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Research Article

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Microvesicle Cargo of Tumor-Associated MUC1 to Dendritic Cells Allows Cross-presentation and Specific Carbohydrate Processing

Aurelia Rughetti[1], Hassan Rahimi[1], Francesca Belleudi[2], Chiara Napoletano[1], Federico Battisti[1], Ilaria G. Zizzari[1], Morena Antonilli[3], Filippo Bellati[3], Hans H. Wandall[4], Pierluigi Benedetti Panici[3], Joy M. Burchell[5], Mara R. Torrisi[2], and Marianna Nuti[1]

Abstract

Tumor-associated glycoproteins are a group of antigens with high immunogenic interest: The glycoforms generated by the aberrant glycosylation are tumor-specific and the novel glycoepitopes exposed can be targets of tumor-specific immune responses. The MUC1 antigen is one of the most relevant tumor-associated glycoproteins. In cancer, MUC1 loses polarity and becomes overexpressed and hypoglycosylated. Changes in glycan moieties contribute to MUC1 immunogenicity and can modify the interactions of tumor cells with antigen-presenting cells such as dendritic cells that would affect the overall antitumor immune response. Here, we show that the form of the MUC1 antigen, i.e., soluble or as microvesicle cargo, influences MUC1 processing in dendritic cells. In fact, MUC1 carried by microvesicles translocates from the endolysosomal/HLA-II to the HLA-I compartment and is presented by dendritic cells to MUC1-specific $CD8^+$ T cells stimulating IFN-γ responses, whereas the soluble MUC1 is retained in the endolysosomal/HLA-II compartment independently by the glycan moieties and by the modality of internalization (receptor-mediated or non–receptor mediated). MUC1 translocation to the HLA-I compartment is accompanied by deglycosylation that generates novel MUC1 glycoepitopes. Microvesicle-mediated transfer of tumor-associated glycoproteins to dendritic cells may be a relevant biologic mechanism *in vivo* contributing to define the type of immunogenicity elicited. Furthermore, these results have important implications for the design of glycoprotein-based immunogens for cancer immunotherapy. *Cancer Immunol Res; 2(2); 177–86. ©2013 AACR.*

Introduction

Changes in glycosylation that occur during tumor transformation can profoundly affect the interactions between tumor cells and the microenvironment with strong impact on the overall antitumor immune response. The mucin MUC1 is one of the most relevant tumor-associated glycoproteins; it is an *O*-linked glycosylated transmembrane protein normally expressed on the apical surface of epithelial cells, but aberrantly expressed in a broad spectrum of carcinomas. Upon malignant transformation, MUC1 loses polarity and becomes overexpressed and hypoglycosylated, revealing an immunogenic region of tandem repeats of 20 residues. The novel MUC1 glycoforms that arise carry shortened glycan moieties: Tn (GalNAc), T (Galβ1,3GalNAc), ST (NeuAcα2, 3Galβ1,3GalNAc), and STn (NeuAcα2,6GalNAc; ref. 1). These changes in the glycosylation pattern impact MUC1 immunogenicity and the interactions of tumor cells with the microenvironment (2). In patients with cancer, anti-MUC1 IgG responses are present and correlate with the outcome of the disease (3). The anti-MUC1 $CD8^+$ T-cell responses in patients with cancer are directed mostly against the peptide epitopes in the MUC1 extracellular domain (4). It is reasonable to think that *in vivo* glycoepitopes displayed by the tumor-associated glycoforms are targets for the antitumor response. In fact, the significant IgG immune response in patients with cancer was shown to be directed against the MUC1 glycoepitopes (5), and the binding of the MUC1 glycopeptides to the MHC groove could induce T-cell activation in mouse models and in human studies *in vitro* (6–8). In addition, the modality of uptake of MUC1 by antigen-presenting cells (APC) *in vivo* can impact antigen presentation, resulting in efficacious $CD8^+$- and IgG-mediated immune responses or in $CD4^+$ tolerizing responses (9). Recently, we have demonstrated that altered MUC1 glycosylation influences its uptake by dendritic cells and modifies dendritic cell functions. We have also demonstrated that MUC1-based

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Tn-peptide interacts with dendritic cells through the C-type Lectin MGL (macrophage galactose-type C-type lectin) and is translocated to the HLA-I compartment, thus indicating that cross-processing for the MUC1 glycoantigen was possible and size dependent (2, 10).

Together, these results offer evidence of the complexity of MUC1 biology in its expression in tumor cells and its relationship with the immune system. Understanding the intracellular processing of the MUC1 glycoprotein and how the immunogenic glycoepitopes can be generated will inform the design of dendritic cell–based vaccines targeting MUC1.

In this report, we show that independent of the glycosylation profile of MUC1, antigen formulation is of crucial importance both for cross-processing and for generating immunogenic glycoepitope array. While the two recombinant soluble glycoproteins of MUC1, the highly sialylated rST-MUC1 and the rTn-MUC1 carrying only Tn glycans, are blocked in the endosomal/HLA-II compartments, the TAA-MUC1 glycoforms, when delivered to dendritic cells by microvesicles, are processed in the HLA-II and HLA-I compartments.

Microvesicles represent a novel vehicle of communication between cells, which has just begun to be explored. Membranes, cytosolic proteins, lipids, and RNA are transferred *in vivo* via microvesicles in body fluids; these cargos possess regulatory and biomarker functions (11). Microvesicles have also been proposed as vehicles for antigen transfer. *In vivo*, tumor cells shed antigens both in soluble form and associated with microvesicles. Microvesicles carrying tumor antigens are valid immunogens in mouse models enhancing the immunogenicity of soluble antigen (12) in *in vitro* human studies (13) and in pilot vaccination studies (14).

Our results support the hypothesis that MUC1 immunogenicity is strictly dependent on the form of the antigen and suggest that microvesicle cargo of MUC1 should be preferred in the design of glycoprotein-based immunogens for cancer therapy.

Materials and Methods

MUC1 recombinant proteins

rST-MUC1 glycoprotein was generated by CHO-K1 cells (ATCC CRL-9618) transfected with a MUC1-murine-IgG_{2a} fusion cDNA construct containing 16 tandem repeats (15). The Fc region was removed by enterokinase treatment. The cells were cultured in Iscove's modified Dulbecco's medium (Hyclone) with 600 μg/mL of neomycin (Invitrogen) and were kept in culture for no longer than 2 months.

rTn-MUC1 glycoprotein was produced in CHO-*ldl*D cells (kindly provided by Prof. Henrik Clausen, University of Copenhagen) transfected with the same construct and cultured as described above.

Exploiting the deficiency of UDP-Gal/UDP-GalNAc4-epimerase in these cells (16), culturing them with 1 mmol/L of GalNAc yielded cells expressing soluble Tn-MUC1. MUC1 glycoproteins were purified from the culture supernatant by anti-MUC1 monoclonal antibodies (mAb) affinity chromatography after cleavage with enterokinase. mAb HMFG2 was used for rST-MUC1 and mAb 5E5 (specifically reacting with MUC1 carrying Tn; ref. 17) for rTn-MUC1. The cells were never kept in culture for more than 2 months. The pattern of glycosylation on MUC1 was indicative of these cells.

Cell lines

DG75 lymphoblastoid cells (provided by Prof. P. Trivedi, "Sapienza" University of Rome, Rome, Italy) were cultured as described by Napoletano and colleagues (13). MUC1-DG75–transfected cells were cultured in neomycin (1 mg/mL, Invitrogen). Before microvesicle production, DG75 and MUC1-DG75 cells were analyzed for the expression of CD22, MUC1, HLA-I-A2, HLA-DR, and CD86 by cytofluorimetry. Cells were also tested for EBV (EBV PCR Kit, Abbott Molecular).

The breast cancer cell line T47D (ATCC-HTB-133) was cultured in Dulbecco's modified Eagle medium (Hyclone) + 10% fetal calf serum (Hyclone). All cell lines were tested for mycoplasma before use (PlasmoTest, Invivogen).

Dendritic cell generation

Peripheral blood mononuclear cells of healthy donors were isolated by Ficoll-Hypaque gradient (1,077 g/mL; Pharmacia LKB). Monocytes were magnetically immunoselected (Miltenyi Biotech). Fifty ng/mL rhGM-CSF and rhIL4 (1,000 U/mL, R&D Systems) were added to the cells in culture at day 0 and 2 to obtain immature dendritic cells (iDC). iDCs were collected at day 5 and matured dendritic cells (mDC) were obtained by incubating iDCs with rhIL-1β, IL-6, TNF-α (10 ng/mL each), and prostaglandin E2 (1 μg/mL; R&D Systems) for 16 hours. iDCs were used for the immunofluorescence studies; mDCs were employed for stimulating $CD8^+$ T cells.

Flow cytometry

MGL receptor on dendritic cells was detected using the mAb anti-human MGL (125A10.03 clone, Dendritics) followed by fluorescein isothiocynate (FITC)-conjugated goat anti-mouse IgG (H+L; Jackson ImmunoResearch Laboratories). After washing, at least 1×10^4 events were evaluated using a FACSCalibur flow cytometer running CellQuest software (Becton Dickinson).

Microvesicle purification

Microvesicles were purified from ascites of patients with ovarian cancer (stages III and IV; obtained after informed consent) and from supernatants of DG75 and MUC1-DG75 cells as described (13). Ascites were centrifuged three times at 4°C and ultracentrifuged (Type 35 rotor, Beckman Coulter) ×2 at 4°C (11,300 rpm/30 minutes; 35,000 rpm/1 hour). The protein concentration was measured by Bradford assay (Bio-Rad Laboratories). To generate microvesicles from the MUC1-DG75 and DG75 cell lines, cells were cultured 3.5×10^5 cells/mL as previously described (13).

MUC1 internalization by dendritic cells

MGL^- iDCs were pulsed with rST-MUC1 or rTn-MUC1 glycoproteins (30 μg/mL; 12 hours; 37°C) or with microvesicles carrying MUC1 purified from ascites [MUC1-Ase-MVs (microvesicles)] or from MUC1-DG75 cells (MUC1-DG75-MVs; 500 μg/mL; 2 and 12 hours; 37°C). For MGL receptor-mediated endocytosis, MGL^+ dendritic cells were incubated with rTn-MUC1 (10 μg/mL; 30 minutes on ice), washed, and incubated (12 hours; 37°C).

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Immunofluorescence microscopy

After MUC1 endocytosis, iDCs were cytospun (8×10^4 cells/sample) and fixed with cold acetone/methanol (1:1; Carlo Erba Reagents). iDCs were incubated (45 minutes; room temperature) with the anti-MUC1 mAb Ma552 (1:20, Monosan), followed by FITC-conjugated goat anti-mouse F $(ab)_2$ (1:100; 30 minutes at room temperature). After washing, the samples were incubated with mAbs anti-Lamp-1 (E-5, Santa Cruz Biotechnology), anti-HLA-DR (L243 clone), and rabbit polyclonal antibody anti-calreticulin (Stressgene). As secondary antibodies, Texas red–conjugated goat anti-mouse (1:100) and anti-rabbit antibodies $F(ab)_2$ (1:300; Jackson ImmunoResearch Laboratories) were used. Unpulsed dendritic cells were used as negative control. Fluorescence signals were visualized with an Axiovert 200 inverted microscope (Zeiss) using a ×63 oil immersion objective (numeric aperture: 1.4); cells were scanned in a series of 0.5 μm sequential sections with an ApoTome System (Zeiss) and images were all acquired by the digital camera Axio CAM MRm (Zeiss; acquisition setting: average 2, filter for background noise: medium). Image analysis was performed by the Axiovision software (Zeiss). Reconstruction of a selection of three central out of the total number of the serial optical sections was shown in each figure. Quantitative analysis of the extent of colocalization of fluorescence signals was performed using the KS300 3.0 Image Processing System (Zeiss). The mean ± SE percent of colocalization was calculated analyzing a minimum of 30 cells for each treatment randomly taken from three independent experiments. To visualize MUC1 glycoepitopes, the following mAbs were used (Table 1): anti-MUC1 (Ma552 clone ref. 18), anti-ST-MUC1 (MY1E12 clone; ref. 19), anti-T-MUC1 (2D9 clone), and anti-Tn-MUC1 (5E5 clone; ref. 20). Quantitative analysis of the percentage of MUC1-positive cells was assessed counting 100 cells per sample, randomly observed in 10 microscopic fields from three experiments. Results were expressed as mean values ± SE. *P* values were calculated using Student *t* test. Significance level was defined as $P < 0.05$ (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$).

CA15.3 assay

CA15.3 levels in total ascites, ultracentrifuged supernatants, and vesicle fractions were determined by chemoluminescence assay (Roche Diagnostic).

Table 1. Anti-MUC1 mAbs employed in the study

mAb	Glycoepitope recognized	Glycan structure
Ma552	All MUC1 glycoforms	
MY1E12	ST-MUC1	◆-○-□-Ser/Thr
2D9	T-MUC1	○-□-Ser/Thr
5E5	Tn-MUC1	□-Ser/Thr

□ GalNAc ○ Galβ ◆ NeuAc

Western blotting

DG75 and MUC1-DG75 were lysed using the NP-40 solution (Biocompare) with phenylmethylsufonylfluoride (PMSF; 1 mmol/L; Sigma) and protease inhibitors (×1; Sigma). Microvesicles and cell lysates (30 μg/sample) were separated on 4%–12% SDS-PAGE and blotted onto nitrocellulose transfer membrane (Schleicher and Schuell). Membranes were incubated with anti-MUC1 Ma552 (1 hour at room temperature), followed by anti-mouse Fc peroxidase-conjugated antibody (1:20,000; Jackson ImmunoResearch; 1 hour at room temperature). Protein bands were detected with enhanced chemiluminescence reagents (ECL Western Blotting Detection, Amersham Biosciences).

Subcellular fractionation

iDCs were incubated with MUC1-Asc-MVs (500 μg/mL) or rST-MUC1 (50 μg/mL; 12 hours; 37°C), washed, and suspended in ice-cold isotonic buffer (0.25 mol/L mannitol, 10 mmol/L HEPES, 1 mmol/L LEGTA, 2% bovine serum albumin), with protease inhibitors (1 mmol/L PMSF, 1 μg/mL aprotinin, and 1 μg/mL Leupeptin; Sigma). Cells were mechanically homogenized and centrifuged at 3,000 rpm. The supernatant was further centrifuged at 11,000 rpm; the pellet, containing endocytic/lysosomal compartments (P10 fraction), was collected and stored at −80°C until use, whereas the supernatant was centrifuged at 33,000 rpm to isolate the cytoplasm (S20 fraction). The protein concentration was measured by Bradford assay (Bio-Rad).

ELISA assay

Flat-bottomed 96-well EIA/RIA plates (Corning Incorporated) were precoated overnight with P10 and S20 fraction (5 μg/well). Samples were incubated with the anti-MUC1 Ma552 (1 hour, 37°C). Peroxidase-conjugated goat anti-mouse IgG (H+L; 1:5,000, Jackson ImmunoResearch) was added to the wells, followed by chromogen addiction (5 mg *O*-phenylenediamine, Citrate phosphate buffer 10 mL, 30% H_2O_2 10 μL; Sigma-Aldrich). The absorbance was measured at 492 nm.

ELISpot

MUC1-enriched $CD8^+$ T cells derived from three $MUC1_{(159\text{-}167)}$ (SAPDNRPAL; ref. 21) vaccinated ovarian cancer patients were cocultured with autologous mDCs pulsed with MUC1-DG75-MVs, DG75-MVs (100 μg/10^5 iDCs), or $MUC1_{(159\text{-}167)}$ peptide (30 μg/mL) in the anti-IFN-γ precoated (1:200; Pharmingen) ELISpot plates (Millipore) overnight. Cytokine release was detected with biotinylated anti-IFN-γ antibody (Pharmingen; 1:250, 2 hours) revealed with streptavidin-alkaline phosphatase (Pharmingen) (1:1,000, 100 μL/well, 1 hour) and chromogen substrate. Spots were counted using the ImmunoSpot Image Analyzer (Aelvis).

Results

Tumor-associated glycoprotein MUC1 is blocked in the endolysosomal/HLA-II compartments in dendritic cells

Uptake of tumor-associated glycoproteins and their intracellular routing in dendritic cells were studied utilizing two distinct glycan forms, Tn (GalNAc) and ST

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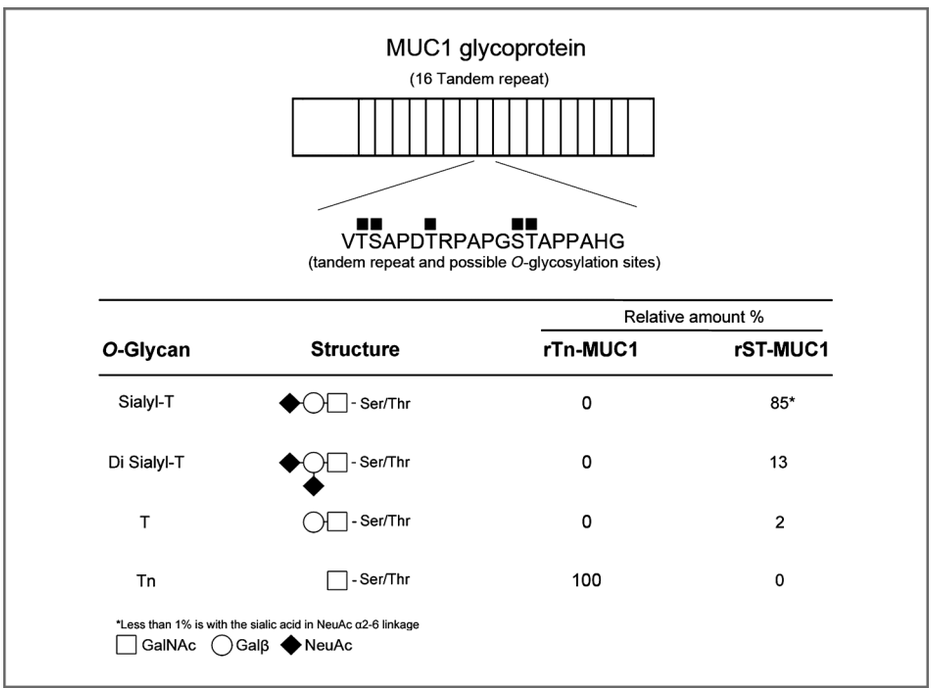

Figure 1. Structure of the recombinant MUC1 glycoproteins. The protein backbone of the MUC1 recombinant protein is depicted indicating the five possible *O*-glycosylation sites for each tandem repeat. The *O*-glycan composition of the rTn-MUC1 produced in CHO-*ldl*D cells and the rST-MUC1 produced in CHO-K1 cells is reported.

(NeuAcα2,3Gal 1,3GalNAc) of the recombinant tumor-associated MUC1 glycoprotein (Fig. 1). The rST-MUC1 was produced by expressing the extracellular domain of MUC1 containing 16 tandem repeats in CHO-K1, whereas the rTn-MUC1 glycoform was produced in CHO-*ldl*D cells. The CHO-*ldl*D cells lack UPD-Gal/UDP-GalNAc4-epimerase that results in their inability to synthesize Galactose and GalNAc. Therefore, in the absence of ST6GalNAc-I, they produce *O*-linked glycoproteins carrying only Tn (16).

To investigate whether diversity of glycan moieties on the MUC1 backbone could modify its processing, the intracellular routing of rST- and rTn-MUC1 glycoproteins via non-receptor–mediated endocytosis was evaluated using MGL-negative iDCs by immunofluorescence after 12 hours (Fig. 2, columns 1–6). As negative control, unpulsed iDCs were employed: no MUC1 staining was detected (data not shown).

rST-MUC1 glycoprotein (green; Fig. 2, column 1–3) appeared to be distributed in discrete granules along the plasma membrane and in the cytoplasm. Colocalization with specific compartment markers (yellow) was performed employing calreticulin, HLA-II-DR, and Lamp-1 molecules as markers for HLA-I, HLA-II, and endolysosomal compartment, respectively (red). A strong association with the endolysosomal compartment (36%) and at a lower extent with the HLA-II compartment (22,6%) could be observed, whereas a rare association with calreticulin staining was quantified (15,8%). Similar to the staining pattern observed for the rST-MUC1, rTn-MUC1 (green) localized into discrete dots distributed at the periphery of the iDCs (Fig. 2, columns 4–6). Quantification of the association between rTn-MUC1 and the compartment markers revealed infrequent colocalization for the calreticulin positive compartment (11%), some association with the HLA-II compartment (29%) and again a strong association with the endolysosomal compartment marker Lamp-1 (41%). These results indicated that following non–receptor-mediated internalization, the glycosylation profile of MUC1 did not influence its intracellular routing in APCs, i.e., both MUC1 glycoforms (rST and rTn) were mostly accumulated in the endolysosomal/HLA-II compartment with no delivery to the HLA-I compartment. The Tn-MUC1 glycoform, also internalized by iDCs upon binding to the C-type lectin MGL (2), was employed as experimental control (Fig. 2, columns 7–9).

rTn-MUC1 endocytated via MGL was distributed to small dotted structures (green) below the plasma membrane. Colocalization studies indicated a significant increase in the association with the Lamp-1 marker (65%) as compared with the non–receptor-mediated internalization, whereas a similar association pattern was found for the HLA-II and calreticulin (32% and 11%, respectively). These results indicated that MGL receptor-mediated endocytosis significantly increased the delivery of the MUC1 antigen to the endolysosomal compartment without affecting the delivery to the HLA-II compartment. A very low level of MUC1 delivery of the antigen to the calreticulin-positive compartment was observed.

MUC1 associated with microvesicles is cross-processed in the HLA-I compartment in dendritic cells

The presence of MUC1 glycoprotein was analyzed in the ascites of 5 ovarian cancer patients using the standardized serum CA15.3 assay that quantifies circulating tumor-associated MUC1 (Fig. 3A). In addition, MUC1 was also quantified in the microvesicles (MUC1-Asc-MVs) and in the supernatants obtained after the ultracentrifugation of the ascites. MUC1 was found at high levels in all the ascites examined and at lower concentrations in the other two fractions. Western blot analysis confirmed the presence of MUC1 on the microvesicles

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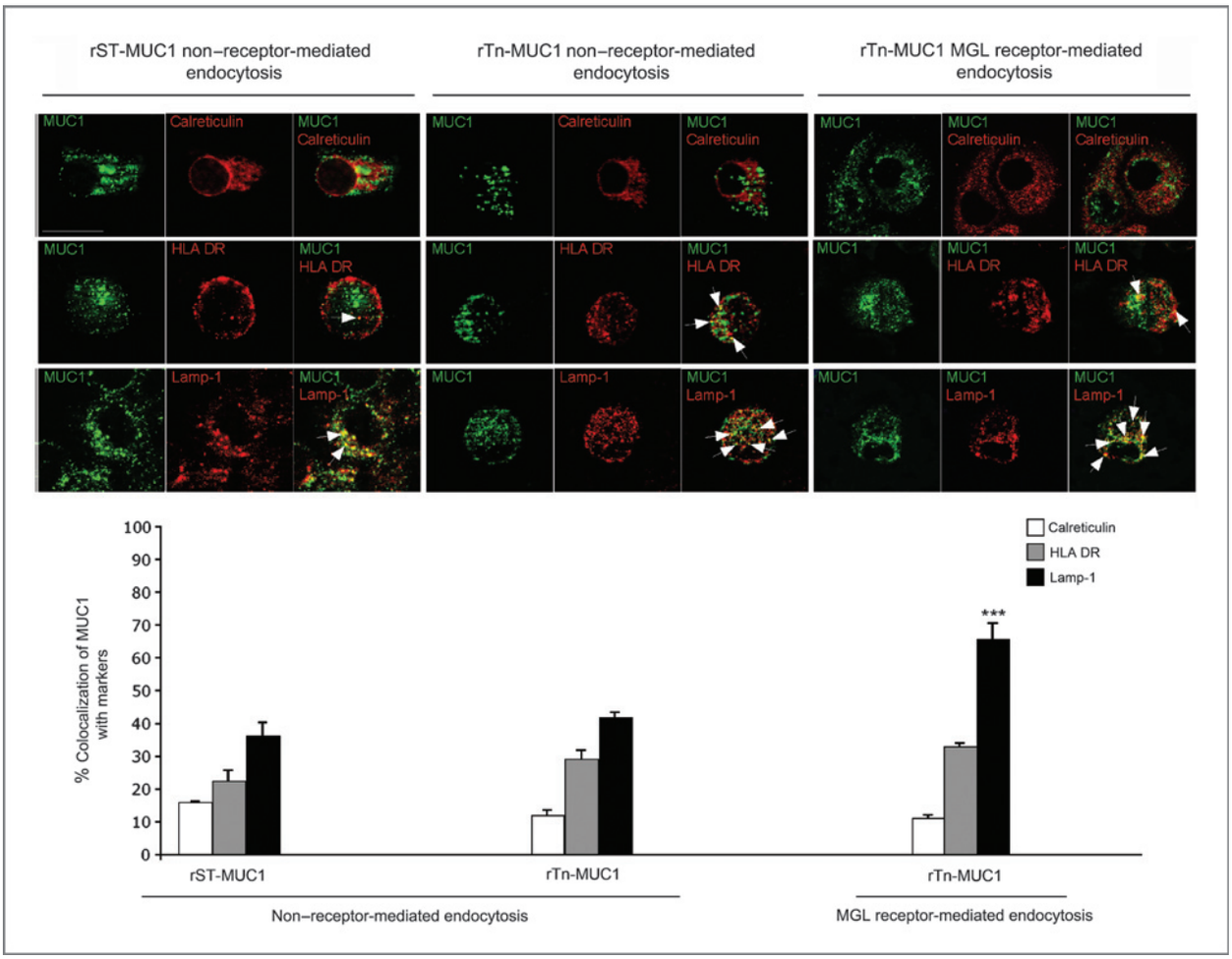

Figure 2. Tumor-associated MUC1 glycoproteins are blocked in the endolysosomal compartment. Intracellular localization of MUC1 in iDCs and its distribution in the intracellular compartments after non–receptor-mediated endocytosis of rST-MUC1 or rTn-MUC1 (columns 1–3 and 4–6, respectively) and MGL receptor-mediated endocytosis of rTn-MUC1 (column 7–9) was visualized by immunofluorescence staining after 12 hours of internalization employing the anti-MUC1 mAb Ma552 (green) combined with antibodies specific for distinct compartment markers (red, in particular, anticalreticulin polyclonal rabbit antibody for ER (first row), anti-HLA-DR (HLA-II compartment, second row), and anti-Lamp-1 (endolysosomal compartment, third row). The percentage of colocalization (yellow) was calculated analyzing a minimum of 30 cells for each treatment randomly taken from three independent experiments. Results are expressed as mean values ± SE in histograms. Magnification, ×63; Bar, 10 μm. ***, $P < 0.005$ versus the corresponding rST-MUC1 and rTn-MUC1 pulsed MGL$^-$ iDCs.

purified from the ascites (Fig. 3B). The purified Asc-MVs carrying MUC1 were used to pulse iDCs, and the MUC1 intracellular routing was analyzed by immunofluorescence after 2 and 12 hours (Fig. 4A). After 2 hours of pulsing, MUC1 carried by microvesicles (green) was found in discrete granules distributed throughout the cytoplasm of cells, whereas no MUC1 staining was detected in the unpulsed iDCs (data not shown). Strong association of MUC1 was found with Lamp-1 and HLA-II-DR staining (70% and 62%, respectively), whereas no colocalization was found with calreticulin (7%). These results clearly showed that MUC1 carried by microvesicles from ascites was delivered to the endolysosomal and HLA-II compartments in a higher percentage compared with the non–membrane-bound forms of MUC1.

After 12 hours, a striking difference was observed: MUC1 staining was mainly cytoplasmic and distributed along the nuclear membrane, with a significant increase in the association with calreticulin (38%) and a significant decrease in the association with Lamp-1 (51%). These results suggested that MUC1 carried by microvesicles was transferred from the endolysosmal to the HLA-I compartment. Surprisingly, MUC1 and HLA-II-DR scarcely colocalized in a significant manner (8%). This result could be explained by the accumulation of MUC1 in the intracellular compartments combined with the translocation of the HLA-II-DR to the plasma membrane after 12 hours of incubation of iDCs (Supplementary Data, Supplementary Fig. S1).

The presence of MUC1 in both the cytoplasm and the vesicle/membrane fractions of iDCs pulsed with MUC1-Asc-MVs (Fig. 4B) confirmed that MUC1 carried by microvesicles was delivered to the HLA-I compartment through the cytoplasm.

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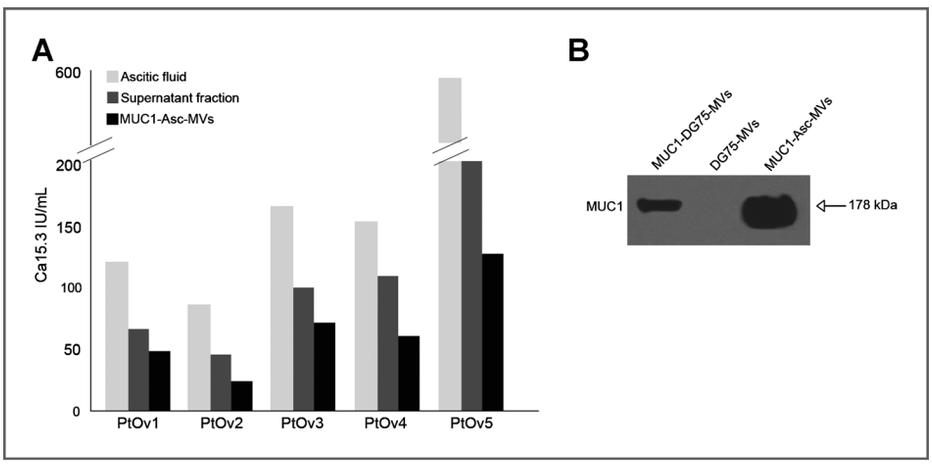

Figure 3. Tumor-associated MUC1 is present in ovarian cancer ascites, both associated with microvesicles and in soluble form. A, MUC1 was detected in ovarian cancer ascites, in the soluble and vesicle fractions after ultracentrifugation by CA15.3 assay. Results (IU/mL) are plotted as histograms (light gray, ascites; dark gray, supernatant fraction; black, microvesicle fraction). B, Western blot analysis of MUC1-Asc-MVs, one representative sample out of five is shown. Microvesicles derived from DG75 and MUC1-DG75 cell lines are also shown as negative and positive controls, respectively.

MUC1 carried by microvesicles is cross-presented by dendritic cells to MUC1-specific CD8$^+$ T cells inducing IFN-γ production

Antigen translocation to the HLA-I compartment in dendritic cells is required but not sufficient for antigen cross-presentation. To evaluate whether MUC1 was cross-presented, mDCs pulsed with MUC1 carried by microvesicles were analyzed for their capacity to activate MUC1-specific CD8$^+$ T cells. The MUC1$_{159\text{-}167}$ peptide was employed to expand MUC1-specific CD8$^+$ T cells from patients with breast cancer, who have received MUC1$_{159\text{-}167}$ peptide vaccination (LITRM/DIMIGE05/01, Visconti and colleagues, Manuscript in preparation).

The lymphoblastoid cell line DG75 was transfected with cDNA encoding the full-length MUC1 containing seven tandem repeats (MUC1-DG75) and microvesicles carrying MUC1 (MUC1-DG75-MVs) were purified from the supernatant of the transfected cell line (Supplementary Data, Supplementary Fig. S2A–S2C). Autologous mDCs were pulsed with MUC1-DG75-MVs or microvesicles purified from the untransfected DG75 cell line (DG75-MVs). These cells were used to stimulate enriched MUC1$_{159\text{-}167}$–specific CD8$^+$ T cells. IFN-γ was highly produced by T cells only in response to mDCs pulsed with MUC1-DG75-MVs, but not to mDCs pulsed with DG75-MVs (Fig. 4C). IFN-γ production was also induced when T cells were stimulated with autologous mDCs pulsed with MUC1$_{159\text{-}167}$ peptide, whereas mDCs pulsed with the rST-MUC1 glycoprotein (30 μg/mL) did not induce any response (data not shown). These results showed that MUC1 carried by microvesicles was cross-presented to T cells.

MUC1 associated with microvesicles is differentially deglycosylated during processing

The evidence that MUC1 associated with microvesicles was delivered to the calreticulin/HLA-I compartments and cross-presented to CD8$^+$ T cells prompted us to further investigate the processing of MUC1 analyzing the exposure of specific glycosylated epitopes. The intracellular processing of MUC1 was studied in iDCs pulsed for 12 hours with rST-MUC1 or the sialylated MUC1-DG75-MVs, both exclusively carrying ST-MUC1 glycoform as probed with the mAbs recognizing the specific MUC1 glycoepitopes, i.e., ST-MUC1, T and Tn MUC1 (mAb My1E12, 2D9 and 5E5, respectively; Supplementary Fig. S2D) and as confirmed by neuraminidase treatment (data not shown).

When dendritic cells were pulsed with MUC1-DG75-MVs (Fig. 5, first row), 95% of the cells showed the presence of ST-MUC1 glycoform localized in granules distributed in the entire cytoplasm. ST-MUC1 carried by microvesicles was further processed exhibiting T (18%) and Tn-glycoforms (45%), which were associated with intracellular small dots in the cytoplasm.

iDCs pulsed with rST-MUC1 glycoprotein (Fig. 5, second row) displayed reduced staining intensity for each mAb tested, indicating a significant decrease in the percentage of positive cells. In fact, only 60% of the cells showed that the ST-MUC1 glycoform was associated with the small vesicles localized just under the plasma membrane. T-MUC1 and Tn-MUC1 glycoforms were totally undetectable or extremely scarce (T-MUC1 < 3%; Tn-MUC1 5–8%). The absence of these glycoforms is probably due to the retention of the rST-MUC1 in the endosomal compartment without further processing. The MUC1-overexpressing breast cancer cell line T47D was used as a positive control (Fig. 5, third row). These results suggested that the processing of MUC1 transferred to dendritic cells by microvesicles was accompanied by the deglycosylation process, thus unmasking novel MUC1 glycoepitopes such as the T and Tn-MUC1.

Discussion

During cancer transformation, several *O*-glycosylated proteins are overexpressed and exhibited aberrant cancer-associated truncated glycans. This change in glycosylation exposes *de novo* tumor-specific and immunogenic glycoepitopes (22). Furthermore, changes in the surface *O*-glycosylation impact the interactions with APCs, as previous studies have shown that selected glycans can be used as efficient tools for dendritic cell targeting in cancer immunotherapy (23, 24).

Several *O*-glycoproteins are used as tumor markers, however, very few of these have been considered for cancer immunotherapy. In fact, it is believed that large *O*-glycoantigens induce mostly humoral and CD4$^+$ responses, and not antigen-specific CD8$^+$ T cells. However, it is clear that in cancer

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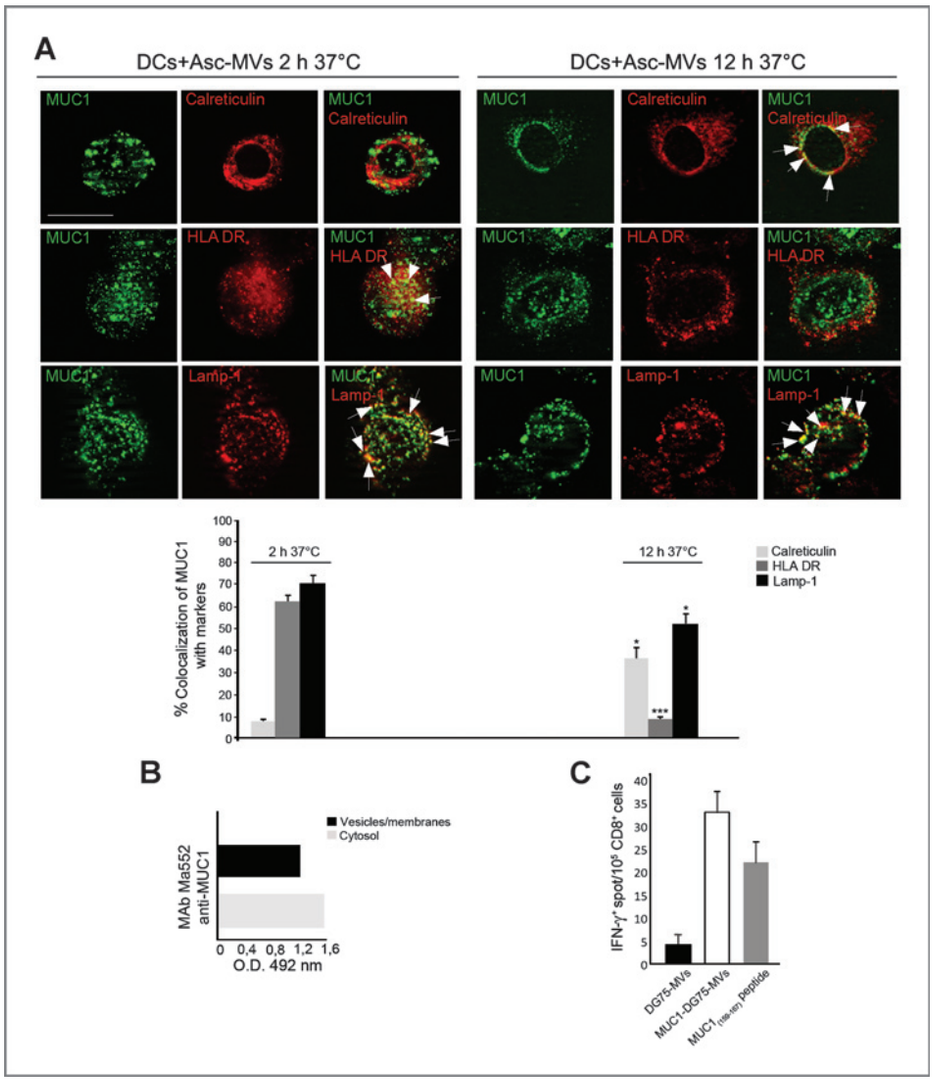

Figure 4. MUC1 carried by microvesicles is transported to the HLA-I compartment in dendritic cells and is cross-presented to MUC1-specific $CD8^+$ T cells. A, intracellular localization of MUC1 in iDCs pulsed with MUC1-Asc-MVs (2- or 12-hour incubation, column 1–3 and 4–6, respectively). MUC1 staining in green (mAb Ma552). Compartment marker staining in red: anti-calreticulin mAb for ER membranes (first row), anti-HLA-II-DR mAb (HLA-II compartment, second row), polyclonal Ab anti-Lamp-1 (endolysosomal compartment, third row). Results are expressed as mean values ± SE in histograms; percentage of colocalization (yellow) was calculated analyzing a minimum of 30 cells for each treatment randomly taken from three independent experiments. Magnification, ×63, Bar, 10 μm. *, $P < 0.05$ or ***, $P < 0.005$ versus the corresponding iDCs pulsed with MUC1-MVs for 2 hours at 37°C; B, Detection of MUC1 molecule in the cytosol and membrane fractions (gray and black histograms, respectively) of iDCs pulsed with MUC1-Asc-MVs by ELISA. C, IFN-γ ELISpot to evaluate the cytokine secretion by MUC1-specific enriched $CD8^+$ T cells in response to mDCs pulsed with DG75-MVs (black histogram); MUC1-DG75-MVs (white histogram) and $MUC1_{(159\text{-}167)}$ peptide (gray histogram). Results are representative of one patient out of three.

patients specific IgG immune responses and $CD8^+$ T-cell–mediated responses have been detected against tumor-associated *O*-glycoantigens such as CEA and MUC1, suggesting that these molecules are cross-presented in humans (25). These immune responses are protective and associated with favorable prognosis (26).

In carcinomas that express MUC1, the tumor-associated glycoforms of MUC1 have been shown to be immune targets *in vivo* (27). In patients with cancer, $CD8^+$ T cells directed against MUC1 peptide epitopes were detected before and after vaccination (28–30), and the IgG auto-antibodies against specific tumor-associated glycoforms were found in patients with cancer with early-stage disease (5, 31).

Early work showed that MUC1 following internalization is blocked in the endolysosomal compartment of dendritic cells (9), suggesting that only a tolerizing $CD4^+$ T-cell response could be generated against this glycoantigen. However, this study lacked the proper information on the glycan composition and, because the carbohydrate moieties decorating the MUC1 peptide backbone affect the uptake by dendritic cells (2), we speculated that the glycosylation profile could also modify the intracellular processing. Two distinct recombinant MUC1 glycoproteins, both tumor associated, were employed to test this hypothesis: the highly sialylated rST-MUC1, mostly carrying ST-*O*–linked glycan (85%) and the rTn-MUC1 carrying only Tn-glycans. rST-MUC1 exerts immunosuppressing effects on dendritic cell differentiation (32), promotes tumor growth (33), and no IgG specific response against the ST-MUC1 glycoform was detected in patients with cancer (34). On the other hand, Tn-MUC1 is immunogenic in mouse models (17, 20) and is a target of a specific IgG immune response in patients with cancer (5).

Despite the different immunogenicity, both rST-MUC1 and rTn-MUC1 were accumulated mostly in the endolysosomal compartment of iDCs after endocytosis. This indicates that the *O*-glycan profile does not influence MUC1 intracellular processing. MGL receptor-mediated endocytosis of the Tn-MUC1 glycoform induced a significant increase of MUC1 accumulation

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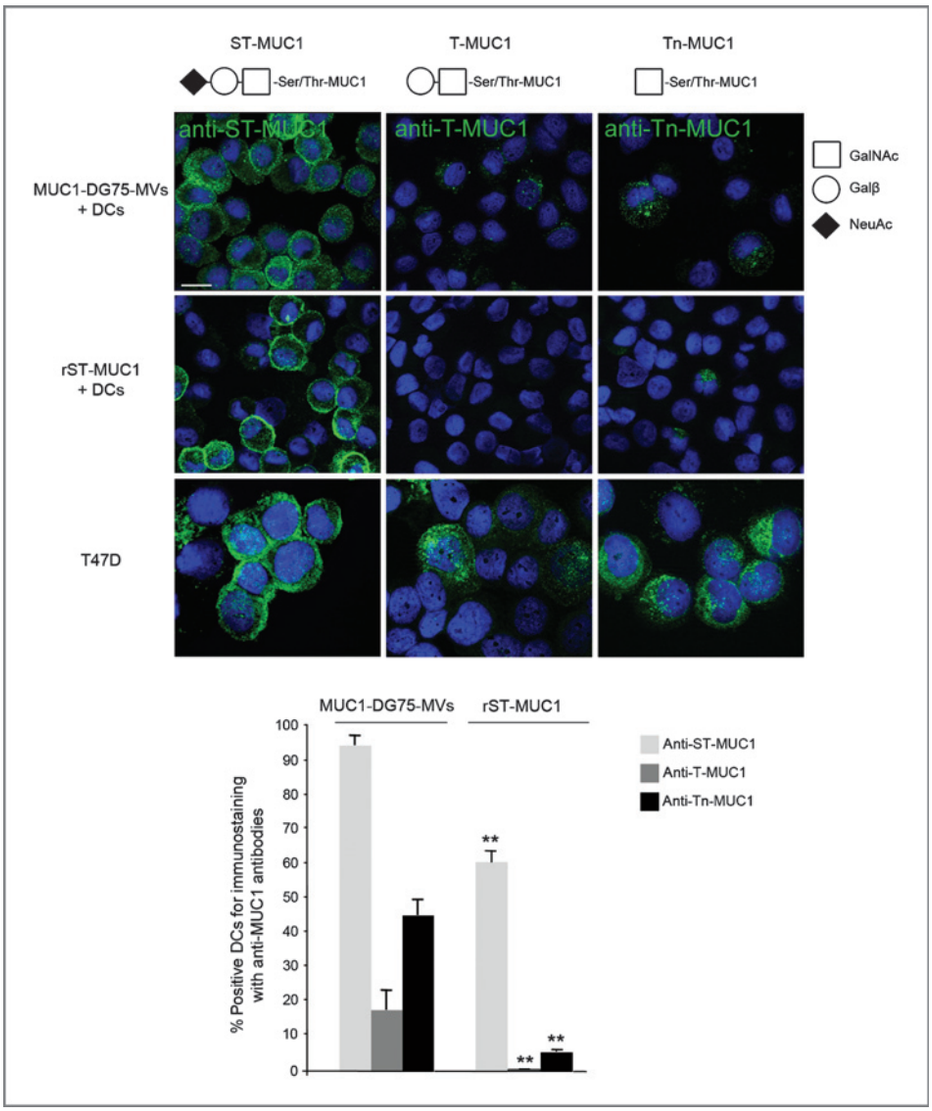

Figure 5. MUC1 carried by microvesicles undergoes deglycosylation in iDCs. MUC1 staining in iDCs pulsed with MUC1-DG75-MVs (first row) or rST-MUC1 glycoprotein (second row; 12-hour incubation). First column, mAb MY1E12 (anti-ST-MUC1); second column, 2D9 (anti-T-MUC1); third column, 5E5 (anti-Tn-MUC1). T47D cells were positive control for mAb reactivity (third row). Magnification, ×63; Bar, 10 μm. Carbohydrate residues are schematically represented. Percentage of positive dendritic cells for each mAb were plotted as histograms (gray, anti-ST-MUC1; dark gray, anti-T-MUC1; black, anti-Tn-MUC1). **, $P < 0.01$ versus the corresponding iDCs pulsed with MUC1-DG75-MVs.

in the endolysosomal compartment but no cross-processing in the HLA-I compartment was observed. These results are in agreement with other studies showing that the presence of Tn residues on the antigen increased antigen uptake by dendritic cells and activation of $CD4^+$-mediated T-cell response, but it failed to induce $CD8^+$ T-cell–mediated response in animal models (35). Cross-processing of MUC1 in dendritic cells was observed only when small synthetic glycopeptides were employed, suggesting that the shortening of the size of the antigen could be a way to override the block in processing (2, 36). However, *in vivo*, only the full-length MUC1 is available to the host immune system and indeed MUC1-specific $CD8^+$ T cells were found in patients with cancer (4, 37). We then hypothesized that other mechanisms of uptake that were glycosylation independent could occur inducing MUC1 epitope cross-presentation.

In vivo, antigens are released by the cells as soluble molecules as well as associated with microvesicles. These organelles are extremely heterogeneous in size, lipid composition, and biogenesis. Release of microvesicles has been regarded as an efficient way to deliver molecular signals into the microenvironment, overcoming cell–cell contact (38, 39). Moreover, tumor antigen-associated microvesicles have been shown to enhance the immunogenicity of soluble antigen (12), and to induce $CD8^+$ T-cell responses in *in vitro* human studies (13) and in pilot vaccination studies (14).

MUC1 bound to the microvesicles is released by cancer cells, as we have detected in ovarian ascites. Uptake of MUC1 carried by microvesicles resulted in strong MUC1 accumulation in the endolysosomal and HLA-II compartments after 2 hours of incubation followed by the translocation of the antigen to the HLA-I compartment after 12 hours of incubation. These results showed for the first time that a large and highly glycosylated antigen such as MUC1 is cross-processed by dendritic cells when delivered through microvesicles. Several cellular mechanisms have been proposed to explain the uptake and

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internalization of microvesicles by dendritic cells: macropinocytosis, and membrane fusion, ligand-receptor–mediated endocytosis. Also, the type of microvesicles and the acceptor cell are critical aspects in deciphering the internalization route of the transported antigen (40). In our system, translocation of MUC1 to the calreticulin-positive compartment was time dependent, implying intracellular transport of MUC1 from microvesicles purified from ovarian ascites and from microvesicles obtained from a MUC1-transfected cell line. These observations prompted us to test whether cross-processing resulted in cross-priming of MUC1 epitopes. Functionally, mDCs primed by MUC1-MVs were able to stimulate IFN-γ production of enriched $CD8^+$ T cells, specific for the $MUC1_{159\text{-}167}$ peptide epitope (21). So far, MUC1-specific $CD8^+$ T cells have been induced employing peptide, RNA pulsed dendritic cells, or tumor cell–dendritic cell fusion (37, 41, 42). This is the first time to our knowledge that the full-length MUC1 glycoprotein is able to activate MUC1-specific $CD8^+$ T cells. Indeed, previous *in vitro* studies have shown that the APC machinery in dendritic cells was competent to generate HLA-I–restricted MUC1 epitopes employed as substrates to the MUC1-based glycopeptides carrying distinct carbohydrate moieties (7, 43). The possibility for a glycoantigen as large as MUC1 to be processed and cross-presented opens the question about the intracellular fate of the carbohydrate moieties associated with the protein backbone. In our study, uptake and processing of ST-MUC1 resulted in the cytoplasmic appearance of T-MUC1 and Tn-MUC1 glycoepitopes as detected by specific mAbs only if the glycoprotein was delivered by microvesicles, suggesting that the processed antigen underwent deglycosylation, thus generating novel immunogenic glycoepitopes.

In summary, we have shown that MUC1 tumor-associated antigen after internalization accumulated in the endosomal/lysosomal compartments independently of its glycosylation profile and by receptor-mediated endocytosis. This block was overridden when MUC1 was delivered to dendritic cells through microvesicles and translocated to the HLA-I compartment, inducing antigen cross-presentation leading to dendritic cells capable of activating MUC1-specific $CD8^+$ T cells. In addition, microvesicle-mediated transfer of the sialylated MUC1 glycoform to dendritic cells mediated the *de novo* appearance of the shorter T and Tn-MUC1 glycoepitopes. Transfer to dendritic cells of large glycoproteins by microvesicles may be a relevant biologic mechanism *in vivo* that contributes to shape the immunogenicity of tumor-associated glycoantigens and to increase the complexity of the tumor glycopeptidome. These results have important implications for the design of glycoprotein-based immunogens for cancer immunotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A. Rughetti, M. Nuti
Development of methodology: A. Rughetti, F. Battisti
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Rahimi, F. Belleudi, C. Napoletano, F. Battisti, M. Antonilli, F. Bellati, H.H. Wandall, J.M. Burchell
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Rahimi, F. Belleudi, C. Napoletano, I.G. Zizzari, P. Benedetti Panici
Writing, review, and/or revision of the manuscript: C. Napoletano, I.G. Zizzari, M. Antonilli, F. Bellati, H.H. Wandall, P.B. Panici, J.M. Burchell, M.R. Torrisi, M. Nuti
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Rahimi, F. Battisti
Study supervision: A. Rughetti, M.R. Torrisi, M. Nuti

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