RESEARCH ARTICLE

Recognition and uptake of free and nanoparticle-bound betalactoglobulin – a food allergen – by human monocytes

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Scope: To improve our understanding of the interaction of food allergens with cells of the immune system, the endocytosis by human monocytes of bovine β-lactoglobulin (BLG) and ovomucoid (OM) – two major food allergens – and human serum albumin (HSA) was studied.

Methods and results: BLG was covalently conjugated to dextran-coated magnetic nanoparticles (MNPs) without affecting its structure and immunoreactivity. BLG-conjugated MNPs were taken up by human monocytes much more efficiently than non-conjugated MNPs, allowing easy magnetic separation of cells that had adsorbed the allergen. BLG, OM, and HSA were conjugated to MNPs also labeled with a fluorescent probe. The uptake of these materials by human monocytes was monitored through flow cytometry, and compared with fluorescent MNPs and the free fluorescently labeled proteins, confirming higher uptake of the BLG-conjugated MNPs versus non-conjugated MNPs. OM but not HSA conjugation to particles enhanced uptake of the MNPs. Confocal microscopy provided direct evidence of the actual internalization of BLG–MNP conjugates into the cytoplasm.

Conclusions: These results contribute to the current understanding of the interaction between food allergens and antigen-presenting cells, and demonstrate that the BLG is readily endocytosed by monocytes both as the single protein and as a conjugate.

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1 Introduction

β-Lactoglobulin (BLG), one of the major whey proteins in cow milk, holds interesting functionalities that can be exploited in food. It belongs to the lipocalin protein-family [1], and is known to bind small hydrophobic molecules, such as retinoic acid, cholesterol, vitamin D, various aromatic compounds, and fatty acids. BLG is a highly stable protein. It is very stable at low pH and resistant to pepsin degradation. Dissociation of its dimeric structure and significant changes in the conformation occur above 65°C, and BLG may undergo various aggregation phenomena upon physical and chemical denaturation in processes that involve partially unfolded intermediates and changes affecting the secondary, tertiary, and quaternary structures of the protein [2–5].

Apart from its potential applications in the food and nutraceutical industry, BLG is also recognized as one of the major allergens in milk [6]. Recently, BLG was demon-

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Abbreviations: **AF,** 5-aminofluorescein; **BLG,** β-lactoglobulin; **CMD,** carboxymethyl dextran; **EDC,** 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; **KSTI,** Kunitz-type soybean trypsin inhibitor; **MNPs,** magnetic nanoparticles; **OM,** hen egg ovomucoid

strated to form nanocomplexes with the $n-3$ long-chain fatty acid docohexanoid acid and low methoxyl pectins with a high colloidal stability and average size of approximately 100 nm [7]. Such particles have a potential as solubility and delivery matrix of high nutritional value fatty acids, e.g. in low-fat drinks, and have furthermore been shown capable as vehicle for delivering and stabilizing hydrophobic compounds, such as vitamin D2 [8] and various drugs [4]. Also, molecules or particles larger than 20–50 nm are endocytosed in a different way than smaller molecules [9, 10]. This may impact the amount of endocytosed antigen as well as the response of the innate immune system and the stimulation of the adaptive immune system [11]. It has been shown in several studies that enterocytes lining the epithelial in the gut take up nanoparticles efficiently [12, 13]. Accordingly, nanoparticles conjugated with BLG may give rise to concerns as to whether the allergic properties of BLG become stronger when present in particles of ~100 nm of size as compared with single molecules (2–4 nm).

Biocompatible magnetic nanosupports with suitable physico-chemical properties are generally synthesized by encapsulating magnetic materials with a polymer layer which affects their colloidal stability, biocompatibility, and extent of cellular uptake [14]. To date, a broad variety of magnetic nanoparticles (MNPs) has been produced, differing in size, nature of the ferromagnetic material (nickel, γ-Fe_2O_3, magnetoferritin, Fe_3O_4), and type of coating used, such as dextran, starch, albumin, and polyethyleneglycol. Dextran has been one of the most common coatings, as it has been proven to have no measurable toxicity [15]. The interest for magnetic nanosupports is not only limited to the obvious ease of their separation under micro- and nanofluidic conditions, or of their entrapment in coatings and films. Most relevant to the biochemist is the fact that nanostructures can be conjugated to biologically active molecules, including hormones, antibodies, and various peptides, taken up by cells, and circulated among tissues expressing their cognate receptors [16]. Therefore, synthetic nano-sized materials, conveniently grafted with high affinity ligands and/or transfection agents to integrate functionally and efficiently into cellular and subcellular structures, can confer magnetic activity to cells, and may represent versatile and non-invasive tools for targeting specific cellular and/or subcellular structures, and for monitoring cellular functions both in vitro and in vivo [17–19].

Here, we present a first report on the preparation and properties of conjugates between MNPs and BLG, a major food allergen, used for the study of BLG interaction with cells of the immune system, as shown here by their specific interaction with human monocytes. Monocytes are actively phagocytosing foreign substances in the body and are able of ingesting large quantities of particulate agents, with subsequent compartmentalization in secondary lysosomes within the cytoplasm [20], making them a suitable cellular model for these studies.

2 Materials and methods

2.1 Proteins and chemicals

All the isolated proteins used in this study (BLG; ovomucoid (OM); bovine and human serum albumin (BSA and HSA, respectively); and Kunitz-type soybean trypsin inhibitor (KSTI)) were from Sigma-Aldrich, and were used without further purification. Chemicals were of reagent grade or better, and were also obtained from Sigma-Aldrich unless otherwise indicated.

2.2 Preparation of nanoparticles and conjugates

Substituted MNPs of general composition $Mn_{0.8}Zn_{0.2}Fe_2O_4$ and a crystallite size (estimated via the Debye–Scherrer formula) of ~14 nm were synthesized by a chemical co-precipitation method [21]. Manganese and zinc were chosen because of their non-toxicity, and because the presence of zinc ions increases the saturation magnetization of magnetite while decreasing its sensitivity to oxidation. To stabilize the MNPs at pH 7, nitrate ions were replaced by citrate. Carboxymethyl dextran (CMD) was synthesized by reacting dextran (Acros, 60–90 kDa) with monochloroacetic acid in the presence of sodium hydroxide and iso-propanol. The degree of substitution was increased by repeating the carboxymethylation step. MNPs were coated with CMD by stirring under heating conditions, and washed several times with water. The characterisation of the different dextran derivates was performed by FT-IR and by Raman spectroscopy. Coated and uncoated MNPs were characterized by X-ray diffraction, by transmission electron microscopy, and by dynamic light scattering. CMD-coated MNPs were conjugated to proteins after 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) activation in the presence of *N*-hydroxysuccinimide [22].

Fluorescent proteins and fluorescent MNPs were prepared by binding fluorescein isothiocyanate (FITC) to proteins and 5-aminofluorescein (AF) to EDC-activated MNPs, respectively, according to Hermanson [23]. The conjugation rates for the different proteins were assessed by spectrophotometry and found to be largely comparable. Proteins (BLG, OM, and HSA) were conjugated to AF-MNPs as reported above for MNPs.

2.3 Immunochemical assays

Dot blotting with specific antibodies, followed by immunoenzymatic detection of the bound antibodies, was performed after immobilizing the conjugates, free BLG, and unmodified MNPs on a nitrocellulose membrane according to published protocols [24, 25]. Membranes were blocked with casein, washed, and incubated with mouse polyclonal antibodies prior to detection with an anti-mouse

IgG-horseradish peroxidase conjugate (P260, DAKO A/S, Denmark) and α-chloro-naphthol. ELISA tests were performed by incubating conjugates between MNPs and BLG with a BLG-specific monoclonal antibody, prior to transfer into 96-well ELISA plates previously coated with BLG. After incubation with antimouse IgG-horseradish peroxidase conjugate, tetramethylbenzidine in peroxide buffer was added, and plates were analyzed after color development by using an automated plate reader and the Bio-Tek's KC4TM software.

2.4 MNP interaction with cells

Cytotoxicity of unmodified and functionalized MNPs was assessed by a colorimetric tetrazolium assay [26] performed on human colon carcinoma cells (HT-29) and Caco-2 cells differentiated in RPMI 1640 medium and incubated with MNP suspensions according to the conditions described by Zhang [27]. Cells were exposed to plain and BLG-conjugated MNPs at concentrations ranging from 0.2 to 2 mg/mL for 12, 24, and 48 h. The same colorimetric method was used to assess, in separate experiments, the viability of Mono Mac 6 human cells after 20–24 h exposure to MNPs in the conditions used for microscopy studies (see below).

Cellular uptake was studied by incubating the human monocyte cell line, Mono Mac 6 (5×10^5 cells/mL) with either plain or BLG-conjugated MNPs (1 mg/mL as MNPs) for various times, followed by separating cells with a MACS® Cell Separation System (Miltenyi Biotec). The magnetically labeled cells were retained on the column, while unlabeled cells passed through. The retained fraction was eluted after detaching the column from the magnet. Both not-retained and retained cells were counted (Fuchs-Rosenthal 0.1 mm cell counting chambers), by using an optical microscope (VWR International). To assess whether MNPs were attached to the outside the cells or located in the cytosol, cells were incubated with either plain AF-MNPs or BLG-conjugated AF-MNPs, and separated as above on a magnetic column at 0 and 60 min incubation prior to analysis by confocal microscopy with a Zeiss LSM710 confocal laser scanning microscope (Carl Zeiss, Jena, Germany). The fluorophore was excited using an argon ion laser and combined with differential interference contrast (DIC) using the ZEN software provided with the microscope. The images were further processed for display using PhotoShop software (Adobe).

2.5 Flow cytometry

To assess the interaction of various proteins with monocytes, Mono Mac 6 cells were plated in microtiter plates (1×10^6 cells/mL, 150 μL/well) to which 25 μL medium and 15 μL of FITC-conjugated proteins (1 mg/mL) were added. Immediately after and after 0.5, 1, or 2 h of incubation, the plates were centrifuged, and washed twice before fixation with 4% formaldehyde. To assess the uptake of MNP-bound proteins, cells were plated in 48-well clustertrays (1×10^6 cells/mL, 0.9 mL/well), added of AF-MNPs (5 mg/mL, 0.2 mL/well) with or without conjugated proteins and incubated for 0–2 h before washing. The cells were analyzed on a flow cytometer, (BD FACSCanto, BD Biosciences, Brondby, DK, Denmark) and the proportion of cells that had taken up the fluorescent label was determined for each protein or MNP at each time point.

2.6 Statistical analysis

Data were analysed by GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA, USA), by performing two-way ANOVA of the uptake of AF- and protein-conjugated MNPs as compared with AF-MNPs without conjugated proteins. Bonnferonis post-test was used to compare the effect of individual proteins, of MNP-conjugated proteins, and of fluorescein-conjugated MNPs (without protein) as a function of time.

3 Results

3.1 MNP-bound BLG retains structure and immunoreactivity

Our conjugation procedure allowed the easy covalent binding of bioactive food proteins to the particles by exploiting, in the case of BLG, the reactivity of surface lysines. Most of the surface lysine residues in BLG are known to be solvent-exposed even in the dimeric form of the protein at neutral pH, and only one of them has been indicated as present in the many reported antigenic sequences of this protein [28–31]. Transmission electron microscopy measurements assessing the MNP size and morphology before and after the functionalization procedure showed that aggregation was induced by the CMD activation procedures – although also unmodified MNPs tend to form aggregates. It was however possible to minimize the extent of MNP aggregation by treating MNP suspension in an ultrasonic bath.

BLG–MNP conjugates were characterized by various immunochemical approaches. Dot blotting with anti-BLG antibodies, followed by immunoenzymatic detection of the bound antibody, demonstrated the actual presence of BLG on conjugates with CMD-coated MNPs, as reported in Fig. 1. Beside assessing the general feasibility of our functionalization approach, the blots in Fig. 1 demonstrate that the polyclonal anti-BLG antibodies used in these experiments were able to recognize epitopes on the bioactive protein also when it was conjugated to MNPs. Thus, our coupling procedure did not negatively affect the protein structure and biological activity. However, dot blots are not suitable for evaluating a coupling ratio for our conjugation strategies, since it was not

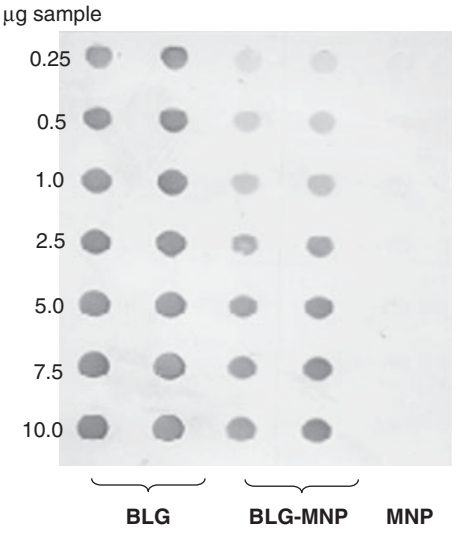

Figure 1. Results of dot-blotting experiments performed with anti-BLG antibodies and BLG-MNP conjugates. A BLG solution was used as a standard, and unmodified MNPs as a control. Amounts are given as micrograms of protein (or of MNPs, as iron) loaded onto the membrane.

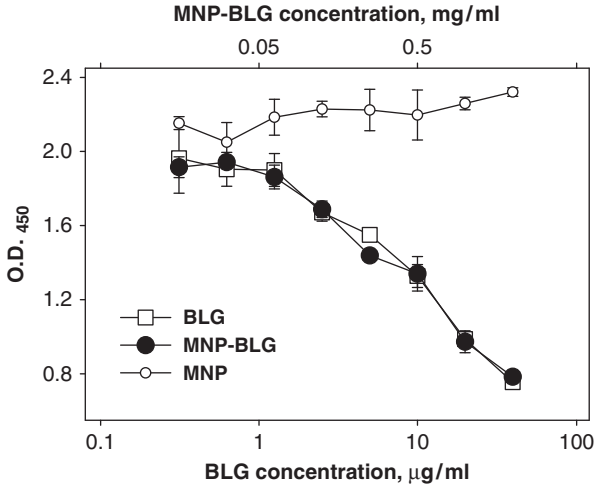

Figure 2. Competitive ELISA for native BLG, MNP–BLG, and unmodified MNPs. Concentrations refer to protein (BLG) or to iron (MNPs and MNP–BLG conjugates).

possible to define a standard curve correlating color intensity and the amount of BLG loaded as a standard. Also, the brownish background of MNP suspensions did not allow a reliable estimation of color intensity by standard image analysis techniques, especially considering the dots with the highest amount of MNPs.

In order to circumvent the limitations outlined above, competitive ELISA was performed by incubating BLG and BLG–MNP conjugates with a mouse anti-BLG monoclonal antibody (Fig. 2). From the parallel curves, we conclude that the monoclonal antibody used does not discriminate between BLG and BLG conjugated to the MNPs, and we could estimate from these data a conjugation yield of 20 μg protein/mg MNPs. Assuming an average molecular weight of 1 000 000 for a particle of the given size and composition, this indicates that each MNP binds one BLG molecule, in accordance with measurements of binding efficiency carried out independently by using trypsin (and the associated proteolytic activity, which was fully retained upon conjugation) as a reporter of conjugation yield (data not shown). These figures compare well with those reported in the literature for similar procedures [32], and are low enough to prevent possible screening effects due to molecular crowding on the MNP surface [33].

3.2 MNP-BLG binds to monocytes

Since uptake of particles – and in particular of those containing redox-active metals – may result either in cell necrosis due to direct cell damage, or in cell apoptosis [34], MNP biocompatibility was tested by incubating different intestinal model cell lines for various times with unmodified and protein-coupled MNPs and by subsequently performing the Mosmann colorimetric MTT assay [26]. In all cases, cell viability was close to that of control untreated cells, indicating the full biocompatibility of the particles and conjugates used in this study with these intestinal cells (data not shown).

The incorporation of either free or BLG-conjugated MNPs was studied in the monocytic cell line Mono Mac 6, which represents a mature human monocytic cell line [35]. Cells were incubated for various times with either plain or BLG-conjugated MNPs and, after incubation, cells were separated on MACS® cell separation columns attached to an external magnet providing a magnetic field strong enough to retain magnetically labeled cells. These are released upon detachment of the column from the magnet. Both non-retained cells and cells retained on the column were counted, and the proportions of retained cells are presented in Fig. 3A.

Cells incubated with either unmodified MNPs or MNPs conjugated to BLG were effectively retained by the magnetic field, whereas this did not happen in the case of cells not exposed to MNPs (not shown). This clearly indicates that monocytes were able to interact with both plain and BLG-conjugated MNPs, thus acquiring magnetic activity, and confirms previous observations about the uptake of MNPs of the same size and composition by human monocytes [18, 20], as well as by mouse macrophages [27], and by human ovarian tumor cells [17]. The number of cells interacting with BLG–MNP conjugates was much higher at each time point as compared with MNPs, suggesting that the presence of BLG on the MNP surface is recognized by the monocytes and thus may favor endocytosis.

Figure 3A also indicates that interaction of monocytes with either plain or BLG-conjugated MNPs is time

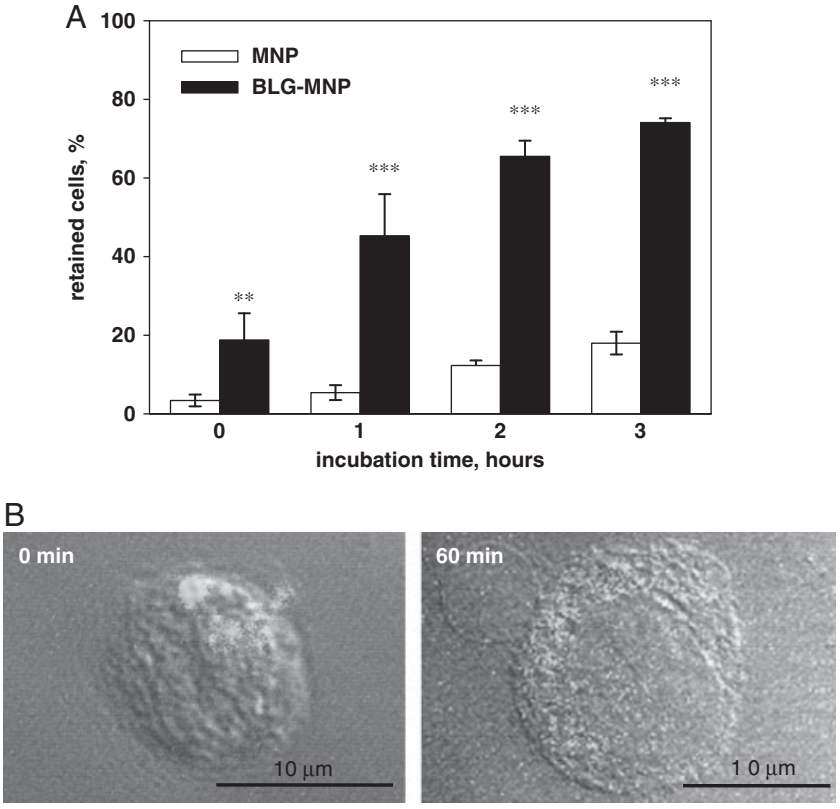

Figure 3. Conjugation to BLG enhances uptake and absorption of MNPs by human monocytes. (A) MNPs and BLG–MNPs were incubated for the given time with cells, prior to transfer onto MACS® columns. Bars show the proportion of retained cells on the column as a function of incubation time with MNPs. The results are the average of three separate experiments. Asterisks indicate the significance of differences between MNPs and BLG–MNPs (**$p<0.01$; ***$p<0.001$). (B) Confocal microscopy images of cells eluted from the MACS® column at the given times of incubation with BLG–MNPs.

progressive. The incubation time was not prolonged because the maximum particle uptake seemed to be reached (at least in the case of BLG-MNPs) at 3 h, and because the conditions used in these experiments may result in artifacts at longer incubation times, as they did not represent optimal living conditions for cells.

The experiments presented in Fig. 3A, although informative, did not establish whether the interaction between monocytes and the various MNPs is associated with their internalization rather than to mere adsorption of the MNPs on the cell surface. In order to address this point, we prepared MNPs conjugated to a fluorophore (aminofluorescein, AF) to be used for direct observation of intracellular uptake by confocal microscopy. In the case of the BLG–MNP conjugates, it was found possible to prepare fluorescent MNPs both by modifying the CMD-coated MNPs prior to protein addition, or to conjugate fluorescein to the bound protein by exploiting the reactivity of the single free thiol of Cys121 in BLG. Although both approaches were proven to be feasible, we decided to bind BLG to AF-labeled MNPs since this procedure allowed to have appropriate controls in the absence of BLG, and to keep protein modification to a minimum. Appropriate ELISA tests carried out along the lines of those reported in Fig. 2 indicated complete retention of immunochemical reactivity in the fluorescein-labeled BLG (Supporting Information Fig. S1) and in BLG conjugates to fluorescent MNPs (Supporting Information Fig. S2).

Figure 3B shows confocal micrographs of cells at 0 and 60 min of incubation with BLG-MNPs that were retained on the column. The distribution of fluorescence in cells after 60 min of incubation is clearly indicative of homogeneous distribution of MNPs inside the cytoplasm, and thus of their uptake and internalization by the monocytes, whereas at 0 h of incubation most of the fluorescent MNPs conjugated to the protein were located on the surface of the cells.

In the frame of the experiments reported in this section, incubation of MNPs with the Mono Mac 6 monocytes for 20–24 h did not reveal any changes in cell viability in comparison to control cells with no addition of MNPs, according to viability staining and microscopic inspection.

3.3 The uptake of protein–MNP conjugates by monocytes is protein-selective

The results presented above raised the question as to whether the BLG-enhanced internalization of MNPs was due to specific interaction between BLG and some receptor on the cell membrane, or if all proteins would enhance the uptake of the MNPs. Accordingly, we FITC-conjugated a number of proteins through their lysine residues to assess their internalization in monocytes by flow cytometry. We included BLG and OM as representatives of major food allergens, along with KSTI and BSA, representing other food proteins. HSA was also used, in what it represents an

endogenous transport protein. Figure 4 shows the proportion of cells that are fluorescence labeled 0–2 h upon addition of proteins to the cell cultures. FITC-labeled HSA and BLG were the two proteins taken up most rapidly and efficiently, followed by OM. Cells added of BSA and KSTI exhibited much lower labeling.

The same flow cytometry-based approach was used to test the specificity of the uptake by monocytes of protein–MNP conjugates. Fluorescent MNPs were prepared, to which various proteins were conjugated in a subsequent and separate step. ELISA measurements carried out along the lines of what reported in Fig. 2 indicated that – in our conditions – all proteins gave conjugates with MNPs containing ~1 molecule protein on each MNP (not shown), and that the fluorescein label on MNPs did not affect the binding stoichiometry (as shown for BLG in Supporting Information Fig. S2).

The various protein–MNP fluorescent conjugates were allowed to interact with monocytes, and the fraction of fluorescent cells was assessed by flow cytometry, again as a function of the time of contact between the cells and MNPs. As shown in Fig. 5, the fraction of cells associated with has–MNP fluorescent conjugates was low, and comparable to that of cells associated with MNPs devoid of any protein. The proportion of cells taking up protein-free fluorescent MNPs also was found to be time-independent, remaining constantly low over the 2 h duration of these experiments.

Conversely, as also shown in Fig. 5, a much larger fraction of cells was able to associate with MNPs conjugated to either BLG or OM. MNP conjugates of these proteins seemed to differ in their uptake time course. In particular, the initial burst in the increase of cells with OM–MNP conjugates was much more evident than the one observed with BLG–MNP conjugates. In this frame, it is also worth noting that the kinetics of uptake of BLG–MNPs and OM–MNPs in Fig. 5 appear different from those obtained when the free, FITC-labeled protein was used (see Fig. 4).

4 Discussion

The processing of food in new and inventive ways that provide new properties in regard to e.g. solubility and delivery may raise new concerns in relation to allergy hazards. A relevant example is food containing nanoparticles made of BLG [7, 8]. Whether BLG in nanoparticles will hold other properties than single BLG molecules as regard their interaction and uptake with cells of the immune system was addressed in the present study. We found that BLG per se was readily endocytosed by human monocytes and that BLG-conjugated nanoparticles were more extensively endocytosed that non-conjugated nanoparticles, indicating that BLG binds to a receptor on human monocytes involved in the endocytosis of particles from the blood or tissue fluids. As not all related proteins were taken up so readily, as demonstrated with BSA and KSTI, it is reasonable to assume that a receptor binding with some specificity is involved.

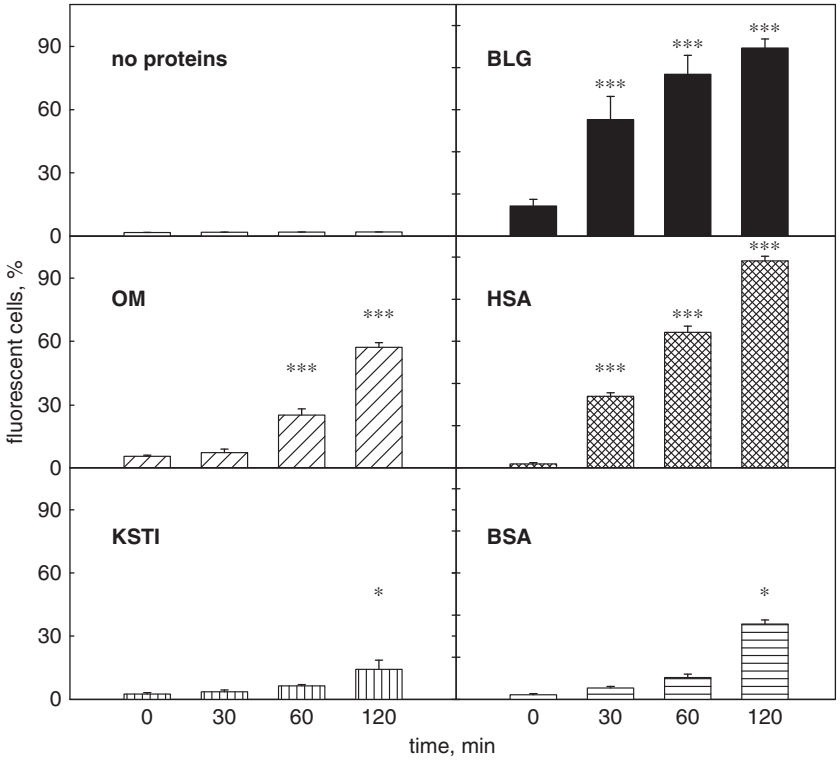

Figure 4. Endocytosis of FITC-conjugated proteins by the human monocytic cell line, Mono Mac 6. Cells were incubated with FITC-conjugated BLG, OM, HSA, KSTI and BSA for the given times, fixed, and analysed by flow cytometry to assess the proportion of cells that have endocytosed FITC-conjugated proteins. For each protein and each time point cells were analyzed in triplicate in two individual experiments. Asterisks indicate the significance of differences (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

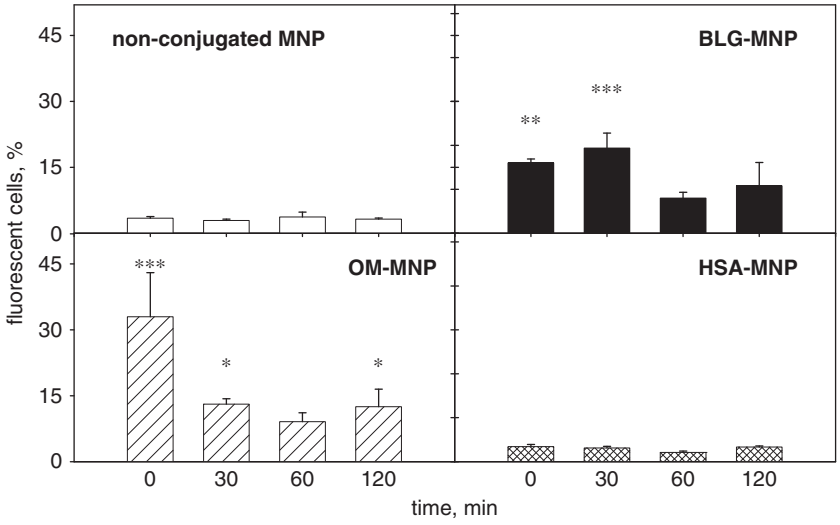

Figure 5. Endocytosis of fluorescent MNPs by the human monocytic cell line Mono Mac 6. Cells were incubated for the given time with AF-labeled MNPs, to which proteins (BLG, OM or HSA) were covalently conjugated where indicated. At the given times, cell were fixed, washed, and analysed by flow cytometry to assess the proportion of cells that have endocytosed AF-conjugated MNPs. Data for each MNP and MNP-protein conjugate and each time point are triplicate replicas from two individual experiments. Asterisks indicate the significance of differences ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

Contrary to BLG, HSA was taken up by monocytes much more quickly and efficiently when it is not bound to MNPs. Since HSA is the most abundant plasma protein serving as carrier of various endogenous and exogenous material [36], it makes sense that it can bind to a receptor on certain cells for the deliverance of its cargo. Many proteins may be able to bind to e.g. scavenger receptors and other highly promiscuous receptors on phagocytosing cells [37] These receptors are often located in cavaeolae, and only allow uptake of proteins and very small particles (< 50 nm), and not that of larger particles, such as the MNP conjugates of HSA in the present study, having a diameter in excess of 60–80 nm. Therefore, BLG-MNPs – but not HSA–MNPs – appear to be taken up through other types of endocytosis, e.g. phagocytosis or macropinocytosis, and this indicates that BLG–MNP conjugates may have specific physiological effects [38].

Whether BLG is taken up by specific receptors remains, however, to be demonstrated. Support for a specific receptor-mediated uptake of BLG was provided by Palupi et al. [39] who demonstrated that FITC-labeled BLG was taken up by a transformed mammalian B-cell line in a specific manner. As BLG is reportedly a carrier of various hydrophobic nutrients, these BLG receptors might be present on all cells in need for such components. Interestingly, another major food allergen, OM, exhibited the same endocytosis pattern as BLG when analysed by flow cytometry, being taken up both as single protein and attached to MNPs, despite the fact that OM has some different properties, e.g. being a glycoprotein [40].

Further mechanistic information may be derived from the time course of some of the events reported here. For instance, the proportion of cells positive for BLG-MNP were highest at time point zero and after 30 min of incubation when analyzed by flow cytometry (see Fig. 5), whereas analysis by separation by MACS® columns (see Fig. 3A) demonstrated a time-dependent increase in the fraction of cells retained on the magnetic column due to their MNP content. This discrepancy can be explained as follows. The protein-conjugated MNPs may be bound to receptors on the surface very rapidly (see the left panel in Fig. 3B), allowing the cells to be detected as fluorescent regardless of whether the MNPs are internalized rather than being just stuck to the cell surface. At later time points the material may have become internalized (see the right panel in Fig. 3B) but may not give rise to further change in the proportion of fluorescent cells in the flow cytometry analysis. Moreover, internalization may give rise to a weaker fluorescence signal, either due to quenching or to the fact that not all the MNPs are endocytosed so that the residual MNPs might be removed during the washing steps. It is likely that the monocytes must bind or endocytose a certain amount of MNPs in order to be retained on the MACS® column, while attachment of even very few particles will be registered by flow cytometry. From this we conclude that the MACS® column-based procedure is quantitatively the most informative.

From the data presented here, it can be concluded that BLG can be taken up by human monocytes both as single proteins and as MNP-conjugated protein, but it is not possible to say right now whether it is taken up by the same mechanism and whether the cellular response ensuing from the uptake is the same. It has been shown that the resulting responses may differ according to the endocytotic mechanism [41], for instance resulting in a pro-inflammatory response as opposed to a non-inflammatory or tolerogenic response. This might be of major importance in the risk assessment of the use of BLG-nanoparticles in food. At present we cannot tell whether the nanoparticle attached to BLG may change the possible responsiveness of the monocytes, but the data we obtained from flow cytometry analysis of BLG and BLG–MNPs indicate different uptake kinetics. In our opinion, monocytes and other phagocytosing immune cells that can serve as antigen-presenting cells represent suitable models for assessing effects of processed

allergens including allergens associated with or included in polymers, as the nature of antigen presentation is believed to be dependent on the endocytotic pathway [42, 43] and to play a key role in determining the type of the subsequent immune response.

Further studies are however required on this peculiar topic by further elucidating protein distribution on MNPs (and thus highlighting any possible endocytosis phenomena), and by testing the effects on particle uptake of other proteins, that have known internalization mechanisms and thus can limit MNP uptake also by other cell lines and primary cells [17]. Yet another possibility for addressing these issues is the use of receptor-specific antibodies that could clarify the details of the recognition and uptake events.

In conclusion, we have shown that the presence of BLG on MNPs enhances the uptake of MNPs in human monocytes, and as not all proteins conjugated to the MNPs result in the same enhancement of endocytosis, this uptake seems to be receptor mediated. Whether this enhanced phagocytosis of particles has physiological implications remains to be investigated.

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The authors have declared no conflict of interest.

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