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Efficient uptake of mannosylated proteins by a human Schwann cell line

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Summary. Complex carbohydrate structures are essential molecules of infectious microbes and host cells, and are involved in cell signaling associated with inflammatory and immune responses. The uptake of mannose-tailed glycans is usually carried out by macrophages, dendritic cells (DCs), and other professional phagocytes to trigger MHC class I- and MHC class II-restricted antigen presentation, and to promote T cell effector responses. Since Schwann cells (SCs) have been proposed as immunocompetent cells, we investigated whether a human cell line (ST88-14 cells) could bind mannosylated ligands in a specific manner. The saturation of uptake of mannosylated molecules by ST88-14 cells and the internalization and distribution pathway of these ligands were tested by cytometry and confocal plus electron microscopy, respectively. This uptake showed a dose-dependent increase, the saturation point being reached at high concentrations of mannosyl residues/240mM mannose. Merging of man/BSA-FITC and S100 labeling showed their partial, but, significant colocalization. Ultrastructural analysis of ST88-14 cells after incubation with HRP-colloidal gold, without or with subsequent chasing at 37°C, showed an initial location on the cell surface and temperature- and time-dependent internalization of the probe. Our findings suggest an efficient mannosylated ligand uptake system through putative lectin(s) that may be operational in inflammatory and immune responses.

Key words: Pattern recognition receptors, Mannose receptor, Endocytic compartments

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Introduction

The uptake of mannose-tailed glycans has been identified as an essential mechanism carried out by phagocytes such as macrophages, dendritic cells (DCs) and other professional antigen presenting cells to enhance MHC class I- and MHC class II-restricted antigen presentation, increase T cell proliferation, and promote T cell effector responses. (for reviews, see Taylor et al., 2005). However, it is currently known that not only macrophages and DCs, but also other cells, such as non-professional phagocytes, are able to perform efficient uptake of mannosylated compounds and/or recognition and internalization of infectious agents (for review, see Stahl and Ezekowitz, 1998; Burudi et al., 1999; Hespanhol et al., 2005).

SC has occasionally been considered as a non-professional phagocyte and immunocompetent cell, able to drive the immune response in the Peripheral Nervous System (PNS) and to display a large repertoire of immune-related properties (Rutkowski et al., 1999; Oliveira et al., 2003; Baetas-da-Cruz et al., 2008; Meyer zu Hörste et al., 2008). Such properties include participation in antigen presentation, at least in some conditions such as in leprosy, in which infected SC process and present antigens of *Mycobacterium leprae* to antigen-specific, inflammatory type 1 T cells (Oliveira et al., 2003). In view of this potential immune competence of SC and, particularly, of interest in their character of antigen-presenting cells, we have studied the uptake characteristics of mannosylated proteins in a human Schwann cell line (ST88-14).

Materials and methods

Cell line cultures

The ST88-14 tumor cell line was isolated from a patient with neurofibromatosis type 1 (Ryan et al.,

1994), and was generously donated by J.A. Flechter (Dana Farber Cancer Institute, Boston, MA). The cells were grown in RPMI 1640 medium supplemented with 5% fetal calf bovine FCS, 1 mM glutamine, 1000U/mL penicillin, and 50 µg/ml streptomycin. All chemicals were from Sigma. The cells, plated in culture dishes or cover slips in 24-well plates (Falcon, Franklin Lakes, NJ), were maintained in a humidified air/CO_2 (95%/5%) atmosphere at 37°C for 24 h.

Flow cytometry-based analysis of the uptake of mannosylated molecules

Flow cytometry was performed according to conventional protocols in order to determine the saturation point of the uptake of mannosylated molecules. Confluent monolayers of ST88-14 cells were harvested by exposure to 10 mM EDTA (Sigma) in pH 7.4 phosphate buffered saline (PBS) at 37°C for 10 min. After repeated pipetting to ensure a single cell suspension, cells were washed three times in Ringer solution and incubated with 6.25, 12.5, 25.0, 50.0, 100.0 and 200.0 µg/ml of the neoglycoprotein mannosyl/bovine serum albumin-FITC-conjugated (man/BSA-FITC, Sigma) diluted in Ringer solution containing 5mM CaCl2 and 1% BSA at 37°C for 1 h. To prevent quenching of fluorescence, 20 mM of NH4Cl was added to the labeling solution. Controls were obtained by the addition of an excess of 250 mM D-mannose (Hespanhol et al., 2005) or with ~ 1.1 µM (50 µg/ml) HRP (a highly mannosylated ligand – Straus, 1981) at 37°C for 40 min, followed by washes and incubation with 50 µg/ml man/BSA-FITC as described above. Samples were analyzed on a Becton Dickinson FACsCalibur (Mountain View, CA).

Detection of mannosylated protein uptake and S100 immunocytochemistry

Cytochemistry assay with man/BSA-FITC binding was performed in order to verify the internalization pattern in ST88-14 cells. Cells adhered on coverslips were washed in Ringer solution and incubated with 50 µg/ml man/BSA-FITC diluted in Ringer solution containing 5 mM $CaCl_2$ plus 1% BSA at 37°C for 1 h. Controls were obtained by incubation of the cells with 250 mM D-mannose or ~ 1.1 µM HRP (Straus, 1981) diluted in the same labeling solution followed by washing and incubation with 50 µg/ml mannosyl/BSA-FITC as described above. To confirm the Schwann-like nature of our ST88-14 cells (Ryan et al., 1994) and also for localization of intracellular pool of internalized man/BSA-FITC, cells were fixed and tagged with a polyclonal brain S100 (Sigma) antibody after previous incubation of living cells with 50 µg/ml man/BSA-FITC.

Ultrastructural cytochemistry of a highly mannosylated ligand

The follow-up of the fate of HRP (Straus, 1981) was done in ST88-14 cells. Living ST88-14 cells adhered to 35 mm Petri dishes were incubated in HRP coupled to 10 nm colloidal gold (HRP/Au) (Sigma) diluted 1:5 (v/v) in Ringer solution containing 5 mM $CaCl_2$ plus 1% BSA at 4°C for 40 min. After incubation, all cultures were rinsed in the same labeling solution to remove non-adhered particles. Some samples were fixed immediately in 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2 at 4°C for 1 h. Other cultures were submitted to chase in labeling solution at 37°C for 1 and 3 h. Negative controls were obtained by preincubating the cells with 250 mM D-mannose at 4°C for 40 min. Cells were scraped out of dishes on ice, centrifuged at 900 g for 10 min, and post-fixed in 1% osmium tetroxide (Sigma) in 0.1 M sodium cacodylate buffer containing 3.5% sucrose at 4°C for 30 min. Cells were then washed in the same buffer, dehydrated in acetone, and embedded in PolyBed 812 (Polyscience, Philadelphia, PA). Thin sections were obtained in an OmU3 Reichert ultramicrotome, stained with aqueous 2% uranyl acetate, and examined in a Zeiss EM 10C transmission electron microscope.

Results

Flow cytometry shows a high percentage of neoglycoprotein-labeled Schwannoma cells

A highly homogeneous region was observed by flow citometry (Fig. 1A). The assays of saturation of the uptake of mannosylated molecules in ST88-14 cells showed a gradual dose-dependent variation in the number of man/BSA-FITC positive cells (Fig. 1B). The saturation point was reached at 100 µg/ml man/BSA concentration (Fig. 1B). Preincubation of ST88-14 cells with ~1.1 µM HRP (data not shown) or 250mM D-mannose inhibited the binding of the man/BSA-FITC (Fig. 1B).

ST88-14 cells uptake a mannosyl neoglycoprotein

To gather information that may confirm the usefulness of a human Schwann-like cell lineage in pathological models, ST88-14 cells were double-labeled with the mannosyl neoglycoprotein and anti-S100 antibody and examined with the Zeiss LSM 510 META confocal microscope. After incubation of fixed cells with the man/BSA-FITC, there was evident labeling with this marker, widely distributed on the cellular surface (Fig. 2A) and also in the intracellular domain (Fig. 2D,G). At the level of the maximal nuclear diameter, there was intense labeling for man/BSA-FITC in perinuclear regions (Fig. 2D). S100 labeling was localized predominantly in the cytoplasm (Fig. 2E,H) and virtually absent from the membrane (Fig. 2B). Merging of man/BSA-FITC and S100 labeling showed that there is significant colocalization of these markers (Fig. 2F,I) suggesting that a major percentage of the mannosyl-binding protein pool is intracellular. Considerable pixel-to-pixel overlap of man/BSA-FITC and S100, which

encompassed several optical sections in the z-series, was observed. Pretreatment of ST88-14 cells with 250 mM D-mannose before the incubation with man/BSA-FITC or ~1.1 μM HRP resulted in virtual absence of this label (not shown). Non-specific labeling due to the secondary antibody was not detected in control sections (not shown).

In order to test whether the mannosylated ligand uptake by ST88-14 is related to the neoplastic transformation, a pilot experiment was performed with human Schwann cells isolated from non-neoplastic tissue (generously donated by P.M Wood - University of

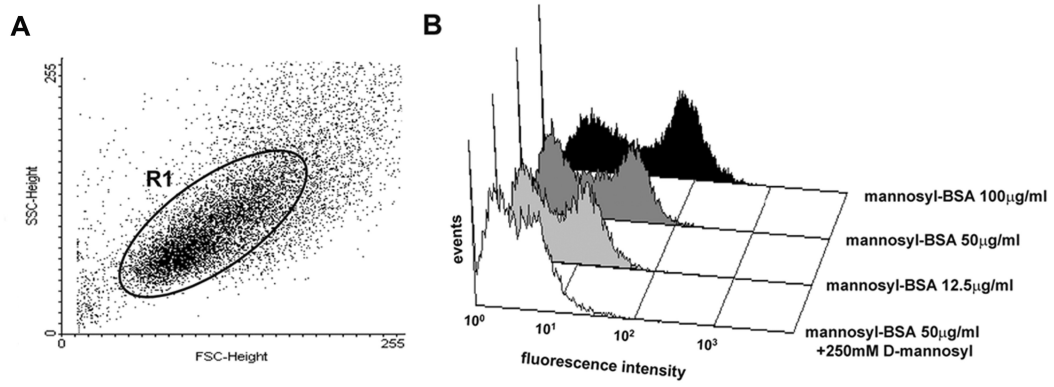

Fig. 1. Flow cytometric analysis of the uptake of man/BSA-FITC in ST88-14 cells. The analysis was performed in the specified region (R1) ST88-14 cells incubated with the man/BSA-FITC in different concentrations (shaded histograms). The saturation point was reached at 100 μg/ml man/BSA-FITC concentration (black histogram). Preincubation of ST88-14 cells with 250mM D-mannose inhibited the binding of the man/BSA-FITC (unshaded histogram). Data are representative of three separate experiments.

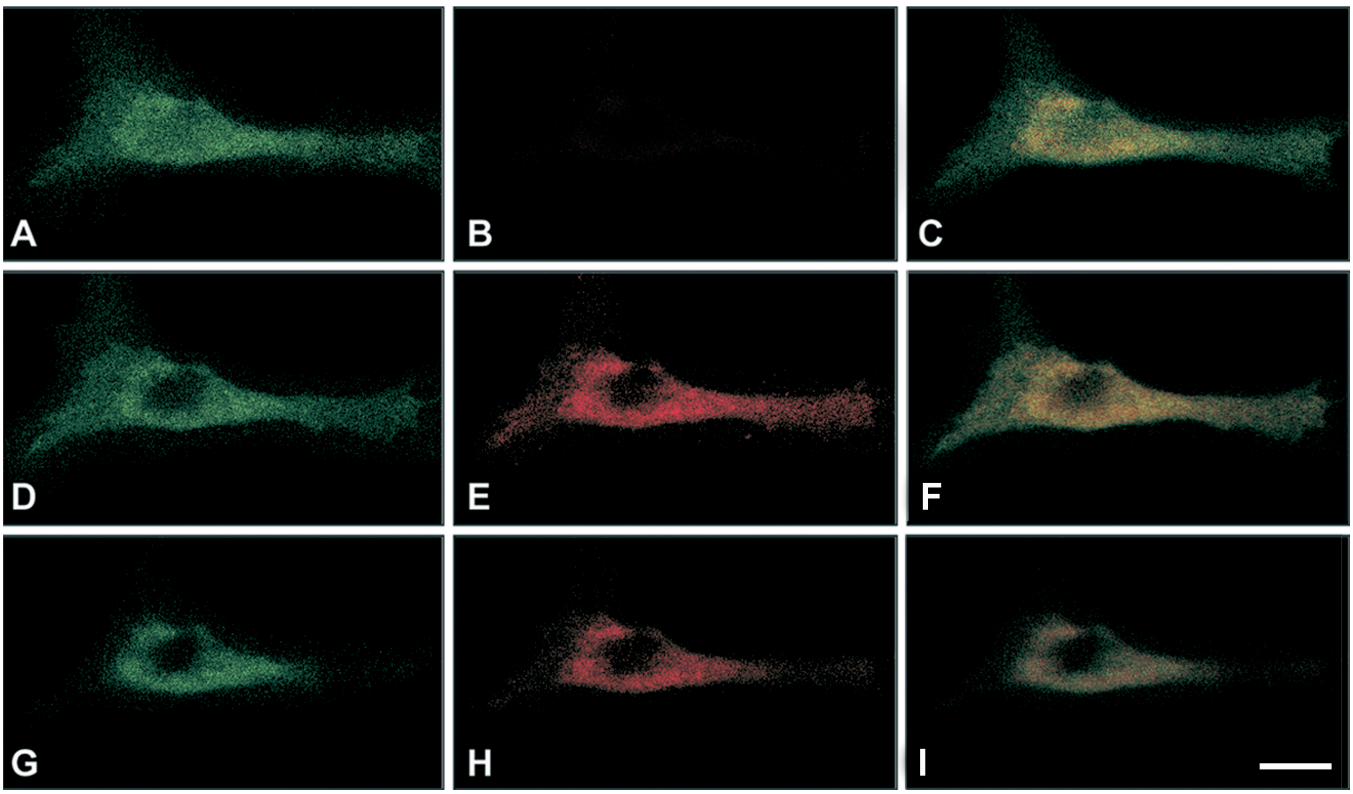

Fig. 2. Confocal microscope images showing uptake of a mannosylated protein and expression of brain S100 by a human Schwann (ST88-14) cell line. The three rows show images of a cell, taken from different optic planes, selected from z-series. Columns (and the three rows) show man/BSA-FITC binding (left-hand column – **A, D, G**), S100 immunoreactivity (middle column – **B, E, H**), and their colocalization (right-hand column – **C, F, I**). The top row represents an optic plane at or near the free surface of the membrane, whereas the two following rows show two planes through the nucleus (nuclear maximal diameter in **D, E, F**). These results are representative of five separate experiments. Bar: 20 μm.

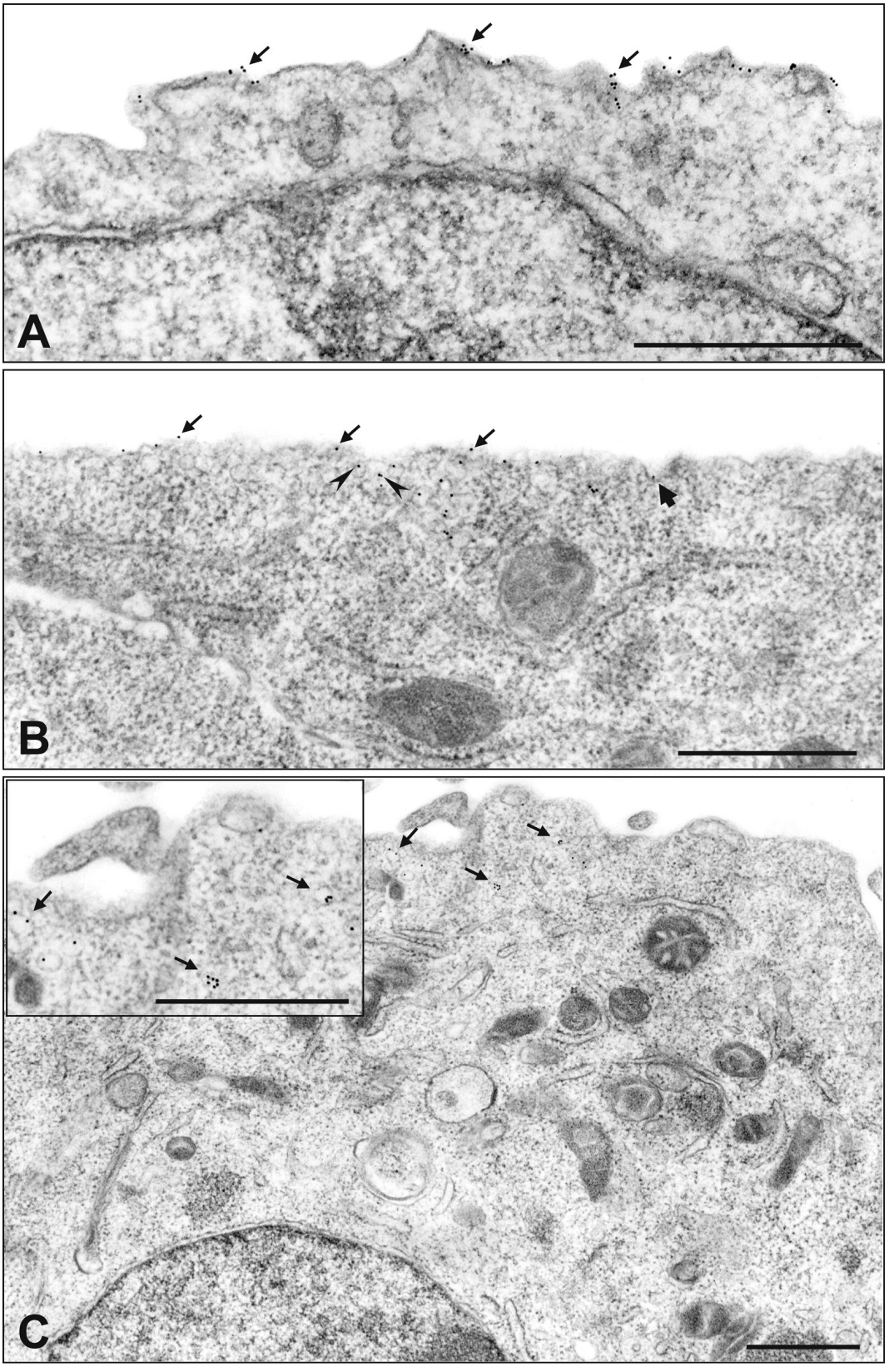

Fig. 3. Ultrastructural analysis showing the localization of MR in ST88-14 cells after incubation with a ligand at different temperatures and time intervals. **A.** Incubation of cells with HRP/Au at 4°C for 40 min reveals numerous gold particles restricted to the cell membrane (arrows). **B.** After washing of HRP/Au and a gradual increase of the temperature to 37°C, maintained for 1 hr, gold particles are still found at the cell surface (arrows) and become abundant in membrane invaginations (thick arrow) as well as within small vesicles (arrowheads) next to the internal face of the plasma membrane. **C.** After chasing for 3 hr at 37°C, virtually all gold particles are found in small vesicles (arrows), indicating MR internalization. The inset is a high magnification view with arrows pointing to some of the numerous HRP/Au-containing vesicles. Bars: 0.5µm

Miami Miller School of Medicine, Miami, FL). In this experiment, similar to that found in ST88-14 cells, normal human Schwann cells, at least from the fixed cultures, showed an intense tagging with man/BSA-FITC (data not shown). Due to limitations on cell yield in cultures from normal human tissue, all other experiments were done on ST88-14 cells only.

ST88-14 cells take up and internalize the mannosylated ligand HRP

To initiate studies of the function of the putative mannosylated ligand uptake system on ST88-14 cells, experiments of HRP/Au internalization were performed. Incubation of the cells with HRP/Au at 4°C for 40 min, followed by washing and fixation, resulted in numerous HRP/Au particles adhered onto the cellular surface (Fig. 3A). In the chasing experiments, in which cells were kept in the labeling solution and the temperature was increased to 37°C for 1 h, some gold particles remained adhered to the cellular surface while others were localized in membrane invaginations, as well as within small vesicles next to the internal face of the plasma membrane (Fig. 3B). After 3 h of chase at 37°C, no adhered gold particles were observed on the cellular surface (Fig. 3C), but vesicles containing one or more gold particles were seen throughout the cytoplasm (Fig. 3C). To discriminate between fluid phase uptake and receptor-mediated uptake, the cultures were co-incubated with HRP/Au and 250 mM D-mannose, which resulted in an obvious reduction in the number of adhered gold particles in agreement with the flow cytometry data.

Discussion

In this work, we show that ST88-14 cells are able to bind specifically and take up mannosylated proteins, thus, suggesting the expression and function of a mannose-specific C-type lectin receptor (ManCTLR) by these cells. We also show that the occurrence of this putative receptor is not linked to neoplastic transformation, since the man/BSA-binding was also detected in SC from human primary cultures.

The prospective functionality of one or more ManCTLR involved in recognition and processing of mannosylated antigens has been inferred from the fate of the mannosylated ligands man-BSA and HRP after uptake by ST88-14 cells. HRP is a glycoprotein devoid of phosphorylated carbohydrates and rich in mannose and N-acetylglycosamine groups (Clarke and Shannon, 1976) that has been usually reported as a label for cytochemical detection of mannosyl binding sites in normal tissue (Straus, 1981) and, more recently, in *Leishmania*-infected dermal cells (Hespanhol et al., 2005). Thus, we used HRP with multiple purposes, namely, as a competitor for man/BSA binding assay and as a probe for the localization and fate of endogenous lectin(s) in an electron microscopic approach.

Both confocal and electron microscopy provided clues to the internalization and distribution pathway of ManCTLR in ST88-14 cells. Double labeling with mannosyl-BSA and S100 showed that there is significant colocalization of these markers suggesting that a major percentage of the ManCTLR cellular pool is intracellular as is the case for the macrophages mannose receptor (Taylor et al., 2005). Although not entirely understood, the colocalization of S100 and of the neoglycoprotein-binding sites seems suitable since both proteins are linked to the Ca^{2+} as well as to the cytoskeleton (Donato, 2001). In HRP/Au ultrastructural experiments, the tracer was found initially adhered to the cellular surface and, after chasing and incubation at 37°C (1–3h), in membrane invaginations and, then, inside small vesicles. Such invaginations and vesicles are probably early endosomes due to their localization near the internal face of the plasma membrane. However, multiple times of incubation are necessary in order to provide additional details on the initial steps of receptor mediated endocytosis in ST88-14 cells.

There is evidence that SC may perform an immune-inflammatory function (for review, see McGreal et al., 2004; Baetas-Da-Cruz et al., 2008) in a process that may involve a role as non-professional phagocytes by engulfing cell debris or microorganisms (Band et al., 1986; Lilje, 2002). This engulfment of cell debris may require the occurrence of a mannosylated ligand uptake system through receptors involved in "clearance" activities such as the killing and ingestion of microorganisms. Furthermore, mannosyl units of lipoarabinomannan from Mycobacterium leprae (Khoo et al., 1995) could serve as a putative ligand for the ManCTLR during bacterial adherence to SC.

Our present results reinforce the notion that SC may have a role in the immune response. Although poorly understood, the presumptive immune function of SC has been demonstrated in several peripheral neuropathies (Harboe et al., 2005; Le and Parada, 2007) for instance, acting as facultative antigen-presenting cells during inflammation, in whose course they process and present endogenous and exogenous antigens (Rutkowski et al., 1999; Lilje, 2002). Thus, the expression of ManCTLR can have important implications, not only for understanding how dermal SC recognize self or non-self molecules, but also for understanding the trigger mechanisms for the production of cytokines by these cells. Nevertheless, at least in the case of professional antigen-presenting cells, uptake by ManCTLR has been considered the major mechanism responsible for the enhanced antigenicity seen with mannosylated proteins (Lam et al., 2007). In this way, a better understanding of the SC mechanism associated with the induction of immune responses, as a result of targeting mannosylated antigens, could be important in exploiting mannose-specific C-type lectin for targeted drug carriers or vaccine design. That could not only mount immune defenses against neoplastic and infectious diseases, but also for specific induction of tolerance in the treatment

of autoimmune disease.

In summary, our current study documents for the first time the presence of a mannose-specific C-type lectin receptor(s) in Schwann and Schwannoma cells, thus reinforcing the importance of these cells as models for the possible role of the peripheral glia in host defense in infectious diseases, neoplasia, and in other degenerative disorders, as well as in a putative role for tolerance in cellular therapy.

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