The influence of the Maillard reaction on the immunogenic properties of food allergens

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Meinen Eltern

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1 Introduction

1.1 Introduction to Allergy

The term allergy was introduced by Clemens von Pirquet in order to differentiate between protective immunity and hypersensitive reactions (von Pirquet, 1906). The World Allergy Organisation now defines allergy as a "hypersensitive reaction initiated by specific immunologic mechanisms" (Johansson et al., 2004). Allergic reactions result from an inappropriate reaction of the immune system to a normally harmless, non-infectious substance, called allergen. Sources of allergens may be grass or tree pollen, animal epithial cells or feathers, house dust mites, drugs, insect venom or food.

The pathologic mechanisms of allergies have been divided into four types (Coombs and Gel 1963). Types I, II and III are antibody mediated and discerned by the antibodies and antigens involved. Type I responses, immediate hypersensitivity reactions, are mediated by IgE antibodies. Type II and III are both mediated by IgG antibodies but type II responses are directed against cell surface or matrix antigens while type III responses are directed against soluble antigens. Type IV is antibody independent and is mediated by T helper cells. In the focus of this thesis is food allergy which is frequently associated with the IgE mediated type I immediate hypersensitivity reaction. Therefore only this mechanism will be described in more detail.

1.2 Mechanism of Type I Allergy

The Type I allergy may generally be divided into two phases, a sensitisation phase and an elicitation phase. In the sensitisation phase the production of allergen specific IgE antibodies is initiated but no symptoms are yet induced. In the elicitation phase repeated contact with the allergen elicits allergic reactions.

During the sensitisation phase allergens enter the body by passing the mucosa, usually in small amounts. The allergens are taken up by antigen presenting cells (APC) such as dendritic cells (DCs) and macrophages. These cells process the allergen by proteolysis and peptide fragments of the allergen are loaded onto MHC class II molecules (MHC II) (chapter 1.10.3). The complex of the allergen peptide and MHC II is presented on the surface of the APCs. $CD4^+$ T cells recognise the complex by a T cell receptor (TCR). The binding of the TCR to the peptide-MHC II complex, in conjunction with further co-stimulatory signals, results in the activation and differentiation of the T cells (chapter 1.12.1). Naïve $CD4^+$ T cells are known as Th0 cells and can differentiate into four subsets; T helper 1 (Th1) cells, T helper 2 (Th2) cells, T helper 17 cells (Th17) and regulatory T cells. In the onset and maintenance of allergic diseases Th2 cells play a critical role (chapter 1.13.1.2). The cytokines IL-4 and IL-13, which are typically secreted by Th2 cells, induce the differentiation of B cells to IgE producing plasma cells (Poo et al., 1988). In contrast to other antibodies which generally bind to Fc receptors only after they have bound to specific antigens,

IgE antibodies are captured by high affinity Fcε receptors (FcεRI) in the absence of bound antigen. Mast cells and basophils express FcεRI, therefore IgE antibodies are bound to the surface of these cells (Murphy et al., 2008).

If the same allergen is reencountered after the sensitisation phase, an allergic reaction with clinical symptoms occurs, this is the elicitation phase. The elicitation phase is divided into an early phase and a late phase reaction. Crosslinking of the specific IgE antibodies by allergens induces the release of inflammatory mediators such as histamine from these cells which induce allergic symptoms such as diarrhoea, conjunctivitis and anaphylaxis. This process is the early phase reaction. In order for cross linking of the IgE antibodies captured on the cell surface to occur, the allergen needs to posses at least two different epitopes recognised by the antibodies.

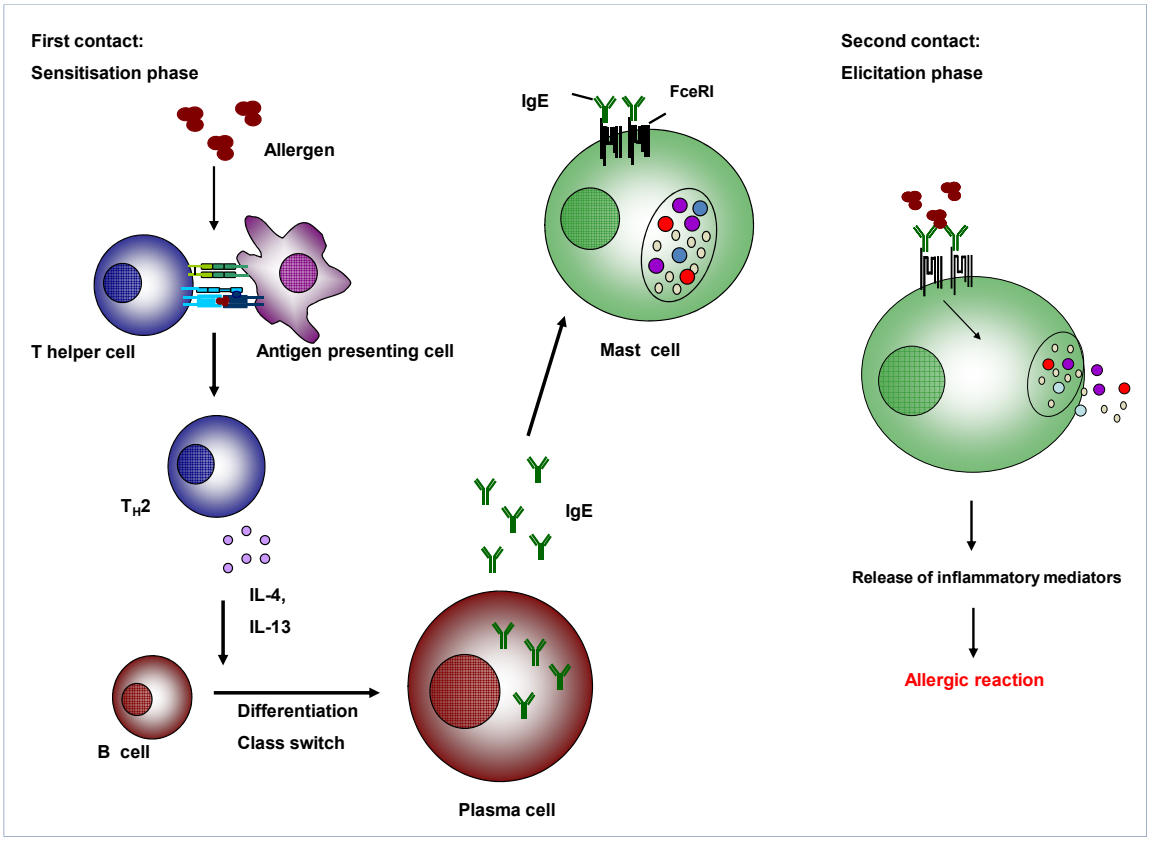

Fig. 1.1 Overview of the mechanism of type I allergy

After the first immediate allergic reaction, the late phase reaction may follow which takes up to 8 to 48 hours to develop. In the late phase reaction, mast cells and basophils are also pivotal players as they are the major source of various cytokines and chemokines. These mediators drive the Th2 immune responses and initiate the influx of leukocytes, for example eosinophils, to the inflammatory sites. Eosinophils produce inflammatory mediators such as leukotrienes, which cause vasodilatation, mucosal swelling and smooth muscle contraction. Symptoms associated with the late phase reaction is a second phase of smooth muscle contraction, edema and swelling due to hypertrophy (increase in cell size) and hyperplasia (increase in cell proliferation) (Murphy

et al., 2008). The late phase reaction occurs in roughly 50% of the patients with an immediate response (Meltzer 1997).

1.2.1 Allergens

In order for a molecule to be defined as an allergen it must display specific properties. It must be able to bind to IgE in order to elicit the clinical symptoms associated with allergy. The IgE antibodies are generally specific for the particular allergen, however cross reactions can occur. Such cross reactions are commonly observed in patients sensitised to the birch pollen allergen Bet v 1. Homologues of this protein are found in fruits and vegetables such as cherries or carrots. These elicit allergic reactions by binding to Bet v 1 specific IgE due to cross reactions. IgE sensitisation to N-glycans (cross-reactive carbohydrate determinants), as they are found on some plant glycoproteins, can also be a cause of cross reactivity.

According to the prevalence of the IgE sensitisation in patients, allergens are divided into major and minor allergens. In order to be classified as a major allergen, the allergen has to be recognised by the serum IgE from more than 50% of the patients in a group of at least 5 allergic patients. From 5-50% prevalence, the allergen is considered minor (Larsen et al., 1996). The binding of allergens by IgE antibodies is dependent on the identification of so called B cell epitopes; these can either be linear or conformational. Linear epitopes consist of a continuous sequence of amino acids while conformational epitopes consist of amino acid sequences which belong to different sections of a protein but have come to lie within close proximity to each other due to the three dimensional structure of the protein. T helper cells recognise allergens only after they have been processed by antigen presenting cells (APC) and are presented as MHC-peptide complexes (chapters 1.9.2 and 1.10). Epitopes recognised by T cells are always linear.

1.3 Food Allergy

For the prevalence of food allergy, only estimated figures exist. Studies claim that 3-7% of the western population suffer from food allergies, some studies even present figures of up to 17% (Rona et al., 2007). Furthermore, the prevalence of food allergy in the western countries has experienced a significant increase in the recent decades (Sicherer 2002; Sampson 2003). Some common allergies include egg (0.5 - 9%), milk (0.2 – 9%), shell fish (2.5%) and peanut (0,5-6%) (Rona et al., 2007). The heterogeneity in the prevalence of food allegy in general and allergies to a specific food stated in different studies is likely to be the result of the different populations being studied and differences in study design or methodology.

The symptoms of food allergy can range from itchiness of the oral cavity and gastrointestinal disorders such as diarrhoea to severe reactions such as dyspnoea or an anaphylactic shock

which can be fatal. Seemingly unrelated symptoms such as itchiness of eyes and rhinitis or contact dermatitis may also be seen in response to aerosolised food particles or handling food.

1.4 Influence of thermal processing on food allergens

Heating makes food safe to eat by inactivating harmful bacteria or toxins. It also enhances the texture, flavour and colour. The thermal processing of foods may decrease or enhance the allergenicity of food allergens as a result of a heat induced change of protein conformation and chemical modifications. The influence of thermal processing on the allergenicity of an allergen depends on temperature, pH, time and on the cooking method. The surrounding food matrix would also have a significant impact. So far, there are no universal rules predicting the consequences of thermal processing on the allergenicity of proteins. A decrease of allergenicity could be the result of the destruction of conformational epitopes by the denaturation of the protein, leaving only linear epitopes available for antibody binding.

Thermal treatment is as likely to not affect or even to enhance the allergenicity of a food protein as it is to reduce it, as has been shown in a considerable number of studies. One of the major allergens of egg, ovomucuid, retains its immunogenicity after being heated to 100 °C (Hirose, 2004). The cherry allergen Pru v 3 remains IgE reactive in thermally processed foods (Scheurer et al., 2004). Allergenicity may increase due to an increased resistance to digestive enzymes, newly available IgE binding sites or better accessibility of these sites due to unfolding of the protein or chemical modification. The latter two can lead to the formation of neoallergens. Neoallergen formation is very likely to play a role in cases where patients react to processed but not to raw food. Interestingly, the first reported case of allergy was a report of a patient who reacted to proteins in cooked but not in raw fish (Prausnitz and Kustner, 1921). A variety of similar cases have been published since then. For instance, it has been reported that a patient with pecan nut allergy had antibodies exclusively to allergenic determinants found in pecan nuts only after longer storage or heating (Malanin, 1995). Another case described a patient with allergic reactions following the consumption of cooked shrimp but not raw shrimp (Rosen et al., 1994). These findings suggest the development of neoallergen formation under the influence of heat.

Protein-sugar interactions occur during thermal processing; these result in the non-enzymatic browning of food. The chemical modifications resulting from this interaction contribute to neoallergen formation. The reaction describing this process is the Maillard reaction and it has been found that the reaction products, in particular the end products of this multistep reaction, may severely influence the immunologic properties of food allergens.

1.5 The Maillard reaction

The Maillard reaction is a major reaction occurring during the thermal processing and storage of food. In 1912 Louis C. Maillard (Maillard 1912) first described systemic studies about the reaction indicating that amino acids and reducing sugars undergo complex reactions during heating which lead to the formation of brown substances. The “Maillard reaction” is a series of subsequent and parallel reactions which can be divided into three stages.

In the early stage, condensation takes place between an amino group of a protein and a carbonyl group of a reducing carbohydrate leading to the formation of a Schiff base. The condensation is followed by an intramolecular rearrangement of the Schiff base, resulting in the first stable reaction product, an aminoketose. The different aminoketoses which can be produced during the first stage of the reaction are collectively called Amadori products (Fig. 1.1). The ε-amino group of lysine represents the primary target for an attack by carbohydrates. Up to 70% of the lysine residues of a protein can react to Amadori products, depending on the time and temperature during heating or storage (Finot et al., 1981). Amadori products are fairly stable in foods with low water activity but during severe heating or prolonged storage they can undergo degradation, this marks the second stage of the Maillard reaction. The degradation reaction of the Amadori products results in the formation of 1,2-dicarbonyls. These are prone for reactions with proteins; this reactivity allows the final stage of the Maillard reaction to occur.

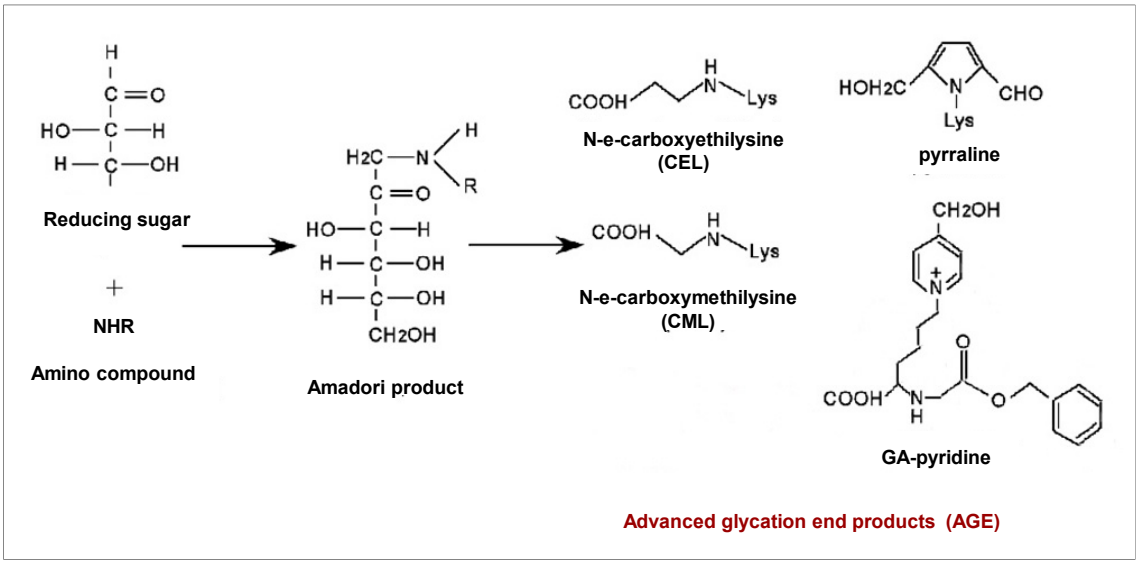

Fig. 1.2 Main steps of the Maillard reaction

The 1,2-dicarbonyls react with further lysine side chains, ones which have not already been modified in the first stage of the reaction, or arginine of the protein to form stable peptide bound amino acid derivatives (Henle, 2005). N-ε-cyrboxymethyllysine (CML), N-ε-cyrboxyethyllysine (CEL), pyrraline, Glycolaldehyde-pyridine (GA-pyridine) and pentosidine are representative structures of amino acid derivatives formed during the final stage of the reaction (Fig. 1.1). Some of the “end products” of the Maillard reaction, namely peptides and proteins modified with these

structures after the reaction are collectively called advanced glycation end products (AGEs). N-ε-carboxymethyllysine (CML) was the first amino-acid derivative of the Maillard reaction detected in food (Hartkopf and Ebersdobler, 1994). A pyrrole derivate of lysine, pyrraline, was quantified in several foods like milk or bakery products (Henle and Klostermeyer 1993; Rufian-Henares et al. 2004). To distinguish the reaction mechanisms of the Maillard reaction from enzymatic glycolsylation the term glycation was introduced (Ledl and Schleicher, 1990).

1.5.1 The Maillard reaction in food allergy

The Maillard reaction may increase the allergenicity of food allergens in thermally processed foods. The glycation structures present on proteins modified by the Maillard reaction could contribute to the formation of new epitopes. When native Ara h 1 and Ara h 2 purified from peanuts (of which they are the major allergens) or recombinant Ara h 2 were subjected to the Maillard reaction by heating with carbohydrates or carbohydrate break down products, the binding of these allergens to serum IgE from peanut allergic patients was increased (Maleki 2001, Gruber 2005). Chung et al showed that the amount of AGE adducts is twofold higher in roasted than in raw peanuts (Chung et al., 2001). A further analysis then showed that mature peanuts, which contain more sugar and protein than immature peanuts, have 20% higher levels of AGE adducts after roasting than identically processed immature peanuts. This increased level of AGE correlated with enhanced binding of IgE to the peanut extract (Chung et al., 2003). Scallop tropmyosin was also found to have increased IgE binding ability after modification by the Maillard reaction (Nakamura et al., 2005).

Another interesting study suggested the generation of neoallergens by the Maillard reaction. The nonallergic protein lectin from peanuts was subjected to modifications by the Maillard reaction. While lectin showed little or no reactivity to serum IgE antibodies from patients with peanut anaphylaxis, the AGE-lectin did bind to the antibodies. Furthermore, AGE-lectin was able to inhibit serum IgE binding to peanut allergens (Chung and Champagne, 1999). IgE is the immunoglobulin isotype involved in the induction of allergic responses (chapter 1.13). The observations suggest that the glycation by the Maillard reaction could enhance the allergenicity of food allergens.

1.5.2 Clinical relevance of the Maillard reaction

Rhabar published in 1968 that the glycosylated part of haemoglobin, HbAIc, was increased in patients with diabetes (Rhabar, 1968). HbAIc is glycated by the binding of glucose to a valine residue in the form of an Amadori rearrangement (Koenig et al., 1977). These studies showed that the Maillard reaction occurs in the body.

Recent studies have shown that various AGEs of endogenous proteins accumulate in the human body during aging, especially in long lived tissues such as collagen, neural myelins or the lens (Simm et al., 2008). The glycation process is dependent on the glucose concentration and is accelerated in hyperglycaemia (a condition in which an excess amount of glucose circulates in the

blood plasma) (Brownlee, 1992, Yamagishi et al. 2003, Yamagishi and Imaizumi, 2005). There is increasing evidence that the accumulation of endogenous AGEs plays a crucial part in the onset of diabetic complications and neurodegenerative disorders (Simm et al 2008; Takeuchi et a., 2008; Gosh and Cooper 2008). A correlation of acute exacerbation of atopic dermatitis with the urinary levels of the AGE pentosidine has also been observed (Tsukahara et al., 2003).

Interaction of AGEs with the signal-transducing receptor RAGE (receptor for advanced glycation end products, chapter 1.6.2) appears to play a role in the onset and exacerbation of diabetic complications and atopic dermatitis. RAGE mediated signalling induces the secretion of inflammatory cytokines (i.e. IL-1α and tumour necrosis factor-α) and growth factors (i.e. vascular endothelial growth factor and connective tissue growth factor) by epithelial cells, macrophages and monocytes. These cyokines and growth factors induce inflammation and oxidative stress, which could contribute to the pathological changes observed in the diabetic complications and atopic dermatitis (Bucula and Vlassare, 1995; Suzuki and Miyata et al., 1999). These observations suggest that AGEs could have an immunostimulatory effect.

Since the *in vivo* Maillard reaction is exceedingly complex, endogenous AGEs are very heterogeneous. So far, only few AGEs generated under physiological conditions have been characterised, such as CML and pentosidine. It is still not known whether the biological activity of endogenous and food derived AGEs is different or not (Koschinsky 1997).

1.5.3 Influence of AGEs on DC function

Studies examining the influence of AGEs on DCs have given conflicting data. AGEs derived from bovine serum albumin (AGE-BSA) were able to induce the maturation of human monocyte derived DCs and augmented their capacity to stimulate T cell proliferation as well as cytokine secretion (Ge et al., 2005). In contrast, adrenocorticotrophic hormone derived AGEs were shown to inhibit maturation and T cell stimulatory capacity of human DCs (Price et al., 2004). Together these studies suggest that AGEs could have an influence on the maturation of DCs as well as on the T cell immunogenicity of antigens. Maturation and antigen presentation in DCs and subsequent activation of T cells are highly critical processes in the onset and maintenance of allergy. Therefore, the glycation of food allergens by the Maillard reaction could contribute to the pathology of food allergy.

1.6 AGE binding receptors

Since it was found that AGEs contribute to the pathology of a variety of diseases, many researchers have tried to identify receptors which mediate the interaction of cells with AGEs. So far, the following receptors have been shown to bind to AGEs: the receptor for advanced glycation end products (RAGE) (Schmidt et al., 1992; Neeper 1992), Galectin-3 (Vlassara et al., 1995) and several receptors belonging to the heterogeneous scavenger receptor family; scavenger receptor

class A type I and II (SR-AI/II) (Suzuki et al., 1997; Araki et al., 1999), scavenger receptor class B type I (SR-BI) (Ohgami et al., 2001, a) and CD36 (Ohgami et al., 2001, b).

1.6.1 Macrophage scavenger receptors

Macrophage scavenger receptors (SR) were originally defined as receptors which could bind to low density lipoproteins (LDL) modified by acetylation (AcLDL) or oxidation (OxLDL) but not unmodified LDLs (Goldstein et al., 1979). Since then, the family of scavenger receptors has vastly expanded. The family is divided into eight classes (Class A, B, C, D, E, F, G and H) according to their multidomain structure (Krieger, 1997). Due to the definition of the receptors, the SR family consists of unrelated distinct gene products with considerable structural heterogeneity amongst the different classes.

1.6.1.1 Class A Scavenger receptors

The scavenger receptor class A has been identified in several species, including humans and mice (Ashkenas et al., 1993; Matsumoto et al., 1990; Tomokiyo et al., 2002). It is highly conserved among the species and is found in three isoforms, SR-AI, SR-AII (both collectively called SR-AI/II) and SR-AIII, which result from alternative splicing of one gene. SR-AIII is non-functional and remains trapped in the endoplasmatic reticulum (Matsumoto et al., 1990; Rohrer et al., 1990; Gough et al., 1998). Further members of class A are the macrophage receptor with collagenous structure (MARCO) (Elomaa et al., 1995), scavenger receptor class A 5 (SCARA-5)(Jain et al., 2006) and scavenger receptor with a c-type lectin domain (SRCL-I/II)(Nakamura et al., 2001). Those receptors have similar domain structures to SR-AI/II.

1.6.1.1.1 Scavenger receptor class A type I and II

SR-AI/II is a trimer consisting of three 80 kDa type II transmembrane proteins with an intracellular N-terminus. It is made up of the transmembrane domain, a spacer region, a helical coiled coil domain, a collagenous domain and a C-terminal cysteine rich domain. SR-AII has a shorter C-terminus than SR-AI (Fig. 1.2). To this date, only the collagenous domain has been identified as a ligand binding domain. SR-AI/II binds to a wide range of anionic ligands including natural carbohydrates, oxLDL and acLDL and chemically modified proteins such as AGEs. The ligand binding specificity of SR-AI and SR-AII is considered to be similar (Kodama et al., 1995; Gough et al., 1998).

The expression of SR-AI/II is restricted to cells of the myeloid lineage (e.g. DCs and macrophages) with the exception of monocytes (Hughes et al., 1995). SR-AI/II is expressed on 26% of splenic $CD11c^+$ DCs (Becker et al., 2006). Expression of SR-AI/II was also found on bone marrow derived murine DCs generated with granulocyte macrophage colony stimulating factor (GM-CSF) where the expression of SR-AI/II was intrinsically correlated with the expression of CD11c (Becker et al., 2006; Amiel et al., 2007).

SR-AI/II mediates the clearance of LDLs and pathogens (Hughes et al., 1995; Platt and Gordon, 2001) and act as an adhesion molecule for macrophages (Santaiago-Garcia et al., 2003). Furthermore, the receptors appear to play a role in antigen presentation. Previous studies have demonstrated that the conversion of antigens to SR-AI/II ligands increased their T cell immunogenicity. For example, maleylation of mouse serum albumin (Mal-MSA) targets the protein for uptake by the scavenger receptors. Mal-MSA was able to induce T cell activation in mice, while native MSA was not. Additionally, T cell activation by Mal-MSA was dependent on the presence of SR-AI/II (Nicoletti et al., 1999). The results indicate that SR-AI/II play a role in the antigen capture and subsequent antigen presentation by APCs.

Araki et al have shown that Chinese hamster ovary cells expressing SR-AI/II take up and degrade AGE-BSA (Araki et al., 1995). Binding of AGE to SR-AI/II was confirmed in peritoneal macrophages from SR-AI/II knock out mice, where degradation of AGE-BSA was considerably reduced compared to that in WT macrophages (Suzuki et al., 1997). It has been demonstrated that BSA modified with glycoaldehyde, which is an intermediate in the formation of CML, is recognised by SR-AI/II (Nagai et al., 2000). However, the exact ligand structure of AGEs which can bind to SR-AI/II remains unknown.

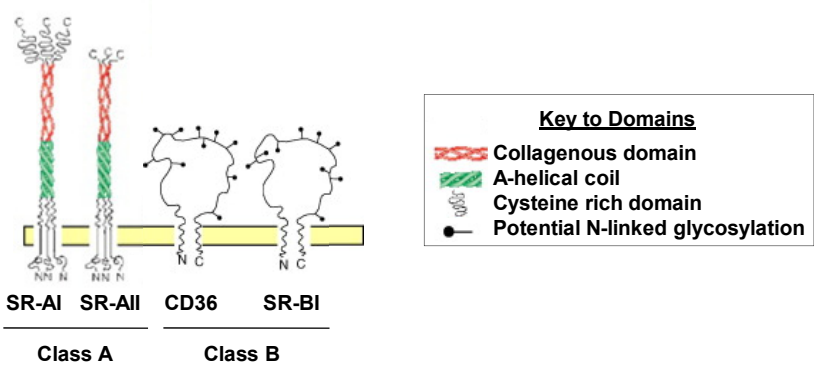

Fig. 1.2 Domain architecture of class A and class B scavenger receptors.
The receptors of one scavenger receptor class are structurally similar but the structures of different classes are very heterogeneous. Figure adopted and modified from Plüddemann et al., 2007.

1.6.1.2 Class B Scavenger receptors

The scavenger receptor class B has two members, CD36 and SR-BI. CD36 is a type II transmembrane receptor consisting of two transmembrane domains, an extracellular loop with glycosylation sites and two short intracellular tails (Greenwalt et al., 1992). The ligand binding site was suggested to be in the central part of the extracellular domain (Fig. 1.2) (Puente et al., 1996). CD36 is expressed on macrophages, platelets, adipocytes and some epithelial and endothelial cells (Terpstra et al., 2000). SR-BI has a similar loop structure as CD36. The receptor exists as two splice variants, SR-BI and SR-BII collectively referred to as SR-BI (Acton et al., 1994). SR-BI

is expressed on monocytes, macrophages and DCs as well as on hepatocytes (Terpstra et al., 2000).
Using Chinese hamster ovary cells expressing CD36, it was found that CD36 mediated the uptake of AGE-BSA which then underwent lysosomal degradation (Ohgami et al., 2001b). SR-BI also mediates the endocytosis of AGE-BSA. AGE-BSA binds to the same sites of SR-BI as AcLDL and OxLDL (Ohgami et al., 2001a).

1.6.2 Receptor for advanced glycation end products (RAGE)

Schmidt and colleagues found that endothelial cells bind to AGE-modified proteins by a lactoferrin like protein (Schmidt et al., 1992). Using molecular cloning, it was shown that this protein was a member of the immunoglobulin superfamily of receptors. The receptor was named the receptor for advanced glycation end products (RAGE) (Neeper et al., 1992). RAGE has an N-terminal extracellular domain, a V-region like and two C-region like domains, one transmembrane domain and an intracellular C terminal domain. The V-region like domain is involved in ligand binding whereas the C-terminus is important for signal transduction (Schmidt and Stern 2000; Neeper et al., 1992; Hofmann et al., 1992; Huttunen et al., 1999).
RAGE expression is observed in various tissues including vascular epithelium, lung, liver and neurons. Endothelial cells, DCs, macrophages, and monocytes are the main cells expressing the receptor (Lin et al., 2009; Cataldegirmen et al., 2005). RAGE contributes to a number of pathological processes in diabetes, atherosclerosis and neurodegenerative disorders (chapter 1.5.2). In addition to cell surface bound RAGE, a soluble secreted form of RAGE has also been identified, so called endogenous secretory RAGE. This form of RAGE results from alternative splicing and appears to protect from AGE-induced injury by capturing AGE ligands (Yonekura et al., 2003). Apart from AGEs, several ligands for this receptor have been identified. One of the ligands is the nuclear protein high-mobility group box 1 (HMGB-1). Stimulation of T cells with HMGB-1 via RAGE was shown to be essential for activation of these cells (Moser et al., 2007).

1.6.3 Galectin-3

Lectins are proteins which can bind to both soluble carbohydrates and carbohydrate moieties which are part of a glycoprotein. They have important functions in the innate and adaptive immune system by sensing glycoconjugates derived from self or pathogens. Galectins are a family of 15 lectins which have the specificity for lactose/galactose in common (Barondes et al., 1995). Galectin-3 was originally described as a cell surface marker for activated macrophages (Ho and Springer, 1982). Later, the receptor was also found on monocytes, eosinophils, natural killer cells, activated T and B cells and DCs (Dumic et al., 2006; Sato and Nieminen, 2004). Galectin-3 interacts with β-galactoside residues of matrix and cell surface proteins via the carbohydrate recognition domain (Pricci et al., 2000). The receptor contributes to a variety of cellular processes, such as phagocytosis by macrophages (Sano et al., 2003), proliferation and

survival of T cells and monocytes (Demetriou et al., 2001; Morgan et al., 2004). It has been shown that Galectin-3 has a high affinity for AGE-BSA (Vlassara 1995).

1.7 Model allergens used in this study

To examine the influence on the immunogenicity and antigenicity of food allergens in this study two model allergens were chosen: chicken ovalbumin (OVA), a major allergen of egg white, and Ara h 2, a major allergen of peanut (*Arachis hypogaea*).

1.7.1 Chicken Ovalbumin, a major allergen of egg white

Egg white allergy is one of the most prevalent allergies in children (Sicherer and Sampson, 2006). Some children become tolerant against egg by the time they reach school age (Wood, 2003). However, the allergy persists in others (Savage et al., 2007) and allergic reactions to egg in those patients are frequently severe. In some cases, onset of egg allergy has also been observed in adults (Nogaard et al., 1992; Escudero et al., 2003). OVA and ovomucoid are the two major allergens of egg white (Mine and Yang, 2008).

OVA is the most abundant protein in avian egg white (Mine and Zhang, 2002). It is a glycoptrotein with a molecular weight of approximately 45 kDa and 386 amino acid residues (Nisbet et al., 1981). The protein contains 3,5% of carbohydrate by weight. There is a single carbohydrate chain attached to the aspargine (Asn) at position 293 which is composed of N-acetyl-glucoseamine (GNac) and mannose (Fig. 1.3). The serine residues at positions 69 and 345 are potential phosphorylation sites (Perlmann, 1952)

Purified OVA is commercially available in large quantities and is cost efficient. It has therefore become a widely used model allergen for immunological analysis. Many immunological tools have been established to study OVA, such as the transgenic mouse strains OT-1, OT-2 and DO11.10, which have T cell receptors specifically recognising OVA peptides. For these reasons, OVA was used as a model allergen in this study.

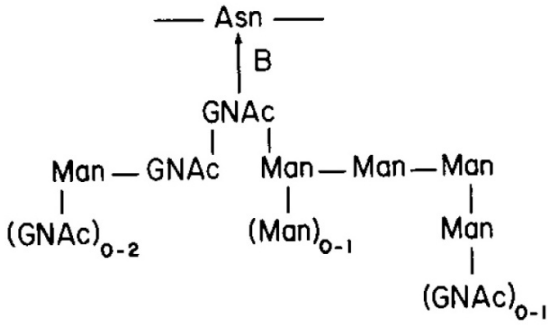

Fig. 1.3 Glycation structures of chicken ovalbumin

The single carbohydrate chain of OVA is attached to Asn293 and is composed of N-acetyl-glucoseamine (GNAc) and mannose (Man). Kiely et al., 1976

1.7.1.1 The Mannose receptor

As OVA has several mannose residues (Fig. 1.3), the allergen binds to the mannose receptor (MR) (Huntington 2001 et al., Burgdorf 2006, Autenrith et al., 2007). The MR is a transmembrane receptor of approximately 180 kDa. It is a c-type lectin. C-type lectins have a highly conserved calcium dependent carbohydrate recognition domain (CRD) of around 120 amino acids. The MR consists of an N-terminal cysteine rich domain, a fibronectin type II repeat domain, eight CRDs, a transmembrane domain and a short intracellular region (Ezekowitz et al., 1990). The CRDs bind to glycoproteins bearing terminal fucose, mannose or glucose, whereas the cysteine rich domain binds sulphated sugar moieties (Largent et al., 1984). The MR is an endocytotic receptor. The receptor also contributes to pathogen defence by the recognition of carbohydrate residues of several microorganisms (McGreal et al., 2005). The MR is expressed by macrophages, DCs, monocytes, dermal microvascular endothelial cells and liver endothelial cells (Harris et al., 1992; Figdor et al.2002).

1.7.2 Ara h 2, a major allergen of peanut

Peanuts (*Arachis hypogaea*) are highly allergenic and cause severe anaphylactic reactions more often than other food known to cause allergic reactions (Yunginger et al., 1989; Sampson et al., 1992). Peanut allergy tends to develop in childhood and often remains throughout life. The prevalence of peanut allergy has risen over the past decade (Burks, 2008). Due to the widespread use of peanut products and the lack of efficient therapies, patients have to accommodate themselves with restrictions on quality of life. Accordingly, peanut allergy is a growing concern in regard to food safety.

Peanuts contain many proteins with allergenic properties. So far, Ara h 1 to 9 have been isolated from peanuts as allergens. Among these, Ara h 1 and Ara h 2 are considered as the major allergens as they are recognised by serum IgE from more than 90% of the patients with peanut allergy (Burks et al., 1992; Burks et al., 1995). The glycoprotein Ara h 2 belongs to the prolamines, a superfamily whose members are frequently found in seeds as storage proteins and amongst which plant food allergens are often found. In SDS-PAGE the protein runs as two bands at approximately 16 kDa and 18 kDa representing the two isoforms, Ara h 2.01 and Ara h 2.02. (Chatel et al., 2003). The amino acid sequence of the isoforms is 98,1% identical. Ara h 2.01 has 160 amino acids, whereas Ara h 2.02 contains an additional insertion of 12 amino acids. Ara h 2 is resistant to acidic conditions and digestion enzymes of the gastrointestinal tract (Astwood et al., 1996, Lehmann et al., 2006), properties which could contribute to the high allergenicity of peanuts. In general, peanuts are thermally treated in some form and Ara h 2 was shown to be highly resistant to temperatures of up to 90°C (Lehmann et al., 2006). Therefore, the influence of thermal processing on the allergenicity of peanut allergens is of great interest.

1.8 Dendritic cells

As professional antigen presenting cells (APCs), dendritic cells (DCs) would be the first immune cells to capture allergens. DCs were first described by Paul Langerhans in 1868, who stained these cells in human skin and mistakenly thought they were nerve cells due to their dendrites (Langerhans 1868). DCs are key players of the immune system in both protection and tolerance. They are widely distributed in most tissues, including non-lymphoid as well as lymphoid tissues and also in the blood. DCs in tissues such as the intestine constantly sample environmental antigens (e.g. food proteins) and migrate into the T cell areas of lymph nodes (LNs). The T cell area is located in the cortex of the LNs and it is within these areas that T cells are found. Within the T cell area the contact between DCs and T cells takes place. When T cell receptors of T cells recognise the antigenic peptide presented by DCs, the T cell is activated (chapter 1.12.1). The migration of DCs to the LNs is greatly enhanced once an inflammatory signal is encountered (Randolph et al., 2008). However, self antigens should not induce an immune response. DCs are able to tolerise T cells to self antigens in the thymus and in the periphery (Bancherau and Steinman 1998). In allergy, DCs play a critical role in the sensitisation to allergens and the induction of the Th2 response (Hammad et al., 2002; Graffi et al., 2002) as well as in antigen presentation to previously primed Th2 cells. The pivotal role of DCs in food allergy was demonstrated by the observation that the adoptive transfer of Payer's patches derived DCs from allergic mice was able to induce antigen-specific IgE responses in naïve mice even without prior antigen challenge of the naïve mice (Chambers et al., 2004).

1.8.1 DC subsets

Since the first description of DCs, it has become apparent that there are many different subtypes of DCs. Each subtype has a particular location and function in the immune system (Shortman and Liu, 2002). In the steady state, DCs can be divided into two major populations in both mice and humans, conventional DCs (cDCs) and plasmacytoid DCs (pDCs). By flow cytometry, murine cDCs can be characterised as $CD11c^{+}CD11b^{+}B220^{-}$ (Wick, 2007), pDCs can be identified by flow cytometry as being $CD11c^{low}$ $CD11b^{-}$ $B220^{+}$ (Brawand et al., 2003). During infection or inflammation, further DCs can develop, such as monocyte derived DCs and tumour necrosis factor (TNF) producing DCs.

cDCs can be further subdivided into migratory DCs and lymphoid tissue resident DCs. Migratory cDCs act as sentinels in the peripheral tissue and migrate to the LN bearing antigens from the periphery where subsequently activates T cells. Examples of migratory cDC would be the Langerhans cells in the skin or the cDCs found in the intestine and airways. Lymphoid tissue resident DCs collect and present antigens in the lymphoid organ itself. Lymphoid tissue resident cDCs include most of the cDCs in the thymus and the spleen.

DCs are of haematopoietic origin. Haematopoietic stem cells are found in the bone marrow and give rise to two lineages of progenitor cells, the myeloid and the lymphoid progenitor. All subtypes

of DCs can develop from either the common lymphoid progenitor (CLP) or the common myeloid progenitor (CMP) populations. Since myeloid precursors are more abundant in the bone marrow than lymphoid precursors, it is assumed that *in vivo* most DCs are of myeloid origin.
pDCs have been mainly identified as important players in viral infections by secretion of large amounts of Interferon (IFN) $-\alpha$ and β. Their potential to take up antigen and initiate T cell responses is still unclear. On the other hand, cDCs are known to be highly sufficient in antigen uptake and are highly potent activators of T cells. These characteristics make them critical players also in allergy. *In vitro* generated bone marrow derived mouse myeloid dendritic cells (mDCs) are regarded as a model for cDCs. Therefore, mDCs were chosen for the present study.

1.9 Antigen uptake by dendritic cells

In most tissues, DCs are in an immature state. In this state they are unable to stimulate T cells but are well equipped to capture antigens. Immature DCs are highly efficient in the uptake of self and foreign antigens. The sampling of their environment by DCs is critical for the regulation of tolerance and the induction of immunity. DCs employ a variety of mechanisms for the uptake, namely pinocytosis, phagocytosis, and receptor-mediated endocytosis.

1.9.1 Pinocytosis and phagocytosis

Pinocytosis describes an uptake mechanism by which fluids are introduced into the cell via the invagination of the cell membrane resulting in the formation of vesicles within the cells. Pinocytosed material is taken up into clathrin coated pits which have the protein chlathrin on the inner side of the membrane which, together with other proteins, forms a basket. The pits are pinched off from the cell membrane and enter the cell as clathrin coated vesicles and fuse with early endosomes and are then processed in the endocytotic pathway (chapter 1.9.2). Pinocytosis is a constitutive process in immature DCs.
For the uptake of large particulates, DCs employ phagocytosis. The particles are usually recognised by receptors on the cell surface. When binding to the particles, the receptors form so called phagosomes by folding of the plasma membrane around the binding area. Within the cells, the phagosome fuses with the lysosome, where the phagocytosed particles are degraded (chapter 1.9.2). Bacteria (Rescigno, 2002), protozoans (Konecny *et al*., 1999) and yeast (d'Ostiani *et al*., 2000), as wells as cell debris or whole dying cells are taken up by phagocytosis. DCs are able to distinguish between necrotic and apoptotic cells and the presentation of cell related antigens may play a role in the maintenance of tolerance (apoptotic cells) and for the triggering of an immune response (necrotic cells) (Steinman *et al*., 2000)

1.9.2 Receptor mediated endocytosis and the endocytotic pathway

To take up soluble exogenous antigens, DCs are equipped with a full array of specialised receptors such as galectins, c-type lectin receptors, Fc receptors (Fanger, 1996; Ravetch, 2001) and scavenger receptors (Becker et al., 2006; Amiel et al, 2007) on the cell surfaces. These receptors bind to conserved structures such as carbohydrate moieties on antigens and pathogen-associated molecular patterns in pathogenic microbes. The captured allergens and pathogenic microbes are endocytosed and targeted into the endocytotic pathway, which consists of various endosomal compartments. Concerning their pH and composition, the endosomal compartments mature gradually along the track which makes it difficult to clearly distinguish them from each other. However, three major compartments are commonly defined, the early endosomes, the late endosomes and the lysosomes.

The membrane and the lumen of the early endosome are similar in design to the cell membrane and the extracellular medium, respectively. In the late endosomes there is already a considerable variety of proteases present which are only found in the endocytotic pathway and the interior is mildly acidic (around pH 6). Late endosomes mature into lysosomes which are highly acidic, with a pH of around 4 to 5 (Poole and Ohkuma, 1981) and have extensive proteolytic activity (Villadangos, 2001). Lysosomes contain around 40 different types of acid hydrolases which depend on the acidic environment of the lysosome for activation (Chapman, 1998). Hydrolases found within the lysosome include proteases, lipases, glycosidases, phosphatases and sulphatases; they are engaged in the antigen processing.

Evidence has accumulated that antigens internalised by receptor mediated endocytosis enter either the MHC class I or the MHC class II loading pathway (chapters 1.10.1. and 1.10.2). The separation of exogenous soluble antigens for MHC I from MHC II restricted presentation appears to depent on the receptor mediating the uptake. Lakadamyali and colleagues have shown that antigens taken up by different receptors are delivered to two different endosomal subsets. One group of endosomes displayed a high mobility along microtubules and matured rapidly towards late endosomes, while the other subset was static and matured much slower (Lakadamyali et al., 2006). The content of the fast maturing endosomes is rapidly degraded in the course of maturation. Ovalbumin (OVA) taken up by the mannose receptor (MR) by mouse DCs and macrophages was delivered to stable endosomes from where it is released for degradation in the cytosol to be processed only for MHC I presentation. In contrast, OVA which was pinocytosed or taken up scavenger receptors (SR) was targeted to the lysosomes (possibly via the fast maturing endosomes) and was exclusively presented on MHC class II (Burgdorf et al., 2007). Ligands of DC-SIGN, dectin-1 and DEC-205 are also targeted to the lysosomes (Engering et al., 2002; Carter et al., 2006; Dudziak et al., 2007)

1.10 Antigen processing by antigen presenting cells

In order for antigens to be made available for presentation to T cells, they have to be processed into short peptides and loaded onto MHC molecules. This is a complex process involving different steps in which mediators of proteolysis, protein sorting and transport, chaperons and other components have to work together in a coordinated fashion. Exogenous and endogenous antigens are presented on MHC I and II which are recognised by $CD8^+$ and $CD4^+$ T cells respectively.

1.10.1 MHC class I restricted presentation

MHC I is a transmembrane heterodimer consisting of an α-chain and a β_2-micorgloblin. The α-chain folds into three domains, α_1, α_2 and α_3. The folding of the α_1 and α_2 domain creates a long groove, which is the site of peptide binding. After assembly of the α chain with the β_2-microglobulin, MHC I is targeted to the peptide-loading complex in the endoplasmatic reticulum (ER). The peptide-loading complex consists of many components, including the chaperon calreticulin and TAP (transporter associated with antigen processing).

Endogenous proteins are degraded in the cytoplasm by the proteasome and other enzymes, generating peptides. The proteasome is the major proteolytic complex and is found in the cytoplasm and the nucleus of the cells. (Kloetzel, 2004; Rock et al., 2004; Shastri et al., 2002). The antigen peptides are then transported into the lumen of the ER by TAP. The final trimming of the peptides takes place in the ER. There, the peptide loading complex conducts the assembly of MHC I with the peptides and the peptide-MHC I complexes are then transported to the cell surface (Elliot and Williams, 2005).

In addition to endogenous antigens, exogenous antigens are also presented on MHC I by a process termed cross presentation. Cross presentation was found to occur in DCs (Dudziak et al., 2007; Pooley et al, 2001), certain macrophages (Burgdorf et al., 2007), B cells (Ke and Kapp, 1996) and, under special circumstances, liver endothelial cells (Limmer et al., 2000). Cross presentation allows APCs to present antigens of viruses which do not infect APCs and antigens of tumours which do not originate from APCs on MHC I. TAP dependent and independent mechanisms have been proposed for the loading of exogenous protein derived peptides to MHC I. In the case of OVA which is taken up via the MR, it was found that after processing of the antigen in the cytosol, the peptides reenter the early endosome in a TAP dependent manner without diversion to the ER, subsequently this would require a MHC class I loading machinery in the early endosomes (Burgdorf et al., 2008).

1.10.2 MHC class II restricted presentation

MHC II is constitutively expressed on professional APCs such as DCs, macrophages and B cells. Cortical and medullary thymic epithelial cells also express the molecules. MHC II consists of two glycoprotein chains, α and β. Both chains have two domains, α_1, α_2 and β_1, β_2 respectively. The

α_1 and β_2 domains form the peptide binding cleft. The β-chain is polymorphic and is responsible for the peptide binding specificity of each MHC class II molecule (Bell at al., 1985; Engelhard, 1994).

The $\alpha\beta$ heterodimers of MHC II are assembled in the endoplasmatic reticulum (ER) together with the invariant chain (Ii) which chaperons the folding of MHC II (Sant and Miller, 1994; Cresswell, 1996). Ii has a targeting motive in the cytoplasmic tail which targets the MHC II-Ii complex to the endocytotic pathway. The compartment where antigen peptides are loaded to MHC II s is termed MHC class II-containing compartment (MIIC).

There has been considerable debate in exactly defining MIIC. Loading of antigen peptide to MHC II has been reported for every location of the endocytotic route. The majority of MHC II-Ii complex is delivered to the transition between early and late endosomes (Geuze 1998; Wattes, 2001) and then enters the late endosomes. There, the MHCII-Ii complex is processed by cysteine proteases. After this processing, only a small peptide remains on the peptide binding groove of MHC II, the class-II associated variant chain peptide (CLIP), which prevents premature binding of antigen peptides. Substitution of CLIP with antigen peptides is catalysed by an accessory molecule, HLA-DM in human and H2-DM in mouse. In B cells, peptide loading is further catalysed by HLA-DO and H2-O in human and mouse respectively (Brocke et al., 2002).

The second location for Ii processing and peptide loading is the early endosome (Villadangos, 2001). Some of the MHC II-Ii complex has been observed in this compartment (Brachet et al., 1999; Pond and Watts, 1997). In the early endosomes, the removal of Ii from MHC II is independent of cysteine proteases and H2-DM, how it is accomplished instead is still unknown. Proteolytic activity in the early endosome is not high. It has been suggested that in the early endosomes MHC II binds to peptides which do not survive the harsher conditions of the later endosomal compartments and are only preserved as part of longer polypeptides. The complex of MHC II and antigen polypeptide could then move to the late endosomes/lysosomes. In these, unprotected regions of the polypeptide on MHC II are clipped off by proteases (Villadangos, 2001). Finally, the antigen peptide - MHC II complex is transferred to the cell surface.

1.11 Maturation of DCs

Before antigen capture or activation by mediators secreted by other cells, DCs in most tissues are immature and unable to stimulate T cells. For an efficient activation of T cells, MHC I or II and co-stimulatory molecules play a pivotal role. Immature DCs express relatively low levels of MHC I and II or co-stimulatory molecules (e.g. CD40 or CD86) on the cell surface. MHC II is synthesised in fairly large amounts but is not transported to the cell surface, instead it is retained in the endocytotic pathway (Pierre et al. 1997).

Maturation transforms DCs from antigen capturing cells into antigen presenting cells. Fully mature cells then express nearly all of their MHC II on the cell surface (Pierre et al. 1997). In parallel, the

cell surface expression of co-stimulatory molecules (e.g. CD40, CD80 or CD86), MHC I and T cell adhesion molecules (e.g. CD48 and DC-SIGN) is upregulated. The morphology of the DCs also changes. The cells lose their adhesive structure, reorganise the cytoskeleton and acquire high cellular motility (Trombetta and Mellman 2005). Membrane folds develop to form long dendrites, increasing the cell surface, thereby increasing the opportunities for T cell interaction. The expression of receptors for antigen capture is significantly reduced. Instead, chemokine receptors which facilitate the migration of the DCs to lymphoid organs are upregulated.

1.12 T cells

Antigen stimulation is passed on to tissues by T cells which initiate tissue cells to increase immune responses. Furthermore, T cells are required for the production of antibodies by B cells (chapter 1.13). The nature of the response depends on the property of the involved T cells (Schmidt-Weber et al., 2007). T cells can be divided into two large groups according to the co-receptors expressed on the cell surface. One group expresses CD4 while the other carries CD8 which bind to MHC II and MHC I respectively. In order to be able to respond to the enormous diversity of antigenic epitopes, an excessively large range of T cell receptors (TCR) must be available for T cells. The antigen recognition domains are encoded by genes which are assembled by somatic rearrangement (Murphy et al., 2008).

1.12.1 Activation of naïve T cells

Naïve T cells are functionally immature. In the cortical region of the lymph nodes, they transiently bind to every APC they encounter. This transient binding is important for the T cells to sample many MHC molecules on the APCs for the presence of the specific antigen peptide their T cell receptor (TCR) recognises. The encounter of a T cell with a peptide-MHC complex which is recognised by the TCR results in the stabilisation of the association between APC and T cell (Bromley et al., 2001). This can last for several days, during which the naïve T cells proliferate and the daughter cells also adhere to the APC and differentiate into effector cells (Murphy et al., 2008).

For the activation of naïve T cells two signals are required. The first signal is the interaction of the TCR with a specific peptide-MHC complex presented on the cell surface of APCs. The second signal required for efficient T cell activation is delivered by co-stimulatory molecules. Both the antigen-specific signal and the co-stimulatory signal have to be delivered by the same cells. Consequentially, only activated APCs, predominantly DCs, can induce T cell responses. This is crucial to prevent responses to self antigens.

The most important co-stimulatory signals for efficient clonal expansion of naïve T cells are provided by members of the B7 family, B7-1 (CD80) and B7-2 (CD86) which interact with CD28 on T cells. Naïve T cells divide and undergo apoptosis on an irregular basis. The binding to the

MHC-peptide complex and CD28 co-stimulation directly trigger the progression through the G1 phase of the cell cycle (Appleman et al. 2000). CD28 co-stimulation also induces the synthesis of IL-2 and the expression of the α-chain (CD25) of the IL-2 receptor (IL-2R). Naïve T cells express an IL-2R made up of the β and γ chains only which has a low binding affinity for IL-2. The α-chain associates with the βγ heterodimer to give rise to a high affinity IL-2R. Binding of IL-2 triggers the entry into the S-phase of the cell cycle (Appleman et al. 2000). The T cells can then proliferate two to three times per day for several days resulting in clonal expansion. IL-2 is an essential survival factor for T cells and also promotes the differentiation into effector cells. Since activated T cells synthesise IL-2, they promote their own growth in an autocrine fashion.

1.12.2 $CD4^+$ T cells

$CD4^+$ T cells are key players of the adaptive immunity. Four main subsets of $CD4^+$ T cells are generally discerned. Namely these are the effector cells Th1, Th2 and Th17, which are opposed by the fourth subset, the regulatory T cells (Tregs), whose function is to suppress effector T cell activation in order to prevent excessive T cell responses and autoimmune diseases. T cell differentiation is mainly determined by the cytokine milieu during the activation of naïve T cells.
In murine models based on artificial immunisation or infection, T cell differentiation is clear cut. In humans, polarisation of $CD4^+$ T cell responses is usually less clear (Romagnani, 1994). An imbalance of the $CD4^+$ subsets in humans is associated with the development of allergies and autoimmune diseases (Farrar et al., 2002; Ho and Glimcher 2002; Neurath et al., 2002)

1.12.2.1 Th1 cells

Th1 cells are characterised by the production of IFN-γ and TNF-β (Mosmann et al 1986, Del Prete et al., 1988). The development of Th1 is driven by IFN-γ, IL-12 and IL-27. IFN-γ and IL-27 activate the transcription factor STAT1 (Signal Transducers and Activators of Transcription protein 1). STAT1 activates T-bet, which is considered to be the key transcription factor in Th1 development, and initiates IFN-γ production (Wilson et al, 2009). T-bet, in association with various co-factors, also antagonises GATA-3, which is the transcription factor controlling Th2 differentiation, thereby silencing the expression of the Th2 associated cytokines IL-4, IL-5 and IL-13 (Dijuretic et al., 2007).
Th1 cells play a critical role in cell mediated immunity. They activate macrophages and control intracellular pathogens such as viruses and certain bacteria, e.g. *Listeria* and *Mycobacterium tuberculosis* (Holland et al., 2007). Th1 cells also stimulate antibody production against extracellular pathogens via co-stimulation of B cells. In human, the antibody subclasses associated with Th1 responses are IgG1 and IgG3, whereas in mouse they are IgG2a and IgG2b (Murphy et al., 2008). The excessive production of Th1 cytokines has been linked with delayed hypersensitivity reactions (Type IV allergies, Abbas et al., 1996).

For Type I allergy it has been demonstrated that Th1 cytokines suppress Th2 responses (Iwamoto et al. 1993; Dow et al. 1999). IFN-γ inhibits Th2 cytokine production and proliferation *in vitro* (Gajewski et al., 1989; Fernandez-Botran et al., 1988). *In vivo*, IFN-γ is capable of reducing the numbers of Th2 cells in the area of allergic inflammation as well as inhibiting the secretion of Th2 cytokines (Li et al., 1996; Iwamoto et al., 1993; Nakajima et al., 1993). Furthermore, IFN-γ can block Th2 induced inflammatory pathways downstream of cytokine production. This effect may occur by the action of the cytokine on inflammatory cells such as eosinophils (Cohn et al. 1999). Subjects with intact oral tolerance to food proteins have reportedly higher levels of IFN-γ and TNF-α than food allergic subjects (Turcanu et al., 2003; Andre et al., 1996). Furthermore, resolution of allergy appears to be related with normalisation of IFN-γ levels (Smart et al. 2002), showing the importance of Th1 cell contribution for a balanced immune response to environmental antigens.

1.12.2.2 Th2 cells

Th2 cells are characterised by the production of IL-4, IL-5, IL-6, IL-9, and IL-13. Th2 differentiation is induced by IL-4. The cytokine activates the transcription factor STAT6, which activates GATA-3. GATA-3 contributes to the expression of IL-4, IL-5 and IL-13 and also in silencing of IFN-γ expression.

In allergic patients, a high frequency of allergen specific Th2 cells in the peripheral blood and inflammatory tissues has been observed. The Th2 response to one or more environmental allergen triggers the recruitment of other cell types, the secretion of a large number of soluble factors and the expression of adhesion molecules. Th2 cytokines are required for the differentiation, maturation, activation and tissue homing of inflammatory cells and the Th2 cells themselves. For instance, IL-4 and IL-13 are required for the production of IgE antibodies by B cells (de Vries et al., 1993, chapter 1.13). IL-5 induces the release of mature eosinophils as well as immature eosinophils from the bone marrow and is important for the terminal maturation and activation of these cells (Palframan et al.,1998). IL-4 induces expression of chemokines (i.e. eotaxin) in epithelial cells and fibroblasts, which induce the migration of eosinophils and Th2 cells into inflammatory sites. IL-4 and IL-9 promote the development and maturation of mast cells. Together, activation of Th2 cells triggers an inflammatory cascade of great complexity which promotes allergic inflammation and the manifestation of clinical symptoms.

1.12.2.3 Th17 cells

Th17 cells are a more recently described subset of $CD4^+$ T cells and are characterised by the production of IL-17, IL-22 and IL-23. Th17 differentiation is induced by IL-6 and TGF-β. Th17 cells contribute to the host defence by acting on various immune and non-immune cells in protecting against extracellular bacteria. However, they also play a critical role in autoimmune diseases such as multiple sclerosis and rheumatoid arthritis. Experimental data suggests that IL-17 coordinates tissue inflammation by inducing the secretion of proinflammatory cytokines such

as IL-6, IL-8 and TNF-α. Involvement of Th17 cells in allergic diseases has also been shown. In atopic contact dermatitis, the frequency of IL-17 producing $CD4^+$ T cells correlated with the severity of the disease (Koga et al., 2008). In asthmatic tissues, upregulation of IL-17 production was found. It was suggested that the cytokine is involved in inflammation and airway remodelling (Matsunaga et al., 2006; Molet et al., 2001; Hashimoto et al., 2007). In some patients suffering from food allergy, enterocolitis (inflammation of the colon and small intestine) and accumulation of neutrohphils in the intestinal tissues was observed. IL-17 seems to recruit neutrophils by inducing IL-8 secretion or mast cell activation in these patients (Oboki et al., 2008), thereby promoting allergic inflammation.

1.12.2.4 Regulatory T cells

Tregs play a critical role in the maintenance of tolerance and suppression of potentially destructive activities of effector T cells and inflammatory cells. Tregs have been classed into naturally occurring Tregs which develop in the thymus (nTregs) and Tregs which emerge in the periphery under various tolerogenic conditions (Sakaguchi et al., 2008), so called inducible Tregs (iTregs). Most nTregs are differentiated in the presence of transforming growth factor - beta (TGF-β), and are characterised by a high expression of CD25 and the co-expression of the forkhead box P3 transcription factor (Foxp3) (Sakaguchi 2005). nTregs produce both TGF-β and IL-10. In iTregs, there are several subsets such as Tr1 cells producing IL-10 and TGF-β producing Th3 cells (Vagnali et al., 2009; Roncarolo and Gregori, 2008). Both nTregs and iTregs reportedly play an important role in suppression of allergic diseases (Akdis, 2009).

The inhibitory cytokines IL-10 and TGF-β produced by Tregs are crucial for their suppressive functions. These cytokines inhibit proliferation and cytokine production of effector T cells. IL-10 also inhibits IgE production of B cells and activation of mast cells. In studies of food allergy, children outgrowing a food allergy seem to have more Tregs than children with persistent allergy (Karlsson et al., 2004). It has also been shown that Tr1 cells consistently represent the dominant T cell subset specific for food allergens in healthy individuals; in contrast, there is a high frequency of allergen specific IL-4 producing T cells in allergic individuals. A quantitative defect of Treg could be involved in the pathogenesis of allergic diseases (Grindebacke H et al., 2004).

1.12.3 $CD8^+$ T cells

$CD8^+$ T cells predominantly differentiate to cytotoxic T cells and play a central role in pathogen defence. The cytotoxic T cells kill target cells which display antigen peptides on MHC I. MHC I is expressed by most nucleated cells. This enables them to present antigens of intracellular pathogens such as viruses to $CD8^+$ T cells, facilitating the elimination of the infected cells and thereby hindering the spreading of the pathogen. APCs can also activate $CD8^+$ T cells with exogenous derived antigens by cross presentation (chapter 1.10.2).

1.13 B cells

The chief function of B cells is the production of antibodies in response to antigens. In mammals, five isotypes of antibodies have been identified; IgM, IgD, IgG, IgA and IgE. They differ from each other in the structure of the constant region of their heavy chains. Mature naive B cells express IgD and IgM on their surface which act as B cell receptors (BCR) and capture and internalise antigens. The BCR have a vast range of antigen specificities, with each individual B cell producing receptors of one specificity which is determined by the variable region of the immunoglobulin. The first antibody isotype secreted upon activation is always IgM, later in the immune response the same veriable region may be expressed in IgE, IgA or IgG antibodies. This process is known as isotype switching and depends on the stimuli which the B cells receive.

For the production of IgE antibodies in response to an allergen, two steps are required. The B cell must first be activated and then a class switch to IgE must be induced. The activation of B cells can be T cell dependent or independent. Antibody production against protein antigens, including allergens, requires antigen specific T cell help (Murphy et al., 2008). Naive B cells can bind antigens (allergens) via their cell surface IgM and IgD. The antigen-Ig complexes are internalised and processed by the B cells. The antigen derived peptides are then loaded onto MHC II and are subsequently presented on the B cell surface. TCRs of activated $CD4^+$ T cells recognising the same antigen as the Ig of the B cells bind to the peptide-MHC II. In addition, the CD40 ligand expressed on T cells interacts with CD40 on B cells, thereby supplying the co-stimulatory signal required for B cell activation and class switching (Rivera et al., 2001). CD40 stimulation by allergen specific T cells upregulates the expression of the co-stimulatory molecules CD80 and CD86 by B cells, which in turn enhances CD40L expression on T cells and increases the stimulation of B cells through the induction of IL-4 (Geha et al., 2003). Proliferating B cells form a so called germinal centre which is present for about three to four weeks after the initial antigen contact. Germinal centre B cells first differentiate into plasmablasts, these are cells which have begun to secrete antibodies but are still dividing and can still interact with T cells. Some of the plasmablasts differentiate further into plasma cells. These no longer express MHC II or CD40. However, they efficiently produce large amounts of antibodies. Other germinal B cells differentiate into memory B cells (Murphy et al., 2008).

In class switching to IgE, the Th2 cytokines IL-4 and IL-13 play a pivotal role. Through the interaction of IL-4 and IL-13 with their receptors on the B cell surface a signal transduction cascade is triggered which results in the transcription of the gene for the epsilon class of the constant region ($C\varepsilon$) of the immunoglobulin heavy chain in B cells, resulting in the production of class E antibodies (de Vries et al., 1993)

2 Aims of this thesis

The Maillard reaction is a chemical reaction between reducing sugars and amino acids which generates protein derivatives modified with glycation structures, collectively called advanced glycation end products (AGEs). The products are important for the brown colour and taste of cooked foods. As 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 interest. The aim of this thesis was to study the influence of the Maillard reaction on the T cell immunogenicity (the ability to induce T cell responses), antigenicity (the ability to induce antibody responses) and the potential allergenicity (an ability to induce allergic responses) of food allergens.

Maillard reaction products have been shown to influence the maturation and T cell activation ability of dendritic cells (DCs). However, the impact of AGEs derived from food allergens on DC function and/or the subsequent activation of allergen specific T cells had not been examined prior to this study. Therefore, in the first part of the thesis, the T cell immunogenicity of the model allergen ovalbumin (OVA) which had been modified by the Maillard reaction was to be examined using *in vitro* murine cell cultures. For this purpose, OVA was to be glycated (AGE-OVA) by thermal processing with glucose and the structural changes induced by this process analysed. For all experiments, native OVA and OVA thermally processed without glucose were to be used as controls. To examine the T cell immunogenicity of AGE-OVA, $CD4^+$ or $CD8^+$ T cells from transgenic mice with OVA specific T cell receptors were to be used and co-cultured with bone marrow derived myeloid DCs (mDCs). Allergen specific T cell activation is preceded by the uptake of allergens by DCs, a process predominantly mediated by endocytotic receptors. In other studies, receptors which can bind AGEs have been identified, e.g. the receptor for AGEs (RAGE), galectin-3 or SR-AI/II (macrophage scavenger receptor class A type I and II). However, the receptor(s) which mediate(s) the uptake of AGEs by mDCs was/were not known. A further aim was therefore the identification of the receptor(s) involved in AGE-OVA uptake by mDCs.

In the second part of this thesis, the immunogenic properties of AGE-OVA *in vivo* were to be examined. For this, mice were to be exposed to AGE-OVA by different immunisation routes. The T cell immunogenicity of the glycated allergen was to be assessed by measuring the proliferation of splenic and lymph node $CD4^+$ T cells of immunised mice in response to *in vitro* re-stimulation with the allergen. In addition, the level of allergen specific antibodies in the serum of immunised mice was to be measured by ELISA in to order elucidate the antigenicity of AGE-OVA.

The prevalence of peanut allergy is increasing. Additionally, peanuts often induce life threatening anaphylactic reactions. Peanuts generally undergo thermal processing (e.g. roasting or frying) before consumption and since peanuts contain high amounts of reducing sugars, the Maillard reaction would occur. Therefore, in the final part of this thesis, the influence of glycation by the Maillard reaction on the immunogenic properties of the major peanut allergen Ara h 2 was to be determined *in vivo*. For this study, recombinant Ara h 2 was used. The recombinant allergen was

to be subjected to glycation by thermal treatment in the presence of carbohydrates and analysed. Unprocessed rAra h 2 and rAra h 2 thermally processed without carbohydrates were to be used as controls for all experiments. T cell imunogenicity and antigenicity of AGE-rAra h 2 *in vivo*, was to be evaluated in a similar way as for AGE-OVA.

3 Material and Methods

3.1 Material

3.1.1 Commonly used equipment.

Special equipment and materials needed for a certain method are listed in the relevant method description.

Tab. 3.1 List of commonly used equipment

Equipment	Model and Company
Magnetic stirrer	IKAMAG® RCT basic (IKA , Staufen)
pH meter	SevenEasy (Mettler Toledo, Giessen)
Power source	PowerPac^{TM} (Biorad, München)
Thermo mixer	Thermomixer Compact (Eppendorf, Hamburg)
Heating block	Digital Heatblock (WVR International, Darmstadt)
Incubator cabinet	Orbital incubator SI50 (Carl Stuart Ltd., Dublin, IRL)
Water bath	Grant GD100 (Grant Instruments Ltd., Cambridge, UK)
Vortex	Vortex-Genie 2 (Scientific Industries, New York USA)
Weighing scales	Explorer Pro, (Ohaus, Pin Brook, NJ, USA) EK-300i (AND, Abingdon, UK)
Centrifuges	Centrifuge 5417C/R (Eppendorf; Hamburg) Multifuge 1S-R (Heraeus, Langenselbold) Sorvall SLA 3000 (Thermo Scientifific, Bonn)
Speed Vac	SPD 111V (Savant, Ramsey, MN, USA)
Seesaw shaker	DUOMAX 1030 (Heidolph Instruments, Schwalbach)
Orbital shaker	KS 130 control (IKA, Staufen)
Laminar flows	Lamin Air HB2448 (Heraeus, Langenselbold) Labotect SG-400 (Labotect, Göttingen)
Incubator (for cells)	Hear cell 150 (Heraeus, Langenselbold)
PCR thermocycler	PCR System 2700 (Applied Biosystem, Weiterstadt)
ELISA reader	Spectra Max 340 PC (Molecular Devices, München)
CD spectrometer	J-810S (JASCO, Groß-Umstadt)

3.1.2 Chemicals

If not otherwise specified, all chemicals were obtained from Merck (Darmstadt), Sigma (Steinheim) or Roth (Karlsruhe).

3.1.3 Buffers

General buffers were obtained from the medium kitchen of the Paul-Ehrlich-Institut (PEI). Special buffers and chemicals are described in the respective methods.

PBS: 1,5 mM $KH2PO_4$
8 mM $Na2HPO_4$
137 mM NaCl
3 mM KCl
pH 7.2

3.1.4 Cell culture media and reagents

Tab. 3.2 Media, additives and reagents for culture of primary mouse cells

	Application	Supplier
RPMI 1640	All cells	Gibco, Fisher Scientific, Schwerte
Glutamine with Penicillin and Streptomycin	All cells	Media kitchen, PEI
Fetal calf serum (FCS), Lot S0115	mDC culture	Biochrom Berlin
FCS	Proliferation assays	Gibco
$C_8H_{18}N_2O_4S$ (HEPES), 1M	mDC culture	Media kitchen, PEI
Recombinant mouse Granulocyte-macrophage colony-stimulating factor (GM-CSF)	mDC culture	R&D Systems, Wiesbaden
Sodium pyruvate	mDC culture	Sigma, Steinheim
10 x lysis buffer	Erythrocyte lysis	BD Biosciences, Heidelberg

3.1.5 Native and recombinant proteins

Tab. 3.3 native and recombinant proteins used in this study

Protein	Supplier
Ovalbumin from chicken egg white, grade V	Sigma, Steinheim
Recombinant OVA	Section 5/4, PEI
BSA	Santa Cruz, Heidelberg
BSA, cell culture tested	Sigma
Recombinant Ara h 2	Section 5/4, PEI
Recombinant cytokines for ELISA	
Recombinant mouse IL-2	Biolegend, San Diego, CA
Recombinant murine IL-4	Peprotech, Hamburg
Recombinant mouse IL-10	BD Biosciences, Heidelberg
Recombinant mouse IL-12	BD Biosciences
Recombinant murine IFN-γ	Peprotech

3.1.6 Antibodies and reagents for immune detection

Tab. 3.4 Antibodies

Antibody	Application	Company
Primary antibodies		
APC Rat IgG2b$_\kappa$ anti-mouse CD4	Flow cytometry	eBioscience, San Diego, CA
FITC rat IgG2b anti-mouse CD4	Flow cytometry	Miltenyi Biotech, Bergisch Gladbach
APC Rat IgG2b$_\kappa$ anti-mouse CD11b	Flow cytometry	eBioscience
PE Rat IgG2b$_\kappa$ anti-mouse CD11b	Flow cytometry	eBioscience
APC Hamster IgG anti-mouse CD11c	Flow cytometry	eBioscience
PE-Cy5.5 Hamster IgG anti-mouse CD11c	Flow cytometry	eBioscience
FITC Hamster IgM$_\kappa$ anti-mouse CD40	Flow cytometry	eBioscience
FITC Hamster IgG anti-mouse CD80	Flow cytometry	eBioscience
FITC Rat IgG2a$_\kappa$ anti-mouse CD86	Flow cytometry	eBioscience

Rabbit polyclonal IgG anti-EEA1	LSM	Calbiochem, Merck, Darmstadt
Mouse IgG2a anti-FITC	ELISA	Chemicon, Millipore, Schwalbach
Rat IgG2a$_\kappa$ anti-mouse Galectin-3	Flow cytometry	eBioscience
Rat IgG2b$_\kappa$ anti-mouse IL-2	ELISA	Biolegend
Rat IgG1$_\kappa$ anti-mouse IL-4	ELISA	eBioscience
Rat IgG2b$_\kappa$ anti-mouse IL-10	ELISA	Biolegend
Rat IgG2a$_\kappa$ anti-mouse IL12 p35/p70	ELISA	eBioscience
Rat IgG2a$_\kappa$ anti-mouse LAMP1	LSM	eBioscience
Rat IgG1$_\kappa$ anti-mouse IFN-γ	ELISA	eBioscience
Rat IgG2a$_\kappa$ isotype control	Flow cytometry	eBioscience
Rat IgG2b isotype control	Flow cytometry	eBioscience
Rat IgG1$_\kappa$ anti-mouse IgE	ELISA	BD Biosciences, Heidelberg
FITC rat IgG2b anti-mouse MHC class II	Flow cytometry	eBioscience
Rat IgG2b$_\kappa$ anti-mouse MHC class II	LSM	BD Biosciences
Rat IgG2a anti-mouse MR (CD206)	Flow cytometry	AbD Serotec, Düsseldorf
Rabbit polyclonal IgG anti-mouse RAGE	Flow cytometry	Abcam, Cambridge
Rat IgG2b anti-mouse SR-A type I (CD204)	Flow cytometry	R&D Systems, Wiesbaden

Secondary antibodies

Biotin rat IgG2b$_\kappa$ anti-mouse Il-2	ELISA	Biolgend
Biotin rat IgG1$_\kappa$ anti-mouse IL-4	ELISA	eBioscience
Biotin rat IgG1$_\kappa$ anti-mouse IFN-γ	ELISA	eBioscience
Biotin rat IgG1κ anti-mouse IgE	ELISA	BD Biosciences
HRP anti-mouse IgG	ELISA	GE Healthcare, München
Cy 3 Goat F(ab') fragment anti-rabbit IgG	LSM	Jackson ImmunoResearch Europe Ltd., Suffolk, Uk
HRP anti-rabbit IgG	ELISA	Sigma
HRP rabbit anti-mouse IgG1	ELISA	Zymed, Invitrogen, Karlsruhe
HRP rabbit-anti-mouse IgG2a	ELISA	Zymed

Alexa Fluor 488 goat anti-rat IgG	FACS, LSM	Molecular Probes, Invitrogen, Karlsruhe
Alexa Fluor 568 goat anti-rat IgG	LSM	Molecular Probes
Alexa Fluor 488 donkey anti-goat IgG	LSM	Molecular Probes

Reagents for immune detection:

Sreptavidin-Horseradisch Peroxidase Conjugate (BD Biosciences)

Tetramethylbenzidine (TMB) substrate reagent set (BD Biosciences)

3.1.7 Mice

Tab. 3.5 Mouse strains used in this study

Genotype	Genetic background	Reference
BALB/c (wild type)		
C57BL/6 (wild type)		
C3H/HeJ (wild type)		
C.Cg-Tg(DO11.10)10Dlo/J (DO11.10)	BALB/c	Murphy et al., 1990
C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-1)	C57BL/6	Hogquist et al., 1994
C57BL/6-Tg(TcraTcrb)425Cbn/J (OT-2)	C57BL/6	Barnden et al., 1998
RAGE$^{-/-}$	C57BL/6	Myint et al., 2006
B6.Cg-*Msr1*^{tm1Csk}/J (SR-AI/II$^{-/-}$)	C57BL/6	Suzuki et al., 1997

C57BL/6 mice were purchased from Harlan-Winkelmann GmbH (Borchen). BALB/c and C3H/HeJ mice were purchased from Charles River Laboratories (Sulzfeld). DO11.10, OT-1, OT-2 and SR-AI/II$^{-/-}$ mice were purchased from Jackson Laboratories (Bar Harbour, ME, USA). RAGE$^{-/-}$ mice were a kind gift from Dr. T. Shoji (Osaka Medical College, Japan). The genetically modified mice were bred in the animal facility of the Paul-Ehrlich-Institut.

3.1.8 Oligonucleotides

Primers were obtained from Sigma Genosys, Sigma-Aldrich, Steinheim

Tab. 3.6 List of primers used

Name	Sequence 5' → 3'
Amplification of SR-A cDNA from mDCs	
SR-A (s)	AGA ATT TCA GCA TGG CAA CTG
SR-A (as)	ACG GAC TCT GAC ATG CAG TG
Amplification of SR-B1 cDNA from mDCs	
SR-B1 (s)	CTC ATC AAG CAG CAG GTG CTC
SR-B1 (as)	GAG GAT TCG GGT GTCATG AA
Amplification of SR-B1 cDNA from mDCs	
CD36 (s)	GTG CTG ATC CTT TCA GAG TCT C
CD36 (as)	CTG AGG AAT GGA TCT TTG TAA CC

The sequence of the sense (s) and antisense (as) primers are given in the standard abbreviations for nucleic acids.

3.1.9 Software

Tab. 3.7 Used EDV programmes

Program	Application
FACS Diva 6.0	Flow cytometric data assimilation and analysis
FlowJo 7.2.4	Data analysis flow cytometry
SigmaPlot 11.0	Graphs
Microsoft Office	Text processing, Tables, Figures
Zeiss LSM Image browser 4.2	Analysis of laser scanning microscopy data

3.2 Methods

3.2.1 Molecular biological methods

3.2.1.1 Total RNA isolation

Total RNA was isolated from myeloid dendritic cells (mDCs, chapter 3.2.3.4) using Trizol reagent according to the manufacturer's instructions. mDCs (5-10x10^6 cells) were lysed with 1 ml of Trizol reagent. The lysed sample was incubated at room temperature for 5 min and then 200 µl of Chloroform was added per 1 ml of Trizol reagent. The sample was shaken vigorously, and incubated at room temperature for 3 min. Subsequently the sample was centrifuged (10,000 rpm, 15 min, 4°C). After centrifugation, the aqueous phase, containing the RNA, was transferred into a

new tube. To precipitate the RNA, 500 µl of Isopropyl alcohol (per 1 ml of Trizol originally added) was added and the sample incubated for 10 min at room temperature. The sample was again centrifuged (10,000 rpm, 10 min, 4°C) and the supernatant removed. To wash the RNA pellet, 1 ml of 75% Ethanol per 1 ml Trizol reagent was added and centrifuged (7,500 rpm, 10 min, 4°C). The RNA pellet was air dried and dissolved with 50 µl of RNAse free water. To determine the amount of isolated RNA, the RNA solution was diluted 1:100 and the OD measured at 230, 260 and 280 nm. The absorption at 230 and 280 nm is measured to calculate the contamination with DNA and protein, respectively. At A_{260} = 1, the RNA concentration is 40 µg/ml.

Trizol reagent (Invitrogen)
Isopropyl alcohol
75% Ethanol (Roth)
RNAse free water (Invitrogen)

3.2.1.2 Reverse transcriptase (RT) reaction

RNA isolated as described in chapter 3.2.1.1 was transcribed to cDNA for the PCR (chapter 3.2.1.3) using the enzyme reverse transcriptase. The reverse transcriptase catalyses the transcription of RNA to DNA. First, RNAse free water, dNTPs, Oligonucleotides and the isolated RNA (chapter 3.2.1.1) was gently mixed and incubated at 65°C for 5 min to break any double stranded RNA sequences. The mixture was immediately placed on ice, where the reverse transcriptase (RT) buffer and the reverse transcriptase were added. The reaction mixture was then incubated at 42°C for 60 min, after which the reaction is stopped by heating to 90°C for 10 min.

Reaction mixture:

RNase free DW	11.0 µl
10 mM dNTP mixture (Invitrogen)	1.0 µl
Oligo(dT)12-18, 0.5 µg/ml (Invitrogen)	1.0 µl
RNA soln. 500 µg/ml	4.0 µl
RT Buffer	2.0 µl
Reverse Transcriptase (New England Biolabs)	1.0 µl

3.2.1.3 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is an *in vitro* application to amplify defined nucleic acid sequences of DNA, using DNA polymerase, which catalyses the polymerisation of deoxyribonucleotides into a DNA strand. Taq polymerase was used, which has the advantage of being heat stable. The PCR programme consists of denaturation, annealing and elongation phases. First, the reaction mixture is heated to 94°C to denature double stranded DNA (denature

phase). Lowering the temperature allows the hybridisation of the oligonucleotide primers to the single stranded DNA templates (annealing phase). Subsequently the temperature is raised to 72°C, which is the optimal working temperature for the Taq DNA Polymerase. The enzyme elongates the primers until the DNA is again double stranded (elongation phase). 35 of these cycles were run, after which a final 8 min elongation phase was added. An aliquot of the reaction mixture was then applied to an agarose gel to analyse the PCR products (chapter 3.2.1.4.)
For one reaction with a total volume of 20 µl the reaction composition below was used. The PCR thermal cycler programmes used in this study are shown in Tab 3.8.

PCR Reaction:

10 x PCR Buffer (Invitrogen)	2.0 µl
50 mM $MgCl_2$ (Invitrogen)	0.6 µl
10 mM dNTP Mix (Invitrogen)	0.4 µl
5-Primer (10 µM)	0.4 µl
3-Primer (10µM)	0.4 µl
Taq DNA Polymerase (Invitrogen)	0.1 µl
cDNA from First Strand Reaction	4.0 µl
DW	12.1 µl

Tab 3.8 PCR-cycler programme conditions for the qualitative analysis of mDC receptor expression

Primer	Cycler programmes
SR-B1	2 min 94°C + 35 cycles (1 min 95°C , 1 min 60°C, 1 min 72°C) + 8 min 72°C
CD36, SR-AI/II	2 min 94°C + 35 cycles (1 min 94°C , 1 min 55°C, 2 min 72°C) + 8 min 72°C

3.2.1.4 Agarose gel electrophoresis

A molecule which posses a net charge will move in an electric field. This phenomenon, electrophoresis, is a powerful tool for separating DNA and other macromolecules such as proteins (SDS-PAGE, chapter 3.2.2.2.). The phosphate residues of DNA are negatively charged. Therefore, DNA molecules move towards the positive anode when in an electric field. Compared to larger DNA fragments, smaller ones can move through the gel matrix quicker, this physical property is exploited for the separation.
In this study agarose gel electrophoresis was employed to detect PCR products (chapter 3.2.1.3). Gels with a concentration of 1.5 % were used. The agarose was heated in 1x TAE buffer until fully dissolved, then this was used to cast the gel. The sample from the PCR which was to be analysed was prepared by adding 1 µl of loading buffer to 5 µl of the sample. The gel was run at 110 V in a Mini Sub-Cell CT chamber (Biorad, München). After 1 h the gel was removed from the running chamber and stained with ethidium bromide for 15 min. Ethidium bromide is an intercalating agent

which is fluorescent under UV light (300 nm), it inserts itself between DNA base pairs making the bands in the dyed agarose gel appear as glowing bands. Excess ethidium bromide was then washed off with water and the gel photographed under UV light.

Agarose (PeqLab, Erlangen)

TAE buffer (20x): 800 mM Tris
2.28% (v/v) CH_3COOH
25 mM EDTA
pH 8.5

Loading buffer (6x): 0,06 % Bromophenol Blue
30 % Glycerol
0.2 mM EDTA

Base pair standard: 100 bp DNA ladder (New England Biolabs, Frankfurt))

Ethidum bromide solution: 10 % Ethidium bromide (w/v) (Fluka, Sigma-Aldrich Steinheim)

3.2.2 Biochemical methods

3.2.2.1 Preparation of proteins modified with AGEs

3.2.2.1.1 Preparation of AGE-OVA and AGE-BSA

Glycated Ovalbumin (AGE-OVA) and glycated bovine serum albumin (AGE-BSA) were produced following a protocol by Gasic-Milenkovic (Gasic-Milenkovic et. al. 2003). 1 mM of OVA or BSA (both from Sigma) was incubated with 1 M Glucose (Sigma) in 100 mM sodium phosphate buffer, pH 7.4 for 6 weeks at 50°C. Excess glucose was removed by extensive dialysis with PBS. OVA or BSA incubated under the same conditions, but without glucose (thermally processed OVA/BSA), and native OVA/BSA were used as controls.

100 mM Sodium phosphate buffer, pH 7,4
Stock solution A: 200 mM monobasic sodium phosphate, monohydrate
27.6g $NaH_2PO_4 \cdot H_2O$ / L
Stock solution B: 200 mM dibasic sodium phosphate
28.4 g Na_2HPO_4 / L

To receive a 100 mM phosphate buffer pH 7,4: mix 19 ml of solution A with 81 ml of solution B and dilute to 200 ml.

3.2.2.1.2 Preparation of AGE-rAra h 2

For the preparation of glycated recombinant Ara h 2 (AGE-rAra h 2) 500 µg/ml of the protein was incubated with 0.1 M of a fructose, galatcose, glucose, lactose, mannose or maltose in PBS. Incubation was for 2 hours at 120°C. Excess carbohydrates were removed by extensive dialysis with PBS. As controls for all experiments, unprocessed recombinant rAra h 2 or rAra h 2 heated for 2 h at 120°C without carbohydrates were used.

3.2.2.2 Measurement of the protein concentration using bicinchoninic acid (BCA)

Protein concentrations in sample solutions were measured using a Bicinchoninic acid (BCA) assay kit (Pierce, Bonn) according to the manufacturer's protocol. The principle of the BCA assay is based on the Biuret reaction. Proteins form complexes with Cu^{2+} when in an alkaline milieu. The Cu^{2+} ions of these complexes are reduced to Cu^{1+} ions by cysteine, tyrosine and tryptophan residues in the proteins. In the second step, BCA reacts with the Cu^{1+} ion. By chelation of two molecules of BCA with one Cu^{1+} ion, a purple reaction product is formed. The BCA/copper complex is water soluble and shows a linear absorbance at 562 nm with increasing protein concentrations. The absorption at this wavelength was photometrically determined using the ELISA reader (Tab. 3.1).

BSA (protein standard): 2 mg/ml bovine serum albumin (Pierce, Bonn)
Micro titre plate: 96 flat bottom well plate (Nunc, Langenselbold)

3.2.2.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (modified after Laemmli, 1970) was used as a method to further analyse proteins after thermal treatment with or without carbohydrates. SDS-PAGE allows the separation of proteins according to their apparent molecular weight. The sample is heated to 95°C with SDS to destroy the secondary and tertiary structures. The negative charge obtained by the protein upon SDS binding is usually much greater than the charge of the native protein, thus the native charge is rendered insignificant. Through the application of voltage, the negatively charged proteins migrate through the gel, the speed depending exclusively on their size after denaturation and treatment with SDS.
In this study, the electrophoresis was performed using 12.5 % acrylamide gel at 100 V in a Mini Protean Cell (Biorad, München). The proteins in the gel were then stained with Coomasie Brilliant Blue (chapter 3.2.2.4).

Running buffer 25 mM Tris
250 mM Glycin
0.1 % (w/v) SDS

2 xLoading buffer:	25 mM Tris
	5 ml Beta-mercaptoethanol
	1 % SDS (w/v)
	10 % Glycerol (w/v)
	0.001 % Bromphenol blue (w/v)

Stacking and separation gel are composed of the following solutions:

Acrylamid:	30% Acrylamid, 0,8% Bisacrylamid (Roth, Karlsruhe)
Solution A :	1.5 M Tris
	0.4 % SDS (w/v)
	240 mM HCl
	pH 8.8
Solution B:	1.5 M Tris
	0.4 % SDS (w/v)
	500 mM HCl
	pH 6.8
APS:	10% Ammoniumpersulfat (Sigma, Steinheim)
TEMED:	N,N,N',N'-Tetramethylethylendiamin (Sigma, Steinheim)
12,5% Separation gel:	7.5 % Acrylamid
	4.5 ml solution A
	80 µl APS
	15 µl TEMED
	6 ml deionised water
4,5 % stacking gel:	0.9 ml Acrylamid
	1.5 ml solution B
	20 µl APS
	15 µl TEMED
	3.5 ml deionised water

3.2.2.4 Coomasie Brilliant blue protein stain

Coomasie brilliant blue (CBB) is a method to stain protein bands in a polyacrylamid gel. CBB R250 is a dye which unspecificaly binds to proteins and can detect 0.5 µg proteins /cm^2 in the gel matrix. After electrophoresis (chapter 3.2.2.3) the gel was incubated in CBB staining solution for 30 min. Subsequently the gel was incubated in unstaining solution over night to make the blue protein bands visible as unbound dye diffuses from the gel into solution.

Staining solution: 1% Coomasie brilliant blue R250 (w/v) (Fluka. Sigma-Aldrich)
25 % Methanol (v/v)
7.5 % Acetic acid (v/v)

Unstaining solution: 25 % Methanol (v/v)
7.5 % Acetic acid (v/v)

3.2.2.5 Structural analysis by circular dichroism spectroscopy

The secondary structure of a protein can be analysed by circular dichroism (CD)-spectroscopy. The sample is irradiated with left and right circular polarised light. The difference of the absorption between the two polarisation directions is measured, giving the CD spectrum of a sample. Using this, the fractions of, for example, alpha helix conformations, beta sheet conformations or random coils of a molecule can be estimated.

For the measurement the salt concentration was not allowed to be over 10 mM. Therefore, the proteins were transferred into a 10 mM KH_2PO_4 buffer by dialysis using a dialysis tube (Float-A-Lyzer, 1 ml volume, 3500-5000 MWCO, Spectra Por, Roth). For the analysis, a spectrum between 185 and 255 nm was measured at 50 nm/min. The resolution of the data points was 0.2 nm. The sample was measured ten times and averaged. Additionally, the spectrum of the protein free buffer was subtracted. The mean molar ellipticity per amino acid residue was calculated by using the following formula:

$$[\Theta]\mathrm{MRW} \frac{100 \times \theta[m\mathrm{deg}]}{c \times d \times N}$$

Where:
$[\Theta]$MRW is the average residual ellipticity
$\theta[m\mathrm{deg}]$ is the measured ellipticity in mdeg
c is the protein concentration
d is the path length of the cuvette
N is the number of the amino acids

Cuvette: quartz cuvettes, 1mm path length (Hellma, Mühlheim)

Potassium-Phosphate buffer: 10 mM KH_2PO_4, pH 7,4

Stock solution A: 100 mM Potassium phosphate monobasic, PH 4,5
13.6 g KH_2PO_4 / L
Stock solution B: 100 mM Potassium phosphate dibasic, pH9
28.4 g K_2HPO_4 / L

To receive a 100 mM phosphate buffer pH 7.4: mix 19.7 ml of solution A with 80.3 ml of solution B and dilute to 1000 ml.

3.2.2.6 Labelling of proteins with Fluorescein isothiocyanate

Labelling proteins with a florescent dye makes it possible to visualise the protein uptake by cells in flow cytometry (chapter 3.4.2.1) or laser scanning microscopy (chapter 3.2.4.3). In this study, native OVA, thermally processed OVA, AGE-OVA, native BSA and AGE-BSA were labelled with Fluorescein isothiocyanate (FITC).

For the FITC labelling of the proteins, the FluoroTag FITC conjugation Kit (Sigma) was used. FITC conjugation occurs through the free amino groups of proteins, primarily of lysine residues, forming a stable thiourea bond. To accurately analyse the uptake of OVA or BSA samples by mDCs (chapter 3.2.3.6), it was crucial that each sample was labelled with a comparable FITC/protein (F/P) molar ratio.

To determine the amount of dye for the optimal and comparable labelling of all three OVAs or BSA, the FITC conjugation was first performed on a small scale using different concentrations of FITC. The proteins were diluted to 5 mg/ml in 0.1M carbonate-bicarbonate buffer, pH 9.0 provided in the kit. One vial of FITC was reconstituted and diluted as shown in Table 3.8. 50 µl of the FITC solution was added drop wise to 200 µl (1 mg) of protein while gently stirring. The reaction vials were covered with aluminium foil to protect FITC from light during the 2 h of incubation at RT.

The labelled proteins were isolated from unreacted dye using a Sephadex G-25 M column A (maximum sample volume 300 µl) provided in the kit. The column was equilibrated with 12 ml PBS. The reaction mixture was applied to the top of the gel bed. The column was eluted with 2.5 ml of PBS, collecting ten 250 µl fractions. Two yellow bands were clearly visible during elution. The lower band consists of the labelled protein, to upper band is the unbound dye. Of each fraction the absorbance at 280 nm (A_{280}, absorbance maximum of proteins) was measured. Fractions with an A_{280} greater than 0.2 were pooled. The conjugate could be stored at 4°C for a short time. For long term storage lypholisation of aliquots and storage at -80°C was most suitable.

After having determined the optimal FITC concentration for each protein, the labelling could be performed on a larger scale. Here, 5 mg of protein was labelled. All other reagents were scaled up accordingly. To isolate the labelled protein from unconjugated FITC, the larger Sephadex G-25 M column B (maximum sample volume 1.5 ml) provided in the kit was used.

Table 3.9 preparation of FITC solution of different concentrations

Label	0,1 M sodium carbonate-bicarbonate buffer	Reconstituted FITC
20:1	2.0 ml	FITC vial
10:1	0.5 ml	0.5 ml of 20:1
5:1	0.75 ml	0.25 ml of 20:1

Determination of the FITC/Protein Molar ratio

The F/P ratio is defined as the ratio of moles FITC to the moles of protein in the conjugate. To determine this ratio, the absorbance of the conjugate at 280 nm and 495 nm (absorbance maximum of FITC) has to be determined.

The conjugates were diluted 1:5 or 1:10 with the same PBS as was used for the elution from the column (this was also used to measure the blank value). The absorbance at 280 and 495 nm was measured using a quartz cuvette with 1 cm path length. From the absorbance readings (A_{280} and A_{495}) of the conjugate sample, the F/P of the FITC-OVA/BSA conjugate was calculated according to the following equation.

Molar F/P =

$$\frac{MW}{389} \times \frac{A_{495}/195}{A_{280} - [(0{,}35 \times A495)]/E_{280}^{0,1\%}} = \frac{A_{495} \times C}{A_{280} - (0{,}35 \times A495)}$$

Where: $C = \frac{MW \times E_{280}^{0,1\%}}{389 \times 195}$

MW is the molecular weight of the protein

389 is the molecular weight of FITC

195 is the absorption $E_{280}^{0,1\%}$ of bound FITC at 490 nm at pH 13

(0,35 x A_{495}) is the correction factor due to the absorbance of FITC at 280 nm

$E_{280}^{0,1\%}$ is the absorption at 280 nm of protein at 1.0 mg/ml

3.2.3 Cell culture Methods

3.2.3.1 Working environment for cell cultures

All work with cell cultures was performed under a sterile laminar air flow cabinet. The surface of all used equipment was disinfected with Terralin Liquid (Schülke und Mayr, Norderstedt). Only sterile disposable or autoclaved plastics and sterile media and buffers were used.

3.2.3.2 Culture conditions

All cells were cultured in CO_2 incubators at 37°C with 5% CO_2 and 95% relative humidity.

3.2.3.3 Counting of cells

The cell number of a cell suspension was determined using a Neubauer counting chamber (LO Laboroptik, Friedrichsdorf). To count only viable cells, trypan blue, a vital stain, is used to exclude dead cells. Trypan blue only stains dead cells as these cells have an altered cell membrane permeability. The cell suspension is given into the counting chamber and four large squares consisting of 16 small squares are counted. The average number of cells counted in each large square is used to calculate the cell number. As each large square has a volume of 0.004 µl, the cell number is multiplied by 10^4 in addition to the dilution factor. The calculation of the cell number is performed using the formula below:

$$C = N \times D \times 10^4$$

C = cells/ml

N = average of the counted cells

D = dilution factor

10^4 = chamber factor

3.2.3.4 Preparation of bone marrow derived myeloid dendritic cells (mDCs)

For the preparation of bone marrow derived myeloid dendritic cells (mDCs) the bone marrow cells of femur and tibia from mice were used.

The mouse was disinfected by bathing it in 70% ethanol. The skin and the muscle were removed from the hind legs and the legs were carefully dislocated from the hip. Any remaining muscle was removed from the bones and the bones disinfected in 70% ethanol. The end of the bones was cut open and the cells were flushed out with 5 ml RPMI medium using a 5 ml syringe (Braun, Melsungen) and a needle (gage 26, Braun). The cell suspension was transferred into a 15 ml centrifugation tube and centrifuged (1,200 rpm, 4°C, 5 min). After discarding the supernatant, red blood cells were lysed using red blood cell lysis buffer (BD Biosciences). The cells were then washed with RPMI containing 10 % FCS three times.

After washing, the cell pellet was resuspended in DC medium and the cell number was determined (chapter 3.2.3.3). The cells were seeded into cell culture flasks (Greiner, Frickenhausen) at $1x10^6$ cell/ml and granular macrophages colony stimulatory factor (GM-CSF) was added according to Tab. 3.10. The bone marrow cells were cultured for eight days. During the culture period the medium was changed three times. The volume of medium changed for each flask size is indicated in Tab 3.11. The last medium change took place 24 h before the cells were used in an assay.

DC medium: RPMI 1640 medium (Gibco)
+ 10% FCS (Biochrom, Lot. S0115)
+ 1 mM Sodium Pyruvate (Sigma)
+ 10 mM HEPES
+ 100 U/ml penicillin
+ 100 µg/ml streptomycin
+ Glutamine
+ 0.1 mM 2-Mercaptoethanol (3.5 µl 14 M in 500 ml medium)
+ 100 ng/ml GM-CSF (added freshly to culture)

Table 3.10 Seeding conditions for mDC

Flask size	Vol. medium	Vol. GM-CSF	Num. cells
T25	5 ml	25 µl	0.5×10^7
T75	15 ml	75 µl	1.5×10^7

Table 3.11 Volume of medium changed of mDC cultures

Flask size	Vol. medium which is changed	Vol. GMSCF
T25	4 ml	20 µl
T75	10 ml	50 µl

3.2.3.5 Assessment of mDC maturation

In order to asses whether AGE-OVA influences the maturation of mDCs, 1×10^6 mDCs were seeded into wells of a 24 well plate (Falcon, BD Bioscience) and stimulated with 10 µg/ml LPS or 20 µg/ml of either form of OVA for 18 hours. Following the incubation, the levels of IL-10 and IL-12p70 in the culture supernatant were measured by ELISA (chapter 3.2.4.5.2.1). Additionally, the cells were stained with PE conjugated anti-mouse CD11b and APC conjugated anti-mouse CD11c mAbs to gate the mDC population. The expression of co-stimulatory molecules and MHC II was assessed by staining with FITC conjugated monoclonal anti-mouse CD40, CD80, CD86 or MHC II antibody (chapter 3.2.4.2.1). FITC intensity of $CD11b^+CD11c^+$ cells was measured by flow cytometry (chapter 3.2.4.1).

3.2.3.6 Assessment of antigen uptake by mDCs

To asses the uptake of different antigens, 5×10^5 mDCs were seeded into wells of a 24 well plate (Falcon, BD Bioscience). The cells were incubated for 15 min with different concentrations of FITC conjugated antigen (3.2.2.5.1). As antigens, AGE-OVA or AGE-BSA and the corresponding controls were used. One experiment was also performed with FITC conjugated human Transferrin (Trf, Invitrogen). Following the incubation with the FITC conjugates, the cells were stained with PE-conjugated anti-CD11b and APC conjugated anti-CD11c (3.2.4.2.1) antibodies and analysed by flow cytometry (3.2.4.1).

To inhibit galectin-3 or mannose receptor (MR) mediated uptake, 150 mM lactose or 3 mg/ml mannan were used respectively. These were given to the mDCs 30 min prior to the addition of

AGE-OVA or the controls. To elucidate the involvement of the receptor for AGEs (RAGE) or the scavenger receptor class A type I and II (SR-AI/II), mDCs derived from the mice deficient of the respective receptor were used.

3.2.3.7 Isolation of $CD4^+$ and $CD8^+$ T cells

$CD4^+$ or $CD8^+$ T cells were isolated from the spleen or lymph nodes (LN) of mice using the magnetic cell sorting systems from Miltenyi Biotec (Bergisch Gladbach). For the isolation of T cells, the spleen or LN were transferred into a Petri dish (Greiner) with RPMI 1640 medium containing penicillin (100 U/ml), streptomycin (100 µg/ml) and β-mercaptoethanol (0.05 mM). The back of a syringe plunger was used to mechanically remove the cells from the tissue. The cells were passed through a cell strainer (70 µm mesh, BD Biosciences) into a 50 ml centrifugation tube and centrifuged at 1,200 rpm at 4°C for 10 min. The supernatant was removed and the cells transferred into a 15 ml centrifugation tube with 10 ml medium and centrifuged again. Lymph node cells were washed two more times with the medium. In the case of splenocytes, red blood cells were lysed by treatment with lysis buffer (BD Biosciences) for 1 min at room temperature. Following lysis, the cells were washed twice with RPMI containing 5% FCS in addition to the above additives. After washing, 10 ml of MACS buffer (see below) was added to the loosened cell pellet and the cell number was determined (chapter 3.2.3.3).

Isolation of $CD4^+$ T cells

To isolate $CD4^+$ T cells, 10 µl of anti-CD4 (L3T4) MicroBeads were added to $1x10^7$ total cells in 90 µl of MACS buffer. The cells were incubated with the beads at 4°C for 15 min. Then the cells were washed by adding 1-2 ml of MACS buffer per $1x10^7$ cells followed by centrifugation at 1,200 rpm for 10 min. The supernatant was taken off and up to $1x10^8$ cells were resuspended in 500 µl of buffer. To separate the labelled from the unlabelled cells, an appropriate MACS column was used (Tab. 3.12). The column was placed in a MACS separator (magnet). The column was equilibrated with MACS buffer and the cell suspension added to the top of the matrix. When inside the separator, the column matrix provides a magnetic field which is sufficient to retain cells labelled with a MicroBead conjugated antibody. Unlabelled CD4 negative cells pass through the column. These were thoroughly washed out of the column with buffer (6x3ml). The retained cells ($CD4^+$ T cells) are eluted with buffer after the removal of the column from the magnet. The purity of the $CD4^+$ T cells was analysed by flow cytometry (chapter 3.2.4.1). The purity of the $CD4^+$ T cells was routinely around 95%.

Isolation of $CD8^+$ T cells

For the isolation of $CD8^+$ T cells, negative selection was used. To label non-$CD8^+$ T cells, 10 µl of a biotin-antibody cocktail against B cells, natural killer cells, $CD4^+$T cells, dendritic cells, granulocytes, macrophages and erythroid cells was added to $1x10^7$ total cells in 40 µl MACS buffer. The cells were incubated with the cocktail at 4°C for 10 min. Following the labelling, 30 µl

of buffer and 20 µl of anti-biotin MicroBeads per $1x10^7$ total cells were added. The cells were incubated for 15 min at 4°C. The remaining procedure was as for the $CD4^+$ T cell isolation, however here the unlabelled cells in the eluate were those of interest as these were the $CD8^+$ T cells.

MACS buffer: PBS, Dulbecco's, low endotoxin (Biochrom, Berlin)
+ 0.5% BSA (cell culture tested)
+ 2mM EDTA

MicroBead conjugated monoclonal rat IgG2b anti-mouse CD4 (L374) antibody (Miltenyi Biotec)

$CD8^+$ T cell isolation kit, mouse (Miltenyi Biotec). Contains:

1) Cocktail of biotin-conjugated monoclonal antibodies against mouse CD4 (L3T4) (rat IgG2b), CD11b (Mac-1) (rat IgG2b), CD45R (B220) (rat IgG2a), DX5 (rat IgM) and Ter-119 (rat IgG2b).
2) MicroBeads conjugated monoclonal anti-biotin antibody (mouse IgG1)

Table 3.12 MACS columns

Column	Max. number of labelled cells	Max. number of total cells
MS	$1x10^7$	$2x10^8$
LS	$1x10^8$	$2x10^9$

3.2.3.8 Assessment of antigen specific $CD4^+$ T cell activation by mDCs

3.2.3.8.1 Activation of antigen specific $CD4^+$ T cells from naïve transgenic mice with OVA specific TCR

Splenic $CD4^+$ T cells were isolated from OT-2 or DO11.10 mice as described (3.2.3.7.). To evaluate the T cell activation, $4x10^4$ $CD4^+$ T cells were co-cultured in a U-bottom 96 well plate (Nunc, Langenselbold) with $8x10^3$ mDCs and stimulated with 0.2, 20, or 200 µg/ml of either form of OVA or BSA for 24, 48, 72 or 96h. In some experiments, $1.25x10^5$ mDCs were first incubated with either form of OVA for 3 h in a 96 well flat bottom plate (Nunc) and then washed with PBS and fixed with 0.008% glutaraldehyde (Roth). Subsequently 2.5×10^5 $CD4^+$ T cells were added and incubated for 21 h. The concentration of IL-2 in the culture supernatants was measured by ELISA (chapter 3.2.4.5.2.1).

3.2.3.8.2 Activation of antigen specific $CD4^+$ T cells from mice after immunisation with OVA

Wild type BL6 mice were subcutaneously immunised with either form of OVA as described in chapter 3.2.5.5.2. The inguinal (groin) and poplitial (knee) lymph nodes (LN) of immunised mice were removed one week after immunisation. $CD4^+$ T cells were isolated from LNs by magnetic

cell sorting system (chapter 3.2.3.7). In a 96 well flat bottom plate (Nunc), $1.6x10^5$ $CD4^+$ T cells were co-cultured with $4x10^4$ BL6 mDCs and stimulated with 0.2, 20, or 200 µg/ml of AGE-OVA or one of the controls. After 24 h, the IL-2 concentration in the co-culture supernatants was measured (chapter 3.2.4.5.2.1).

3.2.3.9 Activation of antigen specific $CD8^+$ T cells from naïve transgenic mice with OVA-specific TCR

mDCs generated from BL6 mice were pre-matured for 2 h with 10 µg/ml LPS and $1.25x10^5$ cells seeded into a 96 well flat bottom plate. The mDCs were then stimulated with 2, 20, or 200 µg/ml of native, thermally processed or AGE-OVA for 3 h. The mDCs were washed with PBS and fixed with 0.008% glutaraldehyde solution. Splenic $CD8^+$ T cells were isolated from OT-1 mice as described (chapter 3.2.3.7) and 2.5×10^5 T cells added to the mDCs. The cells were incubated for 21 hours. After this time the IL-2 concentration in the supernatants was determined by ELISA (chapter 3.2.4.5.2.1).

3.2.3.10 Assessment of T cell proliferation using carboxyfluorescein succinimidyl ester

For the analysis of cell proliferation, staining of cells with carboxyfluorescein succinimidyl ester (CFSE) is a sensitive and convenient method. CFSE is non-fluorescent and colourless until the acetate groups of the agent are cleaved by intracellular esterases. This process yields the highly fluorescent carboxyfluorescin succimidyl ester. The succimidyl ester groups react with intracellular amines, forming fluorescent conjugates. The formed protein-dye adducts remain in the labelled cells throughout meiosis. Daughter cells inherit the label after cell division, however the fluorescence is diluted with each generation, as shown in Fig 3.1.

In this study, the CellTrace CFSE Cell Proliferation Kit (Invitrogen) was used to trace the proliferation (cell division) of $CD4^+$ T cells isolated from spleens of naïve OT-2 mice. For the staining, T cells were resuspended in pre-warmed PBS/0.1% BSA at $1x10^6$ cells/ml. The cells were incubated with 10 µM CFSE at 37°C for 10 min. The staining was quenched by adding 5 volumes of ice cold culture medium to the cells. The CFSE stained $CD4^+$ T cells were then co-cultured with mDCs upon stimulation with antigens as described in 3.2.3.8.1. After 24, 48, 72 and 96 h of stimulation, the cells were harvested from the wells with EDTA/Trypsin and stained with APC-conjugated anti-mouse CD4 antibody (chapter 3.2.4.2.1). The intensity of CFSE in the CD4 T cells were analysed by flow cytometry (chapter 3.2.4.1).

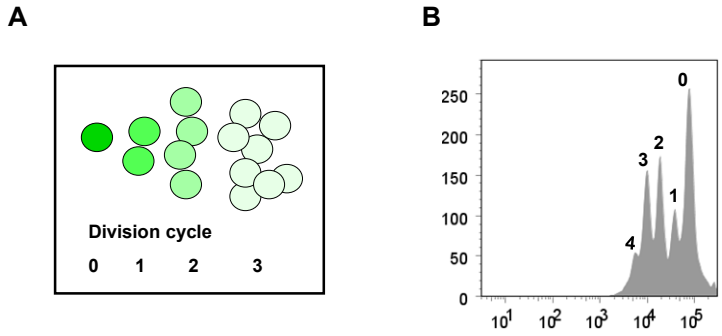

Fig 3.1 Diagrams of dye dilution during proliferation of CFSE stained cells.

A Scheme of the CFSE dilution after each cell division. **B** Example of flow cytometry histogram showing five generations of $CD4^+$ T cells, including the parent generation (0).

3.2.3.11 Assessment of T cell proliferation using tritium [^{3}H] labelled thymidine

Another method for the measurement of cell proliferation is radioactive labelling. This method utilises the principle that cells incorporate radioactive labelled nucleotides into newly synthesised DNA. The level of radioactive labelled nucleotide incorporated by the cells is measured by a scintillation counter. A frequently used nucleotide is thymidine labelled with tritium [^{3}H].

In this study the proliferation of $CD4^+$ T cells isolated from the spleen (chapter 3.2.3.7) of intraperitoneally immunised mice (chapter 3.2.5.5.1) was measured. As antigen presenting cells, (APCs) splenocytes of naïve mice belonging to the same strain as the mice from which the $CD4^+$ T cells were isolated from were used. To suppress proliferation while retaining the antigen presenting ability of splenocytes, the cells were irradiated or treated with mitomycin C (MMC, Sigma). For irradiation, splenocytes received γ-rays from Caesium 137 (1,500 R). MMC is an antibiotic from *Streptomyces caespitosus.* It inhibits DNA synthesis by forming crosslinks between complementary strands of DNA. For the MMC treatment, $1x10^8$ cells were resuspended in 900 µl medium containing 10% FCS to which 100 µl 500 µM MMC was added and then incubated at 37°C for exactly 30 min. The cells where then washed 3 times with medium.

$1x10^5$ $CD4^+$ T cells were co-cultured with $4x10^5$ irradiated or MMC treated splenocytes in 96 well flat bottom plates (Nunc). The cells were stimulated with antigens or Con A (a mitogen which induces unspecific polyclonal activation of T cells). After 72 hours 0.1 µCi of [*methyl*-^{3}H]Thymidine (GE Healthcare) was added to each well. After further 20 h, the cells were harvested.

To harvest the cells, a harvester (Inotech) with vacuum pump was used to trap the cellular DNA on a glass fibre membrane (Perkin Elmer, Rodgau). Medium and unincorporated thymidin was removed by extensive washing with water followed by a wash with 70% ethanol. Once the membranes were dry, a scintillation matrix (Perkin Elmer) was melted on. The level of tritium was then determined using a gamma counter (1450 Microbeta, Perkin Elmer).

To compare the $CD4^+$ T cell proliferation of different samples, the stimulation index was calculated. This index takes into the account the proliferation (as counts per minute) in co-cultures

which were treated with medium only. The Stimulation index is calculated by using the following formula:

Stimluation index = (cpm Ag – cpm NC)/cpm NC

Where:
cpm: counts per minute
cpm Ag is the cpm measured in a well where the cells where stimulated with antigen
cpm NC is the cpm measured in a well where the cells were treated with medium only

3.2.4 Immunological methods

3.2.4.1 Flow cytometry

Flow cytometry is a versatile method which simultaneously measures several characteristics of particles, usually cells, as they flow in a stream through a beam of light. The properties, which can be measured by flow cytometry, are size, granularity and relative fluorescence intensity of the cells. The properties are defined using an optical-to-electronic coupling system which records how a cell scatters laser light and emits fluorescence.

For the analyses, the cells are taken up by a capillary and singly pass a laser beam. Once the cells pass through the laser intercept, they scatter laser light and additionally, if fluorescent molecules are present on the cells, they fluorescence. The forward scatter of the light (FSC) is a measurement of light deflection of a low angle and is dependent on the size of the cell. The sideward scatter (SCC) is a measurement of the light refraction in a right angle and this is dependent on the granularity of the cell, the size and structure of its nucleolus and the amount of vesicles in the cell. With these two parameters, cell populations may be crudely differentiated.

The application of various fluorescent dyes is vital for the flow cytometric analysis. For instance, to identify a particular cell type, the cells are labelled with fluorescent conjugated antibodies against cell surface markers which are characteristic for the cells (e.g. proteins of the CD-classification such as CD11c). The scattered and fluorescent light is steered to the appropriate detectors by a combination of beam splitters and filters. The fluorescent signals are converted to electronic signals in proportion to the fluorescent strength.

By combining the information from the FSC/SSC and the fluorescence intensity of the cells stained with specific markers, identification of target cells and a measurement of the frequency of the target cells in the total cell population is facilitated. The simultaneous use of different fluorophores is possible because even if the fluorophores are excited at similar wavelengths, they have emission spectra which are characteristic for each fluorophore. Overlaps in these spectra can be compensated prior to the sample measurement.

In this study, flow cytometric analysis was performed using an LSRII (Becton Dickinson) in connection with the FACS-Diva Software. The data was analysed using FlowJo software (Tab. 3.7).

3.2.4.2 Antibody staining of cells for flow cytometry

3.2.4.2.1 Direct staining of cell surface molecules for flow cytometric analysis

Cell surface molecules can be labelled directly when fluorescent dye conjugated antibodies against the target molecules are available and staining with these antibodies is strong enough for detection (i.e. does not need to be amplified with an additional step). In this study, the co-stimulatory molecules CD40, CD80, CD86 and MHC II and RAGE on mDCs as well as CD4 on T cells were stained with the direct staining method. To identify the mDCs by flow cytometric analysis CD11c and CD11b were also routinely stained with fluorophore conjugated antibodies specific for these markers.

For the staining, mDCs were harvested from the cell culture plates using a small cell scraper. To stain the $CD4^+$ T cells of co-culture assays, the cells were detached from the wells using EDTA/Trypsin (2 min at 37°C). All cells were spun down (1,200 rpm, 5 min, 4°C) and then washed with FACS buffer. Typically, 0.5 – 1.0 x 10^6 cells in a total volume of 50 to 100 µl (FACS buffer) were incubated with the antibody at 4°C for 20 min. Subsequently, the cells were washed with FACS buffer. The pellet was then resuspended in FACS buffer containing 0.5% PFA and analysed by flow cytometry on the same day.

3.2.4.2.2 Indirect staining of cell surface molecules for FACS analysis

MR, SR-A type I, Galectin-3 and RAGE on mDCs were labelled with an indirect staining method using unlabelled primary unlabelled antibodies. mDCs (2.5 x 10^5 cells) were stained with antibodies specific for MR, SR-A type I, Galectin-3 or RAGE. To prevent unspecific binding of the antibodies to FcγRI (IgG receptors) on the cell surface, the cells were treated with 100 µl blocking buffer for 30 min at 4°C. To this suspension the purified monoclonal rat anti-mouse MR, SR-AI or galectin-3 antibody was given. The cells were incubated with the antibodies for 30 min at 4°C and then washed with PBS. This was followed by 20 min incubation at 4°C with an AlexaFluor 488 goat anti-rat IgG antibody in blocking buffer. Following receptor staining, the cells were washed three times with FACS buffer. Subsequently CD11b and CD11c were then stained as described (chapter 3.2.4.2.1).

EDTA/Trypsin (PEI media kitchen)

FACS buffer: PBS pH 7.2
+ 1% BSA
+ 20mM EDTA
+ 0.75 % (w/v) Sodium azide
+ 0.75 % (w/v) Sodium azide

Fixation solution: 1g of Paraformaldehyde (PFA) are solved in 100 ml PBS at 70°C

Blocking buffer: PBS pH 7.2
+ 5% normal goat serum
Filtered

3.2.4.3 Cell analysis by confocal laser scanning microscopy (LSM)

Confocal laser scanning microscopy is a technique employed to obtain high resolution images of a sample. A conventional light microscope returns an image which is obtained from a sample slice which is as thick as the light will penetrate. The major advantage of a confocal microscope is that it acquires an image from one selected depth. This makes it very suitable for studying the (co)-localisation of proteins in cells because there is no superimposing of image information from multiple depths in the specimen which may lead to false co-localisations. In LSM, a laser beam is focused into a small part of the sample by an object lense. In the specimen, proteins are usually labelled with fluorophore conjugated antibodies or are directly labelled with a fluorescent dye. Emitted light is recollected by the same object lense as is used to focus the laser beam, then passes through a pinhole and before it reaches a detector, usually a photomultiplier or an avalanche photodiode. Excitation and detection focus are confocal to each other. Information outside of the focus depth is excluded in two ways; the light intensity outside of the focus is very weak and additionally it is blocked by the pin hole. The images are acquired point by point, requiring a reconstructing with a computer.

3.2.4.3.1 Cell preparation for LSM

mDCs ($5x10^5$ cells) were seeded into the wells of a 24 well plate (Falcon) containing sterilised round cover slips (12 mm in diameter, Roth) and left to adhere for 1 h. The medium was then removed and replaced with fresh medium containing 10 µg/ml of AGE-OVA. The cells were then incubated with the antigen for 5 to 60 min at 37°C. Subsequently the cells were washed with PBS. In the following staining procedure the cells were washed with PBS (3 times, 10 min) between each step. During all incubations, the cells were kept in the dark at room temperature and were slowly agitated on a seesaw shaker. After washing, cells were fixed with 4% PFA in PBS for 20 min. The cells were then blocked and permeabilised for 1 h. This was followed by incubation with primary antibody (purified anti-SR-A, anti-MHC class II, anti-Lamp1 or anti-EEA1 antibody) for 1 h. Subsequently, the cells were incubated with the appropriate secondary antibody for 30 min. As a counter stain and to facilitate the identification of individual cells under the microscope, the cell nuclei were stained with DAPI (Invitrogen) for 7 min. Following a rinse with deionised water the cover slips were mounted onto SuperFrost microscope slides (Roth) with Fluorescence mounting medium (Dako, Hamburg). The slides were kept in the dark at 4°C until analysis with a laser

scanning microscope LSM 510 (Carl Zeiss, Jena). The images were analysed with the Zeiss LSM Image browser 4.2.

Fixation solution (4% PFA):
16% PFA (Pierce Bonn) diluted with PBS pH 7.2

Blocking/permeabilisation buffer:	PBS, pH 7.2 + 5% skimmed milk powder + 1% normal mouse serum (own production) + 0.1 % Triton X 100 + 1 µg/ml normal mouse IgG (Santa Cruz)
Assay buffer:	PBS, pH 7,2 + 1% normal mouse serum (own production) + 0.1 % Triton X 100

3.2.4.4 Enzyme linked immunosorbent assay (ELISA)

The enzyme linked immunosorbent assay (ELISA) employs antibodies as an exceedingly specific analytical reagent for the detection and quantification of proteins or other antigens with which less than 1 ng of protein can be detected.
There are two types of ELISA commonly used. One is the indirect ELISA, for which the antigen is adsorbed directly to the microtitre plate. The second type is the sandwich ELISA, for which the antigen is captured by an antigen specific antibody which is attached to the plate. In both types the antigen is detected by a (second) antibody. This antibody can be conjugated with an enzyme or it may be unlabelled and is itself detected by a further, enzyme conjugated, antibody. The enzyme reacts with a colourless substrate to give a coloured product. Alternatively, biotin molecules may be linked to the detection antibody to which a strepdavidin coupled enzyme can bind, this additional step has the advantage of amplifying the signal. The optical density of the coloured product indicates the quantity of the antigen or antibody of interest in the samples.

For all ELISAs, the wells were washed three times with washing buffer between each step to remove any proteins or antibodies that were not specifically bound. Incubations at room temperature were performed on an orbital shaker at 80 rpm.

3.2.4.4.1 Indirect ELISA

3.2.4.4.1.1 Measurement of mouse serum Immunoglobulins

To detect antigen (OVA or rAra h 2) specific antibodies in mouse sera, microtitre plates (MaxiSorp F96; Nunc, Langenselbold, Germany) were coated with antigens diluted in coating buffer at 4 °C

over night. The OVAs were coated at 100 µg/ml using 100 µl/well, whereas Ara h 2 was coated at 5 µg/ml using 50 µl/well. The plates were then blocked with 2 % BSA in PBS for 1 h at RT. After blocking, mice sera diluted in assay buffer were applied to the plates and incubated for 2 hours at RT or over night at 4°C. Serum IgG antibodies bound to the antigen in the wells were detected with horse reddish peroxidase (HRP) conjugated rabbit anti-mouse IgG1 or IgG2a antibodies (Zymed), diluted 1:4000 in assay buffer. Serum IgE antibodies were detected by a biotin conjugated anti-mouse IgE antibody (BD Biosciences), diluted 1:1000. The plates were incubated with the secondary antibodies for one hour at RT. For the detection of serum IgE, the plates were further incubated with a streptavidin-HRP conjugate diluted 1:2000 in assay buffer for 30 min at RT. Subsequently, 100 µl of substrate solution were added to the plates, and incubated at RT for 10 to 30 min. The substrate used for the peroxidase was 3, 3', 5, 5'-tetramethylbenzidine (TMB, BD Biosciences). The reaction was stopped with 50 µl of stopping solution. The absorption of each well at 450 nm was measured using an ELISA reader.

3.2.4.4.1.2 Detection of Maillard reaction products

AGEs were detected using an indirect ELISA system. The procedure was as described in chapter 3.2.4.4.1.1. The different OVA samples were added to the first well at 10 mg/ml in 100 µl coating buffer and then a tenfold serial dilution in the subsequent wells was performed. AGE structures were detected with mouse monoclonal antibody (mAb) against CEL, pyrralin or GA-pyridine or rabbit polyclonal antibodies against CML (each used at 0.1 µg/ml). Subsequently, the plates were incubated with HRP conjugates of either anti-mouse IgG (diluted 1:4000, GE Healthcare, Munich, Germany) or anti-rabbit IgG antibodies (diluted 1:5000, Sigma-Aldrich). The substrate was applied to the plates as described in 3.2.4.3.1.1.

3.2.4.4.1.3 Verification of comparable FITC/protein ratio on labelled proteins

To verify whether the FITC-conjugated proteins were labelled with a comparable F/P molar ratio, an ELISA was performed in addition to the calculation based on the absorption at 280 and 495 nm (chapter 3.2.2.6). The procedure was almost identical to that described above (chapter 3.2.4.4.1.1). The FITC conjugated proteins were added to the first well at 100 µg/ml in 100 µl coating buffer and then diluted 1:10 in the consecutive wells. FITC on the proteins was detected by an HRP-conjugated mouse anti-FITC antibody, diluted 1:5000. Subsequently, the substrate was applied to the plates as described in 3.2.4.4.1.1.

3.2.4.5.2 Sandwich ELISA

3.2.4.5.2.1 Sandwich ELISA for the detection of cell supernatant cytokines

Sandwich ELISAs were performed to measure the concentration of cytokines in cell culture supernatants. Microtitre plates were coated at 4°C over night with a primary antibody (eBioscience or Biolegend) against the cytokine of interest, diluted 1:1000 in coating buffer. After blocking, cell culture supernatants were added to the wells. To quantify the cytokine concentration

in the supernatant, recombinant cytokines (Tab. 3.3) with known concentrations were added to the plates in different dilutions as standards. The supernatants and standards were incubated for 2 h at RT or at 4°C over night. The plates were then incubated with a biotin-conjugated secondary antibody (eBioscience or Biolegend) specific for the cytokine, diluted 1:1000 and subsequently with a streptavidin-HRP conjugate, diluted 1:4000. The substrate was then applied to the plates as described in 3.2.4.4.1.1.

Micro titre plate: 96 well, Maxisorb (Nunc, Langenselbold)

Coating buffer (50mM Sodium-carbonate puffer):	1.696g Na_2Co_3 2.856g $NaHCo_3$ ad 1L deionised water pH 9.6 (does not need to be adjusted)
Washing buffer:	PBS pH 7.2 + 0.05 % Tween 20
Blocking buffer:	PBS pH 7.2 + 2% BSA
Assay buffer:	PBS pH 7.2 + 0.05% Tween 20 + 1% BSA

Tetramethylbenzidine (TMB) substrate reagent set (BD Biosciences)

Stopping solution:	1N H_2SO_4

3.2.5 Animal experimental methods

All mice experiments were performed according to the guidelines of animal welfare of the state and the PEI.

3.2.5.1 Housing of mice

Gene modified mice were bred under specific pathogen free (SPF) conditions in the S1 area of the “Zentrale Tierhaltung” of the PEI. For breeding, one male and one female were kept in a cage. For experiments, mice were transferred into the experimental mouse room. In the room, mice were housed in cages with single cage ventilation in same sex groups of up to 5 individuals.

3.2.5.2 Short term mouse anaesthesia

For short term anaesthesia, 1-2 ml of Isofluran were given into a glass with lid and covered with a bit of tissue. The mouse was put into the glass and carefully watched until anaesthesia set in. After taking the mouse out of the glass, it remained in anaesthesia for a further 30 sec.

3.2.5.3 Mouse euthanasia

The mice were anaesthetised with Isofluran (chapter 3.2.5.2) and then sacrificed by cervical dislocation.

3.2.5.4 Blood harvest from the tail vain

The levels of antigen specific antibodies in sera of immunised mice are important indicators for the antigenicity of protein. The sera were won by harvesting blood from the lateral tail vain of immunised mice. To facilitate an easier harvest of blood, the mice were warmed up under an infrared light for 20 sec. The mouse was then fixed in a tube which allowed easy access to the tail. To draw blood, the tail was carefully cut with a thin razor blade and the blood collected.
After the harvest, the blood was kept at room temperature for 30 min to inactivate complement factors. Then it was centrifuged at 4,500 rpm for 15 min at 4°C to separate the serum from the red blood cells. The sera were kept at -20°C until analysed by ELISA (3.2.4.4.1.1).

3.2.5.5 Immunisation

For this study, several immunisation methods were used. For all experiments an adjuvant was used because proteins alone generally induce only weak immune responses in animal models. Adjuvants are non-specific stimulators of the immune response, making a prospective antigen more immunogenic. The effect of an adjuvant may be the result of one or more mechanisms of action: a depot effect, an antigen presenting effect, via CD8 cytotoxic lymphocyte induction or by an immune activation/modulation effect (Cox and Coulter, 1997).

3.2.5.5.1 Intraperitoneal immunisation

For intraperitoneal immunisation (ip), aluminium hydroxide (Imject Alum, Pierce, Bonn) was used as an adjuvant. Aluminium salts adsorb antigens and provide a depot effect (Gupta and Rost 2000). The depot effect is a classic mechanism in which the adjuvant protects the antigen from dilution, rapid degradation and elimination by the host. The intact antigen is localised in the immunised tissues and released slowly, resulting in prolonged exposure of the immune system to the antigen. This results in a continued stimulation of antibody producing cells, leading to a production of high levels of antibodies by the host. Additionally, aluminium adjuvants induce Th2 dominated immune response (Lindbald 2000).
The alum was added to the antigen in PBS and the mixture incubated at RT for 30 min, rotating slowly. For the immunisation, 200 µl PBS containing the antigen (either of the OVAs or rAra h 2) adsorbed to Alum was injected into the peritoneum of mice. Different immunisation protocols were used, refer to the respective results section for these. Seven days after each immunisation, blood

was harvested from the tail to determine the antibody levels in the serum. One week after the last immunisation, the mice were sacrificed and the splenicT cell activation analysed (3.2.3.11).

3.2.5.5.2 Subcutaneous immunisation

Subcutaneous (sc) immunisation was used as a method to obtain high frequency of antigen-specific $CD4^+$ T cells from inguinal (groin) and popliteal (knee) lymph nodes. For the subcutaneous immunisation TiterMax Gold® Adjuvat (Sigma) was found to be an effective adjuvant. This is a water-in-oil emulsion. Emulsions are commonly used depot adjuvants. TiterMax Gold® consists of squalene (a metabolisable oil), an emulsifier, a microparticulate silica and a block copolymer. The pronounced adjuvant activity of the high molecular weight copolymers presumably results through antigen presentation, complement activation, chemotactic properties and macrophage activation (Howerton et al., 1990; Hunter and Bennett 1984).

To prepare the immunisation solution, AGE-OVA or one of the controls was diluted in PBS to 2 mg/ml. This antigen solution was then mixed at a ratio of 1:1 with TiterMax Gold®, adding the antigen solution drop wise and mixing thoroughly in between additions to facilitate the preparation of a homogenous emulsion. The emulsion was then taken up into a 1 ml syringe (Braun) with a needle (25 G, Braun). Each mouse subcutaneously received 100 µg of OVA. One week after the immunisation, the mice were sacrificed and the poplitial and inguinal lymph nodes removed to assess the activation of the $CD4^+$ T cells present in these organs (chapter 3.2.3.7.2).

4 Results

4.1 Analysis of glycated ovalbumin (AGE-OVA)

To investigate the influence of glycation by the Maillard reaction on the immunogenicity of food allergens, OVA was used as a model allergen. First, OVA glycated by the Maillard reaction (AGE-OVA) was prepared by incubation of OVA with glucose for six weeks (chapter 3.2.2.1.1). The glycation structures on AGE-OVA were analysed by ELISA using antibodies recognising the AGE structures N-ε-cyrboxymethyllysine (CML), N-ε-cyrboxyethyllysine (CEL), Glycolaldehyde-pyridine (GA-pyridine) or pyrraline (Fig. 4.1). CML, CEL and GA-pyridine were detected on AGE-OVA, whereas pyrraline was not detected by the used ELISA (Fig. 4.2). A reversed-phase HPLC analysis (Henle 1991, Henle 1997) of AGE-OVA confirmed the presence of high levels of CML (Tab. 4.1, performed by Ms Anne Wellner, TU Dresden). Fructose-lysine, which is an intermediate of the Maillard reaction, was also detected by this method. The reversed-phase HPLC analysis was sensitive enough to detect minimal amounts of pyrraline (Tab. 4.1). The respective glycation structures were not detected on native OVA or OVA thermally processed under the same conditions as AGE-OVA but without glucose (thermally processed OVA) (Fig. 4.2). Tab. 4.1 also shows that the amount of free lysine was considerably reduced after OVA had been glycated by the Maillard reaction. Only 22.1 ± 2.0mmol free lysine/100g protein remained in AGE-OVA as opposed to 50.9 ± 0.1 mmol free lysine/100g protein found in native OVA. Therefore, over 50% of the free lysine residues found in OVA had been modified during thermal processing with glucose. Importantly, different batches of AGE-OVA produced and used for this study were glycated to a similar extent with the same AGE structures (Fig. 4.2).

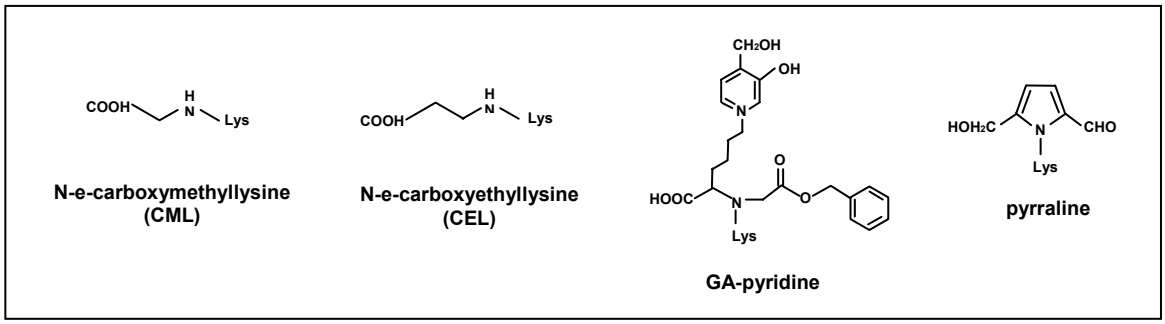

Fig. 4.1 Structural formula of glycation structures.

Structural formulas of the advanced glycation end products which can be detected by specific antibodies in the ELISAs used. Lys: lysine residues of protein

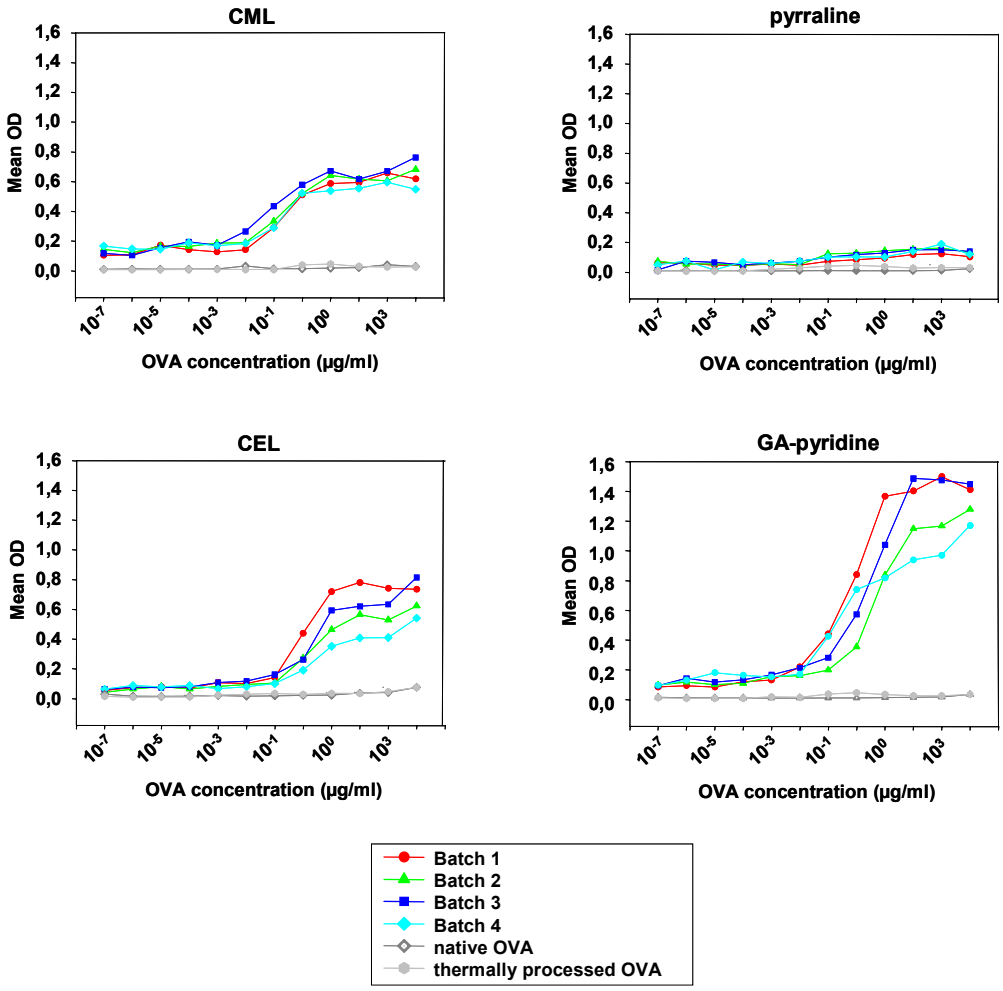

Fig. 4.2 Detection of glycation structures on AGE-OVA produced by the Maillard reaction.
AGE-OVA was prepared by incubating 1 mM OVA with 1 M Glucose at 50°C for 6 weeks. The presence of glycation structures was verified by ELISAs performed with antibodies against different AGE structures. The coloured curves show four representative lots of AGE-OVA. Native OVA and OVA thermally processed without glucose were used as controls.

Table 4.1 Analysis of AGE-OVA

Fructose-lysine in glycated ovalbumin [mmol/100 g protein]	10.7 ± 0.6
Pyrraline in glycated ovalbumin [µmol/100 g protein]	29.4 ± 1.2
Pentosidine in glycated ovalbumin	n.d.
N-ε-carboxymethyllysine in glycated ovalbumin [mmol/100 g protein]	4.55 ± 0.49
Free lysine in glycated ovalbumin [mmol/100 g protein]	22.1 ± 2.0
Free lysine in non-glycated ovalbumin [mmol/100 g protein]	50.9 ± 0.1

The glycation structures found on AGE-OVA and loss of free lysine due to glycation. Analysis performed by A. Wellner, TU Dresden, using biochemical methods.

Besides the modification with glycation structures, any conformational change of OVA after the thermal incubation with or without glucose would be of importance, as this may also alter its immunogenic potential. To analyse the 2D-structures of AGE-OVA and the control OVAs, CD

spectroscopy was performed (chapter 3.2.2.5). The mean molar elipticity and the exact protein concentration were included for the evaluation of the spectra. The spectra measured for native, thermally processed and AGE-OVA were virtually identical (Fig 4.3, A). They have minima at 208 and 220 nm, characteristic for a mostly α-helical protein. The results indicate that the 2D-structure of OVA was not affected by thermal incubation either with or without glucose.

An SDS-PAGE was also performed to examine the nature of AGE-OVA. Native OVA is found in the gel at around 45 kDa (Fig. 4.3, B, *lane 1*), the 35 kDa band below the main band is only very faint. The band at 35 kDa would probably stem from a fraction of OVA which has degraded. For thermally processed OVA, the main band at 45 kDa is reduced and the 35kDa band has increased in intensity (*lane 2*). This suggests that minor degradation occurred during thermal treatment of OVA. The appearance of OVA in SDS-PAGE is completely changed by glycation. AGE-OVA is seen as a smear (*lane 3*) where the majority of the protein is found above 45 kDa and becomes very diffuse indicating, as expected, a significant modification of the protein by the Maillard reaction. This is in accordance with the data in Fig. 4.2.

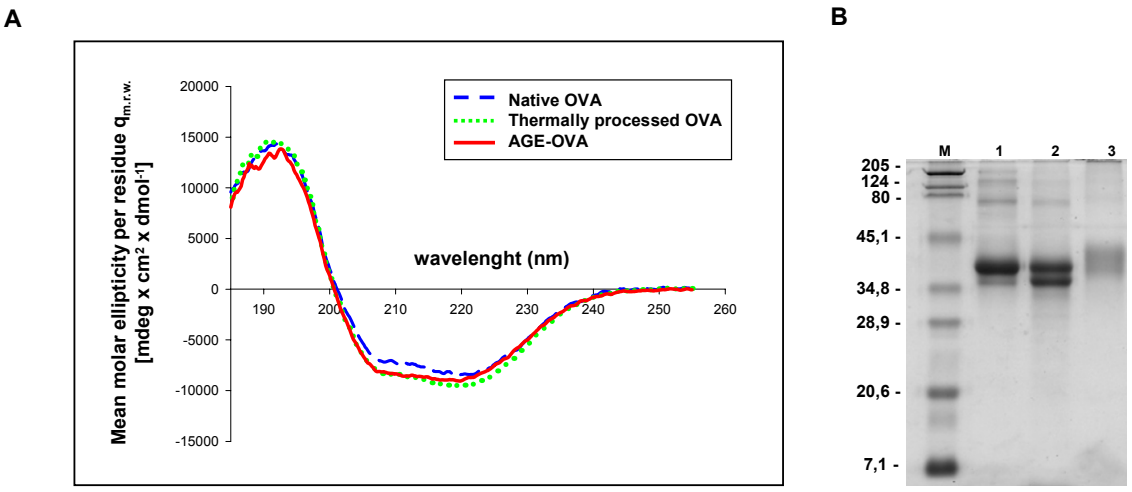

Fig 4.3 Analysis of AGE-OVA by CD spectroscopy and SDS-PAGE

The impact of thermally processing OVA at 50°C for 6 weeks with and without glucose on the 2D-structure and gel running properties of OVA was analysed by CD spectroscopy and SDS-PAGE. **A** CD spectrum of native, thermally processed and AGE-OVA was measured. **B** AGE-OVA and the controls were loaded on a 12.5% Tris-gylcine gel and stained with Coomasie brilliant blue. M: molecular weight standard, Prestained Broad Range (BioRad), *1*: native OVA; *2*: thermally processed OVA; *3*: AGE-OVA.

4.2 Analysis of glycated bovine serum albumin (AGE-BSA)

For some experiments bovine serum albumin (BSA) was used as a control protein. For this purpose glycated BSA (AGE-BSA) and thermally processed BSA were prepared in the same way as OVA (chapter 3.2.2.1.1).

As was observed for AGE-OVA, CML, CEL and GA-pyridine but not pyrraline were detected on the AGE-BSA but not on BSA thermally processed without glucose (Fig. 4.4 A). In SDS-PAGE,

native BSA is found at around 67 kDa (Fig. 4.4 B, *lane 1*). After thermal processing without glucose, the majority of the protein was still found at 67 kDa (*lane 2*). As a result of the modifications by the Maillard reaction, there is no distinct band in the lane where AGE-BSA was applied (*lane 3*) but rather a diffused smear, similar to what was observed for AGE-OVA (Fig. 4.3, B *lane 3*). Collectively the analysis of AGE-OVA and AGE-BSA indicates that these proteins would possess similar glycation structures.

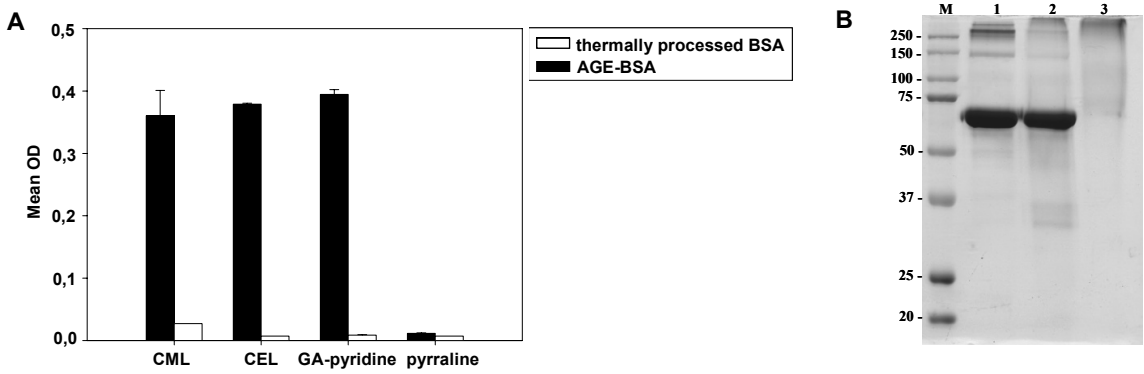

Fig. 4.4 Detection of glycation structures on AGE-BSA produced by the Maillard reaction.
AGE-BSA was prepared by incubating 1mM BSA with 1M Glucose at 50°C for 6 weeks. **A** The formation of glycation structures was verified by ELISA using antibodies against different AGE structures. BSA thermally processed without glucose was used as a control. **B** Native, thermally processed, or AGE-BSA were loaded on a 12.5% Tris-gylcine gel and stained with Coomasie brilliant blue. M: molecular weight standard, Prestained Broad Range (BioRad), *1*: native BSA; *2*:: thermally processed BSA; *3*: AGE-BSA.

4.3 Influence of glycation by the Maillard reaction on the T cell immunogenicity of OVA *in vitro*

$CD4^+$ T cells are key players in the onset and maintenance of allergic diseases. It was therefore of interest how the glycation of OVA influences its T cell immunogenicity.

4.3.1 Activation of OVA specific $CD4^+$ T cells *in vitro* by glycated OVA

4.3.1.1 Comparing the T cell activation ability of AGE-OVA and the unglycated OVAs

First, the T cell immunogenicity of AGE-OVA was examined using an *in vitro* cell culture system. T cells from two transgenic mouse strains, DO11.10 (BALB/c background) and OT-2 mice (BL6 background), were used. Around 80-85% of the $CD4^+$ T cells from these transgenic mice posses a T cell receptor (TCR) which is specific for OVA (peptide 323–339, one letter coded amino acid sequence: ISQAVHAAHAEINEAGR). However, the TCRs of the two strains recognise different haplotypes of the MHC class II molecule. TCRs of OT-2 cells recognise a complex of the peptide and a I-Ab molecule, while TCRs of DO11.10 cells recognise a complex of the peptide and a I-Ad

molecule. Furthermore, the TCRs of these $CD4^+$ T cells bind to some different amino acid residues of the OVA peptide 323–339. In this experiment the $CD4^+$ T cells of OT-2 mice were mainly used because other genetically modified mice used in this study were also generated on a BL6 background. The experiment here was also performed with DO11.10 $CD4^+$ T cells in order to elucidate whether the genetic background may have an influence on the T cell activation by AGE-OVA.

First, $CD4^+$ T cells isolated from the spleen of OT-2 mice were co-cultured with mDCs and stimulated with either AGE-OVA or one of the controls, native and thermally processed OVA, at 2, 20 or 200 μg /ml (chapter 3.2.3.8.1). Upon activation, $CD4^+$ T cells produce the cytokine IL-2. The concentration of IL-2 in the culture supernatant can easily be measured by ELISA and therefore is a valuable marker to assess activation of T cell.

After 24 h, the highest concentration of IL-2 was found in the culture supernatants of T cells stimulated with AGE-OVA (Fig 4.5). It was of interest whether this enhanced T cell activation by AGE-OVA could also be seen at later time points or whether the level of activation becomes aligned for all three forms of OVA. As shown in Fig. 4.5, the level of IL-2 in the culture supernatants was much higher when the T cells were stimulated with AGE-OVA than when the cells were stimulated with the controls also after 48, 72 and 96 hours of co-culture. This result indicates that AGE-OVA activates OVA-specific $CD4^+$ T cells much stronger than native or thermally processed OVA.

To examine whether the genetic background of the mice influences the assessment of the T cell immunogenicity of AGE-OVA, $CD4^+$ T cells isolated from DO11.10 mice were co-cultured with mDCs and stimulated with either form of OVA. As shown in Fig. 4.6, compared to the control OVAs, AGE-OVA induced a higher level of IL-2 production after 24 h in the co-culture of DO110.10 cells with mDCs. This was the case for all concentrations tested and also for incubation times of 48, 72 and 96 hours (data not shown).

Together these results indicate that the glycation of OVA by the Maillard reaction enhances the $CD4^+$ T cell immunogenicity of the allergen. Additionally, the enhanced $CD4^+$ T cell activation by AGE-OVA was found to be independent of the mouse strain used as a source of the OVA specific $CD4^+$ T cells.

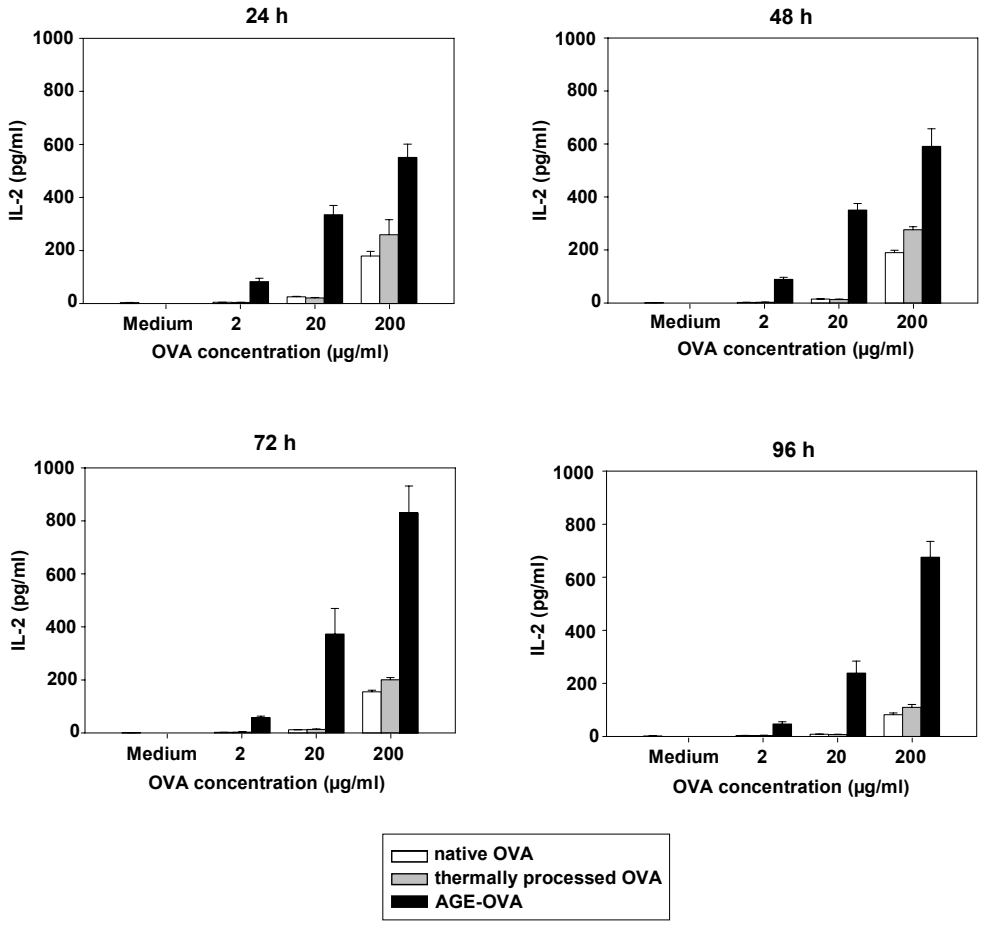

Fig 4.5 Assessment of OT-2 CD4$^+$ T cell activation induced by AGE-OVA

OVA-specific CD4$^+$ T cells isolated from OT-2 mice and BL6 mDCs were co-cultured and stimulated with native, thermally processed or AGE-OVA for 24, 48, 72 or 96 hours. The IL-2 concentration in the culture supernatants was determined by ELISA. Error bars signify the SD of culture triplicates and ELISA duplicates.

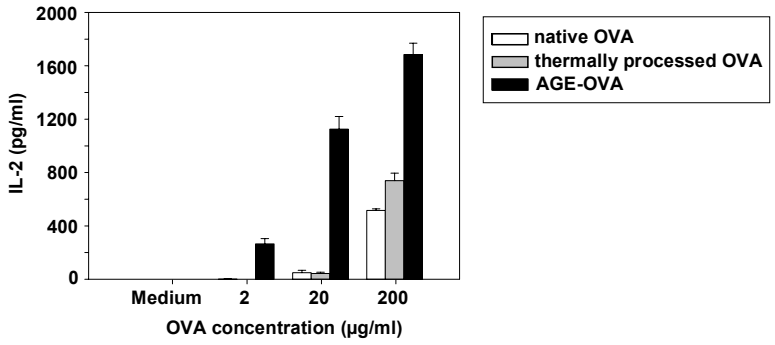

Fig 4.6 Assessment of DO11.10 CD4$^+$ T cell activation induced by AGE-OVA

OVA specific CD4$^+$ T cells isolated from DO11.10 mice were co-cultured with BALB/c mDCs and stimulated with, native, thermally processed or AGE-OVA for 24 h. The IL-2 concentration in the culture supernatants was measured by ELISA. Error bars signify the SD of culture triplicates and ELISA duplicates.

4.3.1.1.1 Verification of mDC involvement in CD4$^+$ T cell activation by AGE-OVA

It was viable to verify whether the CD4$^+$ T cells could be directly activated by AGE-OVA or whether mDCs are required to mediate the enhanced T cell activation. Therefore CD4$^+$ T cells isolated from DO11.10 mice were cultured with or without mDCs and stimulated with native, thermally processed or AGE-OVA.

As shown in Fig. 4.7, the amount of IL-2 produced by CD4$^+$ T cells cultured without mDCs but stimulated with one of the OVAs at 20 or 200 μg/ml was extremely low when compared to the amount of IL-2 produced by the T cells co-cultured with mDCs. This clearly indicates that mDCs play a critical role in the enhanced OVA specific CD4$^+$ T cell activation induced by AGE-OVA.

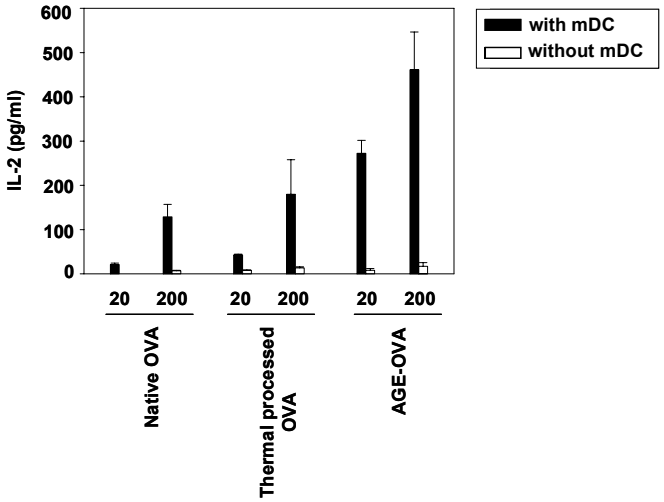

Fig 4.7 Involvement of mDCs in OVA specific CD4$^+$ T cell activation by AGE-OVA

CD4$^+$ T cells isolated from DO11.10 mice were cultured with or without BALB/c mDCs. The cells were stimulated with AGE-OVA or the controls for 24 h. The concentration of IL-2 in the culture supernatants was determined by ELISA. Error bars signify the SD of culture triplicates and ELISA duplicates.

An additional experiment was performed to elucidate whether antigen modified with Maillard glycation structures have the ability to activate CD4$^+$ T cells directly. For this experiment, AGE-BSA was used. The co-cultures of OVA specific CD4$^+$ T cells and mDCs were stimulated with native, thermally processed or AGE-BSA at 2, 20, and 200, μg/ml. The IL-2 concentration in the culture supernatants was below the detection level of the ELISA (2 pg/ml, data not shown). These results indicate that the T cells are only activated via their TCR which recognises the OVA peptide 323–339 presented on MHC class II molecules of mDCs. The glycation structures alone could not activate the CD4$^+$ T cells directly.

4.3.1.2 Assessment of T cell activation by selectively glycated OVA

The AGE-OVA used in this study has a variety of glycation structures, some of which could be detected by ELISA or biochemical methods (chapter 4.1). By selectively modifying OVA with glycation structures, it could be verified that glycation of the allergen does indeed have a significant influence on its T cell immunogenicity. Furthermore, it was of interest to investigate

which of the AGE structures may be responsible for the enhanced $CD4^+$ T cell activation induced by AGE-OVA. The lysine residues of OVA were selectively modified with CML or CEL by Ms Anne Wellner, TU Dresden. Samples in which different numbers of lysine residues were modified were prepared: CML-OVA 1 or 2, modified with 36.77 and 1.64 mmol CML/100g OVA, respectively and CEL-OVA 1, 2 or 3, modified with 45.17, 26.0 and 1.35 mmol CEL/100g OVA, respectively.

$CD4^+$ T cells isolated from the spleen of OT-2 mice were co-cultured with BL6 mDCs, and stimulated with CEL- or CML-OVA (3.2.3.8.1). As comparison, the T cells were again stimulated with native, thermally processed or AGE-OVA. The AGE-OVA was analysed by Ms Anne Wellner and was found to be modified with approximately 4.55 mmol CML/100g OVA (Tab. 4.1).

OVA selectively modified with CEL or CML induced a higher T cell activation than native and thermally processed OVA. (Fig. 4.8). For CEL-OVA as well as CML-OVA the amount of IL-2 produced by the stimulated T cells was dependent on the level by which OVA was modified. When higher levels of CML or CEL were present on OVA, more IL-2 was produced by the stimulated T cells. CEL-OVA 1 and CML-OVA 2, the selectively modified OVA with high levels of CEL and CML respectively, induced a comparable amount of T cell activation as AGE-OVA. These results suggest that selective modification with either CEL or CML alone significantly enhances the $CD4^+$ T cell immunogenicity of OVA. Additionally, the data suggests that CML and CEL detected on crude AGE-OVA would at least in part contribute to the enhanced $CD4^+$ T cell activation.

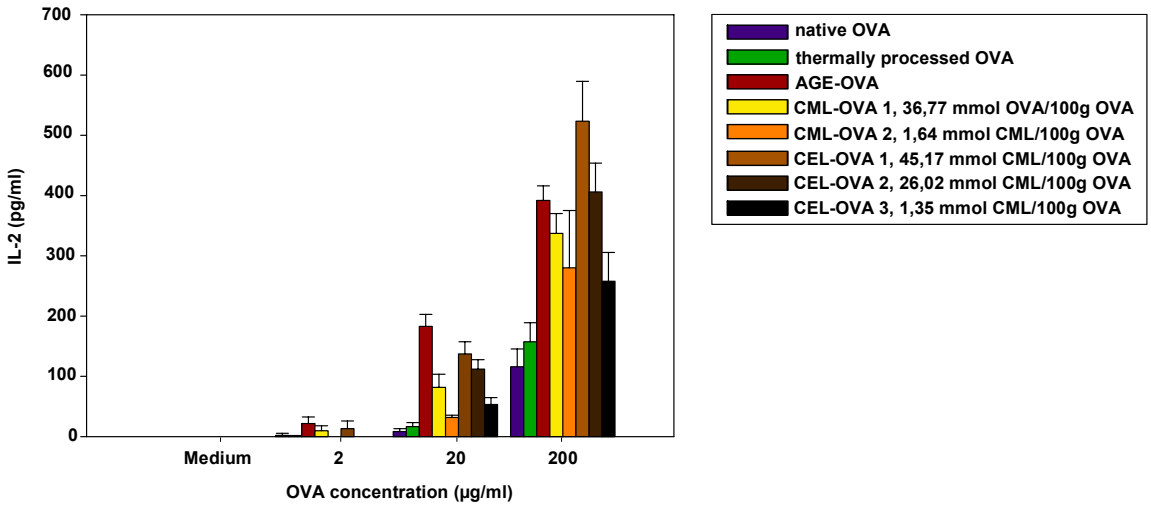

Fig 4.8 Determination of OT-2 $CD4^+$ T cell activation induced by CML- and CEL-OVA after 24 h

$CD4^+$ T cells isolated from OT-2 mice were co-cultured with BL6 mDCs and stimulated with OVA modified with the lysine derivates CML or CEL for 24 h. As comparison, the cells were also stimulated with native, thermally processed or AGE-OVA. The IL-2 concentration in the cell culture supernatants was determined by ELISA. Error bars signify the SD of culture triplicates and ELISA duplicates.

4.3.2 Influence of AGE-OVA on the proliferation of OVA specific CD4$^+$ T cells

As AGE-OVA significantly enhanced the IL-2 production by OVA specific CD4$^+$ T cells in comparison to the control OVAs, it was of particular interest to see whether this also results in enhanced proliferation of the T cells. CD4$^+$ T cells isolated from the spleen of OT-2 mice were stained with CFSE (chapter 3.2.3.10) before being co-cultured with mDCs and stimulated with native, thermally processed or AGE-OVA, at 2, 20, or 200 μg/ml. The proliferation of the T cells was then determined by analysing the CFSE dilution using flow cytometry (chapter 3.2.4.1).

For up to 48 hours no proliferation of CD4$^+$ T cells could be observed (data not shown). After 72 h cell division was clearly detected (Fig. 4.9). The number of dividing cells was significantly higher upon stimulation with 2 or 20 µg/ml of AGE-OVA when compared to cells that were stimulated with either of the control OVAs (Fig. 4.9) at these concentrations. This difference was not observed when the CD4$^+$ T cells were stimulated with the very high concentration of 200 µg/ml of OVA. After 96 h, the number of cells which proliferate upon stimulation with 20 µg/ml of either form of OVA had also become comparable. However, CD4$^+$ T cells which had been stimulated with only 2 µg/ml of allergen had proliferated only if stimulated with AGE-OVA (Fig. 4.9). These results show that AGE-OVA not only induces enhanced IL-2 production but also increases the proliferation of OVA specific CD4$^+$ T cells when co-cultured with mDCs.

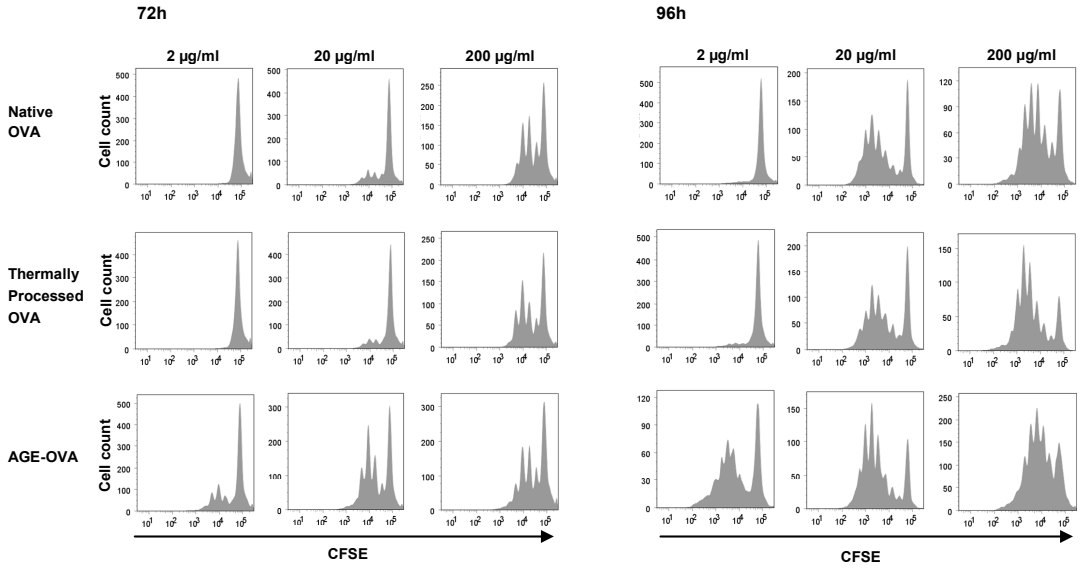

Fig 4.9 OVA specific CD4$^+$ T cell proliferation induced by AGE-OVA analysed using CFSE staining

CD4$^+$ T cells were isolated from OT2-mice and stained with CFSE. The T cells were co-cultured with mDC and stimulated with different concentrations of AGE-OVA, native or thermally processed OVA. After 72 and 96 h the proliferation of the CD4$^+$ T cells was determined by flow cytometry.

4.3.3 Cytokine production by AGE-OVA activated $CD4^+$ T cells

Production of Th2 type cytokines such as IL-4 by allergen specific $CD4^+$ T cells is a critical component in the development and maintenance of food allergy (chapter 1.2 and 1.12.2.2). It was of interest whether glycatated food allergens may preferentially induce Th2 differentiation. Therefore the influence of the glycated OVA on the cytokine production of $CD4^+$ T cells was analysed. As $CD4^+$ T cells from OT-2 mice predominantly produce Th1 type cytokines such as IFN-γ but very little Th2 type cytokines such as IL-4, $CD4^+$ T cells from DO11.10 mice, which efficiently produce Th2 type cytokines, were used. The isolated T cells were co-cultured with BALB/c mDCs and stimulated with native, thermally processed or AGE-OVA for 72h. The concentration of IL-4 and IFN-γ in the culture supernatants was determined by ELISA (chapter 3.2.4.5.2.1).

All three forms of OVA induced a robust secretion of IFN-γ by $CD4^+$ T cells when the cells were stimulated with 200 µg/ml of allergen (Fig. 4.10). However, AGE-OVA induced significantly higher IFN-γ and IL-4 production than the control OVAs when $CD4^+$ T cells were stimulated with only 2 or 20 µg/ml of allergen. Native or thermally processed OVA induced robust secretion of IL-4 by the T cells only at a very high allergen concentration, 200 µg/ml (Fig. 4.10). Collectively, the results show that AGE-OVA, in addition to strongly enhanced $CD4^+$ T cell activation and proliferation (Fig. 4.6), also increased secretion of IFN-γ and IL-4.

Interestingly, AGE-OVA induced the highest secretion of IL-4 by the T cells at the lower allergen concentration of 2 µg/ml. It seems that in $CD4^+$ T cells stimulated with AGE-OVA at 200 µg/ml, the high level of IFN-γ inhibited IL-4 production by the cells. At the allergen concentrations used this could not be observed for native or thermally processed OVA, however, it may be possible that if higher allergen concentrations where used, increased IFN-γ may also inhibit IL-4 in cells stimulated with native or thermally processed OVA. The fact that IFN-γ secretion inhibits production of IL-4 is well established (chapter 1.12.2.1)

However, the ratio of IL-4/IFN-γ in the supernatants of T cells stimulated with native, thermally processed OVA or AGE-OVA seems to be comparable. Therefore the glycation of the allergen would not particularly induce Th2 cell differentiation.

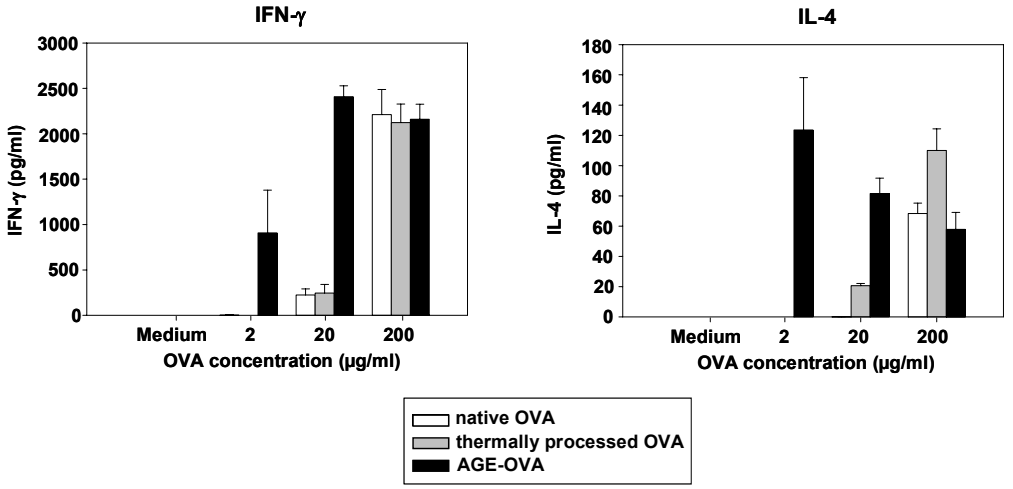

Fig 4.10 Determination of the phenotype of activated OVA specific CD4$^+$ T cells

CD4$^+$ T cells isolated from DO11.10 mice were co-cultured with BALB/c mDCs and stimulated with AGE-OVA or the controls for 72 h. The concentration of IL-4 and IFN-γ in the culture supernatants were measured by ELISA. Error bars signify the SD of culture triplicates and ELISA duplicates.

4.3.4 Influence of AGE-OVA on the activation of OVA specific CD8$^+$ T cells

It was of interest to examine whether the glycation of OVA influences the stimulatory effect of the allergen on CD8$^+$ T cells. To elucidate the CD8$^+$ T cell immunogenicity of AGE-OVA, CD8$^+$ T cells isolated from spleen of the transgenic mouse strain OT-1 were used. These cells have a TCR which specifically recognises OVA residues 257-264 (SIINFEKL). CD8$^+$ T cells were co-cultured with BL6 mDCs, which had been prestimulated with 200, 20 or 20 µg/ml of native, thermally processed or AGE-OVA for 3 h (chapter 3.2.3.9). After 21 h of co-culture, the IL-2 concentration in the culture supernatants was measured by ELISA (chapter 3.2.4.5.2.1).

As shown in Fig. 4.11, AGE-OVA and native OVA induced comparable IL-2 production by the CD8$^+$ T cells. This result indicates that glycation of OVA by the Maillard reaction does not enhance the CD8$^+$ T cell immunogenicity of the allergen. Interestingly, thermally processed OVA induced an enhanced IL-2 secretion by CD8$^+$ T cells. In Fig. 4.3, it is shown that thermally processing without glucose induces partial degradation of OVA. The partial degradation may enhance the processing of thermally processed OVA resulting in more MHC class I-OVA peptide complex to be presented on the cell surface of mDCs.

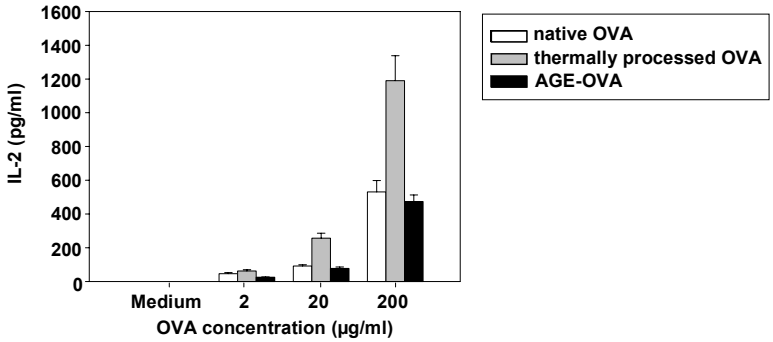

Fig 4.11 Assessment of CD8$^+$ T cell activation after stimulation with OVA

CD8$^+$ T cells isolated from OT-1 mice were co-cultured with BL6 mDCs, which had been preincubated with native, thermally processed or AGE-OVA for 3 h. After 21 h of co-culture, the IL-2 concentration in the cell culture supernatants was measured by ELISA. Error bars signify the SD of culture triplicates and ELISA duplicates.

4.4 Interaction of AGE-OVA with mDCs *in vitro*

Dendritic cells are professional antigen presenting cells which are highly specialised in the uptake of antigens and subsequent activation of T cells. As shown in Fig. 4.7, it was found that mDCs are essential for the enhanced CD4$^+$ T cell activation by AGE-OVA. To obtain insights into the role of mDCs in the enhanced T cell activation by AGE-OVA, (i) the influence of AGE-OVA on mDC maturation and (ii) the mechanisms involved in the uptake of AGE-OVA by mDCs were examined.

4.4.1 Influence of AGE-OVA on the maturation of mDCs

First the influence of AGE-OVA on mDC maturation was assessed. mDCs were stimulated with 20 µg/ml of AGE-OVA or either of the controls, native and thermally processed OVA for 18 h. As positive control in this experiment, LPS, a known inducer of mDC maturation, was used (chapter 3.2.3.5). The cell surface expression of the maturation markers CD40, CD80, CD86 and MHC class II molecules (MHC II) on mDCs was analysed by flow cytometry (chapter 3.2.4.1). Furthermore, the concentration of IL-12p70 and IL-10 in the supernatant of the stimulated mDCs was measured by ELISA (chapter 3.2.4.5.2.1).

As expected, stimulation of mDCs with LPS upregulated the expression of all maturation markers on the cell surface (Fig. 4.12 A). LPS stimulation also induced a significant secretion of IL-12p70 and IL-10 by mDCs (Fig. 4.12 B). In contrast, the expression of the maturation markers by mDCs was not upregulated upon stimulation with AGE-OVA or any of the controls (Fig 4.12 A). In line with this, mDCs stimulated with either of the OVAs did not secrete detectable levels of IL-12p70 or IL-10 (Fig. 4.12, B). Collectively, these results indicate that AGE-OVA is not capable of inducing the maturation of bone marrow derived mDCs.

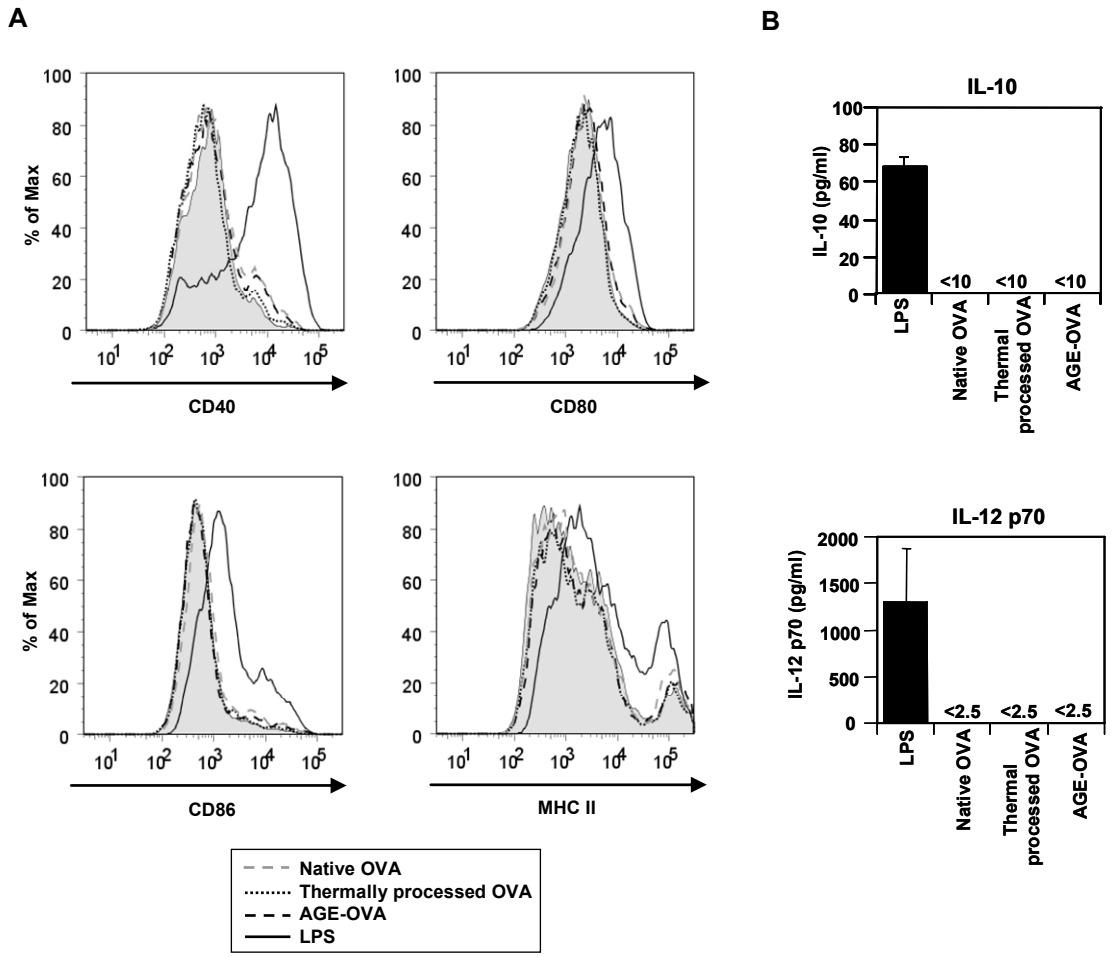

Fig 4.12 Assessment of mDC maturation induced by AGE-OVA

mDCs were stimulated with 20 µg/ml of native, thermally processed or AGE-OVA or 10 µg/ml of LPS for 18h. **A** Expression of the maturation markers CD40, CD80, CD86 and MHC II on mDCs was analysed by flow cytometry. Gray areas represent cells cultured with medium only. **B** The concentrations of IL-10 and IL-12p70 in the culture supernatants were measured by ELISA. Error bars signify the SD of culture triplicates and ELISA duplicates.

4.4.2 Influence of glycation of OVA on the uptake of the allergen by mDCs

Antigen-specific T cell activation is preceded by the uptake of antigens by DCs. To further investigate the mediator function of mDCs in the enhanced $CD4^+$ T cell immunogenicity of AGE-OVA, the uptake of native, thermally processed and AGE-OVA by mDCs was compared.

4.4.2.1 Verifying the comparability of the FITC labelling of the different OVA forms

In order to compare the uptake of AGE-OVA and the controls by mDCs, the OVAs had to be labelled with FITC at comparable FITC/protein molar ratio (chapter 3.2.2.6). The FITC/protein molar ratio was calculated by measuring the absorption of the samples at 280 nm (absorption maximum of proteins) and 495 nm (absorption maximum of FITC) as described in chapter 3.2.2.6.

The proteins were usually labelled with around 2.5 moles of FITC per mol of protein. Additionally, an ELISA using a mouse-anti FITC monoclonal antibody was performed to verify comparable labelling of all three OVA samples (chapter 3.2.4.4.1.3). A representative result of the ELISA is shown in Fig. 4.13. The result shows that the anti-FITC monoclonal antibody bound to FITC labelled native, thermally processed and AGE-OVA equally well at all concentrations. This indicates that the three samples were labelled with a comparable FITC/protein molar ratio. Therefore these FITC-conjugates could be used to compare the uptake of the three forms of OVA since differences in FITC intensity of the cells would not be the result of non-uniform labelling of the proteins.

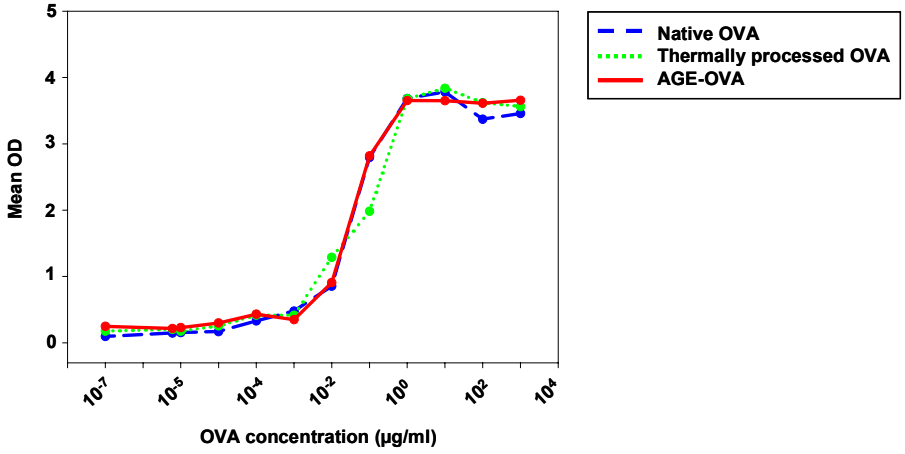

Fig 4.13 Comparability of FITC/protein ratio of the three OVAs

ELISA plates were coated with FITC labelled native, thermally processed or AGE-OVA. The level of FITC on the OVAs was determined using a mouse-anti FITC antibody.

4.4.2.2 Analysis of the uptake of AGE-OVA by mDCs

To assess the level of OVA uptake by mDCs, the cells were incubated with 0.5 or 5 µg/ml of FITC labelled native, thermally processed or AGE-OVA for 15 min (3.2.3.6). Following the incubation, mDCs were harvested and stained with PE conjugated anti-mouse CD11b and APC conjugated anti-mouse CD11c (3.2.4.2.1) antibody. The FITC intensity of the $CD11b^{+}CD11c^{+}$ cells was measured by flow cytometry (3.2.4.1).

When incubated with 5 µg/ml of allergen, the mDC uptake of AGE-OVA is more than fivefold higher than that of native or thermally processed OVA (Fig. 4.14 A). The result was even more striking when only 0.5 µg/ml of the allergens were given to the cells. Here, the mDC uptake of AGE-OVA was nearly tenfold higher than that of the control OVAs (Fig. 4.14 A). The fold increase of the mean fluorescence intensity (MFI) of cells incubated with native, thermally processed or AGE-OVA in relation to the auto fluorescence of mDCs cultured with medium only is shown in Fig. 4.14 B. The MFI of the mDCs incubated with native or thermally processed OVA was only around 5 and 20 fold greater than that of the unstimulated cells at the antigen concentration of 0.5 and 5

μg/ml respectively. For AGE-OVA the fold increase of the MFI was approximately 20 and 60 fold at the antigen concentration of 0.5 and 5 μg/ml respectively. The results strongly indicate that AGE-OVA was taken up by mDCs much more efficiently than native and thermally processed OVA.

The increased fluorescence intensity of mDCs incubated with FITC-AGE-OVA might result from the attachment of the glycated allergen on the cell surface due to electric charges of the glycation structures. To verify whether AGE-OVA was taken up (endocytosed) by mDCs or simply attached to the cell surface, the localisation of AGE-OVA in the cells was analysed by confocal laser scanning microscopy (chapter 3.2.4.3). The mDCs were incubated with AGE-OVA for 15 min under the same conditions as for the flow cytometric analysis. The majority of AGE-OVA was not attached to the cell surface but clearly located within the mDCs (Fig. 4.14, C). This observation indicates that AGE-OVA was indeed endocytosed by the mDCs.

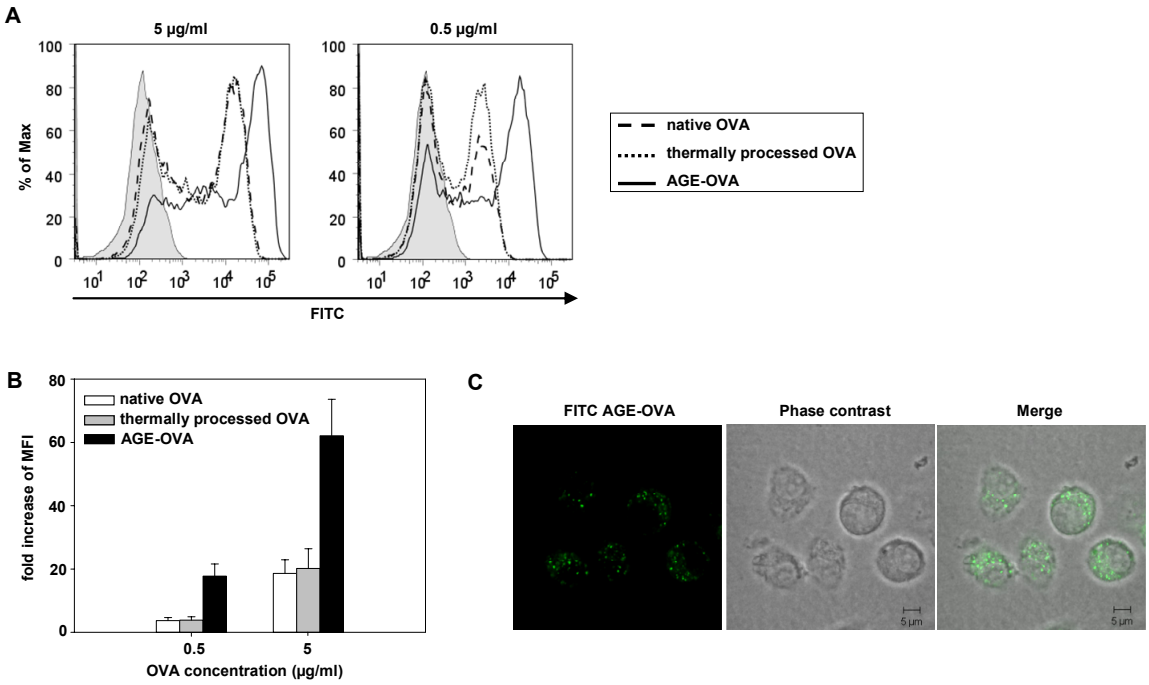

Fig 4.14 Influence of glycation on its uptake by mDCs

Wt mDCs were incubated with 0.5 or 5 µg/ml of FITC conjugated native, thermally processed or AGE-OVA for 15 min. **A** Analysis of uptake by $CD11b^{+}CD11c^{+}$ mDCs using flow cytometry. Gray areas represent cells cultured with medium only. Graphs are representative of three independent experiments. **B** The FITC mean fluorescence intensity (MFI) is shown as fold increase of MFI of cells cultured with medium only. Error bars signify the SD of three independent experiments. **C** The uptake of FITC AGE-OVA by mDCs was analysed by confocal laser scanning microscopy.

4.4.3 Receptors mediating the uptake of AGE-OVA by mDCs

Native OVA has a single carbohydrate side chain composed of N-acetylglucoseamine (GNAc) and mannose which facilitates the uptake of the allergen by the mannose receptor (MR) (Huntington 2001 et al., Burgdorf 2006, Autenrith et al., 2007). AGEs have been shown to bind to a variety of cell surface receptors such as RAGE (receptor for advanced glycation end products) (Schmidt 1992, Neeper 1992), scavenger receptor class A type I and II (SR-AI/II) (Suzuki 1997, Araki 1999) scavenger receptor class B type I (SR-BI) (Ohgami et al., 2001, a), CD36 (Ohgami et al., 2001, b) and Galectin-3 (Vlassara 1995). Following the observation that glycation of OVA enhances its uptake by mDCs, the aim was to identify the receptor(s) involved in the uptake of AGE-OVA by mDCs.

4.4.3.1 Detection of receptors expressed on bone marrow derived mDCs

First, it was verified whether the receptors which have previously been found to bind AGEs and the MR are expressed by the mDCs used in this study. The expression of MR, SR-AI, RAGE and Galectin-3 on the surface of the mDCs was analysed by flow cytometry (chapter 3.2.4.1) using monoclonal antibodies recognising these receptors (chapter 3.2.4.2.2). Expression of SR-B1, CD36, and also SR-AI/II by mDCs was examined by RT-PCR (chapter 3.2.1.2)

As shown in Fig. 4.15 A and B, the majority of mDCs express the MR and Galectin-3. Cell surface expression of RAGE however, could not be detected by flow cytometry (Fig. 4.15, C). Gene and protein expression of RAGE was also not detected when using RT-PCR and Western Blot, respectively (data not shown). For the detection of SR-A a monoclonal antibody against SR-A type I only was used, as this is the only antibody commercially available which was suitable for flow cytometric analysis of BL6 derived mDCs. As shown in Fig. 4.15 D SR-AI was detected on some but not all mDCs. Additionally, the expression of SR-AI/II was confirmed by RT-PCR (Fig. 4.15, E, *lane 3*). Gene expression of SR-B1 and CD36 by mDCs could also be shown by RT-PCR (Fig. 4.1,E, *lanes 1 and 2*). Therefore several receptors which may potentially bind AGE (-OVA) are expressed by the mDCs used in the present study.

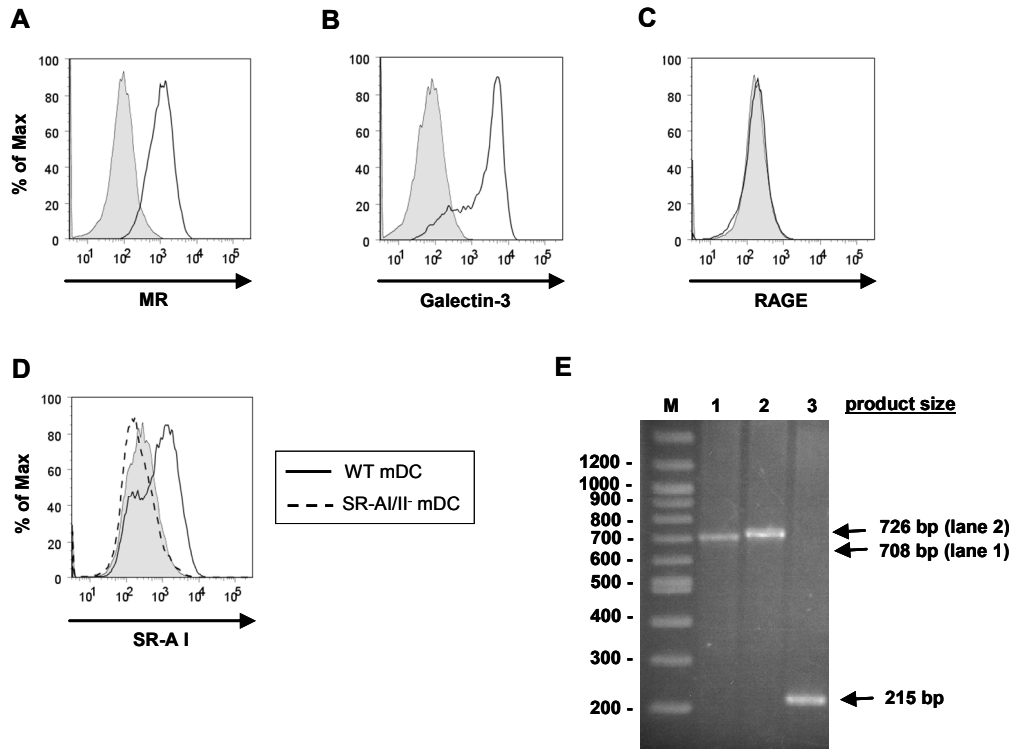

Fig. 4.15 Expression of OVA and AGE-binding receptors expressed by mDCs

Wt mDCs were analysed for their expression of the receptors to which AGE-OVA may bind. **A-D** The cells were stained with the respective monoclonal antibody and the expression of the receptors in $CD11b^+CD11c^+$ cells was analysed by flow cytometry. Gray areas represent the relevant isotype controls. **E** Total RNA of mDCs was isolated and subjected to reverse transcriptase treatment. PCR was then performed using primers detecting the genes for SR-B1, CD36 and SR-A, respectively. PCR products were analysed using a 1.5% agarose gel. *M*: size standard, 100 bp DNA ladder (NEB), *1*: SR-B1; *2*: CD36; *3*: SR-A.

4.4.3.2 Non-involvement of RAGE in the uptake of AGE-OVA

As shown in Fig. 4.15, expression of RAGE by mDCs could not be detected. However, as RAGE is a well known AGE-binding receptor, it remained an attractive potential receptor for the endocytosis of AGE-OVA in this study. It could not be excluded that the methods described above to detect RAGE expression were not optimal. Therefore it was examined whether the uptake of AGE-OVA is attenuated in mDCs generated from RAGE deficient mice.

After incubation of wild type (Wt) mDCs and RAGE deficient mDCs with 5 μg/ml FITC-conjugated native, thermally processed or AGE-OVA for 15 min (chapter 3.2.3.6), the cells were stained with PE conjugated anti-mouse CD11b and APC conjugated anti-mouse CD11c (chapter 3.2.4.2.1) antibodies. The FITC intensity of $CD11^+CD11c^+$ cells was analysed by flow cytometry (chapter 3.2.4.1). The fluorescence intensity of mDCs incubated with AGE-OVA was comparable in Wt and RAGE deficient mDCs (Fig. 4.16). A reduction in the uptake of native and thermally processed OVA was also not observed in RAGE deficient mDCs. The results indicate that RAGE is not

involved in the uptake of AGE-OVA or in the uptake native and thermally processed OVA in the experimental system used in this study.

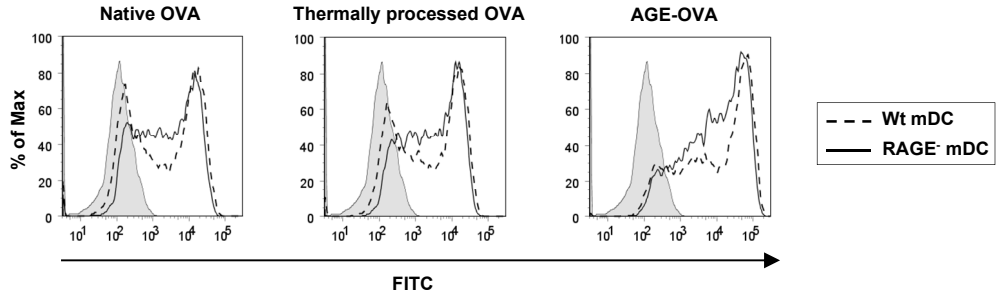

Fig. 4.16 RAGE is not involved in the uptake of AGE-OVA by mDCs

Wt or RAGE deficient mDCs were incubated with 5 µg/ml of FITC conjugated AGE-OVA or the controls. The uptake of the OVAs by $CD11b^{+}CD11c^{+}$ mDCs was analysed by flow cytometry. Gray areas represent mDCs cultured with medium only. Graphs are representative of three independent experiments.

4.4.3.3 Non-involvement of Galectin-3 in the uptake of AGE-OVA

Next it was investigated, whether Galectin-3 is engaged in the uptake of AGE-OVA. Lactose is a ligand of Galectin-3 (Cherayil et al., 1989) and is commonly used to block the interaction of other ligands with this receptor (Li et al., 2008; Karlsson et al., 2009). The uptake of all three OVAs by lactose treated and untreated Wt mDCs was compared. mDCs were treated with 150 mM lactose 30 min prior to the addition of the OVAs (chapter 3.2.3.6). No significant reduction in the uptake of AGE-OVA or of the controls was observed in lactose treated mDCs (Fig. 4.17). Therefore galectin-3, highly expressed on the mDCs (Fig 4.15, B), does not mediate the uptake of AGE-OVA.

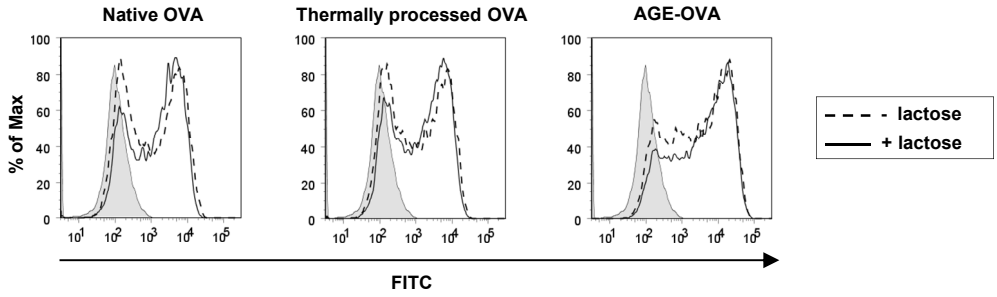

Fig. 4.17 Galectin-3 is not involved in the uptake of AGE-OVA by mDCs

mDCs were pre-incubated with lactose for 30 min to inhibit possible galectin-3 mediated uptake. Subsequently the cells were incubated with 5 µg/ml of FITC conjugated native, thermally processed or AGE-OVA .The uptake of the OVAs by $CD11b^{+}CD11c^{+}$ mDCs was measured by flow cytometry. Gray areas represent mDCs cultured with medium only. Graphs are representative of three independent experiments.

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

Following the findings that RAGE and Galectin-3 are not involved in AGE-OVA uptake by mDCs, the role of a further potential receptor, SR-AI/II, was examined using mDCs generated from bone marrow cells of SR-AI/II deficient mice (chapter 3.2.3.6). Importantly, AGE-OVA uptake by SR-AI/II deficient mDCs was significantly reduced in comparison to that by Wt mDCs (Fig. 4.18). This reduction of uptake by the SR-AI/II deficiency is particularly pronounced when adding the lower concentration of AGE-OVA, 0.5 μg/ml. Surprisingly, native and thermally processed OVA uptake was also reduced in SR-AI/II deficient mDCs (Fig. 4.18). However, the reduction in the uptake of the control OVAs was considerably less than that of AGE-OVA. Additionally, when SR-AI/II deficient mDCs were treated with only 0.5 μg/ml of native or thermally processed OVA, a visible reduction of the uptake was no longer observed. The results indicate that SR-AI/II plays a pivotal role in the uptake of AGE-OVA by mDCs. The uptake of native and thermally processed OVA could also be mediated by SR-AI/II but only to a small extend.

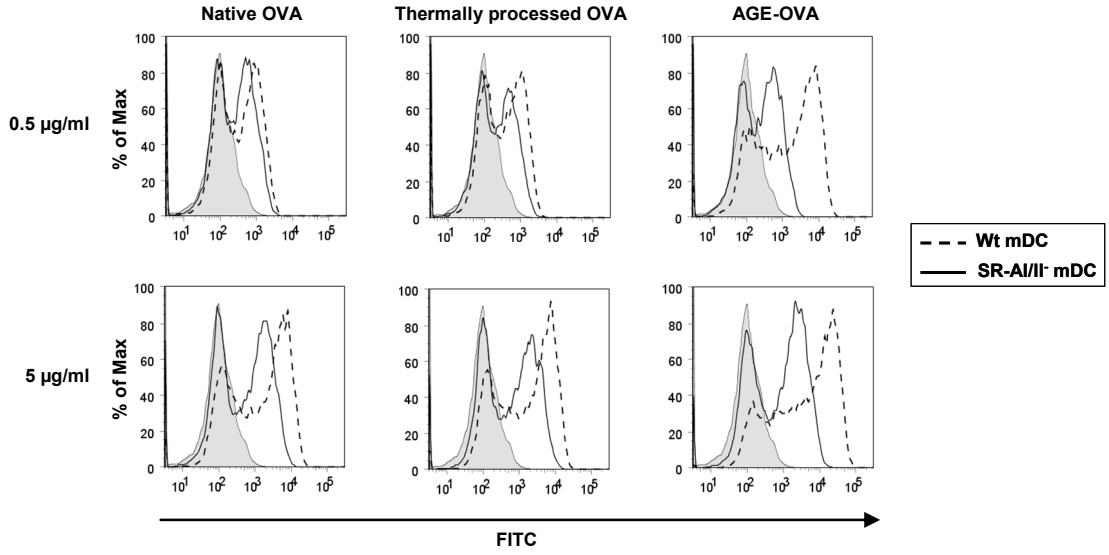

Fig. 4.18 Uptake of AGE-OVA by SR-AI/II deficient mDCs

Wt mDC or SR-AI/II deficient mDCs were incubated with 0.5 or 5 μg/ml of FITC labelled native, thermally processed or AGE-OVA for 15 min. The FITC fluorescence intensity of $CD11b^{+}CD11c^{+}$ cells was measured by flow cytometry. Gray areas represent mDCs cultured with medium only. Graphs are representative of three independent experiments.

4.4.3.5 Involvement of the MR in the uptake of AGE-OVA by mDCs

There was still considerable uptake of all three OVAs by SR-AI/II deficient mDCs (Fig. 4.18). OVA has a single carbohydrate side chain containing mannose, which facilitates the uptake of the allergen by the mannose receptor (MR) (Huntington 2001 et al., Burgdorf 2006, Autenrith et al., 2007). Therefore the remaining uptake observed in SR-AI/II deficient mDCs may be mediated by the MR. To verify this, mannan, a carbohydrate which inhibits MR mediated endocytosis (Sallusto

et al., 1995), was used. SR-AI/II deficient mDCs were treated with 3 mg/ml of mannan prior to the incubation with 0.5 or 5 μg/ml of either of the FITC conjugated OVAs (chapter 3.2.3.6).

Treatment of SR-AI/II deficient mDCs with mannan almost completely abolished the uptake of native and thermally processed OVA (Fig. 4.19). This observation indicates that the MR is the main receptor for the uptake of native and thermally processed OVA. Regarding the mDC uptake of AGE-OVA, the MR inhibition in combination with the SR-AI/II deficiency did not attenuate the uptake completely. Some uptake of AGE-OVA was still observed in SR-AI/II deficient mDCs which had been incubated with 5 μg/ml of OVA even after mannan treatment. The results suggest that in addition to the SR-AI/II, the MR is a mediator for the uptake of AGE-OVA. However, one or more further receptor(s) could be involved in the mDC uptake of AGE-OVA .

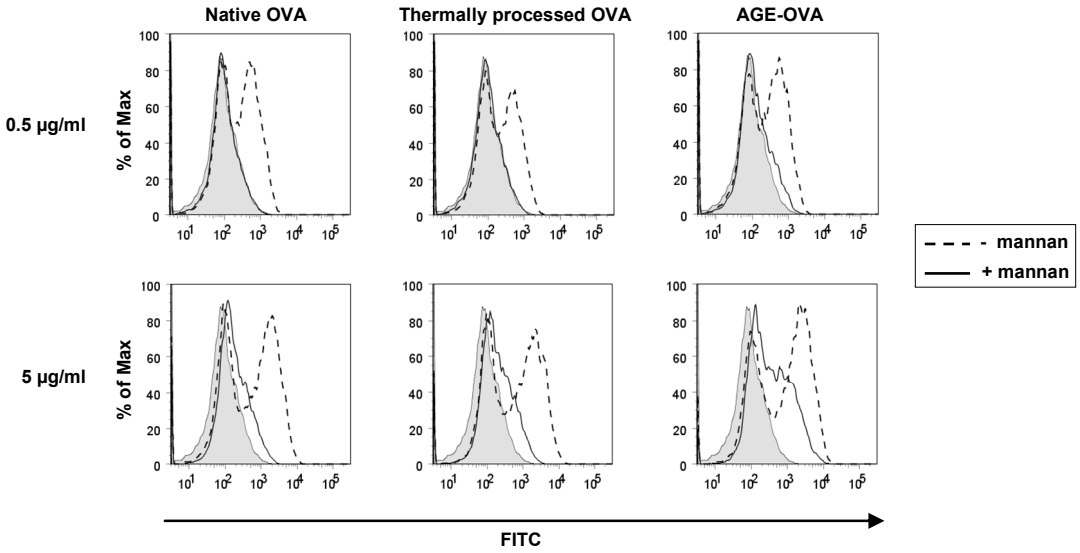

Fig. 4.19 Influence of mannan treatment on AGE-OVA uptake by SR-AI/II deficient mDCs

SR-AI/II deficient mDCs were treated with 3 mg/ml of mannan for 30 min and then incubated with 0.5 or 5 μg/ml of FITC labelled native, thermally processed or AGE-OVA for 15 min. The FITC fluorescence intensity of $CD11b^{+}CD11c^{+}$ mDCs was measured by flow cytometry. Gray areas represent mDCs cultured with medium only. Graphs are representative of three independent experiments.

4.4.4 Verification of interaction of AGEs with SR-AI/II

The experiments in chapter 4.4.3 have demonstrated that the uptake of AGE-OVA is significantly reduced in SR-AI/II deficient mDCs while the uptake of native OVA and thermally processed OVA is only marginally affected. It was therefore very likely that the glycation structures on AGE-OVA enhance the interaction of AGE-OVA with SR-AI/II. To verify the interaction of AGEs with SR-AI/II, AGE-BSA was used. The profile of the glycation structures of AGE-BSA was comparable with that of AGE-OVA as shown in Fig. 4.2 and Fig. 4.4.

4.4.4.1 Uptake of native and AGE-BSA by Wt mDCs

First, it was determined whether the glycation of BSA increases the uptake of the protein by mDCs as was observed for OVA (Fig. 4.14). Native BSA and AGE-BSA were labelled with FITC at a comparable FITC/protein ratio (chapter 3.2.2.6). The level of the uptake of these BSAs was compared by measuring the FITC intensity of mDCs by flow cytometry (chapter 3.2.4.1).

As shown in Fig. 4.20, the uptake of AGE-BSA was markedly greater than that of native BSA at both antigen concentrations used. Similar to the observations made with OVA (Fig 4.14), this difference was most evident at an antigen concentration of 0.5 μg/ml (Fig. 4.20) at which an uptake of native BSA by mDCs could barely be detected, while AGE-BSA is efficiently taken up. This result suggests that glycation structures are indeed the critical component for the enhanced uptake of glycated proteins by mDCs.

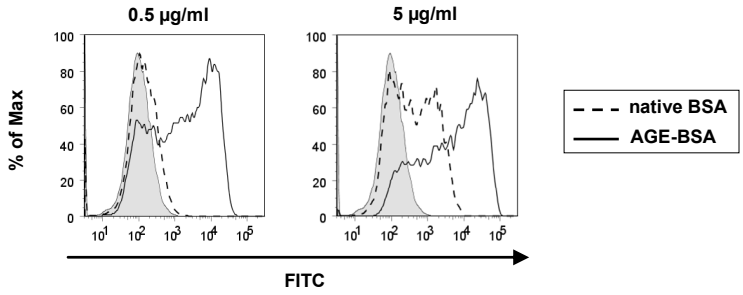

Fig. 4.20 Uptake of native BSA and AGE-BSA by Wt mDCs

mDCs were incubated with 0.5 or 5 μg/ml of FITC conjugated native BSA or AGE-BSA for 15 min. The uptake of these BSAs by $CD11b^+CD11c^+$ mDCs was measured by flow cytometry. Gray areas represent cells cultured with medium only. Graphs are representative of three independent experiments.

4.4.4.2 Inhibition of mDC uptake of AGE-OVA by AGE-BSA

To further verify the involvement of the glycation structures of AGE-OVA in the enhanced uptake by mDCs, an inhibition assay using AGE-BSA was performed. To this end, mDC uptake of FITC labelled AGE-OVA in the presence of native BSA or AGE-BSA was examined (chapter 3.2.3.6). In contrast to OVA, BSA does not possess natural carbohydrate residues which may facilitate binding to SR-AI/II (Wada, 1996). However, AGE-BSA, as an AGE derivative, should also be capable to bind to SR-AI/II.

Native BSA did not inhibit the uptake of AGE-OVA by Wt mDCs, even when added at a very high concentration, 500 μg/ml (Fig. 4.21, A). In contrast, AGE-BSA inhibited the uptake of AGE-OVA by Wt mDCs in a dose dependent manner. High concentrations of AGE-BSA also inhibited the uptake of AGE-OVA by SR-AI/II deficient mDCs, but considerably less than in Wt mDCs (Fig. 4.21 B). Native BSA, again, did not inhibit the uptake of AGE-OVA by the deficient mDCs. These results further support that (i) the glycation structures are the critical components in the uptake of AGE-OVA by SR-AI/II and (ii) (an)other receptor(s) in addition to SR-AI/II interact(s) with AGEs, although the role of the(se) receptor(s) in the uptake of the AGEs would be minor.

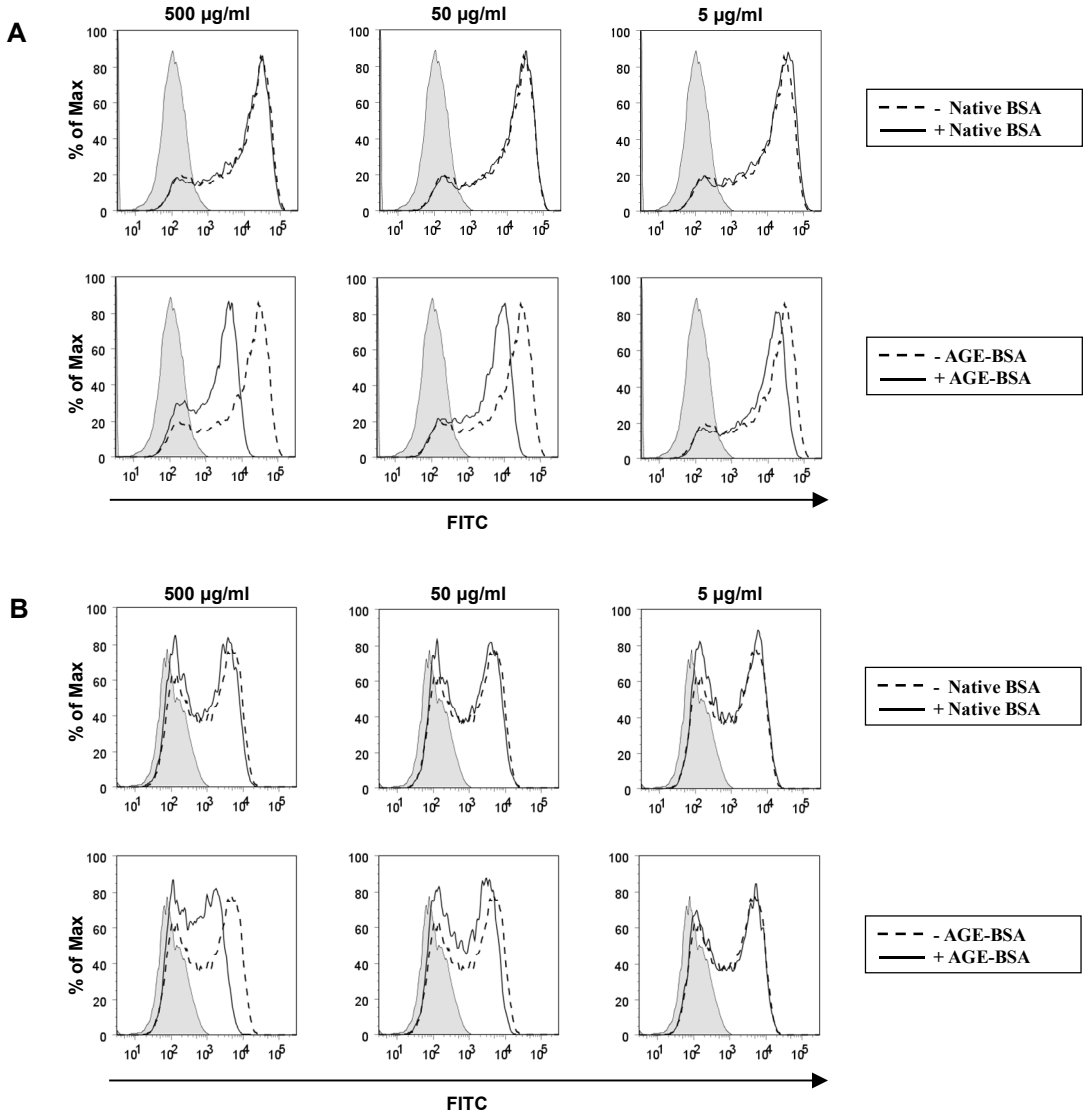

Fig. 4.21 AGE-BSA inhibition of AGE-OVA uptake by Wt and SR-AI/II deficient mDCs

A WT mDCs or **B** SR-AI/II deficient mDCs were incubated with 5 µg /ml of FITC-conjugated AGE-OVA together with native or AGE-BSA at the indicated concentrations for 15 min. The uptake of AGE-BSA by $CD11b^{+}CD11c^{+}$ mDCs was measured by flow cytometry. Gray areas represent cells cultured with medium only. Graphs are representative of three independent experiments.

4.4.4.3 Excluding unspecific interaction of FITC with SR-AI/II

SR-AI/II has a broad ligand binding specificity against polyanionic molecules such as glycoproteins and chemically modified proteins. It was of importance to exclude the possibility of an involvement of FITC in the interaction of the labelled OVAs with SR-AI/II.

To examine the involvement of FITC in SR-AI/II mediated uptake by mDCs, Transferrin (Trf) labelled with FITC was used. This protein is specifically internalised by the Transferrin receptor (TrfR) (Trowbridge and Shackleford, 1986). The endocytosis of Trf should therefore not be

affected by a lack of SR-AI/II on mDCs. As was expected, the uptake of FITC-Trf was comparable in Wt and SR-AI/II deficient mDCs (Fig. 4.22.). This result indicates that FITC-Trf uptake is mediated only by the TrfR and, importantly, excludes the possibility of FITC binding to SR-AI/II.

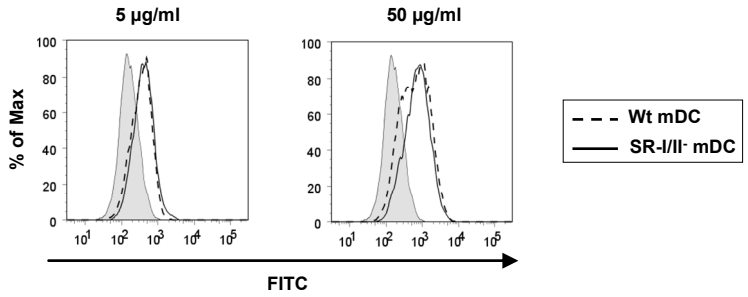

Fig 4.22 Using FITC-Trf to exclude the possibility of unspecific interaction of FITC with SR-AI/II
Wt mDCs or SR-AI/II deficient mDCs were incubated with 5 or 50 µg/ml of FITC-conjugated Trf. The uptake by $CD11b^+CD11c^+$ mDCs was measured by flow cytometry. Gray areas represent cells cultured with medium only. Graphs are representative of three independent experiments.

4.4.5 Uptake of recombinant OVA by Wt mDCs and SR-AI/II deficient mDCs

In general, recombinant proteins which are prepared employing expression systems in *E. coli* do not possess natural carbohydrate residues. By using recombinant OVA it was possible to examine whether the natural single carbohydrate side chain composed of mannose and N-acetyl-glucosamine on native OVA binds to SR-AI/II, or not (Fig. 4.18). The only possible mechanism for recombinant OVA to be taken up by mDCs would be pinocytosis. Here, the uptake of recombinant OVA and native OVA by Wt mDCs and SR-AI/II deficient mDCs was compared. The uptake level of recombinant OVA by Wt mDCs was significantly less than that of native OVA (Fig. 4.23, A). Furthermore, the uptake of recombinant OVA by SR-AI/II deficient mDCs is not reduced in comparison to the uptake by Wt mDCs (Fig. 4.23, B) while the uptake of native OVA was reduced by SR-AI/II deficiency (Fig. 4.18). These results indicate that SR-AI/II is engaged in the uptake of native OVA by association with the single carbohydrate side chain of the allergen.

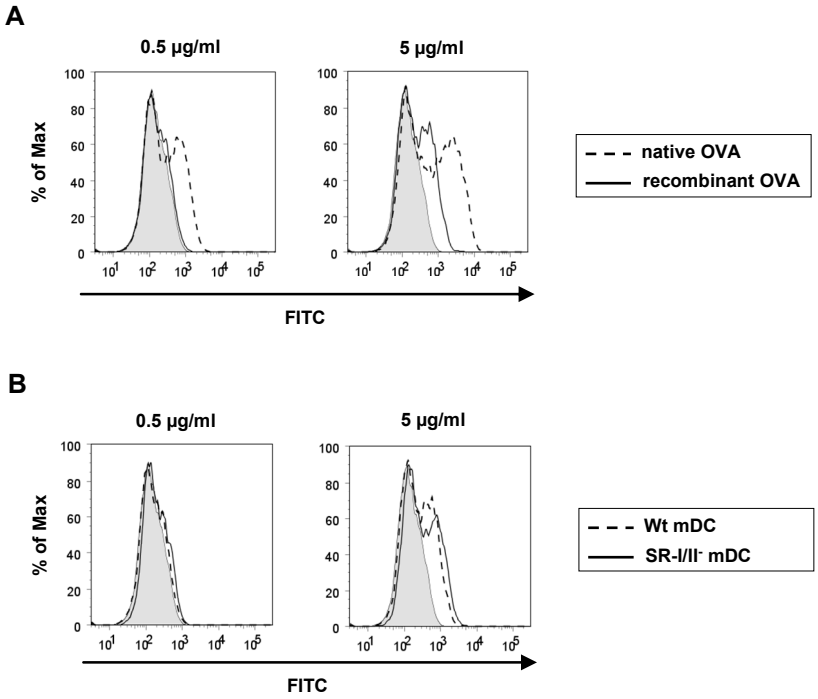

Fig 4.23 Uptake of recombinant OVA by Wt mDCs and SR-AI/II deficient mDCs

Wt mDCs or SR-AI/II deficient mDCs were incubated with 0.5 or 5 µg/ml of FITC-conjugated native or recombinant OVA for 15 min. The uptake by $CD11b^+CD11c^+$ mDCs was measured by flow cytometry. **A** Uptake of native or recombinant OVA by Wt mDCs. **B** Comparison of recombinant OVA uptake by Wt and SR-AI/II deficient mDCs. Gray areas represent cells cultured with medium only. Graphs are representative of three independent experiments.

4.4.5 Role of SR-AI/II mediated OVA uptake by mDCs in the activation of OVA specific $CD4^+$ T cells

In this study it was found that (i) mDC uptake of AGE-OVA is greater than that of native or thermally processed OVA and that (ii) SR-AI/II plays a key role in the increased uptake of AGE-OVA by mDCs. Next, it was of interest whether the SR-AI/II mediated uptake of AGE-OVA contributes to the enhanced $CD4^+$ T cell immunogenicity of the allergen (chapters 4.3.1 and 4.3.2).

4.4.5.1 Verification of antigen uptake and T cell stimulatory capacity of SR-AI/II deficient mDCs

First, it had to be verified that SR-AI/II deficient mDCs have the capacity to express similar levels of co-stimulatory molecules and MHC II as Wt mDCs, since these molecules are essential for an efficient T cell stimulation (chapter 1.12.1). Wt and SR-AI/II deficient mDCs were stimulated with LPS, an inducer of DC maturation, and the expression of CD40, CD80 CD86 and MHC II on the cell surface was measured by flow cytometry (chapter 3.2.4.1). As seen in Fig. 4.24, the co-stimulatory and MHC II were comparably expressed on both Wt and SR-AI/II deficient mDCs. This

result indicates that the T cell stimulatory capacity of SR-AI/II deficient mDCs is not affected by a reduced ability to express surface molecules essential for T cell activation.

Additionally, the assessment of the uptake ability of mDCs using FITC conjugated Trf (Fig. 4.22) showed that the uptake of Trf by SR-AI/II deficient mDCs was comparable to that of Wt mDCs. Collectively these results show that SR-AI/II deficiency would not generally influence the antigen uptake by and the T cell stimulatory ability of mDCs.

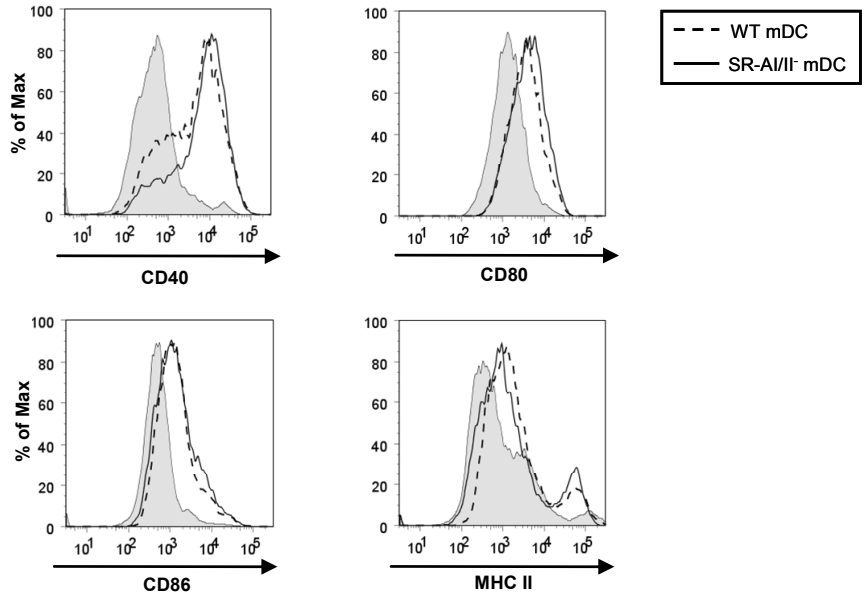

Fig 4.24 Expression of co-stimulatory molecules by Wt and SR-AI/II deficient mDCs after LPS stimulation.
Wt mDCs or SR-AI/II deficient mDCs were incubated with 10 µg/ml of LPS for 18 h. The expression of CD40, CD80, CD86 and MHC II on the cell surface of $CD11b^+CD11c^+$ mDCs was analysed by flow cytometry. Gray areas represent cells cultured with medium only. Graphs are representative of three independent experiments.

4.4.5.2 The role of SR-AI/II in the activation of OVA specific $CD4^+$ T cells

The role of SR-AI/II mediated OVA uptake in the activation of OVA-specific $CD4^+$ T cells was determined by performing a T cell activation assay with SR-AI/II deficient mDCs in addition to Wt mDCs (chapter 3.2.3.8.1). Wt or SR-AI/II deficient mDCs were incubated with 20 or 200 µg/ml of native, thermally processed, recombinant or AGE-OVA for 3 h before the cells were fixed with 0.008% Glutaraldehyde. $CD4^+$ T cells isolated from OT-2 mice were then cultured for 21 h in the presence of the fixed mDCs. The IL-2 concentration in the culture supernatants was measured by ELISA (chapter 3.2.4.5.2.1). Recombinant OVA was included as a further control to verify the importance of the natural carbohydrate residues in the uptake of native OVA by SR-AI/II. The protocol using mDC fixation was chosen to reduce the involvement of pinocytosis in the uptake of these OVAs by mDCs.

Consistent with the previous result (Fig. 4.5), OT-2 $CD4^+$ T cells cultured in the presence of Wt mDCs showed a considerably stronger activation when stimulated with AGE-OVA than when stimulated with native or thermally processed OVA (Fig. 4.25). A remarkable reduction of the IL-2

production by OVA-specific $CD4^+$ T cells in response to the allergens was observed if the T cells were cultured in the presence of SR-AI/II-deficient mDCs instead of Wt mDCs. The decrease was observed for native, thermally processed and AGE-OVA stimulated T cells (Fig. 4.25). The results clearly indicate that SR-AI/II mediated uptake of OVA by mDCs is important for efficient OVA specific $CD4^+$ T cell activation. The $CD4^+$ T cell activation induced by recombinant OVA cultured with Wt mDCs is considerably lower than the activation induced by native OVA and is not affected by the lack of SR-AI/II expression on the mDCs. This suggests that the non-recombinant OVAs interact with SR-AI/II via their carbohydrate side chain as was also indicated by Fig. 4.23. Highly interestingly, for the T cells which were added to SR-AI/II deficient mDCs instead of Wt mDCs an enhanced activation in response AGE-OVA could no longer be observed. The abolishment of the difference in the IL-2 production induced by native, thermally processed and AGE-OVA when using SR-AI/II deficient mDCs suggests that the enhanced $CD4^+$ T cell immunogenicity of AGE-OVA in *in vitro* co-cultures is dependent on the expression of SR-AI/II by the mDCs. The basis for the SR-AI/II dependent enhanced $CD4^+$ T cell activation by AGE-OVA would be an increased affinity of AGE-OVA to SR-AI/II due to the glycation structures as was also indicated in Fig. 4.21. Additionally, the data shown in Fig 4.25 suggests that OVA taken up by mDCs via endocytosis by SR-AI/II enters the MHC class II loading pathway which results in the presentation of the antigen peptide for $CD4^+$ T cells.

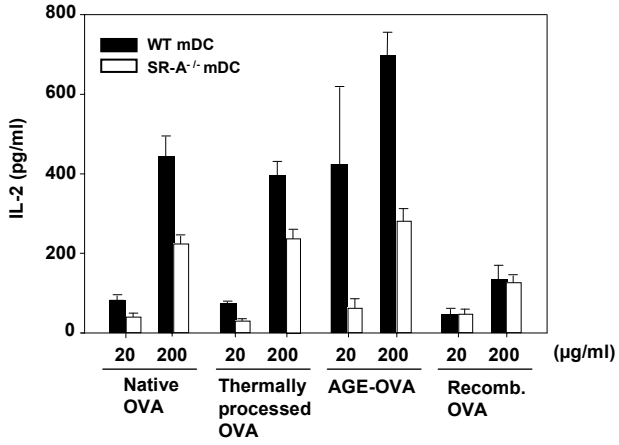

Fig. 4.25 The role of SR-AI/II in the AGE-OVA mediated enhanced $CD4^+$ T cell activation

Wt mDCs or SR-AI/II-deficient mDCs were incubated with native, thermally processed, recombinant or AGE-OVA for 3 h and fixed with 0.008% glutaraldehyde before $CD4^+$ T cells isolated from OT-2 mice were added and cultured for 21h. The concentration of IL-2 in the culture supernatant was measured by ELISA. Error bars signify the SD of culture triplicates and ELISA duplicates.

4.4.6 Localisation of internalised AGE-OVA in mDCs

As shown in Fig 4.25, SR-AI/II mediated uptake of AGE-OVA facilitates OVA specific $CD4^+$ T cell activation. $CD4^+$ T cells are activated by recognition of antigenic peptide bound to MHC II on the cell surface of DCs via the TCR. It was therefore hypothesised that SR-AI/II targets its ligands to the MHC class II loading pathway in mDCs. To elucidate whether AGE-OVA endocytosed by SR-AI/II is indeed targeted to the MHC class II loading pathway, the localisition of endocytosed AGE-OVA in mDCs was analysed. To this end, immunofluorescence staining of cellular compartments for the MHC class II loading pathway in AGE-OVA incubated mDCs was performed (chapter 3.2.4.3.1). The stained cells were then analysed using confocal laser scanning microscopy (LSM, chapter 3.2.4.3).

First, the association of AGE-OVA with SR-AI/II was verified. mDCs were incubated with FITC labelled AGE-OVA for 5 min and immediately fixed. SR-AI/II in the incubated cells was then stained anti-SR-AI/II antibodies. As show in Fig 4.26, AGE-OVA did indeed co-localise with SR-AI/II, confirming the association of AGE-OVA with the receptor.

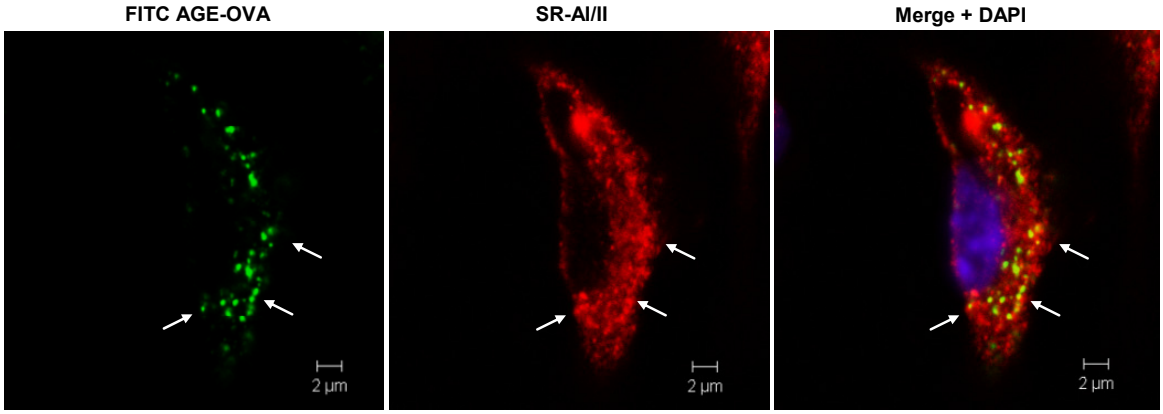

Fig 4.26 Localisation of AGE-OVA and SR-AI/II in mDCs

mDCs were incubated with 10 µg/ml of FTIC labelled AGE-OVA for 5 min and then fixed with 4% PFA. The cells were then stained with polyclonal goat anti-SR-AI/II antibodies and Alexa 568 labelled monoclonal anti-goat IgG antibody. DAPI (blue) was used to stain the nucleus. The arrowheads point to SR-AI/II co-localised with AGE-OVA.

Next, it was examined whether AGE-OVA and MHC II were found in the same cellular compartments. mDCs were incubated with FITC labelled AGE-OVA for 15 min and stained with anti-MHC II antibody. As shown in Fig 4.27 A, AGE-OVA and MHC II co-localised in mDCs. In SR-AI/II deficient mDCs, only small amounts of AGE-OVA were found inside the cells and there is little co-localisation with MHC II (Fig. 4.27 B). In MR-deficient mDCs, the co-localisation of AGE-OVA with MHC class II molecules was, again, observed (Fig. 4.27 C). These results suggest that AGE-OVA taken up by SR-AI/II enters compartments which also contain MHC II.

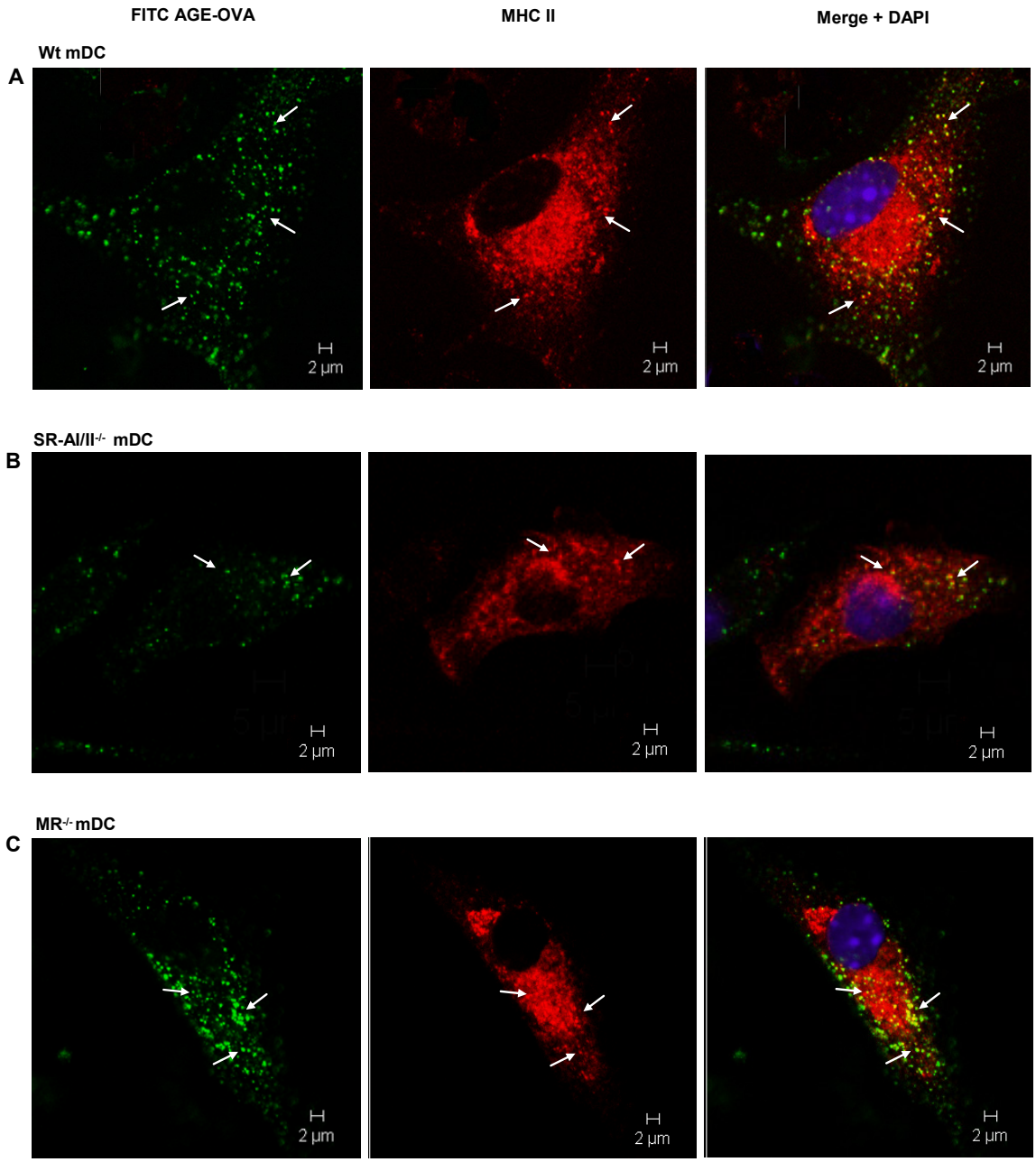

Fig 4.27 Localisation of AGE-OVA and MHC II in Wt, SR-AI/II deficient and MR deficient mDCs

A WT mDCs, **B** SR-AI/II deficient mDCs or **C** MR deficient mDCs were incubated with FITC labelled AGE-OVA for 15 min and then fixed with 4% PFA. The cells were then stained with a monoclonal rat anti-MHC II antibody and Alexa 568 labelled monoclonal anti-rat IgG antibody. DAPI (blue) was used to stain the nucleus. The arrowheads indicate MHC II co-localisation with AGE-OVA.

Multiple pathways regulate the targeting of antigens to MHC class II restricted presentation (Chapter 1.9.2). Burgdorf et al have shown that pinocytosed native OVA is delivered to the late endosomes/lysosomes in DCs, where antigenic peptides are generated by proteases and loaded to MHC II. To elucidate whether AGE-OVA was targeted to the late endosomes/lysosomes in mDCs here, the cells were incubated with FITC AGE-OVA for 15 to 60 min and then stained with

anti-LAMP-1 antibody. LAMP-1 is a marker protein for the late endosomes/lysosomes. Here, co-localisation of AGE-OVA with LAMP-1 could not be observed at any time point of the incubation (Fig 4.28 A, showing 60 min incubation of AGE-OVA).

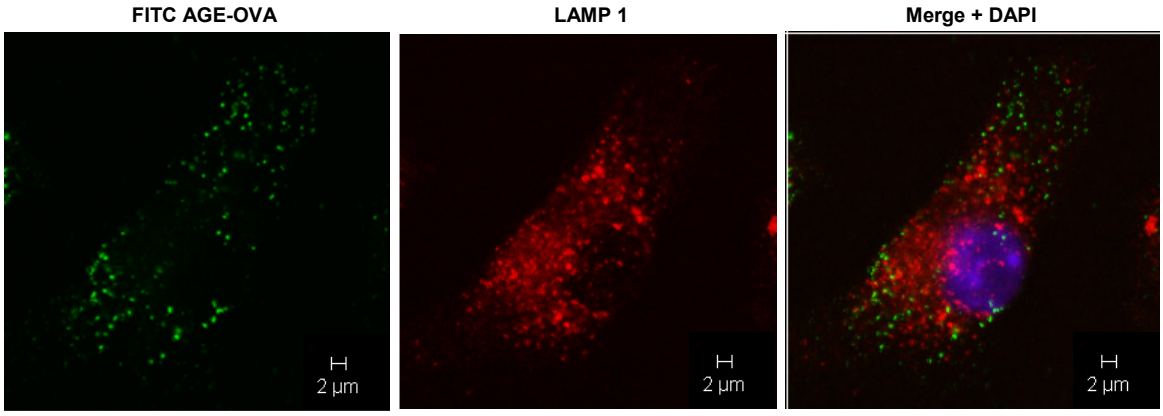

Fig 4.28 Localisation of AGE-OVA and LAMP-1 in mDCs

Wt mDCs were incubated with FITC labelled AGE-OVA for 60 min and then fixed with 4% PFA. The cells were then stained with monoclonal rat anti-LAMP1 antibody and Alexa 568 labelled monoclonal anti-rat IgG antibody to stain the lysosome / late endosome. DAPI (blue) was used to stain the nucleus.

Several studies have shown that nascent MHC II is delivered from the ER, where they are assembled, to the early endosomes (Villadangos et al., 2000; Benaroch et al., 1995) (chapter 1.10.3). To examine whether this may also be the case in mDCs, the cells were stained with anti-MHC II antibody and anti-early endosome associated protein 1 (EEA1) antibodies. Consistent with the previous studies, co-localisation of MHC II and EAA1 was observed in mDCs (Fig. 4.29).

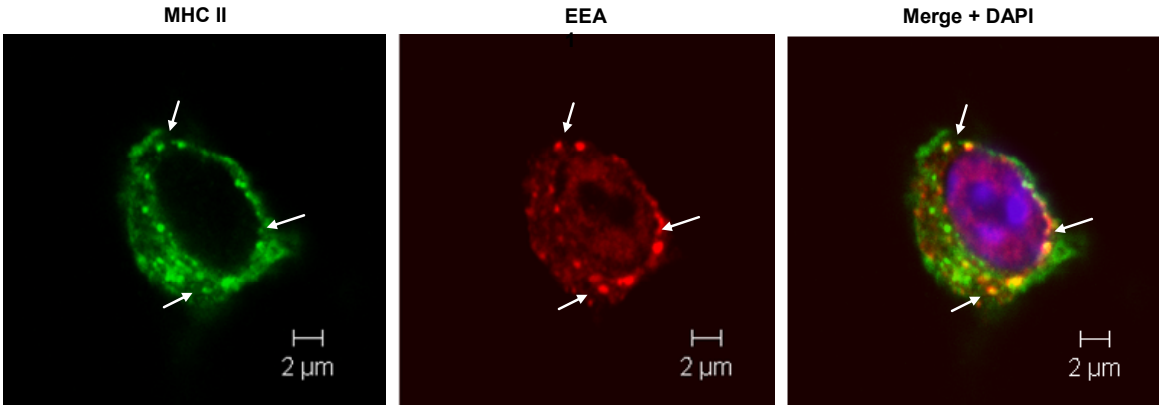

Fig 4.29 Localisation of MHC II and EEA1 in mDCs

Wt mDCs were fixed with 4% PFA. The cells were then stained with a monoclonal rat anti-mouse MHC II antibody and polyclonal rat anti-EEA1 antibodies followed by Alexa 568 labelled monoclonal anti-rat IgG antibody and Cy3 labelled monoclonal anti-rabbit IgG antibody to stain MHC II and the early endosomes, respectively. DAPI (blue) was used to stain the nucleus. The arrowheads indicate MHC II co-localisation with EEA1.

Next it was elucidated whether AGE-OVA had also been delivered to the early endosomes. Therefore mDCs were incubated with FITC conjugated AGE-OVA for 15 min followed by staining with anti-EAA1 antibodies. A clear co-localisation of AGE-OVA with EAA1 was observed (Fig 4.30 A). Burgdorf et al have shown that the MR targets OVA to the early endosomes (Burgdorf et al., 2007). To eliminate involvement of MR mediated targeting to the early endosome, MR deficient mDCs were also stained with anti-EAA1 antibodies after incubation with AGE-OVA. As shown in Fig 4.30 B, AGE-OVA was found in the early endosomes even in MR deficient mDCs. These results suggest that SR-AI/II, as the only other main receptor involved in AGE-OVA uptake, targets AGE-OVA to the early endosomes, in which MHC II was also found (Fig. 4.29).

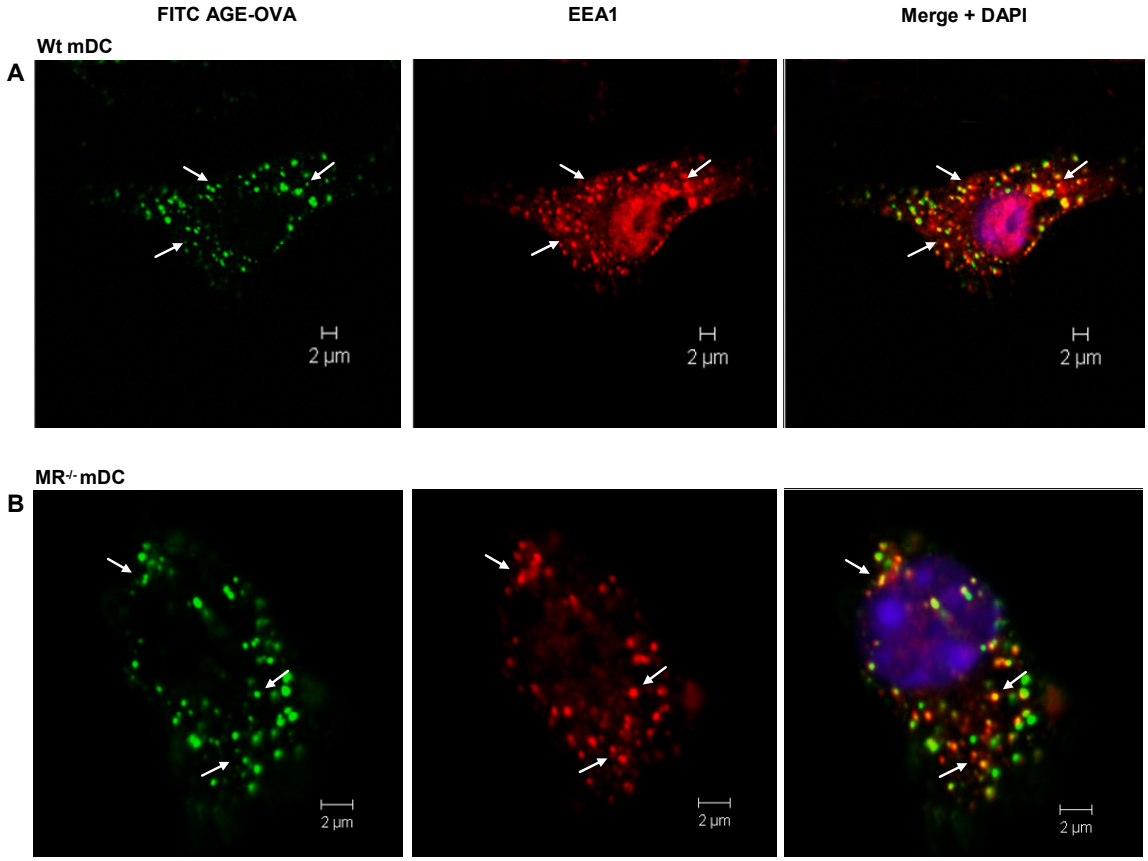

Fig 4.30 Localisation of AGE-OVA and EEA1 in Wt and MR deficient mDCs

A WT mDCs or **B** MR deficient mDCs were incubated with AGE-OVA and then fixed with 4% PFA. The cells were stained with polyclonal rabbit anti-EEA1 antibodies and Cy3 labelled monoclonal anti-rabbit IgG antibody. DAPI (blue) was used to stain the nucleus. The arrowheads indicate co-localisation of EEA1 with AGE-OVA.

4.7 Influence of glycation by the Maillard reaction on the T cell immunogenicity and antigenicity of OVA *in vivo*

As demonstrated in chapter 4.3, the glycation of OVA by the Maillard reaction significantly enhanced the $CD4^+$ T cell immunogenicity of the allergen *in vitro*. Next, it was investigated whether AGE-OVA has a higher $CD4^+$ T cell immunogenicity and antigenicity (the ability to induce antibody production) than native OVA and thermally processed OVA *in vivo*. It was of particular interest to see whether AGE-OVA has a higher potential to induce allergen specific IgE antibodies, as this immunoglobulin is a critical component in the pathophysiology of (food) allergy (chapter 1.2). BL6 mice were used for the *in vivo* experiments since the SR-AI/II and RAGE deficient mice used for the *in vitro* study were generated on this genetic background.

4.7.1 Assessment of the T cell immunogenicity of AGE-OVA by subcutaneous immunisation of mice

First, subcutaneous (sc) immunisation was performed to examine the $CD4^+$ T cell immunogenicity of all three OVAs *in vivo* (chapter 3.2.5.5.2). The method efficiently induces activation of antigen specific $CD4^+$ T cells in the draining lymph nodes (LN) by only one immunisation. Mice were sc immunised with native, thermally processed or AGE-OVA together with the adjuvant TiterMax Gold®. Seven days after immunisation, $CD4^+$ T cells were isolated from the LN using a magnetic cell sorting system (chapter 3.2.3.7). The T cells were co-cultured with mDCs, and stimulated with the OVAs for 24 h. As a positive control for the assay, $CD4^+$ T cells were incubated with concanavalin A (Con A) which is a lectin with mitogenic properties, i.e. it induces antigen unspecific activation and proliferation of T cells. In order to assess the level of $CD4^+$ T cell activation, the IL-2 concentration in the cell culture supernatants was measured (chapter 3.2.4.5.2.1).

The sc immunisation efficiently induced activation of antigen specific $CD4^+$ T cells in the LNs (Fig. 4.31). $CD4^+$ T cells from all three immunisation groups displayed a greater IL-2 production upon *ex vivo* recall stimulation with AGE-OVA compared to the recall stimulation with native or thermally processed OVA. Furthermore, $CD4^+$ T cells isolated from AGE-OVA immunised mice produced considerably higher levels of IL-2 in response to AGE-OVA than T cells from mice immunised with native or thermally processed OVA. The results suggest that AGE-OVA has a higher T cell immunogenicity than native OVA and thermally processed OVA not only *in vitro* but also *in vivo*.

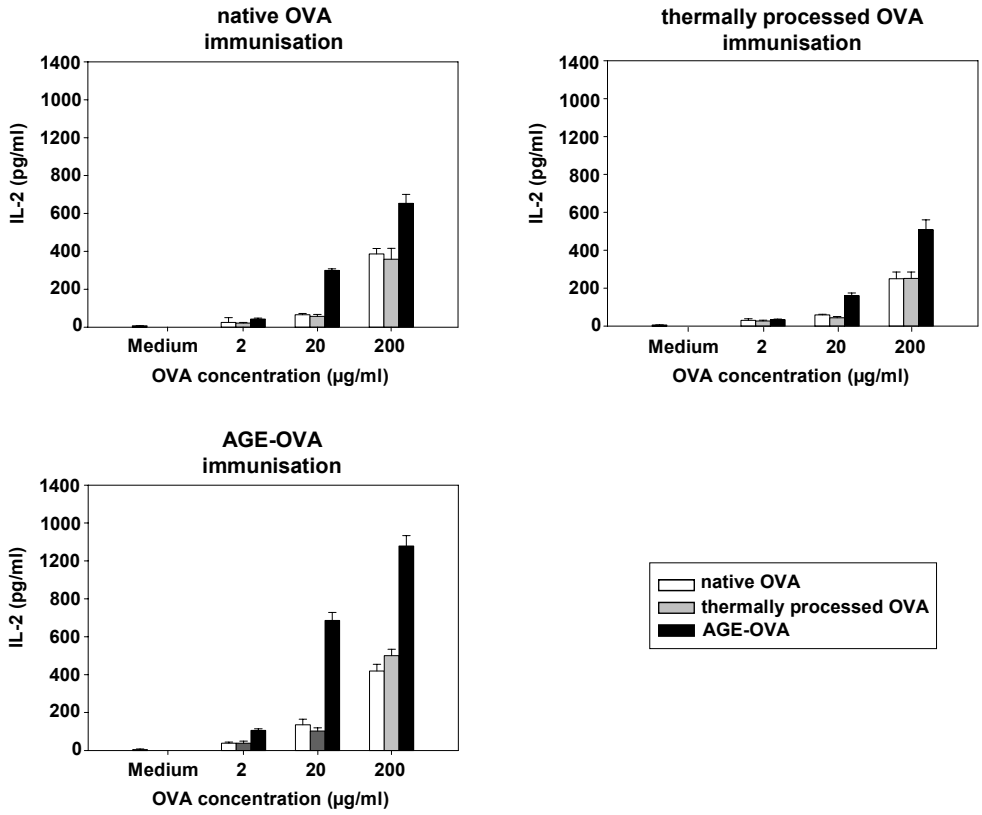

Fig. 4.31 LN CD4$^+$ T cell activation induced by subcutaneous injection of AGE-OVA in BL6 mice

BL6 mice (n=3) were subcutaneously immunised with 100 µg of native, thermally processed or AGE-OVA. Seven days after immunisation, CD4$^+$ T cells were isolated from the LN, co-cultured with mDCs and stimulated with either form of the OVA for 24 h. The IL-2 concentration in the culture supernatants was determined by ELISA. IL-2 production by CD4$^+$ T cells stimulated with Con A (2.5 µg/ml) was comparable among all immunisation groups. Error bars signify the SD of cell culture triplicates and ELISA duplicates.

4.7.2 Assessment of the T cell immunogenicity and antigenicity of AGE-OVA using intraperitoneal immunisation of mice

Next, the T cell immunogenicity and antigenicity of AGE-OVA was assessed by intraperitoneal (ip) immunisation of BL6 mice with the glycated or unglycated forms of the allergen. In mice, the usage of an adjuvant is generally required to induce a robust IgE production. Therefore, for the assessment of the antigenicity of AGE-OVA, the mice were immunised with the allergen in combination with the adjuvant aluminium hydroxide (Alum) which efficiently facilitates the production of antigen specific IgE and IgG antibodies (Cox and Coulter, 1997). BL6 mice were immunised ip with 50 µg of native, thermally processed or AGE-OVA in combination with 2 mg Alum (chapter 3.2.5.5.1). The mice were immunised three times to induce detectable level of allergen specific IgE antibodies. The second and third immunisations were performed two weeks and three weeks after the first immunisation, respectively. As controls, mice were immunised with Alum in PBS only.

4.7.2.1 Assessment of the T cell immunogenicity of AGE-OVA by ip immunisation of mice

First, the T cell immunogenicity of all three OVAs in ip immunised mice was assessed. One week after the final immunisation, $CD4^+$ T cells isolated from the spleen were co-cultured with mitomycin C (MMC) treated splenocytes (used as antigen presenting cells) and stimulated with native, thermally processed or AGE-OVA for 96 hours. Proliferation of T cells during the final 20 h was assessed by measuring the incorporation of [^{3}H-methly]-thymidine into the cells (chapter 3.2.3.11).

$CD4^+$ T cells isolated from mice after three ip immunisations with either of the OVAs showed comparable levels of proliferation in response to native, thermally processed and AGE-OVA (Fig. 4.32). Due to the multiple immunisation, the maximum *in vivo* level of T cell responses to the antigens could have been induced, which may lead to the comparable levels of T cell activation in all immunisation groups.

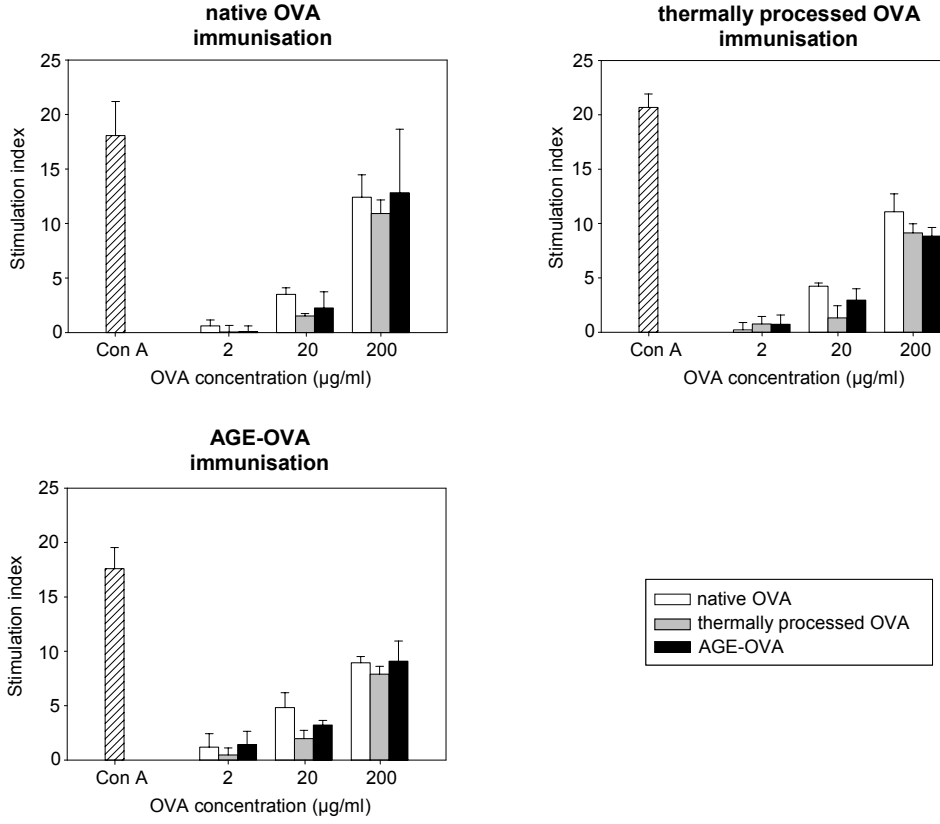

Fig. 4.32 Splenic $CD4^+$ T cell activation induced by ip immunisation of BL6 mice

BL6 mice (n=3) were ip immunised with native, thermally processed or AGE-OVA. Seven days after the third immunisation splenic $CD4^+$ T cells were purified from the immunised mice. The T cells were co-cultured with MMC treated splenocytes from naïve BL6 mice and stimulated with either of the OVAs for 96 h. Proliferation of the T cells during the final 20 h was assessed by measuring incorporation of [^{3}H-methly]-thymidine into the cells. The proliferation is shown as stimulation index. Error bars signify the SD of cell culture triplicates. The data shown represents two independent experiments

4.7.2.2 Assessment of the antigenicity of AGE-OVA by intraperitoneal immunisation of mice

Next, the antigenicity of the OVAs in ip immunised mice was examined. Seven days after each immunisation serum samples were collected (chapter 3.2.5.4.). The levels of IgG1, IgG2a and IgE antibodies specific for native, thermally processed or AGE-OVA in the sera of individual mice were measured by ELISA (chapter 3.2.4.4.1.1).

First, the allergen specific IgG1 and IgG2a antibodies in the sera of the immunised mice were measured. The levels of allergen specific IgG1 antibodies in the sera of the mice which had received only one ip immunisation with the OVAs was very low (data not shown). After the second ip immunisation a robust allergen specific IgG1 antibody production was observed. The level of IgG1 antibodies binding to native, thermally processed, or AGE-OVA was almost comparable in all three immunisation groups (Fig. 4.33).

Small differences were observed in IgG2a production (Fig. 4.34). The production of IgG2a appears to be a bit higher in three mice of the AGE-OVA immunised group. Also, the binding of IgG2a to AGE-OVA from native and thermally processed OVA is a little reduced compared to binding to the unglycated OVA forms (Fig. 4.34,C). Compared to the levels of allergen specific IgG1 antibodies, those of specific IgG2a antibodies were significantly lower in the immunised mice. In mice, IgG antibodies are classified into IgG1, IgG2a, IgG2b and IgG3. IgG1 antibody production is mediated by Th2 cells, whereas IgG2a antibody production is mediated by Th1 cells. As Alum predominantly induces Th2 cell responses, IgG1 antibody production would be dominant in the immunised mice (Cox and Coulter, 1997).

To examine whether glycation structures influence T cell differentiation *in vivo*, the IgG1/IgG2a ratio for each immunised mouse was calculated. AGE-OVA induced a slightly lower IgG1/IgG2a ratio than immunisation with the control OVAs (Fig. 4.35). However, the difference was not considerable. These results suggest that AGE-OVA would not have any influence on the T cell differentiation *in vivo,* which is consistent with the *in vitro* data (Fig. 4.10).

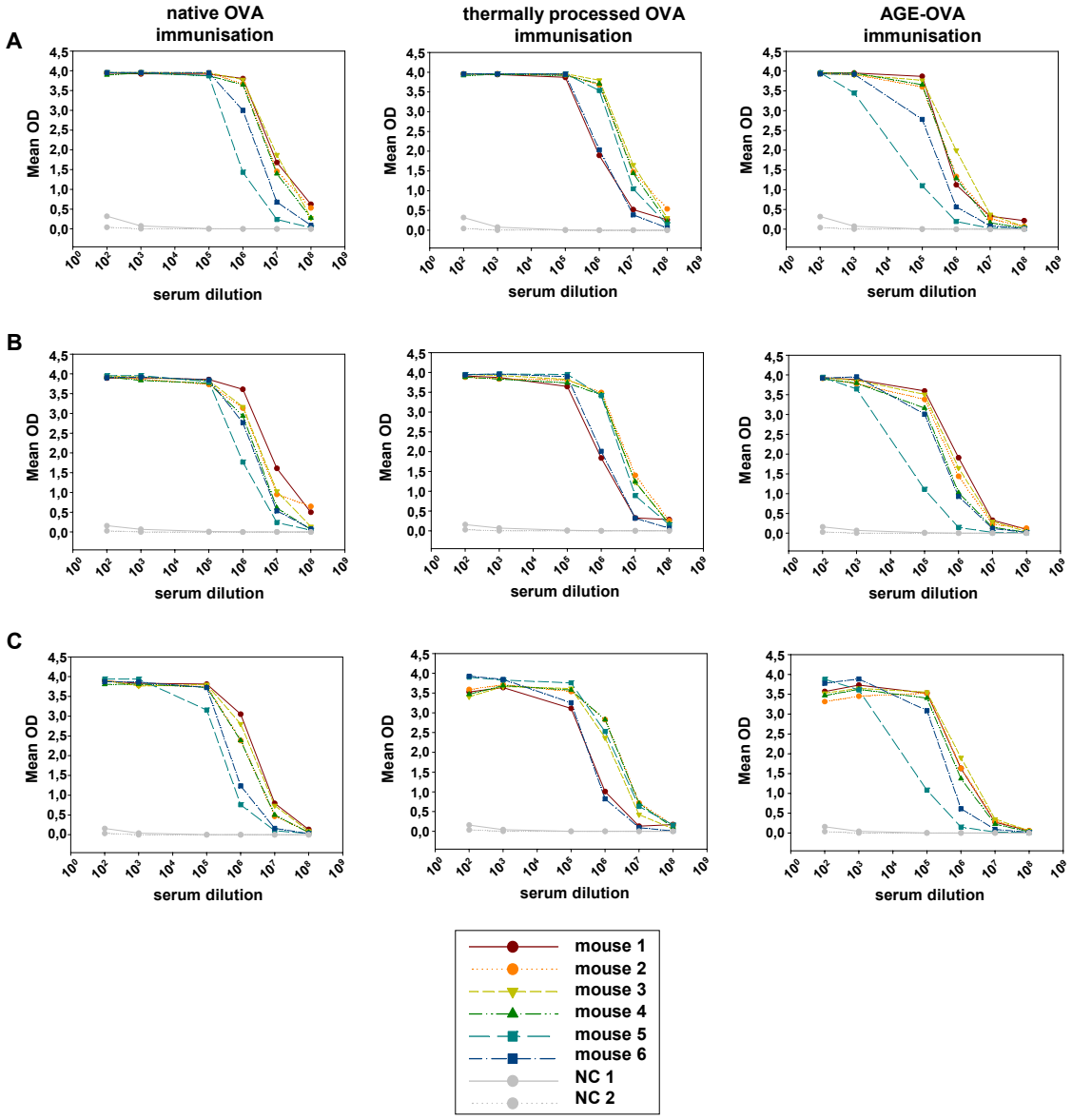

Fig. 4.33 Allergen specific IgG1 production in BL6 mice after the second ip immunisation with native, thermally processed or AGE-OVA

BL6 mice (n=6) were ip immunised with 50 µg of native, thermally processed or AGE-OVA with 2 mg Alum three times. One week after the second immunisation, blood was harvested from the mice. **A** The level of anti-native OVA IgG1 antibodies in the sera was measured using ELISA plates coated with the native allergen. **B** The level of anti-thermally processed OVA specific IgG1 antibodies in the sera was measured using plates coated with thermally processed OVA. **C** The level of anti-AGE-OVA IgG1 antibodies in the sera was measured using plates coated with glycated OVA. NC1 and NC2; mice immunised with PBS and Alum alone.

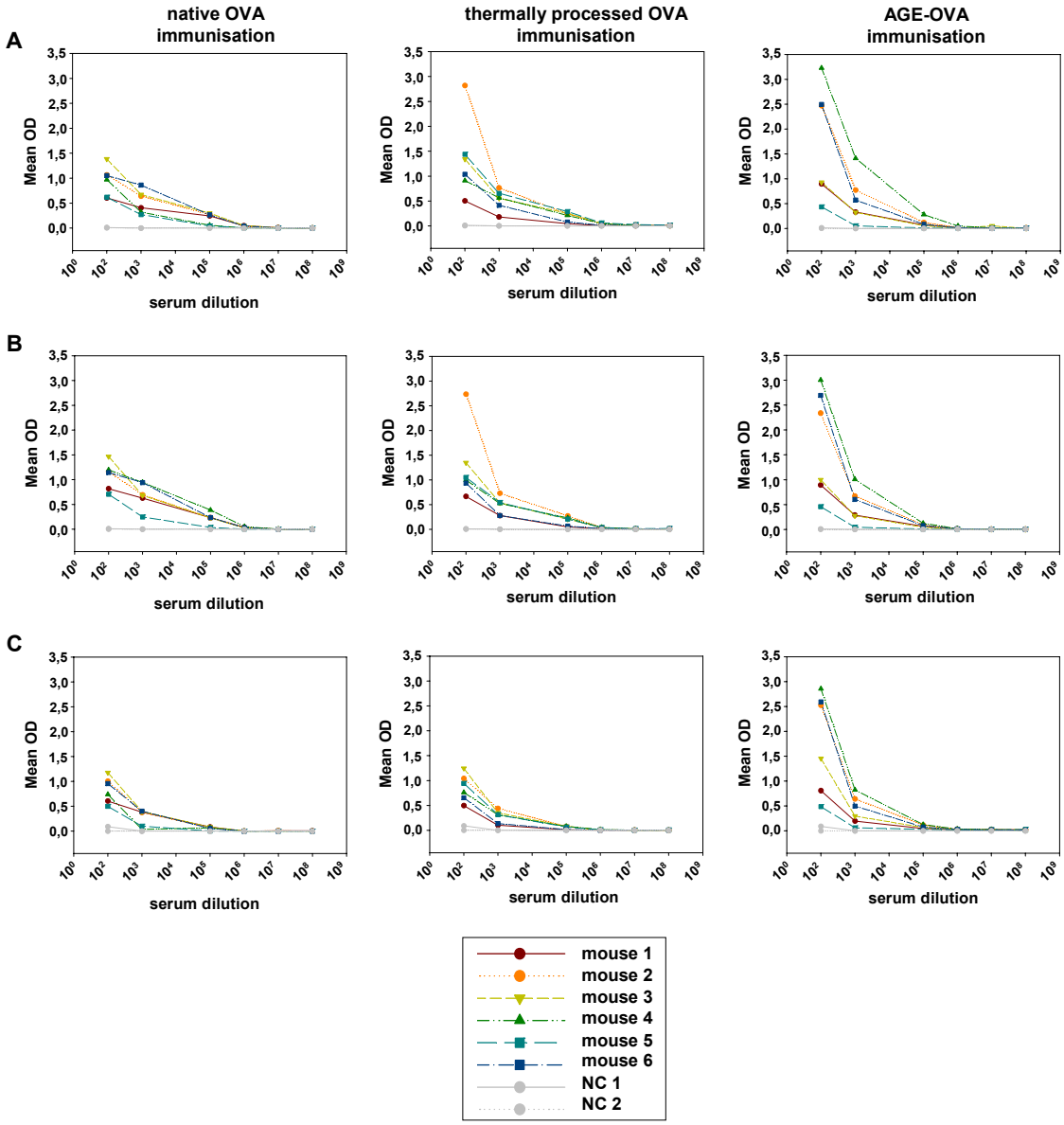

Fig. 4.34 Allergen specific IgG2a antibody production in BL6 mice after the second ip immunisation with native, thermally processed or AGE-OVA.

BL6 mice (n=6) were ip immunised with 50 µg of native OVA, thermally processed OVA or AGE-OVA with 2 mg Alum three times. One week after the second immunisation, blood was harvested from the mice. **A** The level of anti-native OVA IgG2a antibodies in the sera was measured using ELISA plates coated with the native allergen. **B** The level of anti thermally processed OVA specific IgG2a antibodies in the sera was measured using plates coated with thermally processed OVA. **C** The level of anti-AGE-OVA IgG2a antibodies in the sera was measured using plates coated with glycated OVA. NC1 and NC2; mice immunised with PBS and Alum alone.

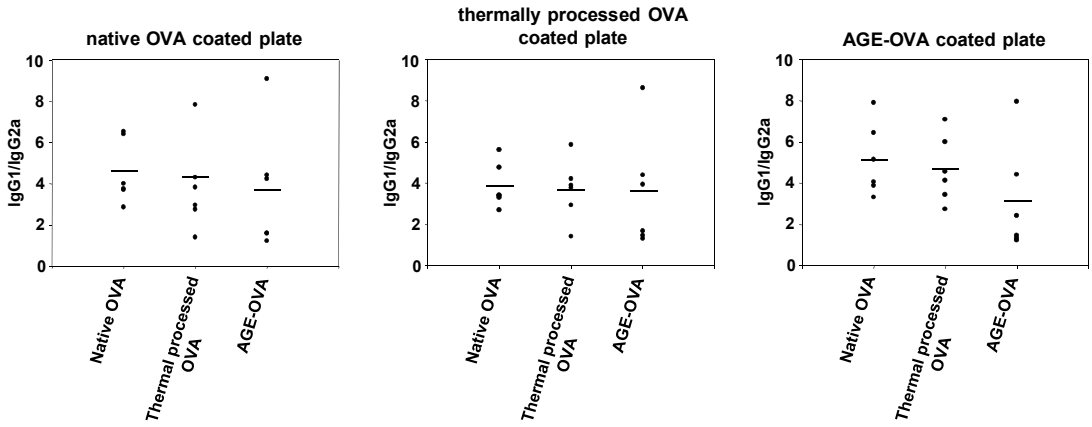

Fig. 4.35 IgG1/IgG2a ratio in BL6 mice after the second ip immunisation with native, thermally processed or AGE-OVA

The IgG1/IgG2a ratio in individual mouse ip immunised with native, thermally processed, or AGE-OVA, was calculated using the allergen concentration at 50% of the maximum absorption in the ELISA for measurement of IgG1 (Fig. 4.33) and IgG2a levels (Fig 4.34). Each dot represents the IgG1/IgG2a ratio of an individual mouse. Horizontal bars represent the mean for each group.

Finally, the allergen specific IgE antibodies in the sera of ip immunised mice were measured. AGE-OVA induced significantly higher levels of allergen specific IgE antibodies than native and thermally processed OVA (Fig. 4.36). Furthermore, the IgE antibodies from AGE-OVA immunised mice bound equally well to native, thermally processed and AGE-OVA. These results strongly suggest that the glycation of OVA by the Maillard reaction could enhance the antigenicity of the egg white allergen to induce allergen specific IgE antibodies and antibodies from mice immunised with AGE-OVA not only recognise the glycated allergen but also the unglycated forms.

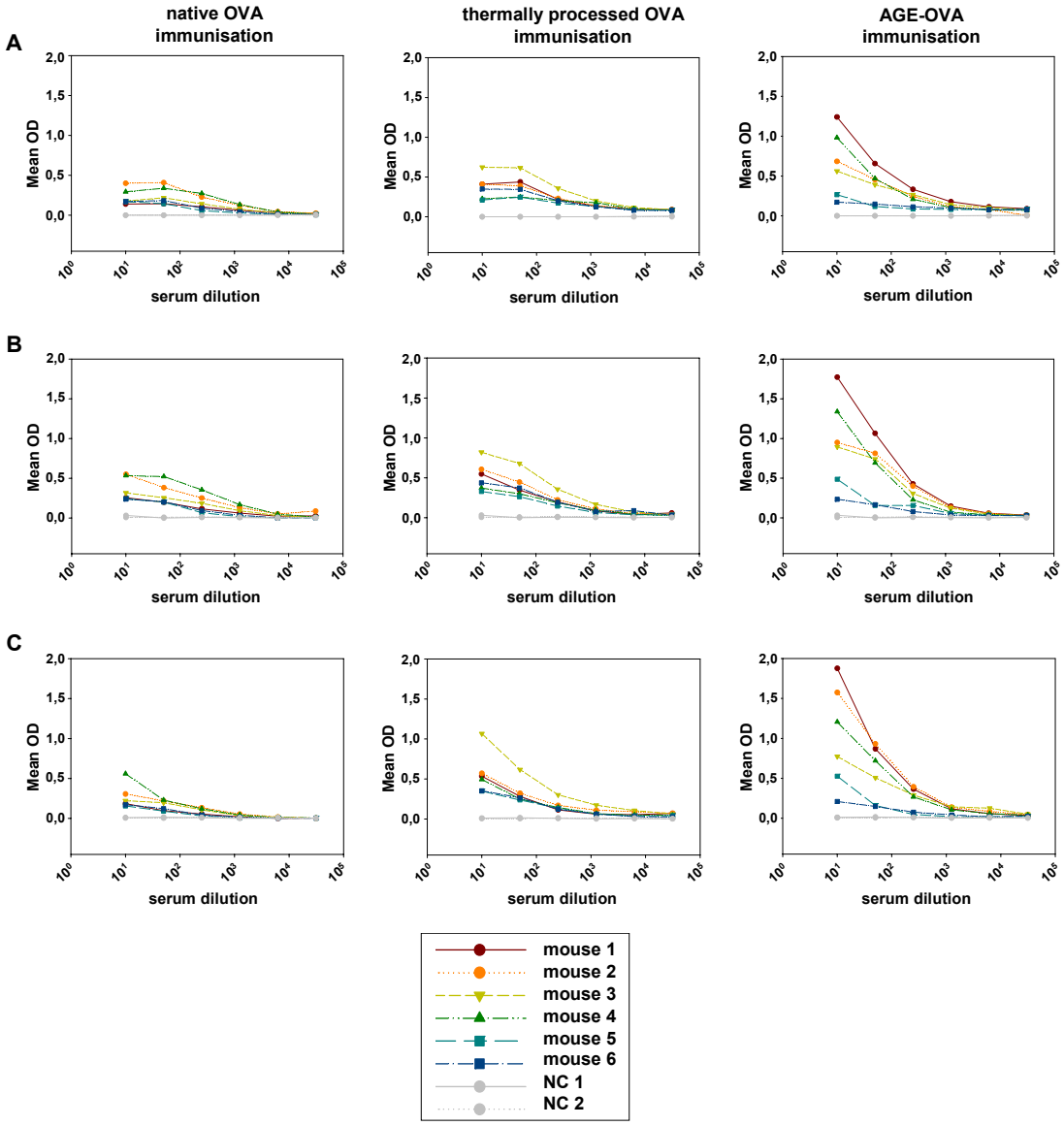

Fig. 4.36 Allergen specific IgE antibody production in BL6 mice after the second ip immunisation with native, thermally processed or AGE-OVA

BL6 mice (n=6) were ip immunised with 50 µg of native OVA, thermally processed OVA or AGE-OVA with 2 mg Alum three times. One week after the second immunisation, blood was harvested from the mice. **A** The level of anti-native OVA IgE antibodies in the sera was measured using ELISA plates coated with the native allergen. **B** The level of anti-thermally processed OVA IgE antibodies in the sera was measured using plates coated with thermally processed allergen. **C** The level of anti-AGE-OVA IgE antibodies in the sera was measured using plates coated with glycated OVA. NC1 and NC2; mice immunised with PBS and Alum alone.

4.8 Analysis of AGE-rAra h 2

Peanuts are one of the most common causes of severe allergic reactions to food. It has been shown that the Maillard reaction occurs during thermal processing of peanuts as the nuts contain many carbohydrates such as glucose and galactose (Maleki et al., 2001, Chung et al., 2003). Since peanuts are usually not eaten raw but roasted, fried or boiled, it is of importance to investigate how the Maillard reaction influences the T cell immunogenicity and antigenicity of peanut allergens such as one of the major allergens Ara h 2. Our collaborator, the Department of Allergology at the Paul-Ehrlich-Institut, has succeeded in producing recombinant Ara h 2 (rAra h 2) using an expression system in *E.coli*. Here, it was investigated whether the Maillard reaction influences the T cell immunogenicity and antigenicity of Ara h 2 by using the recombinant protein.

First, it was examined whether the type of carbohydrate present during thermal processing has an influence on the formation of glycation structures on glycated Ara h 2 (AGE-Ara h 2). rAra h 2 was incubated with glucose, galactose, fructose, lactose, maltose or mannose at 120°C for 2h (chapter 3.2.2.1.1). ELISAs were performed to analyse the glycation structures on rAra h 2 after thermal processing with the different carbohydrates (chapter 3.2.4.4.1.2). Independent of the carbohydrate used for the process, high levels of CML and GA-pyridine were detected on AGE-Ara h 2 (Fig. 4.37), while pyrraline and CEL were not detected by the ELISAs. Galactose, which is naturally contained in peanuts, induced a higher level of GA-pyridine than the other carbohydrates and was also very potent in inducing CML. Therefore, galactose, which is found in peanuts (Rudrapatnam et al., 197), was used in the preparation of AGE-Ara h 2 for the subsequent studies.

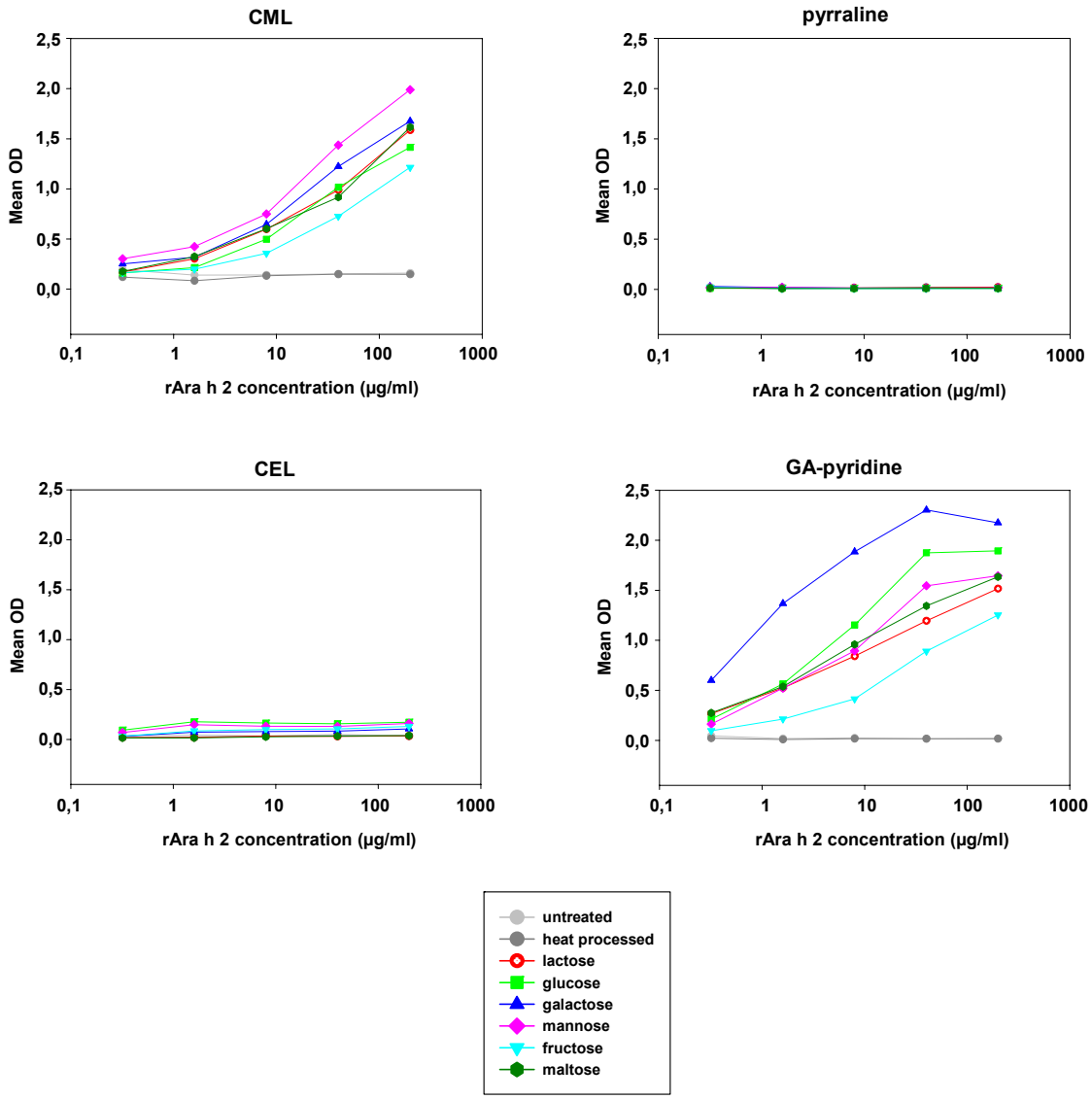

Fig. 4.37 Glycation structures on AGE-rAra h 2 produced by the Maillard reaction.

AGE-rAra h 2 was prepared by incubating 500 µg/ml of rAra h 2 with 0.1M lactose, glucose, galactose, mannose, fructose or maltose in PBS at 120°C for 2h. The glycation structures on rAGE-Ara h 2 were analysed by ELISA. Unprocessed Ara h 2 and Ara h 2 thermally processed without carbohydrates were used as controls.

To examine whether the thermal processing with or without galactose at 120°C influences the secondary structure of rAra h 2, CD spectroscopy was performed (chapter 3.2.2.5). The mean molar elipticity and the exact protein concentration were included for the evaluation of the spectra. Consistent with the CD spectrum of native rAra h 2 isolated from peanut extracts (Lehmann et al, 2006), unprocessed rAra h 2 had its maximum at 190 to 193 nm and its minima at 208 – 210 and 222 nm (Fig. 4.38). The result indicates that α-helices dominate the secondary structure of the protein. The CD spectrum of rAra h 2 incubated at 120°C with or without galactose significantly differed from that of the unprocessed allergen. The minima of thermally processed and AGE-Ara h 2 were at 199 and 198 nm respectively (Fig 4.38). The result suggests that (i) thermal processing

of rAra h 2 at 120°C leads to a destruction of the α-helical structures, and that (ii) the protein structure of the processed allergen is dominated by random coils.

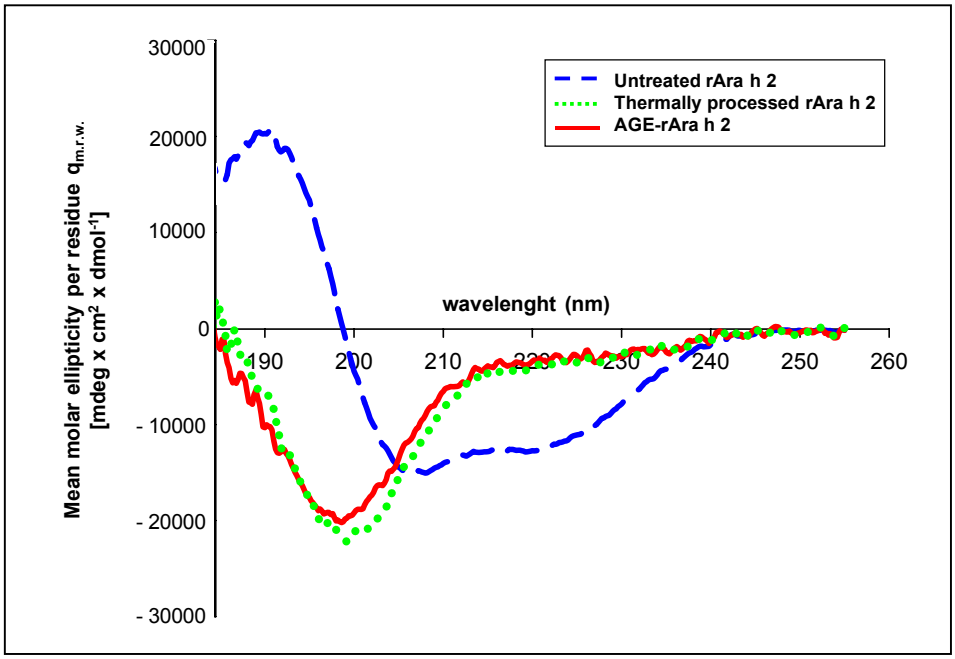

Fig 4.38 Analysis of AGE-rAra h 2 by CD spectroscopy

rAra h 2 was incubated with and without galactose at 120°C for 2h. The CD spectrum of unprocessed, thermally processed and AGE- rAra h 2 was analysed.

4. 9 Influence of glycation by the Maillard reaction on the T cell immunogenicity and antigenicity of rAra h 2

C3H/HeJ mice are a high responder mouse strain for Ara h 2 (Li et al., 2000; Proust et al., 2008). To examine whether the glycation of rAra h 2 by the Maillard reaction influences the T cell immunogenicity and antigenicity of this major peanut allergen, C3H/HeJ mice were ip immunised with 5 µg of unprocessed, thermally processed or AGE-rAra h 2 together with 2 mg Alum in 200 µl PBS (chapter 3.2.5.5.1). Two and three weeks after the first immunisation, the mice were boosted with 1 µg of the allergen together with 1 mg Alum. As controls, mice were immunised with Alum in PBS only.

4.9.1 Assessment of the T cell immunogenicity of AGE-rAra h 2 by ip immunisation of mice

To assess the T cell immunogenicity of AGE-rAra h 2, mice were sacrificed one week after the final immunisation and $CD4^+$ T cells were isolated from the spleen of immunised mice. The

proliferation of splenic CD4$^+$ T cells in response to unprocessed, thermally processed or AGE-rAra h 2 was measured (chapter 3.2.3.11).

As show in Fig. 4.39, stimulation with Con A induced comparable levels of proliferation of CD4$^+$ T cells from all three groups. Therefore it was feasible to compare the levels of proliferation of CD4$^+$ T cells from the mice immunised with the different forms of rAra h 2. The stimulation index was highest for splenic CD4$^+$ T cells from all three groups when re-stimulated with unprocessed rAra h 2. This indicates, that unprocessed rAra h 2 is the best stimulator of proliferation of T cells purified from rAra h 2 immunised mice, independent of the form of rAra h 2 used for immunisation. The data suggests that the CD4$^+$ T cell immunogenicity of AGE-rAra h 2 is lower than that of unprocessed rAra h 2 and thermally processed Ara h 2 in C3H/HeJ mice.

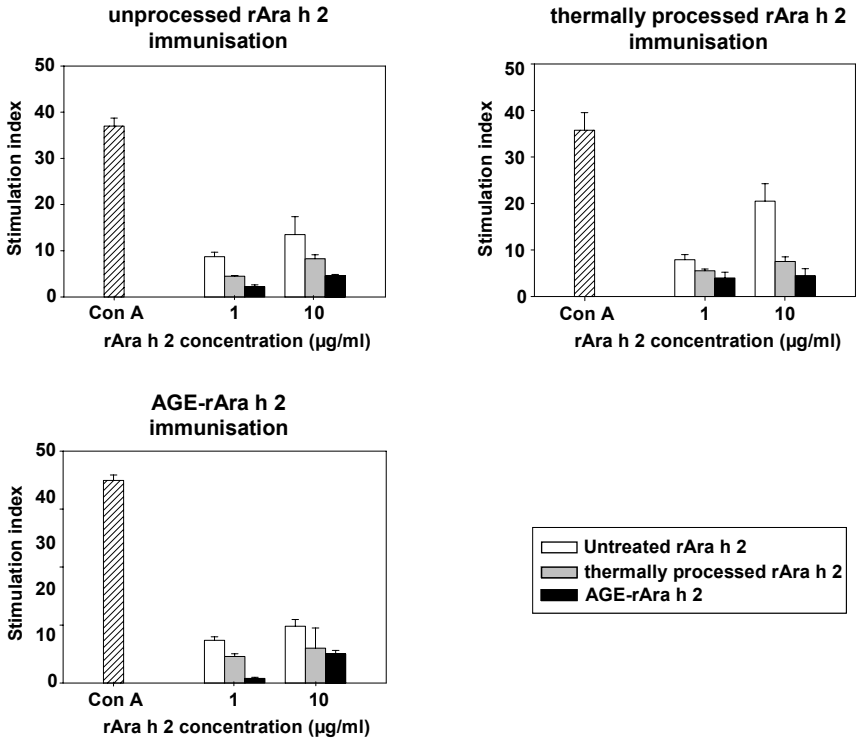

Fig 4.39 Allergen specific proliferation of CD4$^+$ T cells from mice after ip immunisation with unprocessed, thermally processed or AGE-rAra h 2

C3H/HeJ mice (n=4) were ip immunised with unprocessed, thermally processed or AGE-rAra h 2 together with Alum three times. Splenic CD4$^+$ T cells were isolated from immunised mice, co-cultured with irradiated syngeneic splenocytes and stimulated with either form of rAra h 2 for 96 h. Proliferation of T cells during the final 20 h was assessed by measuring the incorporation of [^{3}H-methly]-thymidine by the cells. The level of proliferation is indicated as stimulation index. Error bars signify the SD of cell culture triplicates.

4.9. 2 Cytokine production of CD4$^+$ T cells from the mice after receiving ip immunisation with unprocessed, thermally processed or AGE-rAra h 2

To assess whether AGE-rAra h 2 influences the CD4$^+$ T cell differentiation *in vivo*, the cytokine production of splenic CD4$^+$ T cells from mice immunised with unprocessed, thermally processed

or rAGE-Ara h 2 was examined. The concentration of Th1 type cytokine IFN-γ and Th2 type cytokine IL-4 in the culture supernatant of $CD4^+$ T cells after 72h incubation with either form of rAra h 2 was measured by ELISA (chapter 3.2.4.5.2.1).

Unprocessed rAra h 2 induced the highest secretion of both IFN-γ and IL-4 by $CD4^+$ T cells, independent of which mouse group these were isolated from (Fig. 4.40 A and B). The cytokine production by $CD4^+$ T cells stimulated with thermally processed or AGE-Ara h 2 was comparable for all three immunisation groups. The level of cytokine production and proliferative responses of isolated $CD4^+$ T cells (Fig. 4.39) corresponded well. Collectively these results suggest that the glycation structures on AGE-rAra h 2 do not influence on the T cell differentiation in C3H/HeJ mice.

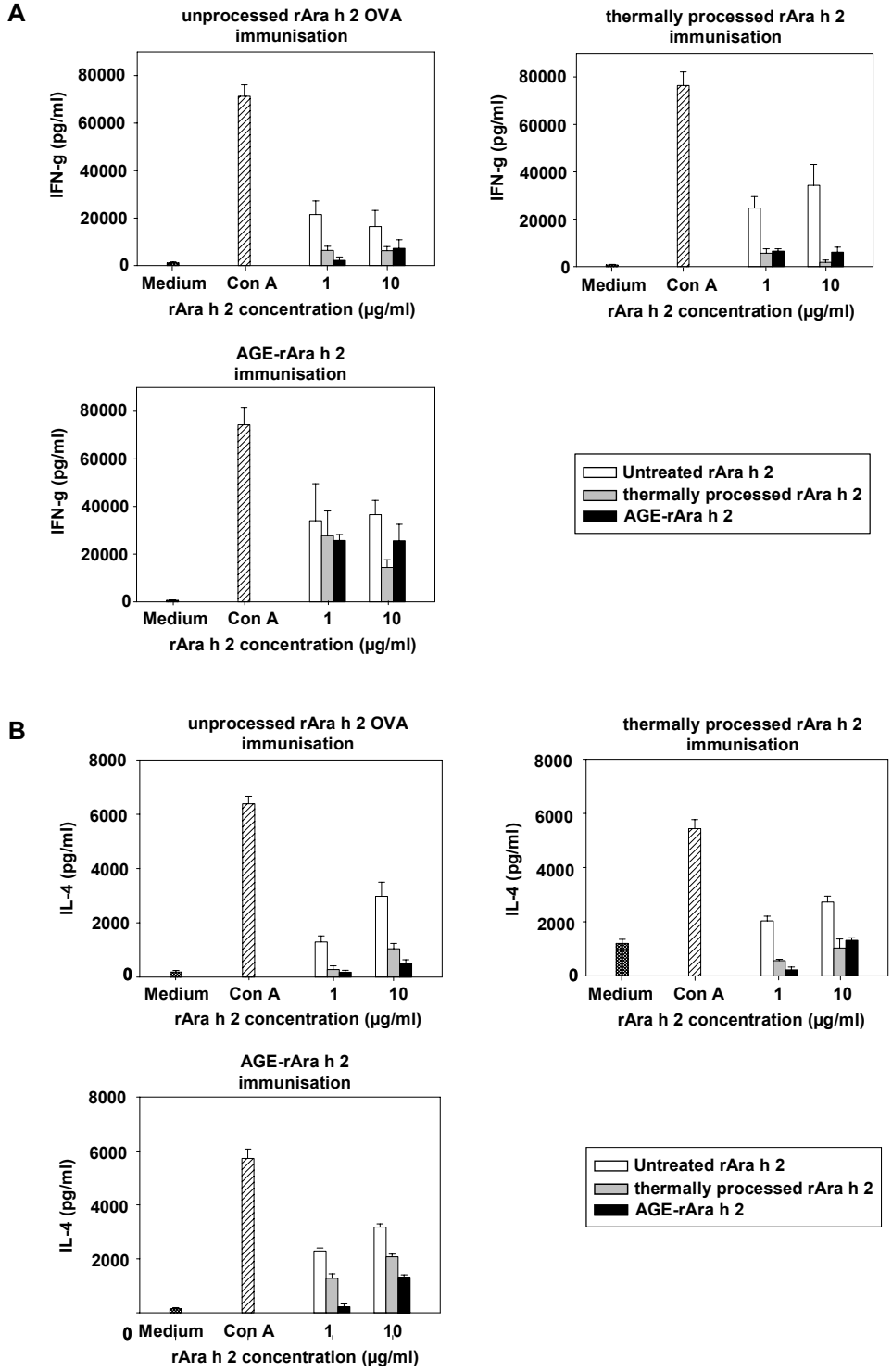

Fig 4.40 Cytokine production of CD4$^+$ T cells from mice immunised with unprocessed, thermally processed or AGE-Ara h 2.

C3H/HeJ mice (n=4) were ip immunised with unprocessed, thermally processed or AGE-rAra h 2 with Alum three times. Splenic CD4$^+$ T cells were isolated from immunised mice. The T cells were co-cultured with irradiated syngeneic splenocytes and stimulated with either form of rAra h 2 for 72h. The concentration of **A** IFN-γ and **B** IL-4 in the culture supernatants was measured by ELISA. Error bars signify the SD of cell culture triplicates and ELISA duplicates.

4.9.3 Assessment of the antigenicity of AGE-rAra h 2 by ip immunisation of mice

For the assessment of the antigenicity of AGE-rAra h 2, serum harvested seven days after the last immunisation was used. The experiments to examine the antigenicity of AGE-rAra h 2 were performed twice.

The levels of IgG1 and IgG2a antibodies specific for unprocessed rAra h 2 in the sera of immunised mice were measured by ELISA (chapter 3.2.4.4.1.1). Both independent experiments showed that immunisation with unprocessed rAra h 2 and Ara h 2 thermally processed without galactose induced higher rAra h 2 specific IgG1 and IgG2a antibodies in mice, compared to immunisation with AGE-rAra h 2 (Fig. 4.41, A and B and Fig. 4.42, A and B). The results indicate that in C3H/HeJ mice the potential of AGE-rAra h 2 to induce allergen specific IgG was lower than that of unprocessed and thermally processed Ara h 2.

Next, the level of IgE antibodies specific for unprocessed rAra h 2 in the sera of immunised mice was measured. In both experiments, the level of anti-unprocessed rAra h 2 IgE antibodies in the sera of AGE-rAra h 2 immunised mice was lower than that in the sera of mice immunised with unprocessed or thermally processed rAra h 2 (Fig. 4.41 C and Fig. 4.42 C). Since the amount of thermally processed rAra h 2 and AGE-rAra h 2 was limited, the levels of anti- thermally processed rAra h 2 and anti-AGE-rAra h 2 IgE antibodies could only be measured for experiment 1. In this experiment, IgE antibodies in the serum of several mice immunised with unprocessed, thermally processed or AGE-rAra h 2 exihibited IgE antibodies binding to unprocessed rAra h 2 but not to AGE-rAra h 2 (Fig. 4.43). Only one mouse immunised with thermally processed rAra h 2 had serum IgE antibodies which had binding activity to the glycated allergen. The results suggest that glycation of rAra h 2 results in a decrease of the antigenicity of the allergen and also reduces recognition of the allergen by IgE antibodies specific for the unglycated forms of rAra h 2.

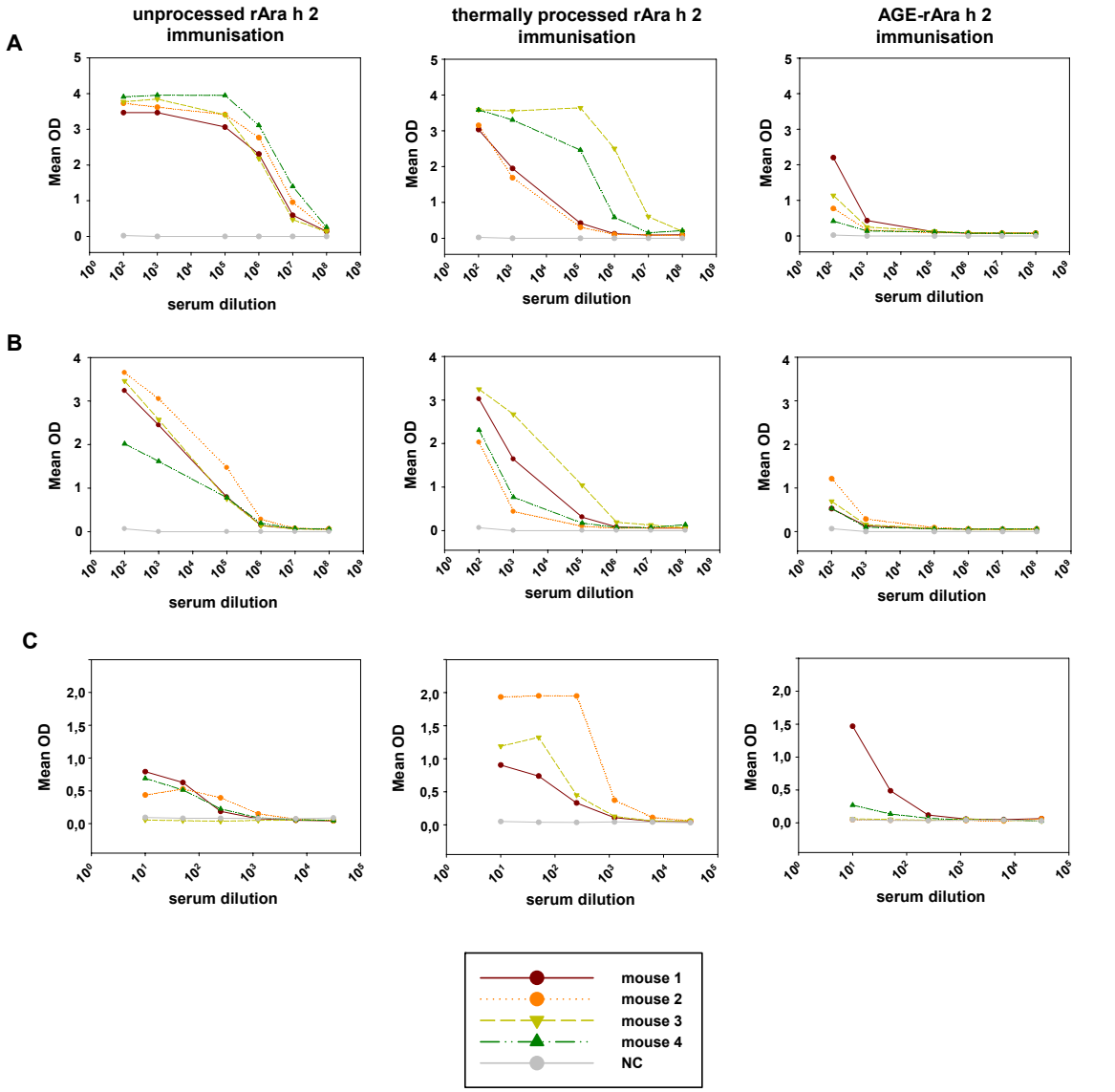

Fig. 4.41 Detection of rAra h 2 specific IgG1, IgG2a and IgE antibody production in mice after ip immunisation with unprocessed, thermally processed or AGE-rAra h 2 in Experiment 1

C3H/HeJ mice (n=4) were ip immunised with unprocessed, thermally processed or AGE-rAra h 2 with Alum three times. The levels of **A** IgG1, **B** IgG2a and **C** IgE binding to unprocessed rArah2 in the sera of immunised mice was determined by ELISA using plates coated with unprocessed rAra h 2. NC; mouse immunised with PBS and Alum only.

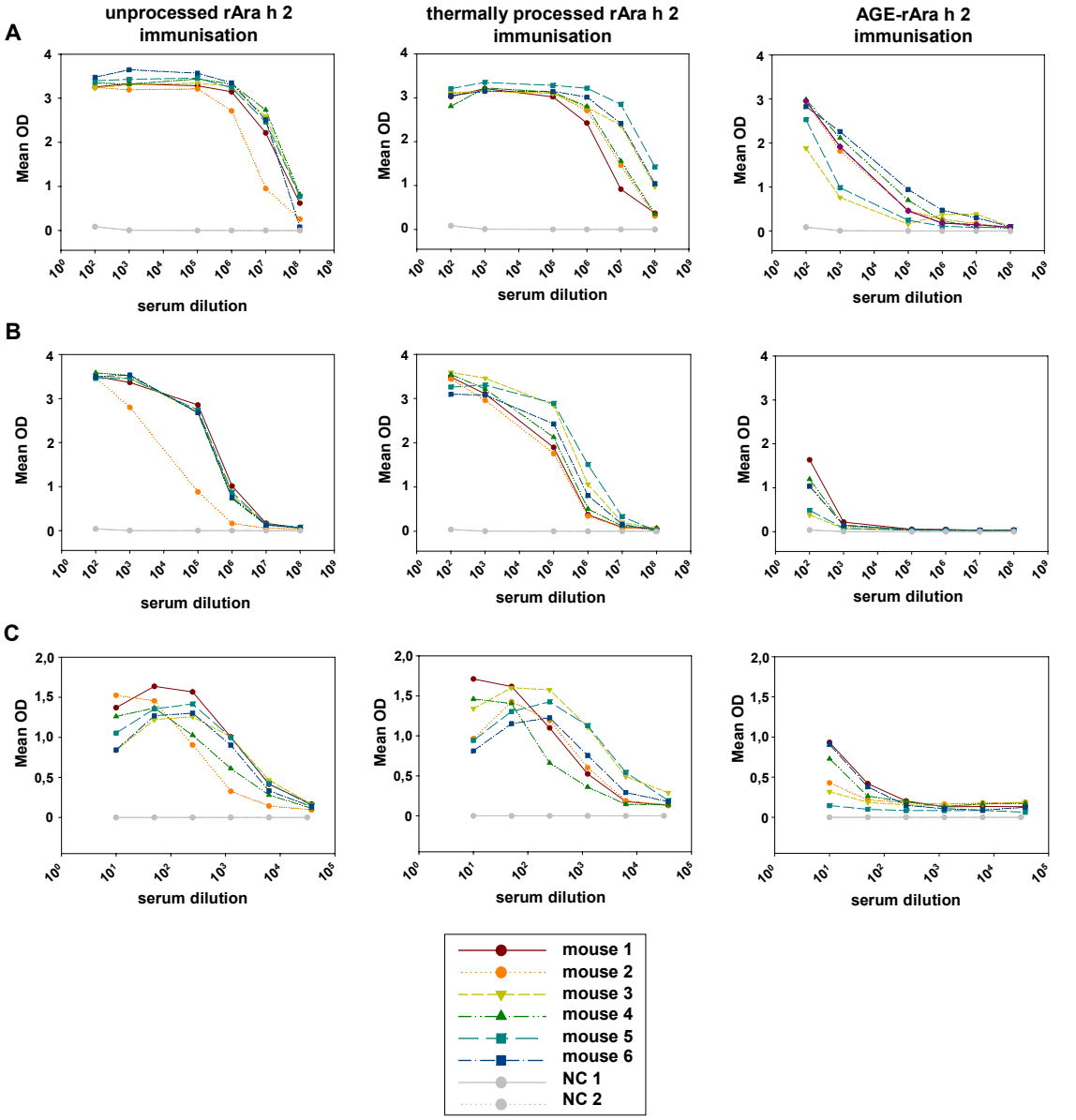

Fig 4.42 Detection of Ara h 2 specific IgG1, IgG2a and IgE antibody production in mice after ip immunisation with unprocessed, thermally processed or rAGE-Ara h 2 in Experiment 2.

C3H/HeJ mice (n=6) were ip immunised with unprocessed, thermally processed or AGE-rAra h 2 plus Alum three times. The levels of **A** IgG1, **B** IgG2a and **C** IgE binding to unprocessed rArah2 in the sera of immunised mice was determined by ELISA using plates coated with unprocessed rAra h 2. NC; mouse immunised with PBS and Alum only.

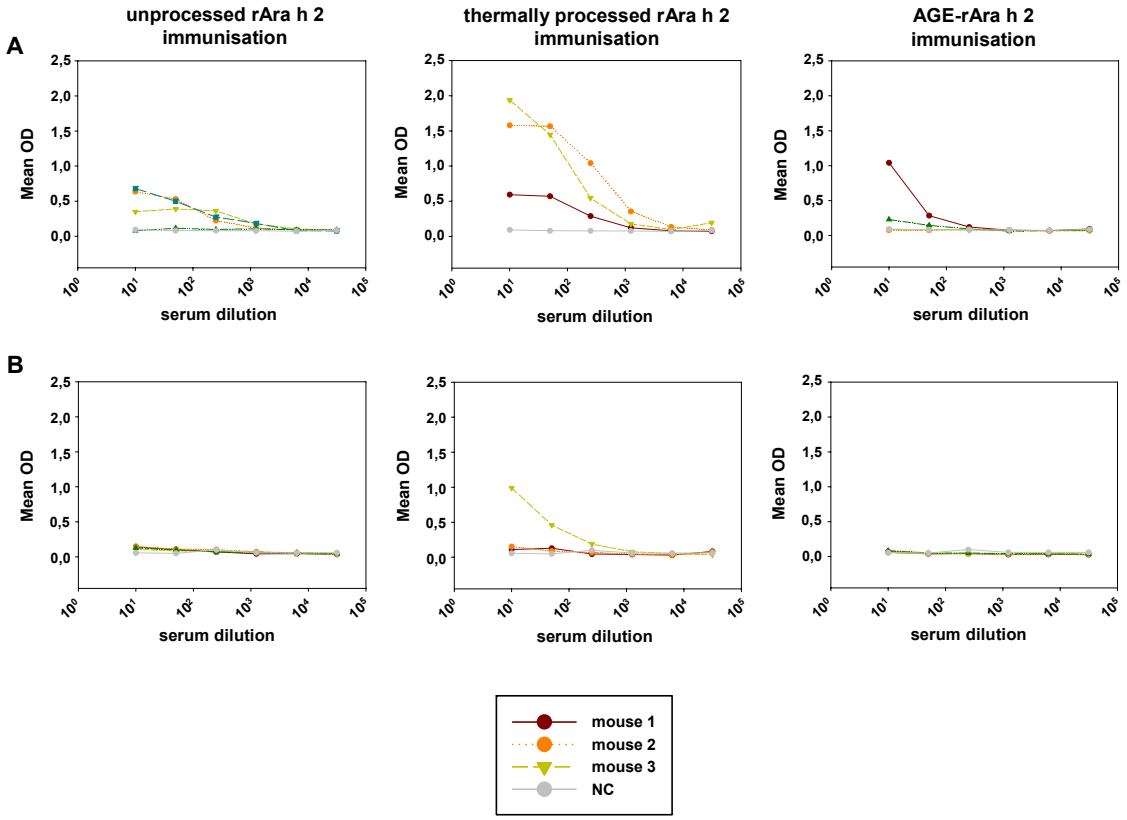

Fig. 4.43 Allergen specific IgE antibody production in C3H/HeJ mice after ip immunisation with unprocessed, thermally processed or AGE-rAra h 2 in Experiment 1

C3H/HeJ mice were ip immunised with unprocessed, thermally processed or AGE-rAra h 2 plus Alum three times. **A** The levels of anti-thermally processed rAra h 2 specific IgE antibodies in the sera of the mice was measured using plates coated with the processed allergen. **B** The levels of anti-AGE-rAra h 2 specific IgE antibodies in the sera of the mice was measured using plates coated with the glycated allergen. NC; mouse immunised with PBS and Alum only.

5. Discussion

The majority of our food is modified by physical and chemical events during storage and processing. The formation of AGEs produced by the Maillard reaction during the thermal processing or storing of food has been suggested to exert important effects on the immunogenicity of food proteins. In this thesis, it was investigated how the T cell immunogenicity and antigenicity of allergens is influenced by glycation by the Maillard reaction using the hen egg white allergen OVA and one of the major peanut allergens, Ara h 2, as model allergens.

5.1 Glycation of OVA enhances the $CD4^+$ T cell immunogenicity of the allergen *in vitro*

First, the impact of AGEs derived from food allergens on DC function and the subsequent activation of allergen specific T cells was studied using AGE-OVA. Here, it was found that AGE-OVA does not trigger the maturation of bone marrow derived mouse myeloid dendritic cells (mDCs) but enhances the activation of allergen specific $CD4^+$ T cells. Moreover, it was demonstrated that the enhanced T cell immunogenicity of AGE-OVA depends on SR-AI/II-mediated uptake of AGE-OVA by mDCs.

5.1.1 Incubation of allergens with sugars efficiently induces modification by the Maillard reaction

For the preparation of AGE-OVA, native OVA was incubated with glucose at 50°C for 6 weeks. On AGE-OVA, high levels of the glycation structures CEL, CML and GA-pyridine were detected (Fig. 4.2). The secondary structure was not affected by the treatment (Fig. 4.3). The preservation of the secondary structure of OVA during the medium temperature used here is consistent with a study which extensively examined the effect of thermal processing and glycation on the biochemical properties of OVA (Sun et al., 2004).

It may be argued that the long incubation time at medium temperature applied in the protocol for AGE-OVA preparation is not appropriate since it does not reflect the conditions found when cooking or frying an egg. A medium temperature and consequently a longer incubation time for the preparation were chosen to limit thermal denaturation and aggregation of OVA. Higher temperatures lead to denaturation and aggregation of OVA due to unfolding. The aggregated OVA is insoluble in any buffer suitable for cell culture or *in vivo* use. Additionally, such medium temperatures are often used in (accelerated) trials mimicking long term storage of food, during which Maillard reaction products are also formed. Here, the aim was to study the influence of glycation structures on the immunogenicity of food allergens. CEL and GA-pyridine detected on AGE-OVA are also found on glycated rAra h 2 (Fig. 4.37) as well as in protein extracts of peanuts roasted using conditions resulting in peanuts similar to industrially processed peanuts in taste,

texture and browning (data not shown). Therefore AGE-OVA, as it was prepared here, is an appropriate model AGE for studying the T cell immunogenicity and antigenicity of food allergen derived AGEs.

5.1.2 AGE-OVA has a higher $CD4^+$ T cell immunogenicity than unglycated OVA

In this study it was found that AGE-OVA induced a higher OVA specific $CD4^+$ T cell activation than native and thermally processed OVA *in vitro*. As shown in Fig. 4.5 and Fig. 4.9, stimulation with AGE-OVA resulted in a higher IL-2 production by OVA specific $CD4^+$ T cells as well as a stronger proliferation of these cells compared to stimulation with native or thermally processed OVAs when the T cells were co-cultured with mDCs. The results suggest that AGE-OVA could have a higher T cell immunogenicity than the unglycated forms of OVA.

If the AGEs were capable of directly stimulating T cell activation in an allergen unspecific manner, this may result in harmful immune reactions in response to any glycated protein. It was found, however, that the glycation structures of AGE-OVA do not directly activate T cells unspecifically, but that $CD4^+$ T cell activation depended on the presence of mDCs. Two different steps in the interaction of AGE-OVA with mDCs may play a role in the enhanced $CD4^+$ T cell activation by the glycated allergen; namely mDC maturation and/or antigen uptake by the mDCs.

5.1.2.1 Increased $CD4^+$ T cell activation does not result from an AGE-OVA induced enhanced maturation of mDCs

The maturation of DCs is characterised by the upregulation of the expression of co-stimulatory molecules (namely, CD40, CD80 and CD86) and MHC class II molecules (MHC II) on the cell surface, all of which are required for efficient T cell priming. Furthermore, activation of mDCs also results in the secretion of cytokines such as IL-10 and IL-12. Incubation of mDCs with AGE-OVA did not increase the expression of the aforementioned molecules nor induce cytokine secretion by mDCs (Fig. 4.12). The results indicate that the enhanced $CD4^+$ T cell immunogenicity of AGE-OVA was not due to a stimulatory effect of AGEs on the maturation and activation of mDCs.

Previous studies have shown that AGEs may have an inhibitory as well as a stimulatory effect on human DC maturation (Price et al., 2004; Ge et al., 2005). The discrepancy among the observations, including those of the present study, may be due to variations in the expression of AGE receptors on the cell surface. Recent findings have shown that engagement of RAGE with its ligands triggers the maturation of DCs (Manfredi et al., 2008; Dumitriu et al., 2005). RAGE was not detected on mDCs in the experimental setting of the present study (Fig. 4.15 C); this may be a reason why AGE-OVA did not induce the maturation of the cells. In the study by Price et al, which also found that AGEs do not induce DC maturation, RAGE was also not detected on the DCs (Price et al., 2004). In contrast, the DCs used in the study by Ge et al expressed RAGE and it was found that AGEs induced enhanced maturation of these DCs (Ge et al., 2005).

As described in chapter 1.8 of the introduction, maturation of DCs is vital for the efficient priming of T cells by these APCs. Although an enhanced expression of co-stimulatory molecules or MHC

II after stimulating with either form of OVA could not be detected, a robust T cell activation was achieved using these mDCs in co-culture with T cells. This can be explained by the fact that the bone marrow derived DCs generated in this study are not fully immature but do express co-stimulatory molecules without needing additional stimulation (Fig. 4.12).

5.1.2.2 Glycation of OVA by the Maillard reaction significantly enhances the uptake of the allergen by mDCs

A very important finding in this thesis is that glycation of OVA by the Maillard reaction enhanced the uptake of the allergen by mDCs (Fig 4.14). This is highly interesting, since the increased uptake of AGE-OVA by mDCs would explain the enhanced $CD4^+$ T cell activation by the glycated allergen. Three mechanisms are responsible for antigen uptake by DCs, namely pinocytosis, phagocytosis and receptor mediated endocytosis (chapter 1.9). It is very unlikely that phagocytosis is responsible for the uptake of soluble OVAs as the mechanism is generally used for particulate antigens. Pinocytosis would take up similar amounts of OVA, independent of the presence of glycation structures and would therefore not contribute to enhancing the uptake of OVA after glycation. Therefore, receptor mediated endocytosis was hypothesised to be the mechanism underlying the enhanced uptake of AGE-OVA by mDCs.

Native OVA is glycosylated with a single carbohydrate side chain composed of N-acetyl-glucosamine and mannose (Tai et al., 1975). It is well established that the endocytosis of native OVA is mediated by the mannose receptor (MR) which binds to the mannose residues of the carbohydrate side chain (Huntington 2001 et al., Burgdorf 2006, Autenrith et al., 2007). AGEs have been found to bind to several cell surface receptors such as SR-AI/II (Suzuki et al., 1997; Araki et al., 1999), RAGE (Schmidt et al., 1992; Neeper et al., 1992) and galectin-3 (Vlassara et al., 1995) which are expressed by DCs. In this study, SR-AI/II and the MR, but not galectin-3 and RAGE, were identified as important receptors mediating the uptake of AGE-OVA by mDCs. Deficiency of RAGE in mDCs (Fig. 4.16) and treatment of Wt mDCs with lactose, an inhibitor for galectin-3 mediated endocytosis, did not reduce the uptake of any form of OVA (Fig. 4.17). In contrast, SR-AI/II deficiency in mDCs significantly attenuated the uptake of AGE-OVA by the cells (Fig. 4.18). Treatment of SR-AI/II deficient mDCs with mannan, an inhibitor for MR mediated endocytosis, further reduced the uptake of AGE-OVA considerably (Fig. 4.19). The results clearly indicate that SR-AI/II and the MR mediate the uptake of AGE-OVA by mDCs. The minor uptake of AGE-OVA still observed in SR-AI/II deficient mDCs after treatment with mannan (Fig. 4.19) suggests a marginal involvement of further receptors in the uptake. As mDCs also express CD36 and SR-BI (Fig. 4.15 E), which too bind AGEs (Ohgami et al., 2001, a; Ohgami et al., 2001, b), these receptors may be the ones engaged in the residual uptake by the mannan treated SR-AI/II deficient mDCs.

Unexpectedly, the uptake of native OVA and thermally processed OVA was also reduced in the SR-AI/II deficient mDCs, although the reduction level was considerably lower than that observed for AGE-OVA (Fig. 4.18). Treatment of SR-AI/II deficient mDCs with mannan almost completely

abolished the uptake of native and thermally processed OVA (Fig. 4.19). The results indicate that, as expected, the MR is the main receptor mediating the uptake of native and thermally processed OVA by mDCs. SR-AI/II would also be involved in the uptake of the unglycated OVAs but its role is only a marginal one.

5.1.2.3 SR-AI/II mediates the enhanced uptake of AGE-OVA by mDCs

As described above, the natural mannose residue found on OVA facilitates the uptake of the allergen by the MR. In macrophages, scavenger receptors, in addition to the MR, were suggested to contribute to the uptake of native OVA (Burgdorf et al., 2007). However, the scavenger receptors constitute a heterogeneous and large receptor family and the specific scavenger receptor(s) participating in the OVA uptake was/were not previously identified. Here, it was found that SR-AI/II is the scavenger receptor involved in the uptake of native OVA by mDCs. SR-AI/II binds to a wide range of anionic ligands including natural carbohydrates and chemically modified residues of proteins (Platt and Gordon, 2001; Plüddemann et al., 2007). To test whether the natural carbohydrate side chain of OVA can bind to SR-AI/II, recombinant OVA was used. Recombinant OVA does not have any carbohydrate residues due to its production in *E. coli* where posttranslational enzymatic glycolsylation generally does not occur. First, the uptake of recombinant OVA by mDCs was less than the uptake of native OVA (Fig. 4.23 A). Furthermore, SR-AI/II deficiency of mDCs did not attenuate the uptake of recombinant OVA (Fig. 4.23 B) in contrast to the observations made with native OVA (Fig. 4.18). These results strongly indicate that the carbohydrate side chain of OVA facilitates the interaction of native OVA with SR-AI/II, resulting in the minor uptake by this receptor (Fig. 5.1).

AGE-OVA would bind to SR-AI/II not only via the natural carbohydrate side chain but also via the glycation structures which result from the modification by the Maillard reaction. This hypothesis is supported by the experiments performed with AGE-BSA. AGE-OVA and AGE-BSA have a similar glycation profile (Fig. 4.2 and Fig. 4.4). The uptake of AGE-BSA by mDCs was considerably higher than native BSA uptake (Fig. 4.20). As BSA does not have any natural carbohydrate residues which may be recognised by the MR (Wada, 1996), the possibility that the MR mediates the uptake of AGE-BSA is therefore excluded. AGE-BSA inhibited the uptake of AGE-OVA by Wt mDCs (Fig. 4.21 A), but only induced very little inhibition of the uptake by SR-AI/II deficient cells (Fig. 4.21 B). In contrast, native BSA could not inhibit AGE-OVA uptake by mDCs at all (Fig. 4.21). Those results strongly indicate that the glycation structures of AGE-OVA and AGE-BSA mediate the binding of the glycated proteins to SR-AI/II. Collectively the results suggest that native and thermally processed OVA interact with SR-AI/II via their natural carbohydrate side chain only, whereas AGE-OVA can interact with SR-AI/II via its glycation structures in addition to the natural carbohydrate residues (Fig. 5.1). Therefore, the presence of the glycation structures enhances the binding of AGE-OVA to SR-AI/II, which increases the overall uptake of the allergen by mDCs and subsequently induces higher allergen specific $CD4^+$ T cell activation.

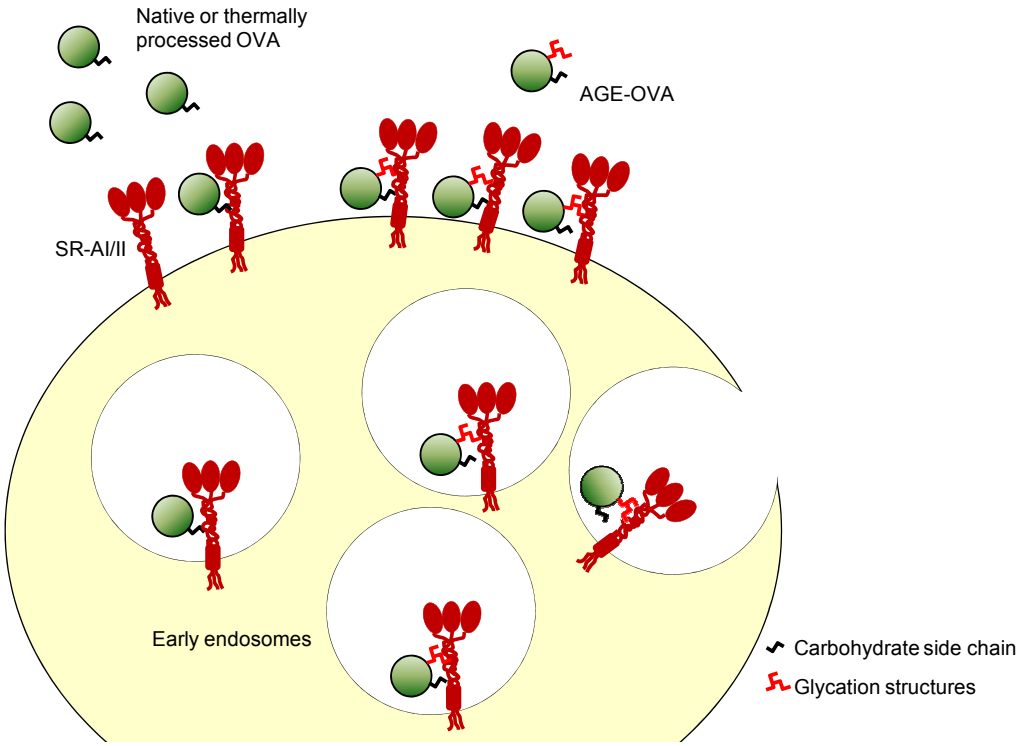

Fig. 5.1 Glycation of OVA enhances the binding affinity of the allergen for SR-AI/II

Native and thermally processed OVA bind to SR-AI/II via their natural carbohydrate side chain. AGE-OVA, in addition to the natural carbohydrate side chain, can bind to SR-AI/II via the glycation structures, resulting in an increased uptake of the glycated allergen by the receptor.

5.1.3 CEL and CML contribute to the enhanced $CD4^+$ T cell activation by AGE-OVA

Thermal incubation of OVA with glucose results in the modification of the protein with a mixture of glycation structures. The glycation profile of AGE-OVA is not fully characterised but high concentrations of CEL and CML were detected (Fig. 4.2 and Tab. 4.1). Here it was found that OVA selectively modified with either CML or CEL only, enhanced the $CD4^+$ T cell activation in a similar way as crude AGE-OVA did (Fig. 4.8). This further supports the conclusion that the glycation structures on AGE-OVA are indeed responsible for the increased $CD4^+$ T cell immunogenicity. The presence of either CML or CEL, or both, on AGE-OVA may mediate the binding of the glycated allergen to SR-AI/II and contribute to the enhanced $CD4^+$ T cell immunogenicity of AGE-OVA.

Contradicting results have now been published concerning the binding of CML to RAGE. Some studies do show CML to be a ligand for RAGE (Kislinger 1999, Hudson 2003). More recently, on the other hand, it was also demonstrated that RAGE was actually unable to bind CML (Buetler et al., 2008; Wilton et al., 2006). The Maillard glycation structures which act as ligands for the other AGE binding receptors, galectin-3, SR-BI, CD36 and SR-AI/II, have not yet been identified. A common denominator found for all SR-AI/II ligands is that they are polyanionic but the exact molecular properties which enable a molecule to bind to SR-AI/II have not yet been fully

elucidated (Platt and Gordon, 1998). In studies characterising AGE-BSA produced by incubating the protein with glucose, glycation increases the number of negative charges on the protein (Wu et al., 1996; Westwood and Thornally, 1995). Therefore, the modification with glycation structures is likely to increase the negative charge of OVA. Although being polyanionic alone is not sufficient in order for a molecule to become a ligand for SR-AI/II (Horiuchi et al., 1986), the increased negative charge could be one factor responsible for enhancing the affinity of AGE-OVA for SR-AI/II. Uptake studies using FITC-labelled CEL- and CML-OVA could help to elucidate whether these glycation structures are able to interact with the AGE-binding receptors.

5.1.4 SR-AI/II plays a critical role in the activation of $CD4^+$ T cells by (AGE-)OVA

To closer investigate the role of SR-AI/II in OVA specific $CD4^+$ T cell activation by mDCs, $CD4^+$ T cells were cultured in the presence of fixed allergen pulsed Wt or SR-AI/II deficient mDCs. $CD4^+$ T cell activation in response to native, thermally processed or AGE-OVA was significantly reduced in cultures with SR-AI/II deficient mDCs (Fig. 4.25). In contrast, SR-AI/II deficiency did not reduce T cell activation in response to recombinant OVA, which does not have a binding moiety for SR-AI/II. The results are a further indicator that SR-AI/II mediates the uptake of all three OVAs by mDCs and that it is a critical component for optimal activation of OVA specific $CD4^+$ T cells. Furthermore, IL-2 production in response to stimulation with native, thermally processed and AGE-OVA was comparable when SR-AI/II deficient mDCs were used. This clearly demonstrates that the enhanced uptake of glycated OVA by SR-AI/II is responsible for the increased $CD4^+$ T cell activation by AGE-OVA when WT mDCs are used as APCs.

The receptors which mediate the endocytosis of a particular antigen dictate the intracellular routing of the endocytosed antigen and consequently on which class of MHC molecule the antigen peptides will be presented (chapter 1.9.2). For example, antigens taken up by the glycan-binding receptor DC-SIGN (CD209) are delivered to the MHC class II loading pathway (Engering et al., 2002). On the other hand, antigens taken up by the MR are targeted to the MHC class I loading pathway (Burgdorf et al., 2007). In the present study it was found that the glycation of OVA does not enhance its potential to stimulate $CD8^+$ T cell activation. OVA-specific $CD8^+$ T cells which were co-cultured with mDCs secreted comparable levels of IL-2 in response to stimulation with native and AGE-OVA (Fig. 4.11). The results suggest that SR-AI/II is a receptor targeting its ligands to the MHC class II, but not to the MHC class I loading pathway.

Since the uptake of AGE-OVA by SR-AI/II is considerably higher than that of native or thermally processed OVA due to the glycation structures, an increased amount of AGE-OVA would be delivered to the MHC class II loading pathway. Subsequently, this would result in a substantial increase of the amount of OVA peptide-MHC class II complex on the surface of the mDCs, facilitating the enhanced OVA specific $CD4^+$ T cell activation. This result is in good accordance with previous publications which have shown that the $CD4^+$ T cell immunogenicity of an antigen is enhanced if it is chemically modified to function as a SR-AI/II ligand (Abraham et al., 1995; Nicoletti et al., 1999).

5.1.5 SR-AI/II targets AGE-OVA to MHC class II loading pathway

The results in the present study allow the hypothesis that SR-AI/II channels OVA to the MHC class II loading pathway (Fig. 4.25, chapter 5.1.4). To further verify this hypothesis, it was attempted to identify the location of AGE-OVA within the mDCs using immunocytochemistry. After the uptake, the majority of AGE-OVA was found in EEA1 positive compartments, which are the early endosomes (Fig. 4.30 A). Previously, the MR has been shown to transport its ligands to the early endosomes. Here, it was observed that AGE-OVA co-localised with EEA1 even in MR deficient mDCs (Fig. 4.30 B). Since SR-AI/II was found to be the only other receptor besides MR which contributes significantly to AGE-OVA uptake by mDCs, it can be assumed, that AGE-OVA, which co-localises with EEA1 in MR deficient mDCs was transported to the early endosomes after uptake by SR-AI/II. These observations suggest that not only the MR but also SR-AI/II transports its ligands to the early endosomes (Fig. 5.2).

As described in the introduction, nascent MHC II can be delivered from the endoplasmatic reticulum (ER) to the early endosomes (Brachet et al., 1999; Pond and Watts, 1997; Villadangos et al., 2000). In the present study MHC II was also found to co-localise with EEA1 (Fig. 4.29). In the early endosomes, there appears to be a H2-DM independent process of Ii degradation and peptide loading (Villadangos et al., 2000). Due to the open conformation of the peptide binding groove, MHC II can bind to peptides of undefined length (Watts, 1997). Therefore, proteins can bind to MHC II even before being processed to short peptides by the proteases in later endocytotic compartments (Lindner et al., 1996). Taken together, the data of the present study suggests that the majority of AGE-OVA could be targeted to the early endosomes by the SR-AI/II (Fig. 5.2). In the early endosomes some of it is processed to polypeptides and loaded onto MHC II (Fig. 4.27). The MHC II - OVA polypeptide complex may then continue along the endocytotic pathway to the increasingly proteolytic compartments where the unprotected regions of the polypeptide are clipped off (Villadangos, 2001). AGE-OVA which is not loaded onto MHC II in the early endosomes would be degraded to peptides in the late endosomes/lysosomes and the peptides loaded onto MHC II in the lysosomes (Fig. 5.2).

In this study, co-localisation of AGE-OVA with the late endosome/lysosome marker Lamp1 or the lysosomal dye lysotracker was not detected (Fig. 4.27 and data not shown). The late endosome/lysosomes are compartments involved in the processing of the majority of antigens and the formation of MHC II - peptide complexes (Fig. 5.2). Inconsistent with the present study, Burgdorf et al have suggested (Burgdorf et al., 2007) that most of the OVA endocytosed by scavenger receptors is transported to the lysosomes for processing and loading onto MHC II. In order to visualise OVA in mDCs, the protein was labelled with FITC. The possibility that FITC was degraded in the lysosomes cannot be excluded. The lysosomes are very acidic compartments with a pH ranging from 4-5 (Poole and Ohkuma, 1981). FITC is sensitive to pH and its fluorescence intensity is quickly reduced in an acidic environment. The degradation of FITC in the lysosomes could be the reason why AGE-OVA could not be observed in this particular compartment. In the previous study, Burgdorf et al identified the cellular location of native OVA in

the lysosomes using commercially available AlexaFluor 488 OVA conjugates (Burgdorf et al., 2007). These are modified with a high amount of AlexaFluor 488, a dye which is more stable than FITC under acidic conditions. A further study using AGE-OVA labelled with AlexaFluor 488 would be necessary to verify whether AGE-OVA is also transported to the lysosomes.

OVA endocytosed by the MR is transported into stable early endosomes before being exported into the cytosol where it is processed by the proteasome (Burgdorf et al, 2008). Subsequently, the processed antigenic peptides are reimported into the same endosomal compartment by TAP (transporter associated with antigen processing) to be loaded onto MHC I (Fig. 5.2) (Burgdorf et al, 2008). In contrast to SR-AI/II binding, glycation of OVA does not appear to alter the binding of OVA to the MR (Fig. 4.18 and 4.19). All three forms of OVA would therefore be taken up by the MR in similar amounts (Fig. 4.19). As a result, AGE-OVA and native OVA induced comparable activation of OVA specific $CD8^+$ T cells (Fig. 4.11). Compared to native OVA, thermally processed OVA induced higher $CD8^+$ T cell activation. Although the CD analysis showed identical spectra between native and thermally processed OVA (Fig. 4.3, A), SDS-PAGE analysis suggests that minor degradation and/or denaturation of the allergen might be induced by the thermal processing without glucose (Fig. 4.3., B). The enhanced $CD8^+$ T cell activation by the thermally processed OVA may therefore be due to slight denaturation, which may facilitate a more efficient processing by the proteasome.

Collectively, the results presented here suggest that SR-AI/II targets its ligands to the early endosomes for MHC class II loading. As described above, the MR also targets the ligands to the early endosomes but for MHC class I loading. It is still not sure whether both receptors target their ligands to the same early endosome population or not. It should however be noted that the early endosomes of a cell are not made up of a uniform pool of endocytotic compartments. Instead, it was recently shown that there are two distinct populations of endosomes, one which is highly mobile and quickly matures to late endosomes and subsequently lysosomes and a second, much more static population (chapter 1.9.2) (Lakadamyali et al., 2006). It appears that OVA endocytosed by the MR is delivered to the stable early endosome population and never enters the late endosomes or lysosomes (Burgdorf et al., 2007). On the other hand, OVA endocytosed by SR-AI/II may enter the rapidly maturing early endosomes. The model of the intracellular pathway of SR-AI/II endocytosed OVA presented here will have to be confirmed in future studies.

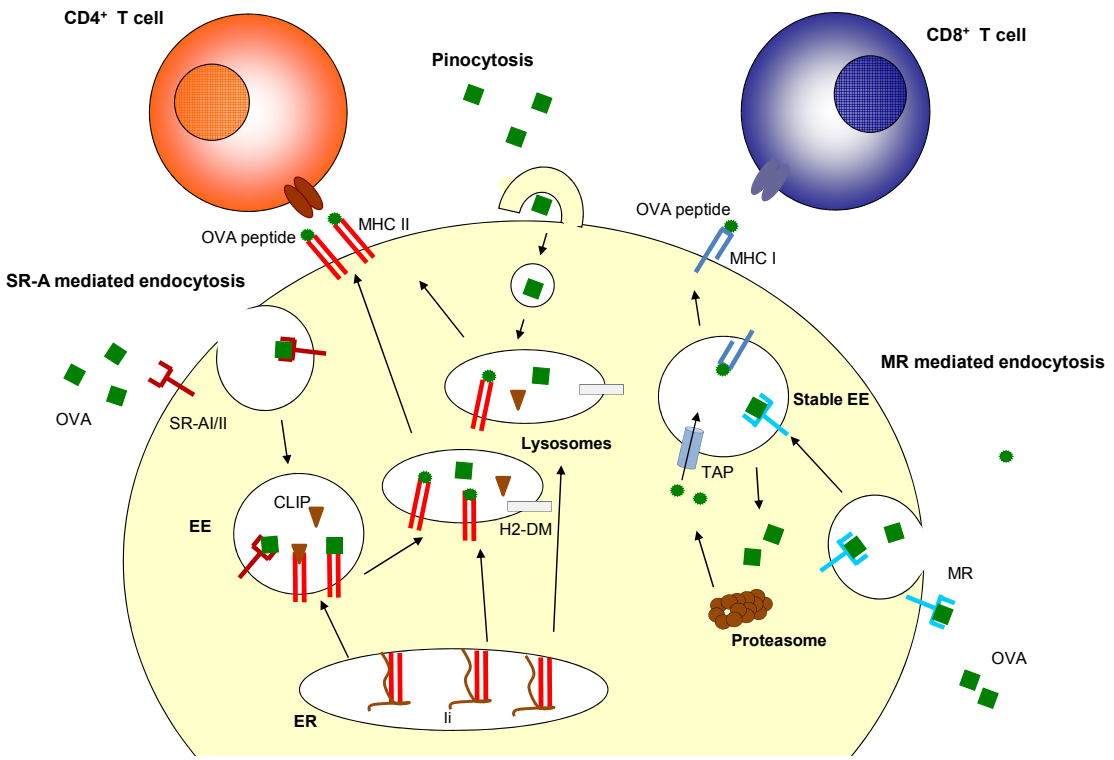

Fig. 5.2 Model for the processing of AGE-OVA taken up by mDCs via SR-AI/II

AGE-OVA taken up by SR-AI/II is delivered to the early endosomes where MHC II is found. Another fraction of the AGE-OVA may progress from the early endosomes to the late endosomes/lysosomes for processing and MHC II loading. AGE-OVA taken up by pinocytosis also enters the MHC II restricted processing and loading. The MHC II-peptide complex is subsequently transported to the cell surface where it is recognised by OVA peptide specific $CD4^+$ T cells. AGE-OVA taken up by the MR enters stable early endosomes from where it is brought to the proteasome for processing. The OVA peptides re-enter the same early endosomes via TAP, are loaded onto MHC I and the MHC I-peptide complex is then directly transported to the cell surface for presentation to $CD8^+$ T cells. Figure partially adopted from Burgdorf et al., 2007.

5.2 Glycation of OVA by the Maillard reaction enhance the antigenicity and T cell immunogenicity of the allergen *in vivo*

Next, the immunogenicity and antigenicity of AGE-OVA was evaluated *in vivo*. Mice were used for the evaluation because access to human materials, e.g. peripheral blood monocytes (PBMC) and sera from patients with food allergy, is generally limited. In addition, many patients with egg allergy are small children and so it is even more difficult to obtain suitable amounts of blood samples for PBMC studies. The usage of mice also has the advantage that it is possible to investigate the sensitisation (priming) effect of AGEs on the induction of allergen specific T cell and antibody responses. Here, it was found that AGE-OVA induced a higher production of allergen specific IgE antibodies in BL6 mice, compared to native and thermally processed OVAs.

5.2.1 Antigenicity of AGE-OVA in intraperitoneal immunisation

Intraperitoneal (ip) immunisation of mice with AGE-OVA resulted in a significantly higher IgE production than immunisation with native or thermally processed OVA (Fig. 4.36). These results indicate that AGE-OVA has a higher antigenicity than the unglycated forms of the allergen. Allergic reactions are induced by the association of allergens with IgE bound to the high affinity IgE receptor, FεcRI, on the surface of mast cells (chapter 1.2). Therefore, glycation by the Maillard reaction may also increase the allergenicity of OVA.

Two components of the immune response could be involved in the enhanced IgE production by AGE-OVA: $CD4^+$ T cell and B cell activation. $CD4^+$ T cells play a crucial role in the induction of IgE production by B cells (chapter 1.13). The results of the experiments using *in vitro* co-culture of $CD4^+$ T cells with mDCs (Fig. 4.5) and subcutaneous immunisation (Fig. 4.31) for the assessment of the $CD4^+$ T cell immunogenicity of the three types of OVA strongly indicate that AGE-OVA has a higher T cell immunogenicity than native or thermally processed OVA. These results suggest that the enhanced IgE production results from increased $CD4^+$ T cell activation by AGE-OVA. Furthermore, the increased $CD4^+$ T cell activation by AGE-OVA also resulted in a higher amount of IL-4 being secreted by the activated T cells (Fig. 4.10). IL-4 is a key cytokine for the production of IgE antibodies by B cells. This would further explain why the production of IgE was considerably enhanced following immunisation with AGE-OVA.

It may be argued that in the case of ip immunisation, all three OVAs had a comparable T cell immunogenicity (Fig. 4.32). The discrepancy may be the result of the immunisation protocol used; three immunisations with a relatively high dose of allergen were performed. Therefore, even if AGE-OVA does have an enhanced $CD4^+$ T cell immunogenicity also with ip immunisation, the enhancing effect may not have been detectable if the antigen dose was so high as to induce a maximum activation even with less immunogenic antigens after three immunisations. The influence of the allergen dose is also supported by the *in vitro* data showing that higher concentrations of the different OVAs could induce comparable proliferation of $CD4^+$ T cells but at lower doses, AGE-OVA was a much more potent inducer of T cell proliferation than native and thermally processed OVA (Fig. 4.9).

When analysing the data of $CD4^+$ T cell activation and the antibody production, it has to be taken into account that the data presented for the antibody production represents the situation after only two immunisations, whereas the *ex vivo* stimulation of $CD4^+$ T cells to asses the T cell immunogenicity of AGE-OVA was performed after three immunisations. The antibody data after three immunisations was not used as the levels of antibody were so high, that no differences could be observed between serum dilutions (data not shown). This supports the notion that the T cell activation had reached a maximum after three immunisations. However, it is likely that after two immunisations, a difference in $CD4^+$ T cell activation between the groups would be observed, with a stronger activation in AGE-OVA immunised mice as was found after subcutaneous immunisation (Fig. 4.31). This would explain the increased IgE production after just two ip immunisations with AGE-OVA. The ip immunisation should therefore be repeated with an altered

protocol to confirm that $CD4^+$ T cell activation was higher in AGE-OVA immunised mice after only two immunisations.

In addition to enhanced $CD4^+$ T cell activation, the second component of the immune response being influenced by the glycated OVA could be the B cell activation. The glycation structures may act as novel immunepitopes for the immunoglobulins of B cells, the BCRs. This could result in the production of antibodies specific for the glycation structures found on OVA. However, the antibodies from AGE-OVA immunised mice bound equally well to native, thermally processed and AGE-OVA (Fig. 4.33, Fig. 4.34 and Fig. 4.36). Therefore, even if some antibodies with specificity for the glycation structures are produced in BL6 mice, their level would be rather low. Instead, the data suggests that antibodies from AGE-OVA immunised mice are likely to recognise the same epitopes as mice immunised with the unglycated forms of OVA. These could be epitopes which remain unglycated or epitopes where the glycation does not affect immunoglobulin binding. Hence, glycation of OVA does not appear to directly alter the interaction of the allergen with B cells. Therefore, the enhanced production of IgE induced by AGE-OVA immunisation would mainly be the result of increased $CD4^+$ T cell activation.

As shown in Fig. 4.19 and Fig. 4.25, it was found that SR-AI/II mediates the uptake of AGE-OVA by mDCs and that this contributes to the enhanced OVA specific $CD4^+$ T cell activation by the glycated allergen. In order to verify the importance of SR-AI/II for the enhanced antigenicity of AGE-OVA *in vivo*, the usage of SR-AI/II deficient mice may be a choice. However, SR-AI/II does not only play a role in antigen uptake but also promotes cell adhesion to matrix molecules and other cells (Khoury et al., 1994; Gowen et al., 2000). This function could contribute to the involvement of SR-AI/II in limiting DC migration from the site of antigen uptake to the draining lymph nodes (Arredouani et al., 2007). Therefore, the absence of SR-AI/II itself can have an enhancing influence on the T cell activation in the lymph nodes. For this reason, SR-AI/II deficient mice were not used to verify the role of SR-AI/II *in vivo*. Further study would be necessary to see whether DCs which express SR-AI/II predominantly take up AGE-OVA *in vivo*. This could for example be done using immunohistochemistry.

5.2.2 The influence of AGEs on $CD4^+$ T cell differentiation

As illustrated in chapters 1.12.2.2 and 1.13, Th2 cells play a pivotal role in the IgE production by B cells and in the maturation and activation of inflammatory cells involved in allergic reactions. Th2 cells are opposed by Th1 cytokines and regulatory T cells which inhibit the differentiation of $CD4^+$ T cells to Th2 cells and suppress the activation of effector T cells respectively. Therefore, it was of utmost interest to see whether the glycation of food allergens influences the differentiation of $CD4^+$ T cells.

In this study it was found that neither AGE-OVA nor AGE-rAra h 2 influences the T cell differentiation. In *in vitro* co-cultures of mDCs and OVA specific $CD4^+$ T cells, AGE-OVA enhanced the production of IL-4 but also IFN-γ (Fig. 4.10), giving no indication of a shift in T cell polarisation. Furthermore, in ip immunised mice, native, thermally processed and AGE-OVA

induced comparable IgG1/IgG2a antibody ratios (Fig. 4.34). These results indicate that AGE-OVA did not alter the $CD4^+$ T cell differentiation towards a particular T helper cell phenotype.

SR-AI/II plays a pivotal role in the uptake of AGE-OVA *in vitro* and possibly also *in vivo*. Previous data regarding the influence of SR-AI/II on T cell differentiation is controversial. Maleyated allergen, a modification which targets the protein for SR uptake, induced a Th1 dominated allergen response, e.g. predominant production of the Th1 cytokine IFN-γ by splenic T cells and increased IgG2a antibody production in response to the antigen (Bhatia et al., 2002). In contrast, other studies showed that OVA treated with formaldehyde, resulting in an aldehyde modified allergen which was taken up by macrophages via the SR, leads to a Th2 dominated immune response marked by higher IL-5 production by T cells and increased IgG1 production in comparison with the unmodified OVA (Moghaddam et al., 2002; Allison and Fearon 2000; Willis et al., 2002). The different observations could be due to different protein modifications which may bind to distinct binding sites of SR-AI/II and subsequently trigger different signalling pathways. The collagenous region of SR-AI/II has been identified as the binding site of the receptor, however within this at least two discrete binding sites exist. For instance, the SR-AI/II ligand acetylated LDL is a poor competitor for binding of oxidised LDL, also a SR-AI/II ligand (Platt and Gordon, 2001, Plüddemann et al., 2007). SR-AI/II ligands, by binding to distinct binding sites of the receptor, differentially modulate specific protein kinase signal transduction pathways (Coller and Paulnock, 2001; Hsu et al., 2001). Therefore, the stimulatory effect of SR-AI/II-ligand interaction on maturation and cytokine production of DCs appears to be ligand dependent. The cytokines which are produced by DCs influence the differentiation of $CD4^+$ T cells (chapter 1.12.2). However, AGE-OVA did not induce maturation of mDCs nor did it stimulate cytokine production by these cells (Fig. 4.12). In summary, binding to distinct binding sites and the triggering of different signalling pathways could offer an explanation for the contrasting immunomodulating effects observed by ligand binding to SR-AI/II. The AGEs may associate with a binding site of SR-AI/II which does not trigger maturation nor results in the release of cytokines which generates a different phenotype than the uptake of unglycated OVA.

5.3 Glycation reduces the allergenicity of rAra h 2

Ara h 2 is one of the major allergens of peanuts (Burks et al., 1992; Burks et al., 1995). Peanuts are usually not consumed raw but roasted, fried or boiled. Since peanuts contain a significant amount of reducing sugars (Vercellotti et al., 1995), the Maillard reaction occurs during the thermal treatment of peanuts (Maleki et al., 2000, Chung et al., 2003). Consequently, it was of major interest to elucidate what effect the Maillard reaction may have on the immunogenicity of this allergen. Several studies have shown that thermal treatment of peanuts or single allergens in the presence of carbohydrates enhances their binding activity for serum IgE from peanut allergic patients as a result of modifications by the Maillard reaction (Maleki et al., 2000; Chung SY et al.,2001; Gruber et al, 2005). However, so far no study has been performed to examine the

influence of the Maillard reaction on the immunogenicity, antigenicity and allergenicity of Ara h 2 *in vivo*. Here, it was observed that glycation of Ara h 2 by the Maillard reaction reduced the immunostimulatory potential of rAra h 2 in C3H/HeJ mice.

5.3.1 Thermal incubation of rAra h 2 with carbohydrates leads to glycation and loss of secondary structure

First, different carbohydrates were tested for the glycation of rAra h 2, namely fructose, glucose, galatcose and mannose, which are naturally found in peanuts (Rudrapatnam et al., 1974). Lactose and maltose were also used; these are not present in peanut but are frequently used in peanut products. In a previous study it was observed that the binding properties of AGE-Ara h 2 for serum IgE from peanut allergic patients is dependent on the carbohydrate used for the preparation of the particular AGE-Ara h 2 (Maleki et al., 2000). The data suggest that the carbohydrate present during the thermal processing could influence the development of the type and/or the amount of glycation structures and subsequently influences the binding properties of the prepared AGEs for serum IgE. It was therefore of interest to see whether the different carbohydrates resulted in different glycation structures on rAra h 2.

CML and GA-pyridine but not CEL and pyrraline could be detected in rAra h 2 incubated with any one of the carbohydrates (Fig. 4.37). Galactose appeared to generate the highest level of GA-pyridine. Whether this difference in the amount of GA-pyridine could be of relevance for the immunogenicity of the allergen was beyond the scope of this study. However, it was interesting, since a particularly high increase in the binding of serum IgE of peanut allergic patients was observed when Ara h 2 had been incubated with galactose (Maleki et al., 2000). CML and GA-pyridine were also detected in extracts of roasted peanuts in our hands (data not shown). The presence of CML in roasted peanut extracts was also confirmed by another study (Chung et al., 2001). As galactose induced the highest level of GA-pyridine as well as inducing high levels of CML and is a carbohydrate found in peanuts (Rudrapatnam et al., 1974), it was used for the preparation of AGE-rAra h 2 in the present study.

Here, it was also observed that rAra h 2 was severely affected by thermal treatment. The CD spectrum of untreated rAra h 2 shows that rAra h 2 is a protein dominated by α-helices (Fig. 4.38). This finding is in consent with the CD spectra published for rAra h 2 by Lehmann et al. and for native Ara h 2 (Lehmann et al., 2006; Maleki et al., 2003). However, the α-helical structure of rAra h 2 was lost after thermal treatment with or without galactose (Fig. 4.38). In contrast, Lehmann et al. found that when heating rAra h 2 up to 90°C, there was little change of the CD spectrum (Lehmann et al., 2006) and Ara h 2 isolated from roasted peanut extracts exhibited only a very minor reduction of the α-helical structure in favour of β-sheets and random coils (Maleki et al., 2003). Intact disulfide bonds were found to be vital for the heat stability of rAra h 2 (Lehmann et al., 2006). The correct disulfide bond formation was not checked for rAra h 2 used in the present study. Although the CD spectrum of rAra h 2 used here resembles that of rAra h 2 and

native Ara h 2 published previously, it is possible that the disulfide bonds of rAra h 2 used for this study were not formed correctly, which would make the protein more susceptible to heat degradation. Therefore, the loss of secondary structure observed for rAra h 2 after thermal incubation with and without galactose may be due to incompletely formed disulfide bonds. The discrepancy to the other publications may also be explained by the different incubation conditions used. In the present study rAra h 2 was heated in solution at 120°C for 2 h. Lehmann et al. heated rAra h 2 to 90°C, a considerably lower temperature, during CD spectroscopy. In the study by Maleki et al. Ara h 2 was isolated after the complete nuts had been roasted at 350°C for 20 min (Lehmann et al., 2006; Maleki et al., 2003). In the latter study the food matrix of the whole peanut may protect the conformation of Ara h 2 during roasting.

5.3.2 The glycation of rAra h 2 by the Maillard reaction reduces the $CD4^+$ T cell immunogenicity of the allergen *in vivo*

In this thesis it was found that glycation of rAra h 2 by the Maillard reaction reduces the $CD4^+$ T cell immunogenicity of the allergen. $CD4^+$ T cells isolated from mice immunised with AGE-rAra h 2 proliferated much better in response to unprocessed rAra h 2 and rAra h 2 thermally processed without galactose (Fig. 4.39) than to AGE-rAra h 2. One factor influencing the $CD4^+$ T cell immunogenicity of the allergen may be the denaturation of the allergen induced by heating; $CD4^+$ T cells isolated from all three immunisation groups responded better to stimulation with unprocessed rAra h 2 than to thermally processed rAra h 2. Since T cells recognise sequential epitopes, the loss of the conformation of rAra h 2 by thermal treatment is unlikely to directly influence the recognition of the allergen by TCRs. Thermal denaturation of proteins is often irreversible and this appears to also be true for the rAra h 2 used in this study as shown by the CD spectrum analysis performed at room temperature after heating (Fig. 4.38, discussed in 5.3.1). Generally, the cause for the irreversibility is aggregation of the heat unfolded polypeptide (Benjwal et al., 2006). Therefore both thermally processed and AGE-rAra h 2 are likely to be aggregated. In order for T cells to be activated by an allergen, the protein has to be processed by APCs and the allergen peptides loaded onto MHC molecules (chapter 1.10). It is possible that the aggregation of rAra h 2 inhibits or alters the processing of the allergen by the proteases in the endosomes and lysosomes where antigens are processed for MHC class II presentation. If this is the case, less MHC II-peptide rAra h 2 complexes would result from the intracellular processing of the aggregated allergen as opposed to processing of the rAra h 2 which had not been subjected to thermal treatment. Consequentially, less peptide would be presented on the cell surface of the APCs resulting in reduced $CD4^+$ T cell activation by thermally processed and AGE-rAra h 2.

The proliferate response was even lower in response to AGE-rAra h 2 than to thermally processed rAra h 2 stimulation. Therefore, the $CD4^+$ T cell immunogenicity of AGE-rAra h 2 may be further reduced by the glycation structures on the allergen. In the amino acid sequence of Ara h 2 arginine is fairly abundant and some lysines are also present (Chatel et al., 2003). It could be

assumed that some of these residues are part of the T cell epitopes recognised by C3H/HeJ mice. Therefore, core amino acid residues recognised by TCRs of Ara h 2 specific T cells may be glycated by the Maillard reaction and this could influence the binding of TCRs to the MHC II – rAra h 2 peptide complex on the surface of APCs. There are two possible ways in which T cells could have been primed in mice immunised with AGE-rAra h 2 if core epitope residues of the allergen where glycated: (i) the T cells in AGE-rAra h 2 immunised mice were primed *in vivo* by Ara h 2 epitopes which remained unglycated but of which less may be available on AGE-rAra h 2 than on the unglycated rArah 2 forms or (ii) they were primed by glycated epitopes but glycation impairs optimal binding to the TCRs. Both possibilities would result in a dampened T cell response to AGE-rAra h 2. In addition, glycation may further impair the intracellular processing of the allergen which may already be affected by aggregation, as discussed above. This could also further reduce the T cell immunogenicity of AGE-rArah 2.

5.3.3 The glycation of rAra h 2 by the Maillard reaction reduces the antigenicity of the allergen *in vivo*

The present study also showed that the glycation of rAra h 2 by the Maillard reaction significantly reduces the antigenicity of the allergen when C3H/HeJ mice were exposed to the allergen by ip immunisation. AGE-rAra h 2 was a poor inducer of rAra h 2 specific IgE as well as IgG1 and IgG2a (Fig. 4.41 and Fig. 4.42). The results suggested that glycation of the Maillard reaction could also reduce the allergenicity in some food allergens.

As described in chapter 5.3.2, the activation of allergen specific $CD4^+$ T cells in response to AGE-rAra h 2 was lower than in response to unprocessed or thermally processed rAra h 2. Since activated $CD4^+$ T cells are essential for the activation of B cells and the subsequent antibody production (chapter 1.14), the reduced activation of $CD4^+$ T cells by AGE-rAra h 2 could be one reason why only very little amounts of antibody were produced in mice immunised with AGE-rAra h 2.

The reduced antigenicity of AGE-rAra h 2 could also be explained by glycation of B cell epitopes of the allergen. It is unlikely that loss of secondary structure alone influences the antigenicity since rAra h 2 thermally processed without glucose induced a similar level of antibody production as unprocessed rAra h 2. Furthermore, the serum IgE from mice immunised with the unprocessed rAra h 2 was able to bind to thermally processed Ara h 2 (Fig. 4.43). In contrast, IgE antibodies from mice immunised with unprocessed or thermally processed rAra h 2 were not able to bind to AGE-rAra h 2 (Fig. 4.43). Ara h 2 has eleven IgE epitopes recognised by C3H/HeJ mice (Li et al., 2000). Some of the epitopes, including two of the three major epitopes, contain lysine and/or arginine residues. Glycation of lysine and/or arginine residues within the B cell epitopes of Ara h 2 may hamper the recognition of AGE-rAra h 2 by B cells and therefore contribute to the reduced antigenicity of AGE-rAra h 2.

The data obtained here *in vivo* in a mouse model is in contrast to studies showing that roasted peanut extracts or single glycated peanut allergens had a higher binding activity for peanut allergic patient serum IgE than extracts from raw peanuts or unmodified single allergens (Maleki et al., 2001; Chung et al., 2001; Gruber et al., 2005). More specifically, the binding activity for human IgE of peptide sequences containing complete sequences or parts of the sequence of two major Ara h 2 epitopes was increased after thermal incubation with carbohydrates, suggesting that the incubation may have transformed these residues into high affinity IgE binding sites (Gruber et al., 2005). One reason why human IgE may show increased binding to glycated allergens may be that peanut allergic patients have some antibodies specific for the glycated form of the allergens as suggested by the work by Chung et al. where AGE-specific IgG antibodies inhibited binding of patient IgE to roasted peanut extracts (Chung et al., 2001). Increased binding of patient serum IgE to peanut allergens after heating may also result from antibodies which were produced in response to recognition of unmodified epitopes by the specific BCRs. That IgE antibodies primarily produced in response to unmodified Ara h 2 can indeed exhibit enhanced binding to the glycated allergen was demonstrated with serum from rabbits immunised with unprocessed Ara h 2 (Gruber et al., 2005). IgE binding to glycated proteins could be enhanced due to the polyanionic character and the alkylated lysines of the glycated protein (Sano et al., 1999). The possible explanations why glycation enhances binding of peanut proteins to human or rabbit IgE do not appear to apply to the particular mouse model used here. The data in Fig. 4.43 suggests that IgE antibodies produced in AGE-rAra h 2 immunised mice recognise only the unglycated allergen, indicating that recognition of unglycated epitopes underlies the antibody production even in mice immunised with the glycated allergen. In addition, antibodies primarily recognising unglycated allergen do not appear to be able to bind AGE-rAra h 2 and show no enhanced binding to rAra h 2 thermally processed without galactose (Fig. 4.43). It was shown that nine out of the eleven epitopes recognised by C3H/HeJ mice are essentially in the same regions as the epitopes identified as human-IgE binding epitopes, including the two major epitopes which displayed enhanced IgE binding after being glycated (Li et al., 2000; Stanley et al., 1997; Gruber et al., 2005). It may therefore be expected, that IgE antibodies from C3H/HeJ mice could also bind to these glycated epitopes, but this does not appear to be the case. The human immune system is able to respond to an enormous variety of antigens. The basis for this is the huge genetic variety of the genes encoding the MHC molecules, the TCRs and the BCRs/Immunoglobulins found in the population. Furthermore, the variability of these receptors is increased by mechanisms such as gene rearrangement and polygeny. In contrast to the enormous versatility of the human immune system, the immune system of an inbred mouse strain, such as the C3H/HeJ mouse, where individuals are homozygous and 99% genetically identical to each other, is rather restricted. Therefore, while Ara h 2 modified with glycation structures may be able to induce allergen specific IgE production in some human individuals and human IgE have been shown to bind glycated peanut allergen (Maleki et al., 2001, Gruber et al., 2005), the considerable more

limited variability of IgE antibodies in the mice may not have yielded IgE antibodies which can recognise glycated Ara h 2 despite recognising similar sequences of the allergen.
One final possibility which has to be considered is that AGE-rAra h 2 was not bound efficiently to the plates used for the ELISAs. If this was the case, this could falsely give the impression that IgE antibodies from C3H/HeJ mice are not able to bind to AGE-rAra h 2. It was aimed to exclude this possibility by using different kinds of ELISA plates with coatings facilitating the capture of proteins with a variety of molecular properties. No difference between the plates used could be observed (data not shown). To further examine this issue, inhibition ELISAs could be performed in a future study.

5.4 Differential effects of glycation on the T cell immunogenicity and antigenicity of OVA and rAra h 2

In this study, the glycation of OVA enhanced the $CD4^+$ T cell immunogenicity and antigenicity of the allergen. In contrast AGE-rAra h 2 was found to have a reduced T cell immunogenicity and antigenicity compared to unprocessed rAra h 2. Previous studies have also suggested that the influence of glycation on the immunostimulatory properties of a particular protein is partially dependent on the protein itself (Price et al., 2004; Ge et al., 2005). This highlights the complexity of the immune reaction against food allergens glycated by the Maillard reaction.
A likely explanation for the differential effect of the glycation on the immunogenicity and antigenicity of the food allergens is that the glycation of amino acid residues within epitopes recognised by TCRs or BCRs could influence the interaction of the allergen with the receptors. In BL6 and BALB/c mice, the dominant epitope for OVA specific $CD4^+$ T cells of OVA are the amino acid residues 323-339 (one letter coded amino acid sequence: ISQAVHAAHAEINEAGR). This region contains one arginine residue which may be glycated by the Maillard reaction. However, the arginine residue is not a core amino acid recognised by the TCR of the transgenic OT-II and DO11.10 $CD4^+$ T cells, which represent a dominant clone of OVA specific $CD4^+$ T cells in BL6 and BALB/c mice (Robertson et al., 2000). As the T cell epitope in OVA is not influenced by the glycation, only the enhanced binding of AGE-OVA with SR-AI/II expressed on mDCs is likely to play a role in the enhanced T cell immunogenicity of the glycated allergen.
In contrast to OVA, T cell and/or B cell epitope(s) in rAra h 2 may be modified by glycation as was described in the previous sections (chapter 5.3.2 and 5.3.3). To assess the immunogenicity and antigenicity of AGE-rAra h 2, C3H/HeJ mice were used. This particular mouse strain was identified as a high responder strain for Ara h 2 by assessing immune responses against “unprocessed native Ara h 2” and raw peanut extract (Li et al., 2000; Proust et al., 2008). $CD4^+$ T cells and B cells in C3H/HeJ mice may be the best responders to the native Ara h 2, but not to the glycated rAra h 2.
While it was shown here that both AGE-OVA and AGE-rAra h 2 had been modified with CML and GA-pyridine by thermal processing with carbohydrates, the glycation profile of the two allergens

would not be exactly the same. For example, on AGE-OVA CEL could be detected (Fig. 4.2) but it was not found on AGE-rAra h 2 (Fig. 4.37). It is therefore possible that the different glycation profiles of the allergens contribute to the contrasting influence which the Maillard reaction has on the immunostimulatory properties of each allergen. The different modifications present could influence the recognition of the allergen by TCRs and BCRs or the antigen processing due to different sizes and molecular properties of the individual glycation structures.

The particular glycation structures present on an allergen would also influence to which endocytotic receptors the allergen can bind. It was not tested whether AGE-rArah 2 binds to, for example, SR-AI/II. It was shown that enhanced $CD4^+$ T cell activation by AGE-OVA is heavily dependent on the interaction of the glycated allergen with SR-AI/II. If AGE-rAra h 2 is not able to bind to SR-AI/II, this could also contribute to the differences seen in the immunogenic properties of the glycated forms of the two allergens.

Collectively the presented data suggests that the influence which the glycation has on the immunogenic properties of a particular food allergen depends on the occurrence of glycation within T cell and/or B cell epitopes of the allergen. The interaction with endocytotic receptors expressed by APCs is also likely to play a role.

5.5. Potential Allergenicity of AGEs of food allergens

In this thesis it could be demonstrated that the Maillard reaction influences the $CD4^+$ T cell immunogenicity of the hen egg white allergen OVA and the recombinant peanut allergen Ara h 2. $CD4^+$ T cells are a critical component for the IgE production by B cells and subsequently the allergic responses. Hence, the results presented here suggest that the Maillard reaction could be capable of influencing the allergenicity of food allergens in allergic individuals. If the glycation by the Maillard reaction does not attenuate T cell and B cell epitope recognition, the glycated food allergens could enhance Th2 cell activation and subsequent IgE production by efficient SR-AI/II mediated uptake of the AGEs by DCs. In fact, AGE-OVA, which is capable of binding to SR-AI/II, induced higher $CD4^+$ T cell activation and IgE production in mice than its unglycated counterparts. An increased amount of IgE could augment the activation of effector cells, e.g. mast cells, resulting in the release of more inflammatory mediators. This would lead to an increase in the severity of allergic symptoms. To our knowledge, the present study is the first to demonstrate that the formation of AGEs enhances the $CD4^+$ T cell immunogenicity, antigenicity and potential allergenicity of a food allergen. The results strongly suggest that the influence of the Maillard reaction needs to be carefully considered in the evaluation of the allergenicity of food allergens.

The results in this thesis show that AGE-OVA was able to enhance the production of Th1 as well as Th2 cytokines by allergen specific $CD4^+$ T cells (Fig. 4.10). Therefore, while AGE stimulation can result in enhanced activation of allergen specific $CD4^+$ T cells and a subsequent increase in cytokine production, the increase of both Th1 and Th2 cytokines in response to AGEs indicates

that they do not alter the differentiation of activated $CD4^+$ T cells. In recent years, studies have been published which suggest that in healthy subjects IL-10 producing regulatory T cells (Tregs) are the dominant T cell subset specific for food allergens, in contrast, patients with allergy have increased numbers of allergen specific IL-4 producing T cells (Akdis M, et al., 2004; Akdis CA, et al., 2005). In healthy individuals, glycated allergens may enhance the activation of Tregs and help to maintain tolerance. In allergic patients, glycated allergens could enhance activation of Th2 T cells and promote allergic symptoms by increased production of cytokines which enhance allergen specific IgE production (mediated by IL-4, IL-13) or activation and infiltration of inflammatory cells such as eosinophils (IL-5).

It has been speculated that the glycation structures in food allergens could act as novel B cell epitopes. The results presented here do not clearly show an induction of antibodies specifically binding to AGE structures in mice. However, this does not exclude the possibility that anti-AGE antibodies are induced in human subjects. Compared to the mouse immune system, the immune system of humans has a much greater diversity in regards to genes encoding MHC molecules, TCRs and BCRs/immunoglobulins. The vast genetic diversity enables the human immune system to respond to the enormous amount of different antigens which it encounters. In the genetic diversity among different individuals some would produce antibodies specific for the glycated form of a particular allergen while others may not. This notion is supported by observation that some but not all allergic patients have IgE antibodies exclusively to determinants found in a particular food only after thermal processing or storage or have allergic reactions only to the cooked form of a food (Prausnitz and Kustner, 1921; Malanin, 1995; Rosen et al., 1994). Furthermore, several studies have shown the existence of T cells specially recognising glycoepitopes (Dudler et al., 1995; Corthay et al., 1995; Michaelsson et al., 1996; Haurum et al., 1994), suggesting that T cells specific for glycation structures could also be induced in humans. Studies with human cell culture systems, preferably employing APCs and T cells from food allergic individuals, would give further insight to how the glycation of food allergens could influence the immune response in food allergy.

In the past decade, the prevalence of food allergy has increased and currently no effective treatment has been established. It is therefore a pressing concern to gain further understanding of the mechanisms underlying food allergy in order to develop new strategies of treatment and prevention. One important aspect to study would be the properties of the food allergens themselves. While many food allergens have been isolated and characterised, the influence which food processing has on the allergenic properties of allergens has not been studied in great detail. In the present study it could be shown that the Maillard reaction, as it occurs during thermal processing and storage of food, can severely alter the T cell immunogenicity and antigenicity of food allergens which could potentially contribute in the enhancement in the severity of allergic reactions. The results of this thesis unequivocally show that the influence of the Maillard reaction is an important factor to be considered when studying the pathology of food allergy. This study can help to increase our understanding of the mechanisms contributing to food allergy.

6 Summary

The Maillard reaction takes place between proteins and reducing sugars during thermal processing and storage of foods. The reaction produces protein derivates with glycation structures such as Nε-carboxyethyllysine (CEL), Nε-carboxymethyllysine (CML), pyrralin and GA-pyridine, collectively called advanced glycation end products (AGE). As many foods are cooked in some form before being consumed, a potential involvement of AGEs in the pathology of food allergy is of great interest. This assumption is corroborated by the fact that some food allergic patients show anaphylactic reactions only against stored or heated foods. The Maillard reaction may create new pathogenesis related immune epitopes in food allergy. The aim of this thesis was to examine the influence of the Maillard reaction on the immunogenic properties of food allergens.

In the first part of the thesis, the influence of the Maillard reaction on the T cell immunogenicity of the egg white allergen OVA was studied. The uptake mechanisms of mDCs for AGE-OVA were investigated using inhibitors of putative cell surface receptors for AGEs as well as mDCs which are deficient of one of these receptors. Here, it was shown for the first time that an AGE of a food allergen has a higher T cell immunogenicity than its native counterpart. AGE-OVA was prepared by thermal processing of OVA in the presence of glucose. Activation and proliferation of OVA specific $CD4^+$ T cells by AGE-OVA was evaluated in co-cultures with mDCs as antigen presenting cells. Compared to the controls (native OVA and OVA thermally processed without glucose), AGE-OVA enhanced the activation of OVA-specific $CD4^+$ T cells upon co-culture with mDCs. The result indicates that glycation of OVA by the Maillard reaction enhances the T cell immunogenicity of the allergen. Antigen specific T cell activation is preceded by the uptake of antigens by DCs. In the present study, it was found that mDC uptake of AGE-OVA was significantly higher than that of the controls. Additionally, the scavenger receptor class A type I and II (SR-AI/II) was identified as a mediator of AGE-OVA uptake, whereas the receptor for AGEs (RAGE) and galectin-3 were not involved. $CD4^+$ T cell activation in response to stimulation with AGE-OVA was significantly inhibited when the T cells were cultured with SR-AI/II deficient mDCs. Furthermore, immunocytochemistry data showed co-localisation of AGE-OVA with SR-AI/II and MHC II in mDCs incubated with the glycated allergen. The presentation of soluble antigens by APCs is governed by endocytotic receptors, which determine the intracellular routing of the endocytosed molecules. The results of this thesis suggest that SR-AI/II targets its ligands to the MHC class II loading pathway in DCs. Taken together, the presented data allows the following hypothesis. The glycation of OVA results in an enhanced association of the allergen with SR-AI/II which leads to the observed increased uptake of the allergen by mDCs. Therefore, larger amounts of the allergen enters the MHC class II loading pathway, leading to a greater amount of OVA peptide-MHC II complex being presented on the cell surface of mDCs. This in turn results in the observed enhanced activation and proliferation of the OVA specific $CD4^+$ T cells by AGE-OVA. Collectively, the data shown in this thesis indicate that mDC expressed SR-AI/II is critical for the enhanced $CD4^+$ T cell immunogenicity of AGE-OVA.

Next, T cell immunogenicity and antigenicity of AGE-OVA was evaluated *in vivo*. C57BL/6 mice received subcutaneous (sc) or intraperitoneal (ip) immunisation with native, thermally processed or AGE-OVA. For the evaluation of the T cell immunogenicity, $CD4^+$ T cells were isolated from lymph nodes of immunised mice and the activation/proliferation of T cells upon recall stimulation with the allergens was measured. To assess the antigenicity, levels of allergen specific antibodies in sera of ip immunised mice were measured by ELISA. Importantly, AGE-OVA showed a higher T cell immunogenicity in mice which had received sc immunisation with either form of OVA. Furthermore, when the mice received OVA by ip immunisation, AGE-OVA induced a higher IgE production than native or thermally processed OVA. These results indicate that AGE-OVA has a higher $CD4^+$ T cell immunogenicity as well as an enhanced antigenicity *in vivo* in comparison to the unglycated forms of OVA. Since $CD4^+$ T cells play a pivotal role in the IgE production by B cells, the enhanced T cell immunogenicity of AGE-OVA would explain the higher IgE production following immunisation with the glycated allergen. IgE antibodies are critical components in the induction of allergic responses. Therefore, the results of this thesis suggest that the Maillard reaction would be capable of enhancing the allergenicity of food allergens.

In the final part of the thesis, the influence of glycation by the Maillard reaction on the T cell immunogenicity and antigenicity of the major peanut allergen rAra h 2 was studied. As peanuts are responsible for the majority of the more severe allergic reactions to food and are usually eaten after thermal processing, it is of major interest to elucidate how the Maillard reaction affects the allergenic potential of the peanut allergen. In order to evaluate the T cell immunogenicity and antigenicity of AGE-rAra h 2, C3H/HeJ mice were ip immunised with unprocessed rAra h 2, rAra h 2 thermally processed without carbohydrate or AGE-rAra h 2. AGE-rAra h 2 showed a lower T cell immunogenicity in mice receiving ip immunisation than unprocessed or thermally processed rAra h 2. Furthermore, the ip immunisation with AGE-rAra h 2 induced a lower IgE production than ip immunisation with its unglycated counterparts. Collectively these results suggest that the glycation of rAra h 2 may reduce the allergenicity of the allergen.

In conclusion, the present study shows that the Maillard reaction alters the allergenic properties of food allergens considerably by influencing the T cell immunogenicity of the allergens. The results of this thesis strongly support the significance of Maillard glycation products as pathogenesis-related factors in food allergy. Here, the immune mechanisms underlying the influence of glycation structures on T cell immunogenicity of food allergens were elucidated. If the glycation by the Maillard reaction does not attenuate the recognition of T cell or B cell epitopes, glycation of food allergen can enhance allergen specific $CD4^+$ T cell activation and subsequent IgE production by efficient SR-AI/II mediated uptake of the allergens by DCs. Together, this study provides valuable information about the allergenicity of glycated proteins in thermally processed foods and helps to increase our knowledge on the respective mechanisms of food allergy.

7 German Summary

Die allergische Reaktion ist eine Überempfindlichkeit des Immunsystems gegenüber harmlosen, nicht-infektiösen Substanzen, sogenannten Allergenen. Stoffe die allergische Reaktionen vom Soforttyp (IgE-vermittelt) hervorrufen können sind z.B. Pflanzenpollen, tierische Epithelzellen, Staubmilben, Insektengifte und Lebensmittel. Die darin sich befindenden Allergene sind meist Proteine. Für die Ausbildung einer allergischen Erkrankung ist ein wiederholter Kontakt mit den Allergenen nötig. Während des ersten Kontaktes mit dem Allergen wird das Immunsystem sensibilisiert, dabei werden Immunzellen wie z.B. dendritische Zellen, T-Zellen und B-Zellen aktiviert und Allergen-spezifische Antikörper gebildet. Insbesondere IgE Antikörper spielen bei der Allergie eine wichtige Rolle. Bei dem ersten Kontakt mit dem Allergen kommt es noch nicht zur Manifestation von Symptomen. Erst bei einem wiederholten Kontakt mit dem Allergen werden Symptome induziert. Die Symptome können lokal sein wie zum Beispiel Juckreiz in Mundhöhle und Pharynx bei Nahrungsmittelallergien oder die allergische Rhinitis bei der die oberen Atemwege betroffen sind. Es kann aber auch zu systemischen Reaktionen wie einem anaphylaktischen Schock kommen, der zum Tode führen kann.

Die Prävalenz von Lebensmittelallergien hat in den vergangenen Jahrzehnten in der Bevölkerung der westlichen Welt deutlich zugenommen (Sicherer 2002; Sampson 2003) und aktuell gibt es keine wirkungsvolle kausale Behandlung. Es ist deshalb von hohem Interesse die Mechanismen der Nahrungsmittelallergie weiter zu untersuchen, um neue Strategien für die Prophylaxe und Therapie entwickeln zu können. Ein wichtiger Faktor dabei ist es, die Eigenschaften der Allergene besser einordnen zu können. Dabei muss bedacht werden, dass Lebensmittel vor dem Verzehr häufig verschiedenen Prozessen, wie z.B. Garen durch Hitze unterzogen werden, die sich erheblich auf die immunogenen Eigenschaften der Allergene auswirken können. Abhängig von den Bedingungen der Prozessierung und der Hitzestabilität des Proteins, kann sich die Allergenität eines Allergens durch Hitze verringern, aber ebenso auch erhöhen. Einer Erhöhung der Allergenität können ein oder mehrere Dinge zugrundeliegen, so kann das Allergen z.B. resistenter gegenüber Verdauungsenzymen werden (Maleki et al., 2001). Durch die Denaturierung des Proteins können möglicherweise IgE-Bindungsstellen leichter zugänglich werden, oder auch neue entstehen. Auch durch chemische Modifikation können neue IgE-Bindungsstellen auftauchen. Durch das Entstehen neuer Bindungsstellen können somit sogenannte Neoallergene gebildet werden. Ein biochemischer Prozess der während der Lagerung bzw. der Verarbeitung von Lebensmitteln unter Hitzeeinwirkung abläuft und zur Bildung von Neoallergenen führen, bzw. die allergenen Eigenschaften von bestehenden Allergenen verändern kann, ist die Maillard-Reaktion.

Die Maillard Reaktion ist verantwortlich für die nicht-enzymatische Bräunung der Lebensmittel. Während der Reaktion werden die Aminosäure-Gruppen der Proteine durch nicht-enzymatische Kondensation mit reduzierenden Zuckern modifiziert. Die dabei entstehenden Glykosylamine bilden durch Umlagerung die ersten stabilen Produkte der Reaktion, die sogenannten Amadori-

Produkte. Diese können zu reaktiven Dicarbonyl-Intermediaten degradiert werden, welche mit weiteren Aminosäureresten reagieren und durch Quervernetzungen oder Umlagerungen stabile Endprodukte bilden (Henle, 2005). Beispiele solcher Endprodukte sind Nε-Carboxyethyl-Lysin (CEL), Nε-Carboxymethyl-Lysin (CML), Pyrraline und Glykoaldehyde-Pyridine (GA-pyridine). Proteine die auf diese Weise durch die Maillard-Reaktion modifiziert wurden, nennt man *advanced glycation end products* (AGEs). Um den Prozess von der enzymatischen Glykosylierung zu unterscheiden, wurde der Begriff Glykierung eingeführt (Ledl and Schleicher, 1990).

Bereits veröffentliche Studien deuten darauf hin, dass die Maillard-Reaktion eine wichtige Rolle im Pathomechanismus der Lebensmittelallergie spielt. So gibt es eine Reihe von Veröffentlichungen die Fälle beschreiben bei denen allergische Reaktionen auf gekochte, aber nicht auf rohe Lebensmittel beobachtet wurden (Prausnitz and Kustner, 1921; Malanin, 1995). Des Weiteren wurde gezeigt, dass die Glykierung von Allergenen durch die Maillard-Reaktion deren Fähigkeit Serum-IgE von Patienten mit einer Sensibilisierung für das entsprechende Allergen zu binden signifikant erhöhen (Gruber et al., 2005; Chung et al., 2001; Chung et al., 2003). Diese Beobachtungen sprechen deutlich dafür, dass durch die Maillard-Reaktion neue, für die Pathogenese der Lebensmittelallergie relevante, Immunepitope entstehen.

Bei der Sensibilisierung und auch bei den allergischen Reaktionen nach wiederholten Allergenkontakt spielen dendritische Zellen und T-Zellen, insbesondere $CD4^+$ T-Zellen, eine zentrale Rolle. Bisherige Studien haben unterschiedliche Auswirkungen von AGEs auf dendritische Zellen gezeigt. AGE-Derivate von BSA induzierten verstärkt die Reifung der dendritischen Zellen und erhöhten auch deren Fähigkeit die Aktivierung von T-Zellen zu induzieren (Ge et al., 2005). Im Gegensatz dazu, zeigte sich, dass AGE-Derivate des Adrenocorticotrophen Hormons die Reifung von dendritischen Zellen inhibiert und deren Kapazität T-Zellen zu stimulieren verringert (Price et al., 2001). Diese Ergebnisse zeigen, dass die T-Zell-Immunogenität von Antigenen durch die Maillard-Reaktion beeinflusst werden kann. Allerdings ist der Einfluss von AGE-Derivaten von Lebensmittelallergenen auf die Funktion von dendritischen Zellen und die Aktivierung von T-Zellen weitgehend unbekannt, insbesondere gab es bisher keine *in vivo* Studien die den Einfluss der Maillard-Reaktion auf die immunogenen Eigenschaften von Allergenen untersucht haben.

Der Aktivierung von T-Zellen geht die Aufnahme des Allergens durch die dendritischen Zellen voran. Häufig vermitteln Rezeptoren auf der Oberfläche der dendritischen Zellen die Aufnahme. Für einige Rezeptoren wurde gezeigt, dass sie AGEs binden können, zu diesen gehört RAGE (Receptor for AGEs) (Schmidt et al., 1992; Neeper et al., 1992), Galectin-3 (Vlassara et al., 1995), macrophage scavenger receptor class A type I/II (SR-AI/II) (Suzuki et al., 1997; Araki et al., 1999) sowie scavenger receptor class B type I und CD36 (Ohgami et al., 2001 a; Ohgami et al., 2001). Welche Rezeptoren für die Aufnahme von AGEs durch dendritische Zellen verantwortlich sind, wurde bisher nicht genau untersucht.

In dieser Dissertation sollte der Einfluss der Maillard-Reaktion auf die immunogenen Eigenschaften von Lebensmittelallergenen *in vitro* und *in vivo* untersucht werden. Dafür wurde zum einen das Modelallergen Ovalbumin (OVA) und zum anderen ein Majorallergen der Erdnuss, Ara h 2, in rekombinanter Form verwendet (rAra h 2). Von beiden Allergenen wurde durch Erhitzen zusammen mit einem Kohlenhydrat ein AGE-Derivat hergestellt, AGE-OVA bzw. AGE-rAra h 2. Für alle Versuche wurden als Kontrollen unbehandeltes Allergen und Allergen welches ohne Kohlenhydrate erhitzt wurde verwendet.

Im ersten Teil der Arbeit wurden die immunogenen Eigenschaften von AGE-OVA *in vitro* in murinen Zellkulturen untersucht. Verwendet wurden dabei aus Knochenmarkzellen der Maus generierte dendritische Zellen. Des Weiteren wurden T-Zellen verwendet die aus der Milz von transgenen Mäusen isoliert wurden. Diese T-Zellen besitzen T-Zellrezeptoren die spezifisch OVA erkennen. Es konnte gezeigt werden, dass die Modifizierung von OVA durch die Maillard-Reaktion die $CD4^+$T-Zell-Immunogenität des Allergens deutlich erhöht: AGE-OVA induzierte eine verstärkte Aktivierung und Proliferation von $CD4^+$ T-Zellen die mit dendritischen Zellen co-kultiviert wurden. Die erhöhte Stimulation der $CD4^+$ T-Zellen durch AGE-OVA wurde nur beobachtet, wenn die T-Zellen mit dendritischen Zellen kultiviert wurden. AGE-OVA kann daher die T-Zellen nicht direkt aktivieren, sondern muss von den dendritischen Zellen aufgenommen, prozessiert und auf MHC Klasse II Molekülen (MHC II) präsentiert werden, um von den T-Zellen erkannt zu werden. Daraus ließ sich schlussfolgern, dass die erhöhte Aktivierung der $CD4^+$ T-Zellen durch AGE-OVA von der Interaktion des glykierten Allergens mit den dendritischen Zellen abhängig ist. Im Folgenden wurde gezeigt, dass AGE-OVA besser von den dendritischen Zellen aufgenommen wird, als natives OVA oder OVA welches ohne Glukose erhitzt wurde. Es war deshalb von Interesse den oder die Rezeptor(en) zu identifizieren welche die Aufnahme von AGE-OVA vermitteln. OVA besitzt durch enzymatische, postranslationale Glykosilierung eine Kohlenhydratseitenkette aus N-Acetyl-Glukosamin und Mannose. Es ist bekannt, dass Mannose die Bindung an den Mannoserezeptor (MR) erlaubt und OVA durch diesen Rezeptor aufgenommen wird (Huntington 2001 et al., Burgdorf 2006, Autenrith et al., 2007). Das konnte in der vorliegenden Arbeit für alle drei OVA-Formen bestätigt werden. Zusätzlich stellte sich heraus, dass SR-AI/II eine elementare Rolle bei der Aufnahme von AGE-OVA spielt. Für die Aufnahme von nativen und ohne Glukose erhitztem OVA spielte er dagegen nur eine untergeordnete Rolle. Es wurde in früheren Publikationen gezeigt, dass Antigene die über Scavenger Rezeptoren aufgenommen werden hauptsächlich auf MHC II präsentiert werden und $CD4^+$ T-Zellen aktivieren (Burgdorf et al., 2007). Auch die Ergebnisse der vorliegenden Arbeit deuten darauf hin, dass AGE-OVA welches von SR-AI/II aufgenommen wird auf MHC II präsentiert wird. Zum einen führte die Kultivierung von OVA-spezifischen $CD4^+$ T-Zellen mit SR-AI/II defizienten dendritischen Zellen zu einer signifikanten Reduktion der $CD4^+$ T-Zell-Aktivierung was darauf hindeutet, dass die SR-AI/II-vermittelte Aufnahme des Allergens zur Aktivierung der $CD4^+$ T-Zellen beiträgt. Zum anderen zeigte sich, dass die Glykierung von OVA nicht zu einer erhöhten $CD8^+$ T-Zell-Aktivierung führte. Das deutete darauf hin, dass OVA welches vom SR-AI/II aufgenommen wurde nicht auf MHC I

präsentiert wurde. In vorhergehenden Studien wurde gezeigt, dass OVA-spezifische $CD8^+$ T-Zellen durch OVA aktiviert werden, welches vom MR aufgenommen wurde (Burgdorf et al., 2007). Da alle drei OVA-Formen ähnlich gut vom MR aufgenommen wurden, entspricht das Ergebnis den Erwartungen. Weitere Hinweise darauf, dass OVA, welches vom SR-AI/II aufgenommen wurde auf MHC II präsentiert wird, stammen von Experimenten mit rekombinanten OVA. Rekombinantes, in *E. coli* hergestelltes, OVA besitzt keine natürlichen Kohlenhydratseitenketten die ein Aufnahme durch Rezeptoren ermöglicht und wird deshalb nur durch Pinozytose aufgenommen. Im Gegensatz zur Aufnahme von nativen, ohne Glukose erhitzten und AGE-OVA, wurde die Aufnahme von rekombinanten OVA deshalb nicht durch die Abwesenheit von SR-AI/II auf den dendritischen Zellen beeinflusst. Gleiches galt für die Aktivierung von $CD4^+$ T-Zellen durch rekombinantes OVA. Zusätzlich war die Aufnahme des rekombinanten OVAs und auch die Aktivierung der T Zellen sehr gering. Durch Pinozytose aufgenommenes OVA wird ebenfalls auf MHC II präsentiert (Burgdorf et al., 2007). Natives, ohne Glukose erhitztes, wie auch AGE-OVA werden zusätzlich zur Pinozytose vor allem durch MR bzw. SR-AI/II vermittelte Endozytose aufgenommen. Die Ergebnisse der vorliegenden Arbeit lassen folgende Hypothese zu: Sowohl natives als auch ohne Glukose erhitztes OVA können durch ihre Kohlenhydratseitenkette an SR-AI/II binden und der Rezeptor vermittelt eine partielle Aufnahme dieser nicht-glykierten OVA-Formen. AGE-OVA hat zusätzlich zu der natürlichen Kohlenhydratseitenkette die AGE-Strukturen auf der Oberfläche, die eine stärkere Bindung an SR-AI/II erlauben und daraus folgt eine erhöhte Aufnahme von AGE-OVA durch die dendritischen Zellen. Das führt dazu, dass eine größere Menge des Allergens für die MHC II-Beladung prozessiert wird. Dadurch erhöht sich die Menge an MHC II-OVA-Peptid-Komplexen auf der Oberfläche der dendritischen Zellen, wodurch wiederum eine stärkere Aktivierung der $CD4^+$ T-Zellen stattfinden kann. Dieses Ergebnis wird durch frühere Publikationen unterstützt, die gezeigt haben, dass die $CD4^+$ T-Zell-Immunogenität erhöht wird, wenn Proteine chemisch so modifiziert werden, dass sie an SR-AI/II binden können (Abraham et al., 1995; Nicoletti et al., 1999).

Ein Einfluss der Glykierung durch die Maillard-Reaktion auf die Fähigkeit von OVA die Reifung der dendritischen Zellen zu induzieren konnte dagegen nicht beobachtet werden. Eine Erklärung dafür könnte das Fehlen von RAGE auf den hier verwendeten Zellen sein, wurde doch in einer früheren Studie gezeigt, dass die verstärkte Reifung der dendritischen Zellen nach Stimulation mit AGE-BSA von der Anwesenheit von RAGE abhing (Ge et al., 2005), dessen Bedeutung für die Reifung von dendritischen Zellen auch in anderen Studien gezeigt wurde (Dimitriu et al., 2005; Manfredi et al., 2008).

Im zweiten Teil der Arbeit wurde der Einfluss der Glykierung auf die T-Zellen Immunogenität und die Antigenität von OVA *in vivo* untersucht. Hierfür wurden C57BL/6-Mäuse subkutan oder intraperitoneal mit AGE-OVA, oder einer der Kontrollen immunisiert. Zur Bestimmung der T-Zell-Immunogenität wurden die $CD4^+$ T-Zellen der Lymphknoten bzw. der Milz isoliert und *in vitro* mit den Allergenen restimuliert. Daraufhin wurde die Aktivierung bzw. die Proliferation der T-Zellen gemessen. Es konnte gezeigt werden, dass bei der subkutanen Immunisierung der Mäuse, AGE-

OVA eine deutlich höhere T-Zellen-Immunogenität besitzt als natives oder ohne Glucose erhitztes OVA. Weiterhin induzierte AGE-OVA in Mäusen denen die Allergene intraperitoneal verabreicht wurden eine höhere allergenspezifische IgE-Produktion, als die Kontrollen. Somit konnte gezeigt werden, dass die Glykierung von OVA nicht nur dessen OVA-spezifische $CD4^+$ T-Zell-Immunogenität *in vitro* und *in vivo* erhöht, sondern auch die Antigenität des Allergens verstärkt. Da die B-Zell-Aktivierung und die IgE-Produktion dieser Zellen maßgeblich von $CD4^+$ T-Zellen beeinflusst wird, ist die erhöhte IgE-Produktion nach Immunisierung mit AGE-OVA mit großer Wahrscheinlichkeit auf die verstärkte Aktivierung der allergenspezifischem $CD4^+$ T-Zellen zurückzuführen.

Schlussendlich sollte der Einfluss der Maillard-Reaktion auf die immunstimulatorischen Eigenschaften eines Hauptallergens der Erdnuss, Ara h 2, untersucht werden. Erdnüsse sind die häufigsten Verursacher von sehr starken allergischen Reaktionen nach dem Verzehr von Nahrungsmitteln, diese können mitunter auch letal sein (Burks et al., 1992; Burks et al., 1995). Erdnüsse werden meist erst nach thermischer Verarbeitung verzehrt. Es wurde gezeigt, dass die Maillard-Reaktion in Erdnüssen stattfindet (Maleki et al., 2001, Chung et al., 2003). Es sollte deshalb auch hier untersucht werden wie die Glykierung des Allergens dessen T-Zell-Immunogenität und die Antigenität *in vivo* beeinflusst. Dafür wurden C3H/HeJ-Mäuse intraperitoneal mit AGE-rAra h 2, oder einer der Kontrollen immunisiert und die Immunogenität und die Antigenität wie für OVA beschrieben analysiert. Es stellte sich heraus, dass die Glykierung von rAra h 2 zu einer deutlichen Reduktion von sowohl der T-Zell-Immunogenität als auch der Antigenität führte. Es scheint möglich, dass durch die Glykierung T-Zellen-Epitope verändert wurden und dadurch eine effektive Aktivierung der $CD4^+$ T-Zellen eventuell durch verminderte Bindung des T-Zellen-Rezeptors verhindert wurde. Es besteht auch die Möglichkeit, dass im Falle von rAra h 2 die Glykierung eine reduzierte Prozessierung und Präsentation des Allergens durch Antigen-präsentierende Zellen zur Folge hat. Die verringerte Antigenität ist höchstwahrscheinlich eine direkte Folge der geringen T-Zell-Aktivierung durch AGE-rAra h 2. Mitunter könnten auch die B-Zell-Epitope glykiert sein und ebenfalls die Bindung des Allergens an die Rezeptoren behindern.

Zwar beeinflusste die Maillard-Reaktion die immunstimulatorischen Eigenschaften sowohl von OVA als auch von rAra h 2, der Effekt für die beiden Allergene war aber gegensätzlich. Auch andere Studien haben gezeigt, dass der Einfluss den die nicht-enzymatische Glykierung auf ein Protein haben kann, nicht zuletzt auch von der Eigenschaft des Proteins selber abhängig ist (Price et al., 2004; Ge et al., 2005). Eine Möglichkeit für den Unterschied kann die Positionen sein, an denen die beiden Allergene modifiziert wurden. Während die Epitope von OVA die für die Bindung des Allergens durch T- und B-Zellen benötigt werden evtl. nicht modifiziert wurden bzw. nur an Stellen wo die Bindung nicht behindert wurde, könnten die Epitope von rAra h 2 so modifiziert wurden sein, dass die Bindungen teilweise blockiert wurden.

Th2-Lymphozyten spielen eine kritische Rolle in der Pathogenese der Allergie. AGE-OVA konnte *in vitro* sowohl die Sekretion des Th1-Zytokins IFN-γ, als auch des Th2-Zytokins IL-4 durch die

$CD4^+$ T-Zellen erhöhen. Es fand dabei aber keine Polarisierung der Zytokin-Produktion statt. Die Zytokine beeinflussen die Antikörperantwort; während Th1-Zytokine die Produktion von IgG2a fördern, führt die Ausschüttung von Th2-Zytokinen zur Produktion von IgG1 und IgE (Kopf et al., 1993; Coffman et al, 1986; Snapper et al., 1987). *In vivo* führte die Immunisierung mit AGE-OVA im Vergleich zur Immunisierung mit nativen oder ohne Glucose erhitztes OVA nicht zu einer nennenswerten Veränderung des Verhältnisse von IgG1 zu IgG2a. Daher zeigen zwar sowohl die *in vitro* als auch die *in vivo* Daten, dass AGE-OVA die Aktivierung der $CD4^+$ T-Zellen erhöht aber keine Polarisierung in Richtung eines bestimmtes Th Phenotypes bewirkt. In gesunden Individuen sind IL-10 produzierende regulatorische T-Zellen der dominierende Allergen-spezifische T-Zellen Subtyp, während bei Allergikern eine erhöhte Anzahl an IL-4 produzierenden Allergen-spezifischen T-Zellen festgestellt werden konnte (Akdis M, et al., 2004; Akdis CA, et al., 2005). In gesunden Menschen könnte es daher zu eine verstärkten Aktivierung der regulatorischen T-Zellen durch glykierte Allergene kommen und die Toleranz gegenüber harmlosen Allergenen fördern, während es womöglich in Allergikern zu einer verstärkten Aktivierung von Allergen-spezifischen Th2 Zellen kommt. Die dementsprechend erhöhte Ausschüttung an Th2-typischen Zytokinen würde zu einem Anstieg der IgE-Produktion (durch IL-4, IL-13) oder zur Einwanderung und Aktivierung von Eosinophilen (IL-5) führen. Das wiederum hätte eine Verschlimmerung der allergischen Symptome zur Folge.

Es wurde gemutmaßt, dass Glykierungs-Strukturen die durch die Maillard-Reaktion bei Lebensmitteln entstehen als neue B-Zellen-Epitope fungieren könnten. Die Ergebnisse der vorliegenden Arbeit zeigen keine eindeutigen Hinweise darauf, dass in den immunisierten Mäusen Antikörper, die spezifisch Glykierungs-Strukturen binden, induziert wurden. Diese Feststellung schließt aber nicht aus, dass in Allergikern anti-AGE-Antikörper gebildet werden. Im Vergleich zum Immunsystem der Maus, besitzt das humane Immunsystem eine wesentlich grössere Variabilität hinsichtlich der Gene welche für die MHC-Moleküle, T-Zell-Rezeptoren und B-Zellen-Rezeptoren/Immunoglobuline kodieren. Die große genetische Variabilität ermöglicht es dem menschlichen Immunsystem auf die enorme Vielfalt an Antigenen zu reagieren. Zieht man die genetische Diversität zwischen einzelnen Individuen in Betracht, so erscheint es realistisch, dass einige Menschen Antikörper spezifisch für AGE-Derivate von Lebensmittelallergenen produzieren, andere dagegen nicht. Diese Ansicht wird auch dadurch unterstützt, dass einige Allergiker nur auf erhitzte Nahrungsmittel reagieren, andere aber nur auf rohe. Zudem haben einige Studien gezeigt, dass es T-Zellen gibt, die spezifisch Glykanepitope erkennen (Dudler et al., 1995; Corthay et al., 1995; Michälsson et al., 1996; Haurum et al., 1994), was darauf hindeutet, dass es auch T-Zellen mit einer Spezifität für Glykierungs-Strukturen geben könnte. Studien in humanen Zellkulturen, vorzugsweise mit Antigen-präsentierenden Zellen von Lebensmittelallergikern könnten dazu beitragen weitere Erkenntnisse darüber zu gewinnen wie die Glykierung von Nahrungsmittelallergenen die humane Immunantwort auf das respektive Allergen beeinflussen kann.

Zusammengefasst konnte in dieser Arbeit gezeigt werden, dass die Maillard Reaktion die $CD4^+$ T-Zellen-Immunogenität des Hühnereiweiss-Allergens OVA und des Erdnuss-Allergens Ara h 2 beeinflussen kann. Da $CD4^+$ T-Zellen ein zentrale Rolle bei der Produktion von IgE-Antikörpern durch B-Zellen spielen, und somit auch für die darauf folgenden Symptome, zeigen die hier präsentierten Ergebnisse, dass die Maillard-Reaktion die Allergenität von Nahrungsmittel-Allergenen in Individuen mit Nahrungsmittelallergie beeinflussen könnte. Es besteht die Möglichkeit, dass die Glykierung durch die Maillard-Reaktion die Erkennung von T- und B-Zellepitope inhibieren kann. Ist diese jedoch nicht der Fall, wie höchst wahrscheinlich bei OVA, so könnten glykierte Lebensmittelallergene die Th2-Akitiveirung und die IgE-Produktion erhöhen indem sie effizienter durch SR-AI/II-vermittelte Endozytose von dendritischen Zellen aufgenommen werden als das native Protein. Diese These wird dadurch unterstützt, dass in dieser Arbeit gezeigt wurde, dass AGE-OVA auch *in vivo* eine erhöhte $CD4^+$ T-Zell-Aktivierung und IgE-Produktion auslösen kann, wahrscheinlich durch Interaktion mit SR-AI/II. Soweit bekannt, ist die vorliegende Arbeit die erste Studie die zeigt, dass die Bildung von AGEs die $CD4^+$ T-Zell-Immunogenität, Antigenität und potentiell die Allergenität von Lebensmittelallergenen erhöhen kann. Schlussfolgernd zeigen die Ergebnisse, dass der Einfluss der Maillard-Reaktion bei der Bewertung der Allergenität von Lebensmitteln bedacht werden sollte. In zukünftigen Studien muss zu einem geklärt werden welche AGE-Strukturen an SR-AI/II binden können, zum anderen wäre es von Interesse, dass in *vitro* Studien mit Immunzellen durchgeführt werden die von Allergikern isoliert wurden.

8 Abbreviations

°C	Degree Celcius
µCi	Microcurie
µg	Microgram
µl	Microlitre
^{3}H	Tritium
A_x	Absorbance at x nm
aa	Amino acid
Ab	Antibody
AD	Alzheimers disease
APC	Antigen presenting cell
APC	Allophycocyanin
AGE	Advanced glycation end products
Ara h	*Arachis hypogaea* (peanut)
BCA	Bicinchoninic acid
BCR	B cell receptor
Bet v	*Betula verrucosa* (common birch)
BL6	C57BL/6 mice, immunocompetent black Wt mice
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CD	Circular dichroism
CEL	N-ε-cyrboxyethyllysine
CML	N-ε-cyrboxymethyllysine
Con A	Concanavalin A
Cor a	*Corylus avellana* (hazelnut)
cpm	Counts per minute
CRD	Carbohydrate recognition domain
CT	Cholera toxin
DC	Dendritic cells
DNA	Desoxyribonucleic acid
dNTP	Desoxyribonucleosidtriphosphat
DO11.10	C.Cg-Tg(DO11.10)10Dlo/J mice, transgenic mice with OVA specific TCR on $CD4^{+}$ T cells
EAACI	European Academy of Allergology and Clinical Immunology
EDTA	Ethylendiamintetraacetat
EEA1	Early Endosome Antigen 1

e.g.	For example
ELISA	Enzyme linked immunosorbent assay
ER	Endosplasmatic reticulum
FACS	Fluorescence-activated cell sorting
FcεRI/II	Receptor for the constant region (Fc) of IgE
FCS	Fetal calf serum
Fig	Figure
FITC	Fluorescein isothiocyanate
g	gram
G	Gauge
GA-pyridine	Glycolaldehyde-pyridine
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GNac	N-Acetylglucoseamine
h	hour
HEPES	N-2-hydroxyethylpiperazin-N2`-ethansulfonic acid
HRP	Horseradisch Peroxidase
i.e.	That is
IL	Interleukin
IFN	Interferon
IgE	Immunoglobulin E
IgG	Immunoglobulin G
ip	intraperitoneal
kDa	Kilodalton
l	Litre
LPS	Lipopolysaccharides
LN	Lymph node
M	Molar
mA	Milliampere
MACS	Magnetic adsorption cell sorting
mDC	Myeloid dendritic cell
mg	Milligram
min	Minutes
MHC	Major histocompatibility complex
MHC I	MHC class I molecule
MHC II	MHC class II molecule
ml	Millilitre
MLN	Mesenteric lymph nodes
mM	Millimolar
MMC	Mytomycin C

MR	Mannose receptor
mRNA	messenger RNA
MW	Molecular weight
NC	Negative control
n.d.	Not detected
Num.	Number
ng	nanogram
nm	nanometre
OAS	Oral allergy syndrome
OD	Optical density
OT-1	C57BL/6-Tg(TcraTcrb)1100Mjb/J mice, transgenic mice with OVA specific TCR on $CD4^+$ T cells
OT-2	C57BL/6-Tg(TcraTcrb)425Cbn/J mice, transgenic mice with OVA specific TCR on $CD8^+$ T cells
OVA	Ovalbumin
PBS	Phosphate Buffered Saline
pDC	Plasmacytoid dendritic cells
PCR	Polymerase chain reaction
PE	Phycoerythrin
PEI	Paul-Ehrlich-Institut
PFA	Paraformaldehyde
pg	picogram
Pru av	*Prunus avium* (cherry)
RNA	Ribonucleic acid
RT-PCR	Reverse trancriptase polymerase chain reaction
SD	Standard deviation
SDS	Sodiumdodecylsulfat
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
sec	Seconds
sc	Subcutaneous
SR-AI/II	Scavenger receptor class A type I and II
SR-BI	Scavenger receptor class B type I
Tab	Tabel
TAE	Tris-Acetat EDTA
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TEMED	Tetramethylethylendiamin
TGF	Transforming growth factor
Th1	T helper cell subtype 1

Th2	T helper cell subtype 2
TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
Treg	Regulatory T cell
Tris	Tris-(hydroxymethyl)-aminomethan
Vol.	Volume
Wt	Wild type

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10 Lebenslauf

Geburtsdatum/Ort	14.09.1981, Dresden
Familienstand	ledig
Staatsangehörigkeit	Deutsch

Berufserfahrung

April 2006 - Juni 2009	Paul-Ehrlich-Institut, Langen (Bundesamt für Sera und Impfstoffe) Wissenschaftliche Mitarbeiterin in der Wissenschaftlichen Nachwuchsgruppe „Experimentelle Allergologie“, NG1
September 1999 - August 2000	Freiwilliges soziales Jahr am Marienhospital, Bonn.

Studium und Promotion

Seit April 2006	Promotion am Paul-Ehrlich-Institut in Langen in der Arbeitsgruppe von Frau Dr. M. Toda (NG1) zum Thema: „The influence of the Maillard reaction on the immunogenic property of food allergens”
Juli 2005 – März 2006	Diplomarbeit bei Frau Prof. G. Klug am Institut für Mikrobiologie und Molekularbiologie der Justus-Liebig-Universität, Gießen zum Thema: „Partielle Aufreinigung des Exosoms aus *Sulfolobus solfataricus* und Studien zur Interaktion des Komplexes mit anderen Proteinen“
Oktober 2000 – März 2006	Studium der Biologie an der Justus-Liebig-Universität, Gießen

Schulbildung

1994 - 1999	Colaiste Muire, Ennis, Co. Clare, Irland Secondary School (Gymnasium)
1991 - 1994	Scoil Chríost Rí, Ennis, Co. Clare, Irland Primary School (Grundschule)
1988 - 1991	106. Oberschule Albert Hensel, Dresden (Grundschule)

Auszeichnungen/Preise

Juni 2007	Stipendium für die Reise zum Congress of the EAACI (European Academy of Allergology and Clinical Immunology), Götheburg, Schweden. Verliehen für den Abstract *"Glycation of ovalbumin by the Maillard reaction enhanced allergen-specific activation of murine T cells"*
Juni 2008	Congress of the EAACI (European Academy of Allergology and Clinical Immunology), Barcelona, Spanien. Posterpreis in der Kategorie *Inflammatory Cells and Mediators I*. Mit dem Poster *"Glycation by the Maillard reaction enhances the uptake of Ovalbumin by murine dendritic cells"*
September 2009	Einladung zum 4. Gemeinsamen Allergie-Kongress in Berlin. Die Präsenentation vom Mainzer Allergie-Workshop der DGAKI 2009 wurde für die Vortragsreihe „Das Beste aus Mainz" ausgewählt.
Oktober 2009	Forschungspreis des Paul-Ehrlich-Instituts, 1. Preis für exzellente Nachwuchsforschung verliehen für die Publikation „*Glycation of a food allergen by the Maillard reaction enhances T-cell immunogenicity: the role of macrophage scavenger receptor class A type I and II*"

11 Publikationen und Präsentationen

11.1 Publikationen

Ilchmann A, Burgdorf S, Nagai R, Waibler Z, Wellner A, Yamamoto Y, Hiroshi Y, Scheurer S, Henle T, Kurts Ch, Kalinke U, Vieths S, Toda M. Glycation of a food allergen by the Maillard reaction enhances T-cell immunogenicity: the role of macrophage scavenger receptor class A type I and II. *J Allergy Clin Immunol.* 2009 Oct 27. [Epub ahead of print]

Hilmenyuk T, Bellinghausen I, Heydenreich B, **Ilchmann A**, Toda M, Grabbe S, Saloga J. Effects of glycation of model food allergen ovalbumin on antigen uptake and presentation by dendritic cells as well as T cell proliferation and cytokine production. *Immunology*. 2009 Nov 17. [Epub ahead of print]

Walter P, Klein F, Lorentzen E, **Ilchmann A**, Klug G, Evguenieva-Hackenberg E. *Characterization of native and reconstituted exosome complexes from the hyperthermophilic archaeon Sulfolobus solfataricus.* Mol Microbiol. 2006 Nov;62(4):1076-89

11.2 Präsentationen

März 2007	Mainzer Allergie-Workshop der DGAKI (Deutsche Gesellschaft für Allergologie und klinische Immunologie e.V) (Vortrag)
Juni 2007	Congress of the EAACI (European Academy of Allergology and Clinical Immunology), Götheburg, Schweden (Vortrag)
März 2008	Mainzer Allergie-Workshop der DGAKI (Deutsche Gesellschaft für Allergologie und klinische Immunologie e.V) (Vortrag)
Juni 2008	Congress of the EAACI (European Academy of Allergology and Clinical Immunology), Barcelona, Spanien (Poster Präsentation)
März 2009	Mainzer Allergie-Workshop der DGAKI (Deutsche Gesellschaft für Allergologie und klinische Immunologie e.V) (Vortrag)
März 2009	World Immnue Regulation Meeting, Davos, Switzerland. (Poster Präsentation)

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