

# A Fully Synthetic Therapeutic Vaccine Candidate Targeting Carcinoma-Associated Tn Carbohydrate Antigen Induces Tumor-Specific Antibodies in Nonhuman Primates

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## ABSTRACT

We recently developed an efficient strategy based on a fully synthetic dendrimeric carbohydrate display (multiple antigenic glycopeptide; MAG) to induce anticarbohydrate antibody responses for therapeutic vaccination against cancer. Here, we show the superior efficacy of the MAG strategy over the traditional keyhole limpet hemocyanin glycoconjugate to elicit an anticarbohydrate IgG response against the tumor-associated Tn antigen. We highlight the influence of the aglyconic carrier elements of such a tumor antigen for their recognition by the immune system. Finally, we additionally developed the MAG system by introducing promiscuous HLA-restricted T-helper epitopes and performed its immunological evaluation in nonhuman primates. MAG:Tn vaccines induced in all of the animals strong tumor-specific anti-Tn antibodies that can mediate antibody-dependent cell cytotoxicity against human tumor. Therefore, the preclinical evaluation of the MAG:Tn vaccine demonstrates that it represents a safe and highly promising immunotherapeutic molecularly defined tool for targeting breast, colon, and prostate cancers that express the carbohydrate Tn antigen.

## INTRODUCTION

Cancerous transformation is very often associated with a dysregulation of the glycosylation processes leading to altered carbohydrate patterns at the surface of cancer cells (1). This results in the expression of various carbohydrate antigens such as blood group-related Tn, T, sialyl-Tn, sialyl-T antigens (family of T antigens) associated with carcinomas (2) or glycolipidic GM2, GD2, and GD3 associated with melanomas (3). Some of these tumor-associated carbohydrate antigens, involved in metastatic processes and associated with a poor prognosis, represent an excellent target for immune intervention after tumor resection and chemotherapy treatments to avoid cancer recurrence (4).

In a large variety of epithelial cancers such as breast, ovarian, colorectal, pancreatic, or prostate cancers, highly *O*-glycosylated mucins are strongly affected in their carbohydrate patterns displaying nonglycosylated as well as abortive glycosylated products such as Tn, T, sialyl-Tn, and sialyl-T antigens (2, 5–8). Naked peptides derived from variable number tandem repeat of MUC1 sequences have been used for the design of immunotherapeutic vaccine to elicit cellular and humoral antitumoral immune responses (9–11). However, some MUC1 peptide sequences showed T-cell immunosuppressive activity (12). Moreover, anti-MUC1 natural antibodies found in cancer patients with a favorable prognosis preferentially recognize glycosylated forms of MUC1 peptides (13). Therefore, the family of T antigens represents a suitable candidate for immune intervention. Moreover, these truncated glycosylation products

are expressed in fetal life and remain mainly cryptic in normal adult tissues, thus limiting risks of autoimmunity.

Up to now, to elicit B-cell responses specific for these carbohydrate tumor antigens, they have been chemically linked to a carrier protein, such as keyhole limpet hemocyanin (KLH), to provide T-cell help required for antibody production. Several gangliosides-KLH glycoconjugates have reached Phase II/III clinical trials (14). For the family of T antigens, the administration of sialyl-Tn-KLH glycoconjugate in DETOX adjuvant was correlated with an increased survival in patients with metastatic breast cancers (15). In contrast to the large ganglioside structures, short haptenic Tn, T, sialyl-Tn molecules require to be associated as cluster of at least two to three units to mimic native forms found on mucins (16–18), whereas a single unit does so poorly (19). This requirement for repetitive carbohydrate units reflects the composition of the mucin substrate, highly enriched in consecutive serine or threonine residues, but also the *O*-glycosylation process occurring in cancer cells. For instance, the *N*-acetylgalactosaminyl-transferase T3, responsible for the glycosylation of consecutive threonine residues, is overexpressed in adenocarcinomas leading to the expression of Tn clusters (20). Therefore, the strategy of carbohydrate clustering has greatly improved the immunogenicity of these short haptenic molecules allowing the recognition of native carbohydrate structures on tumor cells (21–23).

On protein glycoconjugates, the carbohydrate density that can be achieved is highly variable. Advances in the design of appropriate linkers and in conjugation procedures have improved the efficiency of chemical coupling to obtain elevated carbohydrate:carrier protein ratio (24). However, the use of a limited number of carrier proteins to conjugate carbohydrate antigens may limit the efficacy of these glycoconjugates. Indeed, the immune response to the carrier is much more superior to the one directed against the carbohydrate antigens, and this may lead to carrier-induced epitopic suppression (25, 26). In addition, an accurate molecular definition of glycoconjugates in terms of composition and structure can only be achieved by the full chemical synthesis of immunogens.

Following an entirely chemical synthesis process, we developed dendrimeric MAG as an alternative strategy to glycoprotein conjugates. MAG is based on the linking of a high density of carbohydrates to a nonimmunogenic lysine core to focus the immune response to the haptenic moiety (27, 28). We applied the MAG strategy to the Tn antigen ( $\alpha$ -GalNAc-Ser/Thr) with a trimeric form, which was associated with a T-helper peptide to allow the induction of a T cell-dependent IgG antibody response against the Tn tumor antigen. When administered with alum, in either therapeutic or prophylactic protocols, these MAG conjugates increased up to 80% the survival of tumor-bearing mice (23, 28). In previous attempts, fully synthetic lipoglycopeptides with a dimeric Tn associated with a palmitoyl core induced IgM but failed to elicit IgG antibodies required for antibody-dependent cellular cytotoxicity (ADCC) against cancer cells (29). The same strategy developed with the carcinoma-associated Lewis<sup>x</sup> antigen also resulted in the sole IgM antibody production (30). In many

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cases, KLH glycoconjugates also induced IgM antibodies but no or moderate IgG in mice and humans (4). This major issue can be partially overcome by using a strong adjuvant such as QS21. In contrast, the multiple antigenic glycopeptide (MAG) showed its immunological potency and therapeutic effects in mice using the harmless and commonly used vaccine adjuvant, aluminum hydroxide.

In the present study, to optimize the development of MAG:Tn immunogens, we first investigated the influence of the amino acid carrying the GalNAc to design the most appropriate antigenic and immunogenic Tn cluster. The efficacy of the MAG strategy was then tested by comparing the MAG:Tn with its KLH-Tn counterpart in inducing anti-Tn IgG antibodies in mice. Finally, to apply the MAG:Tn for human vaccination we designed two MAG:Tn compounds in which "universal" CD4<sup>+</sup> T-cell epitopes known to stimulate effective T-helper cell responses in human populations with many HLA diversity were introduced. These MAG were tested in nonhuman primates (macaques and green monkeys) and found to induce strong anti-Tn IgG antibodies capable of specifically recognizing Tn-expressing human tumor cells. Moreover, these antibodies were able to mediate ADCC against Tn-positive human tumor cells.

## MATERIALS AND METHODS

**Chemical Syntheses of Linear and Dendrimeric Glycopeptides.** The MUC glycopeptides (SLSYTNPAV and ALGSTTPPA series) were synthesized on an Applied Biosystems Pioneer peptide synthesizer using continuous-flow Fmoc chemistry and 2-(1H-9-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/diisopropylethylamine as the coupling reagents. The protected glycosylated building blocks [Fmoc-Ser( $\alpha$ -GalNAc(OAc)<sub>3</sub>)-OH or Fmoc-Thr( $\alpha$ -GalNAc(OAc)<sub>3</sub>)-OH] were incorporated manually using 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate/1-hydroxybenzotriazole activation. The synthesis of the other peptides and glycopeptides was performed as described previously by solid-chemistry (23, 31, 32). Briefly, the protected amino acids were incorporated manually into the peptide sequence using 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate/1-hydroxybenzotriazole/diisopropylethylamine as the coupling reagents. Fmoc protection was removed with 20% piperidine in dimethylformamide. The glycosylated building blocks [Fmoc-Ser( $\alpha$ -GalNAc)-OH, Fmoc-Thr( $\alpha$ -GalNAc)-OH, or Fmoc-hSer( $\alpha$ -GalNAc)-OH (32)] were incorporated as their crude pentafluorophenyl esters in the presence of 1-hydroxybenzotriazole. The products were cleaved from the resin with aqueous trifluoroacetic acid, triisopropylsilane, H<sub>2</sub>O, and phenol. When necessary, deacetylation of the sugar residue was achieved with a catalytic amount of sodium methoxide in methanol at pH 11. Peptides and glycopeptides were purified by reverse-phase high-performance liquid chromatography using a Perkin-Elmer pump system with a UV detector at 230 nm. The column was a Waters Delta Pak C18 (15  $\mu$ , 300Å, 7.8  $\times$  300 mm), and the gradient was performed with water (0.1% trifluoroacetic acid)/acetonitrile over 20 min. The compounds were characterized by amino acid analysis and mass spectrometry. Mass spectra were recorded by electrospray (electrospray mass spectrometry) in the positive mode on a Quattro-LCZ or LCT of mass spectrometer (Micromass, Manchester, United Kingdom). The sample was dissolved at 10 mM concentration in water:acetonitrile (1:1) with 0.1% formic acid. Amino acids and electrospray mass spectrometry mass analyses were conformed to expected products.

**Immunization of Mice.** BALB/c mice (CER Janvier, Le Genest St Ile, France) were i.p. immunized with 1 or 10  $\mu$ g of MAG:Tn3-PV, MAG:Tn(S)3-PV, MAG:Tn(hS)3-PV, or KLH-Tn(c) together with alum (Serva, Heidelberg, Germany) or QS21. KLH-Tn(c) and QS21 were from previous studies (4, 21). For comparative studies between MAG and KLH conjugates, mice received 0.34  $\mu$ g and 1  $\mu$ g of Tn cluster, respectively, per immunization. Immunostimulatory oligonucleotides containing unmethylated CpG motifs were synthesized by Prologo (Paris, France). CpG 1826 was used in mice. CpG 2006, active on human peripheral blood mononuclear cell, was used for primate immunization. The anti-CD40 mAb (FGK45) was prepared from ascitic fluids.

**Immunization of Primates.** The 9 African Green Monkeys, *Chlorocebus sabaeus*, used in this study were all simian immunodeficiency virus and simian

T-lymphotropic virus negatives. Both male and female juveniles (under 3 years of age) and subadults (over 3–4 years) were included. Animals lived in Senegal (West Africa) and were caught from the wild using nets and baits. After a minimal period of 60 days of adaptation to captivity that included observation and veterinary carefulness, animals entered immunization experiments. Animal care operations were in compliance with the regulations detailed under the Guide for the Care and Use of Laboratory Animals. Immunizations were performed s.c. on the back region of monkeys (injections of 0.2 ml/animal/immunization) after an anesthesia with ketamine. Animals received three to four injections of 500  $\mu$ g of MAG:Tn3-TT or control MAP:TT in the presence of 1 mg of aluminum hydroxide with or without 100  $\mu$ g of ODN 2006. Animals were bled before and after each immunization (5–10 ml of blood/animal). On the day of withdrawals, monkeys were examined and weighed.

The 8 adults, *Macaca mulatta*, were imported from China and were housed at the animal house of Rennemoulin (France) in single cages in accordance with the European Community guidelines for animal care. Macaques were injected previously with noninfectious antigenic formulations containing simian immunodeficiency virus Tat and Nef proteins (# 250, 254, 327, and 340) or simian immunodeficiency virus nef only (#279, 328, 332 and 338) and were divided in two groups, respectively. In each group, 1 animal received adjuvant alone (alum plus ODN 2006) and 3 others received adjuvant (alum plus ODN 2006) together with 500  $\mu$ g of MAG:Tn3-TT or MAG:Tn(S)3-PADRE. Immunizations were performed i.m. (4 injections of 0.5 ml/animal/immunization) after an anesthesia. Animals were bled before and at the time of immunization (10 ml of blood/animal). Blood samples were used to analyze sera for anti-Tn antibodies and proliferative response of peripheral blood lymphocyte to TT and PADRE peptides containing T-cell epitopes.

No adverse reaction or local inflammation was noted at the sites of injection, and the weight of the animals did not vary by >10% during the study. By the end of the immunization procedure, all of the animals were healthy and increased in weight.

**Antibody Detection by ELISA and by Fluorescence-Activated Cell Sorting.** Sera were tested as described previously (28) for anti-Tn antibodies by ELISA using biotinylated synthetic Tn cluster glycopeptides (see Table 1) coated on streptavidin plates. aOSM (kindly provided by Dr. Eduardo Osinaga, Facultad de Medicina, Montevideo, Uruguay) and KLH were directly coated on plates. Goat antimouse IgG or goat antihuman IgG peroxidase conjugate (Sigma, St. Louis, MO) was used. Sera from mice and primates were tested by flow cytometry on Tn-expressing human tumor cell lines, Jurkat and MCF-7, and on Tn-negative tumor cells, T2 and MDA231. Binding of antibodies to the cells was revealed with goat antimouse IgG antibody conjugated to FITC or goat antihuman IgG antibody conjugated to phycoerythrin and paraformaldehyde-fixed cells were analyzed on a fluorescence-activated cell sorter. Statistical analysis was performed by a permutation test using the StatXact software (Cytel Software Corporation, Cambridge, MA).

**Competition Assay with Glycosylated MUC1 Peptides.** Sera from green monkeys were tested for recognition of MUC1 peptide sequence in liquid phase using a competition assay. For this purpose, Tn3-G6KG-biot was plated on streptavidin-coated plates and then incubated for 15 min with sera together with serial concentration of nonbiotinylated Tn3-G6KG or MUC1 glycopeptides (competitors). After washing, IgG bound to Tn3-G6KG-biot/streptavidin plates was detected as described above. After this procedure, the concentration of nonbiotinylated Tn3-G6KG and MUC1 glycopeptides giving IC<sub>50</sub> of the signal obtained with the serum alone were determined. For each experiment, IC<sub>50</sub> for all of the MUC1 glycopeptides were normalized with the IC<sub>50</sub> obtained with the nonbiotinylated Tn3-G6KG in the same experiment. Results are expressed as IC<sub>50</sub> (MUC1 glycopeptide)/IC<sub>50</sub> (nonbiotinylated Tn3-G6KG) for each serum and as a mean of value obtained in two to three experiments.

**ADCC Assay.** Sera from primates were tested by a <sup>51</sup>Cr release assay for their capacity to mediate ADCC of tumor cells performed by a human natural killer (NK) cell clone (kindly given by Dr. Fathia Mami-Chouaib, IGR, Villejuif, France) as effector cell. For the cytotoxic assay, tumor-target cells were labeled with <sup>51</sup>Cr, then incubated with serum for 20 min. at 4°C, washed twice, and plated at 10<sup>4</sup> cells/well. NK cell clone cells were added for 4 h at various E:T ratios. The percentage of specific lysis was calculated as 100  $\times$  (experimental release – spontaneous release)/(maximal release – spontaneous release).

Table 1 List of compounds used in this study

Compound <sup>a,b,c</sup>	Peptidic backbone		Glycosidic moiety				
	Structure	Sequence <sup>b</sup>	Tn structure	Tn copies	Use	Host	Reference
MAG:Tn3	Dendrimer	-	S*T*T*	12	Vacc.	Mouse	This study
MAG:Tn3-PV	Dendrimer	PV	S*T*T*	12	Vacc.	Mouse	(1)
MAG:Tn(S)3-PV	Dendrimer	PV	S*S*S*	12	Vacc.	Mouse	This study
MAG:Tn(hS)3-PV	Dendrimer	PV	hS*hS*hS*	12	Vacc.	Mouse	(2)
MAP-TT	Dendrimer	TT	-	0	Vacc.	Primate	This study
MAG:Tn3-TT	Dendrimer	TT	S*T*T*	12	Vacc.	Primate	This study
MAG:Tn(S)3-PADRE	Dendrimer	PADRE	S*S*S*	12	Vacc.	Primate	This study
KLH-Tn(c)	Linear/protein	KLH	cluster	951	Vacc.	Mouse	(3)
Tn3-G6K(biot)G	Linear	Poly-gly	S*T*T*	3	ELISA	-	(4)
Tn(S)3-G6K(biot)G	Linear	Poly-gly	S*S*S*	3	ELISA	-	(5)
Tn(hS)3-G6K(biot)G	Linear	Poly-gly	hS*hS*hS*	3	ELISA	-	This study
Tn(T)3-G6K(biot)G	Linear	Poly-gly	T*T*T*	3	ELISA	-	This study
SLS*YT*NPAV	Linear	MUC1	S* and T*	2	ELISA	-	This study
ALGS*TT*PPA	Linear	MUC1	S* and T*	2	ELISA	-	This study
ALGS*T*TPPA	Linear	MUC1	S*T*	2	ELISA	-	This study
ALGS*T*T*PPA	Linear	MUC1	S*T*T*	3	ELISA	-	This study
S*T*APPAHG	Linear	MUC1	S*T*	1	ELISA	-	This study

<sup>a</sup> MAP and MAG refer to dendrimeric compounds based on the (Lys)<sub>2</sub>-Lys-β-Ala core; Tn3-G6K(biot)G, Tn(S)3-G6K(biot)G, Tn(T)3-G6K(biot)G, and Tn(hS)3-G6K(biot)G were abbreviated for simplicity as Tn3, Tn(S)3, Tn(T)3, and Tn(hS)3, respectively.

<sup>b</sup> T-helper peptide sequences: PV = KLFVAVKITYKDT is restricted by H-2<sup>d</sup>; TT = QYIKANSKFIGITEL is restricted by HLA-DR1, DR3, DR5, DR7, and DRw52; PADRE = AKXVAAWTLKAAA (X = cyclohexylalanine) is restricted by HLA-DR1, DR4w4, DR4w14, DR5, and DR2w2a.

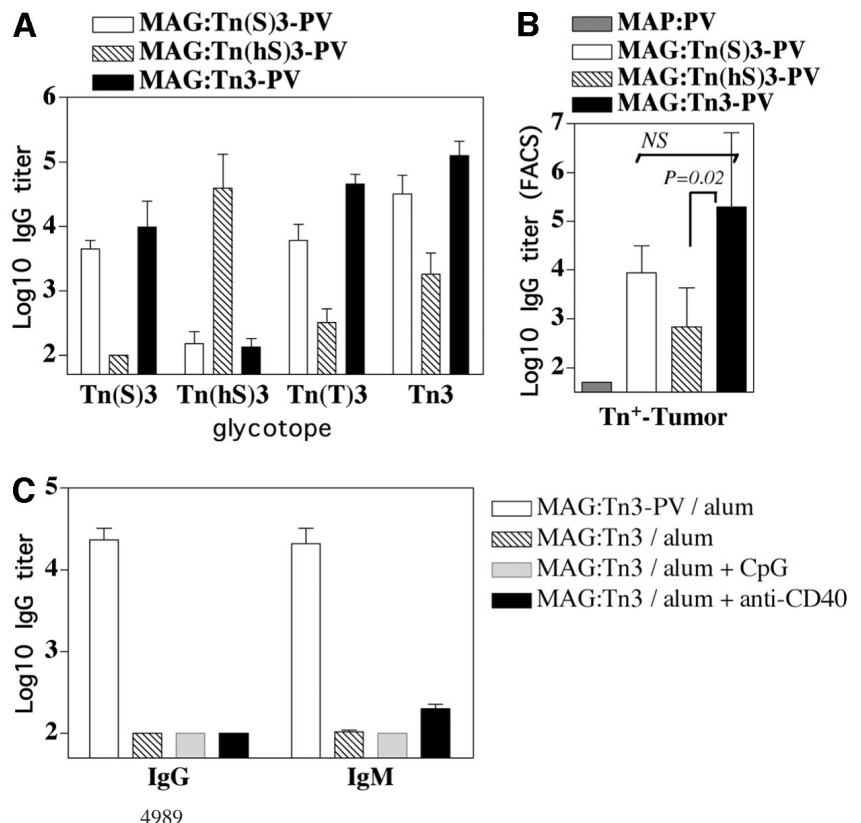
<sup>c</sup> \*: α-DGalNAc; biot, biotine; hS, homoserine.

## RESULTS

**Influence of the Aglyconic Backbone on the Tn Glycotope Recognition.** Haptenic Tn, T, and sialyl-Tn molecules require to be associated as clusters of at least two to three units to mimic native forms found on mucins (16–18). However, thus far the influence of amino acids (Ser or Thr) carrying the α-D-GalNAc within the cluster has never been considered. Therefore, we first investigated the role of the Tn aglyconic backbone for antibody recognition. We immunized mice with three different MAG, MAG:Tn3-PV, MAG:Tn(S)3-PV, and MAG:Tn(hS)3-PV (Table 1), and we analyzed immune sera for the recognition of their homologous synthetic Tn cluster, Tn3, Tn(S)3, and Tn(hS)3. In these condi-

tions, high anti-Tn IgG antibody titers were found in all groups of mice (Fig. 1A). When sera induced by MAG:Tn3-PV were analyzed for the recognition of different heterologous Tn clusters (Table 1), these antibodies also recognized Tn(S)3 and Tn(T)3 motifs but not the non-natural Tn(hS)3 cluster. The same was true for antibodies induced by the MAG:Tn(S)3-PV. In contrast, antibodies induced by MAG:Tn(hS)3-PV recognized efficiently the synthetic Tn(hS)3 cluster, but poorly Tn3, and not Tn(S)3 and Tn(T)3, demonstrating a highly reduced cross-recognition of the GalNAc moiety for non-natural amino acids. In addition to these data, we found that among the Tn-specific monoclonal IgG antibodies we produced, some recognized the synthetic Tn(S)3 but not

Fig. 1. Influence of the glycosidic display on the generation of antibodies that recognize the native form of Tn. A, mice ( $n = 5$ ) were immunized three times with 10 μg of multiple antigenic glycopeptide (MAG):Tn(S)3-PV, MAG:Tn(hS)3-PV, and MAG:Tn3-PV in alum, and sera collected after the last boost were tested for anti-Tn IgG antibodies by ELISA using synthetic Tn(S)3, Tn(hS)3, Tn(T)3, or Tn3 as indicated. B, the same sera were tested for native Tn recognition by fluorescence-activated cell sorter using Tn-positive human tumor cells (Jurkat). Statistical analysis is indicated by the  $P$  or by  $NS$  for nonsignificant statistical difference. C, mice ( $n = 4$ ) were immunized twice, on days 0 and 21, with 10 μg of MAG:Tn3-PV or MAG:Tn3 in alum alone or together with 50 μg of CpG or with  $3 \times 100$  μg of anti-CD40 monoclonal antibody. Sera collected at day 28 were analyzed for anti-Tn IgG and IgM antibodies by ELISA using synthetic Tn3; bars, ±SD.



other Tn clusters based on a different backbone.<sup>6</sup> To additionally evaluate this phenomenon, we compared the capacity of the antibodies induced by the different MAG immunogens to recognize the “native form of Tn” on tumor cells by flow cytometry (Fig. 1B). No significant difference was found for the recognition of the Tn-positive Jurkat cell by antibodies elicited by MAG:Tn3-PV or with MAG:Tn(S)3-PV, whereas MAG:Tn(hS)3-PV induced antibodies displayed low reactivity for native Tn on Jurkat (Fig. 1B). Altogether, these results show that the Tn antigen/antibody interaction does not involve the sole carbohydrate moiety even when it is displayed as a cluster. However, as long as immunogens are built by *O*-glycosylation of natural serine and threonine, there exists sufficient diversity of the polyclonal IgG response to accommodate the large variability of mucin sequences and to allow the efficient recognition of the native forms of the Tn antigen.

**T-Cell Help Requirement to Induce Anti-Tn Antibodies with MAG Glycoconjugate.** We showed previously that *in vivo* depletion of the CD4<sup>+</sup> T-cell compartment abrogates the induction of anti-Tn antibodies and the protection afforded by the MAG:Tn3-PV against the growth of Tn-expressing tumor in mice (23). We sought to determine whether we could bypass the requirement of T-cell help to induce anticarbohydrate antibodies by directly activating B cells. Such direct activation could be achieved by the cross-linking of membrane immunoglobulin by repetitive carbohydrate units (such as those found in bacterial polysaccharides) and/or by toll-like receptor or CD40 triggering. For this purpose, a MAG:Tn3 containing four Tn3 clusters devoided of peptide T-cell sequence was synthesized (Table 1). As shown in Fig. 1C, the multimeric MAG:Tn3 administered in alum did not induce any anti-Tn antibody responses. Neither TLR9 triggering by coinjecting CpG nor an anti-CD40 monoclonal antibody treatment together with MAG:Tn3 immunization led to the induction of anti-Tn antibodies (Fig. 1C) demonstrating the absolute requirement of a CD4<sup>+</sup> T-cell help to induce antibodies against the monosaccharidic Tn antigen.

**Comparison of KLH and MAG Glycoconjugates.** Several KLH glycoconjugates developed with tumor-associated carbohydrates have entered in Phase II/III clinical trials (4, 14). These glycoconjugates were more or less efficient in inducing IgG antibodies required for ADCC. Therefore, we next performed a comparative study in mice of the immunogenicity of MAG:Tn3-PV *versus* its KLH-Tn(c) counterpart using different adjuvant settings. In contrast to the MAG, when KLH-Tn(c) was administered in alum, strong anti-KLH antibodies were induced but no anti-Tn IgG as tested by ELISA (Fig. 2A). In both cases, IgM antibodies specific for Tn were induced (Fig. 2B). We next compared KLH and MAG glycoconjugates injected with a stronger adjuvant such as QS21 (Fig. 2A). In these conditions, both KLH and MAG Tn-conjugates elicited anti-Tn IgG antibodies regardless of the synthetic Tn cluster used for detection. The nonglycosylated backbone was not recognized by any of the mouse sera (data not shown). Anti-Tn titers were significantly higher in the case of MAG and were maintained for >3 months after the last boost, indicating an induction of long-lived plasma cells (Fig. 2C).

When we tested sera from KLH-Tn(c) and MAG:Tn3-PV in QS21 immunized mice for Jurkat cells recognition, again the antibody titers induced by the MAG compound were significantly superior to those induced by the KLH glycoconjugate (Fig. 2D). However, no significant difference was observed when the Tn-positive MCF7 cells were used as a source of native Tn antigen. KLH naturally displays the glycosidic T antigen [Gal(β1-3)-GalNAc; Ref. 33] and, therefore, the reactivity of sera from animals immunized with KLH-Tn(c) most likely reflects the recognition of both Tn and T antigens on MCF7, whereas MAG:Tn3-PV induced antibodies are specifically directed against Tn. In contrast, Jurkat

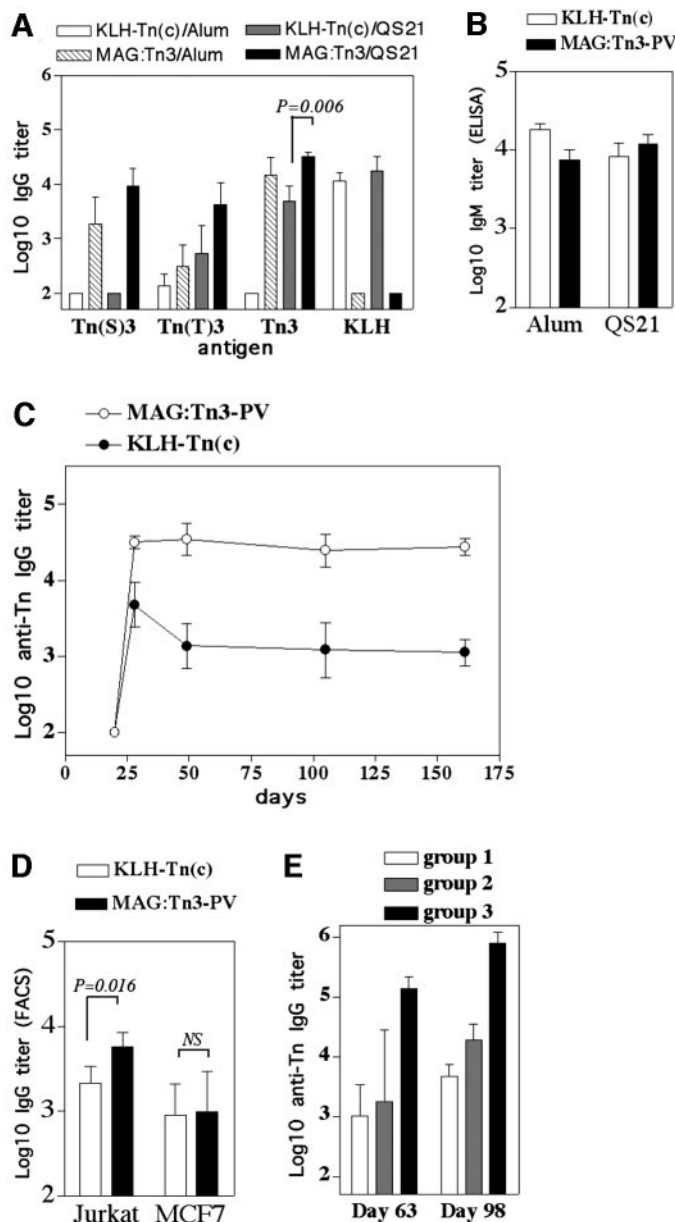


Fig. 2. Superior efficacy of multiple antigenic glycopeptide (MAG) over keyhole limpet hemocyanin (KLH) glycoconjugate in its ability to induce anti-Tn IgG antibodies. (A–D) BALB/c mice were immunized on days 0, 21, and 42 with KLH-Tn(c) (15.6  $\mu$ g, *i.e.*, 1  $\mu$ g of Tn cluster) or MAG:Tn3-PV (1  $\mu$ g, *i.e.*, 0.34  $\mu$ g of Tn cluster) mixed with alum (A and B) or QS21 (A–D). A and B, sera were collected 1 week after the second (QS21 group;  $n = 9$ ) or the third (alum group;  $n = 5$ ) immunization and tested by ELISA as indicated for anti-Tn3 or anti-KLH IgG antibodies (A) or anti-Tn3 IgM (B). C, sera from mice ( $n = 9$ ) immunized with indicated glycoconjugate in QS21 and collected at different time points were tested for anti-Tn antibodies using synthetic Tn3. D, sera from mice immunized with glycoconjugates in QS21 were tested for native Tn recognition by fluorescence-activated cell sorter using the Tn-positive Jurkat and MCF7 tumors. E, on days 0, 7, 14, and 42, mice ( $n = 3$ ) were immunized with 1  $\mu$ g of KLH-Tn(c); groups 1 and 2) or 1  $\mu$ g of MAG:Tn3-PV (group 3) mixed with QS21. On day 70, mice were recalled with KLH-Tn(c); group 1) or MAG:Tn3-PV (groups 2 and 3). Sera collected before (day 63) or after (day 98) the last recall were tested by ELISA for anti-Tn IgG against synthetic Tn3. Statistical analysis is indicated by the *P* or by *NS* for nonsignificant statistical difference; bars,  $\pm$ SD.

cells do not display the T antigen at the cell membrane (34) due to a lack of  $\beta$ 1-3 galactosyl-transferase expression (35).

Finally, we analyzed whether the B-cell response induced by the KLH glycoconjugate could be recalled and be additionally increased by the MAG. After three injections of KLH-Tn(c) in QS21, mice were recalled with MAG:Tn3-PV or KLH-Tn(c). In these conditions, no memory

<sup>6</sup> Unpublished observations.

response was recalled by the KLH or the MAG glycoconjugates boosting injection (Fig. 2E). In contrast, after the same schedule, MAG:Tn3-PV could additionally recall the anti-Tn IgG response induced by previous MAG:Tn3-PV immunizations. Altogether, these data clearly show the qualitative and quantitative superior efficacy of MAG:Tn3-PV over KLH-Tn(c) in inducing an anti-Tn immune response.

**Induction of Tn-Specific Antibodies by “Humanized” MAG in Nonhuman Primates.** We next designed MAG for human vaccination by introducing either a TT (36) or a PADRE (37) peptide that allows a broad coverage of HLA diversity (Table 1). We showed recently that linear Tn(S)6-PADRE induced anti-Tn antibodies in HLA-DR1 and -DR4 transgenic mice (38). Likewise, the new dendrimeric MAG, MAG:Tn(S)3-PADRE, and MAG:Tn3-TT (Table 1), also induced anti-Tn antibodies in these HLA transgenic mice (data not shown). Therefore, we next evaluated the potency of these MAG in two nonhuman primate species. In a first experiment, 2 groups of 3 macaques were immunized with MAG:Tn(S)3-PADRE and MAG:Tn3-TT with alum and CpG oligonucleotide as adjuvants. Control animals received the adjuvant preparation alone. IgG antibodies specific to Tn were detected by ELISA in all of the animals immunized with MAG but not in controls (Fig. 3, A and B). We also assessed the peripheral blood lymphocyte proliferative response to CD4<sup>+</sup> T-cell

peptides in vaccinated macaques after the last boost. TT-specific T-cell responses were detected in all of the animals vaccinated with MAG:Tn3-TT. PADRE-specific T-cell responses were found in 2 of 3 animals vaccinated with MAG:Tn(S)3-PADRE, although of lower intensity as compared with the TT responses (Fig. 3C).

Next, we carried an experiment with the MAG:Tn3-TT in green monkeys, to additionally evaluate the immunogenicity of this MAG when administered with alum alone or with CpG ODN. Control groups consisted of dendrimeric MAP:TT devoid of Tn residues. Anti-Tn IgG titers were already detectable after the first immunization (Fig. 3D). When green monkeys were immunized with MAG:Tn3-TT with alum as the sole adjuvant, 2 animals developed anti-Tn IgG after two immunizations and the third animal required a total of four injections to develop anti-Tn antibodies (Fig. 3E).

Finally, we tested the ability of sera from MAG vaccinated monkeys to bind mucin derived structures. Postimmune sera from all of the macaques and green monkeys vaccinated with MAG:Tn3-TT together with alum plus CpG ODN were able to recognize aOSM (Fig. 3F). Sera from control animals did not react with aOSM. To ensure that antibodies induced in primates will recognize human mucins, we designed several glycopeptides from human MUC1 mucin repeats. As shown in Table 2, three different MUC1 mucin sequences *O*-glyco-

Fig. 3. Immunogenicity of multiple antigenic glycopeptide (MAG) in different nonhuman primate species. A–C, on days 0, 21, and 42, macaques were immunized with MAG:Tn(S)3-PADRE or MAG:Tn3-TT mixed with alum and CpG-ODN as adjuvant or with adjuvant alone. D and E, on days 0, 21, and 42, green monkeys were immunized with MAG:Tn3-TT or nonglycosylated MAP:TT control mixed with alum together with (D) or without (E) CpG-ODN as indicated. A, B, D, and E, at the indicated time points, sera were collected and tested for anti-Tn reactivity against the synthetic homologue, Tn3 or Tn(S)3. C, peripheral blood lymphocyte from macaques collected at day 60 were stimulated for 4 days with the TT or the PADRE peptide, and cell proliferation was measured by [<sup>3</sup>H]thymidine incorporation over the last 16-h. For each monkey, results are expressed as stimulation index (SI) corresponding to the number of cpm obtained with cells incubated with the peptide divided by the number of cpm obtained with cells incubated in medium alone. F, sera from macaques or green monkeys collected just after the last immunization were tested for anti-aOSM reactivity by ELISA.

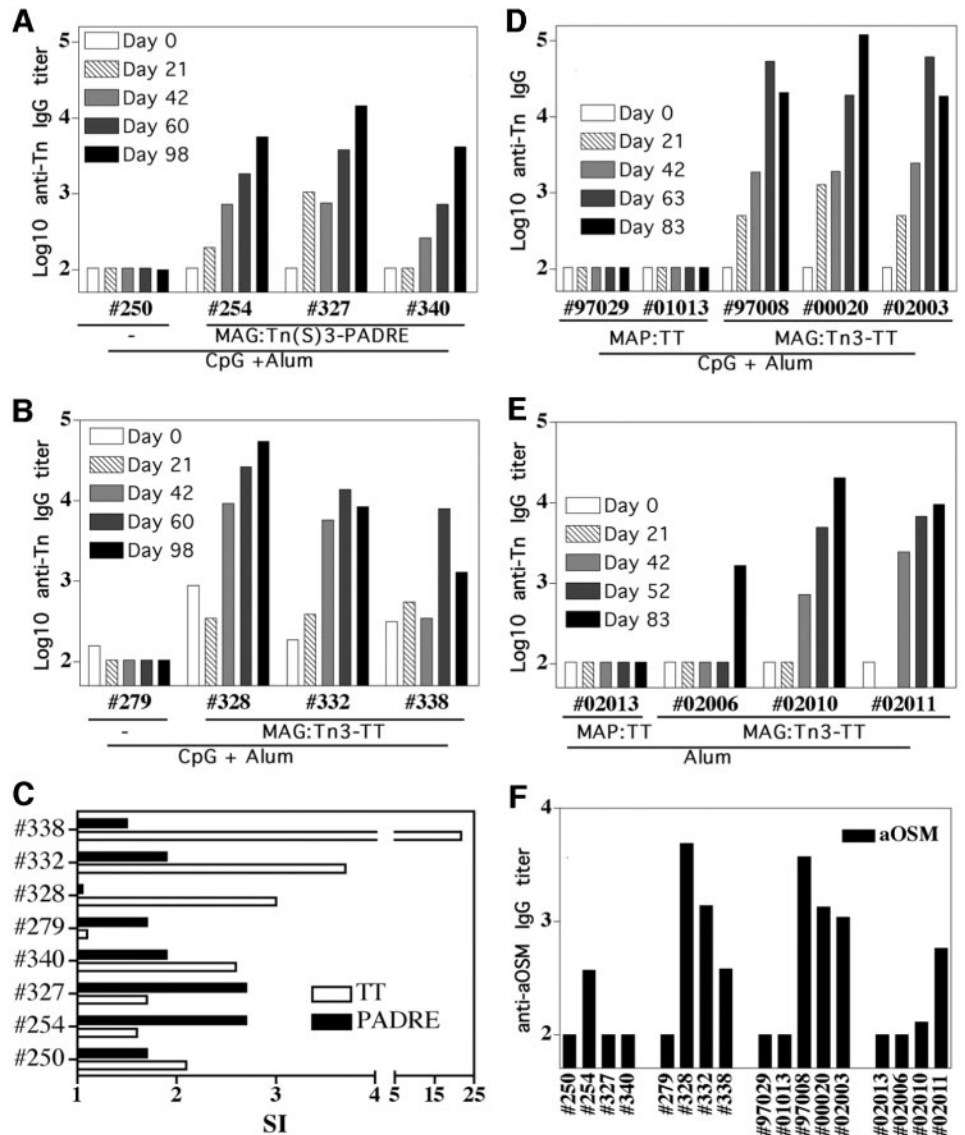


Table 2 Analysis of the recognition of glycosylated mucin-derived peptides by sera from green monkeys

Competitor <sup>a</sup>	Green monkey <sup>b</sup>					
	#97008	#00020	#02003	#02006	#02010	#02011
S*T*T*G6KG	1	1	1 <sub>c</sub>	1	1	1
STAPPAHG	-	-	-	-	-	-
S*T*APPAHG	1.3	0.8	2.2	0.6	0.9	1.5
ALGSTTPA	-	-	-	-	-	-
ALGS*TT*PPA	86.6	275.1	45.5	51.6	16.6	70
ALGS*T*TPA	169	446.5	37.8	37.3	15.9	46.1
ALGS*T*T*PPA	77.5	458.1	34.1	29.9	12.9	31.6
SLSYTNPAV	-	-	-	-	-	-
SLS*YT*NPAV	96.8	233.6	37.5	80.8	30.2	102.7

<sup>a</sup> Peptides or glycopeptides were tested in an inhibition assay to compete with a reference glycopeptide (S\*T\*T\*G6KG) for the binding to each serum (see "Materials and Methods"). The IC<sub>50</sub> value was determined for each competitor and normalized with the IC<sub>50</sub> obtained for the reference glycopeptide to compete with itself. Results are expressed as the mean of two to three experiments.

<sup>b</sup> Sera from green monkeys (see legend Fig. 3) were tested for the recognition of glycosylated or nonglycosylated human MUC1 mucin peptides (\* indicates  $\alpha$ -DGalNAc).

<sup>c</sup> Because no inhibition was obtained with nonglycosylated peptides, IC<sub>50</sub> and ratio could not be calculated (- indicates the lack of inhibition).

sylated with GalNAc residues were positively recognized by sera from green monkeys immunized with MAG:Tn3-TT. Sera recognized Tn when located in the middle of the MUC1 peptide backbone, although less efficiently than the Tn located at the edge of the peptidic chain, showing the capability of MAG-induced antibodies to recognize carcinoma-associated forms of human mucins.

**Recognition and Killing of Human Tumor Cells by Antibodies from Vaccinated Monkey.** To evaluate the antitumor therapeutic potential of antibodies elicited in primates, we assessed their ability to

specifically recognize human tumor cells expressing Tn by flow cytometry. When preimmune sera from green monkeys were analyzed for Tn-positive tumor cell recognition, almost no background reactivity was found for the Jurkat T lymphoma (Fig. 4, A and B), and a low reactivity was found for the MCF7 mammary adenocarcinoma (Fig. 4C). Likewise, postimmunization sera from MAP:TT vaccinated controls did not show any increase of reactivity as compared with preimmune sera. In contrast, after MAG:Tn3-TT vaccination, a strong and specific recognition of Jurkat cells was found (Fig. 4, A and B). MCF7 was also specifically recognized by these postimmune sera (Fig. 4C) but to a lesser extent as compared with Jurkat, which may reflect the level of Tn expression on these two cell types. Positive reactivity of immune sera was also found with other human Tn-expressing tumor cell lines, the T47D breast carcinoma and the LS180 and LSC colon carcinomas (Fig. 4D). Tn-negative T2 thymoma and MDA231 adenocarcinoma did not show any difference of reactivity between pre- and postimmune sera fully confirming the Tn-specificity of antibodies elicited after MAG:Tn3-TT vaccination (Fig. 4D). These results show the high specificity of anti-Tn antibodies induced by the MAG:Tn3-TT and their capacity to recognize the native Tn antigen on tumor cells.

We then analyzed the ability of sera from green monkeys to mediate killing of Tn-positive human tumor cells performed by human NK cells. As shown in Fig. 5, NK cells showed cytotoxicity against Jurkat cells coated with postimmune, but not with preimmune, sera from 5 of 6 animals vaccinated with MAG:Tn3-TT. In contrast, pre- and postimmune sera from the 3 animals immunized with MAP:TT devoid of Tn antigen did not mediate any specific killing of Jurkat cells. Because a direct killing of MCF7 cells by NK

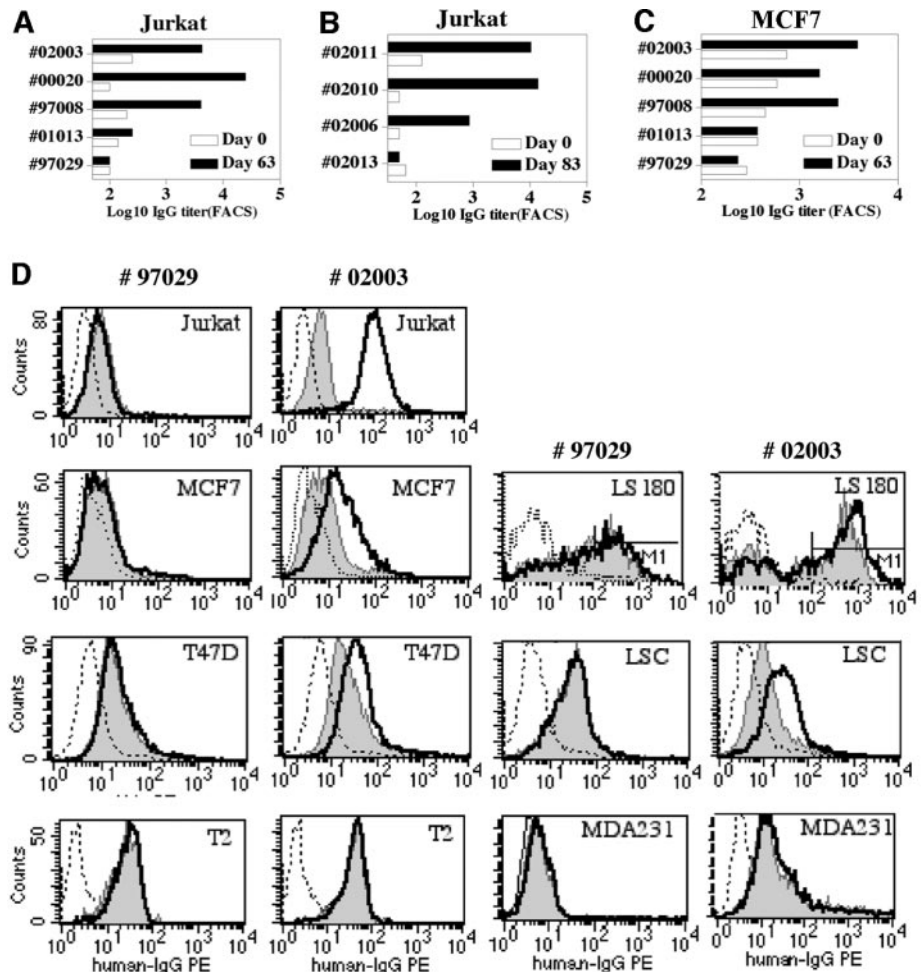


Fig. 4. Multiple antigenic glycopeptide-induced primate antibodies recognize human tumor cells expressing Tn. A–C, preimmune and postimmune sera obtained from the indicated green monkeys after immunization protocol detailed in Fig. 3 were analyzed for antibody titer against Tn-positive Jurkat (A and B) and MCF7 (C) cells. D, fluorescence-activated cell sorting (FACS) histograms for cells stained with secondary reagent alone (----), with preimmune sera (gray histograms), or postimmunization sera (bold line) collected from indicated green monkeys and tested against Tn-positive [Jurkat T lymphoma; MCF7 and T47D mammary adenocarcinomas; LS180 and LSC colon carcinomas or Tn-negative tumor cell lines (T2 and MDA231)]. For LS180, the percentage of positive cells (gated in M1) observed for pre and postimmune sera is 50% and 52%, respectively, for #97029 and 55% and 75%, respectively, for #02003.

cells was observed in the absence of any antibody (data not shown), it was not possible to assess ADCC against this tumor cell line. Altogether, these results show that the MAG is capable to induce antibodies specific for Tn on human tumor cells and to mediate killing of these cells via an ADCC mechanism.

## DISCUSSION

In the present study, we elaborated a candidate vaccine targeting the Tn tumor-associated carbohydrate tumor antigen for immunotherapy of carcinoma cancer. We designed a suitable glycoepitope cluster containing three  $\alpha$ -GalNAc on a STT backbone, and we show that the MAG has superior immunogenic potential over a KLH-Tn glycoconjugate. We also show that the MAG designed for human therapy is able to induce anti-Tn IgG antibodies in two nonhuman primate species that can mediate ADCC against human tumor cells.

The Tn antigen displayed on carcinoma-associated human mucins is preferentially displayed as a cluster of several Tn (16–18), and this configuration is optimal for recognition of Tn by IgG antibodies to mediate ADCC against cancer cells. We showed previously that a cluster of three Tn is very efficient in stimulating anti-Tn antibodies capable of recognizing native tumor forms of Tn and eradicating Tn-expressing tumors in mice (23). The cluster we introduced in the MAG is a tri-Tn cluster (three  $\alpha$ -GalNAc on a STT backbone) corresponding to a glycoepitope recognized by the MLS128 monoclonal antibody that has been obtained after immunizing mice with the human carcinoma cell line LS180 (39, 40). Amino acids flanking the Tn antigen have been shown to modulate antibody recognition (41, 42), but nothing is known about the contribution of the aglyconic part of the Tn structure (Ser or Thr residues) for antibody binding. To design the most suitable vaccine candidate for targeting immune responses to cancer cells, we first investigated the influence of the amino acid backbone displaying the GalNAc residue. We show for the first time that antibody recognition of the  $\alpha$ -GalNAc moiety of Tn is influenced by the aglyconic part of the Tn structure. Indeed, Tn displayed on a non-natural homoserine residue was not the most appropriate to induce anti-Tn antibodies that recognize the native form of Tn. Likewise, antibodies induced by Tn clusters based on natural amino acids (Ser or Thr) failed to efficiently recognize the GalNAc residue display on a homoserine backbone. The influence of the amino acid backbone was less sensitive when *O*-GalNAc residues were displayed by natural amino acids for polyclonal antibodies. However, among Tn-specific monoclonal antibodies we produced, we found some exclusive fine specificity for Ser or Thr. It remains that the diversity of the polyclonal response allows a clear cross-reactivity between anti-Tn antibodies raised by *O*-GalNAc residues carried by a SSS, a STT, or a TTT backbone for heterologous backbones. The STT backbone was found to be the most permissive for induction of anti-Tn antibodies that recognize degenerated Tn clusters. These anti-Tn antibodies recognize a large variety of GalNAc glycosylated MUC-1 peptide sequences allowing a broad spectrum of recognition for native forms of Tn on cancer cells. Antibodies induced by a Tn3 cluster on a STT backbone can efficiently recognize a large variety of MUC-1 peptide sequences with different levels of GalNAc glycosylation. Given the large heterogeneity of mucin sequences, it is critical to build immunogens capable to induce anti-Tn antibodies with a broad spectrum of recognition for native forms of Tn.

The second important point highlighted by the present study is that the full synthetic MAG:Tn immunogen is able to induce anti-Tn IgG antibodies with a mild adjuvant setting (alum), whereas the KLH-Tn conjugate requires the use of a much powerful adjuvantation, such as QS21. Likewise, glycolipopeptides based on dimeric or trimeric Tn cluster induced IgM, but no or low IgG anti-Tn antibodies (21, 29). In contrast,

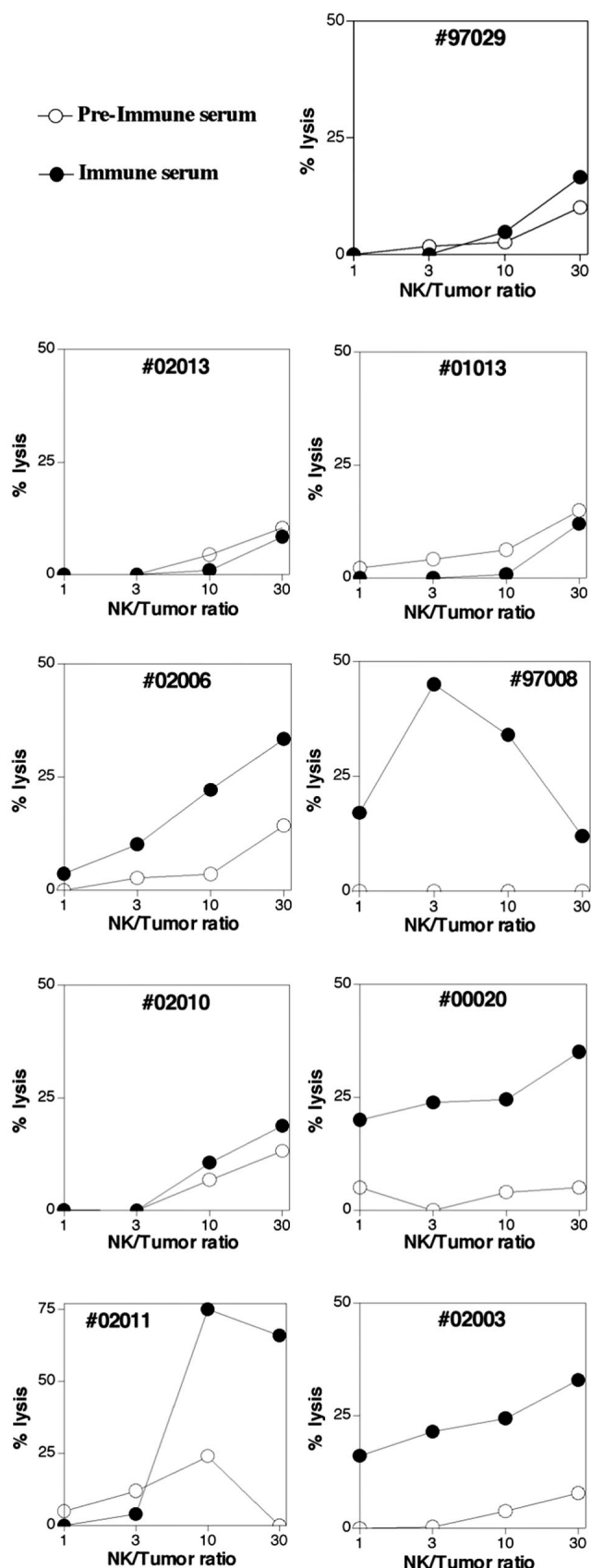


Fig. 5. Multiple antigenic glycopeptide-induced primate antibodies are able to mediate antibody-dependent cellular cytotoxicity against Tn-positive tumor cells. [<sup>51</sup>Cr]-labeled Jurkat cells were incubated with pre- or postimmune sera from green monkeys injected with MAG:Tn3-TT or control MAP:TT as detailed in Fig. 3. These target cells were subjected to lysis by the natural killer cell clone at the indicated natural killer (NK):tumor ratio for 4 h.

the MAG:Tn3-PV mixed with a lipopeptide adjuvant induced strong anti-Tn IgG antibodies (data not shown). Therefore, the MAG system offers the possibility to induce a strong antitumoral immune response with a well-known, commonly used and harmless adjuvant.

The last important point provided by the present study is that two different MAG designed for human therapy by introducing promiscuous T-helper HLA-DR binding peptides are able to induce anti-Tn antibodies in two nonhuman primate species. The two different CD4 T-cell peptides, PADRE and TT, introduced into the MAG were capable to provide help for anti-Tn antibody production in all of the immunized primates. Again, these antibodies could be induced with a mild adjuvant setting (alum), although the addition of CpG oligonucleotides strongly improved the immunogenicity of the MAG by eliciting a quantitatively higher and more rapid response. Importantly, in all of the experimental settings, no adverse reaction was observed in any animal such as local inflammation at the sites of injection or weight loss assessing the safety of the MAG. Tn-specific antibodies elicited in these nonhuman primates were able to recognize glycosylated human mucin sequences as well as Tn-positive human tumor cells. Antibodies specific for tumor-associated antigens are able to mediate tumor cell killing by complement-dependent cytotoxicity or by ADCC. Importantly, these antibodies in the presence of human NK cells could mediate ADCC against tumor cells demonstrating their antitumoral potency. In conclusion, we have designed and validated a fully synthetic vaccine targeting the carbohydrate Tn tumor antigen for immunotherapeutic purposes in humans, opening the way for a new generation of vaccines based on fully synthetic glycopeptides.

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## A Fully Synthetic Therapeutic Vaccine Candidate Targeting Carcinoma-Associated Tn Carbohydrate Antigen Induces Tumor-Specific Antibodies in Nonhuman Primates

Richard Lo-Man, Sophie Vichier-Guerre, Ronald Perraut, et al.

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