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Menopausal Status Influences the Expression of Programmed Death (PD)-1 and its ligand PD-L1 on Immune Cells from the Human Female Reproductive Tract

Zheng Shen, Marta Rodriguez-Garcia, Mickey V. Patel, Fiona D. Barr, and Charles R. Wira*

Department of Physiology and Neurobiology, Geisel School of Medicine at Dartmouth, Lebanon, NH, USA

Abstract

The PD-1/PD-L1 pathway regulates peripheral tolerance, immune responses, and is up-regulated in chronic viral infections, including HIV infection. However, expression of PD-1/PD-L1 on immune cells from the human female reproductive tract (FRT), and possible regulation by menopause and sex hormones are poorly understood. We analyzed PD-1/PD-L1 expression on CD4⁺ and CD8⁺ T cells, CD163⁺ macrophages, and CD11c⁺ dendritic cells (DC) from endometrium (EM), endocervix (CX) and ectocervix (ECX). PD-1 and PD-L1 were constitutively expressed on CD4⁺ and CD8⁺ T cells from EM, CX and ECX. PD-L1⁺CD4⁺ T cells were increased in CX compared to EM and ECX, while no differences were found for PD-1 or between CD8⁺ T cells from different sites. Macrophages and DCs constitutively expressed PD-L1, but not PD-1, with no differences observed between FRT sites. Premenopausal FRT tissues showed increased PD-L1 expression on CD8⁺ T cells but decreased expression on DCs when compared to postmenopausal women. *In vitro* estradiol treatment up-regulated PD-L1 expression on CD8⁺ T cells from CX, but not EM or ECX, and had no effect on PD-1/PD-L1 expression on the other cell types. Our results suggest that PD-L1 may be involved in the differential regulation of FRT immune responses between premenopausal and postmenopausal women.

Keywords

CD8⁺ T cells; CD4⁺ T cells; Dendritic Cells; premenopausal; postmenopausal; sex hormones

INTRODUCTION

The female reproductive tract (FRT) is a unique mucosal environment that balances protective immune responses against microbial challenge with immune tolerance necessary for reproductive success. The mucosal immune system in the FRT consists of immune cells

*Correspondence: Charles R. Wira, Charles.R.Wira@Dartmouth.edu, 603-650-7733.

AUTHORSHIP

Z.S. designed and performed most of the experiments and analyzed the data. M.V.P., and F.D.B. contributed to the tissue processing and analysis. M.R-G. and C.R.W. contributed to experimental design, data analysis. Z.S., M.R-G. and C.R.W. wrote the manuscript.

DISCLOSURES

The authors declare no conflicts of interest.

as well as resident epithelial cells and supportive stromal cells. The immune cell number, distribution and function in the FRT are directly regulated by the sex hormones estradiol and progesterone during the menstrual cycle and indirectly by secretions from other cells, which are also responsive to hormones [1, 2].

We previously demonstrated that 6–20% of all cells in the FRT are immune cells with more cells present in the upper tract than in the lower tract [3]. Immune cells are distributed throughout the FRT, but their phenotype and function are regulated by anatomical location within the FRT, sex hormones and menopausal status [2, 4–9]. For example, we previously found that CD4⁺ and CD8⁺ T ratio is different in EM, CX and ECX and changes with menopausal status [10]. Within the CD4⁺ T cell fraction, Th17 CD4⁺ T cell numbers were reduced in the EM from premenopausal women compared with postmenopausal women. CTL activity is also specifically reduced in the EM of premenopausal women, but increases following menopause [11]. Recognizing that FRT immune cells are responsible for the initial response to a range of sexually transmitted pathogens and are phenotypically distinct from blood immune cells [8, 12, 13], it is important to define their unique characteristics and the role of hormones in regulating their immune responses.

Programmed death 1 (PD-1) is a negative regulator of T cell activity [14, 15]. Under physiological conditions, PD-1 is induced after T cell activation. PD-1 binding to PD-L1 attenuates immune responses and limits immune-mediated tissue damage [16, 17]. The PD-1/PD-L1 pathway is also up-regulated during pregnancy to prevent rejection [18, 19]. Pathological conditions, such as cancer or chronic infections, also exploit this pathway to suppress protective T cell responses [15, 16]. PD-1 is expressed on activated blood T cells, B cells, NK T cells, monocytes/macrophages, and DCs. PD-L1 is one of several ligands for PD-1 and is constitutively expressed on immune cells and on a wide range of non-hematopoietic cells such as epithelial and endothelial cells [20–23]. While the expression levels and function of PD-1 and PD-L1 on immune cells from blood have been intensively studied, the expression and regulation of PD-1 and PD-L1 on immune cells from the FRT are poorly understood.

In this study, we analyzed PD-1 and PD-L1 expression on T cells (CD4⁺ and CD8⁺), macrophages (CD163⁺) and DC (CD11c⁺) from sites in the upper (EM, CX) and lower (ECX) FRT in premenopausal and postmenopausal women to investigate if PD-1/PD-L1 baseline expression changes with anatomical location in the FRT, menopausal status and sex hormones.

MATERIALS AND METHODS

Study subjects

Human subject work was carried out with the approval of the Dartmouth College Institutional Review Board. Approval to use tissues was obtained from the Committee for the Protection of Human Subjects (CPHS).

Source of tissue

Human FRT tissues were obtained immediately following surgery from women who had undergone hysterectomies at Dartmouth-Hitchcock Medical Center (DHMC, Lebanon, NH). Tissues from the EM, CX and ECX were collected from hysterectomy patients with benign conditions such as fibroids and prolapse. Tissue samples were distal from the sites of pathology and were without pathological lesions as determined by a pathologist at DHMC. Menopausal status was determined by a pathologist based on the histological evaluation of sections of the EM. The age, menopausal status and anatomical location of tissues from each patient are shown in Table 1. For the majority of tissues used in this study, matched samples were available from the EM, CX and ECX.

Tissue processing

Tissues were rinsed with 1x HBSS (Hanks balanced salt solution) supplemented with phenol red, 100 U/ml penicillin, 100 µg/ml streptomycin (all Thermo Scientific Hyclone, Logan, UT), and 0.35 mg/ml NaCO₃ (Fisher Scientific, Pittsburgh, PA). Tissues were then minced under sterile conditions into 1–2 mm fragments and digested at 37°C for 1hr using an enzyme mixture consisting of 0.05% collagenase type IV (Sigma-Aldrich, St. Louis, MO) and 0.01% DNase (Worthington Biochemical, Lakewood, NJ) in 1xHBSS (Invitrogen Life Technologies, Grand Island, NY) as previously described [10]. After digestion, cells were dispersed through a 250-µm nylon mesh screen (Small Parts, Miami Lakes, FL) and filtered through a 20-µm mesh filter (Small Parts) to separate stromal cells from epithelial cells, which were retained on the nylon mesh. Stromal cells were washed, erythrocytes lysed, and dead cells removed using the Dead cell removal kit (Miltenyi Biotec, Auburn, CA) according to manufacturer instructions, to obtain a mixed cell suspension, consisting of immune cells and stromal fibroblasts, which was used for our subsequent analysis.

Hormone treatment of cells

17β-estradiol (E₂; Calbiochem, Gibbstown, NJ) and progesterone (P; Calbiochem) was dissolved in 100% ethanol at an initial concentration of 1×10⁻³ M, evaporated to dryness in a glass vial and resuspended in complete media consisting of X-VIVO 15 Media (Lonza, Walkersville, MD) supplemented with 10% charcoal stripped human AB serum (Valley Biomedical, Winchester, VA) to a concentration of 1×10⁻⁵ M. Further dilutions were made to achieve final working concentration of 5×10⁻⁸ M E₂ and/or 1×10⁻⁷ M P. Both hormone concentrations are routinely used by our laboratory and are within the physiological range of hormone concentration [24]. As a control, an equivalent amount of ethanol without dissolved hormone was initially evaporated. Freshly isolated mixed cell suspension were plated at a density of 1×10⁵ cells per well in ultra-low attachment 96-well culture plate (Corning, Corning, NY) in 0.2ml of complete media and treated either with 5×10⁻⁸ M E₂ and/or 1×10⁻⁷ M P at 37°C for 24hr.

Flow cytometry

Mixed cell suspensions, either freshly isolated or hormone-treated, were stained for surface markers with the following mouse anti-human antibodies: CD45-VioletFluor 450, CD3-APC-Cy7, CD8-FITC, CD11b-PE (Tonbo Biosciences, San Diego, CA), CD3-APC, CD163-

APC, CD11c-PerCp-Cy5.5 (BioLegend Inc., San Diego, CA), PD-L1-PE-Cy7, PD-1-PerCp-Cy5.5 (BD Biosciences, San Jose, CA), CD4-PE (eBioscience, San Diego, CA). Analysis was performed on MACSQuant flow cytometer (Miltenyi Biotec) using MACSQuantify software and data were analyzed with FlowJo software (Tree Star, Inc., Ashland, OR). Expression of surface markers was measured by the percentage of positive cells and the mean fluorescence intensity (MFI).

Statistics

Data analysis was performed using the GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA). A two sided P value <0.05 was considered statistically significant. Comparison of three groups with matched samples was performed applying Friedman test with Dunns-post test. Comparison of three or more groups was performed applying the non-parametric Kruskal Wallis test followed by Dunns-post test. Comparison of all the treatment group vs. control group was performed applying Repeated Measures ANOVA test with Dunns-post test.

RESULTS

CD4⁺ T cells from the FRT express of PD-1 and PD-L1

We analyzed the expression of PD-1/PD-L1 by flow cytometry on FRT CD4⁺ T cells in mixed cell suspensions recovered from enzymatic digestion of hysterectomy tissues. As shown in Figure 1, PD-1 (Figure 1A) and PD-L1 (Figure 1B) were expressed on CD4⁺ T cells from EM, CX, and ECX from the same patient. We found that whereas 9–12% of CD4⁺ T cells expressed PD-1 (Figure 1C), approximately 37–49% of these cells expressed PD-L1 (Figure 1D). When EM, CX and ECX were compared, CD4⁺ T cells from CX expressed significantly higher PD-L1, either as % positive cells or MFI (Figure 1D). In contrast, there were no significant differences in PD-1 expression when anatomical sites were compared (Figure 1C).

CD8⁺ T cells from the FRT express of PD-1 and PD-L1

We next analyzed the expression of PD-1 and PD-L1 on FRT CD8⁺ T cells by flow cytometry. In a representative sample (Figures 2A and 2B), PD-1 and PD-L1 were expressed on CD8⁺ T cells from EM, CX, and ECX from the same patient. However, as shown in Figure 2C and 2D, while PD-1 and PD-L1 expression was detected on CD8⁺ T cells in all FRT tissues, no significant differences in PD-1 or PD-L1 (% positive cells and MFI) was observed when anatomical sites were compared. We found that expression of PD-1 was 7–10% (Figure 2C) whereas PD-L1 was 39–48% of CD8⁺ T cells (Figure 2D). Similar to CD4⁺ T cells, expression of PD-L1 on CD8⁺ T cells was greater than PD-1.

CD11c⁺ DC and CD163⁺ Macrophages from the FRT express PD-L1 but not PD-1

Recognizing that DCs and macrophages play a role in immune regulation and tolerance through the PD-1/PDL-1 pathway [2], we examined the expression PD-1 and PD-L1 on CD11c⁺ DC and CD163⁺ macrophages from EM, CX, and ECX. As seen in Figures 3A and 3C, 29–35% of CD11c⁺ DC and 45–54% of CD163⁺ macrophages expressed PD-L1, with no significant differences between anatomical sites, either as % of positive cells or MFI. Interestingly, CD11c⁺ DC expressed higher levels of PD-L1 (MFI) when compared to the

other cell types we investigated. In contrast to CD4⁺ and CD8⁺ T cells, PD-1 expression was undetectable on CD11c⁺ DC and CD163⁺ macrophages from all FRT tissues (not shown).

Menopausal status affects PD-L1 expression on FRT CD8⁺ T cells and CD11c⁺ DC

To investigate whether the menopausal status of patients would affect PD-1 and PD-L1 expression on immune cells from the FRT, hysterectomy tissues were classified as premenopausal or postmenopausal based on EM histology. As seen in Figure 4A and 4B, menopausal status significantly influenced PD-L1 expression on CD8⁺ T cells and CD11c⁺ DC. When measured as MFI, CD8⁺ T cells from premenopausal tissues (EM, CX, and ECX) expressed significantly more PD-L1 than CD8⁺ T cells from postmenopausal tissues (Figure 4A). In contrast, PD-L1 expression on CD11c⁺ DC was significantly lower in EM and CX/ECX from premenopausal tissues compared to postmenopausal tissues (Figure 4B). No significant differences were observed between premenopausal and postmenopausal tissues for PD-L1 expression on CD4⁺ T cells and CD163⁺ macrophages, or for PD-1 expression on CD4⁺ and CD8⁺ T cells (data not shown). Interestingly, no significant differences were seen in % of PD-L1 positive cells when premenopausal tissues were compared to postmenopausal tissues. Overall, these findings indicate that menopausal changes affect MFI expression of PD-L1 on CD8⁺ T cells and CD11c⁺ DC without changing the % of positive cells in the FRT.

Estradiol increases PD-L1 expression on FRT CD8⁺ T cells

To determine whether E₂ and/or P regulate PD-1 or PD-L1 expression in the FRT, freshly isolated mixed cell suspensions were incubated with E₂ (5×10⁻⁸ M), P (1×10⁻⁷ M) or a combination of E₂ and P for 24 hr. As shown in Figure 5A, E₂ treatment significantly increased PD-L1 expression on CD8⁺ T cells from the CX, whereas P, either alone or in combination with E₂ had no effect. In contrast, there was no effect of either hormone on PD-L1 expression on CD8⁺ T cells from the EM (Figure 5B). While PD-1 and PD-L1 expression in samples from individual patients did change with hormone treatment, we found no consistent effect on CD4⁺ T cells, CD11c⁺ DC or CD163⁺ macrophages at the EM, CX or ECX.

DISCUSSION

In this study we report that PD-L1 is broadly expressed on T cells, macrophages and DCs from the human FRT, while PD-1 is selectively expressed on T cells. Importantly, PD-L1 expression changed with menopausal status, anatomical location and sex hormones in a cell-specific manner. To the best of our knowledge, this is the first study to evaluate PD-1 and PD-L1 expression on immune cells throughout the human FRT. These findings are relevant to our understanding of FRT immune regulation and tolerance.

Our results demonstrate that between 20 and 40% of CD4⁺ and CD8⁺ T cells from the FRT constitutively express PD-L1. This is in contrast to blood T cells, in which PD-L1 expression is induced after activation or chronic infection [14, 15]. The functions and mechanisms for broad PD-L1 expression on T cells from the FRT are unknown and may be different depending on anatomical location. For example, T cells in the endometrium may

up-regulate PD-L1 in response to the tolerogenic tissue environment, rich in TGF- β and IL-10 [25–27], and may be up-regulated to mediate potential interactions with PD-1-expressing cells from the trophoblast to prevent rejection [18, 28]. In contrast, T cells in the cervix and specially the ectocervix are exposed to a higher load of commensals and potential pathogens, which may mediate PD-L1 up-regulation [29]. Unexpectedly, we observed significantly increased PD-L1 expression on CD4⁺ T cells from the endocervix compared to the other FRT sites. The reason for this compartmentalization is unclear, but we recently demonstrated that Th17 cells are also increased in the endocervix [10]. Since a negative control between PD-L1 and Th17 has been described [30, 31], it is plausible that PD-L1⁺CD4⁺ T cells are increased in CX to control Th17 cell activation. Future studies will address the influence of the mucosal environment on this possible connection.

A key observation from this study is that menopausal status influences PD-1/PD-L1 expression in the FRT. PD-L1 expression was significantly higher on CD8⁺ T cells and lower on DCs from premenopausal women compared to postmenopausal women. The reason for the differences seen between premenopausal and postmenopausal women remains unclear, but may be due to hormonal fluctuations. Menopause is characterized by decreased levels of E₂ and progesterone in comparison to premenopausal women and the absence of cyclic changes in hormone levels [2]. Previously we found differences in immune cell distribution specific to the EM from postmenopausal compared to premenopausal women, such as reduced numbers of CD4⁺ T cells and NK cells and increased Th17 cells [7, 10]. The present studies extend these findings by demonstrating that menopausal status also regulates PD-L1 on CD8⁺ T cells and DCs, not only in the EM, but also throughout the FRT. These results suggest very fine control of the PD-1/PD-L1 pathway, important in regulating immune responses and peripheral tolerance.

Another fundamental difference between premenopausal and postmenopausal women is the suppression of CD8⁺ CTL activity in the EM of premenopausal women relative to that seen after menopause [11]. Mouse models demonstrate that suppression of CD8⁺ T cell immune responses can be mediated through PD-L1 expressed on the CD8⁺ T cells [32]. This would suggest that immune responses of CD8⁺ T cells from the human FRT might be suppressed through engagement of PD-L1 expressed on the CD8⁺ T cells. Our finding that PD-1 was expressed on 10% of CD8⁺ T cells while PD-L1 was present on 40% of CD8⁺ T cells, suggests that PD-L1, rather than PD-1, is more likely to control T cell function in the FRT. Intriguingly, our studies in the EM show increased PD-L1 expression on premenopausal CD8⁺ T cells at a time when CTL activity was suppressed [11]. Whether PD-L1 reduction after menopause is responsible for the increase in CTL activity in the EM at this time remains to be determined.

We also demonstrate that PD-L1 is susceptible to direct hormonal regulation. We observed that E₂ was able to up-regulate PD-L1 expression on endocervical CD8⁺ T cells. To the best of our knowledge, this is the first demonstration of a direct effect of E₂ on PD-L1 expression on T cells. Just why PD-L1 expression on CD8⁺ T cells from the CX but not the EM increases in response to E₂ remains unclear. As discussed elsewhere [2], in addition to their direct effects, sex hormones regulate the tissue environment in the FRT and indirectly affect immune function through growth factors, cytokines and chemokines. Therefore the

possibility exists that PD-L1 expression in the EM is regulated through the indirect effects of sex hormones. Further studies are needed to determine the extent to which the local environment in combination with sex hormones regulates PD-L1 expression and immune function throughout the FRT.

In conclusion, we characterized the cell surface expression of PD-1 and PD-L1 on CD4⁺ and CD8⁺ T cells, macrophages and DC from the upper and lower FRT. PD-1 and PD-L1 are constitutively expressed within the FRT. PD-L1, but not PD-1, is increased on CD8⁺ T cells from premenopausal women and selectively responsive to estradiol. Future studies are needed to understand the implications of PD-1 and PD-L1 regulation of immune protection in the FRT.

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Abbreviations

CTL	cytotoxic T lymphocyte
CX	endocervix
DC	dendritic cells
E₂	17β-estradiol
ECX	ectocervix
EM	endometrium
FMO	fluorescence minus one
FRT	female reproductive tract
MFI	mean fluorescence intensity
P	progesterone
PD-1	programmed death 1
PD-L1	programmed death 1 ligand

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Figure 1. Expression of PD-1 and PD-L1 in CD4⁺ T cells from the female reproductive tract
 Representative contour plot of PD-1 (A) and PD-L1 (B) expression in CD4⁺ T cells.
 Negative control was established using fluorescence minus one (FMO). (C) Expression of % PD-1 positive cells and mean fluorescence intensity (MFI) in CD4⁺ T cells. (D) Expression of % PD-L1 positive cells and MFI in CD4⁺ T cells. Each dark circle represents a single patient (n=12). Linked circles represent matched endometrium (EM), endocervix (CX), and extocervix (ECX) from each individual patient. *P<0.05, **P<0.01.

Figure 2. Expression of PD-1 and PD-L1 in CD8⁺ T cells from the female reproductive tract
 Representative contour plot of PD-1 (A) and PD-L1 (B) expression in CD8⁺ T cells. Negative control was established using fluorescence minus one (FMO). (C) Expression of % PD-1 positive cells and MFI in CD8⁺ T cells. (D) Expression of % PD-L1 positive cells and MFI in CD8⁺ T cells. Each dark circle represents a single patient (n=12). Linked circles represent matched EM, CX, and ECX from each individual patient.

Figure 3. Expression of PD-L1 in CD11c⁺ dendritic cells and CD163⁺ macrophages from the female reproductive tract

(A and B) Expression of % PD-L1 positive cells and MFI in CD11c⁺ dendritic cells from EM (n=9), CX (n=4), and ECX (n=4). (C and D) Expression of % PD-L1 positive cells and MFI in CD163⁺ macrophages from EM (n=9), CX (n=7), and ECX (n=7). Each dark circle represents a single patient. Horizontal lines represent the mean $\pm$ SEM.

Figure 4. Effect of menopausal status on PD-L1 expression on CD8⁺ T cells and CD11c⁺ dendritic cells from the female reproductive tract

(A) The same samples shown in Figure 1 and 2 were separated into premenopausal (closed circles; secretory phase of the cycle) and postmenopausal (open circles) groups with PD-L1 expression (MFI) on CD8⁺ T cells. (B) The same samples shown in Figure 3A and 3B were separated into premenopausal (closed circles; secretory phase of the cycle) and postmenopausal (open circles) groups with PD-L1 expression (MFI) on CD11c⁺ dendritic cells. For this analysis, the CX and ECX samples were pooled together. Horizontal lines represent the mean $\pm$ SEM. *P<0.05.

Figure 5. Effect of estradiol and/or progesterone on PD-L1 expression on CD8⁺ T cells from the female reproductive tract

Isolated mixed cells suspension from FRT tissues treated with estradiol (E₂, 5×10^{-8} M), progesterone (P, 1×10^{-7} M), either alone or the combination for 24 hr. Data normalized to % of control MFI values defined as 100% (dashed line) from (A) CX CD8⁺ T cells (n=3) and (B) EM CD8⁺ T cells (n=6–7), Each symbol represents a single patient with circles and triangles indicating EM and CX respectively. Horizontal lines represent the mean $\pm$ SEM. *P<0.05.

Table 1

Characteristics of the patients and tissues of the study

	Premenopausal	Postmenopausal
Number of donors	7	21
Age: average (range)	42.1 (34–52)	56.6 (45–78)
Menstrual stage		
Secretory	7	
Atrophic		21
Number of tissues		
Endometrium (EM)	7	19
Endocervical (CX)	4	14
Ectocervical (ECX)	4	12