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MONITORING OF *IN VITRO* BIOAVAILABILITY AND UPTAKE OF GLYCOSYLATED FOOD ALLERGENS USING CELL-BASED MODELS

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PRAĆENjE *IN VITRO* BIOUSVOJIVOSTI I PREUZIMANjA GLIKOZILOVANIH ALERGENA HRANE UPOTREBOM ĆELIJSKIH MODELA

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"Živi kao da ćeš sutra umreti. Uči kao da ćeš večno živeti."

Monitoring of *in vitro* bioavailability and uptake of glycosylated food allergens using cell-based models

SUMMARY

The increasing problem of food allergies in the population worldwide requires the extensive engagement of researchers in the elucidation of the mechanisms underlying processes of food digestion, allergen transport, and uptake by the immune cells and its effector immune responses. The development of the *in vitro* assays and cell-based models has allowed bridging the problem of food allergy research and the respect of the ethical norms in used research procedures.

The red meat allergy is a novel type of food allergy characterized by the production of an IgE antibody against the carbohydrate galactose-α-1,3-galactose (α-Gal). Glycoproteins from non-primate mammals are rich with an α-Gal as post-translational modification. Also, red meat allergy is characterized by the delayed onset of symptoms which may be related to the mechanism and the fate of α-Gal carrying proteins in the human gastrointestinal tract. Furthermore, the uptake, processing, and mechanisms of presentation of α-Gal by the immune cells are still unknown. Therefore this doctoral dissertation aimed to investigate how protein glycosylation by α-Gal affects their susceptibility to gastric digestion, does α-Gal conjugated to proteins affects their transport through the Caco-2 cell monolayer, which mimics the gastrointestinal layer, and to examine the influence of α-Gal epitopes on the protein surface on their uptake and processing by immature monocyte-derived dendritic cells (iMDDCs). The study revealed that the presence of the α-Gal glycosylation on protein surface had an impact on their susceptibility to gastric digestion and the digestion pattern of the obtained protein fragments upon pepsinolysis. Prolonged survival, up to 2h of digestion, was characteristic of the large proteins fragments bearing the α-Gal epitope. Importantly, transport through the Caco-2 monolayer of proteins conjugated to α-Gal was hampered in comparison to unconjugated proteins. Furthermore, differential centrifugation of Caco-2 cell lysates upon transport experiments revealed that α-Gal could be detected on the intact protein in the endosomal fraction of the cells. Also, the level of galectin-3, which is abundantly expressed by intestinal epithelial cells and is

possibly associated with pro-inflammatory properties, was not altered by the presence of α-Gal glycosylated BSA (bovine serum albumin) (BSA-α-Gal) in comparison to BSA. To monitor the influence of α-Gal epitopes on the protein surface on their uptake and processing iMDDCs were prepared from healthy blood donors and red meat allergic patients. Overtime increased internalization of α-Gal carrying proteins in iMDDCs from healthy individuals and red meat allergic patients was noted. The flow cytometric analysis also revealed that the uptake of α-Gal carrying proteins was significantly higher than the uptake of non-α-Gal carrying proteins. For analysis of the spatial distribution of α-Gal carrying proteins inside iMDDCs confocal microscopy was employed and interestingly α-Gal carrying proteins were scattered around the cytoplasm while detection of proteins not carrying α-Gal was negligible. Upon uptake experiments, the iMMDCs lysates were prepared and resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis which showed that α-Gal carrying proteins were processed to a lesser extent compared to non-α-Gal carrying proteins.

As a general conclusion, it can be underlined that glycosylation of food proteins has effects on their digestibility and transport through the intestinal monolayer and on their successive uptake and processing by cells of the immune system. This thesis has established new *in vitro* protocols as well as protocols for setting up cell-based models that, in addition to examining the effects of glycosylated food allergens, may be used in some future studies.

Keywords: α-Gal; bioavailability; transcytosis; glycoprotein; Caco-2 cells; mammalian meat allergy; iMDDCs; uptake; cell-based models.

Scientific field: Chemistry

Scientific subfield: Biochemistry

Praćenje *in vitro* biousvojivosti i preuzimanja glikozilovanih alergena hrane upotrebom ćelijskih modela

REZIME

Alergije na hranu su rastući problem u ljudskoj populaciji širom sveta i rešavanje ovog problema zahteva opsežno angažovanje istraživača u rasvetljavanju mehanizama uključenih u procese varenja hrane, transporta alergena i njihovog unosa od strane imunih ćelija odgovornih za efektorske mehanizme imunoloških odgovora. Ovo je nezamislivo bez razvoja *in vitro* testova i ćelijskih modela koji premošćuju problem istraživanja alergija na hranu uz poštovanja etičkih normi u korišćenim istraživačkim metodama.

Novu vrstu alergije na hranu, alergiju na crveno meso, karakteriše sinteza imunoglobulina E kao odgovor na prisustvo šećera galaktoza-α-1,3-galaktoza (α-Gal), koji je prisutan na površini glikoproteina primata. Takođe, alergiju na crveno meso karakteriše odložena pojava simptoma što može biti rezultat promena u mehanizmu obrade proteina koji nose α-Gal u gastrointestinalnom traktu čoveka. Dalje, unos, obrada i mehanizmi prezentacije α-Gal šećera od strane imunih ćelija još uvek nisu poznati. Stoga ciljevi ove doktorske disertacije su ispitivanje kako α-Gal glikozilacija proteina utiče na njihovu digestiju od strane pepsina, da li α-Gal glikozilacija proteina utiče na njihov transport kroz monosloj Caco-2 ćelija, koji oponaša gastrointestinalni epitel, kao i ispitivanje uticaja α-Gal glikozilacije na površini proteina na njihov unos i obradu od strane nezrelih dendritičnih ćelijama kultivisanih iz monocita (iMDDC). Iz dobijenih rezultata moze se zaključiti da prisustvo α-Gal glikozilacije na površini proteina utiče na njihovu podložnost na digestiju a najviše na obrazac dobijenih fragmenata proteina nakon pepsinolize. Veliki fragmenti proteina koji nose α-Gal prisutni su čak i do 2 sata digestije. Takođe, važno je istaći da je transport α-Gal glikozilovanih proteina kroz Caco-2 monosloj otežan u poređenju sa neglikozilovanim proteinima. Dalje, diferencijalnim centrifugiranjem lizata Caco-2 ćelija nakon transcitoze pokazalo je da je α-Gal prisutan na intaktnim proteinima u endozomalnim frakcijama ćelija. Takođe, nivo galektina-3, koji je obilno eksprimiran na intestinalnim epitelnim ćelijama i za koji se veruje da je u sprezi sa mehanizmima alergijskih reakcija,

ne menja se u prisustu BSA (goveđi serum albumin) glikozilovanog α-Gal-om (BSA-α-Gal) u poređenju sa neglikozilovanim BSA. Da bi se ispratio uticaj α-Gal glikozilacije proteina na njihov unos i obradu od strane ćelija imunog sistema, korišćeni su iMDDC uzeti od zdravih davalaca krvi i pacijenata sa alergijom na crveno meso. Primećena je povećana internalizacija α-Gal glikozilovanih proteina od strane iMDDC i zdravih osoba i pacijenata sa alergijom na crveno meso. Korišćenjem metoda protočne citometrije došlo se do rezultata da je unos proteina, od strane iMDDCs, koji nose α-Gal bio znatno veći od unosa proteina koji nemaju α-Gal. Za analizu prostorne distribucije proteina koji nose α-Gal unutar iMDDCs korišćena je konfokalna mikroskopija i zanimljivo je da su proteini koji nose α-Gal rasuti po citoplazmi, dok je detekcija proteina koji ne nose α-Gal zanemarljiva. Nakon preuzimanja proteina od strane iMMDCs, pripremljeni su lizati ovih ćelija čiji su proteini razdvojeni u gelu elektroforetskom tehnikom, ovo je pokazalo da su proteini koji nose α-Gal procesovani u manjoj meri u poređenju sa proteinima koji ne nose α-Gal.

Kao opšti zaključak, može se navesti da glikozilacija proteina hrane utiče na njihovu digestibilnost i transport kroz intestinalni monosloj kao i na njihovo dalje preuzimanje i procesovanje od strane ćelija imunog sistema. Takođe, kao jedno od postignuća ove doktorske disertacije treba istaći i uspostavljanje novih *in vitro* protokola kao i protokola za uspostavljanje ćelijskih modela, koji se, pored ispitivanja efekata glikozilacije alergena hrane, mogu koristiti u nekim budućim studijama.

Ključne reči: α-Gal; biousvojivost; transcitoza; glikoprotein; Caco-2 ćelije; alergija na crveno meso; iMDDCs; usvajanje; ćelijski modeli.

Naučna oblast: Hemija

Naučna disciplina: Biohemija

List of abbreviations

AF488 – Alexa Fluor 488;

APCs – antigen-presenting cells;

BCA - bicinchoninic acid;

BCIP - 5-bromo-4-chloro-3-indolyl phosphate;

BSA – bovine serum albumin;

BSL - biosafety level risk;

bTG – bovine thyroglobulin;

CBB R-250 - Coomassie Brilliant Blue R-250;

CD – cluster of differentiation;

CLEC9A - C-type lectin domain family 9 member A;

CLSM – confocal laser scanning microscopy;

CX3CR1 - C-X3-C motif chemokine receptor 1;

CytD - cytochalasin D;

DCs – dendritic cells;

DMEM – Dulbecco's Modified Eagle's medium;

DMSO - dimethyl sulfoxide;

FBS – fetal bovine serum;

HSA – human serum albumin;

Ig – immunoglobulin;

IL – interleukin;

iMMDCs – immature monocyte-derived dendritic cells;

MDC – monodansylcadaverine;

MHC - major histocompatibility complex;

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;

NAl – N-Acetyllactosamine;

NBT - nitro blue tetrazolium;

PAA – polyacrylamide;

PBS - phosphate buffered saline;

PBS-T - phosphate buffered saline - Tween 20;

PVDF - polyvinylidene difluoride;

RPMI - **R**oswell **P**ark **M**emorial **I**nstitute;

RT - room temperature;

ScFV - single-chain variable fragment;

SDS PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis;

SGF - simulated gastric fluid;

TBS-T - Tris-buffered saline – Tween 20;

TEER - transepithelial electrical resistance;

Treg – T regulatory cells;

α-Gal - galactosyl-α-1,3-galactose;

Contents

1.   Introduction

Nowadays with the emerging problem of increase of food allergies in the population worldwide, research on the mechanism underlying processes of food digestion, allergen transport, and uptake by the immune cells and its effector immune responses is necessary. The research community, as well as our modern society, agrees that the establishment and improvement of *in vitro* models are required. Therefore efforts of many researchers are directed toward the development and improvement of *in vitro* models such as cell-based models to monitor and examine the physiological and pathological processes which occur in the human gut and are responsible for the development of food allergies.

To better understand and explain processes related to food processing in the human gut it is necessary to define the frequently used term **bioavailability**. In general, bioavailability refers to gastrointestinal digestion, absorption, tissue distribution, metabolism, and bioactivity. Bioavailability is usually defined as "the fraction of ingested nutrient or compound that reaches the systemic circulation and is utilized". Furthermore, the term bioavailability also means that the compound of interest is efficiently digested and absorbed, and as such exhibits effects on human health. However, already mentioned ethical restrictions as well as practical difficulties in the assessment of effects of food compounds on human health narrow the term bioavailability and define it as "the fraction of a given compound or its metabolite that reaches the systemic circulation without considering bioactivity" (Wood 2005, Holst and Williamson 2008).

2. Theoretical part

The theoretical part of this doctoral dissertation provides background and theoretical knowledge essential for understanding the rationale, relevance, and significance of the conducted research.

2.1. Immune system

The immune system represents the defense system enrolled in the protection from the invading pathogens. The origin of the term immunity is Latin, derived from the word *immunitas* which means "exempt from public service" and referred to as a legal exemption from military service in ancient Rome. A science that deals with the study of the immune system is immunology and Louis Pasteur is considered to be the father of modern immunology due to its efforts to firmly establish the germ theory of disease during the 1860s. Interestingly, the practice of variolation was spread throughout England in the 1740s, but vaccination is not introduced before 1798 by Edward Jenner who realized that individuals who had cow's pox do not get smallpox. Although Jenner's observation was correct, it was not supported by an understanding of how immunity develops (Smith 2012). Besides infectious, non-infectious agents can also provoke responses of the immune system. Therefore one of the definitions of the immune response is: "reactions to the components of microbes as well as to macromolecules such as proteins and polysaccharides and low molecular weight chemicals that are recognized as foreign, regardless of the physiologic or pathologic consequence of such a reaction" (Abbas, Lichtman et al. 2007).

The host immunity can be divided into innate and adaptive immunity (Figure 1). The innate immunity or native is characterized by early reactions, during several hours after exposure of an organism to pathogens, and provides an early line of defense against microbes. The principal components of innate immunity are epithelial cells and their antimicrobial substances, phagocytic and natural killer cells, blood proteins (members of the complement system), and cytokines. The adaptive immunity is stimulated by the successive exposure to the "invaders" e.g. microbes and increases in extent and defensive potential with each exposure. There are two types of adaptive immune responses, called humoral immunity in which macromolecules like antibodies produced

in B lymphocytes are involved and cell-mediated immunity mediated by T lymphocytes (Abbas, Lichtman et al. 2007). The immune system fulfills its protective role through several important functions such as detection of the threat, which is referred to as immunological recognition; elimination of the threat by various immune's effector functions, prevention and decrease of damage to the host by immune regulation and development of immunological memory to respond quickly to future threats.
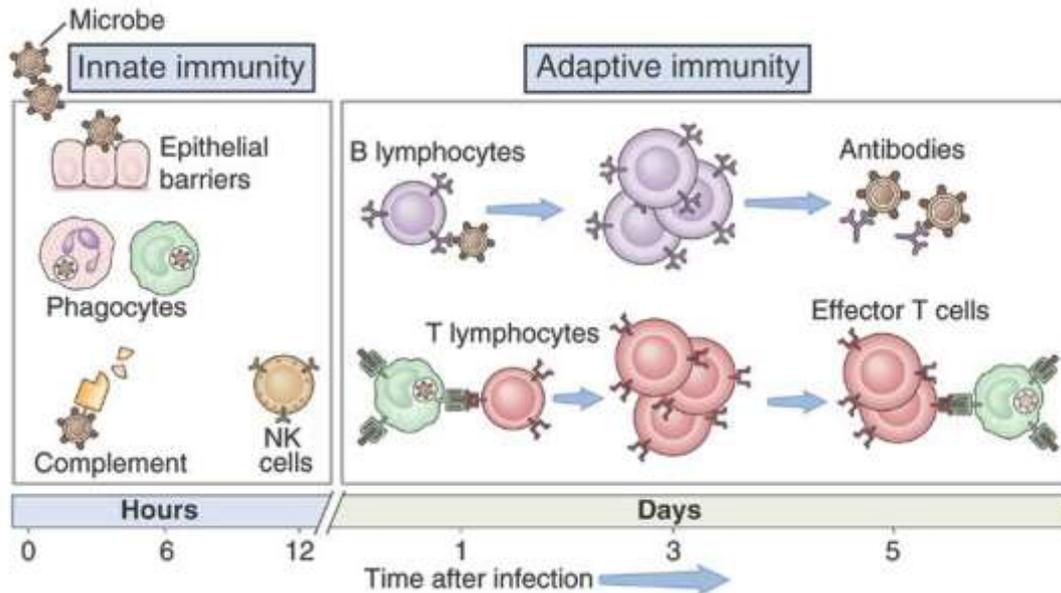
Figure 1. The host immunity – innate and adaptive immunity.[1] NK cells – natural killer cells

Failure and errors in the immune regulation lead to disease states such as allergy and autoimmune disease. The mechanism of allergic reactions can be divided into two phases (a) sensitization and memory and (b) effector phase. During the sensitization phase (Figure 2) allergens that reach and/or pass through the epithelial layer of gut, skin, or airways are being encountered by the resident dendritic cells (DCs), captured, processed, and presented within the major histocompatibility complex (MHC) class II molecules on the cell surface. In the lymph node antigen-presenting, DCs establish communication with naïve CD4^{+} T cells which further differentiate into allergen-

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[1] Reprinted from Abbas, A. K., A. H. Lichtman and S. Pillai (2007). Cellular and molecular immunology,

specific CD4^{+} Th2 cells. Furthermore, naïve B cells under the stimuli of Th2 cell, which produce high levels of interleukins (ILs) IL-4 and IL-13, and by recognition of endogenous antigens via IgM B cell receptor activate IgE isotype class-switching and differentiate into IgE memory B cells which can mature and differentiate into plasma cells. Plasma cells produce allergen-specific IgE antibodies which bind to the high-affinity IgE receptor (FcεRI) on the surface of basophils and mast cell. During the sensitization phase, there are no symptoms of allergy. Interestingly several studies have demonstrated that epithelial cells from resident epithelia are involved in this phase of sensitization, e.g. epithelial cells can produce cytokines such as IL-25, IL-33, IL-31, or thymic stromal lymphopoietin (TSLP or IL-7) upon stimulation contributing to the generation of allergen-specific CD4+ Th2 cells and allergic sensitization. Moreover, epithelial cells are key players in establishing immune tolerance to allergens (Palomares, Akdis et al. 2017).
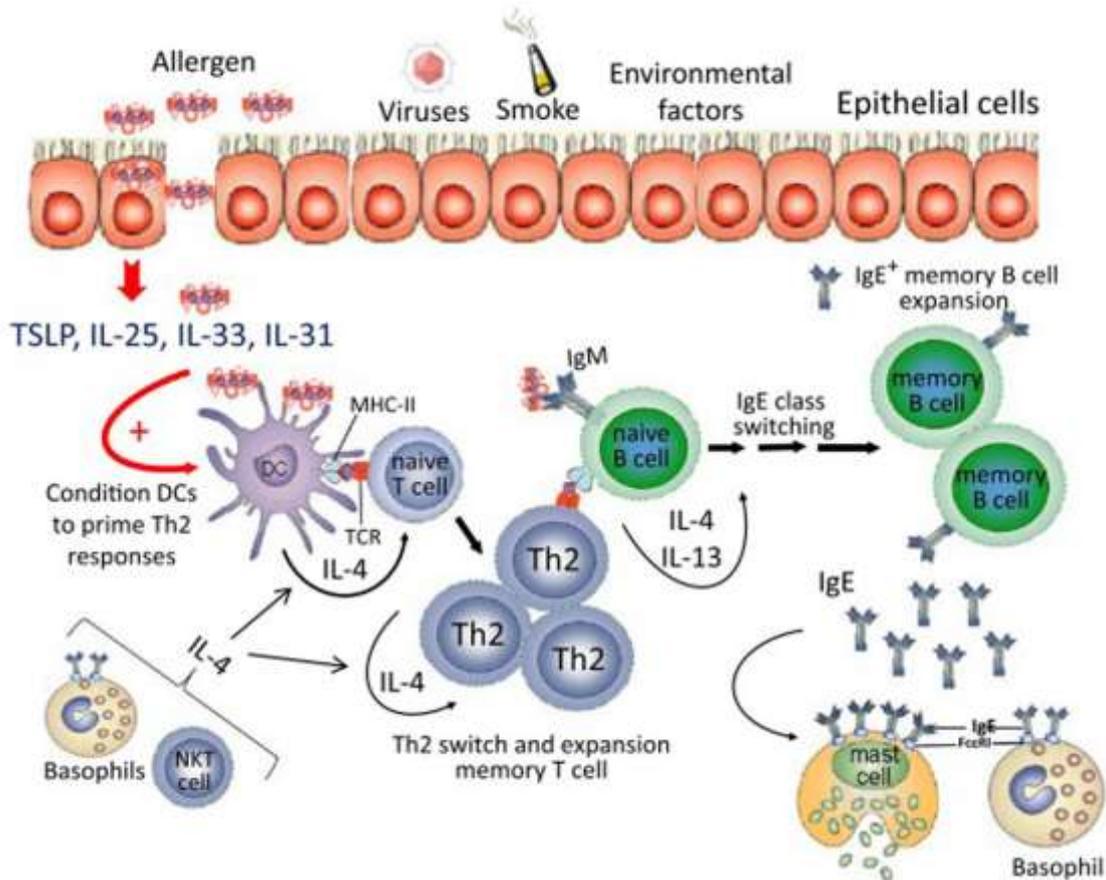
Figure 2. Mechanism of allergic sensitization.[2] TSLP - thymic stromal lymphopoietin, DC - dendritic cell, NKT cell - natural killer T cell, Ig - immunoglobulin, MHC - major histocompatibility complex, TCR – T cell receptor, Th – T helper.

The effector phase of the allergic reactions occurs when re-encountering the allergen leads to crosslinking of the IgE bound to FcεRI on the sensitized basophils and mast cells (Figure 3). This leads to the release of mediators such as histamine, heparin, proteases, prostaglandins, leukotrienes, and cytokines from activated basophils and mast cells referred to as the early stage of the IgE mediated hypersensitivity reaction. Besides the early stage of hypersensitivity, the allergic reaction can be also characterized by a late phase which starts several hours after exposure to the allergen. The release of mediators by basophils and mast cells triggers long-term inflammatory processes that involve attracting various immune cells to allergen sites, including Th2 lymphocytes, eosinophils, basophils, and monocytes. (Palomares, Akdis et al. 2017). During the

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[2] Reprinted from Palomares, O., M. Akdis, M. Martín-Fontecha and C. Akdis (2017). "Mechanisms of immune regulation in allergic diseases: the role of regulatory T and B cells." Immunological Reviews **278**(1): 219-236.

effector phase, individuals can experience various symptoms ranging from mild to severe anaphylactic reactions.
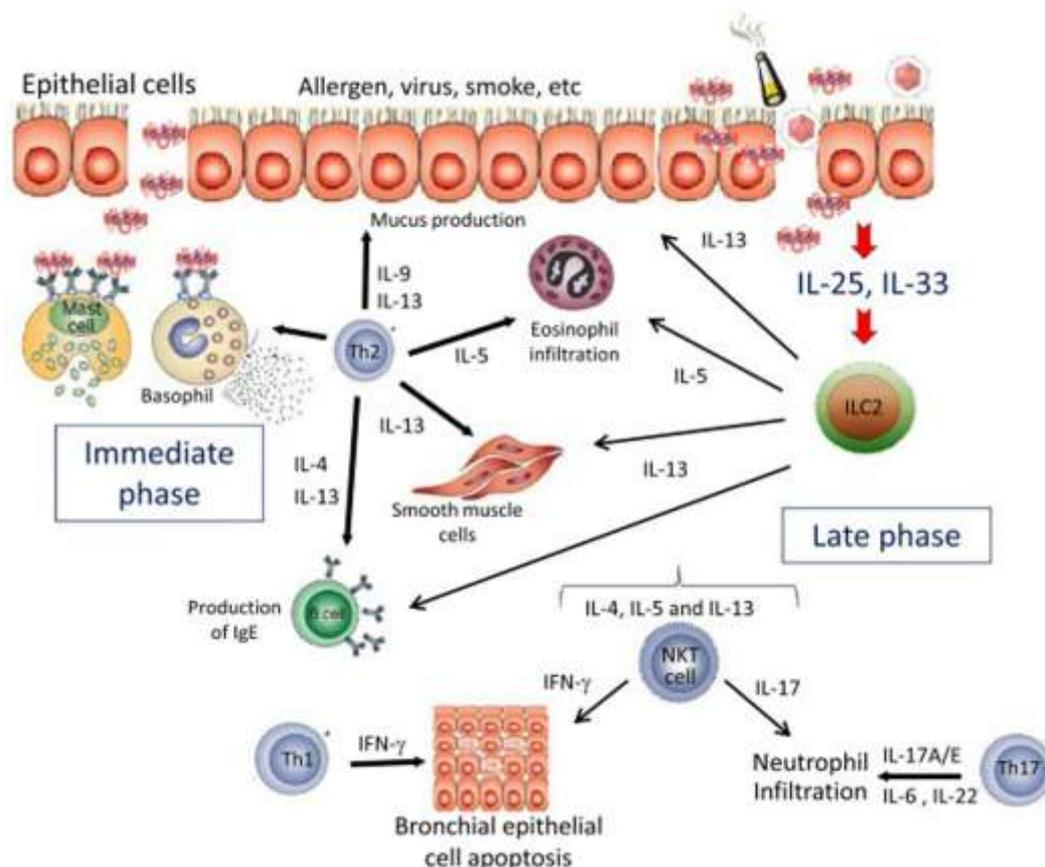
Figure 3. Effector phase of an allergic reaction.[3] IL – interleukin, Ig – immunoglobulin, IFN-γ – interferon γ, ILC2 – innate lymphoid cell type 2, Th – T helper, NKT cell - natural killer T cell.

2.1.1. The immune system underlying the gastrointestinal barrier

The immune system underlying the gastrointestinal tract (GIT) is a part of the common mucosal immune system (Figure 4). The widely used term "common mucosal immune system" should be replaced by the term "integrated mucosal immune system" since there is more and more evidence that immunization in one part of the mucosal immune system does not protect all other mucosal organs (Pabst and Rothkötter 2006). Although originally belonging to the cells of the intestinal epithelia, the cells specialized in sampling and presentation of antigens on the luminal side of GIT are microfold cells (M cells). M cells are located in intestinal lymphoid structures such as Payer's patches

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[3] Reprinted from Palomares, O., M. Akdis, M. Martín-Fontecha and C. Akdis (2017). "Mechanisms of immune regulation in allergic diseases: the role of regulatory T and B cells." Immunological Reviews 278(1): 219-236.

and isolated lymphoid follicles. The most abundant immune cell type underlying intestine are mononuclear phagocytes or macrophages and DCs. The main role of these cells is a sampling of the content in GIT and to orchestrate both innate and adaptive immune responses.

The immune cells are characterized and differ by the expression of some surface markers, macrophages with surface expression of CD11c and CX3CR1 are recognized as the intestinal macrophages involved in the antigen sampling which do not migrate to the mesenteric lymph nodes in physiological conditions (Schulz, Jaensson et al. 2009). Besides macrophages and DCs, lymphocytes are present in the intestinal mucosa. Naïve lymphocytes are primed in Payer's patches and mesenteric lymph nodes and through the expression of the gut-homing chemokine receptor 9 and integrin $\alpha4\beta7$ can migrate within the gut (Mora, Bono et al. 2003). Plasma cells that produce IgA antibodies and CD4$^+$ T cells are mainly distributed in the lamina propria while CD8$^+$ T cells are in the epithelium (Mowat 2003). Different subsets of CD4$^+$ T cells are present such as T helper (Th) cells like Th17, which produce IL-17, Th1, and T regulatory (Treg) cells. Furthermore, Treg cells can be divided into two subsets of cells forkhead box protein P3 (Foxp3) and Type 1 regulatory T (Tr1) cells which are both involved in the tolerogenic immune response. Innate lymphoid cells (ILCs) are recently discovered cells in the gut which also belong to the immune system. The main characteristic of ILCs is that they do not possess receptors and upon stimulation do not undergo clonal selection. Within this cell population three cell subsets, ILC1, ILC2, and ILC3, can be recognized. ILC1 resemble the most the natural killer (NK) cells but unlike them lack cytolytic activity and have a different developmental pathway. It is believed that ILC2 are typically associated with the Th2-response, while ILC3 produce high amounts of IL-17A, IL-22 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Walker, Barlow et al. 2013, Parigi, Eldh et al. 2015, Geremia and Arancibia-Cárcamo 2017). The all stated above demonstrates the complexity of the gut immune system and the complex interplay ongoing among the different subsets of the immune cells which are present in the layers under the intestinal barrier.
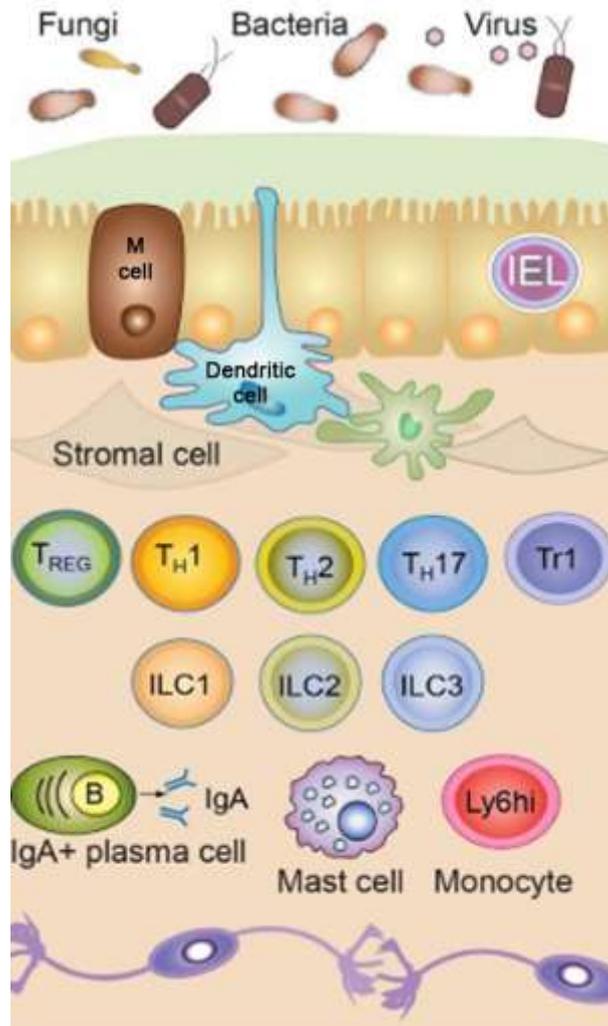
Figure 4. The immune cells underlying the gastrointestinal barrier.[4] IEL - Intraepithelial lymphocyte, M cell - Microfold cell, T_{REG} – T regulatory cell, T_H – T helper, ILC - Innate lymphoid cells, Ig – Immunoglobulin, $Ly6^{hi}$ - lymphocyte antigen 6 "high" monocyte.

2.1.1.1. *Dendritic cells*

Dendritic cells were discovered in the 1970s by two scientists Ralph Steinman and Zanvil Cohn (Steinman and Cohn 1973), but their role in the immune system was discovered 40 years later. Dendritic cells efficiently stimulate B and T lymphocytes. While B cells can directly recognize antigen through their receptors, T cells need antigen to be processed and presented to them by the antigen-presenting cells to get

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[4] Modified and reprinted from Parigi, S. M., M. Eldh, P. Larssen, S. Gabrielsson and E. J. Villablanca (2015). "Breast Milk and Solid Food Shaping Intestinal Immunity." Frontiers in Immunology **6**(415).

activated. Antigen-presenting cells possess on their surface the MHC molecules within which they present fragments of the processed antigens. On the other side, T cells possess T cell antigen receptors (TCRs) which recognize antigen fragments presented by the MHCs. There are two types of MHC molecules, MHC class I which stimulate cytotoxic T cells, and MHC class II which stimulates T helper cells. APCs process and present intracellular antigens within MHC class I molecules to cytotoxic T cells which upon activation directly kill a target cell. On the other hand, extracellular antigens uptaken and processed by the APCs are presented within MHC class II to the Th cells which are involved in the immune-regulation (Abbas, Lichtman et al. 2007).

Dendritic cells can be classified as plasmacytoid DCs (pDCs) and classical DCs (cDCs). The plasmacytoid DCs unlike cDCs express low levels of MHC class II, costimulatory molecules, and CD11c integrin as well as pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs). Upon recognition and uptake of foreign nucleic acid molecules, pDCs produce high amounts of type I interferon and acquire the ability to present foreign antigens (Merad, Sathe et al. 2013). The classical DCs have an important role in capturing foreign antigens and their subsequent processing and presentation to T lymphocytes, and also in sensing tissue injuries. Also, cDCs are involved in the development of tolerance to self-antigens which are cellular proteins, peptides, enzyme complexes, ribonucleoprotein complexes, DNA, and post-translationally modified antigens. In the human body, it is estimated that cDCs contribute from 1 to 5 % to the total cell number of a specific organ, and that depending on the classification of DCs. The estimation of the number of cDCs in some organs can be quite complex, especially in the human intestine.

The cDCs in the gut are organized within lymphoid tissue such as Peyer's patches, draining lymph nodes as well as in the lamina propria of the small intestine and colon. The cDCs have been divided into subsets according to the expression of specific markers or according to the cellular origin. The surface markers according to which DCs can be classified are: CD8α, CD11b (also known as integrin αM), CD24, CD64 (also known as FcγRI), CD103 (also known as integrin αE), CD172a (also known as SIRPα and SHPS1), CX3C-chemokine receptor 1 (CX3CR1), F4/80, XC- chemokine receptor 1 (XCR1), CLEC9A (also known as DNGR1) and E-cadherin (also known as

cadherin 1). The cDCs in the lamina propria can be divided into cDC1 which express XCR1 and cDC2 which express signal regulatory protein α (SIRPα/CD172a). The cDC2 can further be divided to CD103⁻CD11b⁺ cells and a gut-specific $CD103^+CD11b^+$ population although under the influence of transforming growth factor β (TGFβ) $CD103^-CD11b^+$ give rise to $CD103^+CD11b^+$. According to the cellular origin cDCs in the lamina propria originate from pre-cDC progenitor under the stimuli of cytokine Fms-like tyrosine kinase 3 ligands (FLT3L). The main characteristic of the cDCs in the lamina propria is their ability to migrate to the draining lymph nodes in a CCR7-dependent manner and more importantly to interact with recirculating T cells (Banchereau and Steinman 1998, Guilliams, Ginhoux et al. 2014, Stagg 2018).

2.2. Organization of the gastrointestinal tract

The definition of the GIT also called the alimentary canal, is "an open-ended or hollow-like tube, organized into regions and layers with each having peculiar features in structure and functions" (Welcome 2018). The components of the GIT are mouth, pharynx, esophagus, stomach, small intestine, large intestine, rectum, and anus (Figure 5). The components of the upper GIT tract are the mouth, pharynx, esophagus, and stomach, while the lower GIT includes the small and large intestine, rectum, and anus. The important feature of the digestive system is that it connects other systems such as the endocrine, lymphatic, muscular, nervous, circulatory, respiratory, urinary, reproductive, skeletal, and integumentary. The major tissue types which form the GIT and associated organs (liver and pancreas) are epithelial, muscle, nervous, and connective tissue which are formed from different cells associated and with a specific function (Welcome 2018).
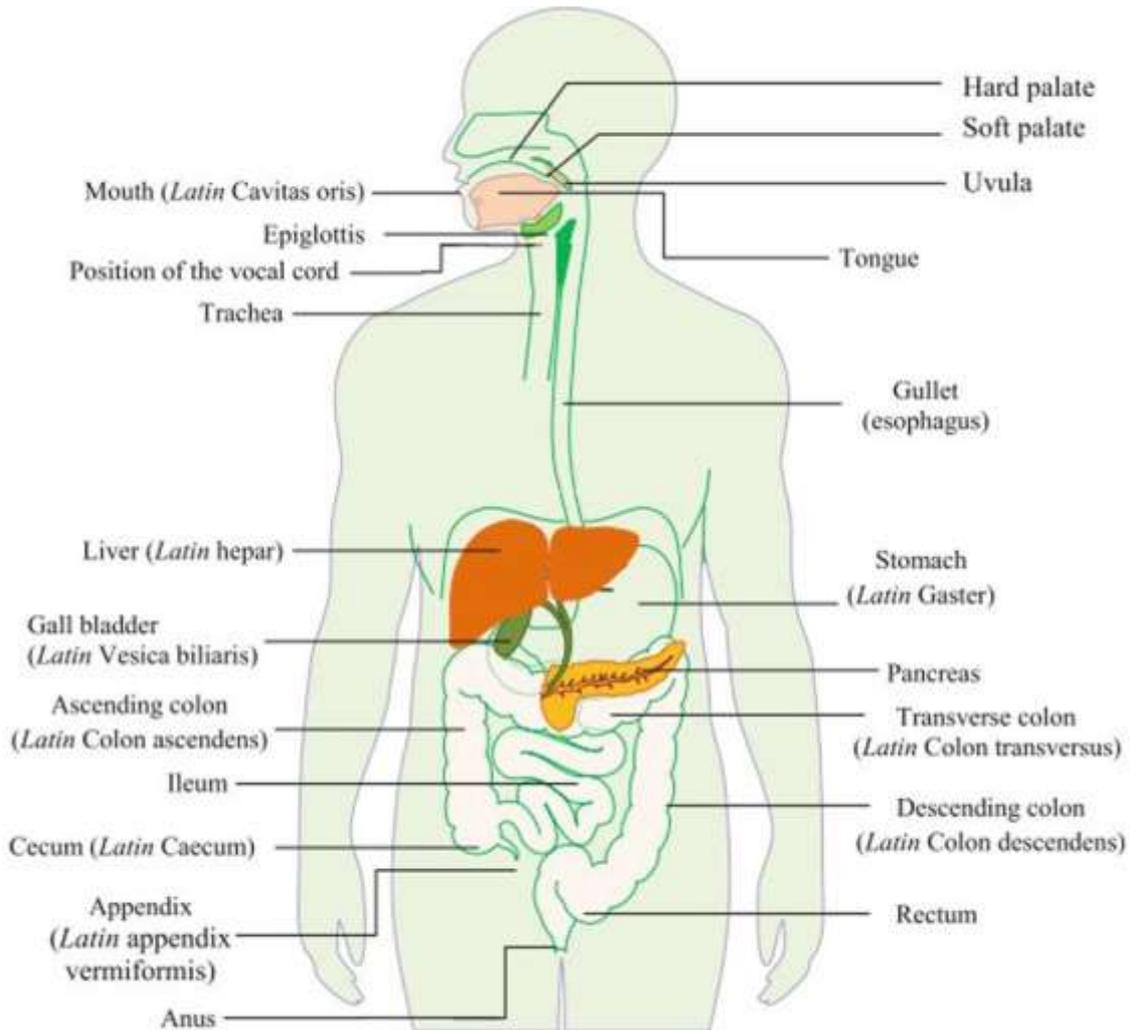
Figure 5. The organization of the gastrointestinal tract in the adult human.[5]

The small intestine (average length 5 – 6 m) consists of the duodenum, jejunum, and ileum and it is the major site for nutrients and drug absorption which is enabled by the great surface area. The average length of the duodenum is 25 cm, the jejunum is around 2.5 m and the ileum is 3 m. The major absorptive cells of the small intestine are enterocytes which on the luminal surface contain microvilli. The great surface area of the small intestine is achieved by the tightly wrinkled interior cell wall which forms the so-called intestinal crypts built from enterocytes organized in the finger-like structures known as the villi which additionally increase the surface area from 0.33 m^2 to 120 m^2 with the villi and microvilli extensions (Figure 6) (Kararli 1995).

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[5]Reprinted from Welcome, M. O. (2018). Gastrointestinal physiology: Development, principles and mechanisms of regulation.
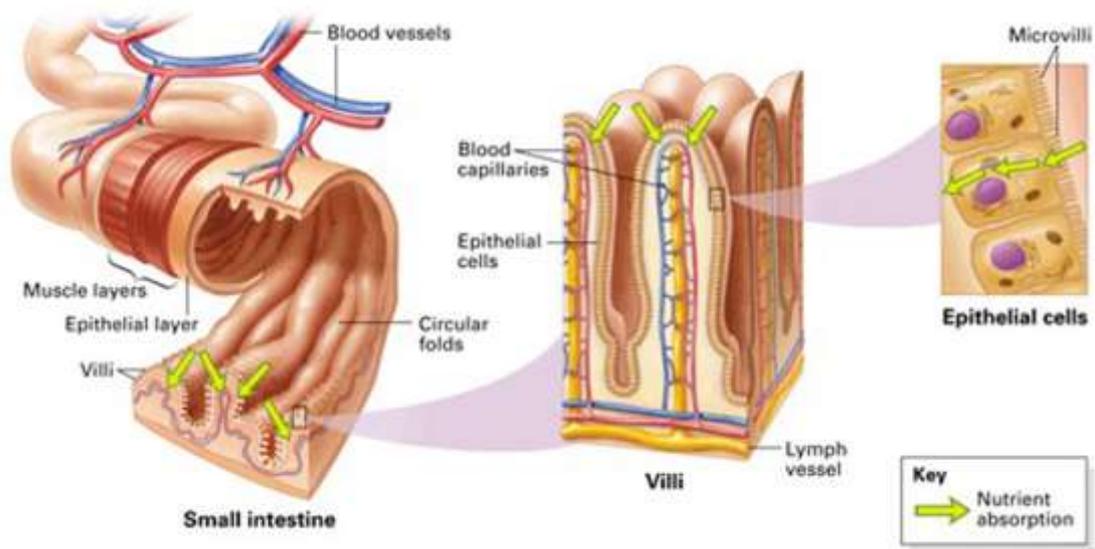
Figure 6. Organization of the small intestine.[6]

2.2.1. The intestinal barrier

The intestinal barrier is composed of the physical, biochemical, and immune elements but the main component is the intestinal epithelial layer which represents the physical barrier between the lumen of the GIT and the body. The lumen of the intestine which is in direct contact with the outer environment is coated with a mucus layer. Besides as a physical barrier, the secreted mucus facilitates the movement of food through the intestine. The rigid structure of the mucus-building proteins and its high cohesion give mucus the sticky gel-forming appearance. The major components of the mucus layer are mucins secreted by the goblet cells (GCs) located in the intestinal epithelial layer among enterocytes (Figure 7). Mucins are high molecular weight glycoproteins characterized by a high content of sugar moieties that are attached to the amino acids side-chains of serine or threonine by O-glycosidic bonds (Johansson and Hansson 2013). Changes in the patterns of mucin secretion are probably the primary event in Crohn's disease or secondary event during inflammation of GIT (Niv 2016).

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[6] Image taken from the site http://www.Mtchs.Org/bio/text/chapter29/concept29.2.html
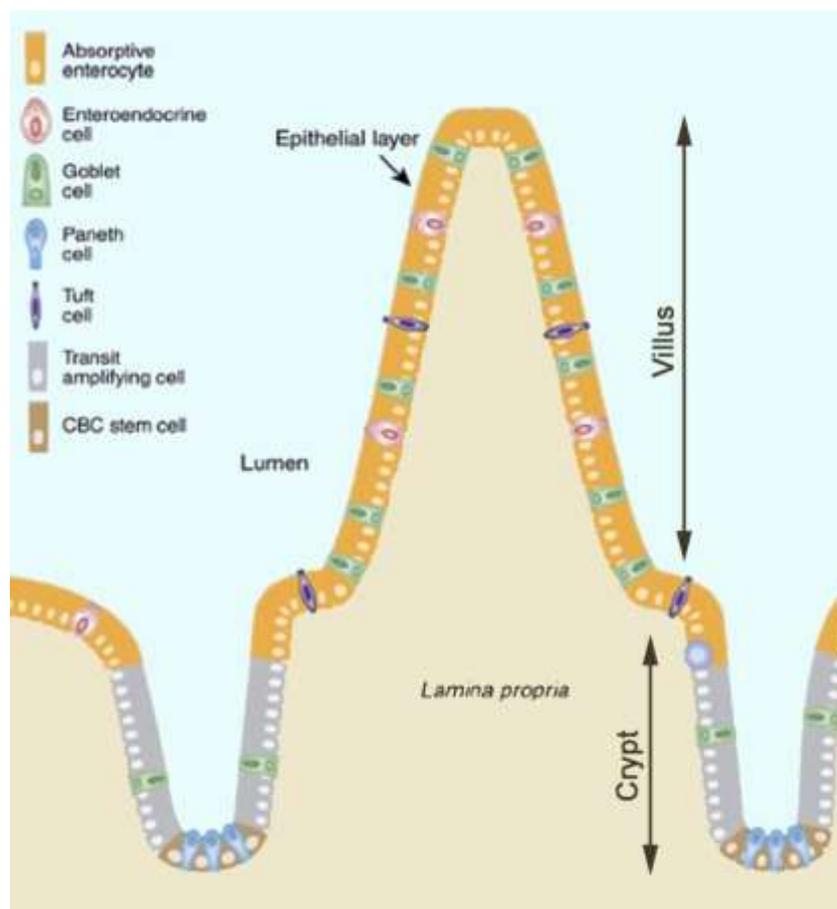
Figure 7. Organization and belonging cells of the intestinal epithelial layer.[7] M cell -
Microfold cell, CBC stem cell - crypt base columnar stem cells.

    The intestinal epithelial layer of villus and crypts consists of enterocytes as major
absorptive cells, goblet cells (GCs), enteroendocrine cells, Paneth cells, stem cells, and
tuft cells (Figure 7) (Okumura and Takeda 2017). The **GCs** are specialized in mucus
secretion and are scattered among enterocytes in the epithelium of the small intestine
and colon. Interestingly, the number of GCs along GIT ranging from relatively few in
the duodenum up to many cells in the colon. Recent findings suggest that except for
their role in the barrier maintenance GCs are involved in innate immunity. These cells
beside mucins can secrete anti-microbial proteins, chemokines, and cytokines, but
furthermore, they are capable of forming the goblet cell-associated antigen passages and
induction of the adaptive immune responses (Knoop and Newberry 2018).
Enteroendocrine cells are localized individually among the enterocytes and other
exocrine cells of the villus. The main function of these cells is the production of gut

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[7] Modified and reprinted from Gerbe, F. and P. Jay (2016). "Intestinal tuft cells: epithelial sentinels
linking luminal cues to the immune system." Mucosal Immunology **9**(6): 1353-1359.

hormones which have key roles in the coordination of processes such as food digestion and absorption, secretion of insulin, and appetite regulation (Gribble and Reimann 2019). **Paneth cells** are located at the ends of intestinal crypts and their role is to secrete antibacterial proteins such as defensins to protect adjacent stem cells inside of the wall of the crypt (Ganz 2000). The **intestinal stem cells** are undifferentiated cells capable of continuous cell division to replenish the intestinal epithelium. Stem cells and approximately once every four days completely replace all the enterocytes and GCs (Umar 2010). The **tuft cells** located in the villus have an important role in promoting immunity against parasitic helminths and protozoa. Studies on the mice showed that tuft cells are capable of producing IL-25 which further promotes the rapid expansion of ILC2. Interestingly, tuft cells possess chemosensory receptors for sweet, bitter, and umami taste and they are involved in the perception of compounds with these tastes in food (Harris 2016).

2.2.1.1. Enterocytes

The enterocytes are the main building blocks of the intestinal epithelial layer of the villus and they have the main role in the digestion process so they are well-studied. Enterocytes are the main absorptive cells responsible for the uptake of ions, water, nutrients, vitamins, and unconjugated bile salts. Besides this role, recently it was discovered that they have an important role in the induction of oral tolerance to food proteins. The establishment and maintenance of oral tolerance is a very complex and demanding process that requires the participation of enterocytes in the numerous mechanisms. Those mechanisms include the constant communication between enterocytes and the intestinal mucosa-associated lymphoid tissue (MALT) to maintain the tolerance to food and microbial antigens (Miron and Cristea 2012). It is demonstrated that enterocytes possess the so-called pattern-recognition receptors (PRRs), including Toll-like receptors (TLRs), by which they can sense the presence of microbes (Hornef and Bogdan 2005).

The transport of the allergens from the lumen of the intestine to the underlying immune system occurs by several routes: by paracellular diffusion, *via* M cells in Peyer's patches, via goblet cell-associated antigen passage, and by enterocyte transcytosis. Uptake of allergens by enterocytes can occur as an active process under

stimuli of the pre-existing allergen-specific IgA and IgE antibodies because enterocytes possess low-affinity IgE receptor designed as CD23 (Tu, Salim et al. 2005). Enterocytes constitutively express a low level of MHC class II molecules and in conditions of mucosal inflammation such as inflammatory bowel disease the expression of the MHC class II molecules increases (Mayer, Eisenhardt et al. 1991). Cathepsin proteases involved in the MHC class II-mediated antigen processing are also constitutively expressed. Furthermore in the enterocytes two distinct pathways of MHC class II antigen processing are noted, one in the presence of inflammation and another in its absence (Hershberg, Framson et al. 1997). Enterocytes are very dynamic cells and in constant contact with adjacent cells and tissues, actively communicating with T-cells, dendritic cells, granulocytes, monocytes, macrophages, and mast cells (Shaykhiev and Bals 2007).

2.3.   Food digestion

The process of food digestion involves mechanical, chemical, and enzymatic food breakdown. Food digestion starts in the mouth where food is chewed and mixed with saliva. The saliva contains the digestive enzyme α-amylase which is secreted by the parotid glands. Alpha-amylase catalyzes the reaction of starch hydrolysis into maltose and other small glucose polymers with three to nine glucose units (Munegumi, Inutsuka et al. 2016). But less than 5 % of the starch is hydrolyzed in the mouth because the food remains there for less than 5 min (Woolnough, Bird et al. 2010). Next, the slippery broken-down food mass named bolus moves to the lower parts of the digestive tract. Through the pharynx, the bolus arrives at the esophagus where peristaltic contractions and the pressure of the bolus stimulate the lower esophageal sphincter to open and food moves into the stomach. The digestion process continues in the stomach which strong muscular contractions additionally mix and mash the food. The lumen of stomach wall which is in the direct contact with outer environment is coated with different types of cells. These cells secrete mucus, hydrochloride acid and enzyme pepsin which additionally contributed to the digestion of bolus, which after mechanical, chemical and enzymatic digestion becomes semiliquid mass named chyme. Importantly, a thick layer of mucus coats the stomach wall in order to protect it from digesting itself. The time which food spends in the stomach depends on the food composition, meals

which have high fat and protein content take longer to break down compared to meals with high carbohydrate content. Usually it takes a few hours to completely empty the stomach content. From the stomach, chyme enters into the duodenum, and digestion is aided by the juices secreted by the liver, pancreas, and gallbladder. An interesting fact is that pancreas secrets up to 1.5 liters of pancreatic juice per day which is composed of water and contains bicarbonate ions which are very important in the neutralization of the acidic chyme and enzymes which are important for a further breakdown of the proteins, lipids, and carbohydrates. Furthermore, bile is excreted in the intestine which is required for lipid digestion. Bile is synthesized in the liver, stored in the gallbladder, and excreted via a duct. In the intestine, bile surrounds the fats and acts as an emulsifier e.g. detergent which emulsifies fats. This process is very important for the digestion of fats because allows fat to move in the intestinal water environment and its digestion by lipase. Because of the peristaltic contraction of a muscle in the intestinal wall, chyme moves in this part of GIT into both directions back and forth this enables further chyme mixing. In the intestine, a big portion of the meal components are completely broken down and chyme is now a mixture of amino acids, emulsified fatty acids, and monosaccharides. The absorbing surface area in the intestine is enormous (120 m^2) and covered with a thick layer of mucus which enables that nutrients such as amino acids, monosaccharides, water-soluble vitamins, and other components reach to enterocytes, the main absorptive cells. These compounds are transported through epithelia by trans-cellular transport and reach blood circulation. On the other hand, the major products of lipid digestion like fatty acids and 2-monoglycerides, as well as, fat-soluble vitamins and other lipids enter the enterocyte by simple diffusion across the plasma membrane. Inside enterocyte fatty acids and 2-monoglycerides are used for the synthesis of triglycerides and packaged with cholesterol, lipoproteins, and other lipids into particles called chylomicrons. They are transported first into lymphatic vessels and after that into blood vessels (Kong and Singh 2008, Boland, Golding et al. 2014).

The process of food digestion is very efficient as reflected in the fact that less than 10 % of food remains undigested. Among the undigested food, the portion is indigestible fibers which together with undigested food move from the small intestine to the large intestine. The main process in the large intestine is water reabsorption because water is present in the food but also in the gastric and the pancreatic juice which in total

leads to a few hundred milliliters of liquid in the intestine. This would be tremendous water lost if we know that in the human body water is highly conserved. Besides water, also, the absorption of minerals such as sodium and potassium occurs. In this part GIT, the number of commensal bacteria is huge (greater than 10^{14}) and this number is bigger than the total cell number in the human body (10^{13}) this insight or fact can be frightening from the aspect that bacteria are disease-causing (Sender, Fuchs et al. 2016). However, the majority of bacteria in the large intestine are not harmful and some are even required and beneficial for human health. Some essential nutrients like short-chain fatty acids, vitamin K and B12 are synthesized by the commensal bacteria in the large intestine from the undigested food and fibers (Koh, De Vadder et al. 2016).

2.3.1. Enzymes involved in food digestion

The food digestive process is impossible without the different enzymes which are spatially distributed. Different parts of the human digestive system are characterized by their specific cocktail of enzymes which operate at different optimal pH range. In the saliva (pH 7.2) the main digestive enzyme is α-amylase whose systematic name is 1,4-alpha-D-glucan glucohydrolase. It catalyzes the rapid hydrolysis of 1,4-alpha-D-glucosidic bonds when the next bond in the sequence is 1,6. There are six isoforms of this enzyme, which the optimal pH range is around 7. Starch is a natural substrate of this enzyme that hydrolyzes into maltose, maltotriose, maltotetrose, and some higher oligosaccharides by the action of this enzyme. (Zakowski and Bruns 1985) Another digestive enzyme found in the saliva is lingual lipase, present in very low levels and secreted by the glands of von Ebner located in the human tongue. Lingual lipase was first detected in the rat and it seems that in humans its role in fat digestion is not so important as in rat (Dawes, Pedersen et al. 2015).

Besides the lingual, a more potent gastric lipase is secreted in the gut. The main enzymes secreted in the stomach are pepsin, which will be discussed later and already mentioned gastric lipase. The main feature of gastric enzymes is the retention of their activity in the acidic environment at pH 2 which classifies them as an extremophile. Gastric lipase is involved in the gastrointestinal lipolysis of dietary fat, catalyzes the hydrolysis of triacylglycerols to free fatty acids, diacylglycerol, monoacylglycerol, and glycerol, and shows preferential hydrolysis at the sn-3 position of triacylglycerol. It is

secreted by the gastric chief cells in the mucosa of the stomach. For its activity, it does not require cofactors like the pancreatic lipase (Aloulou and Carrière 2008, Nomura and Casida 2016).

Pancreatic juice is rich with digestive enzymes like trypsin, chymotrypsin, carboxypeptidase, elastase, pancreatic lipase, sterol esterase, phospholipase, several nucleases, and pancreatic amylase. **Trypsin** is a digestive enzyme secreted in the pancreas in an inactive form (zymogen) as trypsinogen to prevent enzyme activation in the pancreas. In the small intestine, trypsinogen is cleaved by the enteropeptidase from the intestinal mucosa into the active form trypsin. Furthermore, trypsin itself can cleave more trypsinogen and this process is the so-called autoactivation. Trypsin is a serine protease responsible for the cleavage of the protein peptide bonds at the carboxyl end of the amino acids lysine and arginine. This process is crucial for the absorption of dietary proteins because large proteins are broken down into smaller peptides which can further be degraded to amino acids by the action of other proteases and peptidases (Voet and Voet 2011). **Chymotrypsin** is also a serine protease active in the small intestine produced in the pancreas as a zymogen chymotrypsinogen. In the small intestine, trypsin cleaves chymotrypsinogen into its active form chymotrypsin. Chymotrypsin preferentially cleaves peptide amide bonds in which the N-terminal amino acid is tryptophan, tyrosine, phenylalanine, or leucine. **Carboxypeptidase** unlike trypsin and chymotrypsin catalyzes the hydrolysis of the peptide bonds at the carboxy-terminal (C-terminal) end of the proteins or peptides. There are several types of carboxypeptidases classified according to their active site mechanism and substrate preference. Carboxypeptidase A (A-aromatic, aliphatic amino acids) which cleaves peptide bonds of amino acids containing aromatic or branched hydrocarbon chains is present in the small intestine. It is also produced in the pancreas in the zymogen form, procarboxypeptidase, and it is activated in the small intestine by trypsin cleavage. There are two isoforms of carboxypeptidase, A1 and A2. Another pancreatic carboxypeptidase is carboxypeptidase B (B-basic) that cleaves peptide bonds of positively charged amino acids (arginine, lysine). (Laethem, Blumenkopf et al. 1996) **Elastase** is another serine protease that breaks elastin a fiber which together with collagen makes the connective tissue. Also, elastase cleaves the specific peptide bonds on the carboxyl side of small, hydrophobic amino acids such as glycine, alanine, and valine. **Pancreatic lipase**

(pancreatic triacylglycerol lipase or steapsin) hydrolyzes dietary fats in the human digestive system. It converts triglyceride substrates from the ingested fats and oils to monoglycerides and free fatty acids. Together with bile acids, pancreatic lipase is the main digestive enzyme responsible for lipid digestion, lipids are first emulsified by bile salts and then broken down by the lipase. Interestingly, unlike pancreatic proteases, lipase is secreted in its already active form but for its activity requires the presence of another enzyme colipase. The resulting free fatty acids and monoglycerides are moved along the small intestine and absorbed from enterocytes into the lymphatic system by a specialized vessel called the lacteal (Pandol 2010). The initial metabolic transformation of dietary cholesterol and its esters is catalyzed by **sterol esterases**. The cholesterol esterase from pancreatic juice catalyzes the hydrolysis of cholesterol esters to free sterol, which is the form required for absorption in the intestinal lumen. Further, the sterol is re-esterified by mucosal sterol esterase before absorption into the lymphatic system. (Hyun, Kothari et al. 1969) **Phospholipases** are enzymes that catalyze the hydrolysis of the phospholipids from the dietary fats into free fatty acids and hydro-, lipophilic substances. According to the position of bond in phospholipid which catalyze they are divided into four classes A (subclasses A1 and A2), B, C, and D. Pancreatic phospholipase A2 is secreted as a zymogen, pro-phospholipase A2, and it is activated in the small intestine by trypsin cleavage (Gudgeon, Patel et al. 1991). **Nucleases** catalyze the cleavage of the phosphodiester bonds between the nucleotides of nucleic acids. According to the regions of the nucleic acids which they cleave they, are divided into endonucleases, which act on the middle regions, and exonucleases, which act on the end of the target molecule (Whitcomb and Lowe 2007).

2.3.1.1. *Pepsin*

Pepsin is a proteinase secreted by the chief cells of the human gastric mucosa. Inside the chief cells, the enzyme is stored in granules as inactive zymogens. In the stomach, zymogen-pepsinogen is activated by low pH below 4.5 but it is easily inactivated by pH above 7.0. Unlike pepsin, pepsinogen contains 44 additional amino acid residues which "cover" the enzyme's active site and prevent premature enzyme activation. The low pH value (around pH 2) in the stomach causes removal of the additional amino acids which leads to alteration of the protein structure, so the enzyme's active site becomes

uncovered and available to bind substrate. Besides this, for the protein to be active, one of the two aspartate residues in the catalytic site has to be protonated (Asp 215), and the other deprotonated (Asp32). This occurs between pH 1 and 5, so this is the optimal pH range of pepsin activity. Because pepsin has two aspartate residues in the active site it belongs to the family of aspartic protease. Pepsin is a monomer composed of two domains. It has 326 amino acid residues with a high percentage of acidic residues and has a molecular weight of 34.6 kDa. The secondary structure predominates beta-sheet but also has a limited number of alpha-helices. There are four reported pepsin proteins: pepsin A (the predominant gastric protease) and minor forms, pepsin B (parapepsin I), C (gastricsin), and D (an unphosphorylated version of pepsin A) (Lee and Ryle 1967). Pepsins B and C share a higher degree of homology (Narita, Oda et al. 2002). The reaction mechanism of pepsin catalysis is shown in Figure 8. In the first step of the reaction oxygen from the water, the molecule performs the nucleophilic attack on the carbonyl carbon on the substrate. This attack of a water molecule on the peptide bond is facilitated by the attack of deprotonated Asp32 on the water proton whereby oxygen becomes a stronger nucleophile. At the same time, the oxygen of the carbonyl group attacks the proton Asp215, which is protonated, which leads to the formation of a transition state. In the transition Asp32 is protonated, Asp215 deprotonated and the substrate is in the amid dihydrate form. In the next step, there is proton transfer from the Asp residue to the nitrogen of amide bond and from the hydroxyl group of carbonyl carbon to the Asp residue as well as rearrangement of electrons in the peptide bond which leads to its cleavage (Garrett and Grisham 1999).
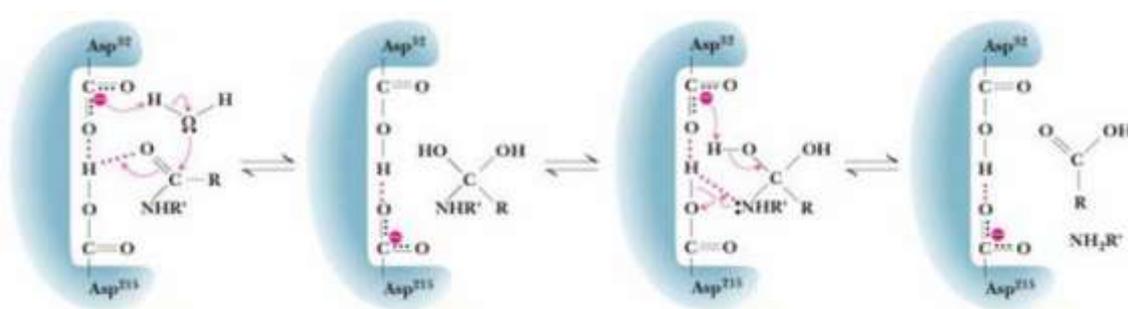
Figure 8. Pepsin reaction mechanism.[8]

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[8] Reprinted from Garrett, R. H. and C. M. Grisham (1999). <u>Biochemistry</u>. Fort Worth, Orlando, Saunders College Publ.

2.4. Food Allergies

The adverse reactions to food (Figure 9) can be immune-mediated, non-immune mediated, and toxic. Immune-mediated reactions can be divided into food allergy and coeliac disease (Turnbull, Adams et al. 2015). Food allergies represent an emerging health problem especially in the developing countries and within the young children population. There is no precise information about its prevalence but it could be stated that ''food allergy affects more than 1 to 2% but less than 10% of the population''. The most common allergens in children are cow's milk (2.2%), peanut (1.8%), and tree nuts (1.7%), while the most common allergens in adults are shellfish (1.9%), fruits (1.6%), and vegetables (1.3%) (Sicherer and Sampson 2014).
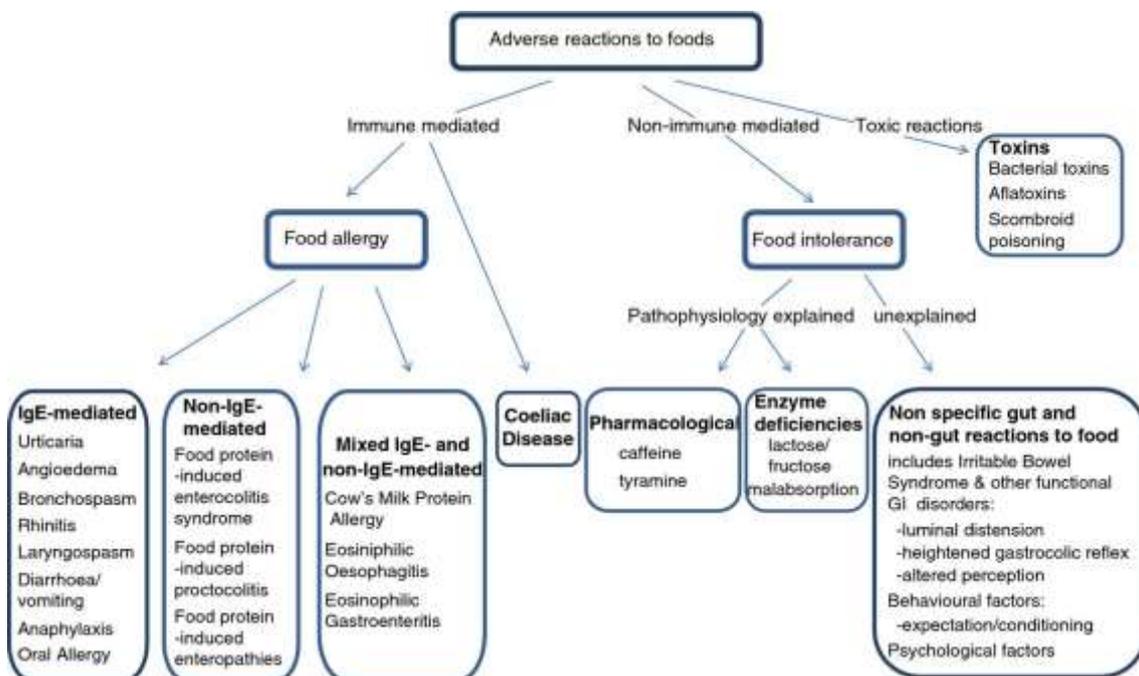
Figure 9. Adverse reactions to foods.[9]

In food allergies, the immune response can be IgE-mediated, non-IgE-mediated, or a mixture of both. During IgE-mediated food allergies the first step is the phase of allergen sensitization when specific IgE antibody to food allergen secrets into the serum. In the second phase or the effector phase, symptoms and signs of food allergy occur after the exposure to the food allergen. Non-IgE-mediated food allergies are

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[9] Reprinted from Turnbull, J. L., H. N. Adams and D. A. Gorard (2015). "Review article: the diagnosis and management of food allergy and food intolerances." Alimentary Pharmacology & Therapeutics **41**(1): 3-25.

characterized mostly by T cell-mediated processes. The third type of food allergies is mixed IgE and non-IgE-mediated food allergy such as eosinophilic inflammation of GIT (Turnbull, Adams et al. 2015).

Sensitization to food allergens occurs in the GIT and oral cavity and those are well-known routes which include class 1 or oral allergens such as milk, egg, or peanut. Another sensitization route is *via* respiratory tract which includes class 2 food allergens or aeroallergens such as the major birch pollen allergen Bet v 1. The immune responses to class 2 food allergens cross-react with homologous food allergens, such as the major apple allergen Mal d 1, and cause oral allergy syndrome. (Valenta, Hochwallner et al. 2015) Furthermore, it is proposed that the third route of sensitization to food allergens (e.g. peanut) is via skin contact, but still, there is an ongoing research of this process (Asero and Antonicelli 2011). During food ingestion in the stomach and intestine proteins from food are digested to peptides by digestive enzymes at appropriate pH levels. However, the remaining intact food proteins and large peptides are capable of reaching the intestinal cells and as such transported from the lumen of the gut to the mucosa by the epithelial cells, M cells localized above the Peyer's patches or by mucosal DCs which extend their dendrites into the gut lumen (Sampson, O'Mahony et al. 2018).

It is well known that the largest secondary lymphoid organ in the human body is gut-associated intestinal lymphoid tissue. This is understandable because, from the one side, ingested antigens are constantly sampled and recognized as harmful or non-harmful by the immune cells of GIT but from the other side some antigens like commensal bacteria must be actively ignored by these cells. All this explains the complexity of intestinal lymphoid tissue as well as the regulatory processes occurring within this tissue (Nowak-Wegrzyn, Szajewska et al. 2017).

To establish and maintain a physiological immune response, it is important to establish tolerance to food proteins transported through the epithelium in the absence of other dangerous signals. Currently, scientists show great interest in the understanding mechanism of establishing tolerance. The study on mice showed that the population of CD103^{+} DCs after the uptake of food antigens in the gut migrates to the lymph nodes to "educate" the naïve T cells (Figure 10a). The characteristic of CD103^{+} DCs is the

induction of Treg cells (Iwata, Hirakiyama et al. 2004, Coombes, Siddiqui et al. 2007). Also, CD103^{+} DCs express aldehyde dehydrogenase which enables the metabolism of vitamin A to retinoic acid, which is important in the regulation of T cells. T-cells possess the retinoic acid receptor and its interaction with retinoic acid induces the expression of the gut-homing receptor α4β7 and FOXP3, the master transcription factor responsible for inducing and maintaining a Treg phenotype (Elias, Laurence et al. 2008). It was shown that αvβ8 integrin expressed on DCs generate the signal which causes the release of immunosuppressive TGFβ which is involved in the further Treg cell induction (Worthington, Czajkowska et al. 2011). According to all above mention, in the GIT there is a very complex interplay between DCs and T-cells.

The food antigens which reach the bloodstream enter the portal circulation and pass through the liver where again encounters the tolerogenic populations of APCs, the resident macrophages (Kupffer cells), and liver sinusoidal endothelial cells. This process is very important for the explanation of an unusually high rate of new-onset food allergy in the individuals with liver-transplantation because there is a mismatch between MHC on their T cells and MHC on the liver allograft (Boyle, Hardikar et al. 2005, Brown, Haringman et al. 2012, Renz, Allen et al. 2018).
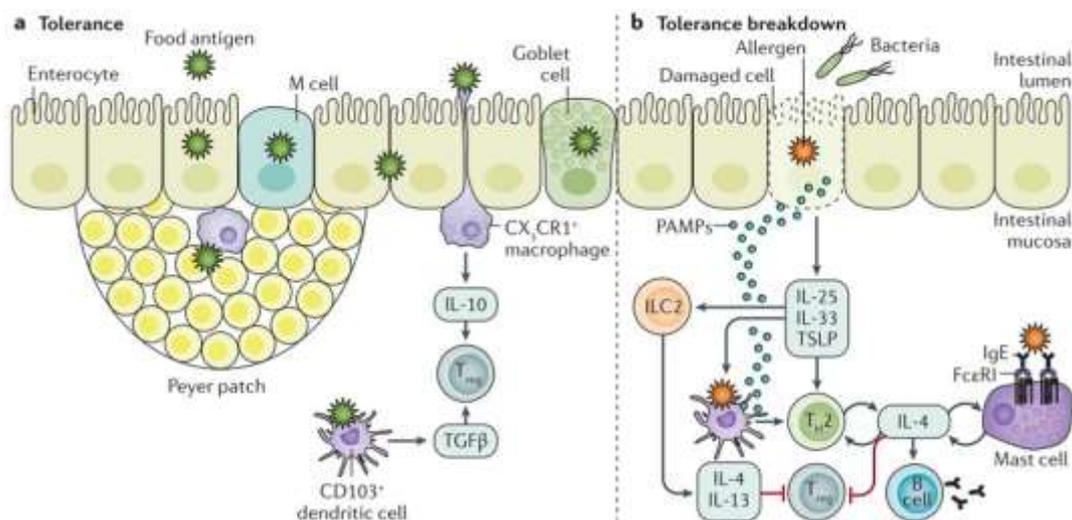
Figure 10. Establishing of immune tolerance to ingested antigens and tolerance breakdown.[10] ILC - Innate lymphoid cells, Treg – T regulatory cell, T_H – T helper, IL – Interleukin, PAMPs - Pathogen-associated molecular patterns.

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[10] Reprinted from Renz, H., K. Allen, S. Sicherer, H. Sampson, G. Lack, K. Beyer and H. Oettgen (2018). "Food allergy." Nature Reviews Disease Primers **4**.

The established tolerance to food antigens can be broken and induction of Treg cells by CD103^{+} DCs can be shifted to the generation of proallergic Th2 effector cells (Figure 10b). The factors which induce the brake of tolerance are various and poorly understood. It is considered that pathogen-associated molecular patterns (PAMPs), products of commensal and pathogen microorganisms, induce injury of the intestinal epithelium but also antigens exposure at other sites, like skin, are the main culprits. The key proallergic cytokine is IL-4, it is produced by the Th2 cell population upon exposure to food allergens. IL-4 further drives IgE switching in B cells and influences mast cell survival and tissue sensitivity to mast cell mediators. (Renz, Allen et al. 2018). Also, the gut epithelial cytokines induce expansion of Th2 innate cells which produce IL-4 and IL-13 cytokines that block Treg cell function (Blázquez and Berin 2008). Another important impact arises from the allergen-specific IgE antibodies which activate mast cells via FcεRI and activated mast cells to generate IL-4 which further promotes Th2 cell induction and suppresses Treg cell induction, this is known as the positive feedback loop (Burton, Noval Rivas et al. 2014). Interestingly, Treg cells can be reprogramed towards the Th2 phenotype to produce IL-4 while maintaining expression of FOXP3^{+} (Noval Rivas, Burton et al. 2015). Beside the intestine, there are other routes and causes of tolerance breakdown. For example, children who have food allergy often have atopic dermatitis and food allergens may enter through scratched and inflamed skin and induce the breakdown of tolerance in the intestine (Brough, Liu et al. 2015). Another example of tolerance breakdown is the oral allergy syndrome in which IgE antibodies are generated in response to some inhaled aeroallergens cross-react with food allergens. Hypersensitivity to latex can also induce sensitivity to food such as banana, avocado, and kiwifruit (Webber and England 2010).

2.4.1. Red meat allergy

The intriguing story of mammalian red meat allergy begun recently in 2007, when Van Nunen (2009) reported that individuals who had been bitten by ticks experienced urticarial or anaphylactic reactions after ingestion of red meat. A few years later Commins (2009) reported anaphylactic and urticarial reactions in individuals who consumed red meat and previously reported large local reactions to a tick bite. (Levin, Apostolovic et al. 2019) The most important in elucidating the causative agent of red

meat allergy was cetuximab, a chimeric mouse-human monoclonal antibody against the epidermal growth factor receptor, used for the treatment of patients with colorectal cancer. It was discovered that these patients have a high titer of IgE antibodies reactive toward the galactosyl-α-1,3-galactose (α-Gal). In cetuximab, α-Gal is present on 2 N-linked oligosaccharide domains on the Fab portion of the cetuximab heavy chain (Qian, Liu et al. 2007, Chung, Mirakhur et al. 2008). Allergic reactions to red meat have been reported in many other countries besides Australia and the USA, such as Sweden, Germany, France, Spain, Japan, and Africa (Jacquenet, Moneret-Vautrin et al. 2009, Commins, Kelly et al. 2012, Jappe 2012, Sekiya, Fukutomi et al. 2012, Hamsten, Tran et al. 2013). The symptoms of red meat allergy are various, ranging from urticaria, angioedema, and the most severe allergic reaction, anaphylaxis (Kiewiet, Apostolovic et al. 2020). Recent findings suggest that abdominal pain is a frequent but often underreported symptom (Commins, Satinover et al. 2009, Mabelane, Botha et al. 2018). One more interesting fact about the red meat allergy is the delayed onset of symptoms, usually 3 to 6 hours after the red meat consumption, but according to some reports, symptoms could also appear within 2 hours (Commins and Platts-Mills 2009, Mabelane, Botha et al. 2018). There seems to be some link between reactions to cetuximab and red meat allergy in areas of high prevalence of the lone star tick, *Amblyomma americanum*. It is believed that tick bites are an important cause of sensitization to α-Gal. This epitope is found in the content of gastrointestinal tract and/or saliva of different species of tick such as *Ixodus ricinus*, *Amblyomma sculptum*, *Heamaphysalis longicornis*, and *Ixodes scapularis* (Hamsten, Starkhammar et al. 2013, Araujo, Franco et al. 2016, Chinuki, Ishiwata et al. 2016, Cabezas-Cruz, Espinosa et al. 2018). Moreover, intradermal injection of tick salivary gland extract from *A. americanum* induced sensitization to α-Gal and allergic responses upon ingestion of pork sausage in the murine model of α-Gal allergy (Commins and Karim 2017).

The concentration and the total amount of α-Gal present in different foods varies but it is the most abundant in the food derived from the animal's internal organs (Morisset, Richard et al. 2012, Apostolovic, Tran et al. 2014). Interestingly some of the identified α-Gal-containing IgE-binding proteins, such as creatine kinase M-type, aspartate aminotransferase, alpha- and beta-enolase were stable to heat treatment (Apostolovic, Tran et al. 2014). Also, it seems that lipids present in meat influence the severity of

symptoms as consumption of meat with higher lipid content induced more severe symptoms in allergic individuals (Steinke, Pochan et al. 2016). Moreover, some cofactors can influence and induce more severe symptoms even after the ingestion of food with a very low content of α-Gal. The identified cofactors are exercise, nonsteroidal analgesics, and alcohol (Fischer, Eberlein et al. 2017). The cause of the delayed onset of symptoms and the cofactor mechanism of action is still unknown and bioavailability and presentation of cofactors and allergen to immune cells can be one of the explanations.

Also, the prevalence of red meat allergy is higher in middle-aged individuals compare to young people, probably because they spend more time in nature and are more exposed to tick bites. Interestingly, in the earliest reports, a higher prevalence of red meat allergy was noted in the male population than in the female population. This observation was attributed to the higher environmental exposure of males regarding their profession, but with the emerging data, a clear line between the frequency of red meat allergy between men and women cannot be drawn (Commins, James et al. 2011, Commins, James et al. 2014, Kiewiet, Apostolovic et al. 2020).

2.4.1.1.   *Alpha-Gal epitope structure and recognition*

Alpha-Gal consists of two molecules of galactose linked by an α-1,3-glycosidic bond and it is the main recognition domain of α-Gal epitope which contains three carbohydrate residues (Galα1-3Galβ1-4GlcNAc-R) (Figure 11). The alpha-Gal epitope is found on proteins and lipids of non-primate mammals. The synthesis of the α-Gal epitope is catalyzed by different enzymes, but in the last step, the enzyme α-1,3-galactosyltransferase (α1,3GT) catalyzes the formation of the α-1,3-glycosidic bond between two Gal residues. It is found that humans, apes, and Old World monkeys have α1,3GT pseudogene on chromosome 9 instead of the normal gene present in non-primate mammals. It is thought that inactivation of this enzyme has occurred 28 million years ago when two point mutations (deletion) happened in the exon that codes the main catalytic domain of the enzyme which causes a frameshift and a premature stop codon (Larsen, Rivera-Marrero et al. 1990). The lower mammals possess α-Gal as a post-translational modification while humans do not, so this is a reason why they produce α-Gal antibodies (Commins 2015).
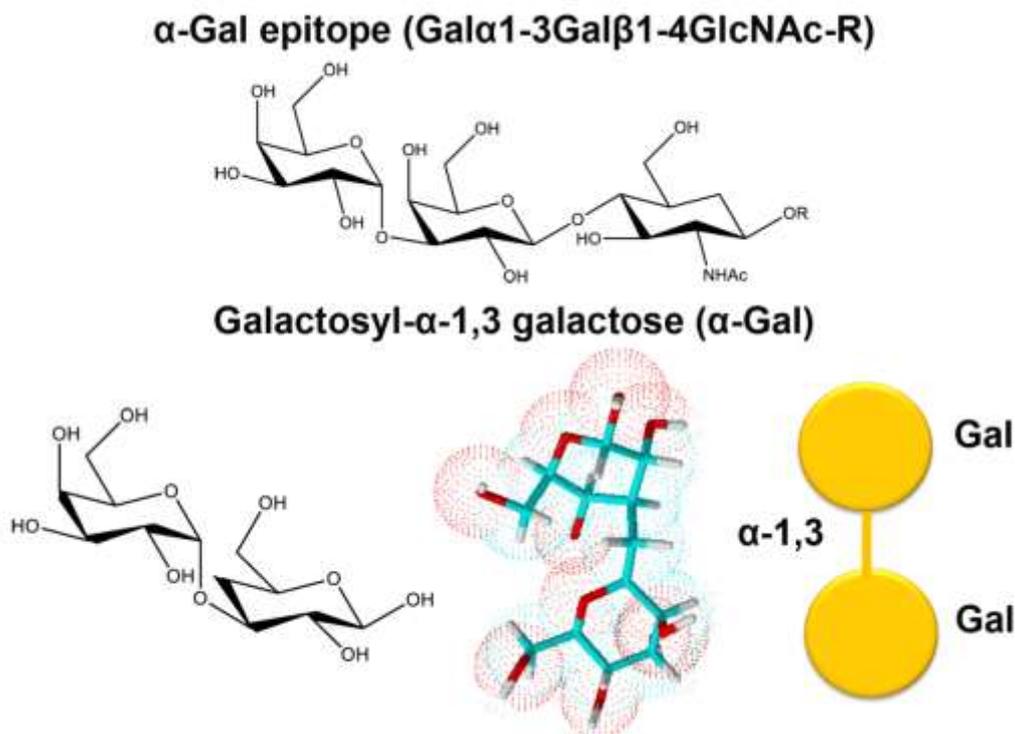
Figure 11. The structural formula of the α-Gal epitope and α-Gal residue.

There are obvious structural similarities among α-Gal and the blood group B-antigen, with B-antigen possessing an extra fucose residue on the glycan core. Because of this, in the individuals with B or AB blood group, the levels of IgE and IgG against α-Gal are measured. The obtained results showed that healthy individuals have almost the non-existing level of IgE and low level of IgG antibodies to α-Gal. The connection between the levels of IgG and IgE antibodies was found, thus in patients with higher IgE levels proportionally higher level of IgG1 was found when compared to the background level of IgG2 specific for α-Gal. These findings suggest that in the normal physiological conditions the low level of IgG2 present probably an immune response against the α-Gal epitope expressed on normal gut bacterial flora. During the immune response to the α-Gal sensitization by Th2 cells elevated levels of IgE, and IgG1 are generated in addition to the existing IgG2 (Oostingh, Davies et al. 2003, Rispens, Derksen et al. 2013). Importantly, the immune response to α-Gal and B-antigen differs among red meat allergic patients and healthy individuals. The immune response to α-Gal in red meat allergic patients is characterized by increased levels of IgE, IgG1, and

IgG4, while the response to the B-antigen is characterized by increased levels of IgG2 (Apostolovic, Rodrigues et al. 2018).

Production of anti-α-Gal antibodies is a response to continuous antigenic stimulation by carbohydrate antigens on GI bacteria of the normal flora (Galili, Mandrell et al. 1988) similar to the production of antibodies to the blood group A and B carbohydrate antigens (Springer and Horton 1969). It was shown that anti-α-Gal antibodies are capable to differentiate structures with variations in the linkage positions of the terminal galactose residues but have the same carbohydrate sequence and anomerity (Galili, Macher et al. 1985). The hydrogen bonds, hydrophobic interactions, and van der Waals' forces are responsible for the binding of anti-α-Gal antibodies to the α-Gal epitopes. These forces are of short distance and require closer interaction and more precise orientation such as the case for the hydrogen bonds which are the main interaction, in this case, compare to ionic bonds between charged amino acids on the binding surface of the antibodies and protein allergens (Commins 2015).

2.5. Development and use of *in vitro* systems for studies of physiological processes such as bioavailability and uptake by immune cells

The use of *in vitro* models is required during the experimental study of complex processes such as food digestion, the release of food nutrients, and their bioavailability. In the past two decades, a lot of effort has been put into developing these models but still, there is not a complete, model that describes in detail all these processes in the organism. Hence, some models mimic conditions in the mouth, stomach, and the small intestine, the so-called digestion models, and those which serve for monitoring the transport of the nutrients and allergens across the intestinal barrier or for monitoring the uptake of allergens by the GIT immune cells. Existing *in vitro* digestion models can be firstly classified as the static or dynamic models and secondly as a monocompartmental, bicompartmental, or multicompartmental model (Guerra, Etienne-Mesmin et al. 2012).

In the literature different *in vitro,* static models are applied depending on the starting material such as whole meal, proteins, carbohydrates, etc. For example one of the static digestion models is developed by Moreno and coworkers for analysis of allergen 2S albumin (Ber e 1) (Moreno, Mellon et al. 2005). The same protocol with slight

modifications is used for the analysis of bovine thyroglobulin, protein carrying α-Gal epitope (Apostolovic, Krstic et al. 2017).

Static digestion models are widely used in the research laboratory setting. In these models, the gastric phase encompasses hydrolysis of the homogenized food or protein of interest, usually allergen, by pepsin under given conditions such as pH and temperature and for a set period. Usually, the given conditions are pH 1-3 temperature 37 ˚C and the duration of digestion is usually 1 to 4 hours. The gastric phase of digestion can be stopped after a predetermined time and obtained so-called "digesta" can be examined and characterized, or followed by the intestinal phase of digestion. During this phase of digestion in the same vessel enzymes with or without bile characteristics for intestinal digestion are added and pH raised to 6 or 7. Besides enzymes, bile can be added in this phase of digestion depending on the starting material used for the analysis (Hur, Lim et al. 2011).

Just a brief look at the literature indicates the diversity of digestion protocols used in the research and this is the starting point for developing a unique protocol that would be used in laboratories to make the obtained results comparable. Such is initiative is proposed by the COST Infogest network in the form of a consensus paper where a general standardized and practical static digestion model was described (Figure 12) (Minekus, Alminger et al. 2014). Although Infogest's statistic digestion model is mostly for the "whole meal situation" it can be easily applied to a single protein/allergen. However, the statistic *in vitro* models has its weaknesses such as no good replacement for the complex peristaltic movements, gastric emptying, and continuous changes in the pH and secretion flow rates. To overcome these dynamic models are developed such as dynamic monocompartmental models (Hoebler, Lecannu et al. 2002), dynamic bicompartmental models (Mainville, Arcand et al. 2005), and multicompartmental models (Minekus, Marteau et al. 1995).
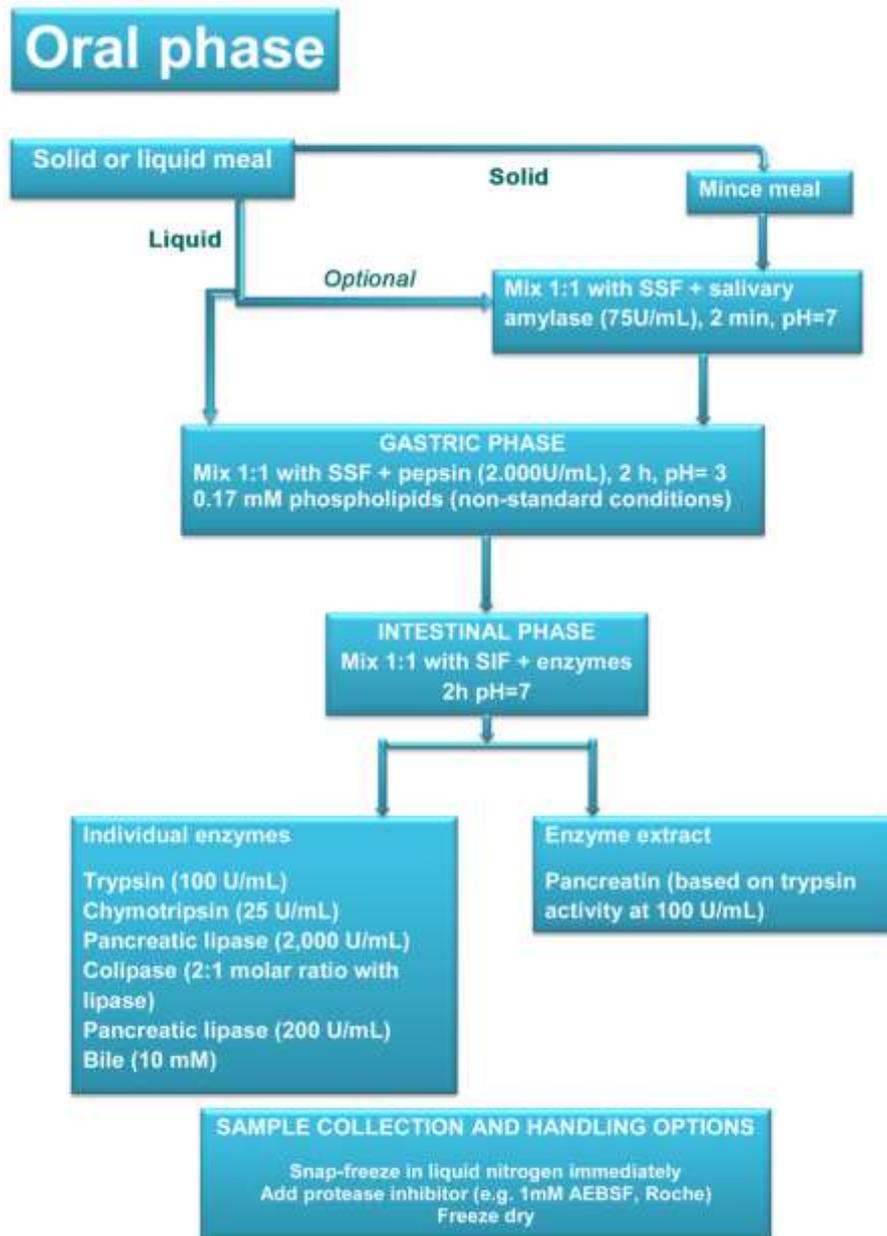
Figure 12. Flow diagram of a simulated *in vitro* digestion method by Minekus and coworkers. SSF-Simulated Salivary Fluid, SGF-Simulated Gastric Fluid, and SIF-Simulated Intestinal. Enzyme activities are in units per mL of the final digestion mixture at each corresponding digestion phase.[11]

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[11] Modified and reprinted from Minekus, M., M. Alminger, P. Alvito, S. Ballance, T. Bohn, C. Bourlieu, F. Carrière, R. Boutrou, M. Corredig, D. Dupont, C. Dufour, L. Egger, M. Golding, S. Karakaya, B. Kirkhus, S. Le Feunteun, U. Lesmes, A. Macierzanka, A. Mackie, S. Marze, D. J. McClements, O. Ménard, I. Recio, C. N. Santos, R. P. Singh, G. E. Vegarud, M. S. J. Wickham, W. Weitschies and A. Brodkorb (2014). "A standardised static in vitro digestion method suitable for food – an international consensus." <u>Food & Function</u> **5**(6): 1113-1124.

The cell-based models for monitoring of transport of food proteins and/or allergens as well as other nutrients, and very important drugs are widely used. These experimental models are crucial in the initial phase of the clinical trials and to help to reduce animal experimentation. Caco-2 cells (cancer cell line), which mimic the intestinal epithelial cells and forming the intestinal-like barrier under certain growing conditions, are a gold standard model for evaluation of transport through the intestinal epithelium (Hubatsch, Ragnarsson et al. 2007). Their phenotype is well characterized and they are widely used for the analyses of drug transport (Sun, Chow et al. 2008) or transport of food allergens (Moreno, Rubio et al. 2006, Thierry, Bernasconi et al. 2009, Price, Ackland et al. 2014). Another cancer cell line widely used in "gut-mimicking" experiments is T-84 (Raffatellu, Wilson et al. 2005, Tran, Gomez et al. 2009). The main characteristic of these two cancer cell lines is forming polarized monolayer and exhibition of microvilli formation. Also for transport experiments these cells are commonly grown on semi-permeable Transwell® inserts. Beside tremendous advantages in the first place easy maintenance, cultivation, and reproducibility, these cell-based model system have some limitations. It is primarily a lack of mucus-secreting goblet cells in the intestinal-like barrier as well as the expression of different transporters and metabolic proteins depending on the applied culture conditions. To overcome some of these obstacles recently the primary human intestinal epithelial cells (hInEpCs) have become recently commercially available. As these cells are more difficult to handle, there is only a limited amount of data based on these cells (Kauffman, Gyurdieva et al. 2013). Also, the hInEpCs line is the basis for the development of novel, *in vivo*-like organoid molecules. The organoids represent the respective native organs in their structure and function features. For this purpose adult intestinal stem cells are directly isolated from human biopsies and this system can be maintained even for several months without significant phenotype changes (Sato, Vries et al. 2009, Sato, Stange et al. 2011, Schweinlin, Wilhelm et al. 2016).

3.   Aims

Within this doctoral dissertation, several aims have been formulated:

a) To examine the effect of the α-Gal epitope on the protein surface with respect to susceptibility to gastric digestion in the pepsin *in vitro* digestion model.

b) To examine the effect of the α-Gal epitope on protein transport through the monolayer of Caco-2 cells, a model system of the intestinal epithelium.

c) To examine the effect of the α-Gal epitope on protein uptake and processing in the immature monocyte-derived dendritic cells.

4. Pepsin digestion pattern of α-Gal glycosylated bovine serum albumin

4.1. Introduction

Although in recent years resistance to gastrointestinal digestion of food allergens as one of the markers of allergenicity was questioned it is still an inevitable tool in assessing the allergenicity, especially for novel food allergens (Bøgh and Madsen 2016). According to our knowledge, there is a lack of data in the literature on the digestibility of proteins carrying the α-Gal epitope. Also, it is very hard to compare results obtained in different studies dealing with resistance to gastrointestinal digestion since different *in vitro* digestion protocols have been applied. To avoid these obstacles Minekus and coworkers (Minekus, Alminger et al. 2014) proposed a consensus model for simulated *in vitro* gastrointestinal digestion which was primarily intended for whole meal digestion simulation but can also be applied to individual proteins/allergens.

The α-Gal-containing proteins from different beef preparations (raw, medium rare, fried, and boiled beef) have been identified and their potential allergenicity was investigated by monitoring the binding of IgE antibodies from meat-allergic patients (Apostolovic, Tran et al. 2014). Although various α-Gal carrying proteins have been identified little is known about their bioaccessibility during gastrointestinal digestion. This is important especially in terms of the food matrix which can have a tremendous impact on the bioaccessibility of proteins from food (Vissers Y.M., Wichers H.J. et al. 2012). Besides α-Gal caring protein, protein without this epitope or deglycosylated protein is required for an in-depth investigation of the influence of α-Gal epitope on protein resistance to digestion and pattern of the released digestion peptides.

Bovine thyroglobulin (bTG) is a naturally occurring protein that possess α-Gal epitope as a posttranslational modification and it is abundantly present in red meat (Rawitch, Pollock et al. 1993). Apostolovic, Krstic et al. (2017) performed the simulated *in vitro* gastrointestinal digestion of bTG using protocols recommended by Moreno, Mellon et al. (2005) and Koppelman, Hefle et al. (2010) and found that α-Gal peptides were present during the whole gastric phase of protein digestion. It was also confirmed that there was not IgE binding to digested peptides if they were

deglycosylated. Besides this, another important finding was that at the acidic pH conditions deglycosylated bTG was insoluble which makes gastric digestion impossible and thus limiting the use of the deglycosylated bTG as the α-Gal negative control (Apostolovic, Krstic et al. 2017).

As previously mentioned studies dealing with the gastrointestinal digestion of red meat as well as individual α-Gal-containing proteins are highly needed. Also, it is very important in the research that some consensus models be adopted and followed in conducting *in vitro* studies of bioaccessibility of food proteins and simulated gastrointestinal digestion of individual proteins/allergens. Taking into account previously mentioned one of the aims of this doctoral dissertation was to check the usability of the simulated *in vitro* pepsin digestion for the monitoring digestion of α-Gal containing food allergens using α-Gal glycosylated bovine serum albumin (BSA-α-Gal), as a model protein.

4.2. Methodology

4.2.1. Reagents

Used proteins were commercially available. BSA and pepsin were obtained from Sigma-Aldrich (Merck, Steinheim, Germany). α-Gal glycosylated BSA (BSA-α-Gal) and N-Acetyllactosamine conjugated BSA (BSA-NAl) were obtained from Dextra Laboratories (Reading, UK). Other chemicals used in the experiments were p.a. grade and obtained from Sigma-Aldrich unless stated otherwise.

4.2.2. Protein sample preparation

Used proteins were fluorescently labeled by conjugating AlexaFluor488 (AF488) fluorescent dye using an AF488 protein labeling kit (Invitrogen, Molecular Probes, Inc., Eugene, Oregon, USA) according to the manufacturer's instructions. For determination of protein concentration commercial kit Pierce Bicinchoninic acid (BCA) Protein Assay Kit (Pierce Biotechnology, Rockford, Illinois, USA) was used.

4.2.3. *In vitro* gastric digestion

"For *in vitro* gastric digestion the statistic digestion model proposed by Minekus, Alminger et al. (2014) was used with slight modification. The carbohydrate conjugated proteins BSA-α-Gal, BSA-NAl, and unconjugated BSA fluorescently labeled with AF488 were pre-incubated at 37 °C for 15 min before pepsin in simulated gastric fluid (SGF) was added in a 1:3 (volume protein/volume SGF with pepsin) ratio. *In vitro* digestion protocol was adjusted to single protein digestion so the final enzyme activity was 8 U pepsin per mg of protein. The recipe and calculation for the preparation of 4 × SGF are presented in Table 1. The pH of the digestion mixture was adjusted to 3 by the addition of 1 M HCl. Simulated gastric digestion was monitored for up to 4 h at 37 °C, and aliquots were withdrawn from a single digestion mixture at different time points for further analysis. The digestion was stopped by raising the pH of the digestion mixture to 7.5 by adding 1 M NaHCO$_3$." (Krstic Ristivojevic M., Grundström J. et al. 2020)

Table 1. Preparation of stock solution of simulated gastric fluid (SGF). The masses of substances are calculated to prepare 200 mL 4 × concentrate SGF solution.

Constituent	SGF pH 3	
	4 × SGF*	Final conc. in SGF
	g	mmol/L
KCl	0.41	6.90
KH$_2$PO$_4$	0.10	0.90
NaHCO$_3$	1.68	25.00
NaCl	2.21	47.20
MgCl$_2$(H$_2$O)$_6$	0.02	0.10
(NH$_4$)$_2$CO$_3$	0.08	0.50
CaCl$_2$(H$_2$O)$_2$	0.02	0.15
For pH adjustment		
	mL (12M[a])	mmol/L
HCl	1.04	15.6

*to prepare 200 mL of 4 × SGF solution
[a] molarity of a stock solution

4.2.4. SDS PAGE analysis

For analysis of digestion products of BSA with or without glycosylation sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) according to Laemmli (1970) under reducing conditions was performed. The digestion products of BSA with or without glycosylation were resolved on the 14% polyacrylamide (PAA) hand-cast gels and analysis were carried out using a Hoefer Scientific Instruments apparatus (Holliston, Massachusetts, USA) with a discontinuous buffer system. The resolved PAA gels were stained using Coomassie Brilliant Blue R-250 and scanned using the Typhoon FLA 7000 laser scanner (GE Healthcare, Freiburg, Germany).

4.2.5. Immunoblot for detection of the BSA and α-Gal epitope

"Bovine serum albumin and α-Gal in BSA-α-Gal digests were detected by immunoblot analysis. Previously resolved proteins by SDS PAGE were transferred to polyvinylidene difluoride (PVDF) membranes (0.2 µm pore size) using an EBU-4000 Semi-Dry Blotting System (Fisher Scientific Company L.L.C., Pittsburgh, Pennsylvania, USA). For the immunodetection of *in vitro* pepsin-digests, peptides of BSA-α-Gal, BSA-NAl, and BSA the membranes were probed with the rabbit polyclonal anti-BSA IgG antibody (Invitrogen, A11133) diluted 1:1000 in 0.1% human serum albumin (HSA) for 1.5 h at room temperature (RT) followed by goat anti-rabbit IgG conjugated to alkaline phosphatase (AP) (ab97072, Abcam, Cambridge, Massachusetts, USAab97072) diluted 1:5000 in 0.1% HSA for 1 h at RT. Immunoreactive bands were visualized using nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrates. For the immunodetection of α-Gal on peptides of BSA-α-Gal obtained upon simulated *in vitro* digestion the membranes were probed with monoclonal mouse anti-α-Gal antibody M86 (Enzo Life Sciences, Farmingdale, New York, USA) diluted 1:5 in 0.1% HSA in Tris-buffered saline – Tween 20 (TBS-T) for 3 h followed by AP-labeled goat anti-mouse IgM antibody (Southern Biotech, Birmingham, Alabama, USA) diluted 1:3000 in TBS-T for 1 h at room RT. Reactive bands were visualized as stated above." (Krstic Ristivojevic M., Grundström J. et al. 2020)

4.3. Results

4.3.1. Pepsin digestion pattern of BSA, BSA-α-Gal, and BSA-NAl

The assessment of the stability of a protein or its proteolytic fragments during pepsin digestion is an important parameter in sensitization in food allergy. Hence, it is of importance to examine whether the α-Gal glycosylation of BSA has any impact on its resistance to pepsin digestion and profile of the proteolytic fragments. In the first experiment of this thesis, the difference in molecular weight between molecules of BSA before and after glycosylation was checked. The pure proteins, BSA (control) and with α-Gal glycosylation (BSA-α-Gal) were separated onto 12 % PAA gel using SDS-PAGE (Figure 13).
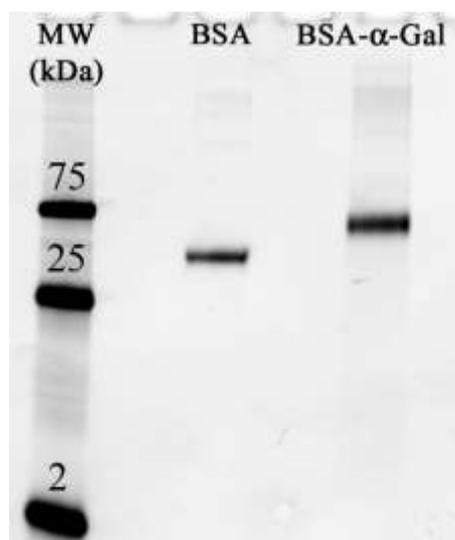
Figure 13. Fluorescent detection of AF488 labeled BSA and BSA-α-Gal resolved on the 12 % PAA gel after SDS-PAGE.

To examine the influence of glycosylation of BSA on its resistance to pepsin digestion, the pepsin digestion profiles of BSA-α-Gal, BSA-NAl and BSA were compared using SDS PAGE under reducing conditions. Obtained gels were analyzed by staining with CBB R250 or by immunoblot using a polyclonal anti-BSA antibody (Figure 14 and 17). Besides this, to investigate the content of the α-Gal epitope on the released peptides during digestion of BSA-α-Gal by pepsin, the obtained gel was additionally analyzed by immunoblot using mouse monoclonal anti-α-Gal antibody M86 (Figure 16). To eliminate the additional bands in the digestion profiles of analyzed BSA which can be originated from pepsin during its auto-digestion or the presence of

impurities, a sample of pure pepsin was also analyzed at 0 and 4 h from the beginning of digestion (Figure 15).
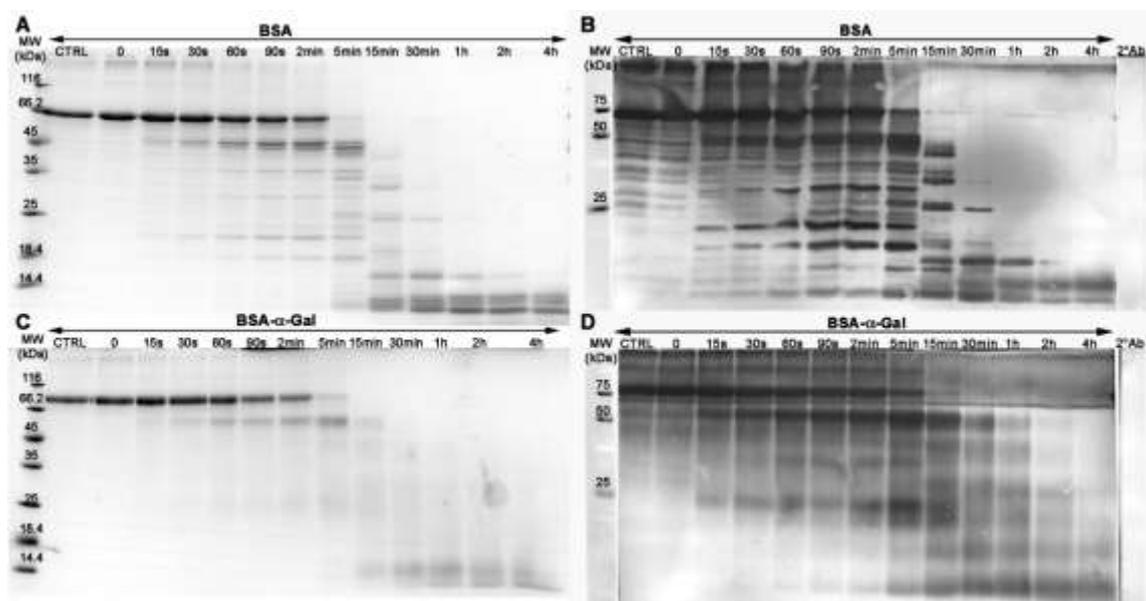
Figure 14. *In vitro* gastric digestion products of BSA and BSA-α-Gal obtained during 4 h of digestion, resolved on SDS PAGE, and analyzed by staining with CBB R-250 (A) and (C), or by immunoblot with polyclonal anti-BSA antibody (B) and (D). 2°Ab = control of unspecific binding of the secondary antibody, MW = Molecular weight markers, and CTRL = undigested protein.
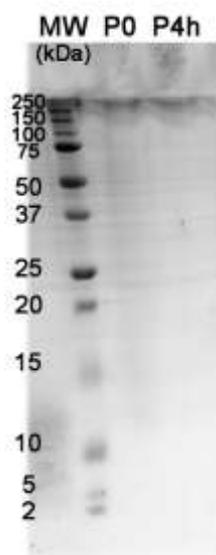
Figure 15. SDS-PAGE analysis of pepsin control solution at 0 and 4 h time point of digestion, stained by CBB R-250. MW = Molecular weight markers.

From the presented results it can be seen that intact BSA and BSA-α-Gal can be detected up to 15 min of digestion (Figure 14B and D) and that the degradation pattern

of BSA and BSA-α-Gal are different. Interestingly, BSA and BSA-α-Gal degraded promptly, even after 15 s of digestion peptides of about 45 kDa for BSA and 55 kDa for BSA-α-Gal appeared and became more intense when digestion was prolonged up to 5 min. The important finding was that the pepsin digestion products of BSA-α-Gal with MW ranging between 25 and 55 kDa were present for up to 2 h of digestion. On the other hand, similar-sized pepsin digestion products of BSA were only detected up to 30 min of digestion. Also, another difference between digestion patterns was that BSA digestion peptides of the MW below 25 kDa were distinct bands whereas BSA-α-Gal digestion peptides below 25 kDa were smeared bands. The obtained results suggest that the presence of the α-Gal epitope on the BSA-α-Gal alters the protein susceptibility to pepsinolysis.

According to the obtained results, it can be concluded that the glycosylation of BSA with the α-Gal epitope can influence the resistance of BSA-α-Gal to digestion by pepsin. To confirm these results, in the next experiment the content of α-Gal epitope on the released pepsin digestion fragments obtained during *in vitro* digestion of BSA-α-Gal were determined by immunoblot analysis using mouse monoclonal anti-α-Gal IgM antibody (Figure 16).
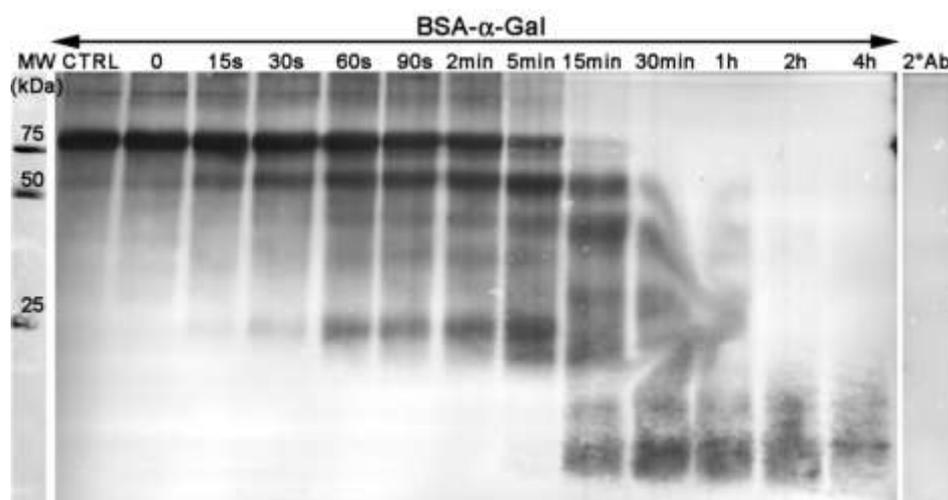
Figure 16. *In vitro* gastric digestion products of BSA-α-Gal during 4 h of digestion analyzed by immunoblot analysis using mouse monoclonal anti-α-Gal antibody M86. 2°Ab = control of unspecific binding of the secondary antibody, MW = Molecular weight markers, and CTRL = undigested protein.

In Figure 16 it can be seen that the large α-Gal carrying peptide with the MW of approx. 55 kDa appeared after only 15 s of digestion and was still detected, although

barely, after 2 h of the *in vitro* pepsin digestion. Also, peptide fragment with MW of approx. 45 kDa follows the appearance of the larger previous peptide. Moreover, peptide fragments with MW of approx. 37, 32, and 25 kDa carrying α-Gal were detected after 1 h.

To gain a better insight into the effect of the α-Gal epitope on the pepsin digestion of the α-Gal containing proteins we performed *in vitro* pepsin digestion of BSA-NAl, which possesses a carbohydrate modification similar in size to α-Gal (Figure 17). According to the obtained results presented in Figure 17 this carbohydrate modification of BSA has a significant impact on its resistance to pepsinolysis. Intact BSA-NAl was still detectable after 15 min of digestion compared to BSA which was, although barely, detectable up to 5 min of digestion (Figure 14). On the other hand, immunoblot detection using a polyclonal anti-BSA antibody revealed that the intact protein was present for up to 2 h of *in vitro* digestion (Figure 17B). Moreover peptide fragments with MW of approx. 55, 45, 37, 32, and 25 kDa were detected even after 4 h of *in vitro* pepsin digestion.
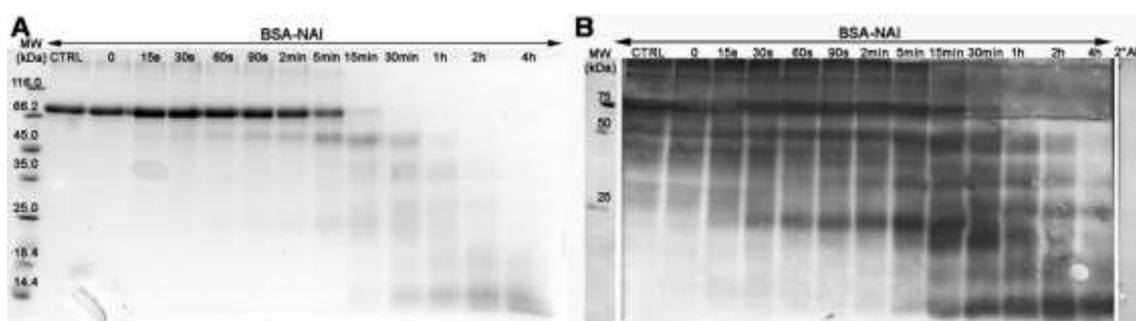
Figure 17. *In vitro* gastric digestion products of BSA-NAl obtained during 4 hours of digestion, resolved on SDS PAGE, and analyzed by staining with CBB R-250 (A), or by immunoblot with polyclonal anti-BSA antibody (B). 2°Ab = control of unspecific binding of the secondary antibody, MW = Molecular weight markers, and CTRL = undigested protein.

Different systems for detection which are more or less sensitive were used for the analysis of obtained peptide fragments during digestion of different BSA molecules by pepsin. In Figure 18 the obtained digestion patterns of the AF488 labeled BSA, BSA-α-Gal, and BSA-NAl using fluorescent detection are shown. Obtained digestion patterns of proteins are in correlation with the results obtained with the above-mentioned detection systems such as CBB R-250 and immunoblot analysis using a

chromogenic NBT/BCIP substrate. The intact BSA was visible up to 5 min of pepsinolysis while BSA-α-Gal and BSA-NAl were visible up to 15 min of pepsinolysis. Also, the presence of the α-Gal epitope on the BSA-α-Gal peptides and NAl on the BSA-NAl peptides is the probable reason for the obtained smeared bands whereas BSA peptides were distinct on the resolved PAA gels.
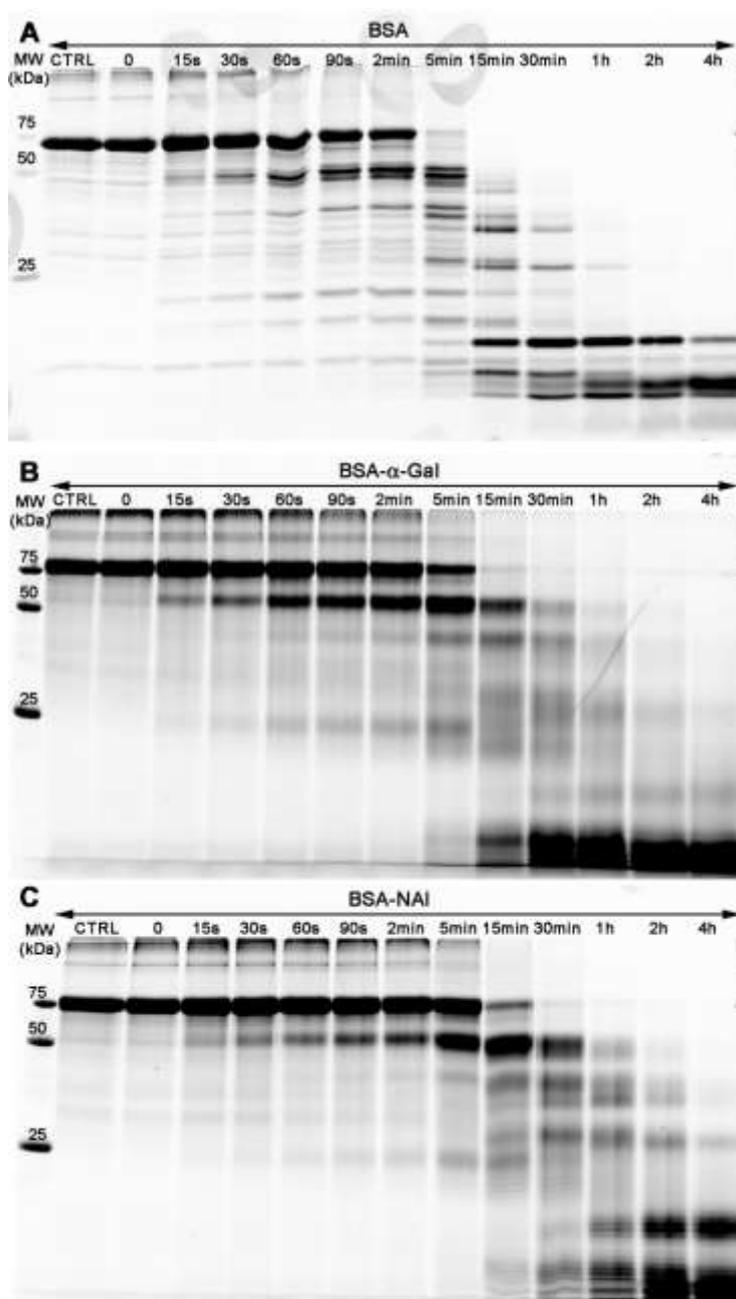

Figure 18. *In vitro* gastric digestion products of (A) BSA, (B) BSA-α-Gal, and (C) BSA-NAl obtained during 4 hours of digestion, resolved on SDS PAGE and analyzed by fluorescent detection using AF488 fluorescent dye. MW = Molecular weight markers, and CTRL = undigested protein.

4.4. Discussion

Glycosylation a posttranslational modification of proteins and lipids contributes to the complexity of the biological structures available in living organisms. This modification is especially important for the proteins which are the key players in a broad spectrum of biological processes (Maureen E. Taylor and Drickamer 2011). Although carbohydrates as such are mainly not considered as potent inducers of allergic reactions, α-Gal carbohydrate proves the opposite. The alpha-Gal epitope is present in beef proteins and even food processing such as cooking does not influence its allergenicity (Apostolovic, Tran et al. 2014).

The obtained results demonstrated that the digestion stability of proteins and the pattern of the resulting peptides was influenced by protein glycosylation, one of the most common posttranslational modifications (Krstic Ristivojevic M., Grundström J. et al. 2020). One of the findings is that the type of glycosylation influence the protein susceptibility to pepsinolysis, α-Gal glycosylation increased the resistance of BSA to pepsinolysis, but NAl glycosylation hampered pepsinolysis even more. "Intact BSA-α-Gal, similar to BSA, could be detected up to 15 min of *in vitro* pepsin digestion, whereas intact BSA-NAl could be detected for up to 2 h. These findings are following a previous study that demonstrated that glycosylation protects the egg white allergen ovomucoid from pepsinolysis (Benede, Lopez-Fandino et al. 2013). The major impact of protein glycosylation was observed in the digestion patterns of BSA-α-Gal compared to BSA, large digestion products of BSA-α-Gal ≥ 25 kDa were present for up to 2 h, which was not seen for BSA. Moreover, in the lower molecular weight region where BSA-α-Gal gave rise to weak smeared peptide bands, BSA generated well-defined peptide bands. Immunoblot analysis of α-Gal containing proteins upon gastric digestion resulted in a rich spectrum of peptides abundant in α-Gal epitopes, where α-Gal epitope was detected on the large peptides even after 2 h of *in vitro* pepsin digestion. Although faintly stained, the major peptides carrying the α-Gal epitope, detected at molecular weights between 25 and 55 kDa provide a prolonged survival of proteins during gastric digestion and a prolonged availability for uptake by intestinal cells." (Krstic Ristivojevic M., Grundström J. et al. 2020) This is of great importance since it was shown that α-Gal containing peptides resulting from pepsinolysis are allergenic (Apostolovic, Krstic et al. 2017).

5. Influence of the α-Gal glycosylation on the protein surface on its transcytosis through the Caco-2 monolayer, bioavailability assessment

5.1. Introduction

"There is an evident lack of literature dealing with the fate of the α-Gal carrying proteins in the gastrointestinal tract, including processing, uptake, and transport of α-Gal carrying proteins by the intestinal epithelium". (Krstic Ristivojevic M., Grundström J. et al. 2020) For consequential examination of bioaccessibility of food proteins and allergens and their peptides except *in vitro* simulated gastrointestinal digestion, there are established cell-based *in vitro* models for monitoring protein transport through the intestine. "Enterocytes of the intestinal epithelium are the most important cells for absorbing nutrients from food and at the same time excluding antigenic macromolecules and microbes from crossing the barrier. The human colon carcinoma cell-line Caco-2 is used as a model system of the intestinal epithelium. When cultured on permeable supports, the cells differentiate into a monolayer that is similar to the small intestinal villus epithelium and this monolayer expresses brush border proteins, tight junctions between cells, and is highly polarized (Hubatsch, Ragnarsson et al. 2007, Sun, Chow et al. 2008). The Caco-2 cell monolayer has been used for investigation of transport of food allergens (Moreno, Rubio et al. 2006, Thierry, Bernasconi et al. 2009, Tordesillas, Gomez-Casado et al. 2013, Price, Ackland et al. 2014). Also, the Caco-2 *in vitro* model system has been applied to show that allergens can be resistant to digestion and maintain their allergenicity after passing the epithelial barrier (Tordesillas, Gomez-Casado et al. 2013)." (Krstic Ristivojevic M., Grundström J. et al. 2020) Importantly, intestinal cells form one of the most important lines of defense which are exposed to microbes daily. Hence, in recent years more and more studies revealed that enterocytes are in constant communication with the surrounding cells such as cells of the immune system which are underlying the intestinal barrier. "Pathogen-derived glycoconjugates can be sensed by mammalian lectins, which are of importance in both innate and acquired immune responses. Galectin-3, the chimera type galectin, can bind to α-Gal (Peterson, Jin et al. 2005, Jin, Greenwald et al. 2006) and is abundantly expressed by intestinal epithelial cells (Dumic, Dabelic et al. 2006). Furthermore, galectin-3 is

upregulated in myeloid and epithelial cells upon microbial stimulation and has mainly pro-inflammatory properties (Diaz-Alvarez and Ortega 2017)".(Krstic Ristivojevic M., Grundström J. et al. 2020)

The main goal of this part of the thesis was to investigate the impact of the α-Gal epitope on the protein surface and for the transport rate through the monolayer of enterocytes using the Caco-2 monolayer model system. BSA and HSA without and with bound α-Gal epitope (BSA-α-Gal and HSA-α-Gal, respectively) were used as the model protein for this study.

5.2. Methodology

5.2.1. Reagents

Proteins were commercially available. BSA and HSA were obtained from Sigma-Aldrich (Merck, Steinheim, Germany). α-Gal glycosylated BSA (BSA-α-Gal), HSA (HSA-α-Gal), and N-Acetyllactosamine conjugated BSA (BSA-NAl) were obtained from Dextra Laboratories (Reading, UK). Other chemicals used in experiments were p.a. grade and obtained from Sigma-Aldrich unless is stated otherwise.

5.2.2. Protein sample preparation

Used proteins were fluorescently labeled by conjugating AlexaFluor488 (AF488) fluorescent dye using an AF488 protein labeling kit (Invitrogen, Molecular Probes, Inc., Eugene, Oregon, USA) according to the manufacturer's instructions. For determination of protein concentration commercial kit Pierce Bicinchoninic acid (BCA) Protein Assay Kit (Pierce Biotechnology, Rockford, Illinois, USA) was used.

5.2.3. Cell culture and viability assay

Caco-2 (HTB37) cell line was obtained from The American Type Culture Collection (ATCC, Manassas, Virginia, USA). Caco-2 cells were cultured in complete Minimum Essential medium (cMEM) supplemented with 10% of fetal bovine serum (FBS), 2 mM glutamine, 1% of nonessential amino acids (NEAA), 100 IU/mL penicillin, 0.1 mg/mL streptomycin, and 50 µg/mL gentamicin. Cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2. Caco-2 cells viability in serum-free media was evaluated by

MTT assay 1, 4, and 24 h after the media exchange, according to a slightly modified procedure of Mosmann (1983). Briefly, Caco-2 cells were seeded in 96-well plates at a density of 10 000 cells per well and cultured for the next 21 days to allow the forming of the cell monolayer. On the 21st day of culturing, cells were thoroughly washed with PBS and incubated with MEM medium supplemented with 2 mM glutamine, 1% of NEAA, 100 IU/mL penicillin, 0.1 mg/mL streptomycin, and 50 µg/mL gentamicin but without FBS for the next 1, 4 and 24 h. MEM with 10% FBS and mentioned supplements were added to the cells in the control wells. The cell culture medium was added to blank wells. After the indicated period, 20 µL of MTT solution (5 mg/mL in phosphate-buffered saline, PBS) were added to each well and incubated for the next 4 h at 37°C. The medium was carefully removed and 200 µL of dimethyl sulfoxide (DMSO) was added to dissolve the formed formazan complexes; absorbance was read at 492 nm. Data are expressed as the percentage of viability with untreated cells taken as 100%.

5.2.4. Transport through the Caco-2 monolayer

"For transport studies, the Caco-2 cells at passages of 23–68 were seeded at 6×10^4 cells/cm^2 (Behrens and Kissel 2003) on cell culture inserts for 12-well plates (ThinCert, Greiner Bio-One GmbH, Frickenhausen, Germany) and cultured for 21 days before the start of the experiments. The medium was replaced three times per week. Before the transport studies and at the end of the experiment, transepithelial electrical resistance (TEER) was measured using a Millicell-ERS VoltOhmmeter (Millipore, Amsterdam, Netherlands). Only cell monolayers with TEER > 300 Ωcm^2 were used and none of the added proteins had any effect on the TEER. Transport studies were started by adding 400 µg of AF488-labeled proteins in 500 µL (0.8 mg/mL) complete MEM (cMEM) to the apical compartment. Aliquots were withdrawn from the basolateral compartment after 1, 2, and 4 h of incubation, and the fluorescence was measured using a FluoroMax-4 spectrofluorometer (Horiba Scientific, Kyoto, Japan). Protein concentration was calculated from standard curves of the corresponding protein, with the limit of detection (LOD) of 0.011 µg/mL (LOD = 3Sa/b, where Sa is the standard deviation of the response and b is the slope of the calibration curve)."(Krstic Ristivojevic M., Grundström J. et al. 2020)

5.2.5. Intracellular visualization of proteins and isolation of the endosomal fraction of the Caco-2 cell monolayer

"To visualize the internalization of AF488-labeled proteins, confocal laser scanning microscopy (CLSM) was employed (Vukojevic, Heidkamp et al. 2008). Isolation of the endosomal fraction of Caco-2 cell lysates was performed as previously described (Mao, Ou et al. 2016) with slight modification. Briefly, Caco-2 cells were cultured in the same conditions as for transport studies in 6-well plates. Before the addition of treatments, the monolayers were washed 3 times with PBS, and the cultivating medium was replaced with a medium without FBS. Caco-2 cell monolayers were incubated with 100 µg of AF488-labeled BSA or BSA-α-Gal for 4 and 24 h, unstimulated cells were used as control. After incubation, the cells were washed 3 times with PBS and detached from the culture inserts using an ice-cold PBS-EDTA solution. The cell pellets were washed 3 times with PBS and lysed with lysis buffer (250 mM sucrose, 50 mM Tris-Cl (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM egtazic acid (EGTA)) with a protease inhibitor cocktail (Sigma-Aldrich). The suspended cell lysates were left overnight at −80 °C. The next day, endosomes were harvested in the third pellet after three centrifugation steps of the supernatant: 1st ($1000\times$ g, 10 min, performed twice and the supernatants were pooled), 2nd (16 000$\times$ g, 20 min), and 3rd (100 000$\times$ g, 1 h) for immunoblot analysis. All centrifugation steps were performed at 4 °C, and for the last ultracentrifugation step a Sorvall WX80 ultracentrifuge (Thermo Electron LED GmbH, Langenselbold, Germany) with T-880 rotor was used."(Krstic Ristivojevic M., Grundström J. et al. 2020)

5.2.6. Statistical analysis

Data are presented as mean ± standard deviation (SD) and were analyzed using GraphPad Prism software version 6.04 software for Windows (La Jolla, California, www.graphpad.com). Each sample was analyzed in duplicate with two biological replicates. The differences were analyzed by two-way ANOVA. The transport rates were calculated by linear regression. Differences were considered significant if $p < 0.05$.

5.3. Results

5.3.1. Parameters which indicate the quality of the Caco-2 monolayer

Till today FBS supplementation is practically inevitable in culture media used for maintenance of almost all cell culture lines. FBS with its very complex composition provides optimal conditions for cell culture growth, BSA is one of the most represented components of FBS. Because BSA present in the cell culture media can interfere with the transport of BSA-α-Gal through the Caco-2 monolayer, it was necessary to reduce its presence in media, but this step can cause the disturbance of optimal Caco-2 cell viability. Hence, the testing of the viability of cells used in *in vitro* Caco-2 cell-based model in conditions of the reduced quantity of FBS in culture media was the first experiment in this part of the thesis. The results of this experiment are presented in Figure 19.
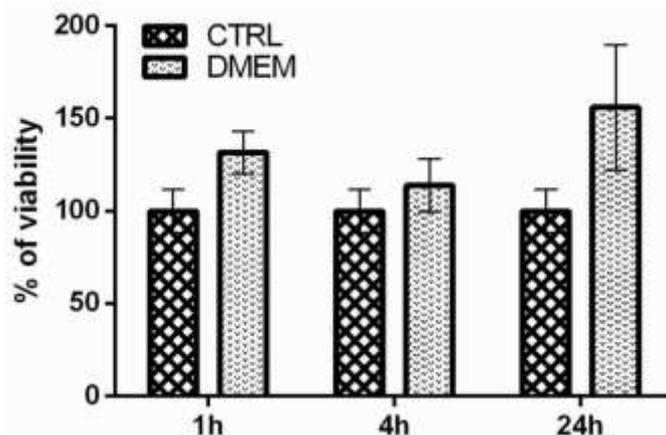
Figure 19. The vitality of Caco-2 cells was determined after 21 days of differentiation and subsequently, Caco-2 cells were maintained for 1, 4, and 24 h in the medium without FBS. CTRL-Cells in the control wells were maintained in the medium with FBS.

From Figure 19 it can be concluded that the maintenance of Caco-2 monolayer in the medium without FBS does not influence the vitality of the established Caco-2 monolayer. This was preferably for the study of the transport of model proteins since any disruption of the monolayer could lead to leakage of the test protein to the basolateral compartment of the transwell insert.

Another important parameter that indicates the quality of established cell monolayer is the value of TEER. It was adopted that the value of TEER bigger than 300 Ω/cm^2 indicates that the Caco-2 monolayer is of satisfactory quality. Also, TEER must be measured during the period of differentiation cell, before and after transport experiments.
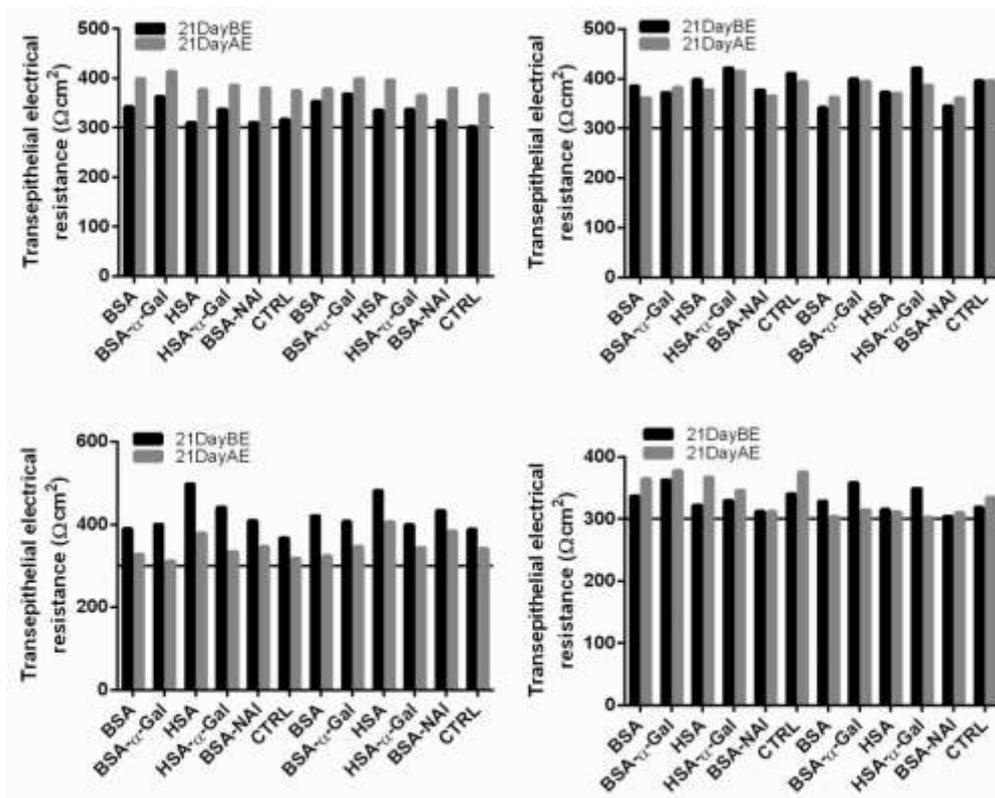
Figure 20. TEER values of Caco-2 monolayer obtained on the 21st day of cell culturing before transport experiment (21DayBE) and after transport experiment (21DayAE), CTRL – untreated Caco-2 monolayer.

Figure 20 presents the TEER values of Caco-2 monolayer obtained before and after transport experiments. The variations in obtained values between 300 and 500 Ω/cm^2 among used monolayers were expected due to the dynamics and complexity of the used system. Interestingly in some transwell inserts the measured TEER values were higher after the transport experiment, while in others were lower than values obtained before the experiment. In all cases obtained values of TEER after transport experiments were higher than 300 $\Omega/cm2$ which indicates that transport of proteins did not disturb the established Caco-2 cell monolayer.

5.3.2. Effect of glycosylation of proteins on their transport through the Caco-2 monolayer

In recent years it becomes evident that protein and allergen transport through intestinal epithelia is also an important parameter in sensitization and food allergy induction. For this purpose as one of the aims of this thesis, *in vitro* model system based on Caco-2 cells was established which was used for the monitoring and comparison of the transport of different proteins (BSA and HSA) which bearing α-Gal epitope (BSA-α-Gal and HSA-α-Gal) through the monolayer of these cells during 4 h of the observation (Figure 21). Their transport rates were compared with the transport rates of appropriate controls (BSA and HSA), and with the BSA-NAl which carbohydrates' residues are similar to α-Gal (Table 2).

Table 2. The average transport rates (ng/h) of the different proteins through the Caco-2 monolayer.

Protein	Transport rate (ng/h)	95 % CI	$r^{2\dagger}$	p-value‡
BSA	142.8	103.9 – 181.7	0.87	< 0.001
BSA-α-Gal	77.2	53.1 – 101.3	0.84	< 0.001
HSA	158	117.2 – 198.7	0.88	< 0.001
HSA-α-Gal	78.8	61.0 – 96.6	0.91	< 0.001
BSA-NAl	135.0	83.4 – 186.6	0.77	< 0.001

† Goodness of fit, ‡ if the slope is different from zero
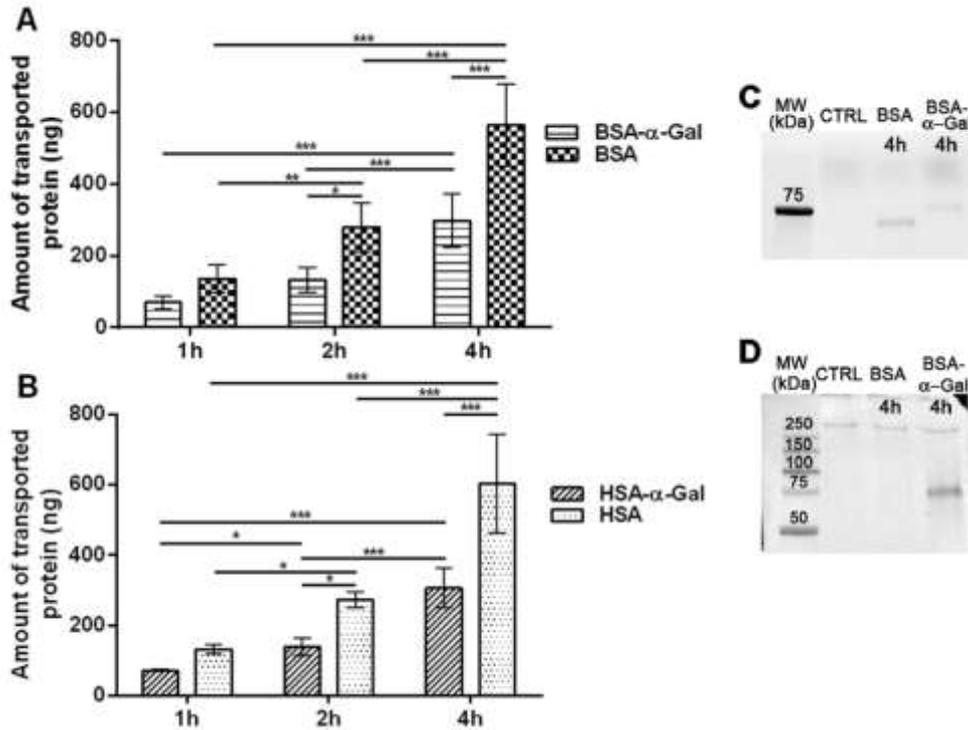
Figure 21. Transport (ng) of proteins carrying α-Gal through the intestinal Caco-2 monolayer. (A) Time-dependent transcytosis of BSA-α-Gal and BSA, n = 4, and (B) human serum albumin (HSA) carrying α-Gal (HSA-α-Gal) and HSA, n = 4. * p < 0.05, ** p < 0.01 and *** p < 0.001 analyzed by two-way ANOVA with Bonferroni's post hoc test; (C) fluorescent detection of BSA-α-Gal and BSA in Caco-2 lysates resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) gel; (D) immunoblot detection of the α-Gal epitope in lysates from Caco-2. MW = Molecular weight markers and CTRL = untreated Caco-2 cells.

Fluorescently labeled BSA-α-Gal in the amount of 400 μg was applied on the apical side of Caco-2 monolayer and after 1 h of incubation 70 ± 19 ng was transported to the basolateral compartment and after 4 h, the amount of transported protein had increased to 299 ± 75 ng (Figure 21A). In comparison to glycosylated protein unconjugated BSA was transported approximately two times more, administrated in the same amount in the apical compartment of Caco-2 monolayer, 136 ± 39 ng had been transported after 1 h and 565 ± 113 ng after 4 h of incubation (Figure 21A). Also, the transport of HSA-α-Gal through Caco-2 cell monolayer was monitored and compared with the transport of unconjugated HSA (Figure 21B). When 400 μg of HSA-α-Gal was applied in the apical compartment 72 ± 3 ng was transported in the basolateral compartment after 1h of incubation and 306 ± 57 ng of protein was transported in the

basolateral compartment after 4 h of incubation. The transport of HSA-α-Gal was similar to that of BSA-α-Gal but significantly lower compared to the transport of HSA. After 1 h of incubation 132 ± 13 ng of HSA had been transported in the basolateral compartment when 400 μg of HSA was applied in the apical compartment and 603 ± 141 ng of HSA was transported in the basolateral compartment after 4 h of incubation. The obtained results showed that the amount of transported proteins was constant over time which enables the calculation of the average transport rates for investigated proteins (Table 2). Unconjugated HSA and BSA have the transport rate almost two times higher (158 and 142.8 ng/h) than HSA-α-Gal and BSA-α-Gal (78.8 and 77.2 ng/h).

The important question was whether transport through the Caco-2 monolayer depends on the type of glycosylation present on the protein. To answer that question we compared the transcytosis of BSA-NAl at different time points (Figure 22). When 400 μg of BSA-NAl was applied in the apical compartment of the transwell inserts, 105 ± 11 ng of protein was transported in the basolateral compartment after 1 h of incubation and 499 ± 167 ng of protein after 4 h of incubation.
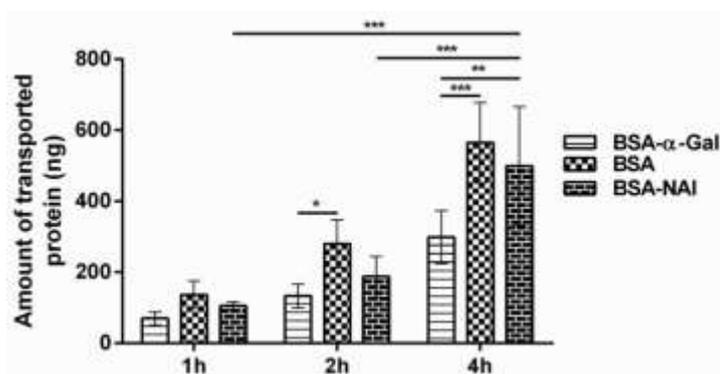
Figure 22. Comparison of transcytosis (expressed as an amount of transported protein) of BSA-α-Gal, BSA, acetyllactosamine (NAl) conjugated to BSA (BSA-NAl) through the Caco-2 monolayer, n = 4, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ analyzed by two-way ANOVA with Bonferroni's post hoc test.

According to the result from Table 2, it can be seen that the calculated average transport rate of BSA-NAl (135.0 ng/h) is almost the same as the transport of BSA (142.8 ng/h). This result indicates that glycosylation alone is not the cause of why BSA-α-Gal and HSA-α-Gal have two times smaller transport rates than BSA and HSA, but also the type of glycosylation.

To confirm the presence of the intact BSA proteins with and without α-Gal epitope in the Caco-2 cells, after transport experiments Caco-2 cell monolayers were collected, cells were lysed and cell lysates were resolved on SDS PAGE. The used PAA gels were illuminated and fluorescently labeled BSA and BSA-α-Gal were visible (Figure 21C). The difference in the MW of the resolved fluorescently labeled proteins suggests that BSA-α-Gal was intact in the Caco-2 cell lysates. For the additional confirmation of the detection of the α-Gal epitope western blot analysis with a chicken single-chain variable fragment (ScFV) anti-α-Gal was performed (Figure 21D). In Figure 21D is a visible band which corresponds to the MW of approx. 75 kDa and it is a strong confirmation that the α-Gal epitope is still present on the intact protein.

According to the results of this experiment, it can be concluded that the *in vitro* developed model system based on Caco-2 cells monolayer can be used for the investigation of the transport rate of different glycosylated food allergens, as well as how specific glycosylation can affect this process.

5.3.3. The α-Gal carrying proteins can be detected in endosomes

In the previous experiment, the presence of intact BSA and BSA-α-Gal was confirmed inside of the Caco-2 cell. In the next experiment, the confocal laser scanning microscopy (CLSM) was applied for visual confirmation of the presence of the fluorescently labeled BSA and BSA-α-Gal inside of Caco-2 cells. This analysis also enables us to get an impression of the spatial distribution of these proteins inside of cells.
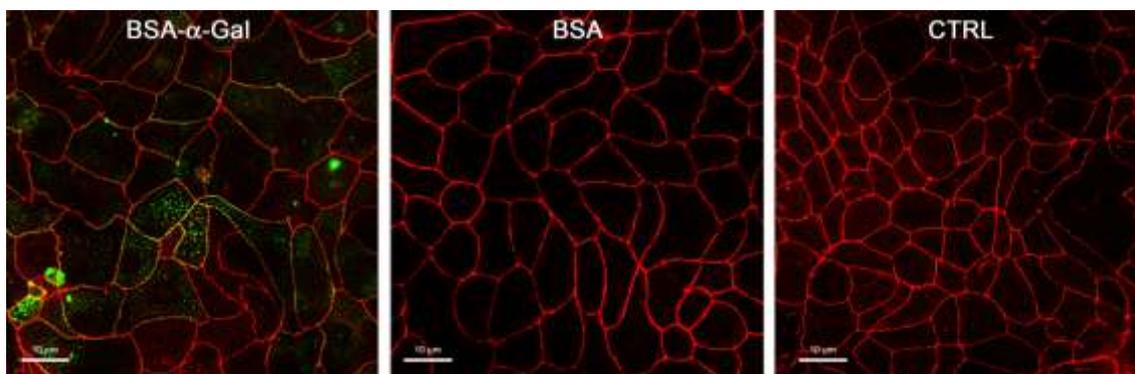
Figure 23. Spatial distribution of fluorescently labeled BSA-α-Gal and BSA with green fluorescent dye AF488 in Caco-2 cells after 4 h of incubation at 37 ˚C analyzed by confocal laser scanning microscopy. CTRL- untreated cells with fluorescently labeled protein.

After 4 h of incubation, CLSM analysis reveals the presence of green fluorescent dye only in the Caco-2 cells which were incubated with   BSA-α-Gal. The lack of the green signal in the control cells as well as in the cells incubated with BSA suggests that there was no accumulation of the BSA inside cells. Scattered green in the cytosol of Caco-2 cell, incubated with BSA-α-Gal implicate that protein is still was present in some small subcellular structures such as endosomes after 4 h of incubation.

Further, for the confirmation of the spatial distribution of proteins inside the cells, differential centrifugation was used to isolate endosomes from Caco-2 cells. Again, using fluorescence detection the presence of intact proteins was confirmed in the cell lysates and subsequently in the endosomal fraction of cell lysates after 4 and 24 h of incubation of Caco-2 cells with BSA and BSA-α-Gal (Figure 24A and 24B). The resolved protein content of the endosomal fraction of Caco-2 cell lysates via SDS PAGE was transferred on the PVDF membrane and probed with a polyclonal anti-BSA antibody (Figure 25). In the corresponding endosomal fraction of Caco-2 cell lysates, both BSA and BSA-α-Gal were detected after 4 and 24 h of incubation. Interestingly BSA was found in both unstimulated Caco-2 cells (CTRL) and BSA-α-Gal stimulated cells, probably due to its presence in FBS supplement in the culturing medium used before the transport experiment.
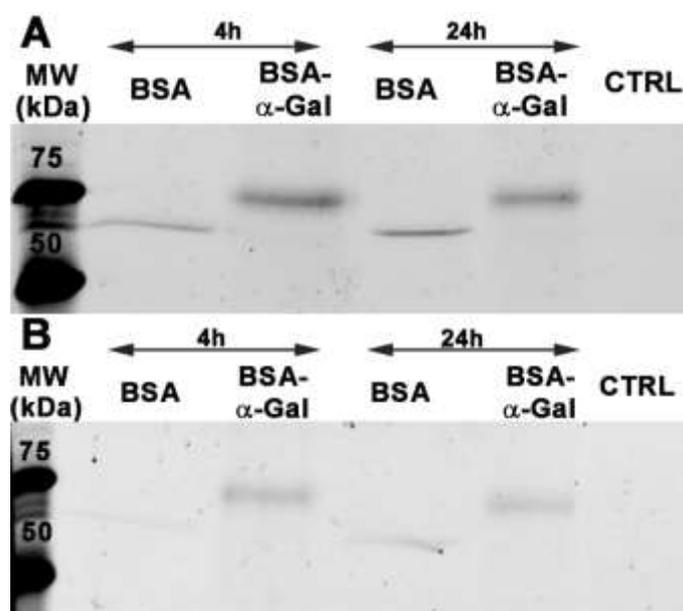
Figure 24. Fluorescent detection of BSA and BSA-α-Gal in (A) Caco-2 lysates resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) gel and (B) endosomal fraction of cell lysates after 4 and 24 h of incubation of Caco-2 cells with BSA and BSA-α-Gal. CTRL-untreated Caco-2cells, MW-Molecular weight markers.
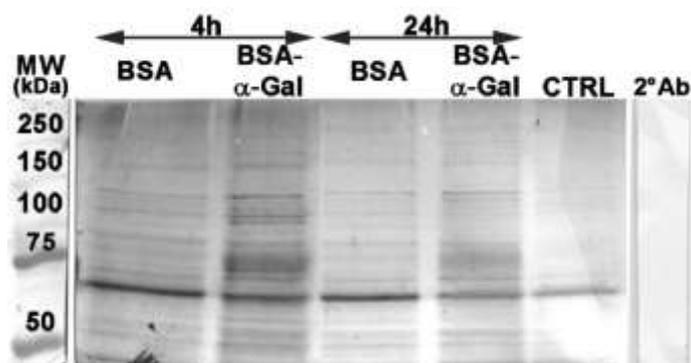
Figure 25. Immunoblot analysis of an endosomal fraction of cell lysates after 4 and 24 h of incubation of Caco-2 cells with BSA and BSA-α-Gal using a polyclonal anti-BSA antibody. CTRL=unstimulated Caco-2cells, MW = molecular weight markers.

The detection of the α-Gal epitope in the endosomal fraction of Caco-2 cell lysates was performed using a monoclonal anti-α-Gal M86 antibody (Figure 26). After 4 h of incubation of Caco-2 cells with BSA-α-Gal, the α-Gal epitope was detected at the MW of approx. 75 kDa which corresponds to the intact protein. Moreover, except intact BSA-α-Gal, an additional band containing the α-Gal epitope was detected at the approx. MW of 150 kDa in the endosomal fraction of the Caco-2 cell lysates. This additional band is probably a dimer of the BSA molecule.
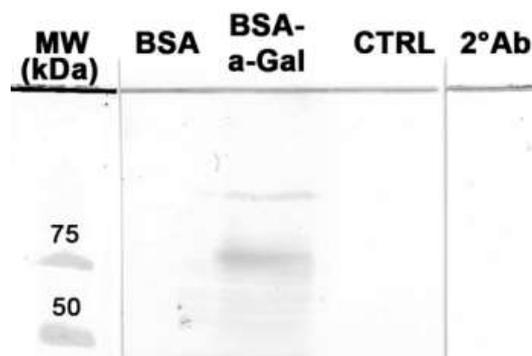
Figure 26. Immunoblot detection of BSA and BSA-α-Gal using monoclonal anti-α-Gal M86 antibody in the endosomal fraction obtained by differential centrifugation of Caco-2 cell lysates after 4 h of incubation, 2°Ab = control of unspecific binding of the secondary antibody, MW = Molecular weight markers, and CTRL = unstimulated Caco-2 cells.

5.3.4. The α-Gal carrying proteins do not influence the level of galectin-3 in Caco-2 cells

Recently the role of galectin-3 is featured in both innate and acquired immune responses. In that sense, it was of importance to examine if there are potential changes in the levels of the galectin-3 in differentiated Caco-2 cells upon incubation with α-Gal conjugated and unconjugated BSA at different time points (Figure 27). According to the results present in this figure, it can be seen that the intensities of galectin-3 bands in stimulated Caco-2 cells are the same as the band in the unstimulated cells, which indicates that the galectin-3 level in Caco-2 cells did not change during transport and uptake of BSA-α-Gal by Caco-2 monolayers.
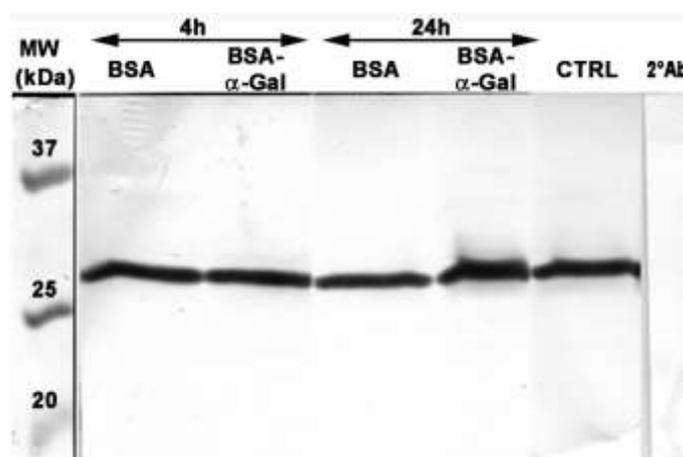
Figure 27. Western blot detection of galectin-3 using the polyclonal anti-human galectin-3 antibody in Caco-2 cell lysates after 4 and 24 h of incubation Caco-2 cells with BSA and BSA-α-Gal. MW = Molecular weight markers, and CTRL = unstimulated Caco-2 cells.

5.4. Discussion

Investigation of complex processes of food digestion, nutrients bioaccessibility, and bioavailability is quite challenging for researchers from the different aspects of research. To solve this puzzle it is necessary to start whit simpler models and aspects and accumulate knowledge to obtain a broader and precise picture of ongoing processes. Since food allergens are transported through gut epithelial cells, it is of importance to investigate the transcytosis of novel allergenic foods.

The results obtained for cell vitality confirmed that culturing conditions did not interfere with Caco-2 cell viability and monolayer integrity. Consequently obtained values of TEER measurements before and after transport experiments confirmed that the Caco-2 monolayer was intact and maintained its integrity after transcytosis. Regarding the transport "it was demonstrated that the presence of the α-Gal epitope on BSA hampers transport through the intestinal Caco-2 monolayer compared to unconjugated BSA, which could be a possible reason why the allergic reaction to α-Gal is delayed. Interestingly, the transport of BSA-NAl was higher than that of BSA-α-Gal, even though the carbohydrates α-Gal and NAl are of similar size, suggesting that the slower transport of BSA-α-Gal is glycosylation specific. In contrast to our results, Román-Carrasco, Lieder et al. (2019) showed that only α-Gal bound to lipids, not to proteins, could be detected in the basolateral medium after transport through the Caco-2 monolayer. However, in both studies different methods for the detection of transported proteins and peptides as well as the amount of α-Gal containing proteins added was different. In this study Krstic Ristivojevic M., Grundström J. et al. (2020) 800 μg/mL of pure α-Gal containing protein was applied compared to 1 mg/mL of digested red meat extract in the study of Román-Carrasco, Lieder et al. (2019), suggesting that the lower amount of α-Gal containing proteins in the extract could not be detected".(Krstic Ristivojevic M., Grundström J. et al. 2020)

Intact proteins with and without α-Gal epitope were detected in Caco-2 cell lysates upon transport studies. Moreover, CLSM revealed that BSA-α-Gal was scattered inside the living cells and its apparent accumulation was notable. On the other hand lack of the green signal arising from BSA suggests that intact protein could not be detected inside of the Caco-2 cells although its presence in Caco-2 cell lysates was confirmed. One explanation for the obtained results is that the CLSM fluorescent signal is collected from max 40 to 50 cells in one confocal plane while for the lysate preparation a minimum of 20 000 cells was used. Also in immunoblots, the signal is amplified by using a primary and secondary antibody. "It has been previously shown that mannosylated BSA is transported by endosomes and lysosomes in alveolar macrophages (Wileman, Boshans et al. 1985). In this study, BSA-α-Gal and the α-Gal epitope on the intact protein were detected in the endosomal fraction of Caco-2 cell lysates, and intact protein was detected even after 24 h of incubation. One of the

possible explanations of this delayed processing is that the presence of α-Gal epitopes on the protein surface leads to steric interference that impedes access to cellular and endosomal proteases involved in protein degradation (Krstic Ristivojevic M., Grundström J. et al. 2020). It has been already demonstrated that protein modifications, such as lactosylation, can influence protein hydrolysis catalyzed by cathepsin D, a protease present in endolysosomal cell compartments (Kastrup Dalsgaard, Holm Nielsen et al. 2007). In the used model of the intestinal epithelium, any effect on the expression of galectin-3 in the Caco-2 cells after stimulation with BSA-α-Gal is not seen. BSA-α-Gal is a harmless protein and since healthy gut epithelium should be tolerant to commensal bacteria of the gut flora, which expresses α-Gal, an inflammatory response cannot be expected." (Krstic Ristivojevic M., Grundström J. et al. 2020) In the shaping of the biological process of immune tolerance to food and microbial antigens besides immune cells also all the cells in the human intestine are participated (Miron and Cristea 2012).

"The main results of this study are that α-Gal carrying proteins are transported through the intestinal Caco-2 monolayer in significantly lower amounts compared to non-α-Gal carrying proteins and that BSA-α-Gal is notably accumulated and retained in endosomes of the intestinal cells. The results, contribute significantly to the understanding of the mechanisms underlying the delayed onset of mammalian meat allergic symptoms. However, further research is needed to elucidate the kinetics of the processing of α-Gal containing proteins." (Krstic Ristivojevic M., Grundström J. et al. 2020)

6. Influence of the α-Gal glycosylation on the protein surface with respect to uptake and degradation in immature monocyte-derived dendritic cells

6.1. Introduction

The cascade of processes leading to an allergic reaction to food starts with the digestion process and bioaccessibility of allergens through their transport across the intestinal barrier and encounter with immune cells. One unusual characteristic of red meat allergy is delayed symptoms, which usually appear from 3 to 6 hours after meat consumption (Nunez, Carballada et al. 2011). It is assumed that the main reason for this is the delayed appearance of the allergen in the bloodstream as well as disturbances in its processing by the immune cells such as dendritic cells and T-cells. "The DCs are professional antigen-presenting cells and the role of immature DCs is sampling the environment to capture antigens and initiate an adaptive immune response by activating and priming naive CD4+ T-cells (Banchereau, Briere et al. 2000). The subsequent outcome of this interaction can be tolerance promotion or induction of active immunity (Steinman 2003). Internalization and degradation of antigens by DCs are important steps in determining whether an antigen will be treated as dangerous or innocuous (Lambrecht and Hammad 2010). Antigens can be actively internalized in several different ways: receptor-mediated endocytosis, macropinocytosis, and phagocytosis (Roche and Furuta 2015). After uptake, the vesicle formed fuses with endosomal compartments where the antigens will be processed into peptides that can be further presented on the cell surface."(Krstic Ristivojevic, Grundstrom et al. 2018) It is very interesting how glycans on glycoprotein were processed by DCs and how the position of the glycan group within MHC on the DCs influences the recognition of the T-cell and antigen-presenting cells. There are two possible scenarios, in the first scenario, glycan is linked to an amino acid serves to anchors the glycopeptide into the MHC and does not participate in MHC binding and as such is nonimmunogenic. In the second scenario when glycan is linked to an amino acid that is pointing away from the MHC molecule and thus participates in MHC in the binding between MHC and T cell, moreover it makes dominant part of recognition by T cell. In that case, the glycopeptide is designated as immunogenic (Werdelin, Meldal et al. 2002). Thus it is very important to

investigate the uptake and the routes of internalization of α-Gal containing proteins by the DCs. Because of this, the last aim of this thesis was to investigate how α-Gal glyco-component on the protein surface influence their uptake and processing by iMDDCs.

6.2. Methodology

6.2.1. Reagents

Used proteins are commercially available. BSA, HSA, bTG, monodansylcadaverine (MDC), and cytochalasin D (CytD) were obtained from Sigma-Aldrich (Saint Louis, MO, USA). α-Gal glycosylated BSA (BSA-α-Gal), HSA (HSA-α-Gal), and N-Acetyllactosamine conjugated BSA (BSA-NAl) were obtained from Dextra Laboratories (Reading, UK). Other chemicals used in experiments were p.a. grade and obtained from Sigma-Aldrich unless is other stated.

6.2.2. Fluorescent labeling of proteins

Used proteins (BSA, BSA-α-Gal, BSA-NAl, HSA, HSA-α-Gal, and bTG) were fluorescently labeled by conjugating of AlexaFluor488 (AF488) fluorescent dye using an AF488 protein labeling kit (Invitrogen, Molecular Probes, Inc., Eugene, Oregon, USA) according to the manufacturer's instructions. For determination of protein concentration commercial kit Pierce Bicinchoninic acid (BCA) Protein Assay Kit (Pierce Biotechnology, Rockford, Illinois, USA) was used.

6.2.3. Donors

In this study, buffy coats from 18 healthy blood donors and four patients diagnosed with red meat allergy were used. Diagnose of red meat allergy was made by a physician experienced in allergic diseases.

6.2.4. Ethics Statement

In this study, all experiments were following relevant guidelines and regulations and approved by the local ethics committee (2014/847-32 and 2016/1447-32). The study participants gave written informed consent for the sample collection.

6.2.5. Cell isolation and culturing

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats by density gradient centrifugation using Ficoll–Paque (GE Healthcare, Uppsala, Sweden). CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were used for CD14+ monocytes isolation following the manufacturer's instructions. The isolated CD14+ monocytes were then cultured for 6 days. The medium for cultivation was 'complete' RPMI (cRPMI) consisting of RPMI-1640 (Gibco) supplemented with 25 µg/mL gentamicin (Gibco), 10% (v/v) heat-inactivated fetal calf serum (FCS; Hyclone, Logan, UT), 2 mM L-glutamine, 100 IU/mL penicillin (Gibco), 100 µg/mL streptomycin (Gibco) and 50 µM β-mercaptoethanol (KEBO-lab, Spånga, Sweden) in the additional presence of 800 U recombinant interleukin-4 (rIL-4; Nordic BioSite, Täby, Sweden) and 10 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; Biosource International, Camarillo, CA). Fresh rIL-4 (800 U/mL) and GM-CSF (10 ng/mL) were added after 3 days of culture. After 6 days when the MDDCs exhibited a typical immature phenotype and uptake analysis were performed. "For uptake analysis, immature MDDCs (iMDDCs) were seeded in multiwell flat-bottom tissue culture plates and incubated at 37 ˚C with 10 µg/10^6 cells of fluorescently labeled proteins for 1, 2, and 4 h. Internalization was inhibited by incubating the iMDDCs at 4°C, or by adding MDC (200µM) or CytD (2µM) 30 min before adding the proteins for each time point. In all experiments, untreated iMDDCs were used as a negative control." (Krstic Ristivojevic, Grundstrom et al. 2018)

6.2.6. Flow cytometry

"Cells were harvested after incubation with AF 488 labeled proteins and stained with LIVE/DEAD Aqua stain (Molecular probes, Thermo Fisher) or fixable viability stain 780 (BD Bioscience, San Jose, Calif., US). After this, the samples were fixed with 2% PFA and surface markers (CD11c-PE-Cy7 (clone B-ly6), CD14-APC (clone M5E2), both from BD Bioscience) were stained. At least 10 000 single cell events were collected on a FACSCanto II flow cytometer using the BD FACS Diva software (BD Bioscience). Data analysis was performed using FlowJo version 10 software (FlowJo LLC, Oregon, US)" (Krstic Ristivojevic, Grundstrom et al. 2018) and used gating strategy is shown in Figure 28.
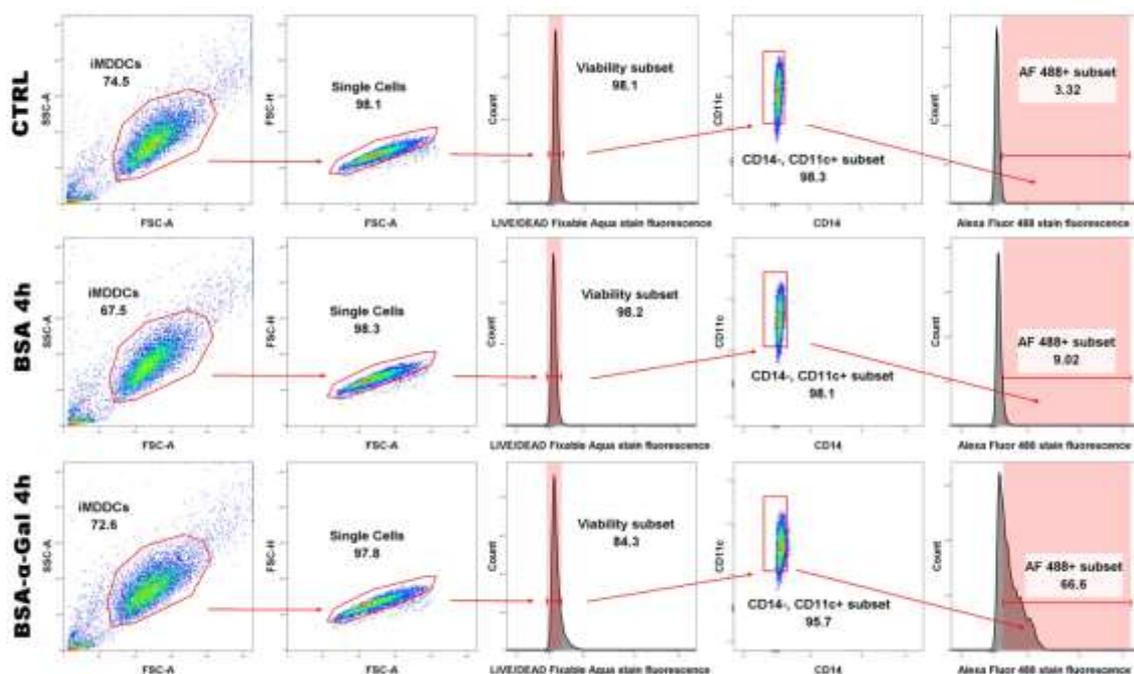
Figure 28. Gating strategy for assessment of protein uptake by immature monocyte-derived dendritic cells after 4h of incubation iMDDCs with BSA and BSA-α-Gal. CTRL= untreated cells. Data analysis was performed using FlowJo version 10 software.

6.2.7. SDS-PAGE and Immunoblot analysis of α-Gal proteins

"After incubation with AF 488 labeled BSA and BSA-α-Gal for 1, 2, 4, 6, 8 and 16 h, iMDDCs were pelleted and the cell pellets were treated with radioimmunoprecipitation lysis buffer. The lysates were clarified by centrifugation and the soluble fractions were separated by SDS-PAGE under reducing conditions. Intracellular AF 488 labeled proteins were visualized on a ChemiDoc system (Bio-Rad, Hercules, CA, US). Following visualization, resolved proteins were transferred to PVDF (0.2 µm pore size) using a Bio-Rad turbo system. The membranes were probed with a previously validated 24 monoclonal anti-α-Gal antibody (M86, Enzo Life Sciences, Farmingdale, NY, USA) followed by alkaline phosphatase labeled goat anti-mouse IgM antibody (Southern Biotech, Birmingham, AL, USA). Immunoreactive bands were visualized using NBT and BCIP substrates (Bio-Rad)." (Krstic Ristivojevic, Grundstrom et al. 2018)

6.2.8. Confocal Laser Scanning Microscopy

"The iMDDCs were cultured for 1 h at 37°C on coverslips coated with poly-L-lysine (Neuvitro Corporation, WA, USA) to allow cell attachment. After 1 and 4 h of incubation with AF 488 labeled proteins ($10\mu g/10^6$ cells), the coverslips were washed with PBS and stained with purified anti-human HLA-DR antibody (clone L243, BioLegend, San Diego, CA, USA), followed by a secondary goat anti-mouse IgG antibody conjugated with Alexa Fluor 555 (AF 555) (clone Poly4053, BioLegend). The coverslips were mounted using Prolong Gold Antifade reagent with DAPI (Molecular probes). The CLSM was performed using the LSM 510 Meta system (Carl Zeiss, Jena, Germany) individually modified to allow imaging with improved detection efficiency using avalanche photodiodes 25. The alpha Plan-Fluar 100x/1.45 oil immersion objective (Zeiss MicroImaging GmbH, Jena, Germany) was used throughout. Images were acquired at 1024×1024 pixel resolution, scanning speed 12.6 µs/pixel, without averaging, and prepared for publication using the Zeiss LSM Image Browser software." (Krstic Ristivojevic, Grundstrom et al. 2018)

6.2.9. Statistical analysis

Data are presented as mean value ± SD. Differences in protein uptake at the same time point were analyzed by two-way ANOVA using Bonferroni post hoc test. Differences in protein uptake at different time points were analyzed by two-way ANOVA using Tukey post hoc test. The effect of inhibition and uptake for red meat allergic patients was analyzed by paired t-test. Differences were considered significant if $P < 0.05$. For statistical analysis, GraphPad Prism version 6.00 software for Windows (GraphPad Software, La Jolla, CA, USA) was used.

6.3. Results

6.3.1. The α-Gal glycosylation of protein influences protein uptake in iMDDCs

As one of the cells of the immune system which underlines the layer of the intestinal cells and one of the most important cells in the initiating of immune responses, DCs were inevitable in the examination of the uptake of the proteins conjugated to α-Gal epitope and unconjugated ones. The iMDDCs differentiated from

healthy individuals were analyzed by flow cytometry after 1, 2, and 4 h of the incubation with fluorescently labeled α-Gal and non-α-Gal carrying BSA or HSA (Figure 29).

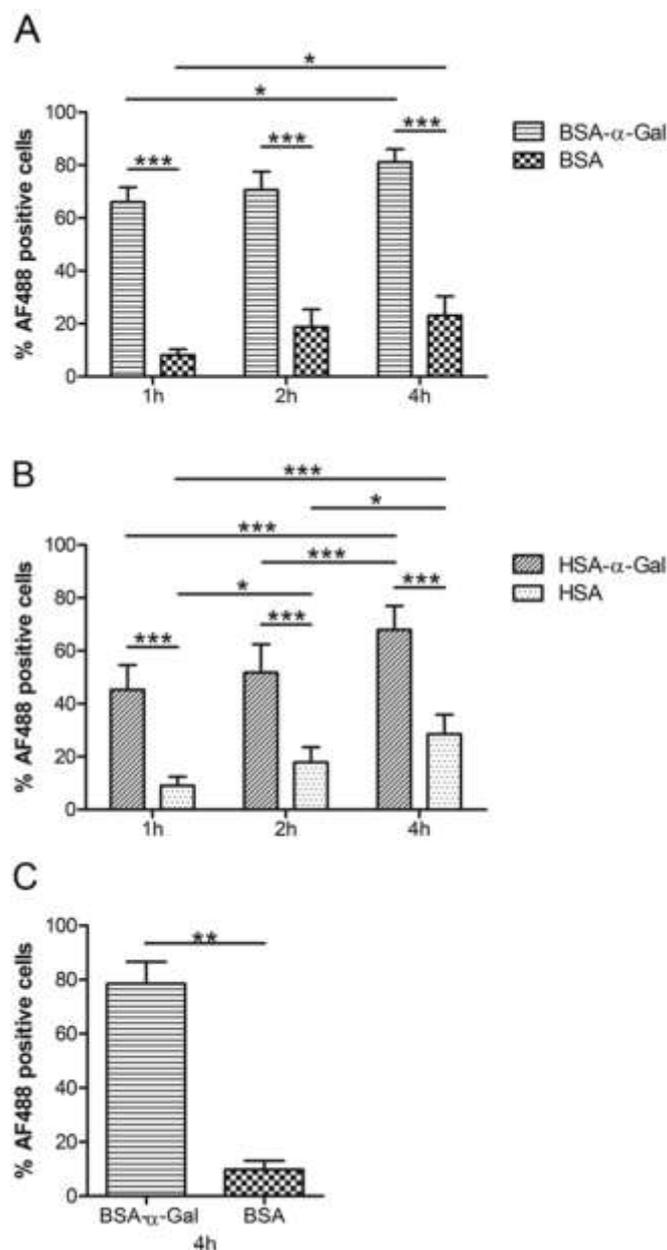**Figure 29.** Time-dependent uptake of (A) BSA-α-Gal and BSA, n = 10, and (B) HSA-α-Gal and HSA, n = 8, by iMDDCs generated from healthy donors. *p < 0.05, and ***p < 0.001 analyzed by two-way ANOVA with Bonferroni's post hoc test. (C) Uptake of BSA-α-Gal and BSA by iMDDCs generated from red meat allergic patients after 4 h of incubation, n = 4. **p < 0.001 analyzed by paired t-test.

From the obtained results it is evident that the internalization of BSA-α-Gal by iMDDCs was significantly higher compared to internalization of BSA at all three time points ($p < 0.0001$, Figure 29A). Also, the accumulation of BSA-α-Gal, inside cells, has been increasing along with the period of incubation ($p = 0.0107$, from 1 h to 4 h). Furthermore, similar results were obtained for the uptake of the HSA-α-Gal and unconjugated HSA (Figure 29B). Uptake and internalization of both proteins increased over time (HSA-α-Gal: $p < 0.0001$ from 1 to 4 h and $p = 0.0002$ from 2 to 4 h, HSA: $p = 0.0441$ from 1 to 2 h, $p < 0.0001$ from 1 to 4 h and 0.0127 from 2 to 4 h). Also, the uptake of HSA-α-Gal was significantly higher compared to the uptake of HSA ($p < 0.0001$ at all three time points).

In the next experiment iMDDCs differentiated from four red meat allergic individuals were used for the monitoring of the uptake and internalization of the α-Gal conjugated and unconjugated BSA after 4 h of incubation with iMDDCs (Figure 29C). Obtained results show that BSA-α-Gal was internalized by these cells a statistically significant in a higher degree than BSA ($p = 0.0013$). This was similar to the result obtained for healthy individuals.

6.3.2. Influence of protein size and the type of carbohydrate modification carried by the protein on the uptake of protein by iMDDC

In the previous experiment, it was proved that iMDDCs very efficiently uptake the α-Gal-proteins. In the next experiment, iMDDCs obtained from the healthy individuals were used for the investigation of whether the molecular size of proteins or the type of carbohydrate modification carried by the protein affect uptake of proteins by iMDDC. For this experiment, besides BSA and BSA-α-Gal as the controls, bTG and BSA-NAl were used. These two proteins were chosen because bTG is naturally carrying α-Gal epitope and has 10 times bigger molecular weight than BSA-α-Gal, while BSA-NAl has a carbohydrate modification N-acetyllactosamine similar size as α-Gal. The uptake of above mentioned all proteins labeled by fluorescence dye AF 488 by iMDDCs after 1 and 4 h of incubation was monitored by flow cytometry.

The obtained results showed that the uptake of bTG was significantly higher compared to BSA after 1 and 4 h of incubation ($p < 0.0001$). The uptake of bTG was

comparable to the uptake of BSA-α-Gal (Figure 30A). The uptake and internalization of BSA-NAl after 1 and 4 h of incubation of iMDDCs was significantly lower compared to BSA-α-Gal ($p < 0.0001$), and it was comparable to the uptake of BSA (Figure 30B).
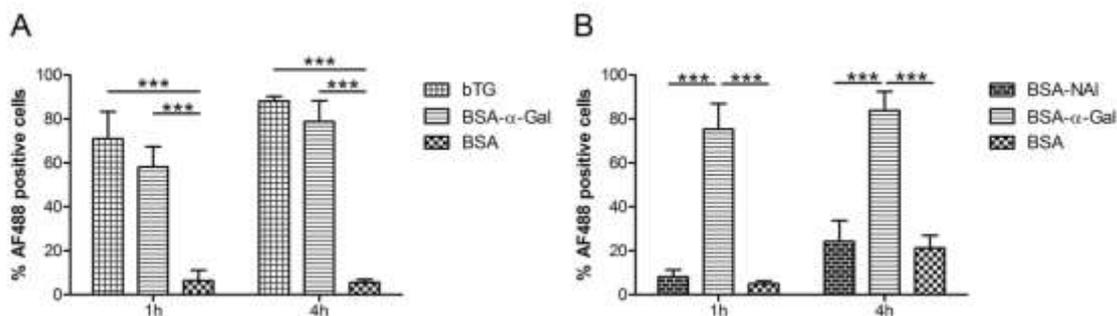
Figure 30. The impact of protein size and conjugation on protein uptake. The uptake of bTG compared to the uptake of BSA-α-Gal and BSA. (B) The uptake of BSA-NAl compared to the uptake of BSA-α-Gal and BSA. iMDDCs were generated from healthy blood donors, n = 4. ***$p < 0.001$ analyzed by two-way ANOVA with Bonferroni's post hoc test.

According to the obtained results, it can be concluded that: the size of protein did not have any effect on the uptake of protein by iMDDC; the type of glycosylation has a big influence even when the size of glycans similar.

6.3.3. Investigation of the internalization routes of proteins by iMDDCs

The uptake and internalization of different proteins (BSA, HSA, bTG) carrying to α-Gal epitope by iMDDCs obtained from healthy blood donors and red meat allergic patients were demonstrated in the previous experiments. For the understanding of these processes, it is very important to examine a potential route of the entrance of those proteins into the cells as well as whether the uptake of proteins into iMDDCs is an active process. In this experiment, the uptake of BSA and BSA-α-Gal in the presence of two inhibitors CytD and MDC and at 4°C by iMDDCs was investigated.

According to the obtained results in Figure 31, it can be seen that the uptake of α-Gal carrying proteins is an active process since internalization of BSA and BSA- α-Gal was significantly lower ($p = 0.0165$; $p = 0.0017$, respectively) during 4h of cells incubation at 4 °C compare to the incubation at 37 °C.

Figure 31. The internalization of BSA-α-Gal and BSA by iMDDCs obtained from healthy blood donors after 4 h of incubation at 37 and 4°C, n = 4 (BSA-α-Gal, p = 0.0017, and BSA, p = 0.0165), * = p < 0.05, ** = p < 0.01 and *** = p < 0.001 analyzed by paired t-test.

For the analysis of the route of the protein internalization, inhibitory agents such as CytD and MDC were added to iMDDCs cultures 30 min before the addition of BSA-α-Gal or BSA. The effect of CytD which inhibits macropinocytosis and MDC which inhibits receptor-mediated endocytosis, on the uptake of BSA-α-Gal or BSA by iMDDCs was analyzed by flow cytometry after 1 and 4 h of incubation of iMDDCs with BSA-α-Gal or BSA (Figure 32).

Incubation of iMDDCs with both CytD and MDC statistically significant inhibited the internalization of BSA-α-Gal and BSA after 1 h (Figure 32A and C) and 4 h of incubation (Figure 32B and D). The effect of both inhibitors on the inhibition of uptake of BSA-α-Gal is more prominent after 1h of incubation than 4h of incubation. According to the number of cells labeled by the fluorescent dye, MDC shows a bigger effect on the inhibition of BSA-α-Gal uptake than CytD after 1h of incubation.
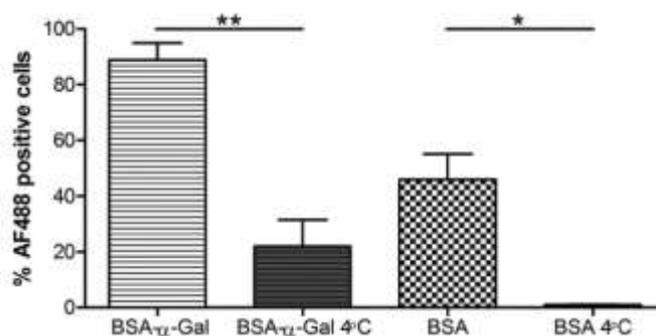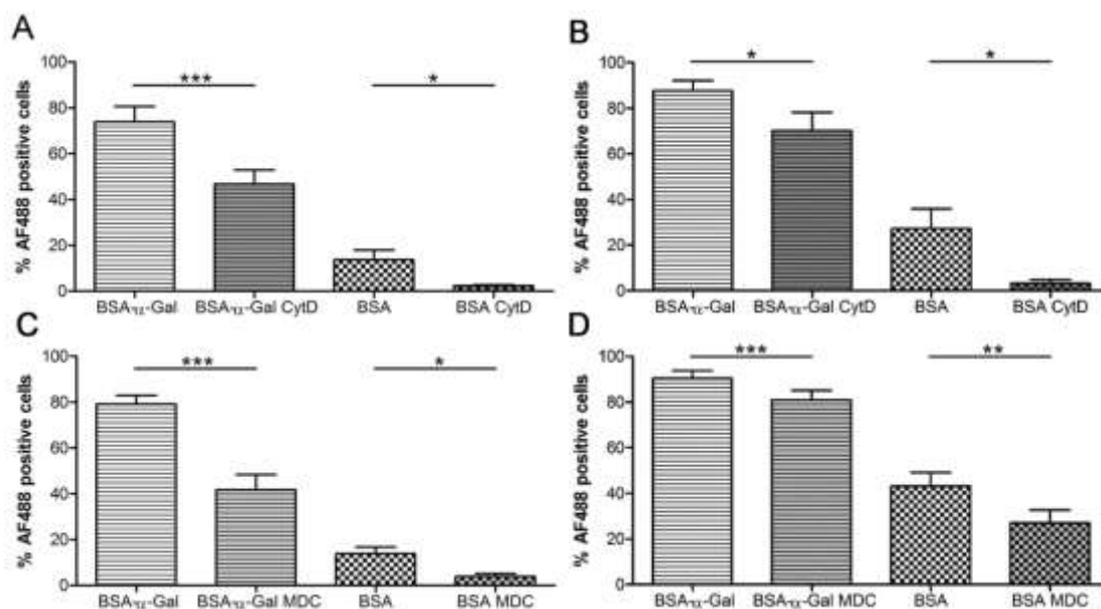
Figure 32. The internalization of BSA-α-Gal and BSA by iMDDCs obtained from healthy blood donors after 1 h (A and C) and 4 (B and D) of incubation of iMDDCs and proteins in the presence of (A) and (B) Cytochalasin D,(n = 6; BSA-α-Gal and n = 5; BSA), and (C) and (D) Monodansylcadaverine (n = 7). *p < 0.05, ***p < 0.001 analyzed by paired t-test.

6.3.4. The processing of proteins with and without α-Gal epitope by iMDDC

Although all previous experiments indicate that proteins, which carrying the α-Gal epitope are internalized by iMDDCs nothing is known about their further fate in these cells. One important question is: in which way proteins that carrying α-Gal epitope are processed by iMDDCs. Are any differences in the rate of processing of proteins with and without the α-Gal epitope? In an attempt to get answers to these questions, in the next experiment, the presence of BSA and BSA-α-Gal labeled by fluorescent dye AF 488, in the lysates of iMDDCs obtained at different time points was analyzed by SDS PAGE. The bands of BSA and BSA-α-Gal in the gel were visualized by fluorescent detection (Figure 33A), while the presence of α-Gal epitope on the intact proteins or their digested fragments was analyzed by immunodetection using monoclonal anti-α-Gal M86 antibody (Figure 33B).

The bands of BSA-α-Gal and BSA were detected at MW which corresponds to approx. MW of their intact form. Interestingly in the case of BSA-α-Gal, a band above 75 kDa, which corresponds to the intact protein was present for up to 16 h of uptake of

BSA-α-Gal. The protein band lower than the intact protein was detected after 4, 6 and 8 h of BSA-α-Gal incubation (Figure 33A). The detected band at lower MW than intact BSA-α-Gal probably represents degraded products of the protein. Regarding the uptake of BSA, a strong protein band, which corresponds to the intact protein was detected for up to 6 h of uptake while after 8 and 16 h of uptake very weak protein band could be noted at a slightly higher MW.

When the presence of the α-Gal epitope is analyzed in iMDDCs lysates, it can be seen from Figure 33B that the α-Gal epitope was present for up to 16 h at MW of the band which corresponds to the intact protein. Besides this band, in all time points of the protein uptake weak protein bands at approx. MW of 150 kDa was detected.
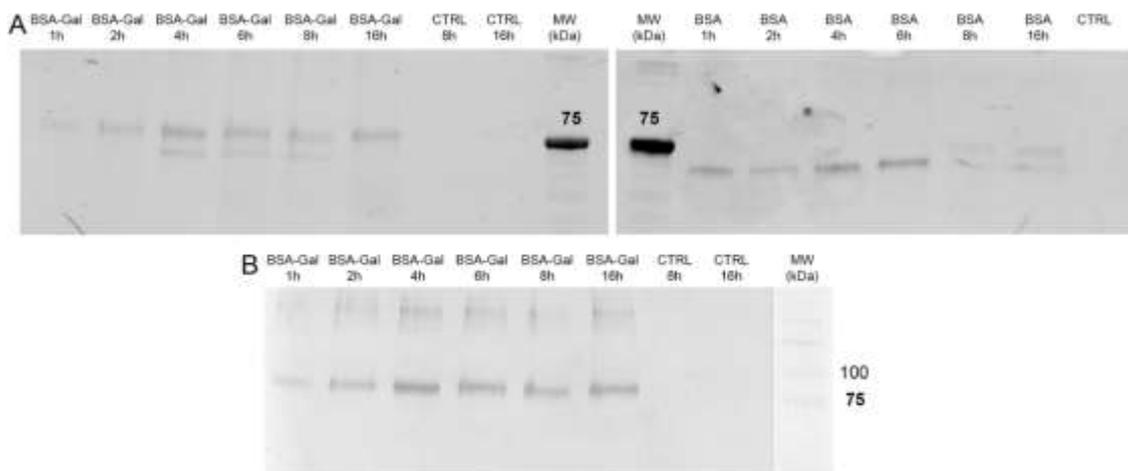
Figure 33. (A) Fluorescent detection of BSA-α-Gal and BSA labeled by fluorescent dye AF 488 and (B) Western blot detection of the α-Gal epitope using monoclonal anti-α-Gal M86 antibody in iMDDCs lysates prepared from iMDDCs after 1 to 16 h of incubation with BSA-α-Gal and BSA, and resolved using SDS polyacrylamide gel electrophoresis. iMDDCs were generated from healthy blood donors, CTRL=unstimulated cells.

6.3.5. The α-Gal containing proteins are scattered in the cytoplasm of iMDDCs

For a better understanding of the processes behind the processing of proteins that carry α-Gal epitopes such as BSA-α-Gal and bTG inside of DCs, CLSM analysis was used. The iMDDCs were incubated with BSA-α-Gal and BSA AF488 labeled for 1 and 4 h at 37 ˚C, after the end of this period iMDDCs were prepared for CLMS to monitor

the uptake of proteins because this analysis enables very accurate prediction of the spatial distribution and localization of proteins inside the cells (Figure 34). Besides these proteins, CLSM was used for the monitoring of the uptake of bTG and BSA-NAl, by iMDDCs. The bTG, which naturally possesses α-Gal epitope as posttranslational modification and has the MW 10 times higher than the MW of BSA-α-Gal and BSA-NAl, which carrying a glycan similar size as α-Gal were used to check how iMDDCs uptake and process these proteins (Figure 35).

The obtained images using CLSM are presented in Figure 34 and fluorescent green signals arising from BSA-α-Gal were detected in almost 50% of the iMDDCs represented in that confocal plane. Furthermore, after 4 h of incubation green fluorescent signal was dominantly localized in the perinuclear region of almost all iMDDCs represented in that confocal plane. On the other hand, there was no detectable green signal arising from fluorescently labeled BSA neither after 1 or 4 h of the monitored uptake. Importantly CLSM imaging was following the results obtained using flow cytometric analysis.
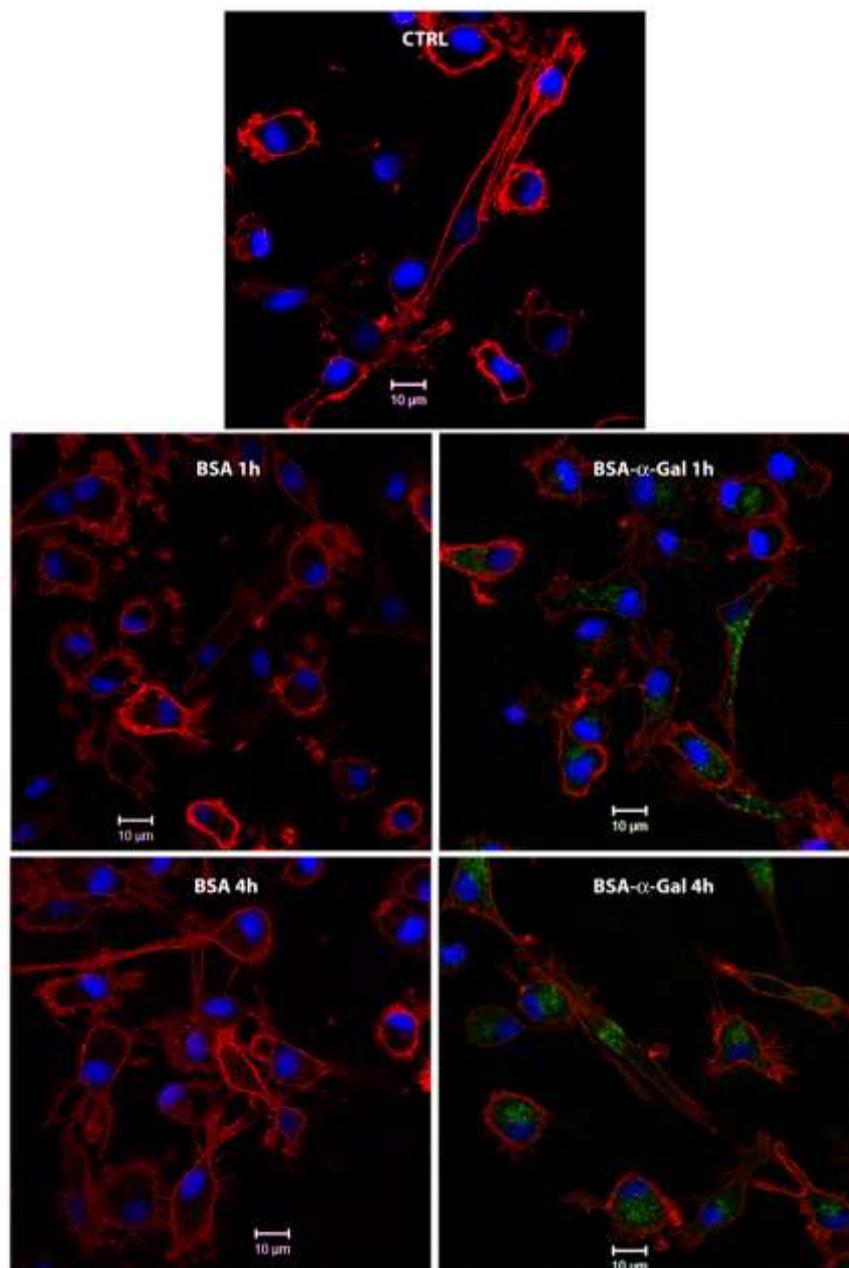
Figure 34. The uptake of BSA-α-Gal and BSA by iMDDCs after 1 h and 4 h of incubation iMDDC with AF488 labeled proteins at 37 °C was analyzed by confocal laser scanning microscopy. CTRL = unstimulated iMDDCs, green = BSA-α-Gal or BSA, red = HLA-DR and blue = DAPI stained nuclei.

It is evident the lack of green fluorescent signals arising from BSA-NAl after 1 and 4 h of incubation of iMDDCs with protein, while strong green signal arising from bTG was detected scattered in cell cytosol of almost all iMDDCs after 1 and 4 h of the uptake. Overall results demonstrated that proteins with α-Gal epitope as its sugar modification were retained and accumulated inside of iMDDCs.
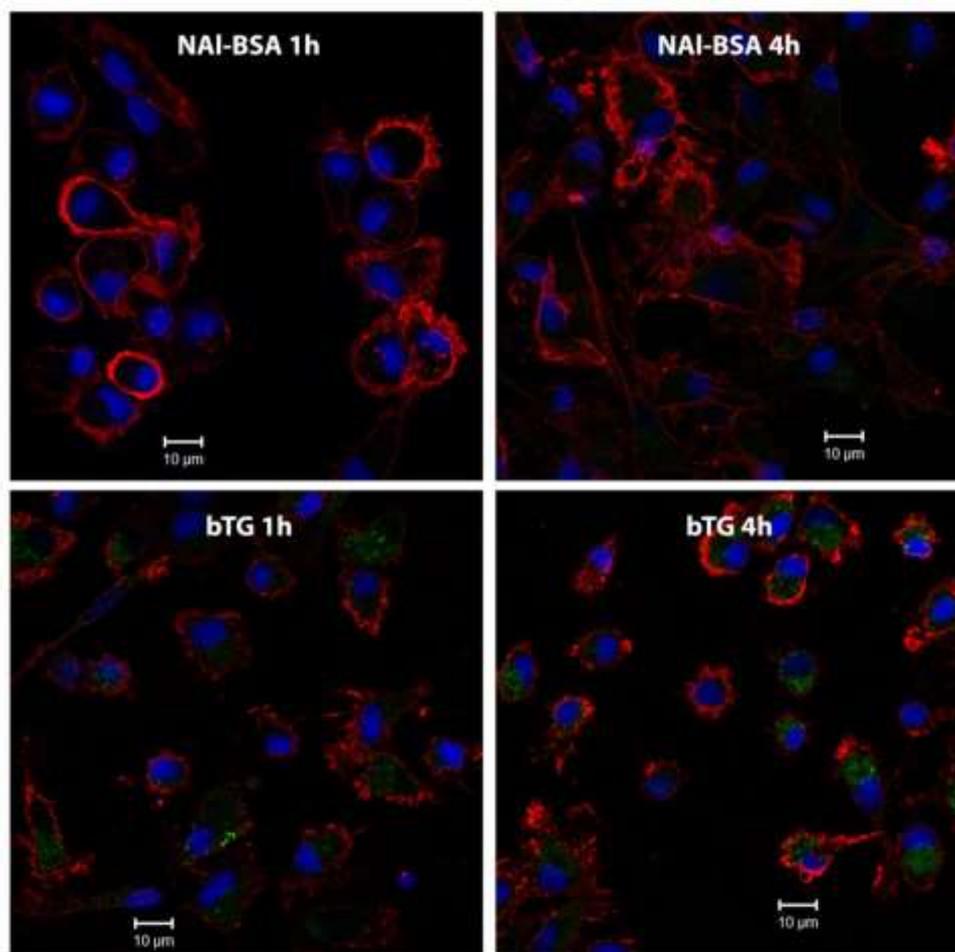
Figure 35. The uptake of BSA-NAl and bTG by iMDDCs after 1 h and 4 h of incubation iMDDC with AF488 labeled proteins at 37 °C was analyzed by confocal laser scanning microscopy. Green = BSA-NAl or bTG, red = HLA-DR and blue = DAPI stained nuclei.

6.4.   Discussion

"The α-Gal rich foods elicit delayed allergic symptoms in red meat-allergic patients, which could be due to uptake and processing by DCs. The present study (Krstic Ristivojevic, Grundstrom et al. 2018), shown efficient uptake of the α-Gal model antigens like BSA-α-Gal and HSA-α-Gal by *in vitro* cultured human iMDDCs. Similar results were obtained for iMDDCs generated from healthy individuals and red meat allergic patients, indicating that altered uptake of α-Gal in DCs *per se* is not a factor contributing to red meat allergy. However, DCs are likely involved in the sensitization phase of red meat allergy where α-Gal containing proteins may be taken up in the close vicinity of a tick bite. In atopic individuals, the DCs may then initiate allergic

sensitization through skewing of CD4+ T cells towards a Th2 response. A recent report showed that the context of allergen uptake is important for the maturation of iMDDCs and that the maturation effect differed between healthy and atopic donors (Aglas, Gilles et al. 2018).

Interestingly, the uptake of BSA and HSA alone was not as efficient as for their α-Gal carrying counterparts. Thus, the presence of α-Gal on the surface seems to enhance the internalization of the protein, suggesting an α-Gal dependent uptake mechanism. The results are in line with previous studies on glycosylated protein uptake, where a 100-fold higher uptake of mannosylated BSA compared with non-mannosylated BSA was demonstrated (Engering, Cella et al. 1997). Furthermore, advanced glycation end products of ovalbumin were taken up more efficiently by iMDDCs compared with ovalbumin (Hilmenyuk, Bellinghausen et al. 2010).

It also investigated if the size and type of conjugation of the proteins have an impact on the uptake. The iMDDCs from healthy individuals showed no difference in the uptake of BSA-α-Gal and bTG even though the MW of bTG is approx. 10 times higher than the MW of BSA-α-Gal. Interestingly, the uptake of BSA-NAl was several times lower compared to BSA-α-Gal, even though the α-Gal and NAl carbohydrates are of similar size. Previous studies have shown that MDDCs internalize BSA with different glycosylation patterns to very varying extent (Avraméas, McIlroy et al. 1996), and for other glycosylated allergens, the mannose receptor plays a major role in glycoallergen recognition and uptake (Royer, Emara et al. 2010). The results of this study strongly suggest that the specific glycosylation of protein antigens predominantly influences the uptake, while the size of the protein is not a decisive factor. This is in line with previous studies which have shown that human DCs, in addition to protein antigens, can internalize large size particles (Foged, Brodin et al. 2005) while the key factor that influences protein uptake is the surface architecture (Engering, Cella et al. 1997).

What was next elucidated in this study, whether α-Gal could be detected in the cytoplasm of iMDDCs. Fluorescence detection confirmed the presence of BSA-α-Gal in iMDDC cytoplasm. Moreover, SDS PAGE analysis showed that BSA-α-Gal was detected after up to 16 h of incubation, while BSA was only detected up to 6 h. Western blot with monoclonal anti-α-Gal antibody confirmed that the BSA-α-Gal still carries the

epitope. These results suggest that the degradation pathway of α-Gal carrying proteins is slower than for non-carrying proteins and that they are stored intact inside of cells. Most likely the α-Gal glycosylation protects the protein from degradation and as such may increase the processing time and exposure on the surface of the cell. Other studies have indeed shown that reduced degradation increases antigen presentation on MHCII (Delamarre, Pack et al. 2005) and that the allergenic Bet v 1a was degraded slower than its hypoallergenic isoform Bet v 1d (Freier, Dall et al. 2015), suggesting that processing time affects allergenicity. This is also in line with the delayed appearance of symptoms in red meat allergic patients.

The route of internalization of proteins was analyzed by adding CytD and MDC to the iMDDC cultures which resulted in partial inhibition of the internalization of BSA, regardless of the presence of α-Gal. The inhibition was stronger after 1 h of incubation than after 4 h, which probably reflects a temporary effect by both inhibitory agents. MDC blocks receptor-mediated endocytosis whereas CytD affects the invagination of the cell membrane by blocking actin polymerization, which inhibits macropinocytosis and phagocytosis (Ivanov 2008). However, the blocking of actin polymerization may also inhibit receptor-mediated endocytosis (Ivanov 2008). Fluorescently labeled BSA can be used for measuring uptake *via* macropinocytosis, but as with chemical inhibition of antigen-uptake, it is difficult to unambiguously distinguish between macropinocytosis and receptor-mediated endocytosis as the uptake mechanism in DCs (Liu and Roche 2015). These results thus indicate that both receptor-mediated endocytosis and macropinocytosis are involved in the uptake of α-Gal containing proteins.

The confocal analysis revealed accumulated BSA-α-Gal and bTG after 1 and 4 h of incubation, whereas no internalization of BSA and BSA-NAl could be detected at any time point. Thus, for antigen uptake, the conjugated sugar seems to be more important than the protein size, in line with the flow cytometric analysis. After 1 h the proteins were found in clusters scattered around the cytoplasm while after 4 h, the proteins were found closer to the nucleus, suggesting that BSA-α-Gal is taken up and processed in endosomes.

In conclusion, α-Gal containing proteins are actively and promptly internalized by iMDDCs in a time-dependent manner, and the mechanism of internalization seems to be α-Gal dependent. Also, the differences in degradation kinetics of proteins carrying α-Gal and those that do not reflect the delayed onset of symptoms that red meat allergic patients experience. Taken together, it is shown that α-Gal is important for antigen processing and these results add new knowledge of α-Gal as a clinically relevant food allergen." (Krstic Ristivojevic, Grundstrom et al. 2018)

7. Conclusions

According to the aims set out in section 3, the following conclusions are drawn:

a) The α-Gal glycosylation hampers the resistance of BSA to pepsinolysis and alters the digestion patterns of BSA-α-Gal compared to BSA, whereas large digestion products of BSA-α-Gal bearing α-Gal were prolonged time present in the digestion mixture.

b) The type of glycosylation influence the protein susceptibility to pepsinolysis, α-Gal glycosylation hampers the resistance of BSA to pepsinolysis, but NAl glycosylation hampers pepsinolysis even more.

c) The presence of the α-Gal epitope on BSA influences their transport through the intestinal Caco-2 monolayer compared to unconjugated BSA and the slower transport of BSA-α-Gal is glycosylation specific.

d) The intact proteins and the α-Gal epitope on the intact protein can be detected in the endosomal fraction of Caco-2 cell lysates which implicates the delayed processing of α-Gal carrying proteins in the intestinal cells.

e) The level of pro-inflammatory factors (galectin-3) in the intestinal cells is not affected by the presence of BSA-α-Gal in comparison to BSA.

f) The presence of α-Gal on the protein surface enhances their internalization by iMDDCs.

g) The types of protein glycosylation of antigens influence their uptake by iMDDCs, while the size of the protein is not a decisive factor.

h) The α-Gal glycosylation protects the protein from degradation in the iMDDCs and as such may increase their processing time and exposure on the surface of the cell.

i) The receptor-mediated endocytosis and macropinocytosis are involved in the uptake of α-Gal containing proteins by iMDDCs.

j) The BSA-α-Gal is taken up and processed the most probably in endosomes in iMDDCs.

8.   Appendix

Biosafety level risk

Biological agents of plant, animal, or human origin are classified according to biosafety level risk (BSL). This means that work with such agents requires the strict following of guidelines for laboratory facilities, safety equipment, and laboratory practices and techniques. There are four levels of biological containment (BSL 1 - 4) and experiments performed in this doctoral dissertation were performed in laboratories that apply BSL-2 containment. This means that agents used in performed experiments pose a moderate risk to personnel and the environment (BSL-2 risk) and BSL-2 containment required lab coats, gloves and face protection, biological safety cabinets (Class 2, type A, BSC), decontamination of waste materials, restricted access to laboratory and laboratory work supervised by a trained scientist who understands the risk associated with used biological agents.

General cell-culturing protocols

The work with cell cultures and cell-based models follows some specific assay protocols, also some general protocols which are routines in cell-cultures laboratories worldwide.

Thawing cells

To ensure the maximum survival of cells upon thawing the next general protocol should be followed:

1. The culturing medium should be preheated to 37 °C.

2. The cryovials containing frozen cells should be inserted into the water bath adjusted to 37 °C immediately after removal from liquid nitrogen containers or deep-freezer to prevent cell lysis.

3. Upon defrosting the content, the cryovials should be transferred to the biological safety cabinet and the cell suspension should be carefully passed on appropriate volume prewarmed culturing, usually 5 mL of culturing medium in a 25 mL flask (T-25).

4. The cells suspension in the medium should be gently mixed and culturing flask should be placed in the incubator for maintaining the optimal conditions for cell culture growth.

5. The following day culturing medium should be changed with a fresh medium to increase the survival of still fragile cells and to allow cell adhesion for adherent cell cultures also the viability of the cells and eventual presence of contaminants such as bacteria should be checked under a microscope.

Maintaining cell culture

The cell cultures and cell-based models are usually grown in an air atmosphere in incubators which provides them a constant temperature of 37 °C and the optimal percentage of CO_2. The cell suspension or monolayers are kept in appropriate sterile dishes such as flasks, Petri dishes, transwell inserts, etc. in an optimal culturing medium usually supplemented with FBS and antibiotics. For optimal cell growth culturing medium should be regularly replaced with a fresh one, usually, mediums contain pH indicators such as phenol red whose color change signals the level of metabolic activity of the cells which results in a change in pH of the culturing medium. The cells grown in laboratory conditions follow the cell growth curve (Figure 36) which consist of *lag* phase which reflects the cells recover from sub-cultivation, attach to the surface and start to spread, *log* phase which reflects exponential cells growth and characteristic doubling rate of cells which defines the cell line's doubling time, plateau phase in which the culture is confluent and cell growth slows or even stops and eventually death phase during which cells start dying and in case of adherent cultures detach from the surface.
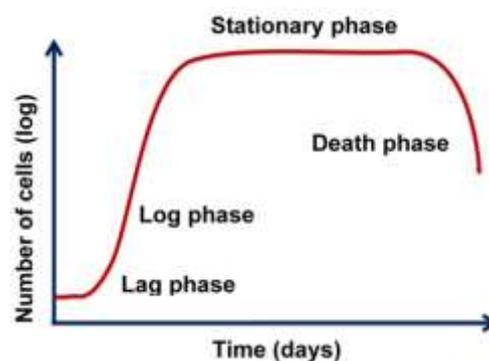

Figure 36. Phases of cell cultures growth.[12]

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[12] Available at https://www.leica-microsystems.com/science-lab/how-to-do-a-proper-cell-culture-quick-check/

It is crucial to ensure viability and stabile proliferation rate, as well as genetic stability of the cultured cells for that purpose cells should be kept in their exponential phase of growth i.e. *log* phase. To ensure the mentioned characteristics the cell cultures should be sub-cultured before reach the stationary phase of growth. For each cell line, the growth curves should be determined since the optimal splitting ratio is evaluated based on it. The number of sub-cultures is designated as passage number which must be regularly recorded and kept as low as possible.

Sub-culturing protocol

For the cell cultures grown in suspension, the sub-culturing protocol is quite simple when the growth curve and subsequently splitting ratio was determined. The cell suspension should be collected from the culturing vessel, transferred to the appropriate tube, and centrifuged to spin down the cells to the bottom of the tube. The residual medium should be carefully discarded and optionally the cells can be washed with PBS which requires second round centrifugation and the remaining PBS should be discarded. The cells should be resuspended in an appropriate volume of fresh medium suitable for use in subsequent cell counting and optimal cell splitting.

For the adherent cells, when cultured cells usually rich 80 – 90% of confluence, which can be determined by simple inspection of the cell culture under the microscope, sub-culturing is recommended. First cell monolayer should be washed with PBS to remove the remaining FBS from the culturing medium, next the cells should be detached from the bottom of the vessel by the addition of trypsin or in case of semi-adherent cells prolonged incubation with PBS and simply tapping the bottom of the vessel is enough. The detached cells should be resuspended in a fresh medium containing FBS to ensure the inactivation of the trypsin since FBS contains trypsin inhibitors. Further, the cell suspension should be centrifuged, a medium should be discarded and the cells should be resuspended in an appropriate volume of fresh medium suitable for use in subsequent cell counting and optimal cell splitting.

Cell freezing

For the long-term storage and preservation of the cell lines, the cells should freeze. After passage and cell counting, a definite number of viable cells usually up to 6 x 10^6 cells in 1 mL of the freezing solution should be left for storage. The used freezing

solution must contain a cryoprotective agent such as DMSO or glycerol to reduce the detrimental effects which freezing can have on cell viability. The cryoprotective agents reduce the freezing point and increase the permeability of the cell membrane to water. The cell suspension in freezing solution should be stored at – 80 °C for up to 6 months and subsequently transferred in liquid nitrogen for long term storage.

Cell counting

Cell counting is performed on regular basis in cell laboratories, for that purpose commercial devices so-called cell-counters can be used but cell counting using cell-counting chambers i.e. hemocytometers is unsurpassed (Figure 37).
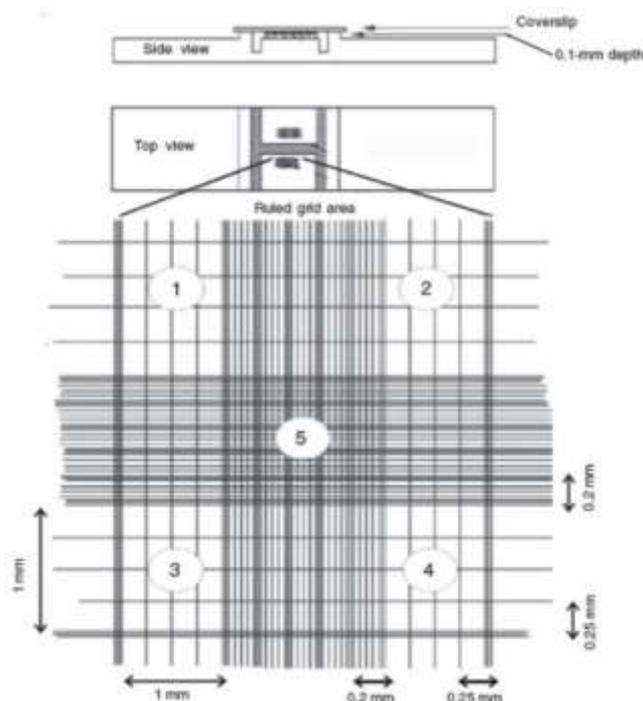
Figure 37. Hemocytometer and dimensions of counting grid chambers.[13]

Before counting to discriminate live and dead cells, cell suspension should be mixed with a 0.1 % Trypan blue solution. Trypan blue is an azo dye which penetrates in death cell due to impaired membrane permeability while live cells with intact membrane are non-colored. The cell suspension mixed with Trypan blue should place on top of

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[13] Available at
https://biocyclopedia.com/index/biotechnology_methods/microbiology/cell_count_by_hemocytometer_or_measuring_volume.php

hemocytometer and covered with cover glass. For example if in a square with dimensions 1 mm x 1 mm (depth 0.1 mm) the next calculation should be applied:

$$\frac{Counted\ cell\ number}{Number\ of\ squares} \times 10^4 \times Dilution = Conc.of\ cells\ in\ mg/mL$$

Measurement of transepithelial electrical resistance (TEER)

The measurement of transepithelial/transendothelial electrical resistance is a sensitive and reliable method for confirming the integrity and permeability of the monolayer. It is the measurement of electrical resistance across a cellular monolayer. For TEER measurement commercial devices are in use (Figure 38).
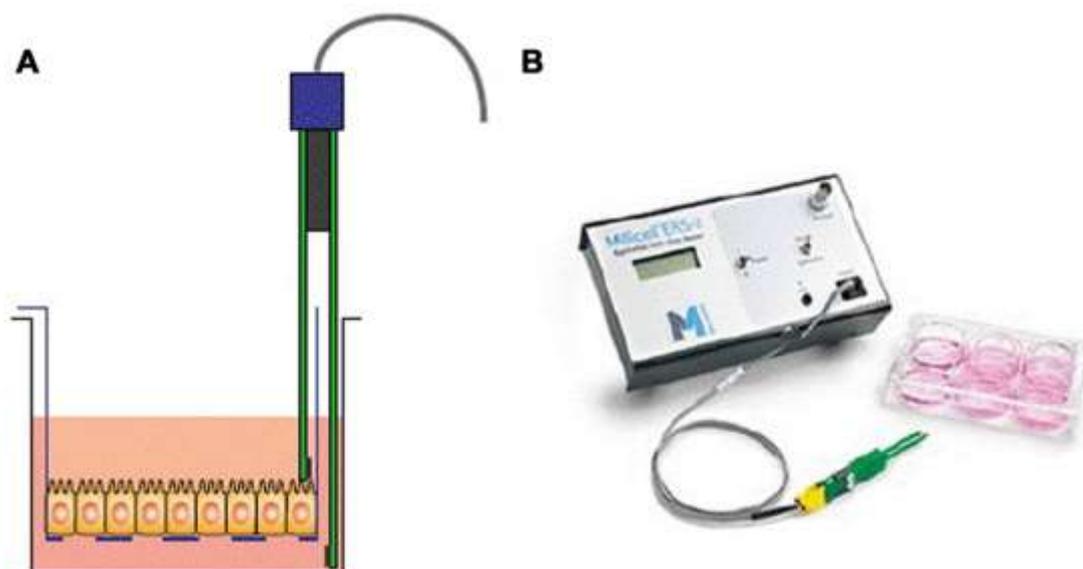
Figure 38. (A) Schematic diagram of TEER measurement, (B) TEER measurement device.[14]

TEER should be monitored during the differentiation and growth of cells on the transwell inserts as well as before and after transport experiments. As blank or control empty transwell insert without seeded cells only with medium should be used. Before measurement electrodes should be sterilized by dipping the electrodes in 70 % ethanol for 15 min, afterward electrodes should be dried on air and dipped in a prewarmed culturing medium before measurement. TEER measurement should be performed in a

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[14] Available at
https://ebrary.net/24380/health/measurement_transepithelial_electrical_resistance_teer

biosafety cabinet to ensure sterile conditions. The average value of three consecutive measurements is taken as the TEER value. Usually, TEER is expressed as measured TEER value divided by the surface of the transwell insert in $\Omega \, / \, cm^2$.

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Maja Krstić Ristivojević is co-author of 11 scientific papers, 2 in the journals of category M21a, 8 in the journals of category M21, and 1 in the journal of category M23, according to the current categorization of international journals.

List of scientific papers that are part of the doctoral dissertation:

1. **M. Krstić Ristivojević**, J. Grundström, T. A. T. Tran, D. Apostolovic, V. Radoi, M. Starkhammar, V. Vukojević, T. Ćirković Veličković, C. Hamsten & M. van Hage. α-Gal on the protein surface affects uptake and degradation in immature monocyte derived dendritic cells. Scientific Reports, (2018), vol. 8(1), Article number: 12684, DOI: 10.1038/s41598-018-30887-8. (Multidisciplinary Sciences; IF 2017: 4.122 (12/64)).
2. **M. Krstić Ristivojević**, J. Grundström, D. Apostolovic, M. Radomirovic, V. Jovanovic, V. Radoi, G. MB Kiewiet, V. Vukojević, T. Ćirković Veličković & M. van Hage. Alpha-Gal on the Protein Surface Hampers Transcytosis through the Caco-2 Monolayer. International Journal of Molecular Sciences, (2020), vol. 21(16), DOI: 10.3390/ijms21165742. (Biochemistry & Molecular Biology; IF 2019: 4.556 (74/297)).

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Maja Krstić Ristivojević je koautor 11 naučnih radova, 2 u časopisima kategorije M21a, 8 u časopisima kategorije M21 i 1 u časopisu kategorije M23, prema trenutnoj kategorizaciji međunarodnih časopisa.

Spisak naučnih radova koji su deo doktorske disertacije:

1. **M. Krstić Ristivojević**, J. Grundström, T. A. T. Tran, D. Apostolovic, V. Radoi, M. Starkhammar, V. Vukojević, T. Ćirković Veličković, C. Hamsten & M. van Hage. α-Gal on the protein surface affects uptake and degradation in immature monocyte derived dendritic cells. Scientific Reports, (2018), vol. 8(1), Article number: 12684, DOI: 10.1038/s41598-018-30887-8. (Multidisciplinary Sciences; IF 2017: 4.122 (12/64)).

2. **M. Krstić Ristivojević**, J. Grundström, D. Apostolovic, M. Radomirovic, V. Jovanovic, V. Radoi, G. MB Kiewiet, V. Vukojević, T. Ćirković Veličković & M. van Hage. Alpha-Gal on the Protein Surface Hampers Transcytosis through the Caco-2 Monolayer. International Journal of Molecular Sciences, (2020), vol. 21(16), DOI: 10.3390/ijms21165742. (Biochemistry & Molecular Biology; IF 2019: 4.556 (74/297)).

Прилог 1.

Изјава о ауторству

Потписани-а _____Маја Крстић Ристивојевић_____ _____

број уписа _____БХ22/2012_____ _____

Изјављујем

да је докторска дисертација под насловом

"Monitoring of *in vitro* bioavailibility and uptake of glycosylated food allergens using cell-based models"

„Праћење *in vitro* биоусвојивости и преузимања гликозилованих алергена хране употребом ћелијских модела"

- резултат сопственог истраживачког рада,
- да предложена дисертација у целини ни у деловима није била предложена за добијање било које дипломе према студијским програмима других високошколских установа,
- да су резултати коректно наведени и
- да нисам кршио/ла ауторска права и користио интелектуалну својину других лица.

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У Београду, _____

_____

Изјава о истоветности штампане и електронске верзије докторског рада

Име и презиме аутора _____Маја Крстић Ристивојевић_____

Број уписа _____ДБ22/2012_____

Студијски програм ____Доктор биохемијских наука _____

Наслов рада "Monitoring of *in vitro* bioavailibility and uptake of glycosylated food allergens using cell-based models"
 „Праћење *in vitro* биоусвојивости и преузимања гликозилованих алергена хране употребом ћелијских модела"

Ментор ____Тања Ћирковић Величковић_____

Изјављујем да је штампана верзија мог докторског рада истоветна електронској верзији коју сам предао/ла за објављивање на порталу **Дигиталног репозиторијума Универзитета у Београду.**

Дозвољавам да се објаве моји лични подаци везани за добијање академског звања доктора наука, као што су име и презиме, година и место рођења и датум одбране рада.

Ови лични подаци могу се објавити на мрежним страницама дигиталне библиотеке, у електронском каталогу и у публикацијама Универзитета у Београду.

Потпис аутора

У Београду, _____

_____

Прилог 3.

Изјава о коришћењу

Овлашћујем Универзитетску библиотеку „Светозар Марковић" да у Дигитални репозиторијум Универзитета у Београду унесе моју докторску дисертацију под насловом:

"Monitoring of *in vitro* bioavailibility and uptake of glycosylated food allergens using cell-based models"

„Праћење *in vitro* биоусвојивости и преузимања гликозилованих алергена хране употребом ћелијских модела"

која је моје ауторско дело.

Дисертацију са свим прилозима предао/ла сам у електронском формату погодном за трајно архивирање.

Моју докторску дисертацију похрањену у Дигитални репозиторијум Универзитета у Београду могу да користе сви који поштују одредбе садржане у одабраном типу лиценце Креативне заједнице (Creative Commons) за коју сам се одлучио/ла.

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(Молимо да заокружите само једну од шест понуђених лиценци, кратак опис лиценци дат је на полеђини листа).

Потпис аутора

У Београду, _____

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