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Recombinant Tumor-Associated MUC1 Glycoprotein Impairs the Differentiation and Function of Dendritic Cells

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Tumors exploit several strategies to evade immune recognition, including the production of a large number of immunosuppressive factors, which leads to reduced numbers and impaired functions of dendritic cells (DCs) in the vicinity of tumors. We have investigated whether a mucin released by tumor cells could be involved in causing these immunomodulating effects on DCs. We used a recombinant purified form of the MUC1 glycoprotein, an epithelial associated mucin that is overexpressed, aberrantly glycosylated, and shed during cancer transformation. The O-glycosylation profile of the recombinant MUC1 glycoprotein (ST-MUC1) resembled that expressed by epithelial tumors in vivo, consisting of large numbers of sialylated core 1 (sialyl-T, ST) oligosaccharides. When cultured in the presence of ST-MUC1, human monocyte-derived DCs displayed a modified phenotype with decreased expression of costimulatory molecules (CD86, CD40), Ag-presenting molecules (DR and CD1d), and differentiation markers (CD83). In contrast, markers associated with an immature phenotype, CD1a and CD206 (mannose receptor), were increased. This effect was already evident at day 4 of DC culture and was dose dependent. The modified phenotype of DCs corresponded to an altered balance in IL-12/IL-10 cytokine production, with DC expressing an \( \text{IL-10}^{\text{high}} \text{IL-12}^{\text{low}} \) phenotype after exposure to ST-MUC1. These DCs were defective in their ability to induce immune responses in both allogeneic and autologous settings, as detected in proliferation and ELISPOT assays. The altered DC differentiation and Ag presentation function induced by the soluble sialylated tumor-associated mucin may represent a mechanism by which epithelial tumors can escape immunosurveillance.


During tumor transformation, genomic instability creates a vast repertoire of tumor cells that are selected by factors present in the microenvironment, such as growth factors, nutrient supply, and immune pressure (1). Tumor cells have multiple strategies to elude both innate and adaptive immune responses. Cancer cells can down-modulate HLA class I expression, generate HLA class I processing-defective variants or down-modulate tumor-specific Ags, all of which decrease their immunogenicity. Tumor cells can also affect the function of the APCs, in particular dendritic cells (DCs) that play a pivotal role in the induction and maintenance of an effective immune response (2, 3). In cancer patients, the number of DCs is reduced, and their functions are impaired. An increased proportion of immature DCs has been associated with the suppression of Ag-specific T cell responses (4, 5).

Transformed cells produce a variety of immunosuppressive cytokines and chemokines such as IL-10, vascular endothelial growth factor, and TGF-β, that can negatively affect the maturation and function of APCs (6–8). High concentrations of some of these molecules are found in the sera of cancer patients and are associated with disease progression and poor response to immunotherapy (9, 10). In addition, tumor cells also secrete other molecules that can down-modulate the Ag presentation process and induce early apoptosis of monocyte-derived DCs (11). High numbers of immature DCs can be found in the tumor bed (12), and the release of cytokines and immunosuppressive factors by the tumor can account for such phenomena (13).

Changes in glycosylation can also modulate interactions of tumor cells with effector cells of the immune system and their products. In this context, glycans carried by tumor Ags can differ profoundly from those found on the surface of normal cells, thus modifying the antigenic profile of these molecules. The MUC1 glycoprotein is a transmembrane epithelial mucin that is normally expressed on the apical surface of most simple glandular epithelial cells. In tumors, MUC1 is overexpressed, aberrantly glycosylated,

¶Abbreviations used in this paper: DC, dendritic cell; T antigen (or core 1), Galβ1,3GalNAc; Tn antigen, GalNAc; disialyl-T, NeuAcα2,3Galβ1,3GalNAc; sialyl-T, NeuAcα2,3Galβ1,3GalNAc; sialyl-Tn, NeuAcα2,6GalNAcβ1,3Galβ1,4GlcNAcβ1,6GlcNAcβ1,2Glc; CHO, Chinese hamster ovary; MUC1-IgG, recombinant fusion gene MUC1-mIgG2a; ST, sialylated core 1; ST-MUC1, recombinant cleaved MUC1; RT, room temperature.

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and shed in the tissue microenvironment (14). MUC1 expressed by breast carcinoma cells carries short O-glycans such as GalNAc (Tn), or Galβ1-3GalNAc (T or core 1), many of which are sialylated to give sialyl-Tn and mono- or disialyl-T. Although sialyl-T structures are expressed by some normal cells, such as resting lymphocytes, the O-glycans carried by MUC1 on normal breast epithelial cells are generally core 2 based and have extended branched poly-N-acetylactosamine chains (15, 16). Moreover, the occupancy of the O-glycosylation sites present in the core of the extracellular domain is increased in malignancy, making the breast cancer mucin more densely glycosylated (17).

The lack of a recombinant MUC1 with a glycoform equivalent to that produced by tumors has impeded studies on the influence of the glycosylation profile on the interaction of MUC1-expressing tumor cells with the immune environment. We have used the Chinese hamster ovary (CHO)-K1 eukaryotic system to produce MUC1 as a secreted glycoprotein (18), carrying the short sialylated O-glycans (mainly monosialyl-T) found on breast cancer cells. The ST-MUC1 glycoform (ST-MUC1) was evaluated on the differentiation and Ag-presenting ability of DC. The results indicate that the ST-MUC1 glycoprotein can contribute to the immunosuppression observed in cancer patients.

Materials and Methods
Production of recombinant MUC1 glycoprotein
Recombinant glycosylated MUC1 was generated and produced as previously described (18, 19). Briefly, cDNA of the MUC1 extracellular domain containing 16 tandem repeats was fused at the 5′ terminus of the nucleotide sequence of exons 1–3 of the murine IgG2a Fc domain. The chimeric DNA construct was subcloned into pcDNA3 vector and transfected into CHO-K1 cell line, and stable transfectants were selected for their resistance to neomycin and level of recombinant protein secretion. The secreted glycosylated MUC1 fusion protein (ST-MUC1-IgG) was concentrated from protein-free perfusion culture supernatant through ultrafiltration using a 100-kDa cutoff.

Unglycosylated recombinant MUC1 was produced in stably MUC1-transfected CHO Ildid cells (20) grown in DMEM-Ham’s F12 medium (1:1), 3% FBS in the presence of selectable marker G418 (400 μg/ml) without addition of galactose or GalNAc. Briefly, cDNA of the MUC1 extracellular domain containing 32 tandem repeats was fused at the 5′ terminus of the nucleotide sequence of exons 1–3 of the murine IgG2a Fc domain. The chimeric DNA construct was subcloned into pcDNA3 vector and transfected into CHO-K1 cell line, and stable transfectants were selected for their resistance to neomycin and level of recombinant protein secretion. The secreted glycosylated MUC1 fusion protein (MUC1-IgG) was purified from culture supernatant after dialysis by nickel-chelating chromatography and analyzed by SDS-PAGE silver stain. MUC1-IgG and ST-MUC1-IgG were cleaved by incubating with enterokinase (Invitrogen), was purified from culture supernatant after dialysis by nickel-chelating chromatography (19).

Purified proteins were tested for the presence of endotoxin contamination using QCL-1000 (Cambrex; BioWhittaker), showing results below 0.5 endotoxin U/μg.

Generation and culture of DCs
Generation of Dendritic Cells (DCs) from PBMC (PBMCs) was performed as described previously (21). In brief, PBMC were isolated by Ficoll-Hypaque (1.077 g/ml, Pharmacia, LKB, Sweden) density gradient centrifugation of anti-coagulated blood obtained from buffy coat preparations of healthy volunteers from the Blood Bank, University of Rome “La Sapienza.” Cells were seeded (6 × 10^6 cells/ml) into six-well plates (3 ml/well; BD Biosciences) in RPMI medium (HyClone) supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine (Sigma-Aldrich), 50 μg/ml streptomycin, 50 IU/ml penicillin (Flow Laboratories). After 2 h of incubation at 37°C, nonadherent cells were removed, and the adherent blood monocytes were cultured in RPMI medium supplemented with 5% human AB Serum (Sigma-Aldrich), antibiotics, and glutamine. The following cytokines were added: human recombinant GM-CSF (50 ng/ml; Molgardin; Schering-Plough) and IL-4 (700 IU/ml; Genzyme) for the generation of immature DCs. When DCs were grown in the presence of ST-MUC1 (ST-MUC1-DCs), control proteins (unglycosylated MUC1, or MUC2), or MUC1 60-mer peptide corresponding to 3 MUC1 tandem repeats, the compounds were added at the onset of the culture and then every 72 h (days 0, 3, and 6). The viability of DCs exposed to the recombinant proteins was monitored by trypan blue exclusion and by propidium iodide staining analyzed in flow cytometry.

Flow cytometry
Expression of cell surface markers before and after exposure to ST-MUC1 glycoprotein was quantified by flow cytometry incubating the cells with fluorochrome-conjugated mAbs to human DC-specific markers at saturating concentration for 1 h on ice. After incubation, the cells were washed with PBS and analyzed using a FACS Calibur (BD Biosciences), and samples were analyzed with CELLQUEST software (BD Biosciences). DCs were gated by side light scatter and forward light scatter parameters. Results plotted as superimposed histograms in which mean fluorescence intensities are reported.

FITC-conjugated mAbs used in this study were isotype controls (MOPC21 and MOPC-11), anti-DR (TU36), and anti-CD86 (2331/FUN-1); PE-conjugated mAbs used in this study were isotype control (MOPC21), anti-CD1a (HI149), anti-CD1d (CD1d12), anti-CD11c (B-ly6), anti-CD83 (HB15e), anti-CD206 (19.2); CyChrome-conjugated mAbs were anti-CD40 (5C3) and anti-CD84 (HA-58). All the Abs used were IgG1, except mAbs MPC-11 and TU36, which were IgG2b. To screen for HLA-A2 expression, 100 μl of blood from healthy donors were incubated with FITC-conjugated anti-HLA-A2 mAb BB7.2 (Cancer Research U.K.), after RBC lysis by ACK (Sigma-Aldrich). Propidium iodide (Sigma-Aldrich) staining (10 μg/ml) was used as viability control.

Statistical analysis
The differences in DC marker expression between monocyte-derived DCs and DCs differentiated in the presence of ST-MUC1 were analyzed by Student’s t test (one tail).

Cytokine measurements
The levels of IL-12 (p40/p70) and IL-10 in the DC culture supernatants were measured using ELISA kits purchased from R&D Systems according to the manufacturer’s instructions. Maturation of DCs was induced by adding 100 ng/ml LPS (Sigma-Aldrich) at day 6 of culture overnight. Culture medium were collected at day 7 of culture and stored at −80°C until use.

Allogeneic MLR
The ability of DCs and ST-MUC1-pulsed DCs to stimulate allogeneic T cells was tested in MLR. At day 6 of culture, DCs were exposed to 20 μg/ml ST-MUC1 for 24 h. At day 7, DC and ST-MUC1-pulsed DCs were harvested, washed to remove cytokines and glycoprotein present in the culture, and irradiated (3 × 10^4 rads). The APCs were then seeded (10^4/ml) with allogeneic T cells (5 × 10^5/100 μl/well) in 96-well round-bottom plates (Costar, Cambridge, MA) in RPMI supplemented with 5% human AB serum, glutamine, and antibiotics for 5 days. Each sample was tested in triplicate. T cell proliferation was measured by the uptake of ^3H]thymidine (ICN) which was added 1 μCi/well during the final 18 h of culture. Cells were harvested onto 96-well GF/C filter plates (Packard BioScience) with an automated 96-well harvester, and ^3H]thymidine incorporation was measured using a TopCount scintillation counter (Packard BioScience). The proliferation index was calculated by dividing the count average obtained for DC + T cell samples by the count average obtained for T cells alone. PHA, 5 μg/ml (Sigma-Aldrich), was added as a positive control on day 2.

Ag-specific CTL induction
DCs and ST-MUC1-DCs generated from monocytes of a HLA-A2 healthy donor were pulsed with 50 μg/ml matrix influenza virus A2 peptide (GIL- GFVFTL, flu peptide; Sigma Genosys), 3 μg/ml β2-microglobulin (Sigma-Aldrich) for 2 h at day 7 of culture. APCs were then washed and plated with CD8+ enriched lymphocytes at a ratio of 1:5 in RPMI with 5% human AB serum, glutamine, and antibiotics. The culture medium was also supplemented with 50 μm IL-2 and 20 μg/ml flu peptide. The CD8+ enriched cells were obtained from the mononuclear cells isolated by Ficoll-Hypaque centrifugation gradient, plated for 2 h to remove the adherent cells. Cells in suspension were harvested, and CD4− lymphocytes were removed by plating cells onto plastic coated with 10 μg/ml purified anti-CD4 mAb RPA-T (Valter Occhiena) for 2 h at 37°C. Enrichment for CD8− cells was monitored by flow cytometry using the anti-CD8 mAb OKT8.
ELISPOT assay

After 1 week in culture with DCs, the T cells were harvested, washed, and plated for the ELISPOT assay to evaluate the production of IFN-γ as a response to the specific viral Ag stimulation. The 96-well multiscriff filter plates (Millipore) were coated with 100 μl of mAb anti-human IFN-γ (10 μg/ml; clone NIB42; BD Pharmingen) diluted in PBS (HyClone). After overnight incubation at 4°C, unbound mAb was removed, and aspecific binding sites were blocked with PBS-1% BSA (Sigma-Aldrich) (200 μl/well) for 2 h at 37°C and then with RPMI 1640 with 5% human AB serum (100 μl/well). The CD8+ cells were plated in quadruplicate onto the coated wells (5 × 10^5 cells/100 μl/well) in RPMI 1640 containing 5% AB serum, glutamine, and antibiotics. Cells were then restimulated with flu peptide (20 μg/ml) or with flu peptide-pulsed autologous irradiated PBMCs (1:1) (PBMCs were pulsed with 50 μg/ml flu peptide and 3 μg/ml β2-microglobulin (Sigma-Aldrich) for 2 h). After 24 h of incubation at 37°C, wells were washed six times with PBS-0.25% Tween 20, three times with PBS, and once with H2O. The plate was then incubated for 2 h at room temperature (RT) in 50 μl/well PBS-1% BSA containing 5 μg/ml biotinylated secondary anti-IFN-γ mAb (clone 4S.B3; BD Pharmingen) and washed three times with PBS-0.25% Tween 20 and three times with PBS. The plate was further incubated with 50 μl/well streptavidin-alkaline phosphatase (BD Pharmingen) at a dilution of 1/2000 in PBS-1% BSA for 2 h. After three washings in PBS, the plate was incubated with 50 μl/well of chromogen substrate (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium; Sigma-Aldrich) in the dark at RT. Spots were counted using the ImmuNoSpot Image Analyzer.

Results

Recombinant ST-MUC1 glycoprotein

A human recombinant MUC1 (ST-MUC1-IgG) was produced in CHO-K1 cells (18), as a fusion protein between the MUC1 extracellular domain (containing 16 tandem repeats) and mouse IgG2a Fc fragment, as indicated in Fig. 1A. The recombinant MUC1 extracellular domain, ST-MUC1, was released from the ST-MUC1-IgG fusion protein by enterokinase treatment and separated from the IgG tail by further purification using anion exchange chromatography.
Biochemical characterization of the purified fusion protein revealed that the amino acid composition is equivalent to the one expected (Fig. 1B). Moreover, O-glycosylation of the ST-MUC1-IgG was similar to that found on the breast cancer-associated MUC1, as expressed by breast cancer cell line T47D, with sialylated core 1 (ST) structures being the dominant O-glycans (see Fig. 1C). Moreover, the tandem repeat domain was densely glycosylated, with an average of 4.3 sites glycosylated per tandem repeat (Fig. 1B).

ST-MUC1 glycoprotein modifies the phenotype of monocyte-derived DCs

Monocytes were differentiated toward DCs in the presence of ST-MUC1 from the onset of the culture (ST-MUC1-DCs), and the cell phenotype was analyzed after 7 days (Fig. 2A). Cell viability was not affected by the addition of the glycoprotein. In all donors tested ($n = 8$), the presence of ST-MUC1 was associated with a more immature phenotype of the DCs, although a variability in the intensity of phenotypic changes could be observed between donors. The expression of CD1a, when present, was increased, whereas the expressions of DR, CD86 (a costimulatory molecule), and CD83 (a DC lineage marker) were all down-regulated (Fig. 2A). Table I summarizes the results of eight donors as mean fluorescent intensity of expression of the above markers. Also, CD86 was similarly affected in DCs differentiated from CD34$^+$ progenitor cells exposed to the ST-MUC1 (data not shown). Control experiments were performed using the unglycosylated MUC1 produced similarly in the CHO Idld cell system and a synthetic peptide corresponding to three tandem repeats. The presence in the differentiating culture of the naked MUC1 both as recombinant proteins and

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** DC phenotype is modified by exposure to the ST-MUC1 tumor-associated glycoform. A, Monocytes were cultured in the presence of GM-CSF and IL-4. ST-MUC1 (20 μg/ml) was added at the onset of the culture. Expression of cell surface molecules was assessed at day 7 by flow cytometry with fluorochrome-labeled mAbs. The results of three representative experiments are shown. Continuous line, control DCs; bold continuous line, ST-MUC1-DCs. Mean fluorescence intensities for each sample, after subtraction of the mean fluorescence intensity for the matched isotype-control, are shown. Mean fluorescence intensities for ST-MUC1-DCs are shown in boldface. B, Phenotype characterization of DC at day 7 of culture grown in the presence of the unglycosylated MUC1 control protein (20 μg/ml) and MUC1 60-mer corresponding to three tandem repeats. Results of one representative experiment are shown. Continuous line, control DCs; bold continuous line, unglycosylated MUC1-DCs/MUC1 60-mer DCs.
as synthetic peptide does not affect DC phenotype as characterized by the cytofluorimetric analysis of the expression of DR, CD1a, CD86, and CD83 markers. Similar results were obtained with the unrelated protein with the MUC2 backbone (22) (data not shown).

To evaluate the kinetics of the effect of ST-MUC1 on the differentiation process, the phenotype of DCs exposed to ST-MUC1 (20 \(\mu\)g/ml) was analyzed, in six different donors, at days 4 and 7 of culture. Fig. 3 shows the results of one representative experiment: at day 4 of culture, a clear down-modulation of DR, CD86, and CD83 (\(p_{CD86} = 0.003\) and \(p_{CD83} = 0.002\), respectively) was noted already at day 4 of culture, whereas CD1a was up-regulated (\(p = 0.038\)). Similar results were obtained also when low amounts of ST-MUC1 (1 \(\mu\)g/ml) were used and were still maintained at day 7 of culture. The expression of some additional molecules involved in Ag capture (CD206 (mannose receptor)), presentation (CD1d), and costimulation (CD40) was also analyzed. These markers were also modified in the presence of ST-MUC1; the expression of CD1d and CD40 was decreased, whereas the CD206 expression was increased, and these changes appeared from day 4 of culture (Fig. 4).

**ST-MUC1 glycoprotein-treated DCs show an impaired production of IL-12 cytokine**

Monocyte-derived DCs can secrete different patterns of inflammatory and immunoregulatory cytokines that can modulate the type of T cell response induced (Th1 vs Th2). The amount of IL-12 and IL-10 was measured in the culture supernatant of DCs alone or differentiated in the presence of ST-MUC1 glycoprotein (Fig. 5). In accordance with the modified DC phenotype, IL-10 secretion is increased in ST-MUC1-DCs as compared with the control DCs (Fig. 5A). Moreover, ST-MUC1-DCs produce lower amount of IL-12, even at low concentrations of the ST-MUC1 (Fig. 5B). Maturation of control DCs by exposure to LPS resulted in an increase

### Table I. Summary of phenotype analysis of DCs differentiated in the presence or absence of ST-MUC1 displayed as mean fluorescence intensity

<table>
<thead>
<tr>
<th>Donors</th>
<th>DR (^a)</th>
<th>CD83</th>
<th>CD86</th>
<th>CD1a</th>
</tr>
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<tr>
<td>D1</td>
<td>237</td>
<td>174</td>
<td>80</td>
<td>33</td>
</tr>
<tr>
<td>D2</td>
<td>205</td>
<td>115</td>
<td>46</td>
<td>22</td>
</tr>
<tr>
<td>D3</td>
<td>363</td>
<td>470</td>
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<td>D4</td>
<td>460</td>
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<tr>
<td>D6</td>
<td>458</td>
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<td>70</td>
<td>33</td>
</tr>
<tr>
<td>D7</td>
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<td>93</td>
<td>63</td>
<td>28</td>
</tr>
<tr>
<td>D8</td>
<td>302</td>
<td>182</td>
<td>109</td>
<td>41</td>
</tr>
</tbody>
</table>

\(^a\) Phenotype was assessed between days 5 and 7 of culture. Results are reported as mean fluorescence intensity values. Mean fluorescence intensity isotype controls ranged from 15 to 25.

\(^b\) ST-MUC1 was added at a final concentration of 20 \(\mu\)g/ml.

**FIGURE 3.** ST-MUC1 impairs DCs differentiation from the beginning of culture, also at low concentrations. Monocyte-derived DCs were cultured in the absence or presence of ST-MUC1 at different concentrations (1 and 20 \(\mu\)g), and their phenotypes were analyzed by FACS at days 4 and 7 of culture. Continuous line, DCs; bold continuous line, ST-MUC1-DCs. Values of M.F.I. for each sample, after subtraction of the M.F.I. for the matched isotype-control, are shown. Mean fluorescence intensities for ST-MUC1-DCs are shown in boldface.
in IL-12 secretion. However, even after LPS stimulation, DCs differentiated from monocytes in the presence of ST-MUC1 were unable to produce IL-12. This effect of ST-MUC1 is dose dependent, as DCs cultured at the low concentration of 1 μg/ml can be induced to secrete IL-12 by LPS (Fig. 5B, middle and left panels).

**ST-MUC1 glycoprotein down-modulates APC function of DCs**

To define whether the immature phenotype induced by ST-MUC1 was accompanied by a reduced function of DCs, allogeneic and autologous MLR were performed. Fig. 6A shows that the proliferative allogeneic response of T cells was reduced when DCs previously exposed to ST-MUC1 were used as stimulus compared with the untreated DCs. This suggested that the ST-MUC1 glycoform can modify the immune response by interfering with the Ag presentation process. The ability to induce CTLs specific for a recall viral Ag such as the influenza matrix epitope was also investigated. ST-MUC1-DCs and control DCs were pulsed with the HLA-A2-restricted matrix peptide of the influenza virus (GILGFVFTL) and used as APCs to stimulate autologous enriched CD8+ lymphocytes. After 1 week of culture, cells were harvested and washed to remove any immunoregulatory factor endogenously present, and the specificity for the flu Ag was tested by measuring the release of IFN-γ, after a second stimulation, in overnight ELISPOT assay (Fig. 6B). The CD8+ cells primed with ST-MUC1-DCs showed a decreased ability to produce IFN-γ, after a second Ag stimulation with either peptide alone or peptide-pulsed autologous fresh PBMCs. Furthermore, lymphocytes primed with ST-MUC1-DCs had a decreased background production of IFN-γ compared with the culture control (Fig. 6B, left side of panel). These results suggest that T cells originally primed with impaired DCs do not fully recover even in the presence of a second stimulation (peptide alone or presented again by autologous freshly thawed APCs).

**FIGURE 5.** Differentiation of monocytes in the presence of ST-MUC1 switches DCs to IL-10↑IL-12↓ phenotype. DCs were differentiated from monocytes with GM-CSF and IL-4 in the presence or absence of ST-MUC1, 1 or 20 μg/ml. After 7 days, supernatants from DCs and ST-MUC1-DC cultures were tested for the presence of the immunomodulatory cytokines IL-10 (A) and IL-12 (B). Maturation of DCs was induced by adding LPS (100 ng/ml) at day 6 of culture. iDC, immature DC; mDC, mature DC.
of ST-MUC1

wk in culture, the cells were harvested, washed, and seeded (10^6 cells/ml; 100 healthy donor were stimulated with autologous DCs pulsed with flu matrix peptide or autologous ST-MUC1-DCs pulsed with flu matrix peptide. After 1 proliferation index. Proliferation was evaluated at day 4 as incorporation of [3H]thymidine for 16 h, and results are plotted as the of the ST-MUC1 glycoprotein (f). Here we demonstrate that a glycosylated recombinant form of

DCs, switching them to a IL-10highIL-12low phenotype that was breast cancers, inhibited the differentiation of monocyte-derived

factors has also been shown to be a relevant mecha-

molecules and the secretion of IL-10-regulatory cytokine resulted in an impaired ability of such APCs to induce allogeneic and autologous immune responses. The ability of DCs secreting high amounts of IL-10 to promote T cell anergy has been shown in other systems (27–28).

It is well established that tumor cells produce and shed several molecules that can negatively affect the maturation and function of immune cells. The majority of molecules that have been identified thus far are chemokines and cytokines such as vascular endothelial growth factor, IL-10, and TGF-β that impair DC function by altering the phenotype or by enhancing spontaneous apoptosis. Some of these findings have been associated with poor prognosis in patients (4, 5). However, the shedding by the tumor of soluble factors distinct from cytokines has also been shown to be a relevant mechanism of immunosuppression in vivo and in vitro (13, 29, 30). Some reports have indirectly addressed the question of MUC1 mucin being involved in the impairment of the immune response. In a clinically relevant mouse model of pancreatic cancer, MUC1-specific CTLs are indeed produced; however, they are anergic and nonfunctional in the tumor microenvironment. CTL tolerance could be reversed in vitro by the use of CD40 costimulation (31). Furthermore, release of MUC1 by human tumor pancreatic cell lines has been correlated with an altered DC phenotype and compromised function (32). In addition, partially purified forms of the shed circulating MUC1 glycoprotein were shown to down-regulate

Discussion

Several reports have described the tumor habitat as an immuno-

Discussion

Several reports have described the tumor habitat as an immuno-

A

B

FIGURE 6. ST-MUC1 glycoprotein impairs Ag-presenting function of DCs. A, ST-MUC1-DCs show a reduced stimulatory function in allogeneic MLR. Proliferative response of freshly isolated PBLs (5 × 10^4/100 μl/well) to allogeneic DCs (1 × 10^4/100 μl/well) (□) or DCs cultured for 24 h in the presence of the ST-MUC1 glycoprotein (●). Proliferation was evaluated at day 4 as incorporation of [3H]thymidine for 16 h, and results are plotted as the proliferation index. B, ST-MUC1-DCs show a reduced capacity to stimulate autologous T cells to response to an influenza peptide. T cells from a HLA-A2 healthy donor were stimulated with autologous DCs pulsed with flu matrix peptide or autologous ST-MUC1-DCs pulsed with flu matrix peptide. After 1 wk in culture, the cells were harvested, washed, and seeded (10^5 cells/ml; 100 μl/well) in a 96-well plate (Millipore) that had been previously coated with anti-IFN-γ Ab. The cells were restimulated with 20 μg/ml peptide (middle panel, + flu) or with peptide-pulsed irradiated PBMC (1:1), 50 μg/10^6 cells/ml (right panel, PBMCs + flu), incubated overnight, and the production of IFN-γ was analyzed by ELISPOT. The first panel (no flu) shows basal IFN-γ production of T cells initially stimulated with flu-pulsed DCs or flu-pulsed ST-MUC1-DCs. Each experimental condition was performed in quadruplicate, and the results are plotted as the mean of number of spots/10^5 cells. ST-MUC1-DCs_A and ST-MUC1-DCs_B correspond to two different purification batches of ST-MUC1.
not seen with recombinant MUC1 produced in CHO ldlD cells and thus lacking O-linked glycosylation or with a peptide corresponding to three tandem repeats as well as with the unrelated recombinant MUC2 protein produced in the CHO-K1 cell system (data not shown). In this context, it is highly significant that the enzyme catalyzing the synthesis of the sialyl-T (ST3Gal-1) is up-regulated in breast cancer, and this increase correlates with grade and increased aggressiveness (35). Moreover, in the MUC1-transgenic mouse, where human MUC1 is expressed as a self Ag, tumors expressing the ST-MUC1 glycoform grew faster than tumors expressing MUC1 carrying the extended core 2-based O-glycans (36). We cannot, however, exclude that other tumor-associated MUC1 glycoforms carrying carbohydrate determinants such as Tn, sialyl-Tn, and T may also affect DC differentiation. Although the unglycosylated MUC1 does not exert a similar effect as well as a MUC1 glycopeptide carrying the Tn Ag (data not shown), further studies are needed to investigate the possible effects of other tumor-associated MUC1 glycoforms. Indeed, glycosylation profile of the MUC1 extracellular domain may play an important role in modulating the interactions of MUC1. On erythroblasts, MUC1 is sialylated, and this particular glycoform can bind sialoidhesin, a sialic acid-binding Ig-like lectin expressed by macrophages, suggesting a possible contribution of MUC1 in cell-cell contacts in a bone marrow microenvironment (37). Similarly, sialylated MUC1 glycoforms expressed by breast tumor cells could contribute to cell-cell contact between cancer cells and tumor-infiltrating macrophages in vivo (38). Mucins can be differentially glycosylated also in nontumoral cells. In T cells, activation leads to expression of membrane mucins carrying core 2-based O-glycans, whereas the resting T cells express sialylated core 1-based structures (39–41). In the change to malignancy in breast, the reverse change is seen, with the core 2-based structures found on the MUC1 mucin produced by the normal epithelial cells become partly replaced by core 1-based O-glycans in the cancer mucin (42). The fact that the sialyl-T O-glycan is expressed on some normal cells suggests that there may be a link of the control of autoimmune responses, possibly through interactions of the carbohydrates with specific lectins expressed by immune effector cells.

Lectins expressed on DCs and other APCs are receptors designed for the host defense from pathogens (43), where the carbohydrate pattern dictates the final routing in the endocytic pathway and eventually the Ag presentation to effector cells. For example, the interactions with DC lectin have been proposed to explain immune evasion by viral pathogens such as HIV (44). It is likely that the effects of ST-MUC1 on the DC phenotype that we describe here are mediated by interactions with lectins expressed by DCs, which lead to a functional change, impairing the overall Ag presentation by DCs.

In particular, the down-regulation of CD86, one of the most prominent ST-MUC1 phenotypic changes, is consistent with the observed altered function of DCs. The significantly down-regulated CD86 expression confers to the APC a weak costimulatory signal 2. It is well established that efficient T cell activation and memory is obtained when the Ag-triggered activation (signal 1) is matched with appropriate costimulatory pathways (signal 2) (45, 46). The balance of the interactions between costimulatory molecules on APCs and their counterreceptor on the T cells steers the immune response toward anergy or activation and furthermore the polarization of this response (47–49). Signal 2 is moreover important for the survival and proliferation of T cells, but also to induce CTL with higher avidity when signal 1 is weak as in the case of tumor self Ags or when Ag concentration and/or MHC-Ag complexes are low (50, 51). The activation of T cells in the absence of CD86/CD28 leads eventually to Th2 response or anergy.

The release of IL-10 in the tumor microenvironment could also contribute to shift the T cell response toward a Th2 phenotype and the generation of regulatory T cells (52, 53).

The presence of ST-MUC1 recombinant glycoprotein that is similar to the tumor-associated glycoform secreted by the tumor cells induces production of IL-10 in the DC culture and prevents secretion of IL-12. LPS stimulation restores IL-12 production, but only when DCs are exposed to minimal amounts of the immunosuppressive glycoprotein. This could indicate that the amount of tumor-associated MUC1 released in the tumor microenvironment (i.e., related to tumor burden and progression) may be an important variable. Experiments are in progress to define the kinetics and the molecules involved in the possible reversibility of this effect. The autocrine IL-10 production by DCs has also been shown to down-modulate the CD86-costimulatory molecule and inhibit spontaneous maturation of DCs in vitro (54). Moreover, IL-10 reduces T cell alloreaction induced by both epidermal Langerhan’s cells and myeloid DCs (55, 56). It is interesting that in our cell culture system, DCs exposed to ST-MUC1 are poor stimulators in both autologous and allogeneic settings.

MUC1 shedding is one of the first steps that follow epithelial cell transformation. The tumor-associated form of the molecule gains access to the circulation of carcinoma patients, and variations in the levels of circulating MUC1 are used to monitor therapy in breast cancer patients and for the early detection of recurrence (57). The results described here suggest that the presence of MUC1-ST glycoforms can promote from the onset of the tumor the progressive induction of immune silencing in synergy with other described immunosuppressive factors. The early impairment of DCs can strongly deviate and compromise the possible immune responses leading furthermore to progression of the disease.

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Disclosures
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