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Tn Glycosylation of the MUC6 Protein Modulates Its Immunogenicity and Promotes the Induction of Th17-biased T Cell Responses*[S]

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The Tn antigen (α-GalNAc-*O*-Ser/Thr) is one of the most specific human cancer-associated structures. This antigen, together with mucins, the major carriers of *O*-glycosylated tumor antigens in adenocarcinomas, are being evaluated as anti-cancer immunotherapeutic targets. In particular, the MUC6 protein, which is normally expressed only in gastric tissues, has been detected in intestinal, pulmonary, colorectal, and breast carcinomas. To develop anti-cancer vaccines based on the Tn antigen, we produced MUC6 proteins with different Tn density by using mixtures of recombinant ppGalNAc-T1, -T2, and -T7. The obtained glycoproteins were characterized and analyzed for their immunological properties, as compared with the non-glycosylated MUC6. We show that these various MUC6:Tn glycoproteins were well recognized by both MUC6 and Tn-specific antibodies. However, Tn glycosylation of the MUC6 protein strongly affected their immunogenicity by partially abrogating Th1 cell responses, and promoting IL-17 responses. Moreover, the non-glycosylated MUC6 was more efficiently presented than MUC6:Tn glycoproteins to specific T CD4$^+$ hybridomas, suggesting that Tn glycosylation may affect MUC6 processing or MHC binding of the processed peptides. In conclusion, our results indicate that Tn glycosylation of the MUC6 protein strongly affects its B and T cell immunogenicity, and might favor immune escape of tumor cells.

Altered glycosylation is an almost universal feature of cancer cells (1). In particular, the incomplete elongation of *O*-glycan saccharide chains leads to the expression of shorter carbohydrate structures, such as the Tn antigen (2). This antigen, defined as a D-GalNAc unit α-linked to a serine or threonine residue (α-GalNAc-*O*-Ser/Thr), is one of the most specific human cancer-associated structures (3). Indeed, Tn is expressed by epithelial tumors and is associated with most carcinomas including breast, lung, colon, prostate, and pancreatic cancers, whereas it is masked in normal tissues (3).

This *O*-linked epitope is usually expressed on mucins as their carbohydrate core structure (4). Mucins are high molecular weight *O*-glycosylated proteins that participate in the protection, lubrication, and acid resistance of the epithelial surface (5). In cancer, mucins influence cell adhesion (6) and contribute to tumor invasiveness (7). The involvement of mucins and their associated carbohydrate antigens (*e.g.* Tn antigen) in the metastatic process of tumor cells makes them relevant targets for the prevention of metastasis and recurrence of cancers by therapeutic vaccination (8, 9).

MUC6 is a mucin that is found at high levels only in the normal stomach and gall bladder (10, 11) but which is aberrantly expressed in various carcinomas and may constitute in itself a target antigen for cancer immunotherapy. Indeed, whereas MUC6 has been detected in intestinal (12), pulmonary (13), colonic (14), and mammary adenocarcinomas (15), it is not expressed in the corresponding normal tissues. It has been recently demonstrated that MUC6 on breast cancer cells displays the Tn antigen (16). In addition, several studies have suggested that mucin-associated carbohydrates (including the core Tn antigen) may be essential for the definition of these tumor antigens (17, 18). Indeed, a mucin peptide containing the Tn antigen was shown to be more immunogenic than the non-glycosylated peptide (19). In this context, glycosylated mucins could represent important targets for the development of efficient immunotherapies.

Using total tumor cell extracts, we recently described the enzymatic preparation of MUC6 glycoproteins carrying the Tn antigen (20). The resulting MUC6:Tn glycoprotein, prepared with breast cancer cell extract as a source of UDP-*N*-acetylgalactosamine:polypeptide *N*-acetylgalactosaminyltransferases (EC 2.4.1.41, ppGalNAc-Ts),[3] was demonstrated to induce

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[3] The abbreviations used are: ppGalNAc-Ts, UDP-*N*-acetylgalactosamine:polypeptide *N*-acetylgalactosaminyltransferases; UDP-GalNAc, uridine diphospho-*N*-acetylgalactosamine; SELDI-TOF MS, surface-enhanced laser desorption/ionization time-of-flight mass spectrometry; BMDC, bone marrow-derived dendritic cells; LN, lymph node; MGL, macrophage Gal/GalNAc lectin; ELISA, enzyme linked immunosorbent assay; BSA, bovine serum albumin.

antibodies that recognized human tumor cells (20). This family of enzymes catalyzes *in vivo* the first step of the mucin-type *O*-glycosylation pathway, *i.e.* the Tn antigen synthesis (21).

In the present study, we designed different mixtures of recombinant ppGalNAc-T1, -T2, and -T7 to carry out GalNAc enzymatic transfer onto the Ser and Thr residues of the MUC6 mucin. Indeed, as opposed to tumor cell extracts, the use of recombinant ppGalNAc-Ts with overlapping and/or complementary substrate specificities enables the production of and access to various protein glycoforms in a reproducible and convenient manner. A series of MUC6:Tn glycoproteins was produced, characterized, and their immunological properties were analyzed using various *in vitro* and *in vivo* assays. This study shows that these MUC6:Tn glycoproteins were well recognized by both MUC6 and Tn-specific antibodies. However, the Tn glycosylation of the MUC6 protein strongly affected its immunogenicity by partially abrogating Th1 cell responses and promoting the production of IL-17. Thus, the design of glycoprotein-based vaccines should take into account the possible immunomodulating properties of glycosylation.

EXPERIMENTAL PROCEDURES

Mice—6- to 8-week-old female BALB/c or C57BL/6 mice were obtained from CER Janvier or Charles River. Animals were kept in the Pasteur Institute animal house in specific pathogen-free conditions, with water and food supplied *ad libitum*, and handled in accordance with institutional guidelines for animal welfare.

MUC6 Protein and Peptides—An 86-amino acid sequence of a half-tandem repeat of human MUC6 was cloned and expressed in *Escherichia coli* and purified as previously described (20). Briefly, a cDNA clone containing a partial sequence of the tandem repeat of human MUC6 was isolated from total cDNA of MCF7 breast cancer cells and expressed in *E. coli* Bli5 by induction with 1 mM isopropyl β-D-thiogalactoside. The recombinant protein was purified over Ni^{2+}-nitriloacetic acid columns under denaturing conditions according to the manufacturer's (Qiagen, Hilden, Germany) instructions. The MUC6 recombinant protein was characterized by amino acid analysis and surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS), and was quantified by quantitative amino acid analysis (net peptide content). These analyses, together with N-terminal sequencing, showed that it lacks the N-terminal methionine residue.

The 15-mer peptides, overlapping by five amino acids and spanning the sequence present in the recombinant MUC6 protein, were synthesized by PolyPeptide (Strasbourg, France). The amino acid sequences of the peptides are shown in Fig. 4*A*.

ppGalNAc-Transferases—Soluble forms of the bovine ppGalNAc-T1 (kindly given by Dr. F. Piller) and human ppGalNAc-T2 and -T7 were used. ppGalNAc-T1 was expressed in the yeast *Pichia pastoris* KM71H strain, as previously described (20). Human ppGalNAc-T2 and -T7 were cloned in pAcGp67 vector and expressed in insect cells, using the baculovirus system, by the Plateforme de Production de Protéines Recombinantes (Institut Pasteur, Paris, France).

Enzymatic Synthesis of MUC6:Tn Glycoproteins—The MUC6:Tn glycoproteins were obtained by enzymatic GalNAc transfer, using ppGalNAc transferases. Optimal conditions for *in vitro* glycosylation of both MUC6 proteins were determined following assays performed at analytical scale, using SELDI-TOF MS (Ciphergen Biosystems, Fremont, CA), as described (22).

ppGalNAc-Ts were incubated individually or together at 37 °C with uridine 5′-diphospho-*N*-acetylgalactosamine (UDP-GalNAc) (2 eq per Thr/Ser eq) and purified MUC6 (40–80 μM) in 50 mM imidazole buffer, pH 7.2, containing 15 mM $MnCl_2$ and 0.1% Triton X-100. After a 24 h incubation, equal amounts of ppGalNAc-Ts and UDP-GalNAc were added and incubated for another 24 h. The resulting MUC6:Tn glycoproteins were purified using nickel-nitrilotriacetic acid-agarose (Qiagen) and then subjected to reverse-phase high-performance liquid chromatography (RP-HPLC) using a PerkinElmer pump system with an UV detector at 230 nm and a Symmetry 300 C18 column (5 μm, 300 Å, 3.9 × 250 mm) (Waters). Elution was carried out with a linear gradient of 10–60% acetonitrile in 0.1% trifluoroacetic acid in water at a flow rate of 1 ml/min (over 30 min). The main peak was collected and lyophilized. The MUC6:Tn glycoproteins were characterized by amino acid analysis and mass spectrometry. All conjugates were quantified by amino acid analysis (net peptide content).

Recognition of MUC6:Tn Glycoproteins by Anti-Tn mAb and Anti-MUC6 Serum—The antigenicity of the MUC6:Tn glycoproteins was analyzed by enzyme-linked immunosorbent assay (ELISA). Microtiter plates (Nunc, Roskilde, Denmark) were coated overnight with the purified glycoproteins (0.1 μg/ml). Plates were washed three times with 0.1% Tween 20 in PBS (PBS/T) and nonspecific binding sites were blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at 37 °C. After washing, the anti-Tn mAb 83D4 (kindly provided by Dr. E. Osinaga, Uruguay) or a polyclonal anti-MUC6 serum (20) was added and incubated for 2 h at 37 °C. After three washes with PBS/T, plates were incubated with goat anti-mouse IgM or anti-IgG peroxidase conjugates, respectively (Sigma), for 1 h at 37 °C. The plates were revealed using *o*-phenylenediamine/H_2O_2 and read photometrically at 492 nm in an ELISA autoreader (Dynatech, Marnes la Coquette, France).

Western Blot Analysis of MUC6-Tn Glycoproteins—The MUC6-Tn glycoproteins were analyzed by Western blotting using an anti-His mAb (Qiagen) and the anti-Tn mAb 83D4. The glycoproteins were separated in a 13% SDS-PAGE and transferred to nitrocellulose sheets (Amersham Biosciences, Saclay, France) at 30 V overnight in 20 mM Tris-HCl buffer, pH 8.3, 192 mM glycine, and 10% ethanol, as previously described (23). Residual protein-binding sites were blocked by incubation with 3% BSA in PBS at 37 °C for 2 h. The nitrocellulose was then incubated for 2 h at 37 °C with either the anti-His mAb or the anti-Tn mAb 83D4. After three washes with PBS containing 0.1% Tween 20 and 1% BSA, the membrane was incubated for 1 h at room temperature with goat anti-mouse immunoglobulins conjugated to peroxidase (Sigma) diluted in PBS containing 0.1% Tween 20 and 1.5% BSA and reactions were developed with enhanced chemiluminescence (ECL) (Amersham Biosciences, Saclay, France). The same procedure was performed omitting the antibodies as a negative control.

Endotoxin Level Determination—The endotoxin level of glycosylated and nonglycosylated MUC6 proteins was determined according to the instructions of the manufacturer, using the Limulus Amebocyte Lysate QCL-1000 kit (Cambrex, Emerainville, France). All MUC6 and MUC6-Tn (glyco)proteins showed very low levels of endotoxins (<2 enzyme units/mg of protein).

Induction of Antibodies by MUC6:Tn Glycoproteins—Mice were immunized intraperitoneally with 10 μg of each glycoprotein in alum (1 mg) and CpG (10 μg) per mouse, at days 0, 21, 42, and 63. Bleedings were carried out at days 20, 28, 49, and 70.

Sera were analyzed by ELISA. Ninety-six-well microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4 °C with 0.1 μg of MUC6:Tn glycoproteins or non-glycosylated MUC6 per well in 50 mM carbonate buffer, pH 9.6. After blocking with 3% BSA in PBS, three washes with PBS containing 0.1% Tween 20 were performed. Then, serially diluted sera in buffer (PBS plus 0.1% Tween 20, 1% BSA) were added to the wells for 1 h at 37 °C. Following three washes, wells were treated 1 h at 37 °C using goat anti-mouse IgG or IgM peroxidase conjugate (Sigma) and *o*-phenylenediamine-H_2O_2 was then added as substrate. Plates were read photometrically at 492 nm in an ELISA auto-reader (Dynatech). The negative control consisted of adjuvant-injected mouse sera diluted 100-fold. ELISA antibody titers were determined by linear regression analysis plotting dilution *versus* $A_{492\ nm}$. The titers were calculated to be the $\log_{10}$ highest dilution, which gave twice the absorbance of control mouse sera diluted 1:100. Titers were given as the arithmetic mean ± S.D. of the $\log_{10}$ titers.

Mouse sera were also tested at a 1:100 dilution by flow cytometry on the Jurkat and MCF7 Tn-expressing human tumor cell lines. Cells were first incubated for 30 min with sera at 4 °C in PBS containing 5% FCS and 0.1% sodium azide and then with an anti-mouse IgM/IgG goat antibody conjugated to FITC or phosphatidylethanolamine, respectively (Sigma). Paraformaldehyde-fixed cells were analyzed on a FACScalibur flow cytometer (BD Biosciences).

T Cell Responses—Mice were subcutaneously immunized at the base of the tail with 10 μg of MUC6:Tn glycoproteins in CFA (Complete Freund Adjuvant). Inguinal lymph nodes (LN) from control or MUC6:Tn-immunized mice were removed after 10 days, and the cells were dispersed manually and centrifuged at 1,500 × *g* for 5 min. Cells were suspended in complete culture medium, consisting of RPMI 1640 with GlutaMAX (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum, 50 μM 2-mercaptoethanol, 100 units/ml of penicillin, 100 mg/ml of streptomycin. Cells (1×10^6/well) were cultured for 72 h at 37 °C and 5% CO_2 in 96-well plates with MUC6 peptides (10 μg/ml), MUC6:Tn glycoproteins or non-glycosylated MUC6 (1–10 μg/ml). They were then pulsed with [^{3}H]thymidine (ICN Biomedicals Inc.) for the last 18 h of culture and harvested by an automated cell harvester (Skatron). Proliferation was determined by incorporation of the radioactivity by the cells and the results (expressed in counts per minute) represent the means of triplicate determinations. Controls were incubated either with culture medium alone or with 0.5 μg of concanavalin A. The negative control group consisted of mice immunized with a peptide corresponding to the T $CD4^+$ epitope of the maltose-binding protein from *E. coli* (NGKLIAYPIAVEALS) (24). Secreted cytokines (IFNγ, IL-5, and IL-17) levels were tested on culture supernatants by interleukin-specific sandwich ELISA. Results are expressed in picograms/ml.

Generation of Bone Marrow-derived Dendritic Cells (BMDC)—BMDC were generated from bone marrow precursors from C57BL/6 mice. Briefly, bone marrow cells from femurs and tibias were harvested and plated at a density of 2×10^5 cells/ml in complete culture medium supplemented with 1% of a GM-CSF-containing supernatant. After 3 days of culture at 37 °C, the medium was replaced. Cells were recovered on days 6 or 7, by flushing the plates with 5 mM EDTA in PBS.

Generation of MUC6 Hybridomas and Antigen Presentation Assay—$MUC6_{33-47}$- and $MUC6_{83-97}$-specific hybridomas were generated in our laboratory, and are IA^b-restricted. C57BL/6 mice were subcutaneously immunized with 100 μg of the relevant MUC6 peptide in CFA. After 10 days, inguinal LN cells were harvested, incubated with the respective peptides, and fused with hypoxanthine/aminopterin/thymidine medium-sensitive BW5147 myeloma cells using polyethylene glycol. Specific hybridomas were selected according to the production of IL-2 after stimulation by the corresponding MUC6 peptide.

For antigen presentation to T cell hybridomas, BMDC (5×10^4/well) were pulsed with serial dilutions of glycoproteins or non-glycosylated MUC6 protein and incubated with the T cell hybridoma (5×10^4/well) for 24 h. The culture supernatants were frozen, and then tested for interleukin 2 (IL-2), measured by a specific ELISA. Results are expressed in picograms/ml.

In Vitro Tn Glycopeptide Internalization Assay—The *in vitro* internalization of MUC6:Tn glycoproteins was analyzed by flow cytometry. BMDC were incubated (2.5×10^5/well) with Alexa 647-labeled antigen for 1 h at 37 °C in complete medium (to assess uptake), or at 4 °C in complete medium (to assess binding). Cells were then washed twice and analyzed by FACS. For inhibition assays, cells were incubated with glycopeptides in complete medium supplemented with 10 mM EDTA or 10 μg/ml of anti-MGL mAb (ERMP23, Cedarlane Laboratoires Ltd.) for 1 h.

RESULTS

Production and Physicochemical Characterization of the MUC6:Tn Glycoproteins—The production of the MUC6:Tn glycoproteins was carried out by enzymatic GalNAc transfer on a recombinant MUC6 protein using recombinant ppGalNAc-Ts. This glycosyltransferase family comprises multiple isoforms that control the initiation of mucin-type *O*-glycosylation in mammals. We selected ppGalNAc-T1 and -T2 because they possess a broad and complementary spectrum of specificity (21). Furthermore, the activity of both transferases accounts for most of mucin glycosylation; ppGalNAc-T1 being the major contributor (25). Additionally, we used the ppGalNAc-T7, a follow-up ppGalNAc-T, because it is able to incorporate GalNAc on threonines and serines of partially GalNAc-glycosylated acceptor substrates (26).

GalNAc transfer was performed onto a recombinant MUC6 protein cloned from the MCF7 breast cancer cell line. This

TABLE 1
Characteristics of MUC6:Tn glycoproteins

Protein or glycoprotein	ppGalNAc-T used for glycosylation	Obtained quantity[a]	Product yield[b]	Experimental molecular mass[c]	Tn amount		
					GalNAc[c]	Sites[d]	M_r[e]
		mg	*%*	*Da*		*%*	*%*
MUC6				12,120.3			
MUC6:Tn(T1)	T1	3.8	71	15,993.1	19	45	24
MUC6:Tn(T2)	T2	1.5	39	15,375.1	16	38	20
MUC6:Tn(T1+T2)	T1 + T2	2.8	69	15,982.6	19	45	24
MUC6:Tn(T1+T7)	T1 + T7	4.0	94	16,994.5	24	57	30
MUC6:Tn(T2+T7)	T2 + T7	1.0	26	16,196.2	20	48	25
MUC6:Tn(T1+T2+T7)	T1 + T2 + T7	7.5	51	17,808.3	28	67	35

[a] Net peptide content as determined by quantitative amino acid analysis.
[b] Isolated product yield refers to the obtained yield after the glycosylation reaction and RP-HPLC purification of the resulting glycoprotein.
[c] The average molecular mass and GalNAc number of the glycoprotein were calculated from the medium peak determined by SELDI-TOF MS (calculated average mass of GalNAc M_r = 203.19, see Fig. 1) (22).
[d] The % of obtained glycosylated sites was calculated taking into account the obtained GalNAc number for each glycoprotein as compared to the predicted number of serine and threonine residues (43) using the NetOGlyc 3.1 Server (see supplemental Fig. S1) in MUC6 (100%).
[e] The Tn amount was calculated by taking into account the total GalNAc M_r compared to the overall glycoprotein M_r, as determined by the medium peak in SELDI-TOF MS (see Fig. 1) (22).

recombinant MUC6 contains a half-tandem repeat with 43 predicted *O*-glycosylation sites, comprising 24 threonine and 19 serine residues (supplemental Fig. S1). The purified MUC6 protein was subjected to *in vitro* transglycosylation reactions from UDP-GalNAc, using selected ppGalNAc-Ts alone or combined (Table 1).

Assay conditions (incubation time, UDP-GalNAc, and enzyme amounts) were optimized at an analytical scale. The course of the transfer was monitored on Chip by SELDI-TOF MS as previously reported (22), and the reaction parameters giving the highest Tn density were selected. After purification by RP-HPLC the MUC6:Tn glycoproteins were obtained at a semipreparative scale (net peptide content: 1–7.5 mg). They were characterized by amino acid analysis and SELDI-TOF MS (Fig. 1*A* and Table 1). In all assays, the starting protein was totally converted into glycoconjugate. The SELDI-TOF MS profiles showed polydispersion regarding the average level of GalNAc incorporated (major peak ± 3 GalNAc) (Fig. 1*A*), as previously reported for other *in vitro* glycosylated proteins (20). All MUC6:Tn glycoproteins were obtained with a purity level >95%, as estimated by RP-HPLC (data not shown).

As expected, the obtained MUC6:Tn glycoproteins presented different Tn content depending on the ppGalNAc-T source (Table 1). ppGalNAc-T1 alone was capable of glycosylating 45% of potential *O*-glycosylation sites, representing 24% of the total molecular weight. Interestingly, simultaneous addition of ppGalNAc-T2 to this reaction mixture did not result in an increased glycosylation site number (Table 1). However, the addition of ppGalNAc-T7 did increase the glycosylation amount, resulting in a glycoprotein with 24 GalNAc (57% of predicted *O*-glycosylation sites).

The MUC6 protein was less glycosylated when incubated with ppGalNAc-T2, as compared with -T1 (16 GalNAc, representing 38% of predicted *O*-glycosylation sites). This indicates that ppGalNAc-T1 possesses a broader substrate specificity than -T2, in agreement with previous results on ppGalNAc-T1 and -T2 mucin specificity studies (25). As with ppGalNAc-T1, the addition of ppGalNAc-T7 resulted in an increase of the number of glycosylation sites (20 GalNAc, corresponding to 48% of predicted *O*-glycosylation sites).

The highest GalNAc incorporation rate was obtained when the three enzymes were used simultaneously (28 GalNAc, representing 67% of predicted *O*-glycosylation sites) (Table 1). In this case, GalNAc accounted for 35% of the total glycoprotein weight. Differential Tn density of the MUC6:Tn glycoproteins was also evidenced by Western blotting using an anti-His mAb (Fig. 1*B*).

Attempts to identify glycosylation sites were performed by MALDI MS and Fourier transform MS after trypsin digestion of MUC6:Tn(T1) (supplemental Fig. S1). However, both the inefficient proteolysis and the huge Tn density probably impeded the full assignment of the sites. Nevertheless, partial glycosylation sites of C- and N-terminal (glyco)peptides were obtained showing: (i) the specific glycosylation of Thr^{101}, Ser^{107}, and Thr^{115} and (ii) that all the other glycosylation sites were located between Ser^{33} and Ser^{90}, *i.e.* in the protein portion with the highest Thr, Ser, and Pro density (supplemental Fig. S1).

Analysis of MUC6:Tn Glycoprotein Antigenicity—The antigenicity of the MUC6:Tn glycocoproteins was analyzed by ELISA using a polyclonal anti-MUC6 serum and an anti-Tn mAb raised against human breast cancer cells (83D4) (27). Fig. 2 shows that all MUC6:Tn glycoproteins were similarly recognized by the anti-MUC6 serum, irrespective of their various glycosylation pattern. However, the non-glycosylated MUC6 protein was better recognized that the various glycosylated MUC6:Tn, probably due to decreased accessibility of the protein backbone in glycosylated proteins. In addition, the 83D4 anti-Tn mAb recognized all MUC6:Tn glycoproteins similarly, with a slightly higher recognition of the glycoproteins displaying higher Tn density. As expected, this mAb did not react with the non-glycosylated MUC6. These results confirmed that all MUC6:Tn glycoproteins displayed the Tn antigen and also showed that glycosylation did not significantly alter the accessibility of the MUC6 B-cell epitopes.

Analysis of Antibody Responses Induced by MUC6:Tn Glycoproteins—Several studies have shown that glycosylation can markedly influence not only the structure or function of a protein, but also its antigenicity and immunogenicity (28, 29). For instance, the extensive glycosylation of the Ebola virus glycoprotein has been shown to play an important role in its conformational integrity, antigenicity, and immunogenicity (30). We thus evaluated the immunological

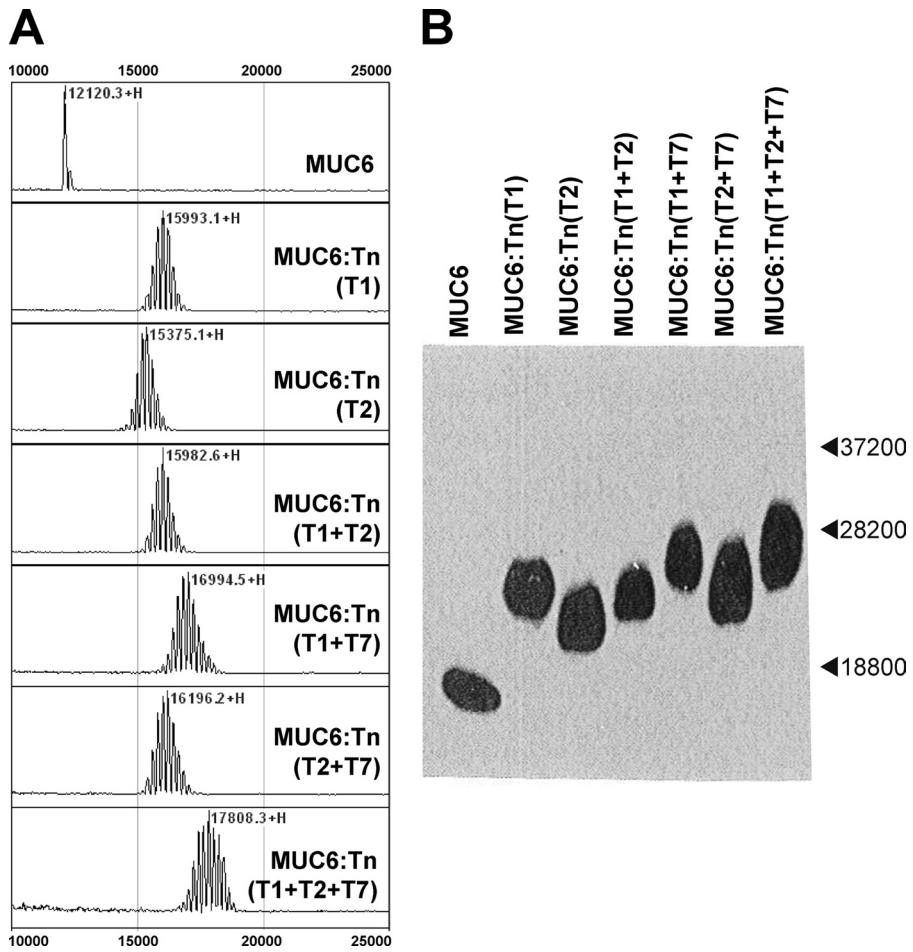

FIGURE 1. **Characterization of the recombinant MUC6:Tn glycoproteins.** MUC6 was glycosylated with different combinations of ppGalNAc-Ts as indicated under "Experimental Procedures," leading to various MUC6:Tn glycoproteins that were sequentially purified by nickel-nitrilotriacetic acid-agarose and RP-HPLC, and analyzed by SELDI-TOF MS (*A*). The medium peak is labeled with the mass/charge (*m*/*z*). Values are expressed in daltons. MUC6:Tn glycoproteins were also analyzed by Western blotting (*B*) using an anti-His mAb followed by an anti-mouse peroxidase conjugate. Molecular markers are expressed in daltons.

properties of the obtained MUC6:Tn glycoproteins presenting different Tn density.

We first analyzed the capacity of the MUC6:Tn glycoproteins to induce anti-MUC6 and anti-Tn antibodies. To this end, BALB/c mice were immunized four times with 10 μg of each glycoprotein every 3 weeks. Sera were collected 1 week after the last immunization and analyzed by ELISA for the presence of specific antibodies against the corresponding glycoprotein and the non-glycosylated MUC6 protein (Fig. 3 and supplemental Table S1). As shown in Fig. 3*A*, high anti-MUC6 antibodies were induced in this strain of mice by the non-glycosylated MUC6 protein and by MUC6:Tn(T2), although at lower titers, but not by MUC6:Tn(T1) or MUC6:Tn(T1+T2). Surprisingly, the addition of ppGalNAc-T7 to -T2 (MUC6:Tn(T2+T7)) totally abolished the induction of anti-MUC6 antibodies. In contrast, MUC6:Tn(T1+T2+T7), but not MUC6:Tn(T1+T7), elicited high responses against this protein.

As expected, the MUC6 protein did not induce anti-glycoprotein antibodies, in contrast to MUC6:Tn(T2) and MUC6:Tn(T1+T2+T7), which stimulated good responses against these antigens. No anti-MUC6 or anti-Tn responses were observed in mice immunized with MUC6:Tn(T1), MUC6:Tn(T1+T2), MUC6:Tn(T1+T7), and MUC6:Tn(T2+T7).

To determine whether the immunogenicity of MUC6:Tn glycoproteins was dependent upon the mouse strain used for the analysis, we also analyzed the antibody responses induced by some of these glycoproteins in C57BL/6 mice (Fig. 3*B*). A similar pattern of responses was obtained, with the exception for MUC6:Tn(T1+T7), which induced detectable antibody responses.

It is worth noting that both MUC6:Tn(T1) and MUC6:Tn(T1+T2), carrying the same Tn antigen density (19 GalNAc), did not elicit any antibodies (Fig. 3*A*), whereas the less glycosylated MUC6:Tn(T2) glycoprotein (16 GalNAc) was immunogenic. Thus, these results could indicate that a high density of GalNAc residues have masked MUC6 B cell epitopes and therefore reduced their immunogenicity. However, MUC6:Tn(T1+T2+T7), carrying 28 GalNAc residues, was immunogenic and induced antibody production in the two mouse strains tested. Thus, the capacity of these glycoproteins to induce anti-MUC6/MUC6:Tn antibodies is under a complex control. Their immunogenicity seems to be linked to general properties of these molecules rather than to

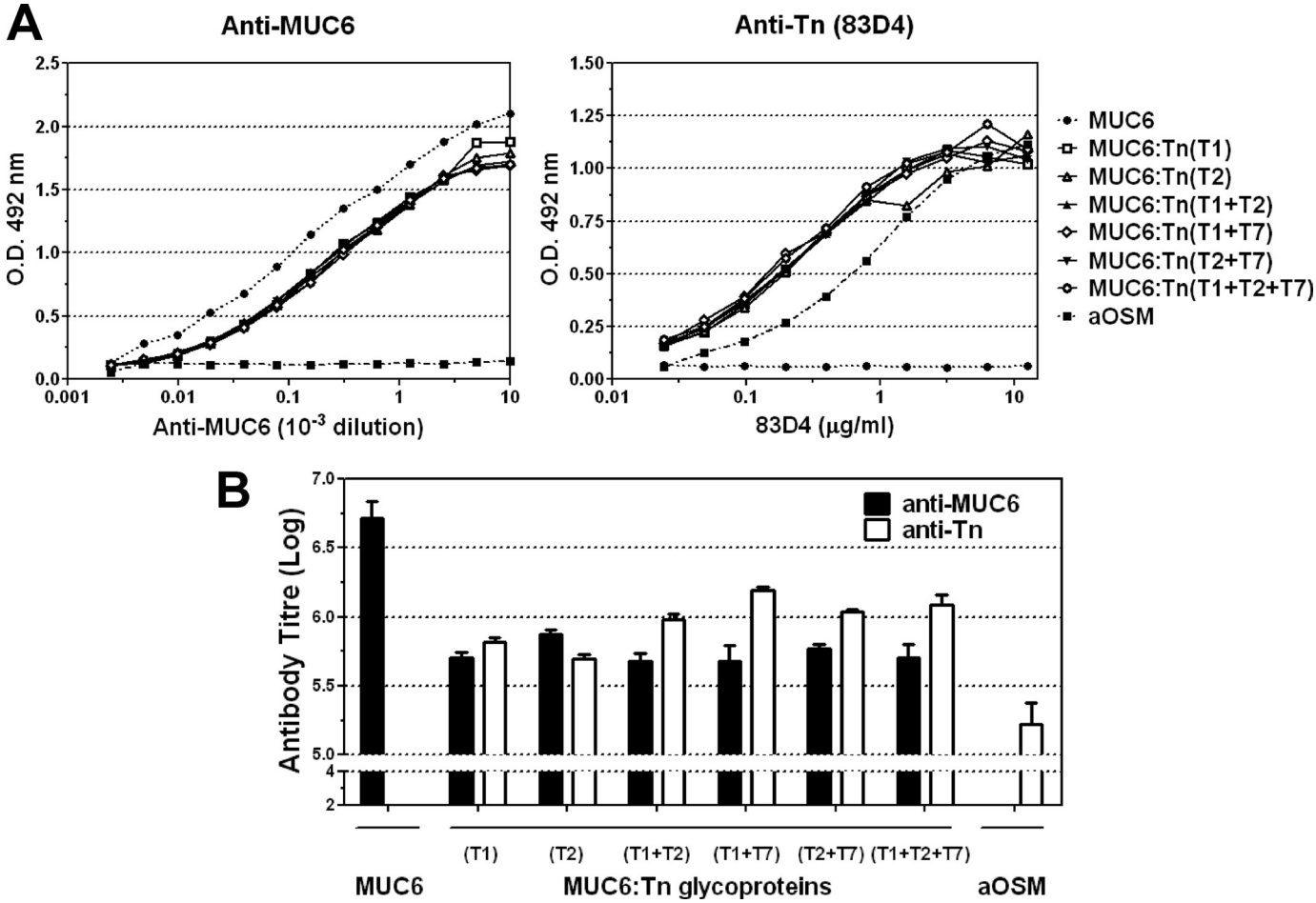

FIGURE 2. **Recognition of the MUC6-Tn glycoproteins by anti-MUC6 and anti-Tn antibodies.** The antigenicity of the MUC6:Tn glycoproteins was evaluated by ELISA using different dilutions of a polyclonal anti-MUC6 serum or anti-Tn mAb 83D4 (*A*). Antibody titers (*B*) were calculated to be the $\log_{10}$ highest dilution, which gave twice the absorbance of normal mouse sera diluted 1:100. asialo-OSM (aOSM), a Tn-rich mucin, was used as a control.

a lack of exposure of B cell epitopes because their proteins displayed comparable antigenicity (Fig. 2).

Only very low levels of IgM antibodies were detectable in sera obtained after MUC6:Tn immunization (data not shown). Moreover, these sera did not react with the aOSM protein, displaying high Tn antigen density.

We also evaluated the capacity of these sera to recognize human tumor cells using the Tn^+ Jurkat and MCF7 cell lines (also expressing MUC6). In 2 of 5 responders, anti-MUC6/MUC6:Tn antibodies were induced in mice immunized with the MUC6:Tn glycoproteins, and bound to Jurkat tumor cells (Fig. 3*C* and supplemental Table S1). Similar results were obtained for the MCF7 breast cancer cell line (data not shown).

Altogether, these results indicate that the immunogenicity of the MUC6:Tn glycoproteins was strongly affected by *O*-glycosylation, despite a comparable recognition by anti-MUC6 and anti-Tn antibodies. Moreover, the capacity to induce anti-MUC6:Tn antibodies was not dependent upon the GalNAc average number and was correlated with the level of anti-MUC6 responses. Importantly, the MUC6:Tn glycoprotein prepared by the combination of ppGalNAc-T1, -T2, and -T7 was capable, in two strains of mice, of inducing anti-MUC6 and anti-MUC6:Tn antibodies, which recognized Tn^+ cancer cell lines. This was also observed for MUC6:Tn(T2), although at lower antibody titers.

The Induction of T Cell Responses by MUC6:Tn Glycoproteins Is Strongly Affected by Tn Glycosylation—The induction of an immune response against an antigen is controlled both by the intrinsic characteristics of the antigen and genetic background of the host. In particular, MHC genes play an essential role in the induction of T-cell-mediated responses because only peptides able to bind to MHC molecules are capable of activating T cells and $CD4^+$ T helper-dependent B cells (31). Additionally, antibody responses can be influenced by the polymorphism of V genes, which may confer individual different capacities to produce antibodies against antigens due to the diversity in the B cell repertoire (32).

The lack of immunogenicity of some MUC6:Tn glycoproteins could be due to the masking or modification of MUC6 T cell epitopes due to GalNAc coupling to certain Thr or Ser of the protein. To evaluate this hypothesis, we first characterized the MUC6 epitopes recognized by $CD4^+$ T cells of C57BL/6 mice immunized by MUC6:Tn glycoproteins, using a panel of 15 overlapping peptides spanning the MUC6 sequence (Fig. 4*A*). The peptides were synthesized as 15-mers and overlapped by 5 residues. The identification of the $CD4^+$ T cell epitopes was performed by the analysis of

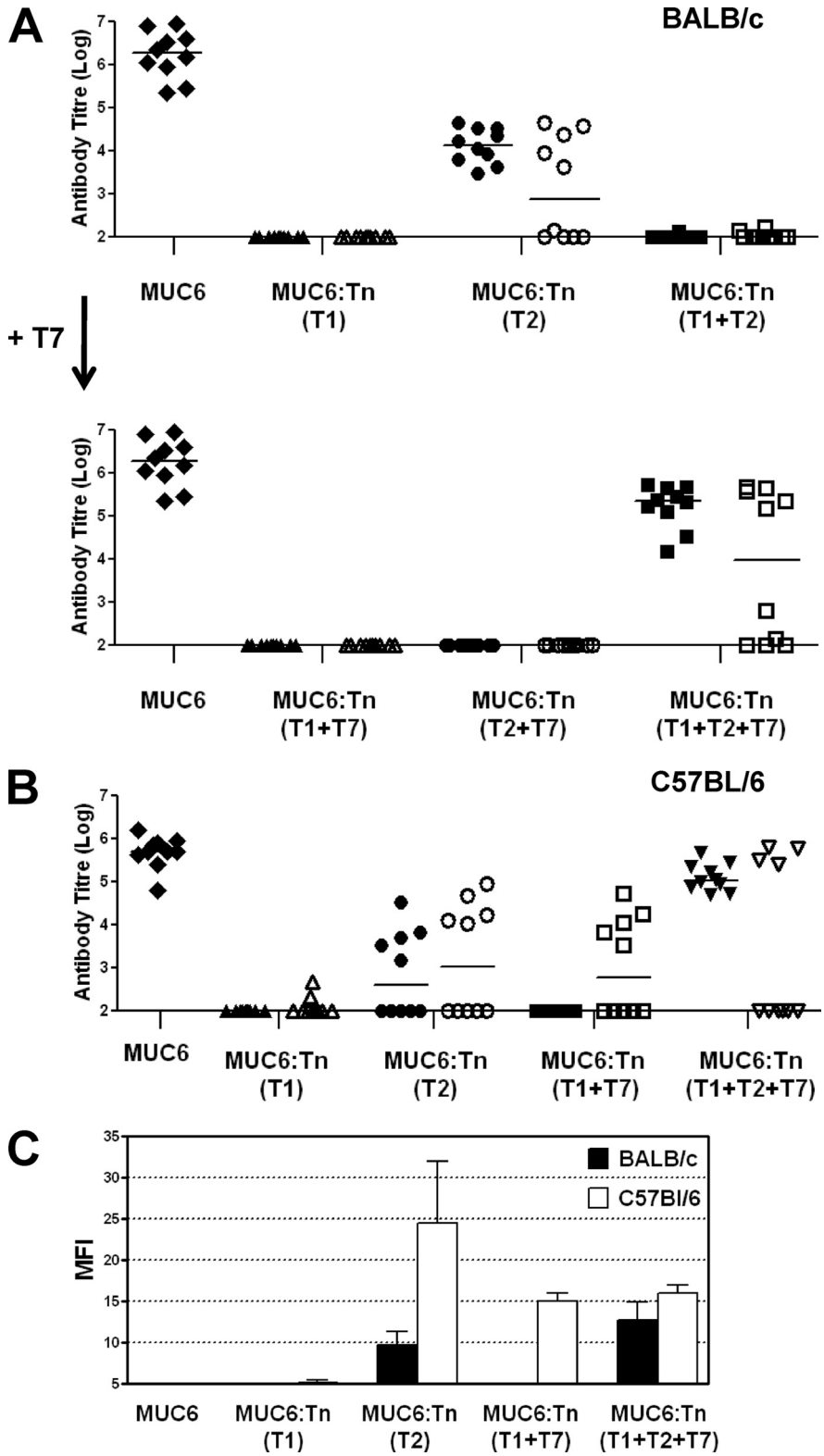

FIGURE 3. **IgG antibody induction by the MUC6:Tn glycoproteins in BALB/c and C57BL/6 mice.** BALB/c (*A*) or C57BL/6 (*B*) mice (10 per group) were immunized intraperitoneally at days 0, 21, 42, and 63 with 10 μg of the various MUC6:Tn glycoprotein or non-glycosylated MUC6, in alum (1 mg) and CpG (10 μg). Bleedings were carried out at days 20, 28, 49, and 70. Specific IgG antibodies against the MUC6 protein (*closed symbols*) and the immunizing glycoprotein (*open symbols*) were detected by ELISA. Individual (*A* and *B*) antibody titers at day 70 are shown. The recognition of human tumor cells was evaluated by flow cytometry using the Tn^+ Jurkat cell line (*C*). In this case, only responder mice are represented.

the proliferation and IFNγ production of draining LN cells, obtained from MUC6-immunized C57BL/6 mice and *in vitro* stimulated by the MUC6 peptides or a negative control MalE peptide. Three peptides, $MUC6_{33-47}$, $MUC6_{83-97}$, and to a lesser level, $MUC6_{98-112}$, stimulated specific responses of these LN cells (Fig. 4*B*). In contrast, LN cells from mice

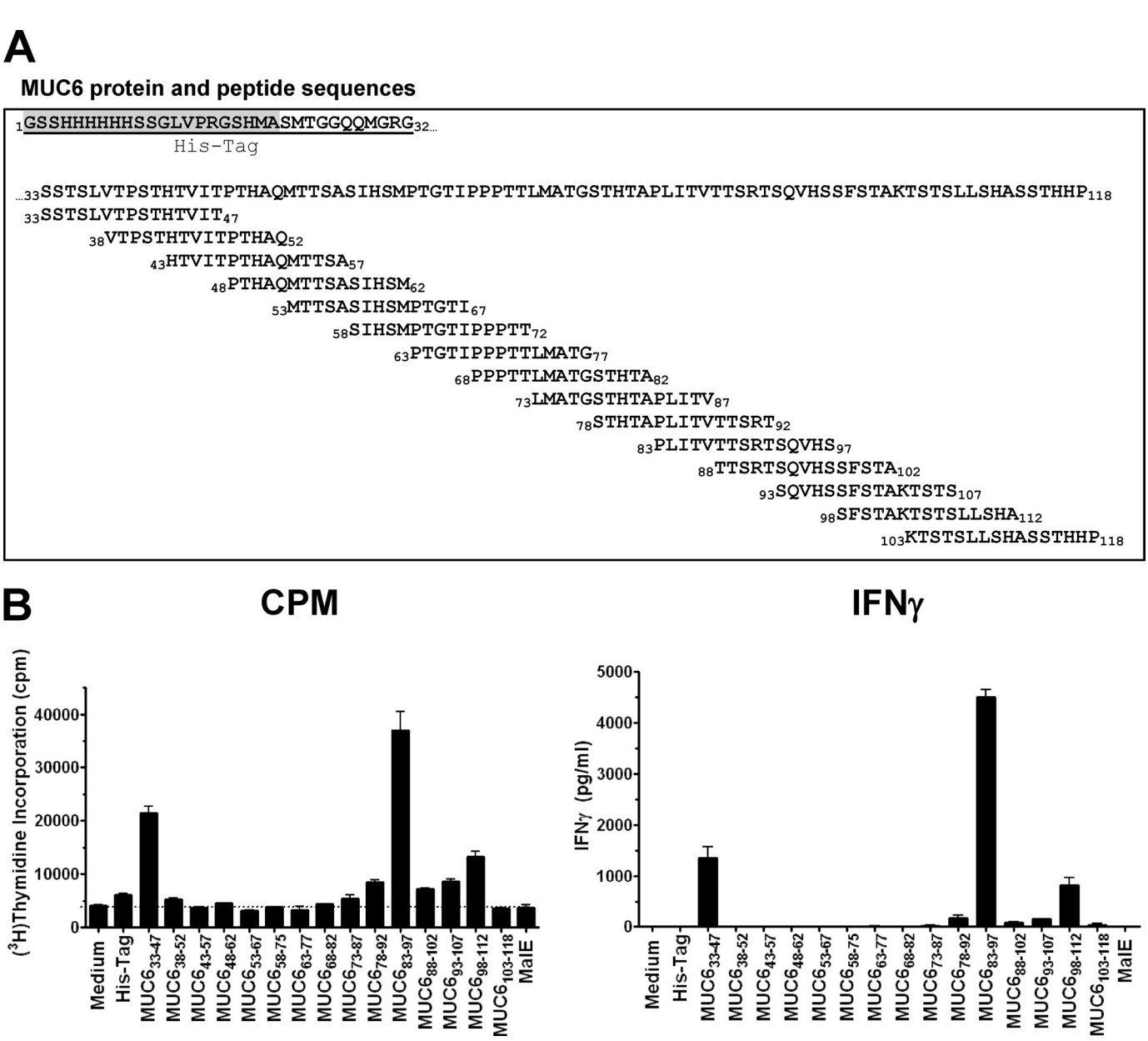

FIGURE 4. **Analysis of proliferative responses of MUC6-primed C57BL/6 draining lymph node cells to 15 peptides spanning the MUC6 sequence of the recombinant protein.** Fifteen peptides, overlapping by 5 residues and spanning the MUC6 recombinant protein sequence, were synthesized as 15-mers and used to identify the T cell epitopes (*A*). The recombinant MUC6 sequence is shown. To detect His tag-specific T cell response, we used a recombinant protein (His tag) that shares the His tag region (shaded in *gray*). The sequence corresponding to the fusion protein is *underlined*. Draining LN cells from five C57BL/6 mice immunized with MUC6 were pooled and cultured in triplicate in the presence of His tag (1 μg/ml) or peptides (10 μg/ml). Peptides containing T cell epitopes were selected according to the incorporation of [^{3}H]thymidine and IFNγ production (*B*). Negative controls consisted of cultures incubated with an irrelevant peptide (*MalE*) or with medium alone. T cells from MalE-immunized mice did not elicit responses to MUC6 peptides (not shown). Results are representative of three experiments.

immunized with the non-related peptide MalE did not proliferate or produce IFNγ following stimulation with these MUC6 peptides (data not shown).

To determine whether MUC6 glycosylation influences the immunodominance of MUC6 T cell epitopes, LN cells from mice immunized with four different MUC6:Tn glycoproteins were stimulated *in vitro* with the $MUC6_{33-47}$ and $MUC6_{83-97}$ peptides. As shown in Fig. 5, the proliferative responses to both the $MUC6_{33-47}$ and $MUC6_{83-97}$ peptides, as well as IFNγ production, of mice immunized with MUC6 glycoproteins was strongly reduced, as compared with the responses obtained with non-glycosylated MUC6-primed T cells. The other MUC6 peptides did not stimulate any responses of these primed T cells, indicating that MUC6 glycosylation did not generate new T cell peptidic epitopes (supplemental Fig. S2). These results suggest that MUC6 glycosylation has affected its processing by antigen presenting cells and/or the capacity of the processed glycosylated peptides to bind to MHC class II molecules, leading to the loss of immunogenicity of the MUC6 $CD4^+$ T epitopes. However, the observation that some of these molecules, such as MUC6:Tn(T2) or MUC6:Tn(T1+T2+T17), were still able to induce antibody responses indicates that the phenomena could be more complex and thus deserves further investigations.

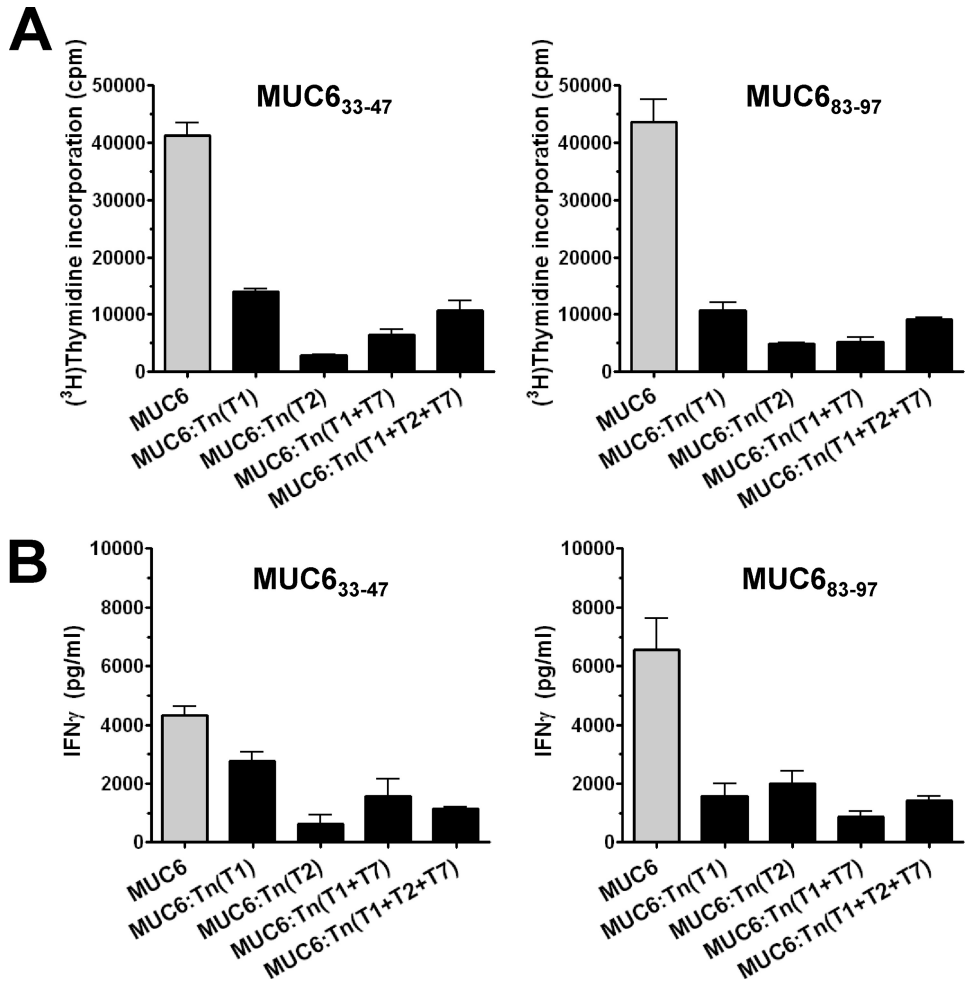

FIGURE 5. **Analysis of T cell responses of MUC6 or MUC6:Tn-primed C57BL/6 mice to MUC6$_{33-47}$ and MUC6$_{83-97}$ immunodominant T cell epitopes.** C57BL/6 mice (5 per group) were immunized with 10 μg of four different MUC6:Tn glycoproteins (*black bars*) or the non-glycosylated MUC6 (*gray bars*) in CFA. Ten days later, draining lymph node cells were pooled and cultured in triplicate in the presence of MUC6$_{33-47}$ or MUC6$_{83-97}$ peptides (10 μg/ml). T cell responses were evaluated by [^{3}H]thymidine incorporation (*A*) and IFNγ production (*B*). The values shown were obtained after subtracting the background responses obtained in cultures with medium alone. Results are representative of two experiments.

MUC6:Tn Glycoprotein Recognition by Specific T Cell Hybridomas—To determine whether the partial loss of T cell epitope immunodominance was due to impaired glycoprotein presentation (33), we generated MUC6-specific CD4$^+$ T cell hybridomas. Several MUC6$_{33-47}$-specific IA^b-restricted T cell hybridomas were selected on the basis of their specific IL-2 secretion following *in vitro* stimulation by this peptide. Representative results are shown for hybridomas IG7 and IG11, which showed dose-dependent responses to the MUC6$_{33-47}$ presented by IA^b-transfected fibroblasts (Fig. 6*A*).

The recognition of non-glycosylated MUC6 and MUC6:Tn glycoproteins by these hybridomas was analyzed by testing their IL-2 production. When incubated with BMDC, both 1G7 and 1G11 hybridomas showed a dose-dependent recognition of the non-glycosylated MUC6. These hybridomas, and in particular the IG11, showed higher responses to low doses of the MUC6:Tn glycoproteins as compared with MUC6. However, at high doses, the four glycoproteins induced a lower IL-2 response than the MUC6 protein (Fig. 6*B*). These results demonstrated that the MUC6:Tn glycoproteins can be efficiently processed and presented by BMDC MHC class II molecules.

C-type lectin receptors on dendritic cells can mediate the uptake of glycosylated antigens. In particular, we and others have recently demonstrated that the macrophage Gal/GalNAc lectin (MGL) specifically recognizes the Tn antigen (34) and mediates enhanced presentation of Tn-glycosylated peptides (35). We thus next evaluated binding and internalization of Alexa 647-labeled MUC6:Tn(T1) by BMDC. As shown in Fig. 6*C*, BMDC were able to bind and internalize this glycoprotein in an efficient and dose-dependent manner, whereas a very low signal was obtained with the unglycosylated MUC6. Moreover, inhibition assays with EDTA and a MGL-specific monoclonal antibody confirmed that the binding and internalization of MUC6:Tn(T1) were mediated by the C-type lectin MGL. This receptor-mediated endocytosis of MUC6:Tn glycoproteins could explain the enhanced responses of MUC6-specific T cell hybridomas to low doses of MUC6:Tn glycoproteins, as compared with MUC6.

In summary, these results show that MUC6 glycosylation did not prevent its processing and presentation, and could even con-

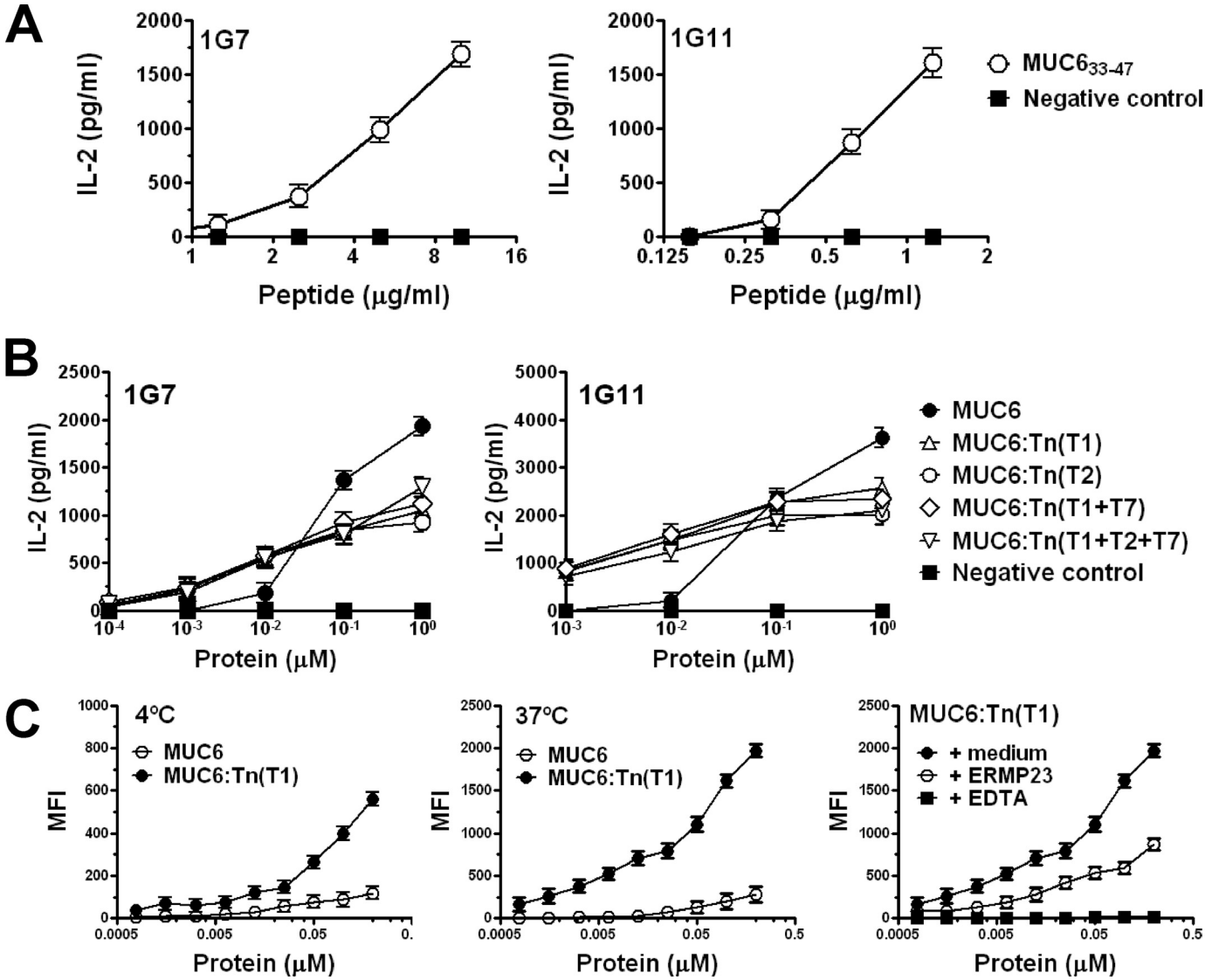

FIGURE 6. **Recognition of MUC6:Tn glycoproteins by MUC6-specific T-cell hybridomas.** The production of IL-2 by MUC6$_{33-47}$ peptide-specific T cell hybridomas 1G7 and 1G11 was analyzed following incubation of fibroblasts L-cells transfected with the IA^b molecule in the presence of various concentrations of this peptide (*A*) or of BMDC with either the nonglycosylated MUC6 or the MUC6:Tn glycoproteins (*B*). Negative controls were stimulated with an irrelevant peptide. IL-2 production was determined by a specific ELISA on cell supernatants harvested at 20–24 h. The results are shown as the mean of triplicates (± S.D., indicated by *standard deviation bars*). Non-glycosylated MUC6 or MUC6:Tn(T1) binding and uptake by CD11c$^+$ BMDC was analyzed by flow cytometry after incubation at 4 or 37 °C with Alexa 647-labeled antigen (*C*). Antigen binding and uptake of MUC6:Tn(T1) was also analyzed in the presence of EDTA or anti-MGL mAb (ERMP23) (*right panel*). Results are representative of three different experiments.

tribute to a better presentation to T cells, by increasing its uptake by dendritic cells. Therefore, the loss of *in vivo* T cell immunogenicity of MUC6:Tn glycoproteins cannot be explained by an impaired T cell antigenicity due to glycosylation.

MUC6:Tn Glycoproteins Preferentially Induce the Activation of Th17 Responses—We then analyzed the type of T-cell response induced by glycosylated MUC6:Tn proteins injected with CFA (Complete Freund Adjuvant). To this end, draining LN cells from C57BL/6 mice immunized with MUC6:Tn glycoproteins were stimulated *in vitro* with MUC6. As depicted in Fig. 7*A*, immunization with MUC6:Tn glycoproteins induced lower proliferative T cell responses than the nonglycosylated MUC6 protein, which was accompanied by a reduced IFNγ production. Remarkably, LN cells from mice immunized by the MUC6:Tn glycoproteins, but not the MUC6 protein, produced strong IL-17 responses after *in vitro* stimulation with MUC6.

A lower proliferative and IFNγ response was also found in draining LN cells from mice primed with MUC6:Tn glycoproteins and stimulated *in vitro* with the corresponding glycoprotein (Fig. 7*B*). However, again a significant level of IL-17 was observed in these LN cell cultures.

We also analyzed the capacity of LN from MUC6-primed mice to respond to *in vitro* stimulation by the glycosylated proteins (Fig. 7*C*). These results demonstrated that MUC6:Tn glycoproteins can stimulate *in vitro* the proliferation and IFNγ response of MUC6-primed T cells, although less efficiently than MUC6, but again demonstrated a preferential production of IL-17. No IL-5 production was observed by LN cells from mice immunized with either MUC6 or MUC6:Tn glycoproteins (data not shown). As expected, LN from control mice did

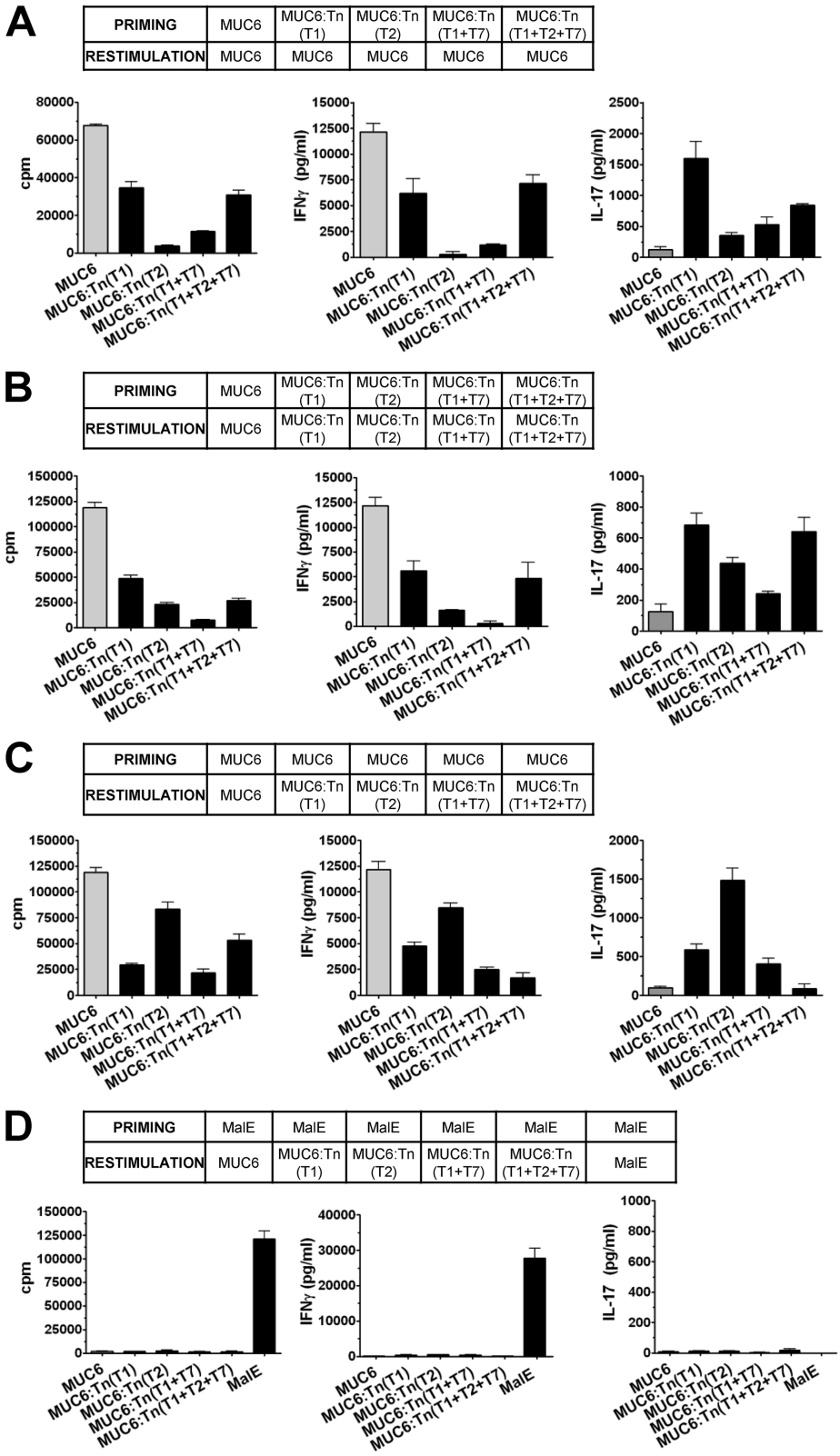

FIGURE 7. **Induction of Th17 T cell responses in mice immunized by MUC6:Tn glycoproteins.** C57BL/6 mice (5 per group) were immunized subcutaneously with 10 μg of the non-glycosylated MUC6 protein or MUC6:Tn glycoproteins in CFA, as indicated in the legends. After 10 days, inguinal LN cells were cultured in triplicate in the presence of non-glycosylated MUC6 protein (1 μg/ml) (*A*) or with MUC6 or the homologous glycoprotein (1 μg/ml) (*B*) for 72 h. Alternatively, cross-reactivity of MUC6-primed T cells was evaluated by immunizing mice with the non-glycosylated MUC6 protein followed by *in vitro* re-stimulation with MUC6 or MUC6:Tn glycoproteins (1 μg/ml) for 72 h (*C*). A control group was immunized with an irrelevant peptide from the MalE protein (*D*). Cell proliferation was evaluated according to the incorporation of [^{3}H]thymidine (counts/min). Culture supernatants were collected and analyzed for IFNγ, IL-17A, and IL-5 levels. Negative controls consisted of cultures incubated with an irrelevant peptide (MalE) or with medium alone. The values shown were obtained after subtracting the background responses of cultures with medium alone, expressed as the mean of triplicates ($\pm$ S.D., indicated by *standard deviation bars*), and are representative of two experiments.

not induce any proliferation or cytokine production when restimulated with MUC6 or MUC6:Tn glycoproteins (Fig. 7*D*).

Altogether, these results strongly suggest that the glycosylation of the MUC6:Tn glycoproteins did not abrogate their capacity to stimulate T cells but induced the preferential stimulation of Th17 responses. This polarization did not require the *in vivo* priming by the glycoprotein but could have been linked to antigen-presenting cells signaling through a lectin receptor.

DISCUSSION

Post-translational modifications occur in a variety of proteins of eukaryotic cells and appear to be significant for a number of cellular functions and the maintenance of homeostasis. Moreover, it is now well established that peptide modifications, such as glycosylation, phosphorylation, methylation, acetylation, or ubiquitination, can be recognized by the immune system (28, 36). Some of these post-translational modifications play important roles in pathogenic processes. In particular, glycosylation of epitopes has been linked to autoimmunity (37), virus infection (30, 38), or cancer (39).

B or T cell recognition of glycopeptides is highly relevant for the immune response against tumors because aberrant glycosylation is a general feature of many cancer cells. Abnormal *O*-glycans expressed by cancer cells have functional importance in cell adhesion, invasion, and metastasis (40). In particular, incomplete elongation of *O*-glycan saccharide chains in mucins can lead to the expression of shorter carbohydrate structures, such as the Tn antigen, one of the most specific human cancer-associated structures (41). In the present study, we describe the production of MUC6:Tn glycoproteins for anticancer immunotherapy and the immunological properties of these tumoral antigens. In particular, we show that the incorporation of the tumor-associated Tn antigen on MUC6 can partially abrogate its capacity to trigger specific T cell proliferation. Furthermore, MUC6:Tn glycoproteins were demonstrated to stimulate T cells that preferentially produced IL-17, demonstrating that MUC6 Tn glycosylation markedly influences its immunological properties.

Glycosyltransferases have been extensively used as tools to perform *in vitro* transglycosylation reactions and represent an attractive alternative to the chemical synthesis of large glycosylated compounds (42). The synthesis of glycopeptides and glycoconjugates with *O*-linked glycans has been reported, especially for the Tn (43, 44), sialyl-Tn (45), and sialyl-Thomsen-Friedenreich (TF) antigens (45, 46). We have previously described the enzymatic synthesis of MUC6:Tn glycoproteins by using ppGalNAc-Ts (20). This large family of enzymes catalyzes *in vivo* the transfer of a GalNAc residue to Ser or Thr (*i.e.* the synthesis of the Tn antigen). To date, 15 ppGalNAc-Ts have been identified in mammals and the functional profile of each member of the family has been established, showing that these enzymes have not only different substrate specificities but also specific tissue expression patterns (21, 47). In this previous study, we have prepared a MUC6:Tn glycoprotein, using a protein extract from the breast cancer cell line MCF7 as the source of ppGalNAc-Ts mixture. This glycoprotein induced IgG antibodies in mice, which were capable of recognizing human tumor cells through a Tn-dependent mechanism (20). However, the use of such cell extracts is not suitable to meet the quality requirements for human vaccine production. In addition, difficulties encountered with reproducibility in the glycosylation rate and immunological properties of the resulting glycoproteins between cell extract batches require the use of a more controlled glycosylation source. Notably, by taking advantage of their different specificities, the ppGalNAcTs can give access to various protein glycoforms. Here, we report the use of different combinations of recombinant ppGalNAc-Ts, as an attractive alternative to the use of cell extracts. We selected ppGalNAc-T1 and -T2 because they possess a broad spectrum of specificity. In addition, ppGalNAc-T7 was used to complement the actions of these two enzymes, because it is able to incorporate GalNAc on threonines and serines of already glycosylated peptides (26). Following this approach, we obtained MUC6:Tn glycoproteins with different average Tn density.

The results obtained using different combinations of ppGalNAc-Ts suggest that the ppGalNAc-T1 and -T2 present not only overlapping, but also complementary MUC6 specificities. Indeed, when ppGalNAc-T7 was added to the glycosylation reaction with -T1 or -T2 alone, the number of glycosylated sites increased by 10–12%. When -T7 was added to the mixture containing both T1 + T2, it increased by 22% the glycosylated sites, suggesting that the sites initially glycosylated by -T1 and -T2 are different.

The fact that ppGalNAc-T2 used with -T1 did not increase the number of glycosylated sites obtained with -T1 alone (19 GalNAc) could be due to an alteration of its peptide specificity caused by prior peptide glycosylation by -T1, as already demonstrated for ppGalNAc-Ts (48, 49). Alternatively, a faster glycosylation kinetic for ppGalNAc-T1 could also mask ppGalNAc-T2 enzymatic efficiency, as previously demonstrated (25).

Prediction of *O*-glycosylation sites by the NetOGlyc3.1 algorithm identified 43 sites (24 threonine and 19 serines) in MUC6. Among the predicted sites, 28 presented a score >0.6, suggesting that these sites are more likely to be glycosylated than the others (supplemental Fig. S1), and might correspond to the glycosylated sites in MUC6:Tn glycoproteins (16–28 GalNAc). The majority of these sites are threonine residues (23 Thr *versus* 5 Ser). This apparent *in vitro* preferential specificity for threonines is in agreement with previous results obtained for -T1 and -T2, which glycosylate serines in a much lower rate than threonines (25).

In conclusion, MUC6 represents a major acceptor substrate for several ppGalNAc-Ts, which was shown to be very useful tools for obtaining glycoproteins carrying the Tn antigen. Moreover, the combined action of several ppGalNAc-Ts improved the efficacy of GalNAc addition on MUC6. However, the addition of GalNAc by different enzymes with distinct substrate specificity may contribute to the complexity of the obtained glycoproteins, concerning both their physicochemical and biological properties.

It has been previously reported that glycosylation may modulate the biological properties of proteins. For instance, carbohydrates can play a role in molecular stability, solubility, activity, antigenicity, and even immunogenicity of proteins (50). Our results regarding the antigenicity of the MUC6:Tn glycopro-

teins demonstrated their recognition by anti-Tn antibody 83D4 raised against human breast cancer cells (27). However, they were slightly less recognized by an anti-MUC6 sera, probably because the GalNAc residues reduce the accessibility of antibodies to the protein core. This would be in agreement with previous reports demonstrating that glycosylation interferes with antibody binding (51).

When injected into mice, MUC6:Tn glycoproteins differed in their capacity to induce specific antibodies: only MUC6:Tn(T2) and MUC6:Tn(T1+T2+T7) were capable of inducing specific antibodies able to recognize tumor cells, demonstrating that GalNAc linkage to the MUC6 protein modified its immunogenicity. Importantly, the capacity to induce MUC6:Tn-specific antibodies correlated with the induction of anti-MUC6 responses. However, we could not find a clear relationship between the GalNAc number and the level of Tn- or MUC6-specific antibodies. This lack of induction of B cell responses could be due to the absence of T cell help, as a consequence of the loss of immunodominance of T $CD4^+$ epitopes on MUC6:Tn glycoproteins. Indeed, both the length and size of carbohydrate chains (33, 52), as well as the position of glycans on the protein (53, 54), could affect the processing of antigens, because the cleavage pattern by proteases are influenced by glycosylation. Thus, glycosylation may convert an immunodominant T cell determinant or epitope into a hidden or cryptic determinant (36). Indeed, the study of the immunodominance of MUC6 epitopes on MUC6:Tn glycoproteins demonstrated a decreased recognition of the MUC6 T cell epitopes by MUC6:Tn glycoprotein-primed T cells. Another explanation to the absence of antibodies may be due to changes of MUC6 conformation depending on GalNAc position and/or density (55). Indeed, the addition of GalNAc residues could induce changes of glycoprotein conformation (56), which may create new conformational B cell epitopes, absent from the unglycosylated protein. Additional experiments to determine the structure of the MUC6:Tn glycoproteins are needed to address this question.

The study of antigen MHC class II presentation of MUC6:Tn glycoproteins by MUC6-specific T cell hybridomas suggested that these glycoproteins are efficiently captured, processed, and presented by dendritic cells. However, at a high antigen dose (>0.1 μM), MUC6 was more effective than the MUC6:Tn glycoproteins in inducing IL-2 production by these hybridoma. In agreement with our results, a MUC1 100-mer peptide devoid of sugars was processed by antigen presenting cells more efficiently than a native glycosylated MUC1 and the level of CTL responses induced correlated inversely with the degree of glycosylation of the priming antigen (57).

Antigen presenting cells are able to process glycoproteins to glycopeptides and present them in association with both MHC class I (53) and class II (58) molecules. However, recent studies have demonstrated that antigen processing or presentation of glycopeptide is highly dependent on the complexity and size of carbohydrate chains, as well as the site where they are attached (33). Glycans on antigens can limit the access of proteolytic enzymes and thereby inhibit the generation of antigenic peptide, as shown for some synthetic glycopeptides that are not immunogenic, despite their binding to MHC class II molecules (59). In fact, short-linked glycans on MUC1, which remain intact during dendritic cell processing in the MHC class II pathway (60), control both the extent and site specificity of proteolysis of MUC1 glycopeptides (33, 61). The presence of some carbohydrate moieties attached to peptides may also modify the binding of processed peptides to MHC molecules. For instance, orientation of the Tn moiety on peptides can determine the affinity with the MHC molecule: when GalNAc is pointed outward of the peptide-binding groove of MHC, glycosylation with Tn at non-anchor positions within the peptide did not significantly affect binding to the MHC molecule (62). On the other hand, if GalNAc is pointed toward the MHC molecule and if it is unable to accommodate in the MHC groove, it can prevent effective binding of the glycopeptide to MHC, being unable to mount a T cell response (62). *N*-Glycosylated peptides have also been shown to greatly reduce binding to MHC (63). In the present study, both T cell hybridomas and *in vivo* MUC6-primed T cells recognized the MUC6:Tn glycoproteins, suggesting that GalNAc residues may be placed outside the TCR binding region, as seen for T cells generated to glycopeptides with high immunogenicity that cross-reacted with the non-glycosylated peptide (63). T cell hybridomas generated to the non-glycosylated peptide reacted equally well, toward the amino-terminal substituted glycopeptides (64).

This study also showed that, at lower antigen doses (<0.01 μM), glycoproteins were more efficiently presented to specific hybridomas than non-glycosylated MUC6, presumably due to increased capture and internalization of the MUC6:Tn glycoproteins by MGL^+ dendritic cells. MGL is a type II transmembrane protein that recognizes terminal GalNAc residues in a calcium-dependent manner (34) and displays a remarkable specificity for the Tn antigen, being able to mediate GalNAc-antigen uptake and presentation by dendritic cells (35, 65). Our results showing inhibition of internalization by dendritic cells of MUC6:Tn(T1) with EDTA and with MGL-specific antibody strongly suggest that this lectin could mediate the MUC6:Tn glycoprotein entry to dendritic cells at low doses. Thus, receptor-mediated endocytosis may be responsible for the higher presentation efficacy of MUC6:Tn glycoproteins at low dose. Indeed, in agreement with this observation, we have recently shown that targeting *in vitro* or *in vivo* MGL^+ dendritic cells enhances uptake and MHC class II presentation of Tn glycopeptides (35).

Importantly, this study demonstrated that, whereas non-glycosylated MUC6 induced a Th1 T cell response, MUC6:Tn glycoproteins induced weak IFNγ responses but high levels of IL-17. Although it is well established that Th17 cells contribute to autoimmunity, their role in cancer is poorly understood and remains controversial. Th17 cells producing IL-17 have been found in tumors of ovarian cancer patients (66) and in mouse tumor models (67) and may contribute to protective tumor immunity by recruiting effector cells to the tumor microenvironment. Indeed, IL-17-deficient mice showed accelerated tumor growth and lung metastasis in several tumor models, and forced expression of IL-17 in tumor cells was shown to suppress tumor progression (66, 68). Thus, the production of IL-17 by T cells induced by MUC6:Tn glycoproteins might have an important role in tumor protection.

The type of the T cell response induced depends on many factors such as the nature of antigen or the mechanisms by which dendritic cells acquire the antigen. Also, innate signals triggered by different receptors, such as C-type Lectin Receptors, may alter T cell polarization. Various studies have shown that antigen targeting to different molecules results in the induction of qualitatively different immune responses. For instance, Dectin-1 targeting favors the induction of IFN-α by both $CD4^+$ and $CD8\ T^+$ cell subsets (69). GalNAc residues on MUC6 proteins may be recognized by a CLR such as MGL on dendritic cells, and induce a polarization toward a Th17 phenotype. However, to our knowledge, no innate signaling pathway has been attributed to MGL on dendritic cells yet.

In conclusion, our results indicate that Tn glycosylation of the MUC6 protein strongly affects its B and T cell immunogenicity. Thus, the design of glycoprotein-based vaccines should take in account the possible immunomodulating properties of glycosylation.

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