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MUC1-specific anti-tumor responses: molecular requirements for CD4-mediated responses

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Abstract

MUC1 was first defined as a tumor antigen in the late 1980s, yet little is known about the types of immune responses that mediate rejection of MUC1$^+$ tumors *in vivo*. MUC1-specific antibodies, T_h cells and cytotoxic T cells can be detected in patients with different adenocarcinomas, yet these tumors usually progress. Thus, there is a need to better understand the *in vivo* mechanisms of antigen-specific tumor rejection. To characterize the nature of MUC1-specific immune responses *in vivo*, rejection of a MUC1-expressing melanoma tumor line (B16.MUC1) was evaluated in mice lacking specific T cell subsets, cytokines, co-stimulatory molecules or molecular effectors of cytolytic pathways. Results demonstrated that rejection of the B16.MUC1 tumor cell line was primarily mediated by CD4$^+$ T cells, and required Fas ligand, lymphotoxin-α, CD40, CD40 ligand and CD28, but not perforin, γδ T cells, IL-4, IL-10, IL-12 or tumor necrosis factor receptor-1. Depletion of NK cells demonstrated that NK cells might also contribute to MUC1 immunity in the B16.MUC1 tumor model. These results demonstrated that the immune response generated against MUC1 does not fit the type 1 or 2 model described for many immune responses. Additionally, multiple cytolytic mechanisms are required for B16.MUC1 rejection.

Introduction

MUC1 is expressed on normal and transformed epithelial cells, including those of the breast, pancreas, lung and gastrointestinal tract. Adenocarcinomas from these organ sites overexpress and aberrantly glycosylate MUC1, which has led to its investigation as a candidate for immunotherapy (1,2). Patients with MUC1$^+$ tumors develop both humoral and cell-mediated immunity against MUC1 as measured by *in vitro* parameters (3–5); however, these responses usually do not eliminate the tumors *in vivo.* Clinical trials that aim to enhance immune responses to MUC1 are in progress (6). Enhancement of endogenous immune responses to MUC1 is predicted to improve survival of patients with MUC1$^+$ adenocarcinomas.

It is clear that immune responses play an important role in preventing tumor growth. Compelling evidence in support of this conclusion comes from findings of increased tumor incidence in immunosuppressed patients as compared to the general population (7). However, *in vitro* investigations of tumor immune responses have mostly failed to clearly define the specificity and nature of immune responses that reject tumors. For example, there were no detectable differences in the anti-MUC1 cytotoxic T lymphocyte (CTL) precursor frequencies of C57BL/6 mice transgenic for human MUC1 (MUC1.Tg) and wild-type C57BL/6 animals (8), even though wild-type animals rejected MUC1-expressing tumors in an antigen-specific manner and MUC1.Tg mice did not, because of immunological tolerance (8,9). In humans, anti-MUC1 responses are frequently detected in patients with advanced and progressing adenocarcinomas (4), but the responses detected *in vitro* are insufficient to protect against tumor progression *in vivo*. Thus, there is a lack of correlation between detectable *in vitro* responses and *in vivo* protection. Therefore, there is a critical need to better understand the nature of antigen-specific anti-tumor immune responses *in vivo* so that improved vaccination strategies can be developed.

We previously investigated, by *in vivo* methods (antibody depletion of specific cell types and passive antibody transfer), the nature of immune responses to MUC1 that mediated rejection of tumors in mice. Wild-type C57BL/6 mice develop

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both humoral and cellular immune responses against human MUC1. Passive transfer of MUC1-specific antibodies did not affect the growth of MUC1-expressing B16 tumors (10). Instead, we found that $CD4^+$ T cells were critical for rejection of B16.MUC1 melanomas, whereas depletion of $CD8^+$ T cells had no effect on tumor rejection (8). Results of other *in vivo* studies suggested that $CD8^+$ T cells were required for MUC1-specific immunity against a different type of tumor, specifically a pancreatic carcinoma (11). One potential limitation of these studies was that they employed antibody depletion as part of the experimental design. Administration of antibodies that deplete $CD4^+$ and/or $CD8^+$ T cells does not completely eliminate the desired cell population (12). Data presented in this report confirm and extend our previous results that $CD4^+$ cells mediate rejection of MUC1-expressing B16 cells by utilizing mice with targeted mutations. Results presented here demonstrate that rejection of the B16.MUC1 tumor cell line is primarily mediated by $\alpha\beta$ $CD4^+$ T cells, NK cells and/or monocytes and required Fas ligand (FasL), lymphotoxin (LT)-α, CD40, CD40 ligand (CD40L) and CD28, but not perforin, IL-4, IL-10, IL-12 or tumor necrosis factor (TNF) receptor (TNFR)-1.

Methods

Mice

Male and female wild-type C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD). Male and female C57BL/6 mice deficient in $\alpha\beta$ T cells ($TCR\beta^{-/-}$) (13), $\gamma\delta$ T cells ($TCR\delta^{-/-}$) (14), IFN-γ (IFN-$\gamma^{-/-}$) (15), CD28 ($CD28^{-/-}$) (16), CD40L ($CD40L^{-/-}$) (17), CD40 ($CD40^{-/-}$) (18), IL-4 ($IL\text{-}4^{-/-}$) (19), IL-10 ($IL\text{-}10^{-/-}$) (20), IL-12 ($IL\text{-}12^{-/-}$) (21), LT-α (LT-$\alpha^{-/-}$) (22) or TNFR-1 ($TNFR\text{-}1^{-/-}$) (23) were purchased from Jackson Laboratories (Bar Harbor, ME). Breeder pairs of C57BL/6 mice lacking CD4 ($CD4^{-/-}$), CD8 ($CD8^{-/-}$), CD4 and CD8 (CD4/$CD8^{-/-}$), perforin ($pfp^{-/-}$) and *gld* mice were purchased from Jackson Laboratories. Age-matched MUC1.Tg (9) mice, and $CD4^{-/-}$ (24), $CD8^{-/-}$ (25), CD4/$CD8^{-/-}$ (26), $pfp^{-/-}$ (27) and *gld* (28) mice were obtained from breeding colonies at the University of Nebraska Medical Center (Omaha, NE). Mice were treated in accordance with the Institutional Animal Care and Use Committee guidelines.

Cell lines

The bladder 6 (BL6) variant of the C57BL/6 murine melanoma, B16, stably expressing *MUC1* cDNA (B16.MUC1) or the control vector (B16.neo) has been described previously (9). In brief, the BL6 variant of the B16 C57BL/6-derived murine melanoma cell line was transfected with human MUC1 flag-tagged cDNA in the pHβApr-1 neo vector (B16.MUC1). Expression was confirmed by Western blotting with the M2 antibody, which recognizes the flag tag. B16 cells were also transfected with the empty pHβAPr-1-neo vector to use as a control (B16.neo). B16 cells were cultured in DMEM (Gibco/BRL, Rockville, MD) supplemented with 10% FBS (Biowhittaker, Walkersville, MD), essential amino acids (Biowhittaker), non-essential amino acids (Biowhittaker), sodium pyruvate (Sigma, St Louis, MO), vitamins (Cellgro, Mediatech, Herndon, VA), penicillin/streptomycin (Biowhittaker) and G418 sulfate (Mediatech, Herndon, VA).

Tumor challenge

Mice were anesthetized (Metofane; Pitman-Moore, Madelein, IL) and challenged by injection s.c. between the scapulae with 2×10^4 viable B16.MUC1 or B16.neo cells. Tumor growth was measured every 2–3 days. Kaplan–Meier survival curves are shown for representative tumor challenge studies. Death was defined as the date on which tumors achieved a diameter of 1.0 cm. Defining death as the point at which the tumor reaches a diameter of 1.0 cm does indeed reflect survival; stabilization or regression is never seen. Mice were euthanized when tumor diameter was >1.2 cm, in accordance with IACUC requirements.

Statistical analyses

Tumor challenge studies were conducted $\geqslant$3 times. Survival data were pooled and the log-rank test was used for statistical analysis of survival. Additionally, the Cox regression analysis was used to confirm statistical differences among experimental groups, and to verify that data from repeated experiments was statistically similar and able to be pooled. $P < 0.05$ was used to determine statistical significance.

Depletion of NK cells

Anti-asialo-GM_1 antibody (Wako, Richmond, VA), diluted to final volume of 400 μl with PBS, was administered by i.p. injection to wild-type C57BL/6 mice 3 days prior to tumor challenge, and 4 and 11 days following tumor challenge. Isotype control rabbit serum was administered to control mice in a similar fashion. Anti-asialo-GM_1 antibody also depletes murine monocytes.

MHC class I and II analysis

B16.MUC1 or B16.neo cells (8×10^5) were cultured in 5 ml of media with or without 25 ng of IFN-γ (R & D Systems, Minneapolis, MN) for 48 h at 37°C and 5% CO_2. Cells were removed from tissue culture flasks with trypsin/EDTA (Gibco/BRL). Cells (1×10^5) were suspended in FACS buffer ($1 \times$ PBS/0.1% FBS and 0.01% sodium azide) and reacted with anti-H-$2K^b$ (IgG2a) (AF6-88.5), isotype control IgG2a, anti-I-A^b FITC (AF6-120.1) or anti-TNP–FITC (isotype control) (G155-178) (PharMingen, San Diego, CA) for 1.5 h at 4°C. Cells were washed 3 times with FACS buffer, and H-$2K^b$-stained cells and IgG2a isotype control cells were detected with a secondary antibody [anti-mouse IgG (Fab_2)–FITC] (Gibco/BRL). Cells were washed 3 times, fixed with 1% paraformaldehyde and analyzed with the FACSCalibur fixed system which had been calibrated with CaliBRITE Beads (Becton Dickinson, Mountain View, CA). No gating was performed. Analysis was performed with CellQuest (Becton Dickinson). Single-parameter histograms are shown.

Results

Involvement of T cells in MUC1 immunity against the B16.MUC1 tumor cell line

Previous studies demonstrated that wild-type C57BL/6 mice produce MUC1-specific immune responses that prolong survival following challenge with MUC1-expressing B16 tumors (9). C57BL/6 mice transgenic for MUC1 (MUC1.Tg) display a degree of immunological tolerance to MUC1, as evidenced by poor survival following B16.MUC1 challenge. In results presented here, the contribution of $CD4^+$ T cells and $CD8^+$ T cells to MUC1-specific tumor immunity was evaluated *in vivo* in C57BL/6 mice deficient in these cell populations.

$CD4^{-/-}$, $CD8^{-/-}$, $CD4/CD8^{-/-}$, wild-type and MUC1.Tg mice were challenged with B16.MUC1 or B16.neo cells s.c. Specific immune responses to MUC1 significantly prolonged survival (Fig. 1) of wild-type mice when compared to $CD4/CD8^{-/-}$, $CD4^{-/-}$ and MUC1.Tg ($P < 0.05$) mice following challenge with B16.MUC1 cells. The B16.MUC1 tumor line grows very aggressively: injection of only 20,000 cells causes palpable tumors to appear within 7–10 days in MUC1.Tg mice. Wild-type mice eliminate most B16.MUC1 tumor cells; however, MUC1 antigen-negative variant tumors appear at a later timepoint and eventually kill most animals. As described previously (9), tumors that appear at delayed times in wild-type animals do not express MUC1 as detected by immunohistochemistry (data not shown). Evidence that the immune response to MUC1 eliminated antigen-positive tumor cells in wild-type mice is seen in the prolongation of survival of the wild-type animals as compared to the MUC1.Tg animals and is substantiated by the fact that tumors in MUC1.Tg mice express MUC1 (9). The results presented in Fig. 1 show that $CD4^+$ cells are required for the MUC1-specific response to B16.MUC1, since $CD4^{-/-}$ animals fail to reject tumors and show survival equivalent to or worse than the immunologically tolerant MUC1.Tg strain.

A specificity control for immune responses to MUC1 is provided by challenging animals with control B16.neo cells, which do not express MUC1, but do express the neomycin resistance gene product. Challenge with B16.neo yielded tumor growth and survival rates that were similar for all groups of mice (Fig. 1B). These data show that differences in survival following tumor challenge observed for the different strains of C57BL/6 mice evaluated here were the result of immune responses to MUC1 and not to the neomycin resistance gene product or other antigens expressed on the B16 tumor cell line. Furthermore, tumor growth rates and survival of MUC1.Tg mice challenged with B16.MUC1 were similar to those seen in all groups of mice following challenge with B16.neo. This indicates that these two cell lines (B16.MUC1 and B16.neo) have similar growth properties *in vivo* and that the observed differences are not due to differences in biological properties of the tumor cell lines.

Survival of $CD8^{-/-}$ mice [median survival time (MST) = 29 days] challenged with B16.MUC1 was statistically indistinguishable when compared to wild-type mice (MST = 29 days, $P = 0.6882$) and improved when compared to MUC1.Tg (MST = 24 days, $P = 0.0159$), $CD4^{-/-}$ (MST = 24 days, $P = 0.0001$) and $CD4/CD8^{-/-}$ (MST = 21 days, $P < 0.0001$) mice (Fig. 1A). These results indicate that $CD8^+$ T cells are not required for

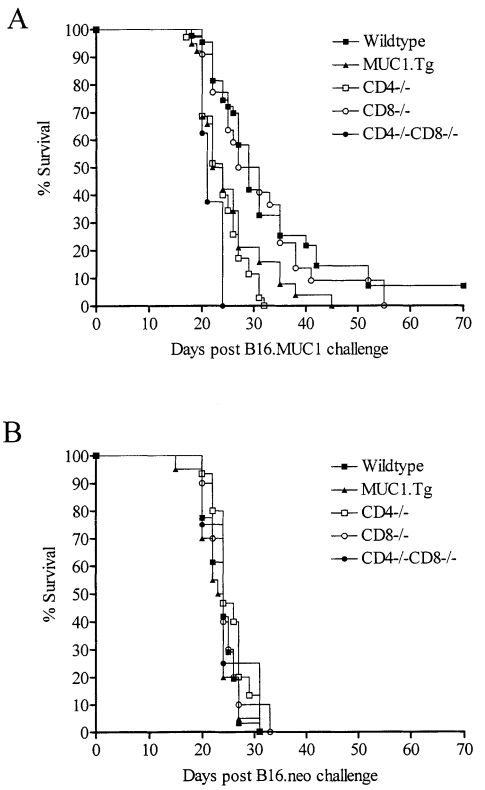

Fig. 1. $CD4^+$ cells are required for MUC1 immunity in the B16.MUC1 tumor model. (A) Wild-type ($n = 42$), MUC1.Tg ($n = 38$), $CD4^{-/-}$ ($n = 35$), $CD8^{-/-}$ ($n = 22$) and $CD4/CD8^{-/-}$ ($n = 8$) mice were challenged with 2×10^4 B16.MUC1 cells s.c. on day 0. (B) Wild-type ($n = 31$), MUC1.Tg ($n = 20$), $CD4^{-/-}$ ($n = 15$), $CD8^{-/-}$ ($n = 10$) and $CD4/CD8^{-/-}$ ($n = 4$) mice were challenged with 2×10^4 B16.neo cells s.c on day 0, and tumor growth was measured over time. Data were pooled from at least three independent experiments conducted at different times. (A) Statistically significant increases in survival were observed in wild-type and $CD8^{-/-}$ mice compared to $CD4/CD8^{-/-}$, $CD4^{-/-}$, MUC1.Tg ($P < 0.05$). (B) No statistically significant differences in survival were observed between any groups of mice ($P > 0.05$).

MUC1-specific immunity in the B16.MUC1 tumor model. In contrast, survival of mice challenged with B16.neo cells was statistically indistinguishable among all strains of mice ($P > 0.05$) (Fig. 1B).

To further evaluate the role of T cells in MUC1-mediated tumor rejection, $TCR\beta^{-/-}$, $TCR\delta^{-/-}$ and $TCR\beta/TCR\delta^{-/-}$ mice were challenged with B16.MUC1 and B16.neo cells. $TCR\beta^{-/-}$, $TCR\beta/TCR\delta^{-/-}$ and MUC1.Tg mice survived for similar periods of time; however, their survival was significantly decreased when compared to wild-type mice and $TCR\delta^{-/-}$ mice (Table 1). Survival of mice challenged with B16.neo cells showed no difference in survival (data not shown). These results support the hypothesis that $CD4^+$ cells are required for B16.MUC1 tumor rejection, since $CD4^+$ cells contain $\alpha\beta$ TCR. Furthermore, the results suggest that $\gamma\delta$ T cells do not play a significant role in B16.MUC1 rejection.

MHC class I and class II expression on B16.MUC1 cells

The functions of $CD4^+$ cells that are required for MUC1-specific tumor rejection are poorly understood. Two non-

Table 1. TCR requirements for MUC1-mediated tumor rejection

Strain	Versus wild-type		Versus MUC1.Tg	
	Δ median survival (days)[a]	Significance[b] (*P* value)	Δ median survival (days)[a]	Significance[b] (*P* value)
TCRβ$^{-/-}$	–11	yes (<0.0001)	–1	no (0.5319)
TCRδ$^{-/-}$	–3	no (0.9931)	+7	yes (0.0007)
TCRβ/ TCRδ$^{-/-}$	–21	yes (<0.0001)	–2	no (0.3495)

[a]Change in median survival compared to non-vaccinated wild-type or MUC1.Tg mice.
[b]Significance ($P < 0.05$) compared to survival of non-vaccinated wild-type or MUC1.Tg mice (log-rank test) exact *P* values listed.

exclusive possibilities are that they act as helper cells or show direct cytotoxic activity. We hypothesized that MHC class II molecules on B16.MUC1 cells would be the target of CD4$^+$ effector cells. MHC class II molecules are not expressed on most tumor cells, including B16.MUC1; however, IFN-γ has been shown to induce class II expression on some tumor cell types (29). To evaluate the potential importance of IFN-γ in the immune response against B16.MUC1, cells were treated *in vitro* and evaluated for expression of MHC class I and II on the cell surface.

MHC class I and II expression was evaluated on B16.MUC1 and B16.neo cells. Cells were stained with antibodies that recognize H-2K^b and I-A^b following culture with IFN-γ or media alone and evaluated using a FACSCalibur. Both cell lines expressed low levels of H-2K^b and no detectable I-A^b in the absence of IFN-γ. Following stimulation with 5 ng/ml of IFN-γ for 2 days, B16.MUC1 and B16.neo cells expressed higher levels of H-2K^b and low levels of I-A^b (Fig. 2). Induction of I-A^b in the B16.MUC1 cell line was reproducibly lower compared to the B16.neo cells. The reason for this difference is unclear. These results are consistent with the hypothesis that the CD4$^+$ T cells responsible for killing B16.MUC1 directly recognize the tumor cells through TCR–MHC class II interactions.

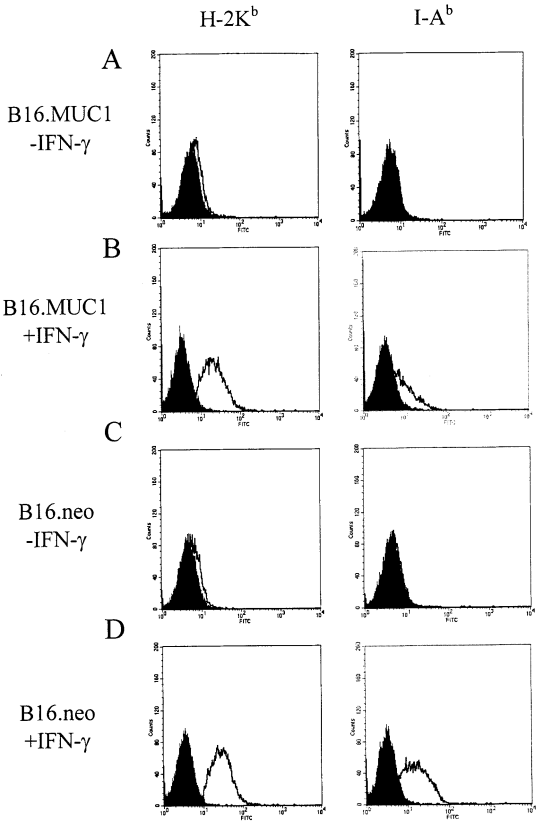

Fig. 2. B16.MUC1 and B16.neo cells up-regulate MHC class I molecules and induce class II expression following stimulation with IFN-γ. B16.MUC1 (A and B) and B16.neo (C and D) cells were cultured in the absence (A and C) or presence (B and D) of 25 ng of IFN-γ for 48 h. Cells were stained with antibodies for MHC class I (H-2K^b) (left panel) and class II (I-A^b) (right panel). Histograms show staining with the isotype control antibody (shaded) and class I/II antibody (solid line).

Involvement of IFN-γ in the B16.MUC1 immune response

The requirement for IFN-γ during rejection of B16.MUC1 tumors was further investigated in mice lacking IFN-γ (15). IFN-γ$^{-/-}$, wild-type and MUC1.Tg mice were challenged s.c. with B16.MUC1 or B16.neo cells. IFN-γ$^{-/-}$ mice showed intermediate results. The majority (16 of 19) of animals showed survival similar to MUC1.Tg mice (Fig. 3A). However, three of 19 mice did not develop tumors, which resulted in the finding that this experimental group was not statistically distinguishable from either MUC1.Tg or wild-type mice. These results indicate that IFN-γ may play a role in the rejection of B16.MUC1, but that there is a stochastic component to IFN-γ's contribution to rejection of B16.MUC1 that is not understood at this time.

Depletion of NK cells decreases immunity against B16.MUC1

Activated NK cells secrete IFN-γ (30) and can recognize tumor cells that express few or no MHC I molecules (31). As there is not currently a C57BL/6 mouse model lacking NK cells, mice were depleted of NK cells with an antibody directed against asialo-GM$_1$. This molecule is expressed on mouse NK cells and monocytes. Depleted mice were challenged with MUC1-expressing B16 cells to determine the role of NK cells in B16.MUC1 tumor immunity. Mice were injected with the antibody 3 days prior to, and 4 and 11 days following, tumor challenge. Survival of wild-type mice treated with anti-asialo-GM$_1$ was significantly worse than isotype control-treated wild-type mice challenged with B16.MUC1 cells (Fig. 4A). Survival of NK-depleted mice was similar to MUC1.Tg mice. In contrast, NK-depleted and wild-type mice challenged with B16.neo cells showed no differences in

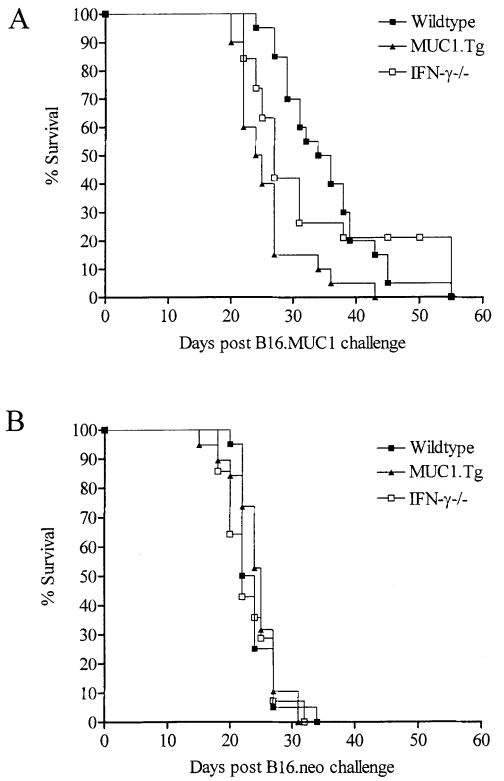

Fig. 3. The role of IFN-γ in MUC1 immunity in the B16.MUC1 tumor model. (A) Wild-type (n = 20), MUC1.Tg (n = 20) and IFN-$\gamma^{-/-}$ (n = 19) mice were challenged with 2 × 10^4 B16.MUC1 cells s.c. (B) Wild-type (n = 20), MUC1.Tg (n = 19) and IFN-$\gamma^{-/-}$ (n = 14) mice were challenged with 2 × 10^4 B16.neo cells s.c on day 0, and tumor growth was measured over time. Data are pooled from at least two independent experiments conducted at different times. (B) no statistically significant differences in survival were observed between any groups of mice ($P > 0.05$).

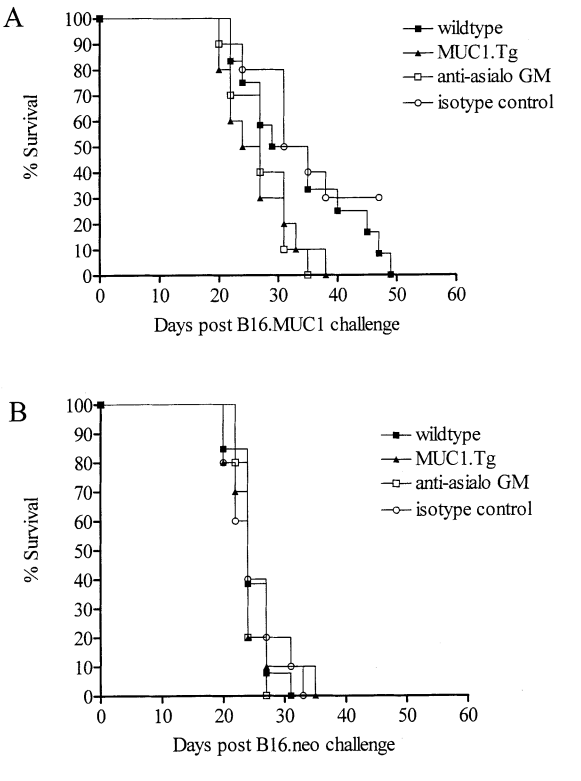

Fig. 4. NK cells contribute to B16.MUC1 immunity. (A) Wild-type (n = 12), MUC1.Tg (n = 10), wild-type mice treated with anti-asialo-GM_1 (n = 10) and wild-type mice treated with control sera (n = 10) were challenged with 2 × 10^4 B16.MUC1 cells s.c. (B) Wild-type (n = 12), MUC1.Tg (n = 10), wild-type mice treated with anti-asialo-GM_1 (n = 10) and wild-type mice treated with control sera (n = 10) were challenged with 2 × 10^4 B16.neo cells s.c. on day 0, and tumor growth was measured over time. Data are pooled from two independent experiments conducted at different times. (A) Significant increases in survival were observed in wild-type and control sera treated mice compared to MUC1.Tg and anti-asialo-GM_1-treated mice ($P < 0.05$). (B) No statistically significant differences in survival were observed between any groups of mice ($P > 0.05$).

survival (Fig. 4B). These data suggest that NK cells or another cell type that expresses asialo-GM_1 plays a role in the antigen-specific immune response against B16.MUC1.

Role of type 1 and 2 cytokines in B16.MUC1 immunity

Immune responses are often described as polarizing towards type 1 or 2. Type 1 responses are characterized by the production of IFN-γ, while type 2 responses produce IL-4, IL-5, IL-6 and IL-10 (32). IL-12 enhances type 1 responses by stimulating the production of IFN-γ by NK and T cells. IL-10 inhibits synthesis of IL-12 by dendritic cells and macrophages, and decreases production of IFN-γ by NK cells, thus generating a type 2 response. To determine whether the immune response against B16.MUC1 is primarily type 1 or 2, mice lacking type 1 [IL-12 (21) or IFN-γ (15)] or type 2 [IL-4 (19) or IL-10 (20)] cytokines were evaluated for survival following challenge with B16.MUC1.

Wild-type, MUC1.Tg, IL-$4^{-/-}$, IL-$10^{-/-}$ and IL-$12^{-/-}$ mice were challenged s.c. with either B16.MUC1 or B16.neo cells. Mice lacking the type 1 cytokine IL-12 challenged with B16.MUC1 cells demonstrated survival similar to wild-type mice and significantly better than MUC1.Tg mice (Table 2). Similarly, mice lacking either IL-4 or IL-10 (type 2 cytokines) challenged with B16.MUC1 survived for times similar to wild-type mice and significantly better than MUC1.Tg mice (Table 2). Survival of mice challenged with B16.neo cells was similar among all groups of mice (data not shown). These data suggest that the immune response generated against B16.MUC1 cannot be strictly classified as either type 1 or 2.

Co-stimulation requirements for the immune response against B16.MUC1

Activation of T cells to become effector T cells requires co-stimulation. Potent co-stimulatory signals result from interactions between B7 on antigen-presenting cells (APC) and CD28 on T cells (33). Co-stimulatory signals can also result from interactions between ICAM-1 on APC and LFA-1 on T cells (34). In addition, interactions between CD40 and CD40L have been described to augment expression of co-stimulatory molecules on many types of APC (35). CD40–CD40L interactions are also required for the migration of dendritic cells to the draining lymph nodes (36). Mice lacking CD28 (16), CD40

Table 2. Cytokine requirements for elimination of B16.MUC1 tumors

Strain	Versus wild-type		Versus MUC1.Tg	
	Δ median survival (days)[a]	Significance[b] (*P* value)	Δ median survival (days)[a]	Significance[b] (*P* value)
IL-12$^{-/-}$	+5	no (0.7584)	+14	yes (<0.0001)
IL-4$^{-/-}$	–8	no (0.3813)	+7	yes (0.0003)
IL-10$^{-/-}$	+1	no (0.2224)	+20	yes (0.0006)

[a]Change in median survival compared to non-vaccinated wild-type or MUC1.Tg mice.
[b]Significance ($P < 0.05$) compared to survival of non-vaccinated wild-type or MUC1.Tg mice (log-rank test) exact *P* values listed.

(18) or CD40L (17) were used to evaluate their role in the activation of MUC1-specific T cells that contribute to tumor rejection.

Wild-type, MUC1.Tg, CD28$^{-/-}$, CD40$^{-/-}$ and CD40L$^{-/-}$ mice were challenged with B16.MUC1 or B16.neo cells. Prolonged survival was observed for wild-type mice compared to survival of CD28$^{-/-}$ and MUC1.Tg mice following challenge with B16.MUC1 ($P < 0.05$) (Fig. 5A), but not with B16.neo cells ($P > 0.05$) (Fig. 5B). This suggests that CD28 is required for immune responses to MUC1 expressed on B16.MUC1 cells, most likely for activation of MUC1-specific T cells. Similarly, CD40$^{-/-}$ and CD40L$^{-/-}$ mice showed decreased survival compared to wild-type mice challenged with B16.MUC1 cells (Table 3). The interaction between CD40–CD40L could be required for migration of APC to draining lymph nodes to activate MUC1-specific T cells. Alternatively, the interaction may be critical for inducing other co-stimulatory molecules on APC.

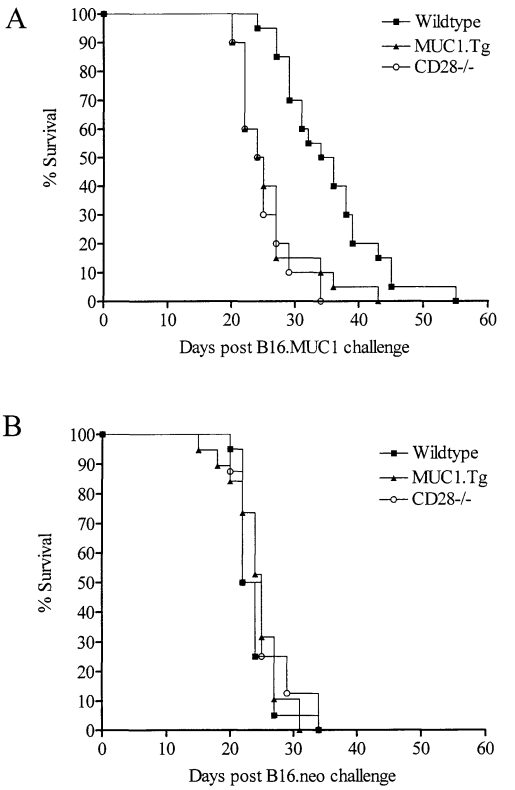

Fig. 5. CD28 is required for MUC1 immunity in the B16.MUC1 tumor model. (A) Wild-type ($n = 20$), MUC1.Tg ($n = 20$) and CD28$^{-/-}$ ($n = 10$) mice were challenged with 2×10^4 B16.MUC1 cells s.c. (B) Wild-type ($n = 20$), MUC1.Tg ($n = 19$) and CD28$^{-/-}$ ($n = 8$) mice were challenged with 2×10^4 B16.neo cells s.c on day 0, and tumor growth was measured over time. Data are pooled from at least two independent experiments conducted at different times. (A) Significant increases in survival were observed in wild-type mice compared to CD28$^{-/-}$ and MUC1.Tg ($P < 0.05$). (B) No statistically significant differences in survival were observed between any groups of mice ($P > 0.05$).

Involvement of cytolytic mediators in B16.MUC1 rejection

We investigated the role of effector mechanisms utilized by cytotoxic T cells specific for MUC1 by evaluating tumor rejection in mice with targeted mutations in molecular mediators of cytotoxicity. Receptor–ligand-induced killing is commonly mediated by FasL (37), membrane-bound TNF-α (38), LT-α (39) and/or TRAIL (40). Cytotoxicty can also be mediated by factors secreted following interaction of the CTL and target cell, including perforin and granzymes (41). Mice with targeted mutations in perforin (27), LT-α (22), TNFR-1 (23) or mice lacking functional FasL (*gld*) (28) were used to evaluate the role of these cytolytic pathways in MUC1-specific immunity.

The pfp$^{-/-}$, *gld*, LT-α$^{-/-}$, TNFR-1$^{-/-}$, wild-type and MUC1.Tg mice were challenged s.c. with B16.MUC1 or B16.neo. Prolonged survival was observed for wild-type mice compared to MUC1.Tg and *gld* mice following challenge with B16.MUC1 cells (Fig. 6A) ($P < 0.05$). These results suggest that MUC1 effector cell populations utilize Fas to lyse MUC1-expressing B16 cells. In contrast, pfp$^{-/-}$ mice showed survival similar to wild-type mice ($P > 0.05$), but improved survival compared to *gld* and MUC1.Tg mice ($P < 0.05$) (Fig. 6A). This indicates that perforin is not required for eradication of B16.MUC1 tumor cells. Challenge of *gld* with B16.neo resulted in small yet significant increased survival compared to wild-type and pfp$^{-/-}$ mice ($P < 0.05$) (Fig. 6B). This indicates that the decrease in survival in *gld* mice seen with B16.MUC1 is indeed due to MUC1. Decreased survival was observed in LT-α$^{-/-}$ mice challenged with B16.MUC1 compared to wild-type mice. In contrast, survival of TNFR-1$^{-/-}$ mice was similar to wild-type mice (Table 4). This suggests that LT-α, but not TNFR-1 is required for anti-MUC1 tumor immunity. Since TNF-α acts through multiple receptors, we cannot exclude a role for TNF-α with these experiments.

Table 3. Co-stimulation requirements for elimination of B16.MUC1 tumors

Strain	Versus wild-type		Versus MUC1.Tg	
	Δ median survival (days)[a]	Significance[b] (*P* value)	Δ median survival (days)[a]	Significance[b] (*P* value)
CD40L$^{-/-}$	–11	yes (<0.0001)	–3	no (0.4)
CD40$^{-/-}$	–5	yes (0.0001)	+1.5	no (0.6363)

[a]Change in median survival compared to non-vaccinated wild-type or MUC1.Tg mice.
[b]Significance ($P < 0.05$) compared to survival of non-vaccinated wild-type or MUC1.Tg mice (log-rank test) exact *P* values listed.

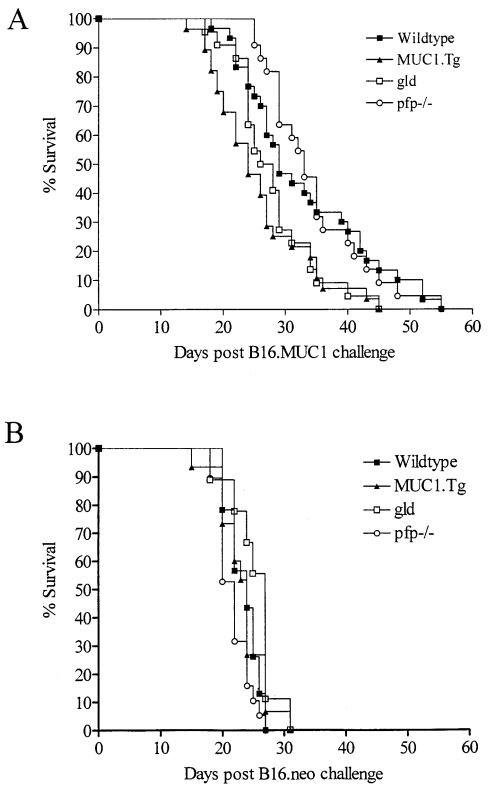

Fig. 6. FasL contributes to MUC1 immunity in the B16.MUC1 tumor model. (A) Wild-type ($n = 30$), MUC1.Tg ($n = 28$), *gld* ($n = 22$) and pfp$^{-/-}$ ($n = 22$) mice were challenged with 2×10^4 B16.MUC1 cells s.c. on day 0. (B) Wild-type ($n = 23$), MUC1.Tg ($n = 15$), *gld* ($n = 9$) and pfp$^{-/-}$ ($n = 19$) mice were challenged with 2×10^4 B16.neo cells s.c on day 0, and tumor growth was measured over time. Data are pooled from at least three independent experiments conducted at different times. (A) Statistically significant increases in survival were observed in wild-type and pfp$^{-/-}$ mice compared to MUC1.Tg and *gld* ($P < 0.05$). (B) statistically significant increases in survival were observed in *gld* mice compared to wild-type and pfp$^{-/-}$ mice ($P < 0.05$).

Discussion

Studies reported here support our hypothesis that CD4$^+$ T cells are critical for rejection of the MUC1-expressing melanoma, B16.MUC1 (8). Historically, CD8$^+$ T cells were thought to be the primary cell type involved in antigen-specific killing of tumor cells (42) and virally infected cells (43). Recently, CD4$^+$ T cells with lytic activity were detected in cancer patients, particularly those with melanoma (44). Cytotoxic CD4$^+$ T cells have also been found to play a role in the elimination of virally infected cells (45), as effectors in autoimmune disease (46), and in the elimination of APC in order to control degrees of immune responses (47). The CD4$^+$ cell required for elimination of B16.MUC1 may be an effector cell that utilizes the CD4–TCR complex and acts directly to cause cytotoxicity, since MHC class II can be induced on B16.MUC1 cells following culture with IFN-γ (Fig. 2). This possibility is further supported by the fact that elimination of IFN-γ decreases MUC1 immunity against B16.MUC1 *in vivo* (Fig. 3) and that knocking out TCRβ cells eliminates the response against B16.MUC1 (Table 1).

Recently, Mukherjee *et al.* demonstrated that adoptive transfer of CD8$^+$ T cells, generated in mice that develop spontaneous MUC1$^+$ pancreatic tumors, eliminates B16.MUC1 tumors in MUC1.Tg mice (48). Hence, it is possible to produce and utilize CD8$^+$ effector cells that are specific for MUC1 and that specifically kill B16.MUC1 tumor cells. Additional results from our laboratory indicate that both CD4$^+$ and CD8$^+$ T cell responses are generated against MUC1-expressing tumors in the pancreas, and that the production of these different responses is in part dependent on the type of tumor cell that expresses MUC1 and on the organ site of challenge (11). These results do not contradict the data presented here. Instead, the results suggest that distinct MUC1-specific immune responses are capable of eliminating B16.MUC1 cells and that different immunization conditions can lead to the production of these responses. Thus, a CD4$^+$ T cell response is generated when the animal is initially challenged with the B16.MUC1 tumor. This is interesting in light of the prediction and finding that a MUC1-specific CD8$^+$ T cell response is equally, if not more, effective in killing tumor cells. Many of the cytolytic CD4$^+$ cells described previously were generated in response to challenge with melanoma cells (49). This suggests that melanoma cells may preferentially stimulate CD4$^+$ responses because of their low level of MHC class I expression and the ability to induce MHC class II expression.

CD4$^+$ T cells are generally thought to exert their activity by acting as T_h cells. In this context, CD4$^+$ T cells function by providing necessary cytokines to stimulate CD8$^+$ CTL activity or antibody production by B cells. Although a helper function for CD4$^+$ T cells cannot be excluded by the results presented here, it is unlikely that lack of helper function alone is the sole explanation for the observation that a lack of CD4$^+$ T cells eliminates effective immunity (tumor rejection) that is specific

Table 4. Cytolytic requirements for elimination of B16.MUC1 tumors

Strain	Versus wild-type		Versus MUC1.Tg	
	Δ median survival (days)[a]	Significance[b] (*P* value)	Δ median survival (days)[a]	Significance[b] (*P* value)
LT-$\alpha^{-/-}$	–13	yes (0.0003)	–0.5	no (0.7582)
TNFR-1$^{-/-}$	+1	no (0.9312)	+12.5	yes (0.001)

[a]Change in median survival compared to non-vaccinated wild-type or MUC1.Tg mice.
[b]Significance ($P < 0.05$) compared to survival of non-vaccinated wild-type or MUC1.Tg mice (log-rank test) exact *P* values listed.

for MUC1. MUC1-specific antibodies are produced in C57BL/6 mice following challenge with B16.MUC1 cells, but antibodies are neither necessary nor sufficient for MUC1 immunity in the B16.MUC1 model (10), suggesting that removal of helper cells (CD4$^+$) required for antibody production would not affect survival. Furthermore, elimination of CD8$^+$ T cells has little effect on survival of mice challenged with B16.MUC1 cells; hence, it is unlikely that CD8$^+$ T cells are the main effector cell population. This suggests that the CD4$^+$ T cells are not just 'helping' CD8$^+$ CTL, but may be playing a more direct role in the rejection of tumor cells in this model.

Alternatively, CD4$^+$ cells may contribute to rejection of tumors by recruiting and providing help to other types of effector cells such as eosinophils or macrophages (50). As of yet no other cytokines have been shown to be required for the rejection of B16.MUC1 cells, although there are many other potential cytokines that the CD4$^+$ cells could be secreting to recruit other non-specific effector cells.

Another possibility is that the CD4$^+$ T cells responsible for MUC1-specific immunity are part of the CD4$^+$ NK T cell population. Although the specific role of these cells in the immune response is controversial, they have been shown to play a role in suppressing the growth of i.p. tumors (51). The CD4$^+$ T cells could also regulate a population of CD4$^-$ NK T or NK cells. Depletion of NK cells in wild-type mice decreased the immune response against B16.MUC1; however, the type of NK cell involved could not be determined in these experiments. Moreover, it remains possible that a different asialo-GM_1^+ cell population, such as macrophages, may be required for this response. Nonetheless, our data suggest that these cells are contributing to an antigen specific anti-tumor response, since depletion of these cell populations did not affect rejection of the B16.neo tumors.

Cytolytic CD4$^+$ T cells can utilize multiple pathways to lyse target cells. Killing by CD4$^+$ T cells is predicted to be induced through interactions between Fas and FasL (37); although CD4$^+$ T cell-mediated cytotoxicity has also been shown to be mediated by perforin (52), perforin and Fas (46), TNF-α (53) or TRAIL (40). The pfp$^{-/-}$, LT-$\alpha^{-/-}$, TNFR-1$^{-/-}$ and *gld* mice were used in our studies to investigate the importance of these cytolytic mediators in MUC1-specific tumor rejection. Our results with pfp$^{-/-}$ and *gld* mice indicate that Fas, but not perforin is utilized in CD4-dependent MUC1-mediated immunity to B16.MUC1 (Fig. 6). This is consistent with published reports showing that the majority of CD4$^+$ cytolytic cells utilize Fas to exert their lytic activity (37). Results with LT-$\alpha^{-/-}$ mice demonstrated that LT-α is required for B16.MUC1 immunity. These mice have a defect in secondary lymphoid tissue development (22). They also have a defect in NK development and recruitment (54–56); thus, LT-α involvement could be associated with an NK cell population. TNFR-1 is not required for B16.MUC1 immunity. These mice have no TNF-induced NF-κB activity (23), although there are other TNFR through which TNF could be acting.

Activation of T cells requires at least two signals: antigen recognition and co-stimulation. The second signal (co-stimulation) usually results from interactions between CD28 and B7-1/B7-2. Initial observations in other experimental systems demonstrated that B7$^+$ B cells and macrophages were lysed by CD4$^+$ CTL, while B7$^-$ B cells and macrophages were not lysed (57). In addition, cloned CD4$^+$ CTL expressed CD28. This led to a hypothesized role for B7–CD28 in CD4$^+$ T cell-mediated cytotoxicity (57). Subsequent experiments determined that the level of B7 expression did not correlate with susceptibility to lysis by CD4$^+$ CTL. Further studies demonstrated that anti-CD28 antibodies did not affect the ability of CD4$^+$ CTL to lyse target cells (57). Thus, we postulate that the contribution of CD28 to B16.MUC1 immunity is at the level of activation and not at the level of effector cell function. Co-stimulation between CD40 and CD40L is required for T cell-dependent antibody production. Signaling that results from interactions between CD40 and CD40L has also been shown to induce expression of co-stimulatory molecules, such as B7, on B cells and other APC. Additionally, CD40 signaling is required for the migration of dendritic cells to draining lymph nodes. Previous studies have demonstrated that MUC1-specific antibodies are not required for B16.MUC1 immunity, thus the requirement for CD40–CD40L is probably not at the level of antibody production. We cannot distinguish in this experimental system whether loss of CD40–CD40L causes a migration defect or a lack of induction of co-stimulatory signals.

Immune responses are generally thought to differentiate into either type 1 or 2 responses. The immune response generated against B16.MUC1 does not appear to fit this paradigm. Elimination of IL-4, IL-10 or IL-12 does not alter the survival of mice challenged with B16.MUC1. It is possible that redundant properties of cytokines have obscured the distinction between a type 1 or 2 response in this experimental system. It is more likely that the type 1 and 2 models that have been previously proposed do not accurately represent the response we have characterized in this report.

Many studies have utilized *in vitro* experiments, including cytotoxicity assays, to quantify immune responses against certain tumors or tumor antigens. These types of responses are frequently utilized as surrogate markers to monitor devel-

opment of immune responses following antigenic challenge. However, *in vitro* detection of cytolytic activity does not always correlate with *in vivo* tumor immunity (8,58). E:T ratios for *in vitro* assays are usually skewed to favor cytolytic activity. Additionally, the effects of suppressive environments, including such factors as local production of cytokines, stromal elements and other factors generated by tumors *in vivo*, may not be accurately reproduced by *in vitro* culture conditions. Thus, improved models that critically evaluate tumor immune responses *in vivo* are required to better understand the nature of effective anti-tumor responses and to give us insight into methods of provoking these responses.

The results presented in this report provide evidence that mice with targeted deletions provide a useful experimental system to dissect the components that contribute to the development of tumor immunity *in vivo*. Further studies with knockout and transgenic mice (immunologically tolerant strains) will provide insights into the intricate requirements for antigen-specific, anti-tumor immune responses and facilitate the development of tumor vaccines that target the specific pathways that are required to produce effective responses against both the target antigen and target cell.

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Abbreviations

APC	antigen-presenting cell
BL6	bladder 6
CD40L	CD40 ligand
CTL	cytotoxic T lymphocyte
FasL	Fas ligand
LT	lymphotoxin
MST	median survival time
Tg	transgenic
TNF	tumor necrosis factor
TNFR	TNF receptor

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