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Comparative analysis of the internalization of the macrophage receptor sialoadhesin in human and mouse primary macrophages and cell lines

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Abstract

Sialoadhesin (Sn) is a surface receptor expressed on resident macrophages with the ability to bind with sialic acids. During inflammation, an upregulation of Sn is observed. Upon binding of monoclonal antibodies to Sn, the receptor becomes internalized and this has been observed in multiple species. The latter characteristic, combined with the strong upregulation of Sn on inflammatory macrophages and the fact that Sn-positive macrophages contribute to certain inflammatory diseases, makes Sn an interesting entry portal for phenotype-modulating or cytotoxic drugs. Such drugs or toxins can be linked to Sn-specific antibodies which should enable their targeted uptake by macrophages. However, the activity of such drugs depends not only on their internalization but also on the intracellular trafficking and final fate in the endolysosomal system. Although information is available for porcine Sn, the detailed mechanisms of human and mouse Sn internalization and subsequent intracellular trafficking are currently unknown. To allow development of Sn-targeted therapies, differences across species and cellular background need to be characterized in more detail. In the current report, we show that internalization of human and mouse Sn is dynamin-dependent and clathrin-mediated, both in primary macrophages and CHO cell lines expressing a recombinant Sn. In primary macrophages, internalized Sn-specific F(ab')₂ fragments are located mostly in the early endosomes. With Fc containing Sn-specific antibodies, there is a slight shift towards lysosomal localization in mouse macrophages, possibly because of an interaction with Fc receptors. Surprisingly, in CHO cell lines expressing Sn, there is a predominant lysosomal localization. Our results show that the mechanism of Sn internalization and intracellular trafficking is concurrent in the tested species. The cellular background in which Sn is expressed and the type of antibody used can affect the intracellular fate, which in turn can impact the activity of antibody-based therapeutic interventions via Sn.

Keywords

Sialoadhesin / Siglec-1 / CD169, internalization, antibodies

Abbreviations

Sialoadhesin (Sn)

Introduction

Macrophages are important cell types within the immune system, in steady state conditions and during inflammation [1, 2]. They have different roles in the innate and adaptive immunity such as phagocytosis of pathogens, antigen presentation as well as tissue remodeling and repair [3-5]. These immune cells also play an important role in some inflammatory diseases and infections [6-10]. During rheumatoid arthritis, macrophages are activated, releasing proinflammatory or regulatory cytokines and chemokines [11]. In cancer, macrophages are present in all stages, for example in primary tumors, macrophages are described to enhance tumor cell invasion [10]. Macrophages are also one of the early targets of HIV-1, which uses macrophages for replication [9]. Because of the role of macrophages in different diseases and infections, targeting macrophages with phenotype-modulating or cytotoxic drugs is an interesting therapeutic strategy. Several macrophage surface receptors, such as CD64 and CD163 are currently being investigated for treatment of inflammatory diseases [12-14].

Another macrophage-specific surface receptor with interesting characteristics for antibody-based targeting is sialoadhesin (CD169 or Siglec-1; Sn). Sn is a member of the Ig superfamily of Siglecs (sialic acid-binding immunoglobulin-like lectins) which are expressed on different cells of the immune system [15]. Like other Siglecs, Sn is able to recognize and bind with sialic acids, a nine carbon sugar found on vertebrates and pathogens [16]. Chronic inflammation, such as rheumatoid arthritis and HIV-1, are associated with an upregulation of Sn, suggesting that Sn plays a role in these diseases [17, 18]. Sn was first thought to be an adhesion molecule since it has no known signalization motif in the cytoplasmic tail [16, 19]. However, further research showed that Sn is involved in cell-pathogen interactions and cell signaling [19, 20]. Moreover, Sn has the ability to internalize after binding with ligands such as monoclonal antibodies (mAbs), suggesting that Sn indeed has signaling functions [21-24]. For HIV-1, it was shown that the interaction with human Sn (hSn) also results in uptake of the virus via Sn. However, detailed analysis showed that this uptake was an invagination of the plasma membrane and not a complete internalization [21]. Whether this peculiar form of uptake is specific for the cell type and hSn is not known and it is not fully clear if mAb binding to hSn also results in plasma membrane invaginations. Even though no known signalization motif has yet been detected in Sn, recent evidence shows that Sn can induce signaling via interaction with DNAX-activating protein of 12 kDa (DAP12). This interaction results in the recruitment of TRIM27 and suppression of antiviral innate immune responses [25]. More specifically, this also results in the inhibition of type I IFN production, which seems to be a negative feedback loop since IFN also upregulates Sn expression [26-28].

Receptor-specific antibodies can be used to target cells during diseases, either resulting in induction of a signalization cascade or by allowing intracellular delivery of drugs or toxins [29]. For instance in cancer, antibodies can activate or antagonize immunological pathways after antibody targeting [29, 30]. Immunotoxins can also be used as an effective treatment for cancer [29]. Different toxins or toxin fragments derived from bacteria, plants- and human enzymes can be fused with antibodies [13]. For these toxins to be active, internalization alone is not sufficient and efficient translocation from endocytic vesicles to the cytoplasm of the cell is usually critical. The efficiency of this translocation depends on the characteristics

of the toxin but can also vary depending on the intracellular route of the internalized immunotoxin [31]. For instance, antibodies binding transferrin are only internalized into endosomes, while CD163-specific antibodies are delivered to the lysosomes [12, 32]. Therefore, an important aspect in the development of immunotoxins is the knowledge on the mechanism of internalization of the receptor, followed by the intracellular route and the final fate.

Some researchers have already evaluated Sn as possible therapeutic target or entry point using liposomes or immunotoxins. Liposomes containing sialic acids on their surface that can bind to mouse Sn (mSn), are selectively internalized after binding and the cargo incorporated in these liposomes was shown to be released for processing [20, 33]. Porcine Sn (pSn) has been used as a model to target vaccines to Sn expressing cells. Fusing peptides to a pSn-specific mAb lead to targeted delivery of the peptide and this resulted in an increased immune response in contrast to non-targeted peptides [34]. Furthermore, targeting of immunotoxins to pSn leads to efficient killing of Sn-expressing macrophages [35]. These interesting observations demonstrate the potential of Sn as a therapeutic target. However, extrapolation of the findings for pSn to hSn is not straightforward. First, no specific sequences involved in internalization, intracellular trafficking or signalization have been identified in the cytoplasmic tail of Sn in any species, so it is not possible to check for conservation of such motifs in hSn. In addition, although the extracellular part of Sn is well conserved in between species, the cytoplasmic tail is very divergent both in length and in amino acid sequence [36]. Since specific amino acid sequences in the cytoplasmic tail of receptors can influence the route of internalization and subsequent intracellular trafficking, the clathrin-dependent endocytosis of pSn and the localization of intracellular pSn-monoclonal antibody (pSn-mAb) complexes in early endosomes, might be different for hSn and mSn [16, 35].

In this study, the internalization mechanism of hSn and mSn and the intracellular fate was analyzed in primary macrophages. Since the background of the Sn-expressing cells may also influence internalization characteristics, the internalization of Sn was also studied in CHO cell lines expressing recombinant Sn (Sn⁺-CHO). This allows a direct comparison of Sn of different species independent of the cellular background. Our results show that antibody-induced Sn internalization is dynamin- and clathrin-mediated for both hSn and mSn in all cell types evaluated. Surprisingly, different cellular localizations of the endocytosed Sn-mAb complexes are observed in primary macrophages and Sn⁺-CHO cell lines. In primary macrophages, the Sn-mAb complexes are observed predominantly in early endosomes. However, Sn⁺-CHO cell lines transport the Sn-mAb complexes more to lysosomes.

Material and methods

All products were purchased from Thermo Scientific, unless stated otherwise.

Ethical Statement

The experiments with BALB/c mice were authorized by the Ethical Committee for Animals of the University of Antwerp; permit number 2014-17. The use of human monocytes was authorized by the Ethical Committee of the Antwerp University Hospital and the University of Antwerp; permit number 16108.

Cells

CHO-K1 cells were kindly provided by Dr. J. D. Esko (University of California, San Diego, CA, USA) and were transfected or transduced to obtain stable cell lines expressing hSn

(hSn⁺-CHO) and mSn (mSn⁺-CHO) [37]. These cells were subcultured in RPMI with 10% heat inactivated fetal bovine serum (iFBS). Primary mouse bone marrow macrophages were collected in RPMI medium. After removing red blood cells with an ammonium-chloride-potassium lysis buffer (VWR and Janssen Chimica), macrophages were seeded in RPMI with 10% iFBS, 1% non-essential amino acids, 1% sodium pyruvate, 1% glutamine and with addition of L929 supernatant and 500 U/ml mouse IFN- α (R&D) in the medium. L929 supernatant was collected from cells kindly provided by Dr. C. Uyttenhove (Ludwig Institute for Cancer Research, Brussels, Belgium). Human monocytes were obtained from blood of healthy volunteers from the Belgian Red Cross-Flanders. The monocellular cells were isolated from the blood using Ficoll (Sigma-Aldrich). CD14⁺ monocytes were isolated using MACS separation following manufacturer's instructions (Miltenyi Biotec). The cells were seeded in RPMI with 10% heat inactivated human serum, 1% non-essential amino acids, 1% sodium pyruvate and 1% glutamine. After two days in culture, 50 ng/ml human IFN- α was added for three days to obtain hSn-positive macrophages.

Production of antibodies and F(ab')₂ fragments

Monoclonal antibodies (mAbs) 12A4 and SySy94, recognizing respectively hSn and mSn, kindly provided by Prof H. Nauwynck (Laboratory of Virology, UGent, Belgium), were produced and purified as previously published [37]. F(ab')₂ fragments lacking the Fc part of the antibody were produced as previously described [38]. Briefly, PNGase F (New England Biolabs) was added to purified mAbs following the manufacturer's instructions. After the deglycosylation step, pepsin agarose beads were added to cleave the mAbs. The F(ab')₂ fragments were purified using a Zeba spin column with a 40 kDa cut off.

Internalization assay

MAB-induced internalization of Sn was performed as previously reported [37]. Briefly, anti-hSn mAb 12A4 and anti-mSn mAb SySy94 or F(ab')₂ fragments, were added to the cells at 4°C during 60 min to allow surface binding. The cells were directly (0 min) fixed with 4% paraformaldehyde (Merck) or were shifted to 37°C and fixed at various time points (15 to 120 min), followed by permeabilization with 0.5% Triton X-100 (Sigma-Aldrich). Alexa Fluor 488-labeled chicken anti-mouse and chicken anti-rat antibodies were added to the cells for the detection of hSn and mSn bound antibodies, together with Texas Red®-X phalloidin to visualize F-actin which is located just beneath the plasma membrane. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). As control antibodies, mAb 40-1a was used for hSn and normal rat IgG control (R&D) for mSn. Hybridoma 40-1a developed by Joshua Sanes was obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242.

Identification of the sialoadhesin internalization mechanism

Different internalization inhibitors were added to the cells prior and during mAb-induced internalization of Sn. Myristoylated dynamin inhibitory peptide (Tocris bioscience), nystatin and amantadine, the latter two purchased from Sigma-Aldrich, were added to the cells in different concentrations. Positive controls were used to analyze the efficiency of the inhibitors. FITC-labeled BSA and biotinylated transferrin were utilized for respectively caveolae- and clathrin-mediated endocytosis [35, 39]. Plasmids encoding wild type proteins dynamin-2 and caveolin-1 were used with their respective dominant negative proteins (DN). DN protein Eps 15 was used with a non-functional double deletion mutant as positive control (Eps 15 DIII Δ 2) instead of a wild type protein. These plasmids were a generous gift from

respectively M. McNiven [40, 41], A. Helenius [42, 43] and A. Benmerah [44, 45] and were further modified as described [39]. The plasmids were transfected in Sn⁺-CHO cells seeded in a 24-well plate (Greiner). Transfection was performed as instructed by the manufacturer using Lipofectamine 2000. The internalization of Sn was induced as stated previously.

Double stainings of Sn with cellular proteins

Internalization of Sn was performed as described above and after fixation and permeabilization, the cells were incubated with the fluorescently-labeled secondary Ab to detect the Sn-bound mAb. A primary antibody against clathrin (Santa Cruz), early endosome antigen 1 (EEA1; Santa Cruz), cation-independent mannose-6-phosphate (CI M6P; Abcam) and cathepsin B (Santa Cruz) was added to the cells for 1 hour at 37°C. Subsequently, Alexa Fluor 555-labeled donkey anti-goat or donkey anti-rabbit antibodies were added for visualization of the cellular proteins. EEA1 is a marker for the early endosomes, CI M6P for the late endosomes and cathepsin B for the lysosomes [46-48].

Statistical analysis and confocal microscopy

Statistical analysis was performed using GraphPad Prism 6 on the results of three independent repeats. For each experiment at least 50 cells and 20 cells were analyzed respectively for the internalization and endolysosomal localization assays. An unpaired, two-tailed student's t-test was performed on the data. Results were considered significant when the p-value was lower than 0.05. Confocal images were obtained using Apotome 2 with an Axio Observer inverted microscope and a Compact Light Source HXP 120C with Filter set 49, 20 and 10 for blue, red and green fluorophores respectively (Zeiss) and using a Nikon Eclipse Ti-E inverted microscope attached to a microlens-enhanced dual spinning disk confocal system (UltraVIEW VoX; PerkinElmer, Zaventem, Belgium) equipped with 405, 488 and 561 nm diode lasers for excitation of blue, green and red respectively. Images were processed using ImageJ software.

Results

Analysis of mAb-induced sialoadhesin internalization

In previous studies, different cells were used to analyze Sn internalization; CHO cells expressing recombinant Sn [23, 37], human THP-1 cells [21], and primary porcine alveolar macrophages [35], making direct comparisons of the results difficult. Therefore, in this study Sn internalization was studied not only in primary Sn-expressing macrophages but also in CHO cells expressing either a recombinant hSn or mSn. Sn⁺-CHO allowed to directly compare the internalization capacity of Sn of different species and to exclude effects specific to the cellular background.

For the primary macrophages, internalization was induced using F(ab')₂ fragments instead of mAbs to avoid Fc-receptor interactions. MAb 12A4 and mAb SySy94 derived F(ab')₂ fragments, which recognize hSn and mSn respectively, were used. For the kinetic studies, antibodies were added at 4°C to allow only binding with surface expressed hSn or mSn. Cells were shifted to 37°C to allow internalization or kept on ice for only surface staining. F-actin was stained to distinguish surface expressed Sn and internalized Sn. Appropriate non-binding control antibodies were used to ensure that internalization and stainings were specific. Human and mouse primary macrophages showed a clear surface staining for respectively hSn and mSn at time 0 min and internalized Sn-mAb complexes were observed after 60 min of internalization. No background staining was observed using the control antibodies. Similar results were recorded when Sn⁺-CHO were used (Supplementary data

Figure S1). To further study the internalization of the different species and cell types, the kinetics of mAb-induced internalization were analyzed. Representative images for each cell type and the corresponding time post shift to 37°C are shown in Figure 1A. Figure 1B shows that the mAb-induced internalization of Sn is time-dependent for each cell type. The amount of intracellular Sn-mAb complexes increased with time until 60 min of internalization and remained constant afterwards. No differences could be observed in between hSn and mSn and different cell types evaluated.

Figure 1: Kinetics of mAb-induced internalization of Sn. After adding anti-Sn mAbs or F(ab')₂ fragments to the cells at 4°C to allow surface staining of Sn, cells were shifted to 37°C to induce internalization during different times. Cells were fixed, permeabilized and Alexa Fluor 488-labeled antibodies (green) were added along with Texas Red®-X phalloidin (red). DAPI was used to stain the nuclei (blue). (A) Representative confocal images were taken at each time for the different species and cell types. (B) For each condition, the intracellular Sn-mAb complexes were counted in 50 cells. Data represents the mean and standard deviation of three independent repeats.

Identification of the mechanism of mAb-induced internalization of sialoadhesin

The mAb-induced internalization of pSn was previously identified to be clathrin-mediated [35]. Since the cytoplasmic tail of Sn contains no known signalization motif and is not conserved between species, it is unclear if this mechanism of internalization is conserved in hSn and mSn [16, 36]. Inhibitors were used to analyze the mechanism of mAb-induced internalization of hSn and mSn in primary macrophages and Sn⁺-CHO cell lines. Dynamin inhibitory peptide was used as a broad inhibitor. Dynamin, as a GTPase, controls initial stages of different mechanisms of endocytosis such as caveolae-dependent endocytosis and clathrin-mediated endocytosis [49-51]. More specific inhibitors were used for these internalization mechanisms, respectively nystatin and amantadine. Nystatin is a sterol-binding drug, preventing the maintenance of caveolae and their release from the plasma membrane [39, 52]. Amantadine inhibits stabilization of the membrane structure and prevents fusion processes by interfering with the interaction between clathrin and adaptors [39, 53]. The inhibitors were added prior and during internalization of Sn. A reduction of the internalization was observed for the primary macrophages as well as the Sn⁺-CHO cell lines using dynamin inhibitory peptide and amantadine. When administering amantadine to hSn⁺-MDMs, cell toxicity was observed at 2,5 mM of amantadine and this concentration was not included for analysis for these cells. Therefore, the effect of lower concentrations of the inhibitor was also analyzed for these cells. No effect was observed using nystatin (Figure 2A). This suggests the mAb-induced internalization of both hSn and mSn is dynamin- and clathrin-dependent.

To confirm this internalization mechanism, Sn⁺-CHO cells were transfected with plasmids overexpressing dominant negative dynamin-2, Eps 15 or caveolin-2. Dynamin-2 is a protein present in dynamin-dependent internalization pathways such as clathrin- and caveolae-mediated internalization. Eps 15 (EGFR pathway substrate 15) and caveolin-1 are proteins in respectively clathrin- and caveolae-mediated endocytosis. Eps 15 binds with the clathrin-adaptor complex AP-2 [54-56]. Caveolin-1 is one of the important proteins used for formation and transportation of caveolae pits [57-59]. For both dynamin-2 and caveolin-1 proteins, a dominant negative (DN) mutant was available and a wild type (WT) as a control. For Eps 15, a non-functional double deletion mutant was used as a positive control. For hSn⁺- and mSn⁺-CHO cells, a significant reduction in Sn internalization was observed for the DN dynamin-2 and Eps 15, compared to the WT proteins (Figure 2B). No significant differences were observed in Sn internalization between the WT and DN caveolin-1. These results further confirm the importance of dynamin and clathrin in Sn internalization.

Figure 2: Effect of inhibitors on human and mouse sialoadhesin internalization. (A) Sialoadhesin internalization was induced in primary cells with antibodies in the presence of dynamin inhibitory peptide, amantadine and nystatin, which inhibit respectively dynamin-dependent mechanisms, clathrin- and caveolin-mediated endocytosis. (B) HSn⁺-CHO and mSn⁺-CHO were transfected with wild type proteins (black) and dominant negative proteins (grey) essential in different internalization mechanism. Dynamin-2 is important in both clathrin- and caveolae-mediated endocytosis; Eps 15 and caveolin-1 respectively in clathrin- and caveolae-mediated endocytosis. The student's t-test was performed to analyze the significance of the results obtained of three independent experiments. Significant differences were observed for dynamin-2 and Eps 15 mutants, as indicated with an *, while for the caveolin mutant no significant effect was observed.

Finally, a double staining between Sn and clathrin was performed. Sn internalization was induced in primary macrophages with F(ab')₂ fragments for 15 min before fixing the cells, followed by further staining of Sn and clathrin. Both for hSn and mSn a close association between Sn and clathrin was observed, confirming the importance of clathrin in antibody-induced Sn internalization (Figure 3).

In conclusion, these results show that hSn and mSn also internalize via clathrin and suggest that the mechanism of Sn internalization is conserved in between species.

Figure 3: Double immunofluorescence staining for Sn and clathrin. hSn and mSn on primary macrophages were allowed to internalize after mAb-binding for 15 min before the cells were fixed and permeabilized. Secondary Alexa Fluor 488-labeled antibodies (green) were added to stain Sn. To visualize clathrin, cells were incubated with a primary anti-clathrin mAb and secondary Alexa Fluor 555-labeled antibodies (red). Nuclei were stained with DAPI (blue). Cells were analyzed by confocal microscopy.

Intracellular trafficking of sialoadhesin-mAb complexes in the endolysosomal system

Knowledge of the intracellular trafficking and localization of internalized Sn-mAb complexes is important for the development of Sn targeted therapeutics, such as immunotoxins. Depending on the intracellular route and the final fate, an immunotoxin will be exposed to different pH values and proteolytic enzymes, which may strongly affect the toxin activity [13]. The localization of intracellular pSn after mAb-induced internalization in primary macrophages was previously shown to be in early endosomes [35]. Because of the differences in amino acid sequence of different Sn species, the localization might be different [36]. Therefore, the location of internalized hSn and mSn was studied in primary macrophages upon stimulation with F(ab')₂ and in Sn⁺-CHO cell lines stimulated with mAb to exclude differences in internalization pathways attributable to the cellular background.

In our study, similar results were obtained for hSn and mSn in primary macrophages. Internalized complexes were localized mostly in early and late endosomes and minimally in lysosomes (Figure 4 and Supplementary data Figure S2). In contrast, in Sn⁺-CHO cell lines an association was observed mainly with lysosomes. For hSn, this was seen at later times (30 min) and for mSn already at early times (15 min).

Figure 4: Analysis of intracellular trafficking and localization of Sn-mAb complexes. HS_n and mS_n internalization was induced in primary macrophages, using F(ab')₂ fragments, and S_n⁺-CHO cell lines, using mAbs for different times. Cells were stained with secondary antibodies to detect the S_n-specific mAbs or F(ab')₂ fragments, with markers for respectively early endosomes, late endosomes and lysosomes, and the presence of the mAbs or F(ab')₂ fragments in these compartments was determined. Data represent means and standard error of the mean of three independent experiments.

Therapeutic use of receptors to target toxins or immunomodulators can be performed with full length mAbs, F(ab')₂ fragments or even smaller constructs [13]. Therefore, and because the interaction of an antibody with the Fc receptor on macrophages could also affect the intracellular trafficking of Sn, the intracellular fate was compared for both F(ab')₂ fragments and full length anti-Sn mAbs. Intracellular Sn-mAb complexes were mainly localized within the endosomes (Figure 5 and Supplementary data Figure S3). Interestingly, no major differences were detected between full length mAbs and F(ab')₂ fragments in human primary macrophages. However, a significant difference was observed in mouse primary macrophages after 90 min of internalization with an increased association with early endosomes. The association with lysosomes was also increased, but not statistically significant.

Figure 5: Comparison of the localization of anti-Sn mAbs and F(ab')₂ fragments in the endolysosomal system of primary macrophages. Anti-Sn mAbs and F(ab')₂ fragments were added to primary macrophages and internalization was allowed for 15 or 90 min. Cells were stained both for the Sn-specific mAbs or F(ab')₂ fragments and markers for respectively early endosomes, late endosomes and lysosomes. The localization of the mAbs or F(ab')₂ fragments in these compartments was determined. Data represent means and standard error of the mean of three independent experiments.

Discussion

Sn, like other Siglecs, can recognize sialic acids, which are terminal nine carbon sugars found on glycans, expressed on vertebrate cells and pathogens [16]. Cell-cell interactions relying on a sialic acid-dependent binding of Sn to CD43 on T-cells and MUC1 on breast cancer cell were previously identified [60, 61]. In addition, for some pathogens it was shown that sialic acid mediated binding to Sn aids in their uptake in macrophages. This has been shown for *Neisseria meningitides* and *Campylobacter jejuni* where Sn binding increases phagocytosis of these bacteria, although Sn does not seem to be directly implicated in the phagocytosis process itself [62-64]. Porcine reproductive and respiratory syndrome virus (PRRSV) was also shown to directly interact with Sn via sialic acids present on the virus particle, resulting in Sn-mediated internalization [35, 65-68]. During HIV-1 infections, an upregulation of Sn is observed, and it was shown that HIV-1 could bind in a sialic acid-dependent manner to Sn [17, 28, 69, 70]. Furthermore, it was shown that Sn mediates HIV-1 trans-infection through T-cells [21, 24, 28]. Interestingly, in these studies, the internalization mechanisms where Sn contributes to, are different. The different mechanisms for pathogens to enter cells upon binding with Sn could be due to the different sizes of the pathogens, since bacteria are bigger, which should lead to phagocytosis. In contrast, viruses are smaller and

can therefore not be phagocytosed since the phagocytosis is defined as the uptake of particles larger than 0,5 μm [71]. More likely, the pathogens and viruses could interact with other receptors besides Sn [72, 73], which results in different internalization mechanisms. In addition, since both primary cells and cell lines were used, differences in cellular backgrounds could also account for the observed differences. Therefore, it is difficult to make conclusions on the internalization of Sn of different species based on these studies. In this study, we showed that both hSn and mSn expressed on primary cells internalize via a clathrin-mediated mechanism. In addition, experiments in CHO cells expressing either hSn or mSn, which allowed a direct comparison of the mechanism of Sn internalization on the same cellular background, confirmed that Sn internalization is, similar to pSn, dynamin-dependent and clathrin-mediated. Because Sn of three different species utilizes the same internalization mechanism, it is most likely that the mAb-induced internalization of Sn in other species is also dynamin-dependent and clathrin-mediated.

Macrophage surface receptors can be used as targets for antibody targeted phenotype-modulating or cytotoxic drugs. This requires the receptor to be expressed homogeneously, persistently and exclusively on specific target cells. Moreover, the surface receptor must induce a signaling cascade or internalize into the cells [29]. Sn possesses these properties. It has been shown for diseases such as arthritis, multiple sclerosis and some cancers, that Sn is upregulated on macrophages and monocytic cells [70, 74, 75]. During arthritis, the expression of Sn is upregulated in parallel with the severity of the disease [76]. In colorectal cancer, an upregulation of Sn is observed on circulating monocytes and tumor infiltrating macrophages [77]. Studies with Sn-deficient mice and Sn-DTR mice show that Sn and Sn-expressing macrophages play a key role in such diseases [78-80]. For instance, Sn-positive macrophages were described as antigen presenting cells in lymph nodes activating tumor antigen-specific T-cells [80]. It was also shown in Sn-deficient mice that the interaction of Sn with pathogens not only aids the uptake of the pathogen but also promotes inflammatory responses [81]. Clearly, this illustrates that Sn could represent a good target for therapeutic interventions. Interestingly, Siglec-2 (CD22; exclusively expressed on B-cells) and Siglec-3 (CD33; expressed on acute myeloid leukemia cells) have already been used as target receptor for immunotoxins in the treatment of cancer [82, 83]. In our study, we confirmed the mAb-induced internalization of hSn and mSn in primary cells and Sn-expressing CHO cell lines, which is a key requirement for the activity of immunotoxins. Our results also showed a clear, time-dependent increase in internalization up to 60 min after induction.

The therapeutic use of mAbs can trigger different outcomes, including the modulation of receptor signaling [29]. Up to now, little is known about the signaling properties of Sn. Our results show that the mAb-induced internalization of hSn and mSn in primary cells and in Sn-expressing CHO cells is dynamin-dependent and clathrin-mediated, suggesting that Sn can induce some kind of signaling. This was also previously shown for pSn [35]. Interestingly, and in contrast with other Siglecs, no amino acid motif that could mediate intracellular signaling has been identified in the cytoplasmic tail of Sn. Moreover, the cytoplasmic tail of Sn is not conserved between the different species [36]. One possible explanation for this apparent discrepancy is that adaptor molecules bind to the transmembrane domain or cytoplasmic tail of Sn. Indeed, recently the association of mSn with DAP12 was described [25] and this could account for similarities in the mechanism of Sn endocytosis in different species. Yet, to our knowledge, no association has been observed between clathrin-mediated endocytosis and DAP12 signaling. Moreover, due to transfection of DAP12 in CHO cells in previous research, we believe no DAP12 is present in wild type CHO cells [84]. The clathrin-mediated endocytosis of hSn, mSn and pSn is therefore probably independent of

DAP12. Interestingly, we observed that the intracellular route upon internalization is different in primary macrophages compared to Sn⁺-CHO cell lines. Due to the difference in cellular background, it is possible that in primary cells, where DAP12 is available, DAP12 rerouts intracellular transport to the endosomes or blocks movement from endosomes to lysosomes.

Another way of using mAbs in therapy is by linking the mAb with bacterial or plant toxins resulting in immunotoxins [29]. The development of these immunotoxins is dependent on the characteristics of the target protein. For such immunotoxins to become activated upon internalization, translocation from the intracellular vesicle to the cytosol is usually required. Depending on the toxin, this translocation can be triggered by acidic endosomes or endoplasmic reticulum-specific enzyme activity as described for respectively diphtheria toxin or pseudomonas exotoxin (PE) [31]. Therefore, PE cannot be used for receptors only localized with the endosomes, like transferrin [32]. Our data shows that upon stimulation of primary macrophages with Sn-specific F(ab')₂, hSn and mSn are mostly located in the early endosomes, somewhat in late endosomes and almost not in lysosomes. This was also previously observed for pSn in primary cells [35]. Interestingly, when a complete mAb was used to stimulate Sn internalization in mouse macrophages, an increased association of internalized Sn-mAb complexes was seen with early endosomes and lysosomes. A possible explanation is that the antibody, in addition to a specific binding to Sn, also interacts with Fc receptors on macrophages, which could influence the intracellular fate of the Sn-mAb complexes towards lysosomes [85]. Possible interactions with Fc receptors can thus clearly affect internalization pathways and the activity of Sn-targeted drugs or toxins.

Besides a role of Fc receptor interactions, our findings also show that the cellular background can affect the fate of Sn-mAb complexes, since in Sn⁺-CHO cell lines, Sn-mAb complexes were clearly localized in lysosomes. These results show that both the type of mAb and the cellular background should be taken into account for the development of Sn-targeted phenotype-modulating or cytotoxic drugs. Previously, pSn-specific immunotoxins linked with the toxin saporin were successfully targeted to porcine macrophages resulting in the killing of these cells [35]. Saporin is different compared to other toxins such as PE in that it does not need to be routed to lysosomes but that it can be translocated from early endosomes to the cytoplasm [86]. Similar immunotoxins could be developed for targeting hSn or mSn.

It should be noted that targeting Sn during inflammation could also target inflammatory monocytes and monocyte-derived macrophages. Indeed, the expression of Sn can be upregulated on these cell types during inflammation [18, 33, 75]. This must be taken into account prior using Sn as target. In contrast, if Sn is targeted to deliver antigens as a vaccination strategy, this normally occurs in steady state conditions, where the expression of Sn is mainly restricted to macrophages. In conclusion, Sn shows some interesting characteristics for using the receptor for therapeutic targeting, which are conserved in between species. Sn has an interesting expression pattern, due to the upregulation in inflammatory conditions and during infection. In addition, Sn shows internalization after mAb-binding and internalized Sn-mAb complexes are routed to endosomes or lysosomes, depending on the type of mAb used.

Conflict of interest

The authors declare no conflicts of interest.

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