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MUC1-specific CTLs are non-functional within a pancreatic tumor microenvironment

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Pancreatic cancer is a highly aggressive, treatment refractory disease and is the fourth leading cause of death in the United States. In humans, 90% of pancreatic adenocarcinomas over-express altered forms of a tumor-associated antigen, MUC1 (an epithelial mucin glycoprotein), which is a target for immunotherapy. Using a clinically relevant mouse model of pancreas cancer that demonstrates peripheral and central tolerance to human MUC1 and develops spontaneous tumors of the pancreas, we have previously reported the presence of functionally active, low affinity, MUC1-specific precursor cytotoxic T cells (pCTLs). Hypothesis for this study is that MUC1-based immunization may enhance the low level MUC1-specific immunity that may lead to an effective anti-tumor response. Data demonstrate that MUC1 peptide-based immunization elicits mature MUC1-specific CTLs in the peripheral lymphoid organs. The mature CTLs secrete IFN-γ and are cytolytic against MUC1-expressing tumor cells *in vitro*. However, active CTLs that infiltrate the pancreas tumor microenvironment become cytolytically anergic and are tolerized to MUC1 antigen, allowing the tumor to grow. We demonstrate that the CTL tolerance could be reversed at least *in vitro* with the use of anti-CD40 co-stimulation. The pancreas tumor cells secrete immunosuppressive cytokines, including IL-10 and TGF-ß that are partly responsible for the down-regulation of CTL activity. In addition, they down-regulate their MHC class I molecules to avoid immune recognition. $CD4^+CD25^+$ T regulatory cells, which secrete IL-10, were also found in the tumor environment. Together these data indicate the use of several immune evasion mechanisms by tumor cells to evade CTL killing. Thus altering the tumor microenvironment to make it more conducive to CTL killing may be key in developing a successful anti-cancer immunotherapy.

Keywords: **CTL, antigens/peptides/epitopes, tolerance, tumor immunity, transgenic models**

Abbreviations: **APC: Antigen presenting cell; CTL: cytotoxic T lymphocytes; DC: dendritic cell; MET: MUC1-expressing pancreatic tumor mouse model; MUC1: human mucin 1; Muc1: mouse mucin 1; MUC1.Tg: MUC1 transgenic; TAA: tumor associated antigen; TILs: tumor infiltrating lymphocytes; TR: tandem repeat.**

Introduction

The 5-year survival of patients with adenocarcinoma of the pancreas remains low at only 4%. In 2000, an estimated 28,300 patients were diagnosed with pancreatic cancer in the United States. Metastatic pancreatic cancer is uniformly fatal because no effective chemotherapy is available. Despite progress in the treatment of cancer with surgery, radiotherapy and chemotherapy, only minimal advances have been made in improving survival rates in patients with pancreatic cancer. The National Cancer Institute has therefore designated research on pancreatic cancer as high priority. Along with research focused on the nature of cells that become transformed in the pancreas and the molecules involved in the process of transformation and metastasis, alternative treatment strategies must be addressed. Cancer immunotherapy is an attractive, non-toxic treatment that should enable the activation of the immune system to attack a developing or metastasizing tumor. Development of preclinical models of spontaneous pancreatic cancer that resemble human cancer is key to progress in this area. One of the major goals of such a therapy is to generate cytotoxic T lymphocytes (CTLs) and memory T lymphocytes directed against tumor associated antigens (TAA), which eventually lead to long lasting anti-tumor immunity. Some of the most promising TAA for immune targeting are conventional cellular proteins that are expressed on both normal and transformed cells, and one such candidate is MUC1.

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Although MUC1 is a self-molecule that is normally expressed on epithelial cells lining ducts and glands at low levels, it is a target for immunotherapy because MUC1 is significantly altered in expression during tumorigenesis. There is a large increase in the amount of MUC1 expressed on cells and in circulation. Its distribution is no longer restricted to the apical surface of the ducts and glands, but it is found throughout the tumor mass and on the surface of tumor cells. Most importantly, the glycosylation is altered. Oligosaccharides are shorter and fewer in number, revealing immunodominant peptide sequences that on normal cell surfaces would be sequestered by glycosylation. In 2000, cancers including pancreatic cancer that express MUC1 accounted for about 72% of new cases and for 66% of the deaths [1]. These observations have prompted clinical vaccination trials aimed at boosting the weak anti-MUC1 immune responses to therapeutic levels.

Human clinical testing should ideally be preceded by extensive animal model studies to show that the concepts can be translated into efficacious therapy for cancer. Until recently, the mouse has not been a suitable preclinical model for testing MUC1-specific immune responses as human MUC1 sequence differs substantially from that of mouse Muc1 and is strongly antigenic in the mouse. In this project, we have utilized a human MUC1 transgenic mouse model (MUC1.Tg) that expresses human MUC1 as a self-molecule. Because the transgene is driven under its own promoter, MUC1 is not over-expressed but is expressed in normal levels, in a tissue specific manner. Mice transgenic for human MUC1 develop B and T cell tolerance and are refractory to immunization with the protein encoded by the transgene [2]. We have crossed the MUC1.Tg mice with mice that carry the first 127 amino acids of the SV40 large T-antigen driven by the elastase promoter. These mice are designated MET and develop spontaneous MUC1-expressing tumors of the pancreas [3]. This experimental model represents an improved model system for evaluating the efficacy of anti-MUC1 vaccine formulations *in vivo* within the context of existing tolerance mechanisms. As previously described, these mice exhibit acinar cell dysplasia at birth, which progresses to microadenomas and single or multiple acinar cell carcinomas. We have previously shown that non-immunized MET mice develop low affinity precursor MUC1-specific CTLs (pCTLs) i.e. the CTLs were cytotoxic only when primed *in vitro* with MUC1 antigen or MUC1 TR peptide at 10^{-6} M for several days. Although these CTLs have minimal effect on the spontaneously growing pancreatic tumors, they were efficient in eradicating injected MUC1 expressing tumors when adoptively transferred [3,4].

In this study, we tested a vaccine formulation comprised of liposomal-MUC1 tandem repeat lipopeptide (L-MUC1-TR) along with liposomal IL-2 (L-IL-2) with the goal of enhancing the already existing MUC1-specific immunity. We compared the immune responses that developed during treatment and at time of sacrifice in the MET mice. Survival and tumor burden were used as end points for determining the effectiveness of the treatment strategy. Data from this study show that MUC1-specific immunization resulted in mature CTLs and that adoptively transferred functional CTLs were rendered non-responsive within the tumor microenvironment, thus interrupting an anti-tumor response. Immunization results in a MUC1-specific CTL response that became tolerized within the growing tumor. Tolerance may be defined as accumulation of antigen-specific T cells, which are hypo-responsive and may have been induced within a non-inflammatory and immunosuppressive tumor micro-environment.

Results

Characterization of immunized MET mice

Tumor burden in immunized MET mice

We immunized mice with L-MUC1-TR ± L-IL-2. A schematic representation of the immunization protocol is illustrated in Figure 1. The first two immunizations administered to young (3 and 5 week old) MET mice utilized syngeneic dendritic cells (DC) loaded with liposomal MUC1 to induce strong immunity as young mice respond well to an antigen when presented in context of DC without the induction of tolerance [5]. From week 7 onwards, mice were given L-MUC1-TR reconstituted in PBS (s.c.) and subsequently boosted with the same formulation every two weeks. L-IL-2 was administered (i.p.) every two weeks to the appropriate groups. Tumor burden was evaluated only at

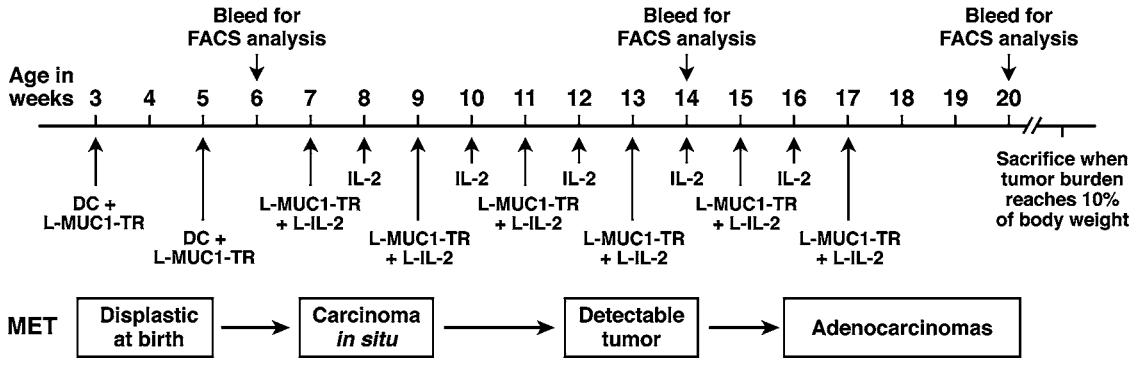

Figure 1. Schematic representation of immunotherapy strategy in MET mice.

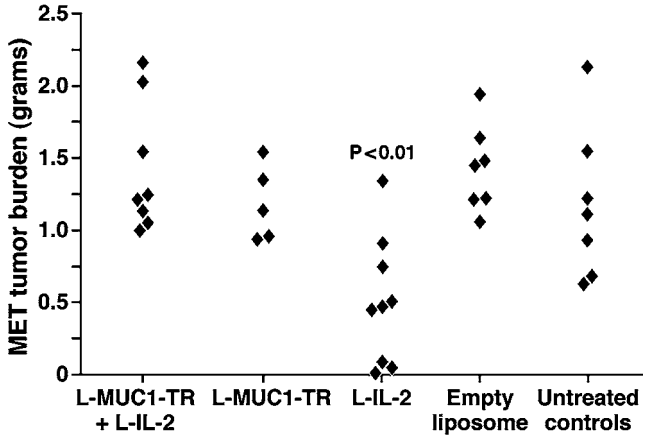

Figure 2. MUC1-specific immunization did not alter pancreatic tumor weight in MET mice, although L-IL-2 treatment had a beneficial effect. At time of necropsy, pancreatic tumors from immunized MET mice were weighed and compared to pancreatic tumor weights from untreated MET mice. No significant differences were observed between untreated control mice and mice immunized with L-MUC1-TR + L-IL-2, L-MUC1-TR, or empty liposome. Significantly lower pancreatic tumor burden (in grams) was observed in L-IL-2 treated mice as compared to untreated control mice ($p < 0.01$). *P* values are from the pair-wise contrasts from a one-way ANOVA model. Individual mice data are shown ($n = 6$ to 9 mice/group).

time of sacrifice, when mice had become morbid and showed weight loss. As tumors are internal and not palpable, tumor progression cannot be readily monitored. Our data demonstrate that treatment with L-IL-2 alone showed significant reduction in tumor burden when compared to untreated controls (Figure 2). All other treatment groups failed to have an effect on tumor burden. No effects on survival or metastasis were observed (data not shown).

T cell immune response in immunized MET mice

The immunization strategy was successful in eliciting strong T cell responses as measured by intracellular cytokine staining and by ^{51}Cr-release assay. We evaluated (a) intracellular IFN-γ expression in T cells and (b) T cells recognizing H-2D^b MUC1 tetramer from peripheral blood lymphocytes (PBL) at 6, 10, and 14 weeks post immunization. MHC class I H-2D^b tetramers containing MUC1 TR APGSTAPPA peptide were used in this experiment. T cells from PBLs were stained with 0.1 mgs/ml MUC1-tetramer directly conjugated to phycoerythrin (PE) for 1 hour on ice, counterstained with fluorescein (FITC)-conjugated anti-CD4 and CD8 antibody for 15 minutes on ice and analyzed by two-color flow cytometry. All treatment groups in immunized mice showed increased numbers of T cells expressing intracellular IFN-γ by 10–14 weeks of age as compared to untreated control or mice treated with empty liposomes (Figure 3A, *p* values shown in the figures). Similarly, we observed an increase in T cells reactive with H-2D^b/MUC1 tetramer in immunized mice as compared to

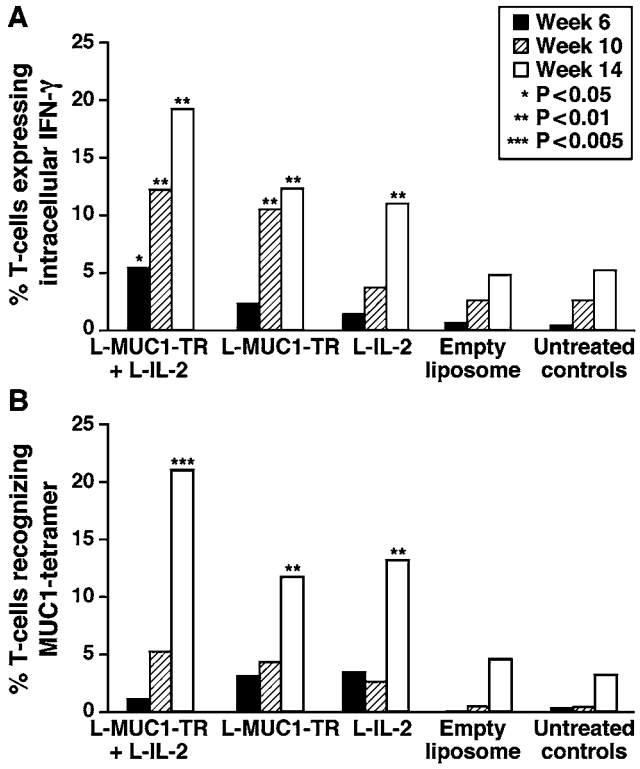

Figure 3. MUC1-specific immunization elicits (A) IFN-γ expressing T cells and (B) T cells that recognize MUC1/D^b tetramer in MET mice. At weeks 6, 10 and 14 post immunization and at necropsy, PBLs were analyzed for presence of T cells expressing IFN-γ and T cells recognizing MUC1/D^b tetramer. (A), Compared to untreated controls only mice in L-MUC1-TR + L-IL-2 treated group showed significant increase in percent of IFN-γ expressing T cells as early as 6 weeks post immunization ($^*p < 0.05$). By 10 weeks post immunization, mice in L-MUC1-TR + L-IL-2 and L-MUC1 TR group showed significant increase in this cell type as compared to untreated controls ($^{**}p < 0.01$). By 14 weeks post immunization, percent T cells expressing IFN-γ increased even more in the L-MUC1-TR + L-IL-2 treated mice ($^{**}p < 0.01$), whereas it stayed the same in L-MUC1-TR treated mice ($^{**}p < 0.01$). At this time, L-IL-2 treated mice also showed significant increase in IFN-γ expressing T cells ($^{**}p < 0.01$) as compared to untreated control mice. (B) Mice in all treatment groups showed no significant increase in percent T cells recognizing MUC1/D^b tetramer at 6 or 10 weeks post immunization as compared to untreated controls. By 14 weeks, mice in all treatment groups except empty liposome group showed increase in percent T cells recognizing MUC1/D^b tetramer ($^{***}p < 0.005$ for L-MUC1 TR + L-IL-2 treated group; $^{**}p < 0.01$ for L-MUC1-TR alone and L-IL-2 alone treated groups). Average of $n = 6$ mice/group is shown. *P* values are from the pair-wise contrasts from a one-way ANOVA model. *indicates comparison of untreated controls versus treatment groups.

control groups (Figure 3B, *p* values shown in the figures). MUC1-specific T cells arise naturally in MET mice as tumors develop [3], thus an increase in IFN-γ-expressing T cells and MUC1 tetramer$^+$ T cells are observed at 14 week time point in untreated and empty liposome treated MET mice.

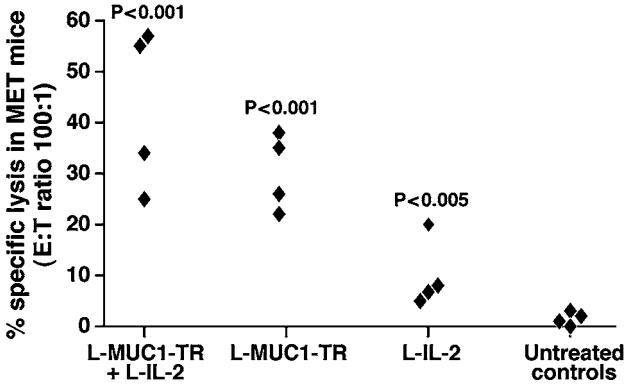

Figure 4. MUC1-specific immunization elicits mature MUC1-specific CTLs in immunized MET mice. CTL activity was determined by a standardized 8 hr ^{51}Cr-release assay using B16 melanoma cells transfected with full-length human MUC1 transgene as targets and splenic T cells from immunized mice with no *in vitro* stimulation with L-MUC1-TR or cytokines. Specific lysis was calculated according to the following formula: (experimental CPM-spontaneous CPM/maximum CPM-spontaneous CPM) $\times$ 100. (A) MET mice in all treatment groups showed significant increase in percent of MUC1-specific mature CTL as compared to untreated mice ($p < 0.001$ for L-MUC1-TR + L-IL-2 treated mice and L-MUC1-TR treated mice; $p < 0.005$ for L-IL-2 treated mice). We were unable to do the CTL assay for mice in the empty liposome treatment group. *Indicates comparison of untreated controls versus treatment groups.

However, the levels are significantly higher in MET mice immunized with L-MUC1-TR and L-IL-2. Data suggest that immunization with L-MUC1-TR elicited MUC1-specific T cells that release IFN-γ and persist in MET mice with high tumor burden.

Mature cytotoxic T cells develop in immunized MET mice

Mature CTLs were observed in L-MUC1-TR + L-IL-2 (4/4 mice) and L-MUC1-TR (4/4 mice) groups but only 1 out of 4 L-IL-2 treated mouse had 20% lytic activity (Figure 4). These CTLs expressed intracellular IFN-γ and were reactive with H-2D^b/MUC1 tetramer (data not shown). Since L-IL-2 alone had a positive effect on MET tumor burden but failed to reproducibly elicit mature MUC1-specific CTLs, we suggest that the beneficial effect of L-IL-2 treatment may be attributed to stimulation of other TAA-specific CTLs that have yet to be identified *in vitro*. To determine specificity of the CTL to MUC1 antigen, B16.neo transfected cells were used as control target cells with lysis of <5% (data not shown).

MUC1 expression decreases in immunized MET mice

A plausible explanation for the failure of MUC1-specific immunization to reduce MET could be that the MUC1-specific CTLs are indeed eradicating MUC1-expressing tumor cells but what eventually grows out are the MUC1 non-expressing tumor cells.

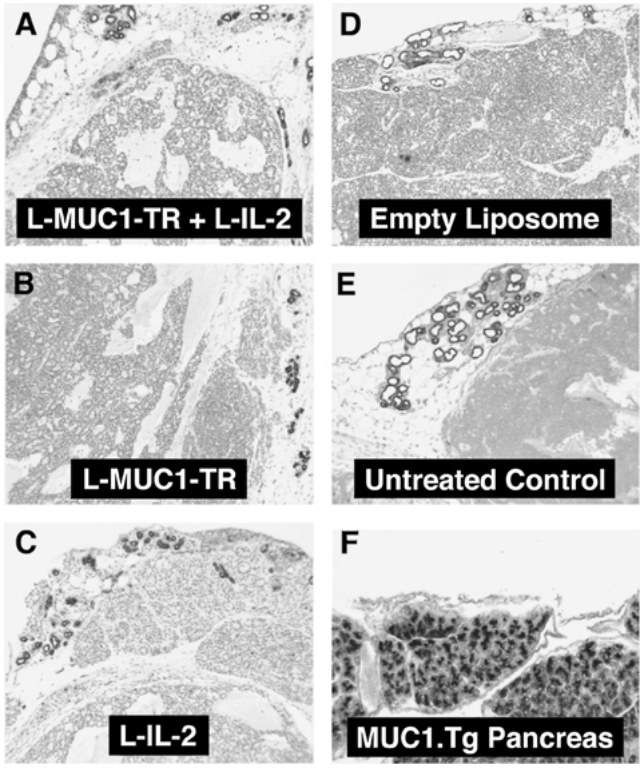

Figure 5. MUC1 expression is significantly reduced in solid undifferentiated tumor sections from MET mice as compared to normal pancreas of MUC1.Tg mice. At time of sacrifice, methacarn fixed and paraffin-embedded sections of tumors from MET mice and normal pancreas from MUC1.Tg mice were stained with B27.29 monoclonal antibody, reactive with MUC1 TR. (A) L-MUC1-TR and L-IL-2 treated tumor; (B) L-MUC1-TR treated; (C) L-IL-2 treated tumor; (D) empty liposome treated tumor; (E) untreated tumor; and (F) pancreas of MUC1.Tg mice. Images were captured at 200X magnification. The large moderately differentiated tumor does not stain for MUC1; however, the more differentiated portions of the pancreas surrounding the tumor stains strongly with the antibody, as does the pancreas from a MUC1.Tg mouse.

To evaluate this, we stained MET tumor sections from immunized and unimmunized MET mice and compared it to normal pancreas. Normal pancreas from MUC1.Tg mice stains very strongly for MUC1 with Mab. B27.29. This antibody is known to react well with highly glycosylated MUC1, in contrast to most other MUC1-specific antibodies [6]. However, solid moderately differentiated tumor sections from treated or untreated MET mice stained very weakly for MUC1 with the same antibody (Figure 5A–E). These results suggest that the naturally occurring MUC1-specific CTLs that exist in the untreated MET mice [3] may be capable of destroying the MUC1$^+$ cells in the tumors and that the tumors grow without MUC1. However, an alternative explanation could be that the tumors antigenically modulate MUC1 antigen by causing changes in glycosylation of MUC1 and therefore evade recognition by MUC1-specific antibodies and CTL.

Table 1. Surface expression of MHC class I molecule on tumor cells decreases as tumors progress in MET mice. Flow cytometric analysis demonstrating percent cells positive for pancytokeratin and MHC class I molecule (H2-D^b/K^b)

Tumor age	*MET*
6 week	35.25 ± 5.1
12 week	15.04 ± 4.1
18 week	3.07 ± 1.0

MHC class I expression decreases in MET mice as tumors progress

A well characterized mechanism by which tumor cells evade CTL killing is by downregulation of MHC class I molecule on their surface. In Table 1, we show that in MET tumors, H2-D^b and K^b positive cells are significantly decreased. In the MET mice, the percent cells that are double positive for pancytokeratin and MHC class I are >30% in 6 week old tumor and decrease to <20% in 12 week old tumor and <5% in 18 week old tumor.

MET tumors release a bioactive form of TGF-ß that down regulates CTL lytic activity

Tumors utilize several mechanisms to escape immune recognition and/or killing. Another such mechanism is the release of immunosuppressive factors such as TGF-ß that are capable of hindering T cell signaling and down regulating their function. We have previously shown that MET tumors express high levels of TGF-ß as detected by immunohistochemistry [3], which does not distinguish between active or latent forms of the protein. We now present data represented in Figure 6A that MET tumor cells grown in culture release an active form of TGF-ß that can inhibit proliferation of Mv1Lu, a mink lung epithelial cell line. Surprisingly, the supernatant from MET tumor cells was as effective in inhibiting MylLu cell proliferation as the standard rHuTGF-ß. Thus, tumors may utilize this mechanism to down regulate CTL activity *in vivo* and escape immune intervention. When MUC1-specific CTLs (1×10^7 cells/ml) were cultured in the presence of supernatant from MET tumor cells for 24 hours, CTL lytic function against B16.MUC1 tumor target was significantly reduced in a dose dependent fashion (Figure 6B). Data presented in Figure 6A clearly demonstrates that MET-derived supernatant contains immunosuppressive factors including TGF-ß that can down-regulate CTL function. CTL clones remained viable and were not apoptotic as determined by trypan blue exclusion and Annexin V staining, post incubation with MET tumor-derived supernatant (data not shown).

Adoptively transferred MUC1-specific CTL clone became tolerized within the pancreas tumor

Mature CTL were observed *in vitro* (Figure 4), from peripheral tissues of MET mice with high tumor burden. These CTLs were

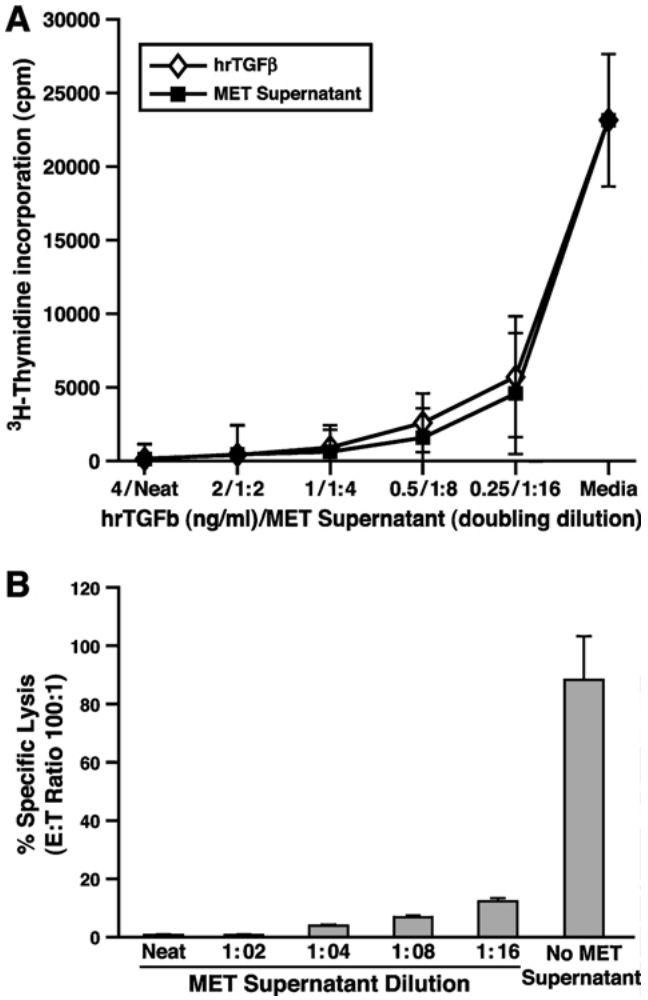

Figure 6. (A) Bioactive form of TGF-ß is secreted by MET tumor cells in culture. Supernatant derived from MET tumor cell culture was tested in a growth inhibition assay with MvlLu cells that are sensitive to TGF-ß inhibition. rHuTGF-ß in DMEM + Nutridoma serum supplement were used as standard (4, 2, 1, 0.5, 0.25, and 0.125 ngs/ml) and serial dilution of the activated supernatant was used as the test sample (neat to 1:16) MvlLu cells were prepared by resuspending at a concentration of 5×10^5 cells/ml in DMEM + Nutridoma serum supplement (no FBS). 100 uls (5×10^4 cells) of cells were added to wells containing the standard rHuTGF-ß or the supernatants. Cells were cultured for 24 h at 37°C in a 10% CO_2 incubator and 1 μCi of ^{3}H-thymidine was added to each well 16 h prior to harvesting. Data are presented as ^{3}H-thymidine incorporation in cpms. All assays were run in triplicate. (B) Supernatant derived from MET tumor cells inhibits cytolytic activity of MUC1-specific CTL. CTLs at 1×10^7 cells/ml in 100 uls were cultured with 100 uls of MET tumor cell supernatant (neat to 1:16 dilution) for 24 hours in a 96 well tissue culture plate. Cells were washed twice prior to being analyzed for lytic activity in a standard ^{51}Cr-release assay at an effector:target (E:T) ratio of 100:1 with the target cells being B16.MUC1 tumors. Titration of E:T ratio was observed in every ^{51}Cr-release CTL experiment, only E:T ratio of 100:1 is presented. Data is presented as % specific lysis.

not effective against the growing pancreatic tumor *in vivo*. To determine the fate of CTL within the growing tumor *in vivo*, we infused 12 week old MET mice with highly cytolytic MUC1-specific clonal CTLs, which were $CD8^+$ T cells expressing specific T cell receptor $V\beta5.1/2^+$ (Figure 7A, left panel). At time of infusion, MET mice have approximately 0.1–0.3 grams of tumor [3]. Two weeks post CTL infusion, pancreatic tumors were removed, tumor infiltrated lymphocytes (TILs) isolated, and percent cells that were double positive for CD8 and $V\beta5.1/2$ determined by flow cytometry (Figure 7A, central panel). As controls, pancreas tumor from MET mice that did not receive CTL infusion was used (Figure 7A, right panel). These control mice did not have T cells that were double positive for CD8 and $V\beta5.1/2$, indicating that the infused CTL enter the pancreas tumor and persist in the tumor for at least 2 weeks. We choose to take TILs at 2 weeks post infusion because in a model of mammary gland cancer, using CFSE (an *in vivo* tracking dye)-labeled CTL we observed that adoptively transferred CTL home to the lymph node and tumor site by 2 days post infusion and are no longer detectable by 21 days (unpublished data). We wanted to determine if the CTLs isolated from MET tumors were tolerant to MUC1 antigen. Tolerance is defined by accumulation of antigen-specific T cells, which are hypo-responsive to the antigen and are cytolytically inactive. We clearly show that the CTLs no longer proliferate to MUC1 antigen presented by syngeneic DC (Figure 7B) nor are they capable of lysing MUC1-expressing tumor cells *in vitro* (Figure 7C). Adding co-stimulatory factors such as anti-CD40 *in vitro* during T cell proliferation assay can reverse the T cell tolerance such that CTLs isolated from TILs can now proliferate to MUC1 antigen (Figure 7B). Anti-CD40 treatment completely restored CTL proliferation, whereas less effect was observed with murine IL-2 (Figure 7B). Isotype control antibodies did not restore proliferation (data not shown). Thus, we were able to demonstrate that, although functionally active CTLs were generated within the periphery of immunized mice, prolonged contact with tumor cells within the tumor environment makes them non-functional. Further analysis of the TIL population revealed the presence of T regulatory or suppressor cells that are defined by $CD4^+CD25^+$ surface markers. These $CD4^+CD25^+$ T cells were sorted, cultured for 24 hours at

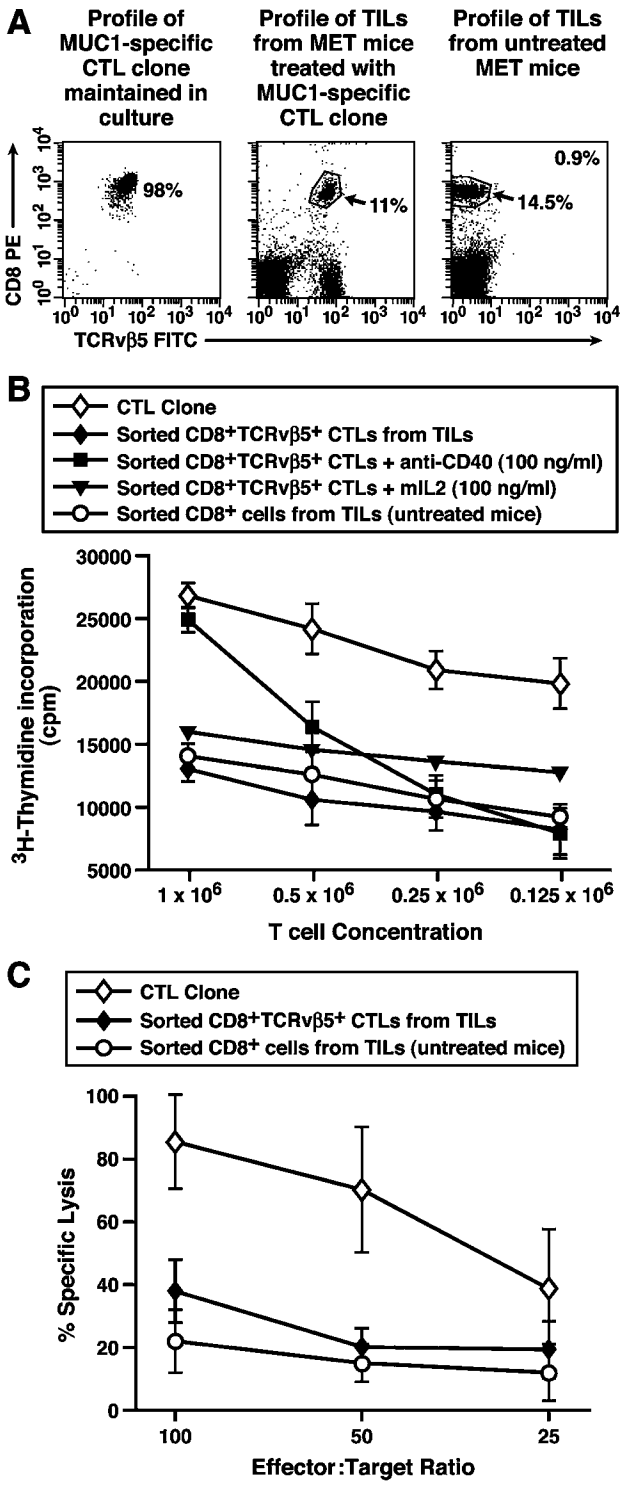

Figure 7. Adoptively transferred CTL become tolerant to MUC1 and are cytolytically inactive after encounter with MET tumor cells. TILs were isolated from tumors of MET mice that received adoptively transferred MUC1-specific CTL clone ($CD8^+/Vb5^+$ cells). (A) The $V\beta5^+/CD8^+$ cells were sorted 15 days post infusion from MET tumors (represented in the central panel). As a positive control, the CTL clone maintained *in vitro* was used (represented in the left panel) and as a negative control, $CD8^+$ T cells were sorted from TILs isolated from tumors of age matched MET mice that were not injected with the CTL clone (represented in the right panel). A box drawn around the cell population represents the sorted cells. CTL-infused MET mice have 11% of their TILs as $CD8^+V\beta5^+$ whereas control mice have 0.9% of their TILs double positive for CD8 and Vß5. Sorted cells were between 90–95% pure. Data representative of one mouse data is shown. The sorted cells were further analyzed for either proliferation in response to DCs pulsed with MUC1 peptide ± anti-CD40 or murine IL-2 (B) or cytotoxic activity against MUC1-expressing B16 melanoma target tumor cells (C). Three CTL-infused MET mice and 3 control MET mice were studied.

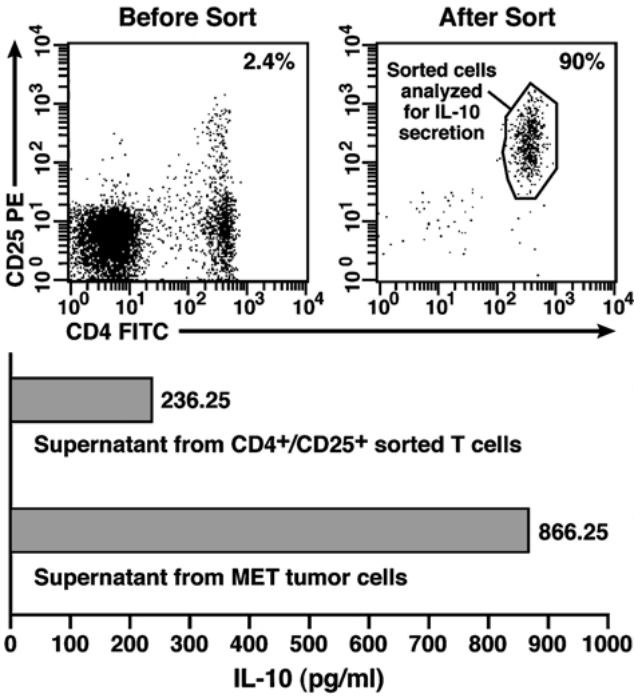

Figure 8. $CD4^+CD25^+$ double positive T regulatory cells were present in MET tumor infiltrate. (A) Unsorted TILs from MET tumors were phenotyped for presence of $CD4^+CD25^+$ double positive cell population and sorted by flow cytometry. Box with the arrow represents sorted population (2.4% of the total MET TIL population was $CD4^+CD25^+$). Sorted cells were 90% pure $CD4^+CD25^+$ T cells. (B) These T regulatory cells were cultured *in vitro* for 24 hours at 1×10^6 cells/ml and analyzed for IL-10 secretion by specific ELISA. MET tumor cell supernatant was also analyzed for IL-10 secretion in the same assay. IL-10 was present in both supernatant derived from T regulatory cells and MET tumor cells, although levels are much higher in the MET tumor supernatant.

37°C in complete DMEM media, supernatant collected and analyzed for IL-10 release. Figure 8 demonstrates that these immunoregulatory cells indeed secrete the immunosuppressive cytokine IL-10, which is known to cause CD8 and CD4 anergy [7,8]. Supernatant from MET tumor cells were also analyzed for presence of IL-10 in the same assay and showed moderate levels of IL-10 (Figure 8).

Discussion

In human cancer patients, the functional status of vaccine-induced, tumor antigen-specific CTL has been questioned due to its inability to inhibit tumor growth [9,10]. In addition, correlation between precursor CTL (pCTL) frequency and clinical outcome could not be established [8,9]. Stimulation of the pCTL with tumor-specific antigen can induce IFN-γ secretion *in vitro*, suggesting that the CTL retain antigen responsiveness [11] and should be capable of lysing tumor cells. However, recently it has been shown that IFN-γ expression may not be the optimal functional parameter to best describe the potential of effector cells [12]. Researchers have used perforin levels in the CTL as a marker for active CTL and combined that with levels of expression of CD27 (a marker for naïve T cells) and CD45RA (marker for effector T cells) to determine the maturation status of the CTL [12–15]. Despite the use of these markers, the standard immune monitoring post immunotherapy is still defined by (a) CTL effector function determined by IFN-γ secretion and ^{51}Cr-release assay and (b) phenotypic analysis determined by Ag-specific tetramer staining. We compared the immune responses that developed during treatment and at time of sacrifice. Our data demonstrate that MUC1-specific immunization elicits MUC1-tetramer reactive CTL that are functionally lytic *in vitro* and express intracellular IFN-γ, but fail to eradicate the spontaneous pancreatic tumors, which is what is observed in human studies. Similar phenomena have been shown in another spontaneous tumor model, the HER-2/neu transgenic mice that develop spontaneous focal mammary carcinomas and demonstrate immunological tolerance to the neu antigen. These mice also were able to elicit neu-specific T cells following neu-specific vaccination but were not protected from the developing mammary tumors [16,17].

Several tumor evasion mechanisms may be employed by the growing pancreas tumor to avoid recognition and killing by functional CTLs. In our previous work, we established that the tumor cells are heterogeneous in nature and can modulate expression of MUC1-epitopes as well as fas ligand epitopes and express high levels of TGF-ß, an immunosuppressive cytokine [3]. In the present study, we demonstrate that cytolytically active CTL become inactive when cultured in the presence of MET tumor cell supernatant (Figure 6B) and that the MET tumor cells secrete a bioactive form of TGF-ß that may be one of the factors responsible for down-regulation of CTL activity (Figure 6A). Other factors secreted by MET cells include IL-10 (Figure 8). Preliminary data showed that treatment of MET mice with neutralizing antibody to TGF-ß did not result in tumor regression (data not shown) suggesting a role for other factors in immune evasion. In addition to immunosuppressive factors released by MET tumor cells *in vitro*, MET tumor cells down-regulate their MHC class I molecule (Table 1) *in vivo* as tumors progress, thus avoiding recognition by class I-restricted $CD8^+$ CTL. We also found immune regulatory T cells that secrete IL-10 in the pancreas tumor microenvironment and may further contribute to the induction of CTL tolerance and/or anergy (Figure 8). These regulatory T cells can inhibit proliferation and function of other T cells and are major players in induction of CD4 and CD8 T cell tolerance [18–22]. Thus, taken together our data suggest that a combination of factors may be employed by the MET tumor cells to evade CTL killing as well as down-regulate CTL function.

It is reported in the literature that CTLs that are detectable in the peripheral lymphoid organs but that do not migrate to the site of the tumor in sufficient numbers may not be active

in destroying the tumor target tissue [23]. Therefore, the fundamental question remains whether the MUC1-specific CTLs in the MET mice infiltrate the pancreas tumor bed and remain active within the solid tumor. To determine the fate of these MUC1-specific CTLs within the growing pancreas tumor, we adoptively transferred functionally active CTLs into MET mice *in vivo* and observed that the CTLs do infiltrate the tumor bed and after an initial period of proliferation, become tolerized to MUC1 antigen and are rendered cytolytically inactive (Figure 7A). This strongly indicates that exposure of CTL to the tumor microenvironment can down-regulate their proliferation and function. Maintenance of the activated state of CTLs is one of the major requirements for effective cancer immunotherapy. This activated state is strongly influenced by inflammatory stimuli as well as the action of $CD4^+$ T helper (Th) cells. Th and CTLs must recognize antigens presented on the same antigen presenting cell (APC) [24,25] and the interaction of Th and APC is sufficient to convert the APC to a state that allows priming and maintenance of CTL. Our data show that treatment with L-IL-2 alone leads to lower tumor burden but not eradication of spontaneous tumors in MET mice (Figure 5). One possibility is that the L-IL-2 may activate the natural killer cells that recognize MHC class I-negative tumor cells. Another possibility is that exogenous L-IL-2 can activate the naturally occurring low affinity MUC1-specific or other TAA-specific CTLs that already exist in the tumor bearing mice and make them effective against the tumor. On the other hand, treatment with MUC1 peptide and liposomes may cause over-activation and induce tolerance of the T cells that ultimately leads to inactivation of effector T cells such that anti-tumor response is interrupted. This result points us to the direction of providing necessary and effective help for the CTL to overcome immunization-induced tolerance or tumor-induced inactivation. In recent years, there is growing evidence that cross-linking of co-stimulatory molecules such as CD40 expressed on APC and CD40L expressed on Th cells as well as OX40 expressed on activated Th cells and OX40L expressed on APC can reverse established T cell tolerance. Both of these molecules are members of the TNF receptor family and signaling through these molecules promotes helper T cell expansion and restores normal functionality of the tumor-specific cytotoxic T cells. Previous data in the literature highlight the potent co-stimulatory capacity of OX40 and CD40, which make them targets for therapeutic intervention in cancer [26–31]. In Figure 7B, we demonstrate that the down-regulation of CTL proliferation (post exposure to MET tumors *in vivo*) to MUC1 peptide-pulsed DC was completely restored by cross-linking CD40 co-stimulatory molecules with specific antibody *in vitro*. Addition of murine IL-2 to these cultures had a beneficial effect although not as significant as treatment with CD40 antibodies. These experiments were conducted *in vitro* and similar experiments are being repeated *in vivo* to determine if an anti-tumor response could be achieved in MET mice with these antibodies and whether CTL activity can be maintained in the presence of the growing pancreas tumor.

The possibility that MUC1-specific CTL can eradicate $MUC1^+$ cells, allowing outgrowth of MUC1 non-expressing cells in the tumor cannot be ignored. In Figure 5, we observed negligible MUC1 staining in pancreatic tumor sections from MET mice as compared to normal pancreas and as compared to dysplastic acinar cell carcinoma or microadenomas [3]. However, considering that the CTLs become tolerized within the tumor microenvironment, it is highly unlikely that the CTLs were active against $MUC1^+$ tumor cells, especially because there was no difference between immunized versus non-immunized MET tumor sections, whereas highly lytic CTLs were detected in immunized MET mice as compared to non-immunized controls. Thus, once again it argues for the possibility of antigenic modulation of MUC1 in which the glycosylation status of MUC1 is altered, thereby hindering antibody reactivity as well as CTL recognition.

In summary, our data suggest that the MUC1-specific CTLs are cytolytically active and are capable of eradicating injectable $MUC1^+$ tumor cells *in vitro* and *in vivo* but that the tumor microenvironment is non-conducive to CTL proliferation and killing. The tumor cells utilize a combination of immune-evasion mechanisms both to down-regulate CTL function and avoid recognition. We also suggest that secondary immunization with antibodies to co-stimulatory molecules such as CD40 may be a useful therapeutic strategy that may provide necessary help to control induction of CTL tolerance and maintain its lytic activity, thus leading to an anti-tumor response. The MET mice appropriately mimic the human situation and serve as an excellent model system in which to study the mechanisms that are involved in inactivation of CTL function as well as to study tolerance versus immunity post vaccination. The tumors arise in an appropriate tissue background and hormonal and stromal milieu as well as in the context of a viable immune system. Future studies utilizing this mouse model may facilitate the design of appropriate immunotherapeutic strategies as well as immune monitoring parameters that may correlate with clinical outcome.

Materials and methods

Mouse model MET

MUC1.Tg mice are bred with oncogene-expressing mice that spontaneously develop tumors of the pancreas and are designated as MET [3]. MUC1.Tg mice were developed in our laboratory [2]. ET mice were obtained as a kind gift from Dr. Judith M. Tevethia (University of Pennsylvania, Hershey, PA) [32]. All mice are congenic on C57BL/6 background at backcross $\geq$10 [33]. All mice were bred and maintained in specific pathogen free conditions in the Mayo Clinic Scottsdale Natalie Schafer Transgenic Animal Facility. All experimental procedures were conducted according to IACUC guidelines.

PCR screening

PCR was used to routinely identify MUC1 and ET positive mice in the colony. PCR was carried out as previously described [2,32]. The primer pairs for MUC1.Tg are 5′-CTTGCCAGCCATAGCACCAAG-3′ (bp. 745 to 765) and 5′-CTCCACGTCGTGGACATTGATG-3′ (bp. 1086 to 1065). Primer pairs for ET are 5′-GCTCCTTTAACCCACCTG-3′ (bp. 4055 to 4072) and 5′-CCAACCTATGGAACTGATGAATG-3′ (bp. 4546 to 4568). The amplification program for MUC1 consisted of one cycle of 5 min at 95°C and 40 cycles of 30 sec at 95°C, 1 min at 61°C, and 30 sec at 72°C followed by one cycle of 10 min at 72°C. The amplification for ET PCR reaction is as above with the exception of annealing temperature of 52°C. The PCR product of each reaction was analyzed by size fractionation through a 1% agarose gel. Amplification of MUC1 resulted in approximately a 500 bp fragment, and of ET in a 491 bp fragment.

Cell lines

B16 melanoma cells transfected with either full-length human MUC1 gene (B16.MUC1) or neomycin resistance gene (B16.neo) were used as targets for CTL assays. These cell lines were originally provided by Dr. Tony Hollingsworth (Eppley Cancer Center, University of Nebraska). B16.MUC1 and B16.neo were maintained in DMEM media with 10% FBS, immglutamax, penicillin (50 units/ml) and streptomycin (50 ugs/ml), supplemented with 300 ugs/ml G418. One day prior to conducting the ^{51}Cr-release assay, cells were treated with 5 ngs/ml IFN-γ (Pharmingen, San Diego, CA). Cells were routinely tested by flow cytometry for the presence of MUC1 and MHC class I. MvlLu cells, a mink lung epithelial cell line, was a kind gift from Dr. E. Akporiaye (University of Arizona, Tucson, AZ). These cells were maintained in DMEM supplemented with 10% FBS, 1 mM glutamax, penicillin (50 units/ml) and streptomycin (50 ugs/ml). FGK 45.5 hybridoma cell supernatant (ATCC, Rockville, MD) was used as a source of CD40 antibody. These cell lines were maintained in the Immunology Core at Mayo Clinic Scottsdale and supernatant immunoglobulins were purified and protein concentration was determined prior to use.

Analysis of MET tumors

The entire pancreas was dissected free of fat and lymph nodes, fixed in methacarn followed by 70% ethanol, air-dried and weighed. Tumor weights plotted represent individual animal data at time of sacrifice. Mice were carefully observed for signs of ill-health, including lethargy, abdominal distention, failure to eat or drink, marked weight loss, and hunched posture.

Immunohistochemistry

Tumors were obtained from control and immunized MET mice at time of sacrifice. Tumors were fixed in methacarn followed by 70% ethanol, paraffin embedded, and sectioned for immunohistochemical analysis. MUC1 antibodies used were B27.29, a mouse monoclonal antibody with epitopes in the tandem repeat extracellular domain of MUC1 [34]. B27.29 is specific for human MUC1 and does not cross react with mouse Muc1. HRP-conjugated B27.29 was obtained from Biomira, Inc., Edmonton, Canada. Antibody staining was blocked with the appropriate peptide.

Adoptive transfer of MUC1-specific CTL and isolation of tumor infiltrated lymphocytes (TILs)

At 12 weeks of age, MET mice received tail vein injection of MUC1-specific CTL clone (2×10^7 cells/mouse/150 uls). MET CTL clonal cells were CD8$^+$ T cells that expressed TCR-Vß5.1/2. Fifteen days post injection, TILs were isolated from MET tumors by manually dissociating the tumor mass with a sterile scalpel, followed by dissociation in 0.5 mM EDTA for 1 hour in a 37°C incubator with constant shaking. Dissociated cells were passed through a nylon sieve and then separated by Ficoll-Hypague (density: 1.077 $\pm$ 0.001 g/ml, Invitrogen Corp., Carlsbad, CA) gradient centrifugation. The buffy coat was removed, washed and counted for further analysis. CD8$^+$Vß5.1/2$^+$ from experimental MET TILs and CD8$^+$ CTL from control MET TILs were isolated by sorting using FACs Vantage (BD instruments, San Diego, CA). Sorted cells were further analyzed for their proliferative capacity to MUC1 TR antigen and cytolytic activity against MUC1-expressing tumor cells. Other sorts from TILs included T cells that were double-positive for CD4$^+$CD25$^+$.

T cell proliferation in response to MUC1 TR peptide

Sorted cells from CTL-infused and control MET mice (various concentrations as illustrated in Figure 7B) were cultured for 5 days in the presence of irradiated dendritic cell (1×10^5/ml) pulsed with 10 μgs/ml MUC1 TR peptide (TAPPAHGVTSAPDTRPAPGSTAPP). MUC1-specific CTL clone was used as a positive control and DC without TILs was used as the negative control. Proliferation was determined by measuring ^{3}H-thymidine incorporation using the beta plate counter (Packard Instruments, Perkin Elmer LifeSciences, Boston MA). In some experiments, anti-CD40 or mouse IL-2 (Pharmingen, San Diego, CA) were added to the co-culture at 100 ngs/ml concentration. Isotype control antibodies at the same concentration were used as negative controls in these assays (data not shown).

CTL assays

Determination of mature CTL activity was performed using a standard ^{51}Cr-release method with no *in vitro* peptide stimulation. Splenocytes from immunized and unimmunized MET mice were harvested by passing through a nylon mesh followed by lysis of red blood cells using Pharmlyze (0.45% ammonium chloride solution purchased from Pharmingen (San

Diego, CA)). Splenocytes or TILs isolated from MET tumors were used as effector cells. The target cell line, B16.MUC1, expressed high levels of MUC1 as determined by flow cytometric analysis using antibodies to the MUC1 conjugated to FITC (Pharmingen, San Diego, CA). For better presentation of MUC1 antigen, B16.MUC1 target cells were treated with 5 ngs/ml IFN-γ one day prior to the assay to up regulate MHC class I surface expression. Specific ^{51}Cr-release was calculated according to the following formula: (experimental CPM-spontaneous release CPM/maximum release CPM-spontaneous release CPM) $\times$ 100. Spontaneous release in all experiments was less than 15% of maximum release. In some experiments, B16.neo transfected cells were used as control target cells with lysis of <5% (data not shown). In some experiments sorted CTLs from CTL-infused and control MET mice were used as effector cells.

Flow cytometry and cell sorting

Single cells from peripheral blood lymphocytes (PBLs) collected from immunized and unimmunized MET mice at various times post immunization were analyzed by two color immunofluorescence for intracellular IFN-γ expression. Intracellular cytokine levels were determined after cells were treated with brefeldin-A [also called Golgi-Stop (Pharmingen)] according to the manufacturer's recommendation (4 uls/1.2 $\times$ 10^7 cells/6 mls for 3 h at 37°C prior to staining). Cells were surface labeled with CD4 and CD8 antibodies conjugated to FITC prior to permeabilizing with the Pharmingen permeabilization kit (Cat # 554722, containing 0.005% saponin and 4% formaldehyde) and staining with anti-IFN-γ conjugated to PE. Tumor cells from freshly dissociated MET tumors were stained with fluorescently labeled anti-pan-cytokeratin (FITC) and anti-MHC class I H2-D^b and H2-K^b (PE). All fluorescently labeled antibodies were purchased from Pharmingen except anti-pan-cytokeratin, clone C-11, purchased from Sigma, St. Louis, MO. Flow cytometric analysis was done on Beckton Dickinson FACscan using the Cell Quest program. TILs isolated from MET tumors were surface labeled with CD25 conjugated to PE and CD4 conjugated to FITC. $CD4^+/CD25^+$ double positive T cell population was isolated by cell sorting using FACs Vantage (BD Instruments).

IL-10 ELISA

$CD4^+/CD25^+$ double positive T cells were cultured for 24 h in DMEM complete media at concentration of 1–2 $\times$ 10^6/ml. Supernatant was assessed for the presence of IL-10 using a murine IL-10 ELISA kit purchased from Biosource (Caramillo, CA). MET tumor cell supernatant was also analyzed for IL-10.

Mv1Lu cell growth inhibition assay

The growth inhibition assay was performed as described [38]. First, latent TGF-ß in supernatant from confluent MET tumor cell culture was activated by adjusting the pH to below 3 with 12 M HC1 and incubating at RT for 1 h. The supernatant was then neutralized to pH 7 with 10 M NaOH. rHuTGF-ß was purchased from R&D Systems (Minneapolis, MN) and standard was prepared in DMEM + Nutridoma serum supplement. rHuTGF-ß was prepared at 8 ngs/ml stock solution and serially diluted 1:2 to obtain standards of 4, 2, 1, 0.5, 0.25, and 0.125 ngs/ml. Serial dilution of the activated supernatant was also prepared from neat to 1:16 and 100 uls added to each well of a 96 well plate. Mv1Lu cells were prepared by resuspending at a concentration of 5 $\times$ 10^5 cells/ml in DMEM + Nutridoma serum supplement (no FBS). 100 uls (5 $\times$ 10^4 cells) were added to wells containing the standard rHuTGF-ß or the supernatants. Cells were cultured for 24 h at 37°C in a 10% CO_2 incubator. 1 μCi of ^{3}H-thymidine was added to each well and incubated for 16 h. Cells were harvested and incorporated radioactivity was measured using a beta plate counter. All assays were run in triplicate.

MUC1-tetramer staining

MHC class I H-$2D^b$ tetramers containing MUC1 TR APGSTAPPA peptide were created by Dr. Larry Pease at Mayo Clinic Rochester. PBLs were stained with 0.1 mgs/ml MUC1-tetramer (directly conjugated to phycoerythrin) for 1 hour on ice. To confirm specificity, a H-$2D^b$ tetramer containing an irrelevant peptide was used as control ($VP2_{121–130}$). The cells were then stained for 15 minutes with CD8 and CD4 antibodies conjugated to FITC prior to analyzing by two-color flow cytometry. Flow cytometric analysis was done on Beckton Dickinson FACscan using the Cell Quest program.

Preparation of dendritic cell-pulsed liposomal MUC1 TR lipopeptide

DCs were derived from C57BL/6 bone marrow cells according to the method described [35]. Briefly, bone marrow cells were flushed from tibia and femur and red blood cells were lysed with ammonium chloride. Cells were cultured in DMEM with 10% fetal bovine serum, 1% glutamax, 50 U/ml penicillin, 50 ugs/ml streptomycin (DMEM complete medium), supplemented with 10 U/ml murine GM-CSF (Pharmingen) and 10 U/ml murine IL-4 (Pharmingen). On day 7, adherent cells were fed with fresh DMEM complete media supplemented with GM-CSF and IL-4 at the same concentration. Between days 10 and 14, we obtained approximately 85 to 90% cells that showed DC phenotype by flow cytometry. DC were fed with MUC1 TR lipopeptide that were enclosed in liposomes according to the method provided by Biomira, Inc. [36]. Briefly, 2 mls of the liposomal MUC1 TR formulation was fed to 200 $\times$ 10^6 DC in a total volume of 20 mls, 24 hours prior to injection. Mice were injected i.p. with 1 $\times$ 10^6 MUC1 TR pulsed DC per mouse in 200 ul volume. For T cell proliferation assays, DCs were pulsed with naked 25 mer MUC1 TR peptide at 10 ngs/ml.

Vaccination strategy

The vaccine formulation consisted of a lipid derivative of a 25 mer MUC1 TR peptide STAPPAHGVTSAPDTR-PAPGSTAPP) which was incorporated into liposomes along with Lipid A as adjuvant. The vaccine was supplied as a sterile powder formulated at Biomira, Inc. (Edmonton, AB, Canada) by a proprietary method. Upon reconstitution with sterile saline for injection, it contained 400 ug/ml of MUC1 lipopeptide, 200 ug/ml of Lipid A (Avanti Polar Lipids, Inc. Alabaster, AL, USA) and 20 mg/ml of carrier lipids in multilamellar vesicles with a mean particle size of 2–3 μm. For the preparation of pulsed dendritic cells, particle size of the vaccine was reduced to <200 nm by ultrasonication. Randomized preclinical trials were performed in MET mice starting at 3 weeks of age.

Five arms in the study include: (i) liposomal MUC1 TR lipopeptide (L-MUC1-TR) (200 ugs/mouse/250 uls s.c.) + L-IL-2 (20,000 Units/mouse/100 uls i.p.), (ii) L-MUC1-TR, (iii) L-IL-2 [37], (iv) empty liposomes, and (v) no treatment. We compared the immune responses that developed during treatment and used survival and tumor burden as the endpoints for determining the clinical effectiveness of the vaccine. A schematic of the immunization protocol is shown in Figure 1.

Statistical analysis

P values are from the one-way ANOVA *F* test for comparing the treatment groups simultaneously and from the pair-wise contrasts from a one way ANOVA model.

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