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Sialyl Tn-expressing bladder cancer cells induce a tolerogenic phenotype in innate and adaptive immune cells

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ABSTRACT

Despite the wide acceptance that glycans are centrally implicated in immunity, exactly how they contribute to the tilt immune response remains poorly defined. In this study, we sought to evaluate the impact of the malignant phenotype-associated glycan, sialyl-Tn (STn) in the function of the key orchestrators of the immune response, the dendritic cells (DCs). In high grade bladder cancer tissue, the STn antigen is significantly overexpressed and correlated with the increased expression of ST6GALNAC1 sialyltransferase. Bladder cancer tissue presenting elevated expression of ST6GALNAC1 showed a correlation with increased expression of CD1a, a marker for bladder immature DCs and showed concomitant low levels of Th1-inducing cytokines IL-12 and TNF-α. *In vitro*, human DCs co-incubated with STn^+ bladder cancer cells, had an immature phenotype ($MHC\text{-}II^{low}$, $CD80^{low}$ and $CD86^{low}$) and were unresponsive to further maturation stimuli. When contacting with STn^+ cancer cells, DCs expressed significantly less IL-12 and TNF-α. Consistent with a tolerogenic DC profile, T cells that were primed by DCs pulsed with antigens derived from STn^+ cancer cells were not activated and showed a $FoxP3^{high}$ $IFN\text{-}\gamma^{low}$ phenotype. Blockade of STn antigens and of STn^+ glycoprotein, CD44 and MUC1, in STn^+ cancer cells was able to lower the induction of tolerance and DCs become more mature.

Overall, our data suggest that STn-expressing cancer cells impair DC maturation and endow DCs with a tolerogenic function, limiting their capacity to trigger protective anti-tumour T cell responses. STn antigens and, in particular, STn^+ glycoproteins are potential targets for circumventing tumour-induced tolerogenic mechanisms.

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1. Introduction

The sialyl-Tn (STn) antigen is one of the most common tumour-associated carbohydrate antigen, expressed by more than 80% of human carcinomas but rarely observed in normal tissues (Cao et al., 1996). STn is a posttranslational modification of cell surface glycoproteins commonly resulting from the overexpression of the ST6GALNAC1 sialyltransferase. This enzyme transfers a sialic acid to the *O*-6 position of *N*-acetylgalactosamine residue linked to a serine or a threonine (GalNAcα-*O*-Ser/Thr) on a given protein, blocking the typical elongation of *O*-glycosidic chains in glycoproteins. STn expression in cancer is associated with adverse outcome and decreased overall survival of the patients (Itzkowitz et al., 1990; Werther et al., 1996). It has been reported that STn expression by cancer cells is associated with many malignant features such as invasiveness (Julien et al., 2012; Pinho et al., 2007), and with epithelial to mesenchymal transition (Lin et al., 2009), a loss of cell differentiation that is an important milestone towards cancer metastasis.

STn is highly expressed in high-grade bladder tumours, which present elevated proliferation rates and high risk of recurrence/progression. In bladder cancer, STn enhances motility and invasive capacity of the cancer cells, thus being associated with malignancy (Ferreira et al., 2013).

The expression of STn is clinically relevant, not only as a marker for diagnosis and prognosis in cancer (the CA72-4 serological test), but also as target for therapeutic strategies. One example is a vaccine consisting of STn-clustered epitopes, Theratope, that has been meaningfully used in clinical trials to immunize breast cancer patients (Adis International, 2003; Holmberg and Sandmaier, 2004; Julien et al., 2012). Other STn-based vaccines have also been developed for application in clinical trial, which includes multi-epitope vaccines containing STn and other tumour-associated carbohydrates and STn-expressing glycopeptides (Heimburg-Molinaro et al., 2011; Madsen et al., 2013; Niederhafner et al., 2008; Slovin et al., 2007). Nevertheless, STn-based vaccines have had limited success (Miles et al., 2011) probably because the pathophysiological role of STn-epitopes in cancer cells remains unclear. One of the factors that has been highlighted by many authors is the low immunogenicity of STn-based vaccines (Julien et al., 2012; Lakshminarayanan et al., 2012; Monti et al., 2004). Thus, the elucidation of the immune mechanisms affected by the expression of STn by cancer cells will contribute to improve anti-STn immunotherapy approaches.

Glycans are involved in multiple biological functions, controlling many features of the immune response. In fact the diversity of glycans and of glycan-recognizing receptors, known as lectins, is highly regulated by the immune cells. One of the examples is the modulation of dendritic cell (DC) functions by changes of their glycan phenotype during differentiation and maturation (Crespo et al., 2009; Videira et al., 2008).

DCs play a unique and decisive role in tumour immunity, being capable of activating antigen-specific T cells against cancer cells (Banchereau and Steinman, 1998). To efficiently prime T cells, DCs undergo maturation, which includes the downregulation of the antigen-uptake machinery, the upregulation of antigen presenting molecules, class I and class II MHC; costimulatory molecules, such as CD80 and CD86 and the synthesis of immune-enhancing cytokines (Langenkamp et al., 2000). However, the degree of DC maturation is dependent on the type of stimulus and tumour cells usually prevent maturation, through many immunosuppressive strategies employed *in situ*. Tumours render DC tolerogenic and bias the immune response in favour of their own progression (Almand et al., 2001; Vicari et al., 2002). The presence of immature DCs at tumour sites is consistent with DC involvement in the tumour progression, most likely by inducing immune unresponsiveness, i.e. tolerance against cancer (Almand et al., 2001). We have previously reported, in bladder cancer tissue, that the lower expression of markers of DC maturation, such as MHC-II, was correlated with risk for recurrence (Videira et al., 2009a). Tumour-residing DCs showing limited maturation or anergy, hold back strategies to induce immune responses and create one important obstacle to the efficacy of immune-based-therapies. Concordantly, in bladder cancer tissue, lower levels of mature DCs are associated with low responses to the *Bacillus Calmette-Guerin* (BCG) immunotherapy, the gold standard treatment for the prophylaxis and management of non-muscle invasive bladder cancer (Beatty et al., 2004; Videira et al., 2009a).

It has been described that STn epitope may confer, to different cancer cell, protection from immune defence thus contributing to malignant phenotype and cancer progression (Monti et al., 2004; Ozaki et al., 2012). Mucins, and in particular STn^+ MUC1 mucins released by cancer cells inhibited DC maturation and modulate DCs towards IL-10^{high} IL-12^{low} regulatory antigen presenting cells with a limited capacity to trigger protective T helper type 1 (Th1) responses (Monti et al., 2004). Interestingly, soluble aberrantly glycosylated MUC1 has also been described to elicit maturation, yet unable to promote Th1 responses (Carlos et al., 2005). While the effect of glycoproteins secreted by tumours is becoming more elucidated, the role of the overall STn expression at tumour cell surface in immunomodulation, remains unknown. Therefore, in this study, we further investigated the influence of STn expression by bladder cancer cells on the immune potency and functionality of human DCs.

2. Material and methods

2.1. Reagents

Fluorescently-conjugated or unlabelled anti-CD14 (M5E2), anti-CD80 (2D10), anti-CD86 (IT2.2) and anti-CD45 (HI30) monoclonal antibodies (mAbs) were purchased from BD Biosciences (San Jose, CA). Anti-MHC-II (L243) and anti-CD1a mAb (HI149) were from Miltenyi Biotec (Bergisch Gladbach, Germany). As anti-STn, we used the HB-STn1 mAb from Dako (Dako Cytomation, Denmark) or clone TKH2 (Kjeldsen et al., 1988). Clone HMFG-2 was used as anti-MUC1 (Griffiths et al., 1986). Interleukin (IL)-4 and Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) were purchased from R&D Systems (Minneapolis, MN). Sialidase from *Clostridium perfringens* was from Roche Diagnostics (Basel, Switzerland) and carboxi-fluorescein diacetate succinimidyl ester (CFSE) from Molecular Probes (Leiden, The

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Netherlands). All other reagents were from Sigma (St. Louis, MO, USA) unless otherwise stated.

2.2. Patient and tissue specimens

This study involved 49 patients, from Hospital São José in Lisbon, who underwent transurethral resection of the bladder tumours. Matched pairs of histologically verified bladder tumours and normal appearing mucosa remote from the tumour were collected and analysed individually. Based on urothelial carcinoma grading and staging criteria of the World Health Organization 132 (WHO), three different groups were considered (Table 1), low-grade (LG, $n = 22$) and high-grade HG non muscle-invasive (NMIBC, $n = 21$) and muscle-invasive (MIBC, $n = 6$) bladder cancers. None of these patients had received prior adjuvant therapy. Patients with *carcinoma in situ* (CIS) were not included, as well as patients with presence of upper tract malignancy, other malignancies, and chronic infections, women expectant or lactating and patients with congenital or acquired immunodeficiency. Prior patient consent and approval from the institute research ethics committee were obtained.

2.3. Histological analysis

The immunohistochemical analysis was performed in automated equipment (Ventana BenchMark® ULTRA). All reagents were from Ventana, USA, unless otherwise stated. Briefly, the slides were heated at 72 °C, deparaffinized with EZ Prep and antigenic recovery at 97 °C. Endogenous peroxidase was blocked with 3% of hydrogen peroxide and the slides were incubated with TKH2 mAb (1:10). This was followed by amplification with HRP UltraView Universal Multimer, revelation with UltraView Universal DAB Chromogen and DAB H202 and the intensification was achieved with UltraView Universal DAB Copper. The nuclear contrast was performed with hematoxylin and bluing. After the immunohistochemical technique, the slides were washed, dehydrated, treated with increasing concentrations of alcohol (75%, 90% and 99%) for 1 min each, cleared in xylene and mounted with synthetic mounting medium (Quick-D-M-Klinipath). A semi-quantitative approach was established to score STn expression based on the percentage of tumour that stained positively in comparison to the tumour bulk. The STn expression was assessed double-blindly by two independent observers and validated by an experienced pathologist. Whenever there was a disagreement, the slides were reviewed, until a consensus was reached.

Table 1 – Information of patients included in this study.

	Number of cancer patients	Age, years median (range)
Total number of cases	49	
Muscle invasive (MIBC)	6	65.1 (56–79)
Non muscle invasive (NMIBC)		
High grade	21	68.7 (47–84)
Low grade	22	69.9 (55–90)

2.4. Cell isolation and culture

Monocytes were isolated by positive selection using anti-CD14 coated magnetic beads (Miltenyi Biotech, Germany) from peripheral blood mononuclear cells (PBMCs) of healthy volunteers, provided and ethically approved by the Portuguese Blood Institute. Monocytes were differentiated into immature mo-DCs (mo-DCs) as described (Videira et al., 2008). Whenever needed, maturation of mo-DCs (mmo-DCs) was induced, at day 5, with 1 μg/ml of lipopolysaccharide (LPS), for 24 h.

2.5. Flow cytometry

Cell purity, differentiation and maturation of mo-DCs was assessed by staining with fluorescein isothiocyanate (FITC)-labelled mAbs against CD14, BDCA-1, CD80 and CD86 or Allophycocyanin (APC)-labelled anti-HLA-DR. Unlabelled mouse anti-STn, -CD44 or -MUC1, followed by anti-mouse Ig-FITC were used to characterize MCR cells. Flow cytometry acquisition was performed, using a FacsCalibur Flow Cytometer. File data was analysed using the CELL QUEST (BD Biosciences) and FLOWING (Turku, Finland) software to discriminate specific populations, and to determine the mean fluorescence intensity (MFI) of the cells.

2.6. Cell lines

The human bladder cancer cell line variants, MCRcont and MCRSTn, were generated as described (Ferreira et al., 2013) and were grown in Dulbecco's modified Eagle medium (DMEM) (Sigma), supplemented with foetal bovine serum, glutamine, penicillin and streptomycin.

2.7. Confocal laser scanning microscopy

Cells were cultured in cover glasses, fixed with 3.7% paraformaldehyde and permeabilized with 0.1% TritonX-100. After blocking with 1% bovine serum albumin (BSA), cells were stained using anti-STn, clone TKH2, or anti-ST6GALNAC1, clone 2C3 (Marcos et al., 2011), followed by fluorescent polyclonal anti-Ig antibody. The cell nuclei were stained with 1 μM TO-PRO-3 dye (Molecular Probes, Leiden, Netherlands). Images were acquired with a Leica TCS SP2 AOBS confocal microscope (Leica Microsystem, Mannheim, GmbH). Representative confocal cross-section images were selected after Z-stacking.

2.8. Establishment of DC: bladder cancer cell lines cocultures

Cancer cell lines were incubated at 0.2×10^6 cells/ml in a 6-well plate, at 37 °C. After 24 h, mo-DCs were added in the proportion of 1:5 (cancer cell: mo-DC) in adhesion buffer (20 mmol/l of trizma hydrochloride, 150 mmol/l of sodium chloride, 1 mmol/l calcium chloride, 1 mmol/l magnesium chloride and 0.5% BSA, pH 8.0) at 37 °C. After 2 h incubation, the non-adherent mo-DCs were washed and the coculture of MCR with adherent mo-DCs was stained with mAbs against MHC-II, which is expressed by DCs, but not by MCR cell lines, thus allowing to assess the percentage of adhering mo-DCs in the coculture. The cell viability was measured by annexin-V

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staining. Some experiments were performed in adhesion buffer without calcium or magnesium, to assess the impact of divalent ions. In other experiments, mo-DCs were incubated with 24 h supernatants obtained from MCR cell cultures to assess the requirement for cell: cell interactions. When testing function blocking mAb, to avoid unspecific Fc receptor-mediated mAb binding to mo-DCs, the Fc receptors from mo-DCs were blocked with 10% human serum. In addition, the blocking antibodies were added to the MCR cells and not to mo-DCs. We used 20 μg/ml of anti-CD44 mAb (clone IM7), 1:100 of anti-STn mAb (clone HB-STn1), 1:20 of anti-MUC1 (clone HMFG-2) or isotype control for 30 min, washed and then cocultured the mAb coated MCR cells with mo-DCs, as described above.

2.9. Gene expression analysis by real-time PCR

RNA extraction from formalin-fixed, paraffin embedded sections was performed after deparaffinization of tissues, using Absolutely RNA FFPE kit (Agilent technologies) while RNA from the cocultures was isolated using the GenElute Mammalian Total RNA Purification kit (Sigma), according to the manufacturer's instructions. After DNAase treatment, 1 μg of total RNA was reverse transcribed with random primers and the real-time PCR was performed with Master Mix, TaqMan probes and primers from Applied Biosystems. The assay ID provided by the manufacturer were: Hs00300842_m1 (*ST6GALNAC1*); Hs00168405_m1 (*IL-12α*); Hs00174128_m1 (*TNF-α*); Hs00174086_m1 (*IL-10*); Hs00372324_m1 (*IL-23*), Hs00203958_m1 (*FoxP3*) and Hs00174143_m1 (*IFN-γ*). The relative mRNA levels were normalized against the arithmetic mean of the *β-actin* and GAPDH expression and calculated by adapted formula $2^{-\Delta Ct} \times 1000$ which infers the number of mRNA molecules of the gene of interest per 1000 molecules of the endogenous controls (Videira et al., 2009b). ΔCt stands for the difference between the cycle threshold of the target gene and that of the endogenous control genes. The efficiency for each primer/probe was above 95% (as determined by the manufacturer). When analysing the gene expression of mo-DCs in the coculture, the contamination with MCR cells was disregarded since the analysis of cytokine gene expression in both MCR cell line variants showed a completely absence of *IL-12α*, *TNF-α*, *IL-10*, *IL-23*, *FoxP3* and *IFN-γ* gene expression.

2.10. Phagocytosis assay

Cell lines were labelled with CFSE according to the manufacturer's instructions and then induced to apoptosis with 10 μM of camptothecin. After 48 h, cells were incubated with mo-DCs in the proportion of 1:2, in a 48-well plate, for 6 h at 37 °C or 4 °C. After incubation, cells were stained with anti-MHC-II mAb and the percentage of MHC-II$^+$/CFSE$^+$ cells (mo-DCs that phagocytosed MCR cells) was calculated by flow cytometry and confirmed by confocal microscopy. The values obtained at 4 °C were subtracted from the 37 °C values.

2.11. T cell activation

Human T cells were obtained during monocyte isolation procedure (CD14$^-$ PBMC fraction) and maintained in complete RPMI medium until complete mo-DC differentiation. Autologous T cells were then incubated with mo-DCs following phagocytosis of MCR cells, as described above, in the proportion of 8:1, in a 96-well round bottom plate, throughout 11 days. As controls, similar experiments were performed with unstimulated mo-DC or with the MCR cell lines (negative controls) or with phytohaemagglutinin (positive control). T cell activation was assessed by the percentage of CD69$^+$ cells within the CD3$^+$ population (T cells) and their MFI values were evaluated by flow cytometry.

2.12. Protein extraction, immunoprecipitation and western blotting

Proteins were isolated from cell lines using RIPA buffer (Sigma–Aldrich) and bladder tumour proteins were extracted from formalin-fixed paraffin embedded tissues using the Qproteome FFPE tissue kit (Qiagen). The amount of protein was estimated with RC protein assay kit (BioRad). CD44 protein was immunoprecipitated from total protein extracts (IP) with anti-CD44 monoclonal antibody (2C5 clone; R&D Systems) using Pierce Direct IP Kit (Thermo Scientific). Protein samples were separated in reducing SDS-PAGE gels, using 20 μg per lane, transferred to 0.45 μm nitrocellulose membrane (GE Healthcare Life Sciences) and blocked with 1% Carbo-Free Blocking Solution (Vector Laboratories). TKH2 and goat anti-mouse IgG1 heavy chain horseradish peroxidase conjugate (Abcam) were used as primary and secondary antibodies, respectively. The Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences) was used as developing reagent. Protein extracts treated with sialidase were used as controls.

2.13. Statistical analysis

Statistical analyses were conducted using GraphPad Prism software, version 5.0 (GraphPad Software, La Jolla, CA). Paired student's *t*-test was used when data was normally distributed, and the results expressed as the mean values ±SDs. Alternatively, Wilcoxon signed-rank test was used in the case of non-normal distribution, and the results were plotted individually or as box and whiskers plot. The correlations were analysed using Spearman and Pearson methods. Tests were considered statistically significant when $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.005$ (***) and marginal significance was considered for $p < 0.1$.

3. Results

3.1. The STn antigen expression and DCs are increased in bladder cancer

We have previously reported that STn is highly overexpressed in bladder cancer tissues, showing a markedly high incidence and intensity only in malignant tissue and was barely detectable in normal mucosa (Ferreira et al., 2013) (Figure 1A). Considering the tumour bulk the maximum STn expression was 30%. The STn expression is highly correlated with the gene expression of the sialyltransferase ST6GALNAC1

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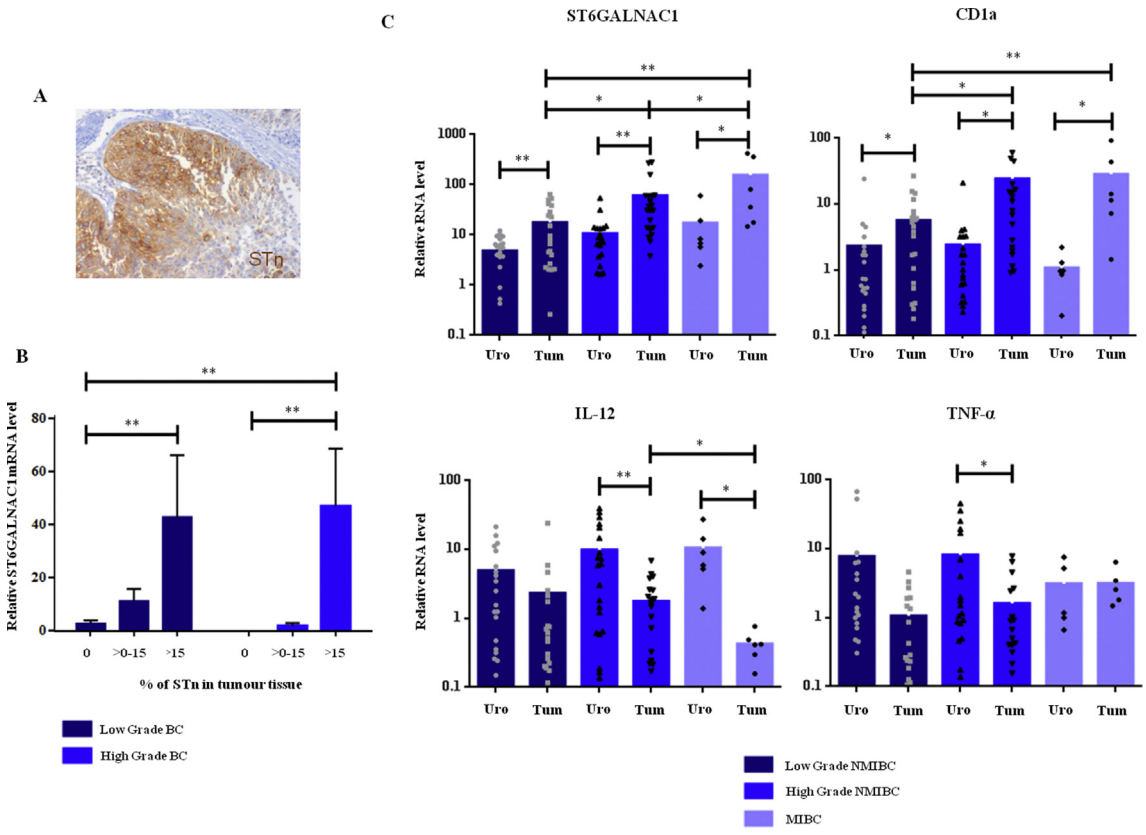

Figure 1 – Bladder cancer tissues show differential expression of STn antigen, CD1a, IL-12 and TNF-α cytokines. A: Analysis of STn expression in high grade tumour bladder cancer tissue. Representative image of a paraffin embedded section, processed for immunohistochemical staining with anti-STn mAb. STn expression was detected in tumour tissue. B: Association between ST6GALNAC1 and STn expression in bladder tumours. STn expression was determined in low and high grade bladder tumours, by immunohistochemistry, using TKH2 mAbs. Specimens were then selected and distributed into three groups, according to their expression of STn related with the tumour bulk: 0%, 0–15% and more than 15% of tissue expressing STn. The relative mRNA levels of *ST6GALNAC1* gene, in the paraffin-embedded sections, was analysed individually, by real time PCR and paired compared with data regarding STn expression. Values infer the number of mRNA molecules of a *ST6GALNAC1* gene, per 1000 molecules of the average of the endogenous controls. C: Gene expression analysis in bladder tissue. The relative mRNA levels of *CD1a*, *IL-12* and *TNF-α* cytokines and *ST6GALNAC1* genes were analysed by real time RT-PCR in specimens from low and high grade non-muscle invasive bladder cancer (NMIBC) ($n = 22$ and $n = 21$, respectively) and muscle invasive bladder cancer (MIBC) ($n = 6$) tissues and also from matched normal urothelium ($n = 49$). Values infer the number of mRNA molecules of each gene per 1000 molecules of the average of the endogenous controls. *CD1a* and *ST6GALNAC1* expression was significantly increased in all tumour tissue, as compared with matched urothelium; while *IL-12* and *TNF-α* cytokines were decreased ($p < 0.05$ (*) and $p < 0.01$ (**)). In general, the differences were more evident in high grade NMIBC.

(Figure 1B) either in low and high grade tumours. To assess whether STn expression was correlated with altered immune function, we quantified the expression of ST6GALNAC1 and CD1a, a marker for immature DC subtype predominant in bladder carcinoma (Figure 1S) (Troy et al., 1999). We also investigated the expression of interleukin (IL)-12, and tumour necrosis factor (TNF)-α, cytokines naturally expressed by DCs and involved in inducing Th1-immune responses. We have analysed tumour tissue and adjacent normal urothelium, and our data showed that both CD1a and ST6GALNAC1 are upregulated in tumour tissue, as compared with corresponding normal urothelium. Interestingly, the upregulation was more pronounced and significantly correlated in muscle invasive and high grade non muscle invasive tumours rather than low grade tumours (Figure 1C). By contrast IL-12 and TNF-α expression, which is associated with DC maturation and Th1-inducing, showed an inverse correspondence with significantly lower expression in high grade tumour tissue than low grade tumour tissue. A correlation was observable between *CD1a* and *ST6GALNAC1* ($r = 0.41$ and $p = 0.003$) expression, pointing out for an association between STn expression by cancer cells and the presence of immature DCs in tumour tissue.

3.2. *Mo-DCs tend to adhere to STn$^+$ bladder cancer cell lines and show significant less mature phenotype*

To further investigate the functional implications of STn-expressing cancer interaction on DC functionality, we established *in vitro* coculture models with STn$^+$ bladder cancer cell lines and human mo-DCs. The transduction of *ST6GALNAC1* sialyltransferase cDNA in MCR bladder cancer cell lines, that natively do not express STn, induced a dramatic expression of STn (Figure 2A), corroborating the role of ST6GALNAC1

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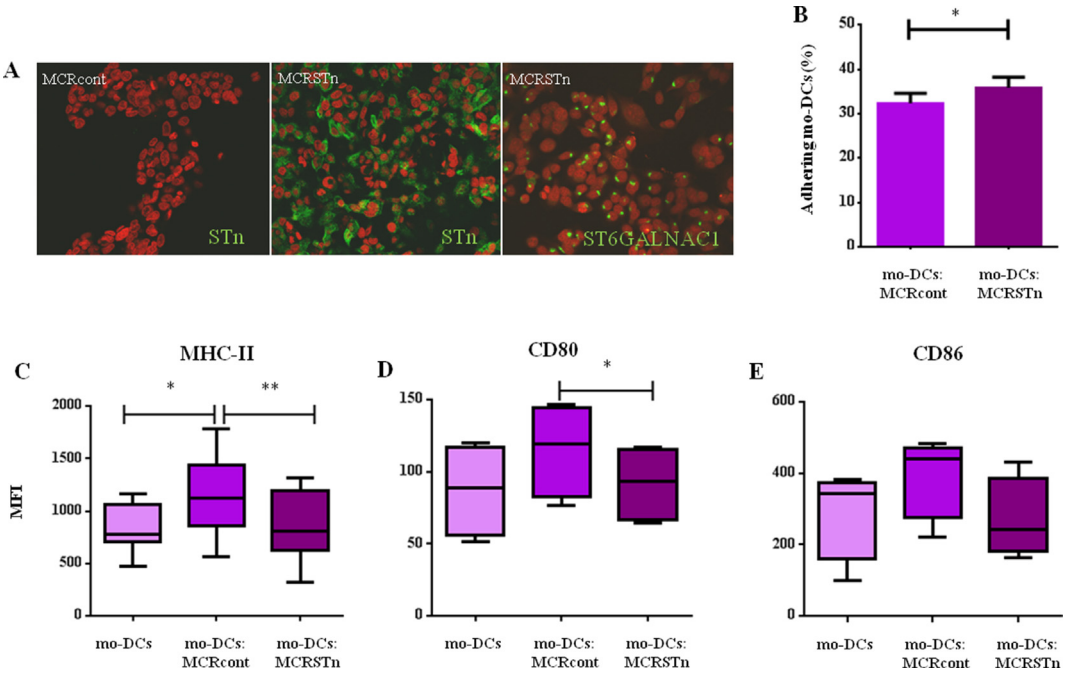

Figure 2 – Mo-DCs adhere preferentially to STn$^+$ MCR cell line and show a less mature phenotype. A: Overexpression of ST6GALNAC1 in MCR bladder cancer cells. MCR cells were transduced or not with a retroviral vector expressing the whole coding region of human *ST6GALNAC1*. Both negative control (MCRcont) and ST6GALNAC1-transduced (MCRSTn) cell lines were stained with anti-STn or anti-ST6GALNAC1 mAbs and then analysed by confocal microscopy. The MCRSTn cell line, but not MCRcont cells expressed the STn antigen and ST6GALNAC1. B: Characteristics of mo-DCs adherent to MCR cell lines. Mo-DCs were cocultured with MCR cell lines and after 2 h incubation, non-adherent mo-DCs were washed and the percentage of mo-DCs adherent to MCR cell lines was estimated by flow cytometry, as the total of MHC-II$^+$ cells in the coculture, following staining with APC-labelled anti-MHC-II mAb. The mean number of mo-DCs adhering to MCR cells was significantly higher in the co-incubation with MCRSTn than with MCRcont ($p = 0.045$ (*), $n = 14$). C-E: Analysis of mo-DC maturation and co-stimulatory profile. Adherent mo-DCs were stained with anti-MHC-II (C), anti-CD80 (D) or CD86 (E) mAbs and then analysed by flow cytometry. The expression level of the antigens was inferred from the mean fluorescence intensity (MFI) of the cells. Values are displayed as box-and-whisker plot with mean and quartiles ± maximum/minimum of 5 independent assays. The expression of MHC-II and CD80 was significantly different in mo-DCs co-incubated with MCRSTn, as compared with MCRcont ($p = 0.0043$ (**) and $p = 0.0438$ (*), respectively). MFI values in mo-DCs alone were comparable to mo-DCs co-incubated with MCRSTn, while mo-DCs co-incubated with MCRcont showed a significant more mature phenotype.

in STn biosynthesis by bladder cancer cells (Ferreira et al., 2013). The obtained STn$^+$ (MCRSTn) and mock transduced STn$^-$ (MCRcont) cell lines were used throughout this study. Co-incubation of human mo-DCs together with MCR cells showed that a significant number of mo-DCs adhered to the cancer cells. The mean percentage of adhering mo-DCs was significantly higher in the coculture with MCRSTn (1.27-fold more) than with MCRcont (Figure 2B). We also investigate whether the observed cellular interactions were dependent on divalent ions, a characteristic of many intercellular interactions. In the absence of divalent ions, mo-DC adhesion to bladder cancer cells was reduced to an average of 10% adherent cells and it was not statistically different between the cocultures with each of two cell line variants (data not shown), suggesting that the differences in adhesion of mo-DCs to MCR cells were dependent on divalent cations.

To characterize the maturation phenotype of the mo-DCs adhering to the bladder cancer cells we assessed the expression of the MHC-II antigen presenting molecule, and co-stimulatory molecules, CD80 and CD86. While the mo-DCs co-incubated with MCRcont cells showed increased expression of MHC-II, CD80 and CD86, mo-DCs adhering to MCRSTn presented an expression level similar to the non-stimulated mo-DCs, suggesting that no maturation was induced (Figure 2C, D and E). To verify whether the immature phenotype of mo-DCs incubated with MCRSTn was dependent on cell contact, parallel experiences were performed where mo-DCs were incubated in the presence of conditioned media, obtained from MCRcont or MCRSTn cell cultures. However, in these conditions the phenotype of mo-DCs incubated with the conditioned medium from either MCR variants was similar (data not shown). The viability of the mo-DCs in culture was approximately 97%, indicating that the observed differences in adhesion and maturation were not due to loss of mo-DC viability. Effects on the MCR cells was also observed, but only when the cocultures were prolonged until 24 h. Specifically, we observed that the proliferative capacity of MCRSTn cells increased 26% ($p = 0.007$), as compared with the proliferative capacity of this cell line in the absence of mo-DCs (Figure 2S).

In order to confirm that the effects on maturation and adhesion to mo-DCs were specifically caused by the enhanced expression of STn, the adhesion assays were performed with a different cell line, namely the breast cancer cell line, MDA-MB-231STn, overexpressing the STn antigen (Julien et al.,

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2001). Similarly to MCRSTn, mo-DCs adhered significantly more to MDA-MB-231STn cells when compared to control cells, and these mo-DCs expressed significantly less MHC-II on their surface, showing a less mature phenotype (Figure 3S). Therefore, the same negative effects is obtained after co-incubation of mo-DCs with STn$^+$ cancer cells, irrespective of the cancer type, corroborating the immunomodulatory role specifically for STn.

We then investigated the effect of STn$^+$ cancer cells in semi-matured mo-DCs. We stimulated mo-DCs with LPS, a canonical inducer of mo-DC maturation, which resulted in a remarkable increase of MHC-II expression by DCs (Figure 4SA). In our conditions, LPS-matured DCs could still respond to secondary maturation stimuli and were therefore referred semi-mature. We observed that semi-matured mo-DCs adhere significantly less to both MCRcont and MCRSTn, when compared with immature mo-DCs (55% and 61% less, respectively, data not shown). After co-incubation of semi-matured mo-DCs with MCRcont, a significant increased expression of MHC-II was observed ($p = 0.043$), while no significant effect was obtained by a parallel incubation with MCRSTn cells (Figure 4SA).

To assess whether the contact with MCR cancer cells influenced further mo-DC responses to maturation stimulus, we stimulated mo-DCs with LPS, after coculture with MCR cells. In the presence of the cancer cells, mo-DCs showed defective LPS-induced maturation, while in their absence mo-DCs underwent maturation (Figure 4SB). The resistance to maturation was more evident when mo-DCs were previously incubated with MCRSTn, as compared with MCRcont cancer cells. Thus, the data suggest that the contact with STn$^+$ bladder cancer cells not only hinders maturation, but also prevents further mo-DC maturation.

3.3. *Contact with STn$^+$ cancer cells compromises mo-DC cytokine expression and phagocytosis*

Next, we investigated the function of mo-DCs following contact with STn$^+$ cancer cells. We analysed the expression of several cytokine genes and found that the expression of IL-12, TNF-α, IL-23 and IL-10 was significantly decreased in mo-DCs co-incubated with MCRSTn, as compared with controls (Figure 3A). Analysis of the level of phosphorylated extracellular signal-regulated kinase (ERK), which is involved in cytokine expression, was reduced by 21% in mo-DCs following contact with MCRSTn cells (data not shown).

Since mo-DCs physiologically phagocyte apoptotic cancer cells, we then compared the capacity of mo-DCs to phagocyte both MCR cancer cell line variants. As shown in Figure 4A and B, the percentage of mo-DCs which phagocytosed MCRSTn is significantly higher (1.25-fold more) than those that phagocytosed MCRcont. Confocal microscopy analysis confirmed the internalization of MCRSTn cells by mo-DCs (Figure 4B).

3.4. *T cells activated with mo-DC loaded with STn$^+$ cancer cells show defective activation profiles*

We next analysed the capacity of mo-DCs to activate autologous T cells by measuring the expression of the T cell activation marker CD69. We observed that the mo-DCs that phagocytosed MCRSTn tended to activate significantly less number of T cells, and more weakly, than mo-DCs phagocytosing MCRcont (Figure 5A and B). T cell activation was also lower in T cells primed by mo-DCs that adhered to STn$^+$ cancer cells (Figure 5S), as compared to control MCR cells. T cells alone and T cells incubated with each apoptotic MCR cell lines (with no mo-DCs) were not significantly activated and lost viability (less than 5% viable cells after 11 days). By contrast, after 11 days, more than 35% of the T cells cocultured with mo-DCs remained viable, suggesting that mo-DCs stimulus is necessary to maintain T cell viability. Interestingly, in the T cell: mo-DC (MCRSTn) coculture, the expression of interferon (IFN)-γ and the transcription factor forkhead box P3 (FoxP3) was significantly affected. Namely, IFN-γ was decreased by 54%, while FoxP3 was increased 39% compared with control (Figure 5C and D). While the differences were marginally significant ($p < 0.1$), they indicated a clear tendency for less Th1-skewed T cell response. These data are in agreement with the above mentioned defective maturation profile of mo-DCs, following contact with STn$^+$ cancer cells.

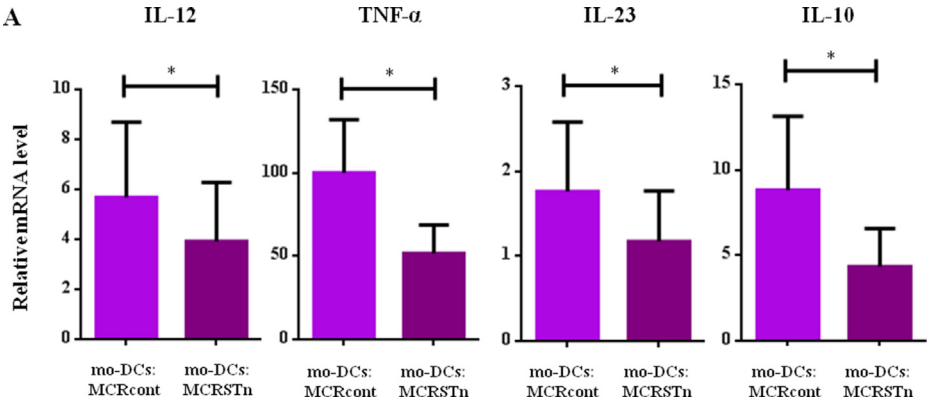

Figure 3 – Co-incubation with STn$^+$ MCR cell line downregulates cytokine expression levels. A: The expression of *TNF-α*, *IL-23*, *IL-12* and *IL-10* cytokine genes was evaluated by quantitative real-time PCR. The relative mRNA levels for each cytokine are expressed as the permillage (‰) of the expression of the endogenous positive controls. Values represent the means of at least 5 independent assays. The expression of *TNF-α*, *IL-12*, *IL-23* and *IL-10* were significantly decreased ($p = 0.015$ (*), $p = 0.031$ (*), $p = 0.034$ (*) and $p = 0.015$ (*) respectively) in mo-DCs co-incubated with MCRSTn as compared with those incubated with MCRcont.

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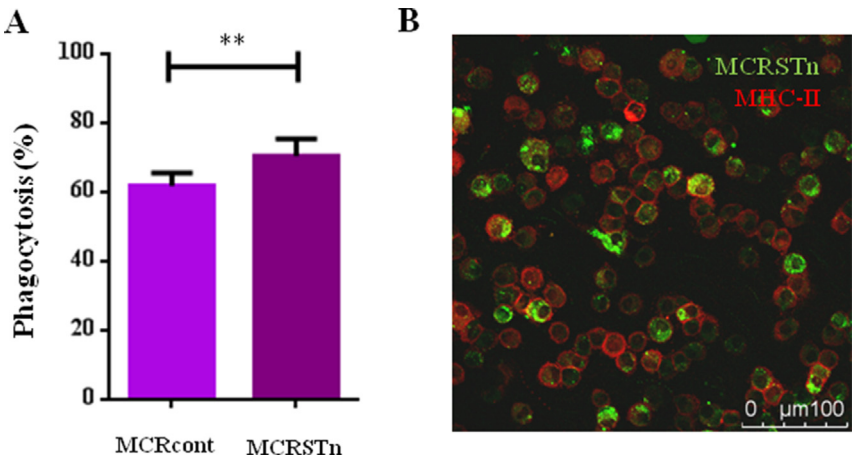

Figure 4 – STn$^+$ MCR cell lines are better phagocytosed by mo-DCs. Both MCRcont and MCRSTn cell lines were labelled with CFSE and then induced to apoptosis. Cells were then incubated with mo-DCs to allow phagocytosis, in the proportion of 1:2 for 6 h at 37 °C or 4 °C and then stained with anti-MHC-II mAb. A: Flow cytometric analysis of percentage of mo-DCs that phagocytosed MCR cells lines. The percentage of mo-DCs that phagocytosed MCR cells was calculated based on the positivity for both MHC-II and CFSE staining. The values obtained at 4 °C were subtracted from the 37 °C values. MCRSTn cells were significantly more phagocytosed than MCRcont ($p = 0.001$ (**), $n = 4$). B: Microscopic analysis of mo-DCs that phagocytosed MCR cells lines. Representative confocal microscopy image showing mo-DCs that phagocytosed MCRSTn cells [MHC-II$^+$ (red)/CFSE$^+$ (green)]. The image is representative of a confocal cross-section image, selected from Z-stack images.

3.5. *STn$^+$ glycoproteins blockade restore DC maturation*

MUC1 and CD44 are glycoproteins equally expressed by both MCR cells (Figure 6A and B) and described as the most likely candidates for being modified by STn by human cancer cells (Julien et al., 2006). The Western blot analysis of MCRSTn cell lysates, using anti-STn mAb, identified one prominent band of approximately 75 KDa, and two weak bands of 150 KDa and 260 KDa each. In all cases, the blot staining was completely abolished after sialidase treatment, proving the STn staining specificity. Immunoprecipitation assays led us to identify the most prominent band (75 KDa) as CD44. Bladder tumours also showed STn in CD44 proteins, as shown by the detection of a STn$^+$ ~75 KDa band in the Western blot analysis (Figure 6C). In order to confirm the role of the STn$^+$ protein scaffold, we blocked STn$^+$ glycoproteins in MCRSTn cells and conducted coculture assays with mo-DCs similar to those described above. In the cocultures, whenever the CD44 was blocked in the MCRSTn cells, they gained the capacity to significantly increase, in mo-DCs, the expression of MHC-II and cytokines, in particular of IL-12 and TNF-α (Figure 6D). Blocking of MUC1, as well as STn antigen in MCRSTn cells also upregulated MHC-II and cytokine expression, indicating a tendency, although not statistically significant (Figure 6D).

4. Discussion

We have recently reported that the majority of high-grade bladder tumours, presenting elevated proliferation indexes and high risk of recurrence/progression and invasion, expressed STn (Ferreira et al., 2013). This glycan is not expressed by normal epithelium and it has been associated with a poor prognosis, and the invasive capacity of the tumours (Ferreira et al., 2013). Similar observations were made for breast and gastric cancers and other solid tumours, supporting the ubiquitous association of STn with malignancy.

The expression of the STn antigen is also accepted to be implicated in the immunogenicity of cancer cells and this has been explored in STn-based vaccines to induce protective immune responses against STn$^+$ cancers. Humoral immune responses against STn have been reported in vaccinated patients and mouse models (Holmberg and Sandmaier, 2004; Julien et al., 2009), demonstrating that the immune system reacts against STn antigens. However, the potency of the immune response is not clinical relevant to provide patients with robust anti-tumour protection.

While, glycans are known to be implicated in several immune responses, the involved biological processes are diverse and complex and thus still poorly understood. Lectin-glycan ligand interactions are implicated not only in mechanisms that establish immune protection, but also in those that establish immunological tolerance. Given the clinical relevance of STn antigens, a deeper investigation on the interaction of STn expressing cancer cells with the immune system is warranted to improve and develop novel STn-based therapeutics.

Here we have studied the influence of STn antigen in the modulation of DCs, which are recognized by their pivotal role in the definition of immune responses. In the tumour tissue, we observed a significant correlation between STn expression and an immature profile of DCs. Indeed, the expression of ST6GALNAC1, which is correlated with the expression of the STn epitopes, in bladder cancer, is correlated with CD1a, a marker of immature DCs. Interestingly, high grade bladder tumours presented not only higher levels of ST6GALNAC1 and CD1a, but also lower levels of the pro-inflammatory, Th1-inducing cytokines, IL-12 and TNF-α, thus supporting the association between STn and DC immature profile.

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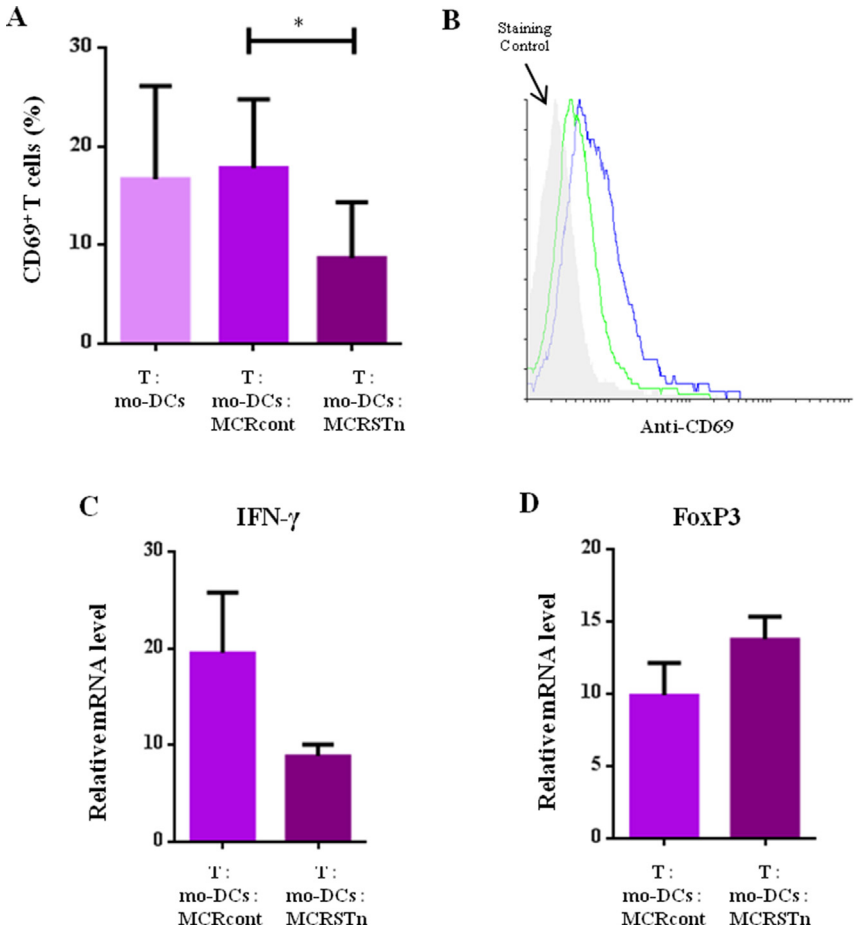

Figure 5 – T cell activation is reduced when primed with mo-DCs that phagocytosed STn$^+$ cancer cells. Mo-DCs were allowed to phagocytose MCR cells and then incubated with autologous T cells (1:8 proportion). A–B: The coculture was analysed by flow cytometry for the expression of the T cell early activation marker CD69. A: Graphical representation of the percentage of CD69$^+$ T cells. Data was determined by the percentage of CD69$^+$, within the CD3 population ($n = 3$). Mo-DCs that phagocytosed MCRSTn cells induce significantly less activation in T cells than mo-DCs that phagocytosed MCRcont cells ($p = 0.026$ (*)). B: A representative CD69 histogram for T cells following priming with mo-DCs that phagocytosed MCRcont (solid line) or MCRSTn (dashed line) cell line. Expression of CD69 by resting T cells is shown as staining control (grey filled peak). C–D: The coculture was analysed by quantitative real-time PCR regarding the expression of *IFN-γ* and *FoxP3* genes. The relative mRNA levels for each gene are expressed as the permillage (‰) of the expression of the endogenous positive controls. C: The expression of IFN-γ was reduced by 54% ($p = 0.074$) in T cells co-incubated with mo-DCs that phagocytosed MCRSTn as compared to controls ($n = 4$). D: T cells co-incubated with mo-DCs that phagocytosed MCRSTn expressed 39% ($p = 0.081$) more FoxP3 than control mo-DCs ($n = 4$).

Maturation is a crucial process that enables DCs to effectively prime T cells to mount responses against malignant cells (Steinman et al., 2003). To confirm a possible inverse association between STn$^+$ cancer cells and DC maturation and to investigate if STn antigen played a role in DC maturation, we used *in vitro* models of STn$^+$ cancer cells cocultured with human DCs. We have found that DCs tend to adhere more to STn$^+$ cancer cells and that this contact inhibits DC maturation and co-stimulation, when compared with DCs cocultured with STn$^-$ control cells. The higher cell adhesion to STn$^+$ cancer cells, which could be due to either stronger or longer cell contacts, was responsible for the defective maturation of mo-DCs. After coculture with STn$^+$ cancer cells, the lower expression of MHC-II and of co-stimulatory molecules could not be rescued by LPS stimulation, suggesting that mo-DC become resistant to further maturation stimuli.

An hallmark of immature DCs is a high phagocytic capacity, which is lowered upon maturation. In agreement with the fact that STn-expressing cancer cells prevents the maturation of mo-DCs, mo-DCs showed better phagocytic capacity for STn$^+$ cancer cells than for control cells. Nevertheless, the effects seen might not only be caused by STn but also some general feature of the MCR cells, since MCRcont cells also induced small maturation arrest.

Mo-DCs incubated with STn$^+$ cancer cells showed defective expression of TNF-α and IL-12, which is consistent not only with DC immature phenotype but also with data observed in bladder tumour tissues. These observations were also in agreement with other reports showing that tumour environment lowers the expression of inflammatory cytokines by DCs (Ishida et al., 2008). We have not detected significantly altered expression of anti-inflammatory

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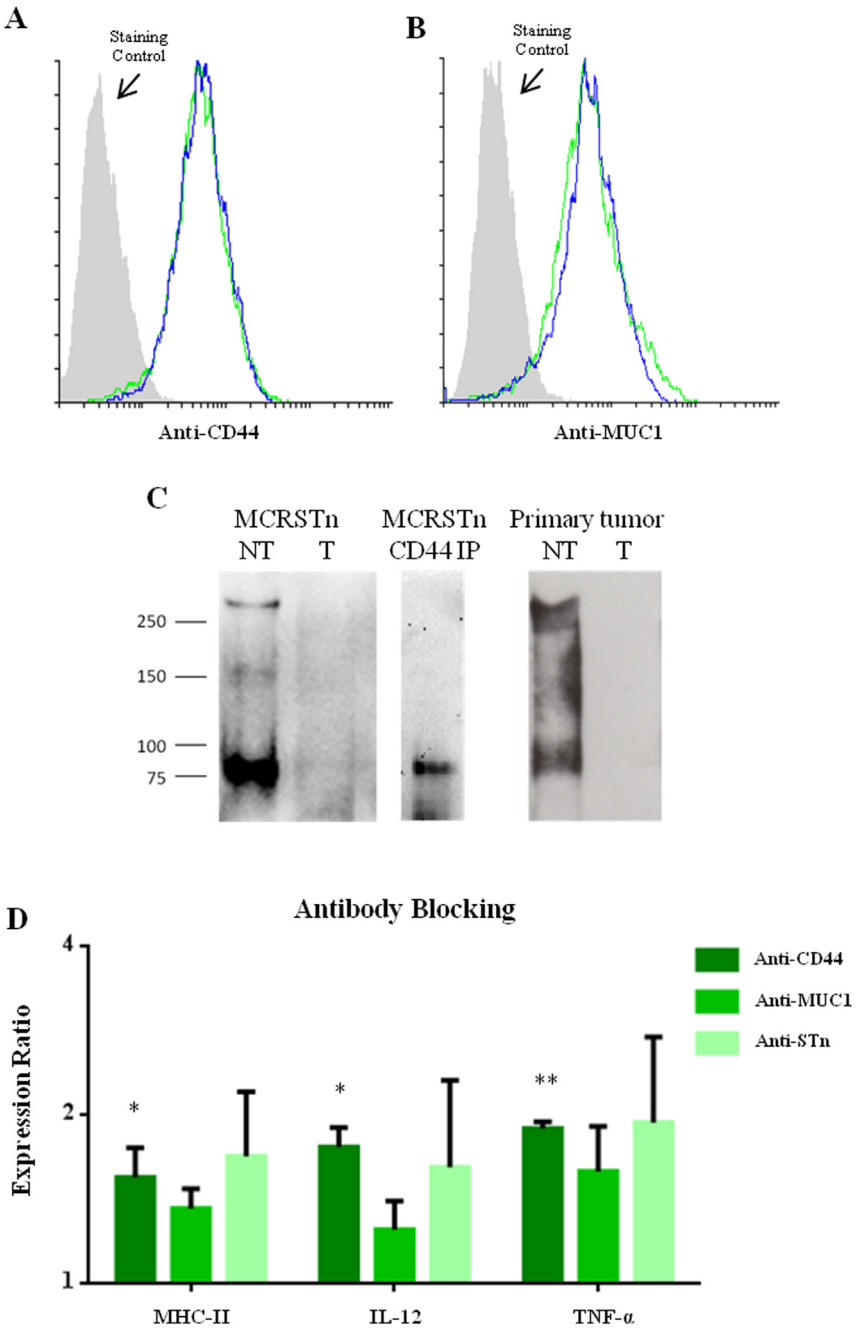

Figure 6 – STn$^+$ glycoproteins blockade restore mo-DCs maturation. A–B: MCR cell lines were analysed by flow cytometry regarding the expression of possible scaffolds of STn, CD44 and MUC1 glycoproteins. MCRcont (dashed line) and MCRSTn (solid line) cell lines were stained with anti-CD44 (A) or anti-MUC1 (B) mAb (not filled peaks) or only with secondary mAb (grey filled peak) as staining control. Both MCR cell lines express similarly CD44 and MUC1 glycoproteins. C: Analysis of STn$^+$ proteins in cancer cells. Total protein lysates from MCRSTn cell line (left image) and primary bladder tumour samples (right image) were treated (T) or not (NT) with sialidase. CD44 immunoprecipitation (IP) from MCRSTn total proteins was performed using Pierce Direct IP Kit (middle image). Cell lysates and CD44 IP were separated and the proteins were transferred to nitrocellulose membrane and stained with anti-STn mAb (clone TKH2). MCRSTn cells showed three proteins (≈75 KDa, 150 KDa and 260 KDa) decorated with STn and the most prominent STn+ protein showed a molecular weight of ≈75 KDa. As control, when the lysate was treated with sialidase, staining with anti-STn was completely abrogated. The CD44 IP analysis showed that CD44 protein is decorated with STn in MCRSTn cells. D: The expression of *MHC-II*, *IL-12* and *TNF-α* is restored when blocking STn and STn$^+$ glycoproteins. Gene expression was evaluated by quantitative real-time PCR in mo-DCs incubated with MCRSTn cell line in presence of anti-CD44, -MUC1 or -STn blocking mAbs. Mo-DC Fc receptors were previously blocked to avoid non-specific Fc receptor-mediated antibody binding. The expression values were calculated as described in the Material and Method section and correspond to the ratio between the expression of mo-DCs incubated with MCRSTn cell line in presence of blocking mAbs and the expression of mo-DCs incubated with MCRSTn in presence of isotypic control. CD44 blockade was able to increase the expression of MHC-II, IL-12 and TNF-α ($p = 0.0294$ (*), $p = 0.0357$ (*), $p = 0.0035$ (**), $n = 3$). Similarly, the expression of MHC-II, IL-12 and TNF-α was also increased by MUC1 and STn blockage.

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cytokines, with the exception of IL-10. IL-10 downregulation may be a balanced consequence of the downregulation of pro-inflammatory cytokines, playing a dual proliferative and inhibitory effect (Ogden et al., 2005).

The negative effect of STn^+ cancer cells on the maturation and Th1-inducing profile of mo-DCs were observable in less than 2 h of coculture. On the other hand, STn^+ cancer cells could themselves be influenced by mo-DCs. This was evidenced by the significant increase proliferation of MCRSTn cells in the presence of mo-DCs that was not observable in MCRcont cells. Yet these differences were only observable after 24 h coculture, and the mechanism behind the altered proliferation may be dissociated from the ones implied on the altered mo-DC phenotype. However, in the future, the effect of mo-DCs in tumour cell proliferation should be further clarified and it might be associated with the fact that STn^+ bladder tumours present elevated proliferation indexes *in situ*.

The fact that using other STn^+ cell line, i.e., the MDA-MB-231STn breast cancer cell line, also rendered mo-DCs with an immature phenotype, with an extent similarly to the phenotype induced by MCRSTn bladder cancer line, strongly suggested that the specific STn overexpression in cancer cell was responsible for the induction of tolerogenic DCs reported here. Nevertheless, we cannot disregard the fact that ST6GALNAC1 overexpression in cell lines also alters other glycan structures beyond the STn (Table IS).

To further confirm the role of STn expression by cancer cells in DC maturation, we used different strategies to abrogate the MCRSTn cancer cell interaction with DCs. Blocking of STn antigen in MCR cells, by means of the HB-STn antibody, described to block STn interactions (Pinho et al., 2007), resulted in tendency of MCRSTn cells to induce DC maturation. This suggested that blocking STn antigen may reverse the propensity of STn^+ cancer cells to induce tolerogenic DCs. The fact that we were not able to obtain statistical significant differences may have to do with the role of the protein scaffolds that also participate in the recognition or interaction of glycans (Padler-Karavani et al., 2008). Therefore, anti-glycan mAbs may not have efficient function-blocking properties, as anti-scaffolds have.

We have observed that the major protein scaffold in the MCRSTn cells and bladder tumours is CD44. Moreover CD44 is also decorated with STn in MDA-MB-231STn breast cancer cells (Julien et al., 2006), which in this work induced the same DC immunomodulation as MCRSTn cells. These findings suggested us a possible involvement of STn^+ CD44 in the acquisition of the immature profile by DCs. Concordantly, blockade of MCR CD44 by means of functional blocking antibodies was able to restore the capacity of the mo-DCs to become matured.

CD44 is a rather ubiquitously expressed adhesion molecule, involved in several processes including migration and cell signalling and recognition. The role of CD44 in governing the progression of tumours, including bladder carcinoma (Golshani et al., 2008), is well documented and its expression by leukocytes also plays a role in modulating immune responses (Hegde et al., 2008; Jacobs and Sackstein, 2011; Weiss et al., 1997). However the role of its recognition by immune cells, such as DCs, is unclear. In our model, it is possible that DC receptor for CD44 may recognize differently the protein due to substitution of normal glycosylation by STn in cancer cells. CD44 is a receptor for hyaluronic acid and other extracellular proteins, such as osteopontin and collagens. Very recently it has been reported that the mannose receptor interacts with CD44 (Salmi et al., 2013). Although, mannose receptor primarily binds mannose and fucose, it is possible that in our model, the STn expression affected the CD44 recognition by mannose receptor, leading to the described immunological implications. Nevertheless, other receptors expressed by DCs may also be involved, such as the Sialic acid-binding immunoglobulin-type lectins (Siglec)-9 that have also been reported to recognize STn antigen (Ohta et al., 2010). In addition, the participation of other STn scaffolds such as MUC1 (Monti et al., 2004) should not be excluded and as matter of fact, we also observed that blockade of MUC1 results in a slight induction of DC maturation. Further studies are therefore necessary to better characterize the mechanisms of STn antigen recognition by DCs.

As mentioned above, previous studies were controversial in demonstrating that STn mucins were implicated in inducing the immature DC phenotype (Carlos et al., 2005; Monti et al., 2004). While this controversy may be due to the complexity of the involved mechanisms, these studies were in agreement, when showing that STn expression has a negative influence on the capacity of DCs to activate T cells, resulting in DCs that do not support T cell commitment to Th1 phenotype, which is important for tumour rejection (Carlos et al., 2005; Monti et al., 2004).

DCs are currently used as anti-tumour cell-based vaccines, where one of the most common strategies is the upload of patient's DCs *ex vivo* with the tumour cell antigens (lysates, cells fusion or apoptotic bodies). Tumour antigens are phagocytosed by DCs and these cells are then used to induce anti-tumour T cells responses and further tumour rejection (Palucka and Banchereau, 2013). It has been reported that DCs pulsed with apoptotic cancer cells are able to activate cytotoxic T cells against poorly immunogenic cancer cells (Goldszmid et al., 2003) and in bladder cancer patients increases survival and cure rate (Nair et al., 1997). In the present study, DCs pulsed with immunosuppressive STn^+ cancer cells showed a tolerogenic/regulatory profile, resistant to further maturation stimuli and limited capacity to activate T cells. In these conditions, mo-DCs express lower levels of Th1-inducing cytokines and preferentially polarize T cells towards a $FOXP3^{high}$ IFN-γ^{low} phenotype, typical of regulatory T cells. On the other hand, it has been recently reported by us that STn expression in bladder cancer is associated with better responses to complementary immunotherapy with Bacillus Calmette-Guérin, a potent inducer of Th1 responses (Lima et al., 2013). Therefore, the combination of Th1-inducing therapies with DC-based therapy against STn-bearing cancer cells could be a promising strategy to induce anti-tumour protective responses. Nevertheless, a better understanding of the mechanisms behind the tolerogenic/regulatory profile of DCs induced by STn^+ cancer cells is still necessary.

5. Conclusions

This study shows that STn, a cancer-associated carbohydrate antigen expressed in high-grade bladder cancers, has the

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ability to down-regulate the anti-cancer immune-response through different mechanisms. First, it hinders the expression of MHC-II and co-stimulatory molecules by DCs, resulting in impaired ability to present cancer-associated antigens to T cells and making DCs unresponsive to successive activation stimuli. Second, it hinders the expression of inflammatory, Th1-inducing cytokines in DCs, which may result in an attenuation of the Th1 microenvironment. Third, DCs pulsed with STn-expressing cancer cells show a reduced ability to activate and polarize T cells towards the Th1 phenotype, resulting in impaired ability to mount an effective anti-tumour response. Finally, blockade of STn antigens and STn protein may reverse tolerance induced by cancer cells. Altogether, these results highlight the expression of STn by cancer cells as a crucial event in the establishment of the tolerogenic microenvironment which allows cancers to escape from the attack of innate and adaptive immunity.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2014.02.008.

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