The Journal of Experimental Medicine

Designer Glycopeptides for Cytotoxic T Cell–based Elimination of Carcinomas

Yanfei Xu,[1] Sandra J. Gendler,[2] and Alessandra Franco[1]

[1]*Torrey Pines Institute for Molecular Studies, San Diego, CA 92121*
[2]*Mayo Clinic Scottsdale, Scottsdale, AZ 85259*

Abstract

Tumors express embryonic carbohydrate antigens called tumor-associated carbohydrate antigens (TACA). TACA-containing glycopeptides are appealing cytotoxic T cell (CTL)-based vaccines to prevent or treat cancer because the same sugar moieties are expressed in a variety of tumors, rendering a vaccination strategy applicable in a large population. Here we demonstrate that by using glycopeptides with high affinity for the major histocompatibility complex and glycosylated in a position corresponding to a critical T cell receptor (TcR) contact, it is possible to induce anti-TACA CTL in vivo. In the current study we show that designer glycopeptides containing the Thomsen-Freidenreich (TF) antigen (β-Gal-[1→3]-α-GalNAc-*O*-serine) are immunogenic in vivo and generate TF-specific CTL capable of recognizing a variety of tumor cells in vitro including a MUC1-expressing tumor. The fine specificity of the TF-specific CTL repertoire indicates that the TcR recognize the glycosylated amino acid residue together with TF in a conventional major histocompatibility complex class I–restricted fashion. These results have high potential for immunotherapy against a broad range of tumors.

Key words: tumor-associated carbohydrate antigens • glycopeptides • CTL • carcinomas • immunotherapy

Introduction

A group of tumor-associated antigens that have been identified and characterized by virtue of their reactivity with antibodies and lectins are carbohydrate in nature and called tumor-associated carbohydrate antigens (TACA; 1). Numerous studies indicate that some TACA are expressed on a variety of neoplastic tissues (2–6) and not normally expressed in differentiated cells, suggesting their suitability as a candidate for vaccine therapy.

The crystal structure of human and murine T cell receptors (TcRs) specific for immunodominant viral peptides presented to cytotoxic T cell (CTL) suggests that the central region of the peptide is critical for TcR contacts and that aromatic rings are accommodated in a small cavity determined by the variable CDR3 region of the TcR (7, 8). These data suggest that small carbohydrate moieties may "fit" in the variable region of the TcR. In fact, our preliminary studies using trinitrophenyl (TNP) as a hapten model, suggested that K^b-restricted peptides haptenated in position 4 in 8 mers or position 5 in 9 mers induced a TNP-specific CTL repertoire whose TcR was poorly dependent on MHC class I contacts and highly degenerate (9). Because of these encouraging results, we applied the same antigenic strategy to TACA-containing glycopeptides because induction of a degenerate T cell response against this class of tumor antigens may be of great advantage in cancer immunotherapy (10–12).

The TACA-based vaccine candidates that we selected to generate glycopeptides are the Thomsen-Freidenreich (TF) antigen (β-Gal-[1→3]-α-GalNAc-*O*-serine) and its immediate precursor Tn (GalNAc-*O*-serine; reference 13). TF is expressed in different human carcinomas as a result of incomplete or aberrant glycosylation (14–18). In a systematic and comparative immunohistochemical study it was shown that the expression of TF and Tn was, with rare exceptions, only found on neoplastic cells and not on normal tissue (19). The importance of the TF antigen expression in carcinomas was also demonstrated by TF-specific antibody responses in cancer patients (20). In fact, TF-specific mAbs have been generated for prognostic and therapeutic applications (21–23).

The TF and Tn carbohydrate are also attractive candidates for targeting immunotherapy against mucin (24), a

Address correspondence to Alessandra Franco, Torrey Pines Institute for Molecular Studies, 3550 General Atomic Court, San Diego, CA 92121. Phone: (858) 455-3885; Fax: (858) 909-5117; email: afranco@tpims.org

Abbreviations used in this paper: CTL, cytotoxic T cell; hs, homoserine; TACA, tumor-associated carbohydrate antigens; TcR, T cell receptor; TF, Thomsen-Freidenreich; TNP, trinitrophenyl.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/2004/03/707/10 $8.00
Volume 199, Number 5, March 1, 2004 707–716
http://www.jem.org/cgi/doi/10.1084/jem.20031865

polymorphic glycoprotein expressed by many epithelial-derived carcinomas. Although interest has focused on development of subunit vaccines against the core peptide unit of mucin (25), the carbohydrate molecules associated with mucin, which included TF and Tn (26), could also be considered for glycopeptides vaccine development. TF and Tn represent neo antigens for T cell surveillance, as was shown previously for humoral immunity against mucin (27).

In this paper we demonstrate that glycopeptides containing small TACA, a disaccharide and a monosaccharide linked to a major TcR contact residue, and having high binding affinity for MHC class I molecules, can serve as vaccines that induce a degenerate carbohydrate-specific CTL repertoire. Furthermore, our study also shows that carbohydrate-specific CTL clones can recognize tumor cells of different origin that express TACA in the context of endogenous glycopeptides of unknown sequences. These findings support the value of the carbohydrate vaccine approach for cancer prevention and treatment.

Materials and Methods

Mice. Female C57BL/6 mice (6–12 wk old) were purchased from The Jackson ImmunoResearch Laboratories.

Peptides and Glycopeptides. The peptides used in this study were synthesized by Fmoc chemistry using a multiple peptide synthesizer (Symphony/Multiplex; Protein Technologies). Peptides were cleaved automatically on the synthesizer using trifluoracetic acid as a cleavage reagent. Peptides were ≥97% pure as assessed by C18 reverse phase high performance liquid chromatography, and the identity of the peptides was verified by mass spectroscopy. Designer alanine-rich peptides with the appropriate MHC anchor and a natural sequence from the Sendai virus NP 324–332 (FAPGNYPAL), modified in position 5 (N→S), were used for carbohydrate conjugation. The designer peptide is a 9-mer that contained the major anchor residues phenylalanine (F) in position 6 and leucine (L) in position 9, together with isoleucine (I) at positions 2 and 3, which we defined to be important for binding to K^b molecules. The glycopeptides containing TF (β-Gal-[1→3]-α-GalNAc-*O*-serine) and Tn (GalNAc-*O*-serine) used in this study were prepared at Carlsberg Laboratories by solid phase synthesis using glycosylated amino acids as building blocks, as described previously (28). Glycopeptides were ≥97% pure as assessed by C18 reverse phase high performance liquid chromatography, and the identity of the peptides was verified by mass spectroscopy.

MHC Binding Assay. EL-4 cells were used as a source of K^b molecules. Nonidet P-40 cell lysates from large scale (10^9–10^{11}) cell cultures were filtered through 0.45-μm filters and purified by affinity chromatography. To measure peptide binding to MHC molecules, MHC binding peptides identified previously were ^{125}I radiolabeled and incubated with 5–10 nM of purified MHC molecules for 48 h in PBS containing 0.05% Nonidet P-40 and protease inhibitors. The K^b binding complexes were subsequently separated from free peptide by gel filtration TSK columns. The binding capacity of peptides to K^b molecules was measured by their capacity to inhibit binding of the radiolabeled ligand. The affinity of the binding was estimated by determining the quantity of peptide required to inhibit 50% of the binding of the radiolabeled peptide.

Immunization Protocols and Characterization of CTL Lines and Clones. Glycopeptides were emulsified in incomplete Freund adjuvant and injected subcutaneously at the concentration of 50 μg, together with 140 μg of an IA^b-restricted Th epitope, the hepatitis B virus core antigen sequence 128–140 (TPPAYRPPNAPIL; 9). Mice were killed 7 d after priming. Splenocytes from each experimental group were pooled and stimulated in vitro in the presence of the glycopeptide that was used as immunogen and, as an APC source, irradiated syngeneic B cell blasts activated in vitro for 48 h by culturing splenocytes with LPS (from *Salmonella typhosa*: Sigma-Aldrich) and dextran sulfate (Amersham Biosciences). Culture medium consisted of RPMI 1640 (Life Technologies) supplemented with 20 mM glutamine, 100 μg streptomycin, 100 U/ml penicillin, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids (Life Technologies), 50 μM 2-ME, and 10% heat-inactivated FCS (Life Technologies). After 5 d in culture, cells were collected, purified over Ficoll gradients, and T cell blasts were cultured in complete RPMI conditioned with supernatant from Con A (Sigma-Aldrich)–activated splenocytes as an IL-2 source. 2 d later (day 7 in culture) T cells were tested for CTL activity. Carbohydrate and peptide specificity were studied in a standard ^{51}Cr release assay using the lymphoma EL-4 ($H\text{-}2^b$) cell line as targets at different E/T in duplicate, with and without antigens. Net specific lysis has been calculated by subtracting the cytolytic response to EL-4 cells in the absence of glycopeptides or peptides defined as background. Data were calculated as % cytotoxicity = [(sample − spontaneous release)/(maximum release − spontaneous release)] × 100.

T cell clones were generated from selected TACA-specific CTL lines at 6 to 7 d after a single in vitro stimulation with the immunizing glycopeptide to avoid in vitro selection. In brief, after Ficoll purification, T cell blasts were plated in 96-well U-bottom plates at 0.5 cell/well in the presence of 1 μg/ml glycopeptide and irradiated LPS/dextran sulfate–activated B cell blasts. Growing wells were expanded with Con A supernatant and restimulated with irradiated B cell blasts and glycopeptide. CTL clones were tested for specificity by using EL-4 targets. Endogenous recognition of the carbohydrate expressed by tumor cells was determined using as targets for the ^{51}Cr release assay the mammary tumor lines TA3/Ha, a well-characterized tumor line known for TF expression, which has been used by other investigators in carbohydrate-based immunotherapeutic studies (3, 29) and TA3/Ha/K^b, the ovary tumor lines MM14 (American Tissue Culture Collection) and MM14/K^b. The melanoma cell line B16 transfected with the human MUC1 gene, namely B16/MUC1 (30), was also included in the study.

Results

Generation of a Peptide Backbone Suitable for Carbohydrate Conjugation. There is a very strong correlation between the affinity of peptide binding to MHC molecules and the capacity of the peptide to induce an immune response (31, 32). This information is critical to the strategy used in this study. The size of a peptide determines its capacity to bind class I MHC. Whereas the optimal peptide length for most murine MHC molecules appears to be 9-amino acid residues, the K^b and K^k molecules prefer 8-residue peptides (33). K^b molecules were initially chosen to define a glycopeptide that: (a) binds with high affinity; and (b) will be of a structure that skews the T cell response toward recognition of the carbohydrate moiety rather than toward either the peptide or the MHC molecule. The critical anchor residues for K^b binding have been canonically defined as phenylalanine (F) or tyrosine (Y) in position 5 and leucine (L) or methionine

(M) at position 8 of an octamer peptide (8). The maintenance of critical hydrogen bonding between main chain atoms in the peptide and atoms in the MHC molecules can be theoretically accomplished with any natural-occurring l-amino acid, avoiding detrimental residues at nonanchor positions that interfere with the "docking" of peptides in the MHC binding groove. Detrimental amino acids tend to be negatively or positively charged amino acids, or in some instances, proline (34, 35). On this basis, alanine (A)-, serine (S)-, and glycine (G)-rich peptides were synthesized, each containing the anchor of phenylalanine (F) in position 5 and leucine (L) in position 8 and their ability to bind to K^b was measured (36). The quantity of peptide needed to achieve 50% inhibition of binding of a reference peptide approximates the affinity of interaction (k_D) between the peptide and MHC. There was considerable difference in the capacity of these anchor residue–containing peptides with different peptide backbones to bind to the K^b molecule as shown in Table I. The glycine-rich peptide bound with very low affinity (53 μM), whereas the serine-rich peptide had ~100-fold higher affinity. The alanine-rich peptide showed the highest affinity for K^b molecules, with a 50% inhibition of 75 nM. On this basis, alanine was chosen as the most appropriate amino acid residue to be used in the peptide backbone for carbohydrate conjugation. Crystallographic analysis of peptides bound to the K^b molecule demonstrated an interesting difference between the conformation of an 8-mer peptide bound to K^b versus a 9-mer peptide (8, 34). Peptides with 8 amino acids had been previously shown to be the optimal length, and the crystallographic analysis indicated that in order for a 9-mer peptide to be accommodated in the peptide binding groove, the first anchor residue had to be shifted from residue 5 to residue 6, thus creating a "bulge" in the NH_2-terminal region of the peptide between residues 1 and 6. It was considered possible that this bulge created to accommodate a 9-mer peptide might be a useful location to attach a carbohydrate residue, to enhance the hapten specificity of the CTL response. For this reason, we identified 9-mer peptides that could bind to K^b with high affinity. Increasing the length of the alanine-rich peptide from 8- to 9-amino acid residues resulted in eightfold reduction in the affinity for K^b molecules from 75 to 610 nM (Table I). To improve this affinity we added isoleucine (I) at position 2 and/or position 3 as previous studies had indicated that these positions might be capable of engaging secondary binding pockets in the MHC molecule (35). High affinity binding (Table I, 10 nM) was achieved when isoleucines were inserted at positions 2 and 3. More significantly, further modification of the peptide with the TF or Tn carbohydrate linked with serine in position 5 (relevant for TcR contact in the K^b model; reference 8) did not alter this high affinity binding (Table I, lines 8–10). Thus, this alanine-rich 9-mer peptide sequence conjugated with TF or Tn through a serine in position 5 was chosen for further studies.

Generation of Anti-TACA CTL In Vivo. To generate anti-TACA–specific CTL, the 9-mer alanine-rich peptides conjugated with either the TF antigen (β-Gal-[1→3]-α-GalNAc-*O*-serine) or the monomeric Tn (GalNAc-*O*-serine) were tested as vaccines in vivo. C57BL/6 mice (H-2^b) were coimmunized subcutaneously with 50 μg CTL glycopeptide and a potent Th epitope (9) in incomplete Freund adjuvant. Mice were killed 7 d after priming and splenocytes were restimulated in vitro with the glycopeptide immunogen. Irradiated, LPS/dextran sulfate–activated B cell blasts were used as APC in culture after pulsing for 2 h with glycopeptides (50 μg/10 × 10^6 cells). CTL lines were expanded with Con A supernatant as an IL-2 source after separation by Ficoll gradient at day 5 in culture. This protocol is very efficient in generating antigen-specific murine $CD3^+$ $CD8^+$ T cells in vitro inasmuch as $CD8^+$ T cells represent ≥75% of the total population at day 7 in culture. Moreover, lack of exogenous IL-2 for such a prolonged time limits considerably the expansion of nonspecific T cells.

Table I. *Construction of High Affinity K^b Binding Peptide Backbones for Carbohydrate Conjugation*

	Peptide	K^b binding capacity IC_{50}
		nM
1.	GGGGFGGL	53,000
2.	SSSSFSSL	509
3.	AAAAFAAL	75
4.	AAAAAFAAL	610
5.	AIAAAFAAL	240
6.	AAIAAFAAL	90
7.	AIIAAFAAL	10
8.	AIIASFAAL	4
9.	AIIA(βGal-[1→3]-α-GalNAc-*O*-ser)FAAL	4
10.	AIIA(GalNac-*O*-ser)FAAL	4

Glycine-, serine-, and arginine-rich peptides with relevant K^b anchor positions were compared for their capacity to bind to purified K^b molecules. K^b binding was measured as the quantity of peptide required to inhibit by 50% (IC_{50}) the binding of a well-characterized radioiodinated K^b binding peptide.

T cell cultures were tested for CTL activity after 7 d in culture in a standard ^{51}Cr release assay. The well-characterized thymoma cell line EL-4 (H-2^b) was used as target cells as they do not express MHC class II molecules, thus avoiding false interpretation of the results due to contaminating $CD4^+$ T cells. Moreover, EL-4 cells do not endogenously synthesize TF, as shown in the left panel of Fig. 4.

Carbohydrate specificity was investigated by comparing the response between the glycopepeptide and its respective nonglycosylated counterpart. As shown in Fig. 1, strong CTL activity specific for the immunizing glycopeptide was generated by either the TF- (Fig. 1 A) or the Tn-(Fig. 1 B) containing glycopeptide(s). In contrast, greatly diminished activity was observed against the same peptide in a nonglycosylated form, demonstrating the generation of carbohy-

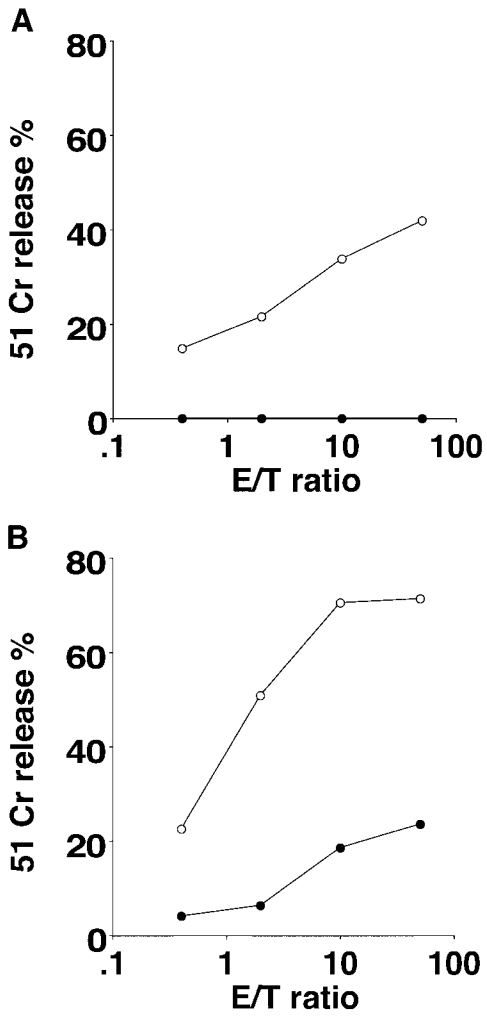

Figure 1. Specificity of primary CTL cell cultures tested in a ^{51}Cr release assay. Labeled EL-4 cells (H-2^b) were incubated with different cell number of T cell blasts in the presence of 1 μg/ml of the immunogen glycopeptide or the unglycosylated peptide backbone. EL-4 cells plated with T cell blasts in the absence of antigens have been assessed in duplicate at every E/T ratio to determine the extent of nonspecific lysis. Cell cultures supernatants were harvested 4 h later and percent ^{51}Cr released was calculated as (sample − spontaneous release)/(maximum release − spontaneous release) × 100. Background lysis has been subtracted and net lysis is shown. Open symbols represent carbohydrate specificity and filled symbols represent peptide specificity. (A) Primary CTL response in a mouse primed with TF (β-Gal-[1→3]-α-GalNAc-*O*-ser). (B) Primary CTL response in a mouse primed with Tn (GalNAc-*O*-ser).

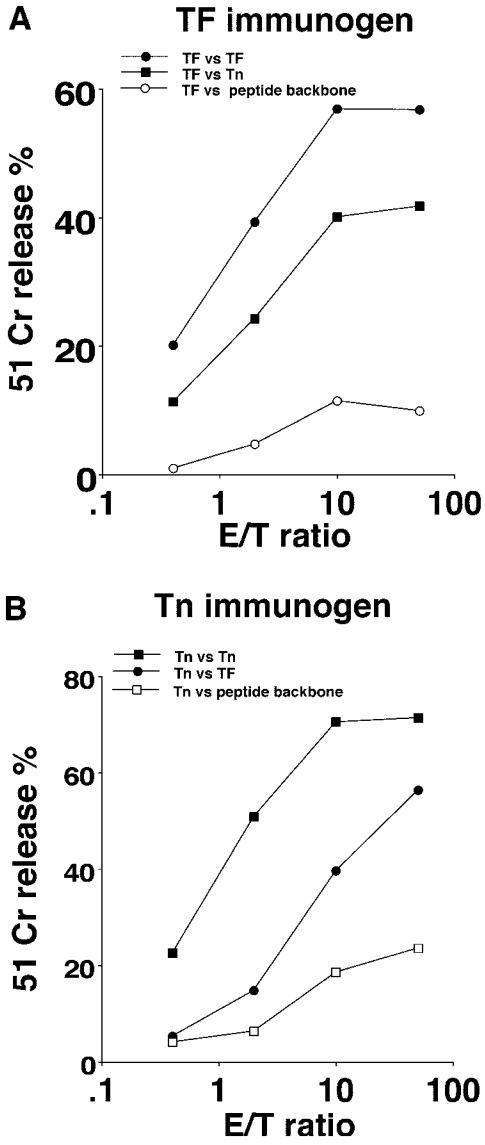

Figure 2. Cross-reactivity between TF and Tn. Splenocytes from immunized mice were pooled in a representative experiment. (A) Average CTL response in 10 mice primed with the TF-serine glycopeptide (filled circles) and the cross-reactive response to Tn (filled squares). (B) Average CTL response of 10 mice primed with the Tn-serine glycopeptide (filled squares) and the cross-reactive response to TF (filled circles). Open symbols represent the specific response to the peptide backbone (AIIASFAAL) in both groups. The background has been subtracted and net lysis is shown.

drate-specific CTLs that either do not use TcR contacts within the peptide backbone or are not activated if such contacts exist. The study has been extended to a large number of mice immunized with the TF-containing glycopeptide. 30 out of 45 analyzed (67%) generated a TF-specific CTL repertoire whereas 13 (29%) generated a repertoire that was carbohydrate specific but cross-reactive to the peptide backbone. None of the animals generated a CTL response that was exclusively peptide specific and two mice (4%) failed to respond to vaccination. Mice were considered responders if their TF-specific recognition at 10:1 E/T ratio was ≥15% after subtracting nonglycosylated peptide recognition and nonspecific lysis (cytolytic activity in the absence of antigen).

Cross-Reactivity between TACA-specific CTL Responses. Carbohydrate chains expressed on the cell surface of tumor cells increase in length during synthesis and modifications in the glycan group may be recognized in a cross-reactive fashion by CTL or they may be recognized as antagonist or null antigens (37). As those possibilities have implications in the design of carbohydrate-based immunotherapeutic vaccines, the reciprocal cross-reactivity between primary CTL lines generated with the disaccharide TF antigen and the

monosaccharide Tn antigen conjugated to the alanine-rich nonapeptide was examined.

The results indicate that mice immunized with the TF-conjugated glycopeptide generated a CTL repertoire that cross-reacted with Tn (Fig. 2 A). Reciprocally, CTL lines from Tn-immunized mice cross-reacted with TF (Fig. 2 B). The results suggest that the GalNAc moiety is highly immunogenic and is recognized by the large majority of the T cell receptors. Differences in the magnitude of the CTL cross-reactive response to Tn by the TF-specific T cells suggest that TcR fine specificities include T cells whose fine specificity is driven toward the β-Gal or the disaccharide β-Gal-(1→3)-α-GalNAc (Fig. 2 A). Similarly, differences in the magnitude of the CTL cross-reactive response to TF by Tn-specific T cells suggest that some Tn-specific TcRs cannot accommodate two glycan groups, although the large majority of the T cells recognize the GalNAc moiety as was the case for cells primed with TF (Fig. 2 B).

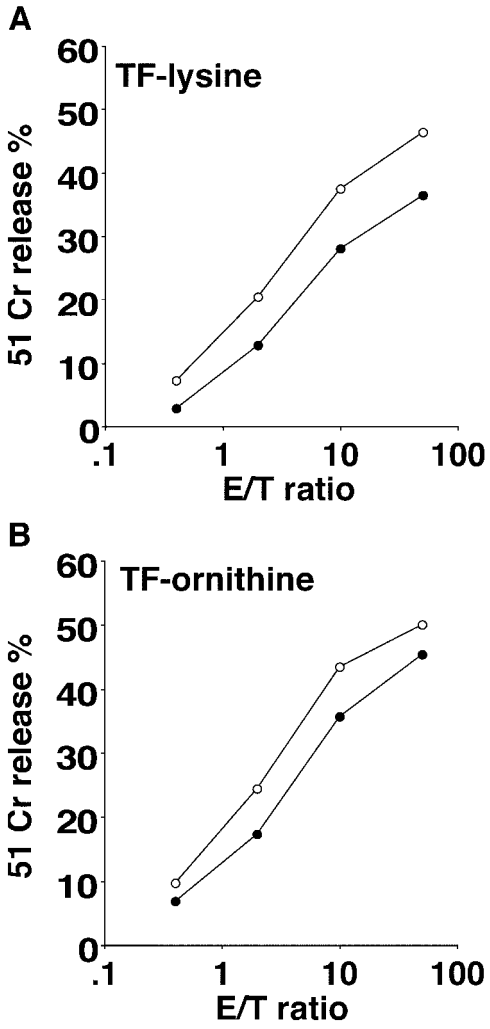

Figure 3. Immunogenicity of glycopeptides containing TF conjugated to lysine or ornithine at position 5. TF-lysine (A) and TF-ornithine (B) were tested and compared for their ability to generate TF-specific CTL. The primary CTL response in six mice primed with each glycopeptide is shown (spleens pooled). The CTL response to the immunizing glycopeptide is represented by open symbols, and the CTL response to the respective peptide backbone is represented by filled symbols in both panels. The background has been subtracted and net lysis is shown.

Taken together the results suggest that Tn is highly immunogenic. Moreover, the higher magnitude of the primary CTL response to the Tn-containing glycopeptide compared with the TF-containing glycopeptides may reflect better presentation of the carbohydrate molecule to TcRs due to its smaller size and reduced flexibility when bonded to K^b molecules.

Immunogenicity of TF Conjugated to Different Amino Acid Linkers. To determine the effect of the amino acid linkers on induction of carbohydrate-specific CTLs, we generated two new TF-containing glycopeptides where instead of serine, bulkier lysine and ornithine residues were chosen for glycosylation in order to enhance the protrusion of the carbohydrate toward the solvent (28). When mice were immunized with TF linked by lysine or ornithine, CTL responses were observed although as shown in Fig. 3, the responses induced by both glycopeptides were skewed toward recognition of the peptide backbone rather than the carbohydrate moiety. These results were somewhat surprising and contrast our previous findings with a different hapten model (9). Overall these results indicate that glycopeptides containing TF linked via a serine residue are the best candidates for immunotherapy.

Recognition of TF-expressing Tumors by Glycopeptide-specific CTL Clones. The recognition of TF-expressing tumor cells by CTL generated from vaccination with the TF-conjugated glycopeptides represents a critical test for the disease relevance of designer glycopeptide-based vaccines.

TF-specific CTL clones were generated from independent T cell lines derived from mice immunized with the TF-serine–containing glycopeptides that were stimulated once in vitro with the glycopeptide immunogen. The T cell clonal repertoire derived from these mice was highly specific for the carbohydrate moiety, supporting results obtained with primary T cell lines.

The T cell clones were tested in vitro for their ability to recognize tumor cells that endogenously synthesize the carbohydrate antigen. Three tumor cell lines have been selected as targets for these experiments: (a) a well-characterized mammary carcinoma that expresses TF, namely TA3/Ha (3, 29); (b) the MM14 ovary carcinoma that also expresses TF, as determined by anti-TF mAbs staining (provided by Dr. B. Jansson, Bioinvent Therapeutic AB, Lund, Sweden; reference 3) the B16 melanoma cell line ($H\text{-}2^b$) transfected with the human MUC1 gene, designated as B16/MUC1 (30). The glycoprotein MUC1 contains TF as determined in our laboratory by specific mAbs staining and reported in the literature (26). The TF expression of these tumors has been evaluated by FACS® analysis using anti-TF mAbs provided by Dr. B. Jansson (38). As shown in the left panel in Fig. 4, the thymoma cell line EL-4 used as target for glycopeptide-specificity studies does not express TF endogenously. However, the mammary carcinoma TA3/Ha and the ovary carcinoma MM14 showed very high TF ex-

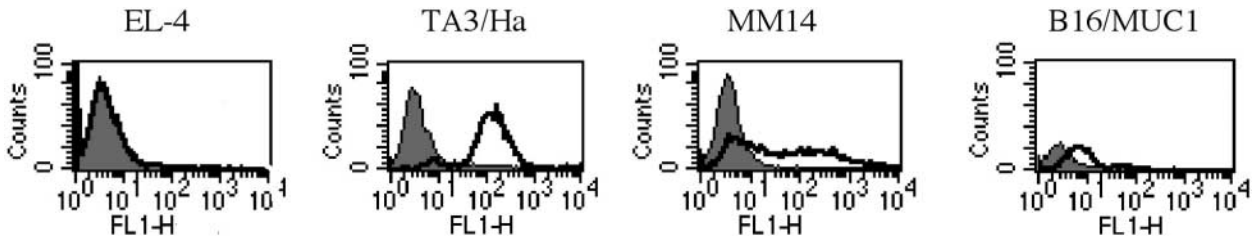

Figure 4. TF expression by different tumor cell lines. IgM TF1 mAb and anti–IgM-FITC have been used in these experiments where the isotype control is also shown.

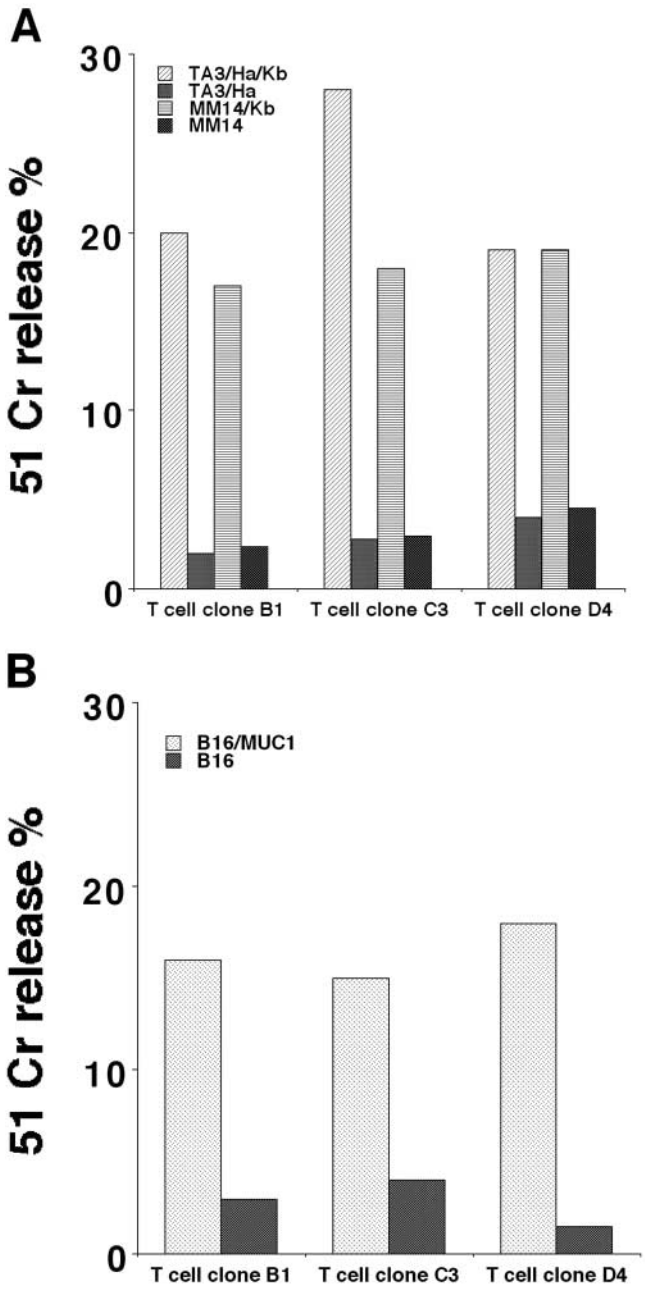

Figure 5. Endogenous recognition of TF-specific CTL clones. (A) Three representative TF-specific T cell clones recognize K^b-transfected carcinomas that express TF, but not the parental tumor lines H-2 mismatched ($H\text{-}2^a$). (B) B16 melanoma ($H\text{-}2^b$) transfected with the MUC1 gene is also recognized by the same T cell clones, but not the parental cell line lacking the TACA antigen (SD ≤1.5 in three repeated experiments).

pression, representing ideal targets to evaluate the ability of glycopeptide-generated CTL to recognize tumors in vitro. Moreover, the melanoma cell line B16 may be induced to express TF when transfected with the MUC1 gene, however, the expression was lower compared with carcinomas as shown in the right panel in Fig. 4.

The two carcinomas cell lines TA3/Ha and MM14 were transfected with a K^b plasmid (donated by Dr. S. Joyce, Vanderbilt University School of Medicine, Nashville, TN) to provide the appropriate MHC class I allele for CTL recognition. Parental cell lines ($H\text{-}2^a$) were also included as control in the same assays to determine the MHC restriction of the CTL clonal repertoire.

The three representative CTL clones shown previously that responded to tumor cells generated from mice primed with the β-Gal-(1→3)-α-GalNAc-*O*-serine glycopeptide showed cytotoxic activity against the TA3/Ha/K^b mammary tumor line transfected with K^b molecules and the K^b-transfected MM14/K^b ovary tumor line but not against the parental tumor lines that expressed the antigen but were MHC class I mismatched. These findings indicate that TF-specific CTL can recognize the TACA endogenously expressed in a MHC class I–dependent fashion (Fig. 5 A). In support of these results, the same T cell clones were also capable of recognizing the melanoma cell line B16 (syngeneic with the T cells) transfected with the MUC1 gene (B16/MUC1) but not the TACA-negative parental cell line (Fig. 5 B). The lysis of this tumor was reduced compared with the two carcinomas cell lines. A possible explanation is the lower expression of TF molecules on B16/MUC1 cell surface compared with the two epithelial tumors as shown in Fig. 4.

Fine Specificity of TF-specific CTL Clones. In the next series of experiments the fine specificity of TF-serine–specific CTL clones was analyzed to address the importance of the glycosylated amino acid linker for TcR recognition. CTLs were tested for their cross-reactivity to TF-lysine, TF-ornithine, and TF-homoserine (hs). TF-hs glycopeptides were included in this analysis because they were shown previously to be highly immunogenic and capable of inducing helper-independent T cell responses (39).

Lastly, we determined the exclusive relevance of position 5 for TcR contact that is critical for the degeneracy of the

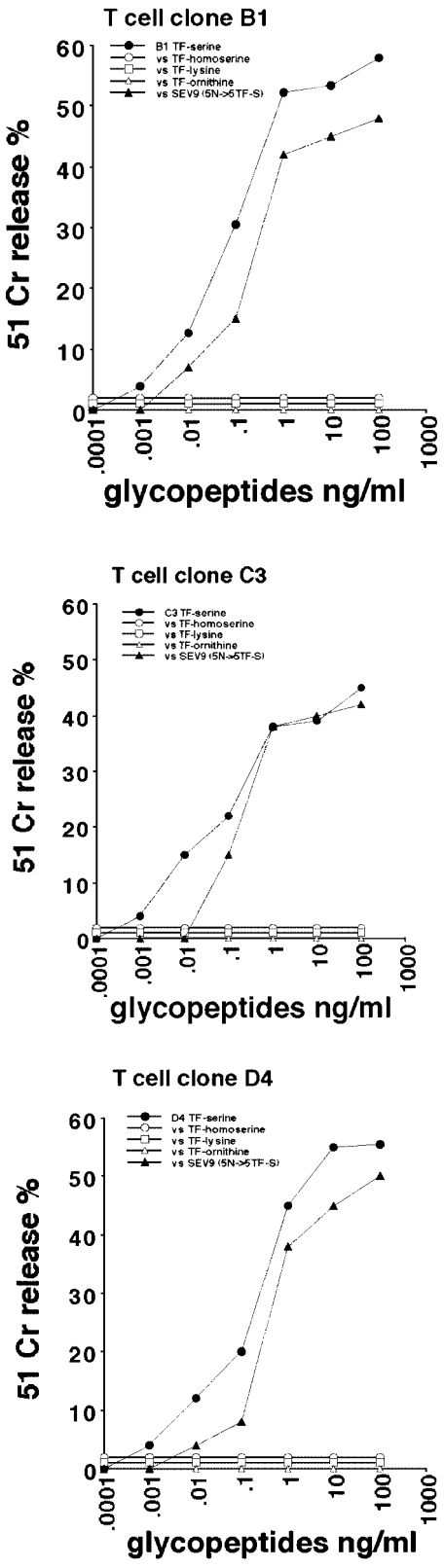

Figure 6. Fine specificity of TF-specific CTL clones. The fine specificity of three representative CTL clones that recognize TF endogenously expressed on tumor cells have been characterized by studying the cross-reactivity to TF-hs–, TF-lysine–, and TF-ornithine–glycopeptides. A modified viral natural sequence from the Sendai virus carrying TF linked to serine in position 5 (5N→5TF-S) was also tested in these experiments (E/T ratio of 5:1).

T cell repertoire. Degeneracy is an important consideration for carbohydrate vaccines as the endogenous peptide presenting TACA in cancer patients is unknown.

We addressed this question by testing the cross-reactivity of TF-specific CTL clones against an unrelated glycopeptide generated from the Sendai virus NP 324–332 epitope sequence that contained TF conjugated at position 5 via substituted serine residue (5N→5TF-S). Previous studies have shown that position 5 of this epitope is a critical TcR contact site (8).

TF-specific T cell clones showed high affinity/avidity for the TF-serine glycopeptides as shown in Fig. 6. However, the results of the cross-reactivity of the T cell clones against glycopeptides carrying different linkers were surprising because TF-serine–specific CTL clones did not cross-react either with the TF-hs–containing glycopeptide, or with the lysine- or ornithine-containing glycopeptides regardless of the concentration. These findings indicate that TF-specific TcRs recognize the natural amino acid linker in the immunogen together with the sugar moiety (Fig. 6). Similarly, none of 45 TF-hs–specific T cell clones derived from mice primed with the TF-hs–linked glycopeptide were capable of recognizing the TF-serine glycopeptides or tumor cells expressing TF (unpublished data).

However, T cell clones showed good cytolitic activity in response to the glycopeptide FAPG(TF-S)YPAL derived from Sendai NP 324–332 (Fig. 6), supporting the importance of the core of the peptide (position 5 in a 9-mer) for TcR contacts and the high degeneracy of the TACA-specific CTL repertoire in immunized mice that generated a TF-specific CTL response (67% out of 45 studied).

Discussion

Here we report that it is possible to generate CTL against two very well-known tumor-associated carbohydrate antigens largely expressed in a variety of epithelial tumors, TF and Tn, by using as vaccines designer glycopeptides with high affinity for MHC class I molecules. The results in the current study are very encouraging because TACA-containing glycopeptides glycosylated in a critical position for TcR recognition to a natural amino acid linker (serine) generated a carbohydrate-specific CTL clonal repertoire in vivo that recognize TACA endogenously expressed in a MHC class I–restricted fashion. We believe that this vaccine approach is particularly interesting for cancer prevention because: (a) TACA are expressed very early during neoplastic transformation (1) rendering possible immunosurveillance by CTL (40); (b) the same TACA-based vaccine can be used in a variety of tumors, circumventing the limitation of epitope mapping; and (c) by designing appropriate peptide(s) backbone sequence(s) it may be possible to target multiple supertype MHC class I alleles (36, 41) and a variety of cancer types, rendering feasible future CTL-based vaccines applicable on a large population scale. In support of this approach is also the fact that the carbohydrate-specific T cell repertoire generated by designer peptide backbones for hapten conjugation is highly degenerate,

as indicated by the recognition of tumors that express the TACA antigen in the context of unknown endogenous peptides and the recognition of a different sequence carrying the carbohydrate antigen in the same position. Degeneracy of the CTL repertoire has been reported previously in a different hapten model relevant in allergy (9), suggesting that hapten-specific CTL may be successfully targeted for different immunotherapeutic approaches.

MHC-restricted, carbohydrate-specific CTL have been generated in different viral models, indicating that peptides can be posttranscriptionally glycosylated once they are loaded in the binding groove of the MHC class I molecule (42–46). One study that characterized the immunogenicity of designer glycopeptides modified a TcR contact(s) within a viral epitope described the induction of non-MHC class I–restricted CTL (47). In another study in allergic patients, pollen carbohydrate–specific CTL have been also detected (48). Altogether, these findings indicate that carbohydrate-specific $CD8^+$ T cells are represented in secondary lymphoid organs and may critically contribute to native immune responses.

The idea of targeting glycoproteins for cancer immunotherapy has been pioneered by Dr. O. J. Finn and her group (for review see reference 25), initially from the observation that human epithelial cells express on the cell surface a polymorphic epithelial mucin, which is encoded by the MUC1 gene, developmentally regulated and aberrantly expressed in tumors (49). Mucin is recognized by CTL in breast and pancreatic cancer (50), as well as by CTL that infiltrate ovarian malignant tumors (51). The highly repetitive sequence of the polypeptide core in mucin may allow simultaneous recognition of many identical epitopes and cross-linking by TcRs (50). A phase I clinical trial in adenocarcinoma patients that were vaccinated with a 105–amino acid synthetic mucin MUC1 peptide that has five repeated immunodominant epitopes demonstrated that antimucin CTL were detectable in 7 out of 22 patients (52). It is worth considering that ~25% of the amino acids within the mucin sequence are serine or threonine (49), potential *O*-glycosylation sites, suggesting that carbohydrate molecules may be as relevant tumor antigens for CTL as they are for humoral responses (27). The data here reported suggest that a glycosylated version of this peptide may be used as a vaccine to potentially increase immunogenicity and antitumor efficacy of the CTL repertoire. Moreover, complex carbohydrates are not removed during processing of glycoproteins by dendritic cells and MUC-1 glycopeptides are presented to MHC class II–restricted T helper cells (53). MUC1-specific T helper cells cross-reacted with TF and Tn, contained within the MUC1 glycoprotein (53) suggesting that either $CD4^+$ or $CD8^+$ T cells accommodate small sugar moiety in their TcRs that represent optimal target for T cell–based immunotherapy. This hypothesis will be explored in the near future in a spontaneous mammary tumor model (MUC1/MMT), which is ideal to test the ability of glycopeptides to prevent or delay tumor onset.

The authors thank Dr. H.M. Gray for critical suggestions and support, Drs. G.Y. Ishioka and D.B. Wilson for critical reading of the manuscript, Dr. T. Yokoyama for K^b binding assay, Dr. M. Meldal for glycopeptides synthesis, Dr. B. Jansson for providing anti-TF mAbs, and C. Auciello for secretarial assistance.

This work has been supported by National Institutes of Health grant R29CA78657 and the Department of Defense grant BC010002 to A. Franco.

Submitted: 28 October 2003
Accepted: 21 January 2004

References

1. Hakomori, S. 1989. Aberrant glycosylation in tumors and tumor associate carbohydrate antigens. *Adv. Cancer Res.* 52: 257–331.
2. Fung, P.Y.S., M. Madej, R.R. Koganty, and B.M. Longenecker. 1990. Active specific immunotherapy of a murine mammary adenocarcinoma using a synthetic tumor-associated glycoconjugate. *Cancer Res.* 50:4308-4314.3.
3. Henningsson, C.M., S. Selvaraj, G.D. MacLean, M.R. Suresh, A.A. Noujaim, and B.M. Longenecker. 1987. T cell recognition of a tumor-associated glycoprotein and its synthetic carbohydrate epitopes: stimulation of anticancer T cell immunity in vivo. *Cancer Immunol. Immunother.* 25:231–241.
4. Singhai, A., and S. Hakamori. 1990. Molecular changes in carbohydrate antigen associated with cancer. *Biol. Assays.* 12: 223–230.
5. Singhai, A., M. Fohn, and S. Hakamori. 1991. Induction of alpha-*N*-acetylgalactosamine-*O*-serine/threonine (Tn) antigen-mediated cellular immune response for active immunotherapy in mice. *Cancer Res.* 51:1406–1411.
6. Zhao, X.-J., and N.-K.V. Cheung. 1995. GD2 oligosaccharide: target for cytotoxic T lymphocytes. *J. Exp. Med.* 182: 67–74.
7. Madden, D.R., D.N. Garbogzi, and D.C. Wiley. 1993. The antigenic identify of peptide-MHC compexes: a comparison of the conformation of five viral peptides presented by HLA A2. *Cell.* 75:693–708.
8. Fremont, D.H., M. Matsumura, E.A. Stura, P.A. Peterson, and I.A. Wilson. 1992. Crystal structure of two viral peptides complex with murine class I H-2Kb. *Science.* 257:919–927.
9. Franco, A., T. Yokoyama, D. Huynh, C. Thomson, S. Nathenson, and H.M. Grey. 1999. Fine specificity and MHC restriction of trinitrophenyl-specific CTL. *J. Immunol.* 162: 3388–3394.
10. Eisen, H.N. 2001. Specificity and degeneracy in antigen recognition: yin and yang in the immune systhem. *Annu. Rev. Immunol.* 19:1–21.
11. Hafler, D.A. 2002. Degeneracy, as opposed to specificity, in immunotherapy. *J. Clin. Invest.* 109:641–649.
12. Schultze, J.L. 2002. Degeneracy instead of specificity: is this a solution to cancer immunotherapy? *Trends Immunol.* 23:343–344.
13. Springer, G.F. 1984. T and Tn, general carcinoma autoantigens. *Science.* 224:1198–1206.
14. Langkilde, N.C., H. Wolf, H. Clausen, T. Kjeldsen, and T.F. Orntoft. 1992. Nuclear volume and expression of T-antigen, sialosyl Tn antigen and Tn antigen in carcinoma of the human bladder. *Cancer.* 69:219–227.
15. Itzkowitz, S., M. Yuan, C.K. Montgomery, T. Kjeldsen, H.K. Takahashi, W.L. Bigbe, and Y.S. Kim. 1989. Expression of Tn, syalosyl Tn and T antigen in human colon can-

cer. *Cancer Res.* 49:197–204.
16. Hull, S.R., and K.L. Carraway. 1988. Mechanism of expression of Thomsen Freidereich (T) antigen at the cell surface of a mammary adenocarcinoma. *FASEB J.* 2:2380–2384.
17. MacLean, G.D., M.B. Bowen-Yacyshyn, J. Samuel, A. Meikle, G. Stuart, J. Nation, S. Poppema, M. Jerry, R.R. Koganty, T. Wong, et al. 1992. Active immunization of human ovarian cancer patients against a common carcinoma (Thomsen-Friedenreich) determinant using a synthetic carbohydrate antigen. *J. Immunother.* 11:292–305.
18. Stein, R., S. Chen, W. Grossman, and D.M. Goldenberg. 1989. Human lung carcinoma monoclonal antibody specific for the Thomsen-Friedenreich antigen. *Cancer Res.* 49:32–37.
19. Cao, Y., P. Stosiek, and G.F. Springer. 1996. Thomsen-Freidenreich-related carbohydrate antigens in normal adult tissues: a systemic and comparative study. *Histochem. Cell Biol.* 106:197–207.
20. Chen, Y.X., R.K. Jain, E.V. Chandrasekaran, and K.L. Matta. 1995. Use of sialyated derivatives and acrylamide copolymers of Gal (beta 1,3) Gal Nac alpha − to determine the specificities of blood group T- and Tn-specific lectines and the copolymers to measure anti-T and anti-Tn antibody levels in cancer patients. *Glycoconj. J.* 12:55–62.
21. Dahlenborg, K., L. Hultman, R. Carlsson, and B. Jansson. 1997. Human monoclonal antibodies specific for the tumor associated Thomsen-Friedenreich antigen. *Int. J. Cancer.* 70: 63–71.
22. O'Boyle, K.P., and K.E. Whrite. 1994. Anti-Tn human monoclonal antibodies generated following active immunization with partially desialylated ovine submaxillary mucin. *Hum. Antibodies Hybridoma.* 5:25–31.
23. O'Boyle, K.P., A.L. Markowitz, M. Khorshidi, P. Lalezari, B.M. Longenecker, K.O. Lloyd, S. Welt, and K.W. Wright. 1996. Specificity analysis of murine monoclonal antibodies reactive with Tn, Sialylated Tn, T and monosialylated (2→6) T antigens. *Hybridoma.* 15:401–408.
24. Gendler, S., J. Taylor-Papadimitriou, T. Duhig, J. Rothbard, and J. Burchell. 1988. A highly immunogenic region of a human polymorphic epithelial mucin expressed by carcinomas is made of tandem repeats. *J. Biol. Chem.* 263:12820–12823.
25. Finn, O.J., K.R. Jerome, R.A. Henderson, G. Pecher, N. Domenech, J. Magarian-Blander, and S.M. Barratt-Boyes. 1995. MUC-1 epithelial tumor mucin-based immunity and cancer vacccines. *Immunol. Rev.* 145:61–69.
26. Dahiya, R., K.S. Kwak, J.C. Byrd, S. Ho, W.H. Yoon, and Y.S. Kim. 1993. Mucin synthesis and secretion in various human epithelial cancer cell lines that express the MUC-1 mucin gene. *Cancer Res.* 53:1437–1443.
27. Liu, X., J. Sejbal, G. Kotovich, R.R. Koganti, M.A. Reddish, L. Jackson, S.S. Gandi, A.J. Mendoca, and B.M. Longenecker. 1995. Structurally defined synthetic cancer vaccines: analysis of structure, glycosylation and recognition of cancer associated mucin, MUC-1 derived peptides. *Glycoconj. J.* 12: 607–617.
28. St. Hilaire, P.M., L. Cipolla, A. Franco, U. Tedebark, D.A. Tilly, and M. Meldal. 1999. Synthesis of T-antigen-containing glycopeptides as potential cancer vaccines. *J. Chem. Soc. Perkin Trans. I.* 1:3559–3564.
29. Hauschka, T.S., L. Weiss, B.A. Holdrige, T.L. Cudney, M. Zumpft, and J.A. Planisken. 1971. Karyotipic and surface features of murine TA3 carcinoma cells during immunoselection in mice and rats. *J. Natl. Cancer Inst.* 47:343–359.
30. Rowse, G.J., R.M. Tempero, M.L. Vanlith, M.A. Hollingsworth, and S.J. Gendler. 1998. Tolerance and immunity to MUC1 in a human MUC1 transgenic murine model. *Cancer Res.* 58:315–321.
31. Shaeffer, E.B., A. Sette, D.L. Jhonson, M.C. Bekoff, J.A. Smith, H.M. Grey, and S. Buus. 1989. Relative contribution of "determinant selection" and "holes in the T cell repertoire" to T cell responses. *Proc. Natl. Acad. Sci. USA.* 86: 4649–4653.
32. Sette, A., A. Vitiello, B. Reherman, P. Fowler, R. Nayersina, W.M. Kast, C.J. Melief, C. Oseroff, L. Yuan, J. Ruppert, et al. 1994. The relationship between class I binding affinity and immunogenicity of potential cytotoxic T cell epitopes. *J. Immunol.* 143:5586–5592.
33. Falk, K., O. Rotzschke, S. Stevanovi, G. Jung, and H.-G. Rammensee. 1991. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature.* 351:290–296.
34. Boehncke, W.-H., T. Takeshita, C.D. Pendleton, R.A. Houghten, S. Sadegh-Nasseri, L. Racioppi, J.A. Berzofsky, and R.N. Germain. 1993. The importance of dominant negative effects of amino acid side chain substitution in peptide-MHC molecules interactions and T cell recognition. *J. Immunol.* 150:331–341.
35. Ruppert, J., J. Sidney, E. Celis, R.T. Kubo, H.M. Grey, and A. Sette. 1993. Prominent role of secondary anchor residues in peptide binding to HLA A2.1 molecule. *Cell.* 74:929–937.
36. Sette, A., J. Sidney, M.F. del Guercio, S. Southwood, J. Ruppert, C. Dahlberg, H.M. Grey, and R.T. Kubo. 1994. Peptide binding to the most frequent HLA-A class I alleles measured by quantitative molecular binding assays. *Mol. Immunol.* 31:813–822.
37. Jensen, T., M. Nielsen, M. Gad, P. Hansen, S. Komba, M. Meldal, N. Odum, and O. Werderlin. 2001. Radically altered T cell receptor signaling in glycopeptide-specific T cell hybridoma induced by antigens with minimal differences in the glycan group. *Eur. J. Immunol.* 31:3197–3206.
38. Jansson, B., and C.A. Borrebaeck. 1992. The human repertoire of antibody specificities against Thomsen-Freidenreich and Tn-carcinoma-associated antigens as defined by human monoclonal antibodies. *Cancer Immunol. Immunother.* 34:294–298.
39. Franco, A., D.A. Tilly, I. Gramaglia, M. Croft, L. Cipolla, M. Meldal, and H.M. Grey. 2000. Epitope affinity for MHC class I determines helper requirement for CTL priming. *Nat. Immunol.* 1:145–150.
40. Ochsenbein, A.F., S. Sierro, B. Odermatt, M. Pericin, U. Karrer, J. Hermans, S. Hemmi, H. Hengartner, and R.M. Zinkernagel. 2001. Roles of tumor localization, second signals and cross-priming in cytotoxic T cell induction. *Nature.* 411:1058–1064.
41. Sette, A., M. Newman, B. Livingston, D. McKinney, J. Sidney, G. Ishioka, S. Tangri, J. Alexander, J. Fikes, and R. Chestnut. 2002. Optimization vaccine design for cellular processing, MHC binding and TcR recognition. *Tissue Antigens.* 59:443–451.
42. Haurum, J.S., G. Arsequell, A.C. Lellouch, S.Y.C. Wong, R.A. Dweck, A.J. McMichael, and T. Elliott. 1994. Recognition of carbohydrate by major histocompatibility complex class I–restricted, glycopeptide-specific cytotoxic T lymphocytes. *J. Exp. Med.* 180:739–743.
43. Otvos, L., G.R. Krivulka, L. Urge, G.I. Szendrei, L. Nagy, Z.Q. Xiang, and H.C.J. Ertl. 1995. Comparison of the effects of amino acid substitutions and beta-*N*- vs. alpha-*O*-glycosylation on the T cell stimulatory activity and conformation

of an epitope on the rabies virus glycoprotein. *Biochim. Biophys. Acta.* 1267:55–64.
44. Haurum, J.S., I.B. Hoier, G. Arsequell, A. Neisig, G. Valencia, J. Zeuthen, J. Neefjes, and T. Elliott. 1999. Presentation of cytosolic glycosylated peptides by human class I major histocompatibility complex molecules in vivo. *J. Exp. Med.* 190:145–150.
45. Ferris, R.L., C. Hall, N.V. Sipsas, J.T. Safrit, A. Trocha, R.A. Koup, R.P. Johnson, and R.F. Siliciano.1999. Processing of HIV-1 envelope glycoprotein for class I-restricted recognition: dependence on TAP1/2 and mechanisms for cytosolic localization. *J. Immunol.* 162:1324–1332.
46. Hudrisier, D., J. Riond, H. Mazarguil, and E. Gairing. 2001. Pleiotropic effects of post-translational modifications on the fate of viral glycopeptides as cytotoxic T cell epitopes. *J. Biol. Chem.* 12:38255–38260.
47. Abdel-Motal, U.M., L. Berg, A. Rosen, M. Bengtsson, C.J. Thorpe, J. Kihlberg, J. Dahmen, G. Magnusson, K.A. Karlsson, and M. Jondal. 1996. Immunization with glycosylated Kb-binding peptides generates carbohydrate-specific, unrestricted cytotoxic T cells. *Eur. J. Immunol.* 26:544–551.
48. Corinti, S., R. De Palma, A. Fontana, C. Gagliardi, C. Pini, and F. Sallusto. 1997. Major hystocompatibility complex-independent recognition of a distinctive pollen antigen, most likely a carbohydrate, by human CD8 α/β T cells. *J. Exp. Med.* 186:899–908.
49. Gendler, S., C. Lancaster, J. Taylor-Papadimitriou, T. Duhing, N. Peat, J. Burchell, L. Pemberton, E.-N. Lalani, and D. Wilson. 1990. Molecular cloning and expression of tumor-associated polymorphic epithelial mucin. *J. Biol. Chem.* 265: 15286–15293.
50. Jerome, K.R., and O.J. Finn. 1993. Tumor-specific cytotoxic T cell clones from paptients with breast and pancreatic adenocarcinoma recognize EBV-immortalized B cells transfected with polymorphic epithelial mucin complementary DNA. *J. Immunol.* 151:1654–1662.
51. Ioannides, C.G., B. Fisk, K.R. Jerome, T. Irimura, J.T. Wharton, and O.J. Finn. 1993. Cytotoxic T cells from ovarian malignant tumors can recognize polymorphic epithelial mucin core peptides. *J. Immunol.* 151:3693–3703.
52. Goydos, J.S., E. Elder, T.L. Whiteside, O.J. Finn, and M.T. Lotze. 1996. A phase I trail of a synthetic mucin peptide vaccine. Induction of specific immune reactivity in patients with adenocarcinoma. *J. Surg. Res.* 63:298–304.
53. Vlad, A.M., S. Muller, M. Cudic, H. Paulsen, L. Otvos, Jr., F.G. Hanisch, and O.J. Finn. 2002. Complex carbohydrates are not removed during processing of glycoproteins by dendritic cells: processing of tumor antigen MUC1 glycopeptides for presentation to major histocompatibility complex class II–restricted T cells. *J. Exp. Med.* 196:1435–1446.