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14. ABSTRACT
This proposal aimed at defining the use of glycopeptides containing tumor associated carbohydrate antigens (TACA), that bind multiple MHC class I alleles, as potential preventive vaccines for carcinomas on a large scale. Our recent results (JEM 2004; 199:707, see appendix) suggest that CD8+ T cells (CTL) are capable of recognizing TACA in a conventional class I MHC-restricted fashion. TF, a disaccharide, and Tn, its immediate precursor, are TACA largely expressed in carcinomas. TF and Tn can be successfully used as T helper (Th)-independent vaccines when conjugated to designer peptide with optimal binding affinity for class I MHC molecules. TF- and Tn-specific CTL generated with this strategy are capable of recognizing TACA-expressing tumors *in vitro,* suggesting that glycopeptides are as effectively presented by class I MHC molecules as non-glycosylated peptides (JEM 2004; 199:707). Because the exact sequences of endogenously synthesized glycopeptides are unknown, the TACA-specific T cell repertoire elicited by carbohydrate-based vaccines is assumed to be degenerate. Here we report that mice genetically manipulated to develop TACA-expressing mammary tumors are not tolerant to glycopeptide vaccination (Figures 1 and 2, Immunol and Cell Biol., 2005; 83:440, see appendix). Moreover, we tested the immunogenicity of designer glycopeptides, capable of binding multiple MHC class I alleles (Table 1, Immunol and Cell Biol., 2005; 83:440, see appendix), as a novel approach for the development of vaccines potentially useful for vaccination of a large fraction of the general population. Our results have suggested that CTL derived from normal donors respond with high efficiency to glycopeptides *in vitro,* opening a new avenue for the design of prospective vaccines for cancer prevention (Table 2, Immunol and Cell Biol., 2005; 83:440, see appendix).

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REPORT MAIN BODY
The goal of this project is to test the ability of glycopeptides that bind multiple Human Leucocytes Antigens (HLA) to serve as vaccines to prevent breast cancer, by inducing the generation of carbohydrate-specific cytotoxic T lymphocytes (CTL) *in vivo*.

Tumor associated carbohydrate antigens (TACA) are a group of tumor antigens identified and characterized by their reaction with antibodies and lectins.

TACA are expressed in the embryo, but not in differentiated cells in adult tissues. In tumor cells, TACA are generated early during tumor progression due to aberrant glycosylation. Because several neoplastic tissues express the same TACA, this approach is very interesting because avoids the need for epitope mapping, a major limitation in cancer immunotherapy.

Moreover, our idea to design TACA-containing glycopeptides with the capacity to bind multiple HLA alleles (the large majority of the A2 subtypes have been studied with the resources provided by this grant) has the potential to indicate a new avenue for low costs cancer prevention in a large scale, and in a broad range of tumors.

Two interesting TACA for CTL-based immunotherapy are the Thomsen- Freidenreich (TF) antigen (α-Gal-(1->3)-β-GalNAc-O-Ser) and its precursor Tn (GalNAc-O-Ser).

In vivo studies in mice indicated that it is possible to generate anti-TF and anti-Tn-specific CTL (*The Journal of Experimental Medicine*, 2004. 199:707, Figure 1). TACA-specific CTL generated with this antigenic strategy are capable of killing a variety of TACA-expressing tumors *in vitro* (*The Journal of Experimental Medicine*, 2004. 199:707, Figure 5).

TACA-specific T cell receptors (TcRs) recognize the amino acid linker together with the TACA (*The Journal of Experimental Medicine*, 2004. 199:707, Figure 6).
The recognition of the amino acid linker by TACA-specific TcR is of the most importance for therapeutic design. In fact, longer amino acid linkers, as lysine or ornitine, although had potentials to better present the TACA antigen to T cells by "bulging" from the HLA binding groove, do not generate TACA-specific CTL, but a strong peptide-specific response (*The Journal of Experimental Medicine*, 2004. 199:707, Figure 3).

The TACA-specific T cell clonal repertoire is very cross-reactive, a great advantage in immunotherapy because the sequences of the endogenous peptides presenting TACA to T cells are unknown (*The Journal of Experimental Medicine*, 2004. 199:707, Figure 6).

Tn was found a better TACA vaccine candidate than TF in these studies (*The Journal of Experimental Medicine*, 2004. 199:707, Figure 1). In fact, within the TF molecule, Tn is the carbohydrate molecule recognized by the large majority of TcRs (*The Journal of Experimental Medicine*, 2004. 199:707, Figure 2). This concept is very relevant to immunotherapy because underlines the possible recognition of Tn by TF-specific CTL and *viceversa*.
Tn is more immunogenic than TF probably because of its small size that suggests a greater, and more stable antigen presentation to TcRs.

To validate this vaccine approach, we demonstrated that the TACA-containing glycopeptides breake immunological tolerance in tumor-bearing mice (namely MUC1/MT transgenic) that express TF and Tn molecules in the context of the MUC1 glycoprotein (*Immunology and Cell Biology*, 2005. 83:440, Figure 1). These preliminary experiments, although not described in the SOW, were required before breeding the double transgenic lineage with the A2/K^b transgenic mice to generate a triple transgenic, "humanized" spontaneous tumor model (MUC1/MT/A2K^b).
Breaking immunological tolerance is very important as a proof of principle for the use of this approach in a clinical setting. Accordingly, TACA-specific T cell clones derived from MUC1/MT transgenic mice recognize Tn-expressing tumors *in vitro*, further supporting that immunological tolerance has been successfully broken (*Immunology and Cell Biology*, 2005. 83:440, Figure 2).

The generation of TACA-specific CTL responses in healthy normal donors has been determined by *in vitro* priming, by using as immunogens the glycopeptide sequences earlier proposed and *in vitro* cultured dendritic cells (DC) as antigen presenting cells.
Glycopeptides have been found capable of binding class I MHC molecules with a higher affinity than corresponding natural peptide sequences (*Immunology and Cell Biology*, 2005. 83:440, Table 1). These results confirm the observation in the murine K^b allele model (*The Journal of Experimental Medicine*, 2004. 199:707, Table 1).
The demonstration that TACA conjugation does not affect MHC binding is very relevant in support of this vaccination strategy.
In terms of immunogenicity, Tn-containing glycopeptides have been proven capable of inducing a high CTL activation following a single stimulation *in vitro* in healthy human donors (*Immunology and Cell Biology*, 2005. 83:440, Table 2).

The breeding of the triple transgenic lineage (MUC1/MT/A2K^b) has been successful.
All the glycopeptides tested, including the designer Tn-containing glycopeptides, were immunogenic in these mice.
However, we were not satisfied on the degree of carbohydrate specificity of the T cell repertoire generated in the triple transgenic lineage, often capable to recognize the peptide backbone used for carbohydrate conjugation with high avidity and affinity.
Unfortunately, although the MUC1/MT mouse model is ideal to study immunotherapy *in vivo*, the "humanized" triple transgenic lineage (MUC1/MT/A2/K^b) is not suitable to test the efficacy of A2-restricted TACA-containing glycopeptides in treating mammary tumors. We believe that the double expression of both, human and murine MHC molecules (A2/K_b) in some instances selects a T cell repertoire profoundly different from A2 human donors.

Because of the success of the anti-tumor responses generated in humans, we decided to expand the clonal repertoire and to transfer T cell clones *in vivo* in the triple transgenic

lineage as passive immunotherapy.

We generated a variety of TACA-specific T cell clones to define the most cross-reactive and select those with the best growing rate *in vitro* (T cell transfer requires a large number of functional T cells).
In support of this vaccine approach we found a large number of TACA-specific T clones capable of recognizing a variety of human A2 tumors *in vitro*.

The studies of T cell transfer are still in progress.

Key Research Accomplishments
1. TACA conjugation does not affect the MHC class I binding affinity of designer, or natural peptide sequences (*The Journal of Experimental Medicine*, 2004. 199:707, Table 1 and *Immunology and Cell Biology*, 2005. 83:440, Table 1).

2. TACA-containing glycopeptides successfully generate anti-TACA responses *in vivo* in mice (*The Journal of Experimental Medicine*, 2004. 199:707, Figure 1) and *in vitro* in healthy human donors (*Immunology and Cell Biology*, 2005. 83:440, Table 2).

3. The glycosilated amino acid linkers (S or Tr for *–O-* glycosylation) are recognized by TACA-specific T cells together with the TACA molecule (*The Journal of Experimental Medicine*, 2004. 199:707, Figure 6).

4. TACA-specific TcRs generated with this strategy are highly cross-reactive and capable of recognizing different glycopeptide sequences, carrying the TACA antigen at the same position (*The Journal of Experimental Medicine*, 2004. 199:707, Figure 6). This is a great advantage in cancer immunotherapy while the sequences of endogenous peptides carrying the TACA antigens are unknown.

5. TACA-containing glycopeptides are capable of breaking immunological tolerance in mice that are genetically manipulated to develop spontaneous mammary TACA-expressing tumors (*Immunology and Cell Biology*, 2005. 83:440, Figures 1and 2)

Reportable Outcomes:
Yanfei Xu, Sandra J. Gendler and **Franco, A**. 2004. Designer glycopeptides for CTL-based elimination of carcinomas. *The Journal of Experimental Medicine*, Vol. 199, pp. 707-716.

Franco, A. 2005. CTL-based cancer preventive/therapeutic vaccines for carcinomas: role of tumor associated carbohydrate antigens. Frontlines review. *Scandinavian J. of Immunol.*, Vol. 61:391-397.

Xu,Y., Sette, A., Sidney, J., Gendler, S., **Franco, A**. 2005. Tumor-associated carbohydrate antigens: a possible avenue for cancer prevention. *Immunol. And Cell Biol.*, Vol. 83:440-448.

Diaz-de-Durana, Y., Mantchev, G.T., Bram, R.J., **Franco, A**. 2006. TACI-BLyS signaling via B cell-dendritic cell cooperation is required for naïve CD8+ T-cell priming in vivo. *Blood.*, Vol. 107:594-601

The Journal of Experimental Medicine

Designer Glycopeptides for Cytotoxic T Cell–based Elimination of Carcinomas

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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

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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.

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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 ($H\text{-}2^b$) 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 ($H\text{-}2^b$) 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-

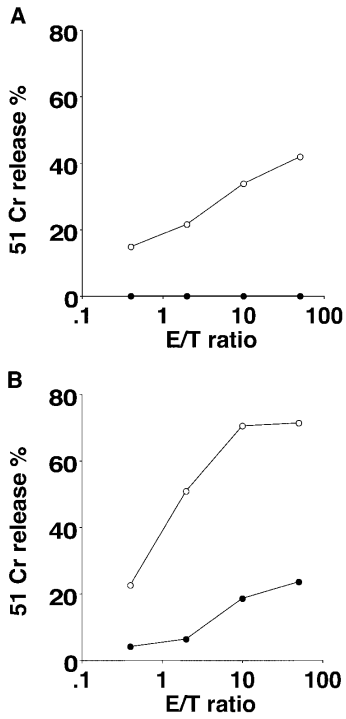

Figure 1. Specificity of primary CTL cell cultures tested in a ^{51}Cr release assay. Labeled EL-4 cells (H-2^b) were incubated with different cell number of T cell blasts in the presence of 1 μg/ml of the immunogen glycopeptide or the unglycosylated peptide backbone. EL-4 cells plated with T cell blasts 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).

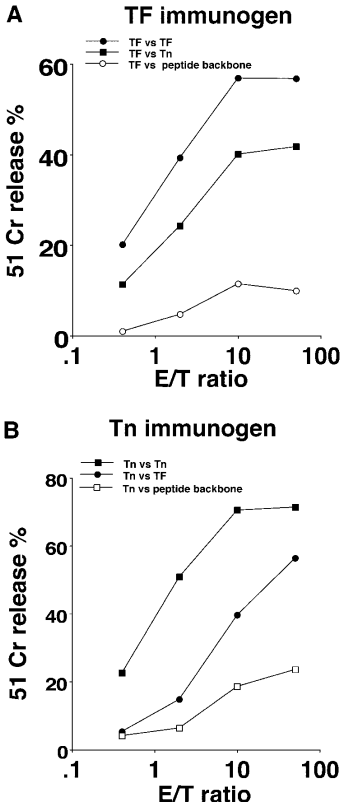

Figure 2. Cross-reactivity between TF and Tn. Splenocytes from immunized mice were pooled in a representative experiment. (A) Average CTL response in 10 mice primed with the TF-serine glycopeptide (filled circles) and the cross-reactive response to Tn (filled squares). (B) Average CTL response of 10 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).

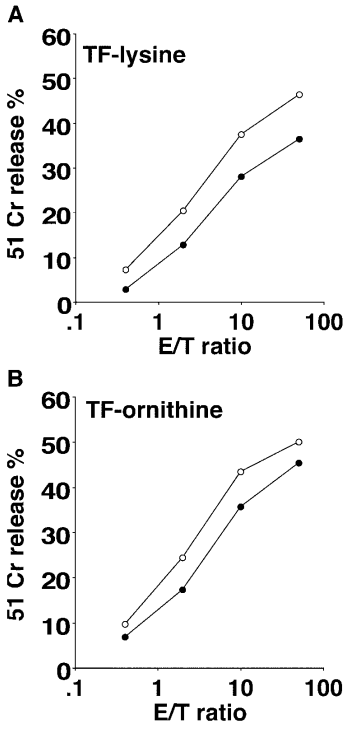

Figure 3. Immunogenicity of glycopeptides containing TF conjugated to lysine or ornithine at position 5. TF-lysine (A) and TF-ornithine (B) were tested and compared for their ability to generate TF-specific CTL. The primary CTL response in six mice primed with each glycopeptide is shown (spleens pooled). 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-

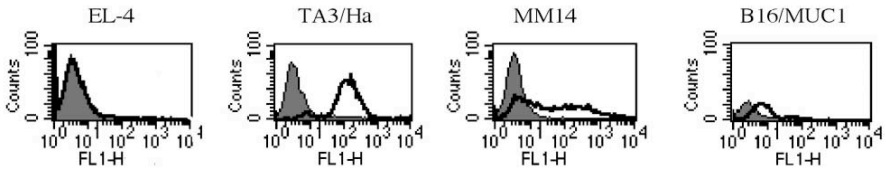

Figure 4. TF expression by different tumor cell lines. IgM TF1 mAb and anti–IgM-FITC have been used in these experiments where the isotype control is also shown.

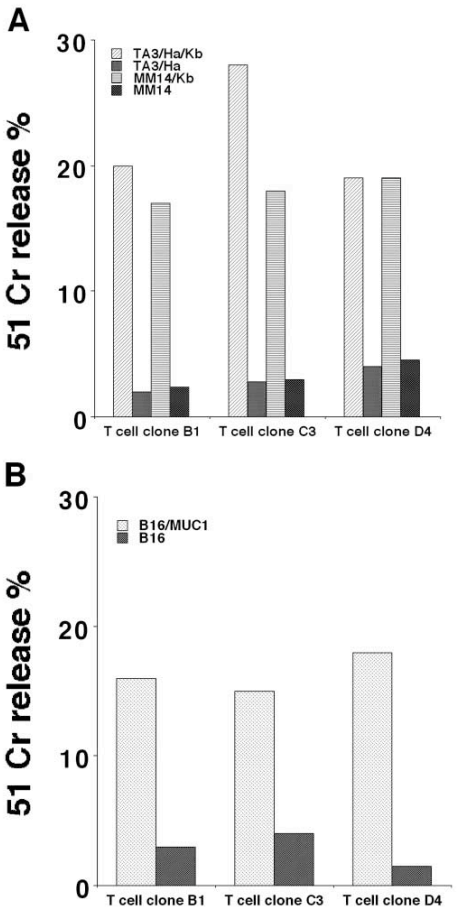

Figure 5. Endogenous recognition of TF-specific CTL clones. (A) Three representative TF-specific T cell clones recognize K^b-transfected carcinomas that express TF, but not the parental tumor lines H-2 mismatched ($H\text{-}2^a$). (B) B16 melanoma ($H\text{-}2^b$) transfected with the MUC1 gene is also 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

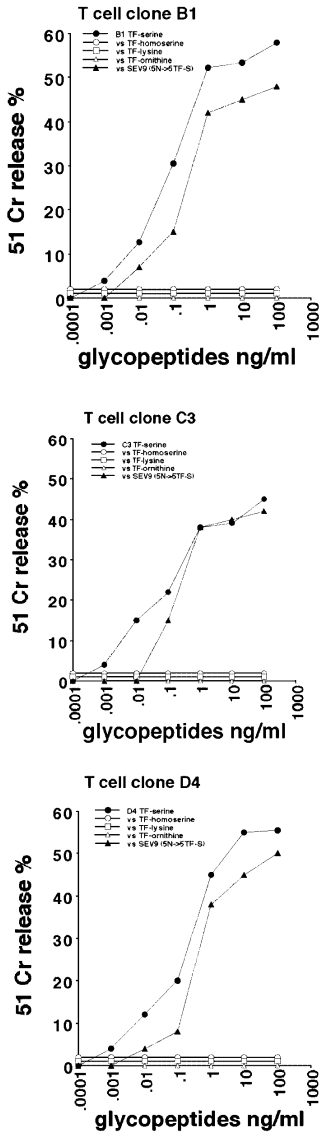

Figure 6. Fine specificity of TF-specific CTL clones. The fine specificity of three representative CTL clones that recognize TF endogenously expressed on tumor cells have been characterized by studying the cross-reactivity to TF-hs–, TF-lysine–, and TF-ornithine–glycopeptides. A modified viral natural 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.

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Special Feature

Tumor-associated carbohydrate antigens: A possible avenue for cancer prevention

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Summary Here we examine the use of glycopeptides containing tumour-associated carbohydrate antigens (TACA) as potential preventive vaccines for carcinomas. Our recent results suggest that CD8+ T cells (CTL) are capable of recognizing TACA in a conventional class I MHC-restricted fashion. The Thomsen–Friedenreich antigen (TF), a disaccharide, and Tn, its immediate precursor, are TACA largely expressed in carcinomas. TF and Tn can be successfully used as Th-independent vaccines when conjugated to designer peptides with optimal binding affinity for class I MHC molecules. TF- and Tn-specific CTL generated using this strategy are capable of recognizing TACA-expressing tumours *in vitro*, suggesting that glycopeptides are as effectively presented by class I MHC molecules as non-glycosylated peptides. Because the exact sequences of endogenously synthesized glycopeptides are unknown, the TACA-specific T cell repertoire elicited by carbohydrate-based vaccines is assumed to be degenerate. Here we report that mice genetically manipulated to develop TACA-expressing mammary tumours are not tolerant to glycopeptide vaccination. Moreover, we tested the immunogenicity of designer glycopeptides capable of binding multiple HLA alleles as a novel approach for the development of vaccines potentially useful for vaccination of a large fraction of the general population. Our results have suggested that CTL derived from normal donors respond with high efficiency to glycopeptides *in vitro*, opening a new avenue for the design of prospective vaccines for cancer prevention.

Key words: glycopeptide, immunotherapy, TACA, Tn.

Introduction

Ehrlich suggested that the immune system 'recognizes' primary developing tumours prior to the discovery of cell types, molecules and effector mechanisms that represent the modern science of immunology.[1] It was not until 50 years later that the concept of 'cancer immunosurveillance' was proposed,[2,3] which described lymphocytes as sentinels that recognize and eliminate continuously arising nascent transformed cells to maintain tissue homeostasis. Today we believe that the immune system controls, but also facilitates, tumour progression by 'sculpting' the immunogenic phenotype of developing tumours. This process has been defined as 'immunoediting' (reviewed in Dunn *et al.*[4]).

The lymphoid cells of acquired immunity play a critical and defined role in the host defence against cancer. In fact, B and T cells have been formally proven to be crucial in cancer surveillance, because mice deficient in the recombination activating gene (RAG-2-deficient) develop spontaneous malignancies,[5] and tumours grown in RAG-2-deficient hosts are less susceptible to immunoediting.[6]

CD8+ T cells (CTL) recognize MHC class I-restricted antigens derived from newly synthesized viral or neoplastic proteins and glycoproteins as a product of an endogenous processing pathway.[7] MHC class I molecules are constitutively expressed on somatic cells including epithelial cells. MHC class II molecules, however, are expressed on lymphoid and myeloid cells, but rarely on somatic cells, and are only induced by inflammatory stimuli, which is the reason why CTL have been preferentially targeted in cancer immunotherapy.

Much time has been expended in an effort to characterize the tumour epitopes recognized by T cells,[8] using diverse technologies.[9] Relevant tumour-specific antigens have been successfully discovered in some tumour types, including melanoma[9] and breast cancer.[10–15] Unfortunately, tumour antigens are often tissue-specific and do not share antigenic patterns, meaning that it is very difficult to generate preventive vaccines for a large population.

A group of tumour-associated antigens have been identified and characterized by virtue of their reactivity with antibodies and lectins that are carbohydrate in nature and called tumour-associated carbohydrate antigens (TACA).[16] Tumor associated carbohydrate antigens (TACA) are broadly expressed as a product of aberrant glycosylation in a large number of tumours and may represent a unique tool for prophylactic or therapeutic vaccination.[17–22] Aberrant glycosylation has been found in all tumour cells examined and the expression of TACA, the result of this process, influences the prognosis and survival of cancer patients in a manner that is proportional to the degree of expression.[23] TACA are present in tumours more

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frequently than oncogene products (e.g. myc, ras^k, HER2/neu) and their association with tumour progression is stronger than the deletion or inactivation of tumour-suppressing genes (e.g. p53, p16).[22] In fact, TACA are not only tumour markers, but constitute part of the machinery that is essential for inducing metastasis and invasiveness.[24]

The Thomsen–Friedenreich antigen (TF or T) (β-Gal-[1-3]-α-GalNAc-*O*-serine)[25,26] and its precursor Tn (GalNAc-*O*-serine)[27] are exclusively expressed in carcinomas but not in normal tissues.[28,29] Their small size and broad expression during the early stages of cancer transformation in several tumour types including bladder, colorectal, gastrointestinal, prostate, ovarian and lung carcinomas[30–34] indicate that TF and Tn are potential targets for CTL-based cancer prevention.

Our previous work demonstrated that it is possible to generate *in vivo* an anti-TF- and anti-Tn-specific CTL repertoire that is capable of recognizing TACA-expressing tumours, including MUC1-expressing tumours, by immunizing mice with designer glycopeptides.[35]

Here we show that mice that have been genetically manipulated to develop spontaneous mammary tumours that express the MUC1 glycoprotein[36] are not tolerant to glycopeptide vaccination. This lack of tolerance is independent from age and tumour progression.

Importantly, conjugation with small carbohydrate molecules to the peptides does not affect binding to the MHC. Moreover, Tn-containing natural viral sequences capable of binding multiple class I MHC alleles are highly immunogenic *in vitro* when tested in normal human donors, indicating that TACA-based CTL vaccines hold great promise for cancer prevention.

Materials and methods

MUC1 transgenic mice and development of MUC1/MT double transgenic mice

The MUC1 transgenic mice were developed by injection of a 10.5 kb genomic *Sac*II fragment containing the entire MUC1 gene sequence, as well as 1.5 kb of 5 sequence and 800 bases of 3 sequence into the pronuclei of fertilized C57BL/6 mouse eggs, which were then transferred to pseudopregnant females.[37] Founder mice were identified by Southern blot analysis and one founder (79.24), which showed appropriate tissue-specific expression of MUC1, was selected for further study. The level and pattern of MUC1 expression in the various organs was similar to that seen in humans. Low levels of expression are observed in normal tissues, with overexpression occurring following the development of tumours in the mammary gland and pancreas.[38,39]

Mice were genotyped by PCR using the following primers: 5′-CTTGCCAGCCATAGCACCAAG-3′ (bp 745–765)[40] and 5′-CTCCACGTCGTGGACATTGATG-3′ (bp 1086–1065).[37,40] MUC1 transgenic mice were maintained continuously on the C57BL/6 background.

Development of spontaneous tumours in MUC1 transgenic mice

To generate spontaneous tumours, MUC1 transgenic mice were bred with mice carrying the MMTV-driven polyoma middle T antigen (MT) to create MUC1/MT mice.[39,41] Prior to generating the double transgenics, the MT mice had been backcrossed 10 generations onto C57BL/6 mice, which made them congenic. The offspring were screened for the polyoma middle T transgene using PCR on 100–500 ng genomic DNA prepared from tail snips with the following primers: 5′-AGTCACTGCTACTGCACCCAG-3′ (bp 282–302) and 5′-CTCTCCTCAGTTCTTCGCTCC-3′ (bp 817–837).[39] PCR reactions (50 (L) contained 2% deionized formamide, 0.2 mmol/L dNTP mix, 1× PCR buffer, 1 (mol/L forward and reverse primers, and 1.75 mmol/L $MgCl_2$. PCR conditions were as follows: a 10 min hot start at 94°C and 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, followed by 72°C for 10 min. Polyoma middle T antigen-positive female mice developed multifocal mammary adenocarcinomas. Tumours were excised when they reached 1 cm in diameter, then they were fixed in methacarn, embedded in paraffin and stained for MUC1 using a humanized HMFG-1 mAb (at 1:5000) and a secondary peroxidase-conjugated antihuman IgG (1:50). This method enabled better differentiation between low and high levels of MUC1 expression while avoiding the background staining that was detected when using an unlabelled murine mAb.

Peptides and glycopeptides

The peptides used in this study were synthesized by Fmoc chemistry using a multiple peptide synthesizer (Symphony/Multiplex, Protein Technologies, Tucson, AZ, USA). Peptides were cleaved automatically on the synthesizer using trifluoroacetic acid as a cleavage reagent. Peptides were ≥97% pure as assessed by C18 reverse phase HPLC, and the identity of the peptides was verified by mass spectroscopy. One designer alanine-rich peptide with the appropriate K^b anchors, and two natural sequences derived from the hepatitis B virus (HBV) and one from HIV were Tn-conjugated either in position 4 or in position 5 to produce the K^b-binding designer alanine-rich AIIA(GalNAc-*O*-S)FAAL, HBV pol 635–643 GLYS(GalNAc-*O*-S)STVPV, HBV core 18–27 FLP(GalNAc-*O*-S)DFFPSV, and HIV gp 120 272–281 TLT(GalNAc-*O*-S)CNTSV.

The Tn-containing glycopeptides (Tn (GalNAc-*O*-)) utilized in this study were prepared by solid phase synthesis using glycosylated amino acids as building blocks, as previously described.[42] Glycopeptides were ≥97% pure as assessed by C18 reverse phase HPLC, and the identity of the peptides was verified by mass spectroscopy.

MHC binding assay

The EBV-transformed cell lines JY (A*0201, B*0702), M7B (A*0202/A*0301, B*3501/B*5301), FUN (A*0203/A*0301, B*4601/B*3501), CLA (A*0206/A*2402, B*0801/B*3502), and AMAI (A*6802, B*5301) were used as the primary sources of HLA A class I molecules. Cells were maintained *in vitro* and HLA molecules were purified by affinity chromatography as previously described.[43] Quantitative assays to measure the binding of peptides to HLA A*0201, A*0202, A*0203, A*0206, and A*6802 molecules were based on the inhibition of binding of a radiolabelled standard peptide.[43,44] Briefly, 1–10 nmol/L of radiolabelled peptide was coincubated at room temperature with 1 (mol/L to 1 nmol/L of purified MHC in the presence of 1 (mol/L human β_2-microglobulin (Scripps Laboratories, San Diego, CA, USA) and a cocktail of protease inhibitors. After a 2 day incubation, binding of the radiolabelled peptide to the corresponding MHC class I molecule was determined by capturing the MHC–peptide complexes on Greiner Lumitrac 600 microplates (Greiner Bio-one, Longwood, FL, USA) coated with the W6/32 antibody, and measuring bound cells using the TopCount microscintillation counter. In addition, following the 2 day incubation, the percentage of MHC-bound radioactivity was determined by size exclusion gel filtration chromatography using a TSK 2000 column.

Immunization protocols and characterization of CTL lines and clones derived from MUC1/MT double transgenic mice

Glycopeptides were emulsified in IFA and injected subcutaneously at a concentration of 50 μg, together with 140 μg of an IA^b-restricted Th epitope, the hepatitis B virus core antigen (HBc) sequence 128–140 (TPPAYRPPNAPIL).[45] Seven days after priming, mice were killed. Splenocytes from each experimental group were pooled and stimulated *in vitro* in the presence of the glycopeptide that was used as an 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, St Louis, MO, USA) and dextran sulphate (DxS; Pharmacia Biotech AB, Uppsala, Sweden). The culture medium consisted of RPMI 1640 (Life Technologies, Gaithersburg, MD, USA) supplemented with 20 mmol/L glutamine, 100 μg streptomycin, 100 U/mL penicillin, 1 mM sodium pyruvate, 0.1 mmol/L non-essential amino acids (Life Technologies), 50 μmol/L 2-ME, and 10% heat inactivated FCS (Life Technologies). After 5 days in culture, cells were collected, purified over Ficoll gradients, and T cell blasts were cultured in complete RPMI conditioned with supernatant from splenocytes activated with ConA (Sigma, St Louis, MO, USA) as an IL-2 source. Two days 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 ratios in duplicate, with and without antigens. Net specific lysis was calculated by subtracting the cytolytic response to EL-4 cells in the absence of glycopeptides or peptides, which was defined as background. Data were calculated as percentage cytotoxicity = [(sample – spontaneous release)/(maximum release – spontaneous release)] × 100.

T cell clones were generated from selected Tn-specific CTL lines at 6–7 days following a single *in vitro* stimulation with the immunizing glycopeptide to avoid *in vitro* selection. Briefly, after Ficoll purification, T cell blasts were plated in 96 well U- bottom plates at 0.5 cells per well in the presence of 1 μg/mL glycopeptide and irradiated LPS/DxS activated B cell blasts. Growing wells were expanded with ConA supernatant and restimulated with irradiated B cell blasts and glycopeptides. CTL clones were tested for specificity by using EL-4 targets. Endogenous recognition of the carbohydrate expressed by tumour cells was determined by using as targets for the ^{51}Cr release assay the mammary tumour line MMT, which is derived from a spontaneous syngeneic tumour in a MMT female (double transgenic MUC1/MT) and a MUC1-transfected B16 melanoma.[37] TACA expression on tumour cells was tested before the experiment in *in vitro* experiments by using anti-MUC1 monoclonal antibodies, the CT2[46] and anti-TF monoclonal antibodies, which were kindly provided by Dr Bo Jansson (Bioinvest, Luden, Sweden).[47]

Donors

In this study, PBMC were derived from volunteer normal donors aged between 40 and 57. Donors were genotyped by PCR to determine their human HLA class I1oci.

Human CTL cultures

Natural peptide sequence and corresponding Tn (GalNAc)-modified glycopeptides were compared side by side for their ability to prime *in vitro* naive cytotoxic T cells derived from PBMC of normal human donors by using autologous, antigen-pulsed, mature dendritic cells (DC) as APC.

Autologous DC were cultured with GM-CSF (kindly provided by Kirin, Gumna, Japan) and IL-4 (Peprotech, Rocky Hill, NJ, USA) and matured with 100 U/mL TNF-α as described previously.[48]

Results

MUC1/MMT are not tolerant to glycopeptide vaccination

TF and Tn are expressed by mucins encoded in humans by the MUC1 gene.[35] MUC1 is a large glycoprotein that is aberrantly expressed in several carcinomas, which makes it an attractive target for immunotherapy.[49] Here we address CTL tolerance to TACA-containing glycopeptides in MUC1/MT mice that spontaneously develop MUC1-expressing mammary tumours within 14 weeks after birth.[39] These mice were exposed in the embryo to TF and Tn TACA antigens. TF and Tn are precursors of the MN blood group substance expressed on glycophorin,[50] which is also expressed on the MUC1 found on tumour cells.[35]

The experiments in MUC1/MT transgenic mice were carried out with the Tn-containing alanine-rich glycopeptide AIIA(GalNAc-*O*-S)FAAL, which has previously been described as more immunogenic than its TF-containing variant AIIA(β-Gal-(1–3)-α-GalNAc-*O*-S)FAAL.[35] In these experiments, MUC1/MT mice were chosen according to age and stage of tumour development. Two mice per group (aged 14, 18, and 24 weeks) were immunized with the glycopeptide emulsified in IFA, coinjected subcutaneously together with an IA^b-restricted Th epitope, the HBc sequence (TPPAYRPPNAPIL) in repeated experiments.

Seven days after priming, the mice were killed. Splenocytes from each experimental group were pooled and stimulated *in vitro* in the presence of the glycopeptide that was used as the immunogen and irradiated B cell blasts as APC. After 5 days in culture, cells were collected, purified in a Ficoll gradient, and T cell blasts were cultured in complete RPMI conditioned with supernatant from ConA-activated splenocytes as an IL-2 source. Two days 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 a target at different E : T ratios in duplicate, with and without antigens.

Carbohydrate specificity was investigated by comparing the responses of the glycopeptide and its respective non-glycosylated counterpart. Net specific lysis was calculated by subtracting the cytolytic response to EL-4 cells alone without antigens, which was defined as background.

A strong CTL activity in response to the immunizing glycopeptide was generated in MUC1/MT transgenic mice, regardless of their age and tumour progression (Fig. 1). In contrast, greatly diminished activity was observed against the same peptide in the non-glycosylated form, demonstrating the generation of Tn-specific CTL that either do not use TCR contacts within the peptide backbone or are not activated if such contacts exist, as previously shown in wild type mice.[35]

Out of 30 MUC1/MT mice studied, 27 (90%) responded to Tn vaccination, suggesting that designer glycopeptides function as 'altered self'. In fact, no response to Tn has ever been detectable in the spleen of unprimed MUC1/MT mice in repeated experiments, proving that TACA-containing glycopeptides have a high potential to break the immunological tolerance to 'self' tumour antigens.

The immunotherapeutic potential of Tn-containing vaccines in this lineage are under investigation in our laboratory.

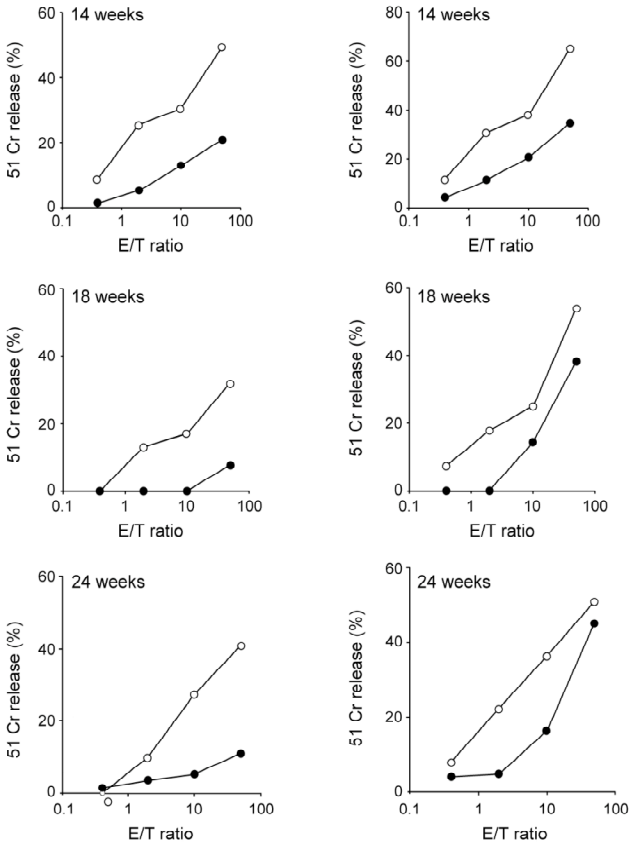

Figure 1 The CTL response to a Tn-containing glycopeptide vaccine in mice affected by Tn-expressing mammary tumours. The specificity of primary CTL cultures tested in a ^{51}Cr release assay is shown. Labelled EL-4 cells (H-2^b) were incubated with different numbers of T cell blasts in the presence of 1 μg/mL immunogen glycopeptide or unglycosylated peptide backbone. EL-4 cells plated with T cell blasts in the absence of antigens were assessed in duplicate at every E : T ratio to determine the extent of non-specific lysis. Cell culture supernatants were harvested 4 h later and the percentage of ^{51}Cr released was calculated as (sample – spontaneous release)/(maximum release – spontaneous release) × #100. Background lysis has been subtracted and net lysis is shown. Empty symbols represent carbohydrate specificity, whereas filled symbols indicate peptide specificity. A representative experiment where two mice at each of the ages 14 weeks, 18 weeks and 24 weeks, which were carrying tumours of different sizes, were studied is shown.

Tn-specific T cell clones kill MUC1-expressing tumours in vitro

The ability to recognize Tn-expressing tumour cells by using CTL generated from MUC1/MT mice immunized with glycopeptides is critical for the design of this vaccine. Tn-specific CTL clones were generated from independent T cell lines derived from MUC1/MT mice immunized with AIIA(GalNAc-*O*-S)FAAL. The T cell clonal repertoire after glycopeptide immunization was predominantly Tn-specific and had high avidity for the carbohydrate moiety, supporting the results obtained with primary T cell lines and confirming the observations obtained in wild type mice.[35]

Two tumour cell lines were selected as targets for these experiments: (i) MMT, a syngeneic mammary tumour carcinoma derived from a double transgenic female MUC1/MT mouse (MMT), that expresses Tn in the context of MUC1; and (ii) a MUC1-transfected B16 melanoma cell line.[37]

The dose–response relationship for the glycopeptide immunogen suggests the generation of high-affinity Tn-specific CTL clones (Fig. 2a). Moreover, because a large percentage of the syngeneic mammary tumour cells and the melanoma cell line transfected with MUC1 were killed, it indicates that Tn-specific CTL can recognize the TACA endogenously expressed in a MHC class I-dependent fashion (Fig. 2b).

Because the sequences of the endogenous glycopeptides that present Tn in the context of the tumour cell surface are unknown, the CTL clonal repertoire is shown to be highly degenerate. Differences in the magnitude of the CTL response against the two tumours by single clones and between clones prove that different glycopeptides are endogenously presented to T cells and that the Tn-specific T cell repertoire is diverse and heterogeneous.

Glycopeptides can be designed to bind multiple class I MHC alleles

To transfer the TACA-containing glycopeptide vaccine strategy from mice to humans, we designed Tn-containing glycopeptides that are potentially capable of binding multiple HLA

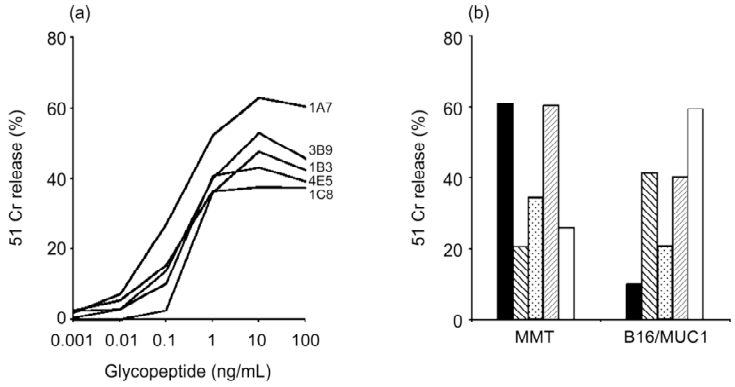

Figure 2 Characterization of Tn-specific CTL clones. (a) Dose–response relationship of five representative anti-Tn CTL clones to AIIA(GalNAc-*O*-S)FAAL measured in a classical ^{51}Cr release assay at a 2:1 E : T ratio. (b) *In vitro* killing of the syngeneic mammary tumour cell line MMT and the melanoma cells transfected with MUC1 and B16/MUC1, by anti-Tn specific CTL clones measured at a 20:1 E : T ratio. (■), 1A7; (▨), 3B9; (⊡), 1B3; (▨), 4E5; (□), 1C8.

Table 1 The HLA binding capacity of A2 binding peptides and their glycopeptide variants

				Binding capacity (IC50 nmol/L)					
Sequence bound	Organism	Protein	Position	A*0201	A*0202	A*0203	A*0206	A*6802	Alleles
FLP (GalNAc-*O*-S)DFFPSV	HBV	Core	18–27	0.19	2.6	0.31	1.0	1063	4
FLPSDFFPSV	HBV	Core	18–27	1.3	53	8.6	0.95	26096	4
GLYS(GalNAc-*O*-S)TVPV	HBV	Pol	635–643	0.25	0.45	0.46	8.1	1315	4
GLYSSTVPV	HBV	Pol	635–643	0.24	0.45	1.4	9.3	3415	4
TLT(GalNAc-*O*-S)CNTSV	HIV-1	gp120	272–281	0.72	15	8.2	24	2627	4
TLTSCNTSV	HIV-1	gp120	272–281	0.29	8.1	13	15	1151	4

HBV, hepatitis B virus.

class I alleles.[51] Class I MHC molecules are expressed at three loci in the human genome and comprise a large number of different molecules, with patterns of sequence conservation and polymorphism.[52] Each of the class I MHC proteins is capable of binding a set of peptides of diverse sequence motifs.[53] The mechanism by which this sequence degenerate binding occurs has been elucidated in the past 10 years.[54–59] Interactions between conserved MHC side chains and polar main chain atoms of the peptide termini provide a universal peptide binding capability. Additional binding energy and limited peptide sequence are selectively introduced by the binding of a few peptide anchor side chains in pockets formed by the MHC side chain that are polymorphic.[53,58,60]

Crystal structures of murine and human class I MHC complexes[54,61] reveal that bound peptides are mainly expressed in the centre of the cleft, where the peptides 'bulge' from the floor of the peptide binding site.

Our experimental design takes advantage of this knowledge. We designed glycopeptides that are potentially capable of binding multiple class I MHC alleles, hoping to skew the fine specificity of the T cells towards the carbohydrate by introducing serine substitutions and the conjugation of *O*-linked Tn at major TCR amino acid residue(s). Two of the peptides that were glycosylated are well-known HBV-derived CTL epitopes previously described in the literature, including the immunodominant HBV core 18–27,[59,62–66] and the HBV pol 635–643.[66,67] Another epitope chosen for glycosylation was the previously described HIV gp 120 272–281.[68,69] Each of the peptides studied had a serine residue at a position predicted to be a major TCR contact and are not likely to interfere with HLA binding capacity, which is ideal for the attachment of sugar residues.

As shown in Table 1, peptides were confirmed to be good A*0201 binders, with binding affinities in the 0.2–72 nmol/L range. Each of the Tn-containing glycopeptide variants also bound with good affinity, and in general bound as well or better than the wild type peptide. The A*02 degenerate binding capacity of the glycopeptides was also retained.

These observations justify the use of the glycopeptide variants in functional experiments to evaluate their immunogenicity and also suggest that they are good candidates for the development of preventive or therapeutic vaccines with broad population coverage.

Table 2 The CTL response to A2 binding peptides and their glycopeptide variants

Sequences	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6	Donor 7	Total
FLP(GalNAc-*O*-S)DFFPSV	5	5	1	3	4	2	4	24
FLPSDFFPSV	1	1	3	6	3	2	4	20
GLYS(GalNAc-*O*-S)TVPV	6	2	1	1	4	4	2	20
GLSYSSTVP	2	0	1	3	5	2	2	15
TLT(GalNAc-*O*-S)CNTSV	7	4	0	1	5	1	5	23
TLTSCNTSV	1	0	0	7	3	5	2	18

Data are the immunogenicity of Tn-containing glycopeptides compared to natural unglycosylated sequences.
The number of specific CTL lines (≥20% specific lysis) out of 12 from 7 individual donors is shown.

Tn-containing glycopeptides prime with high efficiency naive CD8+ T cells derived from normal A2 donors

The three glycopeptides described in Table 1 were studied for their ability to prime *in vitro* naive cytotoxic T cells derived from PBMC of normal donors. Seven HLA-typed A2 donors, aged between 40 and 57 years, were included in this study.

Autologous, antigen-pulsed, mature DC were used as APC for *in vitro* priming. Twelve CTL lines/glycopeptides and 12 CTL lines/peptides were established by coculturing in 48 well flat bottomed plates 5×10^5 fresh PBMC per well and 1×10^5 mature DC per well, which had previously been pulsed for 2 h with glycopeptides or peptides. Each well defined an individual CTL line.

Analysis using FACS to determine the number of CD8+ T cells in the PBMC population indicated that CD8+ T cells represented an average of 25% of the total PBMC, ranging from 13% to 37% in different donors. The small number of CD8+ T cells/T cell lines enabled us to determine the approximate precursor frequency of *in vitro* primed naive CTL.

Each experiment also included 12 CTL lines primed *in vitro* with an irrelevant K^b-restricted peptide derived from chicken ovalbumin (OVA) 57–64[70] as a negative control. The T cell cultures received IL-2 (Peprotech) twice (day 5 and 8) and they were tested for specificity at day 10 in a classical ^{51}Cr release assay.

T2 cells[71] were used as targets in these experiments. Each T cell line was assayed at a 3:1 E : T ratio. All the T cell lines were tested individually for their ability to recognize the glycopeptide immunogens, the peptide backbones and the T2 cell line used as targets in the absence of antigens (background control).

The results of these experiments are summarized in Table 2, where the number of specific lines giving a net specific lysis ≥20% in seven normal donors is reported. Carbohydrate specificity was defined as a specific response to the glycopeptides minus the specific response to the corresponding peptide backbone.

A large number of glycopeptide-specific and peptide-specific CTL lines were derived with a single stimulation *in vitro* in the absence of T cell help, suggesting that antigens that bind HLA molecules with high affinity are Th-independent as previously shown in the K^b model.[35] Moreover, glycopeptides are more immunogenic than peptides, as shown by the number of specific T cell lines obtained by summarizing and comparing the specific CTL responses to Tn-modified and natural peptide sequences. The large number of specific CTL lines obtained, starting from a low number of CD8+ T cells per T cell line, strongly suggest a high precursor frequency of the T cell carbohydrate-specific repertoire in the periphery.

Discussion

We previously demonstrated that TACA-containing designer glycopeptides could generate, *in vivo*, a CTL repertoire that is highly carbohydrate-specific and capable of killing TACA-expressing tumours *in vitro*.[35] Here we show that the same vaccination strategy is successful in generating TACA-specific CTL in mice that develop spontaneous mammary tumours that express the TACA antigen in the context of mucin,[35] namely MUC1/MT.[39]

T cell clones derived from immunized MUC1/MT mice recognize the glycopeptide antigens with high affinity, and kill *in vitro* Tn-expressing tumour cells in the context of the MUC1 glycoprotein,[72] proving that this immunization strategy can break T cell tolerance to 'self' tumour antigens. In fact, Tn-specific CTL are not detectable in unprimed littermates.

In the present study, we found that TACA are presented in the context of endogenous glycopeptides of unknown sequences, showing that the CTL response is very degenerate, which is a clear advantage in immunotherapy.[73]

Because the glycosylated amino acid linker (serine) is also recognized by carbohydrate-specific TCR, we believe that glycosylation of suitable amino acid residues within MHC–peptide complexes may be very common *in vivo*, as suggested by the rabies virus model.[74] Similarly, as glycopeptides are presented to T cells in the thymus, they may contribute to positive selection, shaping the repertoire of T cells that reach the periphery. In fact, the large majority of TACA are embryonic antigens, and are not represented in normal tissues.[22]

Moreover, our results using A2-restricted Tn-conjugated viral sequences indicate that Tn-conjugation does not affect binding to HLA class I molecules and, in fact, increases the immunogenicity of wild type sequences. In support of this assertion is the observation that the TACA conjugation was well tolerated by TCR. The amino acid contacts used for glycosylation in the HBV core 18–27[63,75] and the HIV gp 120 272–281 determinants[76] were shown to be susceptible to mutations due to immune pressure, leading to CTL inhibition. However, serine glycosylation at the same position did not negatively affect, but actually increased the immunogenicity compared with the wild type sequences.

The evidence that normal donors respond to glycopeptide vaccination *in vitro* is very promising for future clinical trials. We have also shown that glycopeptides containing TACA antigens expressed in different tumour types can be designed that bind multiple class I MHC alleles, indicating the possibility for preventive vaccination of a large fraction of the general population.

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