed as previously described [28].

Level of endotoxin was determined by Pierce® LAL chromogenic endotoxin quantification kit (Thermo Scientific, Rockford, USA), according to the manufactures instruction, as 3.42 EU/mg OVA.

2.3 Modification of OVA with MDA

For the protein modification a fresh MDA stock solution was prepared on a daily-base according to the protocol described by Wu et al. [29]. To obtain the MDA stock solution 1,1,3,3-tetramethoxypropane (TMP, Sigma-Aldrich, St. Luis, USA) was hydrolyzed by HCl at 40 °C in the dark for 30 min. After the hydrolysis, the pH of the reaction mixture was adjusted to 7.4 with NaOH. The concentration of MDA solution was calculated by measuring absorbance at 267 nm and by using a molar extinction coefficient of 31500 $M^{-1}cm^{-1}$. One milligram of OVA and OVA treated with increasing concentration of MDA (0.5, 1, 2, 3, 4, 5, and 10 mM MDA) were incubated by continuous shaking for 24 h in the dark at 25 °C. After the treatment samples were dialyzed against deionized water at 4 °C for 72 h and lyophilized. Before further experiments the lyophiylized proteins were stored at 4 °C until downstream analysis.

2.3.1 Measurement of carbonyl group content

For the evaluation of carbonyl group content in control and in modified OVA, the lyophilized protein samples (1 mg) were resolved in 10 mM sodium phosphate buffer pH 7.0 (1 mL). 100 μL of each sample was mixed with 300 μL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) dissolved in 2 M HCl and incubated at room temperature for 2 h. An aliquot of non-treated OVA was mixed with 300 μL of 2 M HCl as a blank. Then, 400 μL of 20% trichloroacetic acid (TCA) was added to each sample and incubated for 20 min at room temperature (RT); the mixture was centrifuged (12000 × g for 10 min) at 4 °C. The supernatant was discarded, and the pellet was washed three times with 500 μL of ethanol/ethyl acetate solution (1:1; v/v). The protein, free of DNPH, was then dissolved in 300 μL of 6 M guanidine hydrochloride in 100 mM sodium phosphate buffer pH 7.0. The absorbance at 367 nm was corrected by the absorbance in the HCl as blank. The results are expressed as nanomoles of carbonyl group per milligram of protein.

2.3.2 Fluorescence spectra of native and MDA modified OVA

Lyophilized protein samples were suspended in 10 mM sodium phosphate buffer pH 7.0. For fluorescence determination, protein concentration was adjusted to 0.04 mg/mL for tryptophan measurement and to 0.2 mg/mL for Schiff base measurement. To determine the fluorescence emission spectrum of tryptophan, the excitation wavelength was set at 290 nm, and the emission spectrum was recorded from 300 to 400 nm. For determination of the fluorescence emission spectrum of the Schiff base, the excitation wavelength was set at 350 nm, and the emission spectrum was recorded from 400 to 600 nm.

2.3.3 Mass spectrometry analysis of native and MDA modified OVA

Mass spectra of intact proteins were obtained on Ultraflextreme MALDI TOF/TOF (Bruker, Bremen, Germany) operating in a linear positive mode using 2,5-dihydroxyactetophenone as a matrix. Spectra were calibrated with Protein standard II (Bruker 207234).

Mass fingerprint analysis was performed as previously described [30]. In brief, intact OVA (control) and three samples: OVA-1MDA (OVA modified with 1 mM MDA), OVA-5MDA (OVA modified with 5 mM MDA), OVA-10MDA (OVA modified with 10 mM MDA) were reduced with 10 mM DTT and alkylated with 50 mM iodoacetamide. Trypsinization was performed with sequencing grade trypsin (Promega, Mannheim, Germany) (w/w 1:50) in 50 mM ammonium bicarbonate, overnight at 37 °C. Prior to MS analysis samples were cleaned over C18 zip tip (Millipore, Massachusetts, USA). Tryptic maps were obtained on Ultraflextreme operating in a reflectron positive mode using α-cyano-4-hydroxycinnamic acid as a matrix and a peptide calibration standard mixture (Bruker 206195) for mass calibration. Samples treated with MDA exhibited almost identical peptide mass fingerprints. The peaks present in control sample were subtracted from peaks present in mass spectra of samples treated with MDA. A resulting list of peptides was searched against list of theoretical modifications generated on an assumption that Lys, Arg, His, Asn and Gln can be modified with MDA [31, 32] and that trypsin will not cleave at Lys or Arg if they are modified with MDA. For resulting candidate peaks amino acid sequence and presence of modification was confirmed by MS/MS spectra obtained in a Lift mode.

2.4 In vitro digestion assay

In vitro digestion assay was performed according to the protocol described by Minekus et al. [33], without addition of pancreatic lipase and bile salts. The final concentration of OVA and a panel of samples: OVA-0.5MDA (OVA modified with 0.5 mM MDA), OVA-1MDA (OVA modified with 1 mM MDA), OVA-2MDA (OVA modified with 2 mM MDA), OVA-3MDA (OVA modified with 3 mM MDA), OVA-4MDA (OVA modified with 4 mM MDA), OVA-5MDA (OVA modified with 5 mM MDA) and OVA-10MDA (OVA modified with 10 mM MDA) was adjusted to 0.2 mg/mL, while the final concentration of pepsin (Sigma Aldrich) in the simulated gastric fluid was 2000 U/mL, and the final concentration of trypsin (Sigma Aldrich) in the simulated intestinal fluid was 100 U/mL. The final volume of each sample was 100 μL and incubation was performed for 2h with shaking (250 rpm) at 37 ºC. Each reaction was stopped by adding 25 μL of 5× loading buffer for SDS-PAGE and incubation at 95 ºC for 30 min before application on SDS-PAGE analysis.

2.5 ELISA inhibition

IgE reactivity of MDA-modified OVA was analyzed in ELISA inhibition. Two patients with a clinical history of allergic reactions to egg white were included in the study. IgE-mediated allergy was documented by case history, skin prick test (SPT), and positive specific IgE (sIgE, CAP-FEIA, Pharmacia-Upjohn, Uppsala, Sweden) to egg white, and positive dot blot with OVA. The patients were sensitized to one or more foods. The study was performed with the approval of the Ethics Committee of the University Children's Hospital of Belgrade, Serbia (Approval No. 017-990/87/2012-01/19).

The assay was performed in ELISA Maxisorp immunoplates (Nunc^{TM}, Reskilde, Denmark). Briefly, each well was coated with OVA (100 μg/mL, 100 μL/well) in 15 mM Na_2CO_3/35 mM $NaHCO_3$, pH 9.5, overnight at 4 °C. The plate was washed (3 × 5 min) with PBS containing 0.01 % (v/v) Tween 20 (TPBS), and blocked with 3% bovine serum albumin (Serva) in TPBS for 3 h at room temperature. After washing,

wells were incubated with the egg white allergic patient's sera (sera 1 was diluted 1:5; sera 2 was diluted 1:3, respectively, 100 μL in TPBS) pre-incubated with a serial dilution (0, 0.01, 0.1, 1, 10, and 100 μg of inhibitor) of OVA; OVA-1MDA (OVA modified with 1 mM MDA), and OVA-10MDA (OVA modified with 10 mM MDA) in TPBS, respectively. The bound IgE was detected using monoclonal alkaline phosphatase labeled anti-human IgE antibody (1:1000, Sigma Aldrich, St. Louis, USA). The reaction was visualized by using *p*-nitrophenylphosphate (1.0 mg /mL) in 100 mM diethanolamine buffer pH 9.6, and the absorbance was measured at 405 nm. All samples were analyzed in duplicates.

2.6 Cell culture

T84 cells (ATCC, Manassas, VA, USA) were grown and maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, USA), supplemented with 10% fetal bovine serum and penicillin (100 IU)/streptomycin (100 μg/mL) (Sigma-Aldrich, St. Louis, USA) at 37 ºC in 5% CO_2 humidified atmosphere. Cells were passaged using 0.25% trypsin solution in phosphate-buffered saline (PBS) without calcium and magnesium. Before each treatment, the nutritive medium was removed, the plates washed with PBS, and the cells incubated in serum-free medium 1 hour prior to treatment. Each experiment contained control monolayers, which allowed for normalization of the data and suitable statistical analysis to be conducted. The standard MTT test was used to ensure that treatments induced no significant cellular death [34]. For MTT test, T84 cells were seeded in flat-bottom 96-well plates (NUNC, Roskilde, Denmark) at a cell density of 1×10^4 cells/well, and for treatments in 6-well plates (NUNC, Roskilde, Denmark), at a cell density of 5×10^5 cells/well. The following treatments were filtered (0.22 μm) and used: OVA,OVA-1MDA (OVA modified with 1mM MDA) and OVA-10MDA (OVA modified with 10mM MDA), all in a concentration of 0.5 mg/mL (which induced no significant cellular death up to 4 hours of treatment as determined by MTT test). After 4h of treatment, the supernatants were collected for analysis of cytokine release by ELISA, and the cells washed with PBS and collected by centrifugation (420 × g, 10 min) for RNA isolation.

2.6.1 RNA isolation and RT-PCR

Evaluation of gene expression was done by reverse-transcriptase polymerase chain reaction (RT-PCR). For the isolation of total RNA, cell pellets were lysed using TRI REAGENT® BD kit (Sigma-Aldrich, St. Louis, USA). cDNA synthesis from total RNA (2 µg) was performed using random primers and MultiScribe™ Reverse Transcriptase from High-Capacity cDNA Reverse Transcription kit (50 U/µL, Applied Biosystems, Carlsbad, USA). PCR reactions (final volume 20 µL) consisted of 10 µL TaqMan Universal Master Mix, 300 ng cDNA and 10 pmol of primers. PCR was performed using the following program: 95 °C for 5 min, 35 cycles at 95 °C for 1min, Tm for 1 min, 72 °C for 30 seconds finally 7 min at 72 °C. The sequence of PCR primers, Tm values and the size of the obtained PCR products are given in Table 1.

The presence, size and quantity of the amplified PCR products were analyzed on 2% agarose/EtBr gels running molecular weight markers alongside (O' Gene Ruler 100 bp DNA Ladder, Fermentas, Vilnius, Lithuania). Quantification of PCR band intensities was performed on samples obtained from three independent experiments, after normalization to the GAPDH signal using Image Studio Lite v5.2 (LI-COR Biosciences), and presented as mean ± SEM. mRNA levels upon treatment were compared to the corresponding non-treated samples (taken as 1), and the graph prepared using GraphPad Prism v6.07 (GraphPad Software).Two way analysis of variance (ANOVA) was used to compare sample means. When an overall statistically significant difference was seen, post-tests were performed to compare treatment pairs, using the Bonferroni method to adjust the p-value for multiple comparisons. Differences were considered significant if $p < 0.05$.

2.6.2 ELISA analysis of IL-33 and TSLP cytokines released in cell supernatants

Detection of cytokines IL-33 and TSLP in cell culture supernatants was performed in ELISA Maxisorp immunoplates (Nunc™, Reskilde, Denmark). For IL-33 detection, each well was coated with goat anti-IL-33 polyclonal antibodies (20 µg/mL, 50 µL/well, BioLegend, San Siego, USA, Poly5163), and for

TSLP detection, anti-human TSLP antibody (20 µg/mL, 50 µL/well, BioLegend, San Diego, USA) in Coating buffer (CB 15 mM Na_2CO_3/35 mM $NaHCO_3$, pH 9.5), overnight at 4 °C. The plates were washed (4 × 5 min) with PBS containing 0.05 % (v/v) Tween 20 - TPBS, and blocking was performed for 2 h at room temperature with 1% BSA in PBS. Serial dilutions of the recombinant human IL-33 (250 pg/mL, 50 µL/well, Biolegend), the recombinant TSLP (500 pg/mL, 50 µL/well, Biolegend, San Diego, USA) or undiluted cell culture supernatants (50 µg/well) were added to the wells and incubated overnight at 4 °C. After washing with TPBS (5 × 5 min), the biotin labeled anti-human IL33 (20 µg/mL, 50 µL/well, BioLegend, San Diego, USA) or the biotin labeled anti-human TSLP (20 µg/mL, 50 µL/well, BioLegend, San Diego, USA) in Blocking buffer was added to the wells and were incubated for 1 h at room temperature. After washing with TPBS (4× 5 min), streptavidin-horseradish peroxidase (1:2000 dilution, 50 µL/well) in TPBS was added and incubated for 30 min at RT. After washing (5 × 5 min) with TPBS, the substrate solution 50 µL/well (ABTS (Sigma-Aldrich) 0.3 mg/mL in 0.1 M citric acid, pH 4.35 and H_2O_2 in final concentration 0.03 % v/v) was added for 1 h at 37 ˚C. The reaction was stopped by the addition of 50 µL of ABTS stop solution (dimethylformamide: ddH_2O; 1:1 (v:v) with 20 % w/v SDS) and the absorbance was measured at 405 nm (reference wavelength 620 nm). One way analysis of variance (ANOVA) was used to compare sample means, and Tukey's Multiple Comparison test was used to compare control with treatments. Differences were considered significant if $p < 0.05$.

3. Results

3.1 Prediction of MDA modified Lys side chains in OVA

MDA primarily forms adducts with lysine residues of proteins [15], and therefore the molecular dynamics simulation was employed for the prediction of Lys side chains that are exposed to the solvent and hence susceptible to MDA modifications. For the MD simulation one subunit of

ovalbumin was used. During analysis of the MD trajectory we considered surface of Lys side-chains exposed to the solvent, percentage of time during which a particular residue is exposed to the solvent, as well as the estimated pK_a value of Lys side-chain amino groups (Table S1, in the Supplementary material). We found the following residues highly exposed to the solvent: Lys46, Lys55, Lys61, Lys92, Lys122, Lys226, Lys277 and Lys285, with surface areas > 100 Å^2 and > 75 % of the time. Lys16, Lys181, Lys186, Lys206, Lys263, Lys289 and Lys321 having > 70 Å^2 and > 50 % of the time. Lys19, Lys189, Lys228, Lys279 were found as residues with side-chains having smallest accessibility to the solvent, while Lys367 did not appear exposed on the surface of the protein in MD trajectory. Position of Lys residues in OVA 3D structure experimentally found as modified by MDA and those for which high or low accessibility to the solvent were found from MD simulations is shown on Figure 1 and Figure S1 (in the Supplementary Material) suggest that terminal amino group of Lys16, Lys181, Lys228, Lys289 and Lys367 will be in its neutral form, therefore less prone to MDA modifications.

3.2.1 Measurement of carbonyl group content in MDA modified OVA

As a bifunctional aldehyde, MDA contains two reactive carbonyl groups which can react with free amino groups of a protein by forming Schiff base. MDA can react with a nucleophilic nitrogen or sulfur atom in side chain of Lys, His, Cys and Arg. Most often the final product of the MDA reaction with Lys is the introduction of one carbonyl group into protein per MDA molecule. In addition, MDA is capable to cross-link proteins via Schiff base formation, and to produce fluorescent adducts such as dihydropyridine(DHP)-Lys [15]. The content of protein carbonyl groups was evaluated by DNPH (Table 2). The level of carbonyl group in OVA was 1.73 nM/mg, while there was gradual increase in the level of carbonyl groups with the increase of MDA concentration ($p < 0.05$). The carbonyl content increased

almost 5-fold after OVA treatment with 1 mM MDA, 15-fold after OVA was treated with 5mM of MDA, and 23-fold after treatment with 10 mM MDA for 24 h at room temperature.

3.2.2 Fluorescence spectra

Tryptophan residue emits fluorescence in the range of 300-400 nm after excitation at 290 nm. DNPH - Schiff base is a conjugated fluorochrome with an excitation maximum about 350 nm and an emission maximum about 460 nm [35]. Profiles of Schiff base and tryptophan fluorescence emission spectra of MDA modified OVAs are shown in Figure 2. With the increasing concentration of MDA the fluorescence from the Schiff base increased (Fig. 2a) while the fluorescence from tryptophan decreased (Fig 2b). This finding is in agreement with findings related to studies on the effects of MDA on proteins fluorescence [36, 37]. The decrease in tryptophan fluorescence could be attributed to the change in the local environment of tryptophan residues due to the binding of the aldehyde to the OVA [37, 38].The increasing fluorescence in 460 nm is attributed to the Schiff formation, as a consequence of MDA interaction with free amino groups in the OVA.

3.2.3 Mass spectrometry analysis

Mass spectrometry analysis of intact OVA and OVA modificates indicated an increase in molecular mass of the protein as the MDA concentration increased (Fig. S2).

OVA and OVA modified with 1 mM, 5 mM and 10 mM MDA were reduced, alkylated and digested by trypsin. By mass spec analysis following OVA peptides with modified Lys were identified: ^{51}TQINKVVR^{58}, ^{85}DILNQITKPNDVYSFSLASR^{104}, ^{111}YPILPEYLQCVKELYR^{126}, ^{187}AFKDEDTQAMPFR^{199}, ^{277}KIKVYLPR^{284}, and ^{278}IKVYLPR^{284}. Two types of MDA adducts on Lys residues were identified: MDA Schiff base (+54 Da) and MDA dihydropyridine(DHP) adduct(+134 Da). Details on the type of modification and Lys position are given in Table 3. Tryptic OVA peptide ^{51}TQINKVVR^{58}was detected with $[M+H]^+$*m/z* value 1091.4 Da (Figure S3a). By MS/MS analysis dihydropyridine type of MDA adduct (+134 Da) was detected at K55. Both types of adducts were

detected for Lys92 in ^{85}DILNQITKPNDVYSFSLASR^{104} with $[M+H]^+$*m/z* values of 2335.2 Da (Fig. S3b) and 2415.22 Da (Fig. 3), respectively. In peptide ^{111}YPILPEYLQCVKELYR^{126} MDA dihydropyridine(DHP) adduct (+134 Da) was in position Lys122,with $[M+H]^+$*m/z* value 2218.1 Da (Fig. S3c). Peptide ^{187}AFKDEDTQAMPFR^{199} with $[M+H]^+$*m/z* value 1609.69 Da (Fig. S3d) bears MDA Schiff base adduct (+54 Da) in Lys189. Interestingly, in peptide ^{277}KIKVYLPR^{284} Lys277 and Lys279 were both modified either in a form of DHP adducts with $[M+H]^+$*m/z* value 1284.72 Da (Fig. 3e), or as a combination with Lys277 in a form of DHP adduct and Lys279 in a form of Schif base, with $[M+H]^+$*m/z* value 1204.69 Da (Fig. S3f). In peptide ^{278}IKVYLPR^{284}Schiff base adduct at Lys279 was detected with $[M+H]^+$*m/z* value 942.55 Da (Fig. S3g). Although the search against list of theoretical modifications was based on the assumption that beside Lys, amino acid residues of Arg, His, Asn and Gln can also be modified, we fail to detect such modifications.

3.3 Simulated gastro-duodenal digestion of MDA-modified OVA

Electrophoretic profiles of OVA modified with increasing concentration of MDA (0.5 mM, 1 mM, 2 mM, 3 mM, 4 mM, 5 mM, and 10 mM) denoted as: OVA-0.5MDA, OVA-1MDA, OVA-2MDA, OVA-3MDA, OVA-4MDA, OVA-5MDA, and OVA-10MDA are shown in Figure 4. No precipitation of OVA protein was observed in the reaction mixtures after MDA modification. Besides monomeric protein band of about 45 kDa, bands above 116 kDa corresponding to OVA oligomers were detected in MDA modified OVA. The intensity of oligomers derived from cross-linking of OVA protein increased with the concentration of MDA. The intensity of the monomeric form was reduced, although it could be a consequence of altered binding of CBB for MDA modified OVA, as it was shown for MDA modified BSA [39].

Digestion stability of MDA modified OVA after being incubated in simulated gastric and simulated duodenal conditions was analyzed by SDS-PAGE. The MDA modified OVA samples were incubated for 2 h in the simulated gastric as well as for 2 h in the simulated duodenal conditions. Under applied experimental conditions considerable amounts of MDA modified OVA remain undigested (Fig. S4).

3.4 ELISA inhibition

Effect of MDA modifications on IgE reactivity of OVA was tested in ELISA inhibition. Two patient's sera were employed in the assay. Intact OVA was coupled to the solid phase, while the egg white patient's sera was preincubated with serially diluted OVA, OVA-1MDA, and OVA-10MDA, respectively before addition to the plate. Figure 5 shows inhibition of IgE binding to non-modified OVA, which is obtained with increasing concentrations of OVA, OVA modified with 1mM MDA, and OVA modified with 10 mM MDA. All the inhibition curves followed the same trend, i.e. the inhibition of IgE binding increased together with the amount of added inhibitor. In both sera the OVA-specific IgE showed higher affinity for non-modified OVA comparing to OVA treated with MDA. Interestingly, OVA and OVA-1MDA revealed comparable IC_{50} with sera 1, while OVA-10MDA revealed lower inhibitory potential. Gradual decline in IC_{50} values was observed for OVA, OVA-1MDA and OVA-10MDA with sera 2.

3.5.1 MDA modified OVA induced mRNA cytokine expression in T84 epithelial cells

To address the question whether OVA and MDA modified OVA can interact with intestinal epithelial cells in a way which may promote the epithelial specific pro-inflammatory immune response, T84 epithelial cells were incubated with the allergen samples (OVA, OVA-1MDA, and OVA-10MDA) for 4 h. In order to assess whether such treatment had any effect on cytokine gene expression, mRNA levels for IL-1β, IL-6, IL-25, IL-33, TSLP and TNFα from treated samples were compared to non-treated controls (Fig. 6). After normalization to the corresponding GAPDH signal, quantitative analysis showed a general up regulation of IL-1β, IL-6, IL-25, IL-33, TSLP and TNFα in cells treated with OVA. OVA-1MDA also induced an up regulation of these genes, except in the case of IL-6, where a down regulation was observed. OVA-10MDA induced a general down regulation of these genes, except in the case of IL-1β where an up-regulation was observed.

3.5.2 MDA modified OVA induced IL-33 expression

Epithelial cells are a potential source of IL-33, a crucial amplifier of innate immunity, and therefore it was interesting to compare the effect of intact OVA and MDA modified OVA on T84 cells in terms of production of IL-33. ELISA was performed to test whether MDA modified OVA besides up-regulation of cytokine gene expression, induced similar effects on the protein level, i.e. production of IL-33 and TSLP cytokines (Fig. 7). The level of IL-33 in the medium of T84 cells treated with OVA was 69.9 pg/mL while OVA-1MDA, and OVA-10MDA induced lower secretion of IL-33 in the medium of 60.4 pg/mL and 47.6 pg/mL, respectively. The level of TSLP that was induced by cell treatment with OVA-1MDA was lower when compared to the TSLP induced by OVA, while induction of TSLP with OVA-10MDA was not statistically significant (Fig. 8).

4. Discussion

The effect of MDA, a secondary polyunsaturated fatty acid oxidation product, on major egg white allergen ovalbumin was studied in terms of structural changes and consequent effects on IgE reactivity, as well as immunomodulatory potential of modified allergen on T84 gut epithelial cells.

From the analysis of the MD trajectory we found Lys46, Lys55, Lys61, Lys92, Lys122, Lys226, Lys277 and Lys285 as good candidates for the MDA derivatization. Lys residues identified by MS/MS analyses as modified with MDA were: Lys55, Lys92, Lys122, Lys189, Lys277 and Lys279. In Figure 1 and in Figures 1S (Supplementary Material), all OVA Lys side chains are shown. In Figure 1S protein is shown in CPK representation, in this way accessibility of different Lys side chains is emphasized. So, our predictions of Lys side-chains prone for modifications with MDA are in agreement with experimental results for Lys55, Lys92, Lys122 and Lys277, while prediction failed for Lys189 and Lys279. The last two residues are close to very flexible OVA *R*-helix, characteristic for the proteins belonging to the serpin family. In our simulations

this helix retained its helical structure, most probably because of constraints imposed on two atoms close to the center of the mass of the protein. Such constraint is imposed in order to preclude movement of the whole system during simulations; in this way the analysis of the MD trajectory is facilitated. In simulations without such constraint *R*-helix appears significantly more mobile and Lys189 appears exposed to the solvent to a significantly higher extent. We cannot offer an explanation for the incorrect prediction of Lys279, but the close proximity of Lys277 and Lys279 should be noted.

Two distinct types of MDA adducts were detected in OVA upon tryptic digestion, with mass increments of 54 Da and 134 Da that correspond to Schiff base and dihydropyridine (DHP)-type adducts, respectively. There were no remarkable differences in digestion stability of OVA and MDA modified OVA in the simulated gastric and duodenal digestion under employed experimental conditions. To get better insight is eventual differences perhaps the test should be performed in the presence of bile salts and lipase, as it has been documented that bile salts can promote the digestion of several dietary proteins [40]. Although IgE reactivity was evaluated with only two patient's sera, the trend of IgE reactivity seems to be declining with the increase of level of MDA modifications in OVA, which should be attributed to the masking and/or steric hindrance of the IgE binding epitopes. In this regard, reduced IgE reactivity of MDA modified OVA is in line with the report for MDA modified shrimp allergen tropomyosin [41]. Schiff base adduct found in position Lys92 might have an impact on IgE binding for the immunodominant epitope AA95-102, while modification of Lys189 could influence IgE binding for epitope AA191-200. However, the modified surface topography of OVA-1MDA revealed no effect on IC_{50} value for sera 1 obtained in ELISA inhibition.

The concept, held for many years, that the surface epithelia contribute to host protection strictly as a physical and chemical barrier is being revised owing to accumulated evidence that epithelial cells can activate tissue-associated lymphocytes and that the epithelial cells′ response to infection and/or stress can strongly influence dendritic cells and subsequent adaptive immune responses [42]. There is increasing evidence highlighting the role of epithelial cells in triggering and modulating immune response to allergens [18]. A range of cytokines including TSLP, IL-33, IL-25, IL-1β, and granulocyte macrophage colony-stimulating factor (GM-CSF) are known to be secreted by airway epithelial cells after allergen challenge [18, 43, 44]. Most of the data which deals with specific interactions of allergens with epithelial cells are based on interactions on respiratory allergens with the airway epithelium. However, the mechanism by which food allergens induce sensitization and afterward specific IgE responses in patients with food allergy are poorly understood [45]. Therefore food allergen research has been focused on the elucidation of structural features of allergens in an effort to delineate the specific characteristics differentiating allergenic from nonallergenic food components, and to determine how food processing can enhance or reduce the ability of food allergens to induce food allergy [46].

In this study we explored the effects of introduced covalent modifications on allergen interactions with epithelial cells. We tested the immunomodulatory effect of MDA modified OVA on epithelial cells by employing T84 colorectal adenocarcinoma cells. Treatment of the T84 cells with OVA allergen induced up-regulation of genes for cytokines IL-1β, IL-6, IL-25, IL-33, TSLP and TNFα. A similar trend was observed for the set of analyzed cytokine genes, except for IL-6, in T84 cells treated with OVA modified with 1mM MDA. OVA modified with 10 mM MDA induced a general down-regulation of these genes, except IL-1β where an up-regulation was observed.

Among the Th2 modulating cytokines produced by epithelial cells IL-33 has been identified as a ligand for ST2, an IL-1R-related protein specifically expressed on the surface of Th2 cells, which belongs to the

Toll-like receptor superfamily [20]. IL-33 can polarize naïve T cells to produce IL-5 and IL-33, while treatment of Th2 cells with IL-33 enhanced IL-5 and IL-13 production. In addition, dendritic cells respond to IL-33 by up regulating cell-surface expression of MHC class II molecules and the co-stimulatory molecule CD86 [47]. Besides IL-33, human epithelial cells can produce TSLP in response to diverse stimuli including microbes, trauma, or inflammation. It seems that TSLP might initiate and aggravate allergic inflammation in the absence of T lymphocytes and IgE, via the innate immune system i.e. activation of mast cells [48].

ELISA was performed to test the effect of OVA and MDA modified OVA on T84 cells in terms of secretion of IL-33 and TSLP. Results indicate that OVA induce secretion of IL-33 by T84 epithelial cells after 4 hours of allergen incubation. Treatment of T84 cells with OVA-1MDA, and OVA-10MDA slightly reduced the level of IL-33 in the medium. However, up-regulation profile of TSLP which was detected at the mRNA level was not confirmed in ELISA at the protein level for the analyzed time-frame (4h of incubation). Interestingly, Tani et al. investigated the immunochemical properties of proteins reacted with MDA [49]. They observed a different profiling of the specific IgE and IgG response after immunization of mice with native OVA and MDA modified OVA. Both the IgE and IgG antibody response to the native OVA was higher than anti-MDA-OVA. The profile of the antibody response was found completely opposite for bovine serum albumin and β-lactoglobulin (β-LG) where IgE and IgG response was significantly higher to anti-MDA-BSA and anti-MDA-β-LG than that of anti-BSA and anti-β-LG. These findings are in line with our results for OVA and MDA modified OVA effects on secretion of IL-33 from T84 intestinal epithelial cells.

In conclusion, MDA modification of food allergen ovalbumin induced structural changes on Lys residues, which contributed to the reduced IgE reactivity of the protein. Ovalbumin induced secretion of pro-allergenic IL-33 cytokine in T84 cells, which can contribute to the development of Th2 immune response. Structural modifications of OVA slightly reduced production of IL-33, which indicates that probably particular molecular features facilitate recognition of this allergen by T84 intestinal epithelial cells. To the

best of our knowledge this is the first evidence that ovalbumin, without the presence of adjuvants induces IL-33 cytokine secretion in a model of intestinal epithelial cell line. Deeper insight into the molecular basis of early events in food allergen sensitization should lead to the development of more effective therapeutic strategies for allergic diseases.

Acknowledgements

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Figure captions

Figure 1. Lys residues mapped on the OVA surface. The following coloring of Lys residues was used: green - predicted as highly reactive and experimentally found as *modified* by MDA; turquoise - experimentally found as modified by MDA, but predicted as poorly reactive; blue - predicted as highly reactive but experimentally found as *unmodified* by MDA; orange - predicted as moderately reactive; red - predicted as poorly accessible to the solvent/poorly reactive.

Figure 2. Fluorescent emission spectra of Schiff base (A) and tryptophan (B) of OVA modified with increasing concentration of MDA: 1) control; 2) 0.5 mM MDA; 3) 1 mM MDA; 4) 2 mM MDA; 5) 3 mM MDA; 6) 4 mM MDA; 7) 5 mM MDA; 8) 10 mM MDA.

Figure 3. MS/MS spectra of OVA tryptic peptide carrying modified lysine residue. Modified lysine residue is marked with an asterisk. Detected y and b ions, with their masses, are shown on peptide sequence.

Figure 4. SDS-PAGE profiles of control and MDA-modified OVA: 1) OVA, 2) OVA-0.5MDA, 3) OVA-1MDA, 4) OVA-2MDA, 5) OVA-3MDA, 6) OVA-4MDA, 7) OVA-5MDA, 8) OVA-10MDA; MM) molecular markers.

Figure 5. IgE ELISA Inhibition: as an inhibitor for IgE binding was used OVA, OVA-1MDA, and OVA-10MDA.

Figure 6. Analysisof gene expression by RT-PCR.C – control (non-treated) cells, OVA – cells treated with ovalbumin, OVA-1MDA – cells treated with 1mM MDA pre-treated ovalbumin, OVA-10MDA - cells treated with 10mM MDA pre-treated ovalbumin.* $p<0.05$, ** $p<0.01$.

Figure 7. Quantification of IL33 in supernatants of T84 cells treated with OVA, 1MDA-OVA, and 10MDA-OVA in ELISA. All the tests were performed in duplicate and results are presented as mean ± SEM.

Figure 8. Quantification of TSLP in supernatants of T84 cells treated with OVA, 1MDA-OVA, and 10MDA-OVA in ELISA. All the tests were performed in duplicate and results are presented as mean ± SEM.

TABLES

Table 1. Sequences of PCR primers, Tms and sizes of the corresponding PCR products of the analyzed genes.

Table 2. Carbonyl group content of control and MDA modified OVA was evaluated after DNPH treatment by measuring the absorbance at 367 nm.

Table 3. Type and position of modified lysines in OVA sequence (UniProtKB AC: P01012)

Table 1.

Gene	GenBank Accession No.	Tm (°C)	Primer sequence (5'-….-3')	PCR product size (bp)
IL-1β	NM_000576.2	49	AACCTCTTCGAGGCACAAGG GGCGAGCTCAGGTACTTCTG	137
IL-6	NM_000600.3	49	ACCCCCAATAAATATAGGACTGGA GAGAAGGCAACTGGACCGAA	145
IL-25	NM_022789.3	49	CCAGGTGGTTGCATTCTTGG TGGCTGTAGGTGTGGGTTCC	51
IL-33	NM_033439.3	51	CACCCCTCAAATGAATCAGG GGAGCTCCACAGAGTGTTCC	115
TSLP	NM_033035.4	51	TATGAGTGGGACCAAAAGTACCG GGGATTGAAGGTTAGGCTCTGG	97
TNFα	NM_000594.3	49	GACAAGCCTGTAGCCCATGT CTCTGATGGCACCACCAACT	107
GAPDH	NM_002046.5	65	AGCAATGCCTCCTGCACCACCAAC CCGGAGGGGCCATCCACAGTCT	132

Table 2.

MDA concentration [mM]	Protein dry mass after MDA treatment [mg]	Absorbance at 367 nm	Carbonyl group content [nM/mg proteins]
0	0.65	0.0247	1.73
0.5	0.65	0.1174	8.21
1	0.65	0.1191	8.33
2	0.6	0.1708	12.94
3	0.6	0.2222	16.83
4	0.55	0.2817	23.28
5	0.55	0.3045	25.17
10	0.5	0.4492	40.84

Table 3.

Position of tryptic peptide in OVA sequence	Sequence of tryptic peptide containing modified lysine (K*)	Modified lysine position in OVA sequence	Mass of tryptic peptide containing modified lysine $[M+H]^+$ (Da)	MS/MS spectra confirming type and position of modification
51-58	TQINK*VVR	K55	1091.40	S1
85-104	DILNQITK*PNDVYSFSLASR	K92	2335.20	S2
85-104	DILNQITK*PNDVYSFSLASR	K92	2415.22	S2a
111-126	YPILPEYLQCamVK*ELYR	K122	2218.10	S3
187-199	AFK*DEDTQAMPFR	K189	1609.69	S4
277-284	K*IK*VYLPR	K277, K279	1204.69	S5
277-284	K*IK*VYLPR	K277, K279	1284.72	S5a
278-284	IK*VYLPR	K279	942.55	S6

K* - malonaldehyde Schiff base adduct (+54 Da)

K* - malonaldehyde dihydropyridine type adduct (+134 Da)

Cam – alkylated Cys (+57.02 Da)

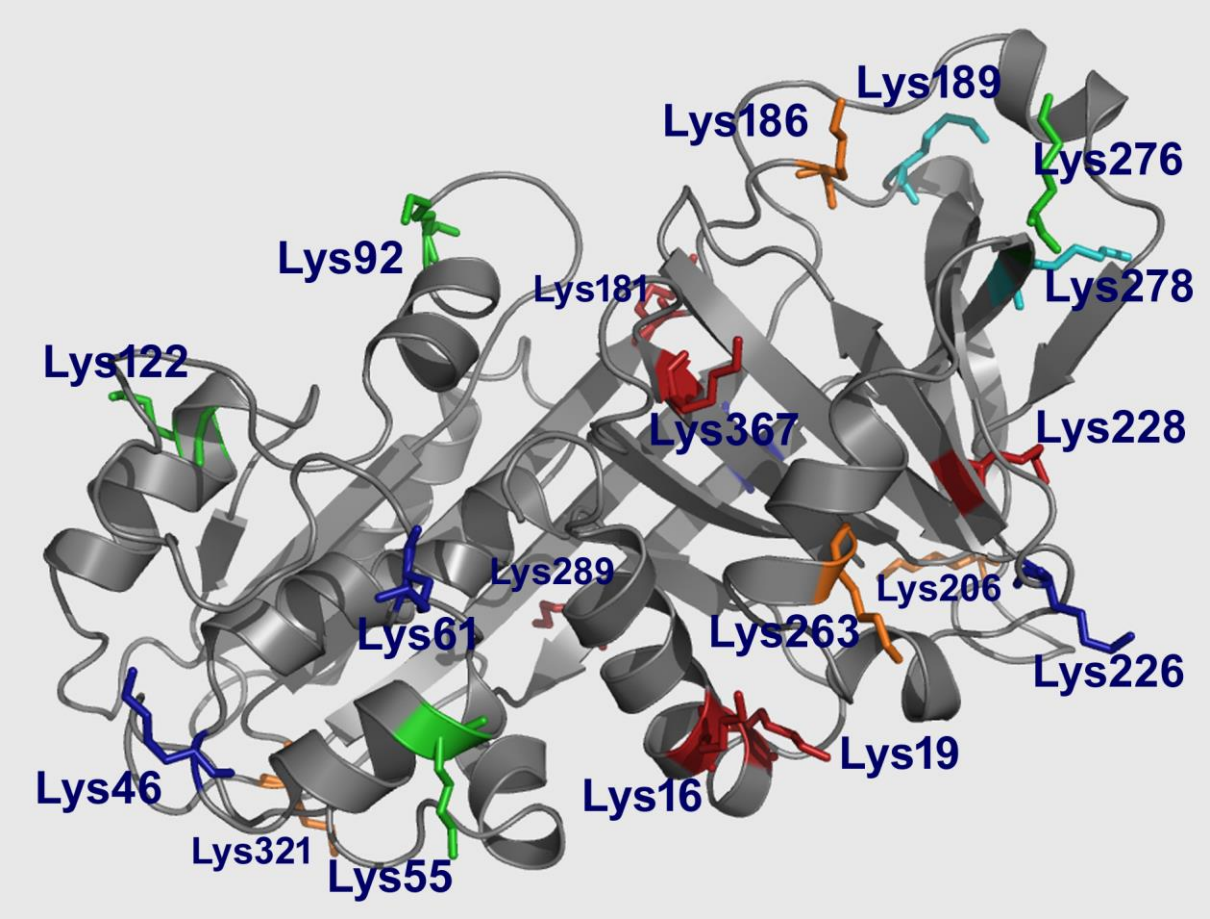


Fig. 1

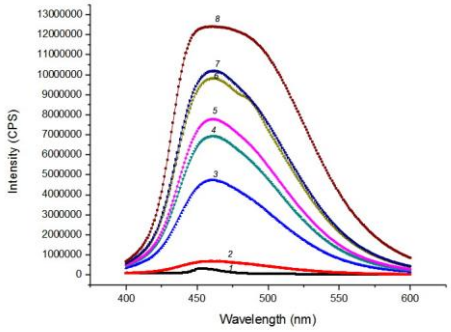


Fig. 2a

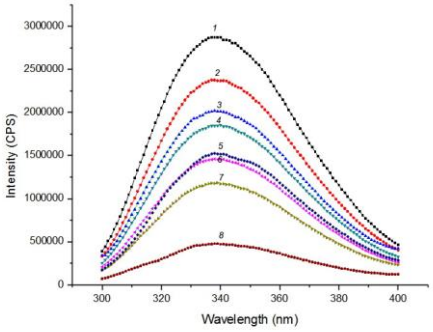


Fig. 2b

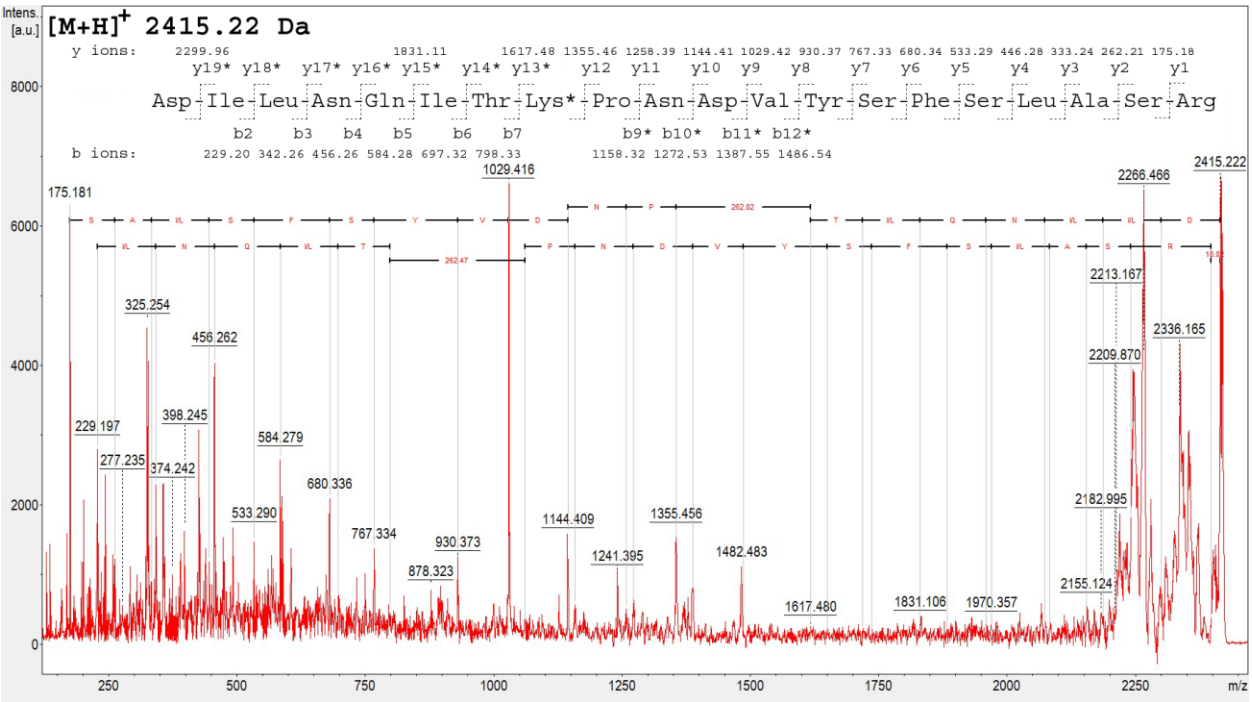


Fig. 3

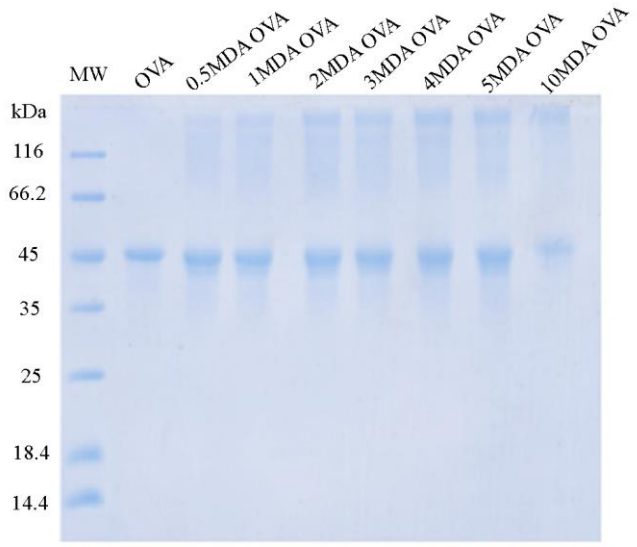


Fig. 4

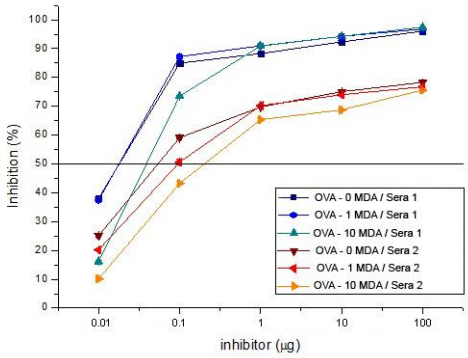


Fig. 5

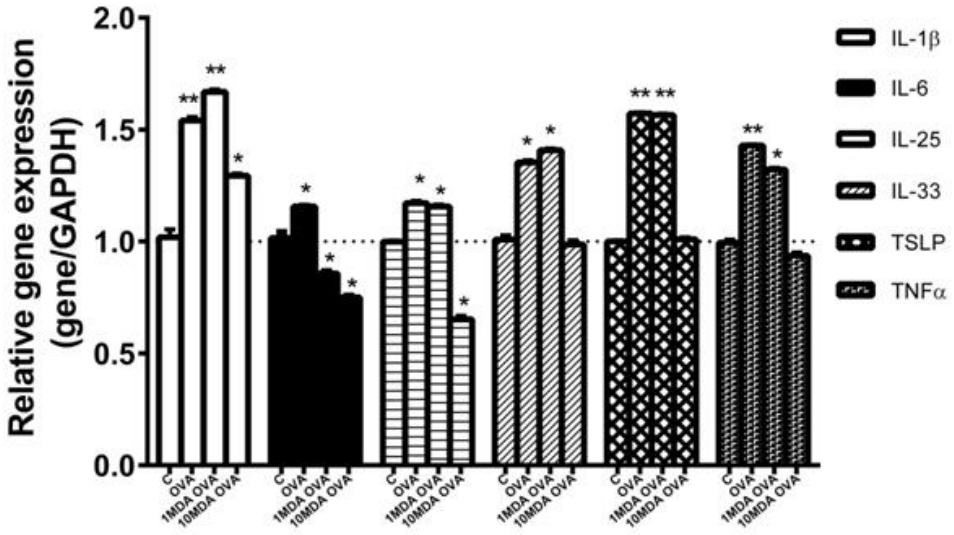


Fig. 6

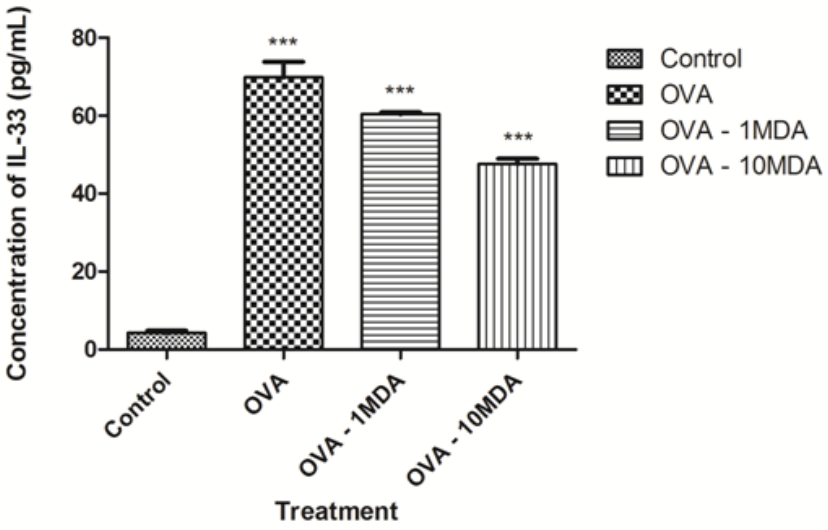


Fig. 7

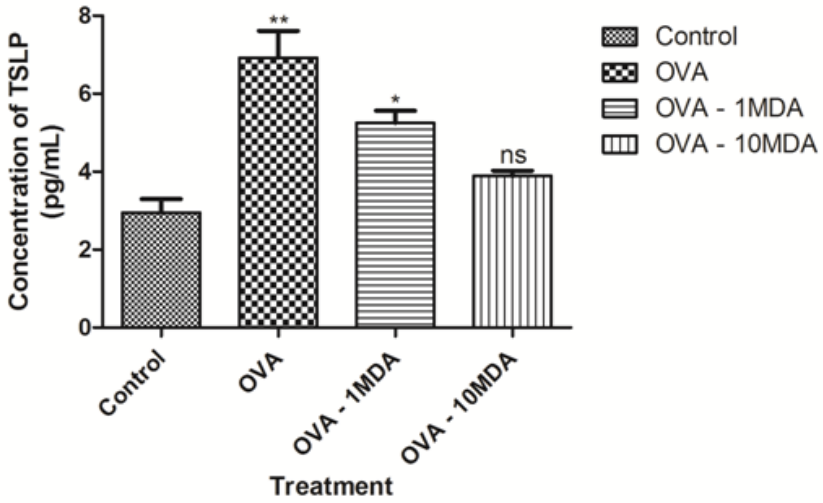
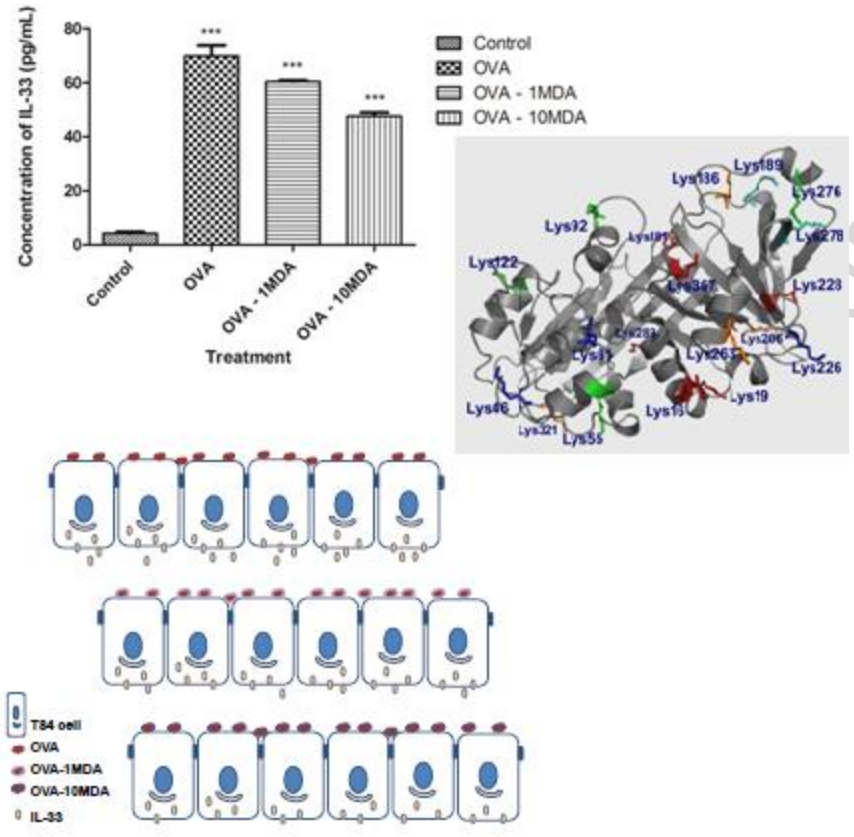


Fig. 8



Graphical abstract

Highlights

Malondialdehyde (MDA) covalently modifies Lys residues in OVA.

MDA modified OVA reveals reduced IgE reactivity.

OVA induced gene expression of pro-inflammatory cytokines in T84 epithelial cells.

OVA and MDA modified OVA induced secretion of IL-33 in T84 epithelial cells.