

Enhanced Uptake of Processed Bovine β -Lactoglobulin by Antigen Presenting Cells: Identification of Receptors and Implications for Allergenicity

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Abstract: Scope: β -lactoglobulin (BLG) is a major cow milk allergen encountered by the immune system of infants fed with milk-based formulas. To determine the effect of processing on immunogenicity of BLG, this article characterized how heated and glycated BLG are recognized and internalized by APCs. Also, the effect of heat-induced structural changes as well as gastrointestinal digestion on immunogenicity of BLG is evaluated.

Methods and results: The binding and uptake of BLG from raw cow milk and heated either alone (BLG-H) or with lactose/glucose (BLG-Lac and BLG-Glu) to the receptors present on APCs are analyzed by ELISA and cell-binding assays. Heated and glycated BLG is internalized via galectin-3 (Gal-3) and scavenger receptors (CD36 and SR-AI) while binding to the receptor for advanced glycation end products (RAGE) does not cause internalization. Receptor affinity of BLG is dependent on increased hydrophobicity, β -sheet exposure and aggregation. Digested glycated BLG maintained binding to sRAGE and Gal-3 but not to CD36 and SR-AI, and is detected on the surface of APCs. This suggests a mechanism via which digested glycated BLG may trigger innate (via RAGE) and adaptive immunity (via Gal-3).

Conclusions: This study defines structural characteristics of heated and glycated BLG determining its interaction with APCs via specific receptors thus revealing enhanced immunogenicity of glycated versus heated BLG.

produced. Heating provokes structural modifications of milk proteins via amongst others denaturation, aggregation and the Maillard reaction (MR),^[1] leading to increased immunogenicity and allergenicity of milk proteins.^[2] β -lactoglobulin (BLG), the most abundant whey protein that is not present in breast milk, has been described as one of the major allergens causing cow milk allergy.^[3] In infants fed with cow milk formulas, BLG is one of the first foreign antigens encountered by the mucosal immune system of the gastrointestinal tract.^[4] Therefore, a number of studies have been performed in order to understand the relative importance of heat-induced changes of immunogenicity and allergenicity of BLG.^[5–10] Recent studies revealed an essential difference between the immune-regulatory, native form of BLG which is able to transport ligands to immune cells, and its' heated form which loses this ability.^[11] While native BLG, loaded with iron-quercetin complexes, was protective

against allergic sensitization, the empty, unloaded BLG resulted in specific allergy.^[11] Denatured BLG, in contrast to the native form, was already shown to induce inflammatory responses.^[12] Also, pasteurization-induced aggregates of BLG were shown to

1. Introduction

Milk is an example of a food that is subjected to specific thermal processing, dependent on the type of product that will be

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play a role in allergic sensitization. Roth-Walter and colleagues showed that aggregated BLG is predominantly taken up in the intestinal lumen by the Peyer's patches instead of via transcytosis through enterocytes.^[13] Similarly, cross-linked BLG was increasingly endocytosed by dendritic cells (DCs), in a food allergy-murine model leading to the development of a Th2-associated environment.^[14] Last, the Maillard reaction (MR, glycation) was also shown to play a role in enhanced immunogenicity of processed BLG. Glycated whey protein increased mRNA expression of the pro-inflammatory cytokines tumor necrosis factor α (TNF- α), interleukin (IL) 1 β and IL-6, as well as increased the phagocytic activity of a murine macrophage cell line.^[15] Furthermore, aggregated BLG appears to be recognized by the receptor for advanced glycation end products (RAGE) expressed on antigen presenting cells (APCs).^[16–18] RAGE has been postulated to play a role in the development of food allergies upon interaction with AGEs formed in the food due to glycation.^[19]

Nevertheless, the relative importance of glycation versus aggregation on immunogenicity and allergenicity of milk proteins, including BLG as one of the major milk allergens, remains unclear. For instance, Perusko and colleagues reported an increased uptake of glycated BLG by mice bone marrow-derived DCs compared to non-treated BLG, but BLG heated in the absence of sugar was not included in their study.^[8] In contrast, the study of Deng and colleagues emphasized the heat-induced formation of amyloid-like structures, aggregates and increased hydrophobicity as the most important features predisposing heat processed proteins to be recognized by APCs.^[20] The processing-related structural changes that enhance immunogenicity of BLG are therefore not fully understood. Moreover, heating and glycation-induced modifications may also affect the digestibility and thus the antigens that encounter the mucosal immune system in the gastrointestinal tract.^[21] For instance it has been shown that both heat induced aggregates and heat-glycation induced aggregates of BLG survive until 60 min in the intestinal phase of simulated adult in vitro digestion, with higher susceptibility to enzymatic hydrolysis of heat-glycation induced aggregates. Whereas, unfolding prior to aggregation in the presence and absence of reducing sugars can facilitate enzymatic hydrolysis, it has been shown that high levels of glycation in infant formula results in impaired digestibility.^[22] Thus, exploring the molecular mechanisms of interaction between heat-treated BLG, as a potential antigen present in infant nutrition, and the human APCs is of particular interest. The aim of the present study was first to evaluate and compare heat and glycation induced changes in immunoreactivity of BLG. Secondly, to specify the receptors involved in binding and internalization of heated and glycated BLG in order to reveal mechanisms responsible for triggering innate and/or adaptive immunity. Last, to evaluate the immunogenicity of heated and glycated BLG after enzymatic digestion in an infant in vitro digestion model.

2. Results

2.1. Chemical Characterization of Processed BLG

Bovine BLG isolated from raw milk was heated: (1) in the presence of glucose (BLG-Glu), (2) or naturally present lactose

(BLG-Lac), and (3) without sugar (BLG-H). WP, isolated from raw bovine milk, was subjected to the same treatment to investigate an influence of all whey proteins simultaneously on the structural and functional changes of BLG. Prior to the heat treatment, both BLG and WP were depleted from LPS contamination. The remaining levels of LPS in the samples used in the functional in vitro assays were below 2 pg mL⁻¹ (Figure S1, Supporting Information).

To describe the influence of heating and glycation on the structure of BLG the exposure of β -sheets and hydrophobic structures were measured. Exposure of both, β -sheets and hydrophobic regions increased strongly already after heating showing significantly higher levels than BLG-NT (Figure 1A,B). Exposure of β -sheets and hydrophobic regions of BLG-Glu did not differ from BLG heated while BLG-Lac showed significantly higher levels of β -sheets and hydrophobic regions than BLG-H. In order to determine the level of glycation, furosine the marker of early MR products and N ϵ -carboxymethyl-L-lysine (CML), a marker of advanced MR products, were quantified in all samples. The level of furosine and CML was significantly higher in the BLG-Lac and BLG-Glu when compared to BLG-NT and BLG-H (Figure 1C). The levels of furosine detected in BLG-Glu were significantly higher than in BLG-Lac, while the CML levels were comparable in both samples. Finally, the post translational modification (nePTM) profile of BLG-Glu versus BLG-Lac was analyzed to screen for modifications, which may be specific for a sample including 15 nePTMs on 25 bindings sites.^[23] Only methylglyoxal-dihydroxyimiazoline on R40 was exclusively detected in BLG-Glu (Figure 1D) while all other nePTMs were either detectable in all or in none of the samples (Figure S2, Supporting Information). Therefore, glycation of BLG in the presence of sugars in the wet heating model was confirmed by detection of furosine, CML and nePTM profiling while the heat-induced structural changes were demonstrated by exposure of β -sheets and hydrophobic patterns.

2.2. Heated and Glycated BLG and WP Bind to sRAGE, Gal-3, CD36 and SR-AI

The next step in our study was to relate the structural changes of BLG to its biological and functional properties, by studying direct interaction with APCs. For that purpose, screening of receptors expressed on APCs, including sRAGE, Galectin-3 and two receptors from scavenger family: CD36 and SR-AI was performed employing a receptor-specific inhibition ELISA. Gal-3, sRAGE and CD36 and SR-AI, gave a positive signal in the inhibition ELISA (Figure 2). The binding of BLG-NT to all four receptors did not differ from the negative control while BLG-H showed a high binding to all studied receptors, which was further increased for the glycated proteins. The differences of receptor inhibition between heated and glycated BLG reached a maximum value of 18% for the CD36 receptor (Figure 2A) suggesting high relevance of heat-induced structural changes in formation of the receptors ligands. The type of sugar used for glycation (glucose vs lactose) had a marginal impact on the level of binding to the studied receptors. Heating of BLG in a presence of other whey proteins resulted in a significantly lower binding to Gal-3, SR-AI and CD36 when compared to pure BLG at the same concentration, suggesting that the

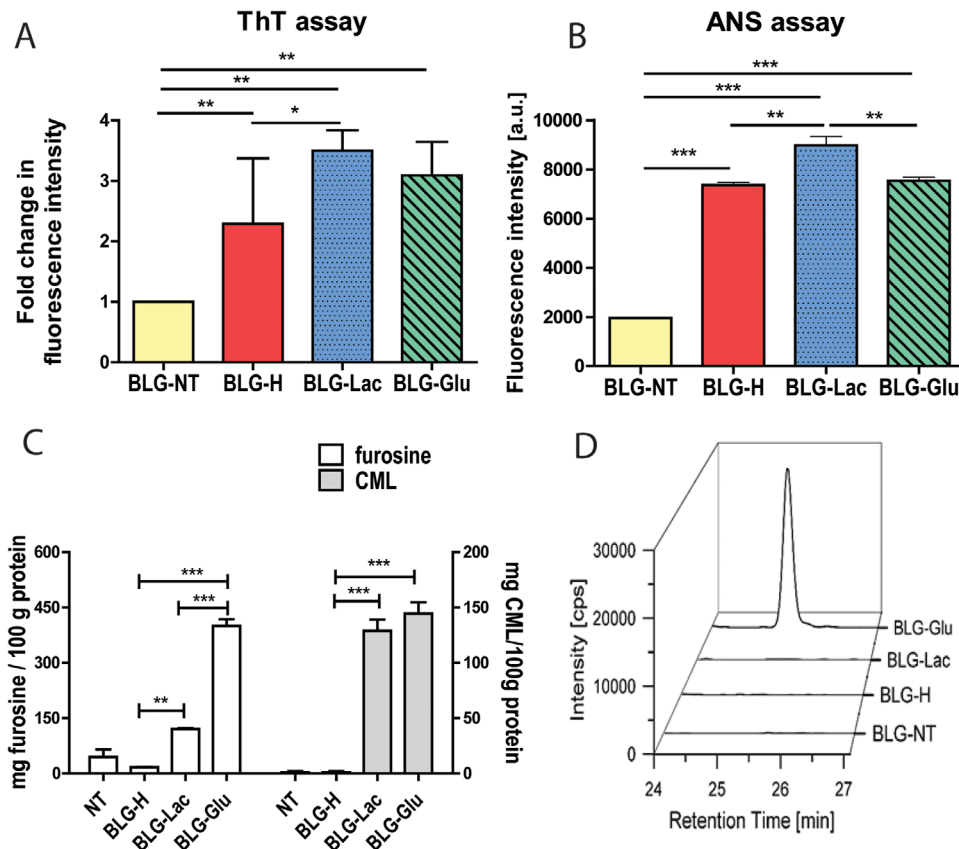


Figure 1. Biochemical characteristic of heated and glycated BLG. A) formation/exposure of the fibril structures measured with ThT-assay; B) changes in hydrophobicity measured with ANS assay; C) level of glycation expressed as amount of formed Carboxymethyllysine (CML) and furosine, (D) microLC-SMRM chromatogram of methylglyoxal-dihydroxyimiazoline on AQSAPLR[+72.0]VYVEE. The transition (m/z 717.4 to 1076.5) of the most intense fragment is displayed for each sample. BLG: non-treated (BLG-NT), heated (BLG-H), heated with lactose (BLG-Lac) or heated with glucose (BLG-Glu). Data shown as mean $\pm$ SD of triplicate wells and are representative of at least three independent experiments. Significant differences analyzed with one-way ANOVA with Tukey post hoc comparison test (GraphPad Prism); * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$.

binding as observed in WP is mainly the result of modification of BLG.

2.3. Heated and Glycated BLG and WP Bind to THP-1 Macrophages and Get Internalized via Both Actin- and Clathrin-Dependent Endocytosis Mechanisms

To confirm the inhibition ELISA results, we performed the receptor binding and uptake assays of non-processed, heated and glycated BLG using THP-1 macrophages and MoDCs (Figure 3). To be able to distinguish the binding of BLG from internalization of BLG, anti-BLG antibodies labeled with two different fluorescent dyes were used, to perform extracellular and intracellular staining. BLG heated and glycated bound to the surface of THP-1 macrophages, and was also detected intracellularly, but non-treated BLG neither bound to nor entered the cells. Moreover, BLG heated in the presence of other WP was equally bound to and internalized by the THP-1 macrophages. The concentration of BLG in WP is approximately 50%; therefore, its binding and internalization by APCs was lower compared to non-treated

BLG but detectable with anti-BLG antibodies (Figure 3A,B). No significant differences between BLG heated and glycated were observed in terms of binding and internalization. The same pattern was noted for both binding to and internalization by MoDCs, although the level of internalization was lower when compared to THP-1 macrophages (Figure 3C,D).

To describe a more detailed mechanism of internalization of heated and glycated BLG we inhibited the major endocytosis pathways. Cytochalasin D was used to block the actin-dependent endocytosis mechanisms, pinocytosis and phagocytosis, while a dynamin inhibitor, dynasore, was used to block the clathrin-dependent endocytosis mechanism. Both cytochalasin D and dynasore significantly reduced the uptake of heated and glycated BLG, indicating that both the actin-dependent endocytosis mechanisms and the receptor-mediated endocytosis mechanisms are involved in the uptake of processed BLG (Figure 4B). Therefore, we blocked the receptors: RAGE, CD36, SR-AI and GAL-3, of which expression on the surface of the PMA-differentiated THP-1 cells was first confirmed by flow cytometry (Figure S3, Supporting Information). We observed the reduced binding of the BLG for the various receptors, demonstrating that the

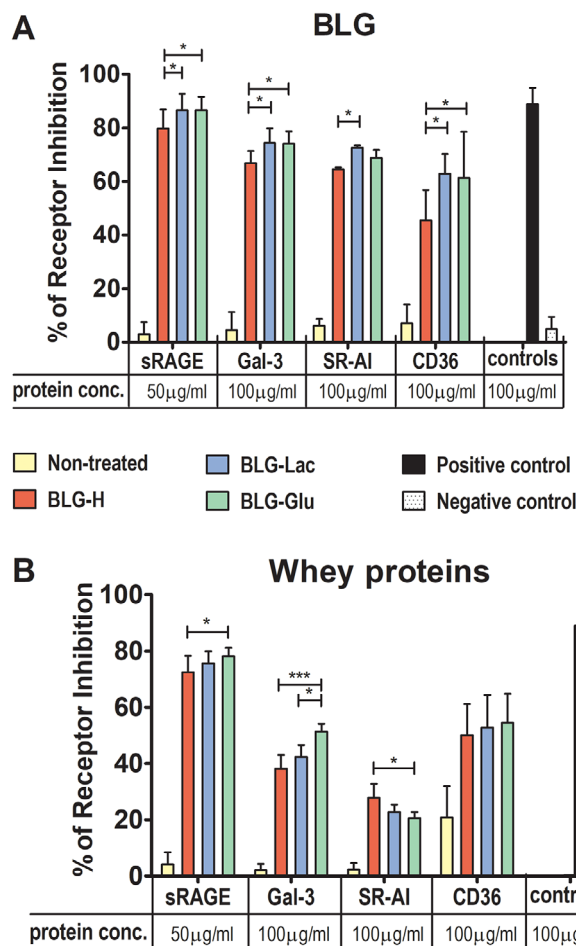


Figure 2. Inhibition of sRAGE, Gal-3, CD36 and SR-AI by BLG and whey proteins measured in competition ELISA. BLG (A) and WP (B), non-treated or heated in the absence of sugar (BLG-H) or in the presence of lactose or glucose (BLG-Lac, BLG-Glu) were pre-incubated with the recombinant forms of sRAGE, Gal-3, CD36 and SR-AI prior adding to the ELISA plate coated with glycated soy protein extract. The data are expressed as percentage of inhibition by the use of non-inhibited signal as the maximum value. Positive control: highly glycated soy protein extract; Negative control: ovalbumin. Data shown as mean $\pm$ SD of triplicate wells and are representative of three independent experiments on three different batches of processed BLG. Significant differences analyzed with one-way ANOVA with Tukey post hoc comparison test (GraphPad Prism); * p < 0.05; ** p < 0.01, *** p < 0.001.

receptor with the highest binding capability was SR-AI followed by RAGE, GAL-3 and finally CD36 (Figure 4A). Finally, we used the anti-BLG antibodies labeled with different dyes for extra- and intracellular staining to distinguish between the receptors directly involved in binding and internalization of heated and glycated BLG. The inhibitors of SR-AI > GAL-3 > CD36 significantly reduced the uptake of both heated and glycated BLG, pointing at the receptors from the SRs family, but also GAL-3, as the main receptors involved in receptor-mediated endocytosis. An inhibitor of RAGE (FPS-ZM1) did not significantly affect uptake of processed BLG (Figure 4B) while it was shown to reduce the external binding to RAGE. No significant differences in the blocking efficiency between heated and glycated BLG were observed.

2.4. Internalization of BLG Does Not Impact the Secretion of Pro-Inflammatory Cytokines by THP-1 Macrophages

To study the physiological consequences of the binding and internalization of heated and glycated BLG by THP-1 macrophages, the levels of the cytokines IL-6, IL-1 β , TNF- α , and IL-10 were determined in the cell culture medium after 24 h incubation with BLG. No significant increase of any of the measured cytokines was observed upon incubation of cells with processed BLG, when compared to the medium control or to non-treated BLG (Figure 4A, Supporting Information). These findings demonstrated that uptake of processed BLG does not result in direct release of pro-inflammatory cytokines. However, when THP-1 cells were first pre-incubated with BLG-Lac followed by the re-stimulation of the cells with LPS, enhanced levels of IL-6 and IL-8 were measured compared to the cells pre-incubated with BLG-H and BLG-NT (Figure 4B, Supporting Information).

2.5. Agglomerates Formed During Heating and Glycation of BLG Show Higher Potential to Bind to the Receptor Present on APCs Than the Low Molecular Weight Fractions

As the next step, we aimed to specify which fractions of heated and glycated BLG are responsible for binding and internalization by THP-1 macrophages. For that reason, we fractionated processed BLG into two fractions with molecular weight (MW) above and below 100 kDa. The purity of the fractions was checked by Native-PAGE, which showed that fraction with MW >100 kDa was not contaminated with monomeric BLG (Figure 5A). Moreover, the protein separation on the gel revealed that heating promotes the formation of aggregates visible on the top of the gel in all the samples, independent of the presence of sugar during heating. Subsequently, both fractions were analyzed in receptor binding ELISA identifying the aggregates as the most potent for binding to all studied receptors (Figure 5A). Finally, the binding assay employing THP-1 macrophages showed more potent binding and internalization of the aggregates (Figure 5A). This shows that heat induced aggregation is a major force promoting internalization of heated and glycated BLG by the receptors present on APCs. Interestingly, aggregates obtained from BLG-Glu were shown to bind and be internalized significantly more efficient than the aggregates obtained from BLG-H and BLG-Lac (Figure 5B).

2.6. Glycated BLG Aggregates are Less Susceptible to Gastrointestinal Digestion than BLG Aggregates Formed Upon Heating Without Sugar

Last, we verified the physiological relevance of BLG aggregates by subjecting them to an in vitro digestion model mimicking gastrointestinal digestion in the infant's stomach and small intestine. At first, the binding of the BLG digest to sRAGE, GAL-3, CD36 and SR-AI was evaluated in an ELISA receptor binding test and compared to the binding of the enzyme matrix and digest of non-treated BLG (Figure 6A). Digests of BLG-Glu and BLG-Lac showed a significantly enhanced binding to sRAGE when compared to digests of BLG-H and non-treated BLG.

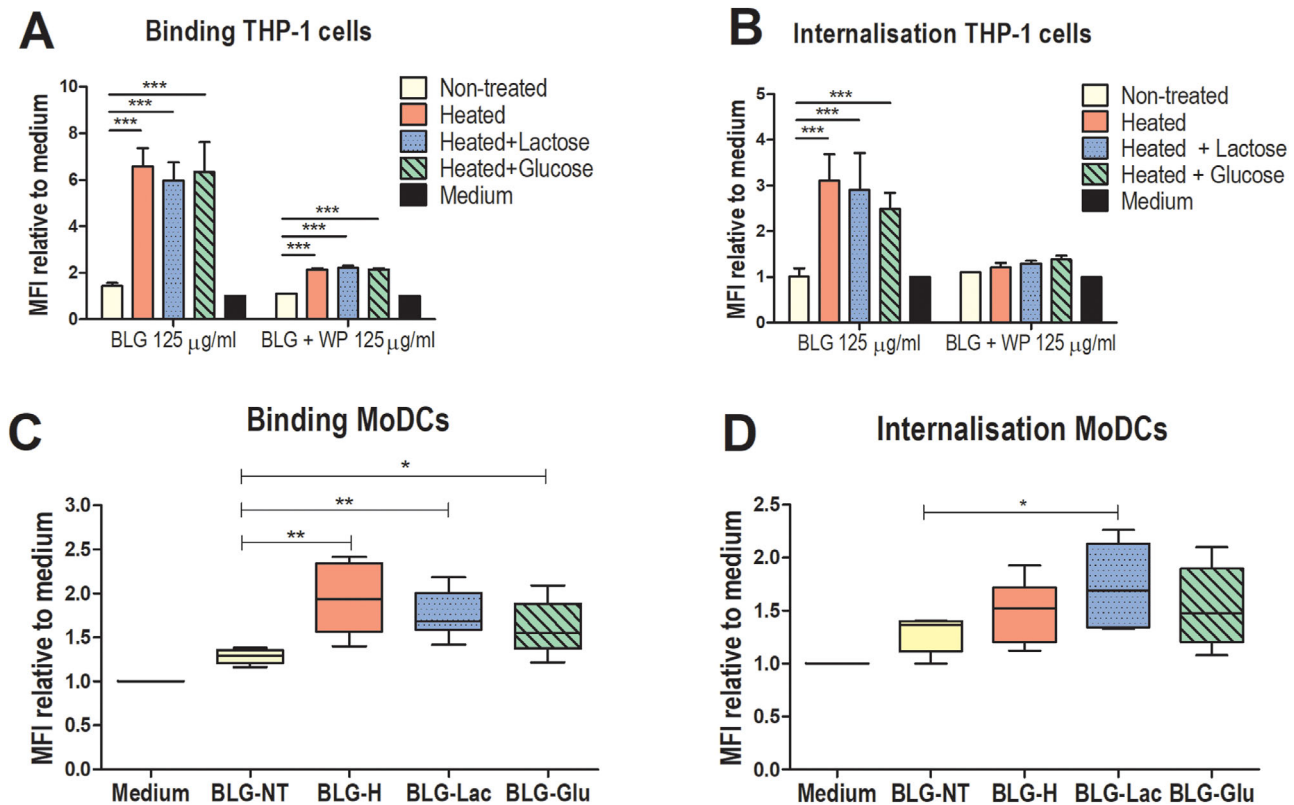


Figure 3. Binding and internalization by human THP-1 macrophages (A, B) and human monocyte derived DCs (C, D) of processed BLG or BLG in the presence of other whey proteins (BLG + WP). PMA differentiated THP-1 macrophages or MoDCs were incubated 2 h in a presence or absence of BLG: non-treated (BLG-NT), heated (BLG-H), heated with lactose (BLG-Lac) or heated with glucose (BLG-Glu) followed by extracellular staining with FITC conjugated anti-BLG antibodies and intracellular staining with Alexa Fluor 647 conjugated anti-BLG antibodies. The level of fluorescence was measured by flow cytometry and expressed as a relative to medium control. Data for THP-1 macrophages (A, B) shown as mean $\pm$ SD of triplicate wells and are representative of three independent experiments. Data for MoDCs shown as mean $\pm$ SD of nine (C) or six (D) healthy donors. Significant differences analyzed with one-way ANOVA with Tukey post hoc comparison test (GraphPad Prism); * p < 0.05; ** p < 0.01, *** p < 0.001.

Gal-3 showed significantly higher binding to digests of BLG-H, BLG-Glu and BLG-Lac as compared to non-treated BLG. The binding of the digests of BLG-H, BLG-Glu and BLG-Lac to the scavenger receptors (CD36 and SR-AI) was on a comparable (BLG-Glu and BLG-Lac) or lower (BLG-H) level than the binding to the digest of non-treated BLG. Next, the binding of all BLG digests to the THP-1 macrophages was studied. The binding of digested BLG was in general significantly reduced when compared to non-digested BLG. However, the digested BLG-Glu was still successfully detected on the surface of THP-1 macrophages by fluorescently labeled anti-BLG antibodies (Figure 6B). Digest of BLG-Glu retained 30% of the binding capacity of non-digested material showing significantly higher binding compared to BLG-NT, BLG-H and BLG-Lac. Digested BLG-Lac showed a similar trend which, although it was not statistically significant. This result shows that aggregates formed especially during heating of BLG in the presence of glucose maintain their immunogenicity after in vitro gastrointestinal digestion. Further attempts to characterize the digests of BLG and to visualize the aggregates with SDS-PAGE, immunoblot staining and glycation staining resulted in a high background from the side of enzymes present in the samples and therefore inconclusive outcomes.

3. Discussion

Cow's milk allergy (CMA) is one of the most common food allergies in early childhood^[24] and heat processing of milk has been implicated in increased allergenicity of cow's milk proteins.^[2,13,14] The first step of allergic sensitization is internalization of antigen by APCs and presentation to T-cells. Heat-treated BLG was already shown in murine in vivo studies to be taken up more efficiently through the Peyer's patches than through absorptive epithelial cells, allowing the protein to interact with APCs.^[13] However, the mechanisms by which both heated and glycosylated BLG is recognized by APCs and the relevance of these interactions to allergic sensitization remain unclear. In this study we described mechanisms by which heat treated and glycosylated BLG is recognized by APCs. We evaluated the relative importance of aggregation versus glycation as well as glycation with glucose versus lactose in terms of receptor binding and internalization. Last, since an immunogenicity of processed BLG in vivo would be modulated by gastrointestinal digestion, we therefore included an infant digestion model of BLG to come closer to the actual in vivo situation.

Cellular binding assays using THP-1 macrophages and human MoDCs revealed enhanced binding and internalization

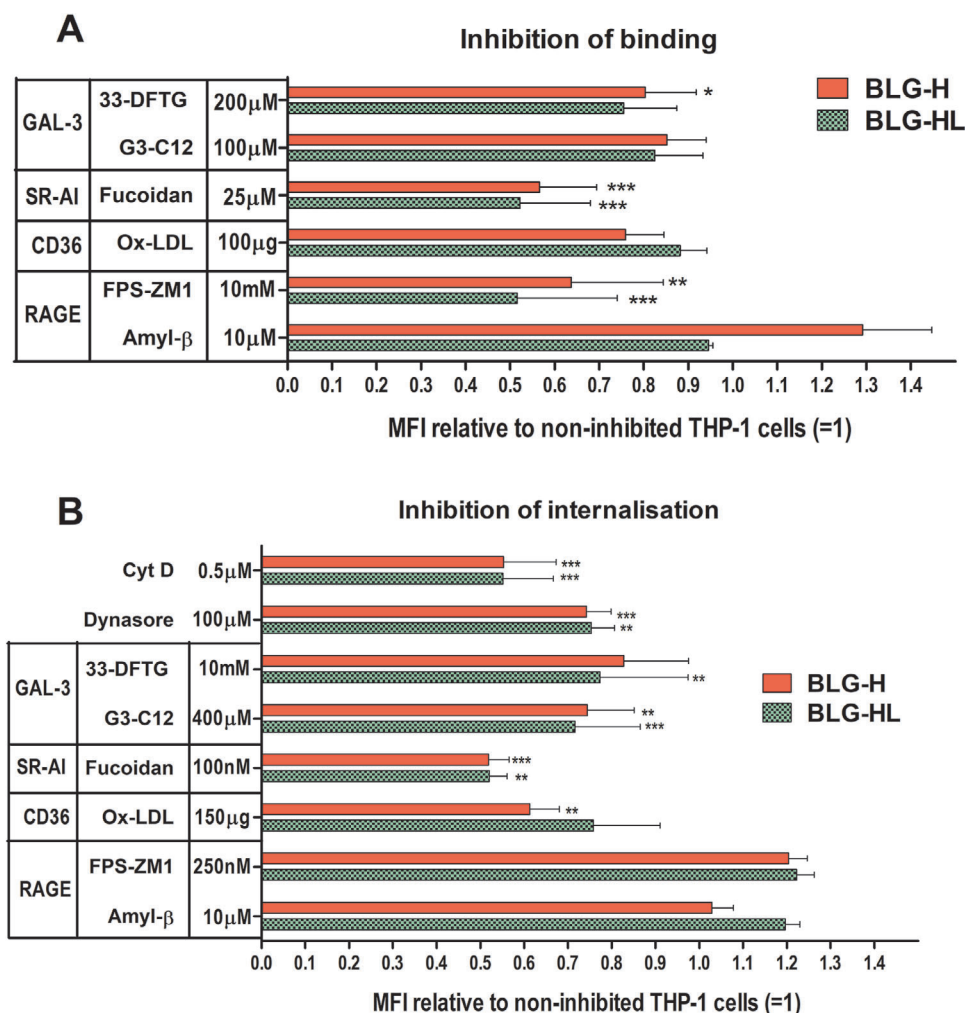


Figure 4. Inhibition of binding and internalization of processed BLG by human THP-1 macrophages. PMA differentiated THP-1 macrophages were pre-incubated with endocytosis inhibitors: dynasore and cytochalasin D (Cyt D) or inhibitors of specific receptors: amyloid- β and FPS-ZM1 for RAGE; Oxidized low-density lipoprotein (Ox-LDL) for CD36; fucoidan for SR-AI, short peptides 33-DFTG and G3-C12 for Gal-3. Followed by pre-incubation with inhibitor the cells were incubated in a presence or absence of BLG: non-treated (BLG-NT), heated (BLG-H), heated with lactose (BLG-Lac) or heated with glucose (BLG-Glu) and stained extracellularly (A) or intracellularly (B) with Alexa Fluor 647 conjugated anti-BLG antibodies. The level of fluorescence was measured by flow cytometry and expressed as a relative to non-inhibited THP-1 cells. Data shown as mean $\pm$ SD of triplicate wells and are representative of three independent experiments. Significant differences analyzed with one-way ANOVA with Tukey post hoc comparison test (GraphPad Prism); * p < 0.05; ** p < 0.01, *** p < 0.001.

of heated and glycosylated BLG but no differences between these two treatments were observed. This suggests that enhanced binding and internalization of heat treated BLG is dominantly caused by heating and to a lesser extent by glycation. Since binding of BLG to the AGE receptors could potentially activate the intracellular pathways and internalization may result in triggering the adaptive immunity, we aimed to identify the receptors involved in both processes. Using receptor specific inhibition ELISA we identified RAGE, Galectin-3, CD36 and SR-AI as the receptors recognizing heated and glycosylated BLG but not BLG-NT. This finding was supported by cellular assays showing that pre-incubation of the cells with receptor-specific inhibitors significantly reduced binding of heated and glycosylated BLG to THP-1 macrophages. These results confirm previous finding which indicated RAGE, Galectin-3 and receptors from

the scavenger family as the main receptors recognizing glycosylated food proteins.^[25–29] Furthermore, BLG heated and glycosylated in the presence of other WPs showed the binding to the studied receptors in ELISA and was detectable on the surface of THP-1 macrophages using anti-BLG antibodies. Therefore, despite the structural changes and protein crosslinking caused by heating in the presence of WPs, the BLG was recognized by studied receptors. This illustrates a major influential role of BLG, as one of the components of the WP fraction, in interacting with APCs. Interaction of glycosylated BLG with APCs was reported previously,^[8,20,25] but has never been directly compared with heated BLG from the perspective of binding and internalization by APCs.

To explore further effects of heating versus glycation on the immunogenicity of BLG, we tested separately the binding and internalization of size-separated aggregates versus low

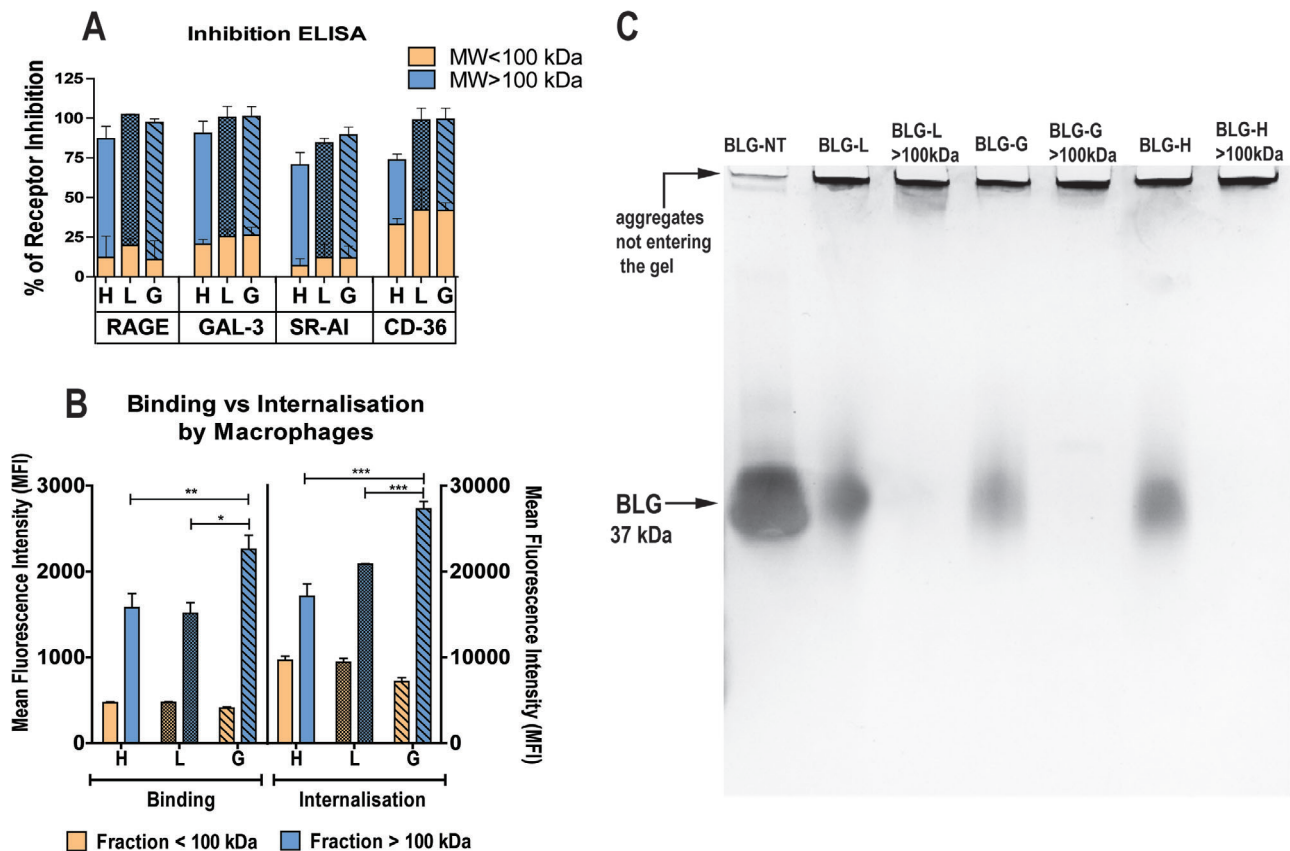


Figure 5. Binding and internalization of aggregates versus low molecular fractions of heated and glycosylated BLG. A) Processed BLG: heated (H), heated with lactose (L) or heated with glucose (G) was fractionated using Amicon-Ultra Centrifugal Filters into two fractions: high molecular weight fraction > 100 kDa and low molecular fraction < 100 kDa. Both fractions were subjected to A) receptor binding assay using inhibition ELISA, B) binding/internalization assay using THP-1 macrophages. Binding to THP-1 macrophages was detected with extracellular staining using FITC conjugated anti-BLG antibodies (B, left Y axis) and internalized BLG was measured by intracellular staining with Alexa Fluor 647 conjugated anti-BLG antibodies (B, right Y axis). C) Composition of the BLG samples as well as the purity of the fraction > 100 kDa was analyzed by native PAGE under non-reducing conditions. Data shown as mean $\pm$ SD of triplicate wells and are representative of three (A) or two (B) independent experiments. Significant differences analyzed with one-way ANOVA with Tukey post hoc comparison test (GraphPad Prism); * p < 0.05; ** p < 0.01, *** p < 0.001.

molecular weight fractions obtained from BLG-H, BLG-Glu and BLG-Lac. Aggregated BLG showed higher binding to all studied receptors as well as more efficient uptake by THP-1 macrophages than lower molecular weight fractions. It shows that the binding of glycosylated food proteins may not solely relate to glycation but also to other modifications that occur upon heating, e.g., aggregation. The formation of aggregates has also been shown previously as a determinant for the binding of sRAGE to heated BLG, independent of the presence of reducing sugars.^[16] Increased exposure of β -sheet structures and hydrophobic patterns detected on BLG-H, BLG-Glu and BLG-Lac suggest that these structures drive the binding of aggregated BLG to studied receptors. The amyloid structure, defined as predominantly containing cross β -sheet patterns,^[30] may promote binding of heated BLG to RAGE and CD36 since both receptors are already known to bind amyloid- β .^[31,32] Next to exposed β -sheets, hydrophobicity is a second physicochemical feature dictating the immune response,^[33] a good example of this being DAMPs like the lipid-A fraction of LPS or peptidoglycan.^[34] Hydrophobic patterns exposed on the surface of a protein due to the heat treatment may interact non-specifically with each other forming

aggregates of different sizes.^[34,35] These processes, as observed also in our study, create all together the immunogenic profile of processed BLG recognized by APCs. The involvement of both hydrophobicity and exposure of β -sheet structures - next to or in combination with glycation of food proteins - in binding to AGE receptors has also recently been demonstrated in other studies.^[20,36]

Although, for non-fractionated BLG, heating seemed to be a driving force for binding to the receptors, the aggregates isolated from BLG-Glu showed enhanced binding and uptake by THP-1 cells compared to the aggregates isolated from BLG-H and BLG-Lac. This indicates that glycation of BLG, especially with glucose, also plays a role in the generation of specific binding ligands for receptors present on APCs. Glucose, having a shorter carbonic chain than lactose, was shown to be more reactive and to modify more amino groups of BLG than lactose.^[37] This was confirmed in our study by higher levels of furosine detected in BLG-Glu compared to BLG-Lac. Furosine indicates the level of early MR products (Amadori products) like fructosyllysine or lactulosyllysine. Therefore, the higher level of Amadori products detected in BLG-Glu may further explain the enhanced binding

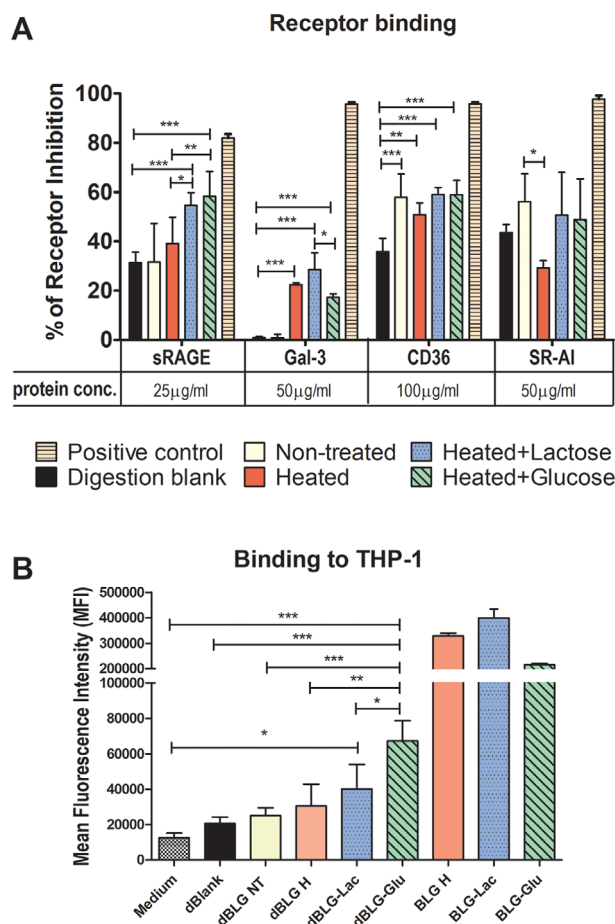


Figure 6. Immunoreactivity of heated and glycosylated BLG after enzymatic digestion in infant gastrointestinal static in vitro model. BLG: Non-treated, heated (BLG-H), heated with lactose (BLG-Lac) or heated with glucose (BLG-Glu) were subjected to gastric and intestinal phase of enzymatic digestion and immunogenicity of digested samples was measured as (A) binding to sRAGE, Gal-3, CD36 and SR-AI in inhibition ELISAs; following positive controls were used: amyloid- β for sRAGE and CD36, fucoidan for SR-AI and soy protein extract glycosylated with glucose for Gal-3. B) binding assay to THP-1 macrophages using Alexa Fluor 647 conjugated anti-BLG antibodies for flow cytometry detection. Digestion blank (dBlank) – sample containing all digestive enzymes but no BLG. Data shown as mean $\pm$ SD of triplicate wells and are representative of at three (A) or two (B) independent experiments. Significant differences analyzed with one-way ANOVA with Tukey post hoc comparison test (GraphPad Prism); * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$.

of BLG-Glu to THP-1 cells, which may be potentially mediated by fructosyllysine-specific receptors.^[38] Another explanation of the enhanced binding of BLG-Glu to THP-1 macrophages can be provided by the methylglyoxal-dihydroxyimide (MGO) modification detected only in BLG-Glu. MGO, a highly reactive compound formed due to the spontaneous degradation of glucose, may further react with lysine and arginine residues forming MGO-modified proteins.^[39] MGO-modified proteins were shown to be highly immuno-reactive^[40] possibly also via interaction with RAGE.^[41] Therefore, MGO-modifications of BLG-Glu may contribute to its' interaction with APCs. This reveals an essential difference between glycation of milk proteins with natu-

rally present lactose during, e.g., milk processing and the glycation with glucose typically used to illustrate an effect of glycation of food proteins.^[8,26,27]

As both heat-induced and glycation-induced aggregates are internalized by APCs via specific receptors, we subsequently evaluated if the aggregates retain their receptor binding capacity after in vitro enzymatic digestion mimicking the infants' gastrointestinal conditions. Although the binding of glycosylated proteins to studied receptors was already shown,^[16,18,26–28] the susceptibility of the binding sites to enzymatic digestion has not been well studied. Our data showed that BLG-Glu and BLG-Lac were still recognized by sRAGE after digestion, demonstrating significantly higher binding compared to BLG-NT and BLG-H. Moreover, digested BLG-Glu was detected on the surface of APCs indicating the binding to the receptors. Blocking experiments with the RAGE antagonist FPS-ZM1 demonstrated that RAGE is not involved in internalization of processed BLG confirming its' role in signal transduction. Internalization may thus occur through Gal-3, which bound to the digests of BLG-H, BLG-Lac and BLG-Glu, suggesting that this may be a potential route for triggering the adaptive immunity through enhanced antigen uptake and presentation to T cells. The binding of digests of glycosylated and heated BLG to scavenger receptors was not higher than BLG-NT and control sample containing enzymes only. This suggests that aggregation is a dominant factor determining the binding capacity of BLG to scavenger receptors, although the role of these receptors after digestion is minor. Summarizing, our results indicate that the molecular mechanisms via which digested glycosylated BLG triggers an immune response are mediated via RAGE on the level of innate immunity and via Gal-3 on the level of the adaptive immune compartment.

RAGE is known to be implicated in a wide range of inflammatory reactions including systemic, but also local intestinal and airway inflammation.^[42,43] In murine models of asthma and allergic airway inflammation, RAGE has been shown to promote the expression of the type 2 cytokines IL-5 and IL-13,^[44] but also IL-33 leading to the accumulation of type 2 innate lymphoid cell in the lungs.^[45] These studies demonstrate an essential role of RAGE in allergic airway inflammation, however the link between RAGE and the development of food allergy is not directly proven yet. Smith et al. in the false alarm hypothesis, proposed that AGEs present in the diet contribute to the development of food allergies via interaction with RAGE consequently priming immune signaling.^[19] RAGE is highly expressed on DCs, macrophages, T lymphocytes, and B cells^[42] regulating such processes as migration of DCs to the lymph nodes^[46] as well as induction of T-cell proliferation.^[47] Therefore, the authors of the false alarm hypothesis suggested that the signaling and priming of immune cells by RAGE-activated APCs is likely important.^[19] Our study supports this hypothesis by showing that RAGE ligands formed as an effect of glycation of BLG retain their activity after enzymatic digestion. This suggests that in vivo, the relative importance of glycation is higher than that of heat-induced aggregation. In the used model, the binding and uptake of BLG did not trigger the direct release of pro-inflammatory cytokines, however the re-stimulation of the THP-1 macrophages with LPS lead to enhanced IL-6 and IL-8 responses suggesting priming of the cells. However, to understand better the role of glycation in allergic sensitization other models, like human DCs, are needed.

In conclusion, this study showed that heat treatment of BLG generates ligands for RAGE, Gal-3, CD-36 and SR-AI as a results of both (1) heat treatment as applied in this study with as consequence increased hydrophobicity, exposure of β -sheets, and aggregation, and (2) glycation (predominantly with glucose) possibly connected to the formation of fructosyllysine and/or methylglyoxal. The importance of glycation in vivo was suggested after gastrointestinal digestion of aggregates, showing that glycated aggregates are less sensitive to digestion and therefore maintain their binding capacity to RAGE and Gal-3. On the basis of these results, we propose that post-digestion retained immunogenicity of glycated BLG may be mediated via RAGE as a signaling pathway, and via Gal-3 as a putative receptor responsible for enhanced antigen uptake and processing, possibly activating the adaptive immune system. However, future studies are needed to confirm this in humans and animal models of allergic sensitization.

4. Experimental Section

Isolation, Treatment and Fractionation of BLG and WP: Raw milk was obtained from the Department of Animal Sciences, Wageningen University & Research (Wageningen, The Netherlands). The whey protein (WP) fraction was purified from the raw bovine milk as described before by Perdijk et al.^[48] BLG was purified and isolated as described by De Jongh et al.^[49] using anion exchange chromatography DEAE Sepharose C-6B (GE healthcare, Chicago, IL, USA). Isolated BLG was lyophilized and of purity >94% was measured as described by Deng et al.^[20] The LPS removal was performed according to the protocol described by Teodorowicz et al.^[50] and the Recombinant Factor C Endotoxin Detection Assay (cat. #609050, Hyglos GmbH, Bernried, Germany) was used to monitor the LPS levels. LPS-free BLG and WP were heated in a wet system above the denaturation temperature and below the boiling point by heating it in phosphate buffer (PBS) at pH 7.4 and applying 100 °C for 90 min in the presence (1:1 w/w) of lactose (BLG/WP-Lac), glucose (BLG/WP-Glu) and in the absence of lactose (BLG/WP-H) to obtain high levels of glycation as well as aggregation. After the heating step BLG and WP were fractionated using Amicon Ultracell 100k and Amicon Ultracell 3k filters (Amicon, UFC910024, Millipore Corporation, Bedford, MA, USA). Proteins were loaded first onto the Amicon Ultracell 100k filter and washed 7 times with PBS followed by centrifugation (3363 g, 10 min, 25 °C). The filtrates were collected, pooled and subsequently loaded onto Amicon Ultracell 3k filters in order to concentrate the collected fraction. The protein concentration in both fractions (above and below 100 kDa) was measured using NanoDrop ND1000. Both fractions were aliquoted before storage at –20 °C.

Structural Analysis: Thioflavin-T (ThT) assay was conducted to monitor the formation of fibril structures during heating according to the previously described protocol.^[16] Hydrophobicity was measured with ANS assay according to the previously described protocol^[20] with the addition that samples were diluted to a protein concentration of 0.5 mg mL⁻¹. All samples were measured in duplicate in a 96-well black polystyrene plate using the Infinite® 200 PRO NanoQuant and i-control software (Tecan, Männedorf, Switzerland). CML and furosine were quantified using uHPLC-ESI-MS/MS according to a method described previously.^[16,51]

Qualitative Screening of Non-Enzymatic Post Translational Modification (nePTM): For the analysis of nePTMs, all samples were dialyzed against demineralized water for 30 h with a molecular cut-off of 3.5 kDa. The samples were freeze dried and reconstituted in tris(hydroxymethyl)aminomethane hydrochloride buffer (100 mmol L⁻¹, pH 7.8). After this, reduction and alkylation of disulfides was conducted with 1, 4-dithiothreitol and iodoacetamide. Enzymatic hydrolysis was performed with Glu-C at 37 °C overnight and stopped by adding formic acid. After dilution to a final concentration of 0.1 µg µL⁻¹, the peptide mixture was analyzed by reversed phase micro liquid chromatography (microLC). For this, an Ultimate 3000 RS system (degasser, binary nano flow pump,

autosampler, column oven; ThermoFisher, Dreieich, Germany) was coupled to a 6500+ QTrap mass spectrometer (AB Sciex, Darmstadt, Germany) equipped with an electrospray ionization source. Analyst 1.6.3 was applied for instrument control as well as for data acquisition. A YMC Triart C18 (500 µm inner diameter, 100 mm length, 3 µm particle size) column was used at 35 °C with the following gradient: A, formic acid (0.1%); B, acetonitrile with formic acid (0.1%), flow rate 30 µL min⁻¹; [time (min)]/%B: -15/2, 5/2, 55/42.5, 55.5/95, 65/95. An aliquot of 5 µL was injected. All flow eluting before 2.0 min and after 55.0 min was discarded by a two-position valve prior to MS analysis. All measurements were performed in positive ionization mode. The ion source was operated at 350 °C with a voltage of +5000 V, the curtain gas set to 40 psi, the nebulizer gas to 40 psi, and the heating gas to 60 psi. All data were acquired in the scheduled multiple reaction monitoring mode (sMRM) with three transitions per peptide. Each sample was analyzed in triplicate. With this method, 12 modified peptides representing 15 different modifications on 25 different amino acid side chains were monitored.^[23] For data evaluation, MultiQuant 3.0 software was used.

Inhibition ELISA with Recombinant Receptors: Inhibition ELISA was conducted as a screening tool to determine the binding strength of the processed BLG to the following receptors: soluble RAGE (# RD172116100, BioVendor, Brno, Czech Republic), galectin-3 (# 1154-DC, R&D Systems, Minneapolis, United States) and two receptors from the scavenger receptor family: SR-AI (# 1154-DC, R&D Systems, Minneapolis, United States) and CD36 (# 1955-CD, R&D Systems, Minneapolis, United States) according to the protocol described by Zenker et al.^[16] The detailed information about antibodies used in the assay are displayed in Table S1, Supporting Information. Positive controls were as follows: amyloid- β for sRAGE and CD36, fucoidan for SRAI and soy protein extract glycated with glucose for Gal-3, while native ovalbumin was used as a negative control.

Native-PAGE Electrophoresis: Gel electrophoresis under non-reducing conditions was performed to monitor protein aggregates that were formed during heat treatment of BLG. Samples were mixed with native PAGE sample buffer (62.5 mM Tris, 25% glycerol, 1% bromophenol blue, pH 6.8) and loaded on Mini-protein TGX precast gels (#4569033, Bio-Rad, Hercules, CA, USA). Gels were run at 100 V for 90 min using a running buffer containing 25 mM Tris and 192 mM glycine and stained with GelCode Blue Stain Reagent (#24590, Thermo Fisher Scientific, Waltham, MA, USA). Images of the stained gels were obtained using a Universal Hood III (Bio-Rad, Hercules, CA, USA) and Image Lab 4.1 software (Bio-Rad, Hercules, CA, USA).

Isolation and Culturing of Monocyte-Derived Dendritic Cells (MoDCs): Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation from fresh blood obtained from healthy anonymous donors as described previously.^[48] Monocytes were differentiated into dendritic cells by culturing them for 6 days in the presence of 20 ng mL⁻¹ IL-4 (Peprotech; 200–04) and GM-CSF (Peprotech; 300–03). After 6 days, immature DCs were used for binding and internalization assays.

Culture of Human Acute Monocytic Leukaemia Cell Line (THP-1): The human acute monocytic leukaemia cell line, THP-1 (American Type Culture Collection, Manassas, VA, USA), was maintained as described before.^[52] Macrophage phenotype was induced by stimulating THP-1 cells with 10 ng mL⁻¹ PMA (Sigma Aldrich, Zwijndrecht, The Netherlands) for 48 h. Differentiated cells were washed twice in supplemented medium and were allowed to rest by incubating them for another 24 h in supplemented medium. Afterwards, the cells were used directly for the binding and/or uptake assay or incubated 24 h in the presence of BLG (25, 125, and 1000 µg mL⁻¹) followed by the detection of cytokines in the supernatant. The supernatant was collected and human cytokine concentrations (IL-6, IL-8, IL-1 β , TNF- α , and IL-10) were determined using the cytometric bead array (CBA) kit (Human Inflammatory Cytokine Kit, #551811, BD Bioscience, Franklin Lakes, NJ, USA), according to the manufacturer's instructions. The samples were analyzed by flow cytometry (CytoFLEX Flow Cytometer, Beckman Coulter, Brea, CA, USA). The results were normalized to cytokine levels of unstimulated macrophages cultured in the medium.

Binding and Internalization Assay: Binding and internalization of BLG was monitored in THP-1 cells and subsequently confirmed in human MoDCs using polyclonal anti-beta-lactoglobulin antibodies (anti-BLG Abs)

produced in rabbit (Abcam, cat. # ab112893) and conjugated with Fluorescein isothiocyanate (FITC, Sigma Aldrich, cat. # F7250) or Alexa Fluor 647 dye using the Alexa Fluor™ 647 Protein Labeling Kit (Thermo Fisher Scientific, cat. # A20173) according to the manufacturer's protocol. Initially, the antibodies were concentrated by ultrafiltration (7000x G) using Microsep Advance Centrifugal Devices with Omega membrane with 3 KDa cutoff (#MCP003C46, PALL Corporation, New York, USA), to a final concentration of 2 mg mL⁻¹. Then, the antibodies were labeled with the FITC and Alexa Fluor 647 dye, following the procedure as described by the manufacturer.

Before the cellular experiments were performed, the binding efficiency of the labeled anti-BLG Abs to non-treated, heated, and glycated BLG was determined using direct ELISA assay. Briefly, white 96-well plates with a clear bottom were coated with non-treated BLG, heated BLG, and BLG glycated with lactose and glucose at concentrations of 10 µg mL⁻¹. The plate was incubated 2 h at room temperature (RT) and washed with 0.05% Tween in PBS (PBST). The coated ELISA plate was blocked with PBS with 3% BSA (v/w) for 1 h at RT followed by washing. Next, anti-BLG Abs labeled with FITC and with Alexa Fluor 647 were added in a series of dilutions (from 40 to 20,480 times). The plate was incubated for 1 h at RT, and washed after which 100 µL of PBS was added to each well. The fluorescence was measured using a Spectromax microplate reader with excitation and emission wavelengths 490/525 nm for FITC and 650/665–715 nm for Alexa Fluor 647. Based on the fluorescence intensity the efficiency of the binding of anti-BLG Abs to non-treated, heated, and glycated BLG was measured, and so then the correction factors were calculated and used for the calculations of binding and internalization in the cellular assays. FITC labeled antibodies diluted 100 times bound on average 1.24 times higher to heated and glycated BLG compared to non-treated BLG. Similarly, Alexa Fluor 647-labeled antibodies diluted 100 times bound on average 1.24 times higher to heated and glycated BLG compared to non-treated BLG.

The binding and internalization of BLG samples was evaluated using PMA differentiated THP-1 macrophages and MoDCs by: (1) extracellular staining using FITC-conjugated anti-BLG antibodies to measure the binding to the surface of the cells, and (2) intracellular staining using Alexa Fluor 647-conjugated anti-BLG antibodies to measure internalized BLG inside the cells. The cells were incubated with BLG or WP samples (125 µg mL⁻¹) for 2 h. Then, the cells were washed with FACS buffer, detached from the plate and transferred to a round-bottom 96-well NUNC plate (#2205, Thermo Fisher Scientific, Waltham, MA, USA), on ice. Subsequently, immunostaining with anti-BLG dye-conjugated antibodies was performed. Initially, the cells were stained extracellularly (30 min, 4 °C, in the dark) with FITC-conjugated anti-BLG antibodies, to assess the extracellular binding of the protein followed by fixation and permeabilization (Intracellular Fixation & Permeabilization Buffer Set, #88-8824-00, eBioscience, San Diego, CA, USA). After centrifugation (3 min, 300 g, 4 °C), the cells were incubated with AlexaFluor 647-conjugated anti-BLG antibodies (30 min, 4 °C, in the dark), to stain intracellularly the internalized BLG. Finally, after two washes with permeabilization buffer, the cells were resuspended in FACS buffer and were analyzed by flow cytometry (CytoFLEX LX Flow Cytometer, Beckman Coulter, Brea, CA, USA). The viability of the cells was monitored using Fixable Viability Dye eFluor 506 (#65-0866-14, eBioscience, San Diego, CA, USA).

Inhibition of Binding and Internalization of BLG by Human Acute Monocytic Leukaemia Cell Line (THP-1): To perform the inhibition of BLG binding and internalization by THP-1 cells, the cells were differentiated with PMA, as described in Section 2.6. After a 24 h resting step, the cells were treated with the following inhibitors: FPS-ZM1 as RAGE inhibitor, oxidized low density lipoprotein (ox-LDL) as CD36 inhibitor; G3-C12 and 33DTFG as galectin-3 inhibitors and fucoidan as inhibitor for the SR-A1. Finally, dynasore was used as inhibitor of clathrin-dependent endocytosis, while macropinocytosis and phagocytosis were inhibited by cytochalasin D (for the details see Table S1, Supporting Information). After 45 min of incubation with the inhibitors, the BLG protein samples were added and the cells were incubated for 2 additional hours. Extracellular and intracellular staining followed, as described in Section 2.8. The samples were analyzed by flow cytometry (CytoFLEX LX Flow Cytometer, Beckman Coulter, Brea, CA, USA).

In Vitro Digestion of β -Lactoglobulin in Infant Gastrointestinal Static in Vitro Model: An infant gastrointestinal static in vitro model was used in order to mimic the digestive conditions of the gastric and intestinal phase of full-term infants as described before.^[53] During the gastric phase, simulated gastric fluids (SGF, 94 mM NaCl; 13 mM KCl; pH 5.3) were added and the pH was adjusted to 5.3 with 0.1 molar (M) hydrochloric acid (HCl) followed by the addition of pepsin (#P6887; Sigma Aldrich, 4036 U mg⁻¹) and lipase (#80612, Sigma Aldrich; 58.3 U mg⁻¹). Enzyme activities were set at 268 U mL⁻¹ of gastric content for pepsin and 19 U mL⁻¹ of gastric content for lipase. The gastric phase was completed with an incubation step at 37 °C for 1 h, while mixing. The intestinal phase was initiated with the addition of simulated intestinal fluid (SIF, 164 mM NaCl, 10 mM KCl, 85 mM of sodium bicarbonate pH 6.6) after which the pH was adjusted to 6.6 with 0.1 M sodium bicarbonate (NaHCO₃). Then, in this order, pancreatin (#P1750, Sigma-Aldrich; 4USP) and bovine bile extract (#B3883, Sigma Aldrich) were added. In the beginning of the intestinal phase the following components were added: (1) calcium chloride (CaCl₂) stock solution to a final concentration of 3 mM; (2) bovine bile extract to a final concentration of 3.1 mM; (3) pancreatin covering the intestinal lipase activity of 90 U mL⁻¹ and the trypsin activity. 16 U mL⁻¹. The intestinal phase was completed with an incubation at 37 °C for 2 h, while mixing. At the start and end of both phases, the pH was measured for all samples. At the end of the intestinal phase, the pH was adjusted to 6.8. The digestion was finalized by inactivating the digestive enzymes with 1 mM Pefabloc (#76307, Sigma Aldrich).

Statistical analysis: Statistical analysis were conducted using SPSS version 23 and GraphPad Prism. For multiple sample comparison, one-way ANOVA was used and Tukey post hoc comparison test. Results were considered statistically different at $p < 0.05$ if not mentioned otherwise.

Dosage information: BLG and WP at the concentration of 125 µg mL⁻¹ was used in all cellular assays including binding and internalization and incubation for a measurement of cytokines. This concentration was chosen as optimal based on the pre-experiments testing a series of concentrations ranging from 25–250 µg mL⁻¹.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

aggregation of β -lactoglobulin, cow milk processing, digestion of β -lactoglobulin, galactin-3 Maillard reaction ligands, glycation of β -lactoglobulin, RAGE Maillard reaction ligands

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