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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
This project is focused on the development of novel tumor vaccines directed at MUC1 and other tumor antigens. Our specific aims are: 1) To assess the effectiveness of vaccine formulations against MUC1 and other tumor antigens in the prevention and treatment of spontaneous breast carcinomas in mice and 2) To translate the most effective vaccine strategies into phase I clinical trials in patients with high and low tumor burden. The model of spontaneous mammary cancer is the MUC1-expressing polyoma middle T antigen mice (MMT). We have tested four vaccines in the preclinical mouse model: 1) liposomal MUC1 tandem repeat peptide, 2) dendritic cells (DCs) pulsed with tumor lysate, 3) DCs fused to MMT tumor cells, and 4) adoptive transfer of MUC1-specific cytotoxic T lymphocytes (CTLs). All vaccines elicited a strong immunological response, delay of tumor growth and development of mature MUC1-specific CTLs. Failure to eradicate the large tumor burden in these mice appears to be due to the development of tolerance in the CTLs within the tumor environment. Tumors also exhibited several known escape mechanisms. Future studies will test strategies that should prevent or over-ride the development of tolerance. The clinical trial protocol is under development.

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INTRODUCTION

This project is focused on the development of novel tumor vaccines directed at MUC1 and other tumor antigens. Expressed on most breast cancers, MUC1 often elicits cellular and humoral immune responses in humans. However, these responses are not sufficiently strong to eradicate tumors, as greater than 90% of breast tumors express MUC1 and these tumors progress. MUC1 is a target for immunotherapy, as there are alterations in expression that make it a tumor-specific antigen. There is a large increase in the amount of MUC1 mucin on cells and in circulation, it is expressed throughout the tumor mass, and glycosylation is altered, revealing immunodominant tumor-specific peptide sequences. Thus, MUC1 core protein is a candidate peptide for novel immunotherapy strategies to strongly activate the immune system to eradicate tumors expressing these epitopes.

Although MUC1 is strongly expressed on mammary (and other) tumors, it is possible that spontaneous mammary tumor heterogeneity of expression may allow MUC1 non-expressing cells to grow out and form tumors. Thus, we are testing dendritic cell (DC)-based vaccines presenting a variety of tumor antigens by using 1) DCs fused with primary tumor cells and 2) DCs pulsed with tumor lysate.

Up to now, all of the MUC1-specific immunotherapy has been tested in mice using injected tumor cells [1-4]. The preclinical model system that we generated develops spontaneous mammary gland cancer that expresses MUC1. MUC1 transgenic mice (MUC1.Tg) were bred with mice carrying the MMTV-driven polyoma middle T antigen (MT) to create MMT mice [5, 6]. The MUC1.Tg mice do not over-express MUC1; rather, the mice express human MUC1 in a developmentally regulated and tissue specific fashion, as the transgene is driven by its own promoter [5]. Mice transgenic for this protein develop B and T cell tolerance and are refractory to immunization with the protein encoded by the transgene. All mice are congenic on the C57BL/6 background to eliminate strain-specific modifier effects. In the MMT mice, mammary gland tumors are induced by the action of a potent tyrosine kinase activity associated with the polyoma virus middle T antigen driven by the mouse mammary tumor virus long terminal repeat (MMTV) [6]. Middle T specifically associates with and activates the tyrosine kinase activity of a number of c-src family members, eliciting tumors when a threshold level of gene product has been attained. This promoter is transcriptionally active throughout all stages of mammary gland development and results in widespread transformation of the mammary epithelium and the rapid production of multifocal mammary adenocarcinomas. Hyperplastic alveolar nodules (HANs) can be detected by whole mount as early as 21 days and palpable mammary gland tumors are detectable from approximately 49 days onwards. Tumor progression is quite rapid, reaching 10% of body weight by about 20 – 24 weeks. All of the female MMT mice get tumors. Tumors arise with synchronous kinetics and are highly fibrotic with dense connective tissue separating individual nests of tumor cells, a pathology that closely resembles scirrhous carcinomas of the human breast [7]. These mice exhibit metastasis in the lungs (30 out of 49 MMT mice tested or 60%) and micro-metastasis in the bone marrow by 4 months of age. The model is described in detail in the paper supplied in the appendix [8]. See Figs. 2 for kinetics of tumor growth and Fig. 3 for a description of MMT tumor cells metastatic to the bone marrow. The MMT mouse appears to be an appropriate model for human cancer and allows us to study the effects of self-tolerance, immunity and auto-immunity to MUC1 as mammary tumors develop spontaneously.

The **hypothesis** of our study is that enhancing MUC1-specific immunity will result in anti-tumor immunity. We propose to develop an optimal cancer vaccine using epithelial cell mucin MUC1 peptides or protein or MUC1-expressing tumors presented by DCs as immunogen. The most successful therapies will be tested in phase I clinical trials. Additional hypotheses are that the immune system is tolerant to MUC1, although immunization strategies can be developed to overcome partial tolerance.

Our **specific aims** are: **1)** to assess the effectiveness of vaccine formulations against MUC1 in the prevention and treatment of spontaneous breast carcinomas in mice and **2)** to translate the most effective vaccine strategies into phase I clinical trials in patients with high and low tumor burden. The clinical trial protocol for aim 2 is to be written during year two of this grant.

RESULTS (BODY)

Specific Aim 1: To assess the effectiveness of vaccine formulations against MUC1 and other tumor antigens in the prevention and treatment of spontaneous breast carcinomas in mice.

Vaccination with Liposomal-MUC1 lipopeptide and liposomal IL-2

We have included with this report our paper entitled "MUC1-specific immunotherapy in a mouse model of spontaneous breast cancer", which has been resubmitted in revised form to the Journal of Immunotherapy. Thus, the results will simply be summarized below.

Liposomal MUC1 peptide vaccine.

Vaccination strategy. The vaccine formulation consisted of a lipid derivative of a 25 mer MUC1 TR peptide (STAPPAHGVTSAPDTRPAPGSTAPP) 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.

Preparation of dendritic cell-pulsed liposomal MUC1 TR lipopeptide. Dendritic cells (DC) were derived from C57BL/6 bone marrow cells according to the method described [9]. Briefly, bone marrow cells were flushed from tibia and femur and red blood cells were lysed with ammonium chloride. Cells ($1x10^6$/ml) were cultured in DMEM with 10% fetal bovine serum, 1% glutamax, 50U/ml penicillin, 50ugs/ml streptomycin (DMEM complete medium), supplemented with 10ngs/ml murine GM-CSF (Pharmingen) and 10ngs/ml murine IL-4 (Pharmingen). At day 7, non-adherent cells were removed, washed, re-suspended ($1x10^6$/ml) in fresh DMEM complete media supplemented with 5ngs/ml GM-CSF. Adherent cells were re-fed fresh DMEM complete media supplemented with 5ngs/ml GM-CSF. Between days 10 and 13, we obtained approximately 70 - 75% cells that showed DC phenotype by flow cytometry. DC were fed with MUC1 tandem repeat lipopeptide that were enclosed in liposomes according to the method

provided by Biomira, Inc. [10]. Briefly, 2mls of the liposomal MUC1 tandem repeat (MUC1 TR) formulation was fed to 2 x 10^8 DC in a total volume of 20 mls, 24 hours prior to injection. Mice were injected i.p. with 1 x 10^6 MUC1 TR pulsed DC per mouse in 200 ul volume. The first two immunizations at weeks 3 and 5 consisted of DC-fed liposomal MUC1 formulation. Thereafter, mice were immunized with liposomal MUC1 vaccine in PBS.

Randomized preclinical trials were performed in MMT mice starting at 3 weeks of age. Treatment arms include: (i) liposomal MUC1 TR lipopeptide (L-MUC1-TR) (200ugs/mouse/250uls s.c.) + L-IL-2 (20,000 Units/mouse/100uls i.p.), (ii) L-MUC1-TR, (iii) L-IL-2 [11], (iv) empty liposomes and (v) no treatment. We compared the immune responses that developed during treatment and used tumor onset, tumor burden and survival as the endpoints for determining the clinical effectiveness of the vaccine. A schematic of the immunization protocol is shown in Figure 1.

Tumor burden and lung metastasis in immunized MMT mice. We immunized mice with L-MUC1-TR $\pm$ L-IL-2. The first two immunizations administered to young (3 and 5 week old) MMT mice utilized syngeneic 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 [12]. 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. Although no significant differences were observed in the onset and progression of the MMT tumors with immunization (Figure 2A), by 18 weeks of age there was significant decrease ($p<0.05$ to $p<0.005$) in tumor burden between immunized and control MMT mice (Figure 2B). To our surprise empty liposomes elicited a similar response suggesting that the lipid A in the formulation may have an effect on the tumor. However, by 20-24 weeks no significant differences in tumor burden between immunized and control mice were observed (Figure 2A). Interestingly, we observed that treatment with L-IL-2 alone had significantly lower numbers of lung metastasis than mice in all other treatment groups and in untreated control mice (Figure 2C). Incidence of metastasis was also lower in mice treated with L-IL-2 as compared to other treatment groups and untreated MMT mice (44% versus 64% in untreated MMT) (Table 2). These results did not reach significance due to low animal numbers. All mice were sacrificed when tumors reached 10% of body weight and therefore difference in survival was not observed. Because MUC1 is expressed on normal cells, we routinely examined mice for signs of autoimmunity post immunization. We monitored weight loss, food intake, general health, hunched back and pathology of various MUC1-expressing organs. No signs of autoimmunity were evident in our study group (data not shown).

T cell immune response in immunized MMT mice. Immunization elicited mature MUC1-specific CTL that were cytotoxic against B16.MUC1 tumor cells in vitro (Figure 3). To determine MUC1 specificity, B16 cells transfected with vector alone (B16.neo) were routinely used as control tumor target and lysis of < 5% was observed making the CTL specific for MUC1 (data not shown). Both MHC-restricted and non-restricted MUC1-specific CTL have been reported in the literature for human cancer [13, 14]; however, in MMT mice, we only detect MHC-restricted CTL. It is possible that these mice do possess unrestricted CTL but these CTLs may be difficult to detect in vitro and therefore have never been reported previously in mice [15-18]. Although the CTLs were cytotoxic in vitro, they had little effect on the growing tumor cells

in vivo in MMT mice. This was determined by immunohistochemistry of MMT tumor sections in which MUC1-expressing tumor cells remained unchanged with immunization, suggesting that the MUC1-specific CTLs were not cytotoxic against the tumor cells in vivo (data not shown). To evaluate the T cell responses during immunization and as the tumors progressed, immunized and non-immunized MMT mice were tail-bled at 6, 14, and 20 weeks, peripheral blood leukocytes (PBLs) were isolated and tested for presence of a) T cells expressing intracellular IFN-γ and b) T cells recognizing H-2D^b/MUC1 tetramer. All treatment groups in immunized MMT mice showed increased numbers of T cells expressing intracellular IFN-γ by 10 -14 weeks of age as compared to untreated control mice (Fig. 4A, p values shown in the figures). By 20 weeks of age, there was a decrease in these cell numbers suggesting that repeated immunization with MUC1 TR peptide or high tumor burden may affect T cell effector function. This observation correlates well with the decrease in tumor burden at 18 weeks in immunized mice, which does not last at 21 weeks (Fig. 2B). Similarly, T cells reactive with H-2D^b/MUC1 tetramer increased by 14 weeks in immunized MMT mice but no further increase was observed at 20 weeks (Fig. 4B, p values shown in the figures). Similar results were observed in the empty liposome group, suggesting that the lipid A present in the liposomal vaccine formulation may be sufficiently immunogenic to elicit an anti-MUC1 response. However, the response seen with the empty liposome group did not translate into MUC1-specific CTL response (Fig. 3) nor did it correlate with elevated serum MUC1 levels in Fig. 5A.

MUC1 Serum levels in immunized MMT mice. As tumors progressed in the MMT mice, MUC1 serum levels increased only slightly as compared to age matched MUC1.Tg mice with maximum reaching to 1500 – 2500 Units/ml of serum at 20 weeks of age. In age matched female MUC1.Tg mice, serum MUC1 levels ranged from 500 to 1200 Units/ml, which is likely to depend upon their estrous cycle status (data not shown). The low levels of circulating MUC1 may explain the lack of an immune response to MUC1 in non-immunized MMT mice (Fig. 3 and Table 1). Immunization, however, significantly increased the serum MUC1 levels compared to untreated MMT mice (Figure 5A), which corresponded directly to the increased CTL activity in these mice. These results suggested that high levels of circulating tumor antigen, MUC1, may activate MUC1-specific CTL that are capable of specifically lysing MUC1-expressing tumor cells *in vitro* (Figure 3). We also detected low levels of circulating antibodies to MUC1 in the L-MUC1-TR + IL-2 treated mice (Fig. 5B) suggesting that immunization and the high level of circulating tumor associated MUC1 has changed the antigenic profile and elicited a low level humoral response to MUC1. Antibodies reactive with MUC1 have been reported in a small number of humans with breast cancer [19, 20]. Although a humoral response is often dismissed as being ineffective against solid tumors, it is still important that the response in the MMT mice once again parallels that in humans.

Results indicated that when compared to untreated mice, immunized mice develop mature T cells that a) express intracellular IFN-γ , b) are reactive with MHC class I H-2D^b/MUC1 tetramer, and c) are cytotoxic against MUC1-expressing tumor cells *in vitro*. The presence of MUC1-specific CTL did not translate into a clinical response as measured by time of tumor onset, tumor burden and survival. Because these immunized mice have developed MUC1-specific CTL responses, these spontaneously arising mammary gland tumors must have evaded the existing CTL. We demonstrate that some of the immune-evasion mechanisms utilized by the tumor cells include

down regulation of MHC-class I molecule (Table 3), expression of TGF-β2 (see below), and decrease in IFN-γ -expressing effector T cells as tumors progress (Fig. 4A).

Tumor lysate pulsed DC were effective in breaking tolerance and preventing tumor formation in MUC1.Tg mice. Another plausible explanation for the failure of MUC1-specific immunization to eradicate MMT tumors is the utilization of a single tumor antigen as immune target. Recent findings suggest that tumor lysate-fed DC generate tumor-specific proliferative cytokine release and cytolytic reactivities *in vitro* as well as effectively prime mice to reject subsequent lethal challenges with viable parental tumor cells [8, 21, 22]. Using an injected tumor model, we show that MUC1.Tg mice immunized with DC pulsed with lysates prepared from C57mg.MUC1 cells were completely protected from subsequent challenge with C57mg.MUC1 tumor cells (Fig. 6). These results are promising, as we were able to break tolerance in the MUC1.Tg mice and protect them against tumor challenge. However, the challenge is to achieve these results in our spontaneous model, which is physiologically more appropriate, and to a large extent, mimics the human situation. These studies using DCs pulsed with MMT tumor lysate are presently underway in our laboratory.

Dendritic cell-tumor cell fusion vaccine

Dendritic cells, the most potent antigen presenting cells, fused with MMT tumor cells should present not only MUC1 but also other antigenic proteins expressed by the tumors. This strategy could alleviate the ability of tumor cells to escape from the immune system by down-regulating MUC1 or by the outgrowth of MUC1-negative cells. The experimental strategy is schematically represented in Figure 7 and includes five treatment arms: a) DC/Tumor cell fusion + IL-2; b) DC/Tumor cell fusion; c) IL-2; d) DC alone and e) no treatment.

Primary resected mammary gland tumors from MMT mice were fused with bone marrow-derived DCs to generate a fusion vaccine that not only expresses MUC1 but also expresses a variety of other tumor antigens. We fused a single cell suspension of $1x10^7$ freshly resected mammary MMT tumor cells with $5x10^7$ freshly prepared DCs with 50% PEG in PBS without Ca^{2+} or Mg^{2+} at pH 7.4. Without any further sorting, the fused cells were irradiated (3000Rads) and injected intradermally into 3 week old MMT mice ($1x10^6$ fused cells/mouse). To test fusion efficiency, tumor cells and DC were labeled with orange and green cell tracker dyes (Molecular Probes, Eugene, Oregon), respectively. Fusion efficiency was determined by percentage of cells that were double labeled using two-color FACs analysis. The phenotype of DC included expression of co-stimulatory molecules B7 and MHC class I, while the phenotype of tumor cells included expression of Pankeratin and MUC1.

Tumor Progression in immunized MMT mice. Although we were unable to eradicate the tumors completely, data demonstrate that treatment with DC-fusion vaccine + IL-2 may have an beneficial effect on tumor growth and progression (Fig. 8) with statistical significance at 21 weeks ($n = 10$ mice/group; $p < 0.05$). In fact, treatment of MMT mice with DC alone had a beneficial effect on tumor growth. Tumor burden was determined by palpation beginning at 9 weeks and calculated by the following formula: tumor weight in gms = (length x width^2) x 0.5 [23]. The results are being written up for publication.

Survival Benefit in immunized MMT mice. Immunized MMT mice had enhanced survival. On average, immunized mice survived 3 weeks longer than untreated control MMT mice (Figure 9A). Although statistical significance was only reached in the DC alone treatment group ($p < 0.01$), clearly mice treated with DC/tumor fusion $\pm$ IL-2 had a beneficial effect. At 17 weeks of age, 100% of mice treated with DC alone or DC/tumor fusion $\pm$ IL-2 remained alive, whereas only 90% were alive in the control group. This trend continued and by 21 weeks of age, only 33% of control mice were alive as opposed to 50 -60% in the treated groups. By 25 weeks, all mice were dead in the control and IL-2 treated group while 40% of mice remained alive in the DC alone group (Fig. 9B). Similar to the previous study, we clearly observe a beneficial effect of IL-2 on tumor progression and survival.

Cellular Immune Response in immunized MMT mice: Peripheral blood from 6, 10, 14, and 18 week old immunized MMT mice were evaluated for presence of activated T cells and DCs by FACs analysis. T cells were evaluated for expression of intracellular IL-2 and IFN-γ. DC activation was evaluated by expression of intracellular IL-12. Compared to untreated controls, immunized MMT mice developed significantly higher percentage of activated T cells as measured by expression of intracellular IFN-γ and IL-2. However, by 18 weeks of age when tumor burden is large, the percentage of T cells expressing IL-2 and IFN-γ decreases suggesting that the tumor environment causes effector T cells to become inactive or anergised (Figure 10A, B). Intracellular IL-12 expression is also enhanced post immunization (Figure 10C) and does not decrease as a function of tumor burden. This suggests that the tumor cells render the effector T cells inactive but do not have a harmful effect on DC. This is an important finding, as future immunotherapy strategies will be designed to prevent and/or reverse the deleterious effects of tumor cells on effector T cells. Cytotoxic T cell activity was measured using B16 melanoma cells expressing MUC1 as tumor targets and splenic lymphocytes from immunized MMT mice as effector cells. These assays were carried out at time of sacrifice when the tumor burden in the MMT mice was approximately 10% of its body weight. Immunized MMT mice develop mature MUC1-specific cytotoxic T cells and no CTL activity was observed in untreated controls (Figure 11). Taken together these results suggest that the DC-based immunotherapy not only elicited a T cell response but also reduced tumor burden as well as enhanced survival.

Circulating MUC1 levels in immunized mice. Similar to the observation with the liposomal MUC1 vaccine study, immunization significantly increased serum MUC1 levels and this correlated with the presence of CTL activity against tumor-specific antigen, MUC1 (Figure 12).

Mechanisms of Tumor Evasion in MMT mice. Similar to the observation made in humans, both immunization strategies elicited mature MUC1-specific CTLs, but were not effective in eradicating the spontaneously arising breast tumors in MMT mice. We therefore postulated that the growing MMT tumor cells evaded immune recognition and killing.

MMT tumors express TGF-β. An effective way a tumor cell evades T cell killing is to render the effector T cells non-functional by releasing immunosuppressive factors. One such factor is TGF-β that is capable of hindering T cell signaling and down regulating their function. We tested whether mammary tumors from MMT mice express TGF-β by specific immunohistochemical staining and demonstrated that MMT tumors expressed TGF-β as early as 6 weeks of age and that the expression increases as the tumors progress (Fig. 13). Both immunizations did not cause a decrease in TGF-β expression, suggesting that the tumors may be utilizing this mechanism to

down regulate T cell activity *in vivo* and escape immune intervention. This phenomenon is also evident from the decrease in the numbers of IFN-γ and IL-2 expressing T cells (Fig. 4 and Fig. 10) as tumor burden increases. However, secondary treatment with TGFβ antibody (four injections with 200ugs/mouse at weeks 7, 9, 11, and 13) did not have any added advantage to the standard DC/tumor fusion vaccine with regards to tumor burden (Figure 8), survival (Figure 9), T cell responses (Figures 10 and 11), or MUC1 serum levels (Figure 12). This suggested that other evasion mechanisms may also be utilized by these tumor cells.

MHC class I expression in MMT mice as tumors progress. We evaluated another well-characterized mechanism by which tumor cells evade CTL killing, which is down-regulation of their surface MHC class I molecules. We observed by two color flow cytometry that percent cells positive for MHC class I and pan-cytokeratin are ~ 13% in 6 week old MMT tumor mice which steadily decreases to < 2% in 18 week old tumor mice (Table 3) and that immunization was unable to up-regulate these levels.

Analysis of the DC/tumor cell fusion immunization is still in progress; hence, the lack of complete statistical analysis and characterization of metastases and other parameters.

Fate of adoptively transferred CTL in MMT mice.

We have published previously that MUC1 transgenic mice with spontaneous tumors of the pancreas (MET mice) naturally develop MHC class I-restricted, MUC1-specific CTLs as tumors progress ([8], see appendix). These CD8+ cells were isolated and cloned. The characteristics of these CTLs are described by Mukherjee et al. ([24], see appendix). We showed that the CTLs isolated from one tumor model have anti-tumor effects on other MUC1-expressing tumors *in vivo*. These data confirm that MUC1 is an important tumor rejection antigen and can serve as a target for immunotherapy.

To determine if MUC1-specific CTLs that are effective in killing MUC1-expressing tumor cells *in vitro* are rendered inactive in a tumor environment, we infused the $CD8^+$ MUC1-specific CTL clone [25] into tumor bearing MMT mice. To test if the infused CTLs can home to the tumor site, we labeled the CTL clone with carboxyfluorescein succinimidyl ester (CFSE, an in vivo tracking dye) and adoptively transferred 1 x 10^7 CTLs intravenously into 12 week old MMT mice. We observed that CFSE-labeled CTL homed to the tumor site and lymph node by 2 days post infusion and could be detected up to 21 days, after which the CTLs were not detectable *in vivo* by flow cytometry (Figure 14). Little to no CTLs were detected within the thymus or the spleen. To determine if the infused CTL were active within the tumor environment, we infused 3 week old MMT mice with 1 x 10^7 CTLs intravenously and boosted every 3 weeks with the same. At 12 to 15 weeks of age we removed the mammary tumors, isolated the tumor-infiltrated lymphocytes (TILs) and sorted for the infused $CD8^+$ CTL clone based on their CD8 expression and T cell receptor (TCR)-Vβ5 expression (Figure 15A, middle panel). TILs isolated from age matched MMT mice that were not infused with CTLs were used as controls. As shown in Figure 15A (right panel), very few $V\beta5^+$ cells were observed in control mice. Therefore, only the $CD8^+$ T cells were sorted and used as a negative control. The CTL clone maintained *in vitro* that had the same profile as the sorted $CD8^+V\beta^+$ cells from the TILs were used as positive control (Figure 15A, left panel). The sorted cells were used in two assays to determine 1) if these cells were hypo-responsive to MUC1 and therefore were tolerant to MUC1 antigen, and 2) if these cells

maintained their cytotoxicity after encounter with the tumor cells *in vivo*. In Figure 15B, we show clearly that in contrast to the CTL clone, the sorted $V\beta5^+/CD8^+$ cells had significantly reduced proliferation in response to MUC1 presented on irradiated DC, suggesting that the infused CTLs became tolerized to tumor antigen MUC1 after encounter with the growing mammary tumor cells. Similarly, the sorted $V\beta5^+/CD8^+$ cells were unable to lyse tumor cells expressing MUC1 *in vitro*, while the same cells that had not encountered tumor cells and were maintained in tissue culture remained highly cytolytic (Figure 15C), suggesting that the infused CTL become cytolytically anergic in the tumor environment. These are extremely significant findings as immunotherapy can now be designed to reverse this established tolerance or prevent the induction of tolerance.

MUC1-specific CTLs are effective at delaying growth of spontaneous mammary tumors.

During our studies on the fate of adoptively transferred CTLs in mice, we noted a substantial effect of these CTLs on the tumor growth. We determined that these MUC1-specific CTLs can delay tumor growth in our spontaneously growing mammary tumors. MMT mice were given injections of 1×10^7 MUC1-specific CTLs intravenously starting at three weeks of age and every three weeks thereafter, up to 12 weeks of age. Starting at week 7 mice were also given injections of IL-2 at the time of CTL injection (20,000 Units/mouse in 100 ul i.p.). Mice were palpated once a week for presence of tumors. Palpable tumors were measured by calipers and tumor weight was calculated. Mice were observed carefully for signs of ill-health, including lethargy, abdominal distention, failure to eat or drink, marked weight loss, or hunched posture. In accordance with IACUC regulations, mice were sacrificed before tumors reached 10% of body weight. Fig. 16 shows tumor progression in 3 MMT untreated mice, in which tumors reached from 1.6 to 2.0 grams by 23 weeks. MMT mice receiving CTL adoptive therapy showed little tumor development until 20 weeks of age, at which time tumors in three of the mice were approximately 0.1 gram. About 6 weeks after the last CTL injection at 12 weeks, tumors began to grow, reaching from 0.2 to 0.6 grams by 23 weeks. One CTL-treated mouse progressed much more rapidly, attaining a tumor of 1.2 grams by 23 weeks. These results are in agreement with the development of tolerance (described above) that occurs by three weeks after adoptive transfer. Tumor growth was suppressed as long as mice received CTL infusions every 3 weeks. Once infusions were halted, tumors resumed growth as early as 3 weeks in one mouse and by 6 weeks post infusion in three other mice. Clearly, adoptive transfer of MUC1-specific CTL early during tumorigenesis can enhance survival in MMT mice that develop spontaneous tumors of the mammary gland. Although these studies are preliminary and performed in small numbers of mice, results are promising. An expanded study will be performed in year two of this grant.

Specific Aim 2: To translate the most effective vaccine strategies into phase I clinical trials in patients with high and low tumor burden.

We proposed to write the clinical trial for this aim during year 2 of this grant. We have already started to do so. In preparation for the clinical trials due to begin August 2003, Mayo Clinic Scottsdale has established a Cellular Immunology Laboratory to monitor patients' responses to immunotherapy. Dr. Pinku Mukherjee has been appointed Director of the Cellular Immunology Laboratory and Teresa Tinder is her Senior Research Technologist. The Cellular Immunology

Laboratory is part of the Mayo Comprehensive Cancer Center. The other participating site is Mayo Clinic Rochester, which is fully equipped for all types of therapies.

KEY RESEARCH ACCOMPLISHMENTS

- Four immunization strategies have been tested in our mouse model of spontaneous mammary gland cancer (MMT mice)
    - Liposomal MUC1 peptide
    - Dendritic cells pulsed with tumor lysate
    - Dendritic cells fused with tumor cells
    - Adoptive transfer of MUC1-specific cytotoxic T lymphocytes

- All vaccines elicited a strong immunological response, consisting of delay of tumor growth and/or development of mature MUC1-specific CTLs
- Liposomal MUC1 peptide vaccine:
    - Vaccine elicited mature MUC1-specific CTLs in the L-MUC1-TR + IL-2 group ($p<0.001$) and in the L-MUC1-TR group ($p<0.05$) compared with untreated controls.

    - MMT tumor burden at 18 weeks was significantly lower in all treatment groups compared with untreated controls.

    - L-IL2 significantly reduced lung metastases ($p<0.005$) compared to other treatment groups and untreated controls.

    - Immunization resulted in significant increases (from $p<0.001$ to $p<0.01$) in T cells expressing intracellular IFNγ at weeks 6 and 12 in treatment groups compared with untreated controls.

    - Immunization resulted in significant increases ($p<0.005$ to $p<0.01$) of T cells recognizing H-2 D^b MUC1 tetramer in treatment groups compared with untreated controls.

    - Circulating levels of MUC1 increased significantly with MUC1-specific immunization and with IL-2 treatment.

    - IL-2 treatment significantly reduced the number of metastases per lung ($p<0.005$) and the incidence of metastasis.

- Dendritic cell/tumor cell fusion vaccine:

    - Vaccine elicited mature MUC1-specific CTLs in all treatment groups compared with untreated controls.

- DC/tumor fusion vaccine reduced tumor burden in MMT mice at 21 weeks ($p<0.05$).
- Immunized mice survived about 3 weeks longer than untreated controls.
- Immunization resulted in increases in T cells expressing intracellular IFNγ, IL-2 and IL-12.
- Circulating levels of MUC1 increased with immunization and with IL-2 treatment.

- Vaccination with dendritic cells pulsed with MMT tumor lysate prevented outgrowth of injected mammary tumors
- MMT tumors express immunosuppressive factors, such as TGFbeta, which may inhibit the effectiveness of the CTLs within the tumor environment.
- MUC1-specific CTLS adoptively transferred into MMT mice (every 3 weeks) homed to the tumor and developed tolerance to MUC1 by 12 to 15 weeks.
- Adoptive transfer of MUC1-specific CTLs into MMT mice delayed tumor growth. Tumors progressed only after infusion of CTLs was stopped.

REPORTABLE OUTCOMES

Mukherjee, P., Madsen, C.S., Ginardi, A.R., Tinder, T.L., Jacobs, F., Parker, J., Agrawal, B., Longenecker, B.M. and Gendler, S.J. (2002) MUC1-specific immunotherapy in a mouse model of spontaneous breast cancer. Journal of Immunotherapy, manuscript under revision.

Pinku Mukherjee, Amelia R. Ginardi, Teresa L. Tinder, Cathy S. Madsen, and Sandra J. Gendler. (2002) Adoptively transferred MUC1-specific CTL home to lymph node and tumors in a spontaneous mouse model of mammary gland cancer. Proceedings of the American Association for Cancer Research 43:976 (#4835).

Gargi D. Basu, Pinku Mukherjee, Judy Bradley, Cathy S. Madsen, Latha Pathangey, Michael Croft, and Sandra J. Gendler. Immunization with OX40 antibody in a spontaneous model of breast cancer. Abstract submitted, Keystone Symposia: Mechanisms of Immunologic Tolerance and its Breakdown

Dr. Pinku Mukherjee, co-investigator on this DOD award, was promoted to Assistant Professor and Director of the Cellular Immunology Laboratory, which is part of the Mayo Comprehensive Cancer Center. Her promotion was effective February 2002.

CONCLUSIONS

All of the vaccine strategies elicited an immune response. Animals developed mature CTLs which were lytic *in vitro* against MUC1-expressing tumor cells. Lytic activity was detected without further *in vitro* stimulation. However, in most cases the spontaneous tumors progressed. The CTLs, while active outside of the environment of the tumor, appeared to become tolerized and unreactive to MUC1 in the vicinity of the tumor. Tumors were found to exhibit several known escape mechanisms, such as the production of immunosuppressive factors and down-modulation of MHC class I molecules on the cells. CTLs adoptively transferred into the tumor-bearing mice were used to follow the development of tolerance, which occurred within about three weeks following injection. These results are similar to what has been detected in humans, where the tumor antigen-specific immune responses are elicited which do not translate into complete clinical response.

Partial clinical responses including lower tumor burden and enhanced survival have been observed with the DC/tumor fusion vaccine supplemented with IL-2. Most importantly, these studies have led us to understand some of the tumor evasion mechanisms utilized by tumor cells to avoid immune recognition and killing. Critical among these are CTL anergy and T cell tolerance induced within the mammary tumor environment. This suggested that co-stimulatory molecules expressed on immune cells that are necessary for complete activation of helper and cytotoxic T cells are not appropriately engaged. We have utilized these important findings to design improved therapies for breast cancer that are described briefly here.

We also demonstrated a complete clinical response in mice that were immunized with DCs pulsed with whole tumor lysate. This method of loading DCs is technically less demanding than the DC/tumor cell fusion and yields exciting results. Using an injected mammary tumor model, we showed that the MUC1.Tg mice immunized with DCs pulsed with lysates prepared from C57mg.MUC1 mammary tumor cells were completely protected from subsequent challenge with tumor cells. These results are extremely promising, as we were able to break tolerance in the MUC1.Tg mice and protect them against tumor challenge.

Most striking was the response to adoptive transfer of MUC1-specific CTLs that we observed. Growth of the MMT spontaneous tumors was inhibited as long as the CTLs were infused every three weeks. Once infusions were stopped, tumors grew out after 3 to 6 weeks. This result suggests that immunotherapy can be effective even against an oncogenic tumor as aggressive as is found in the MMT mice. The MMTV-driven polyoma middle T antigen affects almost every epithelial cell in the mammary gland and the entire mammary tree is burdened with tumors, which may be too aggressive for the immune effector cells to act effectively. Our results with the CTL adoptive transfer suggests that immune effector cells can be extremely effective, as long as there is continual infusion of non-tolerized cells. This result coupled with the observation that the CTLs do become tolerized within the tumor environment suggests that immunotherapy may become very effective once we learn how to prevent or break tolerance. This study, which was performed as a pilot, is being repeated with larger numbers of mice and appropriate study arms.

Future Studies

In recent years, there has been growing evidence that cross-linking of co-stimulatory molecules such as OX40, CD-40, or 4-1BB, all expressed on activated immune regulatory cells, [26-34] can reverse established T cell tolerance and/or prevent induction of T cell tolerance and anergy. 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, CD-40 and 4-1BB, which make them targets for therapeutic intervention in cancer.

We have designed strategies that will include secondary immunization with these agents following conventional anti-tumor vaccination. Primary immunizations will include either 1) DC pulsed with whole tumor lysate or 2) adoptive transfer of MUC1-specific CTL clones. The idea is that this treatment will be able to prevent T cell tolerance and anergy and will generate long lasting anti-tumor immunity. These studies are just about to be started in the laboratory.

REFERENCES

1. Wang, J., Saffold, S., Cao, X., Krauss, J., and Chen, W., Eliciting T cell immunity against poorly immunogenic tumors by immunization with dendritic cell-tumor fusion vaccines. *J Immunol.* **161**(10): 5516-24, 1998.
2. Gong, J., Nikrui, N., Chen, D., Koido, S., Wu, Z., Tanaka, Y., Cannistra, S., Avigan, D., and Kufe, D., Fusions of human ovarian carcinoma cells with autologous or allogeneic dendritic cells induce antitumor immunity. *J Immunol.* **165**(3): 1705-11, 2000.
3. Tanaka, Y., Koido, S., Chen, D., Gendler, S.J., Kufe, D., and Gong, J., Vaccination with allogeneic dendritic cells fused to carcinoma cells induces antitumor immunity in MUC1 transgenic mice. *Clin Immunol.* **101**(2): 192-200, 2001.
4. Gong, J., Koido, S., Chen, D., Tanaka, Y., Huang, L., Avigan, D., Anderson, K., Ohno, T., and Kufe, D., Immunization against murine multiple myeloma with fusions of dendritic and plasmacytoma cells is potentiated by interleukin 12. *Blood.* **99**(7): 2512-7, 2002.
5. Rowse, G.J., Tempero, R.M., VanLith, M.L., Hollingsworth, M.A., and Gendler, S.J., Tolerance and immunity to MUC1 in a human MUC1 transgenic murine model. *Cancer Res.* **58**: 315-321, 1998.
6. Guy, C.T., Cardiff, R.D., and Muller, W.J., Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. *Mol. Cell. Biol.* **12**(3): 954-961, 1992.
7. Cardiff, R.D. and Muller, W.J., Transgenic mouse models of mammary tumorigenesis. *Cancer Surveys.* **16**: 97-113, 1993.
8. Mukherjee, P., Madsen, C.S., Ginardi, A.R., Tinder, T.L., Jacobs, F., Parker, J., Agrawal, B., Longenecker, B.M., and Gendler, S.J., MUC1-specific immunotherapy in a mouse model of spontaneous breast cancer. *J. Immunotherapy*: manuscript submitted, 2002.
9. Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., and Steinman, R.M., Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med.* **176**(6): 1693-702, 1992.

10. Guan, H.H., Budzynski, W., Koganty, R.R., Krantz, M.J., Reddish, M.A., Rogers, J.A., Longenecker, B.M., and Samuel, J., Liposomal formulations of synthetic MUC1 peptides: effects of encapsulation versus surface display of peptides on immune responses. *Bioconjug Chem.* **9**(4): 451-8, 1998.
11. Boni, L.T., Batenjany, M.M., Neville, M.E., Guo, Y., Xu, L., Wu, F., Mason, J.T., Robb, R.J., and Popescu, M.C., Interleukin-2-induced small unilamellar vesicle coalescence. *Biochim Biophys Acta.* **1514**(1): 127-38, 2001.
12. Ridge, J.P., Fuchs, E.J., and Matzinger, P., Neonatal tolerance revisited: turning on newborn T cells with dendritic cells. *Science.* **271**(5256): 1723-6, 1996.
13. Barnd, D.L., Lan, M.S., Metzgar, R.S., and Finn, O.J., Specific, major histocompatibility complex-unrestricted recognition of tumor-associated mucins by human cytotoxic T cells. *Proc Natl Acad Sci U S A.* **86**(18): 7159-63, 1989.
14. Domenech, N., Henderson, R.A., and Finn, O.J., Identification of an HLA-A11-restricted epitope from the tandem repeat domain of the epithelial tumor antigen mucin. *J Immunol.* **155**(10): 4766-74, 1995.
15. Apostolopoulos, V., Xing, P.X., and McKenzie, I.F., Murine immune response to cells transfected with human MUC1: immunization with cellular and synthetic antigens. *Cancer Res.* **54**(19): 5186-93, 1994.
16. Apostolopoulos, V., Karanikas, V., Haurum, J.S., and McKenzie, I.F., Induction of HLA-A2-restricted CTLs to the mucin 1 human breast cancer antigen. *J Immunol.* **159**(11): 5211-8, 1997.
17. Acres, B., Apostolopoulos, V., Balloul, J.M., Wreschner, D., Xing, P.X., Ali-Hadji, D., Bizouarne, N., Kieny, M.P., and McKenzie, I.F., MUC1-specific immune responses in human MUC1 transgenic mice immunized with various human MUC1 vaccines. *Cancer Immunol Immunother.* **48**(10): 588-94, 2000.
18. Pietersz, G.A., Li, W., Osinski, C., Apostolopoulos, V., and McKenzie, I.F., Definition of MHC-restricted CTL epitopes from non-variable number of tandem repeat sequence of MUC1. *Vaccine.* **18**(19): 2059-2071, 2000.
19. Rughetti, A., Turchi, V., Ghetti, C.A., Scambia, G., Panici, P.B., Roncucci, G., Mancuso, S., Frati, L., and Nuti, M., Human B-cell immune response to the polymorphic epithelial mucin. *Cancer Res.* **53**(11): 2457-9, 1993.
20. Kotera, Y., Fontenot, J.D., Pecher, G., Metzgar, R.S., and Finn, O.J., Humoral immunity against a tandem repeat epitope of human mucin MUC-1 in sera from breast, pancreatic, and colon cancer patients. *Cancer Res.* **54**(11): 2856-60, 1994.
21. Fields, R.C., Shimizu, K., and Mule, J.J., Murine dendritic cells pulsed with whole tumor lysates mediate potent antitumor immune responses in vitro and in vivo. *Proc Natl Acad Sci U S A.* **95**(16): 9482-7, 1998.
22. Schnurr, M., Galambos, P., Scholz, C., Then, F., Dauer, M., Endres, S., and Eigler, A., Tumor cell lysate-pulsed human dendritic cells induce a T-cell response against pancreatic carcinoma cells: an in vitro model for the assessment of tumor vaccines. *Cancer Res.* **61**(17): 6445-50, 2001.
23. Simpson-Herrerns, L. and Lloyd, H.H., Kinetic parameters and growth curves for experimental tumor systems. *Cancer Chemother Rep.* **54**: 143-174, 1970.
24. Mukherjee, P., Ginardi, A.R., Tinder, T.L., Sterner, C.J., and Gendler, S.J., MUC1-specific CTLs eradicate tumors when adoptively transferred in vivo. *Clin Can Res.* **7**: 848s-855s, 2001.

25. Mukherjee, P., Ginardi, A.R., Madsen, C.S., Sterner, C.J., Adriance, M.C., Tevethia, M.J., and Gendler, S.J., Mice with spontaneous pancreatic cancer naturally develop MUC1-specific CTLs that eradicate tumors when adoptively transferred. *J Immunol.* **165**(6): 3451-3460, 2000.
26. Gramaglia, I., Weinberg, A.D., Lemon, M., and Croft, M., Ox-40 ligand: a potent costimulatory molecule for sustaining primary CD4 T cell responses. *J Immunol.* **161**(12): 6510-7, 1998.
27. Gramaglia, I., Jember, A., Pippig, S.D., Weinberg, A.D., Killeen, N., and Croft, M., The OX40 costimulatory receptor determines the development of CD4 memory by regulating primary clonal expansion. *J Immunol.* **165**(6): 3043-50, 2000.
28. Bansal-Pakala, P., Jember, A.G., and Croft, M., Signaling through OX40 (CD134) breaks peripheral T-cell tolerance. *Nat Med.* **7**(8): 907-12, 2001.
29. Cella, M., Scheidegger, D., Palmer-Lehmann, K., Lane, P., Lanzavecchia, A., and Alber, G., Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J Exp Med.* **184**(2): 747-52, 1996.
30. Mackey, M.F., Gunn, J.R., Maliszewsky, C., Kikutani, H., Noelle, R.J., and Barth, R.J., Jr., Dendritic cells require maturation via CD40 to generate protective antitumor immunity. *J Immunol.* **161**(5): 2094-8, 1998.
31. French, R.R., Chan, H.T., Tutt, A.L., and Glennie, M.J., CD40 antibody evokes a cytotoxic T-cell response that eradicates lymphoma and bypasses T-cell help. *Nat Med.* **5**(5): 548-53, 1999.
32. Diehl, L., den Boer, A.T., Schoenberger, S.P., van der Voort, E.I., Schumacher, T.N., Melief, C.J., Offringa, R., and Toes, R.E., CD40 activation in vivo overcomes peptide-induced peripheral cytotoxic T-lymphocyte tolerance and augments anti-tumor vaccine efficacy. *Nat Med.* **5**(7): 774-9, 1999.
33. Wilcox, R.A., et al., Cutting edge: Expression of functional CD137 receptor by dendritic cells. *J Immunol.* **168**(9): 4262-7, 2002.
34. Wilcox, R.A., Flies, D.B., Zhu, G., Johnson, A.J., Tamada, K., Chapoval, A.I., Strome, S.E., Pease, L.R., and Chen, L., Provision of antigen and CD137 signaling breaks immunological ignorance, promoting regression of poorly immunogenic tumors. *J Clin Invest.* **109**(5): 651-9, 2002.

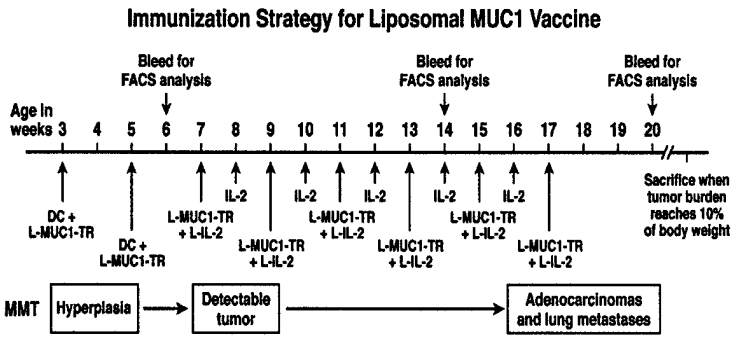

Figure 1. Schematic representation of immunotherapy strategy in MMT mice.

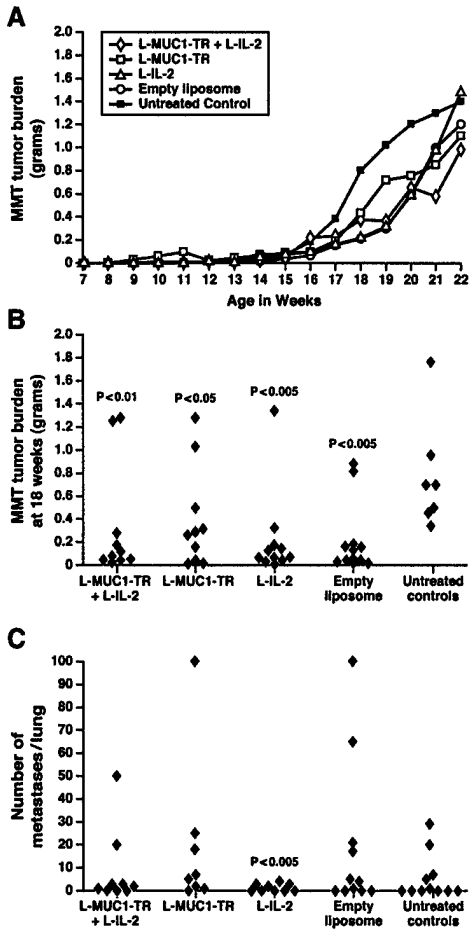

Figure 2 A) MUC1-specific immunization did not alter tumor burden in MMT mice. Tumor growth of immunized MMT mice versus untreated MMT mice. Tumor burden was determined by palpation beginning at 6-8 weeks and calculated by the following formula: gm = (Length x Width^2) x 0.5. No significant difference was observed in mice immunized with MUC1 TR + L-IL-2 as compared to untreated control mice (n = 6-9 mice/group). **B) Tumor burden was lower in immunized MMT mice at 18 weeks of age.** In treatment groups, tumor burden at 18 weeks was significantly lower as compared to untreated control mice. Individual mouse data are shown. *P* values are derived from the pair-wise contrasts from a one-way ANOVA model. **C) L-IL-2 treatment reduces lung metastasis in MMT mice.** The presence of pulmonary metastases was determined by counting gross morphological disease using low power microscopy. Number of metastatic lesions counted in the lungs was significantly lower in mice treated with L-IL-2 as compared to other treatment groups and untreated control mice. Individual mouse data are shown (n = 8 to 11 mice/group). *P* values were from the Pearson chi-square statistic and from Kruskal-Wallis test.

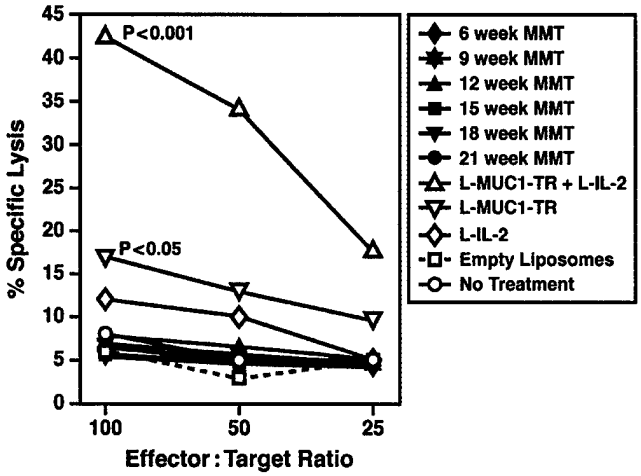

Figure 3. Immunized MMT mice develop MUC1-specific CTLs. CTL activity in 6, 9, 12, 15, 18 and 21 week old untreated MMT mice was determined by a standardized 8hr ^{51}Cr-release assay using B16 melanoma cells transfected with human MUC1 as target cells. Splenic T cells from MMT mice that were stimulated in vitro for 6 days with MUC1 TR peptide (10ngs / ml) and IL-2 (100units / ml) were used as effector cells. In immunized mice, CTL activity was determined at time of sacrifice when tumors reached 10% of body weight. The effector cells were not stimulated *in vitro* with MUC1-TR or cytokines. In untreated mice, no CTL activity was detected at any age. MMT mice in all treatment groups showed increase in MUC1-specific CTL with most lysis detected in mice immunized with L-MUC1-TR + L-IL-2. (*p < 0.001). Specific lysis was calculated according to the following formula: (experimental CPM-spontaneous CPM/maximum CPM-spontaneous CPM) x 100. 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.*

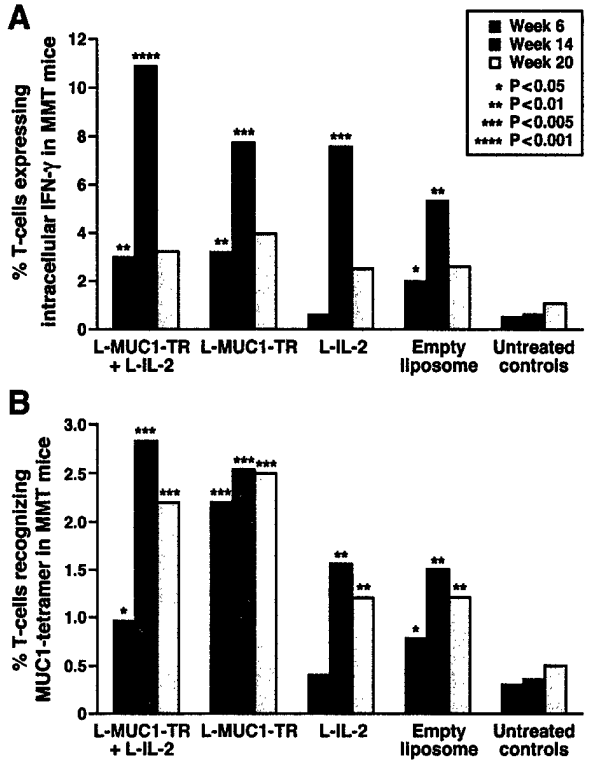

Figure 4. MUC1-specific immunization elicits A) T cells expressing IFN-γ and B) T cells that recognize H-2D^b/MUC1 tetramer. At 6, 14 and 20 weeks post immunization, PBLs were analyzed for presence of T cells expressing IFN-γ and T cells recognizing H-2D^b/MUC1 tetramer. **A)** Six weeks post immunization, mice in all treatment groups showed an increased percent of T cells expressing IFN-γ (** p < 0.01 for L-MUC1-TR groups). No difference in L-IL-2 treated group was observed at 6 weeks, as L-IL-2 treatment only begins at week 7. By 14 weeks post immunization, these T cells further increased. By 20 weeks post immunization, these cells decreased and were at levels similar to that observed at 6 weeks post immunization. **B)** Compared to untreated controls, mice in all treatment groups showed significant increase in percent T cells recognizing H-2D^b/MUC1 tetramer as early as 6 weeks post immunization. By 14 - 20 weeks post immunization, treated mice showed higher percent of circulating T cells recognizing H-2D^b/MUC1 tetramer. 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.*

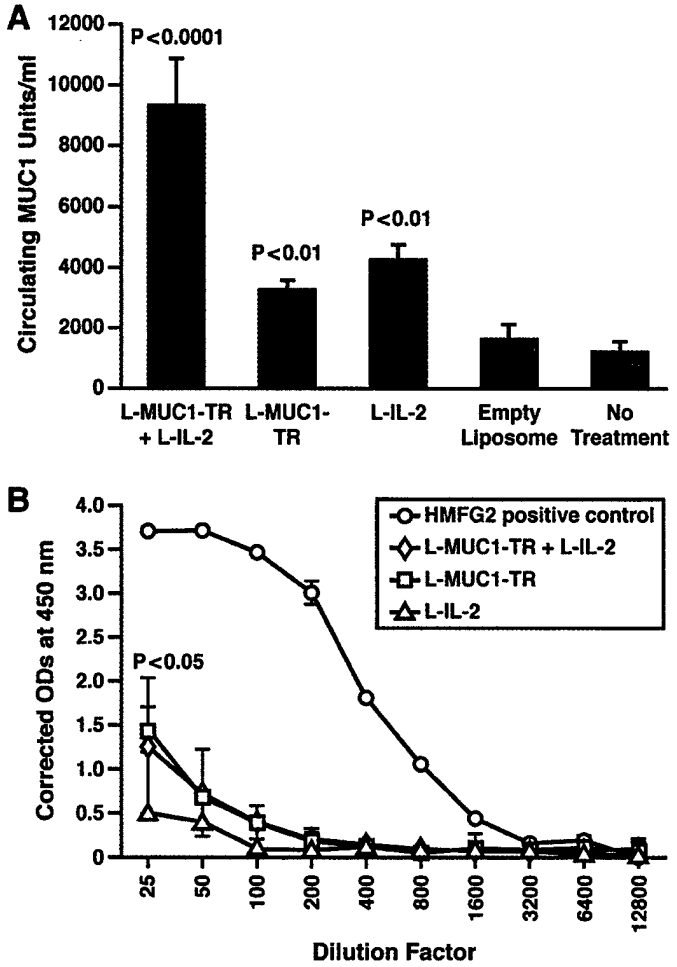

Figure 5. Circulating MUC1 and anti-MUC1 levels in serum of immunized MMT mice. A) Circulating MUC1 levels increase significantly with MUC1-specific immunization as well as with IL-2 treatment. B) Low levels of MUC1 antibodies were detected in MMT mice immunized with MUC1 TR containing vaccine formulation. IL-2 treatment did not elicit MUC1-specific antibodies. Average of n = 6 mice / group is shown. *P* values are from the pair-wise contrasts from a one-way ANOVA model.

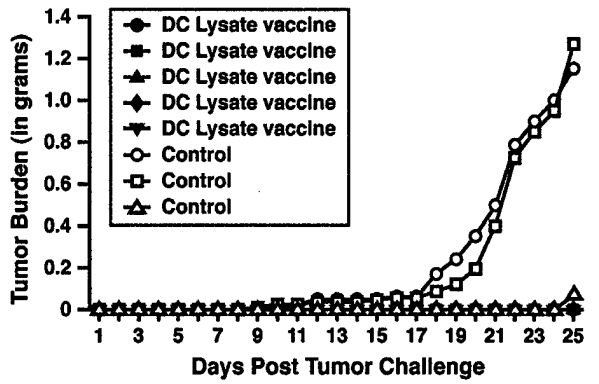

Figure 6. Tumor progression in MUC1.Tg mice immunized with tumor lysate-pulsed DC. MUC1.Tg mice received two intradermal injections, two weeks apart, of C57mg.MUC1 tumor lysate-pulsed DC at 1 X 10^7 DC per mouse. One week after the last injection, mice were challenged with 2 X 10^6 C57mg.MUC1 tumor cells (subcutaneously). All 5 immunized mice were protected from tumor challenge, while all 3 control mice developed tumors. One control mouse had delayed tumor growth.

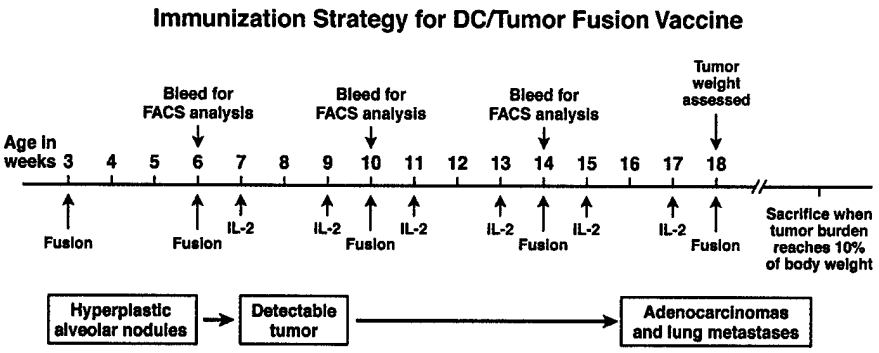

Figure 7. Schematic representation of the DC/Tumor fusion protocol.

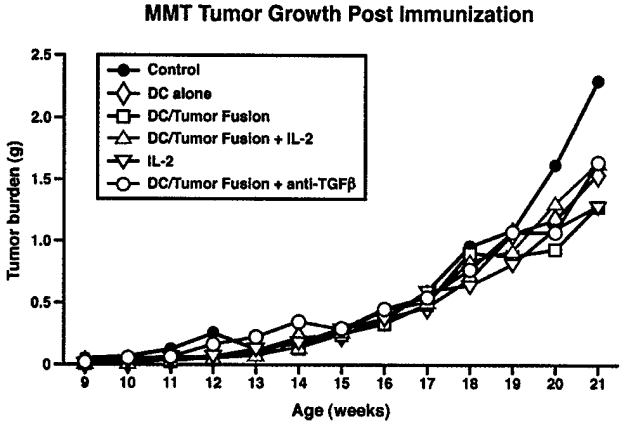

Figure 8. DC/Tumor fusion immunization reduced tumor burden in MMT mice. Tumor growth of immunized MMT mice versus untreated MMT mice. Tumor burden was determined by palpation beginning at 8 weeks and calculated by the following formula: gms = (Length x Width^2) x 0.5. N = 10mice /group.

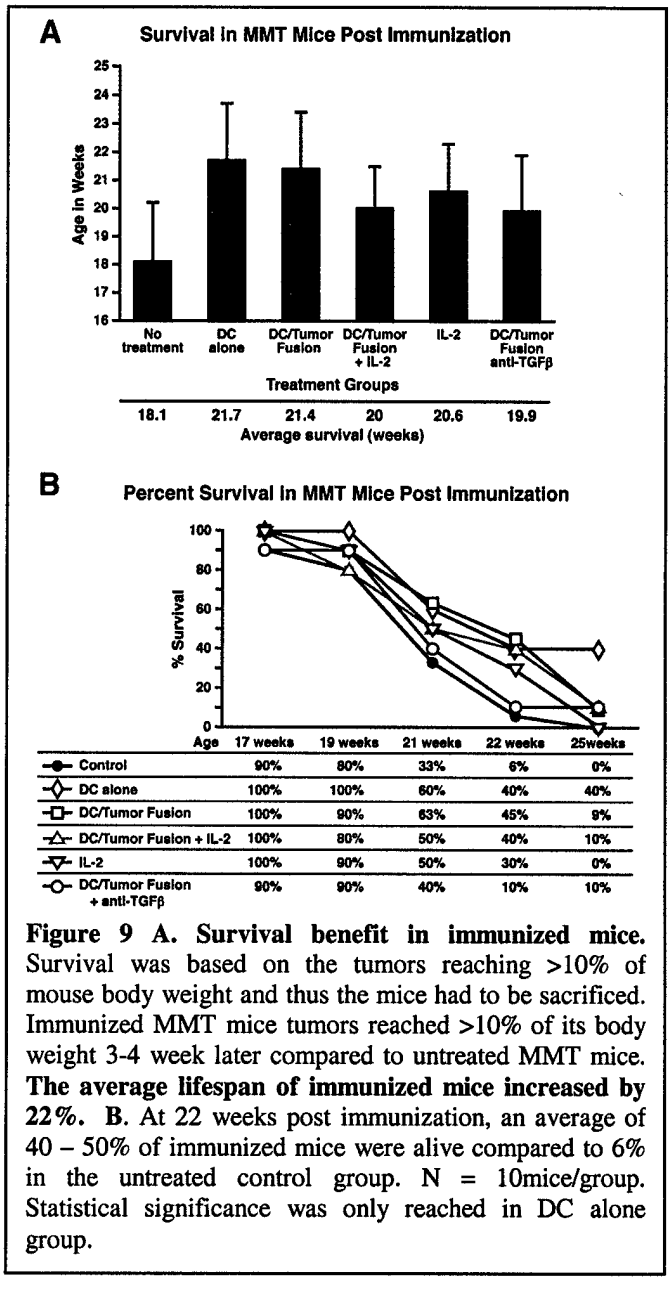

Figure 9 A. Survival benefit in immunized mice. Survival was based on the tumors reaching >10% of mouse body weight and thus the mice had to be sacrificed. Immunized MMT mice tumors reached >10% of its body weight 3-4 week later compared to untreated MMT mice. **The average lifespan of immunized mice increased by 22%. B.** At 22 weeks post immunization, an average of 40 – 50% of immunized mice were alive compared to 6% in the untreated control group. N = 10mice/group. Statistical significance was only reached in DC alone group.

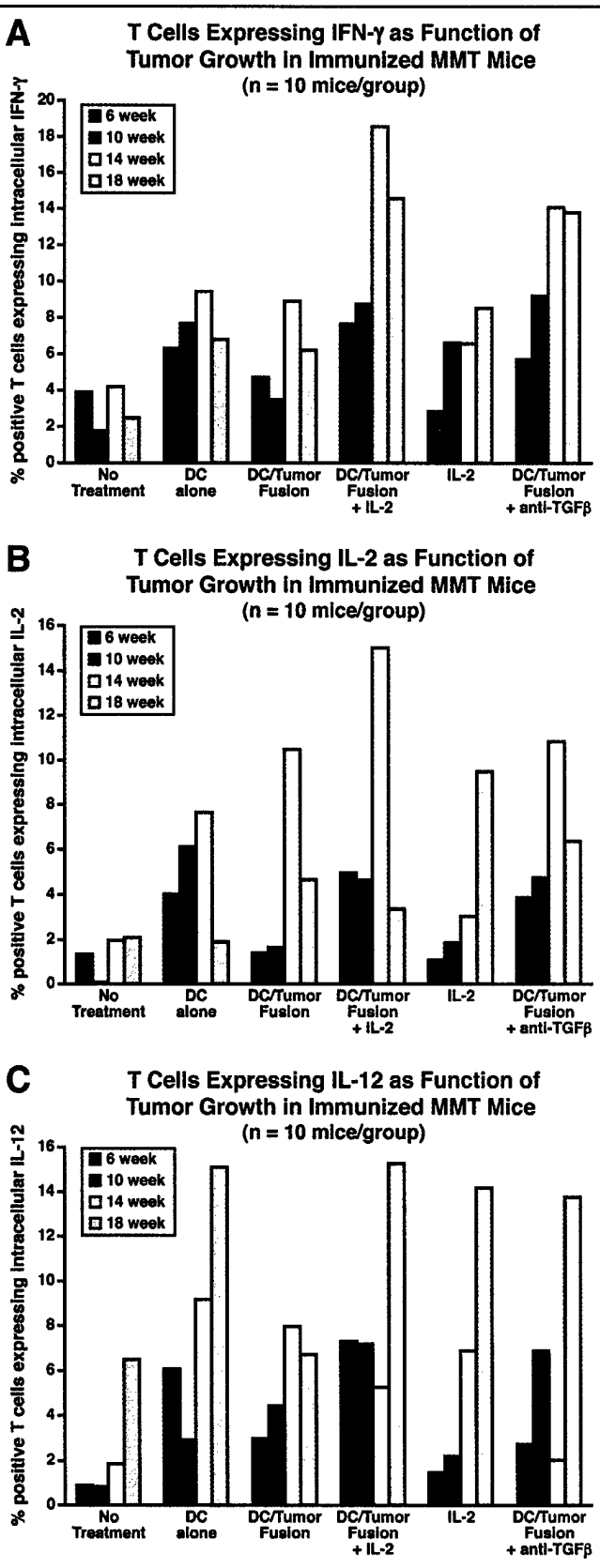

Figure 10. Immunization elicits a cellular immune response as measured by increases in A) IFN-g expressing and B) IL-2 expressing T cells as well as C) IL-12 expressing DC. Intracellular cytokine expression was analyzed by two color flow cytometry. Percent positive CD3+ T cells expressing IFN-g or IL-2 and CD11c+ DC expressing IL-12 is presented. Average of 10 mice/group.

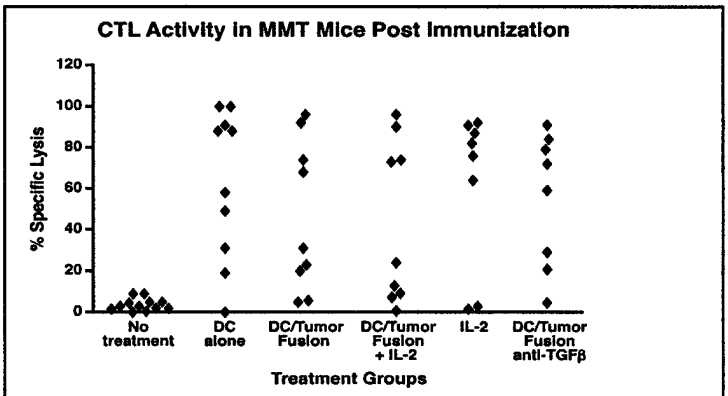

Figure 11. Immunized MMT mice develop MUC1-specific CTLs. CTL activity in immunized MMT mice was determined by a standardized 8hr ^{51}Cr-release assay using B16 melanoma cells transfected with human MUC1 as target cells. Splenic T cells from MMT mice were used as effector cells. CTL activity was determined at time of sacrifice when tumors reached 10% of body weight. The effector cells were not stimulated *in vitro* with MUC1-TR or cytokines. Specific lysis was calculated according to the following formula: (experimental CPM-spontaneous CPM/maximum CPM-spontaneous CPM) x 100. Average of n = 10 mice / group is shown.

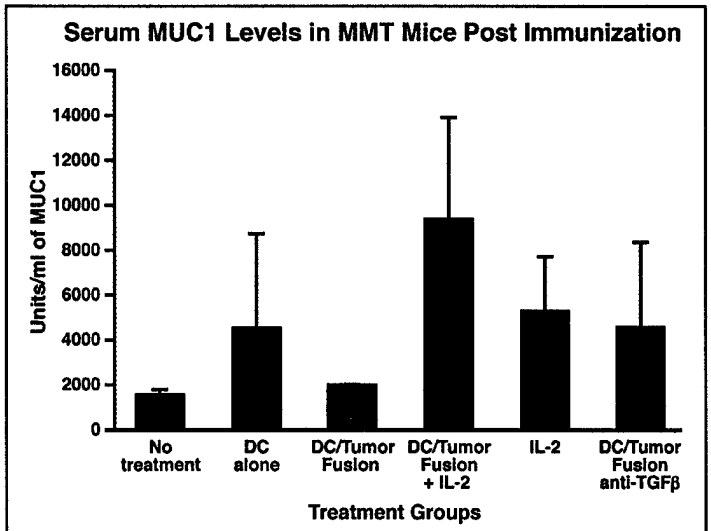

Figure 12. Circulating MUC1 levels in serum of immunized MMT mice. Circulating MUC1 was determined by MUC1-specific ELISA. MUC1 levels increase significantly with immunization as well as with IL-2 treatment. Average of n = 10 mice / group is shown.

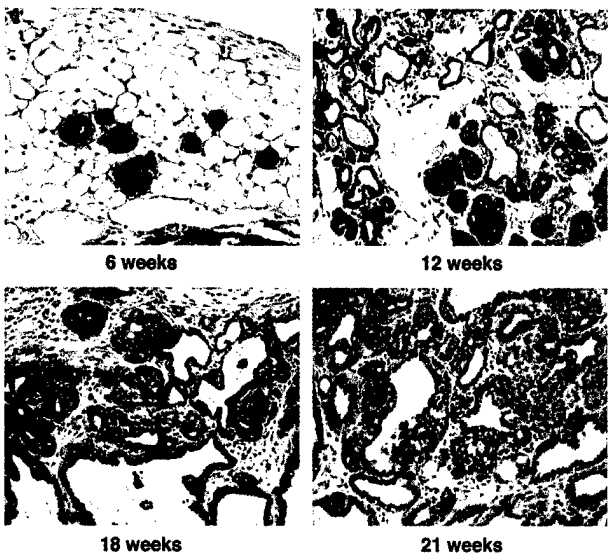

Figure 13. Tumors from MMT mice express TGF-β as early as 6 weeks of age, which increases with time. Methacarn fixed and paraffin-embedded sections of mammary gland tumors from 6, 12, 18 and 21 week old MMT mice were stained with antibody to TGF-β2. MMT tumor at A) 6 weeks; B) 12 weeks; C) 18 weeks; and D) 21 weeks of age. Images were captured at 200X magnification.

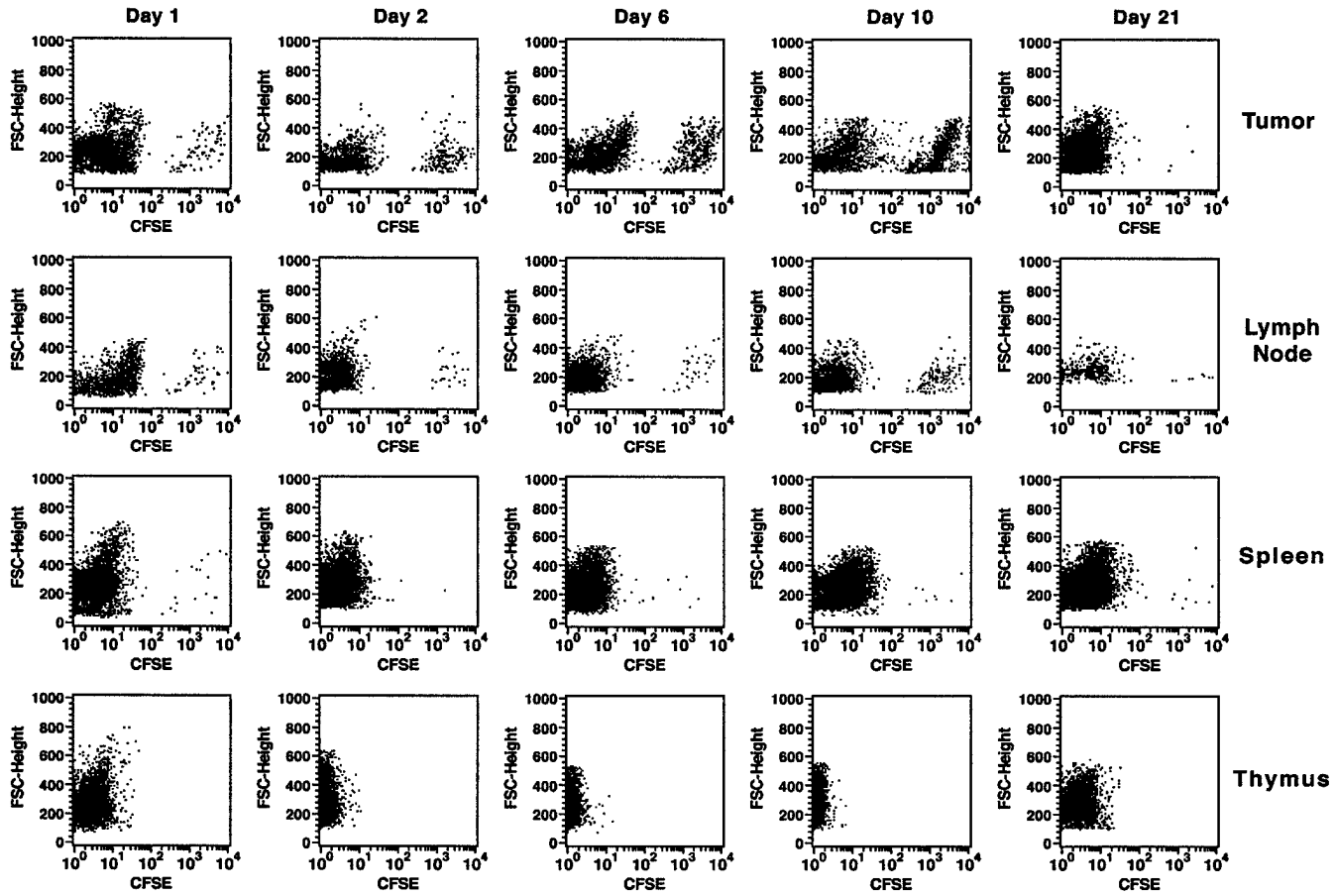

Figure 14. Adoptively transferred CFSE labeled MUC1-specific CTL home and multiply in the lymph node and tumor of tumor-bearing MMT mice. CFSE labeled CTL were infused into 12 week old MMT mice and at various times post CTL infusion, TILs, lymph node cells, spleen cells and thymic cells were isolated and CFSE labeled cells were determined by flow cytometry. N = 5 mice and a representative dot plot is shown. By 21 days post infusion, CFSE labeled cells were undetectable.

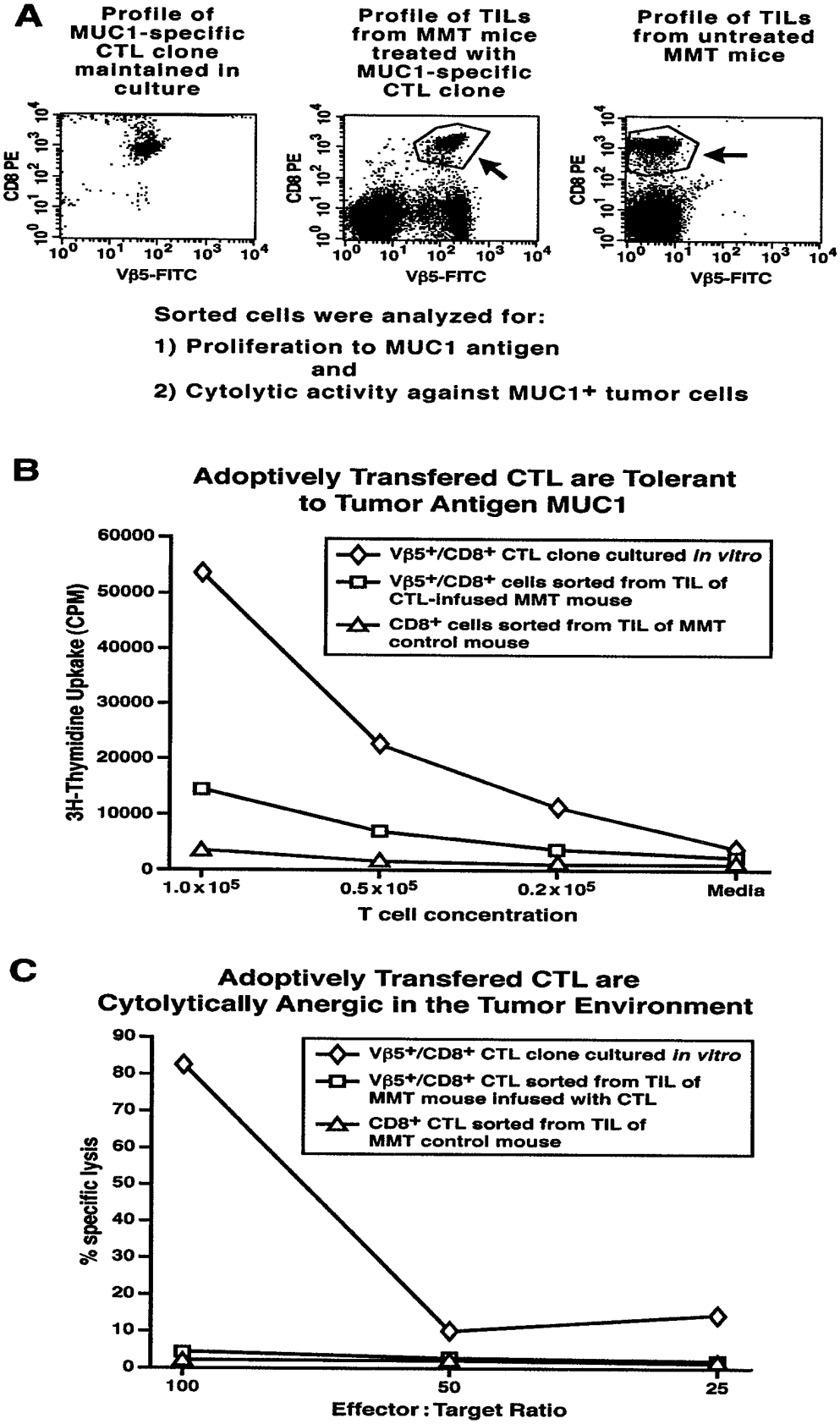

Figure 15. Adoptively transferred CTL become tolerant to MUC1 and are cytolytically inactive after encounter with MMT tumor cells. TILs were isolated from tumors of MMT mice that received adoptively transferred MUC1-specific CTL clone (Vb5+/CD8+ cells). The $V\beta5^+/CD8^+$ cells were sorted (A) and analyzed for either proliferation to MUC1 (B) or cytotoxic activity against MUC1-expressing tumor cells (C). As positive control CTL clone maintained in vitro was used and as negative control $CD8^+V\beta^-$ T cells sorted from TILs isolated from tumor of age matched MMT mice that were not injected with the CTL clone was used. N = 3 CTL-infused MMT mice and 2 control MMT mice. A representative of one mouse data is shown.

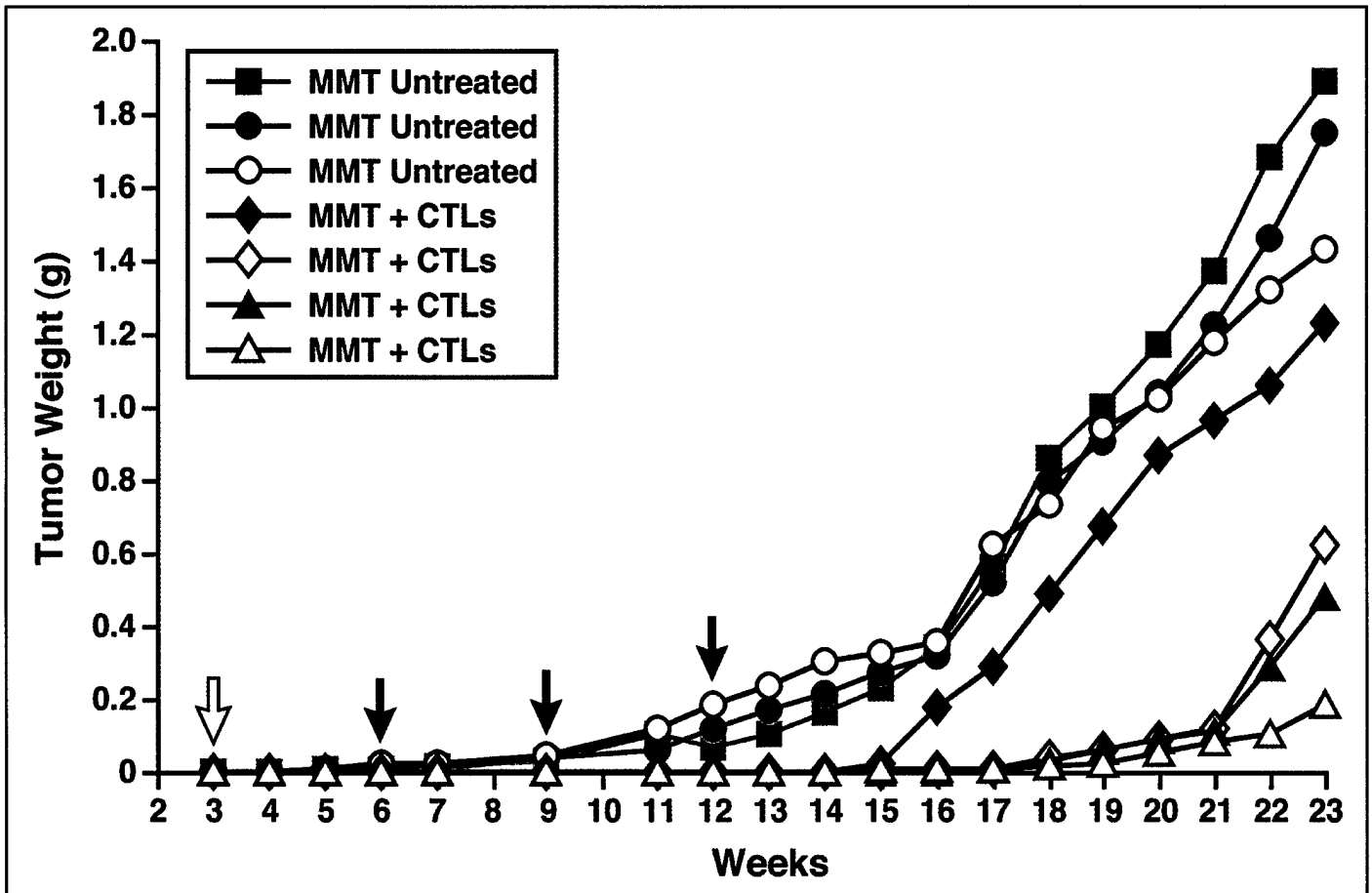

Figure 16. Adoptively transferred MUC1-specific CTL clone inhibits tumor progression in MMT mice. MUC1-specific CTLS (1 x 10^7 cells i.v.) were infused into MMT mice starting at three weeks of age (open arrow). Infusions were given every three weeks and from week 6 onward, were accompanied by IL-2 (20,000 U/mouse i.p.) (filled arrows). Four infusions were performed. Tumor growth was inhibited until 3 to 6 weeks following the cessation of CTL/IL-2 injections, at which time tumors began to progress.

Table 1

Surface and intracellular markers

Markers	6 week MMT	14 week MMT	21 week MMT
IL-2R	1.58	3.28	0.28
CD69	0.75	0.30	0.17
IL-2	1.03	1.29	0.20
γ-IFN	0.50	0.73	0.38
IL-4	0.56	7.67	2.31
IL-5	0.16	2.61	2.28
IL-10	0.24	0.43	0.42
IL-12	0.26	6.43	2.69
B7	4.50	7.70	1.30
CD11c	11.20	12.30	5.50
MUC1-Tetramer	0.31	1.32	0.19

Table 1. Immunophenotype of lymphocytes as tumors progress in MMT mice. We have determined expression of early and late activation markers (CD69 and CD25) on T cells, as well as levels of intracellular cytokines produced by T cells (γ-IFN, IL-2, IL-4, IL-5, IL-10) and dendritic cells (IL-12).

Table 2

Percentage of MMT Mice with Lung Metastasis

Treatment	Number	%
L-MUC1 TR + L-IL-2	6/9	66%
L-MUC1 TR	7/8	87%
L-IL-2	4/9	44%
No treatment	8/12	64%

Table 2. Incidence of lung metastasis in MMT mice. Pulmonary metastases was determined by counting gross morphological disease using low power microscopy. We determined how many mice developed lung metastasis.

Table 3

MHC Class I expression on tumor cells

Tumor age (wk)	MHC I expression
6	12.5 ± 2.0
12	4.1 ± 0.89
18	1.5 ± 0.5

n = 5 MMT mice

Table 3. Surface expression of MHC class I molecule on tumor cells decreases as tumors progress in MMT and MET mice. FACs analysis demonstrating percent cells positive for pan-cytokeratin and MHC class I molecule (H-$2D^b/K^b$).

MUC1-specific Immunotherapy in a Mouse Model of Spontaneous Breast Cancer

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[3] **Abbreviation:** MUC1, human mucin 1; MUC1.Tg, MUC1 transgenic; TR, tandem repeat; DC, dendritic cell; MMT, MUC1-expressing mammary tumor mouse model; CT, cytoplasmic tail; TAA, tumor associated antigen.

ABSTRACT

MUC1 is an epithelial mucin glycoprotein that is over expressed in 90% of all adenocarcinomas including breast, lung, pancreas, prostate, stomach, colon and ovary. MUC1 is a target for immune intervention because in patients with solid adenocarcinomas, low level cellular and humoral immune responses to MUC1 have been observed which are not sufficiently strong to eradicate the growing tumor. The hypothesis for this study is that enhancing MUC1-specific immunity will result in anti-tumor immunity. To test this hypothesis, the authors have developed a clinically relevant breast cancer model that demonstrates peripheral and central tolerance to MUC1 and develops spontaneous tumors of the mammary gland. In these mice, the authors tested a vaccine formulation comprised of liposomal-MUC1 lipopeptide and human recombinant IL-2. Results indicate that when compared to untreated mice, immunized mice develop T cells that a) express intracellular IFN-γ , b) are reactive with MHC class I H-2D^b/MUC1 tetramer, and c) are cytotoxic against MUC1-expressing tumor cells *in vitro*. The presence of MUC1-specific CTL did not translate into a clinical response as measured by time of tumor onset, tumor burden and survival. The authors demonstrate that some of the immune-evasion mechanisms utilized by the tumor cells include down regulation of MHC-class I molecule, expression of TGF-β2, and decrease in IFN-γ -expressing effector T cells as tumors progress. Finally, utilizing an injectable breast cancer model, the authors show that targeting a single tumor antigen may not be an effective anti-tumor treatment, but that immunization with dendritic cells fed with whole tumor lysate is effective in breaking tolerance and protecting mice from subsequent tumor challenge. **Significance:** A physiologically relevant spontaneous breast cancer model has been developed to test improved immunotherapeutic approaches.

Key Words: MUC1-specific CTL, tolerance, immune evasion mechanisms, transgenic mice, spontaneous mouse models of cancer.

INTRODUCTION

Human cancers frequently express abnormal or altered self proteins that are potentially immunogenic and trigger immune recognition. Low-level humoral and cellular immune responses to several antigens, including mucin 1 (MUC1), HER2/neu, p53, and PSA, are present in a significant proportion of early and late stage cancer patients. It is important to determine how to obtain an effective immune response, since the native immune responses fail to eradicate tumors. In this study, human MUC1 is the tumor antigen of choice as it is widely expressed on most solid adenocarcinomas. 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. Underglycosylation of MUC1 reveals peptide epitopes recognized by cytotoxic T cells (CTL) that can kill tumor cells expressing this form of MUC1 (1-4).

The recent description of MUC1 as a target for CTL has raised interest in using this protein as a target for immunotherapy. This year, cancers that express MUC1 accounted for about 72% of new cases and for 66% of the deaths (5). These observations have prompted clinical vaccination trials aimed at boosting the weak immune responses to therapeutic levels. However, 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.

Up until now, all of the MUC1-specific immunotherapy has been tested in mice using injected tumor cells (6-9). In this study, we have characterized the development of a mouse line that expresses human MUC1 as a self-molecule and spontaneously develops MUC1-expressing tumors of the mammary gland. We have developed this line by mating mice that carry the human MUC1 transgene driven by its own promoter (MUC1.Tg mice) (10) with oncogenic mice that carry the polyoma middle T antigen (MTag) driven by the MMTV promoter (11). The MUC1.Tg mice are not a model of MUC1 over-expression, rather the mice express human MUC1 in a developmentally regulated and tissue specific fashion, as the transgene is driven by its own promoter. Mice transgenic for this protein develop B and T cell tolerance and are refractory to immunization with the protein encoded by the transgene (10). These double transgenic mice are designated MMT mice. We have analyzed MUC1 expression in MMT mice as tumor progresses in the mammary gland and characterized MUC1-specific native immune responses that develop during tumor progression. We further tested the efficacy of a MUC1-specific vaccine treatment in MMT mice and determined some of the immune-evasion mechanisms utilized by the tumor cells. Finally, using an injectable model, we demonstrate that targeting multiple tumor antigens rather than a single antigen may be more effective in eliciting an anti-tumor response. Breaking tolerance and eliciting a sustained anti-tumor immune response against the growing tumor are key to developing improved and novel immunotherapies for cancer. Our mouse model provides an improved system for evaluating the efficacy of anti–tumor vaccine formulations *in vivo* within the context of existing tolerance mechanisms.

MATERIALS AND METHODS

Mouse model. MMT: MUC1.Tg mice are bred with MTag oncogene-expressing mice. Double transgenic female mice spontaneously develop tumors of the breast and are designated as MMT. Male mice are discarded from this study. MUC1.Tg mice were developed in our laboratory (10). MTag mice were a kind gift from Dr. W.J. Muller (McMaster University, Hamilton, Ontario, Canada) (11). All mice are congenic on the C57/BL6 background at $N \geq 10$ (12). 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 transgene and MTag oncogene to determine mice that were positive for both transgenes. PCR was carried out as previously described (10,13). The primer pair used for MUC1 transgene are 5'-CTTGCCAGCCATAGCACCAAG-3' (bp. 745 to 765) and 5' CTCCACGTCGTGGACATTGATG-3' (bp. 1086 to 1065). Primer pairs for MTag transgene are 5'-AGTCACTGCTACTGCACCCAG-3' (bp 282 – 302) and 5'-CTCTCCTCAGTTCCTCGCTCC-3' (bp 817 – 837). The amplification program for MTag and 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 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 MTag in a 480 bp fragment.

Cell lines. Bl6 melanoma cells transfected with either full-length human MUC1 gene (Bl6.MUC1) or a neomycin resistance gene (Bl6.neo) were used as target cells for cytotoxic T lymphocyte (CTL) assays. C57mg breast cancer cells transfected with full-length human MUC1 gene (C57mg.MUC1) were used as an injected model of breast cancer. Dr. Tony Hollingsworth from Eppley Cancer Center, University of Nebraska provided the B16.MUC1 cells and our laboratory generated the C57mg.MUC1 cells. Bl6.MUC1 and B16.neo were maintained in DMEM media with 10% FBS, penicillin (50units/ml) and streptomycin (50ugs/ml), supplemented with 300ugs/ml G418. One day prior to conducting the ^{51}Cr-release assay, cells were treated with 5ngs/ml IFN-γ (Pharmingen, San Diego, CA). Flow cytometry was routinely used to test cells for the presence of MUC1 and MHC class I. C57mg.MUC1 was maintained in DMEM with 10% FBS, penicillin (50units/ml) and streptomycin (50ugs/ml), supplemented with 150ugs/ml G418 and 20ngs/ml insulin (Sigma, St. Louis, MO).

Analysis of MMT Tumors: From 8 weeks of age until sacrifice, control and immunized MMT mice were palpated once a week for presence of mammary tumors. Palpable tumors were measured by calipers and tumor weight was calculated according to the formula: grams = (length in cm) x $(\text{width})^2)/2$ (14). In accordance with IACUC regulation, mice were sacrificed when tumors reached 10% of body weight. Mice were carefully observed for signs of ill-health, including lethargy, abdominal distention, failure to eat or drink, marked weight loss, and hunched posture.

Evaluation of pulmonary metastatic lesions. The presence and number of pulmonary metastases was determined by gross morphological assessment using low power microscopy.

Immunohistochemistry. Tumors were obtained from control and immunized MMT mice at time of sacrifice. To determine MUC1 levels in non-immunized MMT mice as tumors progressed, we collected tumors at various time points during tumor progression. Tumors were fixed in methacarn followed by 70% ethanol, paraffin embedded, and step-sectioned for immunohistochemical analysis. MUC1 antibodies used were CT2, an Armenian hamster monoclonal antibody that recognizes mouse and human cytoplasmic tail (CT) region of MUC1 (15) and B27.29, a mouse monoclonal antibody with epitopes in the tandem repeat domain of MUC1 (16). B27.29 is specific for human MUC1 and does not cross react with mouse Muc1. The tandem repeat (TR) antibodies are glycosylation-sensitive in the mammary gland. Antibody to TGF-β2 was purchased from Santa Cruz Biotechnologies (Santa Cruz, California). Secondary antibody was a swine-anti rabbit conjugated to HRP for TGF-β2 (Dako, Carpinteria, CA); and a goat anti-hamster conjugated to HRP for CT2 (Jackson Labs., West Grove, PA.). HRP-conjugated B27.29 was obtained from Biomira, Inc., Edmonton, Canada. Antibody staining was blocked with the appropriate peptides.

MUC1-specific ELISA. Serum MUC1 levels were determined using the Truquant BR EIA supplied by Biomira (17). A MUC1 catcher antibody was used to capture serum MUC1 which was detected using HRP-conjugated secondary antibody and an appropriate substrate.

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 non-immunized MMT 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)). These cells served as effectors. B16.MUC1 that expressed high levels of MUC1 as determined by flow cytometry analysis with B27.29 monoclonal antibody were used as target cells. For better presentation of MUC1 antigen, B16.MUC1 target cells were treated with 5ngs/ml IFN-γ one day prior to the assay to up-regulate MHC class I surface expression. B16 melanoma cells transfected with vector that contains the neomycin resistance gene but no MUC1 (B16.neo) were used as control target cells. Specific ^{51}Cr-release was calculated according to the following formula: (experimental CPM-spontaneous release CPM/maximum release CPM-spontaneous release CPM) x 100. Spontaneous release in all experiments was less than 15% of maximum release. To determine native immune response in non-immunized MMT mice, we performed CTL assays at various time points during tumor progression (6, 9, 12, 15, 18 and 21 weeks). For these studies, splenocytes (1 x 10^6/ml) from non-immunized MMT mice were stimulated for 6 days *in vitro* with 1X 10^5/ml irradiated (3000Rads) bone marrow-derived dendritic cells (DC). Prior to irradiation, DC were pulsed with MUC1 TR peptide (24mer, TAPPAHGVTSAPDTRPAPGSTAPP) at 10 μg/ml for 24 hours. Murine IL-2 (100 units / ml) (Pharmingen) was also added to the culture. At day 6 of culture, effector cells were used in the ^{51}Cr-release assay with B16.MUC1 as target cells. In some experiments, B16.neo cells were used as control target cells.

Flow cytometry. Single cells from peripheral blood lymphocytes (PBLs) collected from immunized and non-immunized MMT mice at various times post immunization were analyzed by two color flow cytometry for lymphocyte activation markers and intracellular cytokines. This included early and late surface activation markers, CD69 and CD25 (IL2-R) on $CD4^+$ and $CD8^+$

T cells. Intracellular cytokine levels were determined after cells were treated with brefeldin-A [also called Golgi-Stop (Pharmingen)] according to the manufacturer's recommendation (4uls / $1.2x10^7$ cells / 6 mls for 3 h at 37°C prior to staining). Cells were surface labeled with CD4, CD8 or CD11c antibodies prior to permeabilizing with the Pharmingen permeabilization kit (Cat # 554722, containing 0.005% saponin and 4% formaldehyde) and staining for intracellular IFN-γ, IL-2, IL-4, IL-5, IL-10 or IL-12. Tumor cells from freshly dissociated MMT tumors were stained with fluorescently labeled anti-pan-cytokeratin and anti-MHC class I H-$2D^b$ and H-$2K^b$. All fluorescently labeled antibodies were purchased from Pharmingen except for 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.

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

Preparation of dendritic cell-pulsed liposomal MUC1 TR lipopeptide. Dendritic cells (DC) were derived from C57BL/6 bone marrow cells according to the method described (18). Briefly, bone marrow cells were flushed from tibia and femur and red blood cells were lysed with ammonium chloride. Cells ($1X10^6$/ml) were cultured in DMEM with 10% fetal bovine serum, 1% glutamax, 50U/ml penicillin, 50ugs/ml streptomycin (DMEM complete medium),

supplemented with 10ngs/ml murine GM-CSF (Pharmingen) and 10ngs/ml murine IL-4 (Pharmingen). At day 7, non-adherent cells were removed, washed, re-suspended ($1X10^6$/ml) in fresh DMEM complete media supplemented with 5ngs/ml GM-CSF. Adherent cells were re-fed fresh DMEM complete media supplemented with 5ngs/ml GM-CSF. Between days 10 and 13, we obtained approximately 70 - 75% cells that showed DC phenotype by flow cytometry. DC were fed with MUC1 tandem repeat lipopeptide that were enclosed in liposomes according to the method provided by Biomira, Inc. (19). Briefly, 2mls of the liposomal MUC1 tandem repeat (MUC1 TR) formulation was fed to 200 x 10^6 DC in a total volume of 20 mls, 24 hours prior to injection. Mice were injected i.p. with 1 x 10^6 MUC1 TR pulsed DC per mouse in 200 ul volume. The first two immunizations at weeks 3 and 5 consisted of DC-fed liposomal MUC1 formulation. Thereafter, mice were immunized with liposomal MUC1 vaccine in PBS.

Vaccination strategy. The vaccine formulation consisted of a lipid derivative of a 25 mer MUC1 TR peptide (STAPPAHGVTSAPDTRPAPGSTAPP) 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 MMT mice starting at 3 weeks of age.

Treatment arms include: (i) liposomal MUC1 TR lipopeptide (L-MUC1-TR) (200ugs/mouse/250uls s.c.) + L-IL-2 (20,000 Units/mouse/100uls i.p.), (ii) L-MUC1-TR, (iii) L-

IL-2 (20), (iv) empty liposomes and (v) no treatment. We compared the immune responses that developed during treatment and used tumor onset, tumor burden and survival as the endpoints for determining the clinical effectiveness of the vaccine. A schematic of the immunization protocol is shown in Figure 1.

Dendritic cells pulsed with C57mg.MUC1 tumor lysate. DC were prepared as described above. Lysates from C57mg.MUC1 cells were made in tissue lysis buffer containing 20mM Hepes, 0.15M NaCl, and 1% Triton X-100. Cells were sonicated using a Branson Sonifier 450 (VWR Scientific) at 20% duty cycle setting with 3 pulses of 10seconds each. Immature 7 day old DC were pulsed with 20ngs/ml of the tumor lysate for 24 hours and then matured with 5ngs/ml TNF-α for 24 hours. MUC1.Tg mice received two intradermal injections, two weeks apart of tumor lysate-pulsed DC at 1 X 10^7 DC per mouse. One week after the last injection, mice were challenged with 2 X 10^6 C57mg.MUC1 tumor cells (subcutaneously).

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. P values for lung metastasis are from the Pearson chi-square statistic and from Kruskal-Wallis test.

RESULTS

MMT mice develop spontaneous mammary gland cancer and lung metastases. MUC1.Tg mice were bred with mice carrying the MMTV-driven polyoma middle T antigen (MT) to create MMT mice. Prior to generating the double transgenics, the MTag mice had been backcrossed 10 generations onto C57BL/6 mice, making them congenic (12). In the MMT mice, mammary gland tumors are induced by the action of a potent tyrosine kinase activity associated with the polyoma virus middle T antigen driven by the mouse mammary tumor virus long terminal repeat (MMTV) (11). Middle T specifically associates with and activates the tyrosine kinase activity of a number of c-src family members, eliciting tumors when a threshold level of gene product has been attained. This promoter is transcriptionally active throughout all stages of mammary gland development and results in widespread transformation of the mammary epithelium and the rapid production of multifocal mammary adenocarcinomas. Hyperplastic alveolar nodules (HANs) can be detected by whole mount as early as 21 days and palpable mammary gland tumors are detectable from approximately 49 days onwards. Tumor progression is quite rapid, reaching 10% of body weight by about 20 – 24 weeks (Figure 2). 100% of the female MMT mice get tumors. Tumors arise with synchronous kinetics and are highly fibrotic with dense connective tissue separating individual nests of tumor cells, a pathology that closely resembles scirrhous carcinomas of the human breast (21). These mice exhibit metastasis in the lungs (30 out of 49 MMT mice tested or 60%) and micro-metastasis in the bone marrow by 4 months of age. Following the observation that epithelial cells were present in several of the 20-24 week old MMT-derived bone marrow cultures, we examined if bone marrow micro-metastasis was occurring in these mice. Bone marrow metastasis was determined by staining bone marrow cells

from MUC1.Tg mice and MMT mice with pan-cytokeratin and MUC1, markers commonly used to detect epithelial tumor cells. Cells positive for both markers were determined by two-color flow cytometry as well as by confocal microscopy and represent micro-metastasis (Figure 3A and B). By flow cytometry, 3-4% of bone marrow cells were positive for both pan-cytokeratin and MUC1. It is important to note that mortality in human breast cancer patient is directly associated with lung and bone metastasis. The MMT mouse appears to be an appropriate model for human cancer and allows us to study the effects of self-tolerance, immunity and auto-immunity to MUC1 as mammary tumors develop spontaneously.

Tumor burden and lung metastasis in immunized MMT mice. We immunized mice with L-MUC1-TR ± L-IL-2. The first two immunizations administered to young (3 and 5 week old) MMT mice utilized syngeneic 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 (22). 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. Although no significant differences were observed in the onset and progression of the MMT tumors with immunization (Figure 4A), by 18 weeks of age there was significant decrease ($p < 0.05$ to $p < 0.005$) in tumor burden between immunized and control MMT mice (Figure 4B). To our surprise empty liposomes elicited a similar response suggesting that the lipid A in the formulation may have an effect on the tumor. However, by 20-24 weeks no significant differences in tumor burden between immunized and control mice were observed (Figure 4A). Interestingly, we observed that treatment with L-IL-2 alone had significantly lower numbers of lung metastasis than mice in all other treatment groups

and in untreated control mice (Figure 4C). Incidence of metastasis was also lower in mice treated with L-IL-2 as compared to other treatment groups and untreated MMT mice (44% versus 64% in untreated MMT) (Table 1). These results did not reach significance due to low animal numbers. All mice were sacrificed when tumors reached 10% of body weight and therefore difference in survival was not observed. Because MUC1 is expressed on normal cells, we routinely examined mice for signs of autoimmunity post immunization. We monitored weight loss, food intake, general health, hunched back and histopathology of various MUC1-expressing organs. No signs of autoimmunity were evident in our study group (data not shown).

MUC1 expression in MMT tumors. Tumor sections from 6, 12 and 21 week old non-immunized MMT mice show strong heterogeneous expression of MUC1 as tumors progress (Figure 5). Paraffin embedded tumor sections from 6, 12 and 21 week old MMT mice were stained with two antibodies, one recognizing the cytoplasmic tail of MUC1 (CT2) and the other recognizing the tandem repeat epitope of MUC1 (B27.29). CT2 antibody recognizes both mouse and human cytoplasmic tail (CT) of MUC1. It is a monoclonal antibody (Mab) raised in Armenian hamster and thus gives very little background when used to stain tissues from mice. Mammary tumors showed moderate expression of human MUC1, using Mab. B27.29 which reacts only with the human MUC1 and not with mouse Muc1 (23). MUC1 was expressed throughout the cytoplasm and around the cells in a pattern similar to that observed in human breast carcinomas. Western blots of tumor lysates using B27.29 for detection showed protein expression (data not shown). Thus, mammary gland tumors that occurred spontaneously in the MMT mice expressed the transgene protein MUC1 in a manner similar to humans. Any alteration in MUC1 expression will be important in the immunological recognition and

presentation of the antigen. Immunization did not alter the MUC1 expression in the MMT tumors (data not shown).

Native immune responses developing in MMT mice as tumors progress. We have determined the native cellular and humoral immune responses in the non-immunized MMT mice as tumors progressed. MMT mice were bled at 6, 14, and 21 weeks of age and PBLs were examined for T cells expressing early and late activation markers (CD69 and CD25), DC ($CD11c^+$ cells) expressing B7 co-stimulatory receptors, T cells and DC expressing intracellular cytokines such as IFN-γ, IL-2, IL-4, IL-5, IL-10 and IL-12. We also examined PBLs for presence of T cells recognizing H-$2D^b$/MUC1 tetramer. The results are tabulated in Table 2. Increases were observed in some of the markers (IL-2R, and intracellular IL-4 and IL-12) at the 14 week time point but the difference was not statistically significantly and was no longer observed at 21 weeks. $CD11c^+$ and $B7^+$ cells were significantly lower at 21 weeks when the tumor burden was high as compared to 6 and 14 weeks, which may indicate a defect in the antigen presenting cells during tumor progression. Thus our data clearly show that no significant alterations in Th1 or Th2 immune phenotype occurs in MMT mice as tumors progressed. Presence of naturally occurring precursor CTL activity against MUC1 in 6, 9, 12, 15, 18, and 21week old MMT mice was also tested and the data are presented in Figure 6. We were unable to detect precursor CTL activity in splenocytes at any time during tumor progression. With regards to humoral immune response, circulating antibody to MUC1 was undetectable by specific ELISA at any time during tumor progression (data not shown). These data taken together clearly indicate that naturally occurring cellular or humoral immune responses in non-immunized MMT mice were non-detectable, which is in sharp contrast to the results reported previously for a similar model of

pancreas cancer (3).

T cell immune response in immunized MMT mice. Immunization elicited mature MUC1-specific CTL that were cytotoxic against B16.MUC1 tumor cells *in vitro* (Figure 6). To determine MUC1 specificity, B16 cells transfected with vector alone (B16.neo) were routinely used as control tumor target and lysis of < 5% was observed making the CTL specific for MUC1 (data not shown). Both MHC-restricted and non-restricted MUC1-specific CTL have been reported in the literature for human cancer (1,24); however, in MMT mice, we only detect restricted CTL. It is possible that these mice do possess unrestricted CTL but these CTLs may be difficult to detect *in vitro* and therefore have never been reported previously in mice (25-28). Although the CTLs were cytotoxic *in vitro*, they had no effect on the growing tumor cells *in vivo* in MMT mice. This was determined by immunohistochemistry of MMT tumor sections in which MUC1-expressing tumor cells remained unchanged with immunization, suggesting that the MUC1-specific CTLs were not cytotoxic against the tumor cells *in vivo* (data not shown). To evaluate the T cell responses during immunization and as the tumors progressed, immunized and non-immunized MMT mice were tail-bled at 6, 14, and 20 weeks, peripheral blood leukocytes (PBLs) were isolated and tested for presence of a) T cells expressing intracellular IFN-γ and b) T cells recognizing H-2D^b/MUC1 tetramer. All treatment groups in immunized MMT mice showed increased numbers of T cells expressing intracellular IFN-γ by 10 -14 weeks of age as compared to untreated control mice (Figure 7A, p values shown in the figures). By 20 weeks of age, there was a decrease in these cell numbers suggesting that repeated immunization with MUC1 TR peptide or high tumor burden may affect T cell effector function. This observation correlates well with the decrease in tumor burden at 18 weeks in immunized mice, which does

not last at 21 weeks (Figure 4B). Similarly, T cells reactive with H-2D^b/MUC1 tetramer increased by 14 weeks in immunized MMT mice but no further increase was observed at 20 weeks (Figures 7B, p values shown in the figures). Although some increase in percent T cells recognizing MUC1-tetramer was observed in the empty liposome treated mice versus untreated controls, significantly higher percent was observed in mice treated with L-MUC1-TR alone or with L-MUC1-TR + L-IL-2 [($p < 0.01$) and ($p < 0.05$) respectively] versus empty liposome group. The increase in empty liposome group suggests that the lipid A present in the liposomal vaccine formulation may be sufficiently immunogenic in eliciting an anti-MUC1 response, albeit not as strong as the vaccine formulation containing MUC1. Furthermore, the response seen with the empty liposome group did not translate into MUC1-specific CTL response (Figure 6) nor did it correlate with elevated serum MUC1 levels in Figure 8A.

MUC1 Serum levels in immunized MMT mice. As tumors progressed in the MMT mice, MUC1 serum levels increased only slightly as compared to age matched MUC1.Tg mice with maximum reaching to 1500 – 2500 Units/ml of serum at 20 weeks of age. In age matched female MUC1.Tg mice, serum MUC1 levels ranged from 500 to 1200 Units/ml which is likely to depend upon their estrous cycle status (data not shown). The low levels of circulating MUC1 may explain the lack of an immune response to MUC1 in non-immunized MMT mice (Figure 6 and Table 2). Immunization, however, significantly increased the serum MUC1 levels compared to untreated MMT mice (Figure 8A), which corresponded directly to the increased CTL activity in these mice. These results suggested that high levels of circulating tumor antigen, MUC1, may activate MUC1-specific CTL that are capable of specifically lysing MUC1-expressing tumor cells *in vitro* (Figure 6). We also detected low levels of circulating antibodies to MUC1 in the L-

MUC1-TR + IL-2 treated mice (Figure 8B) suggesting that immunization and the high level of circulating tumor associated MUC1 has changed the antigenic profile and elicited a low level humoral response to MUC1. Antibodies reactive with MUC1 have been reported in a small number of humans with breast cancer (29,30). Although a humoral response is often dismissed as being ineffective against solid tumors, it is still important that the response in the MMT mice once again parallels that in humans.

MHC class I expression in MMT mice as tumors progress. Since the MUC1-specific CTLs have minimal effect *in vivo,* we postulated that the growing MMT tumor cells evaded immune recognition. We evaluated one of the well-characterized mechanisms by which tumor cells evade CTL killing, down-regulation of their surface MHC class I molecules. We observed by two color flow cytometry that percent cells positive for MHC class I and pan-cytokeratin are ~ 13% in 6 week old MMT tumor mice which steadily decreases to < 2% in 18 week old tumor mice (Table 3) and that immunization was unable to up-regulate these levels.

MMT tumors express TGF-β. Another effective way a tumor cell evades T cell killing is to render the effector T cells non-functional by releasing immunosuppressive factors. One such factor is TGF-β that is capable of hindering T cell signaling and down regulating their function. We tested whether mammary tumors from MMT mice express TGF-β by specific immuno-histochemical staining and demonstrate that MMT tumors express TGF-β as early as 6 weeks of age and that the expression increases as the tumors progress (Figure 9). MUC1-specific immunizations did not alter the expression of TGF-β, suggesting that the tumors may be utilizing this mechanism to down regulate T cell activity *in vivo* and escape immune intervention. This

phenomenon is also evident from the decrease in the numbers of IFN-γ expressing T cells (Figure 7A) as tumor burden increases.

Tumor lysate pulsed DC were effective in breaking tolerance and preventing tumor formation in MUC1.Tg mice. Another plausible explanation for the failure of MUC1-specific immunization to eradicate MMT tumors is the utilization of a single tumor antigen as immune target. Recent findings suggest that tumor lysate-fed DC generate tumor-specific proliferative cytokine release and cytolytic reactivities *in vitro* as well as effectively prime mice to reject subsequent lethal challenges with viable parental tumor cells (31,32). Using an injectable tumor model, we show that MUC1.Tg mice immunized with DC pulsed with lysates prepared from C57mg.MUC1 cells were completely protected from subsequent challenge with C57mg.MUC1 tumor cells (Figure 10). These results are promising, as we were able to break tolerance in the MUC1.Tg mice and protect them against tumor challenge. However, the challenge is to achieve these results in our spontaneous model, which is physiologically more appropriate, and to a large extent, mimics the human situation.

DISCUSSION

We describe a mouse model of spontaneous breast cancer that appropriately mimics human cancer and is an excellent model in which to test novel immunotherapeutic strategies. Some of the important features of this model are the reproducible development of spontaneous mammary gland tumors, the occurrence of invasion and metastasis, the presence of an intact immune system, and presence of a targetable and stable tumor antigen, MUC1. Most importantly, the tumor models resemble human cancer with regards to progression through various development stages of cancer and sensitivity to hormonal and stromal alterations. A recent study has established that MTag-induced hyperplasias, like early proliferative lesions seen in human breast, are heterogeneous with respect to their malignant potential. The study further establishes that MTag gene expression alone is insufficient to induce tumors and that additional events are required for tumorigenesis and metastasis (33).

In the MMT mice, as in humans, the mammary gland develops after birth, tumors are multifocal, grow rapidly and are histologically homogeneous, highly fibrotic with dense connective tissue separating individual nests of cells. Tumors occur in a reasonable time frame to allow for prevention as well as therapeutic studies. Mammary tumors, which can be followed by palpation, are useful for therapeutic studies, as tumor location alleviates the need to sacrifice the animal to determine clinical response and is optimal for prevention therapies, as the tumors develop after birth, and immunizations could precede tumor development. MUC1 is over-expressed in these tumors and immunization with a MUC1-specific vaccine significantly increases MUC1 serum levels and elicits a MUC1-specific cellular and a humoral immune response. Similar to humans,

these immune responses do not translate into a anti-tumor response suggesting that a) the tumor cells successfully evade the immune effector cells using a variety of mechanisms, and b) targeting a single tumor antigen may not be effective. Similar to our study, recent studies showed that the HER-2/neu transgenic mice that are immunologically tolerant to the neu antigen and develop spontaneous unifocal mammary carcinomas, developed neu-specific T cells following vaccination. However, the neu-specific effector T cells were unable to protect these mice from the developing breast tumors (34,35).

MMTV-driven polyoma middle T antigen affects almost every cell in the mammary gland and the entire mammary tree is burdened with tumors, which may be too aggressive for the immune effector cells to act effectively. We have previously shown by adoptive transfer that MUC1-specific CTL developed *in vitro* can eradicate less aggressive, transplantable $MUC1^+$ tumors (4). Whether these CTLs gain access into the growing tumor mass was tested by adoptively transferring carboxyfluorescein succinimidyl ester (CFSE)-labeled MUC1-specific CTL into tumor-MMT mice. Preliminary data suggest that CTLs can enter the tumor mass and multiply for up to 14 days, after which they are undetectable in the tumor mass (unpublished data).

Many reports have suggested that progressing tumors in cancer patients have elaborate means of escaping an apparently effective MHC class I restricted immune response (36,37). Other investigators have found that the CTL response occurs too late to be effective against the tumors (38). Tumors evade host immunity at both the induction and effector phases. Because immunized MMT mice have developed MUC1-specific CTL responses, these spontaneously arising mammary gland tumors must have evaded the existing CTL. We have shown that the immuno-

suppressive factor, TGFβ2 is expressed by MMT tumor cells and may be a factor responsible for rendering the CTLs cytolytically anergic (Figure 9). It has been shown previously that TGFβ may alter TCR sub-component composition and down-regulate CD3ζ, γ, δ but not ε, thereby reducing T cell signaling and CTL responses against tumor cells, and reducing TGFβ2 expression reverses this effect (39). However, including TGFβ2 antibody treatment in our vaccination strategy did not lead to effective anti-tumor response in the MMT mice (unpublished data), suggesting that multiple immune evasion mechanisms may be utilized by the tumors to avoid immune destruction. We have demonstrated that the MMT tumor cells may avoid immune recognition by down-regulating MHC class I expression (Table 3). It has been recently shown that functionally active inhibitory receptors that impart negative signals have been found on T cells. One such inhibitory receptor, CD94-NKG2A, can induce cytolytic anergy in CTLs specific for polyomavirus induced tumors (40). Since the tumors in the MMT mice are induced by the polyomavirus middle T antigen, it is likely that a similar mechanism is occurring these mice. In our preliminary data, we observe that the tumor-infiltrated lymphocytes isolated from MMT tumors do not proliferate in response to MUC1 antigen nor are they cytolytic against MUC1-expressing tumor cells *in vitro,* suggesting that the effector T cells may be tolerant to the antigen or may be anergic (unpublished data). Further experiments need to be conducted to confirm these results.

The beneficial effect of IL-2 treatment may be attributed to stimulation of other tumor antigen-specific CTLs that may exist in these mice, but have never been identified *in vitro.* Analyzing some of the other known tumor antigen-specific CTLs may provide us with better answers and these studies are under way. We show that immunotherapy that targets multiple tumor antigens

elicits an effective anti-tumor response, albeit in an injectable tumor model (Figure 10). Similar studies are under way in the MMT mice.

Finally, targeting a single tumor antigen, such as MUC1, may lead to activation-induced tolerance and/or anergy of CTLs *in vivo,* which eventually results in inactivation of the effector T cells and interruption of an anti-tumor response. A defining feature for a tolerant T cell is its hypo-responsiveness to antigen when compared with either a naïve or a primed T cell and experiments are under way to evaluate if MUC1-specific immunization leads to T cell tolerance. This phenomenon of antigen-specific CTL tolerance following peptide immunization is not new and has been reported by several investigators (38,41,42). Thus, immunotherapy must be effective in breaking the existing tolerance. Recent studies have highlighted the role of two co-stimulatory molecules, OX40 expressed on activated T helper cells and CD40 expressed on antigen presenting cells, as targets for therapeutic intervention in cancer. Both molecules belong to the tumor necrosis factor receptor (TNFR) family and are implicated in preventing tolerance induction as well as reversing established tolerance observed during anti-cancer immunotherapy (43,44). These studies indicate that ligation of these co-stimulatory molecules with specific antibodies may be a useful strategy for enhancing T cell unresponsiveness to anti-cancer vaccination (45).

As is frequently observed with human immunotherapy clinical trials, there is a poor correlation between the ability to induce an *in vitro* cellular T cell response and a clinical response (46,47). The MMT model appropriately mimics the human condition and is an excellent model for testing therapy in a setting relevant to the treatment of human cancer as well as for prevention and

delineation of the mechanisms of tolerance, immunity, and auto-immunity. This study has only touched upon some of the major issues that are critical for designing suitable immune therapies for cancer. Future immunizations in MMT mice will target multiple antigens as well as target the co-stimulatory molecules.

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REFERENCES

1. Barnd DL, Lan MS, Metzgar RS, et al. Specific, major histocompatibility complex-unrestricted recognition of tumor-associated mucins by human cytotoxic T cells. *Proc Natl Acad Sci U S A* 1989; 86:7159-63.
2. Ioannides CG, Fisk B, Jerome KR, et al. Cytotoxic T cells from ovarian malignant tumors can recognize polymorphic epithelial mucin core peptides. *J Immunol* 1993; 151:3693-703.
3. Mukherjee P, Ginardi AR, Madsen CS, et al. Mice with spontaneous pancreatic cancer naturally develop MUC1-specific CTLs that eradicate tumors when adoptively transferred. *J Immunol* 2000; 165:3451-60.
4. Mukherjee P, Ginardi AR, Tinder TL, et al. MUC1-specific CTLs eradicate tumors when adoptively transferred in vivo. *Clin Can Res* 2001; 7:848s-55s.
5. Greenlee RT, Murray T, Bolden S, et al. Cancer statistics, 2000. *CA Cancer J* 2000; 50:7-33.
6. Wang J, Saffold S, Cao X, et al. Eliciting T cell immunity against poorly immunogenic tumors by immunization with dendritic cell-tumor fusion vaccines. *J Immunol* 1998; 161:5516-24.
7. Gong J, Nikrui N, Chen D, et al. Fusions of human ovarian carcinoma cells with autologous or allogeneic dendritic cells induce antitumor immunity. *J Immunol* 2000; 165:1705-11.

8. Tanaka Y, Koido S, Chen D, et al. Vaccination with allogeneic dendritic cells fused to carcinoma cells induces antitumor immunity in MUC1 transgenic mice. *Clin Immunol* 2001; 101:192-200.

9. Gong J, Koido S, Chen D, et al. Immunization against murine multiple myeloma with fusions of dendritic and plasmacytoma cells is potentiated by interleukin 12. *Blood* 2002; 99:2512-7.

10. Rowse GJ, Tempero RM, VanLith ML, et al. Tolerance and immunity to MUC1 in a human MUC1 transgenic murine model. *Cancer Res* 1998; 58:315-21.

11. Guy CT, Cardiff RD, Muller WJ. Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. *Mol Cell Biol* 1992; 12:954-61.

12. Silver LM. in *Mouse Genetics Concepts and Applications* 32-61 (Oxford University Press, 1995).

13. Tevethia MJ, Bonneau RH, Griffith JW, et al. A simian virus 40 large T-antigen segment containing amino acids 1 to 127 and expressed under the control of the rat elastase-1 promoter produces pancreatic acinar carcinomas in transgenic mice. *J Virol* 1997; 71:8157-66.

14. Simpson-Herrems L, Lloyd HH. Kinetic parameters and growth curves for experimental tumor systems. *Cancer Chemother Rep* 1970; 54:143-74.

15. Schroeder JA, Thompson MC, Gardner MM, et al. Transgenic MUC1 interacts with EGFR and correlates with MAP kinase activation in the mouse mammary gland. *J Biol Chem* 2001; 276:13057-64.

16. Price MR, Rye PD, Petrakou E, et al. Summary report on the ISOBM TD-4 Workshop: analysis of 56 monoclonal antibodies against the MUC1 mucin. San Diego, Calif., November 17-23, 1996. *Tumour Biol* 1998; 19:1-20.

17. Reddish MA, MacLean GD, Poppema S, et al. Pre-immunotherapy serum CA27.29 (MUC-1) mucin level and CD69+ lymphocytes correlate with effects of Theratope sialyl-Tn-KLH cancer vaccine in active specific immunotherapy. *Cancer Immunol Immunother* 1996; 42:303-9.

18. Inaba K, Inaba M, Romani N, et al. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* 1992; 176:1693-702.

19. Guan HH, Budzynski W, Koganty RR, et al. Liposomal formulations of synthetic MUC1 peptides: effects of encapsulation versus surface display of peptides on immune responses. *Bioconjug Chem* 1998; 9:451-8.

20. Boni LT, Batenjany MM, Neville ME, et al. Interleukin-2-induced small unilamellar vesicle coalescence. *Biochim Biophys Acta* 2001; 1514:127-38.

21. Cardiff RD, Muller WJ. Transgenic mouse models of mammary tumorigenesis. *Cancer Surveys* 1993; 16:97-113.

22. Ridge JP, Fuchs EJ, Matzinger P. Neonatal tolerance revisited: turning on newborn T cells with dendritic cells. *Science* 1996; 271:1723-6.

23. Sikut R, Sikut A, Zhang K, et al. in *Tumor Biology* (eds. Rye, P. D. & Price, M. R.) 122-26 (S. Karger Medical and Scientific Publishers, 1998).

24. Domenech N, Henderson RA, Finn OJ. Identification of an HLA-A11-restricted epitope from the tandem repeat domain of the epithelial tumor antigen mucin. *J Immunol* 1995; 155:4766-74.

25. Apostolopoulos V, Xing PX, McKenzie IF. Murine immune response to cells transfected with human MUC1: immunization with cellular and synthetic antigens. *Cancer Res* 1994; 54:5186-93.

26. Apostolopoulos V, Karanikas V, Haurum JS, et al. Induction of HLA-A2-restricted CTLs to the mucin 1 human breast cancer antigen. *J Immunol* 1997; 159:5211-8.

27. Acres B, Apostolopoulos V, Balloul JM, et al. MUC1-specific immune responses in human MUC1 transgenic mice immunized with various human MUC1 vaccines. *Cancer Immunol Immunother* 2000; 48:588-94.

28. Pietersz GA, Li W, Osinski C, et al. Definition of MHC-restricted CTL epitopes from non-variable number of tandem repeat sequence of MUC1. *Vaccine* 2000; 18:2059-71.

29. Rughetti A, Turchi V, Ghetti CA, et al. Human B-cell immune response to the polymorphic epithelial mucin. *Cancer Res* 1993; 53:2457-9.

30. Kotera Y, Fontenot JD, Pecher G, et al. Humoral immunity against a tandem repeat epitope of human mucin MUC-1 in sera from breast, pancreatic, and colon cancer patients. *Cancer Res* 1994; 54:2856-60.

31. Fields RC, Shimizu K, Mule JJ. Murine dendritic cells pulsed with whole tumor lysates mediate potent antitumor immune responses in vitro and in vivo. *Proc Natl Acad Sci U S A* 1998; 95:9482-7.

32. Schnurr M, Galambos P, Scholz C, et al. Tumor cell lysate-pulsed human dendritic cells induce a T-cell response against pancreatic carcinoma cells: an in vitro model for the assessment of tumor vaccines. *Cancer Res* 2001; 61:6445-50.

33. Maglione JE, Moghanaki D, Young LJ, et al. Transgenic Polyoma middle-T mice model premalignant mammary disease. *Cancer Res* 2001; 61:8298-305.

34. Reilly RT, Gottlieb MB, Ercolini AM, et al. HER-2/neu is a tumor rejection target in tolerized HER-2/neu transgenic mice. *Cancer Res* 2000; 60:3569-76.

35. Reilly RT, Machiels JP, Emens LA, et al. The collaboration of both humoral and cellular HER-2/neu-targeted immune responses is required for the complete eradication of HER-2/neu- expressing tumors. *Cancer Res* 2001; 61:880-3.

36. Garrido F, Ruiz-Cabello F, Cabrera T, et al. Implications for immunosurveillance of altered HLA class I phenotypes in human tumours. *Immunol Today* 1997; 18:89-95.

37. Hicklin DJ, Marincola FM, Ferrone S. HLA class I antigen downregulation in human cancers: T-cell immunotherapy revives an old story. *Mol Med Today* 1999; 5:178-86.

38. Toes RE, Blom RJ, Offringa R, et al. Enhanced tumor outgrowth after peptide vaccination. Functional deletion of tumor-specific CTL induced by peptide vaccination can lead to the inability to reject tumors. *J Immunol* 1996; 156:3911-8.

39. Pawelec G, Zeuthen J, Kiessling R. Escape from host-antitumor immunity. *Crit Rev Oncog* 1997; 8:111-41.

40. Welsh RM, Stepp SE, Szomolanyi-Tsuda E, et al. Tumor viral escape from inhibited T cells. *Nat Immunol* 2002; 3:112-4.

41. Diehl L, Den Boer AT, van der Voort EI, et al. The role of CD40 in peripheral T cell tolerance and immunity. *J Mol Med* 2000; 78:363-71.

42. Toes RE, Offringa R, Blom RJ, et al. Peptide vaccination can lead to enhanced tumor growth through specific T-cell tolerance induction. *Proc Natl Acad Sci U S A* 1996; 93:7855-60.

43. Bansal-Pakala P, Jember AG, Croft M. Signaling through OX40 (CD134) breaks peripheral T-cell tolerance. *Nat Med* 2001; 7:907-12.

44. Diehl L, den Boer AT, Schoenberger SP, et al. CD40 activation in vivo overcomes peptide-induced peripheral cytotoxic T-lymphocyte tolerance and augments anti-tumor vaccine efficacy. *Nat Med* 1999; 5:774-9.

45. Sotomayor EM, Borrello I, Tubb E, et al. Conversion of tumor-specific CD4+ T-cell tolerance to T-cell priming through in vivo ligation of CD40. *Nat Med* 1999; 5:780-7.

46. Gilboa E. The makings of a tumor rejection antigen. *Immunity* 1999; 11:263-70.

47. Houghton AN. Cancer antigens: immune recognition of self and altered self. *J Exp Med* 1994; 180:1-4.

FIGURE LEGENDS

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

Figure 2. Tumor growth curve in MMT mice as a function of age. Tumor burden was determined by palpation beginning at 8 - 9 weeks of age and calculated by the following formula: gm = (Length x Width^2) x 0.5. Most mice develop palpable tumors by 10 – 11 weeks. Tumors reach 10% of mouse body weight between 20 and 24 weeks at which point mice are sacrificed. Data for individual mice are shown (n = 9 mice).

Figure 3. Bone marrow metastasis is detected in MMT mice. A. Representative histogram for two-color flow cytometric analysis of bone marrow cells derived from 22 week old MMT mouse. The histogram represents percent cells stained positive for pan-cytokeratin that were gated on MUC1$^+$ cells. Two controls include bone marrow cells from age matched MUC1.Tg mice and isotype IgG. **B.** Confocal microscopy showing bone marrow cells from MMT mice positive for pan-cytokeratin (green) and MUC1 (red). Colocalization is shown in yellow. For MUC1 staining, a hamster monoclonal antibody, CT2 is used.

Figure 4. A) MUC1-specific immunization did not alter tumor burden in MMT mice. Tumor growth of immunized MMT mice versus untreated MMT mice. Tumor burden was determined by palpation beginning at 6-8 weeks and calculated by the following formula: gm = (Length x Width^2) x 0.5. No significant difference was observed in mice immunized with MUC1 TR + L-IL-2 as compared to untreated control mice (n = 6-9 mice/group). **B) Tumor burden**

was lower in immunized MMT mice at 18 weeks of age. In treatment groups, tumor burden at 18 weeks was significantly lower as compared to untreated control mice. Individual mouse data are shown. *P* values are derived from the pair-wise contrasts from a one-way ANOVA model. **C) L-IL-2 treatment reduces lung metastasis in MMT mice.** The presence of pulmonary metastases was determined by counting gross morphological disease using low power microscopy. Number of metastatic lesions counted in the lungs was significantly lower in mice treated with L-IL-2 as compared to other treatment groups and untreated control mice. Individual mouse data are shown (n = 8 to 11 mice/group). *P* values were from the Pearson chi-square statistic and from Kruskal-Wallis test.

Figure 5. MUC1 expression increases as tumors develop in MMT mice. Methacarn fixed and paraffin-embedded sections of mammary gland tumors from 6, 12 and 21 week old MMT mice were stained with **A)** CT2, reactive with MUC1 cytoplasmic tail and **B)** B27.29, reactive with MUC1 TR. Specific staining was observed on lumenal surface of mammary epithelial cells and staining pattern is similar to that seen in humans. Staining with these antibodies showed increase in MUC1 expression as tumors developed. Images were captured at 200X magnification.

Figure 6. Immunized MMT mice develop MUC1-specific CTLs. CTL activity in 6, 9, 12, 15, 18 and 21 week old untreated MMT mice was determined by a standardized 8hr ^{51}Cr-release assay using B16 melanoma cells transfected with human MUC1 as target cells. Splenic T cells from MMT mice that were stimulated in vitro for 6 days with MUC1 TR peptide (10ngs / ml) and IL-2 (100units / ml) were used as effector cells. In immunized mice, CTL activity was determined at time of sacrifice when tumors reached 10% of body weight. The effector cells

were not stimulated *in vitro* with MUC1-TR or cytokines. In untreated mice no CTL activity was detected at any age. MMT mice in all treatment groups showed increase in MUC1-specific CTL with most lysis was detected in mice immunized with L-MUC1-TR + L-IL-2. (*$p < 0.001$) as compared to untreated control. L-MUC1-TR immunization alone gave significantly higher CTL activity as compared to empty liposome group and untreated controls ($p < 0.05$). Specific lysis was calculated according to the following formula: (experimental CPM-spontaneous CPM/maximum CPM-spontaneous CPM) x 100. 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.*

Figure 7. MUC1-specific immunization elicits A) T cells expressing IFN-γ and B) T cells that recognize H-2D^b/MUC1 tetramer. At 6, 14 and 20 weeks post immunization, PBLs were analyzed for presence of T cells expressing IFN-γ and T cells recognizing H-2D^b/MUC1 tetramer. **A)** Six weeks post immunization, mice in all treatment groups showed an increased percent of T cells expressing IFN-γ (** $p < 0.01$ for L-MUC1-TR groups). No difference in L-IL-2 treated group was observed at 6 weeks, as L-IL-2 treatment only begins at week 7. By 14 weeks post immunization, these T cells further increased. By 20 weeks post immunization, these cells decreased and were at levels similar to that observed at 6 weeks post immunization. At 14 weeks, % T cells expressing intracellular IFN-γ was significantly higher ($p < 0.06$) in the L-MUC1-TR treated and L-IL-2 treated groups as compared to mice treated with empty liposome. Much higher significance ($p < 0.005$) is observed between L-MUC1-TR + L-IL-2 treated group versus empty liposome group. * *indicates comparison of untreated controls versus treatment groups.*

B) Compared to untreated controls, mice in all treatment groups showed significant increase in percent T cells recognizing H-2D^b/MUC1 tetramer as early as 6 weeks post immunization. By 14 - 20 weeks post immunization, treated mice showed higher percent of circulating T cells recognizing H-2D^b/MUC1 tetramer. At all time points, % T cells recognizing MUC1-tetramer was significantly higher ($p < 0.01$) in the L-MUC1-TR treated group as compared to mice treated with empty liposome. Significant difference ($p < 0.01$) was also observed between the L-MUC1-TR + L-IL-2 treated group versus empty liposome group at 14 and 20 week time point but not at 6 week time point. No significant difference was observed between L-IL-2 treated group versus empty liposome group. * *indicates comparison of untreated controls versus treatment groups.* Average of n = 6 mice / group is shown. *P* values are from the pair-wise contrasts from a one-way ANOVA model.

Figure 8. Circulating MUC1 and anti-MUC1 levels in serum of immunized MMT mice. A) Circulating MUC1 levels increase significantly with MUC1-specific immunization as well as with IL-2 treatment. B) Low levels of MUC1 antibodies were detected in MMT mice immunized with MUC1 TR containing vaccine formulation. IL-2 treatment did not elicit MUC1-specific antibodies. 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.*

Figure 9. Tumors from MMT mice express TGF-β as early as 6 weeks of age, which increases with time. Methacarn fixed and paraffin-embedded sections of mammary gland tumors from 6, 12, 18 and 21 week old MMT mice were stained with antibody to TGF-β2. MMT

tumor at A) 6 weeks; B) 12 weeks; C) 18 weeks; and D) 21 weeks of age. Images were captured at 200X magnification.

Figure 10. Tumor progression in MUC1.Tg mice immunized with tumor lysate-pulsed DC. MUC1.Tg mice received two intradermal injections, two weeks apart, of C57mg.MUC1 tumor lysate-pulsed DC at 1 X 10^7 DC per mouse. One week after the last injection, mice were challenged with 2 X 10^6 C57mg.MUC1 tumor cells (subcutaneously). All 5 immunized mice were protected from tumor challenge, while all 3 control mice developed tumors. One control mouse had delayed tumor growth.

Table 1. Incidence of lung metastasis in MMT mice. Pulmonary metastases was determined by counting gross morphological disease using low power microscopy. We determined how many mice developed lung metastasis.

Table 2. Immunophenotype of lymphocytes as tumors progress in MMT mice. We have determined expression of early and late activation markers (CD69 and IL-2R) on T cells, as well as levels of intracellular cytokines produced by T cells (γ-IFN, IL-2, IL-4, IL-5, IL-10) and dendritic cells (IL-12). The numbers represent percent positive cells stained with the specific antibody and analyzed by flow cytometry. Increases were observed in some of the markers (IL-2R, and intracellular IL-4 and IL-12) at the 14 week time point that declined by 21 week but the difference was not statistically significantly. $CD11c^+$ and $B7^+$ cells were significantly lower at 21 weeks (when the tumor burden was high) as compared to 6 and 14 weeks. * indicate $p < 0.05$.

Average of n = 6 mice / group is shown. *P* values are from the pair-wise contrasts from a one-way ANOVA model.

Table 3. Surface expression of MHC class I molecule on tumor cells decreases as tumors progress in MMT and MET mice. FACs analysis demonstrating percent cells positive for pan-cytokeratin and MHC class I molecule ($H\text{-}2D^b/K^b$).

Figure1.

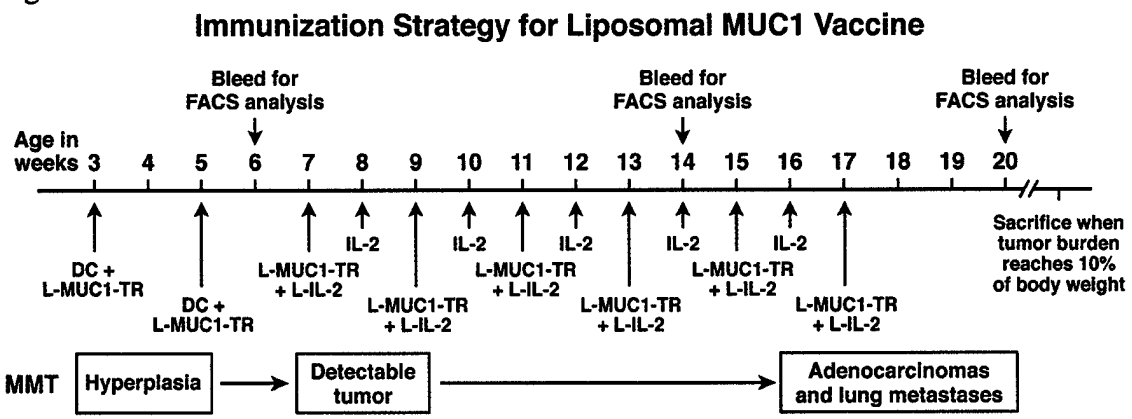
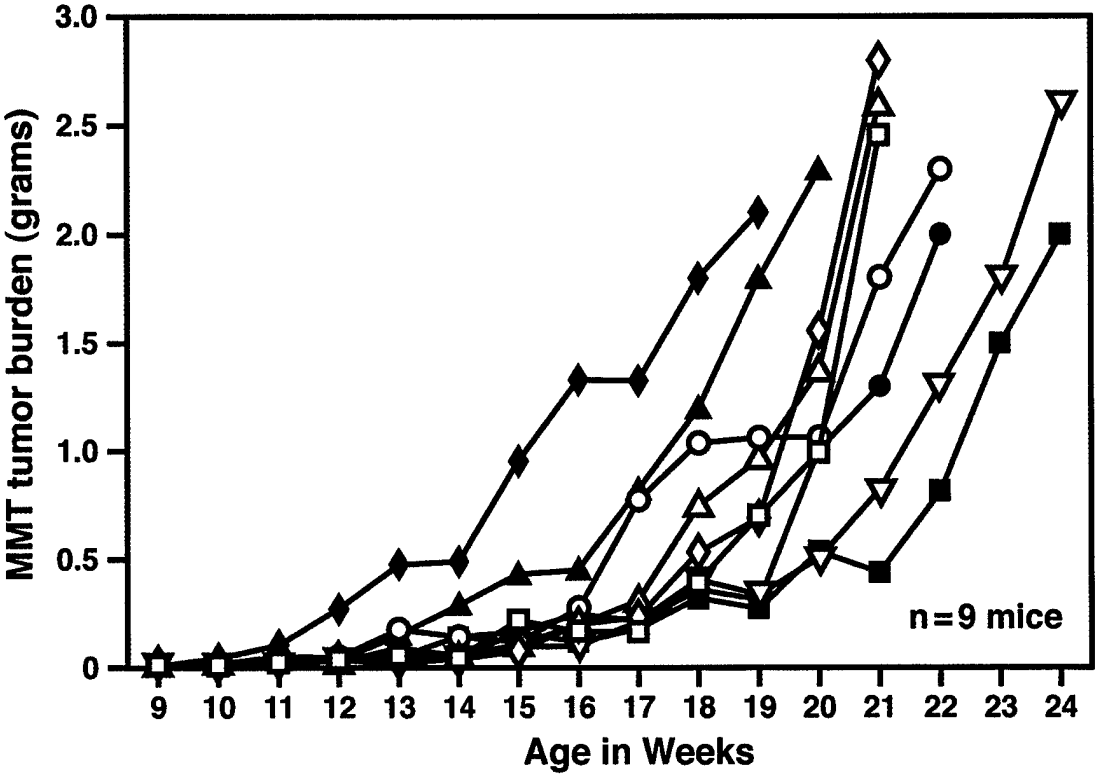

Immunization Strategy for Liposomal MUC1 Vaccine
Bleed for FACS analysis
Bleed for FACS analysis
Bleed for FACS analysis
Age in weeks 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
DC + L-MUC1-TR
DC + L-MUC1-TR
L-MUC1-TR + L-IL-2
IL-2
L-MUC1-TR + L-IL-2
IL-2
L-MUC1-TR + L-IL-2
IL-2
L-MUC1-TR + L-IL-2
IL-2
L-MUC1-TR + L-IL-2
IL-2
L-MUC1-TR + L-IL-2
Sacrifice when tumor burden reaches 10% of body weight
MMT
Hyperplasia
Detectable tumor
Adenocarcinomas and lung metastases

Figure 2.


3.0
2.5
2.0
1.5
1.0
0.5
0
MMT tumor burden (grams)
n=9 mice
9
10
11
12
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14
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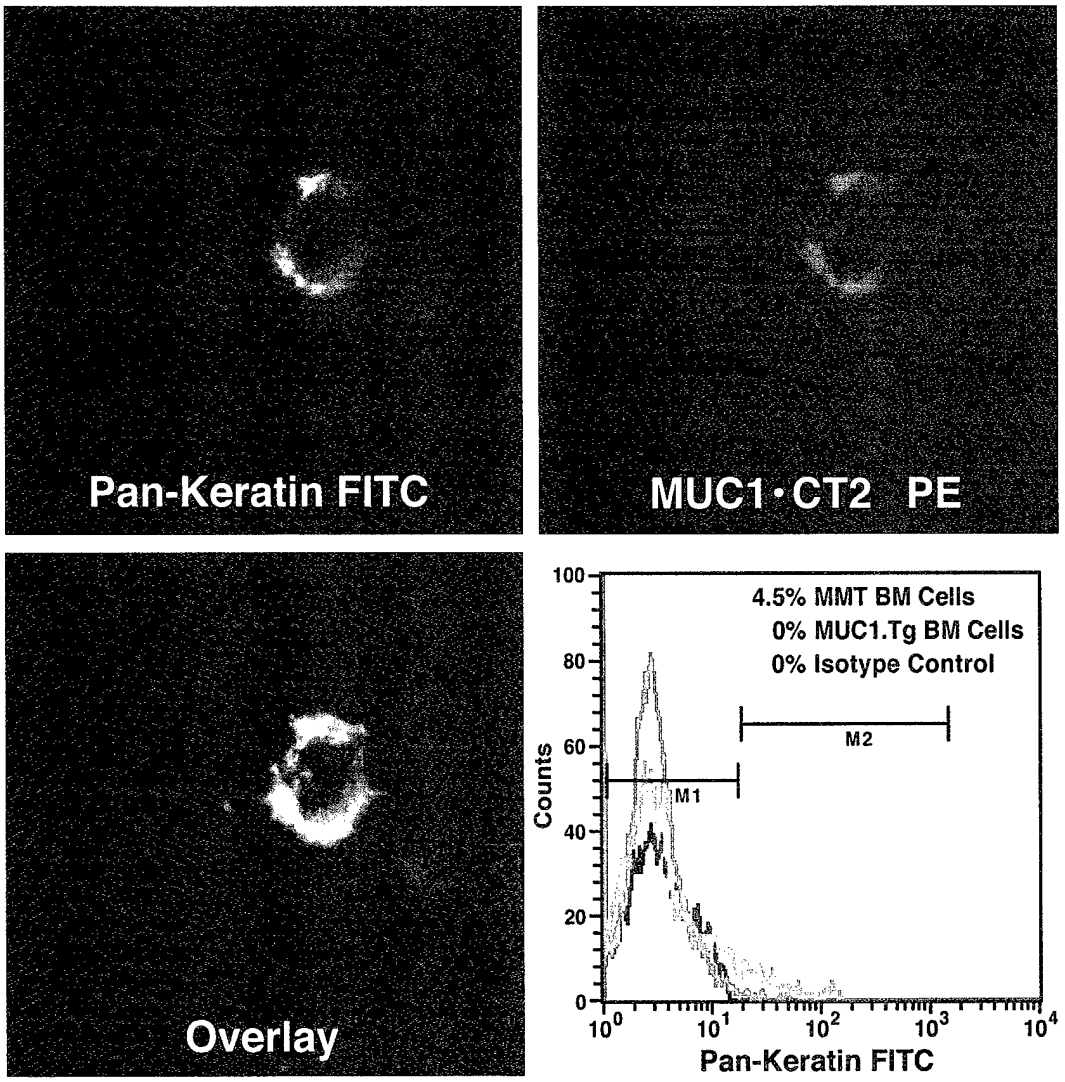
16
17
18
19
20
21
22
23
24
Age in Weeks

Figure3.


Pan-Keratin FITC
MUC1·CT2 PE
Overlay
4.5% MMT BM Cells
0% MUC1.Tg BM Cells
0% Isotype Control
M1
M2
Counts
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
Pan-Keratin FITC

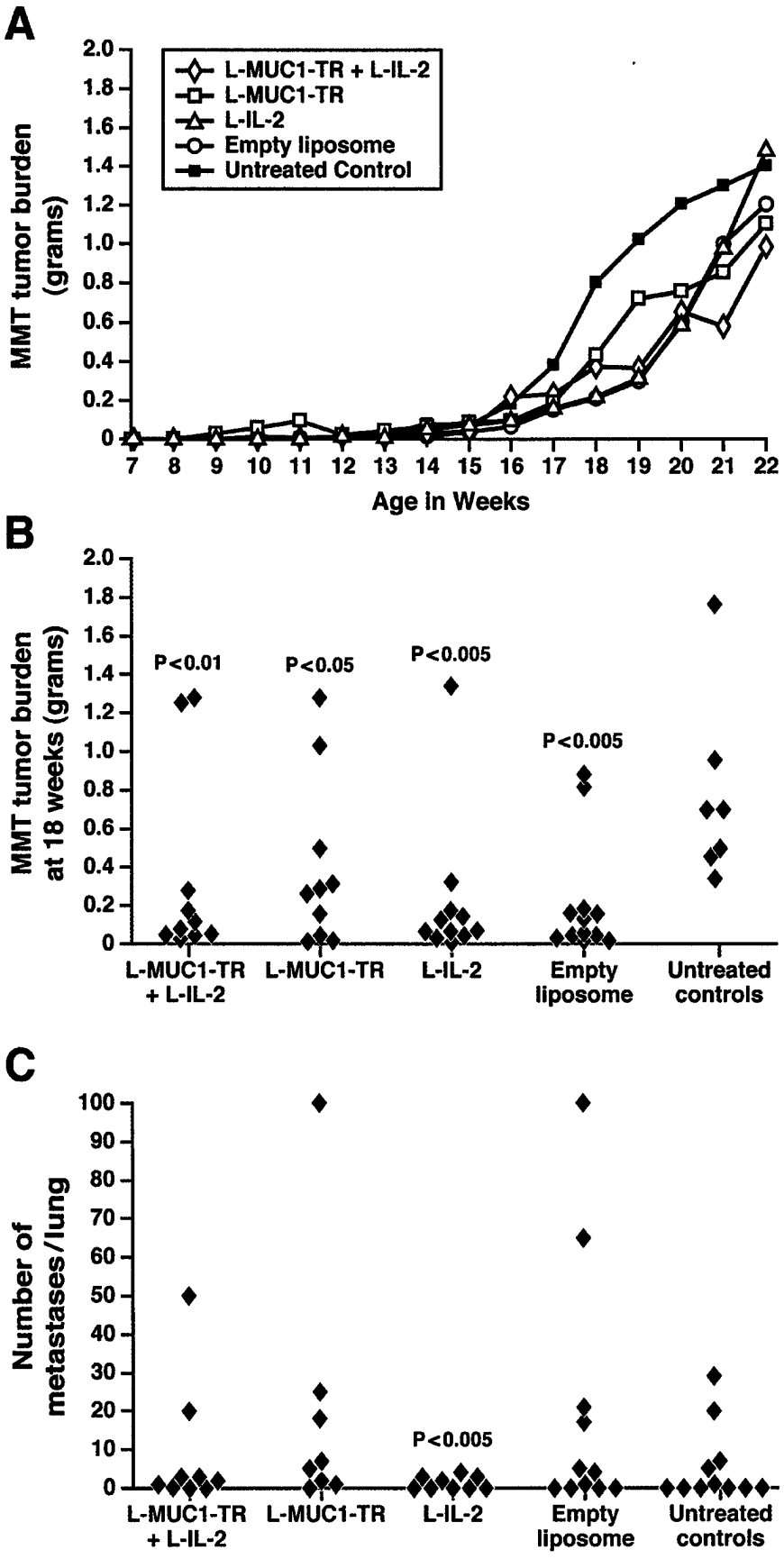

A
L-MUC1-TR + L-IL-2
L-MUC1-TR
L-IL-2
Empty liposome
Untreated Control
MMT tumor burden (grams)
Age in Weeks
B
MMT tumor burden at 18 weeks (grams)
P<0.01
P<0.05
P<0.005
P<0.005
L-MUC1-TR + L-IL-2
L-MUC1-TR
L-IL-2
Empty liposome
Untreated controls
C
Number of metastases/lung
P<0.005
L-MUC1-TR + L-IL-2
L-MUC1-TR
L-IL-2
Empty liposome
Untreated controls

Figure 4.

Figure5.

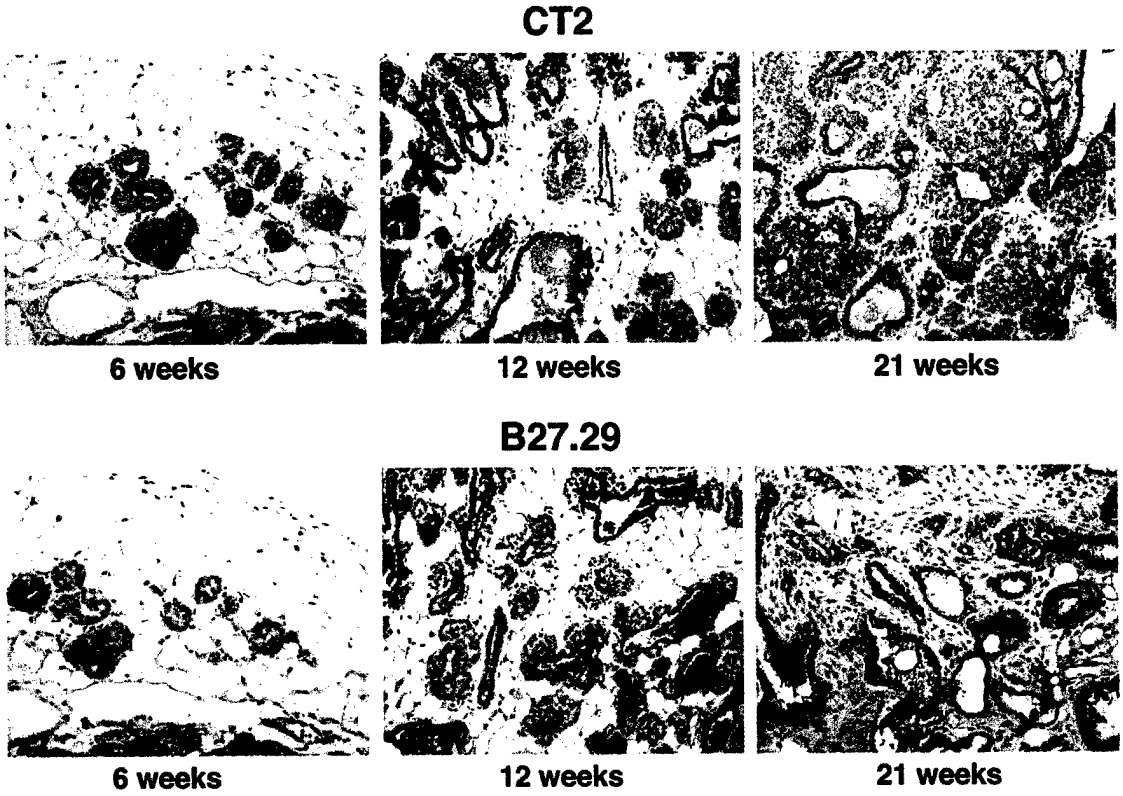
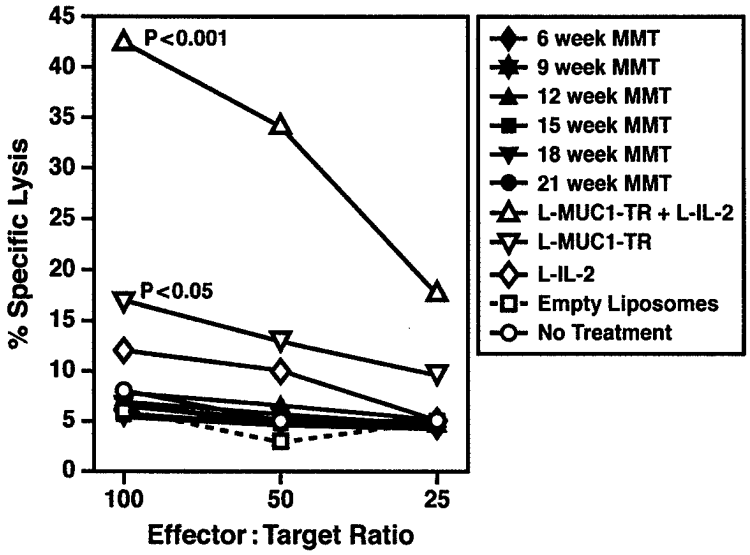


Figure6.


P<0.001
P<0.05
% Specific Lysis
45
40
35
30
25
20
15
10
5
0
100
50
25
Effector : Target Ratio
6 week MMT
9 week MMT
12 week MMT
15 week MMT
18 week MMT
21 week MMT
L-MUC1-TR + L-IL-2
L-MUC1-TR
L-IL-2
Empty Liposomes
No Treatment

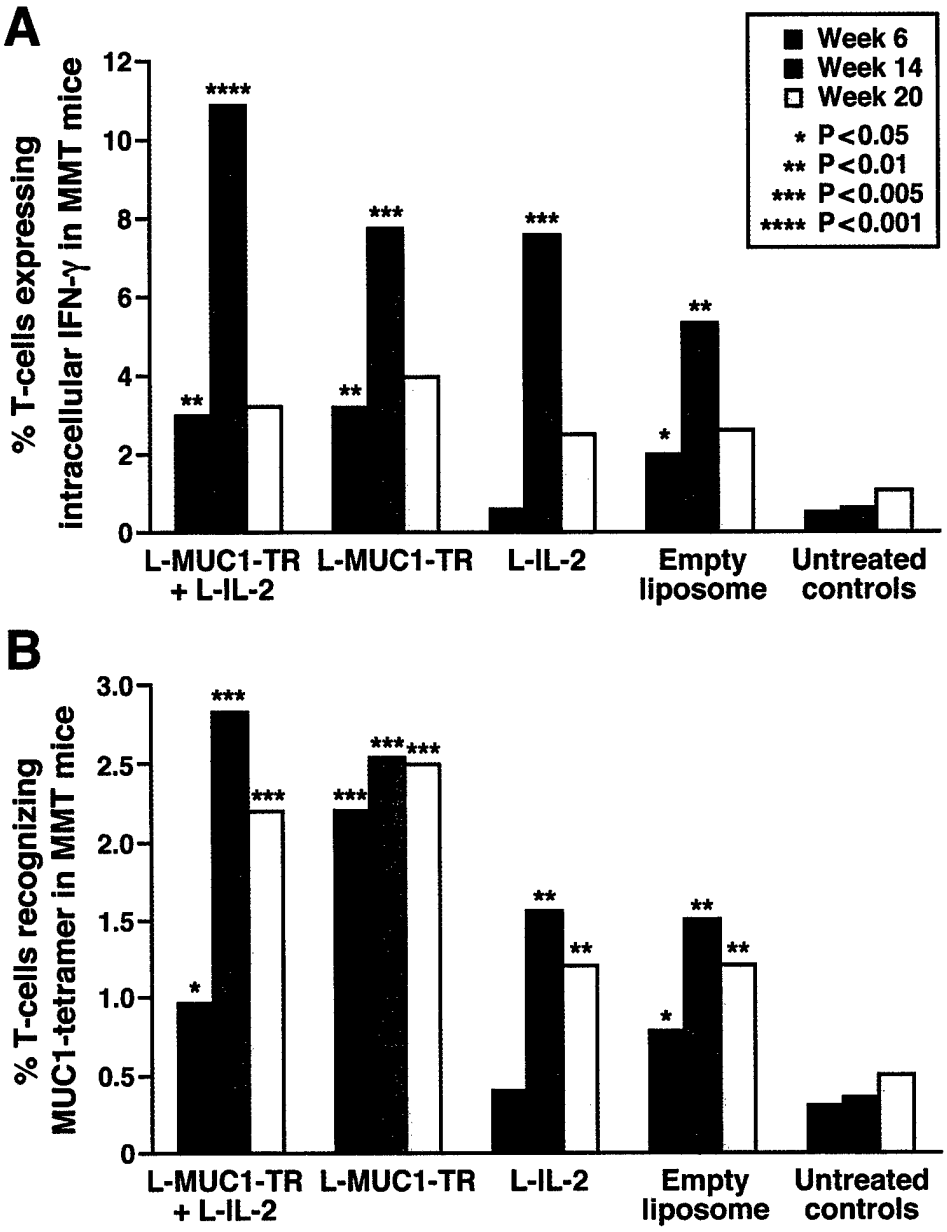

A
% T-cells expressing intracellular IFN-γ in MMT mice
Week 6
Week 14
Week 20
* P<0.05
** P<0.01
*** P<0.005
**** P<0.001
L-MUC1-TR + L-IL-2
L-MUC1-TR
L-IL-2
Empty liposome
Untreated controls
B
% T-cells recognizing MUC1-tetramer in MMT mice
L-MUC1-TR + L-IL-2
L-MUC1-TR
L-IL-2
Empty liposome
Untreated controls

Figure 7.

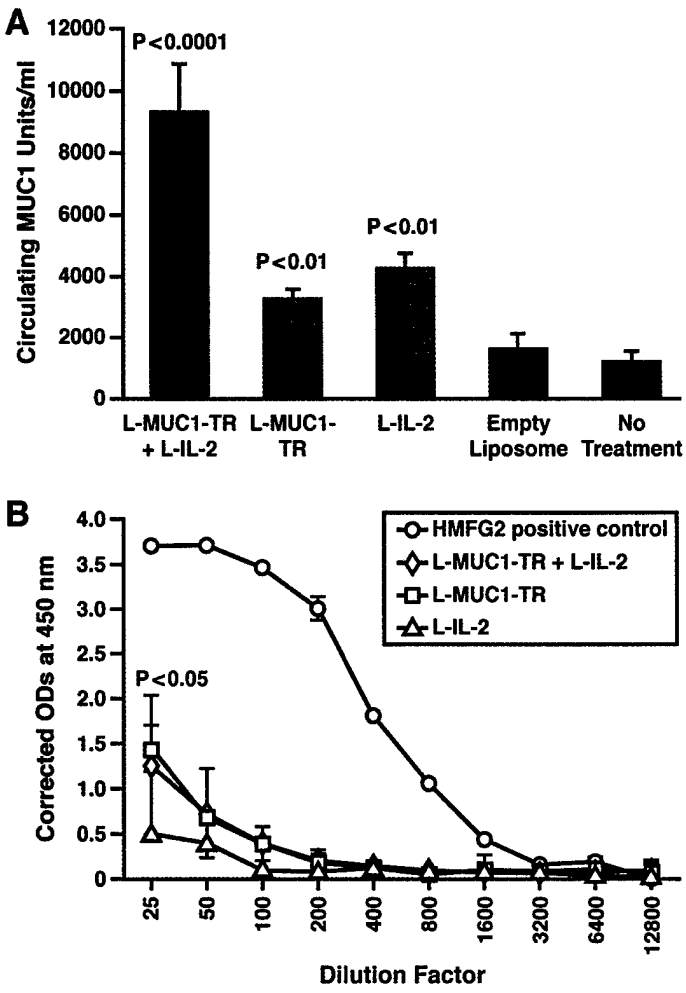

A
Circulating MUC1 Units/ml
12000
10000
8000
6000
4000
2000
0
P<0.0001
P<0.01
P<0.01
L-MUC1-TR + L-IL-2
L-MUC1-TR
L-IL-2
Empty Liposome
No Treatment
B
Corrected ODs at 450 nm
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
P<0.05
HMFG2 positive control
L-MUC1-TR + L-IL-2
L-MUC1-TR
L-IL-2
25
50
100
200
400
800
1600
3200
6400
12800
Dilution Factor

Figure8.

Figure9.

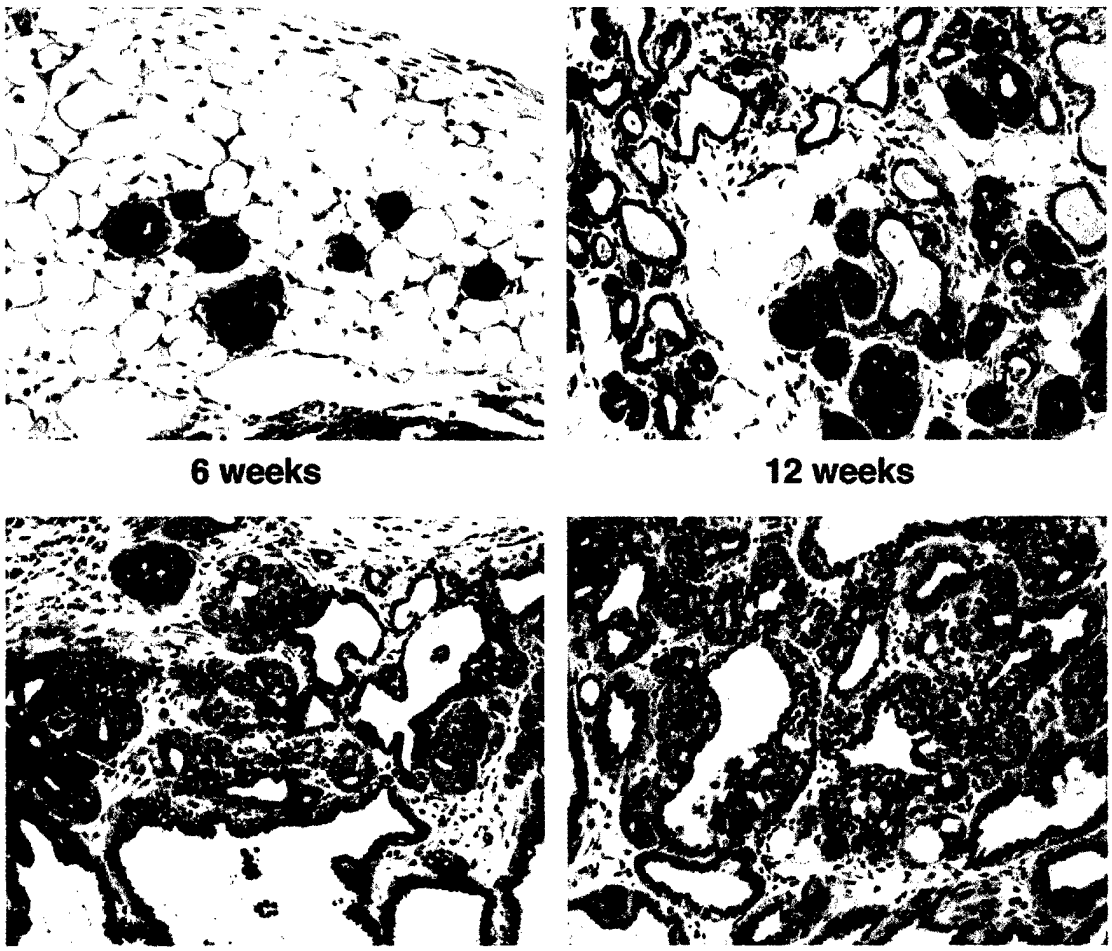


Figure 10.

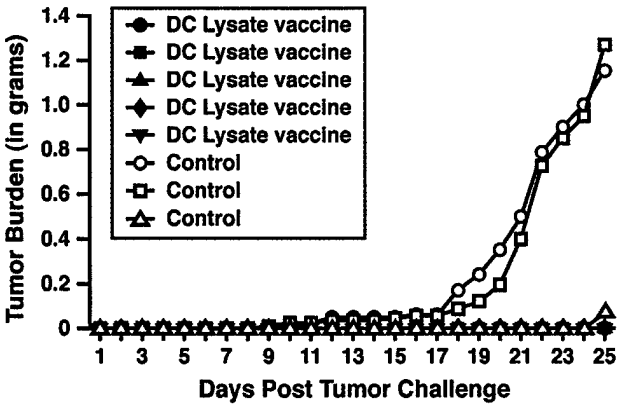

DC Lysate vaccine
DC Lysate vaccine
DC Lysate vaccine
DC Lysate vaccine
DC Lysate vaccine
Control
Control
Control
Tumor Burden (in grams)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
1 3 5 7 9 11 13 15 17 19 21 23 25
Days Post Tumor Challenge

Table 1

Percentage of MMT Mice with Lung Metastasis

Treatment	Number	%
L-MUC1 TR + L-IL-2	6/9	66%
L-MUC1 TR	7/8	87%
L-IL-2	4/9	44%
No treatment	8/12	64%

Table 2

Surface and intracellular markers

Markers	6 week MMT	14 week MMT	21 week MMT
IL-2R	1.58	3.28	0.28
CD69	0.75	0.30	0.17
IL-2	1.03	1.29	0.20
γ-IFN	0.50	0.73	0.38
IL-4	0.56	7.67	2.31
IL-5	0.16	2.61	2.28
IL-10	0.24	0.43	0.42
IL-12	0.26	6.43	2.69
B7	4.50	7.70	1.30*
CD11c	11.20	12.30	5.50*
MUC1-Tetramer	0.31	1.32	0.19

Table 3

MHC Class I expression on tumor cells

Tumor age (wk)	MHC I expression
6	12.5 ± 2.0
12	4.1 ± 0.89
18	1.5 ± 0.5

n = 5 MMT mice

MUC1-specific Cytotoxic T Lymphocytes Eradicate Tumors When Adoptively Transferred *in Vivo*

Pinku Mukherjee, Amelia R. Ginardi, Teresa L. Tinder, Christopher J. Sterner, and Sandra J. Gendler[1]

Department of Biochemistry and Molecular Biology, Mayo Clinic Scottsdale, Scottsdale, Arizona 85259

Abstract

We have reported previously that MUC1 transgenic mice with spontaneous tumors of the pancreas (designated MET) naturally develop MHC class I-restricted, MUC1-specific CTLs as tumors progress (P. Mukherjee et al., J. Immunol., *165:* 3451–3460, 2000). From these MET mice, we have isolated, expanded, and cloned naturally occurring MUC1-specific CTLs *in vitro*. In this report, we show that the CTL line is predominantly $CD8^+$ T cells and expresses T-cell receptor Vβ chains 5.1/5.2, 11, 13, and 2 and Vα chains 2, 8.3, 3.2, and 11.1/11.2. These CTLs recognize several epitopes on the MUC1 tandem repeat with highest affinity to APGSTAPPA. The CTL clone, on the other hand, is 100% $CD8^+$ cells and expresses a single Vβ chain of 5.1/5.2 and Vα2. It recognizes only the H-$2D^b$ class I-restricted epitope of MUC1, APGSTAPPA. When adoptively transferred, the CTLs were effective in eradicating MUC1-expressing injected tumor cells including mammary gland cells (C57mg) and B16 melanomas. These results suggest that MUC1-specific CTLs are capable of possibly preventing, or at least substantially delaying, MUC1-expressing tumor formation. To our knowledge, this is the first evidence that demonstrates that the naturally occurring MUC1-specific CTLs isolated from one tumor model has antitumor effects on other MUC1-expressing tumors *in vivo*. Therefore, our data confirm that MUC1 is an important tumor rejection antigen and can serve as a target for immunotherapy.

Introduction

Human cancer remains a major health problem, and novel therapeutic approaches are necessary to advance patient care. The recent molecular identification of tumor antigens recognized by CTLs derived from cancer patients has initiated a new era in tumor immunology. Thus far, nearly all of the defined tumor antigens known to stimulate CTL responses consist of a short antigenic peptide, either associated or nonassociated with an MHC class I molecule (1). The identification of antigenic peptides and the availability of recombinant proteins and cytokines have given impetus to immunotherapy. One idea is based on the potential of the synthetic peptides to mimic endogenously produced antigenic peptides. Synthetic peptides have been used successfully to expand *in vitro* tumor-reactive CTLs in blood samples from cancer patients (1). This approach is being exploited to generate large numbers of autologous tumor antigen-specific CTLs for adoptive transfer immunotherapy. MUC1 is one such tumor antigen that is being used by several investigators for the generation of CTLs.

MUC1 is a cell-associated mucin that is developmentally regulated and aberrantly expressed by carcinomas, which makes it an important marker in malignancy (2–6). MUC1 is a particularly attractive target for immunotherapy because the expression of MUC1 is altered in cancer. For example, MUC1 is overexpressed, aberrantly glycosylated, and ubiquitously distributed all around the cell rather than being restricted to the apical surfaces of ducts and glands. The recent description of MUC1 as a target for CTLs has raised interest in using this protein as a target for immunotherapy (7, 8). It is expressed by most adenocarcinomas of the breast, lung, stomach, pancreas, colon, prostate, ovary, and cervix and elicits humoral and cellular immune responses in humans. In 1997, cancers that expressed MUC1 accounted for ~72% of new cases and for 66% of deaths (9). However, the presence of these responses in patients with progressive disease suggests that immune recognition is not followed by eradication of tumors. We hypothesize that tumors can be recognized *in vivo* by antigen-specific T cells, but that such encounters occur late in tumor progression and ultimately lead to exclusion, deletion, or dysfunction of responding T cells such that the antitumor response is interrupted. Understanding this phenomenon is essential for the development of effective immunotherapy strategies to restore antitumor immunity in cancer patients. Until recently, the mouse has not been a suitable preclinical model for testing MUC1-specific immune responses because human MUC1 differs substantially in sequence from mouse Muc1 and is strongly antigenic in the mouse.

In this project, we have used a MUC1 transgenic mouse (MUC1.Tg) expressing human MUC1 as a self-molecule crossed with an oncogene-expressing mouse to generate mice that spontaneously develop MUC1-expressing ($MUC1^+$) tumors of the pancreas (MET mice). As tumors progress in the MET mice, low level anti-MUC1 cellular and humoral immune responses develop that are not effective against the spontaneous pancreatic tumors. However, we were able to isolate MUC1-specific CTLs from these mice and develop CTL lines and clones *in vitro*. These CTLs were effective in eradicating $MUC1^+$ tumors *in vivo* when adoptively transferred, suggesting that anti-MUC1 immune responses can be effective against tumors if they occur early during tumor progression. Moreover, these CTLs are effective against $MUC1^+$ tumors and have no effect on $MUC1^-$ tumors. These CTLs are reactive only against MUC1 as expressed by the tumors and do not cause any autoimmune destruction of organs that normally express MUC1.

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Materials and Methods

Mouse Models. MUC1 transgenic (MUC1.Tg) mice were developed in our laboratory and have been described previously. These mice express human MUC1 in a developmentally regulated and tissue-specific fashion because the endogenous MUC1 promoter was used to drive expression. Mice transgenic for this protein develop B- and T-cell compartment tolerance and are refractory to immunization with the protein encoded by the transgene (10). MET mice were generated as described previously (11). MUC1 transgenic mice are bred with oncogene-expressing mice that spontaneously develop tumors of the pancreas (ET mice) and are designated as MET. The ET mice were obtained from Dr. Judith Tevethia (Pennsylvania State University, Hershey, PA) (12) and express the first 127 amino acids of SV40 large T antigen under the control of the rat elastase promoter. Fifty % of the animals develop life-threatening pancreatic tumors by ~21 weeks of age (12). The spontaneous tumors in MET mice arise naturally in an appropriate tissue background and in the context of a viable immune system. The tumors develop more slowly than injected tumors, giving the host immune system time to respond. All mice are on the C57BL/6 background. Mice were bred and maintained in specific pathogen-free conditions in the S. C. Johnson Medical Research Building animal facility at Mayo Clinic Scottsdale. All experimental procedures were conducted according to Institutional Animal Care and Use Committee guidelines.

PCR Screening. PCR was used to routinely identify MUC1.Tg- and ET-positive mice in the colony. PCR was carried out as described previously (10, 12). The primer pairs for MUC1.Tg were 5′-CTTGCCAGCCATAGCACCAAG-3′ (745–765 bp) and 5′-CTCCACGTCGTGGACATTGATG-3′ (1086–1065 bp); for ET, 5′-GCTCCTTTAACCCACCTG-3′ (4055–4072 bp) and 5′-CCAACCTATGGAACTGATGAATG-3′ (4546–4568 bp). The amplification program consisted of one cycle of 5 min at 95°C and 40 cycles of 30 s each at 95°C, 52°C, and 72°C, followed by one cycle of 10 min at 72°C. The PCR product of each reaction was analyzed by size fractionation through a 1% agarose gel. Amplification conditions for MUC1 were the same, except the annealing temperature was 61°C. Amplification of MUC1 resulted in a 500-bp fragment and of ET in a 491-bp fragment.

Cell Lines. Cell lines used in the study included a B16 murine melanoma cell line expressing full-length human MUC1 (B16.MUC1) and B16 transfected with vector only (B16.neo; Ref. 10). These cell lines were kindly provided by Dr. Tony Hollingsworth (University of Nebraska Medical Center, Omaha, NE). B16.MUC1 and B16.neo were maintained in DMEM with 10% fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and 1% Glutamax (DMEM complete medium), supplemented with 300 μg/ml G418 (Life Technologies, Inc., Grand Island, NY). Cells were routinely tested by flow cytometry for the presence of MUC1. C57mg.MUC1 is a mammary gland tumor cell line derived from C57BL/6 mice transfected with full-length human MUC1. These cells were maintained in DMEM complete medium supplemented with 150 μg/ml G418 and 10 μg/ml insulin. L cells transfected with either MHC class I H-2D^b or H-2K^b molecule were a gift from Dr. Larry Pease (Mayo Clinic, Rochester, MN). These cells were maintained in RPMI 1640 supplemented with 10% HAT (Life Technologies, Inc.).

Generation of the CTL Line and Clone. CTLs were obtained from splenocytes of MET mice, 18 weeks of age, and expanded on IFN-γ-treated, irradiated B16.MUC1 cells (20,000 rads for 2 consecutive days). The irradiated B16.MUC1 cells served as feeders. CTLs were maintained on the feeders with IL[2]-2 (100 units/ml) and IFN-γ (150 pg/ml) for 2 weeks prior to removing them from antigenic stimulus and growing on irradiated splenocyte feeders with IL-2 (100 units/ml) for another 2–3 weeks until cells reached confluency. CD8-expressing (CD8$^+$) cells were selected (98% of the CTLs were CD8$^+$) by magnetic activated cell sorting and further maintained on irradiated splenocyte feeders with IL-2 (100 units/ml). When cells reached confluency, their lytic activity was determined by an *in vitro* CTL assay using B16.MUC1 as target cells. These cells were then maintained as a CTL line in DMEM complete medium supplemented with IL-2 (100 units/ml). A portion of these CTLs was used to generate CTL clones by limiting dilution procedure, such that each well in a 96-well plate (containing splenic feeders) received a single cell. We have generated 550 clones with this procedure, and in this report, we present studies completed with the CTL line and one clone (TR clone 1).

Magnetic Activated Cell Sorting. Lymphocytes were stained for 30 min on ice with anti-CD8 antibody conjugated to microbeads (Miltenyi Biotechnologies, Auburn, CA). CD8$^+$ cells were positively selected on an RS-type magnetic column using the Vario MACs magnetic device, following the protocol provided by the manufacturer (Miltenyi Biotechnologies). Purity of the selected cells was verified by flow cytometry and ranged from 92 to 95%. Cells were further cultured for *in vitro* CTL assay and adoptive transfer experiments.

Flow Cytometry. Tumor cell lines were analyzed by immunofluorescence for surface expression of MHC class I and MUC1. The MUC1-specific CTL line and TR clone 1 were analyzed by two-color immunofluorescence for surface expression of CD8$^+$ and TCR Vα chains or TCR Vβ chains as per the manufacturer's recommendations. All fluorescently labeled antibodies were purchased from PharMingen (San Diego, CA). The antibody used for MUC1 staining was HMFG-2, a monoclonal antibody (2). The minimum epitope recognized by HMFG-2 is the immunodominant MUC1 TR epitope, DTR (13). MHC class I H-2D^b tetramers containing MUC1 TR APGSTAPPA peptides were created by Dr. Larry Pease at Mayo Clinic Rochester. CTLs were stained with 0.1 mg/ml MUC1 tetramers (directly conjugated to phycoerythrin) for 1 h on ice. The irrelevant tetramer used was Theiler's virus epitope D^b:VP2$_{121-130}$ (FHAGSLLVFM; Ref. 14). The cells were then stained with anti-CD8 antibody conjugated to FITC for 15 min prior to analysis by two-color flow cytometry. Flow cytometric analysis was done on a Beckon Dickinson FACScan using the Cell Quest program.

[2] The abbreviations used are: IL, interleukin; TR, tandem repeat; TCR, T-cell receptor; DC, dendritic cell; FACS, fluorescence-activated cell sorter.

CTL Assays. Determination of CTL activity was performed using a standard 6-h ^{51}Cr-release method. CTL line and clones served as effectors, and B16.MUC1, B16.neo, and C57mg.MUC1 served as targets. For MHC class I restriction experiments, L cells transfected with either H-2D^b or H-2K^b were used as targets. These cells were pulsed overnight with 10^{-6} M MUC1 TR peptides, STAPPAHGV and SAPDTRPAP, respectively. For L cells, the ^{51}Cr-release assay was done for 8 h instead of 6 h. Bone marrow-derived DCs pulsed with MUC1 TR peptide APGSTAPPA and TR 25-mer STAPPAHGVTSAPDTRPAPGSTAPP (STAPPAH) were used as targets for testing the specificity of the CTL TR clone 1. The use of DCs as targets has been described previously (11). Specific ^{51}Cr-release was calculated according to the following formula: (experimental cpm − spontaneous release cpm/maximum release cpm − spontaneous release cpm) × 100. Spontaneous release in all experiments was <15% of maximum release.

Adoptive Transfer. MUC1.Tg mice were injected (s.c. in the flank) with B16.MUC1 or B16.neo or C57mg.MUC1 tumor cells (1 or 2 × 10^6 cells/mouse/100 μl). Simultaneously, one group of mice received (by i.v. injection) CTLs (5 × 10^6 cells/mouse/100 μl), and the other control group received medium alone. All mice received the tumor cells.

Palpations and Determination of Tumor Weights. Palpations were started 3–5 days after tumor challenge. Tumors were measured using a metric dial caliper (Monostat Corp., Pequannock, NJ), and tumor weights were determined by the formula $(W^2 \times L)/2$.

Results

MET Mice Naturally Develop MUC1-specific CTLs. We have shown previously that by 15 weeks of age when the MET mice already have a large tumor burden in the pancreas, they develop MUC1-specific CTLs that peak at 18 weeks of age and drop off by 21 weeks. We have also shown that these CTLs recognize the immunodominant TR region of MUC1, and that they are MHC class I restricted (11). One of the major goals of immunotherapy is to generate and target CTLs that can effectively eliminate tumor cells. Although the MET mice do develop CTLs, the CTLs develop late in tumor development, when the tumor is of substantial size (1.5–2.0 g) and therefore have no detectable effect on the spontaneous pancreas tumor (11). Thus, we hypothesized that the presence of MUC1-specific CTLs earlier during tumor progression may have an effect against the tumors. To test this hypothesis, we have developed a MUC1-specific CTL line and 550 CTL clones from MET mice 18 weeks of age. Prior to using tumor target cells in a CTL assay to assess lytic activity of the CTL line and TR clone 1, we determined the expression levels of MUC1 and MHC class I on target cells. By FACS analysis, we show that 58% of B16.MUC1 and 44% of B16.neo cells express low levels of MHC class I, and 100% of B16.MUC1 cells express intermediate levels of MUC1 (as assessed by fluorescence intensity). As expected, none of the B16.neo cells expressed any MUC1 (Fig. 1). Similarly, 43% of C57mg.MUC1 cells expressed low levels of MHC class I, and 68% expressed intermediate levels of MUC1 (Fig. 1). The data presented in Fig. 1 are representations of the staining pattern in these cells. This pattern may change

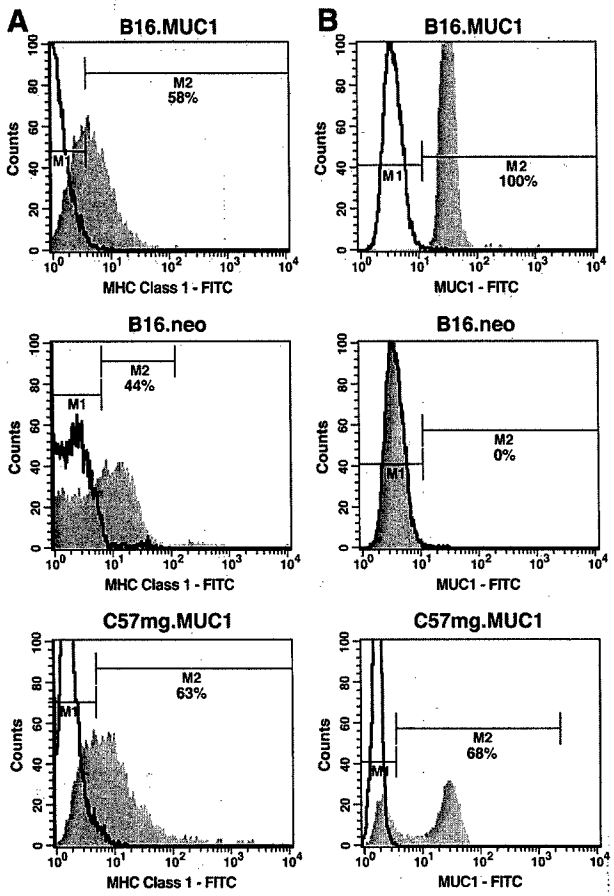

Fig. 1 Flow cytometric analysis of MUC1 and MHC class I expression in B16.MUC1, B16.neo, and C57mg.MUC1 tumor lines. Tumor cell lines were stained with antibody to MHC class I (*A*) and monoclonal antibody to MUC1 (HMFG-2; *B*), and immunofluorescence was analyzed on a Beckon Dickinson FACScan using the Cell Quest program.

with a new batch of cells, time in culture, and with number of passages; we have observed that MUC1 as well as MHC class I levels drop when cells are maintained in culture for extended periods of time (data not shown).

In Fig. 2, we show the *in vitro* lytic activity of the MUC1-specific CTLs against tumor target cells that express MUC1, including B16.MUC1 with 70% lysis and C57mg.MUC1 with 27% lysis at an E:T ratio of 100:1. The CTLs had no effect on tumor cells (B16.neo) that did not express MUC1, showing 7% lysis (Fig. 2*A*), suggesting that the CTLs are specifically directed against the MUC1 antigen. The reason for lower lysis of C57mg.MUC1 cells as compared with B16.MUC1 is not clear, but we do know that C57mg.MUC1 cells grow slower in culture and do not take up ^{51}Cr as effectively as the B16.MUC1 cells (data not shown).

We tested one of the MUC1-specific CTL clones (TR clone 1) for its lytic activity against MUC1. The targets used in this assay were DCs pulsed with either MUC1 TR 25-mer STAPPAH or 9-mer APGSTAPPA peptide. The results in Fig. 2*B* show 50% lysis of DCs pulsed with STAPPAH and 44% lysis of DCs pulsed with APGSTAPPA at an E:T ratio of 100:1. Further characterization of the TR clone 1, using 9-mer over-

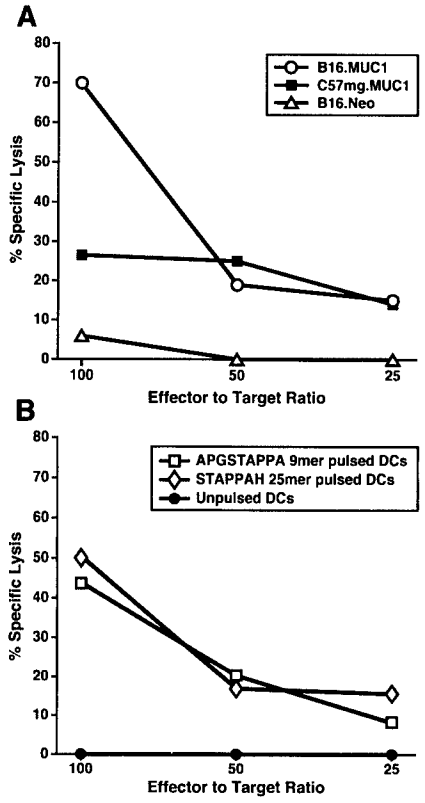

Fig. 2 Cytolytic activity of CTL line and CTL TR clone 1. *A*, CTL activity of the established CTL line against B16.MUC1, B16.neo, and C57mg.MUC1 target cells was determined by a standardized 6-h ^{51}Cr-release assay. *B*, CTL activity of the CTL TR clone 1 was determined against DCs pulsed with the MUC1 TR APGSTAPPA 9-mer peptide, the MUC1 TR STAPPAH 25-mer peptide, and no peptide. Specific lysis was calculated according to the following formula: (experimental cpm − spontaneous cpm/maximum cpm − spontaneous cpm) × 100.

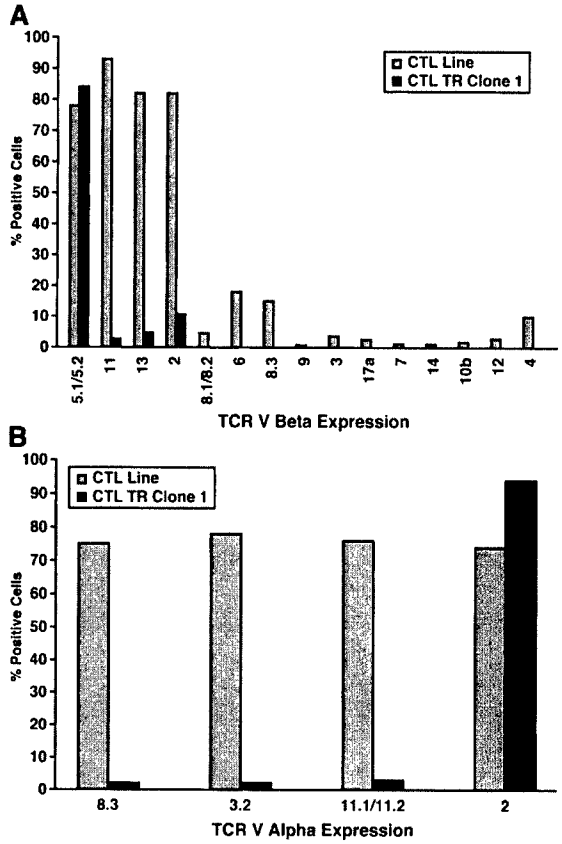

Fig. 3 TCR Vβ and Vα chain expression of the CTL line and TR clone 1. *A*, Vβ expression was determined by staining the CTL line or TR clone 1 with a panel of antibodies directed against 15 different Vβ chains and analyzed by flow cytometry. *B*, Vα expression was analyzed by flow cytometry using four different antibodies to Vα chains.

lapping peptide sequences of the entire MUC1 TR region, suggested that the TR clone 1 exclusively recognized APGSTAPPA epitope and was indeed clonal (data not shown).[4]

MUC1-specific CTL TR Clone 1 Expresses a Single TCR Vβ Chain and a Vα Chain on Its Surface. To further determine the clonality of the CTL TR clone 1, we determined the surface expression of TCR Vα and Vβ chains on the CTL line and TR clone 1. Using a panel of 15 anti-TCR Vβ and 4 anti-TCR Vα antibodies and FACS analysis, we have determined the expression of Vβ and Vα chains of the CTLs. Although the CTL line expresses four TCR Vβ chains 5.1/5.2, 11, 13, and 2 and four TCR Vα chains 8.3, 3.2, 11.1/11.2, and 2, the TR clone 1 expressed a single TCR Vβ chain of 5.1/5.2 and a single TCR Vα 2 chain (Fig. 3).

The MUC1-specific CTL Line Is MHC Class I Restricted. To confirm our previous finding that the MUC1-specific CTLs that naturally develop in the MET mice are MHC class I restricted (11), we tested our CTL line for class I restriction. In this study, we used L cells transfected with either H-2D^b (L-H-2D^b) or H-2K^b (L-H-2K^b) MHC class I molecules and pulsed them overnight with MUC1 TR peptides STAPPAHGV and SAPDTRPAP as target cells. These peptides were chosen because they are known to specifically bind MHC class I H-2D^b and H-2K^b molecules, respectively (15). The CTL line lysed 88% of L-H-2D^b cells pulsed with STAPPAHGV and only 5% of L-H-2K^b cells pulsed with the same peptide. On the other hand, the line showed 30% lysis of L-H-2K^b cells pulsed with SAPDTRPAP as compared with 3% lysis of L-H-2D^b cells pulsed with the same peptide (Fig. 4). This result suggests that the MUC1-specific CTL line recognizes STAPPAHGV presented in context with H-2D^b and SAPDTRPAP presented in context with H-2K^b.

The MUC1-specific CTL Line and TR Clone 1 Bind to the H-2D^b Tetramer Containing APGSTAPPA. Because we had high lysis of DCs pulsed with APGSTAPPA (Fig. 2*B*) and L-H-2D^b cells pulsed with STAPPAHGV (Fig. 4), we were interested in developing H-2D^b tetramers containing one of these peptides. However, previous studies have shown that H-2D^b optimally binds the APGSTAPPA peptide (15); thus, we obtained the H-2D^b tetramer containing the APGSTAPPA peptide, generated by Dr. Larry Pease (14). We show by FACS

[4] Manuscript in preparation.

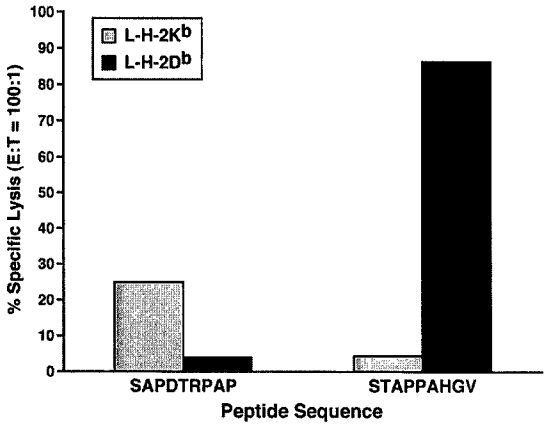

Fig. 4 MHC class I molecule presentation of MUC1 TR epitopes. To determine which MHC class I groove the TR epitopes were presented in and recognized by the CTL line, a standard 8-h ^{51}Cr-release assay was performed. L cells transfected with either the H-2D^b or H-2K^b class I molecule were pulsed overnight with MUC1 TR (10^{-6} M) 9-mer peptides (SAPDTRPAP or STAPPAHGV) and labeled the following day with ^{51}Cr to serve as targets.

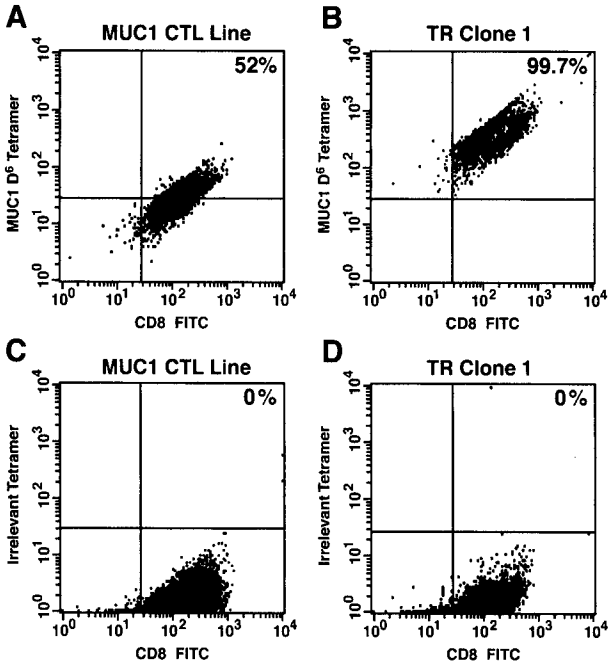

Fig. 5 Tetramer binding assay. MHC class I H-2D^b tetramers containing MUC1 TR peptide APGSTAPPA was analyzed by two-color immunofluorescence for surface expression of CD8 and tetramer binding for the CTL line and TR clone 1. The MUC1-specific tetramer and the irrelevant tetramer ($VP2_{121-130}$) were used at 0.1 mg/ml on 10^6 cells.

analysis that 65% of the CTL line binds H-2D^b tetramer containing APGSTAPPA, whereas 100% of CTL TR clone 1 bind the tetramer (Fig. 5). To complete the analysis of MUC1 epitopes recognized by the CTLs, we used 9-mer overlapping peptide sequences of the: (*a*) TR region; (*b*) extracellular region outside of TR region; (*c*) cytoplasmic tail region; and (*d*) transmembrane region of MUC1 to map the entire MUC1 molecule.[4] These data, taken together, suggest that both the CTL line and TR clone 1 have high lytic activity against MUC1$^+$ tumor cells and recognize specific TR epitopes of MUC1. We therefore wanted to test whether the MUC1-specific CTL line and TR clone 1 are effective against MUC1$^+$ tumor cells when adoptively transferred *in vivo*.

Adoptively Transferred CTLs from MET Mice Are Able to Successfully Reject MUC1$^+$ Tumors in MUC1.Tg Mice. We have shown previously that the MUC1-specific CTL line (5×10^6 cells) could successfully eradicate transplanted B16.MUC1 tumor cells (1×10^6 cells s.c.) from MUC1.Tg mice and that this response was specific to MUC1 antigen (11). In comparison, the CTLs are effective even when tumor burden is increased from 1×10^6 cells to 2×10^6 cells/mouse. Mice that received 2×10^6 B16.MUC1 tumor cells and CTLs at the same time did not develop tumors, whereas mice without CTLs developed tumors by 10 days after injection, and tumors reached 10% of the body weight by 21 days after injection ($P < 0.0001$; Fig. 6*A*). To determine whether the CTLs were effective against established tumors, we injected mice with 1×10^6 tumor cells/mouse 4 and 7 days prior to CTL injections and monitored tumor growth. We show in Fig. 6*B* that the CTLs were effective in substantially delaying the tumor onset in these mice ($P < 0.006$). Once the tumors developed, they grew progressively.

Considering that the CTLs were grown on irradiated B16.MUC1 melanomas, the question arises as to whether the CTLs would be as effective against a different MUC1$^+$ tumor. *In vitro,* the CTLs were effective against the mammary gland tumor cell line C57mg.MUC1 (Fig. 2). We show here that the CTLs were also successful in eradicating C57mg.MUC1 when adoptively transferred *in vivo* (Fig. 6*C*). All mice that received the tumor cells along with the CTLs did not develop tumors ($P < 0.0001$). The eradication of the C57mg.MUC1 mammary gland cells once again shows that the CTLs are specific for MUC1 and that their lytic activity is independent from the B16 cell line on which they were originally expanded.

Thus far, all of the adoptive transfer studies involved the CTL line; thus, in Fig. 7, we determined the effectiveness of the MUC1-specific CTL TR clone 1 in eradicating MUC1$^+$ tumor cells when adoptively transferred. Similar to the CTL line, MUC1-specific CTL TR clone 1 was extremely effective in eradicating B16.MUC1 cells *in vivo* ($P < 0.0001$). Mice injected with TR clone 1 have shown no tumor growth up to 41 days. Although we have not tested the MUC1 specificity of the TR clone 1 in this adoptive transfer experiment, we have shown by *in vitro* CTL assay (Fig. 2*B*) and by tetramer staining (Fig. 5) that TR clone 1 is specific against MUC1 and recognizes a single MUC1 TR epitope, APGSTAPPA.

Discussion

One of the major goals of immunotherapy is to generate and direct CTLs that can effectively eliminate tumor cells. Much of the research has focused on identifying and characterizing proteins expressed on tumor cells that may serve as potential tumor-specific antigens for recognition by CTLs (16). Some of the most promising candidates represent conventional cellular proteins that are expressed in both normal and transformed cells

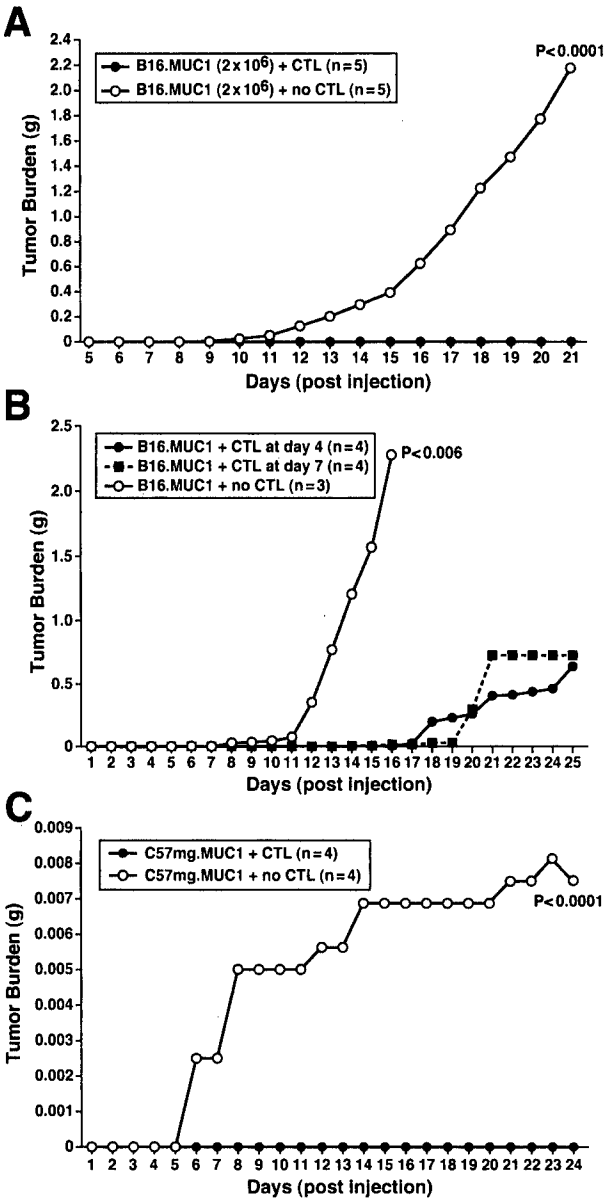

Fig. 6 Adoptive transfer of the CTL line. *A,* tumor growth curve of B16.MUC1 cells at a large tumor burden. Mice were injected with 2×10^6 cells/mouse s.c. with or without simultaneously adoptively transferred CTLs (5×10^6 cells/mouse i.v.). *B,* tumor growth curve of established B16.MUC1 cells. Four days or 7 days after tumor injection (1×10^6 B16.MUC1 cells/mouse s.c.), mice received CTLs at 4 days ($n = 4$) or 7 days ($n = 4$; 5×10^6 cells/mouse i.v.). Control mice received no CTLs. *C,* tumor growth curve of MUC1-expressing C57 mammary gland tumor cells (1×10^6 cells/mouse s.c.) with and without simultaneous administration of CTLs (5×10^6 cells/mouse i.v.). Tumor burden is plotted as an average of the tumor weights of the individual mice. Recipient mice were MUC1.Tg. Tumors were measured using a metric dial caliper (Monostat Corp., Pequannock, NJ), and tumor weight was determined by the formula $(W^2 \times L)/2$.

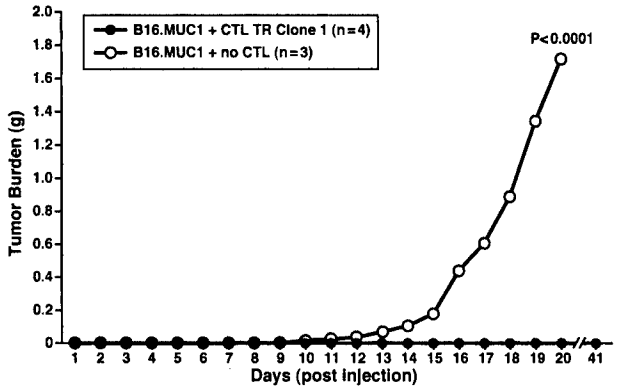

Fig. 7 Adoptive transfer of CTL TR clone 1. The tumor growth curve of MUC1-expressing B16 melanoma cells (1×10^6 cells/mouse s.c.) with and without the simultaneous administration of CTLs from CTL TR clone 1 (5×10^6 cells/mouse i.v.) to recipient MUC1.Tg mice is shown. Tumor burden is plotted as an average of the tumor weights of the individual mice.

(17–28). One such candidate is MUC1. Although MUC1 is a self molecule that is normally expressed in epithelial cells lining ducts and glands in low levels, it is a target for immunotherapy, because during tumorigenesis, MUC1 is significantly altered in expression. There is an 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 is found throughout the tumor mass and on the surface of tumor cells. Importantly, glycosylation is altered. Oligosaccharides are shorter and fewer in number, revealing immunodominant peptide sequences that on normal cell surfaces would be sequestered by glycosylation. Thus, MUC1 peptides may be used in immunotherapy strategies to activate the immune system to kill tumors expressing these epitopes.

From a spontaneous MUC1-expressing pancreatic tumor mouse model (MET), we have successfully isolated, expanded, and cloned naturally occurring MUC1-specific CTLs *in vitro*. These CTLs are $CD8^+$ T cells that express TCR Vβ5.1/5.2 and Vα2 (Fig. 3) and recognize a $H\text{-}2D^b$ MHC class I-restricted immunodominant epitope of MUC1 (STAPPAHGV or APGSTAPPA; Figs. 4 and 5). These CTLs can eradicate $MUC1^+$ tumor cells when adoptively transferred *in vivo* (Figs. 6 and 7). It is important to note that the CTLs characterized in this report differ from those originally described by Dr. Olivera Finn's laboratory (7, 8, 29, 30). The CTLs obtained from the MET mice are class I-restricted CTLs, and we have not seen MHC unrestricted CTLs in these animals. Class I-restricted CTLs have, however, been described recently in humans (31, 32).

Data presented in this study suggest that immunotherapeutic regimens using adoptive transfer of MUC1-specific T cells can eradicate, delay, or substantially reduce the incidence of $MUC1^+$ tumors, which suggest that tumors are somewhat susceptible to immune intervention, at least at early stages. This is the first evidence that demonstrates that the naturally occurring MUC1-specific CTLs isolated from one tumor model has antitumor effects on other MUC1-expressing tumors *in vivo*. Moreover, these CTLs are effective only against $MUC1^+$ tumors and have no effect on $MUC1^-$ tumors. Another important observation is that these CTLs are only reactive against tumor-expressed MUC1 and do not cause any autoimmune destruction of organs that express normal MUC1. Therefore, our

data confirm that MUC1 as expressed on tumors is an important tumor rejection antigen and can serve as an excellent target for immunotherapy.

Although all of the adoptive transfer data presented here are on injectable tumor models, we are most interested in determining whether the CTLs can be as effective in mice that develop spontaneous tumors. Two models of spontaneous tumors that were developed in our laboratory are under study: (*a*) the MET mice from which the CTLs originated; and (*b*) mice that develop spontaneous MUC1-expressing tumors of the mammary gland (designated MMT). Preliminary data suggest that the CTLs may be effective in delaying tumor onset in the MMT mice (data not shown). These studies provide us with the opportunity to develop clinical trials using MUC1-specific, cell-based vaccines.

Human cancers frequently express abnormal or altered self-proteins that are potentially immunogenic and trigger immune recognition. For example, low-level humoral and cellular immune responses to several antigens, including MUC1, HER2/neu, p53, and prostate-specific antigen, are present in a significant proportion of early- and late-stage cancer patients (33). It is important to determine how to obtain an effective immune response, because native immune responses fail to eradicate tumors. Understanding why these low-level immune responses are not effective against the growing tumor is key in developing improved and novel immunotherapeutic strategies for cancer. However, to be successful, these strategies ultimately need to induce reversal of self tolerance without autoimmune destruction and bypass any immune evasion mechanisms used by the tumor cells. The creation of mice bearing transgenic TCRs specific for tumor antigens have revolutionized the study of: (*a*) immune evasion mechanisms by tumors; (*b*) effects of self tolerance on the host's ability to eliminate tumor cells expressing self epitopes; and (*c*) autoimmune destruction. These mice can provide an unlimited source of tumor-specific T cells for *in vivo* studies. Thus, our future direction is to develop transgenic mice that develop $CD8^+$ CTLs that express TCRs specific for the immunodominant epitope of MUC1 so that we may study the above mechanisms as well as the effector functions of CTLs specific for MUC1. Once we identify some of these crucial mechanisms, our ultimate goal is to develop effective immunotherapeutic strategies aimed at preventing and treating human cancers.

Acknowledgments

We acknowledge the following persons and organizations for their contributions to this project: Biomira, Inc. (Edmonton, Canada) for generously providing MUC1 TR peptides; Dr. Larry Pease for providing the MUC1 tetramers and L cells; Dr. Judith Tevethia for kindly providing the original breeding stock of the ET mice; Cathy Madsen and Barbara Reid for technical support; Marv Ruona for the preparation of figures; Carol Williams for assistance with preparation of the manuscript; Suresh Savarirayan and the animal care technicians for excellent animal care; and the radiation oncology department. We also acknowledge Dr. James Lee for providing the magnetic activated cell sorter, Dr. Tony Hollingsworth for providing B16 tumor lines, and Dr. Olja Finn for providing peptides.

References

1. Romero, P., Cerottini, J. C., and Waanders, G. A. Novel methods to monitor antigen-specific cytotoxic T-cell responses in cancer immunotherapy. Mol. Med. Today, *4:* 305–312, 1998.

2. Taylor-Papadimitriou, J., Peterson, J. A., Arklie, J., Burchell, J., Ceriani, R. L., and Bodmer, W. F. Monoclonal antibodies to epithelium-specific components of the human milk fat globule membrane: production and reaction with cells in culture. Int. J. Cancer, *28:* 17–21, 1981.

3. Burchell, J., Gendler, S., Taylor-Papadimitriou, J., Girling, A., Lewis, A., Millis, R., and Lamport, D. Development and characterization of breast cancer reactive monoclonal antibodies directed against to the core protein of the human milk mucin. Cancer Res., *47:* 5476–5482, 1987.

4. Zotter, S., Hageman, P. C., Lossnitzer, A., Mooi, W. J., and Hilgers, J. Tissue and tumor distribution of human polymorphic epithelial mucin. Cancer Rev., *11–12:* 55–101, 1988.

5. Girling, A., Bartkova, J., Burchell, J., Gendler, S., Gillet, C., and Taylor-Papadimitriou, J. A core protein epitope of the polymorphic epithelial mucin detected by the monoclonal antibody SM-3 is selectively exposed in a range of primary carcinomas. Int. J. Cancer, *43:* 1072–1076, 1989.

6. Braga, V. M. M., Pemberton, L. F., Duhig, T., and Gendler, S. J. Spatial and temporal expression of an epithelial mucin, Muc-1, during mouse development. Development (Camb.), *115:* 427–437, 1992.

7. Barnd, D. L., Lan, M. S., Metzgar, R. S., and Finn, O. J. Specific, major histocompatibility complex-unrestricted recognition of tumor-associated mucins by human cytotoxic T cells. Proc. Natl. Acad. Sci. USA, *86:* 7159–7163, 1989.

8. Ioannides, C. G., Fisk, B., Jerome, K. R., Irimura, T., Wharton, J. T., and Finn, O. J. Cytotoxic T cells from ovarian malignant tumors can recognize polymorphic epithelial mucin core peptides. J. Immunol., *151:* 3693–3703, 1993.

9. Parker, S. L., Tong, T., Bolden, S., and Wingo, P. A. Cancer statistics, 1997. CA Cancer J. Clin., *47:* 5–27, 1997.

10. Rowse, G. J., Tempero, R. M., VanLith, M. L., Hollingsworth, M. A., and Gendler, S. J. Tolerance and Immunity to MUC1 in a human MUC1 transgenic murine model. Cancer Res., *58:* 315–321, 1998.

11. Mukherjee, P., Ginardi, A. R., Madsen, C. S., Sterner, C. J., Adriance, M. C., Tevethia, M. J., and Gendler, S. J. Mice with spontaneous pancreatic cancer naturally develop MUC-1-specific CTLs that eradicate tumors when adoptively transferred. J. Immunol., *165:* 3451–3460, 2000.

12. Tevethia, M. J., Bonneau, R. H., Griffith, J. W., and Mylin, L. A simian virus 40 large T-antigen segment containing amino acids 1 to 127 and expressed under the control of the rat elastase-1 promoter produces pancreatic acinar carcinomas in transgenic mice. J. Virol., *71:* 8157–8166, 1997.

13. Price, M. R., Sekowski, M., and Tendler, S. J. Purification of anti-epithelial mucin monoclonal antibodies by epitope affinity chromatography. J. Immunol. Methods, *139:* 83–90, 1991.

14. Johnson, A. J., Njenga, M. K., Hansen, M. J., Kuhns, S. T., Chen, L., Rodriguez, M., and Pease, L. R. Prevalent class I-restricted T-cell response to the Theiler's virus epitope Db:VP2121–130 in the absence of endogenous CD4 help, tumor necrosis factor α , γ interferon, perforin, or costimulation through CD28. J. Virol., *73:* 3702–3708, 1999.

15. Apostolopoulos, V., Chelvanayagam, G., Xing, P. X., and McKenzie, I. F. Anti-MUC1 antibodies react directly with MUC1 peptides presented by class I H2 and HLA molecules. J. Immunol., *161:* 767–775, 1998.

16. Morgan, D. J., Kreuwel, H. T., Fleck, S., Levitsky, H. I., Pardoll, D. M., and Sherman, L. A. Activation of low avidity CTL specific for a self epitope results in tumor rejection but not autoimmunity. J. Immunol., *160:* 643–651, 1998.

17. Cabaniols, J. P., Cibotti, R., Kourilsky, P., Kosmatopoulos, K., and Kanellopoulos, J. M. Dose-dependent, T cell tolerance to an immunodominant self peptide. Eur. J. Immunol., *24:* 1743–1749, 1994.

18. Goverman, J., Woods, A., Larson, L., Weiner, L. P., Hood, L., and Zaller, D. M. Transgenic mice that express a myelin basic protein-specific T cell receptor develop spontaneous autoimmunity. Cell, *72:* 551–560, 1993.

19. von Herrath, M. G., Dockter, J., and Oldstone, M. B. How virus induces a rapid or slow onset insulin-dependent diabetes mellitus in a transgenic model. Immunity, *1:* 231–242, 1994.

20. Miller, J. F., and Morahan, G. Peripheral T cell tolerance. Annu. Rev. Immunol., *10:* 51–69, 1992.

21. Oehen, S. U., Ohashi, P. S., Burki, K., Hengartner, H., Zinkernagel, R. M., and Aichele, P. Escape of thymocytes and mature T cells from clonal deletion due to limiting tolerogen expression levels. Cell. Immunol., *158:* 342–352, 1994.

22. Ohashi, P. S., Oehen, S., Buerki, K., Pircher, H., Ohashi, C. T., Odermatt, B., Malissen, B., Zinkernagel, R. M., and Hengartner, H. Ablation of "tolerance" and induction of diabetes by virus infection in viral antigen transgenic mice. Cell, *65:* 305–317, 1991.

23. Oldstone, M. B., Nerenberg, M., Southern, P., Price, J., and Lewicki, H. Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: role of anti-self (virus) immune response. Cell, *65:* 319–331, 1991.

24. Poindexter, N. J., Landon, C., Whiteley, P. J., and Kapp, J. A. Comparison of the T cell receptors on insulin-specific hybridomas from insulin transgenic and nontransgenic mice. Loss of a subpopulation of self-reactive clones. J. Immunol., *149:* 38–44, 1992.

25. Poplonski, L., Vukusic, B., Pawling, J., Clapoff, S., Roder, J., Hozumi, N., and Wither, J. Tolerance is overcome in beef insulin-transgenic mice by activation of low-affinity autoreactive T cells. Eur. J. Immunol., *26:* 601–609, 1996.

26. Whiteley, P. J., Poindexter, N. J., Landon, C., and Kapp, J. A. A peripheral mechanism preserves self-tolerance to a secreted protein in transgenic mice. J. Immunol., *145:* 1376–1381, 1990.

27. Yule, T. D., Basten, A., and Allen, P. M. Hen egg-white lysozyme-specific T cells elicited in hen egg-white lysozyme-transgenic mice retain an imprint of self-tolerance. J. Immunol., *151:* 3057–3069, 1993.

28. Morgan, D. J., Kreuwel, H. T., and Sherman, L. A. Antigen concentration and precursor frequency determine the rate of CD8+ T cell tolerance to peripherally expressed antigens. J. Immunol., *163:* 723–727, 1999.

29. Jerome, K. R., Barnd, D. L., Bendt, K. M., Boyer, C. M., Taylor-Papadimitriou, J., McKenzie, I. F., Bast, R. C., Jr., and Finn, O. J. Cytotoxic T-lymphocytes derived from patients with breast adenocarcinoma recognize an epitope present on the protein core of a mucin molecule preferentially expressed by malignant cells. Cancer Res., *51:* 2908–2916, 1991.

30. Jerome, K. R., Domenech, N., and Finn, O. J. Tumor-specific cytotoxic T cell clones from patients with breast and pancreatic adenocarcinoma recognize EBV-immortalized B cells transfected with polymorphic epithelial mucin complementary DNA. J. Immunol., *151:* 1654–1662, 1993.

31. Reddish, M., MacLean, G. D., Koganty, R. R., Kan-Mitchell, J., Jones, V., Mitchell, M. S., and Longenecker, B. M. Anti-MUC1 class I restricted CTLs in metastatic breast cancer patients immunized with a synthetic MUC1 peptide. Int. J. Cancer, *76:* 817–823, 1998.

32. Domenech, N., Henderson, R. A., and Finn, O. J. Identification of an HLA-A11-restricted epitope from the tandem repeat domain of the epithelial tumor antigen mucin. J. Immunol., *155:* 4766–4774, 1995.

33. Houghton, A. N. Cancer antigens: immune recognition of self and altered self. J. Exp. Med., *180:* 1–4, 1994.

Mice with Spontaneous Pancreatic Cancer Naturally Develop MUC-1-Specific CTLs That Eradicate Tumors When Adoptively Transferred[1]

Pinku Mukherjee, Amelia R. Ginardi, Cathy S. Madsen, Christopher J. Sterner, Melissa C. Adriance, Mary J. Tevethia, and Sandra J. Gendler[2]

Pancreatic cancer is a highly aggressive, treatment refractory cancer and is the fourth leading cause of death in the United States. In humans, 90% of pancreatic adenocarcinomas overexpress altered forms of a tumor-specific Ag, mucin 1 (MUC1; an epithelial mucin glycoprotein), which is a potential target for immunotherapy. We have established a clinically relevant animal model for pancreatic cancer by developing a double transgenic mouse model (called MET) that expresses human MUC1 as self molecule and develops spontaneous tumors of the pancreas. These mice exhibit acinar cell dysplasia at birth, which progresses to microadenomas and acinar cell carcinomas. The tumors express large amounts of underglycosylated MUC1 similar to humans. Tumor-bearing MET mice develop low affinity MUC1-specific CTLs that have no effect on the spontaneously occurring pancreatic tumors in vivo. However, adoptive transfer of these CTLs was able to completely eradicate MUC1-expressing injectable tumors in MUC1 transgenic mice, and these mice developed long-term immunity. These CTLs were MHC class I restricted and recognized peptide epitopes in the immunodominant tandem repeat region of MUC1. The MET mice appropriately mimic the human condition and are an excellent model with which to elucidate the native immune responses that develop during tumor progression and to develop effective antitumor vaccine strategies. *The Journal of Immunology,* **2000, 165: 3451–3460.**

Mucin 1 (MUC1)[3] is a epithelial cell-associated mucin that is developmentally regulated and aberrantly expressed by carcinomas, which makes it an important marker in malignancy (1–5). This molecule exists as a large extended rod protruding from the apical cell membrane into the lumen of the ducts. MUC1 has an unusual structure, consisting mainly of a 20-aa sequence repeated in tandem on an average of 30–90 times. The tandem repeats (TRs) serve as the scaffold for *O*-linked oligosaccharides that cover the polypeptide core (6). In cancer, there are differences in expression that distinguish this protein as tumor specific. There is a large increase in the amount of mucin expressed on cells and in circulation. Its distribution is no longer restricted to the apical surface of ducts and glands, but it is found throughout the tumor mass and on the surface of tumor cells. Importantly, the glycosylation is altered. Oligosaccharide structures are shorter and fewer in number, revealing immunodominant peptide sequences in every TR that on normal surfaces would be concealed by glycosylation (7). Underglycosylation of MUC1 reveals peptide epitopes recognized by cytotoxic T cells that can kill tumor cells expressing this form of MUC1 (8, 9).

The recent description of MUC1 as a target for CTLs has raised interest in using this protein as a target for immunotherapy. It is expressed by most adenocarcinomas of the breast, lung, stomach, pancreas, colon, prostate, ovary, endometrium, and cervix, which makes MUC1 an attractive therapeutic target. In 1999, cancers that expressed MUC1 accounted for about 72% of new cases and for 66% of the deaths (10). However, expression of the underglycosylated MUC1 is not sufficient to stimulate CTL killing, as >90% of existing carcinomas express MUC1 and these tumors progress. Thus, there is a need for studies to devise effective presentation of MUC1 immunogens to stimulate immune cells to kill tumor cells. The mouse up to now has not been a suitable preclinical model for testing vaccines, as human MUC1 differs in sequence from mouse Muc1 and is a foreign Ag in the mouse. We have developed an inbred C57BL/6 mouse strain that expresses human MUC1 in a tissue-specific fashion, driven by its own promoter. These mice transgenic for a foreign protein develop B and T cell compartment tolerance and are refractory to immunization with the protein encoded by the transgene (11). This experimental model enables us to study the effect of endogenous expression of the MUC1 gene on the ability of mice to produce protective immune responses to tumors, and it represents an improved model system for evaluating the efficacy of anti-MUC1 vaccine formulations in vivo within the context of existing tolerance mechanisms.

Adenocarcinoma of the pancreas is currently the fourth leading cause of death in the United States (12). Metastatic pancreatic cancer is uniformly fatal because no effective chemotherapy is available (13). Most of the models for studying human pancreatic tumors have used either injectable tumor cell lines or xenografts of primary human pancreatic tumors placed directly into the pancreas of mice (14). In this study, we have characterized the development of a double transgenic mouse line that expresses human MUC1 as a self-molecule and spontaneously develops MUC1-expressing tumors of the pancreas. These spontaneous tumors arise naturally in

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[3] Abbreviations used in this paper: MUC1, mucin 1; MUC1.Tg, MUC1 transgenic; TR, tandem repeat; DC, dendritic cell; MET, MUC1-expressing pancreatic tumor mouse model; ET, pancreatic tumor mouse model; FasL, Fas ligand; MACS, magnetic activated cell sorting; HA, hemagglutinin.

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an appropriate tissue background and in the context of a viable immune system. They develop more slowly than injected tumors, giving the host immune system time to respond. Animals experience similar physiological events to humans, which may result in the presence of MUC1 in serum and body fluids and may serve to prime CTLs. Loss of polarized tissue architecture in the tumors and high levels of expression of underglycosylated MUC1 result in circulating MUC1, which may be immunosuppressive (15). Likewise, Abs to the tumor MUC1 may be present, as has been found in humans (16, 17). This model allows us to study for the first time the effects of self-tolerance, immunity, and autoimmunity to MUC1 as the tumors develop spontaneously.

We have analyzed the expression of MUC1 in the bitransgenic mouse model (designated MET) as the tumor develops in the pancreas and characterized the biology of the tumors and the MUC1-specific native immune response that develops during tumor progression. In our model, as in humans, there is overexpression and underglycosylation of MUC1, which in turn elicits cytotoxic T cells against the TR protein core. These naturally occurring CTLs are class I restricted and can be stimulated to kill MUC1-expressing cancer cell lines in vitro. Although these CTLs do not eradicate the spontaneously occurring pancreatic tumors, they are effective in eradicating injectable tumors when adoptively transferred. Thus, the MET model appropriately mimics the human condition and can be used to characterize immunotherapy strategies that will be effective against spontaneous tumors.

Materials and Methods

Mouse model

MUC1 transgenic (MUC1.Tg) mice are bred with oncogene-expressing mice that spontaneously develop tumors of the pancreas (ET mice) and are designated as MET. MUC1.Tg mice were developed in our laboratory (11), and the ET mice were obtained from Dr. Judith Tevethia (18). ET mice express the first 127 aa of SV40 large T Ag under the control of the rat elastase promoter. Fifty percent of the animals develop life-threatening pancreatic tumors by about 21 wk of age (18). All mice are on the C57BL/6 background. Animals were sacrificed and characterized at 3-wk intervals (n = 6 animals/time point) from 3 to 24 wk. Mice were bred and maintained in specific pathogen-free conditions in the S. C. Johnson Medical Research Building animal facility at Mayo Clinic Scottsdale. All experimental procedures were conducted according to the Institutional Animal Care and Use Committee guidelines.

PCR screening

PCR was used to routinely identify MUC1.Tg- and ET-positive mice in the colony. PCR was conducted as previously described (11, 18). The primer pairs for MUC1.Tg are 5′-CTTGCCAGCCATAGCACCAAG-3′ (bp 745–765) and 5′-CTCCACGTCGTGGACATTGATG-3′ (bp 1086–1065) and for ET are 5′-GCTCCTTTAACCCACCTG-3′ (bp 4055–4072) and 5′-CCAACCTATGGAACTGATGAATG-3′ (bp 4546–4568). The amplification program consisted of one cycle of 5 min at 95°C and 40 cycles of 30 s each at 95°C, 52°C, and 72°C followed by one cycle of 10 min at 72°C. The PCR product of each reaction was analyzed by size fractionation through a 1% agarose gel. Amplification conditions for MUC1 are the same except for the annealing temperature, which was 61°C. Amplification of MUC1 resulted in a 500-bp fragment, and amplification of ET resulted in a 491-bp fragment.

Tumor weights

The entire pancreas was dissected free of fat and lymph nodes, weighed, and spread on bibulus paper for photography. Nodules were counted under the dissecting scope. Pancreas was fixed in methacarn, processed for microscopy by conventional methods, step sectioned at 5 μm (about 10 sections per mouse pancreas), stained with hematoxylin and eosin, and examined by light microscopy. Mice were carefully observed for signs of ill-health, including lethargy, abdominal distention, failure to eat or drink, marked weight loss, pale feces, and hunched posture.

Cell lines

Cell lines used included: B16 murine melanoma cell line expressing MUC1 (B16.MUC1) and B16 transfected with vector only (Bl6.neo) (11). These cell lines were kindly provided by Dr. Tony Hollingsworth (University of Nebraska Medical Center, Omaha, NE). B16.MUC1 and B16.neo were maintained in DMEM with 10% FBS, penicillin (50 U/ml), and streptomycin (50 μg/ml), supplemented with 300 μg/ml G418. Cells were routinely tested by flow cytometry for the presence of MUC1.

ELISA

Cytokine levels in culture supernatant samples were determined by specific ELISAs for IFN-γ and IL-2. The IFN-γ assay used a sandwich technique as described by Samuel (19). Abs used were R46A2 as catcher and a second biotinylated Ab, XMG1.2, kindly supplied by Biomira (Edmonton, Canada). The IFN-γ standard was obtained from PharMingen (San Diego, CA). Cytokine levels in the test sample were determined by comparison with reference standards. IL-2 levels were detected using Endogen ELISA kit (Woburn, MA). Serum MUC1 levels were determined using the Truquant BR RIA supplied by Biomira (20). Detection of Ab to MUC1 was conducted by ELISA using synthetic peptides (105 mer) of the 5.25 MUC1 TR as previously described (11).

Immunohistochemistry

Tumors were obtained from MET mice at various time points during tumor progression, fixed in methacarn (60% methanol, 30% chloroform, 10% glacial acetic acid), embedded in paraffin, and sectioned for immunohistochemical analysis. MUC1 Abs used were CT1, a rabbit polyclonal Ab that recognizes mouse and human cytoplasmic tail region of MUC1 (21), HMFG-2, BC2, and SM-3, which have epitopes in the TR domain of MUC1. The TR epitope of HMFG-2 has been mapped to DTR, that of BC2 to APDTR, and that of SM-3 to PDTRP (22). All TR Abs are specific for human MUC1 and do not cross-react with mouse Muc1. All of the TR Abs are glycosylation sensitive in the pancreas. Abs to Fas ligand (FasL) and TGFβ2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary Ab was swine-anti rabbit conjugated to HRP (Dako, Carpinteria, CA). Ab staining was blocked with the appropriate specific peptides supplied by Santa Cruz Biotechnology.

CTL assays

Determination of CTL activity was performed using a standard ^{51}Cr release method after a 6-day in vitro peptide stimulation without additional added cytokines. Splenocytes from individual MET mice were harvested by passing through a nylon mesh followed by lysis of RBC using pharmlyse (0.45% ammonium chloride solution purchased from PharMingen (San Diego, CA) and cultured in DMEM (1×10^6 cells/ml) with MUC1 TR peptide (24 mer, TAPPAHGVTSAPDTRPAPGSTAPP) at 10 μg/ml in a total volume of 5 ml. Target cell lines (B16.MUC1 and B16.neo) were derived from C57BL/6 mice and expressed high levels of MUC1 as determined by FACS analysis using Abs to the TR. Specific ^{51}Cr release at 6 h was calculated according to the following formula: ((experimental release cpm − spontaneous release cpm)/(maximum release cpm − spontaneous release cpm)) × 100. Spontaneous release in all experiments was <15% of maximum release. Ab blocking experiments were performed by preincubating the targets overnight with 1 μg/10^6 cells of H-2K^b/D^b mAb (clone 28-8-6; PharMingen).

Flow cytometry

Single cells from spleens of MET mice were analyzed by two-color immunofluorescence for alterations in lymphocyte subpopulations: CD3, CD4, CD8, Fas, FasL, CD11c, and MHC class I and II. Intracellular cytokine levels were determined after cells were stimulated with MUC1 peptide (10 μg/ml for 6 days) and treated with brefeldin-A (also called GolgiStop; PharMingen) as directed by the manufacturer's recommendation (4 μl/1.2 × 10^7 cells/6 ml for 3 h at 37°C before staining). Cells were permeabilized using the PharMingen permeabilization kit and stained for intracellular IFN-γ, IL-2, IL-4, and IL-5 as described by PharMingen. All fluorescently labeled Abs were purchased from PharMingen. Flow cytometric analysis was done on Becton Dickinson FACscan using the CellQuest program (Becton Dickinson, Mountain View, CA).

Magnetic activated cell sorting (MACS)

Splenic lymphocytes were stained for 30 min on ice with anti-CD8 Ab conjugated to microbeads (Miltenyi Biotechnologies, Auburn, CA). CD8$^+$ cells were positively selected on an RS-type magnetic column using the

Vario MACS magnetic device following the protocol provided by the manufacturer (Miltenyi Biotec, Auburn, CA). Purity of the selected cells was checked by flow cytometry and ranged from 92 to 95%.

Adoptive transfer

Two groups of five MUC1.Tg mice were injected (s.c. in the flank) with B16.MUC1 cells (1×10^6 cells/mouse/200 μl). Simultaneously, one group of mice received (by i.v. injection) $CD8^+$ CTLs (5×10^6 cells/mouse/100 μl) isolated from an 18-wk MET spleen. Splenocytes were grown on irradiated B16.MUC1 cells (20,000 rad $\times$ 2) with IL-2 (100 U/ml) and IFN-γ (150 pg/ml) for 2 wk before sorting for $CD8^+$ cells. The control group received media alone. Palpations were started 5 days after tumor challenge. Tumors were measured using a metric dial caliper (Monostat, Pequannock, NJ), and tumor volume was determined by the formula $(W^2 \times L)/2$. The experiment was repeated one time.

Peptide affinity assay

Bone marrow cells from C57BL/6 mice were isolated and cultured in DMEM with 10% FBS, penicillin (50 U/ml), streptomycin (50 μg/ml), 1% glutamax, stem cell factor (10 ng/ml) (Stem Cell Technologies, Vancouver, Canada), GM-CSF (60 pg/ml) (PharMingen), and IL-4 (200 pg/ml) (PharMingen) for 7–10 days. Murine dendritic cells (DCs) were purified using the DC purification kit (Stem Cell Technologies) and positively selected using the RS-type column on the Vario MACS device using the manufacturer's recommended conditions (Miltenyi Biotec, Auburn, CA). The cells were further cultured for 7 days with GM-CSF (60 pg/ml) and IL-4 (200 pg/ml). At this point, cells were tested for DC-specific and nonspecific cell surface markers by flow cytometric analysis. Abs to CD11c, CD14, Mac1, Gr-1, B7.1, B7.2, and MHC class I and II (PharMingen) were used to determine purity by FACS analysis. DCs were pulsed overnight with various MUC1 TR peptides at concentrations ranging from 10^{-5} to 10^{-7} M. The peptides tested spanned the entire TR region of MUC1 and ranged in size from 9 to 25 mer. Peptides were kindly provided by Dr. M. Longenecker (Biomira). Irrelevant peptides including MUC4, GP100-1 (human melanoma), and KAS6-13 (human multiple myeloma) were used as negative controls. Peptide-pulsed DCs were labeled with ^{51}Cr (100 μCi/10^6 cells) for 2 h in a 37°C incubator with slow shaking and served as targets for CTLs from 18 wk MET mice. B16.MUC1 cells were used as positive targets, and B16.neo cells were used as irrelevant targets. A standard ^{51}Cr release assay was performed using the peptide-pulsed DCs as targets and 18-wk MET splenocytes as effectors.

Statistical analysis

All statistical analyses were performed using the two-sample Student's unpaired t test.

Results

Generation of MUC1.Tg mice with spontaneous pancreatic tumors

We have generated spontaneous pancreatic tumors that express human MUC1 as a self-protein by mating the MUC1.Tg mice with mice that develop tumors of the pancreas (ET mice). The ET mice were developed and kindly donated by Dr. Judith Tevethia (18). They were created by expressing the first 127 aa of SV40 T Ag under control of the elastase promoter. Although the ET mice were made in the B6D2 F_1 mouse strain, they have been backcrossed for >20 generations onto B6 mice. These mice have an interesting advantage over a mouse expressing full-length T Ag. The T1-127 protein does not contain any of the previously identified T Ag CTL epitopes that could be used by the C57BL/6 mice for tumor rejection (18). Although mice expressing full-length T Ag should be tolerant to those epitopes, the absence of the epitopes in the T1-127 ET mice avoids any consideration of SV40-specific immune rejection.

These mice exhibit acinar cell dysplasia at birth, which progresses to microadenomas and single or multiple acinar cell carcinomas. These pancreatic tumors express large amounts of MUC1 similar to the in vivo situation in humans. Although we have not reported the survival data for MET mice, it appears similar to that observed by Tevethia for the ET mice (18), suggesting that the presence of MUC1 protein does not substantially alter tumor progression. We have sacrificed animals at 3-wk intervals to monitor tumor formation and to process the pancreas for microscopy by conventional methods. Tumors in the pancreas were first grossly visualized at week 9. By week 12, as many as nine tumors were observed. Pancreas weights were determined at sacrifice and are shown in Fig. 1. The weights were a general indication of tumor burden. However, in many animals 15 wk or older, the weights did not increase although tumors were present, presumably due to cachexia.

MUC1 is aberrantly glycosylated in pancreatic tumors in MET mice

Histologic analysis showed that the pancreas consisted of dysplastic acinar cells, acinar cell microadenomas, and acinar cell carcinomas. As the pancreatic tumors developed, there was a definite change in the epitopes of MUC1 that were exposed. Normal pancreas expresses MUC1 in acinar and ductal cells as detected by immunohistochemical analysis using CT1, a rabbit antiserum to the cytoplasmic tail of MUC1 (Fig. 2, *A* and *B*). The CT1 reactivity is not affected by glycosylation. MUC1 expressed on normal pancreas is heavily glycosylated (23). This glycosylation masks TR core protein epitopes for the mAbs BC2 (Fig. 2, *C* and *D*), HMFG-2 and SM-3 (not shown). Although BC2 has been described as glycosylation insensitive in the mammary gland, which exhibits a lower level of glycosylation than the pancreas, this clearly is not the case in the pancreas (22). Normal pancreas in humans and mice do not exhibit epitopes for BC2. By 3 wk of age, dysplastic acinar cells had developed, and the glycosylation was altered. All three glycosylation-sensitive Abs, BC2 (Fig. 2, *G* and *H*), as well as HMFG-2 and SM-3 (data not shown), showed strong reactivity with tumor cells. Each of the Abs is shown blocked by an appropriate peptide (Fig. 2, *B*, *D*, *F*, and *H*). These alterations in reactive profiles suggested that in the MET mice, as in humans, MUC1 expressed by tumors is underglycosylated, and the protein core, which is normally covered with carbohydrate, is exposed. This unmasking of the core protein may reveal the peptide epitopes that are recognized by cytotoxic T cells that can kill tumor cells expressing this form of MUC1, similar to what has been observed in humans.

In addition to well-differentiated acinar cell carcinomas shown in Fig. 3, *A–D*, MET mice at 18 and 21 wk developed large solid tumor masses of less differentiated acinar cell carcinomas (Fig. 3, *E–H*). Expression of MUC1 in the large solid tumors appeared to be greatly decreased when we stained with our TR Abs (Fig. 3*G*).

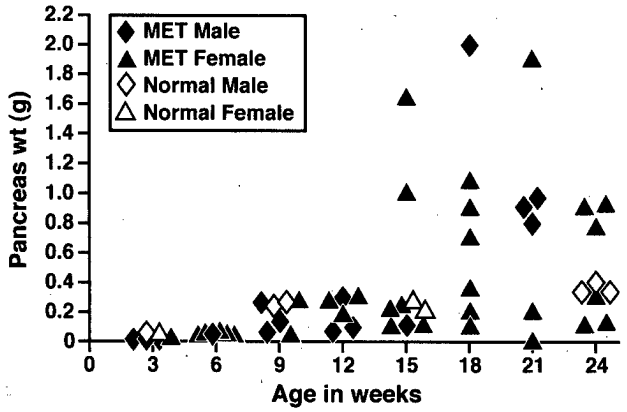

FIGURE 1. Pancreas weight in MET mice as function of age. The entire pancreas was dissected free of fat and lymph nodes, fixed in methacarn, and weighed. Pancreas weights of MET mice are compared with pancreas weights of age-matched normal C57BL/6 mice.

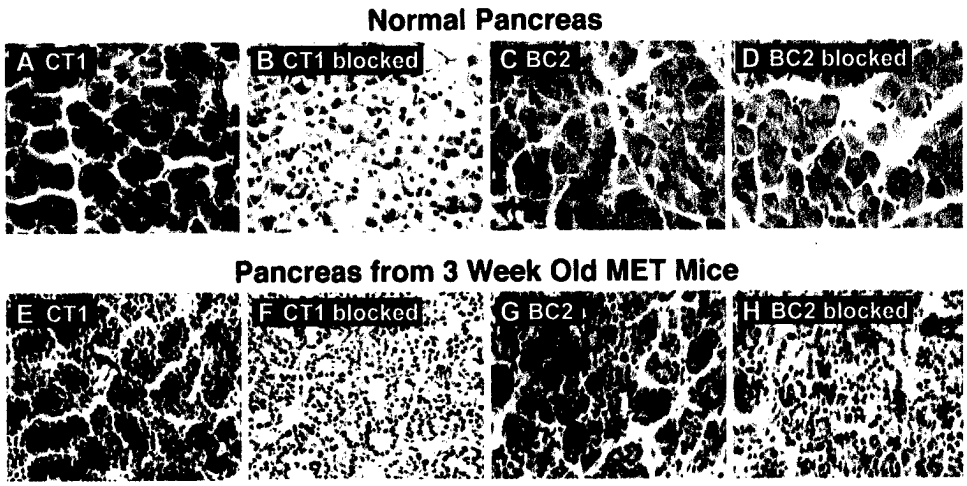

FIGURE 2. Immunohistochemical staining of MUC1-expressing normal pancreas and 3-wk MET pancreatic tumor. Methacarn-fixed and paraffin-embedded sections of normal pancreas showed strong MUC1 expression detected with CT1 antiserum (*A*) and low reactivity with TR mAb BC2 (*C*). Tumors taken at 3 wk show strong reactivity with CT1 (*E*) and BC2 (*G*). Specific staining was blocked by preincubation of mAbs with cytoplasmic tail peptide (*B* and *F*) and TR peptide (*D* and *H*). Images were captured at ×400 magnification.

However, staining with CT1 antiserum, which is against the cytoplasmic tail of MUC1 and is glycosylation insensitive, showed high levels of MUC1 expression (Fig. 3*E*). Specific staining was blocked by appropriate peptides (Fig. 3, *F* and *H*). The staining could represent either mouse or human MUC1 as CT1 Ab cross-reacts with both mouse and human. There is no effective methodology to differentiate between the mouse and human forms of MUC1 using the cytoplasmic tail Ab. The immunogenic TR epitopes appeared to become masked (presumably with carbohydrate) in these later stage tumors. At the same time, there appeared to be decreased amounts of the well-differentiated acinar cell tumors, as the large tumor mass began to dominate physically. It is possible that the CTLs detected at week 18 (see below) that were reactive with underglycosylated MUC1 were eliminating the regions of tumor expressing aberrantly glycosylated MUC1 and allowing outgrowth of tumors expressing nonimmunogenic MUC1. Another possibility is that the CTLs eliminated all tumor cells expressing human MUC1 and tumors that survived expressed only mouse Muc1 as detected by CT1.

The large solid tumors also showed a modulation in their expression of FasL. FasL expression increased as tumors progressed with low level expression at 3 wk (Fig. 4*A*) and strong expression at 18 wk (Fig. 4*E*). These results were confirmed with Western blot analysis of 3- and 18-wk tumor lysates (data not shown). Within the same pancreas, the well-differentiated dysplastic acinar cells showed strong expression of FasL especially in lumenal regions (Fig. 4*E*), whereas the solid undifferentiated tumor mass demonstrated much decreased levels in staining (Fig. 4*I*). Both 18-wk tumor sections shown in Fig. 4 were from the same slide and thus were stained simultaneously. Immunohistochemical staining of TGFβ in MET pancreatic tumors also detected increasing levels of TGFβ as tumors progressed (Fig. 4, *C*, *G*, and *K*). Expression of TGFβ is a known mechanism by which tumors evade immune recognition (24).

Detection of MUC1-specific CTL precursor cells

We have analyzed the native immunological responses in the MET mice as the mice aged and tumors developed. We observed that nonimmunized MET mice developed MUC1-specific cytotoxic T cells that lysed 40% of the MUC1-expressing B16 melanoma target cells in the 12 wk MET mice rising to 80% lysis in 18 wk MET

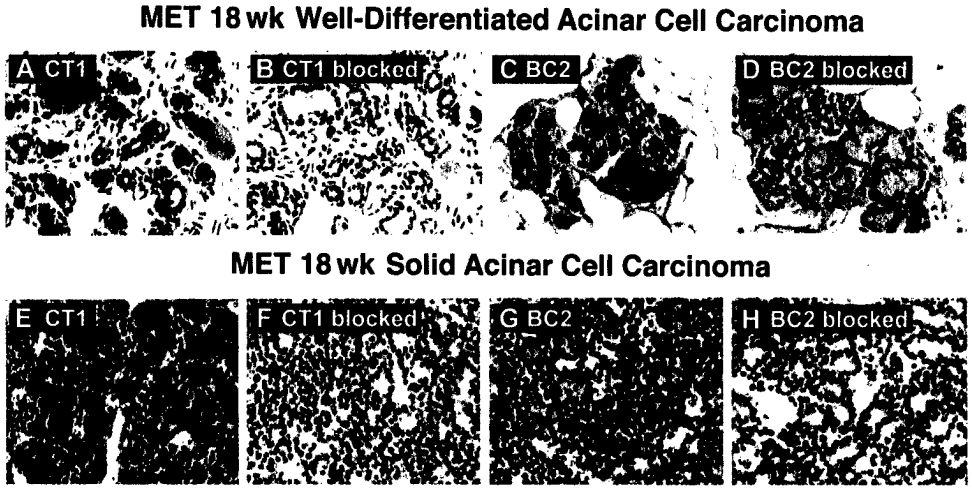

FIGURE 3. Immunohistochemistry of solid acinar cell carcinoma showing decreased expression of TR epitopes on MUC1 core protein. Methacarn-fixed and paraffin-embedded sections of the well-differentiated 18-wk acinar cell carcinoma with TR mAb BC2 (*C*), which is glycosylation sensitive, shows high expression of MUC1 when compared with the 18-wk undifferentiated solid tumor (*G*). Both regions of tumor are present on the same slide and, consequently, underwent the same staining procedure. The same tumor stained with CT1, which is not glycosylation sensitive, shows MUC1 is still highly expressed by the well-differentiated (*A*) and solid (*E*) MET tumors. Staining was blocked by appropriate peptides (*B*, *D*, *F*, and *H*). Images were captured at ×400 magnification.

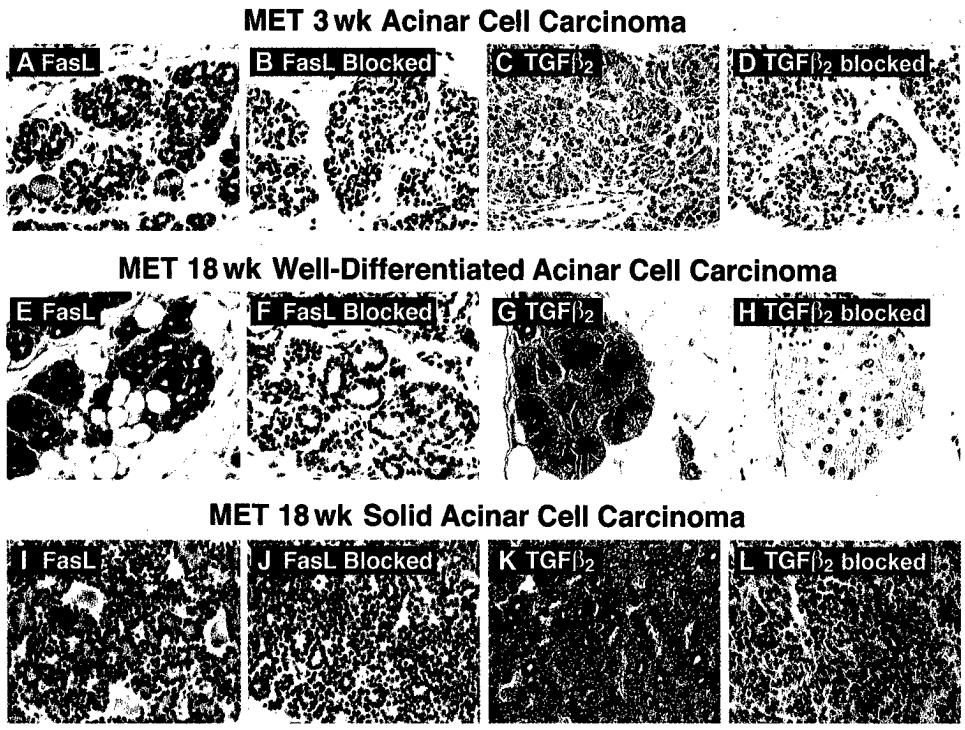

FIGURE 4. Immunohistochemical analysis of FasL and TGFβ2 expression on MET pancreatic tumors. The acinar cell carcinomas were stained with antiserum to FasL and TGFβ2. Low level expression of FasL at 3 wk (*A*) increased as tumors progressed to 18 wk (*E*). The strong staining detected on well-differentiated regions of the tumor (*E*) was much decreased in intensity on the undifferentiated solid tumor portions (*I*). Both tumor sections shown were from the same slide and thus stained simultaneously. Staining of TGFβ2 in MET pancreatic tumors detected increasing levels of TGFβ2 in both the differentiated and undifferentiated portions of the tumors (*C*, *G*, and *K*). Specific staining was completely blocked by the immunizing peptide (*B*, *D*, *F*, *H*, *J*, and *L*). Images were captured at ×400 magnification.

mice (Fig. 5*A*). This lysis compared with 20% lysis of MUC1-negative B16 cells. The level of lysis dropped to about 40% at week 21. Similar lysis was detected using as targets a MUC1-expressing mammary gland cell line C57 MG (data not shown). To our surprise, we detected no CTLs when we used a MUC1-expressing pancreatic tumor cell line (Panc 02) as targets (data not shown). We have found that both the B16 melanoma and the C57 MG cells but not the Panc 02 cells expressed low levels of MHC class I molecules (H-2K^b/H-2D^b) on their surfaces, suggesting that the CTL lysis was MHC restricted. Another clone of B16.MUC1 cells lacking class I expression was not lysed until class I was induced by IFN-γ treatment (data not shown). To confirm this class I restriction, lysis was blocked completely by an Ab to class I (anti-H-2K^b/H-2D^b, clone 28-8-6) (Fig. 5*B*). The appearance of reactive CTLs by 18 wk in eight of eight MET mice is highly significant ($p < 0.001$) and suggests that the high level of aberrantly expressed MUC1 by tumors may be antigenic and can mobilize T cells and elicit a cytotoxic response.

Minimum epitope recognized by MET CTLs and their affinities

We have analyzed CTL reactivity to a selection of MUC1 TR peptides. DCs were loaded with different concentrations of 9-mer and larger peptides and used as targets in a CTL assay. CTLs recognized the MUC1 TR sequences with highest affinity to STAPPAHGV epitope (Fig. 6). These results are similar to those seen in humans, where the CTLs isolated from breast cancer patients also reacted to the STAPPAHGV epitope with low affinity (25). The CTLs were of low avidity as they lost their lytic function by 10^{-8} M. This could be one of several reasons why the CTLs had no obvious effect on the spontaneous growth of pancreatic tumors in vivo. Although our data suggested that the CTLs were recognizing epitopes on MUC1 TR, we have not yet determined whether there are CTL epitopes outside of the MUC1 TR portion, for example, in other regions of extracellular domain or in the cytoplasmic domain.

Cytokine levels in MET mice as tumors progress

The CTLs detected in the MET mice should be fully functional at killing target tumor cells, as they showed increased IFN-γ ($p < 0.05$) and IL-2 ($p < 0.01$) expression in culture supernatants, following stimulation for 6 days with MUC1 TR peptide (Fig. 7, *A* and *B*). IFN-γ levels in the supernatant sample levels ranged from 6,000 to 19,000 pg/ml in 18-wk MET mice (Fig. 7*A*). IL-4 levels showed no significant increase (data not shown), suggesting that the immune response is a type 1 response.

Increased serum MUC1 levels in MET mice as tumors progressed

Increased levels of MUC1 in the sera from mice aged 18 and 24 wk corresponded directly to the increased levels of CTL activity (Fig. 8). These results suggested that high levels of tumor-associated MUC1 may activate MUC1-specific CTLs that are able to lyse MUC1-expressing tumor cells in vitro.

Adoptively transferred CTLs from MET mice are able to successfully reject B16.MUC1 tumors in MUC1.Tg mice

CTLs were obtained from splenocytes of 18 wk MET mice and expanded on irradiated B16.MUC1 cells. CD8$^+$ cells were selected (98% of the CTLs were CD8$^+$) by MACS and adoptively transferred into five MUC1.Tg mice (5×10^6 cells in 100 μl PBS, i.v.). At the same time, these five mice and five control mice received 1×10^6 viable B16.MUC1 cells (s.c. in the flank), and tumor growth was monitored by palpation. By 34 days, all five mice that did not receive the CTLs had tumors of ~1.4 g, whereas mice receiving the adoptively transferred CTLs remained tumor free for 10 wk ($p < 0.05$) (Fig. 9*A*). In vivo adoptive transfer of the CD8$^+$ CTLs from 18-wk MET mice showed that the CTLs were effective in eradicating transplanted B16.MUC1 tumor cells from MUC1.Tg mice. All mice receiving B16.neo tumor cells developed tumors even in the presence of CTLs ($n = 4$) (Fig. 9*A*), providing further evidence that the immune response is specific

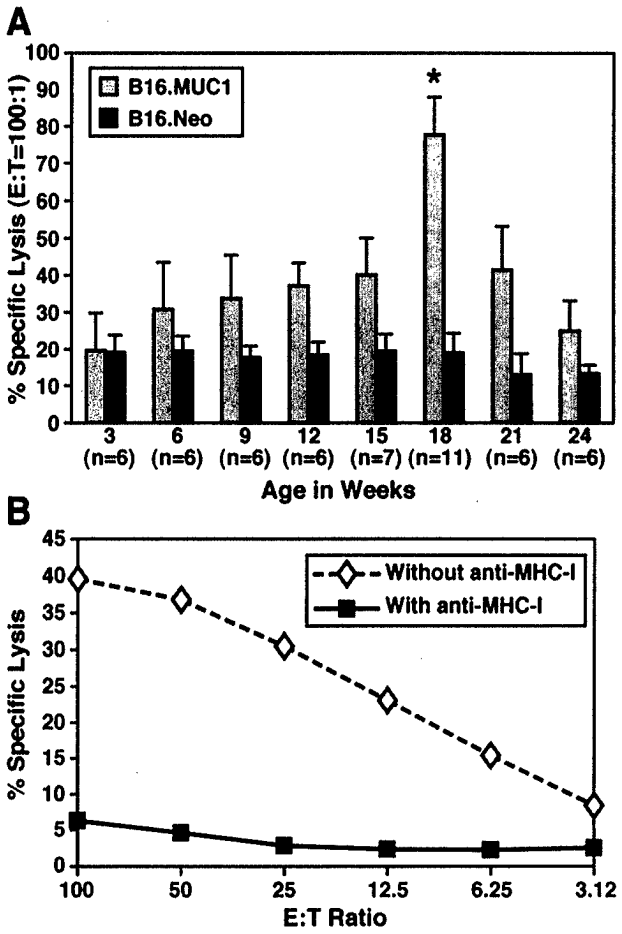

FIGURE 5. CTL precursor activity in MET mice at 100:1 E:T ratio. *A*, CTL activity of splenocytes was evaluated in nonimmunized MET mice of different ages. Determination of CTL activities was performed by a standardized 6-h ^{51}Cr release method after a 6-day in vitro TR peptide stimulation without additional added cytokines. Specific ^{51}Cr release was calculated according to the following formula: ((experimental cpm − spontaneous release cpm)/(maximum release cpm − spontaneous release cpm)) × 100. Spontaneous release in all experiments was <15% of maximum release. *B*, Ab blocking experiments were performed by preincubating the targets overnight with 1 $\mu g/10^6$ cells of H-2K^b/D^b mAb (PharMingen, clone 28-8-6) (*, $p < 0.005$). Error bars represent the SD of the mean; n = number of individual mice assayed.

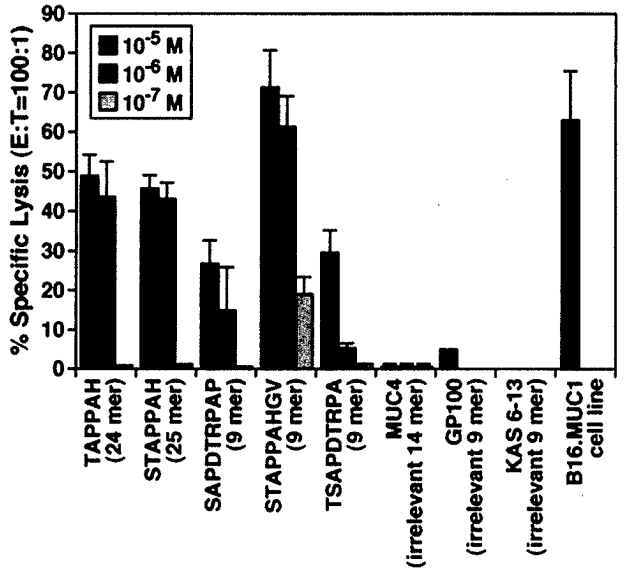

FIGURE 6. Minimum epitope recognized by MET CTLs. Splenocytes were stimulated with MUC1 in vitro for 6 days without added cytokines. DCs were cultured overnight with various concentrations of MUC1 TR peptides ranging from 10^{-5} to 10^{-7} M and used as targets in a standard ^{51}Cr release CTL assay. CTLs from 18-wk MET mice were used as effectors. B16.MUC1 cells were used as a positive control, and three irrelevant peptides, GP-100 (9 mer), KAS 6-13 (9 mer), and MUC4 (14 mer), were used as negative controls. Results are from five MET mice. Error bars represent SD of the mean; n = number of individual mice assayed.

against tumor Ag MUC1. This result suggested that the naturally occurring immunity in MET mice can be transferred. These experiments have been repeated with similar results.

We tested whether some population of effector cells will, upon second adoptive transfer, give rise to memory cells that can be activated upon re-exposure to the same Ag. The hypothesis is that re-encounter with the same Ag can expand the CTL population to a new, stable, higher level of effectors that may be required for long-term protection. CD8$^+$ cells from two of the tumor-free mice were adoptively transferred (5 × 10^6 cells without in vitro expansion) to two additional MUC1.Tg recipient mice to test for memory. These mice also received 1 × 10^6 B16.MUC1 cells (s.c. in the flank). One mouse that received the CD8$^+$ cells had no tumor, whereas the second mouse showed significantly lower tumor burden as compared with mice that did not receive the CTLs (Fig. 9*B*). A third tumor-free mouse was rechallenged with B16.MUC1 cells at week 10 and remained tumor free for an additional 10 wk (data not shown), suggesting once again that the 18-wk MET CTL effectors were able to generate memory T cell effectors that provided long-term protection against MUC1-expressing tumors. These results suggested that the MET mice developed effective cellular responses that eliminated B16.MUC1 tumor cells in vivo. However, these CTLs were ineffective in eradicating the spontaneously occurring pancreatic tumors in the MET mice.

Discussion

The MET mice appear to be an extremely appropriate model for human cancer. In the MET mice, as in humans, MUC1 is aberrantly expressed and underglycosylated in tumors, revealing epitopes on the core protein that are normally masked by carbohydrate structures. With time there appears to be epitope modulation in the larger, progressively growing tumors, with a reduction in TR epitope on MUC1 as detected by specific Ab staining and immunohistochemistry (Figs. 2 and 3). Modulation of antigenic epitopes is one way the host can favor emergence of nonimmunogenic, nonrejectable tumor cell variants (26). Similarly, as has been described in humans, CTLs were identified in nearly all of the MET mice between the ages of 16 and 19 wk (Fig. 5), which effectively lysed MUC1-expressing target cells in vitro. Importantly, these CD8$^+$ CTLs eradicated injectable MUC1-expressing tumors when adoptively transferred in vivo (Fig. 9). The tumor-bearing MET mice, which expressed underglycosylated MUC1 on tumors and presumably also in serum, developed a CTL response to MUC1 that differed from the previously described s.c. response elicited in MUC1.Tg mice bearing B16.MUC1 cells. CTLs from the MET mice were effective in eradicating injectable tumors in vivo, whereas the MUC1.Tg mice with the B16.MUC1 s.c. tumor elicited ineffective CTLs (27). Presumably, the environment in the mice developing spontaneous tumors greatly influences the immune response. In the bitransgenic MET mice as in humans, serum MUC1 levels increased gradually as tumor development progressed, with maximum levels observed at about 16–19 wk of age

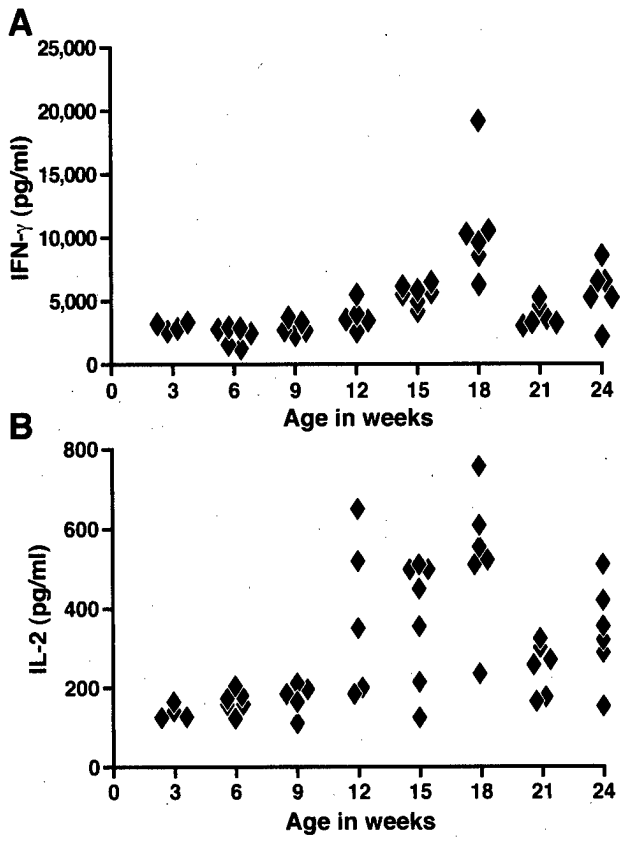

FIGURE 7. IFN-γ and IL-2 levels in MET mice. Splenocytes were derived from individual MET mice and cultured for 6 days with MUC1 TR peptide without added cytokines. Culture supernatants were collected, and cytokine levels determined by specific ELISAs for (*A*) IFN-γ and (*B*) IL-2. IFN-γ levels in the 18-wk MET mice were significantly elevated compared with the other time points ($p < 0.05$), whereas IL-2 levels were significantly elevated in the 12-wk MET mice ($p < 0.01$) and remained elevated until 24 wk of age. The IFN-γ assay used a sandwich technique as described by Samuel (19). Abs used were R46A2 as capture Ab and second biotinylated Ab XMG1.2. The IFN-γ standard was from PharMingen. Cytokine levels in the test samples were determined by comparison with reference standards. All IFN-γ assay reagents were supplied by Biomira (Edmonton, Canada). IL-2 levels were detected using an Endogen ELISA kit (Woburn, MA).

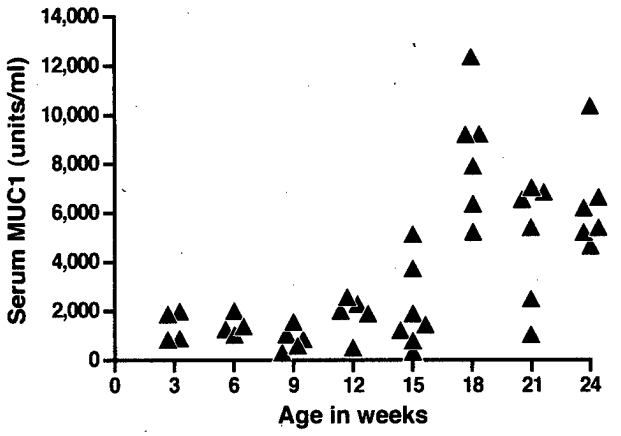

FIGURE 8. Serum MUC1 levels in MET mice. MUC1 levels in serum were determined using the Truquant BR RIA (supplied by Biomira) (20).

(Fig. 8), when mice have a large tumor burden (Fig. 1) and high CTL activity (Fig. 5).

Thus far our data suggest a strong Th1 cellular immune response as determined by high CTL activity and high levels of IFN-γ and IL-2 (Fig. 7, *A* and *B*). Intracellular IFN-γ and IL-2 analyses using FACS confirmed the ELISA results. Interestingly, intracellular IL-5 also showed a 2- to 6-fold increase as tumors progressed. It is of interest to note that IL-5 has been correlated to increased levels of eosinophils, which are known to play a role in tumor immunity (28–31).

Characterization of the MET CTL line revealed an MHC class I-restricted phenomenon (Fig. 5*B*) with highest affinity to the MUC1 TR peptide sequence, STAPPAHGV (Fig. 6). Further characterization of TR peptides is in progress and has revealed that the STAPPAHGV peptide was presented to the CTLs in the D^b but not in the K^b groove of MHC class I molecule (manuscript in preparation). Interestingly, this is the same MUC1 peptide sequence that was recognized by CTL lines isolated from human breast cancer patients (25). Although our data suggest that the CTLs are recognizing epitopes on MUC1 TR, we have not yet determined whether there are CTL epitopes outside of the MUC1 TR portion, for example in other regions of extracellular domain or in the cytoplasmic domain. Although we have isolated lytic CTLs from MET mice, their avidity to the MUC1 TR peptide is low as their lytic activity is lost at 10^{-8} M peptide concentrations. MUC1 is expressed in the transgenic mice under its own promoter, and the pattern and timing of expression of human and mouse MUC1 show

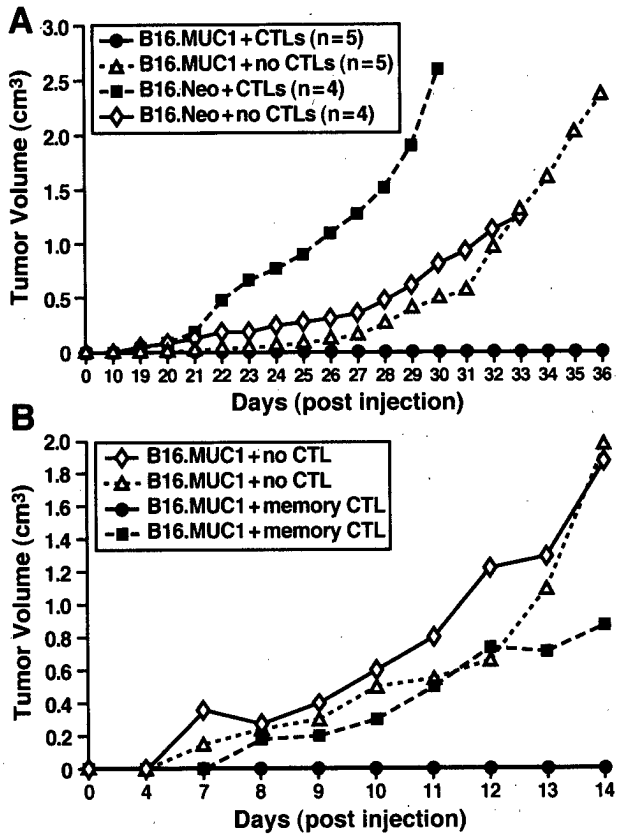

FIGURE 9. Adoptive transfer experiments. *A*, Tumor growth curve of MUC1 expressing B16 melanoma cells and B16.neo control cells (1×10^6 cells/mouse s.c.) with and without adoptively transferred CTLs (5×10^5 cells/mouse i.v.). *B*, Tumor growth curve of MUC1 expressing B16 melanoma and B16.neo control cells in individual mice following adoptive transfer of $CD8^+$ cells from mice that remained tumor free after first adoptive transfer. Tumors were measured using a metric dial caliper (Monostat), and tumor weight was determined by the formula $(W^2 \times L)/2$.

complete concordance. We have detected expression of both mouse and human MUC1 in the mouse thymus in day 18 embryos and in adult thymus (unpublished data). Thus, MUC1 is expressed during the time that central tolerance is established. It is not surprising that high affinity CTLs are not detectable. Studies from other laboratories have shown the generation of CTLs with low avidity for tumor cells expressing influenza virus hemagglutinin (HA) in insulin-dependent HA transgenic mice that express HA as a self molecule on pancreatic islet β cells (32). Significantly, they have shown that vaccination of insulin-dependent HA mice can activate these low avidity CTLs that are able to reject tumor cells expressing high levels of HA, without destruction of pancreatic islet β cells expressing moderate levels of HA. Studies with other transgenic mouse models expressing model proteins as self Ags have shown that T cells with specificity for self proteins can be demonstrated within the peripheral T cell pool (33–43). Often, potentially autoreactive T cells manage to escape deletion by virtue of their lower avidity for self Ag even when the transgene element is present in, or is available to, the thymus (44). The appearance of reactive CTLs by 18 wk in most of the MET mice was highly significant ($p < 0.001$) and suggested that the high level of aberrantly expressed MUC1 by tumors and in serum was antigenic and able to mobilize these low avidity T cells that elicited a CTL response in vitro and were able to eradicate MUC1-expressing injected tumor cells in vivo (Fig. 9). However, these CTLs were unable to eradicate the spontaneous pancreatic tumors. The fact that a tumor Ag like MUC1 elicits a tumor-specific immune response does not necessarily mean that the immune response will cause the rejection of a spontaneously occurring tumor in vivo. Many reports have suggested that progressing tumors in cancer patients have elaborate means of escaping an apparently effective MHC class I-restricted immune response (45, 46). Other investigators have found that the CTL response occurs too late to be effective against the established tumors (47).

Tumors evade host immunity at both the induction and effector phases. Because MET mice have developed strong anti-MUC1 CTL responses, these spontaneously occurring pancreatic tumors must have evaded the existing CTLs. Recent studies have revealed multiple mechanisms by which tumors have avoided immune destruction. Down-regulating their surface expression of MHC class I and costimulatory molecules, such as B7, are a few of the well-studied mechanisms. Preliminary analysis of several lines of primary pancreatic tumor cells generated ex vivo from 19- to 22-wk MET mice revealed very low level surface expression of both B7 (3–5%) and MHC class I (7–9%) by FACS analysis (data not shown). Tumors may evade CTL recognition by changing their antigenic composition, much as viruses. Clearly, in MET mice, as tumors progress there is antigenic modulation of MUC1 (Figs. 2 and 3), thereby allowing escape of MUC1-specific CTL recognition and killing. Other important escape mechanisms are secretion of immunosuppressive substances or induction of suppressor cells or cells secreting inhibitory cytokines in the host immune system and killing of the effector T cells by tumor cells. Factors implicated in this effect include TGFβ (24). It has been shown previously that TGFβ may alter TCR subcomponent composition and down-regulate CD3ζ, γ, and δ but not ϵ, thereby reducing T cell signaling and CTL responses against tumor cells. When TGFβ expression by the tumors was reduced using antisense techniques, CD3 expression was normalized (26). Pancreatic tumor sections from MET mice showed very high expression of TGFβ2 (Fig. 4*C*, *G*, and *K*) and TGFβ3 (data not shown), suggesting again that the tumors are capable of down-regulating the function of existing CTLs and evading host antitumor immunity.

The most radical way for a tumor to induce nonresponsiveness would be to kill the attacking CTLs. One possible mechanism for T cell killing involves the Fas/FasL pathway. Several types of tumors (human and mouse) express FasL, for example primary lung carcinomas, melanomas, colonic adenocarcinomas, hepatic tumors, multiple myelomas, ovarian carcinomas, pancreatic adenocarcinomas, astrocytomas, head and neck squamous carcinomas, and many others (48). Several of these tumor cells expressing FasL can induce apoptosis of Fas-expressing CTLs, thereby evading their own killing (26). In pancreatic tumors from MET mice, we detect high expression of FasL in the well-differentiated dysplastic acinar cells, whereas the solid undifferentiated tumor mass demonstrated much decreased levels in staining (Fig. 4, *A*, *E*, and *I*). This was surprising as this result would indicate reduced ability of tumor cells to kill CTLs via the Fas/FasL mechanism. One could explain this result by presuming that at the undifferentiated stage, the tumor cells need more protection as they are more accessible to the existing CTLs, whereas the well-differentiated solid tumor mass does not need that protection because by this stage they have become inaccessible to the CTLs. Another possible explanation is that in certain circumstances, Fas-mediated costimulation rather than destruction can occur (49). This possibly helps to explain some instances where expression of FasL results in enhanced destruction rather than protection of FasL-bearing cells. In any case, Fas/FasL interactions and apoptosis are complex mechanisms because both T cells and tumors can express Fas and FasL, making the outcome of the interaction difficult to interpret.

To determine whether existing CTLs can successfully access the MET tumors, we stained tumor sections for infiltrating lymphocytes. Hematoxylin and eosin staining showed highly vascularized tumors with vessels filled with lymphocytes. However, we did not observe any lymphocytic infiltration into the tumors (data not shown).

Induction of the cellular response to MUC1 was accompanied by a very modest tumor response in the MET mice. We also detected low levels of circulating Abs to MUC1 in the 18-wk MET mice. Two of the four animals had detectable but low levels of Abs to MUC1 ($A_{450\ mm}$ readings were 1.5 and 1.7 times the negative control mice, data not shown), suggesting that the aberrant glycosylation and the high level of expression has changed the antigenic profile and elicited a low level humoral immune response to MUC1. Mice from all other time points were defined as negative with $A_{450\ mm}$ readings <1.5 times the negative control sera. Abs reactive with MUC1 have been previously reported in a small number of humans with cancer (16, 17). Although a humoral response is often dismissed as being ineffective as eradicating solid tumors it is still interesting that the response in the MET mice once again parallels that in humans.

Several studies in the literature clearly show that there are no simple correlations between the measured CTL responses and clinical responses (50, 51). Clearly, tumor Ags that are capable of eliciting $CD8^+$ CTL responses in vivo will function as important tumor rejection Ags, and their incorporation into effective tumor vaccination protocols is essential. These studies are in progress. Whereas transplantable tumors can be readily cured with immunotherapeutic approaches, similar therapies in cancer patients have been less effective. These apparently contradictory observations between transplantable murine tumor models and cancer patients could be explained in part by the ability of the slowly progressing spontaneous tumor to induce an immune dysfunction as compared with a rapidly growing transplantable murine tumor, which might spare the immune suppression.

The MET model appropriately mimics the human condition and is an excellent spontaneous pancreatic tumor model with which to

elucidate the most successful forms of tumor immunotherapy. In humans, both unrestricted and MHC class I-restricted MUC1-specific CTLs have been reported (8, 25); however, we have only detected MHC class I-restricted CTLs in MET mice. It must be realized that the situation in vivo may be quite different from that in vitro. It is very likely that mice do possess unrestricted CTLs in vivo, but these CTLs may be difficult to detect in vitro and therefore have never been described previously in mice (52–57). Nevertheless, alterations in MUC1 expression and induction of cellular and humoral immune responses in the MET mice recapitulate what has been described in human cancers. These similarities make this an excellent model for testing therapy in a setting relevant to the treatment of human cancer as well as for prevention and delineation of the mechanisms of tolerance, immunity, and autoimmunity.

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References

1. Braga, V. M. M., L. F. Pemberton, T. Duhig, and S. J. Gendler. 1992. Spatial and temporal expression of an epithelial mucin, Muc-1, during mouse development. *Development 115:427.*
2. Burchell, J., S. Gendler, J. Taylor-Papadimitriou, A. Girling, A. Lewis, R. Millis, and D. Lamport. 1987. Development and characterization of breast cancer reactive monoclonal antibodies directed against to the core protein of the human milk mucin. *Cancer Res. 47:5476.*
3. Girling, A., J. Bartkova, J. Burchell, S. Gendler, C. Gillet, and J. Taylor-Papadimitriou. 1989. A core protein epitope of the polymorphic epithelial mucin detected by the monoclonal antibody SM-3 is selectively exposed in a range of primary carcinomas. *Int. J. Cancer 43:1072.*
4. Taylor-Papadimitriou, J., J. A. Peterson, J. Arklie, J. Burchell, R. L. Ceriani, and W. F. Bodmer. 1981. Monoclonal antibodies to epithelium-specific components of the human milk fat globule membrane: production and reaction with cells in culture. *Int. J. Cancer 28:17.*
5. Zotter, S., P. C. Hageman, A. Lossnitzer, W. J. Mooi, and J. Hilgers. 1988. Tissue and tumor distribution of human polymorphic epithelial mucin. *Cancer Rev. 11:55.*
6. Gendler, S. J., and A. P. Spicer. 1995. Epithelial mucin genes. *Annu. Rev. Physiol. 57:607.*
7. Burchell, J., and J. Taylor-Papadimitriou. 1993. Effect of modification of carbohydrate side chains on the reactivity of antibodies with core-protein epitopes of the MUC1 gene product. *Epithelial Cell Biol. 2:155.*
8. Barnd, D. L., M. S. Lan, R. S. Metzgar, and O. J. Finn. 1989. Specific, major histocompatibility complex-unrestricted recognition of tumor-associated mucins by human cytotoxic T cells. *Proc. Natl. Acad. Sci. USA 86:7159.*
9. Ioannides, C. G., B. Fisk, K. R. Jerome, T. Irimura, J. T. Wharton, and O. J. Finn. 1993. Cytotoxic T cells from ovarian malignant tumors can recognize polymorphic epithelial mucin core peptides. *J. Immunol. 151:3693.*
10. Greenlee, R. T., T. Murray, S. Bolden, and P. A. Wingo. 2000. Cancer statistics, 2000. *CA Cancer J. Clin. 50:7.*
11. Rowse, G. J., R. M. Tempero, M. L. VanLith, M. A. Hollingsworth, and S. J. Gendler. 1998. Tolerance and immunity to MUC1 in a human MUC1 transgenic murine model. *Cancer Res. 58:315.*
12. Mohammad, R. M., M. C. Dugan, A. N. Mohamed, V. P. Almatchy, T. M. Flake, S. T. Dergham, A. F. Shields, A. A. Al-Katib, V. K. Vaitkevicius, and F. H. Sarkar. 1998. Establishment of a human pancreatic tumor xenograft model: potential application for preclinical evaluation of novel therapeutic agents. *Pancreas 16:19.*
13. Hwang, R. F., E. M. Gordon, W. F. Anderson, and D. Parekh. 1998. Gene therapy for primary and metastatic pancreatic cancer with intraperitoneal retroviral vector bearing the wild-type p53 gene. *Surgery 124:143.*
14. Hall, P. A., and N. R. Lemoine. 1993. Models of pancreatic cancer. *Cancer Surv. 16:135.*
15. Agrawal, B., M. J. Krantz, M. J. Reddish, and B. M. Longenecker. 1998. Cancer-associated MUC1 mucin inhibits human T-cell proliferation, which is reversible by IL-2. *Nat. Med. 4:43.*
16. Rughetti, A., V. Turchi, C. A. Ghetti, G. Scambia, P. B. Panici, G. Roncucci, S. Mancuso, L. Frati, and M. Nuti. 1993. Human B-cell immune response to the polymorphic epithelial mucin. *Cancer Res. 53:2457.*
17. Kotera, Y., J. D. Fontenot, G. Pecher, R. S. Metzgar, and O. J. Finn. 1994. Humoral immunity against a tandem repeat epitope of human mucin MUC-1 in sera from breast, pancreatic, and colon cancer patients. *Cancer Res. 54:2856.*
18. Tevethia, M. J., R. H. Bonneau, J. W. Griffith, and L. Mylin. 1997. A simian virus 40 large T-antigen segment containing amino acids 1 to 127 and expressed under the control of the rat elastase-1 promoter produces pancreatic acinar carcinomas in transgenic mice. *J. Virol. 71:8157.*
19. Samuel, J., W. A. Budzynski, M. A. Reddish, L. Ding, G. L. Zimmermann, M. J. Krantz, R. R. Koganty, and B. M. Longenecker. 1997. Immunogenicity and antitumor activity of a liposomal MUC1 peptide-based vaccine. *Int. J. Cancer 75:1.*
20. Reddish, M. A., G. D. MacLean, S. Poppema, A. Berg, and B. M. Longenecker. 1996. Pre-immunotherapy serum CA27.29 (MUC-1) mucin level and $CD69^+$ lymphocytes correlate with effects of Theratope sialyl-Tn-KLH cancer vaccine in active specific immunotherapy. *Cancer Immunol. Immunother. 42:303.*
21. Pemberton, L., J. Taylor-Papadimitriou, and S. J. Gendler. 1992. Antibodies to the cytoplasmic domain of the MUC1 mucin show conservation throughout mammals. *Biochem. Biophys. Res. Commun. 185:167.*
22. Price, M. R., P. D. Rye, E. Petrakou, A. Murray, K. Brady, S. Imai, S. Haga, Y. Kiyozuka, D. Schol, M. F. Meulenbroek, et al. 1998. Summary report on the ISOBM TD-4 Workshop: analysis of 56 monoclonal antibodies against the MUC1 mucin. San Diego, California, November 17–23, 1996. *Tumour Biol. 19:1.*
23. Lan, M. S., A. Khorrami, B. Kaufman, and R. S. Metzgar. 1987. Molecular characterization of a mucin-type antigen associated with human pancreatic cancer: the DU-PAN-2 antigen. *J. Biol. Chem. 262:12863.*
24. Wrann, M., S. Bodmer, R. de Martin, C. Siepl, R. Hofer-Warbinek, K. Frei, E. Hofer, and A. Fontana. 1987. T cell suppressor factor from human glioblastoma cells is a 12.5-kd protein closely related to transforming growth factor-β. *EMBO J. 6:1633.*
25. Domenech, N., R. A. Henderson, and O. J. Finn. 1995. Identification of an HLA-A11-restricted epitope from the tandem repeat domain of the epithelial tumor antigen mucin. *J. Immunol. 155:4766.*
26. Pawelec, G., J. Zeuthen, and R. Kiessling. 1997. Escape from host-antitumor immunity. *Crit. Rev. Oncog. 8:111.*
27. Tempero, R. M., M. L. Vanlith, K. Morikane, G. J. Rowse, S. J. Gendler, and M. A. Hollingsworth. 1998. $Cd4^+$ lymphocytes provide MUC1-specific tumor immunity in vivo that is undetectable in vitro and is absent in MUC1 transgenic mice. *J. Immunol. 161:5500.*
28. Fernandez-Acenero, M. J., M. Galindo-Gallego, J. Sanz, and A. Aljama. 2000. Prognostic influence of tumor-associated eosinophilic infiltrate in colorectal carcinoma. *Cancer 88:1544.*
29. Moezzi, J., N. Gopalswamy, R. J. Haas, Jr., R. J. Markert, S. Suryaprasad, and M. S. Bhutani. 2000. Stromal eosinophilia in colonic epithelial neoplasms. *Am. J. Gastroenterol. 95:520.*
30. Nielsen, H. J., U. Hansen, I. J. Christensen, C. M. Reimert, N. Brunner, and F. Moesgaard. 1999. Independent prognostic value of eosinophil and mast cell infiltration in colorectal cancer tissue. *J. Pathol. 189:487.*
31. Hong, S. W., M. Y. Cho, and C. Park. 1999. Expression of eosinophil chemotactic factors in stomach cancer. *Yonsei Med. J. 40:131.*
32. Morgan, D. J., H. T. Kreuwel, S. Fleck, H. I. Levitsky, D. M. Pardoll, and L. A. Sherman. 1998. Activation of low avidity CTL specific for a self epitope results in tumor rejection but not autoimmunity. *J. Immunol. 160:643.*
33. Cabaniols, J. P., R. Cibotti, P. Kourilsky, K. Kosmatopoulos, and J. M. Kanellopoulos. 1994. Dose-dependent T cell tolerance to an immunodominant self peptide. *Eur. J. Immunol. 24:1743.*
34. Goverman, J., A. Woods, L. Larson, L. P. Weiner, L. Hood, and D. M. Zaller. 1993. Transgenic mice that express a myelin basic protein-specific T cell receptor develop spontaneous autoimmunity. *Cell 72:551.*
35. von Herrath, M. G., J. Dockter, and M. B. Oldstone. 1994. How virus induces a rapid or slow onset insulin-dependent diabetes mellitus in a transgenic model. *Immunity 1:231.*
36. Miller, J. F., and G. Morahan. 1992. Peripheral T cell tolerance. *Annu. Rev. Immunol. 10:51.*
37. Oehen, S. U., P. S. Ohashi, K. Burki, H. Hengartner, R. M. Zinkernagel, and P. Aichele. 1994. Escape of thymocytes and mature T cells from clonal deletion due to limiting tolerogen expression levels. *Cell Immunol. 158:342.*
38. Ohashi, P. S., S. Oehen, K. Buerki, H. Pircher, C. T. Ohashi, B. Odermatt, B. Malissen, R. M. Zinkernagel, and H. Hengartner. 1991. Ablation of "tolerance" and induction of diabetes by virus infection in viral antigen transgenic mice. *Cell 65:305.*
39. Oldstone, M. B., M. Nerenberg, P. Southern, J. Price, and H. Lewicki. 1991. Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: role of anti-self (virus) immune response. *Cell 65:319.*
40. Poindexter, N. J., C. Landon, P. J. Whiteley, and J. A. Kapp. 1992. Comparison of the T cell receptors on insulin-specific hybridomas from insulin transgenic and nontransgenic mice: loss of a subpopulation of self-reactive clones. *J. Immunol. 149:38.*
41. Poplonski, L., B. Vukusic, J. Pawling, S. Clapoff, J. Roder, N. Hozumi, and J. Wither. 1996. Tolerance is overcome in beef insulin-transgenic mice by activation of low-affinity autoreactive T cells. *Eur. J. Immunol. 26:601.*
42. Whiteley, P. J., N. J. Poindexter, C. Landon, and J. A. Kapp. 1990. A peripheral mechanism preserves self-tolerance to a secreted protein in transgenic mice. *J. Immunol. 145:1376.*
43. Yule, T. D., A. Basten, and P. M. Allen. 1993. Hen egg-white lysozyme-specific T cells elicited in hen egg-white lysozyme-transgenic mice retain an imprint of self-tolerance. *J. Immunol. 151:3057.*
44. Morgan, D. J., H. T. Kreuwel, and L. A. Sherman 1999. Antigen concentration and precursor frequency determine the rate of $CD8^+$ T cell tolerance to peripherally expressed antigens. *J. Immunol. 163:723.*
45. Garrido, F., F. Ruiz-Cabello, T. Cabrera, J. J. Perez-Villar, M. Lopez-Botet, M. Duggan-Keen, and P. L. Stern 1997. Implications for immunosurveillance of altered HLA class I phenotypes in human tumours. *Immunol. Today 18:89.*

46. Hicklin, D. J., F. M. Marincola, and S. Ferrone 1999. HLA class I antigen down-regulation in human cancers: T-cell immunotherapy revives an old story. *Mol. Med. Today 5:178.*
47. Toes, R. E., R. J. Blom, R. Offringa, W. M. Kast, and C. J. Melief. 1996. Enhanced tumor outgrowth after peptide vaccination: functional deletion of tumor-specific CTL induced by peptide vaccination can lead to the inability to reject tumors. *J. Immunol. 156:3911.*
48. Walker, P. R., P. Saas, and P. Y. Dietrich. 1997. Role of Fas ligand (CD95L) in immune escape: the tumor cell strikes back. *J. Immunol. 158:4521.*
49. Alderson, M. R., R. J. Armitage, E. Maraskovsky, T. W. Tough, E. Roux, K. Schooley, F. Ramsdell, and D. H. Lynch. 1993. Fas transduces activation signals in normal human T lymphocytes. *J. Exp. Med. 178:2231.*
50. Gilboa, E. 1999. The makings of a tumor rejection antigen. *Immunity 11:263.*
51. Houghton, A. N. 1994. Cancer antigens: immune recognition of self and altered self. *J. Exp. Med. 180:1.*
52. Apostolopoulos, V., P. X. Xing, and I. F. McKenzie. 1994. Murine immune response to cells transfected with human MUC1: immunization with cellular and synthetic antigens. *Cancer Res. 54:5186.*
53. Apostolopoulos, V., V. Karanikas, J. S. Haurum, and I.F. McKenzie. 1997. Induction of HLA-A2-restricted CTLs to the mucin 1 human breast cancer antigen. *J. Immunol. 159:5211.*
54. Acres, B., V. Apostolopoulos, J. M. Balloul, D. Wreschner, P. X. Xing, D. Ali-Hadji, N. Bizouarne, M. P. Kieny, and I. F. McKenzie. 2000. MUC1-specific immune responses in human MUC1 transgenic mice immunized with various human MUC1 vaccines. *Cancer Immunol. Immunother. 48:588.*
55. Pietersz, G. A., W. Li, C. Osinski, V. Apostolopoulos, and I. F. McKenzie. 2000. Definition of MHC-restricted CTL epitopes from non-variable number of tandem repeat sequence of MUC1. *Vaccine 18:2059.*
56. Gong, J., D. Chen, M. Kashiwaba, and D. Kufe. 1997. Induction of antitumor activity by immunization with fusions of dendritic and carcinoma cells. *Nat. Med. 3:558.*
57. Gong, J., D. Chen, M. Kashiwaba, Y. Li, L. Chen, H. Takeuchi, H. Qu, G. J. Rowse, S. J. Gendler, and D. Kufe. 1998. Reversal of tolerance to human MUC1 antigen in MUC1 transgenic mice immunized with fusions of dendritic and carcinoma cells. *Proc. Natl. Acad. Sci. USA 95:6279.*