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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

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

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[2] Address correspondence and reprint requests to Dr. Sandra J. Gendler, Mayo Clinic Scottsdale, S. C. Johnson Medical Research Center, 13400 East Shea Boulevard, Scottsdale, AZ 85259. E-mail address: gendler.sandra@mayo.edu

[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 $\times$ 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*).

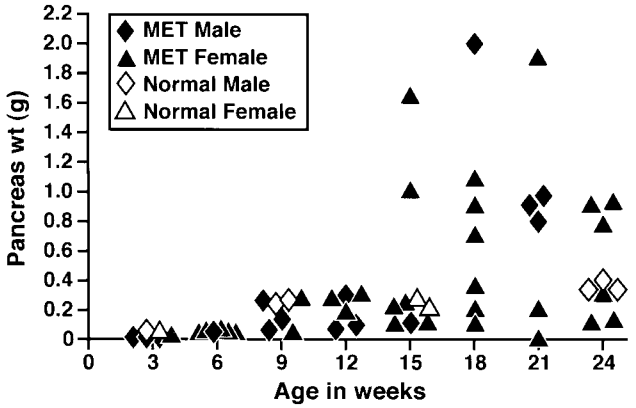

FIGURE 1. Pancreas weight in MET mice as function of age. The entire pancreas was dissected free of fat and lymph nodes, fixed in methacarn, and weighed. Pancreas weights of MET mice are compared with pancreas weights of age-matched normal C57BL/6 mice.

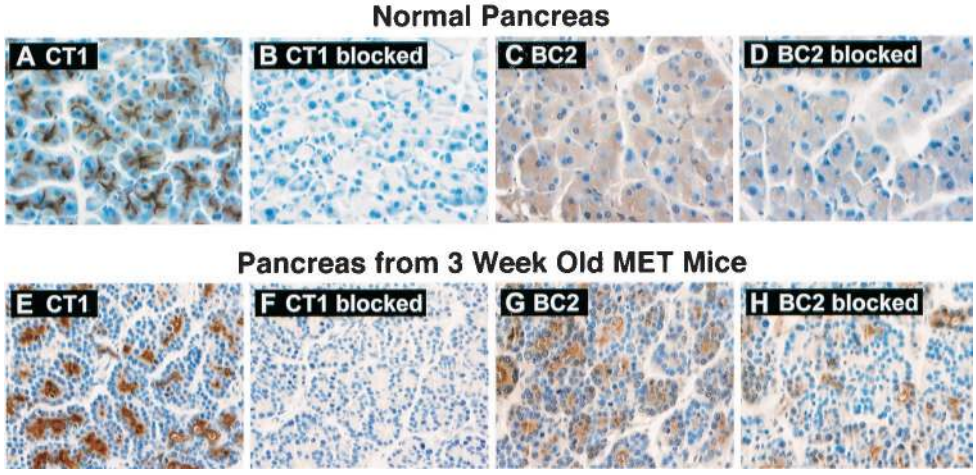

FIGURE 2. Immunohistochemical staining of MUC1-expressing normal pancreas and 3-wk MET pancreatic tumor. Methacarn-fixed and paraffin-embedded sections of normal pancreas showed strong MUC1 expression detected with CT1 antiserum (*A*) and low reactivity with TR mAb BC2 (*C*). Tumors taken at 3 wk show 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

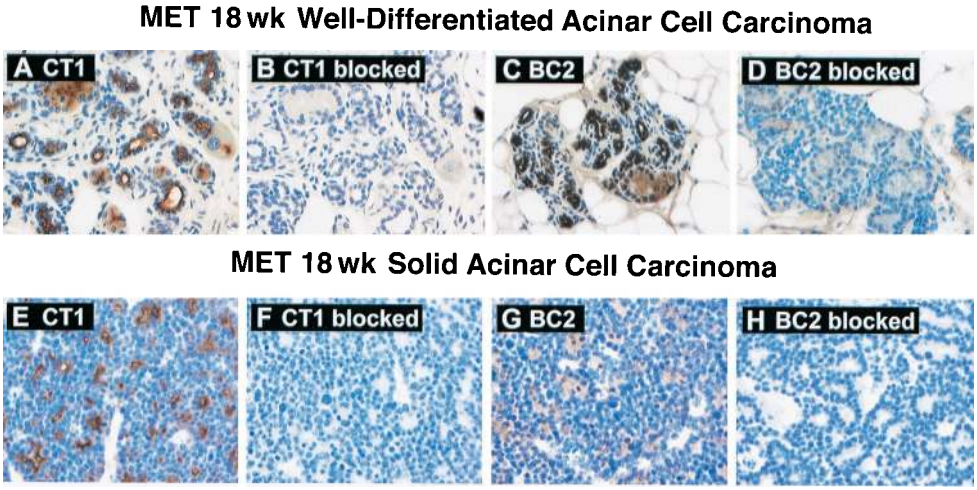

FIGURE 3. Immunohistochemistry of solid acinar cell carcinoma showing decreased expression of TR epitopes on MUC1 core protein. Methacarn-fixed and paraffin-embedded sections of the well-differentiated 18-wk acinar cell carcinoma with TR mAb BC2 (*C*), which is glycosylation sensitive, shows high expression 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.

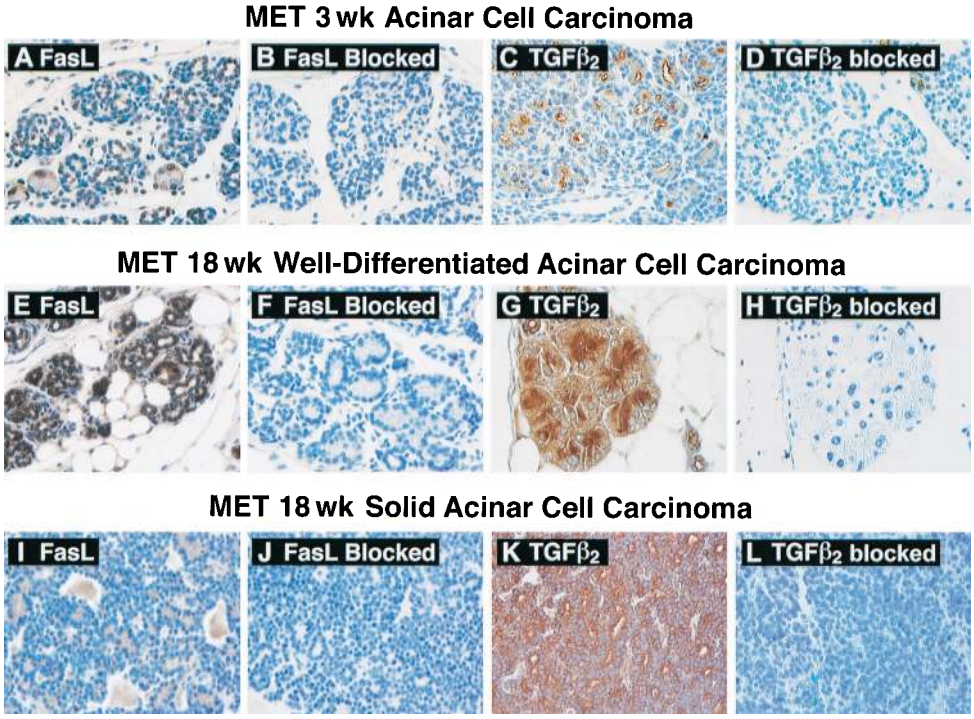

FIGURE 4. Immunohistochemical analysis of FasL and TGFβ2 expression on MET pancreatic tumors. The acinar cell carcinomas were stained with antiserum to FasL and TGFβ2. Low level expression of FasL at 3 wk (*A*) increased as tumors progressed to 18 wk (*E*). The strong staining detected on well-differentiated 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 $\times$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

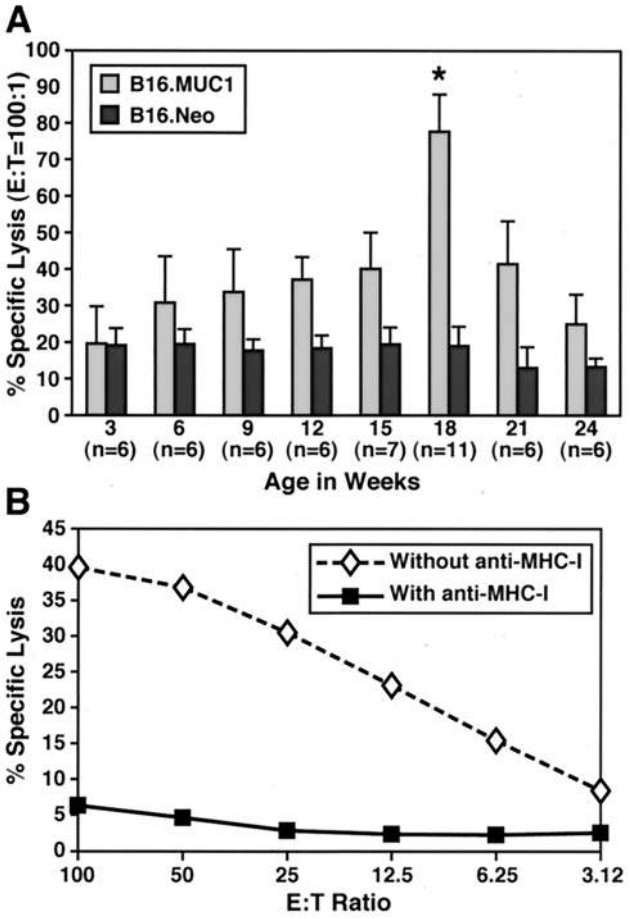

FIGURE 5. CTL precursor activity in MET mice at 100:1 E:T ratio. *A,* CTL activity of splenocytes was evaluated in nonimmunized MET mice of different ages. Determination of CTL activities was performed by a standardized 6-h ^{51}Cr release method after a 6-day in vitro TR peptide stimulation without 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 μg/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.

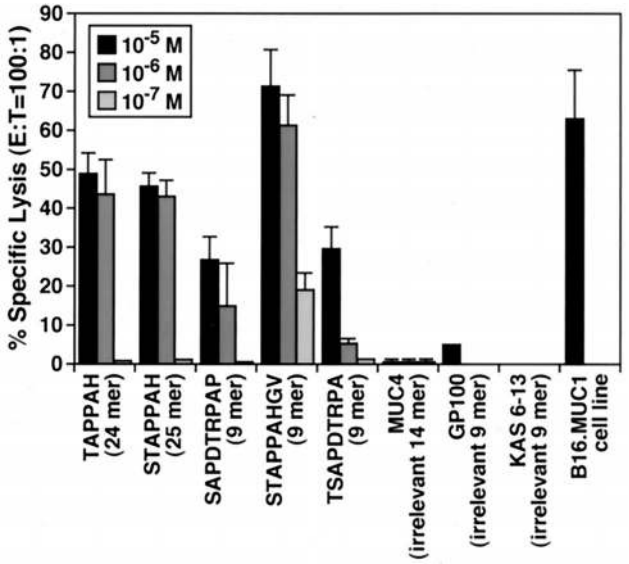

FIGURE 6. Minimum epitope recognized by MET CTLs. Splenocytes were stimulated with MUC1 in vitro for 6 days without added cytokines. DCs were cultured overnight with various concentrations of MUC1 TR peptides ranging from 10^{-5} to 10^{-7} M and used as targets in a standard ^{51}Cr release CTL assay. 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

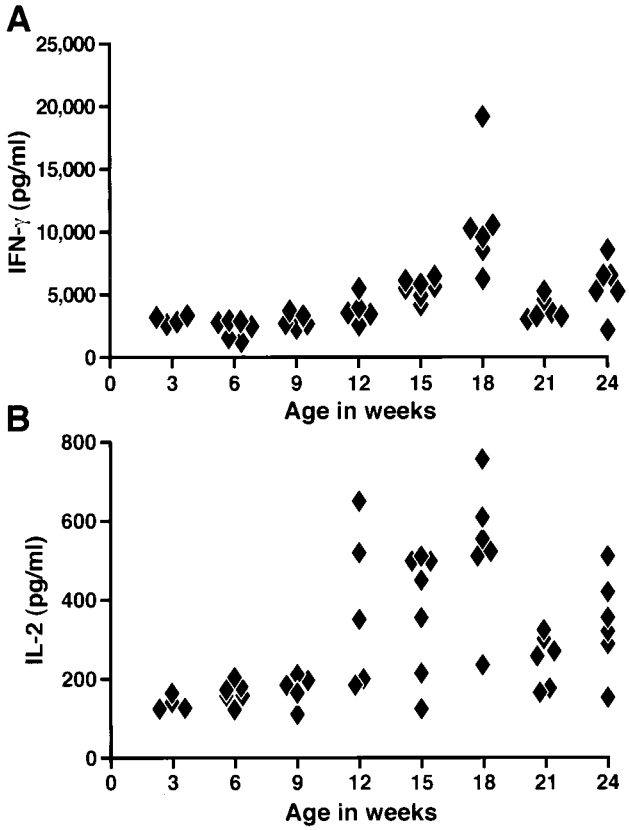

FIGURE 7. IFN-γ and IL-2 levels in MET mice. Splenocytes were derived from individual MET mice and cultured for 6 days with MUC1 TR peptide without added cytokines. Culture supernatants were collected, and cytokine levels determined by specific ELISAs for (*A*) IFN-γ and (*B*) IL-2. IFN-γ levels in the 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).

(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

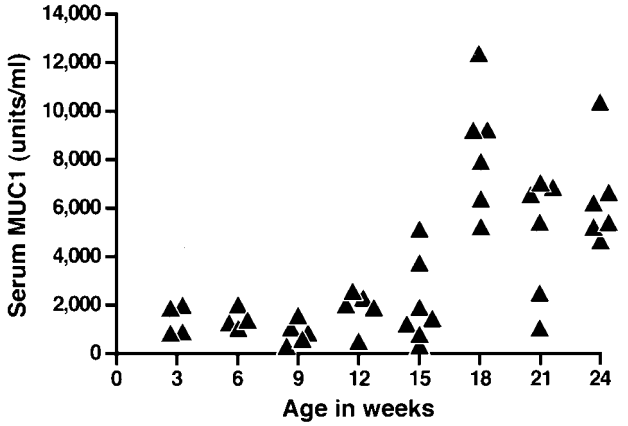

FIGURE 8. Serum MUC1 levels in MET mice. MUC1 levels in serum were determined using the Truquant BR RIA (supplied by Biomira) (20).

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

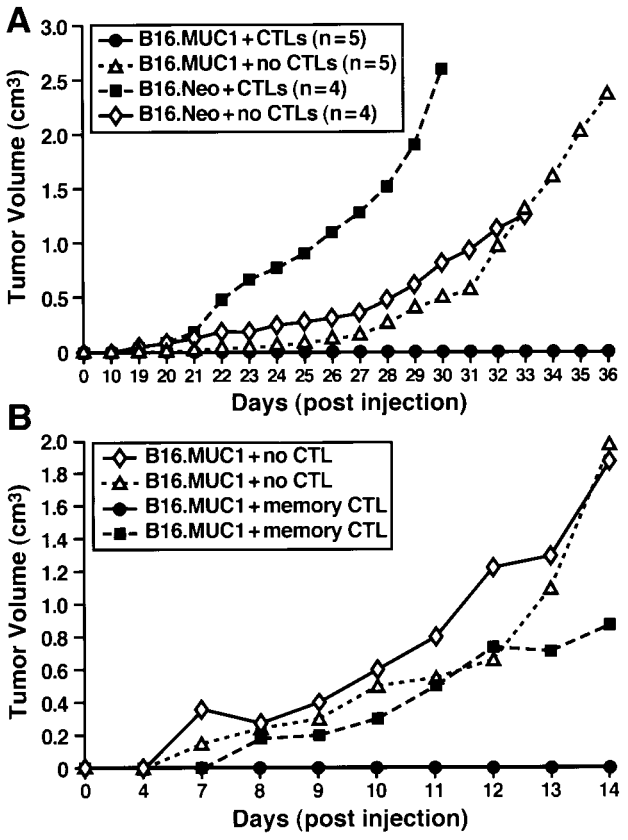

FIGURE 9. Adoptive transfer experiments. *A,* Tumor growth curve of MUC1 expressing B16 melanoma cells and B16.neo control cells (1×10^6 cells/mouse s.c.) with and without adoptively transferred CTLs (5×10^5 cells/mouse i.v.). *B,* Tumor growth curve of MUC1 expressing B16 melanoma and B16.neo control 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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