A conditional mouse model for human MUC1-positive endometriosis shows the presence of anti-MUC1 antibodies and Foxp3+ regulatory T cells

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SUMMARY

Endometriosis is defined by the presence of tissue implants resembling endometrial glands outside of the uterus, at ectopic sites, frequently on the ovarian surface. The ectopic lesions are often invasive, resistant to therapy, and may predispose to endometrioid and clear cell ovarian tumors. The complex mechanisms leading to chronic endometriosis are mediated partly by impaired immune surveillance in the host. Although innate immunity has been addressed previously, the response of adaptive immune effectors to specific antigens has not been characterized, mostly because very few endometriosis antigens have been defined to date. We postulated that the mucin 1 (MUC1) glycoprotein, which is normally present on eutopic human endometrial glands and overexpressed in endometrioid and clear cell ovarian tumors, is also present in ectopic lesions of ovarian endometriosis. Furthermore, changes in MUC1 expression in endometriosis could promote adaptive anti-MUC1 immunity that might play a role in the malignant progression. To test our hypothesis, we crossed MUC1 transgenic mice, which express human MUC1 under the endogenous promoter, with the P-Stop-lox-P-KrasG12D/+ (Kras) mice, in which endometriosis can be induced through Cre-loxP recombination. The double transgenic MUC1Kras mice develop benign, MUC1-positive ovarian lesions, closely resembling human endometriosis. Subsequent to disease induction, the mice generate high titers of IgM and IgG antibodies that are specific for MUC1. Antibodies appear early in disease and the predominance of the IgG1 subclass suggests Th2-driven immunity. Immune phenotyping revealed an accumulation of Foxp3+ regulatory T cells (Tregs) in the draining lymph nodes at late-stage disease. Furthermore, our observations in human endometriosis showed a similar recruitment of FOXP3+ CD4 T cells. Overall, our results reveal a Th2/Treg-dominant natural immunity in endometriosis with potential implications for cancer progression.

INTRODUCTION

Endometriosis is a benign, chronic inflammatory gynecological disorder defined by the presence of tissue implants resembling endometrial glands and stroma outside of the uterus (Bulun, 2009). The ectopic lesions are found most commonly on the ovaries and on the visceral and peritoneal surfaces within the pelvis. As many as 10% of women aged 30-40 can be affected, although many more can have asymptomatic disease (Baldi et al., 2008).

The ectopic lesions often develop as invasive neoplasms and increasing evidence suggests that endometriosis might be a precursor of ovarian epithelial tumors (Brinton et al., 1997; Kobayashi et al., 2007; Melin et al., 2006; Nagle et al., 2008; Olson et al., 2002; Somigliana et al., 2006). Prevention strategies for ovarian cancer, the most lethal gynecologic malignancy, are a research priority, and early identification and treatment of precursor lesions is essential for long-term survival. Current therapy for endometriosis includes surgical and medical approaches aimed at eliminating hormonal imbalances, restoring fertility, reducing the extent of implants and providing pain relief (Baldi et al., 2008; Bulun, 2009). Despite treatment, endometriosis often recurs, leading to renewed pain and infertility. New and improved therapeutic approaches are needed for endometriosis, and the adjuvant potential of immune therapies can be considered.

The pathophysiology of endometriosis is poorly understood. Although the triggering molecular events are yet to be delineated, it has been proposed that impaired immune surveillance (Dunn et al., 2002) in the host may contribute actively to the pathogenesis of endometriosis (Bergqvist et al., 2001; Lebovic et al., 2001; Santanam et al., 2002). Whereas innate immunity has been addressed repeatedly (Maeda et al., 2002; Montagna et al., 2008; Tariverdian et al., 2007; Yamamoto et al., 2008), there have been few studies of the role of adaptive, antigen-specific immunity in this disease (Antsiferova et al., 2005; Podgace et al., 2007; Szyll et al., 2003). Given the potential for the occurrence of ovarian cancer in a subset of endometriosis patients (Brinton et al., 1997; Kobayashi et al., 2007; Melin et al., 2007; Melin et al., 2006; Nagle et al., 2008; Ogawa et al., 2000; Olson et al., 2002; Prowse et al., 2006; Somigliana et al., 2006; Varma et al., 2004; Yoshikawa et al., 2000), the best target antigens to study are those that are present in both endometriosis and ovarian epithelial tumors. Several ovarian tumor-associated antigens have been defined and are currently being tested as antigen-specific immune therapies in ovarian cancer. One such antigen is mucin 1 (MUC1) (reviewed by Vlad et al., 2004), which is normally expressed by glandular and luminal epithelial cells of the human endometrium, and is present only in low levels on the ovarian surface epithelium (Brayman et al., 2004). MUC1 is overexpressed in all subtypes of ovarian epithelial tumors,

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and spontaneous or MUC1 vaccine-induced immunity has been studied extensively in cancer patients (Cramer et al., 2005; Oei et al., 2008; Terry et al., 2007; von Mensdorff-Pouilly et al., 2000). By contrast, much less is known about changes in MUC1 expression and its immunogenic properties in precursor lesions such as endometriosis.

The progression of normal tissue to premalignant and malignant lesions is often a lengthy process leading to complex molecular changes. Whether, and how, these changes are recognized by the adaptive immune system needs to be explored.

This question, which is difficult to address in humans, requires the development of adequate preclinical models. Only a few animal models for endometriosis exist currently, such as immune-deficient animals that have been exposed to transplanted xenogeneic human endometriotic lesions (Awwad et al., 1999; Grummer et al., 2001; Ozawa et al., 2006), and they are not suitable for studies of immunoregulation. Dinulescu et al. (Dinulescu et al., 2005) engineered the first conditional murine model of endometriosis based on the Cre-lox technology (Sauer, 1998). The $loxP$-Stop-$loxP$-$Kras^{G12D/+}$ mice (LSL-Kras, referred to herein as Kras mice) develop de novo, benign ovarian endometriosis-like lesions following injection, to the ovarian bursa, of Cre-encoding adenovirus (AdCre), which mediates DNA recombination and activation of the mutated, floxed, oncogenic $Kras^{G12D/+}$ allele (Dinulescu et al., 2005). The mice show lesions that are similar to human endometriosis, although they do not express any known endometriosis-associated epithelial antigens. The mouse Muc1 homolog (designated ‘Muc1’ to distinguish it from human MUC1) shares little homology with human MUC1 (Spicer et al., 1991) and is not expected to show the same immunogenicity as the human molecule. To explore MUC1 immunobiology in endometriosis, we introduced the human molecule into the Kras model. The newly generated MUC1+/–$loxP$-Stop-$loxP$-$Kras^{G12D/+}$ mice (for brevity, MUC1Kras mice) allowed us to make important observations regarding MUC1 expression and MUC1-specific immune surveillance during the development of endometriosis-like lesions. Our combined data from mice and humans identify MUC1 as a marker of glandular epithelia in endometriosis; show increasing levels of anti-MUC1 antibodies early in the development of lesions; and show an expansion of immune suppressive T cells late in chronic disease. These findings may help explain the role of the adaptive immune system in the pathogenesis of endometriosis and its relationship to cancer.

RESULTS

MUC1 expression in human endometriosis
In the human uterus (Fig. 1A), MUC1 glycoprotein is normally present on the apical pole of epithelial cells lining the lumen of eutopic endometrial glands (Fig. 1B). MUC1 is also expressed by the epithelial cells lining the endometrial lumen (not shown). All glandular and luminal cells in the uterus are positive for cytokeratin 7 (Cyk7) (Fig. 1C), an intermediate filament protein that is important for the proliferation of the endometrial glands during decidualization and a marker for identification of the ectopic endometrium (Norwitz et al., 2000).
MUC1 immunobiology in endometriotic mice

al., 1991). Ovarian endometriosis is defined by the presence in the ovaries of infiltrating glandular lesions resembling uterine glands and stroma (Fig. 1D). Similar to the uterine (eutopic) glands, the ectopic (endometriotic) glands are positive for MUC1 (Fig. 1E) and Cyk7 (Fig. 1F) and are accompanied by focal, periglandular endometriotic stroma and fibrous tissue (Fig. 1F, arrowheads). This is in contrast to normal ovaries, where the only cells that express MUC1, albeit at low levels, are the ovarian surface epithelial (OSE) cells (Fig. 1G).

We confirmed the increase in MUC1 in nine cases of human endometriosis by quantitative real-time polymerase chain reaction (qRT-PCR). All endometriosis specimens (E1-E9) showed significant increases in MUC1 gene transcripts (Fig. 1H) when compared with normal ovaries from women undergoing prophylactic oophorectomies (N1-N3). By contrast, only two out of six cases of benign physiological ovarian cysts showed an upregulation of the MUC1 gene. We acknowledge that, in the absence of cell-specific RNA extraction, some of the variability seen may have been because of the tissue sampling for mRNA extraction. Nevertheless, all endometriosis specimens showed increased levels of MUC1, suggesting that MUC1 could be a marker for epithelial cells in endometriosis lesions.

Anatomical distribution of MUC1 in the gynecological tract of healthy MUC1Kras mice

Dinulescu et al. (Dinulescu et al., 2005) previously reported that activation of the oncogenic KrasG12D mutation in the OSE triggers endometriosis-like ovarian lesions. In order to create a model for the human MUC1 antigen in endometriosis, we crossed the MUC1 mice, which express the human protein under the endogenous promoter, with the Kras mice, in which endometriosis can be induced. The double transgenic MUC1Kras female mice from each litter were screened by PCR and selected based on the heterozygous LSL-KrasG12D/+ allele (supplementary material Fig. S1). All double transgenic mice develop and breed normally and, if unexposed to Cre recombinase, remain healthy throughout their life. The distribution of human MUC1 protein throughout the gynecological tract of healthy MUC1Kras female mice (Fig. 2A-D) closely resembles the distribution in humans (Fig. 1) (Chambers et al., 1994; Rowse et al., 1998). MUC1 expression in the mouse oviduct (Fig. 2A) is similar to that seen in the human fallopian tubes (Brayman et al., 2004). In the eutopic endometrium of MUC1Kras mice (Fig. 2B,D), MUC1 is present on glandular and luminal epithelia, and is confined largely to the apical surface, the facing lumen of the glandular ducts (Fig. 2B), and the endometrial lumen (Fig. 2D). As expected, the ovaries (Fig. 2E) and endometrium (Fig. 2F) from control KrasG12D/+ mice stain negatively for human MUC1. These results illustrate that the tissue distribution of the MUC1 antigen in the gynecological tract of healthy MUC1Kras mice is similar to that seen in MUC1 mice (supplementary material Fig. S2) (Rowse et al., 1998) and in human tissues (Fig. 1B,G), and endorse the suitability of these mice as a model for studying natural or vaccine-induced MUC1-specific immunity in endometriosis and other diseases of the gynecological tract. We note, however, that the normal OSE cells in both MUC1 mice (supplementary material Fig. S2B) and healthy MUC1Kras mice (Fig. 2E) express more MUC1 than the human OSE cells, which show lower, yet detectable, MUC1 levels (Fig. 1G).

Injection of recombinant adenoovirus in the ovarian bursa of MUC1Kras mice leads to effective infection of the OSE

In mice, a bursa (capsule) encloses each ovary, separating it from the abdominal cavity. Kras activation in LSL-KrasG12D/+ (Kras) mice (Dinulescu et al., 2005) requires targeted expression of Cre recombinase in the OSE through a single injection of AdCre under the ovarian bursa. Unlike Kras mice, the OSE cells in MUC1Kras mice express MUC1, a heavily sialylated glycoprotein that can potentially interfere with the ability of the recombinant virus to enter the target cells (Arcasoy et al., 1997). We first confirmed the potential of the adenovirus to infect the surface monolayer of ovarian epithelial cells in these MUC1Kras mice. Intrabursal injection of an adenovirus encoding the lacZ reporter gene (AdLacZ) shows that the virus can successfully infect the MUC1-expressing OSE cells, resulting in blue staining of the surface of the injected ovary upon exposure to X-gal substrate (Fig. 3A). Contralateral uninjected (control) ovaries remained stain-free (Fig. 3B). Furthermore, the efficacy of infection is similar to that seen in single transgenic MUC1 (Fig. 3C) and Kras mice (Fig. 3D). The staining is detectable in the ovaries only, and not in adjacent tissues such as the uterus and/or oviducts (data not shown). Most importantly, the lacZ staining is confined to the OSE cell monolayer.

MUC1Kras mice progressing to disease show increased MUC1-specific humoral immunity

To measure naturally occurring humoral immunity to the MUC1 antigen following disease induction in MUC1Kras mice, we collected blood before, and every 3–4 weeks after, AdCre injection (Fig. 6). The increase of IgM antibodies in experimental mice occurred early after the injection and was maintained throughout the disease (Fig. 6A; and data not shown). The mice also developed MUC1-specific IgG antibodies consisting mostly of the IgG1 subclass (Fig. 6B). At 12 weeks post-injection, some of the MUC1 (control) mice also exhibited an increase in MUC1-specific IgM antibodies, probably because of slight increases in the local expression of MUC1 by the OSE cells in response to transitory adenovirus infection. However, no ectopic lesions were detected in the AdCre-injected MUC1 mice and no MUC1-expressing cells, other than the OSE cells, could be detected in their ovaries (supplementary material Fig. S2). Furthermore, the anti-MUC1 IgG responses in experimental MUC1Kras mice were higher than in control injected MUC1 mice, suggesting that the isotype switch was the result of disease induction rather than local virus-induced early inflammation. Furthermore, the anti-MUC1 IgM antibody titers diminish at late time points, after adenovirus clearance. The isotype switch from IgM to IgG1 is an indirect correlate of in vivo Th2 immunity in mice (Mizoguchi et al., 1999) and our results suggest that a Th2-prone environment may develop in diseased MUC1Kras mice.

MUC1Kras mice with endometriosis show increased CD4 Foxp3 T cells in regional lymph nodes

The balance between the various CD4 T-cell subsets can enhance or limit disease-associated immunity. Our phenotypic analysis of T cells from the draining (para-aortic) lymph nodes showed
increased percentages of CD4+ Foxp3+ regulatory T cells (Tregs) in MUC1Kras- and Kras-diseased mice when compared with uninjected MUC1Kras controls or injected MUC1 mice (Fig. 7A,B; supplementary material Fig. S3; and data not shown). No increases in CD4+ Foxp3+ T cells were detected at early time points, in the absence of lesions (not shown). In addition, the capacity of the T cells in the spleen and regional lymph node to secrete interferon \( \gamma \) (IFN\( \gamma \)) in response to polyclonal stimulation was decreased significantly in diseased mice (Fig. 7C). However, despite the significant increase in the percentage of Tregs in the spleens of diseased mice compared with controls, the increase was less severe when compared with the increase detected in the draining lymph nodes. Overall, these results suggest that when endometriotic lesions are histologically apparent, the immune environment shows skewing towards immune suppression.

To extrapolate our findings to human lesions, we measured the expression of FOXP3 in human endometriosis (Fig. 7D). We detected an increased amount of FOXP3 mRNA in six out of nine patients (E1-E9). Only one of six patients with benign, physiological ovarian cysts (C1-C6) showed FOXP3 transcripts. We confirmed the presence of the FOXP3 protein in lesions by flow cytometry staining of FOXP3+ CD4 T cells that were infiltrating the ovaries of two different endometriosis patients (Fig. 7E). In contrast to the findings in lesions, the systemic FOXP3+ CD4 T cell percentages were within normal limits in both patients, at 2% and 3%, respectively. These results confirm the findings in mice and show, for the first time, the recruitment of FOXP3+ T cells in lesions of endometriosis.

**DISCUSSION**

Very few defined antigens for human endometriosis exist currently and even fewer are confirmed in preclinical animal models. Here, we describe a conditional mouse model of ovarian endometriosis expressing the human MUC1 tumor-associated antigen. The newly generated MUC1Kras mice develop lesions that, in addition to recapitulating the endometriosis-like lesions seen in Kras mice (Dinulescu et al., 2005), are also positive for the human MUC1 antigen, thus mimicking the human disease even more closely. Using the MUC1Kras mice, we detected important changes in ovarian MUC1 expression during disease progression, identified
The report by Dinulescu et al. (Dinulescu et al., 2005) was the first to show that activation of the oncogenic Kras\(^{G12D}\) mutation in the OSE might be responsible for the development of ovarian endometriosis-like lesions in genetically engineered Kras\(^{loxP-Stop-loxP-Kras^{G12D+/−}}\) mice. This model, however, lacks expression of a known antigen with which antigen-specific adaptive immunity can be studied. Although the homology between the mouse Muc1 and the human MUC1 molecules is 87% in the cytosolic tail, the Muc1 extracellular domain (which is normally involved in cell-cell and receptor-ligand interactions, and in immune regulation) is only 34% homologous to its human counterpart (Spicer et al., 1991) and is thus not expected to undergo the same changes before and after disease induction. Furthermore, in vivo and ex vivo studies on murine Muc1 are difficult to perform, mostly owing to the lack of MUC1-specific immune responses, and revealed a Th2/Treg bias in the host.

Nevertheless, our results suggest an apparently paradoxical association between immune reactivity (antibody production) and non-reactivity (potentially owing to immune suppression), similar to findings from Willinsky et al., who demonstrated that tolerance to the antigen occurs at the premalignant stage and induces a default immune response (increased antibodies and T cell unresponsiveness) that is permissive for cancer progression (Willinsky et al., 2008).

In summary, although the preclinical model employed here is not an exact genocopy or immune phenocopy of the human disease, it reproduces closely the histomorphology seen in women and is a valuable in vivo model for ovarian endometriosis. Current studies on the prognostic value of anti-MUC1 antibodies and their relationship to the risk for ovarian cancer are now being explored.

The lower, yet detectable, levels of antibodies seen in MUC1 mice were probably because of the local inflammation associated with AdCre infection. The virus may have triggered the secretion of several pro-inflammatory cytokines (such as IL-1 and IL-6) (Chang et al., 2002) that are responsible for the transitory increase in MUC1 expression (Li et al., 2003; Rowse et al., 1998). Nevertheless, this effect was only transient in MUC1 mice, whose ovaries remained lesion-free throughout the duration of our experiments. By contrast, injection of AdCre in MUC1Kras mice was followed by notable changes in the ovarian surface epithelium and neoformation of endometriotic glands, which triggered persistent high levels of MUC1-specific IgM and IgG1. We postulate that the isotype switch from IgM to IgG1 that was observed during disease development was because of a Th2 bias; this is in agreement with previous reports in humans (Antsiferova et al., 2005; Podgaec et al., 2007) showing increased autoantibody levels (Gleicher et al., 1987) and signs of B-cell activation (Hever et al., 2007). However, it has been argued that chronic exposure to antibodies can promote tumors: antibodies can extravasate into the stroma and form immune complexes that can initiate inflammatory cascades associated with tissue destruction (Johansson et al., 2008). Further studies on the prognostic value of anti-MUC1 antibodies and their relationship to the risk for ovarian cancer are now being explored.
therapies in endometriosis are of limited efficacy, and new and improved venues, including immune-based approaches, are needed. The emergence of MUC1Kras mice will possibly facilitate further studies on the roles of MUC1 in endometriosis, and will allow the in vivo testing of MUC1 vaccines as potential therapies in endometriosis and the prevention of ovarian cancer.

METHODS

Mice

LSL-Kras{G12D/+} mice (B6;129-Kras{tm1Ty}) were obtained from the NIH mouse repository. MUC1 transgenic mice (Rowse et al., 1998) were purchased from Dr S. J. Gendler (The Mayo Clinic, Scottsdale, AZ) and subsequently bred, in house, at the animal facilities of the University of Pittsburgh Cancer Institute (UPCI) and Magee Women's Research Institute (MWRI). All of the experimental procedures described here were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pittsburgh, Pittsburgh, PA.

To obtain MUC1Kras mice, we bred the heterozygous MUC1+/− transgenic mice (MUC1 mice) with the heterozygous LSL-Kras{G12D/+} (Kras) mice. All mice were on the agouti background. PCR genotyping of tail DNA was performed to identify the double transgenic mice in each litter.

PCR genotyping

Mouse tail DNA was isolated using a Puregene DNA purification system (Gentra Systems), according to manufacturer’s instructions. To detect the presence of the wild-type Kras and mutated LSL-Kras{G12D/+} gene, the REDTaq ReadyMix PCR reaction mix (Sigma) was used in a reaction volume of 30 μl. PCR conditions were as follows: 94°C for 3 minutes; 34 cycles of 94°C for 30 seconds, 60°C for 90 seconds and 72°C for 1 minute; followed by 5 minutes at 72°C (Jackson et al., 2001; Tuveson et al., 2004). The sequences of the primers are: forward (KrasG12D/+): 5’-CTCCACGTCGTGGACATTGATG-3’ and (wild-type Kras) 5’-GTCGAC AAG -CTTGCCAGCCA TAGCACCAAG-3’; reverse (universal) 5’-CGCAGACTGTA GACAGCG-3’.

For MUC1 detection, PCR was performed in a reaction volume of 25 μl using AmpliTaq Gold 1.5 U in 10× PCR buffer II, supplemented with 25 mM of MgCl2 and 2.5 mM of deoxynucleotide triphosphates (dNTPs) (all from Applied Biosystems). PCR conditions were as follows: 95°C for 10 minutes; 39 cycles of 94°C for 1 minute, 59°C for 1 minute and 72°C for 1 minute; followed by 10 minutes at 72°C (Beatty et al., 2007). The MUC1 primer sequences follows: 94°C for 3 minutes; 34 cycles of 94°C for 30 seconds, 60°C for 90 seconds and 72°C for 1 minute; followed by 5 minutes at 72°C (Jackson et al., 2001; Tuveson et al., 2004). The sequences of the primers are: forward 5’-CTTGCCAGCCA TAGCACCAAG-3’ and reverse 5’-CTCCACGTCGTGGACATTGATG-3’.

Administration of recombinant adenovirus

Recombinant adenoviruses encoding for either the lacZ reporter gene [Ad5CMVlacz (AdLacZ)] or Cre recombinase [Ad5CMVCre (AdCre)] were injected in vivo under the bursa of surgically exposed ovaries, according to a published protocol (Dinulescu et al., 2005). Both AdLacZ and AdCre were purchased from the University of Iowa Gene Transfer Vector Core. To synchronize ovulation (Flesken-Nikitin et al., 2003), animals were injected intraperitoneally (IP) with 5 μl of pregnant mare serum gonadotropin (PMSG, Sigma) and, 48 hours later, injected IP with 5 μl of human chorionic gonadotropin (hCG, Sigma).

For the lacZ reporter gene experiments, seven different mice (three MUC1Kras, two Kras, two MUC1) were injected under the ovarian bursa with 1.2×107 PFU of AdLacZ, 36 hours after the hCG IP injection. Mice were sacrificed 72 hours post-AdLacZ injection and the organs of the gynecologic tracts (ovaries, oviducts, uteri) were harvested for X-gal staining of intact tissue (whole mounts) (Kiernan, 2007). The organs were fixed for 5 minutes at room temperature in a fixative solution (2% formaldehyde, 0.25% glutaraldehyde in PBS) and then washed three times with PBS. The 0.2% X-gal substrate (5-bromo-4-chloro-3-indolyl-D-galactopyranoside; Invitrogen) was resuspended in N,N-dimethyl formamide and then diluted to the final concentration in 2 mM MgCl2, 5 mM K3Fe(CN)6·3H2O, 5 mM K3Fe(CN)6 in Hank’s buffered salt solution (HBSS) (all from Sigma). The indigo-stained

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**Fig. 6. Natural antibody responses to MUC1 in mice with endometriosis.** At 36 hours after the induction of ovulation, 6–8-week-old female mice were injected with 2.5×107 PFU of AdCre, under the bursa. Blood was collected by tail venipuncture, which was performed at baseline and at every 3 weeks thereafter. Serum anti-MUC1 antibodies were measured by ELISA. The results of the ELISAs for IgM (A) and IgG1 (B) are shown for three different time points: baseline (pre-injection; diamonds), 12 weeks (circles) and 32 weeks (triangles) post-injection. Serum was diluted at 1:80. Each assay was run in triplicate and results from one representative assay are shown. The values shown represent the average optical density (OD) readings after subtracting the readings from control (BSA-coated) wells. Symbols: MUC1Kras (black symbols), Kras (gray symbols), MUC1 (white symbols). For each group, the readings at 12 weeks (circles) and 32 weeks (triangles) were compared with those at baseline (diamonds), and the statistical significance was calculated using the Student’s t-test (Statgraphics). *P<0.0001; **P<0.005; ***P<0.01. (Other statistical calculations: at 12 weeks, IgM is higher in MUC1Kras mice than MUC1 mice, P=0.002; at 32 weeks, both IgM (P=0.05) and IgG1 (P=0.005) are higher in MUC1Kras mice than in MUC1 mice.)
areas indicate β-galactosidase activity in infected cells only and were visualized with a Leica L2 inverted scope and a Canon digital camera. A lack of staining indicates uninfected cells.

For endometriosis-inducing experiments, animals underwent survival surgery and intrabursal injection of AdCre (2.5 × 10⁷ PFU) to one ovary only (with the contralateral ovary serving as a control), at approximately 36 hours after hCG administration. The adenovirus was delivered using a modified calcium phosphate precipitation protocol, as described previously (Dinulescu et al., 2005).

**MUC1-specific antibody detection by ELISA**

Blood samples from experimental (MUC1Kras) and control (Kras and MUC1) mice were obtained by venipuncture of the tail vein before AdCre injections (baseline) and then at every 4 weeks until 8-10 months post-disease induction. Serum was separated by centrifugation and tested for the presence of MUC1-specific antibodies with a MUC1-specific ELISA, as described previously (Soares et al., 2001). Briefly, 96-well Immulon 4 HBX plates (Fisher Scientific) were coated overnight with 10 µg/ml of a 100-amino-acid-long MUC1 peptide in PBS, at room temperature. This peptide comprises five MUC1 tandem repeats: the amino acid sequence of one repeat is GVTSAPDTRPAPGSTAPPAH. The peptide was synthesized at the University of Pittsburgh Cancer Institute Peptide Synthesis Facility. Half of each plate was coated with control 2.5% bovine serum albumin (BSA) to serve as a negative control. The pre-coated plates were incubated with pre-diluted serum (1:80) for 1 hour at room temperature and then with

Fig. 7. Identification of Tregs in diseased mice and in human endometriosis lesions. (A) Abdominal para-aortic (draining) lymph nodes from four different female mice were collected at 32 weeks post-injection and prepared for flow cytometry. Lymph node cells were stained for CD3, CD4, CD8 and Foxp3. The upper dot plots are from two diseased MUC1Kras mice. The lower dot plots are from one diseased Kras mouse (left) and one healthy (non-injected) age-matched MUC1Kras control (right). Percentages were obtained using FACSDiva software, after gating for CD3, subgating for CD4, and subtracting background events from a control gate using an isotype control antibody for Foxp3 (supplementary material Fig. S4). Additional results from two AdCre-injected MUC1 mice, and the gating strategy, are detailed in supplementary material Fig. S3. (B) The percentages of Foxp3 T cells in regional (para-aortic) lymph nodes are higher in diseased mice (MUC1Kras-d, n=4; and Kras-d, n=4) than in healthy age-matched mice (MUC1Kras-h, n=4) (*P<0.02; **P<0.01). (C) Detection of intracellular IFNγ by flow cytometry following phorbol myristate acetate (PMA)-ionomycin stimulation of splenic T cells from healthy age-matched MUC1Kras control mice (upper three dot plots) and from experimental diseased mice (lower three dot plots). Inset dot plots show the percentages of CD3+ CD4+ Foxp3 T cells in spleens from the same mice. Numbers were obtained in FACSDiva using the gating hierarchy described in supplementary material Fig. S4. (D) Quantitation of human FOXP3 gene transcripts by qRT-PCR. mRNA was extracted from normal ovarian tissues (N1-N3, n=3), ovarian endometriosis tissues (E1-E9, n=9) and ovarian cysts (C1-C6, n=6). All results were normalized to GAPDH and presented as the percentage change from N, which is the average of three normal ovaries, rendered as 100%. (E) Detection, by flow cytometry, of human CD3+ CD4+ FOXP3 T cells that were isolated from either endometriosis tissue (left dot plots) or peripheral blood (right dot plots) from the same patients.
goat anti-mouse peroxidase-conjugated secondary antibodies (anti-IgM, anti-IgG and anti-IgG1, Sigma) for 1 hour at room temperature. The plates were washed three times with PBS–Tween 20 (0.1%) and then incubated with the substrate O-phenylenediamine dihydrochloride tablets (Sigma) for 1 hour. The reaction was stopped, and the absorbance was measured at 450 nm with a Multiskan plate reader (Thermo Scientific). Data was represented using the average of triplicate wells, after subtracting the background readings from control wells. Data acquisition and analysis was performed using Ascent Software for Multiskan (Thermo Scientific).

**Flow cytometry**

Immune phenotyping of murine lymphocytes: cells were collected from the para-aortic lymph node and spleen of euthanized mice, and prepared by mechanical disruption and red blood cell (RBC) lysis using RBC lysing buffer (Sigma). The cells were stained within 24 hours. For intracellular cytokine detection, cells were stimulated with PMA (5 μg/ml) and ionomycin (50 μg/ml) for 6 hours in the presence of Golgi Plug (all from BD Biosciences). After stimulation, cells were stained with fluorescent antibodies (all from BD Biosciences) for CD3 (clone 145-2C11), CD4 (clone RM4-5) and CD8 (clone 53-6.7), and then treated with Cytofix/Cytoperm (BD Biosciences). For cytokine detection, anti-mouse IFN-γ (clone XMG1.2) was used. Intracellular staining for Foxp3 (clone FJK-16s) was performed using a mouse Foxp3 staining kit (eBioscience). All antibodies were diluted according to manufacturers’ instructions.

Immune phenotyping of human lymphocytes: lymphocytes infiltrating the endometriotic lesions were isolated using enzymatic tissue digestion, whereby the ovarian tissue was digested for 2-4 hours. For intracellular cytokine detection, cells were stimulated with PMA (5 μg/ml) and ionomycin (50 μg/ml) for 6 hours in the presence of Golgi Plug (all from BD Biosciences). After stimulation, cells were stained with fluorescent antibodies (all from BD Biosciences) for CD3 (clone 145-2C11), CD4 (clone RM4-5) and CD8 (clone 53-6.7), and then treated with Cytofix/Cytoperm (BD Biosciences). For cytokine detection, anti-mouse IFN-γ (clone XMG1.2) was used. Intracellular staining for Foxp3 (clone FJK-16s) was performed using a mouse Foxp3 staining kit (eBioscience). All antibodies were diluted according to manufacturers’ instructions.

**Immunohistochemistry**

Sections with a thickness of 4 μm were obtained from each selected block of formalin-fixed, paraffin-embedded ovary. Antigen retrieval was performed in 0.1 M TRIS buffer (pH 9) for 20 minutes at 100°C. The primary antibodies used were: anti-human MUC1 (clone HMPV, 1:250; BD Pharmingen), anti-Cyk7 (clone RCK105, 1:10; Abcam), anti-ERα rabbit polyclonal IgG (MC-20, 1:100; Santa Cruz Biotechnology). The following secondary antibodies were used (for 30 minutes): biotinylated goat anti-mouse IgGs (1:50; BD Pharmingen) and labeled polymer-horseradish peroxidase (HRP), anti-rabbit (Dakocytomation), ready to use. The antigen-bound antibodies were visualized with a 3,3’-diaminobenzidine (DAB) substrate kit (BD Pharmingen) for 5-10 minutes: positive cells are visualized in brown. Counterstaining was carried out with Mayer’s hematoxylin for 30 seconds, which stains the nuclei blue. Sections were mounted with Permount (Fisher Scientific). Images were acquired using a Canon PowerShot A640 digital camera attached to a Zeiss microscope connected to a Dell workstation, using the AxioVision Rel. 4.6 imaging software.

**Quantitative real-time polymerase chain reaction (qRT-PCR)**

Human specimens were obtained from the University of Pittsburgh Health Sciences Tissue Bank, according to approved Institutional Review Board (IRB) protocols. RNA was extracted from 25 to 100 mg of each homogenized tissue: three normal ovarian tissues (N1-N3), which were used as controls; six ovarian physiological cysts (C1-C6); and nine endometriosis tissues (E1-E9). Total RNA was isolated with TRIzol reagent (Invitrogen) and then purified using an RNeasy Mini kit (Qiagen), according to the manufacturer’s protocol. A High-Capacity cDNA Archive Kit (Applied Biosystems) was used to convert up to 1 μg of total RNA in a single 20 μl reaction
Statistical analyses

Comparisons between ELISA readings from the sera of experimental mice versus control mice were performed with Statgraphics Plus software (Statistical Graphics Corp.) using the Student’s t-test for comparisons of two means.

AUTHOR CONTRIBUTIONS

R.A.B., A.M.V., I.D. and R.C. performed the in vivo experimental work in mice and A.D. contributed reagents. A.M.V. designed all experiments, R.A.B., A.M.V., I.D. and R.C. performed the in vivo experimental work in mice and A.D. performed the qRT-PCR experiments. R.P.E. provided the clinical specimens and A.D. contributed reagents. A.M.V. designed all experiments, and A.D. and R.P.E. participated in the conception, design and coordination of the study. The manuscript was finalized by A.M.V. with assistance from all authors.

SUPPLEMENTARY MATERIAL

Supplementary material for this article is available at dmm.biologists.org using the Statgraphics Plus software (Statistical Graphics Corp.) using the Student’s t-test for comparisons of two means.

REFERENCES


Tube: Endometriosis PBMIC IC

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