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Molecular requirements for CD8-mediated rejection of a MUC1-expressing pancreatic carcinoma: implications for tumor vaccines

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Abstract Previous studies have indicated that different effector cells are required to eliminate MUC1-expressing tumors derived from different organ sites and that different vaccine strategies may be necessary to generate these two different MUC1-specific immune responses. In this study, we characterized molecular components that are required to produce immune responses that eliminate Panc02.MUC1 tumors in vivo by utilizing mice genetically deficient in molecules related to immunity. A parallel study has been reported for a B16.MUC1 tumor model. We confirmed that a $CD8^+$ effector cell was required to eliminate MUC1-expressing Panc02 tumors, and demonstrated that T cells expressing TCR-α/β and co-stimulation through CD28 and CD40:CD40L interactions played critical roles during the initiation of the anti-Panc02.MUC1 immune response. TCR-α/β^+ cells were required to eliminate Panc02.MUC1 tumors, while TCR-γ/δ^+ cells played a suppressive non-MUC1-specific role in anti-Panc02 tumor immunity. Type 1 cytokine interferon-gamma (IFN-γ), but not interleukin-12 (IL-12), was essential for eliminating MUC1-expressing tumors, while neither IL-4 nor IL-10 (type 2 cytokines) were required for tumor rejection. In vitro studies demonstrated that IFN-γ upregulated MHC class I, but not MHC class II, on Panc02.MUC1 tumor cells. Surprisingly, both perforin and FasL played unique roles during the effector phase of immunity to Panc02.MUC1, while lymphotoxin-α, but not TNFR-1, was required for immunity against Panc02.MUC1 tumors. The findings presented here and in parallel studies of B16.MUC1 immunity clearly demonstrate that different effector cells and cytolytic mechanisms are required to eliminate MUC1-expressing tumors derived from different organ sites, and provide insight into the immune components required to eliminate tumors expressing the same antigen but derived from different tissues.

Keywords In vivo animal model · MUC1 · Transgenic/knockout · Tumor immunity

Abbreviations *APC* antigen-presenting cell · *CTL* cytotoxic lymphocyte · *DC* dendritic cell · –/– deficient · *gld* general lymphoproliferative disease · *MHC* major histocompatibility complex · *MUC1.Tg* MUC1 transgenic · *NK* natural killer · *pfp* perforin · *TCR* T cell receptor · *Th* helper T lymphocyte · *WT* wild-type C57BL/6 mice

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Introduction

MUC1 is normally expressed on the apical surfaces of ductal and glandular epithelia. When these tissues become malignantly transformed, MUC1 is overexpressed and aberrantly glycosylated [5, 34, 38, 60]. The ultimate result of these changes is the exposure of normally cryptic portions of the core protein structure to cells of the immune system; therefore, MUC1 may serve as a potential target for vaccine therapies.

Preclinical studies have focused on designing vaccine formulations by which to stimulate anti-MUC1 tumor immune responses. These strategies have included: targeting MUC1 epitopes to antigen-presenting cells [2, 61]; fusing MUC1-postive tumor cells and dendritic cells (DC) [18, 19]; immunizing with admixtures of recombinant vaccinia viruses expressing MUC1 tandem repeats and/or the co-stimulatory molecule B7 [1]; transfecting DC with MUC1 RNA and co-administering interleukin-12 (IL-12) [29]; and delivering synthetic MUC1 epitopes encapsulated in liposomes [51]. The vast majority of these formulations induce robust immune responses, cellular and/or humoral, in wild-type (WT) mice, which are

capable of eliminating MUC1-expressing tumors since human MUC1 is a foreign antigen to these animals.

Since human MUC1 is expressed on the apical surface of normal secretory epithelia and tolerance to self-antigens is a potential obstacle to the development of anti-tumor immunity, mice transgenic for human MUC1 (MUC1.Tg) were developed to provide a relevant model by which to assess the induction of anti-MUC1 immunity in preclinical vaccine studies [50]. These animals express MUC1 in patterns and at levels similar to humans, and they are immunologically tolerant to stimulation with MUC1 antigen and MUC1-expressing tumor cells. Therefore, unresponsiveness to MUC1 by MUC1.Tg mice may closely mimic the status of MUC1 immunity in patients with tumors expressing MUC1. Moreover, immune responses produced in these mice will be required to recognize tumor-associated MUC1 against a background of expression in normal organs and cells. Efforts are currently underway to determine whether or not tolerance to MUC1 can be reversed in MUC1.Tg mice, and what mechanisms induce tolerance to MUC1. A recent study has demonstrated that tolerance to MUC1-expressing tumors can be reversed in MUC1.Tg mice immunized with fusions of DC and carcinoma cells, as detected by both in vitro and in vivo analyses [19]. Although these studies indicate a potential for reversal of tolerance to MUC1, the mechanisms by which tolerance is induced and maintained in MUC1.Tg mice remain unclear.

It has been assumed that the primary effector cell that mediates MUC1 tumor rejection is the $CD8^+$ cytotoxic T lymphocyte (CTL). However, recent studies in C57BL/6 mice have indicated that a $CD4^+$ effector is required for the rejection of a syngeneic MUC1-expressing melanoma cell line (B16.MUC1), while a $CD8^+$ effector is required for the elimination of a syngeneic MUC1-expressing pancreatic carcinoma cell line (Panc02.MUC1) in C57BL/6 mice [41, 62]. These studies indicated that different effectors were required to eliminate MUC1-expressing tumors derived from different organ sites, and raised the possibility that different vaccine strategies were required to generate these two different types of MUC1-specific effectors. In the current report, we explored in vivo the molecular immune components required to eliminate MUC1-expressing tumors derived from pancreatic tissues by utilizing mice harboring mutations that inactivate different molecular components of the immune system. We confirm and extend our previous observation that a $CD8^+$ effector is required to eliminate MUC1-expressing Panc02 tumors in C57BL/6 mice, and discuss similarities and differences in the immune components required to reject Panc02.-MUC1 tumors compared to B16.MUC1 tumors.

Materials and methods

Mice

C57BL/6 mice (five to six weeks old) were purchased from the National Cancer Institute (Frederick, Md.). MUC1.Tg C57BL/6 mice have been described previously [50]. C57BL/6 mice deficient (–/–) in interferon-gamma, IFN-γ (IFN-γ –/–) [10], CD28 (CD28 –/–) [54], CD40L (CD40L –/–) [49], CD40 (CD40 –/–) [27], IL-12 (IL-12 –/ –) [35], IL-4 (IL-4 –/–) [31], IL-10 (IL-10 –/–) [30], lymphotoxin-α (LT-α –/–) [11], tumor necrosis factor receptor-1 (TNFR-1 –/–) [46], T cell receptor-beta,TCR-β (TCR-β –/–) [39], TCR-δ (TCR-δ –/–) [25], or TCR-β/δ (TCR-β/δ –/–) [39] were purchased from Jackson Laboratories (Bar Harbor, Me.). Breeder pairs of C57BL/6 mice lacking CD4 (CD4 –/–) [47], CD8 (CD8 –/–) [14], CD4 and CD8 (CD4/8 –/–), perforin (pfp –/–) [26], and FasL (*gld*) [59] were obtained from breeding colonies at the University of Nebraska Medical Center (UNMC). Mice were treated in accordance with the Institutional Animal Care and Use Committee guidelines.

Tumor cell lines and preparation for tumor challenge

The pancreatic carcinoma cell line Panc02, which is syngeneic to C57BL/6 mice, was obtained from J. Nelson (University of Texas, M.D. Anderson Cancer Center, Houston, Tex.) [8] and transfected with a recombinant expression vector to stably express human MUC1 cDNA (Panc02.MUC1) or the empty control vector (Panc02.neo), as has been described previously [42]. This cell line was maintained in complete medium consisting of McCoy's 5A medium (Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum (Biowhittaker, Walkersville, Md.), penicillin/streptomycin (Biowhittaker), and G418 sulfate (Mediatech, Herndon, Va.) in a humidified incubator at 37°C and with 5% CO_2. On the day of tumor challenge, adherent Panc02.MUC1 and Panc02.neo cells were removed from flasks with trypsin/EDTA (Gibco), washed once in complete medium, counted, and resuspended in HBSS (Gibco) at a concentration of 10×10^6 cells/ml. Cells were then placed on ice until tumor challenge.

Tumor challenge

Subcutaneous (s.c.) tumor challenge was performed as described previously [50]. Briefly, mice were anesthetized with metofane (Pitman-Moore, Madelein, Ill.) or halothane (Sigma) and challenged s.c. between the scapulae with 1×10^6 viable Panc02.MUC1 or Panc02.neo cells in a volume of 100 µl. Tumor growth was measured every two to four days. The experimental endpoint (death) was defined as the time point at which tumor volume reached 1 cm^3, whereupon the animals were euthanized. Data from these studies were represented by Kaplan–Meier survival curves, and statistical differences between survival for all groups of animals were calculated using the log-rank test.

Natural killer cell depletion

Wild-type C57BL/6 mice were depleted of natural killer (NK) cells by injecting mice i.p. with anti-asialo-GM1 (75 µl stock diluted in HBSS to 400 µl per mouse; Wako Chemicals, Richmond, Va.) three days prior to tumor challenge and days 4 and 11 following tumor challenge. Control mice were similarly injected with diluted rabbit sera. Tumor growth was measured as described previously.

MHC class I and II staining and flow cytometry

Approximately 8×10^5 Panc02.MUC1 or Panc02.neo tumor cells were cultured in 5 ml of complete medium in the absence or presence of 25 ng IFN-γ (R&D Systems, Minneapolis, Minn.) for 48 h at 37°C. Cells were removed from culture flasks with trypsin/EDTA, washed, counted, and resuspended in FACS wash solution (1×PBS containing 1% heat-inactivated FBS and 0.01% sodium azide). Approximately 1×10^5 cells were reacted with murine anti-mouse H-$2K^b$ (IgG2a) or murine IgG2a (isotype control); murine anti-mouse I-A^b-FITC (IgG2a) or murine anti-TNP-FITC (isotype control) for 1.5 h at 4°C. Stained cells were then washed three times

with FACS wash solution. H-2K^b- and IgG2a-stained cells were subsequently reacted with a FITC-labeled secondary antibody [goat anti-mouse IgG (Fab_2)-FITC; Gibco BRL], and washed as previously described. Stained cells were then fixed in FACS fixative (1×PBS containing 1% paraformaldehyde) and analyzed by FACScalibur (Becton Dickinson, Mountain View, Calif.). Analysis of MHC class I or II surface staining was performed using CellQuest software provided by Becton Dickinson.

Results

CD8$^+$ cells are the primary effectors in tumor immunity directed against MUC1-expressing Panc02 tumor cells

Previous studies in our laboratory using in vivo antibody depletion of CD4$^+$, CD8$^+$, CD4$^+$ and CD8$^+$ cells in WT C57BL/6 mice demonstrated that CD8$^+$ cells were the primary cell type required for immune rejection of Panc02.MUC1 tumor cells, and that CD4$^+$ cells contribute to tumor rejection when CD8$^+$ cells are present [41]. These results were in stark contrast to earlier findings that CD4$^+$ cells were the primary effectors against B16.MUC1 tumor cells, a melanoma cell line genetically engineered to express human MUC1 [62]. Taken together, these results suggested that distinct immune effectors and possibly different immune mechanisms were required to eliminate MUC1-expressing tumors derived from different tissues. We sought to further define integral parts of the MUC1-specific adaptive immune responses that are required to eliminate Panc02.MUC1 tumors from C57BL/6 mice by utilizing mice genetically deficient in various molecular components of the immune system.

CD4 –/–, CD8 –/–, or CD4/8 –/– mice, in addition to WT C57BL/6 (positive controls) and MUC1.Tg (negative controls) mice, were challenged with Panc02.MUC1 or Panc02.neo tumor cells. A substantial proportion (>60%) of WT mice rejected Panc02.MUC1 tumors, whereas MUC1.Tg mice, which are immunologically tolerant to human MUC1 [50], were unable to reject these tumors, as evidenced by significantly decreased survival ($P<0.0001$) when compared to WT mice (Fig. 1A). Tumors that progress in WT mice are MUC1-negative, while tumors that grow progressively in MUC1.Tg mice are MUC1-positive [42]. If an immune component is not required to reject MUC1-expressing Panc02 tumors, then the survival curve for those immune-deficient mice is similar to that for WT mice, which are immunologically reactive against human MUC1. However, if the immune component being tested is critical for rejecting MUC1-expressing Panc02 tumors, then the survival curve for these immune-deficient mice is worse than that for WT mice, and more closely resembles the survival curve for MUC1.Tg mice, which were unable to reject MUC1-expressing tumors. Fig. 1A demonstrates that CD4 –/– mice experienced significantly prolonged survival ($P=0.0037$) when compared to MUC1.Tg mice, while CD8 –/– mice ultimately

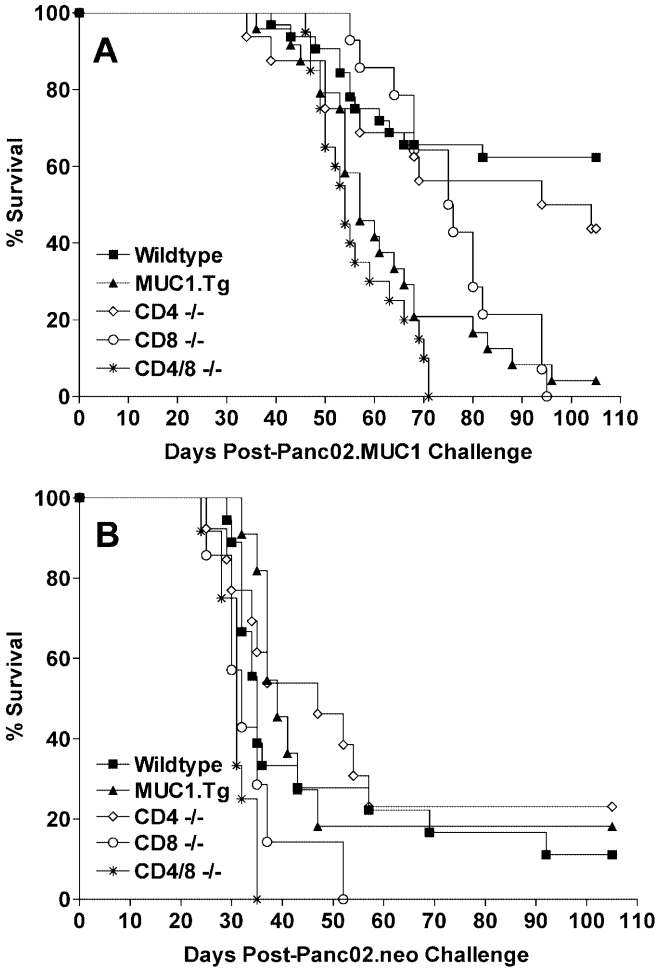

Fig. 1A, B. CD8$^+$ T cells but not CD4$^+$ T cells are the primary effectors for anti-Panc02.MUC1 tumor immunity in C57BL/6 mice. **A** WT ($n=32$), MUC1.Tg ($n=24$), CD4 –/– ($n=16$), CD8 –/– ($n=14$) and CD4/8 –/– ($n=20$) C57BL/6 mice were challenged with 1×10^6 Panc02.MUC1 tumor cells s.c. between the scapulae on day 0. Statistically significant increases in survival were observed in WT and CD4 –/– mice compared to CD8 –/–, CD4/8 –/–, and MUC1.Tg mice ($P<0.003$). **B** WT ($n=18$), MUC1.Tg ($n=11$), CD4 –/– ($n=13$), CD8 –/– ($n=7$), and CD4/8 –/– ($n=12$) C57BL/6 mice were challenged with 1×10^6 Panc02.neo tumor cells s.c. between the scapulae on day 0. No statistically significant differences in survival were observed between CD4 –/– and CD8 –/– mice and WT or MUC1.Tg mice ($P>0.05$). CD4/8 –/– mice consistently experienced significantly decreased survival when compared to wild-type and MUC1.Tg mice ($P<0.004$). Data were pooled from at least two independent experiments conducted at different times

experienced significantly decreased survival ($P=0.0029$) when compared to WT mice. Survival of CD4 –/– mice was statistically indistinguishable from WT survival, while survival of CD8 –/– mice was similar to MUC1.Tg survival. Mice deficient in both CD4$^+$ and CD8$^+$ cells also experienced significantly decreased survival ($P<0.0001$) when compared to WT, CD4 –/– and CD8 –/– mice (Fig. 1A), suggesting a role for CD4$^+$ cells in the presence of CD8$^+$ cells. The prolonged survival of WT mice and CD4 –/– mice seen in Fig. 1A was due

to MUC1-specific immune responses directed against Panc02.MUC1 tumor cells, since all strains of mice tested were unable to efficiently reject Panc02.neo control tumors (Fig. 1B). Taken together, these data confirmed our previous findings that $CD8^+$ cells were primarily required for the elimination of Panc02.MUC1 tumor cells, and that $CD4^+$ cells contributed to tumor rejection when $CD8^+$ cells were present.

NK cells may play a critical role in the development of anti-Panc02.MUC1 tumor immunity

We evaluated the contribution of NK cells to immune responses against Panc02.MUC1 tumors. WT C57BL/6 mice were depleted of NK cells by administration of anti-asialo-GM1, an antibody that is reactive with mouse NK cells and monocytes, and that has been shown to eliminate NK activity in various strains of mice three days prior to and at days 4 and 11 following challenge with Panc02.MUC1 or Panc02.neo tumor cells. Control mice were similarly treated with normal rabbit serum. As shown in Fig. 2A, mice depleted of NK cells and challenged with Panc02.MUC1 tumors showed significantly decreased survival ($P = 0.0135$) as compared to WT mice and survival that was similar to MUC1.Tg mice. The survival of control mice treated with normal rabbit sera was similar to that of WT mice ($P = 0.7509$). The prolonged survival of WT mice and control mice in this study was due to MUC1-specific immune responses, since all strains of mice tested lacked evidence of immunity to Panc02.neo control tumors (Fig. 2B). These results suggest that NK cells or another asialo-GM1-expressing cell population, such as monocytes or NK T cells, may play a critical role during the development of anti-Panc02.MUC1 tumor immunity.

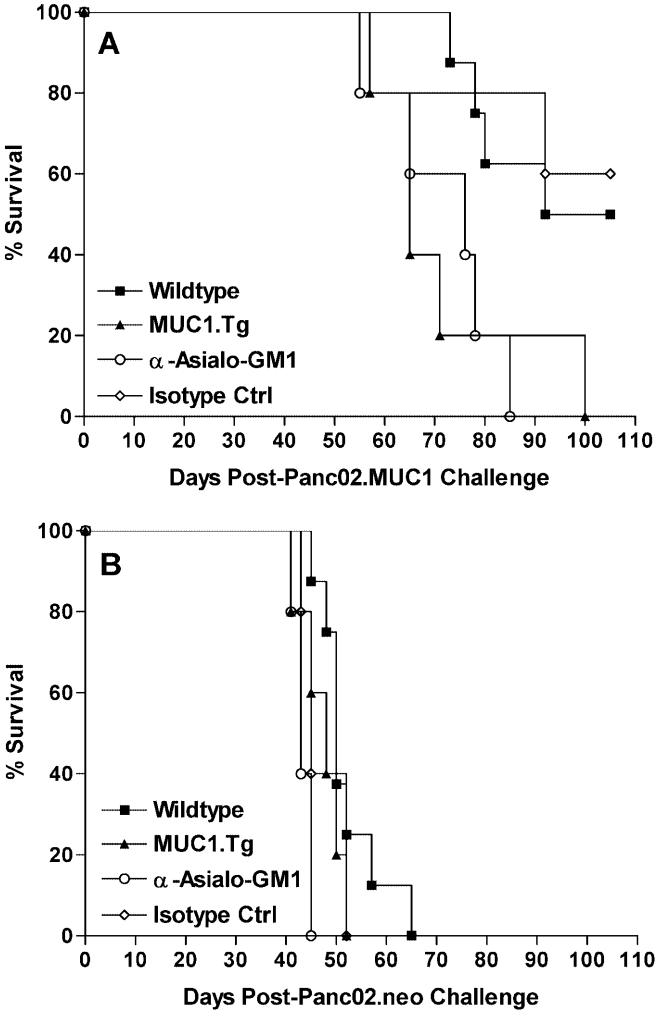

Fig. 2A, B. NK cells may be required to eliminate MUC1-expressing Panc02 tumors. **A** WT ($n = 8$), MUC1.Tg ($n = 5$), anti-asialo-GM1-treated ($n = 5$), and isotype control-treated ($n = 5$) C57BL/6 mice were challenged with 1×10^6 Panc02.MUC1 tumor cells s.c. between the scapulae on day 0. Statistically significant increases in survival were observed in wild-type and isotype control-treated mice compared to anti-asialo-GM1-treated and MUC1.Tg mice ($P \leq 0.0135$). **B** WT ($n = 8$), MUC1.Tg ($n = 5$), anti-asialo-GM1-treated ($n = 5$), and isotype control-treated ($n = 5$) C57BL/6 mice were challenged with 1×10^6 Panc02.neo tumor cells s.c. between the scapulae on day 0. No statistically significant differences in survival were observed between all strains of mice tested ($P > 0.05$)

TCR-α/β^+ cells are required for anti-Panc02.MUC1 tumor immunity, while TCR-γ/δ^+ cells play a suppressive non-antigen-specific role in anti-Panc02 tumor immunity

Having determined that a $CD8^+$ effector cell was required to eliminate Panc02.MUC1 tumors, we evaluated the immune components that had been predicted to play critical roles during the priming phase of anti-MUC1 tumor immunity. We first examined whether T cells expressing TCR-α/β or TCR-γ/δ were required for Panc02.MUC1 immunity by utilizing mice deficient in both TCR-α/β and TCR-γ/δ T cells (TCR-β/δ –/–). As shown in Fig. 3A, TCR-β/δ –/– mice challenged with Panc02.MUC1 tumors showed survival that was significantly decreased ($P < 0.0001$) when compared to that of WT mice, and that was similar to the survival of MUC1.Tg mice, indicating that T cells expressing TCR-α/β or TCR-γ/δ played a critical role during the priming of anti-Panc02.MUC1 immunity. We then investigated whether TCR-α/β or TCR-γ/δ T cells played a unique role during this phase of the anti-MUC1 immune response by evaluating the survival of mice deficient in either of these T cell subpopulations. Mice lacking TCR-α/β T cells were unable to reject Panc02.MUC1 tumors and experienced significantly decreased survival ($P < 0.0001$) compared to WT survival, while mice lacking TCRγ/δ T cells experienced significantly prolonged survival when compared to both WT ($P = 0.01$) and MUC1.Tg ($P < 0.0001$) survival (Fig. 3A). The prolonged survival observed in mice lacking TCR-γ/δ T cells was not due to a MUC1-specific immune response, since these mice also experienced significantly prolonged

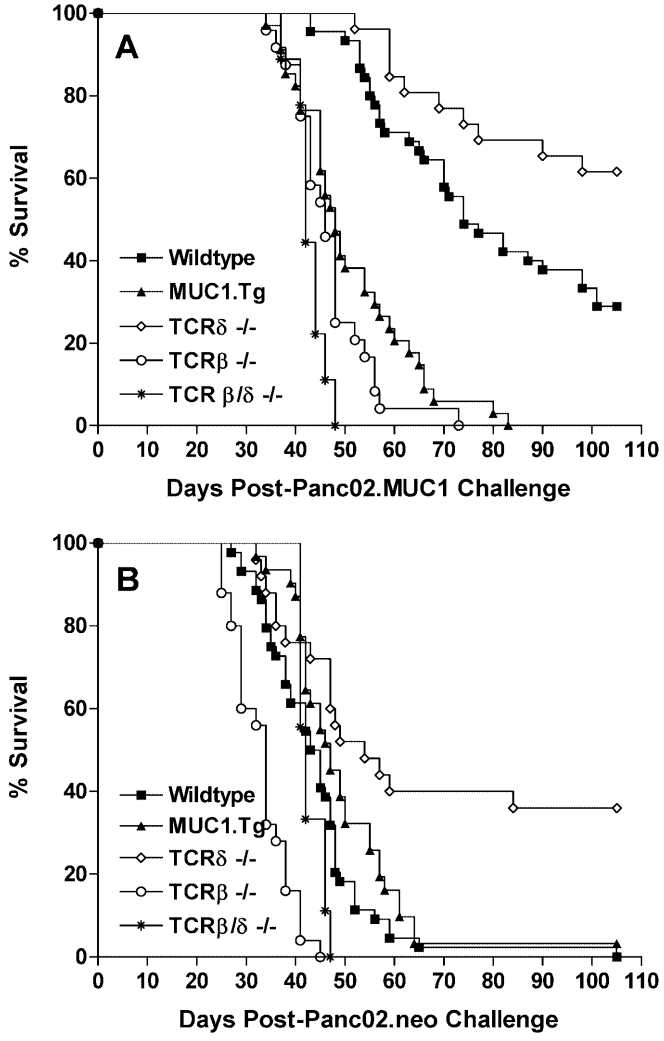

Fig. 3A, B. T cells expressing TCR-α/β are required for anti-Panc02.MUC1 tumor immunity in C57BL/6 mice, while T cells expressing TCR-γ/δ appear to have a suppressive effect on anti-Panc02 tumor immunity. **A** WT ($n=40$), MUC1.Tg ($n=27$), TCR-α/β –/– ($n=25$), TCR-γ/δ –/– ($n=25$) and TCR-β/δ –/– ($n=9$) C57BL/6 mice were challenged with 1×10^6 Panc02.MUC1 tumor cells s.c. between the scapulae on day 0. Statistically significant increases in survival were observed in WT and TCR-γ/δ –/– mice compared to TCR-α/β –/–, TCR-β/δ –/–, and MUC1.Tg mice ($P<0.0001$). **B** WT ($n=39$), MUC1.Tg ($n=26$), TCR-α/β –/– ($n=25$), TCR-γ/δ –/– ($n=25$), and TCR-β/δ –/– ($n=9$) C57BL/6 mice were challenged with 1×10^6 Panc02.neo tumor cells s.c. between the scapulae on day 0. No statistically significant differences in survival were observed between TCR-β/δ –/– and WT or MUC1.Tg mice ($P>0.05$), while TCR-γ/δ –/– mice experienced significantly prolonged survival compared to that of WT or MUC1.Tg mice ($P<0.006$) and TCR-α/β –/– mice experienced significantly decreased survival compared to that of WT or MUC1.Tg mice ($P<0.0001$). Data were pooled from at least two independent experiments conducted at different times

survival compared to that of all other mouse strains challenged with the control Panc02.neo tumor (Fig. 3B). These results suggested that T cells expressing TCR-α/β or TCR-γ/δ exhibit non-redundant roles during the priming phase of the anti-Panc02.MUC1 immune response. T cells expressing TCR-α/β were absolutely required for the elimination of Panc02.MUC1 tumors, while T cells expressing TCR-γ/δ appeared to play a suppressive role in anti-Panc02 immunity that was not antigen (MUC1)-specific.

Co-stimulation through CD28 engagement and CD40:CD40L interactions play a unique role in cell-mediated immunity against Panc02.MUC1 tumors

In order to determine whether signaling via CD28 engagement (signal two) was necessary for the activation of MUC1-specific T cells, we challenged CD28 –/–, WT, and MUC1.Tg mice with Panc02.MUC1 or Panc02.neo tumors. CD28 –/– mice challenged with Panc02.MUC1 tumors experienced significantly decreased survival when compared to that of WT mice ($P=0.0003$), and survival similar to MUC1.Tg mice ($P=0.9063$; Fig. 4A), indicating that signaling through CD28 was critical for the induction of the anti-Panc02.MUC1 T cell-mediated immune response. We also evaluated the importance of co-stimulation through CD40:CD40L interactions during the initiation of MUC1-specific tumor immunity by challenging CD40 –/– and CD40L –/– mice with Panc02.MUC1 or Panc02.neo tumors. Mice deficient in either CD40 or CD40L experienced significantly decreased survival ($P<0.024$) when compared to that of either WT or MUC1.Tg mice (Fig. 4A). Thus, in addition to co-stimulation through CD28 engagement, induction of anti-Panc02.MUC1 T cell-mediated immunity required co-stimulation via CD40:CD40L interactions. The prolonged survival of WT mice in this study was due to MUC1-specific immune responses, since all strains tested were unable to efficiently eliminate Panc02.neo control tumors (Fig. 4B).

IFN-γ but not IL-12 is critical for anti-Panc02.MUC1 immunity

During the proliferation, differentiation, and maturation phase of the antitumor immune response, Th1- and Th2-type cytokines are predicted to greatly influence the overall commitment to tumor-specific cell-mediated and/or humoral immunity. We investigated the roles of Th1- and Th2-type cytokines in anti-Panc02.MUC1 immunity by challenging mice deficient in either Th1 cytokines (IL-12 or IFN-γ) or Th2 cytokines (IL-4 or IL-10) with Panc02.MUC1 or Panc02.neo tumor cells. IL-12 –/– mice challenged with Panc02.MUC1 tumors experienced survival similar to that of WT mice and which was significantly prolonged ($P=0.0008$) when compared to the survival of MUC1.Tg mice; in contrast, IFN-γ –/– mice experienced survival similar to MUC1.Tg mice and which was significantly decreased ($P=0.0004$) when compared to that of WT mice (Fig. 5A). IL-4 –/– or IL-10 –/– mice showed no statistically significant differences in survival compared to that of WT mice ($P>0.1030$), although IL-4 –/– mice appeared to have slightly better

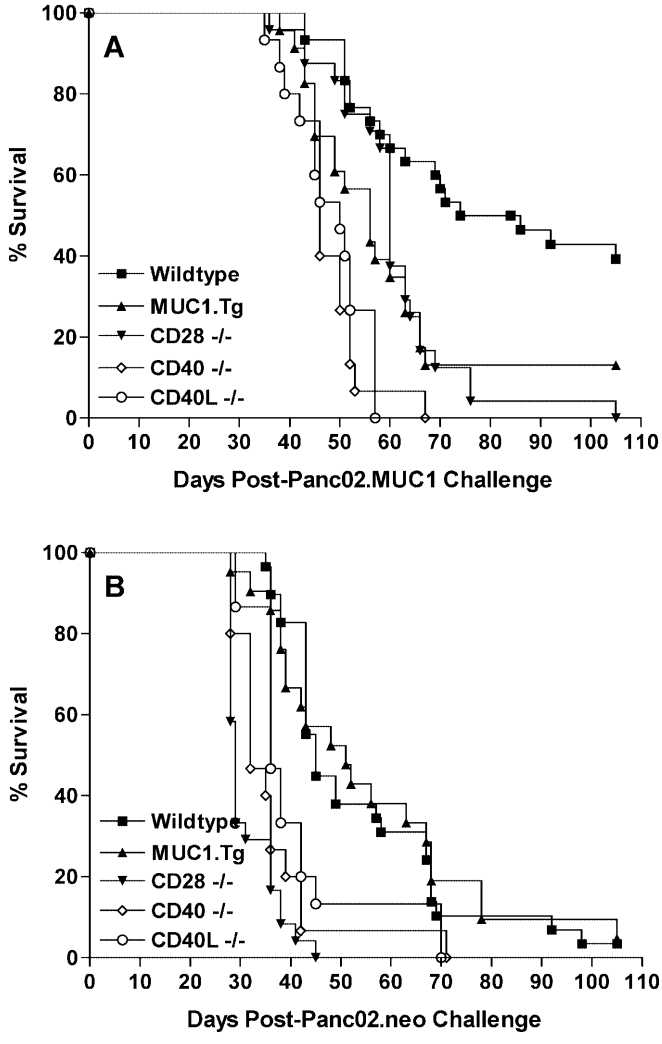

Fig. 4A, B. Co-stimulation through CD28 engagement and CD40:CD40L interaction plays a unique role in cell-mediated immunity against Panc02.MUC1 tumors. **A** WT ($n=30$), MUC1.Tg ($n=23$), CD28 –/– ($n=24$), CD40 –/– ($n=15$) and CD40L –/– ($n=15$) C57BL/6 mice were challenged with 1×10^6 Panc02.MUC1 tumor cells s.c. between the scapulae on day 0. Statistically significant increases in survival were observed in WT mice compared to CD28 –/–, CD40 –/–, CD40L –/–, and MUC1.Tg mice ($P<0.003$). **B** WT ($n=30$), MUC1.Tg ($n=22$), CD28 –/– ($n=24$), CD40 –/– ($n=15$), and CD40L –/– ($n=15$) C57BL/6 mice were challenged with 1×10^6 Panc02.neo tumor cells s.c. between the scapulae on day 0. No statistically significant differences in survival were observed between WT or MUC1.Tg mice ($P>0.05$), while CD28 –/–, CD40 –/–, and CD40L –/– mice experienced significantly decreased survival compared to WT or MUC1.Tg mice ($P<0.02$). Data were pooled from at least two independent experiments conducted at different times

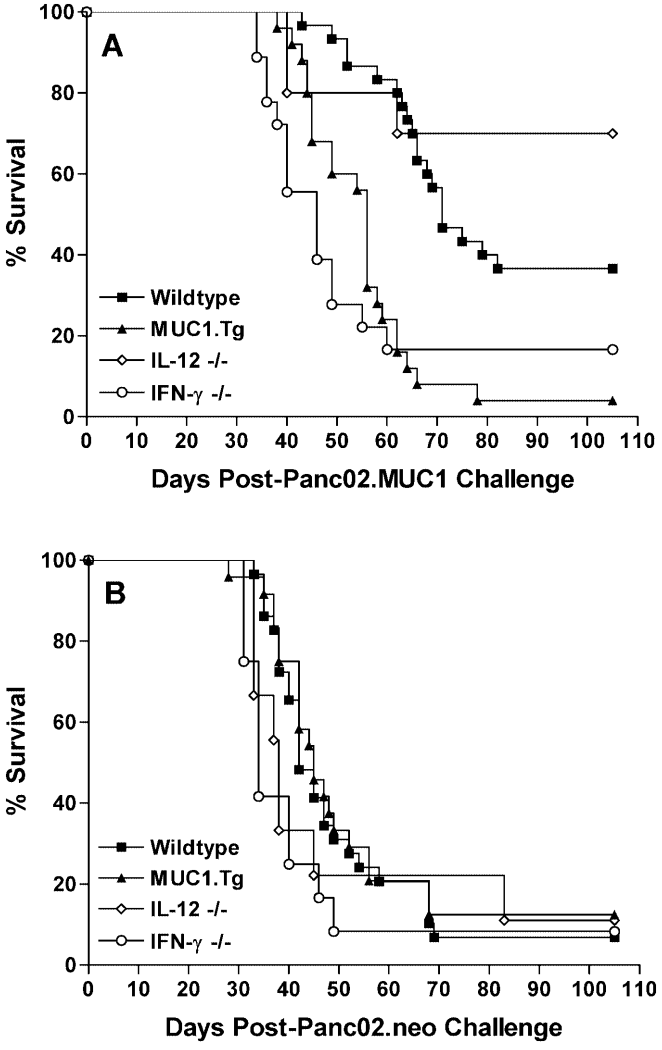

Fig. 5A, B. IFN-γ but not IL-12 is critical for anti-Panc02.MUC1 immunity. **A** WT ($n=25$), MUC1.Tg ($n=25$), IL-12 –/– ($n=10$), and IFN-γ –/– ($n=15$) C57BL/6 mice were challenged with 1×10^6 Panc02.MUC1 tumor cells s.c. between the scapulae on day 0. Statistically significant increases in survival were observed in WT and IL-12 –/– mice compared to IFN-γ –/– and MUC1.Tg mice ($P\le0.0008$). **B** WT ($n=20$), MUC1.Tg ($n=24$), IL-12 –/– ($n=10$) and IFN-γ –/– ($n=12$) C57BL/6 mice were challenged with 1×10^6 Panc02.neo tumor cells s.c. between the scapulae on day 0. No statistically significant differences in survival were observed between any strains of mice ($P>0.05$). Data were pooled from at least two independent experiments conducted at different times

survival than WT mice (Fig. 6A). Both strains showed significantly prolonged survival ($P\le0.0008$) compared to MUC1.Tg mice (Fig. 6A). The prolonged survival observed in WT, IL-12 –/–, IL-4 –/– and IL-10–/– mice was due to MUC1-specific immunity, since all strains evaluated in this study lacked immunity to the control tumor, Panc02.neo (Figs. 5B and 6B). Thus, IL-4 and IL-10 (Th2-type cytokines) were not required for the development of effective anti-Panc02.MUC1 immunity, while IFN-γ (a Th1-type cytokine) but not IL-12 played a critical role in the generation of cell-mediated immune responses directed against Panc02.MUC1 tumors.

Pfp, FasL, and lymphotoxin-α play a unique role during the effector phase of anti-Panc02.MUC1 immunity

During the effector phase, tumor-specific killer cells exert cytotoxic effects on tumor targets primarily by means of two cytolytic pathways, one involving the secretion of granzymes and pfp and the other involving interactions

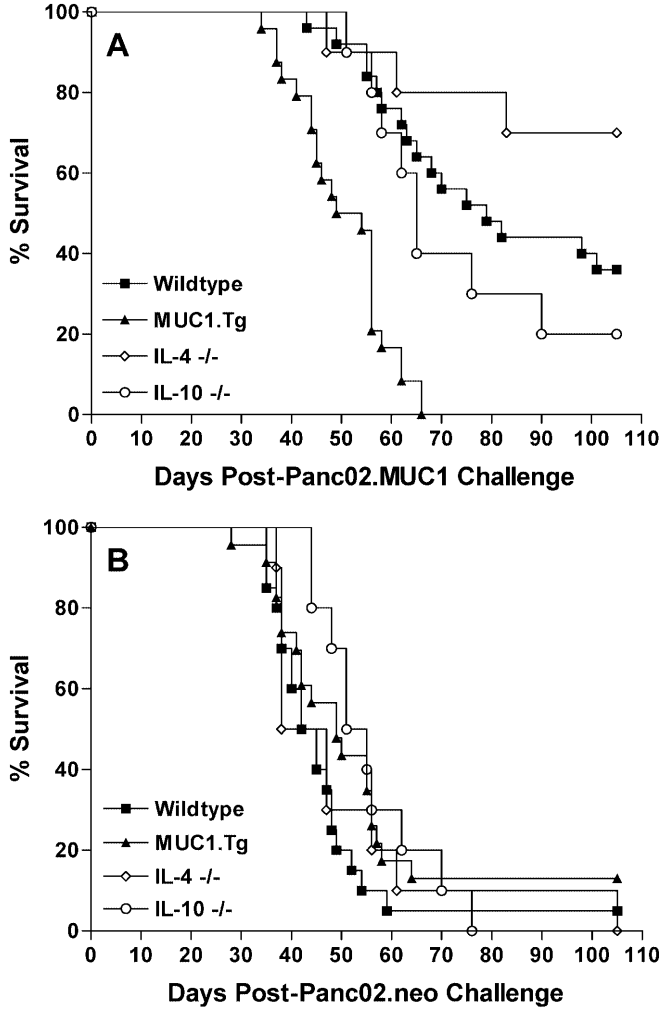

Fig. 6A, B. IL-4 and IL-10 are not required for anti-Panc02.-MUC1 tumor immunity. **A** WT ($n=25$), MUC1.Tg ($n=24$), IL-4 –/– ($n=10$) and IL-10 –/– ($n=10$) C57BL/6 mice were challenged with 1×10^6 Panc02.MUC1 tumor cells s.c. between the scapulae on day 0. Statistically significant increases in survival were observed in WT, IL-4 –/– and IL-10 –/– mice compared to MUC1.Tg mice ($P<0.0008$). **B** WT ($n=25$), MUC1.Tg ($n=23$), IL-4 –/– ($n=10$) and IL-10 –/– ($n=10$) C57BL/6 mice were challenged with 1×10^6 Panc02.neo tumor cells s.c. between the scapulae on day 0. No statistically significant differences in survival were observed between any strains of mice ($P>0.05$). Data were pooled from at least two independent experiments conducted at different times

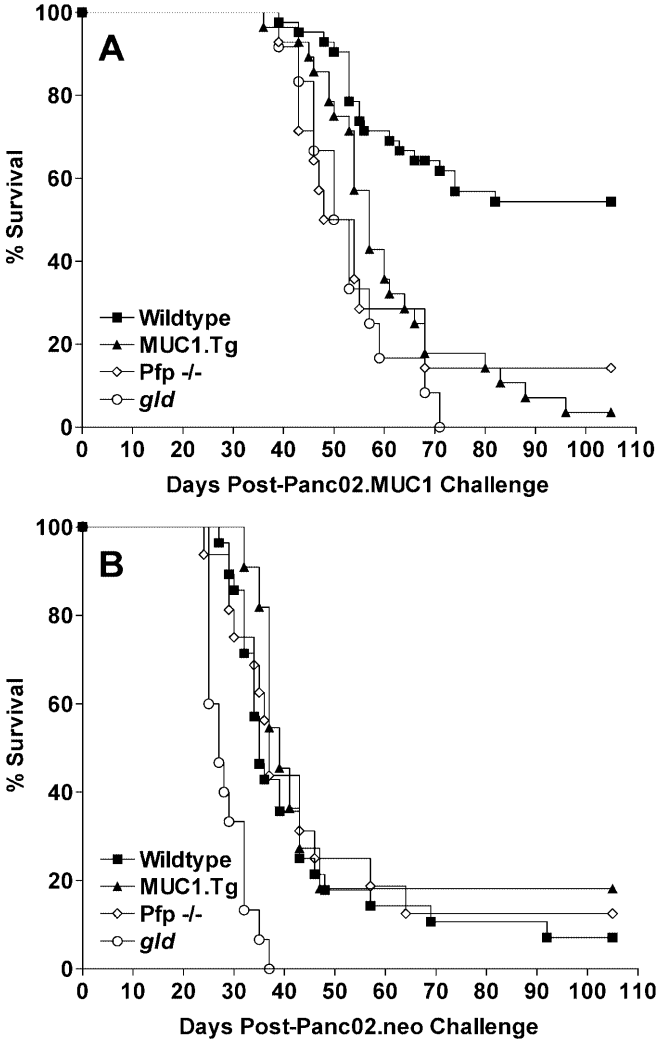

Fig. 7A, B. Both pfp and FasL are required for anti-Panc02. MUC1 tumor immunity in C57BL/6 mice. **A** WT ($n=42$), MUC1.Tg ($n=28$), pfp –/– ($n=14$), and *gld* ($n=12$) C57BL/6 mice were challenged with 1×10^6 Panc02.MUC1 tumor cells s.c. between the scapulae on day 0. Statistically significant increases in survival were observed in WT mice compared to pfp –/–, *gld*, and MUC1.Tg mice ($P\leq0.02$). **B** WT ($n=28$), MUC1.Tg ($n=14$), pfp –/– ($n=13$), *gld* ($n=15$) C57BL/6 mice were challenged with 1×10^6 Panc02.neo tumor cells s.c. between the scapulae on day 0. No statistically significant differences in survival were observed between pfp –/– mice and WT or MUC1.Tg mice ($P>0.05$), while *gld* mice experienced significantly decreased survival compared to WT or MUC1.Tg mice ($P<0.0001$). Data were pooled from at least two independent experiments conducted at different times

between FasL expressed by T cells with Fas expressed by some tumor cells. Activation of either of these mechanisms ultimately results in the death of the tumor target either by apoptosis or cell lysis. In addition to these two primary killing pathways, cytolytic effectors may induce apoptosis of tumor targets through the secretion of cytokines such as LT-α or TNF-α. In order to identify cytolytic mechanisms responsible for rejection of Panc02.MUC1 tumors, we challenged mice deficient in pfp, FasL (*gld*), LT-α, or TNFR-1 with Panc02.MUC1 or Panc02.neo tumors. Pfp –/– and *gld* mice challenged with Panc02.MUC1 tumors experienced survival similar to that of MUC1.Tg mice which was significantly decreased ($P=0.0003$; $P<0.0001$, respectively) when compared to WT survival (Fig. 7A), indicating that both pfp- and FasL-mediated killing pathways played critical non-redundant roles in the rejection of Panc02.MUC1 tumors. The prolonged survival of WT mice compared to that of MUC1.Tg, pfp –/– and *gld* mice seen in Fig. 7A was due to MUC1-specific immune responses since all strains of mice tested in this study showed similar inefficiency in the rejection of Panc02.neo control tumors (Fig. 7B). Challenge of LT-α –/– and TNFR-1 –/– mice with Panc02.MUC1 tumors revealed that the

survival of LT-α –/– mice was similar to that of MUC1.Tg mice and significantly worse ($P=0.0006$) than that of WT mice. TNFR-1 –/– mice experienced survival which was similar to that of WT mice, and which was significantly prolonged ($P=0.001$) when compared to the survival of MUC1.Tg mice (Fig. 8A). The prolonged survival seen in WT and TNFR-1 –/– mice was due to specific immune responses directed against MUC1, since all strains of mice tested were deficient in their ability to reject the control tumor, Panc02.neo (Fig. 8B). Data from these studies suggest that, in addition to perforin- and FasL-mediated cytolytic pathways, LT-α, but not TNFR-1, plays a critical and non-redundant role in cell-mediated rejection of Panc02.MUC1 tumor cells.

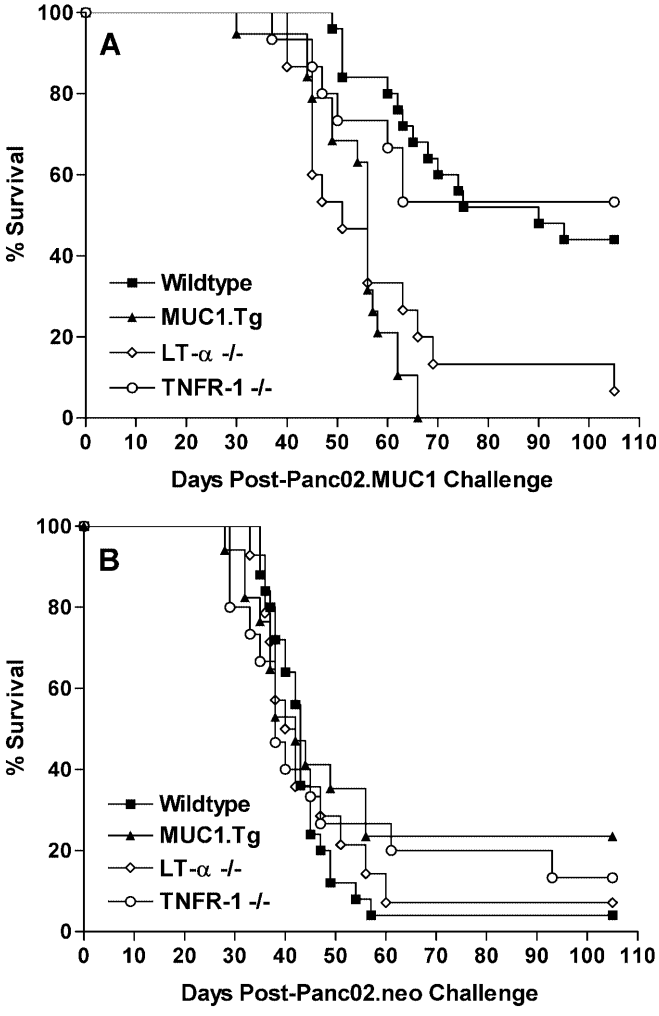

Fig. 8A, B. LT-α, but not TNFR-1, is required for anti-Panc02.MUC1 tumor immunity in C57BL/6 mice. **A** WT ($n=25$), MUC1.Tg ($n=19$), LT-α –/– ($n=14$), and TNFR-1 –/– ($n=15$) C57BL/6 mice were challenged with 1×10^6 Panc02.MUC1 tumor cells s.c. between the scapulae on day 0. Statistically significant increases in survival were observed in WT and TNFR-1 –/– mice compared to LT-α –/– and MUC1.Tg mice ($P<0.02$). **B** Wild-type ($n=25$), MUC1.Tg ($n=17$), LT-α –/– ($n=14$), TNFR-1 –/– ($n=15$) C57BL/6 mice were challenged with 1×10^6 Panc02.neo tumor cells s.c. between the scapulae on day 0. No statistically significant differences in survival were observed between any strains of mice ($P>0.05$). Data were pooled from at least two independent experiments conducted at different times

IFN-γ upregulates MHC class I but not MHC class II on Panc02.MUC1 tumor cells in vitro

Having determined that IFN-γ is critical to the elimination of Panc02.MUC1 tumors, we evaluated whether IFN-γ might have a local effect on Panc02 tumor cells by upregulating MHC class I and/or class II molecules, thereby increasing their ability to display MUC1 peptides in the context of class I or class II molecules for recognition by MUC1-specific T cells. We investigated the effects of IFN-γ on the expression of MHC class I and II by the tumor cells used in these studies. Panc02.MUC1 and Panc02.neo tumor cells were cultured in the presence or absence of IFN-γ in vitro for 48 h and subsequently analyzed by flow cytometry for surface expression of H2-K^b (MHC class I) and I-A^b (MHC class II). In the absence of IFN-γ, both Panc02.MUC1 and Panc02.neo expressed low (Panc02.MUC1) or undetectable (Panc02.neo) levels of H-2K^b and no detectable levels of I-A^b (Fig. 9A–D). However, when Panc02.MUC1 or Panc02.neo cells were cultured in the presence of IFN-γ, both tumor cell lines demonstrated upregulation of H2-K^b surface expression in the presence of IFN-γ (Fig. 9E, G). There were no appreciable effects of IFN-γ on the surface expression of I-A^b (Fig. 9F, H). These results support the hypothesis that IFN-γ has a local effect on Panc02.MUC1 tumor cells by increasing MHC class I expression. This would be expected to enhance availability and presentation of MUC1 peptides in association with MHC class I to MUC1-specific T cells, thereby augmenting the potential interactions of cytolytic $CD8^+$ effectors with Panc02.-MUC1 tumor targets. These studies also suggest that class II-mediated immune mechanisms are of little benefit to rejection of Panc02.MUC1 tumors.

Discussion

The overall goal of the studies reported here was to elucidate the molecular components that play critical and non-redundant roles during the developmental phases of anti-MUC1 immunity in C57BL/6 mice. We utilized mice genetically deficient in selected molecules to evaluate their importance in vivo to immune responses directed against a human MUC1-expressing murine pancreatic carcinoma cell line, Panc02.MUC1.

Previous in vivo cellular depletion studies determined that a $CD8^+$ effector was critical for eliminating Panc02.MUC1 tumors [41]. We confirmed this finding by challenging mice that were genetically deficient in CD4, CD8, or CD4 and CD8 cells with MUC1-expressing Panc02 tumors or neo control Panc02 tumors (Fig. 1). The results stand in contrast to a parallel study that

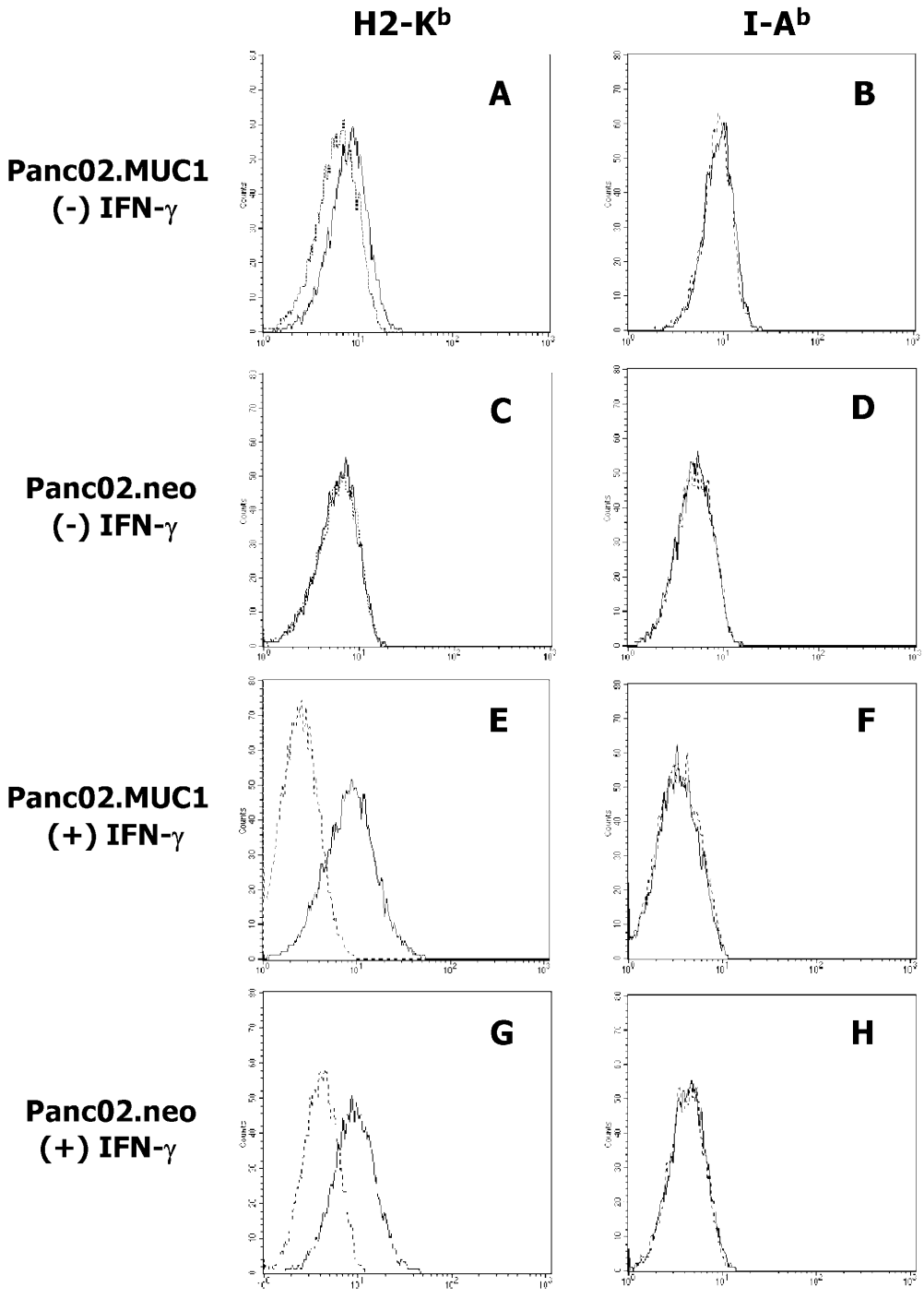

Fig. 9A–D. IFN-γ upregulates MHC class I, but not MHC class II, on Panc02.MUC1 tumor cells in vitro. Panc02.MUC1 (**A**, **B**) or Panc02.neo (**C**, **D**) tumor cells were cultured in vitro in the absence (**A**, **C**) or presence (**B**, **D**) of 25 ng of IFN-γ for 48 h at 37°C. Tumor cells were then reacted with monoclonal antibodies directed against H2–K^b (MHC class I; *left panel*) or I–A^b (MHC class II; *right panel*) and analyzed by flow cytometry. Histograms show reactions with isotype control antibodies (*dotted lines*) and MHC class I or II antibodies (*solid lines*)

evaluated immune responses to a MUC1-expressing murine melanoma cell line, B16.MUC1. In these studies, a $CD4^+$ effector cell was required to eliminate syngeneic B16.MUC1 tumors [62]. Additional studies using the B16.MUC1 tumor model and mice genetically deficient in $CD4^+$, $CD8^+$ or $CD4^+$ and $CD8^+$ cells confirmed that a $CD4^+$ effector was essential for eliminating MUC1-expressing B16 tumors [68]. These results suggested that different effectors were required to eliminate MUC1-expressing tumors derived from different organ sites, and that different mechanisms might be required to generate these two different MUC1-specific effectors. The development of different types of MUC1-specific effectors for eliminating MUC1-expressing tumors derived from different tissues probably results in part from differences in the presentation of MUC1 epitopes to responding cells of the immune system. MUC1 peptides would be predicted to be presented to $CD8^+$ effectors in the context of MHC class I, while peptides presented to $CD4^+$ effectors would be in the context of MHC class II.

It is unclear at this time why the expression of the same antigen by different cell types leads to dramatic differences in the molecular characteristics of immune responses to that antigen; however, this finding is highly significant to the field of tumor immunology and may impact studies of immunity to intracellular pathogens such as viruses and bacteria, that require cell-mediated responses for effectiveness. Two implications from our findings are: (1) that the same types of immune response may not be effective against different cell types that display the same antigen; and (2) that characteristics of the cells that express the antigen play a role in determining the type of response that is produced. This finding has direct implications for future immunization strategies that target single antigens by immunizing with recombinant products expressed by cell types that are different from the cell type that is targeted by the vaccine. Our findings may also explain in part the variability in success and failure of some vaccine strategies, which would be predicted to fail if they provoked an immune response that was not capable of rejecting the appropriate cell type.

Presumably, the production of different immune responses (CD4- versus CD8-mediated) results in large part from differences in antigen processing and presentation. Differences in antigen processing by the tumor cells or antigen-presenting cells (APC) may result in the production of different epitopes that associate with either MHC class I or class II in the tumor cells or in APC that process antigens from tumors. Differences in MUC1 peptide presentation may be due to the peptide sequences themselves, or to differences in post-translational processing such as glycosylation. Studies are currently underway to determine if there are differences in MUC1 epitopes presented by MHC class I or II molecules to elicit rejection of MUC1-expressing Panc02 or B16 tumor cells, and whether these differences can be utilized in designing vaccines that induce or augment immunity directed against Panc02.MUC1 or B16.MUC1 tumors.

We continued our study by evaluating immune components that are predicted to play critical roles during the initial antigen recognition/priming phase of the anti-MUC1 immune response. During antigen recognition and priming, antigenic peptides are recognized in the context of MHC class I or class II molecules expressed on the target cells or on professional APC such as DC or macrophages, by T cells expressing a complementary TCR. This recognition results in the generation of a signal that initiates T cell activation and is referred to as signal one. Signal one may result in the upregulation of CD40L on the T cell, followed by the interaction of CD40L with CD40 expressed by APC. These interactions may lead to the upregulation of B7 molecules on APC, followed by interactions of B7 with CD28 expressed by T cells, referred to as signal two. One result of these interactions is the production of IL-12 by DC or macrophages; B cells are unable to produce IL-12. Using C57BL/6 mice that were deficient in both α/β and γ/δ T cells, we determined that T cells expressing TCR-α/β or TCR-γ/δ were critical in generating immunity against Panc02.MUC1 tumors (Fig. 3A). We further determined that T cells expressing TCR-α/β played an important and non-redundant role in eliminating MUC1-expressing Panc02 tumors. Both of these findings were similar to those requirements for the rejection of B16.MUC1 tumors [68]. Surprisingly, T cells expressing γ/δ TCR played a suppressive role in tumor immunity against Panc02 that was not MUC1-specific (Fig. 3A). These findings contrasted with those from the B16.MUC1 study, which demonstrated that TCR-γ/δ T cells had no effect on the elimination of B16.MUC1 tumors [68]. Although in vitro studies have demonstrated that γ/δ T cells isolated from cancer patients are capable of recognizing both diffuse and solid tumors and exerting cytotoxic effects [13, 36, 63, 67], additional studies have clearly demonstrated that γ/δ T cells can exert suppressive effects against both NK cells and CTL by producing soluble factors such as IL-10 and TGF-β, which abrogate tumoricidal immunity [52, 53]. The 1999 study by Seo et al. also demonstrated that the depletion of γ/δ T cells could result in tumor regression via the augmentation of cytolytic activities exerted by CTL and NK cells. Our results corroborate their findings, and additional studies are underway to further define the suppressive role of γ/δ T cells in our Panc02 tumor model. Taken together, these studies suggest that depletion of γ/δ T cells in WT mice might enable these animals to mount more effective immune responses against Panc02 tumors, while a similar treatment in mice challenged with B16 tumors would not be predicted to have an advantageous effect.

The role of co-stimulation during T cell activation against Panc02.MUC1 tumors was investigated with mice deficient in CD28, CD40, or CD40L. Our findings indicated that co-stimulation through CD28 (signal two) and CD40:CD40L interactions played a crucial non-redundant role in the generation of effective immunity against MUC1-expressing Panc02 tumors (Fig. 4A), which paralleled results in the B16.MUC1 tumor model [68]. Our in vivo findings also correlated with previously reported in vitro requirements for proliferation of lymph node cells from MUC1.Tg mice immunized with fusions of MUC1-positive tumor and DC [18]. The results reported here suggest that CD40:CD40L interactions are required to upregulate B7 and/or other co-stimulatory molecules on APC that are needed for the full activation of MUC1-specific T cells, as has been reported for other antigen systems [3, 20, 21, 22, 65]. Lack of CD40:CD40L interactions or co-stimulation through CD28 may result in anergy or clonal deletion of MUC1-specific T cells, ultimately resulting in the progression of Panc02.MUC1 tumors. The requirement of CD40L for Panc02.MUC1 tumor rejection suggests that inclusion of soluble CD40L in MUC1 tumor vaccine formulations could augment MUC1-specific immunity via the activation of APC functions critical for MUC1 tumor immunity. The requirement for CD28 co-stimulation during production

of MUC1-specific responses supports the use of vaccine strategies that increase expression of B7 molecules on APC via proinflammatory cytokines such as IL-1, IL-6, IL-12, or IFN-γ [15, 16, 37, 45, 48, 56], or genetic manipulations [1]. Since induction of immunity to B16.MUC1 tumors also requires co-stimulation via CD28 and CD40:CD40L interactions [68], similar strategies might be employed to augment MUC1-specific immune responses in this tumor model.

Fully activated MUC1-specific T cells begin the process of becoming fully functional MUC1-specific effectors during the proliferation, differentiation, and maturation phase. This process requires cytokines initially produced by NK cells or monocytes (IFN-γ), and subsequently by other APC (IL-1, IL-12, or IL-6) and T helper (Th) cells. Th cells have been divided into two functional subsets based on their patterns of cytokine secretion [40]. Th1 cells differentiate under the influence of IL-12, secrete IL-2, IFN-γ, and LT-α, and are primarily responsible for the development of cell-mediated ($CD4^+$ or $CD8^+$) immune responses. In the absence of IL-12 (and perhaps in the presence of IL-6), Th2 cells differentiate under the direction of IL-4, secrete IL-4, IL-5, IL-10, and IL-13, and are primarily responsible for development of humoral immune responses. Recent studies have also identified populations of $CD8^+$ effectors that can develop under the direction of Th2 cytokines [58, 64, 66]. In the light of our findings that $CD8^+$ cells were critical for the elimination of Panc02.MUC1, whereas $CD4^+$ cells were required to eliminate B16.MUC1 tumors, we examined whether classical Th1 (IL-12 or IFN-γ) and/or Th2 (IL-4 or IL-10) cytokines played differential roles in the development of these unique MUC1-specific effectors. To our surprise, IFN-γ but not IL-12 was critical for eliminating MUC1-expressing Panc02 tumors (Fig. 5A), while neither IL-4 or IL-10 were required for rejecting these tumors (Fig. 6A). Similar requirements were also noted for the responses that eliminated B16.MUC1 tumors [68]. Previous reports have indicated that IL-12 plays a critical role in enhancing antitumor immunity by increasing IFN-γ levels [12, 15, 44], and therefore may serve as a third signal implying "danger" during $CD8^+$ T cell activation [9]. Our studies indicated that IL-12 alone was not required for the rejection of Panc02.MUC1 tumors; this finding suggests that IL-12 is not required for antitumor CTL responses in this tumor model or that an additional factor(s) compensates when IL-12 is absent [9].

The timing and nature of the requirement for IFN-γ is not known. IFN-γ may be required during the initiation of the immune response as part of NK cell activity and/or APC activation. Additionally, augmented IFN-γ production by Th1 cells may contribute to increased APC activation, and increased proliferation, differentiation and maturation of MUC1-specific $CD8^+$ effectors [6, 17, 33]. Our studies also indicated that IFN-γ upregulated MHC class I expression on Panc02 tumor cells (Fig. 9). Increased MHC class I expression on Panc02.MUC1 tumor cells might result in augmented expression of MUC1 epitopes to $CD8^+$ effectors, thus potentially increasing the likelihood of CTL recognition of MUC1-expressing targets. Taken together, these findings suggest that IFN-γ may serve as a critical adjuvant in MUC1 tumor vaccine protocols designed to provoke MUC1-specific immunity to either Panc02.-MUC1 or B16.MUC1 tumors.

Upon completion of the maturation process, antigen-specific $CD8^+$ effectors are armed to destroy tumor cells upon recognition of tumor peptides in the context of MHC class I molecules presented on the tumor cell surface. Two primary cytolytic pathways have been identified in cytolytic effectors, CTL, and NK cells: the pfp/granzyme pathway and the Fas/FasL pathway. It is also possible for cytolytic effectors to secrete cytokines, such as TNF or LT-α, which can ultimately lead to tumor cell death by apoptosis. In the studies presented here, we determined that both the pfp/granzyme and Fas/FasL cytolytic pathways played critical non-redundant roles in the rejection of Panc02.MUC1 tumors (Fig. 7A). In contrast, parallel studies in the B16 tumor model demonstrated that only the Fas/FasL pathway was required for eliminating B16.MUC1 tumors[4]. Although it is unclear why both pathways are required for eliminating Panc02.MUC1 tumors and that the presence of one does not compensate for the lack of the other, it is intriguing to speculate that one pathway, such as the pfp/granzyme pathway, may be required early in the immune response against MUC1-expressing Panc02 tumors, while the Fas/FasL pathway may be required during later events.

It is also possible that NK cells, whose specific role in the Panc02 tumor model has yet to be elucidated, may preferentially use the pfp/granzyme pathway while the $CD8^+$ effectors use the Fas/FasL pathway, or vice versa. Recent studies have demonstrated that newly synthesized FasL is stored in specialized secretory lysosomes along with pfp and granzymes in both $CD4^+$ and $CD8^+$ T cells and NK cells, and that polarized degranulation controls the delivery of FasL to the cell surface [7]. In this way, FasL-mediated apoptosis may be controlled by receptor-mediated tumor target-cell recognition. Therefore, it is possible that the requirement for both pfp/granzyme-mediated and FasL-mediated pathways is due to the dependence of FasL expression on the cell surface of cytolytic effectors on granule secretion by activated CTL or NK cells. Since pfp, granzymes, FasL and Fas expression can be upregulated by IFN-γ [4, 32, 43, 55], our findings further substantiate the use of IFN-γ as an adjuvant in MUC1 tumor vaccine protocols to augment immune responses against MUC1-expressing tumors.

We also determined that LT-α, but not TNFR-1, was critical in eliminating Panc02.MUC1 tumors (Fig. 8A). Similar requirements were observed for the rejection of B16.MUC1 tumors [68]. LT-α can cause tumor cell death via the induction of apoptotic pathways. Moreover, mice that are deficient in LT-α have no morphologically detectable lymph nodes or Peyer's patches and

show profound defects in the development of follicular DC networks, germinal center formation, and T/B cell segregation in the spleen. Thus, LT-α is required for the normal development of peripheral lymphoid organs [11]. The requirement for LT-α, therefore, might be due to the absolute requirement for the functions that occur in these tissues during the developmental phases of the anti-Panc02.MUC1 immune response. Additionally, recent studies have identified defects in NK cell development, recruitment, and effector function in mice that lack LT-α [24, 57]. Therefore, the role of LT-α in the rejection of Panc02.MUC1 may relate to a specific requirement for NK cells during the initial stages of the immune response against these tumors, as was suggested by the results of our studies using anti-asialo-GM1 (Fig. 2A). In an effort to clarify the role of LT-α in eliminating Pan02.MUC1 tumors, future studies will evaluate the role of NK cells in eliminating Panc02.-MUC1 tumors and whether Panc02.MUC1 tumors are susceptible to apoptosis induced by LT-α. If NK cells are found to play a critical role in eliminating MUC1-expressing Panc02 tumors, as has been strongly suggested for B16.MUC1 tumors, then tumor vaccine formulations designed to increase NK cell activity, such as LT-α, IL-15 and/or IFN-γ administration, might be used to induce or augment immunity against tumors that express MUC1. If Panc02.MUC1 tumors are susceptible to apoptosis signals induced by LT-α, this would suggest that the full rejection of Panc02.MUC1 tumors requires at least three unique cytolytic mechanisms, which play critical and nonredundant roles during the process of tumor rejection. Vaccine strategies that target MUC1 should be designed to induce or augment the expression of these cytolytic mediators.

The studies presented here using the Panc02.MUC1 tumor model and a parallel study using the B16.MUC1 tumor model clearly demonstrate that different effectors ($CD8^+$ versus $CD4^+$, respectively) and cytolytic mechanisms (both pfp and Fas/FasL versus Fas/FasL alone, respectively) are required to eliminate MUC1-expressing tumors derived from different tissues. In addition to these differences, we noted a number of similarities in the immune components required to eliminate these tumors: TCR-α/β T cells, NK cells/monocytes, CD28 and CD40:CD40L co-stimulation, IFN-γ, and LT-α. This information provides insight into the immune components required to eliminate distinct tumors derived from different organ sites but expressing the same antigen, in this case MUC1. These findings should be taken into consideration when formulating vaccines designed to induce or augment immune responses against tumors that express MUC1.

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