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CD4⁺ Lymphocytes Provide MUC1-Specific Tumor Immunity In Vivo That Is Undetectable In Vitro and Is Absent in MUC1 Transgenic Mice

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A C57BL/6 mouse transgenic for human MUC1 (MUC1.Tg) was developed to evaluate MUC1-specific tumor immunity in an animal that expresses MUC1 as a normal self protein. Previous studies showed that MUC1.Tg mice, challenged with syngeneic tumors expressing MUC1 (B16.MUC1), developed progressively growing MUC1-positive tumors, whereas wild-type C57BL/6 (wt) mice developed MUC1-negative tumors at a significantly slower rate. The results of a limiting dilution CTL frequency assay were not informative, in that similar numbers of MUC1-specific CTL precursors (CTL) were detected in MUC1.Tg and wt mice. Tumor immunity in vivo was characterized by an adoptive transfer method to evaluate the degree of MUC1 or non-MUC1 tumor immunity in wt or MUC1.Tg mice. The results revealed that wt mice developed protective tumor immunity mediated by MUC1-specific CD4⁺ lymphocytes, while MUC1.Tg mice were functionally tolerant to MUC1 in vivo. The potential of adoptive immunotherapy to provide immunity to tumors expressing MUC1 and to produce undesirable autoimmunity in recipient MUC1.Tg mice expressing MUC1 as a self Ag was evaluated. Adoptive transfer of immune cells from wt mice primed in vivo with B16.MUC1 tumor cells into MUC1.Tg recipients resulted in significant increases in the survival of MUC1.Tg recipients compared with unmanipulated control MUC1.Tg mice challenged with B16.MUC1 tumor cells. This response was specific for MUC1 since control tumors developed at equivalent rates in recipient or control MUC1.Tg mice. No gross or histologic evidence of autoimmunity was observed in recipient MUC1.Tg mice, indicating that tumor immune responses mediated by MUC1-specific CD4⁺ lymphocytes spare nontransformed epithelia-expressing MUC1.


The human glycoprotein MUC1 is of interest for tumor immunotherapy. MUC1-specific CTLs have been detected in some patients with breast, ovarian, or pancreatic adenocarcinomas (1–3). MUC1 is frequently overexpressed in human adenocarcinomas, in which normal apical expression is lost, and posttranslational modifications are dysregulated compared with nontransformed cells of the same origin (4, 5). MUC1 is also a self protein expressed neonatally and postnatally on nontransformed secretory epithelia (6, 7). Thus, the human T and B cell repertoires are postulated to be immunologically tolerant to MUC1, a feature that may influence immunity to tumors expressing MUC1, and should be considered in the design and testing of MUC1-based immunotherapy.

C57BL/6 mice transgenic for human MUC1 (MUC1.Tg) were developed to investigate anti-MUC1 tumor immunity in an animal that expresses MUC1 as a self Ag on nontransformed epithelia (8–10). MUC1.Tg mice challenged with MUC1-expressing tumors (B16.MUC1) developed progressively growing, MUC1-positive tumors, whereas wild-type C57BL/6 (wt) mice developed MUC1-negative tumors at significantly slower rates. The degree of dissimilarity between MUC1 and its murine homologue, Muc-1, enables the immune system of wt mice to recognize MUC1 as a foreign protein and to eliminate MUC1-expressing tumors. In contrast, MUC1.Tg mice are immunologically tolerant to MUC1 and do not reject B16.MUC1 cells.

A limiting dilution CTL frequency assay was used to quantify the cellular cytotoxic responses to MUC1 or non-MUC1 Ags in wt or MUC1.Tg mice during the course of progressive B16.MUC1 tumor growth. To complement the in vitro results, an in vivo adoptive transfer system was used to evaluate the degree of MUC1 tumor immunity in wt or MUC1.Tg donor mice. Both experimental systems were used to analyze the time course in which MUC1-specific immune responses developed during the course of B16.MUC1 tumor progression.

The MUC1.Tg mice are a preclinical model to evaluate immunotherapies designed to overcome tolerance to MUC1 by immunization with MUC1 vaccine formulations or by adoptive transfer of MUC1-specific cells. Similar to cancer patients, MUC1-specific responses in MUC1.Tg mice have the potential to result in autoimmunity since these mice express MUC1 on epithelia of many organs. To investigate the relationship between MUC1-specific tumor immunity and autoimmunity, MUC1-specific immune cells primed in vivo were adoptively transferred to MUC1.Tg mice, and the degree of MUC1-specific tumor immunity and autoimmunity was investigated.

Materials and Methods

Mice

Female wild-type C57BL/6 (wt) mice, 6 wk of age, were purchased from National Cancer Institute (Frederick, MD). Age-matched female MUC1.Tg
C57BL/6 (MUC1.Tg) mice were obtained from a breeding colony at University of Nebraska Medical Center (Omaha, NE). Mice were treated in accordance with IACUC Institutional Animal Care and Use Committee guidelines.

**Cell lines**

B16.MUC1 or B16.Neo are murine (C57BL/6) melanoma tumor lines transfected with a MUC1 cDNA or control expression vector, respectively (9). Both B16.MUC1 and B16.Neo cell lines express MHC class I in vitro, as detected by flow-cytometric analysis (data not shown).

**Limiting dilution CTL frequency assay**

Anesthetized (Metofane; Pitman-Moore, Madelein, IL) MUC1.Tg or wt mice were injected s.c. between the scapulae with 2 × 10^6 viable B16.MUC1 tumor cells. Splenocytes and brachial LN cells were mechanically separated to a single cell suspension, and RBCs were lysed by hypotonic shock. Efferent lymphocytes were derived from draining LN, and splenocytes obtained from the same mouse were used as APCs. Limiting dilutions (6, 6 × 10^3, 6 × 10^4, 6 × 10^5, or 6 × 10^6) of brachial LN cells were incubated with 4 × 10^4 irradiated (6 × 10^6 rad) B16.MUC1 cells and 3 × 10^4 irradiated splenocytes (2 × 10^5 rad) in flat-bottom 96-well plates (Becton Dickinson, Franklin Lakes, NJ) with 250 μM DMEM high glucose (Life Technologies, Grand Island, NY) supplemented with 20% heat-inactivated FBS (Biowittaker, Walkersville, MD), 10% T-stim (Collaborative Biomedical Products, Bedford, MD), nonessential and essential amino acids (Mediatech, Hernden, VA), vitamins (Life Technologies), 4 mM glutamine (Life Technologies), 625 μM HEPEs (Sigma, St. Louis, MO), 50 μM β-mercaptoethanol (Sigma), 100 μg/ml gentamicin (Life Technologies), and 1 mM sodium pyruvate (Sigma). Cocultures were incubated at 37°C and 5% CO_2 for 7 days and then split equally into triplicate microtiter wells and incubated with 4 × 10^4 freshly irradiated B16.MUC1 cells and 3 × 10^4 freshly irradiated APCs obtained from naive wt mice. Four days later, cytotoxicity was determined in each microculture by using a standard 31Cr-release assay with MUC1-expressing EL-4 cells (EL4-MUC1) (a kind gift of Dr. Olivera Finn) and control EL-4 cells as targets. Briefly, target cells were labeled with 250 μCi of 31Cr (ICN, Costa Mesa, CA) at 37°C for 75 min and washed. Microcultures in one set of plates were incubated with 10^5 ELA-MUC1 targets, and microcultures in the second set of plates were incubated with 10^5 control EL-4 targets. The microcultures in the third set enabled the derivation of the MUC1 or non-MUC1 CTL frequency, as described elsewhere (9). Four treatments at −6, −4, −2, and 7 days (tumor challenge occurred on day 0) of 0.5 mg of control Ab (SFR3-DR5), depleted rat anti-mouse CD4 (clone GK1.5), CD8 (clone 53.6-72), or both were administered i.p. to wt mice. The lymphocyte-depleted condition of representative mice was confirmed by flow cytometry at day 0. Splenocytes and LN cells obtained from representative depleted mice were stained with anti-mouse CD4, anti-mouse CD8, anti-mouse CD3, or anti-mouse CD19 Abs labeled with FITC. Compared with mice administered control Ab less than 2%, the depleted lymphocytes were detected in the CD4⁺, CD8⁺, or CD4⁺/CD8⁺-depleted groups (data not shown). Following initial Ab administration, administered control Ab wt, depleted wt, unmanipulated wt, or MUC1.Tg mice were challenged with 2 × 10^6 B16.MUC1 tumor cells, and tumor growth was evaluated over time.

**Results**

C57BL/6 mice transgenic for human MUC1 (MUC1.Tg) have been developed to investigate anti-MUC1 tumor immunity in an animal that expresses MUC1 as a self Ag on nontransformed epithelia (8–10). MUC1.Tg mice challenged with B16.MUC1 tumor cells (expressing MUC1) develop progressively growing MUC1-positive tumors. Wild-type C57BL/6 mice reject the MUC1-positive tumors (9). Recent results have shown that MUC1-specific Abs do not provide immunity to tumors expressing MUC1 in this model. It was hypothesized that a MUC1-specific cellular response was responsible for eliminating B16.MUC1 tumors in wt mice. In contrast, this response was not produced in the MUC1.Tg mice because MUC1 expression as a self protein in these mice induced MUC1-specific immune tolerance (9).

**MUC1.Tg or wt mice develop similar numbers of MUC1-specific CTLs following B16.MUC1 tumor challenge**

A modified limiting dilution CTL frequency assay was used to quantify in vitro MUC1-specific CTLs or CTLs reactive with Ags other than MUC1 (referred to as non-MUC1-reactive CTLs) in MUC1.Tg or wt mice challenged s.c. with 2 × 10^6 viable B16.MUC1 tumor cells. No MUC1-specific and few (3.3/10^6) non-MUC1-reactive CTLs were detected in naive wt mice (Fig. 1, A and B). Wild-type mice were challenged with 2 × 10^6 B16.MUC1 tumor cells, and CTL frequencies were quantified over the course of progressive tumor growth (42 days). The frequency of MUC1-specific or non-MUC1-reactive CTLs at different time points in these mice was between 0–26/10^6 and 14.6–123/10^6, respectively (Fig. 1, A and B).

MUC1-specific CTLs were also detected in MUC1.Tg mice at frequencies that were similar to and statistically indistinguishable...
LYMPHOCYTES PROVIDE MUC1 TUMOR IMMUNITY

FIGURE 1. MUC1-specific CTLs are detectable in wt and MUC1.Tg mice. MUC1.Tg (●) or wt (■) mice were challenged with 2 × 10^5 B16.MUC1 cells. At the indicated time after B16.MUC1 challenge, a limiting dilution assay was designed to determine the A, MUC1-specific, or B, non-MUC1-reactive CTL frequency (reactive cells/10^6). Symbols represent the determined CTL frequency from individual mice. No statistically significant differences in MUC1-specific or non-MUC1-reactive CTL frequency were observed between wt or MUC1.Tg mice.

from wt mice (Fig. 1A). Both MUC1-specific (0–12.9/10^6) and non-MUC1-reactive CTLs (4–55/10^6) were detected in naive MUC1.Tg mice (Fig. 1, A and B). MUC1.Tg mice were challenged with 2 × 10^4 viable B16.MUC1 cells, and over the course of 28 days the frequency of MUC1-specific or non-MUC1-reactive CTLs was between 12.9–38/10^6 and 14.6–36/10^6, respectively (Fig. 1, A and B).

Results of these CTL frequency studies indicated that the MUC1-specific or non-MUC1-reactive CTL responses detected in vitro were similar between the wt and MUC1.Tg mice. In contrast, results obtained from in vivo challenge with B16.MUC1 cells showed that MUC1.Tg mice developed progressively growing MUC1-positive tumors, while wt mice rejected MUC1-positive tumors and developed MUC1-negative tumors at a significantly slower rate (9). The tumor growth data provided in vivo evidence for a MUC1-specific tumor immune response in wt mice that was absent or ineffective in MUC1.Tg mice.

Cellular adoptive transfer indicated that wt but not MUC1.Tg mice develop MUC1-specific tumor immunity

The in vitro assays were not effective in detecting immune cells that mediated the MUC1-specific tumor immune response. A cellular adoptive transfer system was designed to evaluate MUC1-specific and non-MUC1-reactive tumor immunity in vivo. Splenocytes and LN cells from wt or MUC1.Tg mice previously challenged with 2 × 10^4 viable B16.MUC1 tumor cells (donor mice) were adoptively transferred to nonirradiated wt mice (recipient mice). One day later, recipient or unmanipulated control wt mice were challenged with 2 × 10^4 viable B16.MUC1 or B16.Neo tumor cells, and tumor growth was evaluated over time. Evidence of immunity to MUC1 is provided by an increase in recipient survival compared with control mice following challenge with B16.MUC1 tumor cells.

Adaptively transferred cells from naive wt mice showed no evidence of tumor immunity in wt recipients (data not shown). Splenocytes and LN cells from wt donors challenged with B16.MUC1 tumor cells for different periods of times (15–42 days) were evaluated for immunity to B16.MUC1 or B16.Neo tumors in the adoptive transfer system. Wild-type donors developed strong immunity to B16.MUC1 by day 28 after tumor challenge; survival of wt recipients was increased dramatically compared with control mice (p < 0.05) (Fig. 2A). Immune cells with this phenotype are hereafter referred to as tumor immune cells, and were used in adoptive transfer studies, unless otherwise indicated. Approximately 60% of these wt recipients challenged with B16.MUC1 tumor cells were tumor free for as long as 60 days. Survival of wt recipients challenged with B16.Neo tumor cells was increased compared with control wt mice, although this was not statistically significant (p > 0.30) (Fig. 2B). The adoptive transfer of immune cells that eliminate B16.MUC1 tumor cells, but not B16.Neo tumor cells, indicates that the immunogenicity of the B16.MUC1 tumor is largely due to the expression of MUC1. In some experiments, the CTL frequencies of wt recipients of tumor immune cells or control wt mice were quantified at 21 days after B16.MUC1 tumor challenge. Compared with control mice, the recipient mice had increased MUC1- and non-MUC1-reactive CTL frequencies detected in vitro, although the difference was small and not statistically significant (data not shown).

Interestingly, adoptive transfer of immune cells from wt donors bearing B16.MUC1 tumors for 42 days did not increase the survival of wt recipients compared with control mice after challenge with B16.MUC1 or B16.Neo tumor cells (data not shown). These findings indicate that the wt MUC1-specific tumor immunity is acquired between 0 and 21 days, maintained during days 21 to 35, and not detectable at 35 days post-B16.MUC1 tumor challenge. The in vitro results shown in Fig. 1 suggested that MUC1-specific CTLs exist in MUC1.Tg mice, yet the progressive growth of B16.MUC1 tumors in the MUC1.Tg mice indicated that the CTLs detected in vitro were unable to kill tumors expressing MUC1 in vivo. The wt and MUC1.Tg mice are syngeneic, with the exception of immunity to MUC1.

FIGURE 2. Wt mice develop protective MUC1-specific tumor immunity in vivo. Wild-type tumor immune cells were adoptively transferred to nonirradiated wt recipients. One day later, matched sets of recipient wt (■, n = 5), unmanipulated wt (■, n = 18), or MUC1.Tg (●, n = 5) mice were challenged with 2 × 10^5 B16.MUC1 (A) or B16.Neo (B) tumor cells, and tumor growth was evaluated over time. A, Statistically significant differences in survival were detected between wt mice and MUC1.Tg mice or wt recipient mice (p < 0.05). B, Statistically significant differences in survival were not detected between any group (p > 0.50). The results shown are representative of three independent studies.
of the MUC1 gene, which usually is not expressed on cells of hemopoietic origin (7). Therefore, it was possible to use cellular adoptive transfer into recipient wt mice to evaluate MUC1.Tg tumor immunity in vivo. Spleen and LN cells adoptively transferred from naive MUC1.Tg donors or MUC1.Tg donors challenged with B16.MUC1 tumor cells for 0 to 28 days did not increase the survival of wt recipients challenged with B16.MUC1 or B16.Neo tumor cells compared with control wt mice (p > 0.50) (data not shown and Fig. 3). Collectively, these results demonstrate that wt mice develop effective MUC1-specific cellular responses that eliminate B16.MUC1 tumor cells in vivo; in contrast, MUC1.Tg mice are functionally tolerant to MUC1 since they are unable to eliminate B16.MUC1 tumors in vivo.

**Ab-mediated T cell depletion and adoptive transfer studies demonstrate that CD4+ lymphocytes provide MUC1-specific tumor immunity**

Tumor immune cells from wt donors completely eliminated B16.MUC1 tumor cells in approximately 60% of wt recipients (Fig. 2). In vivo Ab-mediated T cell depletion was used to determine the phenotype of the effector cell responsible for providing MUC1 tumor immunity in wt mice. CD4+, CD8+, or both T cell subsets were eliminated in vivo in wt mice by treatment with depleting Abs. Groups of depleted wt, control wt, or MUC1.Tg mice were challenged with 2 × 10⁶ B16.MUC1 tumor cells, and tumor growth was evaluated over time. The results showed that CD4+ but not CD8+ lymphocytes were required for MUC1-specific tumor immunity (Fig. 4). CD4+ /CD8+ wt, CD4− wt, and MUC1.Tg mice developed progressively growing tumors at similar rates. In contrast, CD8+ wt or untreated wt mice developed tumors that grew at significantly slower rates (p < 0.05) (Fig. 4). The observation that MUC1.Tg mice developed tumors at rates similar to the CD4+ /CD8+ wt mice is consistent with the hypothesis that MUC1 is the dominant Ag responsible for in vivo tumor rejection and that non-MUC1 Ags are not sufficient for tumor rejection in this system.

Adoptive transfer of purified CD4+ but not CD8+ cells from tumor immune wt mice increased the survival of wt recipients following challenge with 2 × 10⁶ B16.MUC1 tumors. The B16.MUC1 tumor growth rate was equivalent between recipients of CD8+ cells and control wt mice. In contrast, compared with wt control mice, recipients of CD4+ cells developed tumors more slowly (Fig. 5). Although the differences did not attain statistical significance, the results shown in Fig. 5 are consistent with the results of the T cell depletion study shown in Fig. 4, and further support hypothesis that MUC1-specific CD4+ T cells protect against B16.MUC1 tumor growth.

**Adoptive transfer of tumor immune cells significantly increased the survival of MUC1.Tg recipients following B16.MUC1 challenge**

The MUC1.Tg mice provide a preclinical model to evaluate strategies to overcome immunologic tolerance to MUC1. Cellular adoptive transfer is one in vivo strategy to analyze the antitumor efficacy and autoimmune consequences of developing MUC1-specific effector cells in MUC1.Tg mice. We hypothesized that adoptive transfer of the MUC1-specific immune response from the wt mice (Figs. 2 and 4) to MUC1.Tg mice would increase the survival of recipient MUC1.Tg mice challenged with B16.MUC1 tumor cells. Cells adoptively transferred from naive wt mice did not increase the survival of MUC1.Tg recipients after B16.MUC1 or B16.Neo tumor challenge (data not shown). Interestingly, MUC1.Tg recipients that received tumor immune cells from wt donors showed increased survival compared with control MUC1.Tg mice following challenge with B16.MUC1 tumor cells (p < 0.05) (Fig. 6A), indicating that it was possible to transfer the anti-MUC1 tumor immune response into these mice. There was no alteration in survival of MUC1.Tg recipients challenged with B16.Neo tumors compared with controls (p > 0.50) (Fig. 6B).

![Figure 3](http://www.jimmunol.org/)

**Figure 3.** MUC1.Tg mice are tolerant to MUC1 in vivo. Splenocytes and LN cells from MUC1.Tg mice challenged with B16.MUC1 for 21 days were adoptively transferred to wt recipient mice. Matched sets of recipient wt (△, n = 4), unmanipulated wt (□, n = 5), or MUC1.Tg (●, n = 4) mice were challenged with 2 × 10⁶ B16.MUC1 (A) or B16.Neo (B) tumor cells, and tumor growth was evaluated over time. A, No statistically significant differences in survival were observed in wt recipients compared with control wt mice (p > 0.30). B, Statistically significant differences in survival were detected between wt recipient mice and control wt mice (p < 0.05). The results shown are representative of three independent studies.

![Figure 4](http://www.jimmunol.org/)

**Figure 4.** CD4+, but not CD8+, T lymphocytes are required for MUC1 tumor immunity in vivo. Depleting Abs were administered to selectively eliminate CD4+, CD8+, or both T cell subsets. Groups of seven CD4−/CD8− wt (△), CD8− (□) wt, CD4−/CD8− (●) wt, unmanipulated (■) wt, or MUC1.Tg (n = 5) (●) mice were challenged with 2 × 10⁶ B16.MUC1 tumor cells on day 0, and tumor growth was evaluated over time. Statistically significant differences in survival were observed in MUC1.Tg, CD4−/CD8− wt, and CD4− wt mice, compared with unmanipulated wt mice (p < 0.05). No difference in survival (p > 0.50) was detected between CD8− wt and unmanipulated wt mice, nor between MUC1.Tg and CD4−/CD8− wt mice.
Adoptive transfer of MUC1-specific T cell responses to MUC1.Tg mice did not produce any overt signs of autoimmune disease. A fraction of MUC1.Tg recipient mice with or without B16.MUC1 tumors was sacrificed, and the organs expressing MUC1 were evaluated for signs of autoimmunity (n = 8). No cellular infiltrate or architectural damage was observed in either group of recipient MUC1.Tg mice (data not shown).

Discussion

In this work, we report that a MUC1-specific CD4+ lymphocyte population is responsible for the in vivo elimination of tumors expressing the tumor-associated Ag MUC1. This activity developed in wt mice, but not MUC1.Tg mice, following challenge with B16.MUC1 tumor cells. The MUC1-specific CD4+ T cell-dependent mechanism of B16.MUC1 tumor protection could be adoptively transferred to wt mice or MUC1.Tg mice, albeit with differential protective effects. This activity was not detected in conventional in vitro assays of cell-mediated cytotoxicity.

A limiting dilution CTL frequency assay was used to quantify, over time, MUC1-specific or non-MUC1-reactive effector cells in recipient mice and provides direct evidence of tumor immunity in vivo. In this system, evidence of the transfer of tumor immunity is provided by an increase in the survival of recipient mice compared with control mice following tumor challenge. Adoptive transfer of immune cells is thought to increase the frequency of MUC1-specific or non-MUC1-reactive effectors in recipient mice and provides direct evidence of tumor immunity in vivo.

The adoptive transfer system was used to investigate the time course of the wt immune response to MUC1 and non-MUC1 tumor Ags following B16.MUC1 tumor challenge. Naive wt donors were unable to provide B16.MUC1 tumor protection. Wild-type mice challenged with B16.MUC1 tumor cells for 21 to 35 days developed tumor immunity that, upon adoptive transfer, provided complete protection in approximately 60% of wt recipients challenged with B16.MUC1 tumor cells. The 60% survival is the result of the tumor dose that we selected for these studies. A relatively high dose of this tumor was selected to clearly detect the differences between the untransferred wt animal response to B16.MUC1 cells and the response in the mice that received an adoptive transfer. Modest increases in survival were observed in wt recipients challenged with B16.Neo tumor cells, suggesting that reactivity to non-MUC1 tumor Ags was of minimal importance to the tumor immune response. Tumor immunity was not detectable by the adoptive transfer system in wt mice bearing B16.MUC1 tumors for greater than 35 days, suggesting that immunity to MUC1 and non-MUC1 tumor Ags had been down-regulated by that time point. The down-regulation of the wt tumor immune response at 35 days is most likely due to anergy (14–16), clonal exhaustion (17), or the effects of inhibitory substances produced by the tumor cells (18, 19), such as MUC1 (20). Coincident with the down-regulation of the immune response, wt mice began to develop tumors at a more progressive rate (Fig. 2).

Different results were obtained when the adoptive transfer system was used to evaluate tumor immunity in the MUC1.Tg mice over the course of B16.MUC1 tumor growth. Adoptive transfer of spleen and LN cells from MUC1.Tg mice challenged with B16.MUC1 tumors for 0 to 28 days provided no significant increase in wt recipient survival following challenge with either

FIGURE 5. Adoptively transferred CD4+ T cells provide tumor protection to wt mice challenged with B16.MUC1 tumor cells. Purified CD4+ (○, n = 4), CD8+ (□, n = 4), or nonpurified (▲, n = 4) tumor immune cells were adoptively transferred to wt recipients. Control wt (■, n = 6) or recipients were challenged with 2 × 104 B16.MUC1, and tumor growth was evaluated over time. At 28 days, statistically significant differences were detected between recipients of nonpurified immune cells and wt control or recipients of purified CD8+ cells (p < 0.05). The difference between wt controls and recipients of CD4+ cells was not significant (p < 0.10).

FIGURE 6. Adoptively transferred MUC1.Tg mice have increased survival following B16.MUC1 tumor challenge. Tumor immune cells were adoptively transferred to nonirradiated MUC1.Tg recipients. One day later, recipient MUC1.Tg (■, n = 5) or control MUC1.Tg (○, n = 5) mice were challenged with 2 × 104 B16.MUC1 (A) or B16.Neo (B) tumor cells, and tumor growth was evaluated over time. A, Statistically significant differences in survival were observed between recipient MUC1.Tg and control MUC1.Tg mice (p < 0.05). B, No differences in survival were detected (p > 0.30). The results shown are representative of three independent studies.
B16.MUC1 or B16.Neo tumor cells. This result indicates that MUC1.Tg mice are functionally tolerant to MUC1 in vivo. Wild-type recipients compared with unmanipulated control wt mice reproducibly developed more progressively growing tumors following challenge with B16.Neo tumor cells (Fig. 3B). One interpretation of these results is that MUC1.Tg mice develop immune suppression during tumor progression that inhibits the development of non-MUC1 immunity in wt mice.

The MUC1.Tg mice maintain immune responsiveness to non-MUC1 tumor Ags (9, unpublished results), yet adoptive transfer of their primed immune cells did not result in increased wt recipient survival after challenge with B16.Neo tumor cells. This finding, in part, may be explained by concept of xenogenization in which strong immune responses to a foreign protein confer immunity to otherwise less immunogenic or nonimmunogenic proteins (21). The immune response of wt mice to the foreign Ag MUC1 and the resulting inflammatory response may have enhanced immune surveillance of non-MUC1 tumor Ags, thus allowing the development and transfer of non-MUC1 immunity and an increase in wt recipient survival after challenge with B16.Neo tumor cells.

The results obtained from the two experimental systems used to evaluate MUC1-specific responses in the MUC1.Tg mice were profoundly different. Results from the limiting dilution CTL frequency assay indicated that MUC1.Tg mice challenged with B16.MUC1 tumor cells produce MUC1-specific CTLs at frequencies similar to wt mice. However, the adoptive transfer system showed that wt but not the MUC1.Tg mice developed protective MUC1-specific immunity.

In vitro assays of cell-mediated cytotoxicity are sometimes difficult to interpret, in part because they are conducted under nonphysiologic conditions that include high concentrations of Ag and stimulatory cytokines. To kill tumor cells in vivo, lymphocytes must function within the tumor microenvironment, an environment that is likely to be distinct from the conditions encountered in vitro. The microenvironment of the tumor presents many challenges to effector lymphocytes, including changes in soluble Ag concentrations with increased tumor development (22), antigenic heterogeneity of tumor cells (9, 23, 24), production of immunoregulatory cytokines by the tumor cells (18, 19), the immunosuppressive effect of tumor burden (25, 26), and physical barriers that preclude tumor cell-lymphocyte interaction (27, 28). Furthermore, it remains unclear whether CTL activity measured in vitro can provide tumor protection in vivo since these assays are at best only correlative. Indeed, the findings presented in this work and by others (29) demonstrate that mice transgenic for human MUC1 develop cell-mediated responses that are detectable in vivo; however, a more germane question is whether these responses eliminate tumors in vivo. The findings presented in this work raise questions regarding the use of the in vitro CTL lytic assay to quantify immunity against tumors. The results underscore the need for the development of other experimental systems to evaluate tumor immunity in vivo.

Results of in vivo, Ab-mediated, T cell depletion studies and the adoptive transfer of purified T cell subsets showed that CD4+ but not CD8+ lymphocytes were responsible for providing MUC1-specific tumor immunity in wt mice. Failure to detect immunity to B16.MUC1 tumor cells in the MUC1.Tg mice indicates that tolerance to MUC1 exists in the CD4+ cell population. Results of previous studies also suggest that MUC1.Tg CD4+ T lymphocytes were tolerant to MUC1: MUC1.Tg mice immunized with MUC1 peptides produced IgM, but only weak IgG Ab responses, which is consistent with a lack of CD4+ Th cell function (9). It is possible that tolerance to MUC1 expressed by the B16 transfectants is maintained by an anergic state deficient in the production of cytokines necessary for MUC1-specific CD8+ CTL priming/activation in vivo (30). While this is an attractive hypothesis, there are other possibilities. CD4+ T cell effectors have been reported in clinical and preclinical models to react with several tumor-associated Ags (31–34), including MUC1 (35). B16.MUC1 cells cultured in vitro express MHC class I, but do not express MHC class II molecules within the detectable limits of flow cytometry (data not shown). However, it is not known whether B16.MUC1 cells up-regulate MHC class II molecules in vivo as a response to inflammatory cytokines such as IFN-γ. MUC1-specific Abs have been shown to play little, if any, role in protection against B16.MUC1 tumors; this lessens the likelihood that CD4+ T cells mediate B16.MUC1 tumor immunity by facilitating the production of high affinity MUC1-specific IgG Abs. The finding that CD8+-depleted mice rejected B16.MUC1 tumors at rates similar to wt controls suggests that CD4+ lymphocytes may play a direct role in tumor rejection or function to help a non-CD8+ immune cell population in the rejection process.

MUC1-specific T cells have been detected in some cancer patients with adenocarcinoma of the breast or ovary (1–3). It is difficult to know whether this immune response detected in vitro destroys MUC1-bearing tumors in vivo. It is likely that these responses are ineffective, since these patients have a poor clinical prognosis. Immunologic tolerance to MUC1 prevents effective MUC1-specific immune responses in both cancer patients and the MUC1.Tg mice. In this respect, the MUC1.Tg mice are a reasonable preclinical model to evaluate immunization strategies designed to overcome tolerance to MUC1.

The ability of MUC1-specific CD4+ effector cells produced in wt mice to provide tumor immunity or provoke autoimmune reactions in normal epithelia-expressing MUC1 was evaluated by the adoptive transfer system. A statistically significant increase in survival was detected in MUC1.Tg recipients after challenge with B16.MUC1 tumor cells. There were no obvious or histologic signs of autoimmunity in recipient MUC1.Tg mice. The observed tumor protection and lack of detectable autoimmunity in the recipient MUC1.Tg mice are consistent with findings in other experimental models. For example, adoptive transfer of anti-Friend leukemia virus envelope or anti-p53 T cells into Friend leukemia virus transgenic mice or p53+/− mice, respectively, eliminated tumor cells expressing these self Ags with no detectable autoimmunity (36–38).

The lack of a detectable autoimmune response may be explained by several different hypotheses. One simple explanation is that levels of MUC1 protein expression in nontransformed cells may not be sufficient to stimulate immune reactivity. Alternatively, the glycosylation patterns may be altered in tumor-associated MUC1 compared with MUC1 expressed by nontransformed epithelium, obscuring immunodominant epitopes. It is not known whether the MUC1-specific CD4+ cells described in this work recognize MUC1 in the context of MHC class II molecules. However, the targeted stimulation or adoptive transfer of MUC1-specific CD4+ cells with MHC class II restriction may promote MUC1-specific tumor immunity with little autoimmunity, since epithelial tissues normally do not express MHC class II molecules.

Peripheral mechanisms of tolerance have been described, including lack of T cell costimulation (14–16, 39), activation-induced cell death (40), and suppressor T cells (41, 42). It remains possible that suppressor T cells are present in the MUC1.Tg mice and function to prevent autoimmune T cells from inducing autoimmunity. In vitro data indicate that both MUC1.Tg and wt mice have equal abilities to respond to MUC1; however, the antitumor responses in the MUC1.Tg mice may be suppressed by immunoregulatory mechanisms that mediate peripheral tolerance to
MUC1. An alternative hypothesis is that regulatory cytokines or chemokines produced by normal epithelial cells inhibit T cell function.

In summary, MUC1.Tg mice develop MUC1-specific CTLs that are detectable in vitro; however, they remain functionally tolerant to MUC1 in vivo, as indicated by their inability to reject tumors expressing MUC1 and the failure of adoptively transferred cells to confer MUC1-specific immunity to wt mice. In contrast, wt mice challenged with B16.MUC1 tumor cells develop a MUC1-specific CD4+ effector population that is capable of rejecting tumor cells expressing MUC1 in vivo. Adoptive transfer of the wt tumor immune cells to MUC1.Tg recipients significantly increased the survival of MUC1.Tg recipients challenged with B16.MUC1 tumor cells without induction of autoimmune. The MUC1.Tg mice provide a useful preclinical model for evaluating novel vaccine formulations and their translation into effective anti-MUC1 tumor responses.

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References