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Surface α2-3- and α2-6-sialylation of human monocytes and derived dendritic cells and its influence on endocytosis

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Abstract Several glycoconjugates are involved in the immune response. Sialic acid is frequently the glycan terminal sugar and it may modulate immune interactions. Dendritic cells (DCs) are antigen-presenting cells with high endocytic capacity and a central role in immune regulation. On this basis, DCs derived from monocytes (mo-DC) are utilised in immunotherapy, though many features are ignored and their use is still limited. We analyzed the surface sialylated glycans expressed during human mo-DC generation. This was monitored by lectin binding and analysis of sialyltransferases (ST) at the mRNA level and by specific enzymatic assays. We showed that α2-3-sialylated *O*-glycans and α2-6- and α2-3-sialylated *N*-glycans are present in monocytes and their expression increases during mo-DC differentiation. Three main ST genes are committed with this rearrangement: ST6Gal1 is specifically involved in the augmented α2-6-sialylated *N*-glycans; ST3Gal1 contributes for the α2-3-sialylation of *O*-glycans, particularly T antigens; and ST3Gal4 may contribute for the increased α2-3-sialylated *N*-glycans. Upon mo-DC maturation, ST6Gal1 and ST3Gal4 are downregulated and ST3Gal1 is altered in a stimulus-dependent manner. We also observed that removing surface sialic acid of immature mo-DC by neuraminidase significantly decreased its endocytic capacity, while it increased in monocytes. Our results indicate the STs expression modulates the increased expression of surface sialylated structures during mo-DC generation, which is probably related with changes in cell mechanisms. The ST downregulation after mo-DC maturation probably results in a decreased sialylation or sialylated glycoconjugates involved in the endocytosis, contributing to the down-regulation of one or more antigen-uptake mechanisms specific of mo-DC.

Keywords Sialic acid · Dendritic cell · Monocyte · Endocytosis · Sialyltransferase · Immunotherapy

Abbreviations

DC	Dendritic cell
Mo-DC	monocyte derived DC
KO	knockout
ST	Sialyltransferase
SNA	*Sambucus nigra* lectin
MAA	*Maackia amurensis* lectin
PNA	*Arachis hypogaea* lectin

Introduction

Dendritic cells (DCs) are professional antigen presenting cells with a unique capacity to withdraw antigens at sites of infection and present them to lymphocytes. These immune sentries have a unique ability to uptake and process

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antigens found in the peripheral blood and tissues. Immature DCs uptake particles and microbes by several systems: receptor mediated, which involves the binding to membrane receptors; phagocytosis of apoptotic and necrotic cells, viruses, bacteria and also by fluid phase uptake, which includes macropinocytosis, a constitutive process in DC consisting in ingestion of large vesicles formed by membrane ruffling [1]. The endocytosed antigens are presented on both MHC class I and class II to resting lymphocytes in secondary lymphoid organs to where antigen-loaded DCs migrate during their maturation process [2]. Due to their potential in initiating the specific immune response, DCs, obtained after differentiation of monocytes or bone marrow cells, are used as vaccines against cancer, infectious agents or alergens [3]. Yet, the wealth of information regarding DC biology and pathophysiology, brought in the last years has shown us the enormous complexity of the system. Many DC properties are still poorly understood, thus complicating the standardization of clinical and immunological criteria to allow the use of DC vaccines with safety [4].

Almost all the proteins involved in specific immune mechanisms are glycosylated and sialic acid is typically the terminal sugar of the glycans carried by these proteins. Sialylation is performed by several sialyltransferases (ST), whose gene expression is regulated in a cell type manner [5]. Various sialylated structures are known to be involved in the innate and adaptive immune responses. As an example, sialylated Lewis X (LeX) antigen, is involved in the selectin binding during the leukocyte migration to sites of inflammation and secondary lymphoid organs [6]. In addition, the upregulation of ST6Gal1 ST expression and consequent augment of α2-6-linked sialic acid in serum proteins is considered an integral part of the systemic inflammatory response [7]. Mouse gene knockout (KO) studies have also provided an irrefutable evidence of the immune-relevant function of the α2-3 and α2-6-sialylation [8–10]. *ST6Gal1* KO mice are reported as having an impaired humoral immune response, evidenced by reduced levels of circulating and surface IgM, impaired B lymphocyte proliferation in response to various activation signals and impaired antibody production in response to antigens [10]. On the other hand, *ST3Gal1* KO mice have an almost total absence of peripheral CD8β T lymphocytes, which are lost by apoptosis [8].

Since the role played by the surface sialylation on DCs is almost unknown, we analysed the α2-3- and α2-6-sialylation profile of human DCs during the *in vitro* differentiation from monocytes and during its maturation upon different stimulus. We used staining with *Sambucus nigra* lectin (SNA) for recognizing α2-6-linked sialic acid; *Maackia amurensis* lectin (MAA) for α2-3-sialylated chains and *Arachis hypogaea* lectin (PNA) for nonsialylated Galβ1-3-GalNAc (core 1 structure of the *O*-linked chains). The expression of α2,3- and α2,6-ST genes and activity of specific ST was also assessed. The ST6Gal1 was found to be specifically correlated with the increased content of α2-6-sialylated *N*-glycans during differentiation. Finally, we compared the effect of removing surface sialic acid from monocytes and mo-DCs on their capacity for uptaking different endocytic agents.

Materials and methods

Media and reagents

The cell culture medium used throughout was RPMI 1640 supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 1% pyruvate, 100 μg/ml penicillin/ streptomycin from Gibco BRL and 50 μM 2-mercaptoetanol and 10% foetal calf serum (FCS) from Sigma (St. Louis, MO). Human recombinant IL-4, GM-CSF, IFN-γ, IL-1β and TNF-α were purchased from R&D Systems, (Minneapolis, MN). LPS from *Escherichia coli* was purchased from Sigma and neuraminidase from *Clostridium perfringens* was obtained from Roche Diagnostics, Basel, Switzerland. Fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated human antibodies against CD14 (M5E2), CD86 (Fun-1) or HLA-DR (L243) were purchased from PharMingen (San Diego, CA) and BDCA-1 (AD5-8E7) from Miltenyi Biotec (Bergisch Gladbach, Germany). Lucifer yellow CH potassium salt, FITC-dextran (DX, Mr=40,000) and FITC-ovalbumin were purchased from Molecular Probes (Leiden, The Netherlands). FITC-lectins (MAA, SNA and PNA) were purchased from EYLaboratories (San Mateo, CA).

Cell culture

To obtain monocytes or monocyte derived DCs (mo-DCs), mononuclear cells were isolated by Ficoll gradient centrifugation, from blood buffy coats of healthy volunteers provided by the Portuguese Blood Institute. Monocytes were purified by positive selection using CD14 antibody coated magnetic beads (Miltenyi). One fraction was processed for analysis and another was cultured in complete RPMI medium supplemented with 1,000 U/ml of IL-4 and GM-CSF to give rise to immature mo-DC. The maturation was induced at 6th day culture, by the addition of 5 μg/ml of LPS or 1,000 U/ml of IL-1β, IFN-γ or TNF-α.

Flow cytometry analysis of antibody and lectin cell staining

Staining with mouse IgG anti-human CD14, BDCA-1, CD86 and HLA-DR antibodies was used to monitor monocyte isolation, mo-DC derivation and maturation processes.

10^5 cells were washed, resuspended in serum free RPMI medium and incubated with 10 μl antibodies for 15 min at room temperature, in the dark. Cells were subsequently washed, resuspended in FACS Flow (Becton-Dickinson, Heidelberg, Germany) and analysed in a FacsCalibur Flow cytometer using CellQuest software (Becton Dickinson). For the lectin staining, 10^5 cells were washed, resuspended in serum free RPMI medium and incubated with 50 μg/ml of FITC-lectins for 15 min, at 18°C in the dark. Cells were then washed and resuspended in FACS Flow supplemented with 0,1% BSA.

Sialyltransferase genes expression

Expression of ST genes was analysed by Real-Time PCR. Briefly, total RNA was extracted from 1×10^6 cells, using GenElute Mammalian Total RNA Purification kit and DNAase treatment (Sigma), according to the manufacturer's instructions. 1 μg of total RNA was reverse transcribed with random primers, using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). PCR was performed in a 7500 Fast system (Applied Biosystems) using TaqManFast Universal PCR Master Mix, primers and Taqman probes provided by Applied Biosystems and following the manufacturer's instructions. The reference sequences detected by each primer/probe set and the Assay ID provided by the manufacturer were the following: *ST6Ga1l* (NM173216.1 NM003032.2/Hs00174599_m1); *ST6Gal2* (NM032528/Hs00293264_m1); *ST3Gal1* (NM173344 NM003033/Hs00161688_m1); *ST3Gal2* (NM006927.2/Hs00199480_m1); *ST3Gal3* (NM174969 NM174968 NM006279 NM174972 NM174964 NM174971 NM174963/Hs00196718_m1); *ST3Gal4* (NM006278/Hs00272170_m1); *ST3Gal5* (NM003896.2/Hs00187405_m1); *ST3Gal6* (NM006100.2/Hs00196086_m1); *ST6GalNAc1* (NM018414.2/Hs00300842_m1); *ST6GalNAc2* (NM006456.1/Hs00197670_m1); *ST6GalNAc3* (NM152996.1/Hs00541761_m1); *ST6GalNAc4* (NM175039.1 NM17 5040.1 NM014403.3/Hs00205241_m1); *ST6GalNAc5* (NM030965.1/Hs00229612_m1); *ST6GalNAc6* (NM013443.3/Hs00203739_m1). Data were normalized against the geometric mean of *β-actin* and *GAPDH* genes expression. The relative mRNA levels were expressed in ‰ of the *β-actin*/*GAPDH* expression and were calculated by the formula $2^{-\Delta Ct}*1000$ [11]. The relative expression for each gene was calculated by using the formula $2^{-\Delta\Delta Ct}$, according to the method described by Livak and Schmittgen [12]. ΔCt stands for the difference between the cycle threshold (Ct) of the amplification curve of the target gene and that of the *β-actin*/*GAPDH*. The efficiency of the amplification reaction for each primer-probe is above 95% (as determined by the manufacturer).

Sialyltransferase assay

Total ST activity was assayed in whole homogenates as previously described [13] with minor modifications, using asialotransferrin and Galβ1-3-GalNAcα1-O-benzyl (Sigma) as acceptors. Cell pellets were homogenized in ice-cold water and the protein concentration assessed by Lowry method [14]. The assay mixture contained 50 mM sodium cacodylate buffer, pH 6,2, 0,1%Triton X-100, 4 μM CMP-[^{14}C]Neu5Ac, 6 μM CMP-Neu5Ac, one acceptor substrate (8 mg/ml of glycoprotein or 2 mM of Galβ1-3-GalNAcα1-O-benzyl) and 50 μg of the protein homogenate, in a final volume of 25 μl. The enzyme reaction was performed at 37°C for 2 h. Controls without exogenous acceptors were run in parallel and their incorporation was subtracted. Mice liver homogenates were used as positive controls.

For the glycoprotein acceptor, the reaction was ended with the addition of 1% phosphotungstic acid in 0,5 M HCl (PTA). Acid insoluble material was collected by centrifugation. Precipitates formed were washed twice with PTA and once with methanol, boiled in HCl 1 M for 20 min and then transferred to liquid scintillation vials and processed for scintillation counting. For the benzyl-glycoside, the reaction was stopped with the addition of 0,5 ml H_2O and products were isolated by hydrophobic chromatography on C18 SepPak cartridges (Millipore Corp., Milford, MA, USA). After washing with water, the benzyl-glycoside was eluted with acetonitrile and counted by liquid scintillation. The activity was measured as the amount of radioactive sialic acid transferred from the donor to the acceptor substrate per hour and per amount of protein (nmol/h*μg protein).

Neuraminidase treatment

Cells were resuspended in RPMI (5×10^6/ml) and treated with 200 mU/ml of neuraminidase in serum-free RPMI for 90 min at 37°C. In parallel, identical samples were treated in the same conditions with heat-inactivated neuraminidase (mock treatment). At the end of the incubation, cells were washed and submitted to endocytosis analysis as described below.

Endocytosis assay

Endocytosis assays were performed with monocytes obtained by CD14 antibody positive selection, as described above or immature mo-DCs cultured until 7th day. 3×10^5 cells in RPMI+10% FCS were incubated with 1 mg/ml of FITC-ovalbumin, FITC-dextran or Lucifer Yellow, for 45 min, on ice or at 37°C. Fifty microliters of trypan blue was added at the end of the incubation to quench the surface attached fluorescence. Cells were then washed twice with PBS+1% FCS and analysed by flow cytometry as described.

Statistical analysis

Data was analysed for statistical significance using the Student's *t*-test and ANOVA One-way (GraphPad Prism Version 4.0). Correlations between variables were tested using the Spearman correlation analysis. p values < 0,05 were considered as significant.

Results

Dendritic cells and monocytes exhibit distinct surface sialylation patterns

In order to analyse the modulation of cell surface sialylation during the *in vitro* mo-DC generation, we have examined the surface sialylation pattern in monocytes and respective derived immature DCs, at 2 days interval.

Binding studies of SNA, a lectin which recognizes mainly sialic acid α2-6-linked to lactosamine (6′ sialyl Galβ1-4-GlcNAc), show that monocyte surface has a low density of such a structure, which increases during differentiation into mo-DC, reaching a maximum at day 6 (Fig. 1a). MAA lectin recognizes mainly sialic acid α2-3-linked to lactosamine (3′ sialyl Galβ1-4-GlcNAc) or, to a lesser extent, linked to Galβ1-3-GalNAc. MAA binding increases dramatically during the beginning of the mo-DC differentiation (maximum at day 2), decreasing slightly until mo-DCs are fully differentiated (Fig. 1a), suggesting that monocytes present a low density of α2-3-linked sialic acid, when compared with mo-DC. On the other hand, the overall reactivity of PNA, a lectin specific for the Core 1 structure of the *O*-linked chains (Galβ1-3-GalNAc, also known as T antigen) was much lower than that shown by previous lectins and it increased slightly during the differentiation process (Fig. 1a). PNA binding could be hindered by sialylation of the galactose or of the GalNAc residues in the T antigen.

Sialyltransferase expression is altered during mo-DC differentiation

To investigate the molecular bases of the sialylation changes accompanying the mo-DC differentiation, we studied the expression of relevant STs at the mRNA. When a marked upregulation of the ST gene expression was observed, we also analysed the enzyme activity level, using specific substrates. For the gene expression two parameters were considered, the relative mRNA levels (Fig. 2a, d) and the expression relative to the one expressed by monocytes for differentiation experiments (Fig. 2b, e) or immature DCs at day 8 for maturation experiments (Fig. 2c, f).

ST involved in α2-6-sialylation of N-linked chains

The most relevant enzyme involved in α2-6-sialylation of lactosamine, giving rise to 6' sialyllactosamine (6′ sialyl Galβ1-4-GlcNAc) is by far ST6Gal1. Although a second ST6Gal has been cloned [15, 16], we didn't find any level of expression and ST6Gal2 was not further considered.

As revealed by real time PCR analysis, the level of ST6Gal1 mRNA expression increases during mo-DC differentiation (p<0,01), closely paralleling the increase of SNA reactivity (Fig. 2d, e). Nevertheless, the major correlation is found between the ST6Gal1 expression and the ST activity (r=0.98, p=0.017), measured with the ST6Gal1-specific acceptor, asialotransferrin [17] (Fig. 3). Altogether, these results indicate that mo-DC differentiation is accompanied by a marked increase of *ST6Gal1* gene expression which results in increased enzyme activity and increased α2-6-sialylation of cell surface lactosaminic termini.

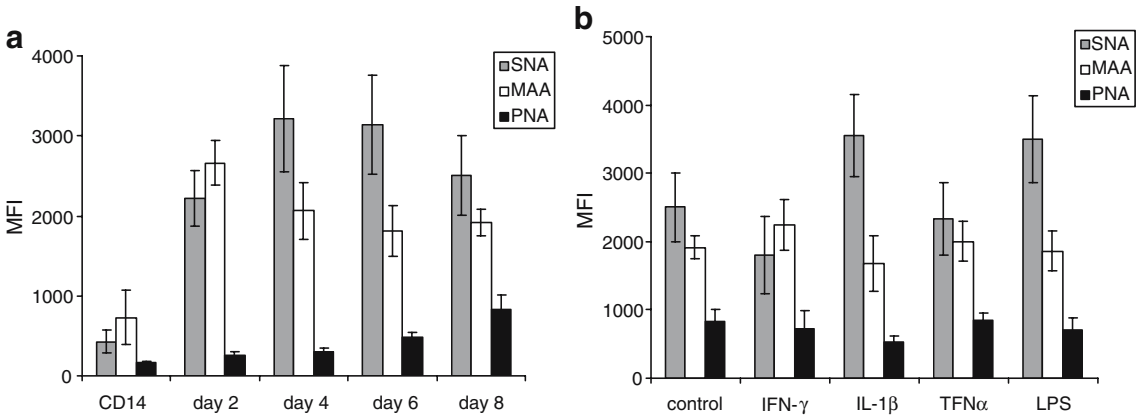

Fig. 1 Lectin binding to cell surface of monocyte (CD14) along its differentiation into DC (**a**) and to DC maturated with IFN-γ, IL-1β, TNF-α and LPS (**b**). Results are the mean fluorescence intensity (*MFI*) obtained by flow cytometry of at least four independent assays. SNA lectin results are represented in *grey*, MAA in *white* and PNA in *black*

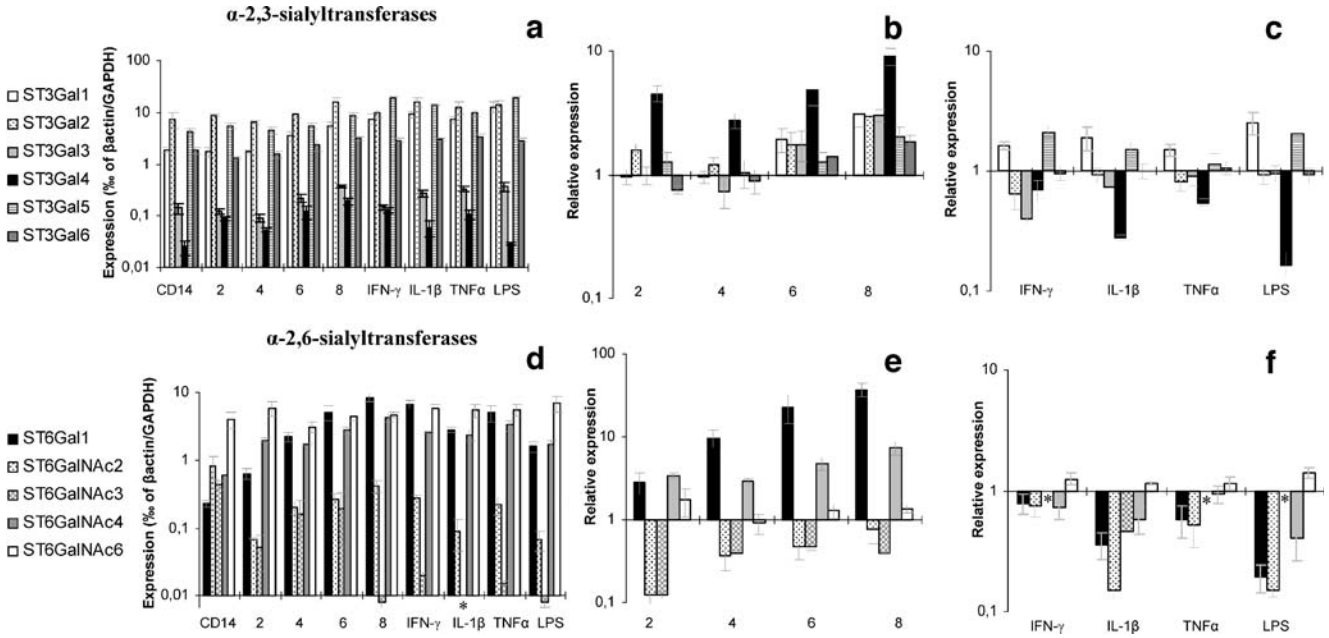

Fig. 2 Relative mRNA level and relative expression of α2,3-sialyltransferases (**a, b, c**) and α2,6-sialyltransferases (**d, e, f**) genes. The mRNA expression level of each gene relative to the endogenous controls, βactin and GAPDH, expression (a, d) was measured from samples collected each 2 days during the differentiation of monocytes (CD14) into DCs (day 2, 4, 6 and 8) and during DC maturation with IFN-γ, IL-1β, TNF-α and LPS. The relative expression compares the expression of a given gene at day 2, 4, 6 and 8 relative to the respective expression in monocytes (B, E) and upon DC activation with IFN-γ, IL-1β, TNF-α and LPS, relative to control, immature DCs at day 8 (**c, f**). Results are the mean of 4 independent assays. *Data from *ST3GalNAc3* expression at minimum and omitted from the graphs

ST involved in α2,3 sialylation of N-linked chains

The superficial α2-3-sialylated lactosaminic chains, which increase during mo-DC differentiation, according to the MAA reactivity, can be sialylated by at least three different STs, namely ST3Gal3, 4 and 6.

The pattern of *ST3Gal4* expression (Fig. 2b) resembles the changes of MAA reactivity, although this correlation is not statistically significant (p=0.360) and the *ST3Gal4* mRNA appears to be expressed at a low level (Fig. 2a). On the other hand, the expression of *ST3Gal3* and *6* mRNA is higher, but shows little changes during differentiation. This finding suggests that ST3Gal4 contributes to the α2-3-sialylation of lactosaminic chains in differentiating monocytes, but the contribution of ST3Gal3 and 6 remains open.

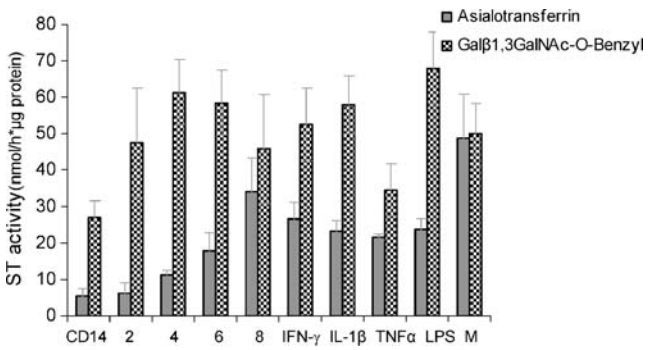

Fig. 3 Level of sialyltransferase activity using asialotransferrin and Galβ1,3GalNAcα1-O-Benzyl glycoside as acceptor substrates. The activity was assessed in sample homogenates collected at two days intervals during the differentiation of monocytes (CD14) into DCs (day 2, 4, 6 and 8) and during DC maturation with IFN-γ, IL-1β, TNF-α and LPS. Mice liver homogenates (*M*) were used as assay control

STs involved in α2-3-sialylation of O-linked chains

The sialylation of galactose residues present in core 1 *O*-glycans is mediated by ST3Gal1, 2 and 4 transferases. According to the real time PCR analysis, besides the above mentioned *ST3Gal4* upregulation, both *ST3Gal1*and *2* genes are also upregulated during mo-DC differentiation with *ST3Gal1* showing a significant increased expression (p<0.05) (Fig. 2b). To corroborate the increased expression of the corresponding enzymes, we have tested the ST activity using Galβ1-3-GalNAc-O-benzyl as an acceptor. The ST activity toward the core 1 structures increases during the mo-DC differentiation process (Fig. 3). These observations could justify somehow the observed low reactivity of PNA, but not its slight increase. It is possible that, at that stage, cells are already saturated with sialylated Galβ1-3-GalNAc *O*-glycans (sialyl T antigens) and, in this context, any increase in α2-3-ST expression will have a negligible influence. In fact, we have observed that mo-DCs dramatically increase their reactivity with PNA lectin after neuraminidase treatment (data not shown), making

evident a significant content of *O*-linked Galβ1-3-GalNAc masked by sialic acid in these cells.

ST involved in α2-6-sialylation of O-linked chains

As shown in Fig. 2d, amongst the genes coding for STs able to catalyze the α2-6- sialylation of the GalNAc residues present on *O*-glycans (*ST6GalNAc1*, *2*, *3* and *4*), *ST6GalNAc4* is the highest expressed, followed by *ST6GalNAc2* and *3*. *ST6GalNAc1* relative mRNA level was very low (<0,01‰ of *βactin*/*GAPDH*) and was not considered further (Fig. 2d). *ST6GalNAc3* was fairly expressed in monocytes, but its level decreases during mo-DC differentiation. Given the *ST6GalNAc4* high mRNA expression (Fig. 2e) and its restricted substrate specificity, recognizing only sialylated Galβ1-3-GalNAc *O*-glycans (sialyl T antigen) [18], it is likely that di-sialyl T antigens are expressed by monocytes and mo-DC.

Maturation induces changes in the sialylation profile of mo-DC

To test the influence of maturation in the sialylation profile, we stimulated mo-DC with TNF-α, IL-1β, LPS or IFN-γ, separately. The mo-DC maturation was confirmed by the increased expression of HLA-DR and the CD86 costimulatory molecule, assessed by flow cytometry (data not shown).

LPS and IL-1β stimuli lead to an increased SNA reactivity, contrarily to IFN-γ and TNF-α, which promote a lower SNA reactivity, suggesting the additional expression of α2-6-sialylated lactosamines in the former (Fig. 1b). Nevertheless, the *ST6Gal1* gene expression (Fig. 2f) and ST activity towards asialotransferrin (Fig. 3) decrease upon all the stimuli and both parameters are highly correlated (r= 0.98, p=0.017). MAA reactivity is not significantly affected upon maturation (Fig. 1b), although the expression of *ST3Gal2* and *4*, which are involved in the synthesis of MAA ligands, decreases with maturation (Fig. 2e). This apparent inconsistency can be explained if one considers that the glycosyltransferases-product relationship is not necessarily linear. In fact, the level of an oligosaccharide product can be regulated at multiple levels, including the action of glycosyltransferases competing for the same acceptor and the action of glycosidases.

On the other hand, the *ST3Gal1* expression increases with all maturation stimuli, except TNF-α (Fig. 2c), and it is significantly correlated with ST activity for Galβ1-3-GalNAc-O-benzyl acceptor (r=0.97, p=0.040). Accordingly, after IL-1β, LPS or IFN-γ stimulation, the PNA reactivity decreases slightly (Fig. 1b), suggesting mo-DC carry out *de novo* synthesis of sialylated T antigens. As expected, the neuraminidase treatment increased PNA binding, revealing the presence of T antigens masked by sialic acid (data not shown).

Neuraminidase treatment affects the endocytosis process in monocytes and in mo-DCs in an inverse manner

DCs use several pathways to capture antigens including macropinocytosis, receptor mediated endocytosis and phagocytosis. The former is described as exclusive of DCs and activated macrophages and is not present in monocytes [19]. In order to verify whether the presence of sialic acid was critical for the endocytosis performed by monocytes and mo-DCs, we have tested the uptake capacity for three endocytic agents after treating both types of cells with neuraminidase. Ovalbumin, Lucifer Yellow and dextran are all described as being uptaken by macropinocytosis by DCs, although a number of receptors have also been identified as involved in the internalization of ovalbumin [20] and dextran is uptaken mainly through mannose receptor [19].

Monocytes treated with neuraminidase present an increased capacity to uptake ovalbumin and dextran, as determined by an higher mean fluorescence intensity, MFI, (1,3 and 2 fold, respectively) when analysed by flow cytometry (Fig. 4, Table 1). The uptake of Lucifer Yellow was almost negligible and unaffected after neuraminidase treatment. By contrast, when compared with the controls, neuraminidase treated mo-DCs showed a decrease in the capacity to uptake all the endocytic tracers tested (Fig. 4). According to the MFI analysis, after neuraminidase treatment, the uptake of ovalbumin, Lucifer Yellow and dextran is decreased to 76, 75 and 85%, respectively and the results have a statistical significance (Table 1). We have also determined the number of cells which underwent endocytosis in the assays performed with the different endocytic tracers and found no significant differences after treating the cells with neuraminidase (Table 1).

Discussion

The importance of sialic acid in the innate and adaptive immune responses appears more and more evident. It is known that the surface α2-6- and α2-3-sialylation of B and T lymphocytes is modulated according to the cell differentiation and maturation [9, 21]. DCs are essential to orchestrate the immune response mediated by lymphocytes, but due to its complex immunobiology, many of its features, such as the sialylation role, are still poorly understood.

The analysis of the ST gene expression demonstrated that all α2,3- and almost all α2,6-ST genes are expressed. Clearly, the α2-3- and α2-6-sialylation profile of monocytes and mo-DCs is a commitment of several STs encoded by different genes.

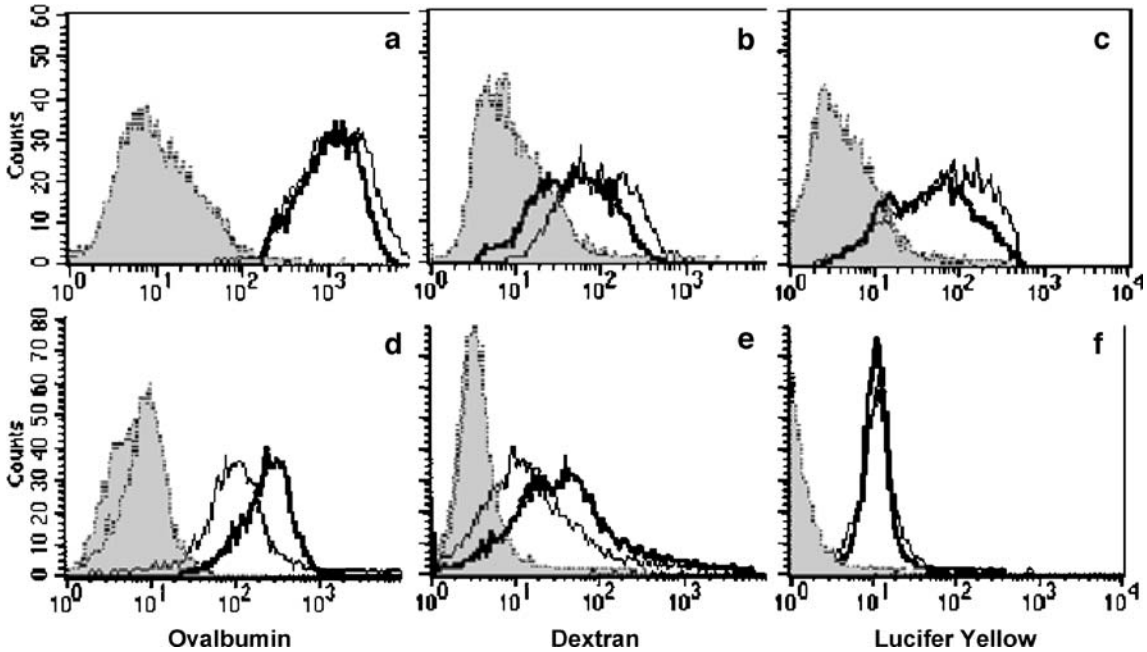

Fig. 4 Effect of neuraminidase treatment on the uptake capacity of immature mo-DC (*upper panels*) and monocytes (*lower panels*). Cells were incubated for 1 h with ovalbumin (**a, d**), dextran (**b, e**) and Lucifer Yellow (**d, f**), as described in Materials and methods and analysed by flow cytometry. Bold and solid lines represent, respectively, neuraminidase treated and non-treated cells incubated at 37°C. *Grey* histograms represent control cells incubated at 0°C. Data shown are a representative of one of different experiments, whose statistical analysis is represented in Table 1

We have observed an increased expression of α2-3-sialylated *O*-glycans and α2-6- and α2-3-sialylated *N*-glycans during the differentiation of monocytes into mo-DCs. It is known that during mo-DC generation, the cell suffers a profound mechanism changing, including the acquisition of specific features such as, increased capacity of antigen uptake and the expression of antigen presenting molecules (MHC class I, MHC class II and CD1 molecules), adhesion and costimulatory molecules (reviewed in [22]). It is, therefore, likely the observed sialylation changes are related with the mechanisms changing during mo-DC.

The expression of sialylated molecules in some cases appears to be under the control of a single ST, in other cases their biosynthesis appears to be more complex. For example, the increase of α2-6-sialylated lactosamines, we detect with SNA during the mo-DC differentiation is clearly related to the increased sialyltransferase activity on asialotransferrin and to the increase of ST6Gal1 transcript. ST6Gal1 is highly expressed in B lymphocytes and one

Table 1 Statistical analysis of the effect of neuraminidase treatment on the endocytosis capacity of mo-DC and monocytes

	Neuraminidase treated		Non treated		Ratio[a]	
	MFI	Percent of fluorescent cells	MFI	Percent of fluorescent cells	MFI	Percent of fluorescent cells
Mo-DC						
Ovalbumin	1239.3±150.4	68.1±8.4	1638.0±231.1	70.1±5.2	0.76**	0.98
Lucifer Yellow	203.3±34.0	32.4±7.1	285.9±45.2	37.6±7.6	0.75*	0.86
Dextran	319.7±45.2	35.4±3.8	372.1±46.2	35.6±5.9	0.85***	0.99
Monocyte						
Ovalbumin	134.3±20.5	79.0±1.7	98.9±18.5	80.0±2.7	1.35*	0.98
Lucifer Yellow	11.0±1.9	87.0±2.1	10.1±1.8	90.0±1.2	1.10	0.96
Dextran	94.7±1.9	80.0±6.2	46.5±1.6	86.5±3.3	2.00***	0.92

[a] The ratio corresponds to the values from neuraminidase treated cells divided by the values of non treated cells

Values are the mean fluorescence intensity (MFI) and percentage of cells which underwent endocytosis (% of fluorescent cells) and represent the mean ± SD of at least four independent assays. The values from the corresponding control cells incubated at 0°C were subtracted

*$p<0.05$; **$p<0.01$;***$p<0.005$

of its best known products is the counter receptor of CD22 [23] but in DC, its specific substrates are still not identified. It is possible that, similarly to B lymphocytes, this 6′ sialyllactosamines are ligands for inhibitory receptors on the surface of DCs or effector T cells, during the antigen presentation.

The increased reactivity with MAA, we observed during mo-DC differentiation, could be attributed to ST3Gal3, ST3Gal4 and/or ST3Gal6. The transcripts of all these three enzymes show a tendency toward upregulation during differentiation, but ST3Gal4 high expression at the beginning of mo-DC differentiation resembles more the level of MAA reactivity. It is reasonable to propose that although ST3Gal4 may have a higher contribution; all the three enzymes contribute to the biosynthesis of MAA ligands.

The low PNA lectin reactivity together with Galβ1-3-GalNAc-*O*-benzyl acceptor enzymatic assays indicate that differentiating mo-DCs express the Core 1 structure of the *O*-linked chains (T antigen) masked by sialic acid. The T antigen can be sialylated in α2-3-linkage to Gal by at least three different transferases, namely ST3Gal1, 2 and 4, yielding the sialyl-T antigen which, in turns, can be further sialylated by ST6GalNAc1, 2, 3 or 4, generating the disialyl T-antigen (reviewed in [24]). According to the real time PCR analysis, all the genes coding for enzymes involved in the α2-3-sialylation of T antigen undergo upregulation during mo-DC differentiation, suggesting the expression of this structure. During mo-DC generation, the *ST3Gal1* expression changes is more similar to the ST activity towards Galβ1-3-GalNAc-O-benzyl acceptor and, the respective enzyme is known to exhibit much more acceptor substrate preference for *O*-linked oligosaccharides of glycoproteins [25] than ST3Gal2 and ST3Gal4 which uses preferentially glycolipids and *N*-glycans, respectively [25, 26]. Therefore, it is likely that the *ST3Gal1* gene is the major responsible for the α2-3-sialylation of T antigens in mo-DC. In addition, the increased expression of *ST3Gal1* during the stimulation of mo-DCs is significantly correlated with the ST activity against the T antigen, indicating an enhanced content of these sialylated structures. Nevertheless, masking by sialylation does not appear to be the major mechanism of regulation of T antigen expression in differentiating mo-DC, as the PNA reactivity does not decrease (as one could expect) but, rather, slightly increases. This means that in mo-DCs probably the main regulation of the T antigen occurs at the level of peptide: GalNAc transferases and/or of β1,3-galactosyltransferase [27].

The STs expressed in differentiating mo-DC, involved in the α2-6-sialylation of the GalNAc residue of the sialyl T structure, ST6GalNAc2, 3 and 4, show opposite behaviours, being the first two downregulated, but poorly expressed and the second slightly upregulated during differentiation. On this base, it is expected that the mo-DC differentiation process affects poorly the expression of the disialyl-T antigen.

The biosynthesis of sialyl LeX, the functional motif of selectin ligands, requires the α2,3 sialylation of lactosamine, followed by the α1-3-fucosylation of 3′ sialyllactosamine. The sialylation reaction may be mediated by ST3Gal4, 6 and, to a lesser degree, ST3Gal3 [28]. The expression of selectin ligands has been demonstrated in DCs derived from bone marrow [29] and we have also observed sialyl LeX antigens in mo-DCs (unpublished data). In neutrophils, ST3Gal4 was found to play the major role [6], although, in this work, *ST3Gal4* exhibited a relative low mRNA level (Fig. 2a) in monocytes and in mo-DCs and ST3Gal6 is probably the main ST involved in the biosynthesis of sialyl LeX in these cells. Nevertheless, further studies are necessary to investigate this assumption.

We have analysed the influence of maturation stimuli in the sialylation pattern of mo-DC. We used independently, TNF-α, IL-1β, LPS and IFN-γ, known for activating DC to polarize Th1 cells, although with slight phenotypical or functional differences [30]. According with the lectin reaction patterns, the major mo-DC phenotype difference after maturation was found for the expression of α2-6-sialylated lactosamines, with each stimulus leading to distinct expression of these structures. Regarding the ST gene expression, all these stimuli lead to a downregulation of *ST6Gal1* and *ST3Gal4* while upregulating *ST3Gal1*. Another research group has recently reported that tolerogenic, immature DCs have a high α2-6-linked sialic acid density, which is decreased after stimulation with a cytokine cocktail (IL-6, IL-1β, TNF-α and PGE_2) [31]. Concomitantly with our results, these authors have also found that *ST6Gal1* is downregulated after stimulation, indicating that this is probably a common pattern of the mo-DC maturation. It is known that the DC maturation leads, generally to the downregulation of the antigen-uptake machinery, upregulation of adhesion and costimulatory molecules and peptide-MHC complexes density on DC cell surface and ultimately the polarization of different T-cell subsets [22]. On this basis, it is possible that the observed expression changes of ST3Gal1, 4 and ST6Gal1 are related with the expression or regulation of specific molecules involved in the maturation.

In our experiments, the endocytic capacity of both monocytes and mo-DCs is affected when the surface sialic acid was removed by neuraminidase treatment. In the case of monocytes, the treatment gives rise to an increased fluorescence intensity of the cells, suggesting an increased amount of endocytosed particles by cell; contrarily to the mo-DCs, whose treatment decreases slightly the amount of endocytosed particles. Either monocytes or mo-DCs are

able to endocytose dextran and ovalbumin through several mechanisms, which include phagocytosis and endocytosis via different groups of receptor families, such as Fc receptors, Toll-like receptors and C-type lectin receptors [32]. Nevertheless, both cells present distinct distribution of endocytic receptors with DC presenting an higher content and also specific receptors such as DC-SIGN and DEC-205 [33, 34]. In DC there is also the additional contribution of macropinocytosis, the actin-dependent formation of large vesicles for fluid-phase solutes uptake. Macropinocytosis is absent in monocytes, which is in accordance with the negligible Lucifer Yellow endocytosis, observed in the monocyte endocytosis assays (Fig. 4). Since the sialic acid removal affects the uptake capacity in an opposite manner in monocytes and mo-DCs, it could be suggested that the reduction in sialylation affects negatively an uptake mechanism exclusive of mo-DC, such as the macropinocytosis, but on the other hand, it promotes a further uptake mechanism probably shared by monocytes and mo-DCs. Taking into account these results and the fact that during the maturation of DCs, where its endocytic capacity decreases, ST6Gal1 and ST3Gal4 enzymes are significantly downregulated, it could be also suggested that these enzymes may be involved in the DC endocytosis. The downregulation of these STs could be associated with the decreased expression of sialylated molecules involved in the endocytosis or to the decreased sialylation of such molecules leading to its lower activity. Nevertheless, the information regarding the glycosylation of molecules involved in the antigen uptake is still scarce and further studies remain to be performed.

Our findings prompt the sialylation pattern as a useful tool to manipulate the antigen uptake in mo-DCs, which should be considered when using mo-DC in cell- based vaccines. However, further studies are still necessary to better understand the role of sialic acid in signaling a specific endocytosis mechanism and in the DC capacity to modulate the immune response.

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