MUC1 Enhances Tumor Progression and Contributes Toward Immunosuppression in a Mouse Model of Spontaneous Pancreatic Adenocarcinoma

Teresa L. Tinder, Durai B. Subramani, Gargi D. Basu, Judy M. Bradley, Jorge Schettini, Arefayene Million, Todd Skaar and Pinku Mukherjee

*J Immunol* 2008; 181:3116-3125; doi: 10.4049/jimmunol.181.5.3116

http://www.jimmunol.org/content/181/5/3116

---

**References**

This article cites 59 articles, 29 of which you can access for free at:
http://www.jimmunol.org/content/181/5/3116.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
MUC1 Enhances Tumor Progression and Contributes Toward Immunosuppression in a Mouse Model of Spontaneous Pancreatic Adenocarcinoma

Teresa L. Tinder,* Durai B. Subramani,2* Gargi D. Basu,2,3* Judy M. Bradley,* Jorge Schettini,* Arefayene Million,† Todd Skaar,† and Pinku Mukherjee4*

MUC1, a membrane tethered mucin glycoprotein, is overexpressed and aberrantly glycosylated in >80% of human ductal pancreatic adenocarcinoma. However, the role of MUC1 in pancreatic cancer has been elusive, partly due to the lack of an appropriate model. We report the characterization of a novel mouse model that expresses human MUC1 as a self molecule (PDA.MUC1 mice). Pancreatic tumors arise in an appropriate MUC1-tolerant background within an immune-competent host. Significant enhancement in the development of pancreatic intraepithelial preneoplastic lesions and progression to adenocarcinoma is observed in PDA.MUC1 mice, possibly due to increased proliferation. Tumors from PDA.MUC1 mice express higher levels of cyclooxygenase-2 and IDO compared with PDA mice lacking MUC1, especially during early stages of tumor development. The increased proinflammatory milieu correlates with an increased percentage of regulatory T cells and myeloid suppressor cells in the pancreatic tumor and tumor draining lymph nodes. Data shows that during pancreatic cancer progression, PDA.MUC1-mediated mechanisms enhance the onset and progression of the disease, which in turn regulate the immune responses. Thus, the mouse model is ideally suited for testing novel chemopreventive and therapeutic strategies against pancreatic cancer. The Journal of Immunology, 2008, 181: 3116–3125.

Approximately 30,000 Americans develop pancreatic cancer each year and nearly as many die from the disease annually (1). Surgical resection remains the only potentially curative intervention for pancreatic cancer but is contraindicated in most patients because their disease is either locally inoperable or metastatic at presentation (2). Among the minority of patients who undergo surgical resection, the median survival is only 20 mo, with a 5-year survival rate of 8–20% (3). Despite some improvements in outcome, pancreas cancer remains a lethal diagnosis for the vast majority of patients. Greater understanding of the disease and development of new strategies to improve patient outcome are in dire need, but progress in these areas has been limited by the lack of an appropriate model that recapitulates the human disease.

Recently, a mouse model of preinvasive and invasive ductal pancreatic cancer has been developed that recapitulates the full spectrum of human pancreatic intraepithelial preneoplastic lesions (PanINs),5 putative precursors to pancreatic cancer (4). These mice, designated PDA, were generated using P48-Cre (5) to drive the KRASG12D mutation in pancreatic ductal precursor cells (4). We have further crossed the PDA mice to the human MUC1 transgenic (Tg) (MUC1.Tg) (6), which express MUC1 in a pattern and level consistent with that in humans. These mice are called PDA.MUC1.

MUC1 is a highly glycosylated type I transmembrane glycoprotein (7), which is overexpressed in ~70–80% PDA and elevated in the pancreatic juice of pancreatic cancer patients (8–11). MUC1 can function as an enhancer of tumor progression (12, 13), as an oncogene (14), and as a target for therapeutic intervention (7). The antigenic profile of MUC1 on malignant cells is different from normal cells due to changes in its glycosylation and expression levels, making MUC1 immunogenic in tumor-bearing hosts. Patients with pancreatic, breast, and ovarian tumors exhibit increased serum MUC1 levels and spontaneous immune responses including development of Abs and T cells specific for MUC1 (15–19). Generation of the PDA.MUC1 mouse model that expresses human MUC1 as a self molecule enables examination of MUC1 function during pancreatic cancer progression and evaluation of novel MUC1-targeted immune therapies.

Immune-based therapies, though promising, have not been as successful as hoped, in part due to the immune evasion tactics used by tumors to escape immune recognition and/or killing. One such evasion mechanism activated in pancreatic cancer is the arachidonic acid/cyclooxygenase 2 (COX-2) pathway (20). COX-2 is an enzyme that is induced during various pathologic conditions including inflammation and cancer; it converts arachidonic acid to PG. It is now well recognized that tumor-associated COX-2 and its product, PGE2, are highly immunosuppressive. PGE2 directly down-regulates CTL and Th lymphocyte functions (21, 22).
addition, PGE2 reverses the ability of dendritic cells (DCs) within tumors to effectively present Ags to T cells, inducing the generation of T regulatory cells (Tregs) and myeloid suppressor cells (MSCs) (23, 24).

We have recently shown that inhibiting COX-2 significantly enhances cancer vaccine efficacy by reducing the activity of another enzyme, IDO, a major player in inducing immune tolerance (25). IDO catalyzes tryptophan to kynurenine (26) to create a tumor microenvironment that is dangerously low in tryptophan. Immune effector cells, in particular CTLs and Th cells, are highly sensitive to low tryptophan levels and fail to proliferate and function effectively (27–29); however, little is known about IDO function in pancreatic tumors. Herein, we use the PDA.MUC1 model to assess the role of MUC1 in immune modulation in the context of COX-2 and IDO activity in pancreatic tumorigenesis.

Materials and Methods

Generation of PDA.MUC1 mice

PDA mice were generated by breeding P48Cre-expressing mice obtained from Dr. Chris Wright (Vanderbilt University, Nashville, TN) to the LSL-KRASG12D mice obtained from Dr. Tyler Jacks (Massachusetts Institute of Technology, Boston, MA) (4); PDA mice were then mated to heterozygous human MUC1.Tg mice that were maintained as heterozygotes.

For P48Cre, the primers were 5'-AGCTAGCCACCATGGCTTGAGTAAGTCT and 3'-GCGGGTG; and 5'-CCTTTACAAGCGCACGCAGACTGTAGA, 3'-CAGATTCTCAATCTGGACACTG as the samples to control for any errors in handling and/or metabolite carryover; and were backcrossed onto C57BL/6 for 10 generations. The P48-Cre mice (5) were obtained on the FVB background and were backcrossed onto C57BL/6 for 10 generations.

For KRASG12D, primers were 5'-GTCGACAAGCTCAT and 3'-GCGGCGG; 5'-H9262 and cell lysate. In brief, 50-μl of serum was added to a specific ELISA using BC2 (IgG) Ab that recognizes the extracellular MUC1 domain; or scrambled or luciferase non-targeting (scCONTROL) siRNA (fall from Dr. McGuckin, Queensland, Australia); Armenian hamster Abs used were mouse anti-MUC1 tandem repeat (TR), BC2 (1/1000 dilution; gift from Dr. McGuckin, Queensland, Australia); Armenian hamster anti-MUC1 cytoplasmic tail (CT), CT2 (1/50; own), goat anti-COX-2 (1/100; Santa Cruz Biotechnology); goat anti-IDO (1/50; Santa Cruz Biotechnology), mouse anti-PNCA (5 μg/ml at 4°C; BD Biosciences). Secondary Abs were anti-mouse (1/100; Dako), anti-hamster (1/250; Jackson ImmunoResearch Laboratories), and anti-goat (1/100; Dako) IgGs conjugated to HRP. Immunopositivity was assessed using light microscopy and images taken at ×100 or ×200 magnification.

Cell Culture, retroviral infection, and small interfering RNA (siRNA) transfection

Human pancreatic cancer cell lines, BxPC3 and MiaPaCa2 cells (American Type Culture Collection), were cultured in DMEM (Invitrogen) plus 10% FCS, 1% Glutamax (Invitrogen), and 1% penicillin/streptomycin. The retroviral infection protocol was previously described (31). In brief, GP2-293 packaging cells (stably expressing the gag and pol proteins) were cotransfected with full-length MUC1 and vector expressing the VSV-G envelope protein (BxPC3.MUC1) or vector alone (BxPC3Neo). Stable cell lines were selected with 0.5 μg/ml G418 beginning 48 h post infection then sorted by flow cytometry. Two independent infections were done with similar results. Transient siRNA transfection was performed with Lipofectamine2000 (Invitrogen) and 100 nM siRNA oligonucleotides as previously described (32–34). In brief, MiaPaCa2 (high endogenous MUC1) cells were plated at 225,000 cells/well in 6-well plates and grown to 50% confluence. Cells were transfected with MUC1-specific (siGENOME small hairpin), or scrambled or luciferase non-targeting (scCONTROL) siRNA (fall from Dr. McGuckin) according to the manufacturer’s instructions. Post-transfection, MUC1 protein expression was determined at 24, 48, 72, and 96 h by flow cytometry and Western blotting; MUC1 knockdown was maintained for at least 96 h. Data are reported for 48 h post-siRNA treatment. FITC-conjugated anti-MUC1 (BD Pharmingen; clone HPMV) was used at 1 μg/106 cells for flow cytometry.

Serum PGE2, metabolite (PGEM), MUC1, and anti-MUC1 Ab ELISA

PGE2 levels in the sera were assessed using a specific ELISA (Cayman Pharmaceuticals) for the PGEM (13,14-dihydro 15-keto PGE2) according to the manufacturer’s recommended protocol. Results are expressed as µg of PGEM per ml of serum. Serum MUC1 levels were determined using the CA15-3 ELISA (Genway Biotech) (35). Detection of Ab to MUC1 was conducted by a specific ELISA using BC2 (IgG) Ab that recognizes the extracellular MUC1 TR as the standard. The plate was coated with the 24-mer TAPARVTSAAPTRAPPGASTTAP peptide as the capture Ab (6, 36).

Measurement of IDO activity by HPLC analysis of kynurenine and tryptophan

Using a published HPLC assay for IDO enzymatic activity measurement (37) as a starting point, we have optimized and validated a sensitive HPLC assay with UV and fluorescence detection that allows effective chromatographic separation of tryptophan and its metabolite, kynurenine, in serum and cell lysate. In brief, 50-μl sample diluted in 150 μl PBS was added to 50 μl of the internal standard, 3-NT. Proteins were precipitated with 50 µl of TCA; samples were then spun at 14,000 × g for 5 min and 200 µl of supernatant transferred to glass tubes for HPLC analysis. All samples were run in duplicate. Calibrators were prepared and frozen in the same fashion as the samples to control for any errors in handling and/or metabolite degradation.

IFN-γ ELISPOT

Cells from tumor draining lymph nodes (TDLNs) were isolated during tumor development and used as responders in IFN-γ ELISPOT assays. The stimulators were irradiated autologous DCs prepared as previously described (38) and pulsed with either a human MUC1 peptide (for PDA.MUC1 mice) or a mouse Muc1 peptide (for PDA mice). The peptides...
used were human MUC1 TR, STAPAHGVTAPDPTRPGSTAPP; and mouse Muc1 CT, SSLSYTNPAVAATSANL. A responder to stimulator ratio of 10:1 was used. Negative control wells contained T cells stimulated with DCs pulsed with an irrelevant peptide (vesicular stomatitis virus peptide, RGYKYQGL). Spot numbers were determined using computer-assisted video image analysis by Zellnet Consulting. Splenocytes from C57BL/6 mice stimulated with concavalin A were used as a positive control.

**CTL assay: ⁵¹Cr-release assay**

CTL activity was determined by a standard ⁵¹Cr-release method using T cells from TDLNs as effector cells and autologous irradiated DCs pulsed with MUC1 TR peptide (same as the ELISPOT) as stimulator cells. Effectors and stimulator cells were coincubated at a 10:1 ratio for 48 h; effectors were then recovered and incubated with ⁵¹Cr-labeled tumor target cells at a 50:1 ratio for 6 h. Target cells included the MUC1-negative melanoma cell line B16, transfected with either full-length MUC1 (B16.MUC1) or vector alone (B16.neo) (36). Target cells were treated with 5 ng/ml IFN-γ (Amersham Biosciences) 1 day before the assay to upregulate MHC class I surface expression and loaded with 100 µCi ⁵¹Cr (Amersham Biosciences) per 10⁶ target cells for 3 h before incubation with effectors. Radioactive ⁵¹Cr release was determined using the Topcount Microscintillation Counter (Packard Biosciences) and specific lysis was calculated: (experimental cpm – spontaneous cpm)/complete cpm × 100. Spontaneous ⁵¹Cr release in all experiments was 10–15% of complete ⁵¹Cr release.

**Isolation of tumor-infiltrating lymphocytes and flow cytometry**

The pancreas was dissected free of fat in DMEM complete medium, rinsed, and cut in small pieces in serum-free DMEM with 1 mg/ml collagenase. Tumor chunks were incubated at 37°C for 30 min, then mashed using frosted glass, filtered through a sieve, and collected. The tumor cells were washed twice in PBS and resuspended in 2 ml of serum-free DMEM. A percoll (Pharmacia) gradient was prepared in a glass tube by layering 3 ml of percoll on top of the tumor cell suspension and centrifuging at 1000 g for 30 min. The interface was carefully aspirated and the resulting pellet was resuspended in 2 ml of serum-free DMEM.
of 80% Percoll, 3 ml of 40% Percoll, and finally 2 ml of cell suspension; this was spun at 2000 rpm for 20 min ambient without brakes. The cells suspended between 40 and 80% Percoll were collected, washed twice with cold PBS, and resuspended in cold FACs buffer (PBS plus 1% FBS).

Flow cytometry Abs included: for Tregs, allophycocyanin-labeled anti-FoxP3 (eBioscience; clone FJK-16s), PE-labeled anti-CD25 (BD Pharmingen; clone pc-61), and FITC-labeled anti-CD4 (BD Pharmingen; clone GK1.5); for MSCs, FITC-labeled anti-CD11b (BD Pharmingen; clone M1/70) and PE-labeled anti-Gr1 (BD Pharmingen; clone RB6–8c5). Cells were acquired on a BD Pharmingen Cyan flow cytometer and analyzed with BD Biosciences FlowJo version 8. For Treg and MSC analyses, the lymphocyte and granulocyte/macrophage populations were gated, respectively.

Statistical analysis

All statistical analyses were performed by the Mayo Clinic Biostatistics Core Facility. A two-factor ANOVA was used to determine significant differences between experimental groups. For the ELISPOT analysis, data were adjusted for operator (different days at which assays were conducted).

Results

Generation of the PDA.MUC1 mouse

To create the PDA.MUC1 line, PDA mice were mated to heterozygous human MUC1.Tg mice (6) (Fig. 1), which express human Muc/MUC1 expression increases with tumor progression. Staining was done using (A and B) CT2, a mAb that recognizes the CT of both mouse Muc1 and human MUC1, and (C and D) BC2, a mAb directed against the TR of only human MUC1. Representative images were captured at ×200 magnification. n = 15 mice per time point have been evaluated with similar results. For comparison, pancreas sections from age-matched MUC1.Tg mice are shown.
MUC1 in addition to the endogenous mouse Mucl. MUC1.Tg exhibit B and T cell compartment tolerance and are refractory to immunization with MUC1 (6). Since the human transgene is driven by its own promoter, MUC1 expression levels are tissuespecific and appropriate.

Significantly enhanced tumor development in PDA.MUC1

Both PDA and PDA.MUC1 mice developed progressive PanINs ranging from PanIN-IA to PanIN-3, eventually resulting in adenocarcinoma (Fig. 2). However, the kinetics of PanIN development and progression differed significantly between the two lines. In PDA.MUC1 mice, PanIN appeared as early as 6 wk, though none were detected in age-matched PDA mice (Fig. 3A). Larger numbers and higher grade of PanINs continued throughout tumor progression in PDA.MUC1 mice (Fig. 3, A–E). At 26 wk, invasive adenocarcinomas were observed in 8 of 10 PDA.MUC1 mice, whereas only 1 of 10 PDA mice had invasive disease at this age (Fig. 3F). In agreement with this,
PDA.MUC1 mice had significantly greater pancreas weights compared with PDA mice at all time points (Fig. 3G). Tumor metastasis was also more prevalent in the PDA.MUC1: at 34 wk, 4 of 10 PDA.MUC1 mice had lung and liver metastases as compared with 0 of 10 PDA mice; by 48 wk, 6 of 10 PDA.MUC1 had metastases compared with only 1 of 10 PDA (Fig. 4C and data not shown).

**Greater MUC1 expression and mucus accumulation in PDA.MUC1 mice**

Analysis of PanIN lesions using the PAS mucus stain revealed much more mucus accumulation in the PDA.MUC1 pancreas compared with PDA pancreas (Fig. 4A). PAS-positive PanINs are generally more aggressive with increased proliferation; (4) these were detected as early as 6 wk of age in the PDA.MUC1 mice. Similarly, invasive adenocarcinoma was clearly visible in the PDA.MUC1 pancreas, whereas age-matched PDA pancreas did not display the same degree of invasiveness (Fig. 4B and data not shown).

Increased proliferation in PDA.MUC1 tumors

High mucus accumulation and high MUC1 expression correlate with a highly proliferative tumor cell phenotype (4, 9, 39). We therefore examined the expression of proliferating cell nuclear Ag (PCNA), a marker of proliferative cells. Pancreas sections from PDA.MUC1 mice showed considerably higher levels of PCNA-positive cells than do PDA tissues (Fig. 6, A and B). Pancreas sections from age-matched MUC1.Tg mice are shown for comparison (Fig. 6C).

Increased circulating MUC1 but stable anti-MUC1 levels with tumor progression

A considerable rise in circulating MUC1 levels is observed at each time point in PDA.MUC1 mice as PanINs progress to adenocarcinomas (Fig. 7A). In contrast, MUC1 levels were undetectable in the non-tumor-bearing MUC1.Tg mice (data not shown). Similarly, circulating anti-MUC1 IgG levels were significantly higher in PDA.MUC1 mice than that found in MUC1.Tg mice (161 ± 45 μg/ml vs <20 ± 10 μg/ml, respectively). However, the Ab levels did not increase significantly with tumor progression (data not shown).

Early detection of naturally occurring MUC1-specific T cell responses

T cells from TDLNs of PDA and PDA.MUC1 mice were analyzed for MUC1-specific immune responses, namely producing IFN-γ in response to MUC1 Ag and killing MUC1-expressing tumor cells.
In both lines, high levels of MUC1-specific IFN-γ-spot producing cells were present at early stages (6–16 wk) but decreased progressively at later time points (Fig. 7B). Note that the IFN-γ production is more dramatic in PDA mice; this may reflect differential response between the mouse Muc1 peptide use as a stimulant in the PDA assay as compared with the human MUC1 peptide used for the PDA.MUC1 assay. Similar to the decline in IFN-γ, specific CTL-mediated lysis of MUC1-expressing tumor cells (B16.MUC1) was seen at early times during PanIN development (6–16 wk) but disappeared by 26 wk of age (Fig. 7C). PDA.MUC1 CTLs did not kill MUC1-negative B16.neo cells; CTL activity was not analyzed for the PDA mice due to lack of a relevant tumor target.

In both lines, high levels of MUC1-specific IFN-γ-spot producing cells were present at early stages (6–16 wk) but decreased progressively at later time points (Fig. 7B). Note that the IFN-γ production is more dramatic in PDA mice; this may reflect differential response between the mouse Muc1 peptide use as a stimulant in the PDA assay as compared with the human MUC1 peptide used for the PDA.MUC1 assay. Similar to the decline in IFN-γ, specific CTL-mediated lysis of MUC1-expressing tumor cells (B16.MUC1) was seen at early times during PanIN development (6–16 wk) but disappeared by 26 wk of age (Fig. 7C). PDA.MUC1 CTLs did not kill MUC1-negative B16.neo cells; CTL activity was not analyzed for the PDA mice due to lack of a relevant tumor target.

### Table 1. Levels of PGEM, kynurenine, and tryptophan in BxPC3 and MiaPaCa2 human pancreatic cancer tumor cell lines

<table>
<thead>
<tr>
<th>Pancreatic Cancer Cell Lines</th>
<th>Percent MUC1&lt;sup&gt;+&lt;/sup&gt; Cells by Flow Cytometry</th>
<th>PGEM Levels in Supernatant (ngs/ml)</th>
<th>PGEM Levels in Lysate (ngs/ml)</th>
<th>Tryptophan Levels in Lysate (ugs/ml)</th>
<th>Kynurenine Levels in Lysate (ugs/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BxPC3 (wild type)</td>
<td>&lt;0.5</td>
<td>255 ± 30</td>
<td>1025 ± 112</td>
<td>18.3 ± 5.5</td>
<td>312 ± 35</td>
</tr>
<tr>
<td>BxPC3 (neo)</td>
<td>&lt;1</td>
<td>312 ± 55</td>
<td>1242 ± 96</td>
<td>15.6 ± 6.8</td>
<td>437 ± 52</td>
</tr>
<tr>
<td>BxPC3 (MUC1)</td>
<td>85*</td>
<td>623 ± 73*</td>
<td>4037 ± 134*</td>
<td>6.2 ± 2.6*</td>
<td>865 ± 105*</td>
</tr>
<tr>
<td>MiaPaCa (wild type)</td>
<td>95</td>
<td>1025 ± 213</td>
<td>2456 ± 421</td>
<td>29.1 ± 6.3</td>
<td>1022 ± 112</td>
</tr>
<tr>
<td>MiaPaCa (luciferase siRNA)</td>
<td>93 (48 h post-siRNA)</td>
<td>1546 ± 178</td>
<td>3021 ± 265</td>
<td>33.8 ± 12.1</td>
<td>1189 ± 216</td>
</tr>
<tr>
<td>MiaPaCa (MUC1 siRNA)</td>
<td>&lt;10* (48 h post-siRNA)</td>
<td>735 ± 98*</td>
<td>964 ± 123*</td>
<td>10.3 ± 4.8*</td>
<td>3045 ± 534*</td>
</tr>
</tbody>
</table>

*Results are shown for uninfected (wild-type), vector (neo)-, or MUC1-infected BxPC3 cells, and for untransfected (wild-type), control siRNA (luciferase)-, or MUC1 siRNA-transfected MiaPaCa2 cells. Average values from n = 3 samples in two separate experiments are reported. For MiaPaCa2 cells, all data are reported for 48 h post-siRNA treatment. Similar results were obtained within 72 h post-treatment. * Represents significant difference between MUC1-expressing and MUC1-depleted cells from wild-type and control cells.

FIGURE 9. Increased percent of Tregs and MSCs in pancreas and TDLNs of PDA.MUC1 mice. Flow cytometric analysis evaluating percentages of CD4<sup>+</sup>/FOXP3<sup>+</sup>/CD25<sup>+</sup> Tregs in (A) tumor and (B) TDLNs of the PDA.MUC1 and PDA mice as a function of age. n = 10 mice per time were evaluated. Significant increase (*, p < 0.05; **, p < 0.01; and ***, p < 0.001) in Tregs are noted. Representative images are shown of (i) forward and side scatter; box represents the gate around the lymphocyte population; (ii) CD4<sup>+</sup> T cells on the gated lymphocyte population, and (iii) FL1 and FL2 scatter plot; box represents the CD25<sup>+</sup> and FoxP3<sup>+</sup> double-positive cells gated on the CD4<sup>+</sup> T cell population. C, Flow cytometric analysis evaluating percentages of CD11b<sup>+</sup>/Gr1<sup>+</sup> MSCs in tumors of the PDA.MUC1 and PDA mice as a function of age. n = 10 mice per time are evaluated. **, p < 0.0001 at 26 wk; *, p < 0.01 at 34 and 48 wk. Representative images are shown for (i) forward and side scatter of the entire pancreas; box represents the gate around the granulocyte population; (ii) CD11b<sup>+</sup>/Gr1<sup>+</sup> double-positive cells gated on the granulocyte population.
MUC1-associated augmentation of COX-2/PGE2 and IDO/kynurenine pathways

Studies indicate that certain forms of tumor-associated MUC1 are immunosuppressive to T cell and DC function (40, 41). To determine the mechanism of immune regulation by MUC1, we assessed some of the known immune-regulatory pathways activated during carcinogenesis. In PDA.MUC1 mice, expression of both COX-2 and IDO increases with age; the highest expression is observed at 48 wk when almost all mice have developed adenocarcinoma (Fig. 8, A and B). COX-2 and IDO expression levels were comparatively lower in PDA pancreatic tumors (data not shown) and absent in the MUC1.Tg normal pancreas (top left panels, Fig. 8, A and B).

To confirm the effect of MUC1 on COX-2 and IDO activities, we analyzed three measures of these tumor-enhancing pathways. The first, PGE2, is a marker of the COX-2 product PGE2 and was significantly higher in the serum of PDA.MUC1 mice compared with PDA mice, especially at later time points (Fig. 8C; $p < 0.05$ for 26 wk, and $p < 0.001$ for 34 and 48 wk). IDO catalyzes tryptophan to kynurenine, thus high kynurenine and low tryptophan levels are indicative of IDO activity. Significantly higher levels of serum kynurenine were detected in PDA.MUC1 mice compared with PDA mice (Fig. 8D; $p = 0.005$ for 26 wk and $p = 0.0001$ for 34 and 48 wk). Circulating kynurenine was significantly higher in PDA.MUC1 mice than in non-tumor-bearing MUC1.Tg by 16 wk ($p < 0.05$ for 16 wk, $p < 0.0001$ for $\geq 26$ wk). In contrast, the levels of kynurenine in the PDA mice were not significantly altered compared with MUC1.Tg (Fig. 8D). In agreement with the kynurenine results, circulating tryptophan levels were significantly decreased in PDA.MUC1 mice at all time points as compared with MUC1.Tg (Fig. 8E; $p < 0.0001$ for all), whereas in PDA mice, the decrease in tryptophan levels was significant only at later stages ($p < 0.05$ for 26 wk, $p < 0.0001$ for 34 and 48 wk). Importantly, PDA.MUC1 mice had significantly lower levels of tryptophan compared with PDA mice ($p < 0.01$ for 26 wk and $p < 0.001$ for 34 and 48 wk), suggesting increased IDO activity in the MUC1-expressing tumors.

To further analyze whether MUC1 regulates COX-2 and IDO function, we stably infected a MUC1-negative human pancreatic cancer cell line, BxPC3, with full-length MUC1. Two separate stable clones were generated; both culture supernatants and cell lysates were analyzed for PGE2, kynurenine, and tryptophan levels. To complement these studies, MUC1 was depleted from a strongly MUC1-positive human pancreatic cancer cell line, MiaPaCa2, using MUC1-specific siRNA (33). Supernatant and lysates from these cells were analyzed 48 h after MUC1 knockdown. BxPC3 cells expressing MUC1 had significantly increased PGE2 and kynurenine levels and decreased tryptophan levels compared with wild type or vector-infected BxPC3 (Table I). In line with this, knockdown of MUC1 from MiaPaCa2 cells resulted in significantly lower PGE2 and kynurenine, with increased tryptophan (Table I).

Increased expression of Treg and MSC in the PDA.MUC1 pancreas

PGE2 is known to induce the generation of Tregs (23) and recruitment of MSCs within the tumor (24). Therefore, we tested the levels of these immune regulatory cells within the tumor microenvironment and in TDLNs. The PDA.MUC1 mice exhibit significantly higher numbers of Tregs (CD4+CD25+FoxP3+ cells) in the tumor and in the TDLNs compared with PDA mice at all ages (Fig. 9A; $p < 0.03$, $p < 0.01$, and $p < 0.05$ for 6, 26, and 34–48 wk, respectively). Correspondingly, MSCs (CD11b+Gri1+ cells, also defined as Mac1+Ly6G+/PDL1+) were significantly higher in the tumors of PDA.MUC1 mice compared with tumors from PDA mice (Fig. 9B; $p < 0.0001$ for 26–34 wk).

Discussion

Many studies have attempted to elucidate the role of MUC1 in pancreatic cancer progression and explore MUC1 as a target for therapeutic intervention, but lack of appropriate models have made this challenging. We describe a model of spontaneous pancreatic adenocarcinoma that expresses human MUC1 as a self molecule. This mouse model is unique in that the pancreatic tumor arises spontaneously in an appropriate tissue background, within a suitable stromal and hormonal milieu, and in the context of MUC1 tolerance and a viable immune system.

We report that the presence of human MUC1 in the PDA mice significantly enhances the development of PanINs and progression to adenocarcinoma in the presence of KRAS mutation. Muc1/MUC1 expression and mucus accumulation in the PDA.MUC1 pancreas was significantly higher than in PDA mice, a clinically significant observation as higher expression of MUC1 has been associated with greater aggressiveness of PanINs and poorer overall survival in pancreatic cancer (4, 10, 42–45). These findings correlated with the severity of the disease: 80% of PDA.MUC1 mice developed invasive adenocarcinoma by 26 wk with greater proliferation in situ; in contrast, only 10% of PDA mice developed adenocarcinoma. The results strongly implicate MUC1 as an enhancer of PanIN progression and development of invasive adenocarcinoma in the setting of KRAS mutation.

Circulating MUC1 levels in the PDA.MUC1 mice increased with tumor progression, supporting the ability of the model to recapitulate the human disease. This suggests that the PDA.MUC1 model may be an appropriate setting for exploring the use of serum MUC1 as a prognostic and diagnostic marker for pancreatic cancer. In the past, Abs to MUC1 have not been specific enough to differentiate aberrantly glycosylated, tumor-derived MUC1 from other sources of elevated MUC1 such as pancreatitis. However, some success has been shown recently using a PAM4-based immunoassay for circulating MUC1 in diagnosis of pancreatic cancer (46); such assays warrant further investigation in preclinical models.

The PDA.MUC1 model offers an appropriate system to study anti-MUC1 immune responses and MUC1-associated immunosuppression during progression to invasive adenocarcinoma. Robust MUC1-specific T cell responses were detected at early time points. This ties in well with previous studies showing that, though MUC1.Tg non-tumorigenic animals are tolerant to MUC1, very early changes in submicroscopic lesions drive MUC1-specific immune responses, likely through aberrant glycosylation of MUC1. However, anti-MUC1 responses diminished over time, suggesting the existence of immunosuppression with tumor progression. This is supported by a different model of spontaneous pancreatic cancer of acinar origin (36) in which MUC1-specific T cell responses were observed early but not late in oncogenesis. MUC1-specific CTLs from the acinar model were subsequently cloned and used successfully in adoptive transfer experiments (36, 47). The high levels of Tregs and MSCs in the PDA.MUC1 tumors may contribute to the reduction in MUC1-specific immune responses at later times. In humans, MUC1-specific responses have been detected in early stage cancer patients (15–17, 48), but as in the mouse models, anti-MUC1 immunity in humans does not result in antitumor immunity, providing evidence of immunosuppression (49, 50). These immunological characteristics lend credence to the PDA.MUC1 model and create an opportunity to study mechanisms of enhancing pre-existing anti-MUC1 immune responses against the growing tumor in an MUC1-tolerant host.
In addition, mucins produced by cancer cells play a critical role in the induction of COX-2 in the tumor microenvironment (51, 52). Tumor-associated carbohydrate Ags and single mucin-type O-glycans such as Tn and sialyl-Tn Ags (which may be found on MUC1) correlated with COX-2 overexpression and low CD8+ T cell infiltration in endometrial cancer; strong expression of sialyl-Tn was associated with poor prognosis (52–54). However, few reports address MUC1 as an immune modulator within the pancreatic cancer microenvironment. We show that PDA.MUC1 tumors have higher COX-2 and IDO activity than PDA tumors, possibly a result of MUC1 enhancing tumorigenicity and/or accumulation of acidic mucins. COX-2 and IDO are major players not only in immune tolerance but also in tumor progression, metastasis, and angiogenesis. Thus, it is feasible that MUC1 expression may contribute toward a highly tolerogenic tumor microenvironment by influencing the COX-2/PGE2 and the IDO/tryptophan pathways. We recognize that the effect of MUC1 may not be direct and that increased COX-2 and IDO activities may themselves enhance MUC1 expression.

Taken together, the data suggest that MUC1-specific immune responses are present early in tumor development and are eventually suppressed, possibly by the growing tumor. Immune suppression during tumor progression is a major impediment to successful immune therapies, but the importance of MUC1 remains unclear. It is known that as tumors progress, neutral mucins are replaced by acidic mucins that negatively regulate T cells and DCs to favor tumor cell proliferation (40, 52, 55–59). We have preliminary evidence for the increased accumulation of acidic mucins in PDA.MUC1 tumors compared with PDA, though further analysis is needed. Thus tumor-associated MUC1 may suppress immunity in part through accumulation of acidic mucins.

These studies demonstrate that MUC1 speeds development of PanIN and fosters progression to adenocarcinoma in the presence of oncogenic KRAS. It is clear that MUC1 by itself (in the MUC1.Tg mice) does not initiate PanIN or adenocarcinoma, suggesting that MUC1 may play a role in immune modulation during existing oncogenesis. This makes MUC1 an even more attractive target for immune-based therapies for pancreatic cancer. To further elucidate the role of MUC1 in this setting, we are currently creating the PDA model in a Muc1 null background. These models will advance our understanding of the importance of MUC1 in immune regulation in pancreatic cancer and may foster use of MUC1 in diagnosis and therapy for this devastating disease.

Acknowledgments
We thank Dr. Sandra Gendler for gifting the MUC1.Tg mice and the MUC1-specific Abs as well for critical comments; Dr. Tyler Jacks and Chris Wright for willingness to discuss and share the LSL-KRasG12D and P24-Cre mice; pathologists, Dr. Giovanni De Petris and Dr. R. Marler, for critical comments on the histology slides; August J. Klug for contributions to genotyping; all members in the animal, histology, and biostatistics cores; and S. J. Gendler. 1998. Tolerance and immunity to MUC1 in a human MUC1 transgenic murine model. Cancer Res. 58: 315–321.


