

Glycation of a food allergen by the Maillard reaction enhances its T-cell immunogenicity: Role of macrophage scavenger receptor class A type I and II

Anne Ilchmann, BSc,^a Sven Burgdorf, PhD,^b Stephan Scheurer, PhD,^c Zoe Waibler, PhD,^d Ryoji Nagai, PhD,^e Anne Wellner, BSc,^f Yasuhiko Yamamoto, MD, PhD,^g Hiroshi Yamamoto, MD, PhD,^g Thomas Henle, PhD,^f Christian Kurts, MD,^b Ulrich Kalinke, PhD,^h Stefan Vieths, PhD,^c and Masako Toda, PhD^a *Langen, Bonn, Dresden, and Hannover, Germany, and Tokyo and Kanazawa, Japan*

Background: The Maillard reaction occurs between reducing sugars and proteins during thermal processing of foods. It produces chemically glycated proteins termed advanced glycation end products (AGEs). The glycation structures of AGEs are suggested to function as pathogenesis-related immune epitopes in food allergy.

Objective: This study aimed at defining the T-cell immunogenicity of food AGEs by using ovalbumin (OVA) as a model allergen.

Methods: AGE-OVA was prepared by means of thermal processing of OVA in the presence of glucose. Activation of OVA-specific CD4⁺ T cells by AGE-OVA was evaluated in

cocultures with bone marrow–derived murine myeloid dendritic cells (mDCs) as antigen-presenting cells. The uptake mechanisms of mDCs for AGE-OVA were investigated by using inhibitors of putative cell-surface receptors for AGEs, as well as mDCs deficient for these receptors.

Results: Compared with the controls (native OVA and OVA thermally processed without glucose), AGE-OVA enhanced the activation of OVA-specific CD4⁺ T cells on coculture with mDCs, indicating that the glycation of OVA enhanced the T-cell immunogenicity of the allergen. The mDC uptake of AGE-OVA was significantly higher than that of the controls. We identified scavenger receptor class A type I and II (SR-AI/II) as a mediator of the AGE-OVA uptake, whereas the receptor for AGEs and galectin-3 were not responsible. Importantly, the activation of OVA-specific CD4⁺ T cells by AGE-OVA was attenuated on coculture with SR-AI/II-deficient mDCs.

Conclusion: SR-AI/II targets AGE-OVA to the MHC class II loading pathway in mDCs, leading to an enhanced CD4⁺ T-cell activation. The Maillard reaction might thus play an important role in the T-cell immunogenicity of food allergens. (*J Allergy Clin Immunol* ■■■■;■■■:■■■-■■■.)

Key words: Food allergy, food allergen, Maillard reaction, T-cell immunogenicity, dendritic cells, macrophage scavenger receptor

From ^aJunior Research Group 1 “Experimental Allergology,” ^dJunior Research Group 2 “Novel vaccination strategies and early immune responses,” and ^cthe Division of Allergology, Paul-Ehrlich-Institut, Langen; ^bthe Institute for Molecular Medicine and Experimental Immunology, Rheinische Friedrich-Wilhelms-Universität, Bonn; ^ethe Department of Food and Nutrition, Laboratory of Biochemistry & Nutritional Science, Japan Women’s University, Tokyo; ^fthe Institute of Food Chemistry, Technische Universität Dresden; ^gthe Department of Biochemistry and Molecular Vascular Biology, Kanazawa University Graduate School of Medical Science; and ^hTWINCORE, Centre for Experimental and Clinical Infection Research, Hannover.

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Reprint requests: Masako Toda, PhD, Junior Research Group 1 “Experimental Allergology,” Paul-Ehrlich-Institut, Paul Ehrlich St 59, Langen 63225, Germany. E-mail: todma@pei.de.

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The Maillard reaction is a chemical reaction between reducing sugars and proteins and generates the so-called advanced glycation end products (AGEs; ie, protein derivatives with glycation structures, such as Nε-carboxyethyl-lysine [CEL], Nε-carboxymethyl-lysine [CML], pyrraline, and GA-pyridine).¹ Because the Maillard reaction occurs during storage and thermal processing of foods, a possible involvement of AGEs in the pathology of food allergy is of great concern. This assumption is corroborated by the fact that some patients with food allergy show anaphylactic reactions only against stored or heated foods.^{2,3} Moreover, the presence of AGEs in food allergens could be linked to an increased binding ability of IgEs from patients allergic to the respective allergen.⁴⁻⁷ These observations suggest that the Maillard reaction creates new pathogenesis-related immune epitopes in patients with food allergy.

Several studies have shown diverse effects of AGEs on dendritic cells (DCs). For instance, AGEs derived from BSA augmented maturation of human DCs and increased their capacity to stimulate allogeneic T-cell activation.⁸ In contrast, adrenocorticotrophic hormone–derived AGEs were shown to inhibit the

Abbreviations used

AGE:	Advanced glycation end product
APC:	Antigen-presenting cell
CEL:	N ϵ -carboxyethyl-lysine
CFSE:	Carboxyfluorescein succinimidyl ester
CML:	N ϵ -carboxymethyl-lysine
DC:	Dendritic cell
FITC:	Fluorescein isothiocyanate
GA:	Glycolaldehyde
mDC:	Myeloid dendritic cell
MR:	Mannose receptor
OVA:	Ovalbumin
RAGE:	Receptor for AGEs
SR-AI/II:	Scavenger receptor class A type I and II

maturation and T-cell stimulatory capacity of the human DCs.⁹ Together, these observations suggest that T-cell immunogenicity of antigens could be influenced by the Maillard reaction. However, the effect of AGEs derived from food allergens on DC function, the subsequent activation of allergen-specific T cells, or both is poorly understood.

Antigen-specific T-cell activation is preceded by the uptake of antigens by DCs. Receptors expressed on the cell surface mediate the majority of antigen uptake by DCs.^{10,11} Importantly, antigen-presenting cells (APCs), such as DCs and macrophages, express several receptors known to bind AGEs, such as the so-called receptor for AGEs (RAGE),^{12,13} galectin-3,¹⁴ macrophage scavenger receptor class A type I and II (SR-AI/II),^{15,16} scavenger receptor class B type I,¹⁷ and CD36.¹⁸ These receptors have been identified by investigating endothelial cells,^{12,13} macrophages,^{15,16} or Chinese hamster ovary cells transfected with putative receptors for AGEs.^{14,17,18} However, the receptors that mediate the uptake of AGEs by DCs remain to be identified.

The aim of this study was to define the influence of the Maillard reaction on the T-cell immunogenicity of food allergens. We used AGE-ovalbumin (OVA; ie, the Maillard reaction products of glucose and the egg white allergen OVA) as a food allergen model of AGEs. We found that AGE-OVA does not trigger the maturation of bone marrow–derived murine myeloid dendritic cells (mDCs) but enhances the activation of allergen-specific CD4⁺ T cells. Moreover, we demonstrated that the enhanced T-cell immunogenicity of AGE-OVA depends on a SR-AI/II–mediated uptake of AGE-OVA by mDCs. Our findings support the significance of AGEs as pathogenesis-related factors in food allergy.

METHODS

Mice

C57BL/6 J (B6) mice and SR-AI/II–deficient mice on a B6 background were purchased from Jackson Laboratories (Bar Harbor, Me).¹⁹ RAGE-deficient mice on a B6 background were kindly provided by Dr T. Shoji (Osaka Medical College, Osaka, Japan).²⁰ OT-II mice expressing a T-cell receptor specific for the peptide OVA_{323–339} were kindly provided by Professor H. Schild and Dr S. Sudowe (Johannes-Gutenberg-University, Mainz, Germany).²¹ Mice were housed under pathogen-free conditions, and animal experiments were performed in compliance with German legislation.

Preparation of AGE-OVA and AGE-BSA

AGE-OVA and AGE-BSA (ie, the Maillard reaction products) were prepared as described previously.²² Briefly, 1 mmol/L OVA or BSA (Sigma-

Aldrich, Steinheim, Germany) was incubated with 1 mol/L glucose in 100 mmol/L sodium phosphate buffer (pH 7.4) at 50°C for 6 weeks. OVA incubated under the same conditions but without glucose and native OVA were used as controls. Protein concentrations of the final samples were measured by using a bicinchoninic acid assay kit (Pierce, Rockford, Ill). The protein concentration was further verified by analyzing valin concentrations using ion-exchange chromatography with Ninhydrin postcolumn derivatization after acid and enzymatic hydrolysis because valin is not modified by the Maillard reaction.²³

Verification of glycation structures in AGE-OVA

A protocol is described in the Methods section of this article's Online Repository at www.jacionline.org.

Preparation of recombinant OVA

A protocol is described in the Methods section of this article's Online Repository.

Fluorescein isothiocyanate labeling of OVAs

A protocol is described in the Methods section of this article's Online Repository.

Generation of bone marrow–derived mDCs

A protocol is described in the Methods section of this article's Online Repository.

Assessment of mDC maturation

A protocol is described in the Methods section of this article's Online Repository.

Assessment of T-cell activation and proliferation

Splenic CD4⁺ T cells were isolated from OT-II mice using an isolation kit from Miltenyi Biotec (Bergisch Gladbach, Germany). CD4⁺ T cells (8.0×10^5 cells/mL) were cocultured with mDCs (1.6×10^5 cells/mL) and stimulated with either form of OVA for 24 hours to evaluate T-cell activation. In the experiment with SR-AI/II–deficient mDCs the APCs (2.5×10^6 cells/mL) were first incubated with either form of OVA for 3 hours and then fixed with 0.008% glutaraldehyde before 21 hours of coculturing with CD4⁺ T cells (5.0×10^6 cells/mL). After coculturing, the concentration of IL-2 in the supernatants was measured by means of ELISA (eBioscience, San Diego, Calif). CD4⁺ T cells were first stained for 15 minutes with 10 μ mol/L carboxyfluorescein succinimidyl ester (CFSE; Invitrogen, Karlsruhe, Germany) and then cocultured with mDCs stimulated with either form of OVA to evaluate T-cell proliferation. Cell proliferation was evaluated by measuring the intensity of CFSE in the CD4⁺ T cells with a flow cytometer, LSR II (BD Bioscience, Heidelberg, Germany). Data were analyzed with FlowJo version 7 software (Treestar, Inc, Ashland, Ore).

Assessment of the uptake of AGE-OVA by mDCs

mDCs (1.0×10^6 cells/mL) were incubated for 15 minutes with fluorescein isothiocyanate (FITC) conjugates of AGE-OVA or of native OVA and OVA thermally processed without glucose as controls. Lactose (150 mmol/L; Sigma-Aldrich) was added to the mDCs 30 minutes before the addition of AGE-OVA or the controls to inhibit a possible galectin-3–mediated uptake.²⁴ Only samples with a comparable FITC/protein molar ratio were used to evaluate the uptake level of AGE-OVA and the controls. After incubation with FITC conjugates of AGE-OVA or the controls, mDCs were stained with both phycoerythrin-conjugated anti-mouse CD11b and allophycocyanin-conjugated anti-mouse CD11c mAbs. The FITC intensity of CD11b⁺CD11c⁺ cells was then analyzed by using flow cytometry. mDCs were first fixed with 4% paraformaldehyde solution (Pierce) after incubation of

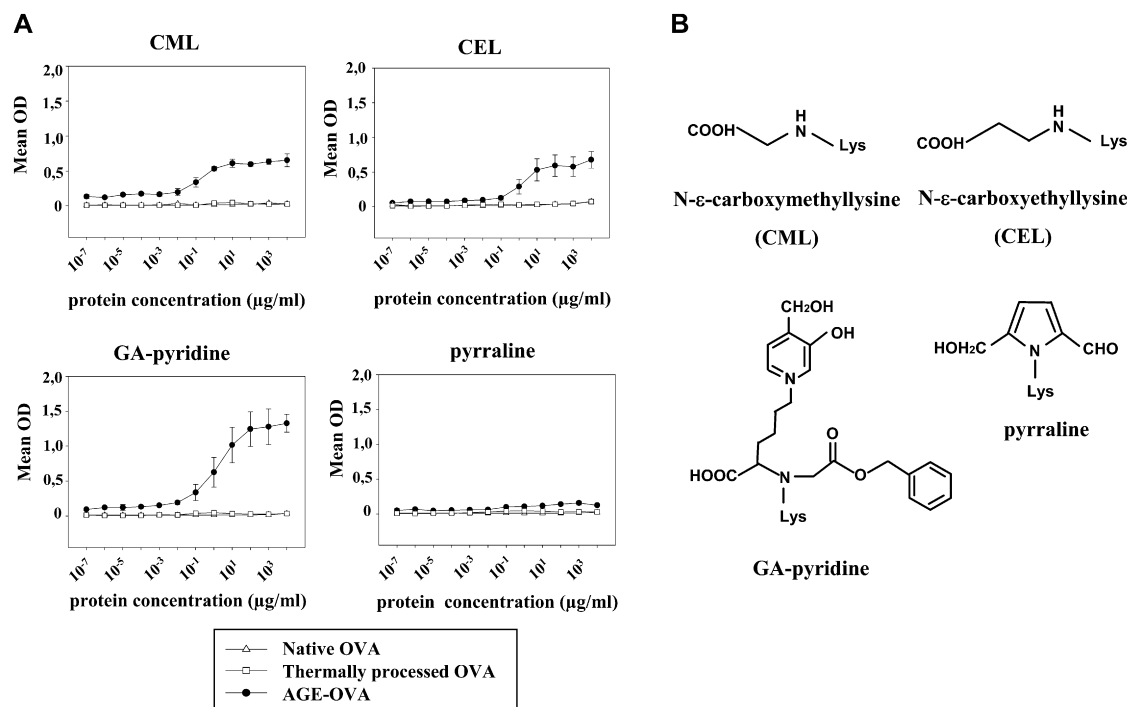


FIG 1. Glycation structures in AGE-OVA produced by the Maillard reaction. **A**, AGE-OVA was prepared by means of incubation of 1 mmol/L OVA with 1 mol/L glucose at 50°C for 6 weeks. The formation of glycation structures was verified by means of ELISA. Native OVA and OVA thermally processed without glucose (thermally processed OVA) were analyzed as controls. The data represent means $\pm$ SEMs of 3 independent experiments. **B**, Structural formula of the glycation structures. Lys, Lysine residues of proteins.

FITC-conjugated AGE-OVA for 15 minutes and then stained with 4',6-diamidino-2-phenylindol (Invitrogen) to verify endocytosis of AGE-OVA by receptor-mediated uptake. Localization of FITC-conjugated AGE-OVA in the cells was analyzed with a laser scanning microscope (LSM 510; Carl Zeiss, Jena, Germany).

Statistical analysis

Significant differences between mean values were assessed by means of ANOVA, followed by the Tukey honestly significant difference multiple comparison test. A *P* value of less than .05 was considered significant.

RESULTS

Identification of AGEs in AGE-OVA

First, we investigated the AGEs of OVA (AGE-OVA) resulting from incubation with glucose at 50°C for 6 weeks.²² As shown in Fig 1, the presence of CEL, CML, and GA-pyridine glycation structures was observed, whereas pyrrole could not be detected. High levels of CML (6.79 ± 0.08 mmol/100 g protein) and lower levels of pentosidine (10.2 ± 0.7 μmol/100 g protein) could be verified by means of reverse-phase HPLC analyses.^{23,24} The respective glycation structures were not detected in native OVA or OVA thermally processed without glucose. SDS-PAGE analysis showed that the protein bands of glycosylated proteins were very diffuse, indicating significant modification of the protein caused by the glycation procedure (see Fig E1, A, in this article's Online Repository at www.jaiconline.org). Analyses of the secondary structures by means of circular dichroism spectroscopy revealed highly similar spectra for either form of OVA (see Fig E1, B), which indicates that OVA

thermally processed without glucose, as well as AGE-OVA, retained the same or a highly similar secondary structure as native OVA.

Influence of AGE-OVA on the activation and proliferation of OVA-specific CD4⁺ T cells

To examine the T-cell immunogenicity of AGE-OVA, we cocultured OVA-specific CD4⁺ T cells derived from OT-II mice with mDCs and stimulated them either with AGE-OVA or with native OVA or OVA thermally processed without glucose as controls. Subsequently, the IL-2 concentration in the cell culture supernatants was determined as a measure of CD4⁺ T-cell activation. Compared with the controls, AGE-OVA induced a higher production of IL-2 (Fig 2, A). In the absence of mDCs, CD4⁺ T cells did not produce detectable levels of IL-2 on AGE-OVA stimulation (data not shown).

Next, we investigated whether AGE-OVA affects the proliferation of OVA-specific CD4⁺ T cells. Therefore CFSE-stained CD4⁺ T cells were cocultured with mDCs and stimulated either with AGE-OVA or with native OVA and OVA thermally processed without glucose as controls. Cell division was detectable after 72 hours. The number of dividing CD4⁺ T cells was significantly higher on stimulation with 2.0 or 20 μg/mL AGE-OVA when compared with that seen in cells stimulated with the controls (Fig 2, B). This effect was not significant if T cells were stimulated with 200 μg/mL protein. Together, these results indicate that glycation of OVA by using the Maillard reaction increases the CD4⁺ T-cell immunogenicity of the allergen and that this effect is mediated by mDCs.

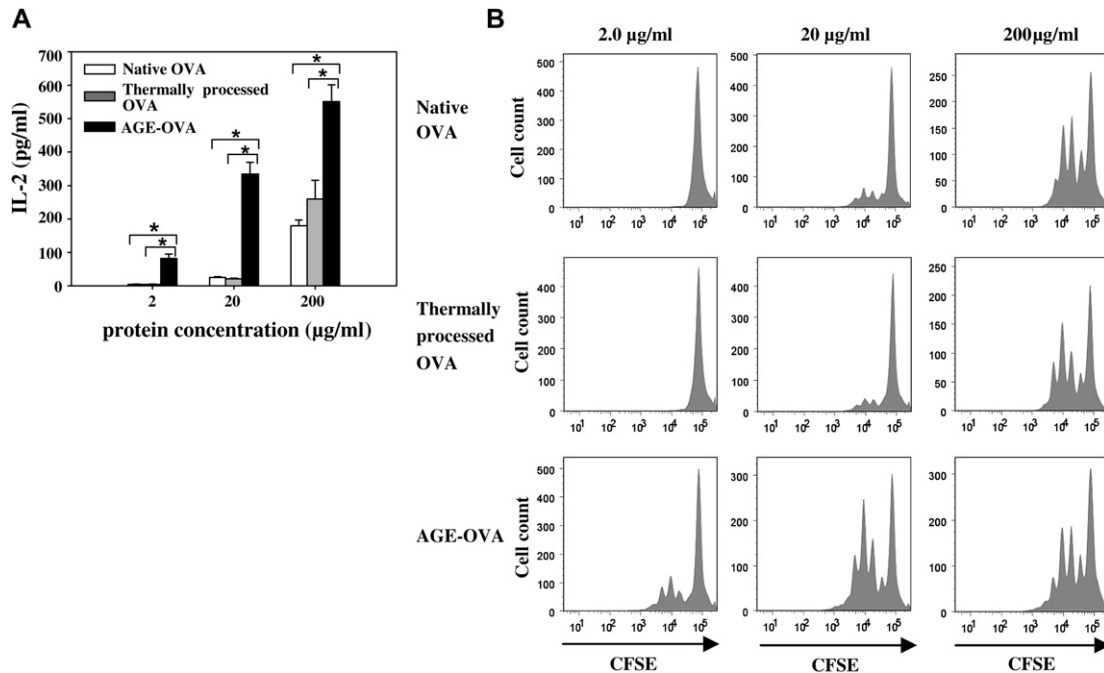


FIG 2. AGE-OVA enhances the activation and proliferation of OVA-specific CD4⁺ T cells. **A**, CD4⁺ T cells isolated from OT-II mice were cocultured with mDCs and stimulated with native OVA, OVA thermally processed without glucose, or AGE-OVA for 24 hours. Concentration of IL-2 in the culture supernatant was measured by means of ELISA. **P* < .001. **B**, CFSE-stained CD4⁺ T cells were cocultured with mDCs and stimulated with either form of OVA. After 72 hours, CFSE intensity of CD4⁺ T cells was measured by means of flow cytometry. The data are representative of 3 independent experiments.

Next, we examined the influence of glycation structures on cytokine production by CD4⁺ T cells. OVA-specific CD4⁺ T cells from DO11.10 mice were cocultured with mDCs and stimulated with either form of OVA. Production of T_H2-type cytokines, such as IL-4, by allergen-specific CD4⁺ T cells is a critical component in inducing food allergy. Because CD4⁺ T cells from OT-II mice predominantly produce T_H1-type cytokines, such as IFN-γ, but hardly any T_H2-type cytokines, for the cytokine assay, we used CD4⁺ T cells from DO11.10 mice, which efficiently produce T_H2-type cytokines. Compared with the controls, AGE-OVA induced higher production of IL-2, IL-4, and IFN-γ (see Fig E2 in this article's Online Repository at www.jacionline.org). The results suggest that glycation structures enhance production of both T_H1- and T_H2-type cytokines.

Influence of AGE-OVA on the maturation of mDCs

To investigate the mediator function of mDCs, we first examined the influence of AGE-OVA on the maturation of these APCs. Expectedly, LPS-stimulated mDCs (LPS is a known inducer of mDC maturation) displayed enhanced expression of the maturation markers CD40, CD80, CD86, and MHC class II molecule. In contrast, the expression of these markers was not enhanced by either form of OVA (Fig 3, A). Moreover, these OVAs also did not induce a detectable secretion of IL-12 p70 or IL-10, which was observed when mDCs were stimulated with LPS (Fig 3, B). Together, these results suggest that the OVA allergens are not capable of stimulating the maturation of mDCs.

Uptake of AGE-OVA by mDCs

To further elucidate the mediator function of mDCs, we next investigated whether glycation through the Maillard reaction influences the uptake of antigen by mDCs. Therefore these cells were incubated with FITC conjugates of AGE-OVA or native OVA, OVA thermally processed without glucose, and recombinant OVA without any natural carbohydrate residues or glycation structures as controls. The FITC intensity of mDCs was analyzed by means of flow cytometry as a measure of antigen uptake. If compared with native OVA and thermally processed OVA, we observed a fluorescence shift of approximately 5- to 10-fold in mDCs incubated with AGE-OVA, indicating higher uptake of the AGE (Fig 4, A). Importantly, the mDC uptake of recombinant OVA was lower than that of native OVA (see Fig E3 in this article's Online Repository at www.jacionline.org). Subsequent confocal microscopic analyses showed that the majority of AGE-OVA was not merely attached on the cell surface of mDCs but was endocytosed into the cells (Fig 4B).

Involvement of SR-AI/II in the uptake of AGE-OVA by mDCs

Native OVA has natural mannose residues, and its uptake is known to be mediated by the mannose receptor (MR).^{25,26} AGEs are known to bind to several cell-surface receptors, such as RAGE, SR-AI/II, and galectin-3.¹²⁻¹⁶ Hence we hypothesized that the uptake of AGE-OVA by mDCs is mediated by 1 or more of these receptors for AGEs in addition to the MR for natural mannose residues. To identify the responsible receptor or receptors for the AGE-OVA uptake by mDCs, we investigated

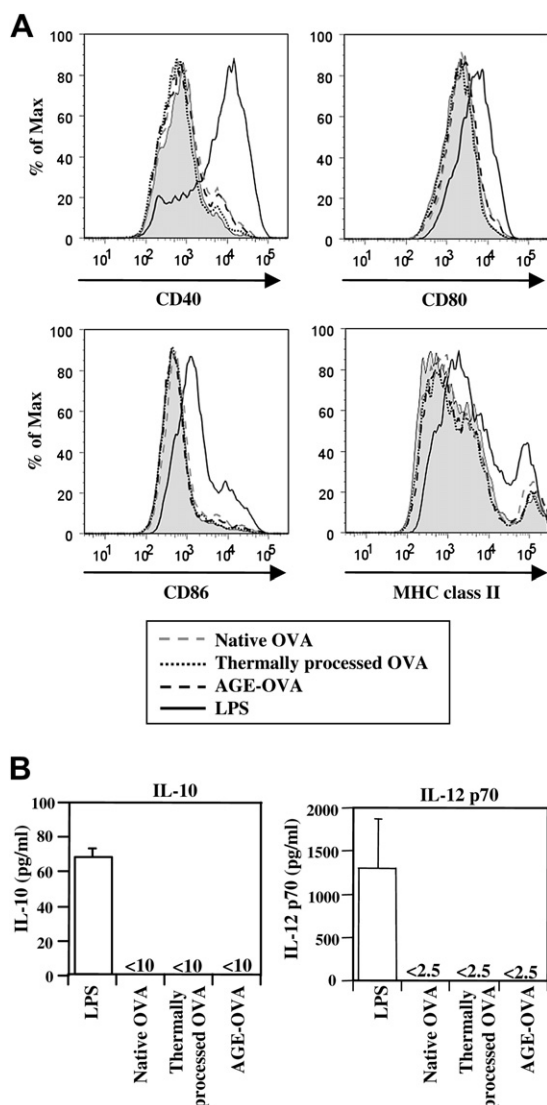


FIG 3. AGE-OVA does not induce the maturation of mDCs. mDCs were stimulated with 20 μ g/mL native OVA, OVA thermally processed without glucose or AGE-OVA or with 10 μ g/mL LPS. **A**, Expression of CD40, CD80, CD86, and MHC class II molecules on mDCs was analyzed with flow cytometry. Gray areas represent mDCs cultured without stimulation. **B**, Levels of IL-10 and IL-12 p70 in the culture supernatants of mDCs were measured by means of ELISA. The data are representative of 3 independent experiments.

RAGE- and SR-AI/II-deficient mDCs, as well as the galectin-3 inhibitor lactose and the MR inhibitor mannan.^{26,27}

We observed almost identical uptake of FITC-conjugated AGE-OVA in wild-type mDCs, RAGE-deficient mDCs, or lactose-treated wild-type mDCs (Fig 4, C and D), indicating that RAGE and galectin-3 would not be involved in the uptake. Likewise, the uptake of native OVA and OVA thermally processed without glucose was not affected in RAGE-deficient mDCs or in lactose-treated wild-type mDCs (see Fig E4 in this article's Online Repository at www.jacionline.org). In contrast, SR-AI/II deficiency resulted in a significant reduction of the AGE-OVA uptake by mDCs (Fig 5, A). The additional treatment of SR-AI/II-deficient mDCs with mannan led to a further reduction of the uptake of AGE-OVA (see Fig E5 in this article's Online

Repository at www.jacionline.org). Together, these results suggest that SR-AI/II and the MR, but not RAGE and galectin-3, are essential mediators of the mDC uptake of AGE-OVA. Unexpectedly, SR-AI/II deficiency also led to a slight reduction of the mDC uptake of native OVA and OVA thermally processed without glucose (Fig 5, A, and see Fig E6 in this article's Online Repository at www.jacionline.org). The additional treatment of SR-AI/II-deficient mDCs with mannan resulted in a complete suppression of the uptake of the non-AGE forms of OVA (see Fig E5). The SR-AI/II deficiency, however, did not inhibit the mDC uptake of recombinant OVA (see Figure E5, A).

Unlike OVA, BSA does not possess natural carbohydrate residues.²⁸ However, BSA should also be capable of binding to SR-AI/II as an AGE derivative. In accordance with this, we show that the uptake of AGE-BSA was significantly attenuated in SR-AI/II-deficient mDCs (see Fig E7 in this article's Online Repository at www.jacionline.org). Consequently, we hypothesized that AGE-BSA should act as an inhibitor of the SR-AI/II-mediated uptake of OVA allergens. To prove this, we incubated wild-type mDCs with native BSA or AGE-BSA together with the different forms of OVA. In accordance with our hypothesis, AGE-BSA, but not native BSA, was capable of inhibiting the uptake of either form of OVA (Fig 6). Together, the results suggest that SR-AI/II plays an important role in the enhanced mDC uptake of AGE-OVA.

SR-AI/II deficiency reduces the activation of OVA-specific CD4⁺ T cells by mDCs

To further verify the obvious function of SR-AI/II in the mDC-mediated enhanced activation of OVA-specific CD4⁺ T cells by AGE-OVA, we examined whether the expression of SR-AI/II is a prerequisite for this effect. Therefore we cocultured OVA-specific CD4⁺ T cells with SR-AI/II-deficient or wild-type mDCs that were pretreated with either form of OVA. As shown in Fig 5, B, we observed significantly reduced production of IL-2 by OVA-specific CD4⁺ T cells in response to native OVA, OVA thermally processed without glucose, and AGE-OVA if the T cells were cocultured with SR-AI/II-deficient mDCs instead of wild-type mDCs. However, the reduction was not observed for recombinant OVA. The capacity to stimulate T cells appears not to be affected in SR-AI/II-deficient mDCs because SR-AI/II-deficient and wild-type mDCs express comparable levels of costimulatory CD40, CD80, CD86, and MHC class II molecules (see Fig E8 in this article's Online Repository at www.jacionline.org). Wild-type and SR-AI/II-deficient mDCs induced comparable IL-2 production of OVA-specific CD4⁺ T cells against recombinant OVA, also indicating the intact T-cell stimulatory capacity of the deficient mDCs. In accordance with this, the results suggest that mDC-expressed SR-AI/II is critical for the activation of OVA-specific CD4⁺ T cells by AGE-OVA.

DISCUSSION

The majority of foods are modified by storage or processing. The formation of AGEs produced by the Maillard reaction during thermal processing of foods is suggested to exert important effects on the immunogenicity of food proteins. To our knowledge, the present study is the first to demonstrate that the formation of AGEs enhances the CD4⁺ T-cell immunogenicity of a food

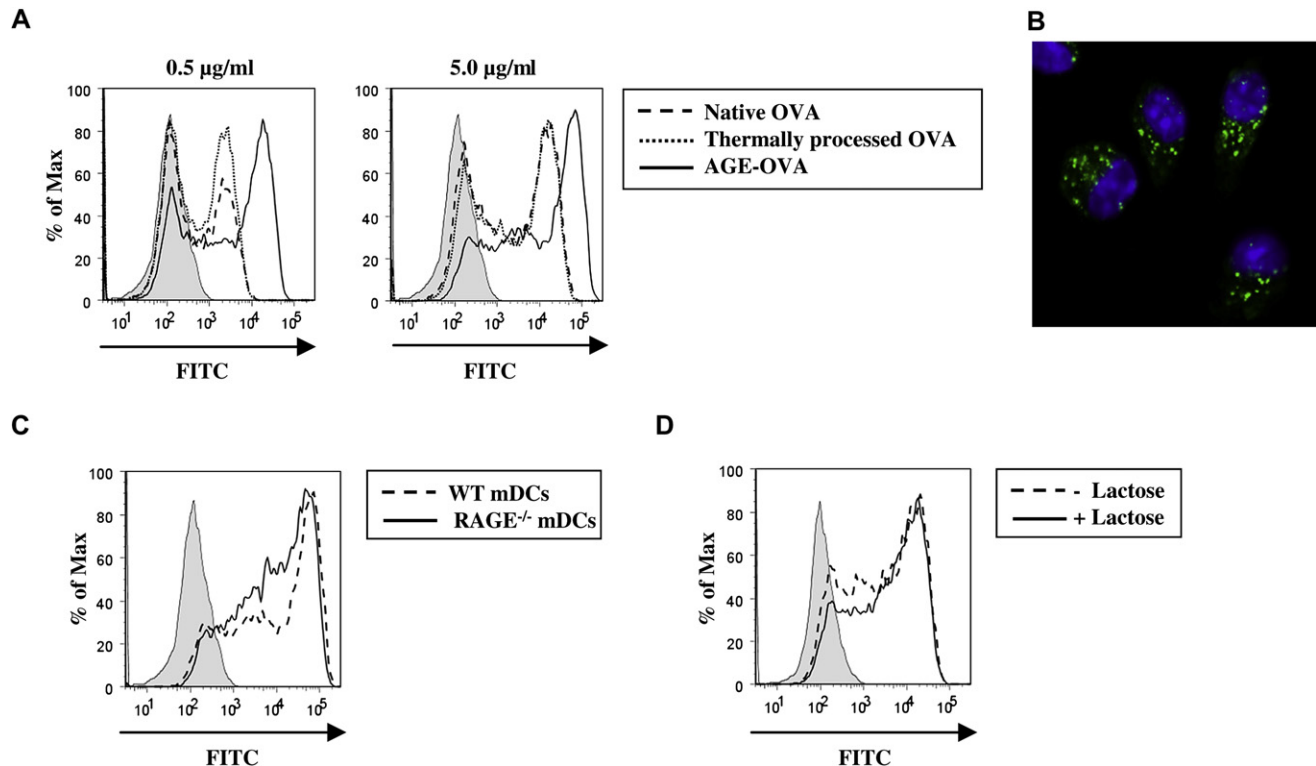


FIG 4. The uptake of OVA by mDCs is increased by means of glycation. RAGE and galectin-3 are not involved. **A**, Wild-type mDCs were incubated with 0.5 or 5.0 $\mu\text{g/mL}$ FITC-conjugated native OVA, OVA thermally processed without glucose, or AGE-OVA for 15 minutes. The mDC uptake of the samples was analyzed by means of flow cytometry. **B**, The mDC uptake of FITC-conjugated AGE-OVA was verified by using confocal microscopy. **C**, Wild-type or RAGE-deficient mDCs were incubated with 5.0 $\mu\text{g/mL}$ FITC-conjugated AGE-OVA for 15 minutes. The uptake of AGE-OVA was analyzed by using flow cytometry. **D**, Wild-type mDCs were treated with or without 150 mmol/L lactose for 30 minutes before incubation with 5.0 $\mu\text{g/mL}$ FITC-conjugated AGE-OVA to prevent galectin-3-mediated uptake. The uptake of AGE-OVA by mDCs was analyzed by means of flow cytometry. Gray areas represent mDCs cultured with medium only. The data are representative of 3 independent experiments.

allergen. The results strongly suggest that the influence of the Maillard reaction needs to be carefully considered in the evaluation of the immunogenicity of food allergens.

Our results are in good accordance with previous studies demonstrating an increased CD4^+ T-cell immunogenicity of antigens chemically modified to function as SR-AI/II ligands.²⁹⁻³¹ Moreover, it could be shown that native OVA endocytosed through scavenger receptors in macrophages predominantly activates the allergen-specific CD4^+ T cells.²⁶ However, the specific scavenger receptor or receptors participating in this OVA uptake were not identified. Our results now substantiate that (1) SR-AI/II is an important mediator of the mDC uptake of native OVA and AGE-OVA and (2) the subsequent activation of OVA-specific CD4^+ T cells occurs through SR-AI/II-mediated uptake (Fig 5).

SR-AI/II belongs to a large and diverse group of scavenger receptors and functions as an endocytic receptor.^{32,33} The expression of SR-AI/II is mostly restricted to myeloid cells.³⁴ The presentation of soluble antigens by APCs is governed by endocytic receptors, which determine the intracellular routing of the endocytosed molecules.^{11,26,35} For example, antigens endocytosed by the MR in DCs were shown to be targeted to the MHC class I loading pathway,²⁶ whereas those endocytosed by the glycan-binding receptor DC-SIGN (CD209) were targeted to the MHC class II loading pathway.³⁵ We found that the glycation

of OVA does not enhance CD8^+ T-cell immunogenicity of the allergen. OVA-specific CD8^+ T cells cocultured with mDCs secreted comparable levels of IL-2 in response to AGE-OVA and native OVA (see Fig E9 in this article's Online Repository at www.jacionline.org). Our results now suggest that SR-AI/II is a receptor targeting its ligands to the MHC class II loading pathway.

Importantly, the activation of OVA-specific CD4^+ T cells by recombinant OVA was lower than that of native OVA and was not attenuated by SR-AI/II deficiency in the mDCs (Fig 5). Recombinant OVA has no natural carbohydrate or glycation structures for SR-AI/II and other endocytic receptors. It could previously be shown that pinocytosed antigens are transferred to the lysosome compartment, where antigens enter the MHC class II loading pathway.²⁶ The recombinant OVA would be taken up by pinocytosis in mDCs, whereas native OVA and AGE-OVA would be taken up not only by pinocytosis but also by SR-AI/II-mediated endocytosis. Because uptake of AGE-OVA by SR-AI/II is higher than that of native OVA as a result of the glycation structures, higher amounts of AGE-OVA would be targeted into the MHC class II loading pathway. This would increase the amount of OVA peptide/MHC class II complex on the surface of mDCs and subsequently induce enhanced OVA-specific CD4^+ T-cell activation.

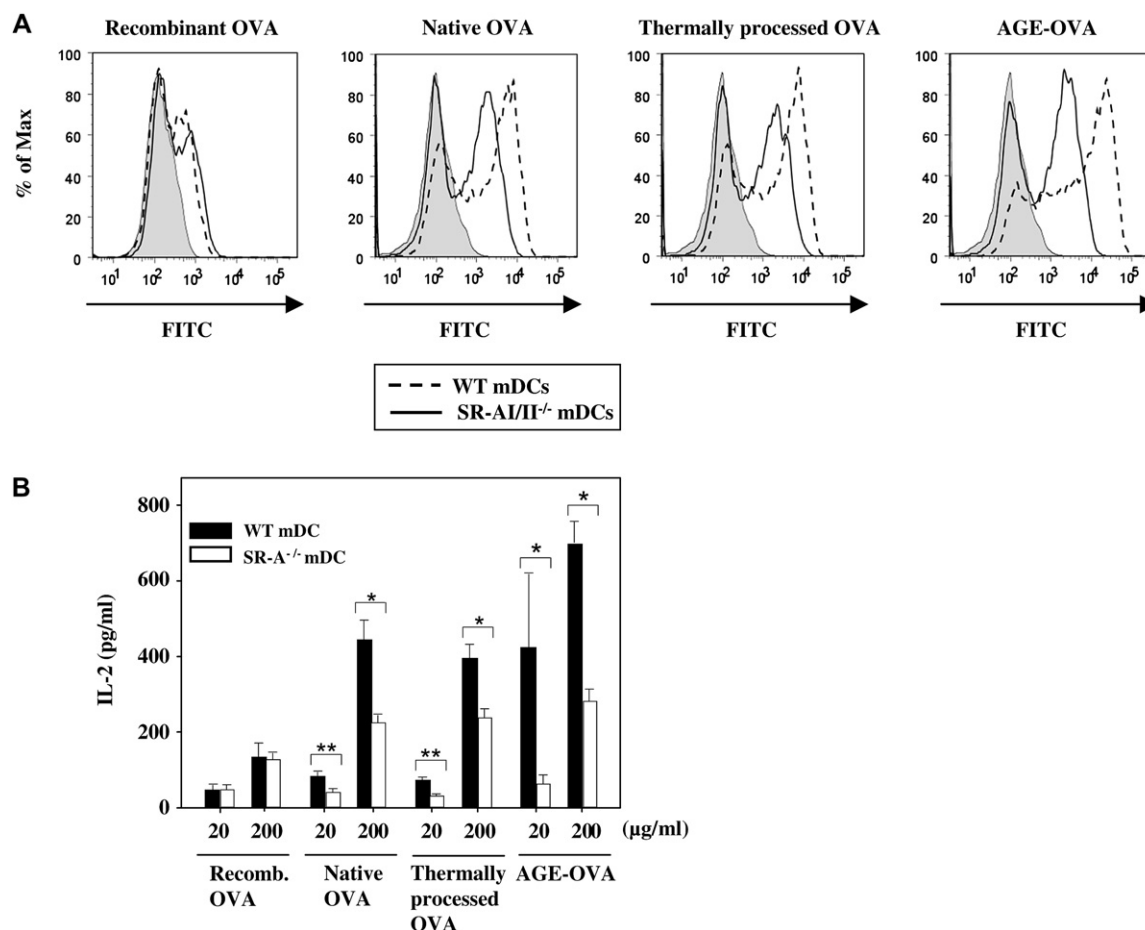


FIG 5. SR-AI/II is involved in the uptake of AGE-OVA by mDCs. **A**, Wild-type or SR-AI/II-deficient mDCs were incubated with 5.0 µg/mL FITC-conjugated recombinant OVA, native OVA, OVA thermally processed without glucose, or AGE-OVA for 15 minutes. The mDC uptake of the samples was analyzed by means of flow cytometry. Gray areas represent mDCs cultured with medium only. **B**, Wild-type or SR-AI/II-deficient mDCs were incubated for 3 hours with either form of OVA and then fixed with 0.008% glutaraldehyde before 21 hours of coculturing with CD4⁺ T cells isolated from OT-II mice. The concentration of IL-2 in the culture supernatant was measured by means of ELISA. * $P < .01$. ** $P < .001$. The data are representative of 3 independent experiments.

Despite the enhanced uptake of AGE-OVA by mDCs, the allergen did not stimulate the maturation of these cells in our experimental setting (Fig 3). Previously, other studies revealed both stimulatory and inhibitory effects of AGEs on the maturation of human DCs.^{8,9} The different observations might be explained by variations in the expression profiles of receptors for AGEs on these DCs. The previously detected *in vitro* stimulation of human DC maturation by AGE-BSA was ascribed to the expression of RAGE.⁸ This is consistent with reports providing evidence that RAGE acts as a receptor triggering the maturation of DCs.^{36,37} In our experiments RAGE expression was not detectable on the cell surface of the mDCs (see Fig E10 in this article's Online Repository at www.jacionline.org). The absence of RAGE in mDCs might explain why AGE-OVA did not stimulate the maturation of the cells.

We found high levels of CEL, CML, and GA-pyridine glycation structures in AGE-OVA (Fig 1, A), which we recently also detected in roasted peanuts (ie, an important food allergen; manuscript in preparation). CEL and CML are also representative AGEs in thermally processed foods.¹ Hence AGE-OVA appears to be an appropriate model for studying the influence of food

allergen-derived AGEs on T-cell immunogenicity. Currently, it is still unknown which glycation structures bind to SR-AI/II or galectin-3, whereas CML has been suggested as a major glycation structure of RAGE ligands.³⁸ The future identification of the exact glycation structures responsible for ligand binding to the different AGE receptors will help to further decipher the influence of AGEs on DC function and the T-cell immunogenicity of food allergens.

In addition, we found that AGE-OVA could enhance production of both TH1- and TH2-type cytokines by allergen-specific CD4⁺ T cells (see Fig E2). The AGEs would enhance the activation and subsequent cytokine production of allergen-specific CD4⁺ T cells but not induce polarization of cytokine production by the T cells. Recent findings suggest that regulatory T cells producing IL-10 consistently represent the dominant T-cell subset specific for food allergens in healthy subjects; in contrast, there is a high frequency of allergen-specific IL-4-producing T cells in allergic subjects.^{39,40} The glycosylated food allergens might enhance activation of allergen-specific regulatory T cells for maintaining tolerance against the allergens in healthy subjects but might promote activation of allergen-specific TH2 cells inducing allergic responses in allergic subjects.

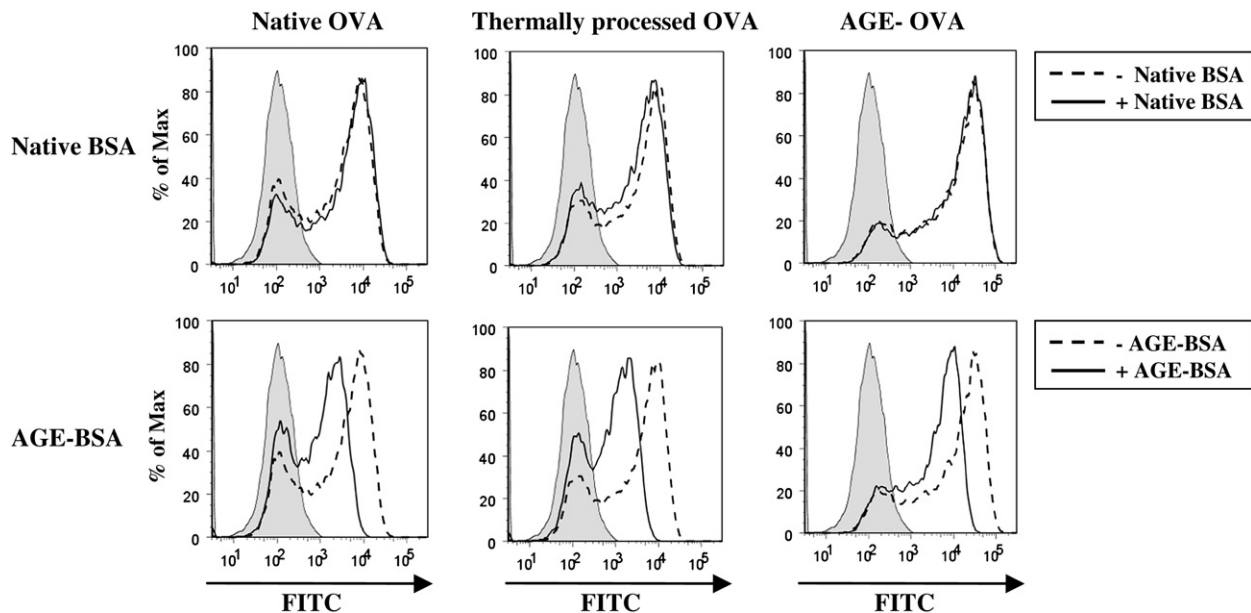


FIG 6. AGE-BSA, but not native BSA, inhibits the uptake of AGE-OVA by mDCs. mDCs were incubated with or without 50 μ g/mL native BSA and AGE-BSA together with 5.0 μ g/mL FITC-conjugated, native OVA, OVA thermally processed without glucose or AGE-OVA. After incubation for 15 minutes, the mDC uptake of either form of OVA was analyzed by means of flow cytometry. Gray areas represent mDCs cultured with cell culture medium only. The data are representative of 3 independent experiments.

In conclusion, we show that the Maillard reaction enhances the CD4⁺ T-cell immunogenicity of the OVA food allergen. It appears that AGEs of food allergens can be endocytosed by mDCs through SR-AI/II, enabling the subsequent presentation of the allergen to CD4⁺ T cells. We also show that IL-4 production by allergen-specific CD4⁺ T cells is enhanced by glycation of a food allergen. Because IL-4 produced by CD4⁺ T cells is a critical component for IgE production by B cells and subsequent allergic responses, our results suggest that the Maillard reaction could be capable of enhancing the allergenicity of food allergens in allergic subjects. Future *in vivo* studies on the T-cell immunogenicity and allergenicity of AGEs will further elucidate the importance of the Maillard reaction in food allergy.

We thank Dr Takuhito Shoji (Osaka Medical College, Osaka, Japan) for providing RAGE-deficient mice; Doreen Werchau, Laura Sandner, and Annette Jamin (Paul-Ehrlich-Institut) for technical assistance; and Stefan Schülke for preparation of recombinant protein. We also thank Professor Av Mitchison (University College London, London, United Kingdom) and Dr Stephan Steckelbroeck (Paul-Ehrlich-Institut) for helpful comments on this study.

Key messages

- The T-cell immunogenicity of food allergens can be enhanced by the Maillard reaction, indicating a critical role for thermal food processing in modulating or enhancing the allergenicity of food proteins.

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METHODS

Mice

C57BL/6 J mice and BALB/c mice were purchased from Jackson Laboratories. OT-I mice expressing a T-cell receptor specific for the peptide OVA_{257–264} were kindly provided by Professor H. Schild (Johannes-Gutenberg-University, Mainz, Germany). DO11.10 mice expressing a T-cell receptor specific for the peptide OVA_{323–339} were purchased from Jackson Laboratories.

Verification of glycation structures in AGE-OVA

The glycation of AGE-OVA was verified by using ELISAs with different available AGE antibodies, as described previously.^{E1–E3} Briefly, microtiter plates (MaxiSorp F96; Nunc, Langensfeld, Germany) were coated with the sample proteins in sodium carbonate buffer (pH 9.6) at 4°C overnight. After blocking with 2% BSA in PBS, AGE structures were detected by incubating the ELISA plates with murine mAb against CEL, pyrralin, or GA-pyridine^{E1E2} or rabbit polyclonal antibodies against CML.^{E3} Subsequently, the plates were incubated with horseradish peroxidase conjugates of either anti-mouse IgG (GE Healthcare, Munich, Germany) or anti-rabbit IgG antibodies (Sigma-Aldrich). The substrate used for the peroxidase was 3, 3', 5, 5'-tetramethylbenzidine (BD Bioscience). The concentration of the glycation structures in AGE-OVA was measured by using reverse-phase HPLC after acid hydrolysis.

Analysis of protein structure of OVAs

SDS-PAGE was performed under reducing conditions in a Mini Protean cell (Bio-Rad, Munich, Germany). The total acrylamide content of the gels was 12.5% (wt/vol), and the cross-linker concentration was 2.7% (wt/wt). The sample load on the gel was 1.0 µg of protein per analytic slot. Protein bands were visualized by means of Coomassie Brilliant Blue R250 staining.

The secondary structure of AGE-OVA, native OVA, and thermally processed OVA without glucose was analyzed by using circular dichroism spectroscopy. The OVAs were dialyzed against 10 mmol/L KH₂PO₄/K₂HPO₄ buffer (pH 7.4), and protein concentrations were adjusted to 5.2 µmol/L. Circular dichroism spectroscopy was performed on a J-810S spectropolarimeter (Jasco, Easton, Md) with constant nitrogen flushing at 20°C.

Preparation of recombinant OVA

Amplified cDNA of OVA was purified from agarose gels by using a gel extraction kit (Qiagen, Hilden, Germany), ligated into a pET15b plasmid (Novagen, Darmstadt, Germany), and transformed into *Escherichia coli* BL 21 Star DE3 (Invitrogen). The cells were grown at 37°C in LB medium containing 50 µg/mL carbenicillin to a cell density of 0.5 at a wavelength of 600 nm. Expression was induced with 0.75 mmol/L isopropyl-D-thiogalactopyranoside. The cell pellet was dissolved in 50 mmol/L phosphate buffer containing 0.5 mol/L NaCl and 0.5 mg/mL lysozyme and physically lysed by means of sonication and 2 freeze-thaw cycles. After centrifugation, recombinant OVA was purified by using Ni-chelate affinity chromatography, and size exclusion chromatography was performed.

Fluorescence conjugation of OVAs

AGE-OVA, recombinant OVA, native OVA, and thermally processed OVA without glucose were conjugated with FITC by using a FlouroTag FITC conjugation kit (Sigma-Aldrich, Steinheim, Germany), according to the manufacturer's instructions. Briefly, FITC was added to 10 mg/mL protein, and the mixture was incubated for 2 hours at room temperature. After the reaction, unconjugated dye was removed with a size exclusion column. The absorption of the conjugated samples was measured at 280 nm and 495 nm, and the fluorescence/protein molar ratio was calculated. Additionally, the degree of FITC conjugation was verified by using ELISA with an mAb against FITC (Millipore, Schwalbach, Germany).

Generation of bone marrow–derived murine DCs

Bone marrow cells were flushed from the femurs and tibias of mice with RPMI 1640 medium (Invitrogen, Karlsruhe, Germany). After lysis of red blood cells with 0.15 N ammonium chloride, the cells were seeded at 1×10^6 cells/mL in RPMI 1640 supplemented with 10% FCS, 1 mmol/L sodium pyruvate, 10 mmol/L HEPES, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.1 mmol/L 2-mercaptoethanol, and 100 ng/mL recombinant GM-CSF (R&D Systems, Wiesbaden-Nordenstadt, Germany) and cultured for 8 days to generate bone marrow–derived mDCs. In the cultures more than 80% of the cells were CD11c⁺ and CD11b⁺ mDCs.

Assessment of mDC maturation

mDCs (1×10^6 cells/mL) were stimulated with 10 µg/mL LPS or 20 µg/mL of either form of OVA for 18 hours. The levels of IL-10 (eBioscience) and IL-12 p70 (BD Bioscience) in the culture supernatant were measured by means of ELISA to assess mDC maturation. In parallel, mDCs were collected and stained with FITC-conjugated anti-mouse CD40, CD80, CD86, or MHC class II molecule mAbs (eBioscience). Additionally, the cells were stained with phycoerythrin-conjugated anti-mouse CD11b and allophycocyanin-conjugated anti-mouse CD11c mAbs to gate the mDC population. FITC intensity of CD11b⁺CD11c⁺ cells was measured by means of flow cytometry with an LSR II (BD Bioscience). Data were analyzed with FlowJo version 7 software (Treestar, Inc, Ashland, Ore).

Assessment of cytokine production by CD4⁺T cells

Splenic CD4⁺ T cells were isolated from DO11.10 mice by using an isolation kit from Miltenyi Biotec. The T cells (8.0×10^5 cells/mL) were cocultured with mDCs (1.6×10^5 cells/mL) and stimulated with either form of OVA for 72 hours. After coculturing, the concentrations of IFN-γ and IL-4 in the supernatants were measured by using ELISA (eBioscience).

Assessment of CD8⁺T-cell activation

Splenic CD8⁺ T cells were isolated from OT-I mice with an isolation kit from Miltenyi Biotec. CD8⁺ T cells (8.0×10^5 cells/mL) were cocultured with mDCs (1.6×10^5 cells/mL) and stimulated with either form of OVA. The concentration of IL-2 in the supernatants was measured by means of ELISA.

Detection of receptors expressed on mDCs

To detect SR-AI, galectin-3, and RAGE expressed on mDCs, the cells were stained with rat anti-mouse SR-AI mAb (clone 268318, R&D Systems), rat anti-mouse galectin-3 mAb (clone M3/38, eBioscience), and rabbit anti-mouse RAGE polyclonal antibodies (Abcam plc, Cambridge, United Kingdom), followed by Alexa Fluor 488 goat anti-rat IgG (H + L) polyclonal antibodies (Invitrogen) and Cy3 goat anti-rabbit IgG (Fc fragment) polyclonal antibodies (Jackson ImmunoResearch Europe Ltd, Suffolk, United Kingdom), respectively. Fluorescence intensity of the stained cells was measured by means of flow cytometry.

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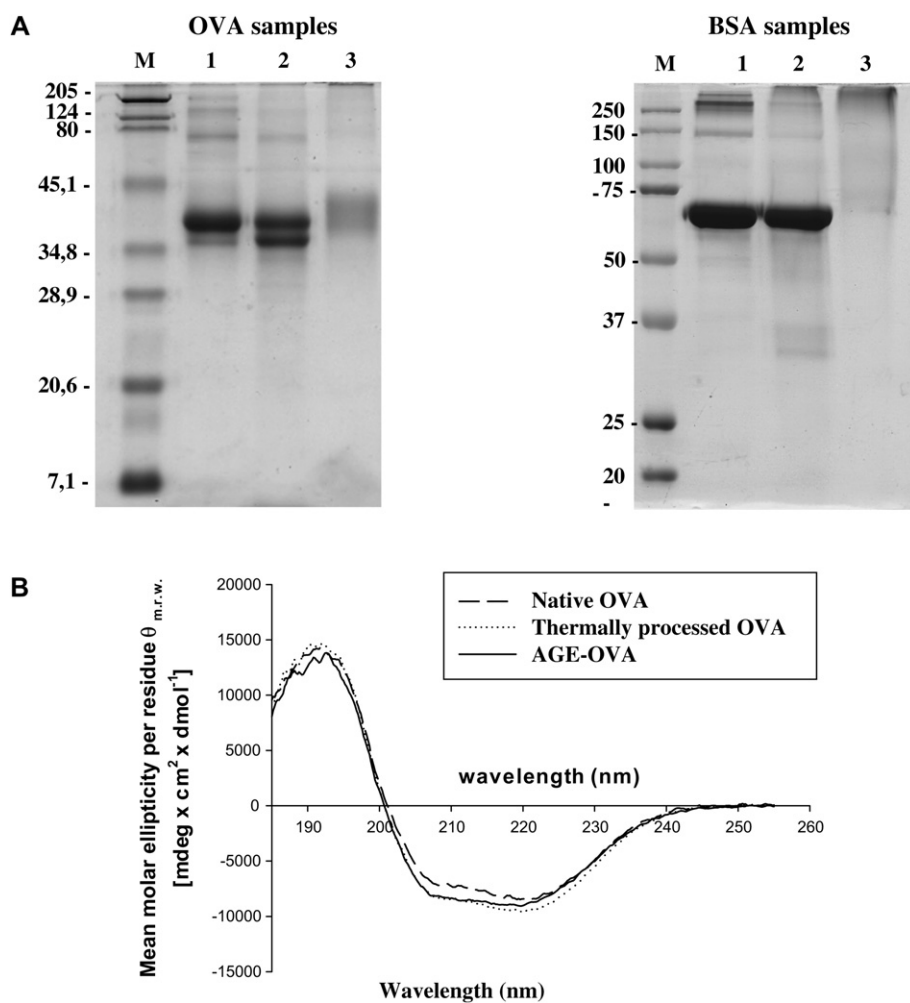


FIG E1. Structural analysis of glycosylated proteins. **A**, SDS-PAGE profiles of OVA and BSA before and after thermal processing at 50°C for 6 weeks with or without glucose. *Lane 1*, Native protein; *lane 2*, protein thermally processed without glucose; *lane 3*, AGE product. **B**, The secondary structure of the OVAs was analyzed using circular dichroism spectroscopy.

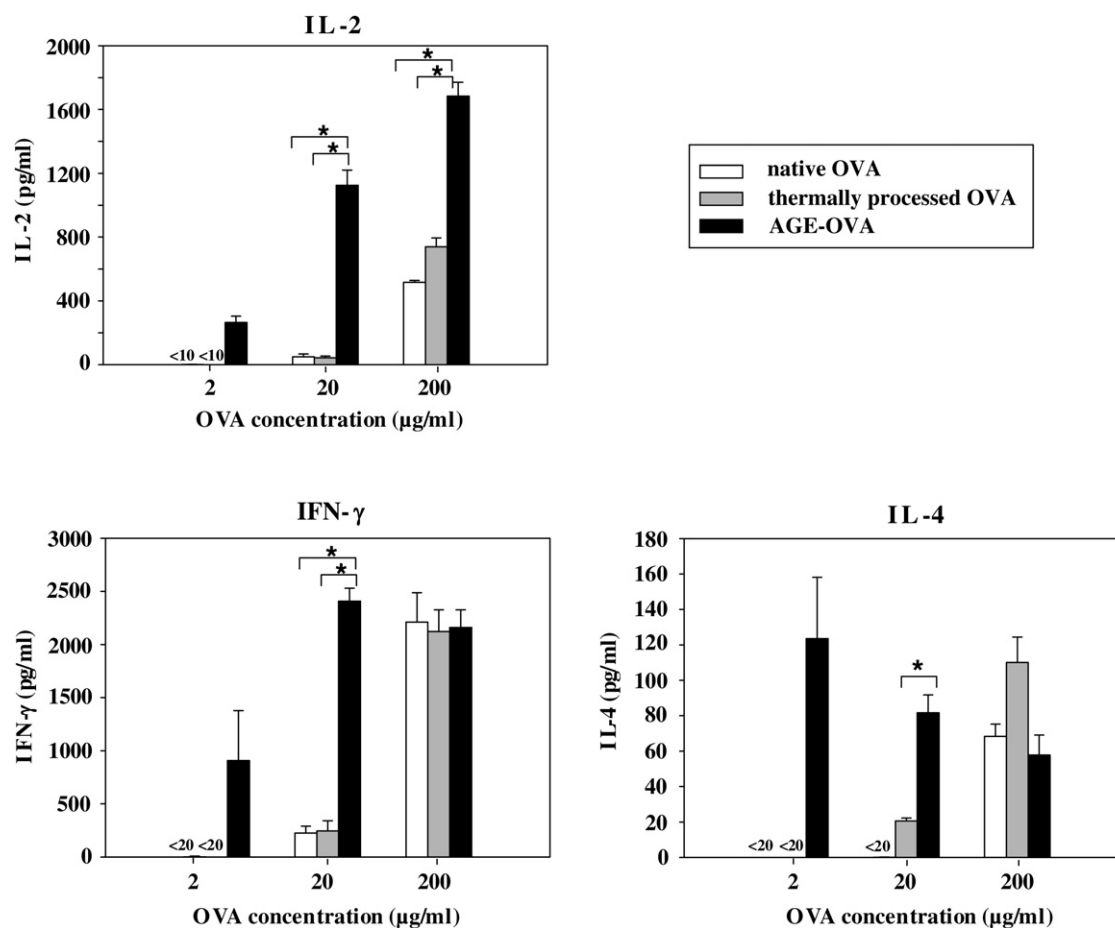


FIG E2. AGE-OVA enhances cytokine- γ production of OVA-specific CD4⁺ T cells. CD4⁺ T cells isolated from DO11.10 mice were cocultured with mDCs and stimulated with AGE-OVA, native OVA, or OVA thermally processed without glucose for 72 hours. The concentration of IL-2, IL-4 and IFN- γ in the culture supernatant was measured by means of ELISA. * $P < .001$. The data are representative of 2 separate experiments.

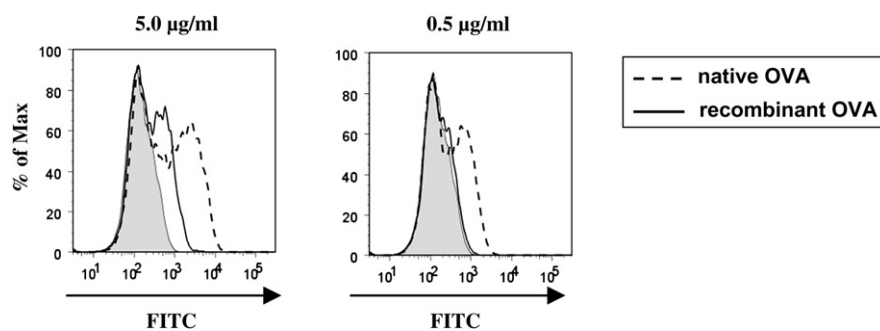


FIG E3. The uptake of native OVA by mDCs is higher than that of recombinant OVA. Wild-type mDCs were incubated with 0.5 or 5.0 µg/mL FITC-conjugated native OVA or recombinant OVA for 15 minutes. The uptake of OVAs by the mDCs was analyzed by means of flow cytometry. *Gray areas* represent cells cultured with medium only. The data are representative of 2 separate experiments.

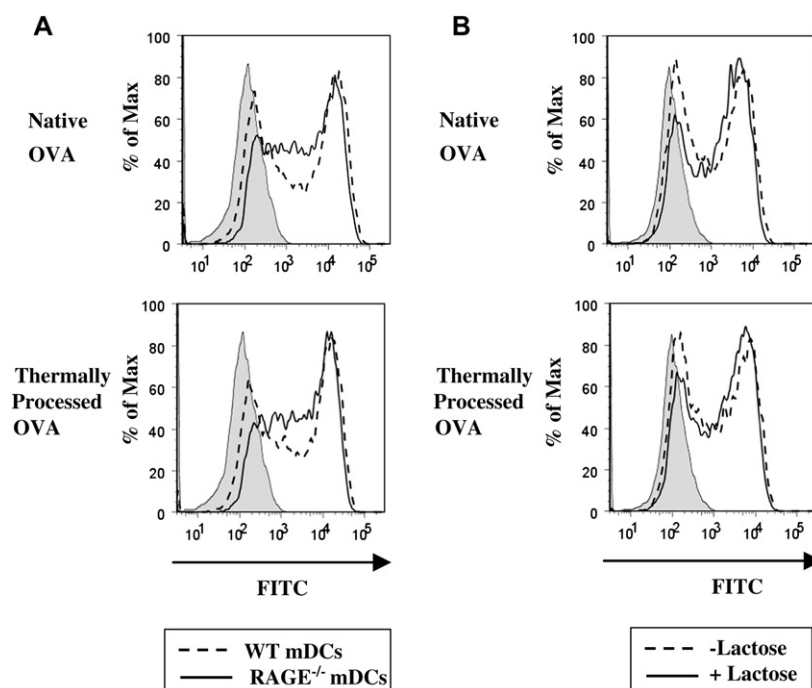


FIG E4. The uptake of native OVA and OVA thermally processed without glucose is not attenuated by the absence of RAGE or blockade of galectin-3. **A**, Wild-type or RAGE-deficient mDCs were incubated with 5.0 μ g/mL FITC-conjugated native OVA or thermally processed OVA without glucose for 15 minutes. **B**, Eild-type mDCs were treated with or without 150 mmol/L lactose for 30 minutes before incubation with the FITC-conjugated OVAs to inhibit galectin-3-mediated uptake. The uptake of OVAs by the mDCs was analyzed by means of flow cytometry. Gray areas represent cells cultured with medium only. The data are representative of 3 separate experiments.

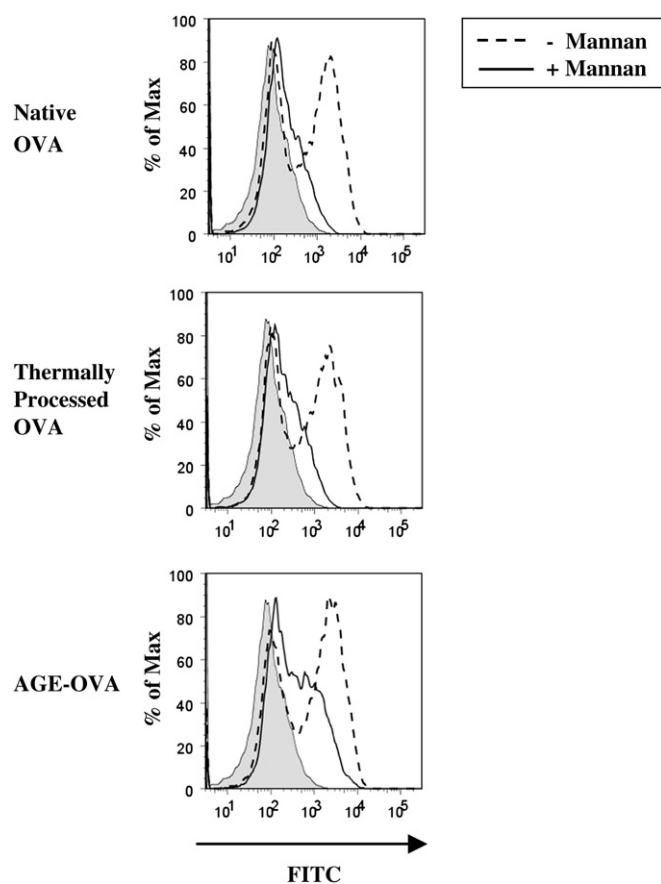


FIG E5. Mannan inhibits the uptake of AGE-OVA in SR-AI/II-deficient mDCs. SR-AI/II-deficient mDCs were incubated with or without 3 mg/mL mannann for 30 minutes before incubation with 5.0 μ g/mL FITC-conjugated native OVA, OVA thermally processed without glucose, or AGE-OVA to inhibit the MR-mediated uptake. The uptake of OVA by the mDCs was measured by means of flow cytometry. Gray areas represent cells cultured with medium only. The data are representative of 3 separate experiments.

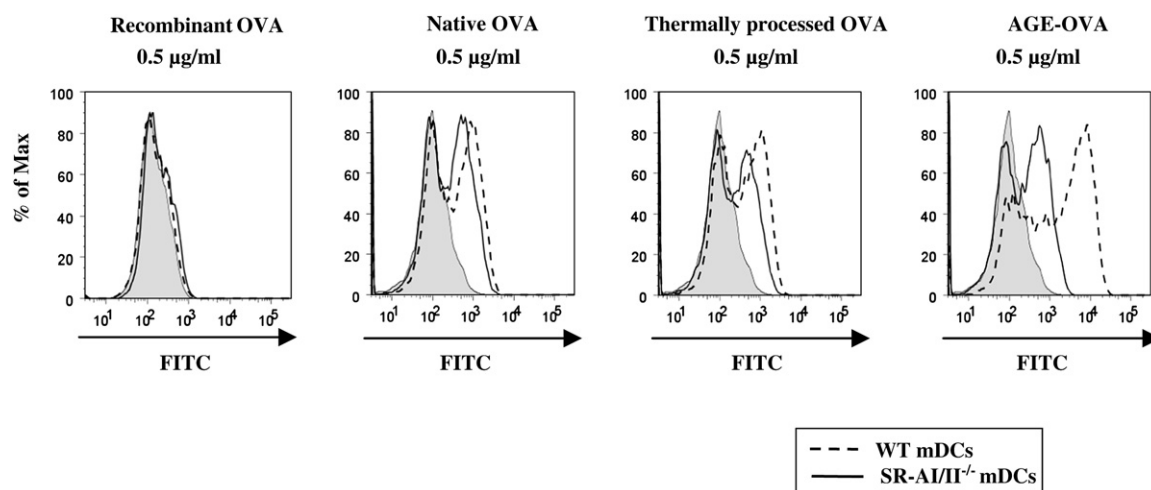


FIG E6. SR-AI/II is involved in the uptake of AGE-OVA by mDCs. **A.** Wild-type or SR-AI/II-deficient mDCs were incubated with 0.5 μ g/mL FITC-conjugated recombinant OVA, native OVA, OVA thermally processed without glucose, or AGE-OVA for 15 minutes. The mDC uptake of the samples was analyzed by means of flow cytometry. Gray areas represent mDCs cultured with medium only. The data are representative of 3 separate experiments.

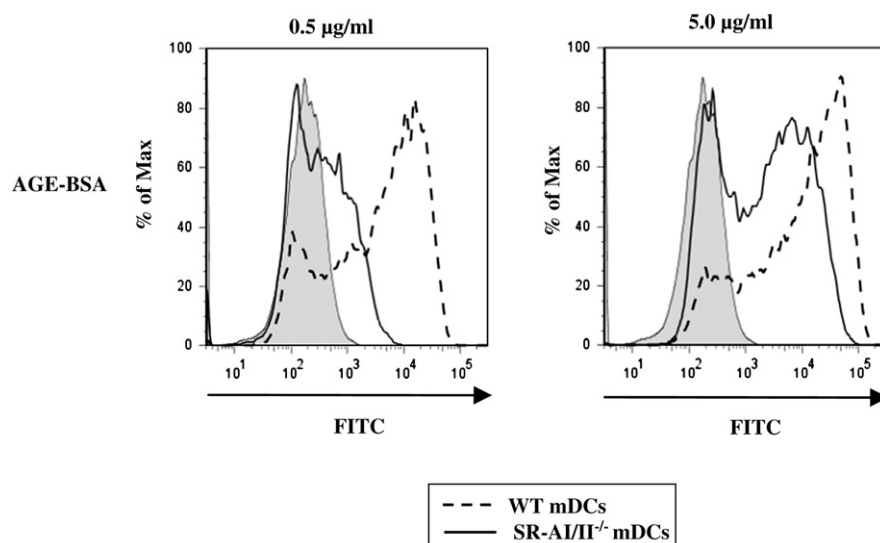


FIG E7. Uptake of AGE-BSA is attenuated in SR-AI/II-deficient mDCs. Wild-type or SR-AI/II-deficient mDCs were incubated with 0.5 or 5.0 µg/mL FITC-conjugated AGE-BSA for 15 minutes. The uptake of AGE-BSA by the mDCs was measured by means of flow cytometry. Gray areas represent cells cultured with medium only. The data are representative of 3 separate experiments.

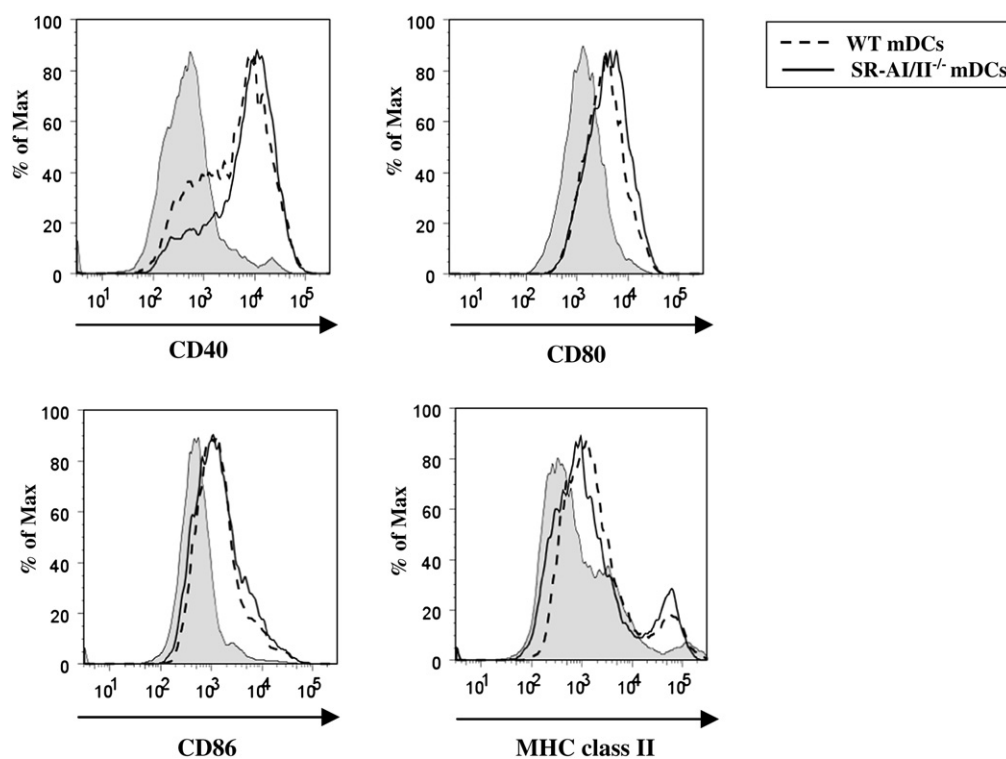


FIG E8. SR-AI/II deficiency does not affect expression of costimulatory molecules and the uptake ability of mDCs. Wild-type or SR-AI/II-deficient mDCs were incubated with 10 mg/mL LPS for 18 hours. Expression of CD40, CD80, CD86, and MHC class II molecules on the mDCs was analyzed by means of flow cytometry. Gray areas represent mDCs cultured without LPS stimulation. The data are representative of 3 separate experiments.

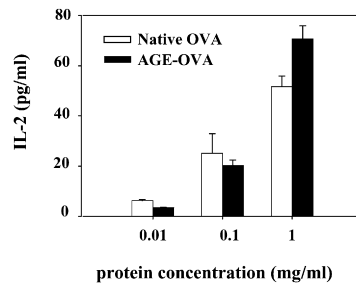


FIG E9. AGE-OVA and native OVA induce comparable activation of OVA-specific CD8⁺ T cells. CD8⁺ T cells isolated from OT-I mice were cocultured with mDCs and stimulated with native OVA and AGE-OVA for 24 hours. The concentration of IL-2 in the culture supernatant was measured by means of ELISA. The data are representative of 3 separate experiments.

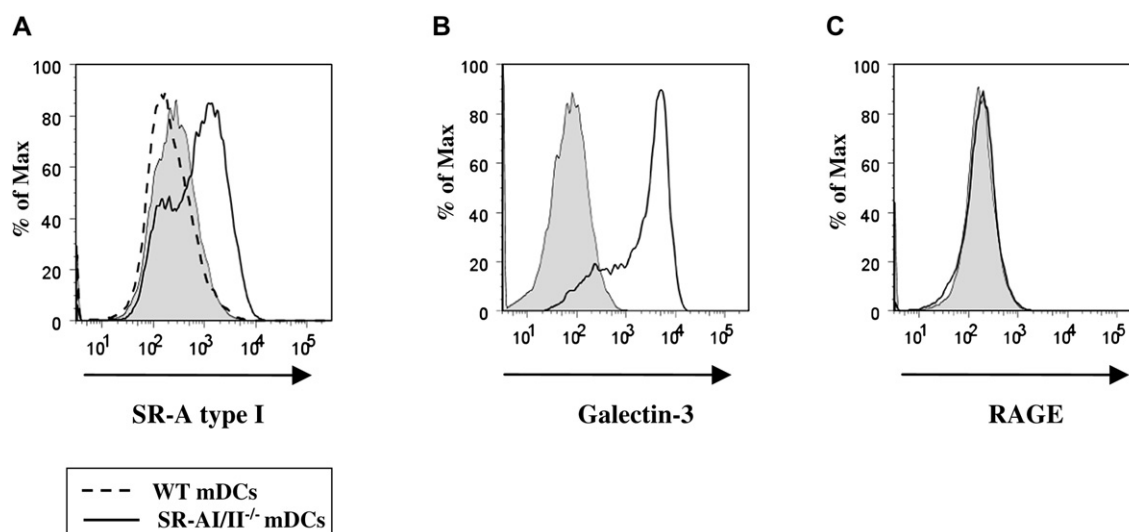


FIG E10. Expression of receptors for AGEs on mDCs. SR-AI, galectin-3, and RAGE expression on the surface of mDCs was analyzed by means of flow cytometry. **A**, SR-AI expression on wild-type and SR-AI/II-deficient mDCs. **B**, Galectin-3 expression on wild-type mDCs. **C**, RAGE expression on wild-type mDCs. Gray areas represent mDCs stained with the isotype control for the respective antibody.