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PD-1 regulates T cell proliferation in a tissue and subset specific manner during normal mouse pregnancy

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Abstract

The regulation of T cell homeostasis during pregnancy has important implications for maternal tolerance and immunity. Evidence suggests that Programmed Death-1 (PD-1) participates in regulation of T cell homeostasis and peripheral tolerance. To examine the contribution of PD-1 signaling on T cell homeostasis during normal mouse pregnancy, we examined T cell number or proportion, PD-1 expression, proliferation, and apoptosis by flow cytometry, BrdU incorporation, and TUNEL assay in pregnant mice given anti-PD-1 blocking antibody or control on days 10, 12, and 14 of gestation. We observed tissue, treatment, and T cell-specific differences in PD-1 expression. Both pregnancy and PD-1 blockade increased T cell proliferation in the spleen while this effect was limited to CD4 T cells in the uterine- draining nodes. In the uterus, PD-1 blockade markedly altered the composition of the T cell pool. These studies support the idea that pregnancy is a state of dynamic T cell homeostasis and suggest that this state is partially supported by PD-1 signaling.

Keywords

Immunity; tolerance; homeostasis; pregnancy; T lymphocytes

INTRODUCTION

A logical corollary of the issues raised by maternal tolerance of the fetus is the question of whether the adaptive immune system is fundamentally different during pregnancy. A valid potential line of specific questioning therefore involves asking whether or not the basic mechanisms that regulate T cell signaling, activation, function, memory, survival and death are altered simply because the host is carrying a fetus, is exposed to pregnancy-related hormones, and is in contact with fetal antigens. We have observed that during syngeneic pregnancy in the C57BL/6 mouse, the maternal T cell pool undergoes enhanced homeostatic turnover. This is likely related to increased expression of receptors to cytokines, such as interleukin 7 and interleukin 15, that are known to support homeostatic proliferation. This

turnover is also likely driven by increased expression of both pro and anti apoptotic genes (Norton et al., 2010) and related to exposure and recognition of male-specific antigens (Bonney et al., 2011; Lissauer et al., 2012). Homeostatic proliferation in response to chronic antigen stimulation can lead to “exhaustion”, marked by expression of a discrete set of genes and decreased immune function (Wherry et al., 2003). It can also lead to death mediated by Fas/Fas ligand signaling (Fortner et al., 2010; Fortner et al., 2011). Proliferation induced exhaustion can also be prevented and/or broken (Barber et al., 2006). There has been increased attention to regulation of homeostatic proliferation as a mediator of T cell development and function, not only of the exhaustion phenotype, but also in the maintenance of naïve and memory T cell pools, and potentially in the generation of autoimmunity. This attention, coupled with our observation of enhanced homeostatic turnover during pregnancy enticed us to further examine this mechanism.

Programmed death 1 (PD-1) is a regulatory receptor (Freeman et al., 2000) which is expressed in low levels on naïve T cells, but is rapidly up-regulated within 24hr of activation (Agata et al., 1996). After binding to its ligands (PD-L1 or PD-L2), phosphatases are recruited that antagonize proximal kinases in the T cell receptor (TCR) signaling cascade (Parry et al., 2005) thus disrupting CD3-dependent gene transcription. A multifunctional protein, PD-1 is known to regulate T cell homeostasis, as the PD-1 pathway is important for limiting the expansion of activated antigen-specific T cells (Zhang et al., 2008), and the amount of PD-1 expressed on T cells may contribute to their sensitivity to negative regulation through this pathway (Carter et al., 2002).

Negative signaling through PD-1 is involved in regulating the homeostatic proliferation of T cells in lymphopenic conditions (Lin et al., 2007; Thangavelu et al., 2011b) and also plays an important role in peripheral tolerance and in the prevention of autoimmunity (Fife and Pauken, 2011; Thangavelu, et al., 2011b). For example, disruption of this pathway in PD-1 knockout mice facilitates the accumulation of auto-reactive T cell clones and the development of strain-specific autoimmune disease (Nishimura et al., 1999; Nishimura et al., 2001). Additionally, more than 30 single nucleotide polymorphisms of the PD-1 gene have been described in humans and some have been linked to autoimmune diseases (Okazaki and Honjo, 2007). In transplantation models, the loss of PD-1 enhances T cell proliferation and causes accelerated graft destruction (Koehn et al., 2008; Wang et al., 2008).

The two PD-1 ligands are differentially expressed by antigen presenting cells (Yamazaki et al., 2002), as well as non-immune cells throughout the body (Latchman et al., 2004). The loss of PD-1 ligand 1 (PD-L1) and PD-L2 causes an accumulation of chronically activated lymphocytes and the development of tissue specific T cell mediated autoimmunity (Keir et al., 2006; Liang et al., 2003). In addition, PD-L1 knockout mice infected with lymphocytic choriomeningitis virus (LCMV) clone 13 die from severe immune-mediated tissue destruction (Mueller et al., 2010). Other studies demonstrated that disruption of PD-L1 enhances graft rejection (Tanaka et al., 2007; Wang, et al., 2008). In contrast, peripheral tolerance can be induced when high levels of PD-1 ligands are expressed during antigen presentation such that negative signals through the PD-1 pathway outweigh positive costimulatory signals (Okazaki and Honjo, 2006b). Over-expression of PD-1 ligands

promotes tolerance of transplanted tissue (Koehn et al., 2008; Ozkaynak et al., 2002; Thangavelu et al., 2011a) and tumor cells (Iwai et al., 2002).

While PD-1 signaling is important to prevent excessive T cell activation (Freeman et al., 2000) its over-expression can cause T cell dysfunction and exhaustion. T cell exhaustion can be mediated through repeated antigen presentation or by chronic antigen exposure in the peripheral tissues (Mueller and Ahmed, 2009). In CD8 T cells, exhaustion is characterized by the progressive loss of effector function (Fuller et al., 2004) in which target cell lysis and IL-2 secretion is impaired first, then TNF α and IFN γ production decreases (Wherry et al., 2003). The level of PD-1 up-regulation in exhausted cells correlates with declining IFN γ secretion (Blackburn et al., 2009). Chronic viral infections including LCMV in mice (Barber et al., 2006; Okazaki and Honjo, 2006a) and HIV infection in humans (D'Souza et al., 2007) induce PD-1 up-regulation and signify T cell exhaustion. Blockade of PD-1 or PD-L1 in these viral infections can improve CD8 T cell proliferation, cytokine production, and cytotoxicity (Barber et al., 2006; Sauce et al., 2007; Yao et al., 2011).

The aim of this study was to determine the contribution of PD-1 signaling to T cell homeostasis during pregnancy. We found that during pregnancy CD4 and CD8 T cells exhibited tissue and subset-specific differences in the gene expression of PD-1. The effect of PD-1 blockade on proliferation was also dependent on the tissue and T cell subset. Although PD-1 blockade was associated with an accumulation of CD8 T cells in the uterus this was not associated with increased resorption or decreased litter size. These data further highlight the complexity of immune cell homeostasis during normal pregnancy.

MATERIALS AND METHODS

Mice and breeding

All mice were housed and bred at the University of Vermont in animal facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care. Mice were maintained under specific pathogen-free conditions. Eight to ten week old C57BL/6 (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Timed mating to same-strain males was performed as described previously (Norton, et al., 2010). The presence of a copulation plug was denoted day 0 of pregnancy. All animal studies were conducted in accordance with the policies of the Institutional Animal Care and Use Committee at the University of Vermont.

Antibodies and reagents

The following anti-mouse monoclonal antibodies were purchased from BD Biosciences (San Jose, CA, USA): PE-conjugated TCR β , APC-conjugated CD8, and FITC-conjugated CD4, FITC-conjugated TCR β , APC-conjugated CD44, APC-Cy7-conjugated CD25, APC-Cy7-conjugated CD4, FITC-conjugated anti-BrdU. The following monoclonal antibodies were purchased from Invitrogen Corporation/Caltag Laboratories (Carlsbad, CA, USA): PE-Texas-Red (PETR)-conjugated CD4, PE-Cy5.5-conjugated CD8, PE-Cy5.5-conjugated TCR β . The following monoclonal antibodies were purchased from eBioscience Inc. (San Diego, CA, USA): PE-conjugated PD-1 and FITC-conjugated PD-1 (Clone J43(Agata, et al.,

1996)). Fluorescein-12-2'-deoxy-uridine-5'-triphosphate (FITC-dUTP) was purchased from Roche Applied Science (Indianapolis, IN, USA).

PD-1 blockade

Plugged females and naïve age-matched littermates were divided into treatment groups so that half received anti-PD-1 antibodies and half received Rat IgG2a K isotype control antibodies (IgG control). On days 10, 12, and 14 of gestation pregnant mice received intraperitoneal injections of 150µg anti-PD-1 or 150µg IgG control antibodies in 150µl total volume. Unmated controls received treatment group specific injections at the same time as their corresponding pregnant littermates. LEAF-purified anti-PD-1 (clone RMPI-14(Yamazaki et al., 2005)) and LEAF-purified Rat IgG2a K isotype control (clone RTK2758) antibodies were purchased from Biolegend (San Diego, CA, USA) for these studies.

FACS sorting and RT-QPCR of sorted cells

Individual spleens and pools of uterine draining lymph nodes (2 femoral and 2 para-aortic nodes from 3 mice) were isolated from unmated controls and pregnant mice on gestational days 5, 8, and 15. Single-cell suspensions were generated by mechanical dissociation and aliquots of 10–30 million cells were treated with 5µM Fcγ III/II receptor (BD Biosciences) for 10 min to block non-specific antibody binding. Samples were then incubated with antibodies to CD4, CD8, and TCRβ in sterile phosphate buffered saline (PBS) (Mediatech Inc., Manassas, VA, USA) containing 0.1% bovine serum albumin fraction V (BSA) (Sigma, St. Louis, MO, USA) (PBS-0.1%BSA) for 30 minutes at 4°C. After washing twice with sterile PBS, cells were resuspended in sterile IMDM medium (Invitrogen) and sorted using the 70µm tip of the BD FACSAria cell sorter (BD Biosciences). Forward and side scatter gating selected nucleated cells, and aggregates were eliminated by doublet discrimination. TCRβ⁺ cells were selected then CD4⁺ and CD8⁺ cells were sorted to greater than 90% purity. Total RNA was extracted from greater than 500,000 cells using Trizol reagent (Invitrogen) per manufacturer's guidelines. Samples were quantified by UV absorbance at 260 nm on a NanoDrop spectrophotometer (ThermoScientific, Wilmington, DE, USA) and RNA integrity was tested using the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

The iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) was used to synthesize cDNA from 250ng of RNA template. The following target genes were amplified: programmed death 1 (*Pd-1*, forward 5'GCTCGTGGTAACAGAGAGAATC-3', reverse 5'-CAGCAGCAGCAATACAGGGATAC-3'); hypoxanthine guanine phosphoribosyl transferase 1 (*Hprt1*, forward 5'-CAGTCCCAGCGTCGTGAT-3', reverse 5'-CAAGTCTTTCAGTCCTGTCCATAA-3'); and succinate dehydrogenase complex subunit A (*Sdha*, forward 5'ATGCCAGGGAAGATTACAAAGTGC-3', reverse 5'-GTAACCTTGCCAGTCTTGATGTCC-3'). At least one primer in each set was designed over an exon-exon junction or spanned an intron region and negative water controls were run for each primer. Each reaction contained 1µl of cDNA, 150nM of each primer, and 12.5µl of Power Sybrgreen Master Mix (Applied Biosystems, Carlsbad, CA, USA) and was performed on an ABI Prism 7000 (Applied Biosystems). Cycling conditions included an

initial denaturation of 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. Each sample was run in triplicate and averaged. PCR products were analyzed by melt curve analysis and were checked for correct size on 2% agarose gels.

The relative quantities of each sample were determined from standard curves generated for each target gene. The expression of the *hprt1* and *sdha* housekeeping genes were confirmed to be stable throughout gestation using the geNorm program (Vandesompele et al., 2002). The relative expression of PD-1 in each sample was then determined by dividing by the geometric mean of the housekeeping genes.

Tissue preparation for flow cytometry

Spleen and uterine draining lymph nodes (para-aortic and femoral) of pregnant mice and unmated controls were isolated in IMDM medium containing 10% fetal bovine serum (Invitrogen) and 1μM beta-mercaptoethanol (Bio-Rad Laboratories) and single cell suspensions were generated by mechanical dissociation. The uteri of non-pregnant mice were removed by cutting at the cervix and ovaries, and then uteri from 3–4 mice were pooled together. The uteri of pregnant mice were isolated by bisecting each uterine horn and peeling away fetal-placental units from the decidual attachment sites. Using a modification of a published methods (Tilburgs et al., 2006) uteri were cut into small pieces and enzymatically digested with 200 U/ml hyaluronidase (Sigma), 0.2 mg/ml DNase I (Sigma), and 0.28 U/ml Liberase Blendzyme 3 (Roche Applied Science) in Hanks Balanced Salt Solution (Mediatech Inc.) containing 10% BSA (Sigma) for 20 minutes at 37°C. Samples were washed twice with PBS-0.1% BSA, then pressed through 100μm mesh and passed through a MACS pre-separation filter (Miltenyi Biotec. Inc., Auburn CA, USA) to remove cell clumps.

In vivo BrdU assay

T cell proliferation was determined using the previously described bromodeoxyuridine (BrdU) incorporation assay (Norton et al., 2009). Pregnant mice and unmated controls received four intraperitoneal injections of 1 mg BrdU (100ul of 10mg/ml BrdU in sterile PBS) (Sigma) in the 24 hours prior to euthanasia. One million spleen and uterine draining lymph node cells were treated with 0.5μM Fcγ III/II Receptor (BD Biosciences), and then stained with antibodies against CD4, CD8, and TCRβ in PBS-0.1% BSA for 30 min at 4°C. Samples were washed with sterile PBS (Mediatech Inc.), then fixed with PBS containing 1% methanol-free formaldehyde (Ted Pella Inc., Redding, CA, USA), and permeabilized overnight in PBS-1% methanol-free formaldehyde containing 0.01% Tween 20 (Sigma). The following day DNA was digested by treatment with 50U/ml of deoxyribonuclease I (Sigma) in buffer containing 0.15M NaCl, 4.2mM MgCl₂ (Sigma) at pH 5.0 for 15 min at 37°C. Cells were washed with PBS and stained with FITC-conjugated anti-BrdU for 30 minutes at 4°C. Samples were then washed with PBS-0.1% BSA and fixed with PBS-0.1% BSA-1% methanol-free formaldehyde. BrdU incorporation in TCRβ⁺CD4⁺ and TCRβ⁺CD8⁺ cells was detected by using a BD LSRII flow cytometer (BD Biosciences) and quantified using FlowJo software analysis (Tree Star, Inc. Ashland, OR, USA).

TUNEL assay to detect apoptosis

The terminal deoxynucleotidyl-transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) flow cytometric assay was used to detect the nicked DNA in apoptotic cells as described previously (Norton, et al., 2009). Briefly, single cell suspensions of spleen and uterine draining node cells were treated with 0.5 μ M Fc γ III/II Receptor and stained with the same antibodies as described in the BrdU assay. Cells were fixed in PBS-1% methanol-free formaldehyde (Ted Pella Inc.) for 15 minutes, washed with PBS (Mediatech Inc.), and then permeabilized by treatment with ice-cold 70% ethanol in PBS for 15 minutes. After washing with PBS, cells were incubated with 10U of terminal deoxynucleotidyl transferase (TdT) and 6.25 μ M FITC-dUTP in 1X TdT reaction buffer with 2.5mM cobalt chloride (all from Roche Applied Science) for 1 hour at 37°C. Samples were then washed with PBS- 0.1% BSA and fixed with PBS-0.1% BSA-1% methanol-free formaldehyde. TUNEL positive TCR β ⁺CD4⁺ and TCR β ⁺CD8⁺ cells were detected by flow cytometry (BD LSRII, BD Biosciences) and quantified with FlowJo software analysis (Tree Star, Inc.).

Mean Fluorescence intensity

Single cell suspensions of spleen, uterine draining node, and uterus were treated with 0.5 μ M Fc γ III/II receptor (BD Biosciences) for 10 min to block non-specific antibody binding. Cells were then incubated with antibodies to CD4, CD8, TCR β , CD44, CD25, and PD-1 for 30 minutes at 4°C. Samples were washed with PBS-0.1% BSA and fixed with PBS-0.1% BSA-1% methanol-free formaldehyde. The geometric mean fluorescence intensity of each molecule on TCR β ⁺CD4⁺ and TCR β ⁺CD8⁺ cells was determined by flow cytometry (BD LSRII, BD Biosciences) and FlowJo software analysis (Tree Star, Inc.).

Data Analysis & Statistics

Flow cytometric data was analyzed using FlowJo software analysis (Tree Star, Inc.) by first selecting lymphocytes by forward and side scatter gating. Histogram analysis was used to select TCR β ⁺ cells, and then CD4⁺ and CD8⁺ T cells were gated. Histogram analysis was used to select CD4⁺ and CD8⁺ T cells that were positive for BrdU and TUNEL staining. Then the number of proliferating or apoptotic cells was calculated by multiplying the number of TCR β ⁺CD4⁺ or TCR β ⁺CD8⁺ cells by the proportion of these cells that were BrdU⁺ or TUNEL⁺ respectively.

Data was graphed and statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). For analysis of splenic RT-QPCR data, one-way analysis of variance (ANOVA) with Newman-Keuls multiple comparison test was used to compare mice in each group. Two-way ANOVA with Bonferroni multiple comparison test was performed to compare pregnant mice and unmated controls treated with IgG control or anti-PD-1 blocking antibodies. In all cases, p values of less than 0.05 were considered significant.

RESULTS

Expression of PD-1 in T cells during normal pregnancy

We have previously observed that pregnancy in non-transgenic syngeneic matings induces multi-lineage expansion and contraction within cells of the spleen (Norton et al., 2009). Within the T cell pool, we also found that both splenic CD4 (Bonney et al., 2011) and CD8 T cells (Norton et al., 2010) undergo a period of enhanced proliferation during normal gestation. Because PD-1 signaling is thought to play an important role in T cell homeostasis by limiting the expansion of activated cells, we hypothesized that pregnancy should alter the expression of *Pd-1* in the spleen. To test this hypothesis, CD4 and CD8 T cells were purified from the spleens of day 5, 8 and 15 timed-pregnant mice and unmated age-matched littermates. The relative gene expression of *Pd-1* was determined by quantitative RT-PCR. Moreover, surface expression of PD-1 was examined by flow cytometry.

In this tissue the relative mRNA expression of *Pd-1*, calculated after normalization to two housekeeping genes (*Sdha* and *Hprt1*), was not significantly elevated or depressed in CD4 T cells sorted from day 5, 8 and 15 pregnant mice compared to unmated (UM) controls (Figure 1A). In CD8 T cells, the relative expression of *Pd-1* was significantly elevated on day 8 of pregnancy (Figure 1C). We also observed that the relative expression of *Pd-1* was consistently higher in splenic CD4 T cells as compared to CD8s (Figure 1A and C), regardless of pregnancy status. CD4 T cells expressed nineteen times more *Pd-1* in unmated mice, and on day 5, 8, and 15 of gestation, the expression was ten, nine, and twenty times higher than CD8s, respectively.

Surprisingly, surface protein expression of PD-1, did not reveal a significant increase or decrease in PD-1 in CD4 or CD8 T cells with respect to pregnancy (Figure 1B and D). Moreover, PD-1 was equivalently expressed on the surface of both T cell types.

The T cells residing in the uterine draining lymph nodes might be exposed to a higher concentration of placental cytokines, or other factors and fetal antigens, including the male antigen, H-Y. Such antigens can be presented in maternal MHC molecules and generate specific immunity during pregnancy (Bonney and Matzinger, 1997; James et al., 2003; Moldenhauer et al., 2009) even if the mother is syngeneic to the fetus (Bonney E.A. and Matzinger, 1997), as in these studies. To examine the effect of pregnancy, we isolated CD4⁺ and CD8⁺ T cells from the para-aortic and femoral nodes of 3–4 mice and determined the relative expression of *Pd-1*. In pooled uterine draining lymph nodes (uterine DN), the relative expression of *Pd-1* was similar in CD4 and CD8 T cells isolated from unmated controls and pregnant mice on each gestational day examined (Figure 1E and G). As in the spleen, the relative expression of *Pd-1* was higher in CD4 than CD8 T cells. However, the relative expression of *Pd-1* in uterine DN CD4 T cells was 50% less than splenic CD4s from the same mice (Figure 1A and E). In contrast, CD8 T cells from the uterine DN expressed equivalent or slightly higher amounts of *Pd-1* when compared to their splenic counterparts (Figure 1C and G). While surface expression of PD-1 in CD4 and CD-8 T cells, was not altered by pregnancy (Figure 1F and H), surface expression of PD-1 was similar overall in spleen and uterine DN. Taken together, the data suggests tissue and cell-specific transcriptional regulation of PD-1.

CD4 T cell homeostasis in the context of PD-1 blockade during pregnancy

T cell number is maintained in lymphoid tissue through a balance of homeostatic proliferation and apoptosis (Fortner et al., 2010). Because PD-1 signaling is an important negative regulator (Freeman et al., 2000), we asked whether CD4 or CD8 T cells were more sensitive to regulation through the PD-1 pathway, and if PD-1 blockade would disrupt their homeostasis during pregnancy. To address this, pregnant mice and unmated controls were treated with IgG control or PD-1 blocking antibodies and CD4 T cell homeostasis was investigated by determining the total cell number, proliferation, and apoptosis in the spleen and pooled uterine draining lymph nodes. Mice in each group were then compared by 2-way ANOVA. Representative flow cytometry is shown (Figure 2A).

First, CD4 T cells in the spleen were analyzed. CD4 cell number was calculated from the total number of cells in the tissue and the proportion of TCR β^+ CD4 $^+$ cells by flow cytometry. In the spleen, the number of CD4 T cells was significantly higher by gestational day 15 as the result of pregnancy ($p=0.001$) but was not altered by PD-1 blockade ($p=0.6$, Figure 2B).

Elevated cell number may represent either enhanced proliferation or an accumulation of cells over time. CD4 T cell proliferation was assayed by an *in vivo* bromodeoxyuridine (BrdU) incorporation assay, which identifies cells proliferating during the 24hr period of BrdU administration. In the spleen, the number of proliferating CD4 T cells was significantly increased as the result of pregnancy ($p<0.0001$) and PD-1 blockade further increased the number of proliferating cells ($p=0.03$, Figure 2C).

Increased proliferation in the context of PD-1 blockade could drive enhanced T cell death in order to maintain homeostasis. To test this, CD4 T cell apoptosis was determined by a flow cytometric terminal deoxynucleotidyl-transferase (TdT)-mediated dUTP nick-end labeling (TUNEL assay). We observed that number of apoptotic CD4 T cells in the spleen was similar in day 15 pregnant and unmated mice ($p=0.7$) and we were unable to detect a statistically significant effect of PD-1 blockade ($p=0.6$, Figure 2D).

Next, we determined the effects of PD-1 blockade on CD4 T cell number, proliferation and apoptosis was determined in the pooled uterine DN. In this tissue, pregnancy alone significantly increased the total number of CD4 T cells on gestational day 15 ($p=0.0002$) but PD-1 blockade did not significantly alter CD4 T cell number ($p=0.8$, Figure 2E). While the number of proliferating CD4 T cells was significantly higher on day 15 of pregnancy ($p<0.0001$, Figure 2F) as compared to unmated mice, PD-1 blockade enhanced proliferation in both unmated and pregnant mice ($p=0.02$). We were unable to detect a difference in the number of apoptotic CD4 T cells in the uterine DN under the influence of pregnancy ($p=0.7$) or PD-1 blockade ($p=0.6$, Figure 2G). Together, these data suggest that CD4 T cell number and proliferation are elevated in pregnancy and this is regulated in part by PD-1 signaling.

CD8 T cell homeostasis in the context of PD-1 blockade during pregnancy

Because CD4 and CD8 T cells may respond to different homeostatic signals (Fortner et al., 2010; Oelert et al., 2010) we next investigated the role of PD-1 signaling in CD8 T cell

homeostasis in normal pregnancy using the same method we employed for study of CD4 T cells. Representative flow cytometry is shown (Figure 3A).

CD8 T cell number was calculated as described above and a change in cell number may reflect homeostatic changes over the period of PD-1 blockade. In the spleen, the total number of CD8 T cells was elevated on day 15 of pregnancy compared to unmated mice ($p=0.03$), but we did not detect a further increase due to anti-PD-1 blockade ($p=0.8$, Figure 3B). We also observed that PD-1 blockade significantly increased CD8 T cell proliferation ($p=0.02$) in addition to the proliferation related to pregnancy ($p=0.003$, Figure 3C). Using the TUNEL assay, we observed that the number of splenic CD8 T cells undergoing apoptosis at that time point was similar to unmated mice ($p=0.6$) and was not significantly altered by PD-1 blockade ($p=0.9$, Figure 3D).

In the uterine DN, the number of CD8 T cells was significantly increased as a result of pregnancy ($p=0.004$) and was not affected by PD-1 blockade ($p=0.9$, Figure 3E). There was a trend towards increased proliferation in the CD8 T cell pool as the result of pregnancy ($p=0.051$), and this was not altered by PD-1 blockade ($p=0.06$, Figure 3F). The number of apoptotic CD8 T cells found at the time of harvest was not significantly increased by either pregnancy ($p=0.3$) or blocking PD-1 ($p=0.7$, Figure 3G). Thus CD8 T cells in the spleen are potentially more responsive to PD-1 signaling than those in the uterine draining lymph node.

Blockade of PD-1 alters T cell surface phenotype

In these studies, PD-1 blockade increased T cell proliferation and this was not abrogated by pregnancy and was in some cases enhanced by it. We would expect that the increased proliferation during PD-1 blockade results from T cell activation and clonal expansion and that these cells would express activation markers. Therefore, we compared the mean fluorescence intensity of candidate molecules in T cells isolated from the spleen and uterine draining lymph nodes of pregnant mice and unmated controls treated with IgG or anti-PD-1 antibodies.

First, since activation of T cells can result in the upregulation of PD-1 (Agata et al., 1996; Vibhakkar et al., 1997) we investigated the effect of PD-1 blockade on its cell surface expression by using flow cytometry and labeled antibodies that do not cross react with the blocking antibodies used in vivo. Figure 4A and 4B show sample flow cytometry. In spleen CD4 T cells, we observed PD-1 expression increase as the result of both pregnancy ($p=0.001$) in the IgG control animals and in response to PD-1 blockade ($p=0.03$ Figure 4C). In the uterine DN, pregnancy did not alter the expression of PD-1 on CD4 T cells ($p=0.8$), however it was elevated in the context of PD-1 blockade ($p=0.011$, Figure 4D). CD8 T cells isolated from the spleen (Figure 4E) uterine DN (Figure 4F) expressed comparable amounts of PD-1 in pregnancy.

In these same experiments, we examined surface expression of the alpha chain of IL-2 receptor (CD25), as it is both an early marker of T cell activation and potentially a marker of regulatory CD4 T cells. In the spleen, CD4 T cells expressed significantly more CD25 protein as the result of pregnancy ($p=0.009$) but not PD-1 blockade ($p=0.3$ Figure 4G). Similarly, CD4 T cells from the uterine DN of pregnant mice expressed higher CD25

($p=0.004$) that was not altered by PD-1 blockade ($p=0.16$, Figure 4H). In contrast, the expression of TCR β , CD4 and CD44 on splenic and uterine DN CD4 T cells was not altered by either pregnancy or PD-1 blockade (data not shown).

Similar to CD4 T cells, neither pregnancy nor PD-1 blockade altered the surface expression of TCR β , CD8, and CD44 on splenic and uterine DN CD8 T cells (data not shown). In contrast to CD4 T cells, CD25 expression was negligible (data not shown) in splenic CD8 T cells and neither pregnancy ($p=0.09$) nor PD-1 blockade ($p=0.4$) significantly altered PD-1 expression. These findings suggest that during pregnancy there exists a hierarchy of responsiveness to PD-1 signaling that is based on tissue and cell type.

PD-1 Blockade alters T cell populations in the uterus during pregnancy

Because of evidence that T cells at the maternal-fetal interface may support tolerance as well as participate in antigen specific immune responses, we sought to examine the effect of PD-1 blockade in the uterus. We observed that the proportion of T cells that were CD4⁺ was similar between MNP and day 15 pregnant mice, and was not increased by PD-1 blockade (Figure 5A). Although we observed a sharp decline in the proportion of CD4 T cells expressing high levels of CD44 (CD44^{hi}) in pregnant mice undergoing PD-1 blockade (Figure 5B) as compared to mice receiving IgG control, the proportion of CD4 T cells that were CD25⁺ in the uterus was not overtly altered by either pregnancy or PD-1 blockade (Figure 5C).

In the CD8 T cell population PD-1 blockade appeared to produce an expansion (Figure 5D), regardless of pregnancy. Like their CD4 counterparts, uterine CD8 T cells expressing high levels of CD44 decreased in response to PD-1 blockade (Figure 5E). This was not observed in the spleen or uterine draining lymph nodes (not shown).

It has been suggested that the reproductive tract contains unique population(s) of T cells that do not express either the CD4 or CD8 co-receptor and which may play a regulatory role (Johansson and Lycke, 2003). Moreover, loss of CD4 or CD8 co-receptor is a sign of prolonged homeostatic proliferation (Fortner et al., 2010). In the uterus the proportion of CD4⁻ CD8⁻ T cells is modestly increased on day 15 of pregnancy (Figure 5F). However, in the context of PD-1 blockade, pregnancy produces a greater relative increase in the proportion of CD4⁻ CD8⁻ population (Figure 5F). Interestingly, the proportion of CD4⁻ CD8⁻ T cells that express high levels of CD44 is elevated in pregnant mice but decreases as the result of PD-1 blockade (Figure 5G). In contrast, PD-1 blockade in MNP mice appears to increase the proportion of CD4⁻ CD8⁻ T cells that are CD44^{hi}. Thus PD-1 blockade produces alterations in the T cell constituents of the uterus (Figure 5G).

Uterine T cell expression of PD-1

The PD-1 pathway may play an important role in the negative regulation of T cells responsive to specific or unspecified environmental antigens. Within the uterus, these antigens may include those expressed uniquely by the fetus. In some systems, PD-1 plays a potentially important role in regulation of fetal antigen specific T cells at the maternal-fetal interface (Taglauer et al., 2009). We thus further investigated PD-1 regulation in pools of uterine CD4 and CD8 T cells in normal pregnancy. We used flow cytometry to determine

the expression of PD-1 on gestational days 5, 8 and 15 and compared it to unmated littermates. These gestational days were chosen because implantation occurs on day 5, placental remodeling occurs around day 8, and day 15 is an important time-point for sensitivity to LPS induced preterm delivery (Bizargity et al., 2009). In these experiments the uteri of antibody treated mated non-pregnant (MNP) mice were used as controls because there is evidence that exposure to male antigens in seminal fluid during mating can induce proliferation of antigen-specific T cells (Moldenhauer et al., 2009).

In un-manipulated pregnancy, we observed that CD4 T cells purified from pooled uteri upregulated surface PD-1 on gestational day 5 as compared to unmated mice (Figure 6A). In uterine CD8 T cells, surface expression of PD-1 was modestly increased on day 5 and 15 of pregnancy (Figure 6B).

The surface expression of PD-1 on uterine T cells led us to question what effect PD-1 blockade would have on T cells at the maternal-fetal interface. PD-1 expression in uterine CD4 T cells was increased as the result of PD-1 blockade (Figure 6C), while the expression of PD-1 on CD8⁺ T cells was markedly increased (Figure 6D). In contrast, pregnancy increased surface PD-1 expression on CD4⁺ CD8⁺ T cells (Figure 6E), with no effect of PD-1 blockade was observed. Interestingly, the uterine CD4⁺ CD8⁺ T cells express more PD-1 than either CD4⁺ or CD8⁺ cells isolated from the same mice. While loss of PD-1-PD-1 ligand signaling has lead to fetal loss in some models (Guleria et al., 2005; Wafula et al., 2009) others have not shown this effect (Taglauer, et al., 2009). We observed that pregnant mice undergoing PD-1 blockade from gestational days 10 to 14 had similar litter sizes and no increase in resorptions as compared to IgG control treated mice (Figure 6F). These results suggest that although PD-1 expression on uterine T cells can be regulated during pregnancy, blockade of this pathway at mid gestation does not cause fetal loss.

DISCUSSION

In this study, the intact T cell pool was investigated in C57BL/6 mice during normal pregnancy and we found that *Pd-1* gene expression was subset-specific with higher levels detected in CD4 compared to CD8 T cells. Furthermore, *Pd-1* expression was tissue dependent in the CD4 population such that spleen cells expressed twice as much *Pd-1* as their cohort in the uterine draining lymph nodes. The expression of *Pd-1* was altered in spleen CD8 T cells in a pregnancy specific way, with increased expression detected on gestational day eight. The marked changes in *Pd-1* were not as evident in surface PD-1 expression as analyzed by flow cytometry. This however may reflect regulation of intracellular stores of PD-1 including control of trafficking to the cell surface in response to T cell activation signals (Pentcheva-Hoang et al., 2007), and does not negate the possible link between increased gene expression the sensitivity to blockade.

We targeted PD-1 signaling by the administration of PD-1 blocking antibodies, which preserves the expression of the PD-1 ligands in peripheral tissues and antigen presenting cells. We found that PD-1 blockade during normal pregnancy altered T cell homeostasis in subset and tissue specific ways. Because *in vitro* assays of T cell proliferation have yielded conflicting results during pregnancy, we utilized bromodeoxyuridine (BrdU) incorporation

to determine *in vivo* proliferation. In the CD4 population, PD-1 blockade augmented proliferation in both the spleen and uterine draining lymph nodes while increased proliferation was only detected in splenic CD8 T cells. The apparent discrepancy between the elevated T cell proliferation we observed after PD-1 blockade and the lack of detected increase in cell number is likely related to the fact that we administered BrdU only during the 24 hours prior to harvest. Thus, only the most recently dividing cells were labeled. Using the TUNEL assay to label nicked DNA, we did not detect a change in T cell apoptosis as a result of either pregnancy or PD-1 blockade. However, due to the rapid clearance of apoptotic cells *in vivo* by phagocytes, any method to analyze apoptosis in freshly isolated cells would be limited to the subset dying at the moment of harvest.

Of interest is that administration of a blocking antibody against PD-1 generated increased surface expression. This is not likely related to overlapping specificities in the anti-PD-1 antibodies or the methods used in these studies as such an overlap would likely *decrease* the detected surface expression. It is however formally possible that administration of specific blocking antibodies or nonspecific immune globulins could directly or indirectly activate T cells. This could in turn lead to increased PD-1 on the cell surface (Agata et al., 1996; Vibhakkar et al., 1997).

Combined with our previous findings, these data suggest that normal pregnancy supports enhanced mid/late-gestation T cell proliferation, which may be the result of increased presentation of fetal and placental antigens (Erlebacher et al., 2007). PD-1 blockade may play a role in regulation of this proliferative state. Due to their higher expression of PD-1, CD4 T cells may be more sensitive to inhibition through this pathway, and in the context of PD-1 blockade activated CD4 T cells are released from this inhibition and undergo enhanced proliferation. This is supported by data in the non-pregnant state showing that PD-1 signaling decreases IL-2 production (Carter et al., 2002). Although it is said that CD4 regulatory T cells play an important role in normal pregnancy (Aluvihare et al., 2004; Kahn and Baltimore, 2010), our studies do not indicate which subset(s) of CD4 T cells are more likely sensitive to PD-1 blockade in the context of pregnancy.

We observed that in the uterus, both CD4 and CD8 T cells retained, and likely up-regulated surface expression of PD-1 during pregnancy. Both T cell subsets were sensitive to PD-1 blockade, in that this treatment increased PD-1 expression. Compared to control, anti PD-1 treatment appeared increase the proportion of CD4-CD8- T cells in the pregnant uterus and moreover decrease the expression of CD44 in this population. A highly speculative model to incorporate these findings might suggest that for some T cells, potentially specific for a subset of as yet unknown antigens highly present in the uterus, stimulation through PD-1 blockade results in increased PD-1 expression, down regulation of the CD4 or 8 co-receptor, and decreased activation. This might be a functional correlate of exhaustion (Bucks et al., 2009; Day et al., 2006; Fourcade et al., 2010; Rocha et al., 1995; Sauce, et al., 2007; Trautmann et al., 2006).

The PD-1 pathway has been implicated in the maintenance of maternal tolerance of the fetus. Estrogen supports the expression of PD-1 (Polanczyk et al., 2006), and the ligands of this molecule are differentially expressed at the maternal-fetal interface in human pregnancy

(Petroff et al., 2002; Petroff, 2005; Petroff et al., 2003). In mouse pregnancies susceptible to early failure, disruption of PD-1/PD-Ligand signaling has been associated with decreased pregnancy success (Wafula et al., 2009). Data also exists suggesting that disruption of the PD-1 pathway during pregnancy could facilitate the expansion of allo-reactive T cells and the generation of an immune response against the fetus (Guleria et al., 2005) which is enhanced in Th17 effector cells (D'Addio et al., 2011) and is also associated with pregnancy loss (D'Addio et al., 2011). In contrast, PD-L1 deficient mice reproduce normally (Nishimura et al., 1998) and deficiency of PD-1/ligand interaction in TCR transgenic OT-1 mice enhanced antigen-specific T cell accumulation but did not enhance fetal loss (Taglauer et al., 2009). Moreover, these and other investigators (Petroff and Perchellet, 2010) did not find an adverse effect of deficiency of PD-1/ligand interaction in allogeneic pregnancies. In our studies, pregnant C57BL/6 mice (responders to the male antigen H-Y and possibly other fetus-specific antigens) undergoing PD-1 blockade from gestational days 10 to 14 had similar litter sizes and no increase in resorptions as compared to IgG control treated mice (Figure 6F). The differing results not only reflect experimental variation, but also the potential importance of differences in antigen-specific T cell frequency, mode and strength of T cell signaling in T cell function. Moreover, the balance between T cell immunity and tolerance involves the integration of multiple positive and negative signals (Nurieva et al., 2006).

Current thinking suggests that full activation of T cells may require three distinct signals delivered through T cell receptor binding of peptide-MHC complexes on antigen presenting cells (Haskins et al., 1984), costimulation (Jenkins and Johnson, 1993), and other survival or proliferation signals (Becker et al., 2002). Finally, the local environment plays an important role modulating these signals (Matzinger, 2007).

We believe that during gestation the same signaling pathways and their integration are utilized as in the non-pregnant state. Previous studies have implicated several mechanisms in maternal tolerance that can control T cell function in the non pregnant state, e.g., MHC expression (Ishitani et al., 2003; Le Bouteiller et al., 1996), costimulation (Petroff, 2005; Petroff et al., 2002) apoptosis (Vacchio and Hodes, 2005), anti-inflammatory cytokines (Bowen et al., 2002) activity of regulatory T cells (Aluvihare et al., 2004; Darrasse-Jeze et al., 2006; Kahn and Baltimore, 2010; Polanczyk et al., 2007; Polanczyk et al., 2004). Our studies here and elsewhere (Norton et al., 2009; Norton et al., 2010) suggest that T cells in the pregnant host, as in the non pregnant state, respond to signals leading to homeostatic proliferation, thus potentially rendering them susceptible to exhaustion and relief from exhaustion as further mechanisms of control. Examination of the intricacies of T cell regulation is likely to continue to reveal basic similarity between T cells in the pregnant and non pregnant host and enhance our understanding of how the pregnant immune system is capable of making an immune response leading to clearance of infections (Constantin et al., 2007), damaged tissue (Chaouat et al., 1995) and circulating fetal antigens (Bonney and Onyekwuluje, 2003) while supporting successful pregnancy.

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Fig. 1. PD-1 gene and protein expression in T cells during normal pregnancy

CD4 and CD8 T cells were purified from the spleens and pooled uterine draining lymph nodes of unmatd B6 mice (UM) and syngeneically pregnant B6 mice on gestational days 5, 8, and 15 (D5, D8, and D15) by FACS sorting. The relative expression of *Pd-1* was determined by RT-PCR and PD-1 cell surface expression by flow cytometry staining. (A-B) Splenic CD4 T cells. *Pd-1* relative gene expression (A) and protein expression (B) is not statistically different between UM controls and pregnant mice on D5, D8, and D15 of gestation. (C-D) Splenic CD8 T cells. *Pd-1* relative gene expression (C) is significantly higher on gestational day 8 than in UM mice [$*p < 0.05$], without any change in PD-1 surface protein expression (D). (E-F) Uterine draining node CD4 T cells. There is no significant difference in *Pd-1* relative gene expression (E) or protein expression (F) between UM controls and pregnant mice. (G-H) Uterine draining node CD8 T cells. *Pd-1* relative gene expression (G) and protein expression (H) is similar between unmatd and pregnant mice. Data shown are mean and SEM for individual spleen samples and pooled lymph node samples. The number of individual/pooled samples in each group are shown in parenthesis. Statistical significance determined by one-way ANOVA with Bonferroni post-test.

Fig. 2. CD4+ T cell homeostasis in the context of PD-1 blockade during pregnancy
 Syngeneically pregnant B6 mice and unmated controls were treated with IgG control or anti-PD-1 blocking antibodies on gestational days 10, 12, and 14 and on day 15 spleen and uterine draining lymph nodes were harvested. CD4 T cell homeostasis was investigated by calculating total CD4 T cell number, proliferation by BrdU incorporation, and apoptosis using flow-cytometric TUNEL assay. (A) Representative flow cytometry showing lymphocyte gating, CD4+ TCRβ+ subset selection, and BrdU+ and TUNEL+ histogram gating. (B–D) CD4 T cell homeostasis in the spleen during PD-1 blockade. (B) The total number of CD4 T cells is elevated on day 15 (D15) of pregnancy in both IgG control (light bars) and PD-1 blockade (dark bars) groups compared to unmated controls [**p<0.01] with no significant effect of PD-1 blockade. (C) The number of proliferating CD4 T cells is significantly higher in day 15 pregnant mice compared to UM controls in both groups [****p<0.0001], and PD-1 blockade independently increased the number of BrdU+ cells in

both unmated and pregnant mice compared to IgG controls [$p < 0.05$]. (D) The number of apoptotic CD4 T cells in the spleen was not significantly changed by either pregnancy or PD-1 blockade. (E–G) CD4 T cell homeostasis in the pooled peri-aortic and femoral uterine draining lymph nodes (uterine DN) during PD-1 blockade. (E) The total number of CD4 T cells in the uterine DN is elevated in day 15 pregnant mice compared to UM controls in both IgG control and anti-PD-1 treated mice [$***p < 0.001$], and this number is unchanged by PD-1 blockade. (F) The number of proliferating CD4 T cells was significantly higher on day 15 pregnant mice compared to UM mice in both treatment groups [$****p < 0.0001$], and PD-1 blockade increased proliferation in both UM and pregnant mice [$p < 0.05$]. (G) The number of apoptotic uterine DN CD4 T cells was not significantly altered by pregnancy or PD-1 blockade. Shown is the mean and SEM with number of mice shown in parentheses. Statistical significance determined by two-way ANOVA with Bonferroni post-test.

Fig. 3. CD8+ T cell homeostasis in the context of PD-1 blockade during pregnancy

CD8 T cells were isolated from the spleens and pooled uterine draining lymph nodes of pregnant and unmaternal mice treated with IgG control or anti-PD-1 antibodies as described previously. CD8 T cell homeostasis was investigated by calculating total CD8 T cell number, proliferation by BrdU incorporation, and apoptosis using flow-cytometric TUNEL assay. (A) Representative flow cytometry showing lymphocyte gating, CD8+ TCRβ+ subset selection, and BrdU+ and TUNEL+ histogram gating. (B–D) CD8 T cell homeostasis in the spleen during PD-1 blockade. (B) The total number of CD8 T cells in the spleen is elevated on day 15 of pregnancy in both IgG control (light bars) and PD-1 blockade (dark bars) groups compared to unmaternal controls [$*p < 0.05$], and PD-1 blockade had no significant effect on cell number in either treatment group. (C) The number of proliferating CD8 T cells

is significantly higher in day 15 pregnant mice compared to UM controls in both treatment groups [$**p<0.01$], and PD-1 blockade increased the number of proliferating cells in both pregnant and unmated mice [$\#p<0.05$]. (D) The number of apoptotic CD8 T cells in the spleen was unchanged by either pregnancy or PD-1 blockade. (E–G) CD8 T cell homeostasis in the pooled peri-aortic and femoral uterine draining lymph nodes (uterine DN) during PD-1 blockade. (E) The total number of CD8 T cells in the uterine DN is elevated in both IgG control and PD-1 blockade treated pregnant mice compared to unmated mice [$**p<0.01$] with no effect of PD-1 blockade. (F) The number of proliferating uterine DN CD8 T cells was not significantly altered by pregnancy or PD-1 blockade. (G) The number of apoptotic uterine DN CD8 T cells was unchanged by pregnancy or PD-1 blockade. Shown is the mean and SEM with number of mice shown in parentheses. Statistical significance determined by two-way ANOVA with Bonferroni post-test.

Fig. 4. Cell surface expression in CD4 and CD8 T cells is altered by PD-1 blockade

Protein expression of PD-1 was determined in CD4 and CD8 T cells from the spleen and uterine draining lymph nodes (uterine DN) by geometric mean fluorescence intensity (Geo MFI) obtained by flow cytometric analysis. (A) Representative flow cytometry showing lymphocyte gating, CD4⁺ TCRb⁺ or CD8⁺ TCRb⁺ subset selection, and PD-1 histogram gating. (B) PD-1 expression by histogram analysis of splenic CD4 T cells from unstained control (1), unmated IgG treated mouse (2), unmated PD-1 treated mouse (3), Day 15 pregnant IgG treated mouse (4), and Day 15 pregnant PD-1 treated mouse (5). (C–D) Protein expression of PD-1 in CD4 T cells from the spleen and uterine DN. (C) The expression of PD-1 in splenic CD4 T cells is elevated by both pregnancy [$**p<0.01$] and PD-1 blockade [$#p<0.05$]. (D) The expression of PD-1 in uterine DN CD4 T cells is not altered by pregnancy but is significantly elevated in PD-1 blockade regardless of pregnant status [$#p<0.05$]. (E–F) Protein expression of PD-1 in spleen and uterine DN CD8 T cells. (E) There is a non-significant increase in PD-1 expression in splenic CD8 T cells due to pregnancy and PD-1 blockade [$p=0.51$ and $p=0.6$ respectively]. (F) CD8 T cells from the uterine DN expressed similar amounts of PD-1 protein regardless of pregnancy [$p=0.4$] or anti-PD-1 treatment [$p=0.4$].

(G–H) Protein expression of CD25 was determined in CD4 T cells from the spleen and uterine draining lymph nodes (uterine DN) by geometric mean fluorescence intensity (Geo MFI) obtained by flow cytometric analysis. (G) Splenic CD4 T cells express more CD25 on day 15 of pregnancy in both IgG control and PD-1 blockade groups compared to UM controls [$**p<0.01$] with no effect of PD-1 blockade. (H) Uterine DN CD4 T cells from

pregnant mice express more CD25 regardless of PD-1 blockade [$**p<0.01$]. Shown is the mean and SEM with number of mice shown in parentheses. Statistical significance determined by two-way ANOVA with Bonferroni post-test.

Fig. 5. Effects of PD-1 blockade during pregnancy on T cell subsets in the uterus

T cells were isolated from pooled uteri of unmated/untreated, mated non-pregnant (MNP) and day 15 pregnant mice by enzymatic dissection and T cell proportions were determined by flow cytometry. **Numbers in parentheses refer to the number of uteri in the pooled sample.** (A–C) Uterine CD4 T cells. (A) The proportion of CD4⁺ T cells in the uterus decreases in pregnant mice as the result of PD-1 blockade. (B) The proportion of CD25⁺ CD4 T cells is similar in MNP and day 15 pregnant mice and PD-1 blockade has no effect. (C) PD-1 blockade increases the proportion of uterine CD4 T cells expressing high amounts of CD44 (CD44^{Hi}) in MNP mice, but decreases this subset in day 15 pregnant mice. (D–E) Uterine CD8 T cells. (D) The proportion of CD8⁺ T cells in the uterus is slightly lower in IgG control pregnant vs. unmated mice and increases in both groups of mice undergoing PD-1 blockade. (E) The proportion of CD44^{Hi} CD8⁺ T cells was slightly decreased in pregnant mice undergoing PD-1 blockade. (F–G) Uterine CD4[−] CD8[−] T cells. (F) The proportion of CD4[−] CD8[−] T cells is elevated in the uterus on day 15 of pregnancy but is unchanged by PD-1 blockade. (G) PD-1 blockade increases the proportion of CD44^{Hi} CD4[−] CD8[−] T cells in MNP mice, but decreases these cells in pregnant mice. Statistical analysis of pooled uterine samples was not performed.

Fig. 6.

PD-1 expression on uterine T cells is altered by PD-1 blockade

(A–B) PD-1 expression in uterine T cells during pregnancy. T cells were isolated from the pooled uteri (3 per pool) of un-manipulated syngeneic pregnant mice on gestational days 5, 8, and 15 and unmated mice by enzymatic dissection. PD-1 expression on uterine CD4 and CD8 T cells was determined by geometric mean fluorescence intensity (Geo MFI) obtained by flow cytometric analysis. (A) Uterine CD4 T cells from pregnant mice had a higher expression of PD-1 on day 5 of pregnancy compared to UM controls. (B) The expression of PD-1 on uterine CD8 T cells was increased on day 5 and day 15 of pregnancy compared to UM controls. (C–E) The effect of PD-1 blockade on uterine T cell expression of PD-1.

Numbers in parentheses refer to the number of uteri in the pooled (p) sample. CD4+, CD8+, and CD4-CD8- T cells were isolated from the pooled uteri of unmated untreated controls (UM UT- black bars), and groups of mated non-pregnant (MNP) and day 15 pregnant mice treated with IgG control or anti-PD-1 antibodies by enzymatic digestion. The cell surface expression of PD-1 was determined by geometric mean fluorescence intensity obtained by flow cytometric analysis. (C) PD-1 expression is elevated in uterine CD4+ T cells in day 15 pregnant mice compared to UM UT controls, and PD-1 blockade further enhances PD-1 expression in pregnancy. (D) Compared to UM UT controls, PD-1 expression on uterine CD8+ T cells is elevated in pregnant mice, and considerably higher in both MNP and Day 15 pregnant mice undergoing PD-1 blockade. (E) The expression of PD-1 on CD4- CD8- T cells is elevated in pregnant mice, with no change due to PD-1 blockade. Statistical analysis of pooled uterine samples was not performed.

(F) The number of live fetuses and resorption sites in pregnant mice was determined by visual examination of the fetal units on day 15 of gestation. There was no difference in the number of fetuses or resorptions between pregnant mice undergoing PD-1 blockade (dark circles) and pregnant IgG control treated mice (light circles). Line represents mean of each group.