



Galectin-3 endocytosis by carbohydrate independent and dependent pathways in different macrophage like cell types

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ABSTRACT

Background: Galectin-3 (the Mac-2 antigen) is abundantly expressed in both macrophage like cells and certain non-macrophage cells. We have studied endocytosis of galectin-3 as one important step relevant for its function, and compared it between variants of a macrophage like cell line, and non-macrophage cells.

Methods: Endocytosis of galectin-3 was observed by fluorescence microscopy and measured by flow cytometry. The endocytosis mechanism was analysed using galectin-3 mutants, galectin-3 inhibitors and endocytic pathways inhibitors in the human leukaemia THP-1 cell line differentiated into naïve (M0), classical (M1) or alternatively activated (M2) macrophage like cells, and the non-macrophage cell lines HFL-1 fibroblasts and SKBR3 breast carcinoma.

Results: Galectin-3 endocytosis in non-macrophage cells and M2 cells was blocked by lactose and a potent galectin-3 inhibitor TD139, and also by the R186S mutation in the galectin-3 carbohydrate recognition domain (CRD). In M1 cells galectin-3 endocytosis could be inhibited only by chlorpromazine and by interference with the non-CRD N-terminal part of galectin-3. In all the cell types galectin-3 entered early endosomes within 5–10 min, to be subsequently targeted mainly to non-degradative vesicles, where it remained even after 24 h.

Conclusions: Galectin-3 endocytosis in M1 cells is receptor mediated and carbohydrate independent, while in M2 cells it is CRD mediated, although the non-CRD galectin-3 domain is also involved.

General significance

The demonstration that galectin-3 endocytosis in M1 macrophages is carbohydrate independent and different from M2 macrophages and non-macrophage cells, suggests novel, immunologically significant interactions between phagocytic cells, galectin-3 and its ligands.

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1. Introduction

Macrophages are part of the mononuclear phagocytic system (MPS) and arise by differentiation of a specific subpopulation of monocytes, as do dendritic cells, osteoclasts, microglia and other macrophage like cells [1–3]. Macrophages can then be further activated by components of the tissue environment in different ways [4,5]. As typical examples, classical activation by e.g. lipopolysaccharide (LPS) or interferon- γ (IFN- γ) leads to inflammatory macrophages (M1) posed to fight acute infection and clean up injured tissue, whereas alternative activation by e.g. IL-4 and/or IL-13 leads to macrophages (M2) that secrete anti-inflammatory cytokines and induce tissue rebuilding.

Galectin-3 (the Mac-2 antigen) is one of the markers usually present on activated macrophages and macrophage like cells [6,7], but almost 30 years after its discovery its function in these cells remains unclear [8]. Mac-2 was identified, together with Mac-1 and Mac-3, as an antigen in a screen of monoclonal antibodies raised against a membrane fraction from thioglycollate elicited mouse peritoneal macrophages [9]. Cloning and sequencing showed that Mac-1 and Mac-3 are membrane glycoproteins (β -integrin and LAMP-2, respectively) [1]. Mac-2 however, is a lectin, later named galectin-3, with characteristics of a small soluble cytosolic protein but with affinity for β -galactosides typically presented on glycoproteins separated from the cytosol by a membrane (extracellularly, at the cell surface or in the lumen of intracellular vesicles). These unusual dual properties of galectin-3 have made it difficult to define its biological roles and a multitude of effects have been described, both in macrophages and in other cells [10].

In non-MPS cells the roles of cytosolic galectin-3 include an anti-apoptotic effect [11] and activation of K-RAS [12] and β -catenin [13]. The non-cytosolic effects include a variety of signals induced

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by extracellular galectin-3 such as cytokine release, induction of apoptosis [14] or immune evasion by cancer cells [15]. Endogenous galectin-3 has also been shown to regulate intracellular trafficking of specific glycoproteins [16,17], and their residence time and fine organization at the cell surface, with consequent effects of cell sensitivity to e.g. growth factors, immune stimulation [18] or apoptosis inducers [19], highly relevant in cancer development. So far it has been thought that all the non-cytosolic effects of galectin-3 require its carbohydrate binding activity residing in the ~130 amino acid (aa) carbohydrate recognition domain (CRD) and its cross-linking ability residing in the ~120 aa N-terminal non-CRD part [10]. An emerging view linking cytosolic and non-cytosolic galectin-3 is as follows: galectin-3 is synthesized in the cytosol where no β -galactosides are present. Upon a so far unknown signal some of the galectin-3 crosses a membrane and translocates into intracellular vesicles and/or directly onto the cell surface. Here it interacts with selected membrane glycoproteins by recognizing and binding β -galactosides in specific constellations. By cross-linking these glycoproteins, which is mediated by N-terminal domain, galectin-3 regulates their trafficking, plasma membrane residence time and organization. Furthermore, cell surface bound and extracellular galectin-3 may re-enter the cell by endocytosis and take part in a recycling loop, and is then found in the vesicular non-cytosolic compartments [17]. It does not re-enter the cytosol as far as is known.

In MPS cells, the requirement of galectin-3 for a several functions has been suggested based on their induction by externally added galectin-3, such as chemotaxis [20], signal induction and opsonisation [21,22], or their loss in galectin-3 depleted cells, such as efficiency of phagocytosis [23] and alternative activation [24]. However, very little is known about the molecular mechanisms of these events. The immediate effects of galectin-3 in non-MPS cells, like entry into vesicles and binding of glycoproteins, occur on a time scale of minutes, whereas most of the consequent effects in both non-MPS and MPS cells have been measured after hours or days. Therefore, we have started to characterize an immediate effect of galectin-3 in macrophages, endocytosis, and compared it between classically and alternatively activated macrophages. Here we show results with the leukemia cell line THP-1, which develops macrophage functions following the addition of cytokine stimulators, produces a homogenous group of cells during different stages of maturation, and has been used as a macrophage model in many studies [25,26–31]. Surprisingly, galectin-3 uptake appears to be by both carbohydrate dependent and independent pathways, different for the different macrophage like cells and also compared to other cell types.

2. Materials and methods

All chemicals were from Sigma Aldrich (St. Louis, MO, USA) unless stated otherwise.

2.1. Cell cultures

THP-1 cells were grown in RPMI-1640 + L-glutamine (HyClone, Cramlington, UK). SKBR3 cells were grown in RPMI-1640 + L-glutamine supplemented with 1x sodium pyruvate and 1x non-essential amino acids. HFL1 cells were grown in DMEM (Invitrogen, Carlsbad, USA) culture medium. All culture media were supplemented with 10% foetal calf serum and 1% penicillin–streptomycin (Invitrogen, Carlsbad, USA)—complete medium. Humidified 37 °C incubators with 5% CO₂ were used for all cell incubations.

2.2. THP-1 cell line differentiation into M0, M1 and M2 macrophage like cells

To obtain M0 macrophage phenotype, THP-1 cells were treated with 50 ng/ml phorbol-12-myristate-13-acetate (PMA) [32] in

complete medium for 3 days, followed by 3 days rest in complete medium. To obtain M1 macrophage phenotype, THP-1 cells were treated with 50 ng/ml PMA in complete medium for 3 days, followed by treatment with 100 ng/ml *Escherichia coli* LPS in complete medium for 3 days [33]. To obtain M2 macrophage phenotype, THP-1 cells were treated with 50 ng/ml PMA with addition of 10 ng/ml IL-4 in complete medium for 6 days (two 3 day pulses) [34].

2.3. Cytokine measurement

Supernatants were collected after the second stage of THP-1 cells differentiation (last 3 days), filtered through 0.45 μ m filters and cytokine concentrations measured by a solid-phase enzyme-labelled chemiluminescent immunometric assay (Immulite 1000, Siemens, Upplands Väsby, Sweden).

2.4. Western blot

1×10^6 of M0, M1 and M2 cells were dissolved in lysis buffer (0.9% NaCl, 2.4% Tris, 0.08% EDTA, 0.01% V/V NP-40) with addition of 2 μ g/ml protease inhibitors: leupeptin, aprotinin and pepstatin. After cell lysis, supernatants containing equal protein amounts were separated by denaturing SDS-PAGE on 4–20% polyacrylamide gel following company instructions (Pierce, Rockford, IL, USA). Subsequently, the proteins were transferred to a PVDF methanol-activated membrane at 100 V for 1 h. In order to avoid unspecific binding, the membrane was incubated with blocking buffer (5% dry milk in PBS-T (1/15 M PBS pH 7.2 + 1% Tween)) for 24 h. The membrane was incubated with rat anti-mouse galectin-3 antibody (1:1000) (anti Mac-2, [9]) in PBS-T for 1 h at room temperature (RT). After washing 3 times (10 min) goat anti rat Horseradish peroxidase (HRP) conjugated antibody in PBS-T was added (1:4000) for 1 h at RT. Proteins were then detected using an ECL Plus Western Blotting Detection System (GE Healthcare, Uppsala, Sweden) for 5 min, and protein bands were scanned using a GelDoc imager (BioRad, Sundbyberg, Sweden), analyzed with ImageJ software [35] and compared to GAPDH as a control. Mouse anti-human antibody raised against GAPDH (Novus Biologicals, Cambridge, United Kingdom) (1:4000) and rabbit anti-mouse HRP conjugated (1:4000) (DAKO, Stockholm, Sweden) for GAPDH detection.

2.5. Galectin-3 real time quantitative PCR (rt-q-PCR)

After differentiation, aliquots of discrete macrophage subtypes were centrifuged at 400 \times g for 5 min at +4 °C and supernatants were discarded. Total RNA was extracted from 10^6 cells with RNeasy® Mini Kit (Qiagen, GmbH, Hilden, Germany) following the manufacturer's protocol. Total RNA (~0.8 μ g) was reverse transcribed using random hexamer primers (5 ng/ μ l) and murine leukaemia virus (M-MLV) reverse transcriptase (10 U/ μ l), in a final volume of 20 μ l containing 1 mM dNTPs, 2 U/ μ l RNase inhibitor, 10 mM DTT in first strand buffer (50 mM KCl, 75 mM Tris/HCl, 3 mM MgCl₂ pH 8.3) during 1-hour incubation at 37 °C. The samples were heated for 5 min at 95 °C and cooled down to 4 °C.

Galectin-3 mRNA expression level was measured using the TaqMan technology on an ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. TaqMan probes and primer pairs for galectin-3 and GAPDH were Assay on Demand gene expression products (Microsynth, Balgach, Switzerland). To avoid amplifications of genomic DNA, probes were designed to span at exon–exon–boundary. The sequences were as follows: GAPDH forward primer: 5'-ACCCACTCCTCCACCTTTGAC-3', GAPDH reverse primer: 5'-CATACCAGGAAATGAGCTTGACAA-3', GAPDH probe: 5'-CTGGCATTGCCCTCAACGACCA-3', galectin-3 forward primer: 5'-GCGGAAAATGGCAGACAATT-3', galectin-3 reverse primer: 5'-CTTGAGGGTTTGGGTTTCCA-3', galectin-3 probe: 5'-ACCCAGATAACGCATCATGGAGCGA-3'. PCRs were prepared in a final volume of 25 μ l,

with 1 μ l of cDNA and a final concentration of 1 \times TaqMan Universal PCR master mix, No AmpErase UNG (Applied Biosystems), 0.1 mM primers and 0.1 mM probes. Reaction mixtures were assembled at 4 °C, followed by PCR consisting of AmpliTaq Gold activation at 95 °C for 10 min followed by 95 °C for 15 s and 60 °C for 1 min for 40 cycles. Calibration curve included four points of the 10-fold diluted cDNA for both galectin-3 and human GAPDH. The expression of galectin-3 mRNA was normalized to the amount of GAPDH in the same cDNA. Relative fold ratio of treated/control samples between 0.5 and 2 was considered not to show significant changes in galectin-3 mRNA levels. The analyses of two independent experiments were performed in triplicate.

2.6. Galectin-3 and galectin-3 mutant production and characterization

Mutants were made using QuickChange® II Site-directed Mutagenesis Kit (Stratagene, Amsterdam, The Netherlands), as previously described [36], with template-DNA isolated from *E. coli* XL1Blue.

Galectin-3 CRD 251C gene in the PET9a vector [37] was kindly provided by Professor James H. Prestegard (Complex Carbohydrate Research Center, University of Georgia, Athens, GA). The additional cysteine at position 251 in that construct was removed using the following mutagenic PCR primers; 5'-GCTTCATATACCATGATATGATGAGGATCCGGCTGC-3' and 5'-GCAGCCGGATCCTCATCATATCATGGTATATGAAGC-3'. To obtain R186S mutant of galectin-3 CRD in the PET 9a plasmid, the same primers as for R186S on intact galectin-3 described in Ref. [36], were used. Successful mutagenesis was confirmed by sequencing by GATC Biotech (Konstanz, Germany), in forward direction from the T7 promoter primer and in reverse direction from the pET-RP primer.

Recombinant human galectin-3 (rh-gal-3) and mutants were produced in *E. coli* BL21Star (DE3) (Invitrogen, Carlsbad, USA) as described by Salomonsson [36]. All produced proteins were tested for LPS content using *Limulus Amebocyte Lysate Endochrome™* kit (Charles River Endosafe, Charleston, SC, USA). In all experiments LPS content was less than 73 pg (12.2 ng per mg of protein), inefficient for macrophage activation in the short-time experimental setup used here [38,39].

The carbohydrate specificity of galectin-3 wt and mutants as tested on glycan array and by a fluorescence anisotropy assay has been described before [36], and here inhibitory potency of asialofetuin and fetuin tested by the FA-assay as for galectin-1 [40] was added as indicated in the text.

2.7. Labelling of proteins

15 fold excess of labelling dye, NHS-fluorescein or NHS-sulphorhodamine (Thermo Scientific, Erembodegem, Belgium) dissolved in dimethyl formamide (DMF) was added to a 2 mg/ml (~80 μ M) protein solution containing lactose (~1 mM) in 100 mM carbonate/bicarbonate buffer. The solution was mixed well and incubated for 1 h at RT. Labelled proteins were separated from the unreacted dye by a buffer exchange to PBS on a PD10 column (Amersham Biosciences, Uppsala, Sweden). Labelled proteins remained intact as tested by SDS-PAGE. Also, carbohydrate binding activity was preserved as tested by binding to a lactosyl-sepharose column and elution with lactose.

2.8. Galectin-3 endocytosis in differentiated THP-1 cells and SKBR3 and HFL1 cells

The cells were grown on coverslips in 24-well plates. 50 000 THP-1 cells per well were differentiated as described in Section 2.2 while SKBR3 and HFL1 cells were grown to 80% confluence. 0.2 μ M NHS-fluorescein or NHS-sulphorhodamine conjugated galectin-3 (wt or mutants; intact or CRD only) in complete medium was added to the cells and incubated for the desired time at 37 °C. Endocytosis was stopped by removing the medium followed by addition of ice cold PBS and

fixation in 2% paraformaldehyde. Coverslips were mounted using Mowiol, after nucleus staining with 0.01 mg/ml Hoechst 33342. Images were captured using Nikon eclipse TE2000-U fluorescence microscope with a CFI Plan Apochromatx100 oil objective: numeric aperture 1.40, working distance 0.13 mm, and equipped with a digital still camera DS-Qi1MC. 3D stacks were acquired from necessary filters (DAPI, FITC or TEXAS RED). Images (RGB, 8 bit, 1280 \times 1024) were deconvolved from a 3D stack (x, y, z dimensions: 129.82 \times 103.85 \times 21.6 μ m) with NIS-elementAR software. Five images of each individual experiment were examined using NIS-elementAR software colocalization application and average values of overlap coefficients according to Manders were calculated after background correction.

2.9. Flow cytometry analysis of galectin-3 endocytosis in differentiated THP-1 cells

500 000 THP-1 cells per well were grown in 24-well plates and differentiated as described in Section 2.2. 2 μ M NHS-fluorescein conjugated galectin-3 (wt or mutants) in complete medium was added to the cells and incubated for the desired time points (5, 15 and 30 min) at 37 °C. After the incubation the cells were washed with PBS prior to 15 min trypsinisation at 37 °C. 100 μ l of 2.5% trypsin (GIBCO, Carlsbad, USA) was used not only to detach the cells from the plate surface but also to remove any residual fluorescent galectin-3 bound on the cell membrane (Figure S5). The samples were then diluted in PBS up to a final volume of 500 μ l and approximately 5 000 cells per duplicate samples were analysed by flow cytometry (Beckman Coulter Cytomics FC 500 MPL, Brea, CA, USA).

2.10. Immunofluorescence

After the desired treatment the cells were fixed in 2% paraformaldehyde, permeabilized with 1% Triton-X100 in PBS for 10 min and washed with PBS. Unspecific antibody binding was prevented using blocking buffer (0.1% Tween, 1% foetal bovine serum (FBS) in PBS) for 10 min at RT. Immunostaining was performed using mouse anti human early endosome specific protein (EEA1) (BD Bioscience, Stockholm, Sweden) (1:250) or mouse anti-human lysosome specific protein (LAMP1) (Santa Cruz, CA, USA) (1:100) for 1 h at RT in a moist chamber. Cells were washed with PBS and then incubated with corresponding secondary antibody, goat anti mouse Alexa Fluor594 (1:400) (Invitrogen, Carlsbad, USA). Rat anti-mouse galectin-3 antibody (anti Mac-2, [9]) (1:100) followed by secondary antibody, rabbit anti-rat FITC (1:500) were used for galectin-3 immunostaining. All antibodies were dissolved in blocking buffer. Control staining using only secondary antibody was run in parallel.

2.11. Endocytosis inhibitors treatment

Differentiated THP-1 cells have been pre-incubated with inhibitors for 2 h before endocytosis experiment as described in Ref. [41]. Inhibitors used were amiloride (1.5 mM), methyl- β -cyclodextrin (30 mM) and chlorpromazine (50 μ M). As controls, fluorescein-labeled dextran at a concentration of 0.1 mg/ml, Filipin-III at a concentration of 10 μ g/ml and fluorescein-labelled transferrin at a concentration of 0.15 μ M were incubated with cells (inhibitor treated or not) for 30 min at 37 °C and subsequently analysed with Nikon eclipse TE2000-U fluorescence microscope.

2.12. Galectin-3 inhibitors treatment

In an attempt to inhibit or modulate galectin-3 endocytosis in differentiated THP-1 cells or SKBR3 and HFL1 cells, the following treatments were used.

For endocytosis inhibition, the compounds were pre-incubated for 30 min in complete medium with the protein to be endocytosed, the

mixture was subsequently added to the cells, and incubated for 30 min at 37 °C. Cells were washed, fixed for 10 min in 2% paraformaldehyde and analysed with Nikon eclipse TE2000-U fluorescence microscope. Compounds used were: 10 mM lactose; 2, 4, 8, 20 or 100 μ M TD139 [42]; 0.6 μ M anti-mouse galectin-3 antibody (anti Mac-2, [9]); 2 μ M recombinant galectin-3 (wt) or R186S mutant.

To obtain the deglycosylated antibody, GST-EndoS enzyme was incubated with the antibody in 1:10 ratio at RT overnight. GST-EndoS was subsequently removed from the mixture using Glutathione sepharose and the cleavage of the glycans was confirmed by lectin blot [43].

For the endocytosis modulation, M0, M1 and M2 macrophages were treated with 10 mM lactose or with 2 μ M recombinant galectin-3 for 30 min on ice. Cells were then thoroughly washed with ice cold PBS before 0.2 μ M fluorescein labelled galectin-3 was endocytosed for 5, 10, 15 and 30 min at 37 °C. The cells were then fixed and analysed.

2.13. Pulse-chase endocytosis

Differentiated THP-1 cells, cultured on cover slips in 24-well plates, were incubated for 5 min with 200 μ l of 0.2 μ M fluorescently labelled protein in complete medium on ice (pulse). After extensive wash with ice-cold PBS, fresh complete medium was added, and the cells were further incubated at 37 °C for the desired time periods (chase). Cells were then washed, fixed, permeabilized and immunostained for EEA1 or LAPM1 as described in Section 2.9.

3. Results

3.1. Carbohydrate dependent uptake of galectin-3 in non-macrophage cell types

To establish a reference point and experimental system, we first studied the endocytosis of galectin-3 in non-macrophage cell types. In previous studies in MDCK cells [17], CHO cells [41], Jurkat and other cells, cell surface-binding and rapid uptake (within ~10 min) of galectin-3 into intracellular vesicles has been shown to be dependent on its carbohydrate binding activity by two main criteria: the uptake was inhibited by a competitive ligand (e.g. lactose), and there was no uptake of a galectin-3 mutant (R186S), which has much lower affinity for LacNAc (~70 fold lower compared to galectin-3 wt) and most N- and O-glycans commonly found in cell surface glycoproteins [36], and the model glycoprotein asialofetuin (>100 fold lower). The carbohydrate dependence has also been shown by deficient binding [44] and uptake of galectin-3 in the mutant CHO cell line Lec1, lacking LacNAc moieties in the N-glycans in cell surface glycoproteins.

Here we analysed this further in SKBR3 cells, a breast carcinoma cell line, and HFL1 cells, a non-transformed human lung fibroblast cell line (Figure S1). Both cell lines endocytosed galectin-3 wild type (wt) and endocytosis was completely prevented by competitive inhibitors, lactose and TD139, when added at concentrations of about 50-fold the K_d for their interaction with galectin-3 in solution, i.e. 10 mM for lactose and 2 M for TD139, a designed thiodigalactoside derivative that binds galectin-3 with low nM affinity [42]. The mutant R186S, deficient in binding to LacNAc, was also not taken up by the cells, while, another mutant, K176LN180T, with enhanced affinity for repeating LacNAc-residues and decreased affinity for 2–3 sialylated galactosides [36] was taken up equally well as galectin-3 wt. A truncated form of galectin-3, containing only the CRD, was also endocytosed about equally well as galectin-3 wt and its uptake was prevented by the inhibitors. This indicates that the cross-linking conferred by the N-terminal domain of galectin-3 is not required for endocytosis in these cells.

3.2. Three different macrophage-like phenotypes

Since we were interested in how galectin-3 endocytosis is carried out in different macrophages, first we had to obtain different macrophage like phenotypes. THP-1 cells were induced to differentiate with PMA for three days. This treatment alone has already been shown to induce galectin-3 expression on both mRNA and protein level [45]. After initial PMA treatment the cells were then cultured for three more days; with or without addition of LPS for classical activation or PMA and IL-4 for alternative activation. In all cases, this resulted in adherent macrophage-like cells, showing different phenotype details that we referred to as M0, M1 and M2, respectively. Morphologically the alternatively activated M2 cells had wide and elongated filopodia and were larger (Figure S4) and more spread on the growth surface than M0 and M1. M1 cells also had filopodia, which appeared narrower than for M2 cells (Fig. 1A). The profiles of secreted cytokines also differed as expected [46], with high IL-6 and TNF- α for M1 cells, high IL-10 for M2 cells, about equal IL-8 for M1 and M2 cells, and low levels of all cytokines measured for M0 (Fig. 1B).

Galectin-3 expression was induced from an initially low level in THP-1 cells (not shown) by PMA treatment, as also found for peripheral blood monocyte derived macrophages [47]. Immunostaining, western blot and rt-q-PCR analysis showed marked expression of galectin-3 in all three differentiated macrophage like THP-1 cells, with slightly higher levels in the M1 and M2 cells on both protein and mRNA level (Fig. 1C). Galectin-3 was found in vesicles, in the nucleus and in the cytosol. All the cell types also released galectin-3 into the culture medium (Figure S2), and M1 cells secreted about 1.5 to 2 times more galectin-3 (~29 ng/ml) than M0 and M2. All this is in agreement with previously shown induction of galectin-3 secretion by LPS in mouse peritoneal macrophages [48] and macrophage like cell lines [49].

3.3. Carbohydrate dependent and independent mechanisms for galectin-3 uptake in THP-1 derived macrophage-like cells

The uptake of galectin-3 in the different macrophage like cells was studied in the same way as for non-macrophage cells described above. Fluorescein-labelled wild type or mutant galectin-3 was added to the cells with or without inhibitors and uptake assessed by fluorescence microscopy. Galectin-3 was rapidly internalized and appeared in vesicles within minutes in all three macrophage types, with the result after 30 min shown in Fig. 2A. The uptake mechanisms were analysed by a series of experiments, and each experiment type was done in parallel for the M1 and M2 cells, and in some case also the M0 cells as shown in Figs. 2–6. Below, however, all experiments are presented for each cell type, one at a time, and the results are summarized in Table 1, to make the combined interpretation clearer.

3.4. Receptor mediated, carbohydrate independent galectin-3 uptake in M1 cells

Surprisingly, galectin-3 uptake in M1 macrophages was not prevented by neither lactose nor the more potent inhibitor TD139 (Table 1 and Fig. 2A, middle row of panels), not even when their concentration was increased to ~1000 times K_d in solution (Fig. 2B). Thus, endocytosis of galectin-3 in M1 macrophages appears to be carbohydrate independent.

This suggested the possibility that the N-terminal domain of galectin-3 conveyed its uptake in M1 macrophages. To analyse the role of the N-terminal domain we used a monoclonal IgG2a anti-Mac-2 antibody [9], directed towards a specific site within this domain [50]. The antibody did not interfere with carbohydrate-dependent CRD-mediated binding of galectin-3, as tested with a small saccharide probe and a model glycoprotein (asialofetuin) in a fluorescence

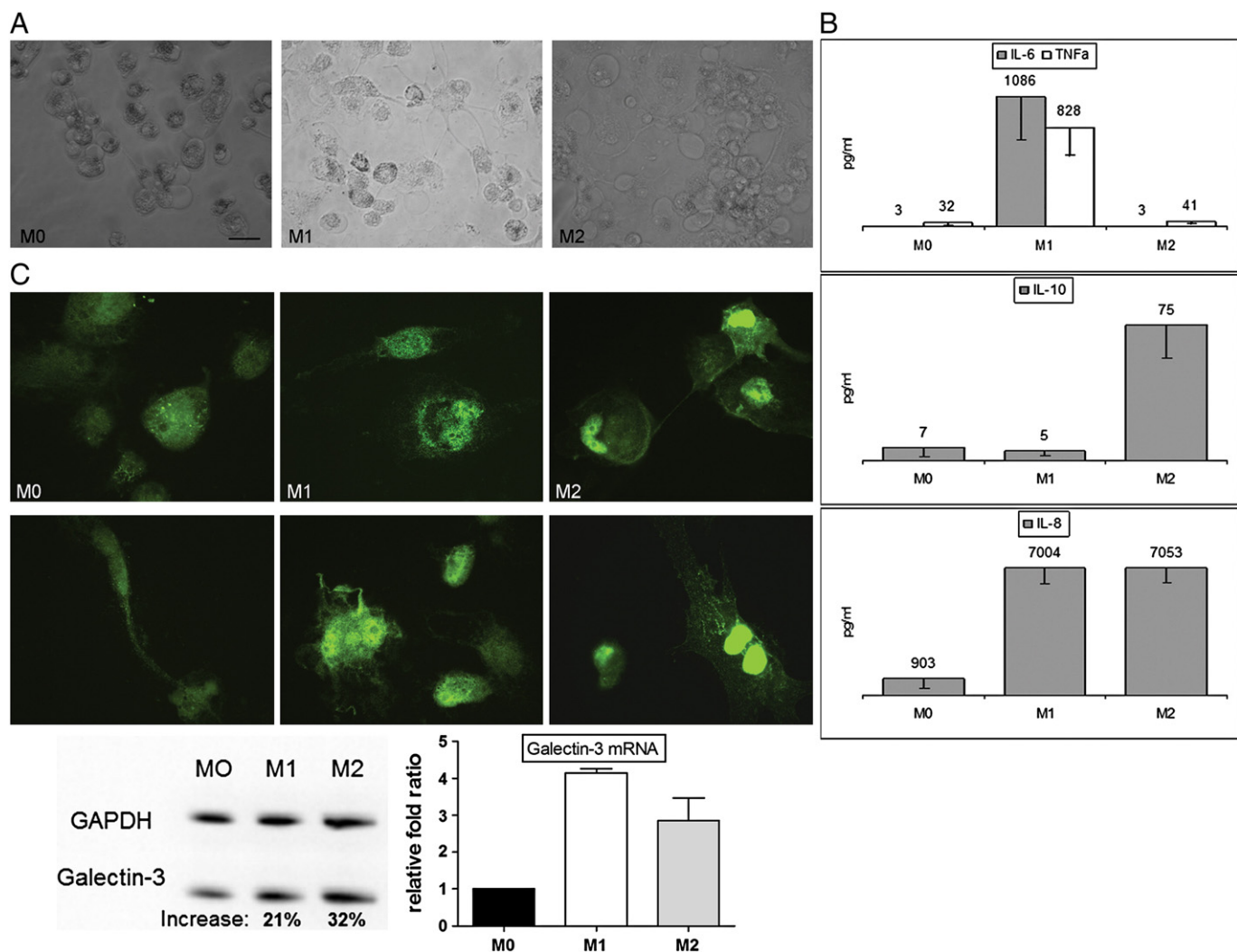


Fig. 1. Differentiation of THP-1 cells into M0, M1 and M2 macrophage like cells. THP-1 cells were treated with PMA (3 days) and then left in complete medium for additional 3 days (M0); treated with PMA (3 days) followed by LPS (3 days) (M1); treated with PMA and IL-4 (6 days) (M2). A. Light microscope images of M0, M1 and M2 macrophages B. Supernatants collected after the second stage of THP-1 cells differentiation (last 3 days) were filtered and subjected to luminometric measurement of IL-6, TNFα, IL-10 and IL-8 concentrations. C. Galectin-3 Alexa488 (green) immunostaining. Galectin-3 immunoblot in M0, M1 and M2 macrophages. Protein bands were quantified using ImageJ software. Galectin-3 mRNA levels normalized to GAPDH mRNA in the same cDNA. Data are representative (A and C) or shown as mean \pm SD (B and galectin-3 mRNA) of at least three independent experiments. Bar represents 20 μ m.

anisotropy assay (Figure S3). We also made a deglycosylated aliquot of the antibody, using the enzyme EndoS, which in turn have lost binding to Fcγ receptors [51]. The native antibody did not inhibit uptake of fluorescein-labelled galectin-3 in M1 cells, whereas the EndoS treated antibody did (Fig. 3, left set of panels, bottom two rows). This suggests that both antibodies bind galectin-3, but that as the native antibody binds Fcγ receptors, this results in uptake of the antibody-galectin-3 complex. The EndoS treated antibody apparently inhibits uptake of galectin-3 by itself, suggesting a role for the N-terminal domain of galectin-3 in its uptake. Thus, M1 macrophages have a carbohydrate independent mechanism for uptake of galectin-3 that involves the N-terminal non-CRD domain. This mechanism has relatively high capacity, since uptake of the labelled galectin-3 was not inhibited by admixture of a 10-fold excess of unlabelled galectin-3 (Fig. 3, left panel, second row) (in contrast to the case with M2 cells described in Section 3.5).

The galectin-3 CRD, lacking the N-terminal domain, was also readily endocytosed in M1 cells (Fig. 4, middle panels), and this uptake was partially inhibited by lactose and almost completely by TD139. This shows that there is also a carbohydrate mediated contribution to the uptake of galectin-3, which, however, is not rate limiting for intact galectin-3.

The R186S galectin-3 mutant that lacks the affinity for LacNAc and common glycoproteins, but has a retained affinity for lactose [36], was also endocytosed in M1 macrophages, and its endocytosis was not inhibited by the galectin-3 inhibitors lactose and TD139 (Fig. 5, top row of panels). As the lack of inhibition could be explained by the lower affinity of R186S for the inhibitors (a factor of about 5 for lactose and >10 for TD139), the uptake was also tested with extra high concentration of TD139 (>300 times its K_d in solution) but again there was no inhibition (Fig. 5). These data are consistent with the carbohydrate independent uptake of galectin-3 wild type described above. However, surprisingly, the CRD of the R186S mutant was also endocytosed and this uptake was again not prevented by the inhibitors (Fig. 4). This suggests that even at high concentration, the inhibitors are not potent enough to inhibit the mutant and/or an additional uptake mechanism of the mutant, different than of galectin-3 wild type (see Section 4 Discussion). In either case, the uptake of the galectin-3 R186S both intact and CRD is clearly different from the case in non-macrophage cells, where these proteins are not taken up at all.

Pharmacological inhibitors of endocytosis were used to profile the mechanism of galectin-3 uptake in relationship to known endocytic pathways—chlorpromazine for clathrin-mediated pathway [52],

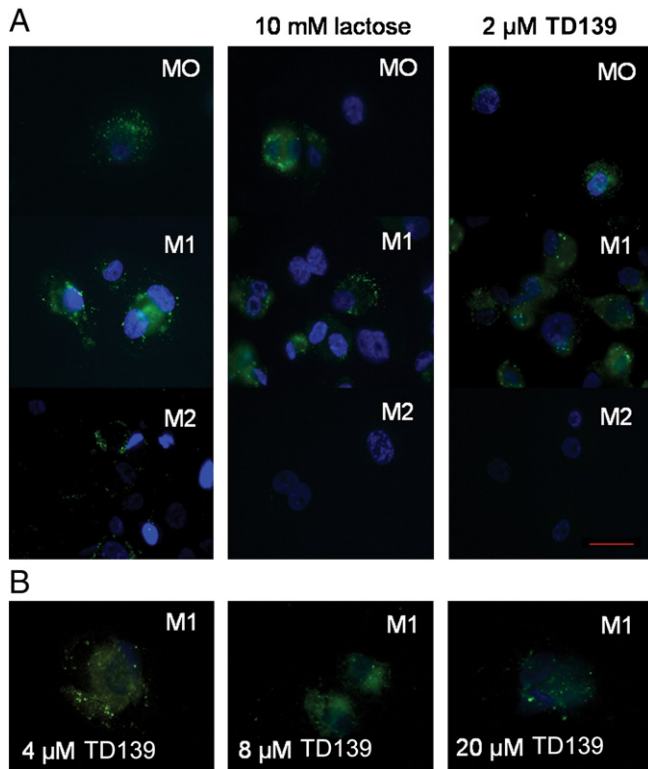


Fig. 2. Galectin-3 endocytosis inhibition by lactose or TD139 in M0, M1 and M2 macrophages. **A.** Uptake of 0.2 μ M, fluorescein-labelled galectin-3 after 30 min in M0, M1 and M2 cells (first column). Fluorescein-labelled galectin-3 was pre incubated with 10 mM lactose (second column) or 2 μ M TD139 galectin-3 inhibitor (third column) for 30 min and then added to the differentiated THP-1 cells and incubated for 30 min. **B.** Fluorescein-labelled galectin-3 was pre incubated with 4, 8 and 20 μ M TD139 for 30 min and then added to the M1 cells. Bar represents 20 μ m.

methyl- β -cyclodextrin for lipid raft pathway [53] and amilorid for micropinocytosis [54], as were previously applied to galectin-3 and -8 uptake in CHO and Jurkat cells [41]. In M1 cells, chlorpromazine inhibited the uptake of both galectin-3 wild type and R186S mutant (Fig. 6) and transferrin used as a control (not shown). Methyl- β -cyclodextrin had no effect on uptake of the galectins (Fig. 6) but inhibited uptake of filipin used as a control (not shown). Amilorid did not inhibit the uptake of the galectins (Fig. 6), as well as the uptake of the control molecule, fluorescein-labelled dextran (not shown). These data suggest that the endocytosis of galectin-3 wild type and R186S mutant in M1 cells is receptor-mediated, and follows a clathrin dependent pathway.

3.5. Carbohydrate dependent and independent mechanisms for galectin-3 uptake in M2 cells

In M2 cells, the uptake of galectin-3 was completely prevented by both lactose and TD139, same as found for non-macrophage cell types (Table 1 and Fig. 2A, bottom row). However, the R186S mutant was endocytosed in M2 cells (Fig. 5, bottom row of panels), and this uptake could not be prevented by galectin-3 inhibitors, even at high concentration, perhaps because both lactose and TD139 bind the mutant with lower affinity as explained above. Thus the M2 cells are similar to non-macrophage cells since galectin-3 uptake could be inhibited by carbohydrates, but similar to macrophage-like cells since the R186S mutant was also taken up.

The galectin-3 CRD, lacking the N-terminal domain, was also readily endocytosed in M2 cells (Fig. 4, right panels), but uptake of the R186S CRD was hardly noticeable compared to wild type CRD. These data indicate a role for both the carbohydrate binding activity and the N-terminal domain of galectin-3 for uptake in M2 macrophages.

Further evidence was the fact that interference with the N-terminal domain, by using the monoclonal anti-Mac-2 antibody, as described in Section 2.12 and in supplementary material, almost completely inhibited endocytosis of both galectin-3 wt and R186S in M2 cells (Fig. 3). This was seen also with the intact antibody, perhaps because IL-4 activated macrophages preferentially express the uptake inhibitory Fc γ -IIb receptor [55].

The uptake of labelled galectin-3 wt and R186S mutant in M2 cells was completely inhibited by the presence of 10-fold excess of unlabelled protein, providing evidence for a specific uptake mechanism, and of lower capacity compared to the case with M1 macrophages (Fig. 3).

The endocytosis of galectin-3 wt and R186S in M2 was inhibited by chlorpromazine, like in M1 cells, but unlike in M1 cells, it was also inhibited by amilorid and partially by methyl- β -cyclodextrin (Fig. 6). In M2 cells all the controls used (including dextran) were inhibited by their respective inhibitors (not shown).

3.6. Galectin-3 uptake in M0 cells

In M0 cells the uptake of galectin-3 was partially, but not fully, prevented by both lactose and TD139 (Fig. 2A). The galectin-3 CRD was also endocytosed in M0 cells with equal inhibiting effects of lactose and TD139 as observed for M1 cells (Fig. 3). The R186S mutant and its CRD were also taken up by M0 cells and this uptake was not prevented by the inhibitors, as described for M1 cells. Thus, the M0 cells interact with galectin-3 in a way intermediate between inflammatory, classically activated (M1) and alternatively activated (M2) macrophage-like cells, but with more similarity to the M1 type.

3.7. The role of endogenous galectin-3 for uptake of exogenous galectin-3

Next we examined the possible role of endogenous galectin-3 in the initial uptake of externally added galectin-3, since endogenous galectin-3 may regulate surface expression of glycoprotein receptors, and might also self-associate with externally added galectin-3. Moreover, all the different macrophage subtypes express (Fig. 1C) and secrete (Figure S2) galectin-3. Macrophages were treated (30 min on ice) either with 10 mM lactose to possibly wash away surface bound endogenous galectin-3, or 1 μ M recombinant galectin-3 (r-galectin-3) to increase the level of surface bound galectin-3. Then the cells were thoroughly washed and warmed to 37 $^{\circ}$ C, before the endocytosis of fluorescein-labelled galectin-3 was examined as described above, but now for 5 and 10 min to capture the initial uptake. In the lactose treated cells, galectin-3 uptake was similar or possibly slightly lower at both time points, whereas in galectin-3 treated cells, the uptake was clearly more prominent especially after 10 min (Fig. 7). Thus, in all three macrophage-like cell types, the presence of galectin-3 at the cell surface enhances uptake of additional galectin-3. This effect appears to require the N-terminal domain as it was not seen at all if labelled galectin-3 CRD was added instead to the cells (not shown).

3.8. Intracellular route of endocytosed galectin-3

A detailed analysis of intracellular trafficking of endocytosed galectin-3 in macrophages is beyond the scope of this paper. Here we analysed the relationship of the endocytosed galectin-3 to early endosomes, as visualized by staining with anti-EEA1, and late endosomes/lysosomes as one potential late target, as visualized by staining with anti-LAMP-1. In M1 cells (Fig. 8), galectin-3 wt appeared as small dots (green) within 5 min accumulated near the larger early endosomes (red) after ~10 min, and within ~15 min it entered the endosomes. After 20 min, galectin-3 appeared again outside the early endosomes, and after 60 min it was completely separated

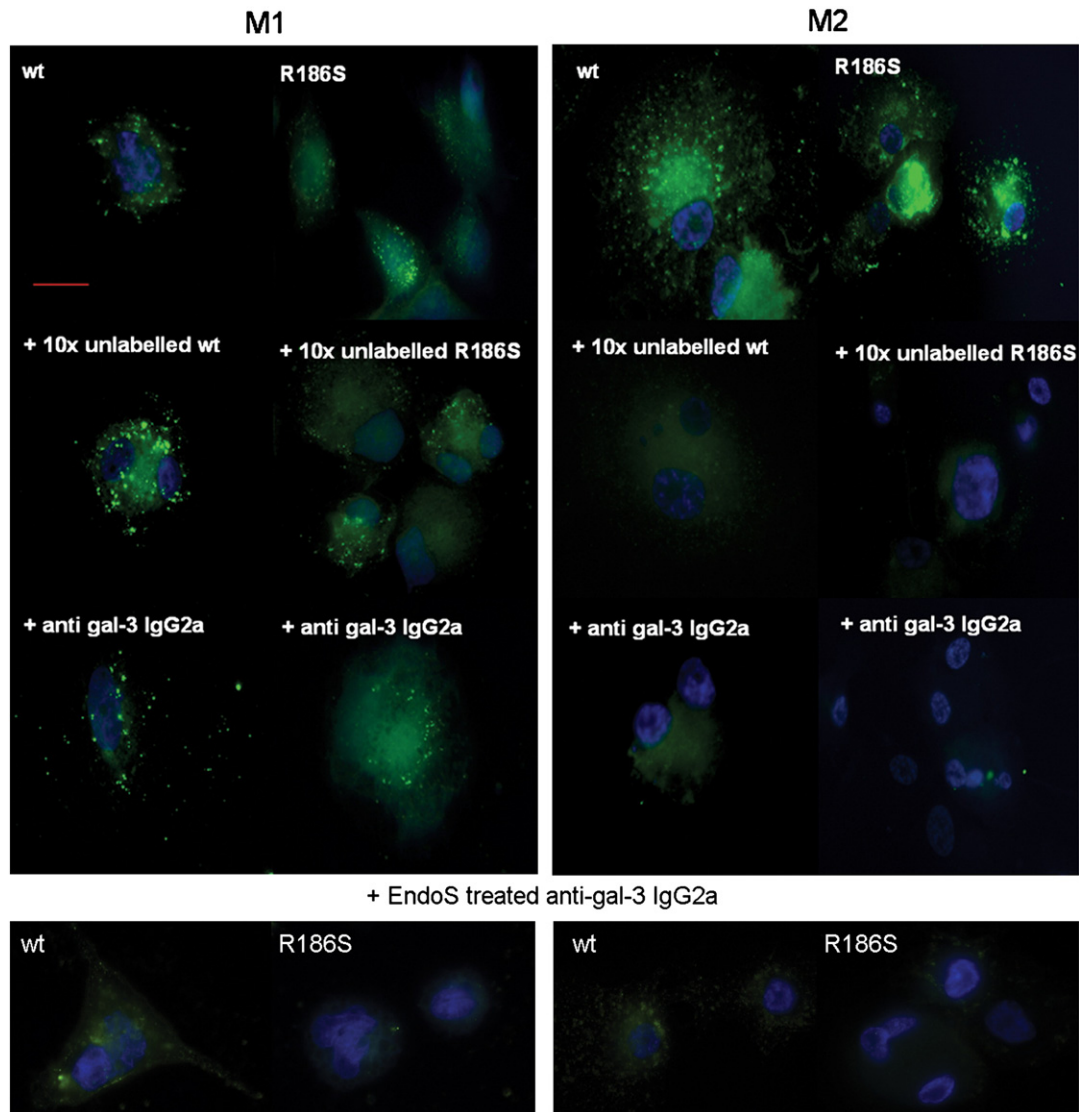


Fig. 3. Inhibition of galectin-3 wt and R186S endocytosis by competition with the unlabelled protein or by anti-galectin-3 IgG [9] (EndoS treated or not) in M1 and M2 cells. 0.2 μ M of fluorescein-labelled galectin-3 wt or R186S were incubated for 30 min in M1 and M2 macrophages and endocytosis was inspected by fluorescence microscopy. To saturate the supposed surface endocytosis-mediating receptor, labelled wt or R186S were incubated with 10 times more unlabelled wt or R186S, respectively and endocytosis inspected after 30 min. Fluorescent galectin-3 wt or R186S mutant were also incubated with 0.6 μ M IgG2a antibody, native or deglycosylated (does not bind Fc γ receptors), directed against galectin-3N-terminus for 30 min. Bar represents 20 μ m.

from early endosomes. R186S mutant followed a similar route, but appeared slightly delayed. More galectin-3 containing vesicles accumulated near the endosomes after 10 min, and there was still clear co-staining after 20 min. Galectin-3 uptake in relation to early endosomes was similar in M2 cells (not shown).

To analyse the intracellular route in more detail, a pulse of 0.2 μ M fluorescein-labelled galectin-3 was added to M0, M1 and M2 cells held on ice. Cells were incubated for 5 min, washed and chased at 37°C for 1 to 60 min (Fig. 9). To quantify co-localization of galectin-3 with the early endosome marker (EEA1) or lysosome marker (LAMP-1), microscope pictures were deconvolved, corrected for background staining and then compared according to the software derived Manders colocalization coefficient, which ranges from 0 to 1.0 and represents the true degree of colocalization [56]. The same was done for galectin-3 wt, R186S mutant deficient in LacNAc binding and K176LN180T mutant with enhanced affinity for repeating LacNAc-residues and decreased affinity for 2–3 sialylated galactosides [36]. In M1 cells, a fraction of wild type galectin-3 colocalized with early endosomes after 10 min and lysosomes after 50–60 min. In M2 macrophages however, a larger fraction remained near early

endosomes after 10 and also 50 min, whereas much less was found near lysosomes after 50 min. The K176LN180T mutant shared a similar fate as galectin-3 wt but showed increased targeting to lysosomes (Fig. 9C). The Manders colocalization-coefficient, varied between galectin-3, mutants and macrophage-like cell type, but was in most cases lower than 0.4 at all measured time points, both with EEA1 or LAMP-1. Taken together, these results suggest that galectin-3 passes early endosomes fairly fast and that most of it are not targeted for degradation in lysosomes. In fact, endocytosed galectin-3 was visualised inside the cell even after 24 h (Figure S6).

3.9. Dynamics of galectin-3 uptake in different macrophages

To quantify the amount of endocytosed galectin-3 in the different macrophages, we used a flow cytometry assay. Fluorescein-labelled galectin was added to differentiated surface grown cells, as described in Section 3.3. At different time points, cells were harvested using trypsinisation and analysed by flow cytometry. The detached cells became round and M2 cells remained considerably larger than M1 cells as measured by forward scatter (Figure S4). Trypsination removes cell

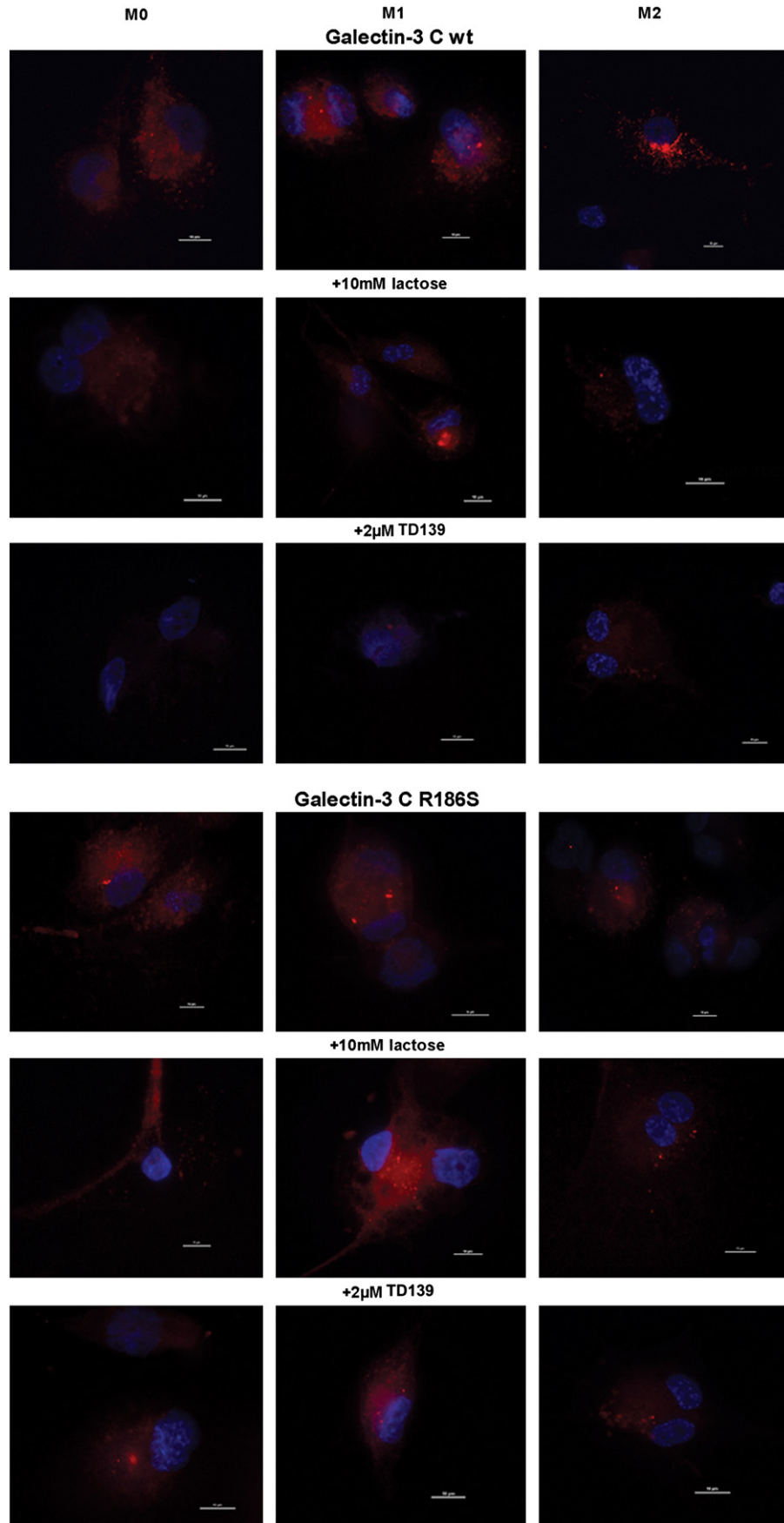


Fig. 4. Inhibition of truncated galectin-3 wt and R186S endocytosis by lactose or TD139 in M0, M1 and M2 cells. Uptake of 0.2 μM of truncated, only CRD containing fluorescein-labelled galectin-3 wt or R186S in macrophages for 30 min after the proteins were pre incubated with 10 mM lactose or 2 μM TD139 galectin-3 inhibitor for 30 min. Bar represents 10 μm.

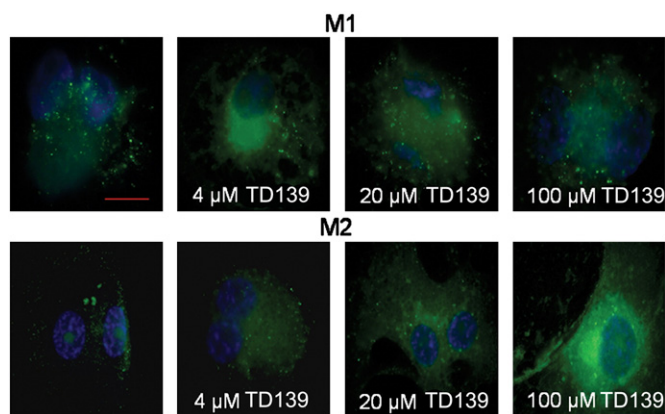


Fig. 5. Inhibition of R186S galectin-3 mutant endocytosis by TD139 in M1 and M2 cells. 0.2 μ M fluorescein-labelled R186S was pre incubated with 4, 20 or 100 μ M TD139 galectin-3 inhibitor for 30 min and added for 30 min to M1 and M2 cells. Bar represents 20 μ m.

surface bound galectin-3 (Figure S5), and, hence, the measured mean fluorescence intensity (MFI) represents endocytosed fraction of added galectin-3. Different galectin-3 concentrations were analysed to generate a dose response curve. After 30 min of incubation with galectin-3 wt, the curve reached a plateau at \sim 2 μ M added galectin in both M1 and M2 cells (Fig. 10). In contrast, the dose response for

the R186S mutant did not reach a plateau. This difference may be related to their difference in route of endocytosis described above and/or a different rate of intracellular degradation (see Section 4 Discussion).

To quantify the rate of galectin-3 uptake, 2 μ M fluorescein-labelled galectin was added to macrophage cell types and incubated for 5, 15 and 30 min, followed by trypsinization and flow cytometry analysis. The uptake continued to rise with time for both macrophage cell types and galectin-3 mutants (Fig. 11). There was an indication that the uptake of R186S mutant was slower initially, which agrees with the delayed uptake described above, but at later time points the rate of uptake was equally high or even higher than of the wild type.

4. Discussion

Here we show that galectin-3 (Mac-2) is not only abundantly expressed and secreted by different THP-1 derived macrophage like cells, but also binds and enters these cells in specialized ways not shared by other cell types. Moreover we show that these mechanisms differ between classically (M1) and alternatively (M2) activated macrophage like cells. This invites the possibility that these mechanisms are related to macrophage specific functions of galectin-3. The results cannot be generalized, however, to all macrophages. Emerging evidence indicate that macrophages *in vivo* are much more variable than previously thought, depending on their different environments [5]. Moreover, they may vary between individuals and human

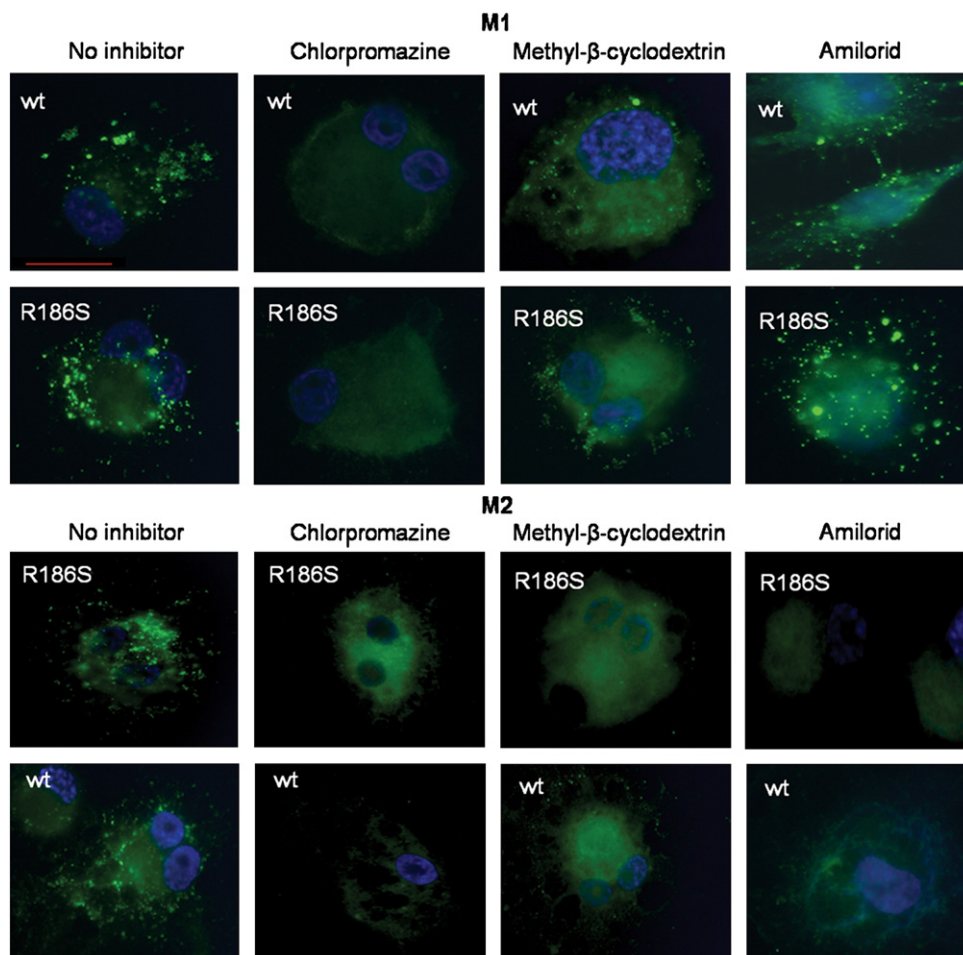


Fig. 6. Inhibition of galectin-3 wt and R186S mutant endocytosis in M1 and M2 cells using different endocytosis pathways inhibitors. M1 and M2 macrophages were pretreated for 2 h with known endocytic pathways inhibitors: 50 μ M chlorpromazine (inhibits clathrin associated, receptor mediated endocytosis), 30 mM methyl- β -cyclodextrin (inhibits lipid raft mediated endocytosis) or 1.5 mM amilorid (inhibits micropinocytosis). Subsequently 0.2 μ M galectin-3 wt or R186S were added to the cells and uptake examined after 30 min by fluorescence microscopy. Bar represents 20 μ m.

Table 1
Summary of galectin-3 and mutant uptake into macrophage like THP-1 cells.

Galectin	Inhibitor	THP-1 cells				Other cells		
		M1	M2	M0	Fig.	SKBR3	HFL1	Fig.
Gal-3	—	+	+	+	2, 3, 6	+	+	S1
Gal-3	Lac	+	—	+/-	2	—	nt	
Gal-3	TD139	+	—	+/-	2	—	—	S1
Gal-3	10× unlabelled R186S	+	—	nt	3	—	—	S1
Gal-3	Anti-Mac-2	+	—	nt	3	nt	nt	
Gal-3	Deglycosylated anti-Mac-2	+/-	—	nt	3	nt	nt	
Gal-3	Chlorpromazin	—	—	nt	6	nt	nt	
Gal-3	Amiloride	+	—	nt	6	nt	nt	
Gal-3	Cyclodextran	+	—	nt	6	nt	nt	
Gal-3C	—	+	+	+	4	+	+	S1
Gal-3C	Lac	+/-	—	+/-	4	nt	nt	
Gal-3C	TD139	—	—	+/-	4	nt	nt	
R186S	—	+	+	+	3, 5, 6	—	—	S1
R186S	TD139	+	—	—	5	nt	nt	
R186S	10× unlabelled R186S	+	—	nt	3	nt	nt	
R186S	Anti-Mac-2	+	—	nt	3	nt	nt	
R186S	Deglycosylated anti-Mac-2	—	—	nt	3	nt	nt	
R186S	Chlorpromazin	—	—	nt	6	nt	nt	
R186S	Amiloride	+	—	nt	6	nt	nt	
R186S	Cyclodextran	+	—	nt	6	nt	nt	
R186S-C	—	+	+/-	+	4	nt	nt	
R186S-C	Lac	+	+	+	4	nt	nt	
R186S-C	TD139	+	+	+	4	nt	nt	
K176LN180T	—	+	+	+	9	+	+	S1
K176LN180T	Lac	nt	nt	nt	—	—	—	S1
K176LN180T	TD139	nt	nt	nt	—	—	—	S1

+ = clearly detected uptake into vesicles, — = no uptake, +/- = low uptake, nt = not tested. Data are shown for the different cell types and from the different figures as indicated.

macrophages are different from those of mice. Nevertheless, the studies here establish in one extensively used macrophage model system [26–31], the THP-1 cell line, that galectin-3 uptake can be cytokine regulated and occur with different mechanisms, carbohydrate independent or dependent, and unlike those in non-macrophage like cells. Further studies will have to establish in which of all different

natural macrophages and other macrophage like cell lines, these mechanism also occur.

Fig. 12 shows some simplified possible mechanisms of galectin-3 interaction with the cell surface or interior membrane of vesicles. In non-macrophage cells, galectin-3 uptake has characteristics consistent with the binding of the galectin-3 CRD to the membrane surface carbohydrates (Fig. 12A), based on inhibition by competing small ligands, lack of binding of the LacNAc deficient binding galectin-3 mutant, R186S, and independence of the N-terminal domain (Table 1, Figure S1). In contrast, in M1 cells the uptake of galectin-3 was carbohydrate independent but could be prevented by interference with the N-terminal part of the protein, and had the characteristics of being mediated by a clathrin associated receptor (Table 1). This suggests the model of Fig. 12C as the major mechanism, where a membrane receptor binds the N-terminal part of galectin-3 (blue, unfilled) leaving the CRD (blue filled) free to interact with carbohydrates in other glycoconjugates. The CRD of galectin-3 R186S was also endocytosed in M1 cells suggesting the presence of some additional receptor, which is not found in M2 or non-macrophage cells.

In M2 cells, galectin-3 endocytosis was mainly carbohydrate dependent since it could be completely inhibited by lactose and TD139, a small synthetic inhibitor of carbohydrate binding, but the non-CRD N-terminal part also contributes as uptake was inhibited by the antibody directed to this domain, and also the intact R186S mutant was endocytosed while its CRD was not (Table 1). One explanation could be a simultaneous combined binding of the two galectin-3 domains to the cell surface, where the affinity of only one is too low to mediate the uptake. An alternative explanation is that one of the domains binds first, and induces cell surface exposure of receptors binding the other. The inhibition of galectin-3 uptake in M2 cells by several pharma also suggests involvement of more than one pathway, perhaps in sequence.

We also demonstrate that galectin-3 enhances its own uptake and that this effect depends on the non-CRD part in all the macrophage like cell types (Fig. 7). The apparent cross linking of galectin-3 by the non-CRD domain, and the resulting cooperative binding to glycoproteins, agglutination of cells and induction of signalling is well known since long [57,58,21,10], but the effect of this in uptake into cells has not been shown before. The proposed mechanism where the non-CRD parts [59] interact with each other is easy to reconcile with the

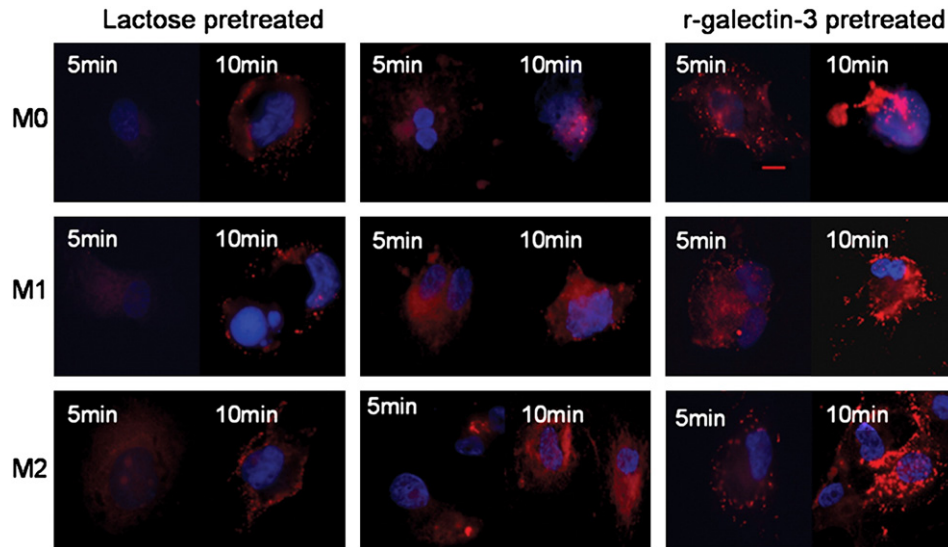


Fig. 7. Pretreatment with lactose or recombinant, unlabelled galectin-3 modulates galectin-3 endocytosis in M1 and M2 cells. M0, M1 and M2 cells were pretreated for 30 min on ice with 10 mM lactose to remove surface bound endogenous galectin-3 or with 1 μ M recombinant galectin-3 for cell surface galectin-3 receptor saturation. M0, M1 and M2 cells without pretreatment served as a comparison (middle column). After thorough wash, 0.2 μ M fluorescein-labelled galectin-3 was added to the cells and endocytosis inspected by fluorescence microscopy after 5 and 10 min. Bar represents 10 μ m.

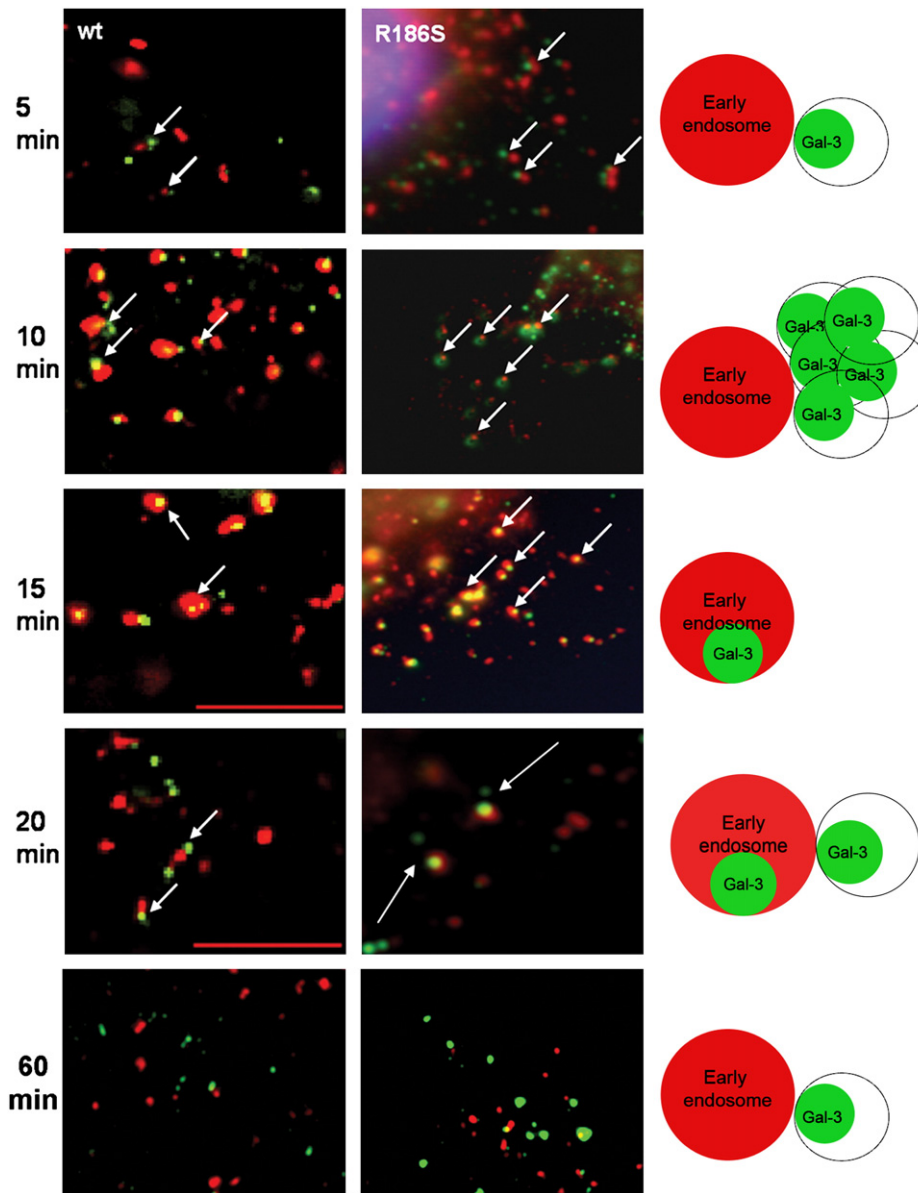


Fig. 8. Galectin-3 wt and R186S endocytosis in relation to early endosomes. The uptake of 0.2 μ M, fluorescein-labelled galectin-3 wt and R186S was examined in M1 cells after 5, 10, 15, 20 and 60 min, followed by immunostaining with Alexa594 labelled anti EEA1 antibody. Third column is a schematic representation proposing the endocytic events taking place. Bar represents 5 μ m.

carbohydrate dependent cell surface binding (Fig. 12A) resulting in model of Fig. 12B, but it is not with the carbohydrate independent model of Fig. 12C occurring in M1 cells. Thus, in these cells the models of Fig. 12B and C occur simultaneously or there is some alternative/additional mechanism for galectin-3 self-enhancement as has been proposed [60,61].

Finally we demonstrate that galectin-3 uptake in macrophage like cells is rapid (occurring within minutes), and that the endocytosed galectin-3 is mainly not targeted to degradative compartments, but to other types of vesicles where it remains even after 24 hours. This suggests that it may enter a pool of recycling galectin-3 as shown to exist in MDCK cells [17]. Other galectins may also be endocytosed [62], but the mechanism and rate have not been studied much. If, as found here, most of the cell bound galectin is found intracellularly within minutes, this has to be taken into account when analysing mechanism for galectin induced effects.

It is not possible to connect the present results directly to previous studies on galectin-3 in most cases, because these have often ascribed galectin-3 to processes occurring over much longer times than

studied here [21,63,64,65]. Moreover, previous studies have used a wide variety of macrophage sources differentiated, with or without further activation, in different ways. The results here, from one system of well-defined model macrophages, highlight the fact that galectin-3 at the macrophage surface may act in multiple ways, carbohydrate dependent and independent, which must be defined and considered for each system and effect.

One is in the uptake of external molecules or particles (phagocytosis) that is a major function of M1 macrophages. The effect of externally added galectin-3 to enhance phagocytosis of e.g. apoptotic neutrophils [66] may occur by the model proposed in Fig. 12B, since the galectin-3 effect and binding to either neutrophil or macrophage were completely carbohydrate dependent; moreover, the self-association of galectin-3 that is part of this model, is readily inhibited by competing saccharides in the same concentration range that inhibits galectin-3 binding to the simple ligands [58,61]. The effect of endogenous galectin-3 on phagocytosis, as detected using macrophages from galectin-3 $-/-$ mice or siRNA, was assumed to be intracellular as the effect could not be inhibited by lactose [23]. However, an alternative explanation shown in

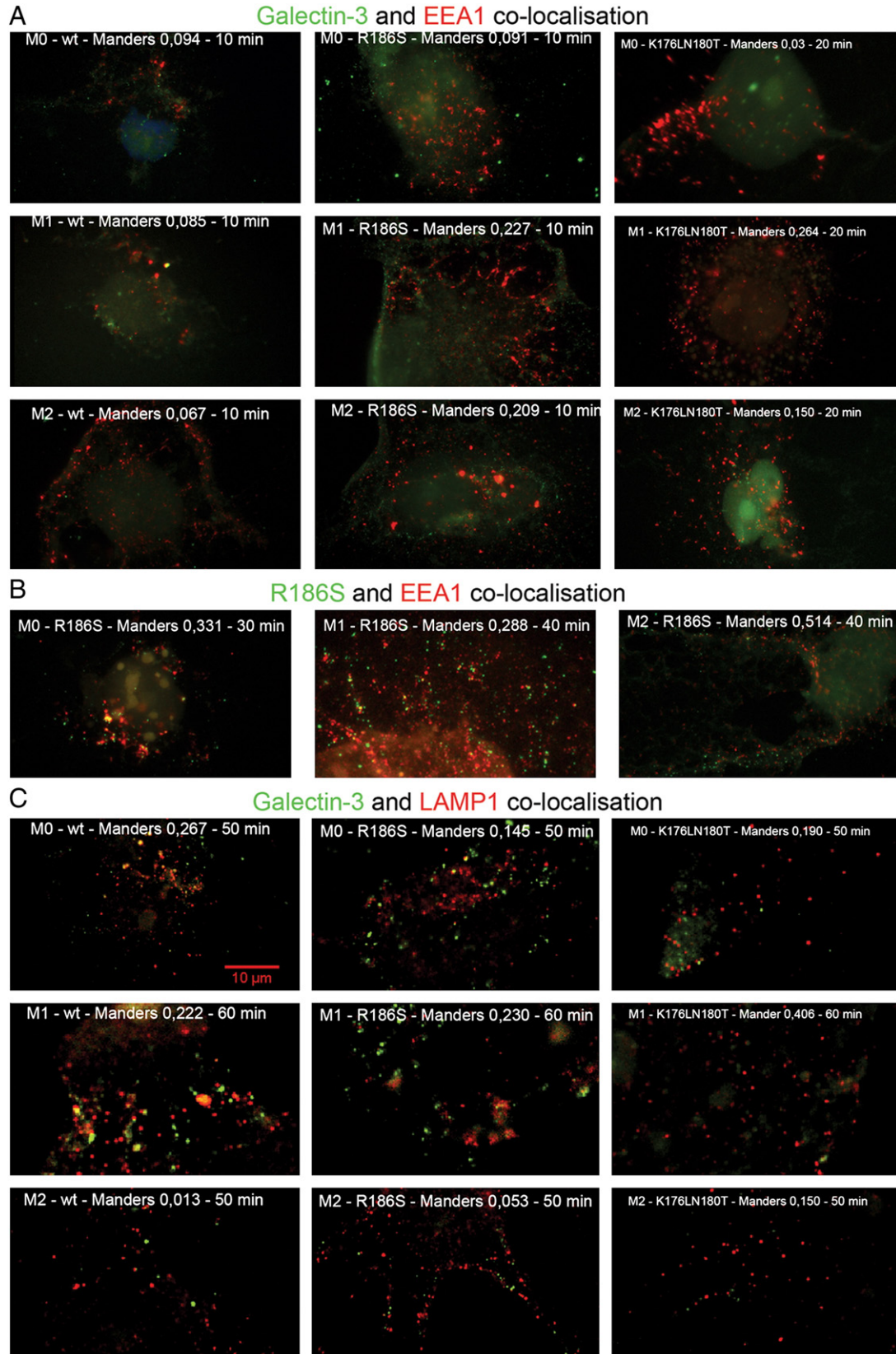


Fig. 9. Galectin-3 wt, R186S and K176LN180T endocytic pathway in relation to early endosomes and lysosomes. The pulse of 0.2 μ M, fluorescein-labelled galectin-3 was chased for 1 to 60 min and examined in M0, M1 and M2 cells, followed by immunostaining with Alexa594 labelled anti EEA1 or anti LAMP1 antibody and co-localisation was quantified by Manders coefficient. A. Co-localisation of galectin-3 (wt, R186S, K176LN180T) with EEA1 after 10 min (for wt and R186S) or 20 min (for K176LN180T). B. Co-localisation of R186S with EEA1 after 30 min (for M0) or 40 min (for M1 and M2). C. Co-localisation of galectin-3 (wt, R186S, K176LN180T) with LAMP1 after 50 min (for M0 and M2) or 60 min (for M1). Bar represents 10 μ m.

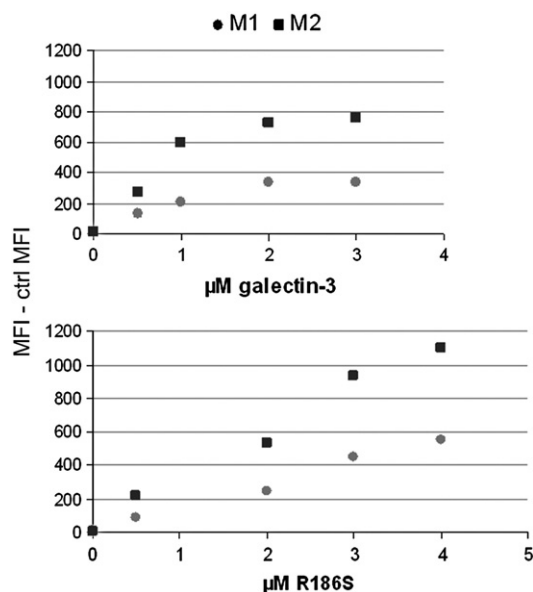


Fig. 10. Mean fluorescence intensities (MFI) after 30 min of galectin-3 wt or R186S endocytosis in M1 and M2 cells measured by flow cytometry. Fluorescent gal-3 wt (0.5, 1, 2, 3 μM) or R186S (0.5, 2, 3, 4 μM) was added to M1 and M2 cells and incubated at 37 °C for 30 min. The cells were then washed, trypsinated to remove surface bound galectin-3 (not endocytosed) and to detach the cells. Samples were then diluted in PBS and analysed by flow cytometry. Approximately 5000 cells were analysed per duplicate samples. The curve shows average MFI with increasing galectin-3 concentrations corrected for macrophage auto fluorescence MFI (ctrlMFI).

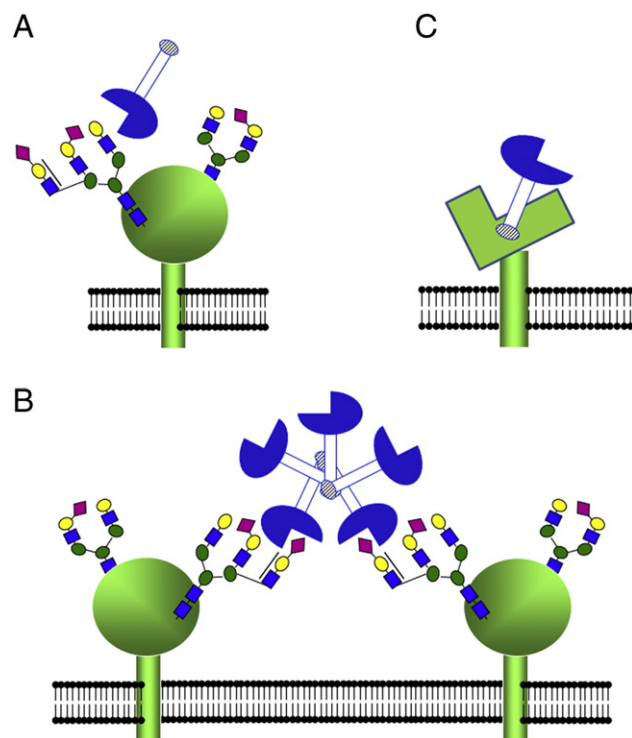


Fig. 12. Schematics of galectin-3 interaction with glycoproteins at the cell surface or luminal side of vesicle membranes. A. Simple carbohydrate mediated interaction as found in non-macrophage cell types. B. Carbohydrate mediated interaction combined with galectin-3 cross linking. C. Carbohydrate independent interaction, mediated by interaction between galectin-3 N-domain and receptor. Galectin-3 is shown blue with CRD filled and N-domain unfilled. Membranes are black and glycoproteins green with attached glycans shown according to color code recommended by Consortium of Functional Glycomics. In panel B, galectin-3 is shown as a pentamer as suggested by Ahmad et al., [59], but other oligomeric forms are also possible.

Fig. 12C, where galectin-3 interaction with the macrophage is carbohydrate-independent and interaction with the phagocytosed particle, even if carbohydrate-dependent may be so tight that it could not be inhibited by the lactose concentration used. The amount of lactose required to inhibit static galectin-3 cell surface binding by 50% is often in the 1–10 mM range and depends on the affinity and density of the cell surface receptors, as studied in detail in Carlsson et al. [41]. Therefore, inhibition by 10 mM lactose, as is often used to prove carbohydrate dependence of a galectin-3 effect does not represent a large excess and may be insufficient to inhibit e.g. phagocytosis. A more potent inhibitor, as the TD139 used here, will provide a more reliable tool.

Another possible role of galectin-3 endocytosis in macrophages is taking part of a recycling loop between intracellular vesicles and the cell surface, and thereby direct trafficking of glycoproteins and endocytosed material, membrane organization, and/or the induction of regulatory signals as suggested for other cell types [17] including M2 macrophages [24]. The present results are consistent with this in that galectin-3 is rapidly endocytosed (in minutes) and is not degraded intracellularly. The effect of galectin-3 on intracellular

trafficking has been assumed to be carbohydrate-dependent, as shown in some cases, but the present results show that also carbohydrate-independent mechanisms have to be considered. The endocytosis of galectin-3 in M2 cells could be inhibited by lactose and the TD139, which agrees with previous results where effects of galectin-3 in differentiation and signalling in M2 macrophages could be prevented by another potent galectin-3 inhibitor [24]. A functionally important targeting effect on TLR2 of galectin-3 in conjunction with phagocytosis has also been proposed, but the role of carbohydrate binding was not studied [64]. Galectin-3 takes part in specific functions of different cells, e.g. endocytosis, trafficking, signalling. Providing evidence for carbohydrate independent involvement in

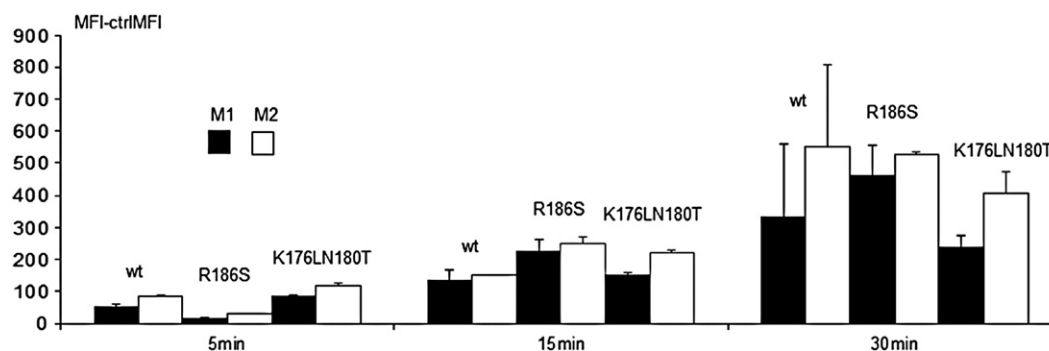


Fig. 11. Galectin-3 wt, R186S or K176LN180T endocytosis quantification in M1 and M2 cells by flow cytometry. The uptake of 2 μM, fluorescently labelled galectin-3 wt, R186S and K176LN180T was examined after 5, 15 and 30 min. After incubation, the cells were trypsinated to remove residual surface bound protein (Figure S5), diluted in PBS and analysed by flow cytometry. Shown are MFI values after correction for macrophage auto fluorescence MFI (ctrlMFI).

some of these functions helps analysing galectin interactions in general.

Supplementary materials related to this article can be found online at [doi:10.1016/j.bbagen.2012.02.018](https://doi.org/10.1016/j.bbagen.2012.02.018).

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