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UNRAVELING A NOVEL TYPE OF SEVERE FOOD ALLERGY

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Unraveling a novel type of severe food allergy

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Stockholm 2016

“Start by doing what's necessary; then do what's possible;

and suddenly you are doing the impossible.”

- Francis of Assisi

ABSTRACT

Allergy belongs to the chronic inflammatory diseases affecting up to 30% of the population in westernized countries where food allergy has become a substantial and evolving public health issue. In the last decade, a novel type of food allergy presenting with severe allergic reactions several hours after consumption of red meat has been described. The response is caused by IgE antibodies directed against the carbohydrate epitope galactose-α-1,3-galactose (α-Gal) found in mammalian meat.

The overall aim of this thesis was to generate new insight into the red meat allergy syndrome, scrutinizing the humoral response to α-Gal and other glycans, as well as the impact of tick bites as the sensitization route. Moreover, the influence of thermal processing on the allergenicity of beef proteins was addressed and finally the involvement of immune cells was investigated.

We showed for the first time that the α-Gal epitope is present in the European tick *Ixodes ricinus*, supporting previous data reporting a strong relationship between tick bites for the production of IgE to α-Gal and subsequent development of red meat allergy. All studied patients described urticaria as the dominant symptom following consumption of red meat and around half of the patients had experienced anaphylactic reactions, emphasising the severity of this novel type of food allergy. After having characterized the patients an immunoproteomic approach was utilised and seven novel α-Gal-containing beef proteins that bound IgE from red meat allergic patients were identified, where four of these proteins were stable to thermal processing. Furthermore, we showed that their IgE antibodies did not recognize the other major mammalian carbohydrate, N-glycolylneuraminic acid, or common cross-reactive carbohydrate determinants from plants and venoms. In addition, we demonstrated that these patients had a selective IgE reactivity against the α-Gal glycan structure unrelated to the whole glycoprotein. Finally we investigated the α-Gal uptake with the model α-Gal-containing antigen bovine thyroglobulin and showed that it was actively internalized by human immature monocyte derived dendritic cells in a dose and time-dependent manner. The similarity in internalization kinetics between the human and bovine thyroglobulin indicated an α-Gal-independent mechanism, presumably via macropinocytosis. After endosomal processing it was possible to specifically detect α-Gal expression on the plasma membrane derived only from bovine thyroglobulin.

The work in this thesis has contributed to increased understanding of the role of IgE antibodies to α-Gal, the allergenicity of α-Gal-containing beef proteins after thermal processing, the importance of tick bites as the primary sensitization pathway and the involvement of immune cells in this delayed allergic response. Such knowledge is vital for the improvement of current treatment strategies. However, a deeper understanding about the mechanisms behind red meat allergy syndrome is needed and warrants further studies.

LIST OF INCLUDED PUBLICATIONS

I. C. Hamsten, M. Starkhammar, T.A.T. Tran, M. Johansson, U. Bengtsson, G. Ahlén, M. Sällberg, H. Grönlund & M. van Hage. **"Identification of galactose-α-1,3-galactose in the gastrointestinal tract of the tick *Ixodes ricinus*; possible relationship with red meat allergy."** Allergy 2013 Apr; 68(4):549-52.

II. Carl Hamsten*, Thi Anh T. Tran*, Maria Starkhammar, Annelie Brauner, Scott P. Commins, Thomas A. E. Platts-Mills, Marianne van Hage. **"Red meat allergy in Sweden: Association with tick sensitization and B-negative blood groups."** J Allergy Clin Immunol 2013 Dec; 132(6):1431-1434.e6.

III. D. Apostolovic*, T.A.T. Tran*, C. Hamsten, M. Starkhammar, T. Cirkovic Velickovic & M. van Hage. **"Immunoproteomics of processed beef proteins reveal novel galactose-α-1,3-galactose-containing allergens".** Allergy 2014 Oct; 6(10): 1308-15.

IV. D. Apostolovic, T.A.T. Tran, S. Sánchez-Vidaurre, T. Cirkovic Velickovic, M. Starkhammar, C. Hamsten[#] & M. van Hage[#]. **"Red meat allergic patients have a selective IgE response to the α-Gal glycan."** Allergy 2015 Nov; 70(11):1497-500.

V. Thi Anh Thu Tran, Jeanette Grundström, Maja Krstic, Vladana Vukojević, Danijela Apostolovic, Carl Hamsten, Guro Gafvelin & Marianne van Hage. **"*In vitro* uptake of α-Gal containing proteins by human monocyte derived dendritic cells".** Manuscript, 2016.

RELATED PUBLICATIONS

Review

Danijela Apostolovic, Thi Anh Thu Tran, Maria Starkhammar, Sara Sánchez-Vidaurre, Carl Hamsten[#], Marianne van Hage[#]. **"The red meat allergy syndrome in Sweden."**Allergo J Int 2016: 25;49-54.

*: Equal contribution; [#]: Shared last authorship

CONTENTS

LIST OF ABBREVIATIONS

α-Gal	Galactose-α-1,3-galactose
A. americanum	*Amblyomma americanum*
APC	Antigen presenting cells
BCR	B-cell receptors
B. taurus	*Bos taurus*
bTG	Bovine thyroglobulin
CCDs	Cross-reactive carbohydrate determinants
CRD	Component resolved diagnostic
CTL	Cytotoxic T-cells
DBPCFC	Double-blind placebo-controlled food challenge
DC	Dendritic cells
FcR	Fc-receptor
GALT	Gut-associated lymphoid tissue
GI	Gastrointestinal
GM-CSF	Granulocyte macrophage colony-stimulating factor
hTG	Human thyroglobulin
IECs	Intestinal epithelial cells
Ig	Immunoglobulins
IgE-abs	IgE antibodies
IL	Interleukin
iMDDC	Immature monocyte derived dendritic cells
I. ricinus	*Ixodes ricinus*
kU_A/L	Kilounits of allergen per liter
M	Microfold
MDDC	Monocyte derived dendritic cells
MHC	Major histocompability complex
Neu5Gc	N-glycolylneuraminic acid
PAMP	Pathogen–associated molecular patterns
PR	Pathogenesis-related
PRR	Pattern recognition receptors
sIgE	Serum-specific IgE
SPT	Skin prick test
TCR	T-cell receptors
TGF-β	Transforming growth factor-β
Th	T helper
Treg	Regulatory T-cells

1 INTRODUCTION

1.1 THE IMMUNE SYSTEM

The immune system has evolved to protect the host against microbial infections through two types of defence mechanisms; the innate and the adaptive immunity. Innate immune recognition is mediated by pattern recognition receptors (PRR), which have broad specificities for conserved and invariant features of microorganisms[1]. In contrast, adaptive immune recognition is mediated by antigen receptors that enable the generation of a tremendously diverse antigen-specific repertoire[2]. Although the innate and adaptive immune responses are fundamentally different in their mechanisms of action, synergy between them is essential for an intact, fully effective immune response. The immune system's ability to eliminate foreign microorganisms also implies it has the potential to harm the body by reacting towards harmless antigens or tissues from self. Such misdirected responses can cause allergic reactions and autoimmune diseases.

1.1.1 Innate immunity

The innate immunity is the first line of defence including the physical barrier of the skin and mucosa lining the surfaces of the body preventing entry of pathogens. Recognition of microbial pathogens by innate immune cells are mediated by PRR that detect conserved pathogen–associated molecular patterns (PAMP), such as bacterial and fungal cell-wall components lipopolysaccharide or viral double stranded RNA. Detection of PAMP leads to a rapid induction of inflammatory response and effective clearance of most invading pathogens[3]. Tissue-resident macrophages and dendritic cells (DC) are some of the first innate immune cells to sense invading pathogens and upon phagocytosis they release signalling molecules, which recruit other immune cells to take part in the inflammatory response[4]. In most cases, neutrophils arrive within hours followed by a later influx of monocytes which differentiates into additional tissue macrophages[5].

1.1.2 Dendritic cells – bridging the innate and adaptive immunity

Dendritic cells are the link between innate and adaptive immunity. They are professional antigen presenting cells (APC) surveilling the body's periphery for potential intruders.

Several subtypes of DC exist based on their functions and locations. The two main subpopulations found in human blood are myeloid DC expressing the marker CD11c and plasmacytoid DC that are CD11c negative and produce type I interferons in response to viral infections[6]. Additionally, a heterogeneous population of tissue-resident DC can be found in different anatomical locations including skin epidermal langerhans cells, interstitial DC, splenic marginal DC, germinal-center DC, thymic DC etc.[7]. Myeloid DC can be generated *in vitro* by culturing monocytes isolated from blood with interleukin (IL)-4 and granulocyte macrophage colony-stimulating factor (GM-CSF). These monocyte-derived DC (MDDC) are a good experimental tool for studying DC that otherwise are difficult to obtain because of their low number in blood[8].

1.1.2.1 Antigen uptake

Immature DC are able to sample the environment and capture foreign antigens to initiate the adaptive immune response against pathogens[6]. There are several mechanisms in which immature DC acquire and process antigens including receptor mediated endocytosis, macropinocytosis and phagocytosis[9]. Particulate (bacteria, viruses and latex beads) and soluble antigens are efficiently internalized by phagocytosis and macropinocytosis, respectively. Both of these processes are actin-dependent, require membrane ruffling and result in the formation of large intracellular vacuoles. Phagocytosis is generally receptor-mediated, whereas macropinocytosis is mediated via fluid-phase endocytosis[10]. Receptor-mediated endocytosis involve interactions of antigens with cell surface receptors, such as C-lectin receptors, scavenger receptors or through Fc-receptors (FcR)[6]. C-type-lectins (mannose receptor and DC-SIGN) recognize specific carbohydrate structures found on pathogens as well as self-proteins[11]. Scavenger receptors are glycoproteins that bind to chemically modified low-density lipoproteins (e.g. apoptotic bodies)[12], while FcR bind the Fc part of immunoglobulins (Ig) and take up antigen complexed with antibodies[13].

1.1.2.2 Antigen presentation

After antigen uptake, the formed vesicles fuse with endosomal compartments where the antigens will be processed into peptides that can be loaded onto major histocompatibility complex (MHC) II molecules. The low pH conditions in these endosomes allow cleavage of antigen by proteolytic enzymes into 13-17 amino acid long peptides that are loaded onto MHC II molecules in the late endosomes[14]. The

peptide:MHC II complexes are then transported to the cell surface[15]. Once DC have internalised microbial antigens they maturate and modify their homing properties, migrating towards secondary lymphoid organs to present peptide-derived antigen to naïve $CD4^+$ T-cells. This results in T-cell stimulation and priming and the initiation of the adaptive immune response[6].

1.1.3 Adaptive immunity

When innate immunity is insufficient in clearing pathogens the adaptive immune system is activated. The adaptive response mediated by T and B lymphocytes is slower but highly antigen-specific. A key mechanism of adaptive immunity is the presentation of peptides on MHC molecules of APC. This leads to proliferation and clonal expansion of antigen-specific T-cells. Furthermore, B cells binding antigen to membrane B cell receptors (BCR) and present peptide for $CD4^+$ T-cells will be licensed to switch and produce soluble BCR e.g. Ig that can be secreted upon activation. An important feature of the adaptive response is the production of long-lived memory cells. These cells are kept in a dormant state and can quickly be activated upon encounter with their antigen, to yield a more rapid and robust protective response[2].

1.1.3.1 T lymphocytes

T-cells are equipped with T-cell receptors (TCR) that recognise peptides presented on MHC molecules by APC. TCR on $CD8^+$ cytotoxic T-cells (CTL) recognize MHC class I peptide complexes, whereas MHC class II peptide complexes are responsible for the activation of $CD4^+$ helper T-cells. Once activated, the T-cells migrate to the sites of infection and eliminate invading pathogens or infected host cells.

$CD8^+$ T-cells

$CD8^+$ CTL recognize peptides presented by MHC class I, derived from cytosolic proteins that are loaded on MHC I in the endoplasmatic reticulum. For the CTL to become fully activated the peptide needs to be presented on an APC with sufficient co-stimulatory molecules present. The activated CTL can induce apoptosis of target cells through a variety of mechanisms, including perforin, granzymes, granulysin or membrane-bound molecules such as FasL or TRAIL. During apoptosis the cells destroy themselves and any intracellular viruses or bacteria present, preventing further spreading of the infection[16].

CD4⁺ helper T-cells

CD4⁺ helper T-cells can be categorised into three main lineages as T helper (Th) 1, Th2 or Th17 cells based on their effector cytokine profiles and functional roles. Th1 cells are characterized by their capacity to produce interferon-γ and are involved in immunity against intracellular bacteria and viruses through activation of macrophages and B-cells[17]. In contrast, Th2 cells produce e.g. IL-4, IL-5 and IL-13 where the response is mainly directed towards extracellular pathogens such as fungal and helminth parasites. The Th2 associated cytokines further activate B-cells to produce IgE antibodies (IgE-abs), that are also involved in allergic diseases and asthma[18]. Th17 cells produce IL-17 and are effective in the protection against extracellular bacteria, but they also play a role in the amplification of autoimmune disorders[19]. In addition, Th17 cells also have a role in the protection against systemic fungal infections[20]. In recent years additional T-cell subpopulations have been discovered, such as IL-22 producing Th22 that infiltrate the skin of patients with inflammatory skin disorders[21]. IL-22 has also been shown to be an important cytokine in the gut[22]. IL-9 producing Th9 cells have been shown to be involved in allergic asthma and autoimmunity[23] and interestingly also have potent anti-tumour functions[24]. It has become increasingly clear that cells belonging to these differentiated lineages are not exclusively terminally differentiated cells, but some maintain a certain degree of plasticity and can acquire characteristics of alternative lineages upon antigenic re-stimulation[25].

Regulatory T-cells

Regulatory T-cells (Treg) maintain immune tolerance, prevent autoimmune disorders and allergic diseases as well as control responses to microbes and tumours. The suppressive function can be mediated through contact-dependent mechanisms and/or the production of IL-10 and transforming growth factor-β (TGF-β)[26]. Currently three major types of Treg have been proposed; natural Treg, which develop in the thymus and constitutively express the high affinity receptor for IL-2 (CD25) and the transcription factor forkhead box protein 3[27,28]. According to one type of classification the adaptive Treg Tr1 cells when induced can exert their suppressive activity through IL-10[29], and finally Th3 cells that are induced by oral antigen administration and exert their suppressive activity via the production of TGF-β[30].

1.1.3.2 Humoral responses

The humoral responses are featured by the activation of B lymphocytes and production of antibodies via BCR recognition of specific antigens. BCR signalling does not require the presence of MHC peptide complexes, but can directly recognize microbial surfaces. Upon recognition and activation the B-cells undergo class-switching from an IgM phenotype to more specialized isotypes such as IgG, IgA or IgE with the license from $CD4^+$ helper T-cells. This also results in proliferation of B-cells with pathogen specific BCR that promote their maturation into antibody-producing plasma cells. The increased antibody concentrations enable binding to the pathogens and result in clearance through antibody-mediated cellular cytotoxicity and FcR-mediated endocytosis. In addition, B-cells are equipped with MHC and co-stimulatory machinery that can activate and amplify antigen specific T-cells[31].

1.2 ALLERGIC DISEASE

Globally more than 300 million people are infected with helminth and other parasitic infections or suffer from allergic disorders including asthma, allergic rhinitis, food allergies and eczema. Allergic diseases are one of the most frequent causes of chronic illnesses and affect 25-30% of the population in westernized countries[32,33]. A common feature of this inflammatory condition is the "type 2 immune response" that protects against helminth infections, but also promotes pathologic responses associated with allergic inflammation. This response is characterized by the production of allergen-specific IgE-abs and expansion of allergen-specific T-cell populations, both of which are reactive against harmless environmental substances (allergens)[33]. The clinical symptoms can vary from allergen-induced rhinitis of the upper airways to asthma of the lower airways, eczema of the skin and gastrointestinal (GI) symptoms caused by food intake or systemic anaphylactic reactions[34].

1.2.1 Aetiology

Allergy is a multifaceted disease resulting from a combination of genetic susceptibility and environmental influences. The interplay between genes and environment is pivotal for the development of the disease. Several observations have clearly shown that changes in the infectious environment and microbial exposure of children associated with a westernized lifestyle are critical factors for the prevalence of allergic diseases.

These observations gave rise to the "hygiene hypothesis" introduced by epidemiologist D. Strachan in 1989[35-37]. Studies supporting the hygiene hypothesis have compared the prevalence in rural and urban areas. Growing up in a farming environment results in protection against the development of atopy, wheezing, and asthma[38,39]. Furthermore, early life exposure to antibiotics may be positively associated with the development of allergic diseases and asthma. Previous studies have shown that antibiotics can induce a predominant Th2 response by suppressing the Th1 response via cytokine inhibition or by disturbing the microbiota of the GI tract, jeopardizing the establishment of oral tolerance and Treg responses[40]. In recent years the importance of reduced immune suppression by Treg has been emphasized in explaining the increased prevalence of allergy[41]. Although results from several epidemiological and experimental studies are supporting the hygiene hypothesis, it is not the sole explanation for the rapid increase in allergic diseases during recent decades[37].

1.2.2 IgE-mediated responses

The immunological basis of IgE-mediated allergic disease can be observed in two phases: the sensitization phase (development of memory T-cell and B-cell responses and production of IgE antibodies to allergens) and the effector phase, comprising of the acute-phase response (within 5-15 minutes) that may be followed by a late-phase response several hours after allergen exposure[42,43].

1.2.2.1 Allergic sensitization

During the sensitization process the allergen enters the body through different mucosal surfaces e.g. skin, nose, lung or via the gut. Here the allergen is taken up by APC, internalized and processed. Activated DC mature and migrate to draining lymph nodes or to the local mucosal sites, where they present peptides, in the context of MHC derived from processed allergen to naïve $CD4^+$ T-cells[42,44]. Priming of allergen-specific Th2 cells leads to their differentiation and clonal expansion, which promotes the production of cytokines such as IL-4, IL-5, IL-9 and IL-13. IL-4 and IL-13 regulates B-cell responses, promoting a switch to IgE antibody production and clonal expansion of naïve and IgE memory B-cell populations (Fig. 1A)[43,45]. Additionally IL-13 can affect the smooth muscle cells, epithelial cells and fibroblast of the lungs to promote airway hyperresponsiveness[46]. IL-5 plays a critical role in eosinophil haematopoiesis,

maturation, activation and prolonged survival while IL-9 stimulates the activation of basophils and mast cells[45-47].

1.2.2.2 Acute-phase response

After the sensitization process, re-exposure to the allergen induces an acute-phase response activating blood basophils and tissue mast cells by cross-linking high-affinity IgE receptors (FcεRI) on the surface of these cells. This result in degranulation and secretion of preformed inflammatory mediators e.g. histamine, heparin, and serine proteases (Fig. 1B), causing symptoms such as bronchoconstriction, urticaria, increased mucus production, vomiting, diarrhoea and anaphylaxis[42,48]. Together with newly synthesized cytokines (IL-4, IL-5 and IL-13) and chemokines these inflammatory mediators promote the local recruitment and activation of leukocytes contributing to the development of the late-phase response[43,45,48,49].

1.2.2.3 Late-phase response

The late-phase response has many common features with the acute-phase response but is typically developed 2-6 hours after allergen exposure in some sensitized patients. The inflammatory mediators released during the acute-phase response promote the recruitment of T-lymphocytes, DC, basophils and other leukocytes to the site of allergen encounter through increased blood flow and vascular permeability[48]. These cells, activated by IgE and allergen-dependent FcεRI aggregation at the surface, increase the uptake of allergen and recognition of allergen-derived peptides by specific memory $CD4^+$ T-cells, causing their reactivation and clonal expansion (Fig. 1C). The crosstalk between different cell types participating in the type 2 immune responses through the production of Th2 related cytokines results in a powerful feedback loop driving this late-phase response of the allergic reaction[45,49].

1.2.2.4 Chronic allergic inflammation

Persistent inflammation induced by prolonged or repetitive exposure to specific allergens leads to development of chronic allergic inflammation. This is typically characterized by the presence of many innate and adaptive immune cells and substantial changes in the extracellular matrix and alterations in the number, phenotype and function of structural cells at the affected site[48].

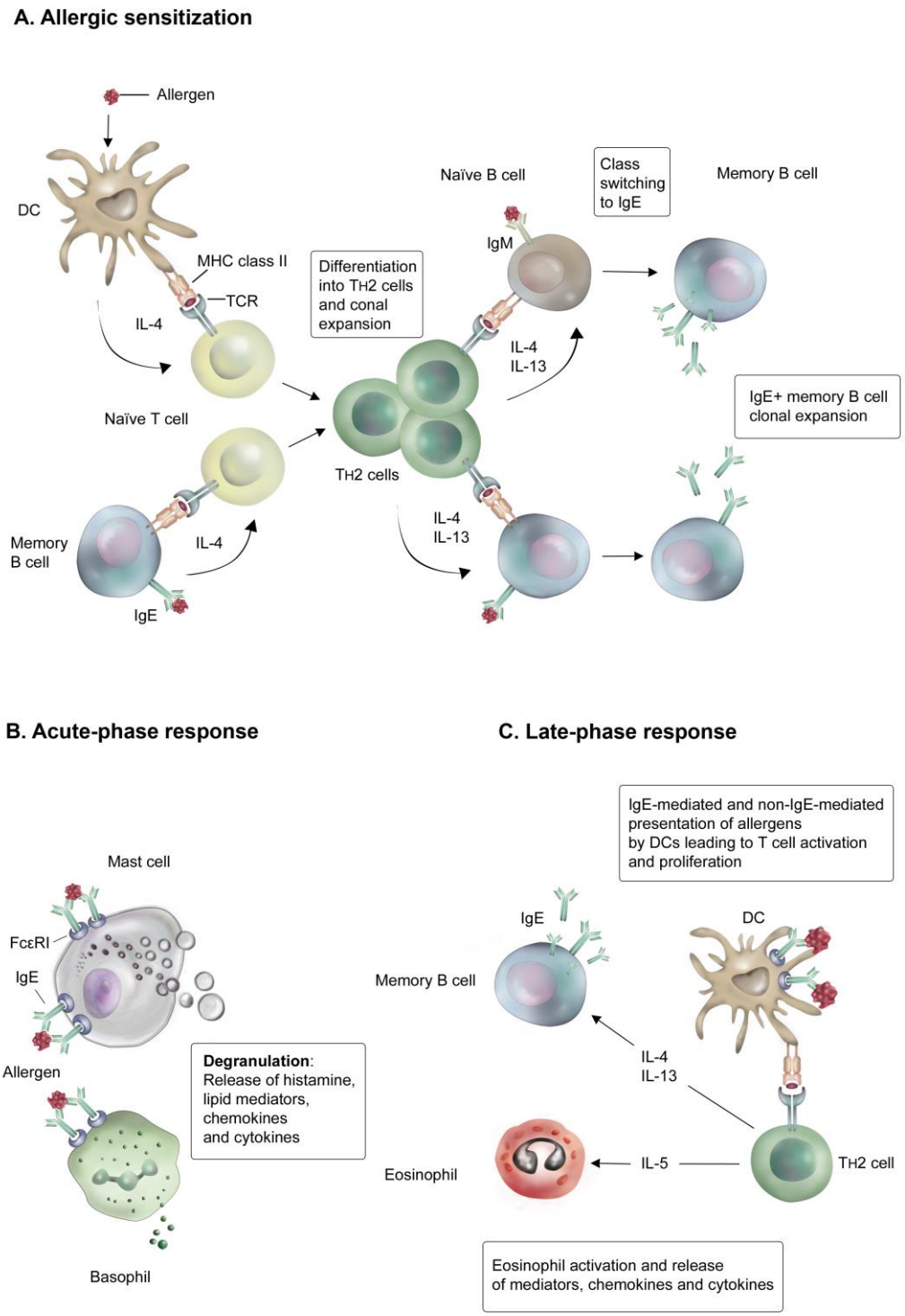

Figure 1. Overview of the allergic immune response. **(A)** Sensitization process where the allergen is taken up by mucosal DC and presented to naïve $CD4^+$ T-cells. Differentiation and clonal expansion of allergen-specific Th2 cells lead to production of IL-4 and IL-13, which can induce class switch of naïve B-cells to IgE-producing B-cells and memory B-cells. **(B)** The acute-phase reaction induced when allergen cross-link surface FcεRI bound IgE on mast cells and basophils, leading to their degranulation of preformed inflammatory mediator. **(C)** The late-phase response is initiated by migration of allergen-specific Th2 cells to allergen exposure site, where they are reactivated and clonally expanded. This leads to production of inflammatory cytokines with IL-5 promoting the recruitment of eosinophils and IL-4 and IL-13 stimulating IgE production by memory B-cells. DC, dendritic cell; IL, interleukin. *(Illustration by Anna Zoltowska Nilsson and modified from Larche, M., Akdis, C.A. & Valenta, R. Nature reviews. Immunology, 2006. 6(10): p. 761-771[43]).*

1.2.3 Food allergy

Food allergy has become a substantial and evolving public health issue in the last decade, emerging as a 'second wave' of the allergy epidemic[50]. It has been reported to affect up to 5% of young children and possibly up to 10% of the population[51], with growing evidence of increase in prevalence[52]. Although there are non-IgE-mediated food allergic responses, the focus of this thesis will be on IgE-mediated reactions, which are the most common type[53]. IgE-mediated reactions to food allergens are characterized by an acute onset of symptoms generally within 2 hours after ingestion or exposure to the eliciting food[54]. They typically involve symptoms affecting the skin (urticaria, angioedema, erythema, and pruritus), GI tract (vomiting and abdominal pain), and respiratory tract (persistent cough, wheeze, respiratory distress, and nasal congestion) or less often anaphylactic reactions (severe life-threatening hypersensitive responses)[54].

During children's first years of life the prevalence of food allergy is the highest and directed at cow's milk, egg, peanut, soy, fish and shellfish[55]. However, most children outgrow their allergy to milk, egg, wheat or soy during their first decade of life while allergies to peanut, tree nuts, fish and shellfish often are persistent[53]. The apparent increase in prevalence of food allergy in infancy may result in an increase in food allergy in adulthood that we now are seeing the consequence of. It has also been proposed that food allergy may be the first step of the allergic march, leading to hay fever and asthma[56,57]. Currently no particulate genes have been associated with development of food allergy, but a family history of allergic disease is a strong risk factor[58]. Other suggested risk factors are mutations in the filaggrin gene, which encodes an epidermal protein that plays a key role in maintaining epithelial barrier function[59], human leukocyte antigens single-nucleotide polymorphisms is associated with increased risk for nut allergy[60] and vitamin D insufficiency has been reported to associate with higher prevalence of food allergy[61]. But there is also increasing evidence that environmental exposure during critical stages of development can alter gene expression and disease predisposition through epigenetic mechanisms[55].

The diagnosis of food allergy can be most problematic as a consequence of inadequate diagnostic procedures. Food allergy can sometimes be over-diagnosed or often not recognized or incorrectly treated[62]. Current approach to manage food allergy substantially relies on educating patients to avoid ingesting the eliciting allergen and how to recognise and promptly treat allergic reactions[52].

1.2.4 Carbohydrate determinants in food allergy

Many allergens that we encounter are glycosylated with one or several carbohydrates. Because carbohydrate moieties can share significant structural homologies beyond that of proteins, they are prone to extensive cross-reactivity, known as cross-reactive carbohydrate determinants (CCDs). These glycans are very important and can modify the structure, function, activity and immunogenicity of proteins and lipids[63]. Not only proteins but also carbohydrates can stimulate the production of IgE-abs and induce type 2 immune responses[64] and about 20% of allergic patients generate a specific anti-glycan IgE response[65,66]. The most clinical relevant form is the pollen-food allergy syndrome where prior sensitization to a cross-reacting inhalant allergen gives rise to an allergic reaction after eating raw fruits, vegetables and tree nuts[67,68]. The allergenic proteins implicated have been classified as pathogenesis-related (PR) proteins, where PR-10 is the most important protein family associated with the pollen-food allergy syndrome. The major birch allergen Bet v 1 is a member of this family of proteins, which can cross-react with the major allergen in apples Mal d 1[69].

Lipid transfer proteins found in peals of e.g. peaches are allergens associated with severe allergic reactions. This severity is attributed to their stability to proteolysis and thermal processing[70,71]. Storage proteins of several nuts and seeds have been identified as important allergens, but cross-reactivity between storage proteins of different foods appears to be limited[72].

The clinical relevance of carbohydrate-specific IgE responses remains controversial, due to conflicting results. A study by Mari *et al.* showed poor biological activity of glycan-specific IgE and strongly argues for their limited clinical relevance[73]. Another study, however, showed that anti-glycan IgE from allergic patients have the capacity to induce IgE-mediated histamine release by human basophils[74]. Despite the conflicting observations, due to different experimental setups and required dosages for histamine release, there are strong *in vitro* indications that glycoproteins can trigger IgE-mediated degranulation of granulocytes and/or mast cells. But still the overall impression is that the effect seen *in vitro* of glycan-specific IgE-abs are of limited clinical relevance[65].

1.2.5 Cross-reacting mammalian antigens

For mammalian meat, the extensive protein homology across mammalian species decreases the likelihood of creating specific IgE-mediated antibody responses[75]. When

clinical relevant reactivity to meat occurs it may be due to cross-reactivity with e.g. serum albumin and actin. In the pork-cat syndrome patients have pre-existing IgE antibodies against cat serum albumin that cross-react with porcine albumin, which can lead to severe or even fatal allergic reactions when pork is consumed[76,77]. In addition, clinical cross-reactivity between beef and cow's milk due to the known beef allergens such as bovine serum albumin, bovine IgG and bovine actin, has previously been reported[78-81].

1.2.6 Galactose-alpha-1,3-galactose (α-Gal) in allergy

In humans and higher primates, the gene encoding α-1,3-galactosyltransferase (α-1,3-GT) is not functional and therefore no α-Gal oligosaccharide is synthesized[82]. Although IgG antibodies to α-Gal are widely expressed in humans[83], presumable in response to continuous exposure to the α-Gal epitope via gut microorganisms[84], IgE-abs to α-Gal on the other hand are uncommon. Adedoyin *et al.* reported in 2006 that IgE-abs to cat IgA were directed to a glycan moiety localized on the α-chain, which might be present in serum of cat-sensitized patients[85]. Additionally, these carbohydrates were shown to be present on IgM antibodies from cat, as well as on IgM from many different mammalian species, but not on human Ig[86]. Further investigations showed that the IgE-binding oligosaccharide on cat IgA was indeed α-Gal[87]. In 2008 an IgE-mediated response to α-Gal was recognized when patients treated with the drug cetuximab, a chimeric mouse–human IgG1 monoclonal antibody against the epidermal growth factor receptor, used for treatment of colorectal cancer and squamous-cell carcinoma of the head and neck, experienced anaphylactic reactions upon the first injection[88]. Further investigations revealed that the reactions were related to pre-existing IgE-abs directed to the α-Gal epitope on the Fab portion of cetuximab[89].

1.2.7 Red meat allergy

Allergy to mammalian meat is rare even though meat is the main protein source in western societies, due to the fact that allergy to mammalian proteins likely approaches the limit of the human immune system to discriminate self from foreign antigens[90]. Initially, the identification of meat derived allergens focused on protein antigens recognized by patients who reported allergic reactions occurring rapidly after exposure[78,91]. Coincident with the rising in food allergy prevalence there has been

reports of anaphylaxis induced by the carbohydrate epitope α-Gal. α-Gal is a newly discovered mammalian carbohydrate epitope commonly expressed on non-primate mammalian proteins[92], with significance in food allergy[93] (Fig.2). Patients with IgE to α-Gal report severe allergic reactions occurring several hours after red meat intake[94,95]. The skin prick test results are disproportionately small or negative compared to what would be expected for a protein allergen[93]. It soon became clear that the novel food allergy syndrome was not only present in USA, Australia and Sweden, but in several other European countries as well[95-99]. The mechanism behind this novel type of food allergy seems to be strikingly different from common IgE-mediated reactions. Commins *et al.* have reported that *in vivo* basophil activation occurs in the same time frame as the late manifestation of symptoms during red meat challenge[100]. Current evidence suggests that the sensitization pathway is associated with tick bites caused by *Ixodes (I.) holocyclus* in Australia[101]*, Amblyomma (A.) americanum* in the US[102] and *I. ricinus* in Europe[95].

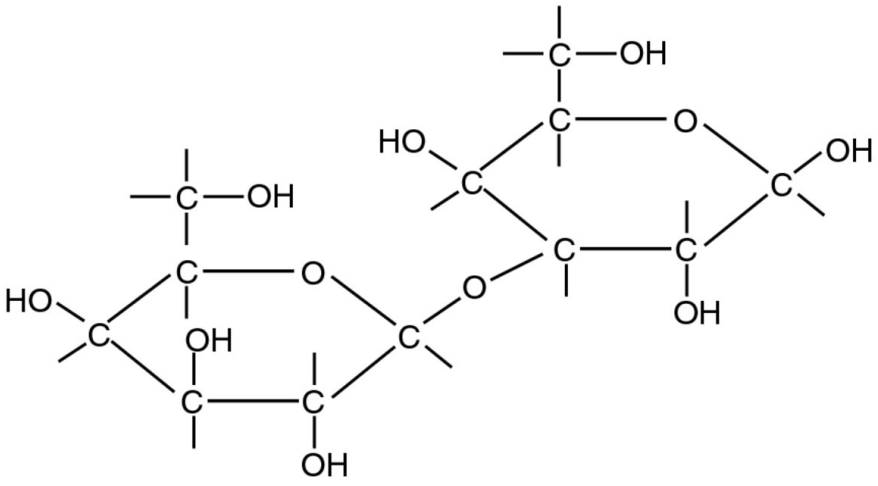

Figure 2. Structure of galactose-α-1,3-galactose (α-Gal). α-Gal is an oligosaccharide expressed on mammalian cells of non-primates. The α-Gal epitope is present in e.g. beef, pork, lamb, and cat dander, but absent in chicken and fish. α-1,3-galactosyltransferase, the enzyme needed for formation of α-Gal is inactivated in humans and higher primates*. (Adapted from Saleh, H. et al. Clin Mol Allergy, 2012. 10(1): p.5[103]).*

1.3 THE GASTROINTESTINAL SYSTEM

The GI tract is one of the largest immunological systems in the body and particularly adapted for environment-host interactions. As the entry for nutrients, the gut is constantly flooded with antigens derived from non-self such as food antigens, invasive and non-invasive pathogens or environmental toxins. Additionally, the intestinal mucosa harbours approximately 1 x 10^{14} microbiota that peacefully colonize the human GI tract in a mutually beneficial state of co-existence[104,105]. Structurally the intestinal mucosa consists of a mucosal layer forming the first line of defence against commensals and pathogenic microorganisms. The second line of defence is the physical barrier consisting of the intestinal epithelial cell (IEC) monolayer. Below the IEC lining reside the intraepithelial lymphocytes and then the lamina propria forming the gut-associated lymphoid tissue (GALT), which can be considered the fourth and final barrier before systemic immunity is required[104,105](Fig. 3). A tight management of the mucosal immune response is critical for host function and survival. On one side maintaining tolerance to harmless compounds and commensal microorganism while on the other efficiently clearing pathogens and their toxins[106].

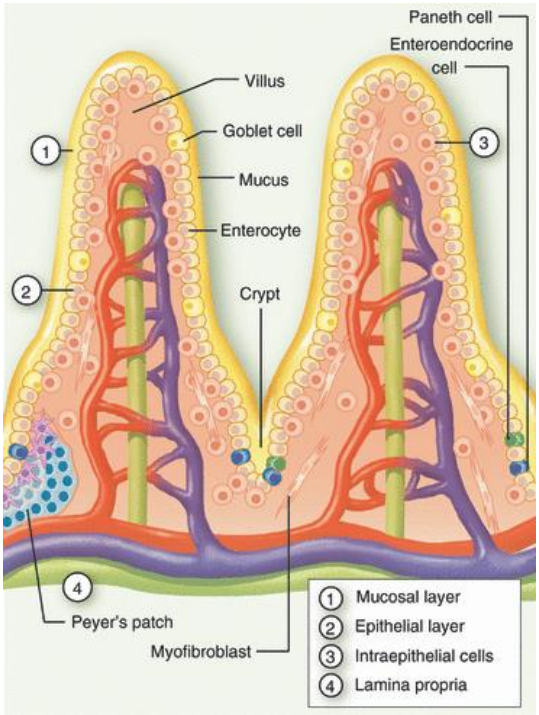

Figure 3. Structural organisation of intestinal mucosa. Representation of the intestinal mucosal barrier, consisting of a mucosal layer **(1)** and gradients of IgA and antimicrobial factors, the epithelial cells **(2)** made up of enterocytes, Paneth cells, goblet cells and enteroendocrine cells, intraepithelial lymphocytes **(3)**, and the lamina propria **(4)**. Additional secondary lymphoid structures, such as and Peyers' patches are present in the lamina propria. *(Adapted from Moens, E. & Veldhoen, M. Immunology, 2012. 135(1): p. 1-8[104]).*

1.3.1 Intestinal epithelial cells

The internal surface of the intestine epithelium is organized in a single layer consisting of many crypts and villi creating a huge surface area, which makes it perfectly suited for digesting and absorption of all necessary dietary nutrients[107]. The diversity of functions that the intestinal epithelium carries out is reflected by additional specialized IEC lineages that arise from pluripotent epithelial stem cells. These cells consist of the absorptive enterocytes, mucus-producing goblet cells, hormone-producing enteroendocrine cells and the microbicidal factor-producing Paneth cells[104,108]. The first line of defence is through secretion of mucins, secretory IgA and antimicrobial peptides that prevent bacterial adhesion and contact with the epithelial surface and underlying immune cells[104,108]. The second line of defence is the physical barrier formed by the intestinal epithelial cells (IECs), interconnected by adherence and tight junctions that keep the integrity of the IEC monolayer[104,107]. At the same time, tight junctions divide the IEC membrane into an apical and basolateral part and help establish and maintain IEC polarity, which is crucial for IEC function[105]. Central to the capacity of IECs to maintain barrier and immuno-regulatory functions is their ability to act as frontline sensors of microbial encounters. IECs express PRR including Toll-like receptors, Nod-like receptors and Rig-I-like receptors to detect common microbial ligands and directing the mucosal immune response[109]. Beside these functions the IECs also regulates transportation of nutrients and antigens, which will be explained in more detail in section 1.3.4.

1.3.2 Gut associated lymphoid tissue

Below the intestinal epithelium lies densely populated resident immune cells forming GALT, which carry out the induction phase of immune response. Specific locations where antigen-specific cellular and humoral immune responses are first generated include Peyer's patches, isolated lymphoid follicles and mesenteric lymph nodes (MLN)[105,110].The MLN also serve as a crossroad between the peripheral and mucosal recirculating pathway[111]. The more diffuse effector site of GALT is represented by T-cells, B-cells, macrophages, DC, neutrophils, other granulocytes and mast cells, residing in the lamina propria as a network of innate and adaptive effector cells[105,110]. The high numbers of DC, macrophages and T-cells within the lamina propria likely reflect the augmented antigen processing and presentation to $CD4^+$ helper T-cells[110]. Additionally, along the surface lining of the epithelium the intraepithelial lymphocytes reside, which are directly involved in host defence as well as barrier maintenance. They are a heterogeneous

population mainly composed of both conventional T-cells expressing TCR-αβ and unconventional T-cells consisting of TCR-γδ[112]. The latter subset is highly enriched in the intestinal epithelium compared to the peripheral blood, but their function is still poorly understood[104]. The intraepithelial lymphocytes share many properties with conventional T-cells but they express antigen receptors with a limited diversity[113] and are in a state of heightened activation, which circumvent their need for priming before becoming fully activated[114]. Additionally, large amounts of IgA antibodies are secreted into the intestine that contribute to shaping the diversity of the bacterial community in the gut and dampens the innate immune response[115].

1.3.3 Antigen modification and allergenicity

It is well-known that the proteolytic activity, stability including resistance to enzymatic digestion and post-translational glycosylation can affect both the immunogenic potential and allergenicity of an antigen[116]. Allergens possess a wide range of intrinsic properties such as protease activity that can lead to enhanced allergenicity. The proteolytic activity of the major mite allergen Der p 1 and kiwifruit cysteine protease (actinidin d 1) can compromise epithelial barrier function by reversibly disrupting tight junctions adjacent IECs[117,118]. Also Cor a 14 from hazelnut has recently been shown to be thermostable and resistant to GI digestion[119]. For Der p 1 it has been shown that after facilitating the access to the immune cells, several studies indicate that the proteolytic activity also enables cleavage of CD23, the low affinity IgE receptor, and indirectly through cleavage of CD25 and CD40, leads to enhanced IgE production[120,121]. Generally, complete food allergens, those able to induce an IgE production and elicit allergic reaction[122], such as cow's milk (caseins and serum albumins), egg white (ovomucoid), peanut (Ara h 2) and soybean (Gly m 4) are very stable proteins able to resist thermal effects and gastrointestinal digestion[123]. In contrast, incomplete food allergens, e.g. Bet v 1 related food allergens such as the apple (Mal d 1), hazelnut (Cor a 1.04) and celery (Api g 1)[124] or proteins from profilin family cross-reacting with pollen related food allergens such as pear (Pyr c 4), cherry (Pru av 4) and celery (Api g 4)[125], are food allergens that lack the capacity to induce IgE sensitization via the GI tract due to less stability under thermal and digestive processes[123]. The processing of food allergens is an additional dimension that must be considered, because it can significantly alter structural characteristics of allergens and thereby increase or attenuate their antigenic potential[126]. Processing may destroy existing allergen epitopes or generate new ones (neoepitopes) as a result of changes in

the protein conformation[127]. Furthermore, Maillard reaction products may affect the allergenicity of food proteins[128]. In general, allergenicity of fruit allergens is not enhanced by glycosylation, which is shown for Pru av 1[129]. In contrast, it has been shown that Maillard modifications can cross-link the peanut allergens Ara h 1 and Ara h 2 to form aggregates that are more resistant to gastric digestion and able to bind IgE more effectively than unmodified allergens[130]. Also tropomyosin in shellfish has been found to increase the IgE binding capacity after Maillard modifications[131].

1.3.4 Transport of antigens

The intestinal epithelial monolayer forms a highly selective barrier where transportation of nutrients, dietary antigens and microbes are tightly controlled by membrane pumps, ion channels and tight junctions, adapting permeability to physiological needs. This is accomplished through two distinct mechanisms; paracellular diffusion through tight junctions between adjacent IECs and transcellular transport involving non-receptor mediated (fluid phase) or receptor mediated transcytosis[110,132]. Paracellular permeability is an indiscriminate mechanism of antigen uptake utilising the gap between tight junctions (Fig. 4) and deliver antigens to the basolateral surface of the IECs directly to APC. More important are the transcellular mechanisms where the larger antigenic molecules are transported either degraded or intact across the IECs (Fig. 4). The fluid phase uptake is predominantly of degradative antigens where only a minor fraction crosses the cell intact. Receptor-mediated mechanisms on the other hand serve to transport intact molecules. During transcytosis macromolecules are partly degraded in acidic and lysosomal compartments and released in peptide associated MHC II antigen presentation at the basolateral side of IECs[132,133].

1.3.5 Antigen sampling and uptake

Antigen uptake is the initial step required for the induction of the immune response. In the intestinal mucosa specialised microfold (M) cells, DC and enterocytes can sample and take up antigens[110]. Interspersed between the epithelial monolayer are the specialized M cells that contribute to selective transfer of antigens via pinocytosis to Peyer's patches[134]. M cells are believed to pass on intact antigens to professional APC but it remains unknown whether they actively participate in the processing and presentation of antigens[110]. As a major population of IECs, the absorptive intestinal enterocytes play an

important role in antigen sampling. Enterocytes are able to sense pathogens through Toll-like receptors or Nod-like receptors[135] and present antigens on MHC I and MHC II[136]. Enterocytes take up soluble antigens from the intestinal lumen via vesicular transport (fluid phase or receptor mediated transcytosis), which are then released basolaterally[133]. Beside M cells and enterocytes, DC are able to extend dendrites through the epithelial tight junctions into the lumen and sample lumenal content without disturbing the epithelial barrier function or permeability[137] (Fig. 4). Once the antigen has been transported to the basolateral side it is taken up by DC, processed and presented to surrounding T-cells present in the lamina propria. Alternatively the DC migrate to MLN or spleen and present antigens to T-cells for the induction of systemic responses[138].

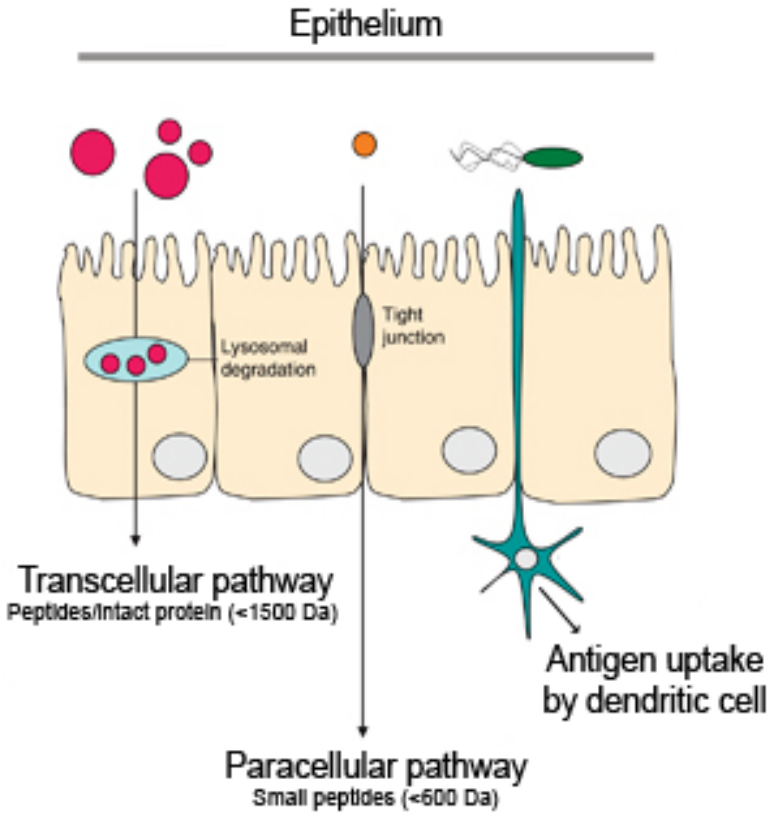

Figure 4. Differential pathways of antigen sampling in the intestinal epithelium. The intestinal epithelial cells create a physical barrier with filtering properties against entry of harmful pathogenic products and harmful molecules (toxins and allergens). Via paracellular diffusion small peptides and molecules are able to enter through the tight junctions between intestinal epithelial cells. Another mechanism is transcellular transport of partially degraded and in lesser extent intact protein across the erythrocytes with degradation along the phago-lysosymol pathway. Direct intestinal sampling of bacterial antigens can also be performed by dendritic cells extending their dendrites across the epithelium. *(Modified from Perrier, C. & Corthesy, B. Clinical and experimental allergy, 2011. 41(1):p 20-28[139]).*

1.3.6 Oral tolerance *vs.* food allergy

Oral tolerance is undoubtedly one of the first and most significant challenges that the immune system encounters. During immune homeostasis in the GI tract innate immune signals, provided by innocuous or commensal bacteria, play important roles in stabilizing this non-inflammatory microenvironment and suppressive functions of Treg. Thus oral tolerance consists of two phases: the maintenance of homeostasis and the suppression of immune responses mediated by antigen-specific Treg[140]. Intestinal DC play a central role in both of these processes, producing immunomodulatory cytokines such as IL-10 and TGF-β that suppress local inflammation in an antigen nonspecific manner. DC also promote differentiation of antigen-specific Treg and initiate antigen-specific secretory IgA antibody production that both are essential for systemic immune surveillance and tolerance[50,141].

However, in some genetically predisposed individuals a breakdown in the development or loss of oral tolerance is believed to lead to food allergy. Currently it is still unclear where and when this occurs, but increased intestinal permeability, decreased oral tolerance and defects in regulation of T-cell activity are some suggested causes[53]. Recent studies have indicated that CD23 (FcεRII) on epithelial cells play a central role in transporting allergen-IgE complexes across the epithelial barrier, bypassing epithelial lysosomal degradation, resulting in the entry of a large amount of intact allergens to the mucosa[142]. IL-4 not only increases the production of IgE from B-cells, but also upregulate the expression of CD23 on IECs[143]. The presence of IgE/CD23 opens a gate for intact allergens to transcytose across the epithelial cells capable of binding and activating mast cell degranulation and induce an allergic inflammatory cascade, which in turn influence ion secretion and modify paracellular permeability[139].

1.4 DIAGNOSTIC METHODS IN FOOD ALLERGY

For managing of food allergies, proper diagnosis is necessary. The conventional examination for IgE-mediated food allergy includes a detailed clinical history, physical examination, skin prick test (SPT) and measurement of serum-specific IgE (sIgE) antibodies[53,144]. Although larger SPT wheal sizes and higher levels of allergen-specific IgE-abs are associated with increased likelihood of allergic reactions, they still lack precision and do not predict severity of allergic reactions[53]. In the last decades sIgE tests

involving purified or recombinant allergens from the most common allergen sources, designated component-resolved diagnostic (CRD), have been established. CRD testing, which can distinguish between genuine sensitization to the allergen source or cross-reactivity and allows for risk assessments, is a valuable tool in the diagnosis of food allergy[145]. Furthermore, the next generation of IgE testing, using a chip based technology covering allergen components from more than 50 allergen sources, is helpful in the diagnosis of food allergy. In addition, the basophil histamine release assay can provide an additional tool in the assessment of food allergy to confirm allergen activation of basophils[54]. The double-blind placebo-controlled food challenge (DBPCFC) is the most specific test for diagnosing food allergy; however, it is not frequently performed due to inherent risk, inconvenience and cost. To access the clinical symptoms related to ingestion of implicated food oral food challenges can be part of the investigation[54,144].

1.4.1 Skin prick test

Skin prick testing is routinely performed in the diagnosis of food allergy since it is minimally invasive, quick, inexpensive and sensitive. SPT has a positive and a negative predictive value of more than 90%[51], although the specificity is lower. The test utilizes the degree of cutaneous reactivity as a marker for sensitivity. When an allergen is inserted into the skin, IgE-abs bound on mast cell receptors are cross-linked leading to release of histamine and other inflammatory mediators. This produces a wheal and flare response where a mean wheal diameter ≥3 mm is defined as a positive result[146] The accuracy of SPT depends on the commercial food extract used. Different batches of commercial allergen extracts may contain different amount of proteins affecting the allergenicity and potency of the extracts[146,147]. In some instances, a prick-prick testing with fresh fruits and vegetables may be more accurate than using commercial extracts given the availability of allergenic proteins[148].

1.4.2 IgE-abs assay

The *in vitro* serum immunoassays measuring circulating allergen-specific IgE-abs are valuable methods in the evaluation of IgE-mediated food allergy. Higher concentration of food specific IgE levels usually correlate with increased risk of clinical symptoms, but the predictive value of specific IgE levels varies depending on age and populations[149-151]. Currently different systems for measuring sIgE antibodies are available such as

ImmunoCAP (Phadia), Immulite (Siemens) and HYTEC-288 (HycorBiomedical)[152]. The assays involve a surface-fixed allergen that is incubated with the serum from suspected allergic individuals. Any occurring sIgE antibodies will bind to the fixed allergen and remain after washing. Bound IgE is visualized by the binding of labelled anti-IgE[152]. The amount of bound IgE-abs is reported in arbitrary mass units (kilo international units of allergen-specific antibodies per unit volume of sample [kU_A/L]). The analytical sensitivity is set to be 0.1 kU_A/L in all three systems, but the result from one system is not equivalent in another system[153].

1.4.3 Component resolved diagnostic

In recent years several studies on food allergens have demonstrated that CRD can improve the specificity of allergy testing. CRD measures specific IgE-abs to purified, native or recombinant allergenic proteins instead of crude extract consisting of a mixture of components[154]. Several studies have reported the usefulness of CRD testing for different food allergies and in the cases of peanut, cow's milk, egg, and shrimp component testing, CRD offered increased specificity but decreased sensitivity compared to SPT and serum sIgE testing[155,156]. CRD for specific food allergen sources is usually added to currently available allergy tests to avoid performing oral food challenges[157].

1.4.4 Basophil reactivity assay

Basophil activation test is an *in vitro* assessment of allergic response that closely resembles food challenges. This test only requires a small amount of whole blood and allows for measurement of a functional response beyond just the presence of antibody. Basophil activation can be assessed using two predominant approaches: investigating secretion of histamine release from basophils and detecting expression of cellular markers after stimulation with flow cytometry[158,159]. CD203c is constitutively expressed at low levels on the surface membrane of resting basophils and is quickly up-regulated upon cell activation[160]. In a resting basophil CD63 is present primarily inside the cell granules, but upon activation, the granules fuse with the cell membrane and CD63 will be exposed on the cell surface, thus expression of CD63 is closely associated with degranulation[161]. The two parameters often defining the response are the concentration of allergen needed for 50% of the maximum response (basophil sensitivity) and the maximum level of secretion (basophil reactivity). Any use of basophil activation test also

needs to consider the issue of 'non-responders', which are individuals whose basophils are not responsive to FceRI cross-linking[162]. The basophil activation assay creates new opportunities in the diagnostic management of IgE-mediated food allergy, as the technique closely mirrors the *in vivo* pathway resulting in symptoms[163].

1.4.5 Oral food challenge

The golden standard of diagnosing an IgE-mediated food allergy is the DBPCFC. In this test, the food is masked and the tested food or placebo is randomly administered. However, since the DBPCFC testing is time- and labour intensive, open challenges or single-blind challenges are frequently performed for clinical purposes[164-166]. In an open challenge, the individual is aware that he/she is consuming the potentially problematic food, which can bias the test from symptoms of anxiety and emotional distress[164]. In these cases a single-blind challenge can be useful when the individual is suspected to have a psychological response[157]. For a diagnosis of IgE-mediated food allergy graded dosing is recommended because it minimizes the risks of severe allergic reactions and identifies the lowest provoking dose (dose threshold)[164,166]. Due to risks for adverse allergic reactions and anaphylaxis, food challenges should always be conducted under the supervision of trained medical staff in a health care facility equipped to treat anaphylaxis[164,166]. Several factors should be considered before performing an open food challenge such as the patient's medical history, age, past adverse food reactions, SPT and food allergen–specific sIgE results[164]. Open food challenges can be used to determine clinical reactivity when the history is uncertain and results of specific IgE testing is negative or when specific IgE test results are positive but below established positive predictive cut-offs for the suspected food[167]. In some individuals food challenge is not the best option, e.g. due to high probability to severe anaphylaxis, confounding medical conditions and medications that might interfere with allergic reactions[164].

1.4.6 Clinical diagnosis of red meat allergy

The clinical symptoms of red meat allergy are similar to other food allergies but have several unique characteristics, which have made the diagnosing process difficult. This is due to several factors, including the often late in life onset of disease, long interval of reaction after eating, and negative SPT, therefore many or most of these patients have been dismissed by physicians and allergologists. However, a few years back children

started too also be diagnosed with red meat allergy, presented primarily with urticaria rather than acute episodes of anaphylaxis seen in adults[168]. This thesis will focus on the adult patient group. In general SPT or prick-prick test with commercial meat extracts produce negative or weak positive reactions (2-5 mm) in these patients, but intradermal tests have been used with better outcome of results[93,169]. Skin testing with freshly prepared meat extracts has been shown to give more reliable reactions, but serum sIgE titres to α-Gal, beef, lamb, and pork are most consistently elevated in this group of patients[169]. Variation has also been noted in the progression of symptoms, with some patients experiencing a much more rapid course once symptoms have appeared. It has been shown that pork kidney is able to elicit faster allergic reactions due to the higher levels of accessible α-Gal epitopes compared to muscle meat[97,98]. Interestingly, the variability of the response, including time of symptom onset, speed of symptom progression, and severity, does not appear to be related to the titre of soluble IgE to α-Gal[170]. In these patients there is often a positive history of prior tick bites. Bites from *A. americanum* (adult, larval or seed ticks) can be severely pruritic, a symptom that can last for weeks, which is accompanied with high levels of IgE-abs to α-Gal in serum[102].

In this patient group it is not feasible to perform either graded food challenge or placebo-controlled food challenges to investigate the delayed occurrence of clinical symptoms (2-8 hours). Therefore, the challenge protocol involves a single dose of meat on patients reporting only episodes of urticaria, to reduce the need for epinephrine treatment of anaphylaxis[98,100].

For diagnosing suspected red meat allergic patients a detailed history, including type of ingested foods, time to onset of symptoms, the geographic area of residence as well as history of tick bites, can help in achieving the correct diagnosis[103].

2 THE PRESENT STUDY

2.1 OBJECTIVE

The overall aim of this thesis was to generate new insights into red meat allergy, a novel type of severe food allergy. The specific objectives were:

Paper I: To investigate whether Swedish patients with suspected red meat allergy have IgE antibodies to α-Gal and whether they also have an IgE antibody response to the European tick *Ixodes ricinus* that can be linked to the α-Gal epitope.

Paper II: To characterize Swedish patients with suspected red meat allergy and explore the significance of sensitization to the European tick *Ixodes ricinus* and the relevance of the B-antigen for the disease.

Paper III: To characterize the proteomic profile of different beef preparations and to investigate their α-Gal reactivity and potential allergenicity.

Paper IV: To investigate whether IgE from red meat allergic patients recognizes other mammalian glycans or glycans derived from plants and insects related to allergic disease. Moreover, we scrutinized whether IgE responses to α-Gal target the whole glycoprotein or the glycan structure only.

Paper V: To demonstrate how human monocyte derived dendritic cells take up the α-Gal model antigen bovine thyroglobulin (bTG), process it and subsequently present α-Gal on membrane bound MHC II molecules.

2.2 METHODOLOGY

2.2.1 Study population

Study subjects from three different populations were included in this thesis. Papers **I-IV** are based on suspected red meat allergy patients from the Allergy Unit at Södersjukhuset, Stockholm, Sweden (study population 1), where all had IgE antibodies against α-Gal. These patients were examined by a physician experienced in allergic diseases and responded to a detailed questionnaire regarding symptoms of meat intake and exposure to ticks. Blood samples were drawn for analysis of IgE antibodies to a panel of different food and inhalant allergens and were also blood typed according to routine methods. In paper **II,** two additional populations were included: healthy blood donors from Blood Center, Karolinska University Hospital (study population 2) and patients with confirmed Borrelia infection from the Department of Clinical Microbiology, Karolinska University Hospital (study population 3). Subjects in these 2 groups were tested for IgE antibodies to α-Gal. Those with α-Gal levels of ≥0.1 kU_A/L were blood typed and analysed for IgE against a panel of food and inhalant allergens.

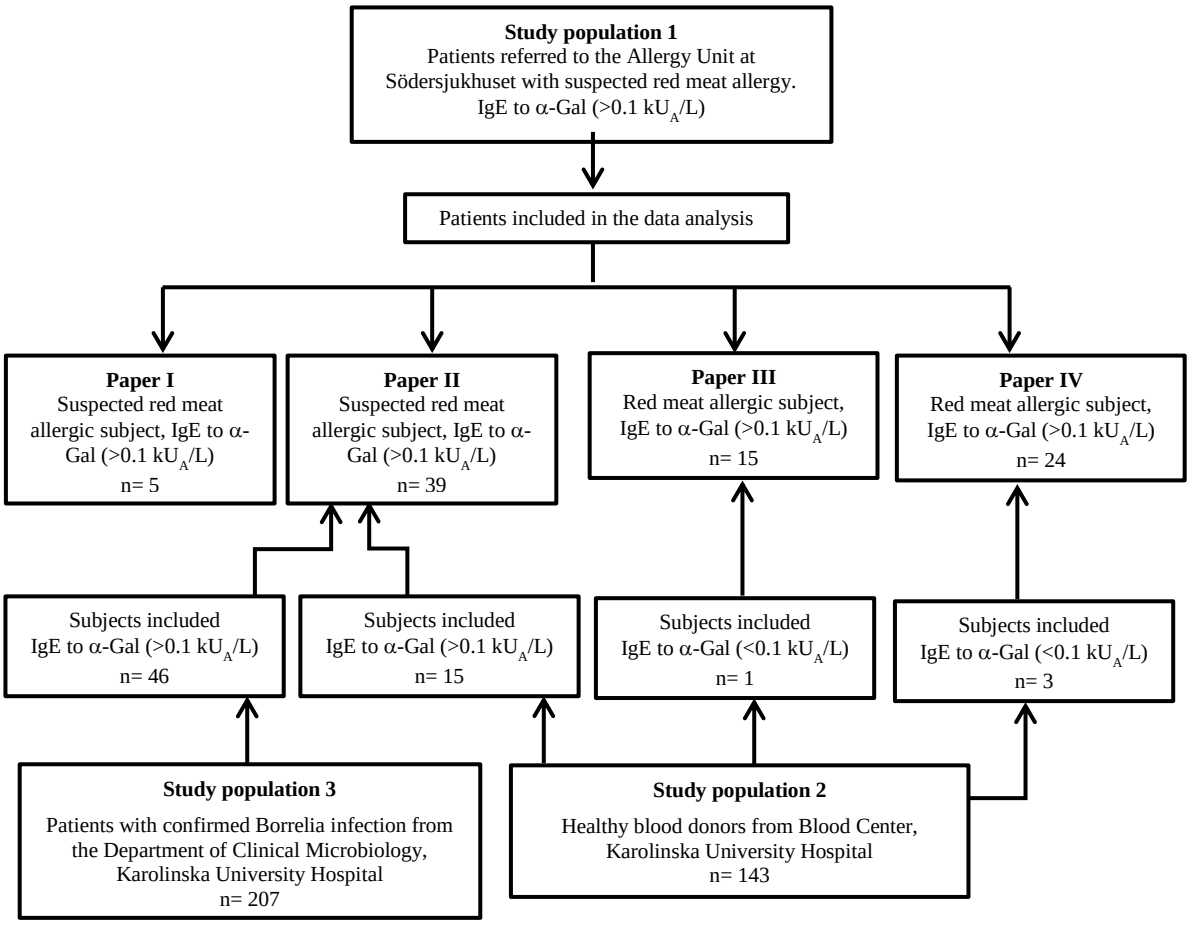

Figure 5. Study population in relation to the papers I-IV.

Paper **III** and **IV** were based on selected red meat allergic subjects (population 1) and healthy blood donors (population 2), who served as controls. The numbers of patients in the different studies are presented in Figure 5. In paper **V** buffy coats from healthy donors, Department of Blood Center, Karolinska University Hospital were used.

2.2.2 Study methods

The methods used for papers **I-V** are described in more detail in the Materials and Methods section for each paper. Table I alphabetically lists the methods applied in the thesis, together with a short description of each method.

Table I: Summarized descriptions of methods used in papers I-V

Method	**Description**
Circular dichroism spectrocospy **[IV]**	Spectrophotometric determination of secondary structure motifs of proteins.
Competition ELISA	Preincubation of sera with dilutions of *I. ricinus* extract followed by detection of IgE binding to a-Gal.
Confocal laser scanning microscopy (CLSM) **[V]**	Microscopy technique used to scan the z-levels of MDDC.
Deglycosylation assay **[III, IV]**	Enzymatic removal of glycans using PNGase F.
Enzyme linked Immunosorbent assay (ELISA) **[I]**	Allergen-specific antibodies in serum were detected by alkaline phosphatase conjugated antibodies.
Fluorescence labelling of proteins **[V]**	Labelling of protein with AF488 and FITC. The conjugated protein was purified by using desalting column to remove unbound fluorophore and protein content was measured with BCA assay.
Flow cytometry **[V]**	Surface and intracellular expressed markers were detected by flourochrome conjugated antibodies.
Generation of monocyte derived dendritic cells (MDDC) **[V]**	Monocytes were isolated from PBMCs and cultured for 6 days in the presence of IL-4 and GM-CSF to obtain immature MDDC.
Immunoblot **[I, III]**	Proteins were separated by SDS-PAGE and analysed

	by immunoblotting under reducing conditions. Detection was performed using anti-α-Gal antibody or serum from red meat allergic patients.
ImmunoCAP **[I-IV]**	Total IgE and allergen-specific IgE antibody measurement in serum where a value of 2.0 kU/L and 0.1 kU_A/L were considered positive, respectively.
ImmunoCAP inhibition **[I-IV]**	Serial dilutions of antigens mixed with sera from allergic patients before allergen-specific IgE measurement. The soluble antigen competes with a similar solid phase-bound antigen for binding to IgE.
Immunofluorescence staining **[V]**	MDDC were cytospun onto glass slides and stained with anti-CD11c for visualization with CLSM.
Immunohistochemistry **[I]**	Detection of α-Gal in tissue sections from ticks probed with α-Gal antibodies and patient serum samples.
Magnetic activated cell sorting (MACS) **[V]**	Separation of $CD14^+$ monocyte using MACS anti-CD14 conjugated microbeads.
Mass spectrometry **[III]**	Protein spots of interest from 2D gel were manually excised and subjected to in-gel digestion with trypsin from porcine pancreas. Obtained peptides were analysed by nano-liquid chromatography tandem mass spectrometry (LC-MS/MS).
PBMC isolation **[V]**	PBMCs were isolated from whole blood by density gradient centrifugation using Ficoll.
Protein extraction (beef) **[III]**	Protein extract was prepared by homogenizing one piece of meat with 30 ml of PBS (pH 7.4) with a POLYTRON homogenizer. Supernatant was collected and filtered through 0.8-lm filter, and protein content was measured with BCA assay.
Protein extraction (tick) **[I, III]**	Whole body tick extract was obtained by crushing frozen ticks in PBS (pH 7.4) using a tissue homogenizer followed by collection of supernatant. Protein content was measured with BCA assay.
SDS-PAGE **[I, III]**	Proteins separation by SDS-PAGE and stained with Coomassie Brilliant Blue.

2D-PAGE **[III]**	Protein from different beef extracts were separated on two different properties, the protein mass and isoelectric point.
Sequence analysis **[III]**	A homology search of proteins identified from the amino acid sequences was performed with BLASTp on the NCBI nonredundant protein sequence database. For prediction of N-glycosylation sites, identified proteins were analysed using the online software Structure Feature Analysis Tool (http:hive.biochemistry.gwu.edu).

2.2.3 Statistical analysis

The statistical analyses were performed using GraphPad Prism software (version 6; GraphPad Software, La Jolla, CA, US). In study **II,** quantitative measurements of IgE antibodies were compared by using Spearman's rank correlation and Mann-Whitney U tests. Categorical measures of Lyme disease status and IgE positivity to α-Gal were compared with the χ^2 test for trend. The frequency of B-positive subjects compared to a theoretical outcome was performed by using the binominal test. A P value lower than 0.05 was considered significant. In study **V,** data are presented as median ±range and the difference in uptake of bTG and human thyroglobulin (hTG) at different time points was analysed by Wilcoxon matched pairs signed rank test where $p < 0.05$ was considered significant.

2.2.4 Ethical statement

The research conducted in this thesis was approved by the Regional Ethical Review Board at Karolinska Institutet in Stockholm, Sweden (Identification number 2011/1604-31/2 and 2011/2085-31/4).

2.3 RESULTS AND DISCUSSION

2.3.1 Identification of galactose-α-1,3-galactose in the gastrointestinal tract of the tick *Ixodes ricinus* (Paper I)

Severe allergy to mammalian meat had been highlighted in USA, Australia and several European countries, but not yet in Sweden. In the greater Stockholm area, which is in the southern part of Sweden, the common European tick *I. ricinus* is frequent, and we have previously identified IgE-mediated responses to α-Gal in this region[87]. Current evidence suggests that the allergic response of patients with severe red meat allergy is induced by tick bites. In this paper we wanted to elucidate whether Swedish patients with suspected red meat allergy have IgE antibodies to α-Gal and whether they also have IgE to the tick *I. ricinus* that could be linked to the α-Gal epitope.

Five Swedish patients reporting anaphylaxis or urticaria following consumption of red meat and having a history of tick bites were IgE positive to α-Gal and beef. They were also IgE negative to chicken due to the absence of α-Gal, while the atopic control was IgE negative to α-Gal, beef and chicken. Investigation of IgE to the tick *I. ricinus* by western blotting showed patterns of strong IgE binding to several tick proteins in the patients but not in the control. Inhibition with α-Gal resulted in a significant reduction of IgE binding in two patients, indicating the presence of α-Gal on several tick proteins. The results also demonstrated that the remaining patients had a strong α-Gal-independent IgE response to tick proteins.

Next competition ELISA was performed with tick extract and a dose-dependent inhibition of IgE to α-Gal was obtained in all red meat allergic patients, but not in the atopic control. A maximum inhibition of 68–97% was obtained using 600 μg/ml of tick extract. Two patients had extremely high IgE levels to α-Gal (≥100 kU_A/L), which might explain the lower degree of inhibition observed in these patients. Additionally, immunohistochemistry staining of cryostat-cut sections was used to further explore serum responses to *I. ricinus*. Both a monoclonal and a polyclonal antibody specific for the α-Gal epitope stained the GI tract of the tick. The same pattern was seen when staining with patient sera IgE positive to α-Gal (Fig. 6), providing the first evidence that α-Gal is present within ticks. This potentially explains the relationship between tick exposure and sensitization to α-Gal, with development of red meat allergy as a secondary phenomenon.

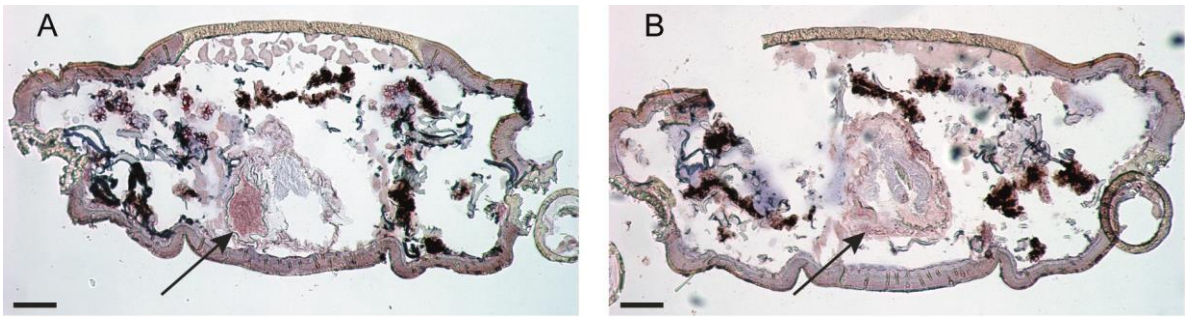

Figure 6. Cryostat-cut lateral sections of Ixodes ricinus nymph probed with (A) patient serum IgE positive to α-Gal, **(B)** a polyclonal mouse anti-α-Gal antibody. *(Figure from paper I).*

2.3.2 Red meat allergy in Sweden: Association with tick sensitization and B-negative blood groups (Paper II)

In this study we characterized 39 Swedish patients with a history of delayed anaphylaxis, angioedema, urticaria or GI symptoms following consumption of red meat. Except for 2 patients they all described urticaria as being the dominant symptom, but as many as 45% had experienced anaphylactic reactions. All had reported IgE responses to α-Gal (median 20 kU_A/L; range 1.3-130 kU_A/L; Table II) and α-Gal-containing allergen sources, such as beef and pork, and the majority also to moose, cow's milk, dog and cat. Strong correlations between IgE antibodies to α-Gal and total IgE (r = 0.70, $P < .001$), beef (r = 0.76, $P < .001$), pork (r = 0.73, $P < .001$) and cow's milk (r = 0.75, $P < .001$) were observed.

In the questionnaire and medical examination, all red meat allergic patients reported tick bites (range of 1 – ≥100 bites, Table II). Tick specific IgE was assayed using whole-body extract of *I. ricinus* and all but two patients were IgE positive. Although IgE titres to *I. ricinus* were lower compared to α-Gal, we observed a significant correlation (r = 0.58, $P <$ .001; Fig. 7A), supporting the association between tick bites and sensitization to α-Gal. This is in line with previous reports showing an association between red meat allergy and tick bites from *I. holocyclus* in Australia[101], *A. americanum* in United States[102] and *I. ricinus* species in northern Spain[95]. To elucidate whether the Swedish red meat allergic patients recognized allergens in the American tick *A. americanum,* the patients were also IgE tested and more than 35% were sensitized to this tick species. However, the IgE levels were much lower than those to *I. ricinus*, suggesting that *I. ricinus* is the species to which they were primarily sensitized. Despite different antibody levels the IgE levels against the two tick species correlated significantly (r = 0.65, P <.001; Fig. 7B).

Table II. Characteristics of red meat allergic patients

Patient no.	Age(y) /sex	Reaction	Time to reaction‡	IgE*					Tick bites§	Blood group
				Total	α-Gal	Beef	*I. ricinus*	*A. americanum*		
1	67/M	AE, GI, U	6	260	54	5.0	30	5.2	+++	A
2	40/F	ANA, GI, U	3-4	120	16	3.5	0.46	<0.10	++	A
3	63/F	GI, U	2	20	13	5.5	0.60	<0.10	++	A
4	69/M	AE, GI, U	4	280	23	11	40	6.0	+++	A
5	39/M	AE, GI, U	4-5	33	6.4	4.4	0.72	<0.10	++	A
6	74/M	U	3-4	210	30	10	1.9	<0.10	+	A
7	51/M	ANA, U	4-5	25	2.2	1.4	0.17	<0.10	+++	A
8	48/F	AE, ANA, GI, U	0.25-1	150	16	7.9	4.7	<0.10	+++	O
9	43/M	AE, ANA, GI, U	8	320	88	11	3.1	0.15	+++	O
10	69/M	GI, U	6	87	18	4.9	1.5	<0.10	+	A
11	46/M	GI	2	90	4.4	2.2	0.35	<0.10	+	O
12	70/M	AE, U	6	420	130	16	37	1.5	+++	O
13	48/F	GI, U	2-6	870	6.2	11	2.8	0.87	++	A
14	44/M	AE, ANA, GI, U	4-7	1800	24	4.5	10	2.6	++	O
15	33/F	AE, ANA, GI, U	6-7	270	46	7.2	0.34	<0.10	+	O
16	65/F	AE, ANA, U	6	550	31	22	13	11	+	O
17	69/M	AE, U	10-12	130	25	20	8.1	0.13	+	A
18	63/F	AE, ANA, GI, U	3-4	140	20	3.0	0.17	<0.10	+	A
19	38/F	GI, U	6	140	19	3.2	2.3	0.23	++	A
20	54/M	ANA, GI, U	2	20	3.6	1.1	0.27	<0.10	+	O
21	74/F	AE, GI, U	8	30	1.6	0.53	0.3	<0.10	++	A
22	36/M	GI, U	6-7	80	22	3.3	2.1	0.11	+++	O
23	60/M	AE, ANA, U	6-7	2200	76	6.2	54	5.9	+++	O
24	54/F	ANA, U	5-6	130	19	3.8	2.9	<0.10	+++	A
25	37/F	AE, ANA, GI, U	6-7	110	12	2.8	1.2	<0.10	+	O
26	57/F	GI, U	2-8	50	29	6.3	0.61	<0.10	+	A
27	69/M	AE, ANA, GI, U	6	48	6.6	0.49	<0.10	<0.10	+++	O
28	49/M	ANA, U	7	48	10	1.8	0.36	<0.10	++	A
29	57/M	U	3	33	2.4	0.25	0.11	<0.10	++	O
30	52/F	AE, ANA, U	1-2	87	12	5.4	2.7	<0.10	++	O
31	73/M	GI	ND	340	37	6.0	1.4	1.3	+	O
32	45/F	AE, U	5	44	5.0	0.3	<0.10	<0.10	++	AB
33	36/F	AE, GI, U	4-5	120	37	9.9	6.9	0.32	+++	O
34	44/F	U	2-7	240	110	9.7	0.26	<0.10	++	O
35	40/M	GI, U	6	270	84	28	3.3	<0.10	++	O
36	41/F	GI, U	3	360	61	12	1.5	<0.10	+	A
37	18/F	AE, ANA, GI, U	4-8	180	1.3	0.93	0.56	0.64	+	O
38	55/M	GI, U	4-12	150	22	1.9	4.4	<0.10	+++	B
39	70/M	AE, ANA, GI, U	0.25-3	150	23	1.9	3.6	<0.10	++	O

M, male; F, female; AE, angioedema; ANA, anaphylaxis; GI, gastrointestinal symptoms; U, urticaria; ND, not determined

* ImmunoCAP IgE results: Total IgE levels are expressed in kU/L and allergen-specific IgE levels in kU_A/L

‡ Time to reaction expressed in hours

§ Estimated total number of tick bites; + 1-10, ++ 10-50, +++ 50-100

(Table from paper II)

To investigate how common IgE antibodies against α-Gal are in the general population, we screened 143 healthy blood donors from the greater Stockholm area. We found that as many as 10% had IgE antibodies to α-Gal compared to 0.7% (1/150) of teenagers from a prospective study on asthma in northern Sweden, where tick bites are rare[102,171]. For further comparison we also screened 207 patients with serologically confirmed Lyme disease, who were enrolled as an established recent tick-bitten population, and found 22% to be IgE positive to α-Gal. We noted that the IgE titres to α-Gal did not differ between healthy blood donors and patients with Lyme disease and were significantly lower (median titre of α-Gal–positive subjects: 0.16 kU_A/L and 0.25 kU_A/L, respectively) than the levels of the red meat allergic patients (median 20 kU_A/L, P <.001; Fig. 7C). These low levels probably only reflect sensitization and are not predictive of an allergic reaction. However, the frequency of α-Gal–sensitized subjects was significantly higher in the group with Lyme disease compared with the healthy blood donors (46/207 *vs.* 15/143; Fig. 7D; χ^2 = 8.09, *P* = .005), which strengthens the role of tick bites for the induction of IgE to α-Gal.

When comparing red meat allergic patients to the α-Gal-positive subjects with Lyme disease, we found that the median IgE titre to α-Gal was significantly higher and that the correlations between α-Gal and total IgE, as well as *I. ricinus*, were significantly stronger in red meat allergic patient group. Similarly, IgE responses to *I. ricinus* were significantly higher in both frequency (37/39 *vs.* 21/46, χ^2 = 23.59, P < .001) and median levels (1.49 *vs.* <0.10 kUA/L, P < .001) in patients with red meat allergy compared with those seen in α-Gal–positive patients with Lyme disease.

Since the α-Gal epitope is a major blood group structure of non-primate mammals and structurally related to blood group B[84], we investigated the blood type of our red meat allergic population. The results showed that the number of B-positive patients in the red meat allergic group (2/39; 5%) was significantly lower compared to the expected frequency in the general Swedish population (18%, *P* =0.04). The findings are in accordance with a recent study on the relationship between IgG and IgE responses to α-Gal and blood group B, where they detected that none of the B-positive subjects expressed IgE antibodies to α-Gal[172].

Taken together, the data from paper I and II showed that the α-Gal epitope was localized in the GI tract of tick *I. ricinus* and there is a strong relationship between tick bites and

the production of IgE to α-Gal in the Swedish red meat allergic patients. This was further supported by the fact that the number of subjects expressing IgE to α-Gal was significantly higher among patients with tick borne Lyme disease compared to healthy blood donors. Additionally, we reported for the first time that red meat allergy is strongly associated with the B-negative blood groups.

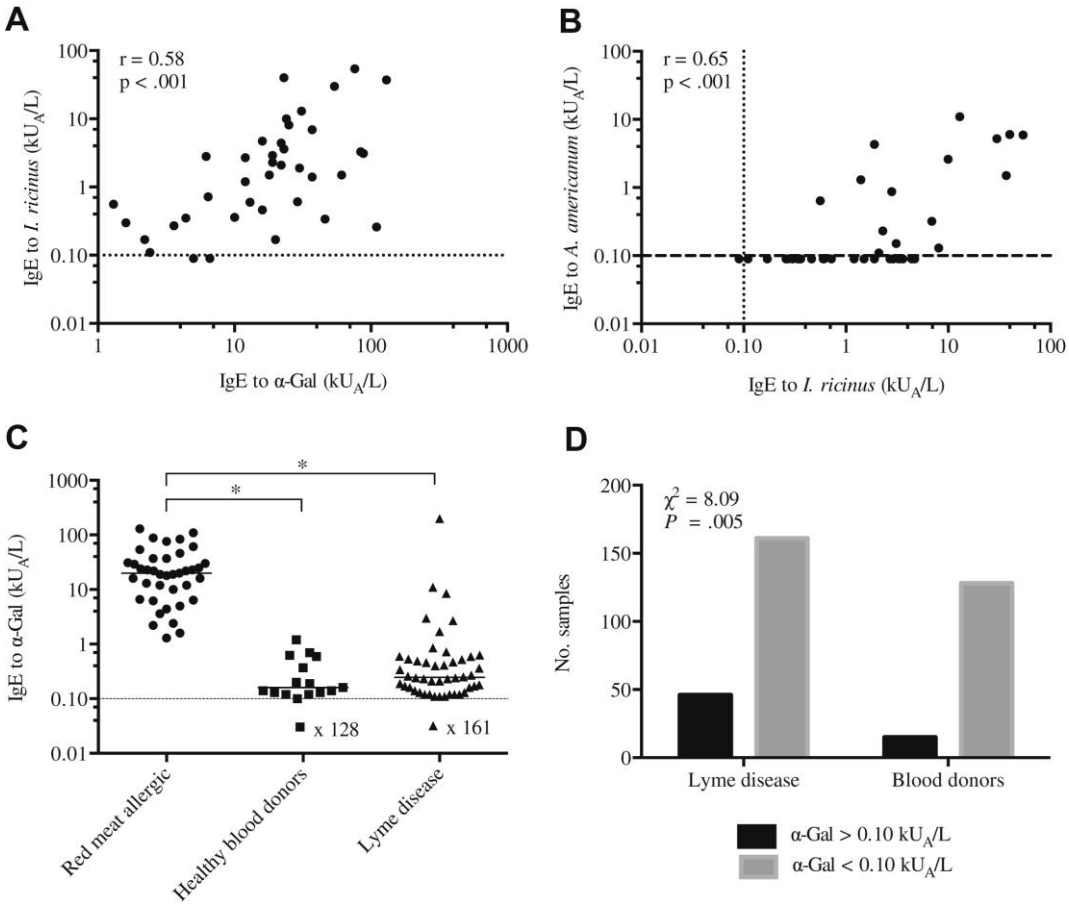

Figure 7. Correlations between IgE responses to α-Gal and *I. ricinus* **(A)** and *I. ricinus* and *A. americanum* **(B)** in Swedish patients with red meat allergy. **(C)** IgE reactivity to α-Gal in patients with red meat allergy compared with that in healthy blood donors and patients with Lyme disease expressed as kilounits of allergen per liter. Mann-Whitney U tests were performed to assess statistical significance: *P < .001. Solid bars denote median values. **(D)** Prevalence of IgE reactivity to α-Gal in healthy blood donors compared with that in patients with Lyme disease. *(Figure from paper II).*

2.3.3 Immunoproteomics of processed beef proteins reveal novel galactose-α-1,3-galactose-containing allergens (Paper III)

Food preparation and processing can influence the allergenicity of protein by either destroying conformational IgE-binding epitopes or sometimes it can produce new epitopes and enhance the allergenicity[173]. In this paper we characterized the proteomic profile of different beef preparations (raw, medium rare, fried, and boiled) and investigated their potential allergenicity among red meat allergic patients.

By immunoblot, patterns of IgE binding to several soluble proteins from different processed beef extracts (25–250 kDa) were detected in patients' sera, which is in line with previous research[78], while IgE binding was not detected in the negative control (Fig. 8A). To compare the IgE binding properties between differently processed beef, serum pool 1 from red meat allergic patients was used and multiple reactive bands from 25 to 150 kDa were detected for all the different beef preparations (Fig. 8B). In raw and medium rare meat, stronger reactive bands were seen between 30 and 100 kDa while boiled and fried meat showed similar reactive protein bands, from 37 to 75 kDa. Their IgE binding pattern compared with raw and medium rare beef extracts was weaker, which could be an indication of reduced capacity to bind IgE (Fig. 8B). By inhibition of patient serum with α-Gal several IgE-binding protein bands disappeared (Fig. 8B). Additionally, a separate immunoblot was developed with anti-α-Gal antibody (Fig. 8C), displaying similar binding patterns to the different processed beef extracts as the patient serum pool. This indicates that most of the proteins still preserve their α-Gal reactivity after heat treatment, which fits with data from our red meat allergic patients who often report allergic reactions after ingestion of cooked meat. To verify that the detected IgE-binding beef proteins indeed contained α-Gal, a deglycosylation assay was used to remove the N-linked glycoproteins from the raw meat extract. When the resulting deglycosylated beef proteins were visualized on immunoblot with anti-α-Gal antibody, all protein bands besides one band around 37 kDa were removed (Fig. 8D).

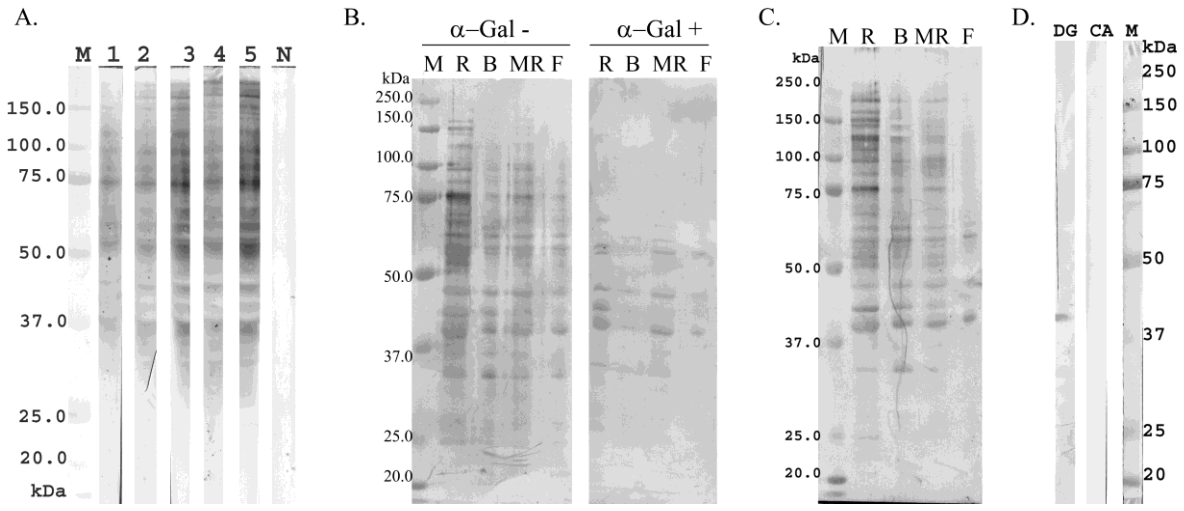

Figure 8. Immunoblot reactivity to meat proteins. **(A)** IgE binding to raw beef extract in serum from five red meat allergic patients (no. 1-5) and a negative control (N). **(B)** IgE-binding profile of different processed beef extracts in serum pool 1 (from red meat allergic patients) without or with preincubation of α-Gal (100 μg/ml). **(C)** α-Gal-binding profiles of different processed beef extract using monoclonal anti-α-Gal antibody. **(D)** Deglycosylated raw beef extract with monoclonal anti-α-Gal antibody. M, molecular weight marker; N, negative control; R, raw; B, boiled; MR, medium rare; F, fried; DG, deglycosylated; CA, control antibody. *(Figure from paper III).*

To identify IgE-binding proteins carrying the α-Gal epitope and examine whether they survive thermal processing, all beef extracts were analysed by powerful immunoproteomic tools: high-resolution 2D PAGE, 2D immunoblot, and MS/MS analysis. When the processed beef preparations were separated on SDS-PAGE (Fig. 9A) they showed overall similar profiles, however some differences were noted. Boiled beef extract contained bands with higher molecular weight, ≥250 kDa, probably originating from thermally induced aggregation of proteins, which were not observed in raw and medium rare meat extracts. Furthermore, when comparing the 2D PAGE protein profiles of raw and medium rare beef extracts (Fig. 9B, C), we were able to identify 53 matched protein spots. Boiled and fried beef extracts also showed similar profiles on 2D PAGE (Fig. 9D, E), where 35 protein matches were identified. Takahashi *et al.* recently demonstrated α-Gal epitopes on two IgE-binding proteins from beef in the high molecular weight area (laminin γ-1 and collagen α-1 (VI) chain). Due to unclear separation of low molecular weight proteins, they reported that other proteins could not be analysed[174]. Our results showed not only clearly separated beef proteins in the lower molecular weight area (20–100 kDa), but also high similarity between raw and medium rare meat, as well as high similarity between boiled and fried meat.

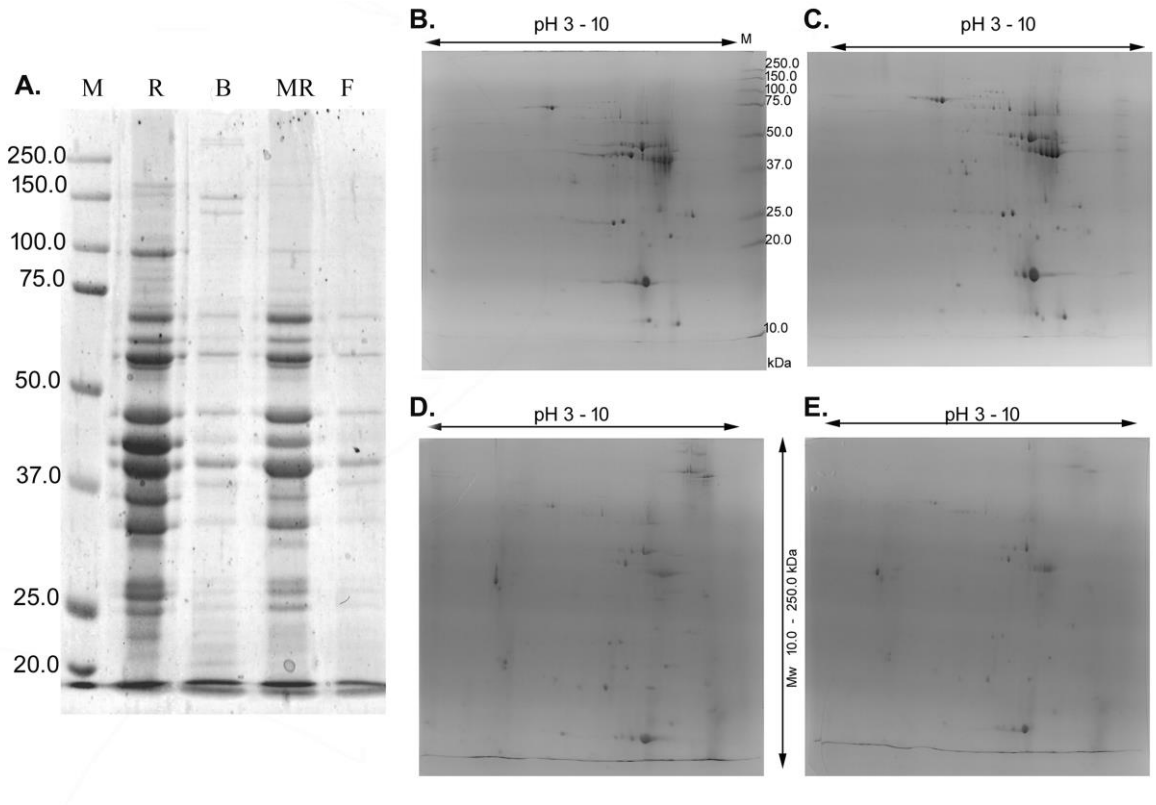

Figure 9. 1D PAGE and 2D PAGE protein profile of different processed beef extracts. **(A)** 1D PAGE and **(B)** 2D PAGE of raw beef extract, **(C)** medium rare beef extract, **(D)** boiled beef extract and **(E)** fried beef extract. M, molecular weight marker; R, raw; B, boiled; MR; medium rare; F, fried. *(Figure from paper III).*

To identify proteins with IgE-binding capacity, a 2D immunoblot was developed using patient serum pool 2. Twenty-nine IgE-binding protein spots around 15–100 kDa were detected (Fig. 10A) and further analysed by peptide mass fingerprint. MS/MS spectra of these proteins gave high identification scores for 18 proteins from the *Bos taurus* (*B. taurus*) database. Sequence analysis showed high similarity (≥85%) between the proteins identified from *B. taurus* and the corresponding proteins from *Homo sapiens*. This suggested that α-Gal, a post-translational modification of non-primate mammals, is an important epitope of these proteins. However, highly homologous proteins without α-Gal were also identified. Prediction of N-glycosylation sites for the identified proteins revealed that 10 of the 18 proteins contained glycosylation sites and some of them more than one. Some of the identified proteins were well-known beef allergens (myoglobin and bovine serum albumin)[80], but the majority had not previously been reported as beef allergens or α-Gal-containing proteins. Seven of the 18 IgE-binding proteins were novel α-Gal-containing proteins, whereof four survived heat treatment. The new identified proteins are cytoplasmic proteins, playing essential roles in the metabolic pathways[175,176]. Some of them have multiple functions; e.g. enolase takes part in processes such as growth control, hypoxia tolerance, allergic responses and may also stimulate Ig production[177].

To detect α-Gal-containing proteins, a 2D immunoblot was developed with the anti-α-Gal antibody, with separation on 10% PA gel to enable investigation of a wider molecular mass range (Fig. 10B). Comparing the immunoblots developed with the serum pool and with anti-α-Gal antibody revealed many IgE-binding proteins containing the α-Gal epitope (Fig. 10A and 10B). According to isoelectric point and molecular weight values on 2D PAGE and immunoblots as well as prediction of N-glycosylation sites, seven proteins (triosephosphate isomerase, carbonic anhydrase 3, lactate dehydrogenase A, creatine kinase M-type, aspartate aminotransferase, β-enolase and α-enolase) were identified as IgE reactive α-Gal-containing proteins (Fig. 10C). Comparison of protein profiles between raw and cooked beef extracts revealed that four of the identified α-Gal-containing allergens (creatine kinase M-type, aspartate aminotransferase, β-enolase and α-enolase) were stable to heat treatment.

We noticed that the α-Gal-containing high molecular weight IgE-binding proteins were in low abundance compared to lower molecular weight α-Gal proteins recognized by the monoclonal anti-α-Gal antibody. Other proteins known to carry α-Gal are thyroglobulin,

fibrinogen, IgG, fibronectin, and integrin (β1 subunit)[83,178]. These proteins were not detected with this experimental setup, presumably due to their low solubility and very high molecular size. Denatured type I bovine collagen has been identified as the major allergenic component of gelatin[179], and red meat allergic patients have been reported to react to gelatin[180].

In conclusion, we demonstrated that the α-Gal epitope is commonly present in beef proteins recognized by red meat allergic patients' IgE. We identified 18 IgE-binding proteins with high identification score from *B. taurus*. By immunoproteomics and MS/MS analysis, seven novel α-Gal-containing beef allergens were described, of which four were stable to heat treatment. Thus, the allergenicity of red meat proteins was preserved even upon thermal processing. Furthermore, we demonstrated that red meat allergic patients also have IgE responses to non-α-Gal-containing proteins.

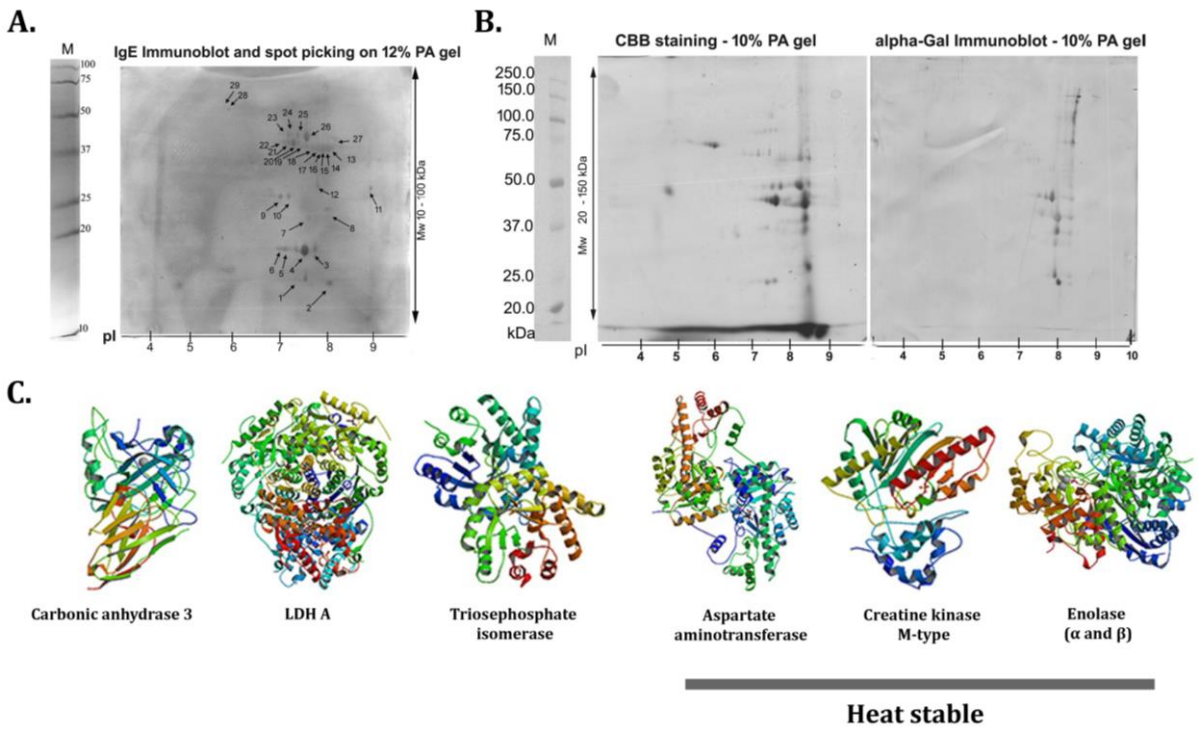

Figure 10. 2D immunoblot analysis of raw beef extract developed with red meat allergic serum pool. **(A)** Protein spots labelled 1–29 were used for peptide mass print analysis and protein identification. **(B)** 2D immunoblot analysis of raw beef extract developed with monoclonal anti-α-Gal antibody (left) and CBB staining of 10% PA gel (right). **(C)** Structures of the seven novel α-Gal-containing protein with the four resisting thermal processing underlined as heat stable, from left to right (carbonic anhydrase 3, lactate dehydrogenase A, triosephosphate isomerase, aspartate aminotransferase, creatine kinase M-type, enolase(α and β). M, molecular weight marker. *(Figures from paper III modified).*

2.3.4 Red meat allergic patients have a selective IgE response to the α-Gal glycan (Paper IV)

The most common carbohydrate epitopes recognized by human IgE are glycans with α1-3 fucose and xylose cores that are present in plants, insects, ruminant nematode, and trematodes[66]. The only mammalian glycan besides α-Gal present in high amounts in beef, lamb, pork, and cow's milk is the sialic acid N-glycolylneuraminic acid (Neu5Gc)[181]. Neu5Gc is present in most mammals including primates[182] but not in humans due to an irreversible mutation in the gene encoding the enzyme responsible for Neu5Gc synthesis[183].

In this study 24 patients with α-Gal-induced red meat allergy and three healthy controls IgE negative to α-Gal were included. They were tested for IgE reactivity against bTG (heavily decorated with α-Gal), the α-Gal and Neu5Gcα glycan, the CCDs-containing allergens MUXF3 (α1,3-fucose and α1,2-xylose core), nCup a 1 (horseradish peroxidase-based carbohydrates), and nArt v 1 (arabinogalactan-rich protein). We found that none of the red meat allergic patients had an IgE antibody response against Neu5Gcα or against the CCD-containing proteins (MUXF3, nCup a 1 and nArt v 1). One patient had a very low IgE response to nArt v 1, but pre-inhibition with bTG did not affect the IgE reactivity to nArt v 1. The result confirmed that the immune response against nArt v 1 was independent of the α-Gal response. The healthy controls did not have IgE reactivity against the tested components except one patient who was sensitized to *Artemisia vulgaris* (nArt v 1 IgE level 1.1 kU_A/L). These data revealed that cross-reactions to glycans, common in other allergic diseases, are not an issue in the pathogenesis and diagnosis of red meat allergy. Even though the glycan Neu5Gcα is present in mammalian meat and milk alongside α-Gal, it is not recognized by IgE from red meat allergic patients. The specificity of the α-Gal response could be due to the route of sensitization through the skin via tick bites. We have recently shown that α-Gal is present within ticks, thus explaining the relationship between tick exposure and sensitization of α-Gal, leading to the development of red meat allergy[184,185].

To enable a thorough investigation of the IgE responses to the α-Gal glycan *vs.* an α-Gal-containing glycoprotein, bTG was deglycosylated. Native and deglycosylated bTG were analysed by circular dichroism spectroscopy and similar secondary structures were observed, indicating that the protein structure was unaffected by the enzymatic procedure. The removal of glycans was visualized by SDS-PAGE where the

deglycosylation of bTG resulted in a shift of the major protein band to below 250 kDa compared to untreated bTG (band above 250 kDa). This was further supported by immunoblot where a significant reduction in IgE binding to deglycosylated compared to untreated bTG was noted. Comparable results were obtained when using a monoclonal anti-α-Gal antibody. Next, IgE levels to deglycosylated bTG were measured and in two-thirds of the patients, deglycosylation of bTG reduced the IgE levels as much as 100-fold and 50% of the cases to below the cut-off (<0.10 kU_A/L). The eight patients that still had a low IgE reactivity to deglycosylated bTG (median 0.20 kU_A/L; range 0.1–2.8 kU_A/L) had high IgE levels to untreated bTG. Hence, this probably reflected an incomplete glycan removal by PNGase F, which could have been due to steric hindrance on the glycoprotein. This was further investigated by pre-incubation with 500 μg/ml of α-Gal (α-Gal-sp-biotin) prior to the measurement of deglycosylated bTG-specific IgE. This resulted in a reduction to below cut-off (<0.10 kU_A/L) in all patients, indicating that their IgE responses were indeed specific for the α-Gal glycan with no binding to the protein structure.

Taken together, the red meat allergic patients have a selective IgE response to the α-Gal glycan that is unrelated to the carrier protein. Common CCDs from plants or venoms were not targets of the IgE response in these patients. Even though the glycan Neu5Gcα was present in mammalian meat and milk alongside α-Gal, it was not recognized by IgE from red meat allergic patients. The specificity of the α-Gal response could be due to the route of sensitization through the skin via tick bites.

2.3.5 *In vitro* uptake of α-Gal containing proteins by human monocyte derived dendritic cells (Paper V)

Carbohydrates have been considered to be T-cell independent antigens, but studies in mice with APC uptake of glycoconjugates revealed that glycan-peptides presented on MHC II can generate specific T-cell responses[186]. Similarly, mice deficient of α-1,3-GT, the enzyme needed for expression of the oligosaccharide α-Gal, produce an α-Gal-specific antibody response when immunized with rabbit red blood cells[187]. Furthermore, mice with concurrent T-cell receptor deficiency do not develop such an immune response. Thus, the antibody response to α-Gal seems to be T-cell dependent[188]. The unusual long delay of allergic symptoms suggests a mechanism dependent on adaptive immunity,

involving DC and T-cells. However, the role of immune cells in the sensitization process of α-Gal is still not known.

In this paper we investigated whether human MDDC take up the α-Gal model antigen bTG, process it and subsequently present α-Gal on membrane bound MHC II. We showed that *in vitro* cultured human iMDDC efficiently take up α-Gal containing proteins in a dose-dependent manner. Immature MDDC were incubated with various concentrations of FITC-labelled bTG for 4h and showed increased uptake with higher concentration of bTG-FITC from 10% at 2μg/ml, 44% at 10μg/ml to 56% at 25μg/ml. Previous studies have shown dose-dependency for protein allergens such as Der p 1 (major allergen for house dust mite)[189] and Phl p 5 (major grass pollen allergen)[190]. When we investigated the internalization process of bTG with confocal microscopy it was possible to clearly visualize much larger accumulation of bTG at 37°C compared to at 4°C, indicating that the uptake of bTG was an active process. Similar results were observed using flow cytometry, showing no increase in uptake after 1h incubation with labelled molecules at 4°C. However, we could not detect any surface binding of bTG, which suggest that bTG is not internalized by receptor-mediated endocytosis but rather macropinocytosis. This result differs from studies of the protein allergens Bet v 1 and Phl p 5 where the uptake is a receptor-mediated process with a clear distinction between binding on cell surface (4°C) and internalization (37°C)[190,191].

We then evaluated the internalization kinetics for bTG and hTG where iMDDC were treated with 2-10 μg/ml bTG-AF488 or hTG-AF488 at 37°C and collected data at various time points. Even though different concentrations were used, the same trend of uptake was observed for both bTG and hTG in all experiments. The percentage uptake of bTG and hTG increased over time from 30 min to 7 hours (Fig. 11A), with slightly higher internalization of hTG, but there was no statistical difference in the uptake of the two antigens. The results further support that the iMDDC do not have a receptor specifically recognising the α-Gal glycan but rather utilise macropinocytosis for internalization. This is in contrast to other studies showing that DC-SIGN and mannose receptor are able to recognise glycosylated allergens[192]. Moreover, allergens from house dust mite (Der p 1 and Der p 2), cockroach (Bla g 2), dog (Can f 1), peanut (Ara h 1) and cat (Fel d 1) have been shown to be recognized by the mannose receptor on human MDDC through their carbohydrate moieties[193,194]. DC-SIGN on MDDC also recognise Ara h 1[194], Der p 2, and Cyn dBG-60 (Bermuda grass pollen)[195]. Analysis by confocal

microscopy revealed that at the 1h time point, bTG had entered the cell and was scattered around the cytoplasm. After four hours, the internalized proteins were clustered together, possibly in endosomal compartments (Fig. 11B). Similar results were obtained for hTG (data not shown).

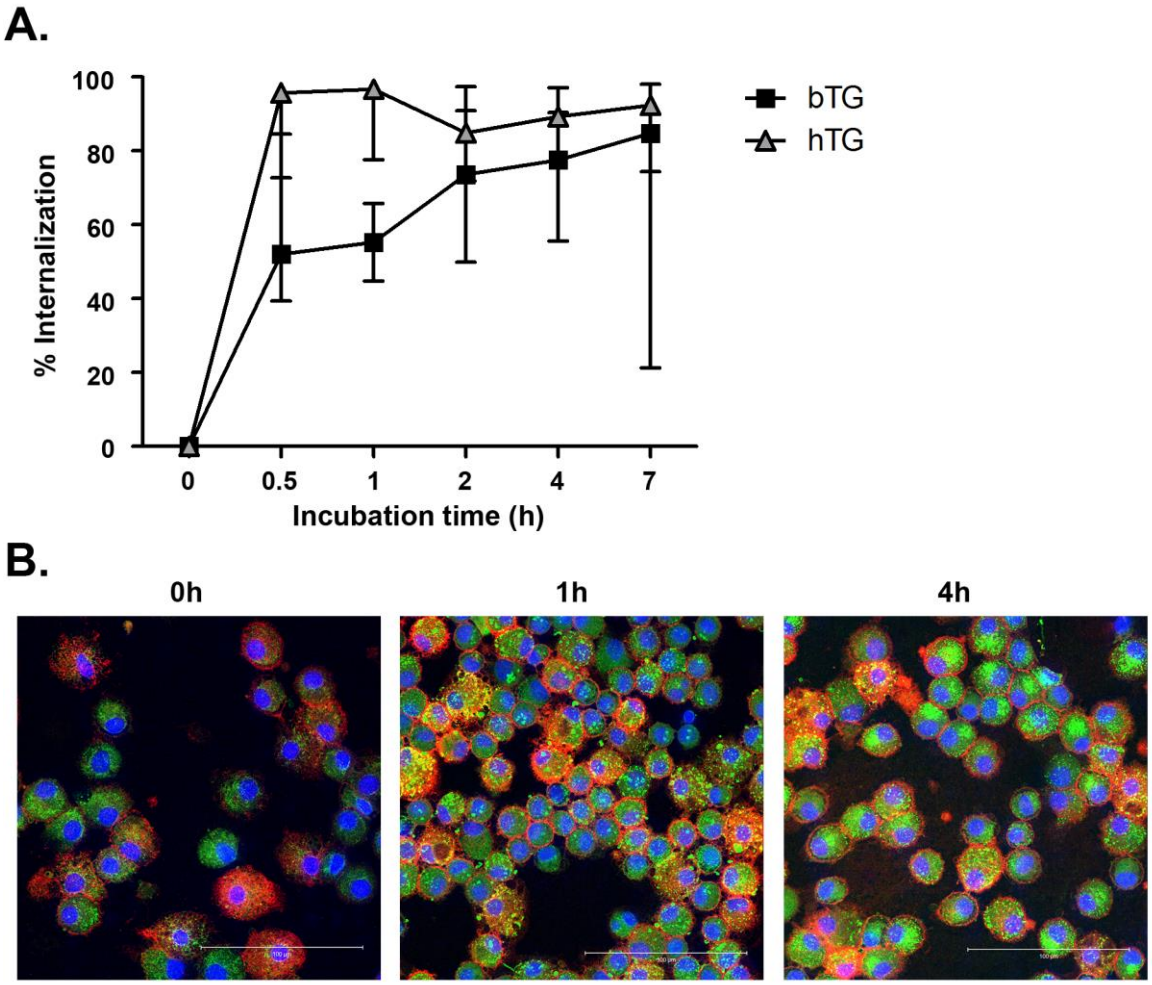

Figure 11. Time dependence of iMDDC uptake of bTG and hTG by **(A)** flow cytometry and **(B)** confocal microscopy. Representative images of iMDDC incubated with bTG for 0, 1 and 4h are shown. The scale bars represent 100 µm. Green = bTG, red = CD11c and blue = DAPI. *(Figure from paper V).*

As it is still unknown how the allergic immune-response to α-Gal is initiated and whether DC and T-cells are involved, we investigated if DC can present α-Gal. Since both bTG and hTG were internalized by iMDDC, we used isolectin GS-IB4, a lectin that has previously been shown to recognize α-Gal residues[196], for cell surface detection of the epitope. We found that α-Gal could be detected on the surface of iMDDC incubated with bTG already after 1h with a median increase of 10% by 2h, 20% by 4h and 45% after 7h compared to 0h which was not seen for hTG (Fig. 12). This finding indicated that the processing of antigen glycosylated with α-Gal within the cell endosomal compartment does not destroy

the α-Gal glycan. A previous study showed similar results for other glycosylated allergens and demonstrated that the mannose receptor plays a major role in glycoallergen recognition, as well as in the development of Th2 response[193]. By blocking antigen processing with monensin, which works by accumulating protein at the endoplasmic reticulum, surface expression of α-Gal was diminished by 33% and 66% compared to iMDDC incubated with bTG for 1 and 4 hour without monensin, respectively (Fig. 12). However, the internalization of bTG and hTG was not affected by monensin (data not shown). Thus, the surface expression of α-Gal is dependent on the antigen being processed intracellularly and transported to the surface, possibly by loading of α-Gal peptides on MHC II where it can be presented to T cells.

To conclude, the data showed that bTG is actively internalized by iMDDC in a dose- and time dependent manner. After endosomal processing in iMDDC it was possible to detect presentation of α-Gal expression on the plasma membrane derived only from bTG. This finding indicates that the processing of an antigen glycosylated with α-Gal within the cell endosomal compartment does not destroy the α-Gal glycan. Our data support that the IgE-mediated antibody response against α-Gal could be T-cell dependent.

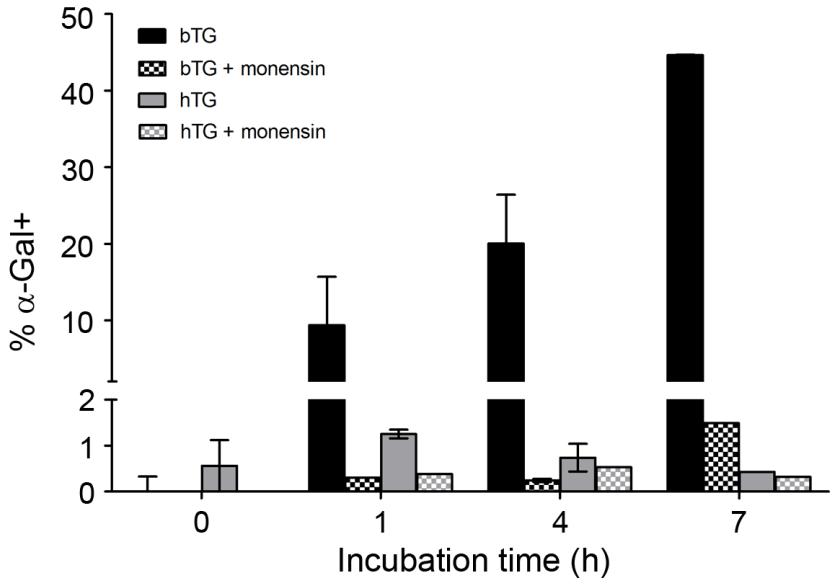

Figure 12. Surface expression of α-Gal in iMDDC after 0, 1, 4 and 7h incubation with bTG, bTG + monensin, hTG and hTG + monensin. *(Figure from paper V).*

2.4 CONCLUDING REMARKS AND FUTURE PERSPECTIVE

Red meat allergy is a novel type of food allergy with increased recognition worldwide in the last decade. The α-Gal carbohydrate has been shown to be a clinically relevant allergen that should be taken into account in the diagnosis of food allergy. The work in this thesis has contributed to increased understanding of the mechanisms behind red meat allergy, including the role of IgE antibodies to α-Gal, the allergenicity of α-Gal-containing beef proteins after thermal processing, the importance of tick bites as the primary sensitization pathway and the involvement of immune cells in this delayed allergic response.

A crucial question is why some individuals produce IgE to α-Gal. Although genetic factors may predispose an individual to develop an allergic reaction to mammalian meat, several studies show strong association between tick bites as an initiator for the development of red meat allergy[95,101,102,184,185]. We showed that the α-Gal epitope was localized in the GI tract of tick *I. ricinus* and that there is a strong relationship between tick bites and the production of IgE to α-Gal. Exposure to *I. ricinus* has increased over the last decade in Sweden and ticks are extending to the north as well as to the west of the country where they have become prevalent (Public Health Agency of Sweden, www.folkhalsomyndigheten.se). This is probably due to expanding deer and rodent populations (major tick vectors) as well as climate changes. In the future it would be interesting to observe whether an increase in the development of red meat allergy in the Swedish population will occur and also study the regional differences.

The red meat allergy syndrome presents several features that are strikingly different from established food allergy with a delayed IgE-mediated systemic reaction occurring several hours after intake. We have recently revealed that the α-Gal epitope is commonly present in IgE-reactive beef proteins, some of which are stable to heat treatment (creatine kinase M-type, aspartate aminotransferase, β-enolase, and α-enolase). Since it is the first time that these four proteins are identified as α-Gal-containing, further studies to elucidate their functionality and potential to elicit an allergic reaction would be of great interest.

It has been postulated that for sensitisation via the GI tract, the implicated allergen must have properties that preserve its protein structure from in the intestine (resistance to low pH, bile salts and proteolysis). Thus, allowing the allergen to remain in a sufficiently intact form to be taken up by the gut is essential to sensitise the mucosal immune system[197-199]. Consequently, it has been proposed that resistance of proteins to pepsin digestion in the stomach is a marker for potential allergenicity[200]. How the digestion process affects the allergenicity of α-Gal-containing proteins is still an open question. Investigating the impact of the digestion process by mimicking gastro-duodenal conditions, where produced peptides from the digests can be characterized and the allergenicity of the α-Gal epitope further studied.

It has also been speculated that the digestion and absorption of lipids could be the rate-limiting step in this delayed allergic reaction, possibly by the appearance of oligosaccharides in a form that can trigger histamine release from mast and/or basophils[169]. Generally, lipid absorption is slower compared to carbohydrates and proteins absorption, because a significant portion of the lipids are formed and transported via very low density lipoproteins and/or chylomicrons[201,202]. α-Gal is known to be abundantly present on glycolipids and glycoproteins of non-primate mammals, including red meat from beef, pork, and lamb[92]. Glycolipids and glycoproteins need to be digested in the intestinal lumen before they can be taken up by enterocytes. The delay in the allergic reaction could be explained by this slower process. It would be very interesting to determine presence of the α-Gal glycan in human plasma and which lipoproteins are involved after a meal containing red meat. This will give an indication of the timeframe when α-Gal occurs in the circulation.

We found that none of the red meat allergic patients investigated had an IgE antibody response against the other abundant mammalian glycan Neu5Gcα or against CCDs from plant or venom sources (nCup a 1, nArt v 1, and MUXF3). Since it is well-known that carbohydrate structures with broad diversity are common in nature[203] the possibility that other allergenic CCDs could be relevant in red meat allergy cannot be excluded. To address this question glycan microarray technology in combination with large libraries of complex carbohydrate can be utilized. Glycan arrays are a new technology that has enabled high-sensitivity and rapid analysis of carbohydrates[204]. With this method information about a broader range of reactivity from many different carbohydrate

structures could be obtained[205], and by comparing red meat allergic patients and healthy donors differences in glycosylation patterns can be detected.

Transport of proteins through the gut epithelial monolayer is the first step in the cascade of events during an immune response to food allergens. Investigation of how the allergenicity of α-Gal-containing proteins is affected by the transport via gut epithelial layer is warranted. For this purpose, the human Caco-2 cell culture model can be used. When in culture these cells differentiate and form a monolayer of cells that express several morphological and biochemical characteristics of small intestinal enterocytes, working as a model of the intestinal barrier that can be used for absorption experiments[206]. This approach will reveal how the α-Gal glycan is modified (e.g. digested, removed from protein core, crosslinked) when passing through the gut epithelial monolayer, which could affect the allergenicity and IgE binding properties.

The unusual long delay of symptoms likely reflects the appearance of the antigen in the bloodstream and suggests T-cell involvement. We have recently shown that iMDDC internalize bTG and after processing present α-Gal on the plasma membrane of cells. Additional investigation of the expression of maturation markers by the iMDDC and the cytokine profile upon internalization of glycoprotein would be very interesting. The result from our study indicates that bTG is taken up by an α-Gal-independent mechanism, presumably via macropinocytosis, and by blocking different uptake pathways in MDDC this can be clarified. Further studies are needed to confirm that α-Gal peptides are presented on MHC II molecules and able to activate T-cells. Additionally, α-Gal-containing peptides derived from the digestion process can be used in uptake experiments with MDDC, which is a more physiological relevant experimental setup compared to uptake of whole glycoprotein by APC.

To explore in more detail the mechanism of α-Gal specific IgE production *in vivo,* a knockout mouse model of the α-1,3-GT gene could be employed. Through evolution the α-1,3-GT gene has been inactivated in humans resulting in the production of anti-α-Gal antibodies by environmental stimulation. In healthy adults 1-5% of the circulating repertoire of IgM and IgG is directed against this glycan[92]. The knockout mouse model also lacks the α-1,3-GT gene required for biosynthesis of α-Gal epitopes, therefore these mice produce anti-α-Gal IgG antibodies levels comparable to humans by immunization

with rabbit red blood cell membrane[187]. This could provide a useful experimental model for investigating the mechanisms of anti-α-Gal antibodies *in vivo*.

The many new findings obtained during the last decade have significantly increased our knowledge in the field of red meat allergy. However, further studies are needed to elucidate the process of digestion, absorption, and delivery of α-Gal-containing molecules to the circulation. More data on the T-cell response to α-Gal (how the antigen is presented, which T-cell subsets are involved and how the response is regulated) will be additional valuable knowledge. Every new piece of information obtained is adding in deciphering the mechanisms behind the red meat allergic syndrome, and will have a major clinical impact on the patients and future treatment strategies.

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