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Cancer Research

Tumor Antigen Epitopes Interpreted by the Immune System as Self or Abnormal-Self Differentially Affect Cancer Vaccine Responses

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Abstract

Epitope selection is an important consideration in the design of cancer vaccines, but factors affecting selection are not fully understood. We compared the immune responses to peptides and glycopeptides from the common human tumor antigen MUC1, a mucin that is coated with O-linked carbohydrates in its variable number of tandem repeats (VNTR) region. MUC1 expressed on tumor cells is characteristically underglycosylated, creating peptide and glycopeptide neoepitopes that are recognized by the immune system. The response to VNTR peptides is weaker in MUC1-transgenic mice (MUC1-Tg mice) than in wild-type (WT) mice, whereas the response to VNTR glycopeptides is equally strong in the two strains. Thus, glycopeptides seem to be recognized as foreign, whereas peptides, although immunogenic, are perceived as self. To explore this further, we generated MUC1 peptide– and glycopeptide–specific T-cell receptor transgenic mice and studied the function of their CD4 T cells when adoptively transferred into MUC1-Tg or WT mice. Peptide-specific T-cell precursors were not centrally deleted in MUC1-Tg mice and did not acquire a T regulatory phenotype. However, their response to the cognate peptide was reduced in MUC1-Tg mice compared with WT mice. In contrast, glycopeptide-specific CD4 T cells responded equally well in the two hosts and, when simultaneously activated, also enhanced the peptide-specific T-cell responses. Our data show that the immune system differentially recognizes various epitopes of tumor-associated antigens either as self or as foreign, and this controls the strength of antitumor immunity. This represents an important consideration for designing safe and effective cancer vaccines. *Cancer Res; 70(14); OF1–9. ©2010 AACR.*

Introduction

Transformed cells express many self-derived tumor-associated antigens (TAA; ref. 1) that can elicit antibody and T-cell responses in cancer patients. However, studies in transgenic and knockout mice indicate that antitumor immunity may be hindered by central and/or peripheral self-tolerance through self-tumor antigen expressed on normal tissues (2–4). This may explain why attempts to boost these responses have been met with limited success.

Abnormal expression of many self molecules through pre- and post-translational modifications generates a spectrum of tumor-specific epitopes. These provide potential targets for eliciting tumor immunity without the risk of autoimmunity that is associated with breaking tolerance to self (5). The transmembrane glycoprotein Mucin 1 (MUC1) is overexpressed by most human adenocarcinomas in an aberrantly glycosylated form containing characteristic short O-linked sugar chains and exposed nonglycosylated protein backbone in the variable number of tandem repeat (VNTR) region. Each VNTR tandem repeat is a 20-amino-acid sequence, HGVTSAPDTRPAPGSTAPPA, which can vary in number from fewer than 25 to more than 125 repeats per allele, effectively dominating the extracellular domain of MUC1 (6). MUC1 VNTR peptide–specific $CD8^+$ CTL are found in patients with $MUC1^+$ tumors, indicating that an immune response can be generated against them (7, 8). However, clinical trials using MUC1 VNTR peptide–based vaccines to boost this immunity in cancer patients have resulted in only marginal increases in CTL activity and ineffective anti-MUC1 antibody class switching (9, 10). The MUC1-transgenic (MUC1-Tg) mouse, which displays the same tissue-specific expression of human MUC1 seen in healthy and diseased human tissues (11, 12), mounts only low antibody and CTL responses to MUC1 VNTR peptides, due in part to reduced responses of MUC1 peptide–specific CD4 T cells compared with those in transgene-negative (wild-type; WT) littermates (13–15). This highlights the critical role of CD4 T-helper cells in promoting antitumor immunity, effective B-cell priming,

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antibody isotype switching, CTL expansion, and CD8 T memory cell responses (16).

Tumor-associated glycoprotein antigens that do not show mutations in their peptide sequence could also be targeted via aberrantly glycosylated and thus tumor-specific glycopeptide epitopes. For MUC1, those are peptides carrying Tn (GalNAc-O-S/T) and T (Gal-GalNAc-O-S/T) glycans (17). On normal cells, these core carbohydrates are further glycosylated to form complex oligosaccharides and thus are not exposed. Aberrant glycosylation in ~90% of adenocarcinomas reveals the Tn and T antigens (18) and allows tumor-associated MUC1 glycopeptides carrying these core glycans to be processed by antigen-presenting cells (APC) and presented on class I and class II MHC, making them targets for T-cell recognition and antitumor immunity (19–21). Indeed, responses to MUC1 glycopeptide in the MUC1-Tg mouse are stronger than those obtained against the MUC1 peptide (22).

To study the mechanisms that cause this disparity in CD4 T-cell immunity to MUC1 peptide versus glycopeptide epitopes, we generated two new MUC1-specific T-cell receptor (TCR) transgenic mice on the WT background. One mouse (RFT) expresses a TCR that preferentially recognizes the MUC1 glycopeptide carrying the tumor-associated Tn glycan (TnMUC1); the other (VFT) is specific for the unglycosylated MUC1 peptide (MUC1p). We show that MUC1p-specific VFT CD4 T cells are not deleted during thymic development or in the periphery of MUC1-Tg mice. However, on antigen-specific stimulation, their proliferation in MUC1-Tg mice was attenuated compared with proliferation in WT mice. TnMUC1-specific RFT CD4 T cells, however, respond equally well in WT and MUC1-Tg mice, thereby mimicking the behavior of OTII CD4 T cells specific for the foreign antigen ovalbumin (ova). Furthermore, coactivation of TnMUC1-specific T cells in the MUC1-Tg mouse confers "help" to MUC1p-specific T cells, raising their activation to levels obtained in WT mice.

Materials and Methods

Mice and cell lines

Mice were bred and maintained in specific pathogen-free conditions at the University of Pittsburgh and treated under Institutional Animal Care and Use Committee–approved guidelines in accordance with approved protocols. C57BL/6, B6.PL-Thy1a/Cy, B6.SJL-PtprcaPepcb/BoyJ, and BALB/c mice were purchased from The Jackson Laboratory, and MUC1-Tg mice (12) were purchased from Dr. S. Gendler (Mayo Clinic, Scottsdale, AZ). MUC1-transgene–positive and MUC1-transgene–negative (WT) mice from heterozygous breeding were identified by PCR analysis. VFT and RFT TCR-transgenic mice were generated at the University of Pittsburgh Transgenic Mouse Facility.

VF5 and RF6 T-cell hybridomas were generated as previously described (20, 22). Briefly, WT C57BL/6 mice were immunized three times with dendritic cells (DC) loaded with either MUC1p or TnMUC1. Seven days after the final immunization, splenocytes and lymph node cells were restimulated *in vitro* and fused with the BW5147 lymphoma using polyethylene glycol 1500 (Roche). $CD3^+CD4^+$ hybridomas were screened by fluorescence-activated cell sorting (FACS), then selected for interleukin-2 production in response to TnMUC1 or MUC1p. Hybridomas were subsequently cloned by limiting dilution.

TCR cloning

TnMUC1-specific TCRα/β cDNAs from the RF6 hybridoma (22) were amplified by 5′ rapid amplification of cDNA ends (5′-RACE) using a Generacer Kit (Invitrogen) in combination with reverse primers specific for TCR-Cα and TCR-Cβ constant regions. MUC1p-specific TCRα cDNA from the VF5 hybridoma (20) was similarly amplified by 5′-RACE. VF5 TCRβ cDNA was amplified using degenerate Vβ primers (23) and reverse primers specific for TCR-Cβ. The amplified TCR chains were sequenced at the University of Pittsburgh Sequencing Facility. MUC1p-specific VF5 TCR contains Vα2.5-Jα49 and Vβ6-Jβ2.5. TnMUC1-specific RF6 TCR contains Vα4.1-Jα16 and Vβ15-Jβ1.3. These rearranged TCR gene segments, including ~150 bp (VF5) or ~100 bp (RF6) of intron downstream of the J gene segments, were cloned from genomic DNA of the VF5 and RF6 hybridomas into pcDNA3.1/V5-His (Invitrogen; VF5) or TOPO TA (Invitrogen; RF6), then subcloned into the TCR cassette vectors pTα or pTβ (a generous gift from Drs. Diane Mathis and Christophe Benoist, Harvard Medical School, Boston, MA; ref. 24). TCR expression constructs were resequenced and tested for functional expression by transfection into DO11.10 or 58a-b- (25) hybridomas.

Generation of TCR-transgenic mice

Linear pTα-VF5α and pTβ-VF5β constructs were microinjected into B6.PL-Thy1a/Cy ($CD90.1^+$) embryos. Linear pTα-RF6α and pTβ-RF6β constructs were microinjected into B6.SJL-PtprcaPepcb/BoyJ ($CD45.1^+$) embryos. VF5α and VF5β founder mice were identified by PCR of tail tissue DNA using primers specific for the Vα2-Jα49 and Vβ6-Jβ2.5 rearrangements. RF6α and RF6β founder mice were identified by PCR for the Vα4.1-Jα16 and Vβ15-Jβ1.3 rearrangements. Founder mice were crossbred to produce double-transgenic $CD90.1^+$VFT and $CD45.1^+$RFT mice.

VFT and RFT TCR transgene expression is controlled by the natural TCRα and TCRβ promoter/enhancer elements included in the cassette vectors (24). Antigen recognition in VFT-Tg and RFT-Tg mice is mediated by $CD4^+$ T cells (Supplementary Fig. S1). Supplementary Fig. S2 shows the preferential recognition of the MUC1 peptide by VFT-Tg CD4 T cells and of the MUC1 glycopeptide by RFT-Tg CD4 T cells.

Flow cytometry

Cells were labeled with the indicated antibodies at 1:50 dilution in FACS buffer (PBS, 5% fetal bovine serum, 0.01% sodium azide) for 30 minutes on ice. Intracellular labeling was done with the BD Cytofix/Cytoperm Solution Kit (BD Biosciences). Labeled cells were analyzed on a LSR II Flow Cytometer using FACSDiva software (BD Biosciences).

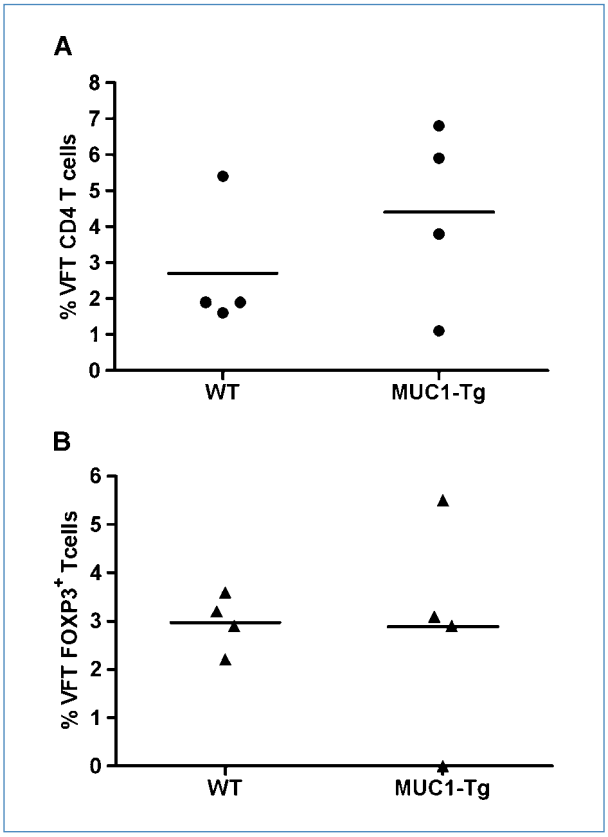

Figure 1. VFT precursors develop through the thymus and enter the periphery at equal levels in WT and MUC1-Tg mice. Recipient mice (WT, $n = 4$; MUC1-Tg, $n = 4$) were lethally irradiated before bone marrow transfer. Five weeks after VFT bone marrow transfer, the presence of mature donor VFT CD4 T cells in the spleens of recipient mice was assessed by flow cytometry. A, percent of donor cells ($V\alpha2^+CD90.1^+$) in the $CD3^+CD4^+$ gated population of each recipient mouse. B, intracellular Foxp3 expression in donor cells. Horizontal bars, mean values. Representative data of two independent experiments.

Bone marrow transplantation

Lineage-negative bone marrow precursors were purified from VFT-Tg mice using a Lineage Cell Depletion Kit (Miltenyi Biotech). WT or MUC1-Tg recipient mice were irradiated (900Rad) 4 hours before i.v. injection with 10^5 lineage-negative cells, plus 2×10^5 host-type whole bone marrow cells to ensure recipient survival (26). The presence of VFT T cells and intracellular Foxp3 expression in spleens from recipient mice 5 to 6 weeks after transfer was determined by flow cytometry.

T-cell adoptive transfer

T cells were purified from spleens of VFT-Tg, RFT-Tg, or OTII-Tg donor mice by CD3 negative selection using magnetic antibody cell sorting microbeads (Miltenyi). Where indicated, T cells were labeled with 5 μmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) before transfer. T cells (3×10^6–5×10^6) were transferred into recipient mice by lateral tail vein injection. For experiments involving *in vivo* stimulation of donor T cells, recipient mice were vaccinated with antigen-loaded or control (no antigen) DCs administered by lateral tail vein (i.v.) injection.

MUC1 peptides and glycopeptides

A 100-mer MUC1 peptide represents 5 repeats of the 20-amino-acid sequence HGVTSAPDTRPAPGSTAPPA from the MUC1 VNTR region and was synthesized as described previously (13). GalNAc-100mer (Tn100mer/TnMUC1) was prepared by enzymatic addition of GalNAc to synthetic peptide substrate using recombinant human UDP-GalNAc:polypeptide *N*-acetyl-galactosaminyltransferase rGalNAc-T1 as previously described (20, 27). The final reaction product contained a heterogeneous mixture of 9 to 15 GalNAc residues per 100-mer peptide molecule, incorporated within the threonine of the VTSA region and adjoining serine and threonine within the GSTA region as defined previously (20, 27). MUC1p and TnMUC1 were synthesized and analyzed at the University of Pittsburgh Genomics and Proteomics Core Laboratories.

Generation of bone marrow–derived DCs and vaccination

DCs were generated as previously described (20) with a few modifications. Briefly, RBC-lysed bone marrow cells from C57Bl/6 mice were plated at 1×10^6 cells/mL in serum-free AIM-V medium (Invitrogen) containing sodium pyruvate, nonessential amino acids, and 2-mercaptoethanol, supplemented with 10 ng/mL granulocyte macrophage colony-stimulating factor (GM-CSF; R&D Systems). Cells were fed on day 3 by replacing half the medium with fresh AIM-V plus 10 ng/mL GM-CSF. DCs were purified on day 6 of culture using Nycoprep 1.068 (Accurate Chemical) gradient. For vaccinations, DCs were loaded with peptides and/or glycopeptides overnight at 37°C in the presence of 10 ng/mL GM-CSF, then washed with PBS and injected i.v. (3×10^6–5×10^5 cells per mouse) with soluble MUC1p or TnMUC1.

Results

MUC1 peptide–specific CD4 T cells are not deleted in the thymus of MUC1-Tg mice, but their response to antigen is inhibited in the periphery

Previous work suggested that MUC1-specific tolerance in the MUC1-Tg mouse might be a reason for hyporesponsiveness of MUC1-specific CD4 T cells *in vivo* (12, 13, 28). More recent studies in MUC1-Tg mice revealed the existence of MUC1p-specific CD4 T cells *ex vivo* using alternative vaccination strategies (14, 15) and *in vitro* using T-cell cloning techniques,[3] suggesting that anti-MUC1p CD4 T cells have not simply been deleted. Similarly, although MUC1p-specific CD4 T cells have been difficult to detect directly *ex vivo* from cancer patients, they have been detected *in vitro* using T-cell cloning techniques (29). To better address the function of MUC1p-specific T cells in an environment where MUC1 is present as a self molecule, we generated MHC

[3] Our unpublished data.

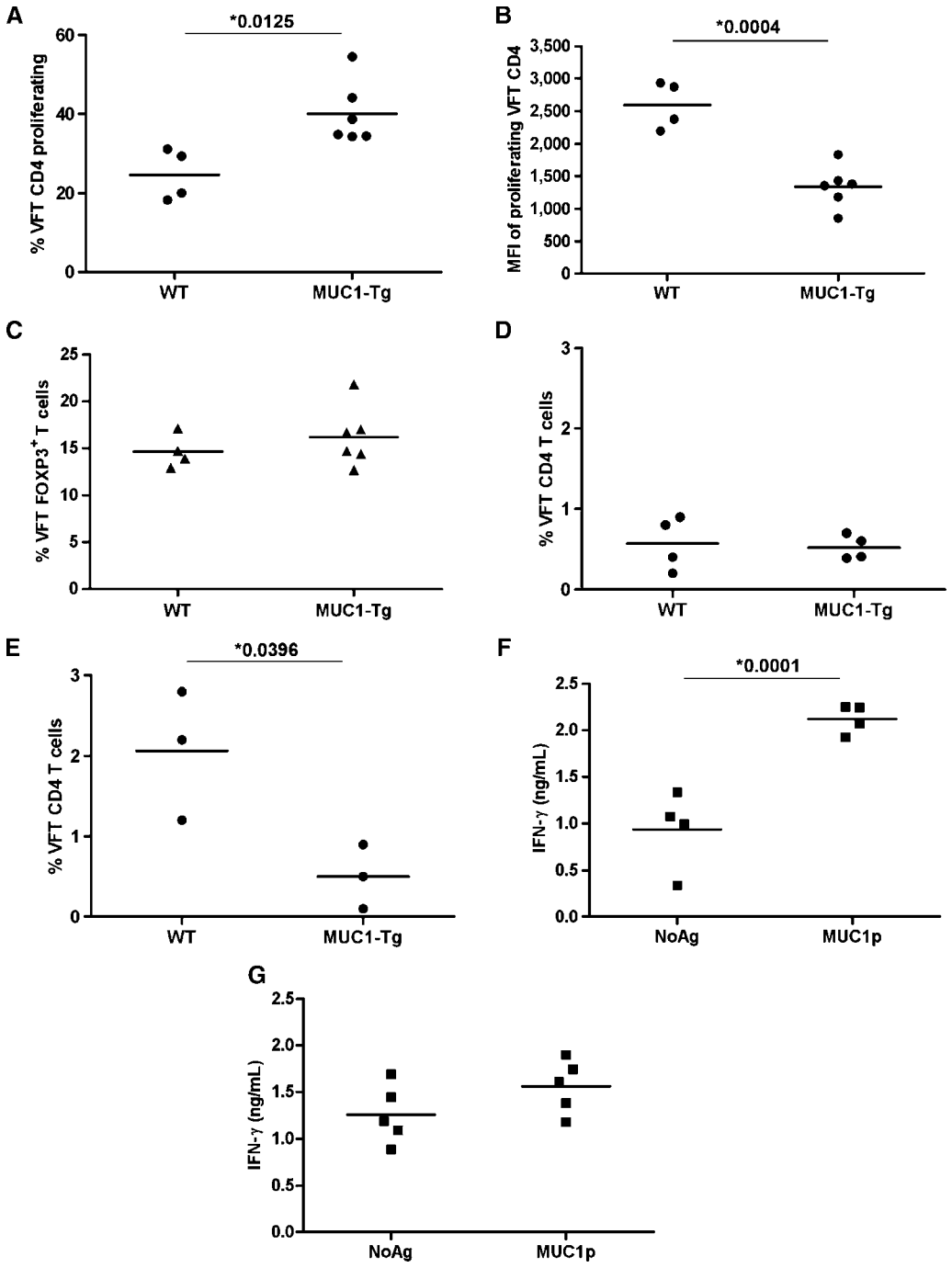

Figure 2. MUC1p-specific VFT CD4 T cells proliferate in the periphery with endogenous stimulation but are hyporesponsive to MUC1p vaccination in MUC1-Tg mice. CFSE-labeled VFT T cells (3×10^6–5×10^6) were transferred (i.v.) into recipient mice. After 5 to 7 d following adoptive transfer, some recipient mice (WT, $n = 4$; MUC1-Tg, $n = 6$) were sacrificed and the presence of donor VFT CD4 T cells ($V\alpha2^+CD4^+CD90.1^+$) in the spleen was determined by flow cytometry. T-cell proliferation is shown as (A) percentage of VFT CD4 T cells with decreased CFSE fluorescence and (B) CFSE MFI of proliferating cells, calculated using the gating strategy in A. C, percent VFT CD4 T cells that are $Foxp3^+$. D, percent VFT CD4 T cells with respect to the recipient splenic CD4 T-cell population before vaccination, 5 to 7 d after adoptive transfer (WT, $n = 4$; MUC1-Tg, $n = 4$), and (E) following two doses of the DC-MUC1p vaccine (i.v.) separated by a 2-wk interval (WT, $n = 3$; MUC1-Tg, $n = 3$). Following the primary DC-MUC1p dose, some recipient mice, (F) WT ($n = 4$) and (G) MUC1-Tg ($n = 5$), were sacrificed and splenocyte IFN-γ production was measured by ELISA after a 72-h *in vitro* MUC1p stimulation (NoAg, no antigen control). Each IFN-γ production data point is the mean of triplicate wells per condition for individual mice. Horizontal bars, mean values for each group of data points. Representative data of two independent experiments. The *P* values were calculated using an unpaired *t* test.

class II (I-A^b)–restricted, MUC1 peptide (MUC1p)–specific VFT TCR (Vα2.5-Vβ6) transgenic (VFT-Tg) mice. The VFT TCR recognizes the peptide epitope HGVTSAPDTRPAP (MUC1p) and was cloned from the VF5 $CD4^+$ hybridoma derived from WT mice that were immunized with MUC1p (20). Although not on a $RAG^{-/-}$ background, more than 60% of VFT CD4 T cells are $V\alpha2^+$.[4] The VFT-Tg mice were generated on a congenic C57BL/6 $CD90.1^+$ background, allowing us for the first time to follow the fate of MUC1p-specific CD4 T cells (VFT) in MUC1-Tg mice ($CD90.2^+$) where MUC1 is a self-antigen.

We transferred lineage-negative VFT-Tg bone marrow precursors to lethally irradiated WT and MUC1-Tg mice, along with bulk syngeneic bone marrow to ensure survival of the irradiated recipients (26). Five weeks later, we found no significant difference between WT and MUC1-Tg recipient mice in the percentage of VFT CD4 T cells that had matured and migrated to secondary lymphoid organs (Fig. 1A; Supplementary Fig. S3A). This indicated that MUC1p-specific CD4 T cells were not deleted in the thymus of MUC1-Tg mice and, at this time after reconstitution (5 weeks), were not subject to peripheral deletion.

To address the possibility that transferred MUC1p-specific CD4 T-cell precursors might encounter MUC1p in the thymus and develop into natural T regulatory cells (Treg), we examined Foxp3 expression in the VFT CD4 T-cell thymic emigrants. Although $Foxp3^+$ VFT CD4 T cells were present, there was no significant difference in their percentage in WT versus MUC1-Tg recipient mice (Fig. 1B; Supplementary Fig. S3B; representative analysis in Supplementary Fig. S4).

VFT T cells provided an excellent tool to answer the long-standing question of whether endogenous MUC1p epitopes are presented to the immune system in MUC1-Tg mice. Naive CFSE-labeled VFT CD4 T cells were transferred into WT and MUC1-Tg mice. After 5 to 7 days, recipient mice were sacrificed and examined for evidence of T-cell activation. $CD90.1^+TCRV\alpha2^+$ VFT T cells were readily detected in both WT and MUC1-Tg recipients. However, a significantly higher percentage of VFT CD4 T cells had proliferated in MUC1-Tg recipients compared with WT recipients (Fig. 2A; Supplementary Fig. S5; representative analysis in Supplementary Fig. S6). Proliferating VFT CD4 T cells in MUC1-Tg mice had lower CFSE mean fluorescence intensity (MFI) than those in WT mice, indicating that they had gone through more cell divisions (Fig. 2B). The proliferating cells in the MUC1-Tg mice did not seem to have converted into $Foxp3^+$ Tregs, as the percentage of these cells at 5 to 7 days after transfer was comparable between WT and MUC1-Tg mice (Fig. 2C). Furthermore, although the percentage of $Foxp3^+$ cells in proliferating versus nonproliferating cells was elevated, as previously reported (30, 31), Foxp3 expression in proliferating and nonproliferating VFT CD4 T cells was the same in WT and MUC1-Tg mice (Supplementary Fig. S7). There was also no significant expansion of VFT CD4 T cells in the MUC1-Tg mice as shown by the same overall percentage of transferred cells in WT and MUC1-Tg recipients (Fig. 2D).

In the next set of experiments, WT and MUC1-Tg mice receiving VFT T cells were vaccinated twice with MUC1p-loaded DCs plus soluble MUC1p (DC-MUC1p) as previously described (28). Significant expansion of VFT CD4 T cells was observed in WT recipients, indicating effective T-cell stimulation (Fig. 2E). In contrast, VFT CD4 T cells failed to expand in response to the vaccine in MUC1-Tg recipients (Fig. 2E). Several recipient mice from each group were sacrificed 5 days after the primary DC-MUC1p vaccination and their splenocytes restimulated with DC-MUC1p *ex vivo* to assess the recall responses. *In vivo* primed T cells from WT recipients produced significantly

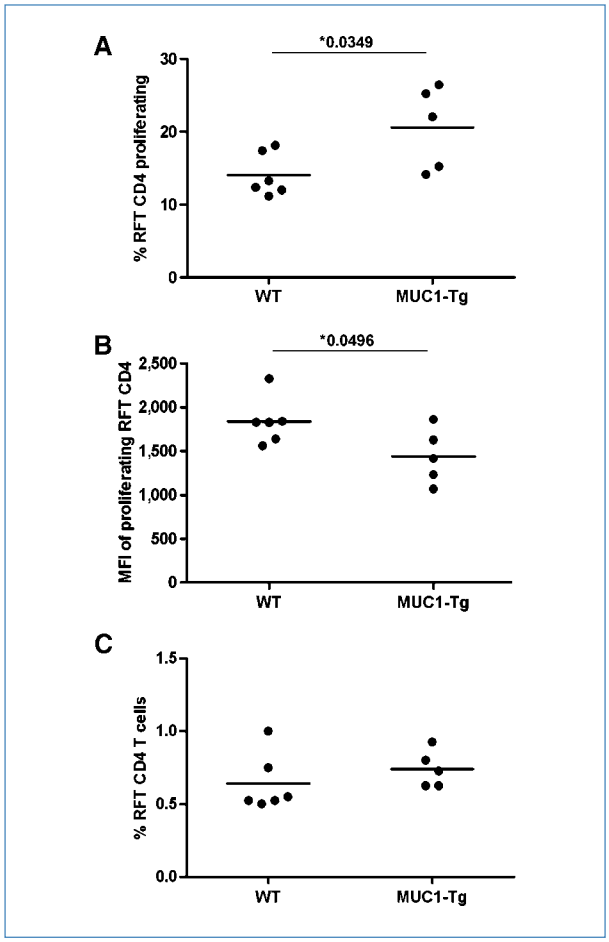

Figure 3. Glycopeptide-specific RFT CD4 T cells proliferate in the periphery with endogenous stimulation in MUC1-Tg mice. CFSE-labeled T cells (3×10^6–5×10^6) were transferred (i.v.) into recipient mice (WT, $n = 6$; MUC1-Tg, $n = 5$). After 5 to 7 d following adoptive transfer, recipient mice splenocytes were analyzed by flow cytometry for the presence of RFT CD4 T cells ($CD3^+CD4^+CD45.1^+$). T-cell proliferation is shown as (A) percent of RFT CD4 T cells with decreased CFSE fluorescence and (B) CFSE MFI of proliferating cells, calculated using the gating strategy in A. C, percent of RFT CD4 T cells with respect to the recipient mouse CD4 T-cell population. Horizontal bars, mean values. Representative data of two independent experiments. The *P* values were calculated using an unpaired *t* test.

[4] Unpublished data.

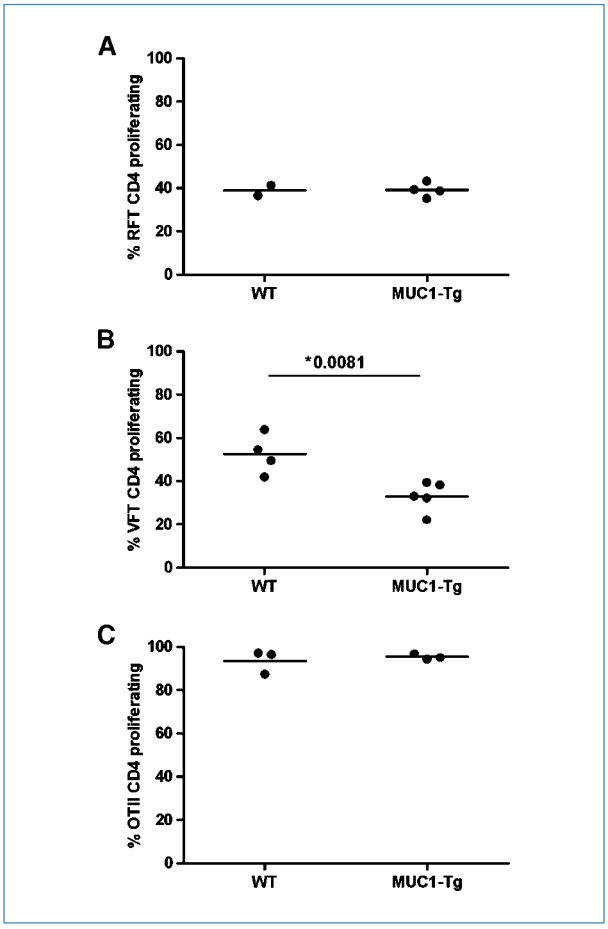

Figure 4. MUC1p-specific hyporesponsiveness of VFT CD4 T cells, but not RFT or OTII CD4 T cells. CFSE-labeled RFT, VFT, and OTII T cells (3×10^6–5×10^6) were transferred (i.v.) into WT and MUC1-Tg recipient mice. One day later, mice were vaccinated (i.v.) using DC-TnMUC1, DC-MUC1p, or DC-ova, respectively. After 4 to 5 d following vaccination, the spleens of recipient mice were analyzed by flow cytometry for the presence of donor (A) RFT CD4 T cells ($CD45.1^+$; WT, $n = 2$; MUC1-Tg, $n = 4$), (B) VFT CD4 T cells ($V\alpha2^+CD90.1^+$; WT, $n = 4$; MUC1-Tg, $n = 5$), or (C) OTII CD4 T cells ($CFSE^+$; WT, $n = 3$; MUC1-Tg, $n = 3$) as a percentage of total $CD3^+CD4^+$ cells. Proliferation of donor cells was determined by a decrease in CFSE fluorescence intensity. Horizontal bars, mean values. The *P* value was calculated using an unpaired *t* test.

higher levels of IFN-γ in response to secondary MUC1p stimulation (Fig. 2F) compared with those primed in the MUC1-Tg recipients (Fig. 2G).

MUC1 glycopeptide–specific CD4 T cells respond equally well in WT and MUC1-Tg mice

To compare MUC1p-specific T-cell responses with tumor-associated MUC1 glycopeptide–specific CD4 T-cell responses, we generated MHC class II ($I\text{-}A^b$)–restricted (Supplementary Fig. S8), TnMUC1-specific TCR-transgenic mice (RFT-Tg) on the congenic C57BL/6 $CD45.1^+$ background. The RFT TCR (Vα4.1-Vβ15) recognizes the TnMUC1 (HGVTSAPDTRPAPGS(GalNAc-O-)TAPPA glycopeptide epitope and was cloned from the RF6 hybridoma derived from CD4 T cells isolated from TnMUC1-immunized WT mice (22). CFSE-labeled RFT CD4 T cells were transferred into WT and MUC1-Tg recipients ($CD45.2^+$). At 5 to 7 days after transfer, we detected a slight but nonetheless statistically significant increase in RFT CD4 T-cell proliferation in MUC1-Tg recipients compared with WT recipients (Fig. 3A and B; representative analysis in Supplementary Fig. S9), yet there was no significant difference in the overall percentage of RFT CD4 T cells between WT and MUC1-Tg recipients (Fig. 3C). The minimal proliferation of RFT CD4 T cells observed in unvaccinated MUC1-Tg mice was expected because of their low-level cross-reactivity with MUC1p originally seen with the RF6 hybridoma (22).

We next addressed if there was an *in vivo* functional difference between RFT and VFT CD4 cells in the WT and MUC1-Tg environments, especially the possibility that glycopeptide-specific RFT cells might respond normally in MUC1-Tg mice. Unlike VFT CD4 T cells that recognize a normal (self) peptide, RFT CD4 T cells recognize a tumor-specific glycopeptide that should not be viewed as self antigen by the immune system. We transferred CFSE-labeled RFT or VFT T cells into WT and MUC1-Tg recipients 1 day before a single vaccination with DCs loaded with MUC1p or TnMUC1 and analyzed *in vivo* proliferation 4 to 5 days later (representative analysis in Supplementary Fig. S10). In response to TnMUC1, RFT T cells proliferated to the same extent in the WT and the MUC1-Tg environment (Fig. 4A). In contrast, VFT CD4 T-cell responses to MUC1p were again significantly inhibited in MUC1-Tg mice (Fig. 4B), as previously shown in Fig. 2.

As a control for T-cell responses to an antigen foreign to both WT and MUC1-Tg mice, we used CD4 T cells from OTII TCR-transgenic mice specific for chicken ova (32). Mice receiving CFSE-labeled OTII CD4 T cells were vaccinated with ova peptide–loaded DCs. Similar to the RFT CD4 T cells, there was no difference in OTII T-cell responses between WT and MUC1-Tg mice (Fig. 4C). Additional control groups composed of WT recipient mice that received unloaded DCs alone showed no T-cell proliferation.[5]

Hyporesponsive peptide-specific T cells are rescued by simultaneous activation of glycopeptide-specific T cells

Peptide-specific VFT and glycopeptide-specific RFT CD4 T cells were mixed at 1:1 ratio, labeled with CFSE, and transferred into WT and MUC1-Tg recipients. One day later, recipient mice were vaccinated with DCs loaded with both MUC1p and TnMUC1 with the expectation that at least some of the DCs would process and present both glycopeptide and peptide epitopes simultaneously. When RFT and VFT T cells were concurrently activated *in vivo*, we saw for the first time equal responses of VFT CD4 T cells in MUC1-Tg and WT recipients (Fig. 5A). Importantly, RFT CD4 T cells were themselves not negatively affected by the presence of VFT cells (Fig. 5B). The hyporesponsiveness of VFT CD4 T cells in MUC1-Tg recipients was also overcome by coactivation of ova-specific OTII CD4 T cells

[5] Unpublished data.

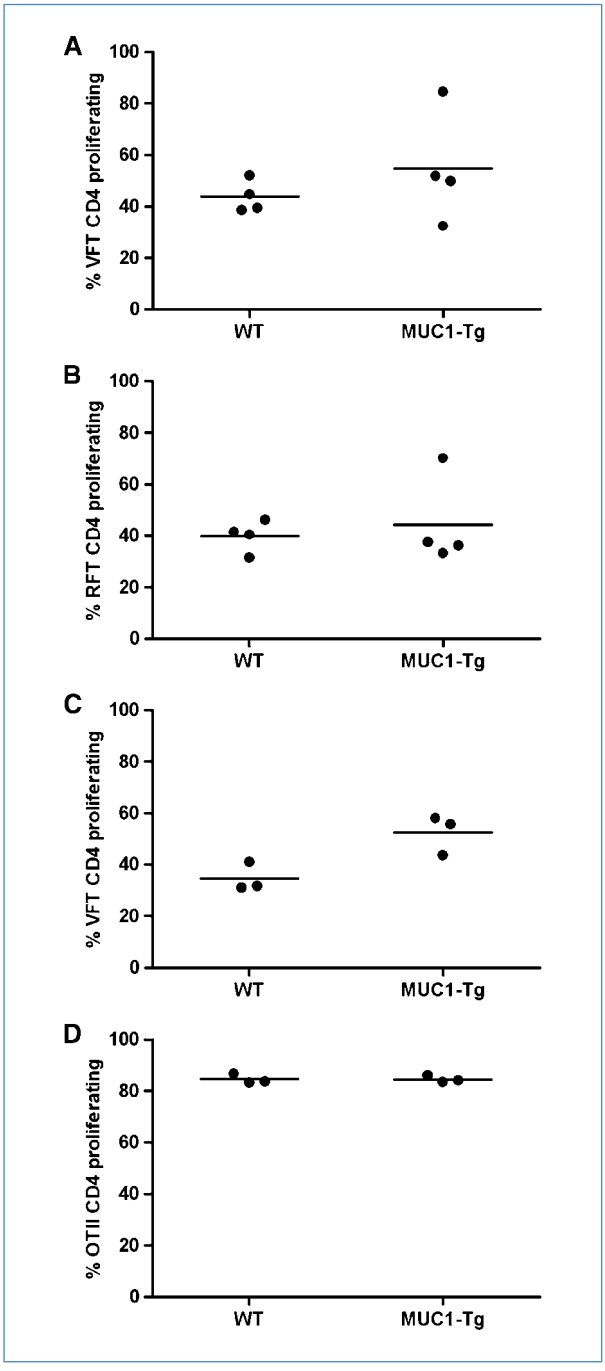

Figure 5. Stimulation of foreign antigen–specific CD4 T cells can help break endogenous MUC1p-specific CD4 T-cell tolerance. CFSE-labeled (A and B) VFT and RFT T cells (WT, $n = 4$; MUC1-Tg, $n = 4$) or (C and D) VFT and OTII T cells (WT, $n = 3$; MUC1-Tg, $n = 3$) were cotransferred (3×10^6–5×10^6; i.v.) into recipient mice (WT and MUC1-Tg). One day later, mice were vaccinated (i.v.) using coloaded (A and B) DC-(MUC1p + TnMUC1) or (C and D) DC-(MUC1p + ova). After 4 to 5 d following vaccination, the spleen of each recipient mouse was analyzed by flow cytometry for the presence of either (A) VFT CD4 T cells ($V\alpha2^+CD90.1^+$) and (B) RFT CD4 T cells ($CD45.1^+$) or (C) VFT CD4 T cells and (D) OTII CD4 T cells ($CFSE^+$) as a percentage of total $CD3^+CD4^+$ cells. The proliferation of donor cells was determined by a decrease in CFSE fluorescence intensity. Horizontal bars, mean values.

(Fig. 5C). Like RFT T cells, OTII CD4 T cells responded at similar levels in the two environments (Fig. 5D).

Discussion

Past and present studies in the MUC1-Tg mouse model have indicated that in the context of that "self" environment, immune responses to unglycosylated MUC1 VNTR peptides are greatly reduced, thus compromising anti-MUC1 tumor immunity (4, 13–15, 28). As a strategy to increase the potency of MUC1 vaccines, we added tumor-associated Tn glycans to the peptide immunogen to more closely represent epitopes that are displayed on all $MUC1^+$ tumors and on APCs that cross-present tumor MUC1 to T cells in patients.

To study the potential differences in T-cell responses to the MUC1 peptide (self) and the glycopeptide ("foreign") epitopes, we generated two TCR-transgenic mice, one (VFT) bearing a peptide-specific TCR and the other (RFT) a glycopeptide-specific TCR. By adoptively transferring TCR-transgenic T-cell precursors or mature T cells into WT and MUC1-Tg mice, we found that the reduced responses of MUC1 peptide–specific CD4 T cells were not due to their deletion during thymic development in MUC1-Tg mice. Rather, their activation was inhibited in the periphery at the time of antigen stimulation. On the other hand, MUC1 glycopeptide–specific CD4 T-cell stimulation did not seem to be subject to that same inhibition, behaving more like the CD4 T cells specific for the foreign antigen ova. We further showed that the hyporesponsiveness of VFT T cells in MUC1-Tg mice can be overcome in the presence of activated RFT or OTII T cells.

The best known mechanism of self-tolerance is thymic deletion of self-reactive T cells (negative selection; ref. 33). More recently, it was shown that developing thymocytes could also differentiate into CD4 Tregs due to positive signals received by self-antigen recognition in the thymus (34, 35). Normal, fully glycosylated MUC1 is expressed by human medullary thymic epithelial cells (mTEC; ref. 36) and could theoretically influence the MUC1-specific T-cell repertoire. Ectopic expression of other peripheral-tissue antigens by mTECs has been reported to result in central tolerance (reviewed in ref. 37), including that to tumor-associated carcinoembryonic antigen (2). However, we previously reported that complete glycosylation of the MUC1 VNTR region as expressed in healthy tissues prevents processing and presentation of peptide epitopes by DCs (38). Thus, the extent to which the MUC1p-specific T-cell repertoire might be affected by self-tolerance was not clear. By comparing MUC1p-specific T-cell development in WT versus MUC1-Tg mice, we did not detect any difference in the efficiency of emigration from the thymus, suggesting that the MUC1-HGVTSAPDTRPAP peptide epitope is either not presented to developing thymocytes or presented at levels insufficient to induce clonal deletion or conversion to naturally occurring Tregs.

Although peripheral tolerance is not well understood, studies have shown that autoreactive T cells specific for model self-antigens (39) and TAAs (3, 40) that are expressed in peripheral tissues can be tolerized at peripheral sites. We observed proliferation of transferred VFT CD4 T cells

in unvaccinated MUC1-Tg mice and not in WT mice, suggesting that peptide epitopes were being presented in the periphery. Lowering the level of T-cell responses to those epitopes in vaccinations might prevent autoimmunity. Similar levels of Foxp3$^+$ VFT CD4 cells in the periphery of WT and MUC1-Tg mice, as well as the lack of suppression of RFT T-cell responses when coactivated with VFT T cells, imply that this is not mediated by induced Tregs, although a more detailed analysis is needed to rule out or reveal preexisting endogenous MUC1-specific Tregs.

There have been no reports of MUC1p vaccine–induced autoimmunity in MUC1-Tg mice (4, 15, 28), primates (41), or human clinical trials (9, 10). Thus, it is likely that MUC1p epitopes are presented at very low levels on normal tissues in the absence of costimulation, which could induce anergy or peripheral deletion of high-affinity T cells. Recently, populations of nonhematopoietic cells that express and present peripheral tissue antigens have been characterized in secondary lymphoid organs (42, 43). These cells could be the source of MUC1 peptides being presented to CD4 T cells in the periphery, rather than the nonlymphoid ductal epithelial tissue (non–MHC class II expressing) where MUC1 is normally expressed. In addition, secondary lymphoid tissues may contain circulating DCs that have captured peripheral tissue antigens, such as MUC1, from dying cells and are presenting the antigens in the steady state to maintain self-tolerance (44, 45).

Gerloni and colleagues (14) previously reported that, by providing a nonself determinant (heterologous help) together with MUC1p, they could activate previously hyporesponsive MUC1p-specific CD4 T cells. We have confirmed that by simultaneously activating ova-specific OTII T cells and showing that they provide heterologous help to VFT T cells. More importantly, however, we show that TnMUC1 can provide similar help by activating glycopeptide-specific T cells, thus leading to improved responses of MUC1p-specific T cells. Although both ova and TnMUC1 can serve the helper function and improve responses at the time of priming, only TnMUC1 would be present and available to perform that function during recall responses when the tumor recurs or a new MUC1$^+$ tumor arises. Although this was not directly tested in our experiments, the ability of VFT T cells to respond in MUC1-Tg mice under some circumstances as robustly as RFT T cells shows that their hyporesponsivness is not simply due to a potentially lower-affinity TCR but rather the environment in which their TCR is activated.

Beyond traditional peptide-specific T-cell responses, recent studies have shown that T cells (via the TCR) can respond to MHC class I– and class II–restricted peptides that contain posttranslational modifications, such as phosphorylation and glycosylation (46). Abnormal glycosylation has been related to autoimmune diseases where abnormally glycosylated proteins efficiently activate effector T cells resulting in autoimmune cytotoxicity (47, 48). In the case of tumor immunity, the ability to direct such cytotoxic responses against abnormal molecules on malignant cells would be beneficial. The fact that the majority of cell proteins are glycosylated and that protein glycosylation is known to be dysregulated in cancer cells (49, 50) should encourage more effort on targeting of tumor-specific glycopeptides (19, 20, 22), a viable alternative to nonmutated peptide targets that have been shown to face self-tolerance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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