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Human Tumor Antigen MUC1 Is Chemotactic for Immature Dendritic Cells and Elicits Maturation but Does Not Promote Th1 Type Immunity[1]

Casey A. Carlos,* Hui Fang Dong,† O. M. Zack Howard,‡ Joost J. Oppenheim,‡ Franz-Georg Hanisch,§ and Olivera J. Finn[2]*

The immunostimulatory outcome of the interactions of many pathogens with dendritic cells (DCs) has been well characterized. There are many fewer examples of similar interactions between DCs and self-molecules, especially the abnormal self-proteins such as many tumor Ags, and their effects on DC function and the immune response. We show that human epithelial cell Ag MUC1 mucin is recognized in its aberrantly glycosylated form on tumor cells by immature human myeloid DCs as both a chemoattractant (through its polypeptide core) and a maturation and activation signal (through its carbohydrate moieties). On encounter with MUC1, similar to the encounter with LPS, immature DCs increase cell surface expression of CD80, CD86, CD40, and CD83 molecules and the production of IL-6 and TNF-α cytokines but fail to make IL-12. When these DCs are cocultured with allogeneic CD4$^+$ T cells, they induce production of IL-13 and IL-5 and lower levels of IL-2, thus failing to induce a type 1 response. Our data suggest that, in vivo in cancer patients, MUC1 attracts immature DCs to the tumor through chemotaxis and subverts their function by negatively affecting their ability to stimulate type 1 helper T cell responses important for tumor rejection. ***The Journal of Immunology,*** **2005, 175: 1628–1635.**

Unique molecular patterns expressed by pathogens are recognized by APCs through pattern recognition receptors (PRRs)[3] such as TLRs (1), C-type lectins (2), and I-type lectins (3). Immature dendritic cells (imDCs) respond to signals from the PRRs by undergoing maturation and turning on the production of specific sets of cytokines typical for individual receptors and the pathogens they recognize. Although it has been assumed that these receptors evolved to signal the presence of invaders from the outside (i.e., pathogens), there is evidence that PRRs also recognize some self-molecules, suggesting that they may have evolved to alert the immune system to changes in the body that threaten the integrity of the organism that might require its involvement (4).

A tumor is an example of an invader from within, and although derived from the host, tumor cells display molecular patterns that distinguish them from normal cells. The presence of tumor-specific immune responses in cancer patients is clear evidence that the immune system has been alerted to the presence of the tumor, but the progressive growth of the tumor suggests that the process of immune activation is not usually conducted successfully.

We have studied the ability of dendritic cells (DCs) to signal to the adaptive immune system the presence of malignant epithelial cells expressing abnormal forms of an epithelial cell glycoprotein, MUC1. This molecule is normally expressed on the apical surfaces of ductal epithelial cells and in the lumen of the ducts (thus, outside of the body) but gains access to the inside of the body during epithelial cell transformation and the growth of epithelial adenocarcinomas (5). The extracellular portion of MUC1 is largely composed of a region of multiple tandem repeats of a 20 aa sequence. On normal epithelia, MUC1 is expressed at low levels and is complexly *O*-glycosylated, but on tumor cells, it is greatly overexpressed and markedly hypoglycosylated with more simple and shorter chains. Reduced glycosylation exposes the peptide backbone resulting in novel peptide epitopes as well as novel truncated carbohydrate epitopes characteristic of tumor cells, such as T (Gal-GalNAc-*O*-Ser/Thr), Tn (GalNAc-*O*-Ser/Thr), and sialyl-Tn (NeuAc-GalNAc-*O*-Ser/Thr) (6). We have published previously that MUC1, either purified from tumor cells or synthesized as a long synthetic tandem repeat peptide with and without *O*-linked carbohydrates, can bind to imDCs and be internalized through an active endocytic process (7). In a subsequently published study, we addressed what type of epitopes, peptides, and/or glycopeptides, are generated and could be presented by the DC that bind these different forms of MUC1, and we showed that both peptides and glycopeptides can be found bound to MHC class II (8).

In this study, we describe an unexpected finding that the exposed unglycosylated epitopes in the tandem repeat region of MUC1 are potent chemoattractants for immature human myeloid DCs. Furthermore, MUC1 bearing short sialylated carbohydrates, which we previously showed binds to and is internalized by DCs (8), induces DC activation and maturation phenotypically similar to that induced by LPS. Both molecules induce an increased expression of CD40, CD80, CD86, and CD83. Importantly, whereas

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[3] Abbreviations used in this paper: PRR, pattern recognition receptor; DC, dendritic cell; imDC, immature dendritic cell; mDC, mature dendritic cell.

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LPS-treated imDCs turn on production of proinflammatory cytokines, DCs exposed to tumor forms of MUC1 produce IL-6, TNF-α, and IL-10 but extremely low levels of IL-12. When naive T cells are stimulated with allogeneic DCs that were exposed to MUC1, they produce higher levels of IL-13 and IL-5 compared with T cells activated with LPS-treated DCs, which make low levels of these cytokines and high levels of IFN-γ.

Th1 type immunity is considered to be important in tumor rejection (9). DCs attracted to the site of the tumor through the chemotactic activity of MUC1 after further interactions with it would not be expected to induce a strongly polarized Th1 response either against MUC1 itself or other Ags simultaneously expressed on MUC1$^+$ tumors. High levels of aberrantly glycosylated MUC1 are expressed on all human adenocarcinomas as well as on the known premalignant lesions, precursors to some of these tumors (10). Our data show a remarkable ability of DCs to be alerted to the existence of tumors, in this case responding to the chemotactic activity of a tumor Ag, but also the ability of some Ags, like MUC1, to subvert their function and prevent development of efficient Th1 type antitumor immunity.

Materials and Methods

Reagents

Human GM-CSF and IL-4 were obtained from Schering-Plough. All other chemokines and cytokines were obtained from the National Institutes of Health cytokine repository or PeproTech. Reagents were purchased from Sigma-Aldrich, unless noted otherwise. Anti-MUC1 Ab MF11 used in the chemotaxis blocking studies was obtained from the International Society for Oncodevelopmental Biology and Medicine, Tumor Diagnosis Workshop 4 International Workshop on mAbs against MUC1 (11). It is specific for the PPAH sequence in the 20-aa-long MUC1 tandem repeat sequence GVTSAPDTRPAPGSTAPPAH. mAbs used to phenotype DCs were purchased from BD Biosciences, unless indicated otherwise.

MUC1 Ag preparations

The MUC1 100mer synthetic peptide (GVTSAPDTRPAPGSTAPPAH) $\times$ 5 represents five unglycosylated tandem repeats from the variable number of tandem repeats region comprising most of the extracellular domain of MUC1. MUC1 100mer was synthesized on a Chemtech 200 automated peptide synthesizer (Advanced ChemTech) with *N*-(9-fluorenyl)methoxycarbonyl chemistry and purified by HPLC at the University of Pittsburgh Cancer Institute Peptide Synthesis Facility. MUC1 Tn-100mer is a glycosylated 100mer peptide that was prepared as described previously (8, 12). It contains 15 GalNAc residues bound to the threonines in VTSA regions and to the serines and threonines in GSTA regions of the tandem repeat sequence GV**T**SAPDTRPAPG**ST**APPAH (glycosylated residues are indicated in bold), consistent with the site-specificity of the recombinant glycosyltransferase rGalNAc-Ts used to glycosylate the peptide. Ascites MUC1 was purified from ascitic fluid obtained from cancer patients and characterized as described previously (13). Generation and purification of recombinant tumor forms MCF-7 MUC1 and HEK MUC1 were described previously (14). In brief, a mammalian episomal expression vector, pCEP-PU (15), encoding six tandem repeats of MUC1 (120mer peptide) under the control of the CMV promoter was used to transfect the breast cancer cell line MCF-7 and the EBV-transformed human embryonic kidney cell line HEK293/EBNA (American Type Culture Collection). This vector contains the secretory signal peptide of BM40, an extracellular matrix protein also known as SPARC or osteonectin, followed by a hexahistidine sequence and a Myc tag used for affinity purification of the MUC1 protein produced by the transfected cells. HEK MUC1 and MCF-7 MUC1 were purified from the conditioned supernatant of confluent cell layers by passage through a Ni^{2+}-nitrilotriacetic acid Superflow column (Qiagen). They were also purified by HPLC on a C8 silica column (Vydac 208TP1015; MZ Analysentechnik). The quality of purified recombinant MCF-7 MUC1 and HEK MUC1 was checked by SDS-PAGE, followed by blotting onto a nitrocellulose membrane. Peptides were detected with an anti-Myc mAb (14) (Santa Cruz Biotechnology). Specific *O*-glycosylation profiles were determined by hydrazinolysis and normal-phase chromatography of 2-aminobenzamide-labeled glycans as described previously (14). The major sialylated glycans on HEK MUC1 were chemically desialylated by treatment of the fusion protein with 0.1 M aqueous trifluoroacetic acid for 1 h at 80°C, followed by drying in a vacuum centrifuge. Sialidase treatment of HEK MUC1 was accomplished with *Clostridium perfringens* sialidase (Biolabs) using 5 U in a total volume of 50 μl of reaction buffer (0.1 M sodium acetate (pH 5.5)) for 18 h at 37°C. The effectiveness of desialylation was checked by gel electrophoresis and desalted on a NAP-5 column. All MUC1 peptides were tested for LPS with the *limulus* amebocyte lysate assay and were found to have $<$0.05 EU/ml by Cambrex BioScience Walkersville.

DC and T cell isolation and purification

Primary human leukocytes were isolated from fresh normal donor leukapheresis packs under an approved human subjects protocol as reported previously (16). In some studies, Percoll-purified lymphocytes or monocytes were cultured at 1×10^6 cell/ml in RPMI 1640 (BioWhittaker) containing 10% FBS (HyClone), 2 mM glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin (Quality Biologicals), with 100 U/ml recombinant human IL-2 for 16 h or 7 days in a 5% CO_2 humidified tissue culture incubator. Human imDCs were generated from purified human peripheral blood monocytes ($>$95%) as described previously (17), and their phenotype was confirmed by flow cytometry. imDCs were CD1a$^+$ (Ab clone H1149), CD14$^-$ (Ab clone M5E2), CD40^{low} (Ab clone 5C3), CD83$^-$ (Ab clone HB15ϵ), CD86^{low} (Ab clone 2331 (FUN-1)), and HLA-DR^{medium} (Ab clone G46-6 (L243)). Mature DCs (mDCs) were generated by culturing imDCs with 1 μg/ml LPS (L-9764; Sigma-Aldrich) for 48 h. The phenotype of mDCs was CD83^{high}, CD86^{high}, and HLA-DR^{high}. Human CD1c$^+$ DCs were immunomagnetically purified with BDCA-1/CD1c microbeads (Miltenyi Biotec) from primary human leukocytes, as described previously (18). In brief, Percoll-purified mononuclear cells were washed and resuspended in 2 mM EDTA containing 0.5% heat-inactivated FBS (Invitrogen Life Technologies). Cells were incubated with anti-CD19 microbeads and anti-CD1c-biotin Ab. Cells were then washed and passed through a column to remove CD19$^+$ B cells. The remaining cells were then incubated with strepavidin microbeads for 15 min. Cells were washed and passed through a second magnetic column to capture CD1c$^+$ cells. This purification resulted in $>$90% CD11c$^+$ cells that were also uniformly phenotypically immature being HLA-DR^{low}, CD83$^-$, and CD86^{low}.

CD4$^+$ T cells were purified from PBMCs using the CD4$^+$ T cells isolation kit (Miltenyi Biotec). In brief, non-CD4$^+$ T cells were removed by labeling cells with a mixture of biotinylated mAbs to CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR $\gamma\delta$, and glycophorin A and mixing labeled cells with anti-biotin microbeads. Labeled cells were removed by passing cells through a magnetic separation column in which the labeled cells became trapped in a magnetic field. The negative cells contained in the column effluent were $>$90% CD4$^+$CD3$^+$ by flow cytometry. The CD4$^+$ fraction was then labeled with CD45RO microbeads (Miltenyi Biotec) and depleted of CD45RO$^+$ cells by magnetic separation.

Chemotaxis assay

Cells were resuspended in chemotaxis medium (RPMI 1640 containing 1% BSA and 25 mM HEPES (pH 8.0)) at 1–5 $\times$ 10^6 cells/ml. Chemokines diluted in chemotaxis medium were placed in the bottom wells of a microBoyden chemotaxis chamber (Neuroprobe). When primary leukocytes were analyzed, 5-μm polycarbonate membranes were placed over the chemokines. Lymphocytes required that the membranes be precoated with 50 μg/ml fibronectin. After the microchemotaxis chamber was assembled, 50 μl of cells were placed in the top wells. The filled chemotaxis chambers were incubated in a humidified CO_2 incubator for 60 min (neutrophils), 90 min (monocytes, immature human DCs), or 3 h (lymphocytes). After incubation, the membranes were removed from the chemotaxis chamber assembly, followed by gently removing cells from the top side of the membrane. The cells on the bottom side of the membrane were stained using Rapid Stain (Richard Allen). The number of migrated cells in three high-powered fields ($\times$200) was counted by light microscopy after coding the samples. In many cases, counting was computer assisted using the BIOQUANT program (R & M Biometrics). Additional chemotaxis experiments done with CD1c$^+$ DCs were conducted similarly but with chemotaxis plates (5.7-mm diameter, 30 ml, 5-mm pores; Neuroprobe). Results are expressed as the mean value of the migration of triplicate sample with the SD shown by bars.

Assessment of DC phenotype and cytokine production

The DC phenotype was assessed by flow cytometry. In brief, cells were washed and counted before incubation with normal mouse serum (The Jackson Laboratory) for 15 min at 4°C to block nonspecific Fc receptor binding. Cells were then stained with specific Abs for 30 min at 4°C in the

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dark and washed extensively before fixation in 1% paraformaldehyde solution. Cytometry was performed with either a Becton Dickinson FACSCalibur cytometer or LSR II cytometer. Flow data were analyzed using CellQuest software.

DC supernatants were collected and stored at −80°C until testing. Cytokines were measured with the Luminex multiplex system (Luminex Corporation) (19). Luminex combines the principle of a sandwich immunoassay with the fluorescent bead-based technology, allowing multiplex analysis of several different analytes in a single microtiter well (20). Microspheres were obtained from BioSource International to measure a panel of DC cytokines (IL-12p70, IL-10, IL-6, IL-1β, and TNF-α). Assays were performed in a 96-well microplate format according to the protocol by BioSource International. A filter-bottom, 96-well microplate (Millipore) was blocked for 10 min with PBS/BSA. To generate a standard curve, 5-fold dilutions of appropriate standards were prepared in media diluent. Standards and supernatants were pipetted at 50 μl/well in duplicate and mixed with 50 μl of the mixture. The microplate was incubated for 1 h at room temperature on a microplate shaker. Wells were then washed three times with washing buffer using a vacuum manifold. A PE-conjugated secondary Ab was added to the appropriate wells, and the wells were incubated for 45 min in the dark with constant shaking. Wells were washed twice, an assay buffer was added to each well, and samples were analyzed using the Bio-Plex suspension array system (Bio-Rad).

MLR and assessment of T cell cytokine production

A total of 1×10^5 allogeneic $CD4^+CD45RO^-$ T cells were cocultured with 1×10^4 DCs/well in complete RPMI 1640 in triplicates in a 96-well plate. On day 6, the plates were spun down and supernatants were collected. Cytokines in the supernatants were measured by Luminex as described above for assessment of DC cytokine production, with a panel of microspheres specific for T cell cytokines IL-5, IL-4, IL-13, IL-10, and IFN-γ (BioSource International).

Results

Tumor Ag MUC1 is chemotactic for imDCs

Using a highly purified form of tumor MUC1 obtained from the ascitic fluid of cancer patients (ascites MUC1), we set up chemotaxis assays with monocytes, lymphocytes, NK cells, immature myeloid DCs, and mature myeloid DCs. We found that ascites MUC1 induced migration of imDCs (Fig. 1*A*) and not of the other four cell types (data not shown). The abrupt dose response to increasing concentrations of MUC1 in Fig. 1 is a result of the accepted way of performing the assays in log dilutions. Ascites MUC1 is large in size (>100 tandem repeats, on average) and presents to the DC a wide variety of unglycosylated as well as complex glycosylated epitopes in the tandem repeat and the N-terminal region (13). To determine what may be the nature of the epitopes responsible for the chemotactic activity, we examined two well characterized recombinant MUC1 molecules made by the cell lines MCF-7 (breast cancer cell line) and HEK293/EBNA (EBV-transformed human embryonic kidney cell line). MCF-7 MUC1 has a lower overall level of glycosylation compared with other breast cancer cell lines, with a large number of polylactosamine-type *O*-glycans with neutral and fucosylated sugars. HEK MUC1 has a greater density of carbohydrates with a high number of glycans terminating in sialic acid residues (14). Both fucosylation and sialylation can drastically change the binding affinity of ligands to receptors and influence the masking or exposure of epitopes (21). As shown in Fig. 1*B*, the less glycosylated MCF-7 MUC1 was chemotactic for imDCs, whereas the more glycosylated and sialylated HEK MUC1 was not. The difference in active concentration between MCF-7 MUC1 and ascites MUC1 is likely due to the difference in glycosylation and size between the two forms described above. Because, unlike ascites MUC1, the recombinant molecules are composed of only tandem repeats and lack the N-terminal segment, we concluded that the chemotactic activity seen in the native and the recombinant forms resides in the tandem repeat region of MUC1.

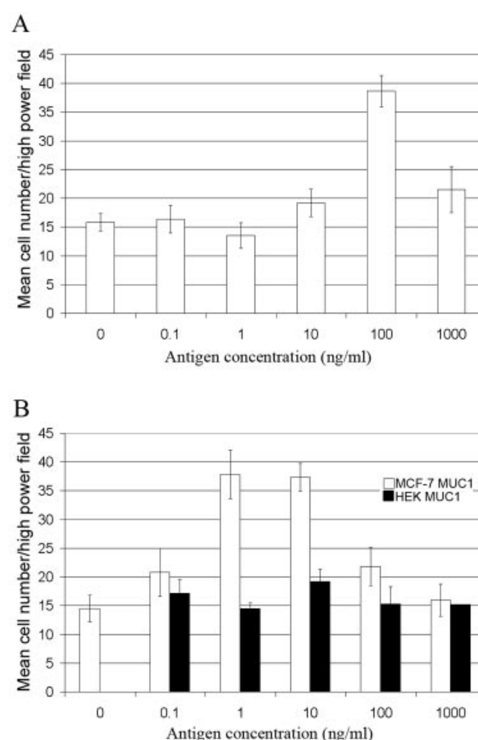

FIGURE 1. Tumor forms of MUC1 are chemotactic for immature myeloid DCs. *A* and *B*, Concentrations of MUC1 in the bottom chamber are shown on the *x*-axis. Increasing concentrations of ascites MUC1 (*A*) and MCF-7 MUC1 and HEK MUC1 (*B*) were added to the bottom chamber. Immature human monocyte-derived DCs were then added to the top wells. After 90 min, the numbers of DCs in the bottom chambers were counted and triplicate wells were averaged. Data are representative of three independent experiments.

The MUC1 chemotactic function is conducted by its peptide backbone

Because our data above also showed that MUC1 with fewer sugars had better chemotactic activity (ascites MUC1 and MCF-7 MUC1), we repeated the chemotactic assays with two synthetic MUC1 peptides. Although the purity of ascites MUC1 and MCF-7 MUC1 preparations was high and documented by several analytical methods (13, 14), there remained a small possibility that some chemokine might have contaminated the preparations and is responsible for the chemotactic activity. Therefore, we used the synthetic unglycosylated 100mer MUC1 peptide and the same 100mer peptide containing a single GalNAc (Tn) attached to 15 of the possible 25 serines and threonines (Tn-100mer) on the five repeats of the MUC1 tandem repeat backbone. The unglycosylated 100mer MUC1 induced chemotaxis (Fig. 2*A*), but the Tn-100mer MUC1 did not (Fig. 2*A*). To further confirm that MUC1 100mer is chemotactic and not simply chemokinetic (increasing random mobility of DCs), we performed a so-called checkerboard analysis. The 100mer MUC1 was added to both the top chamber (horizontal row) and the bottom chamber (vertical column) in increasing concentrations. Migration of imDCs was observed only when there was a true concentration gradient between the two chambers. When, however, concentrations of MUC1 were equal or greater in the top chamber, cells did not migrate to the bottom chamber (Table I). The Tn-100mer is glycosylated in vitro using recombinant glycosyltransferases. This provides great uniformity in the glycosylation of the tandem repeats, such that all three potential glycosylation sites on each repeat are occupied by sugar, leaving very few unglycosylated peptide segments. MCF-7 MUC1, in contrast,

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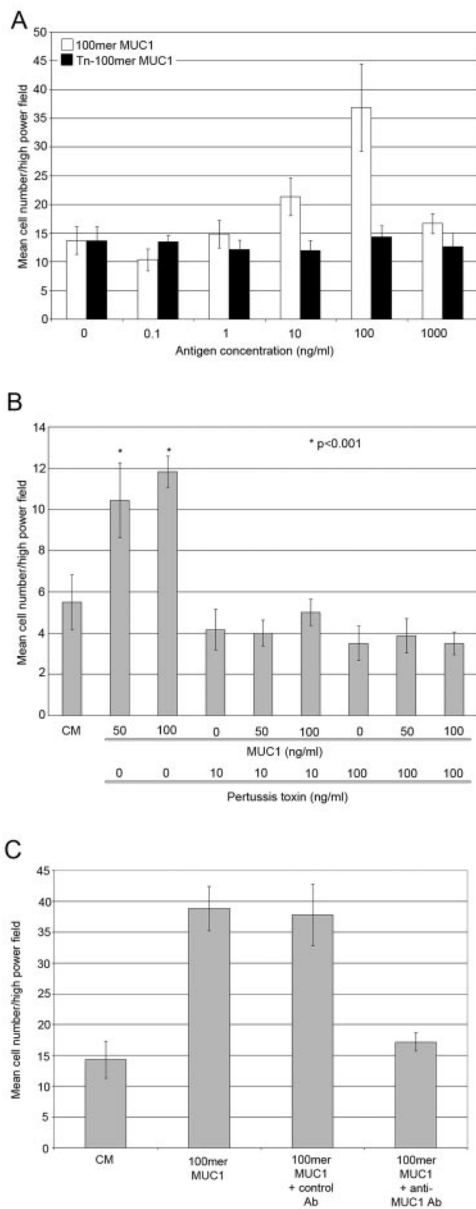

FIGURE 2. The unglycosylated tandem repeats of tumor MUC1 are the chemoattractive regions and function through a G protein-linked receptor. Experiments were conducted as described in *Materials and Methods*. *A*, Unglycosylated 100mer MUC1 and glycosylated Tn-100mer MUC1 were added in increasing concentrations in the bottom chamber. *B*, Pertussis toxin inhibits ascites MUC1-induced chemotaxis. Chemotaxis chambers were set up as in Fig. 1*A* in the presence or absence of the indicated concentrations of pertussis toxin. ∗, $p < 0.001$. There were no significant differences between control media and pertussis toxin-treated cells. *C*, MUC1 100mer (100 ng/ml) was added to the bottom chamber with an Ab specific for the unglycosylated form of MUC1, MF11, or an isotype control Ab. Data are representative of three independent experiments. CM, control media.

retains chemotactic activity due to less uniform in vivo glycosylation from one repeat to another, leaving repeats that are either unglycosylated or underglycosylated, thus exposing larger unglycosylated peptide segments.

Because the majority of chemokine receptors are G protein-linked receptors that are sensitive to inhibition by pertussis toxin, we looked at the effect of pertussis toxin on MUC1-induced chemotaxis. As shown in Fig. 2*B*, the migration induced by ascites

Table I. *Checkerboard analysis of DC migration in response to MUC1 100mer*

Bottom Compartment (ng/ml)	Top Compartment (ng/ml)			
	0	1.0	10	100
0	34.5 (2.6)	36.7 (3.5)	36.7 (3.8)	40.7 (4)
1.0	52.3 (10)	42.2 (6.3)	45 (2.8)	51.2 (5.1)
10	80.7 (5.5)	61.7 (3.3)	43 (5.2)	44.8 (4.3)
100	105.7 (15)	64.8 (7.4)	49 (9.2)	45.8 (9)

Chemotaxis assays were done as described in *Materials and Methods*. The mean number of imDCs that migrated to the bottom chamber are shown with SDs in parentheses.

MUC1 was sensitive to pertussis treatment, thus implicating one or several G protein-linked receptors in this process. Chemotaxis mediated by the synthetic 100mer MUC1 was also sensitive to pertussis inhibition (data not shown). In Fig. 2*C*, we show that the chemotactic effect was specific for the MUC1 peptide sequence and could be blocked with MF11 Ab but not by control Ab. MF11 Ab is specific for the peptide epitope PPAH in the MUC1 tandem repeat sequence GVTSAPDTRPAPGSTAPPAH. Together, these data indicate that the addition of even short *O*-glycans, such as Tn/GalNAc, can block the sites responsible for chemotaxis and that, like chemokines, MUC1 induces chemotaxis through a G protein-linked receptor(s). A growing number of studies indicate that there are distinct differences in cell surface receptors expressed by cultured monocyte-derived DCs and myeloid DCs purified from PBMCs. Monocyte-derived DCs may be more representative of tissue-resident DCs. Human primary DCs, purified from peripheral blood, are the pool of circulating DCs that are poised to respond to changes in the host environment and would be exposed to the chemotactic activity of MUC1 in a tumor-bearing patient. We determined that DCs directly purified from peripheral blood are also chemoattracted to MUC1 (data not shown). Thus, MUC1 is chemotactic to both tissue-resident and circulating DCs and therefore would be capable of affecting both populations in cancer patients.

Tumor forms of MUC1 induce cell surface maturation markers on DCs

The observation that tumor MUC1 is chemotactic for imDCs raised the question of the immunological consequences of the interaction of MUC1 with the DCs once they are recruited from peripheral blood to the tumor site. To model this in vitro, we purified myeloid DCs from PBMCs of normal donors and exposed them to various forms of MUC1. LPS treatment served as the positive control for DC activation and maturation. DCs were immature at the time of purification and responded strongly to LPS by up-regulation of surface markers CD83, CD80, CD86, CD40, and MHC class II (Fig. 3*A* and data not shown). No change in the expression of these markers was seen after interaction with the chemotactic forms, the unglycosylated 100mer MUC1, Tn-100mer MUC1, or MCF-7 MUC1 (data not shown). However, HEK MUC1, the form without chemotactic activity but with high levels of sugars and terminal sialic acid residues, induced increased expression of all of the maturation markers (Fig. 3*A*).

Our observations that tumor MUC1 can activate mDCs, thus presumably making them more-potent APCs, appeared to be in contrast to several reports that terminal sialic acid residues on other tumor-derived molecules and on mucins from other organisms have immunosuppressive effects on DCs and T cells (22–24). Because up-regulation of maturation markers does not always guarantee an immunostimulatory DC (25), we also examined the production of cytokines by DCs that have interacted with various

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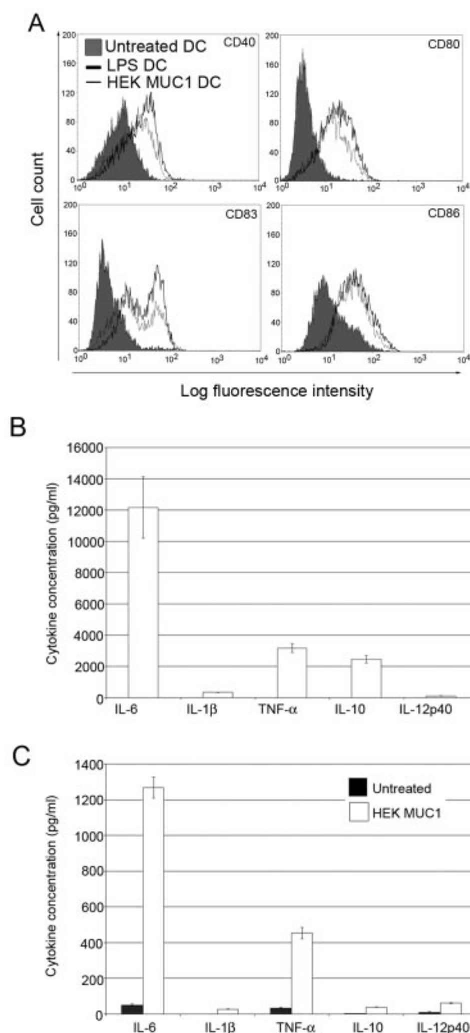

FIGURE 3. Tumor MUC1 induces the maturation and cytokine production of human peripheral blood DCs. CD1c$^+$ human myeloid DCs were purified from the PBMCs of normal donors. *A*, Expression of maturation and costimulatory markers CD40, CD80, CD83, and CD86 on peripheral blood DCs. DCs were untreated or treated with 1 μg/ml LPS or 50 μg/ml HEK MUC1 for 18 h before staining. Filled histogram, Untreated DCs; thick line, LPS DCs; thin line, HEK MUC1 DCs. *B*, Cytokine production by LPS DCs at 18 h as measured by multiplex analysis. *C*, Cytokine production by untreated DCs and HEK MUC1-treated DCs at 18 h as measured by multiplex analysis. *B* and *C*, Triplicate wells were averaged and SDs were calculated. Results are representative of three independent experiments with different donors.

forms of MUC1 and compared them with the DCs activated and matured by LPS. We were especially interested in the production of cytokines that would make the DCs effective in priming naive CD4$^+$ T cells to become Th1 cells. Culture supernatants were collected from DCs left untreated or treated with LPS or tumor MUC1. The presence of various cytokines was assessed by multiplex analysis with Luminex. LPS-treated DCs produced high levels of TNF-α, IL-6, IL-10, and IL-1β and a low but detectable level of IL-12p40 that correlated with increasing concentrations of LPS (Fig. 3*B*). We found that human CD1c$^+$ DCs isolated directly from peripheral blood produced ~100-fold lower amounts of IL-12p40 than monocyte-derived DCs, even when treated with large amounts of LPS with and without other DC maturation signals, such as CD40L (Fig. 3*B* and our unpublished data). Tumor MUC1-treated DCs produced lower overall levels of cytokines than LPS-treated DCs but significantly greater amounts of IL-6, TNF-α, and IL-10 than untreated DCs (Fig. 3*C*). IL-12p40 expression by tumor MUC1-treated DCs was extremely low. Different donors differed in the overall magnitude of response but not in the profile of cytokines produced.

Terminal sialic acid residues on MUC1 induce altered DC maturation

Sialylation has been shown to be important in many receptor-ligand interactions (3, 21), and thus we tested its involvement in MUC1-induced DC maturation. We show in Fig. 4*A* that desialylation of HEK MUC1 ((S$^-$) HEK MUC1; protein with sialic acid residues removed), through chemical removal of terminal sialic acids, abolished its ability to increase expression of maturation markers. Removal of sialic acid residues with bacterial sialidase, although not as complete as chemical removal, also inhibited the ability of HEK MUC1 to induce up-regulation of maturation markers (Fig. 4*A*). Combined with the data above, these data suggested that in vivo, imDCs could be attracted to the tumor site through the chemotactic activity of the unglycosylated peptide epitopes on the tumor MUC1 tandem repeats, and through the interaction with the sialylated carbohydrate epitopes, acquire a more mature phenotype (i.e., up-regulate their cell surface levels of Ag-presenting and costimulatory molecules). Similar to the results that we saw for up-regulation of maturation markers, the induction of cytokine production was dependent on the terminal sialic acids and, with the more efficient desialylation, with trifluoroacetic acid treatment, the better the prevention of cytokine induction (Fig. 4, *B* and *C*).

MUC1-matured DCs fail to promote T cell commitment to Th1

Because DCs showed increased levels of cytokine production and costimulatory molecules after interaction with tumor MUC1, we examined how effective these DCs would be in stimulating and polarizing naive CD4$^+$ T cells. As shown in Fig. 5, *A* and *B*, T cells stimulated with untreated immature allogeneic DCs produced low levels of IL-5, IL-13, and IFN-γ, indicating that they did not have the capacity to skew the development of CD4$^+$ T cells toward either the Th1 or Th2 phenotype. LPS-treated DCs were fully capable of inducing Th1 T cells that produce IFN-γ and repressing IL-5- and IL-13-producing T cells (Fig. 5*A*). HEK MUC1-treated DCs did not skew CD4$^+$ T cells toward either the Th1 or Th2 type but instead induced IL-5-, IL-13-, as well as IFN-γ-producing T cells and suppressed IL-2 production (Fig. 5, *A* and *B*). Compared with DCs treated with HEK MUC1, DCs treated with desialylated HEK MUC1 performed like untreated DCs by failing to induce T cell cytokine production (Fig. 5*B*).

Discussion

The consequence of MUC1 hypoglycosylation and overexpression on tumor cells has been explored in a variety of disease-related situations (5). This includes alteration of the adhesive capabilities of tumor cells and suppression of the function of T cells and in vitro generation of DCs (26–30). This is the first time that MUC1 has been shown to be chemotactic for circulating imDCs and to be capable of subverting their immunostimulatory function. Although certain chemokines have been identified that contain mucin domains (31, 32), no chemotactic regions have been described previously within those domains. Our experiments showing that chemotaxis is induced by peptide epitopes that are only exposed on the tumor-derived molecule suggest that MUC1, which is a secreted as well as a transmembrane molecule, may be acting as a tumor-specific chemokine.

Previous studies have shown that there is a large number of DCs at sites of MUC1$^+$ tumors (33, 34). Our results suggest that the

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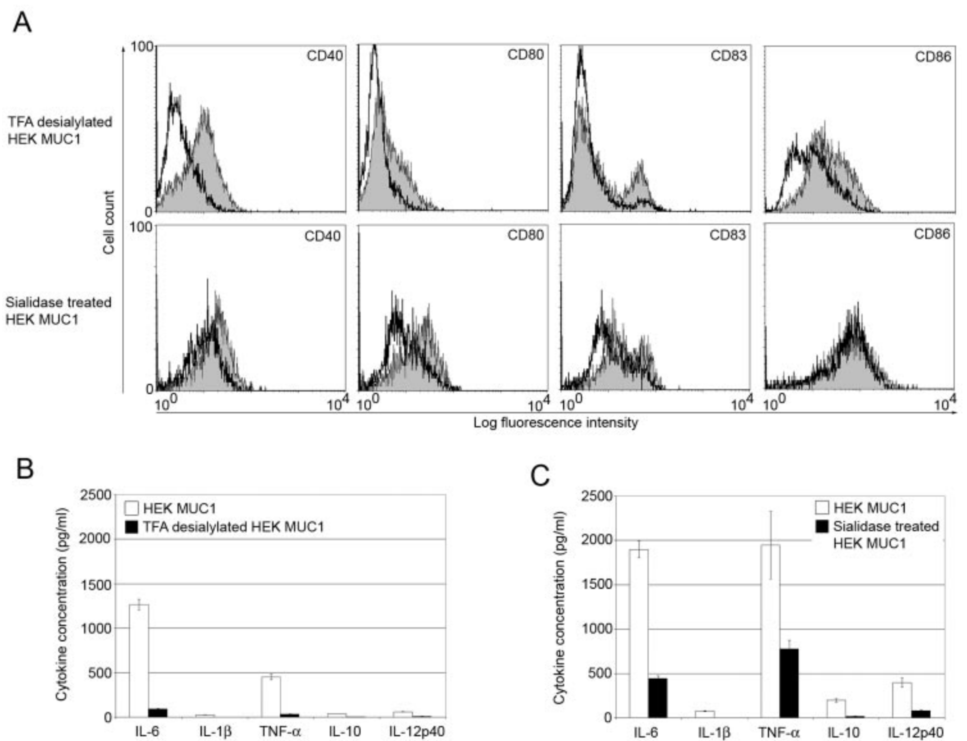

FIGURE 4. The removal of terminal sialic acids from tumor MUC1 prevents the induction of maturation and cytokine production by DCs. CD1c$^+$ human myeloid DCs were purified from the PBMCs of normal donors. DCs were treated with 50 μg/ml HEK MUC1, trifluoroacetic acid (TFA) chemically desialylated HEK MUC1, or sialidase-treated HEK MUC1 for 18 h before flow analysis. Filled histogram, HEK MUC1 DC; thick line (*top panels*), TFA-treated HEK MUC1; thick line (*bottom panels*), sialidase-treated HEK MUC1. *B*, Cytokine production by HEK MUC1 DCs and TFA desialylated HEK MUC1 DCs at 18 h as measured by multiplex analysis. *C*, Cytokine production by HEK MUC1 DCs and sialidase-treated HEK MUC1 DCs at 18 h as measured by multiplex analysis. Results are representative of three independent experiments with different donors.

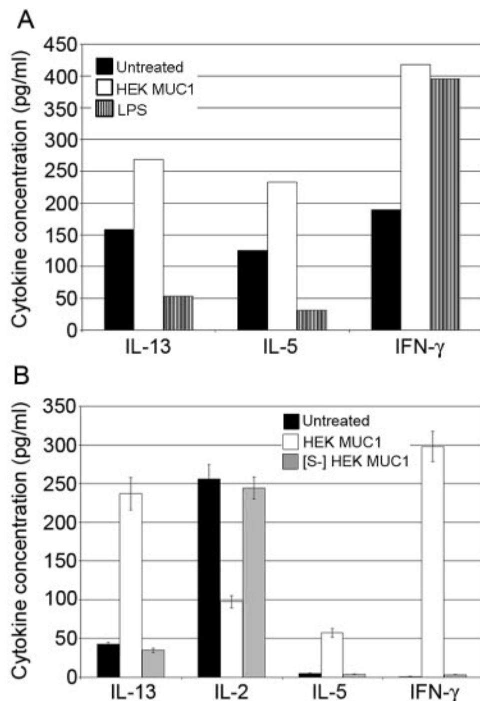

FIGURE 5. DCs that have interacted with MUC1 fail to induce strong Th1 responses in T cells despite expressing costimulatory molecules. *A*, CD1c$^+$ DCs were treated with nothing, HEK MUC1 (50 μg/ml), or LPS (1 μg/ml) for 12 h before washing and plating with naive CD4$^+$ T cells. *B*, CD1c$^+$ DCs were treated with nothing, HEK MUC1 (50 μg/ml), or (S$^-$) sialidase-treated HEK MUC1 (50 μg/ml) for 12 h before washing and plating with naive CD4$^+$ T cells from either the same donor (syngeneic) or an unrelated donor in triplicate wells. Cell-free supernatants from day 6 mixed leukocyte reactions were collected, and cytokine levels were measured by multiplex analysis. Supernatants from DCs or T cells cultured alone did not produce significant levels of any of the cytokines tested. Data are representative of three independent experiments.

overexpression of the tumor form of MUC1 may be the signal that brings those imDCs from the circulation to the tumor site. Unlike the structure of normal MUC1, the unglycosylated peptide backbone of tumor MUC1 has a characteristic shape and highly stable tandem repeat structure that may be highly effective at binding receptors on DCs (35–37). The ability to inhibit chemotaxis with pertussis toxin suggests that this is a G protein-linked receptor. Efforts are underway to identify this receptor. However, because MUC1 is a complex molecule with many epitopes that could potentially interact with not one but several receptors on DCs, the identity of these receptors may be difficult to determine.

DC migration induced by the chemotactic stimulus from MUC1 has similarities to DC migration during inflammation rather than the steady-state migration. It is the proinflammatory chemokines that characteristically induce migration in only the imDC population. imDCs express chemokine receptors such as CCR1, CCR5, and CXCR2 and are primed to respond to proinflammatory chemokines such as CCL2 (MCP-1), CCL5 (RANTES), and CXCL8 (IL-8). After maturation, they down-regulate these receptors and instead up-regulate other receptors, such as CCR7, that are required for migration toward the secondary lymphoid tissues (38, 39). Underglycosylated MUC1 is produced only by abnormal cells, and therefore it should not be encountered during normal DC migration in healthy tissues. More likely, when MUC1$^+$ premalignant cells or small tumors are growing and have not yet gained the mutations to make large amounts of other chemokines, such as CXCL8 (IL-8) and CXCL1–3, MUC1 can attract imDCs (40).

Our experiments with sialic acid removal suggest that receptors that signal the DCs to mature in response to MUC1 are likely to be sialic acid-recognizing receptors of the I-type lectin family known as siglecs (3). MUC1 has been shown to bind to siglec-1/sialoadhesin, but because sialoadhesin lacks a signaling motif, it could not be responsible for the effects that we see (41). The identification of the responsible receptor(s) awaits additional data on the expression

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of various receptors on human DCs and reagents for their characterization. HEK MUC1 has tandemly repeated sialic acids in bothα2-3 and α2-6 linkages that could bind to more than one member of the sialic acid-binding receptor family confounding their identification.

Overexpression of MUC1 and its glycosylation with sialylated core-type glycans are seen on a large number of human cancers (42, 43). Our data suggest that the hypoglycosylated MUC1 draws imDCs to the tumor site where they could pick up tumor Ags for presentation to T cells in the lymph node. Through interactions with the highly sialylated MUC1, these DCs acquire a surface phenotype of activated and matured DCs that are fully expected to promote T cell activation and to skew the response to the Th1 type important for tumor rejection. On closer examination, however, these DCs show instead a state of semimaturation, produce IL-6 and TNF-α that have been implicated in tumor metastasis and progression, and in the lymph node do not support T cell commitment to Th1. In fact, the production of TNF-α and IL-6 has been linked to cancer and, in some cases, to a worse clinical prognosis (44–46). They are also key to the migration of cells through the endothelium and therefore could ease the escape of metastatic cells from the tumor site. Therefore, imDCs that are drawn to the MUC1$^+$ tumor could, through the production of these cytokines, enhance the local immunosuppression and increased tumor invasion. In addition, these DCs promote expansion of T cells that produce large amounts of IL-13, another cytokine that has been implicated in preventing immunosurveillance of tumors and facilitation of tumor outgrowth by repressing the function of tumor-specific CTLs (47, 48). IL-13 has also been implicated in facilitating the growth and spread of tumors by direct effects on tumor cells expressing the IL-13 receptor, type II IL-4R (47).

There has been little information to date on factors produced by malignant or premalignant cells that can affect fully differentiated DCs. Tumor MUC1, along with other tumor-derived molecules, has recently been shown to inhibit the differentiation of monocytes into DCs in vitro (30), and it was suggested that this may contribute to the maintenance of tumor immunosuppression. More importantly, the data presented here show that even normally differentiated DCs that encounter abnormal cells expressing MUC1 will be affected and help start the process of tumor-induced DC dysfunction.

Understanding interactions between tumor Ags and the immune system will help in predicting the potential of certain immunotherapeutic approaches. For example, in choosing the form of MUC1 Ag to use in a cancer vaccine, one can select epitopes that maintain beneficial interactions with DCs and eliminate those that negatively affect their function. In the case of MUC1$^+$ tumors, it has to be taken into consideration that the cancer patient's T cells are continuously exposed to DCs that have interacted with MUC1 in the tumor environment. Therefore, a cancer vaccine administered in the therapeutic setting may not be able to substantially and qualitatively change the ongoing immune response. A more promising scenario would thus be to use the vaccine in a prophylactic setting to elicit a desired immune response and not allow the tumor to determine the fate of tumor-specific T cells.

Disclosures

The authors have no financial conflict of interest.

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