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Original Article

Correlation of CD8 infiltration and expression of its checkpoint proteins PD-L1 and PD-L2 with the stage of cervical carcinoma

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Abstract: The importance of CD8 infiltration for cancer prognosis has been underscored lately by the increased use of checkpoint inhibitors in patients with invasive tumors. The objective of this study was to determine if CD8 infiltration and the expression of two key checkpoint proteins, PD-L1 and PD-L2, varied between early stage (FIGO IA-IIA) and advanced stage (FIGO IIB-IVA) cervical cancer. A tissue micro-array with 61 cervical specimens was analyzed through immunohistochemistry for PD-L1, PD-L2 and CD8 using CD1a (antigen presenting cells) as an internal control. Infiltration of both CD8 and CD1a was evident in control, benign tissues that showed little to no PD-L1 and PD-L2 reactivity. In samples of invasive cervical cancers, there was a three-fold increase in the number of CD8 cells with an increase in the expression of both PD-L1 and PD-L2 ($P<0.001$ for each vs. control). A slight decrease in the number of CD1a cells was observed in malignant tissues compared to controls. No significant difference was evident for the infiltration of $CD8^+$ cells or the expression of either PD-L1 and PD-L2 between the samples of early stage (FIGO IA-IIA) and advanced stage (FIGO IIB-IVA) cancers. Cytotoxic T cell infiltration and expression of two of its key checkpoint proteins (PD-L1 and PD-L2) remained constant as cervical cancers advance from early stage to late stage tumors. This suggests that the immune response may be equivalent in early and late stage cervical cancers.

Keywords: Invasive cancer, PD-L1, PD-L2, CD8, CD1a, HPV

Introduction

HPV infection is a prerequisite for the development of cervical cancer [1]. Given that cervical cancer is the classic example of a malignant tumor associated with a productive viral infection, it is not surprising that the host immune response, especially as it relates to the cytotoxic CD8-mediated response, has received much attention in the pathogenesis and evolution of both cervical intraepithelial neoplasia (CIN) and cervical cancer [2, 3]. The correlation of the $CD8^+$ T cell infiltration and the regression of intraepithelial lesions have been documented by immunohistochemical studies [2, 3]. $CD8^+$ cells are the dominant inflammatory cell type in cervical cancer and their activation by HPV associated vaccines can reduce tumor growth [4, 5]. Much attention has been focused on the activation of $CD8^+$ cells that are commonly found amongst invasive cancer cells. Two recent studies have highlighted that about one third of late stage cancers contain $CD8^+$ cell infiltrates, yet these cytotoxic cells appear to be predominantly quiescent due to the concomitant expression of checkpoint proteins such as PD-1and PD-L1 [6, 7]. Importantly, "unblockage" of $CD8^+$ cell quiescence by inhibitors of these checkpoints have been observed to promote regression of metastastic lung, renal cell and colorectal cancers as well as melanomas [6]. However, little is know about the role of PD-L1 and PD-L2 in invasive cervical cancer.

Programmed cell death protein 1 (PD-1) is a transmembrane glycoprotein expressed on B

and T cells that can serve as a checkpoint to $CD8^+$ cell activation. Through binding to PD-1, programmed death ligand 1 (PD-L1) and ligand 2 (PD-L2) are members of the B7 family receptors that can each inhibit CD8 activation [7]. Previously, our group showed that there was an increased expression of both PD-1 and PD-L1 in CIN and cervical cancer [8]. However, little is known about the relationship of $CD8^+$ cell infiltration to the different stages of cervical cancer. There are data suggesting that there may be a decreased immune response in advanced stages of cervical cancer based on the documented decrease in $CD4^+$, Natural Killer and Langerhans cells in these tumors [9]. The Langerhans cells are sentinels of mucosal immunity that can activate $CD8^+$ T cells [7] and highly express CD1a, a molecule similar to MHC Class I specialized in the presentation of lipid antigens to T cells [9, 10].

The purpose of this study was to investigate PD-L1, PD-L2, and CD1a expression as well $CD8^+$ cell infiltration in invasive cervical cancer with a correlative analysis to the tumor staging diagnosis.

Materials and methods

Study design, study population and tissue samples collection

This case-control study consisted of 61 cervical samples randomly selected from the archives of Fernandes Figueira Institute, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil. Invasive cervical cancer was diagnosed in 39 of these patients via cervical biopsy or conization between 2003 and 2008. Socio-demographic, clinical and behavioral information was extracted from the archives for each patient. The following variables were retrieved: age, age of first sexual intercourse, number of pregnancies, abortions, race, schooling including degree, smoking status, alcohol status, history of oral cancer and whether patient was still alive at the time of this study.

The Institutional Review Board (IRB) from Oswaldo Cruz Foundation (Fiocruz), (CAE 0024.0.0-11.000-09, Rio de Janeiro, Brazil) approved this study.

Cervical biopsies and TMA construction

The cervical specimens were used to construct two tissue microarray (TMA) paraffin blocks. The TMA blocks were constructed as previously described by Pires et al, 2006 [11]. The specimens were reviewed by a senior pathologist and confirmed as invasive cervical cancer (ICC). The cervical specimens from 22 patients undergoing hysterectomies for benign leiomyomatosis disease and no history of concurrent cervical intraepithelial neoplasia (CIN) or cervical cancer were used as negative controls.

Immunohistochemistry for PDL-1, PDL-2, CD1a and CD8

The protocol for immunohistochemistry was as previously published [8]. Briefly, 4 µm sections were cut from a paraffin-embedded TMA block and the paraffin removed by xylene. Next, sections were dehydrated with ethanol, incubated in 3% hydrogen peroxide for 10 min and boiled at 95ºC for 30 min in an antigen retrieval solution (Dakocytomation), followed by a 60 min incubation primary antibody. An automated Leica Bond Max platform was used with one modification: the HRP Polyview conjugate from Enzo Life Sciences (Farmingdale NY) was used as we documented that it was associated with less background (Nuovo GJ, unpublished observations). The dilutions and source of the antibodies used in the study were: anti-CD8 (Ventana Medical Systems, ready to use); anti-CD1a monoclonal (Dakocytomation 1:100); PDL-1 (ABCAM, 1:600) and PDL-2 (Sigma Ald-rich, 1:600). As an additional negative control, some sections were treated identically except that the primary antibody was replaced with non-specific IgG.

DNA extraction and HPV type

HPV DNA extraction, PCR amplification and HPV genotyping were performed as previously published [12]. Briefly, 4 slices of 5 µm were cut from each paraffin embedded cervical biopsy, dewaxed and the pellet suspended in solution with proteinase K (100 µg/mL) with 10% SDS. DNA isolation carried out with Phenol: Chloroform: Isoamyl Alcohol (25:24:1) (Sigma Chemical Co. St. Louis, MO, USA).

HPV detection and genotyping was performed by amplification of HPV L1 consensus region (~150 base pairs) with generic primers $GP5^+$ and $GP6^+$ (synthesized by Invitrogen, Sao Paulo, SP, Brazil), following by purification with GF-1 DNA Recovery Kit (Vivantis, Oceanside, CA, USA) and Sanger sequencing using Applied Biosystems 3730 capillary sequencers. PCR

Table 1. Clinico-histopathologic correlation in the patients included in this study

Characteristics		Patients-N (%)	Controls ICC	Total	*P*	
Age (Years)	Mean (SD)*	47 (8.6)	54.5 (13.9)	51.9 (12.8)	0.01	**
Race or Ethnicity	White	6 (31.6)	11 (34.4)	17 (33.3)	0.838	****
	Non-white	13 (68.4)	21 (65.6)	34 (66.7)		
	Total	19 (100)	32 (100)	51 (100)		
Schooling degree	Some/Completed primary/secondary	13 (76.5)	21 (80.8)	34 (79.1)	1,000	***
	Some/Completed high school	4 (23.5)	5 (19.2)	9 (20.9)		
	Some/Completed college	0 (0)	0 (0)	0 (0)		
	Total	17 (100)	26 (100)	43 (100)		
First sexual intercourse	Mean (SD)	18.7 (3.5)	17.7 (3.5)	18.2 (3.5)	0.263	**
Number of previous pregnancies	Mean (SD)	3.1 (1.9)	6.4 (4.3)	4.9 (3.8)	0.001	**
Abortions	Mean (SD)	0.9 (1.8)	1.3 (2.6)	1.1 (2.3)	0.657	**
Ever smoked	No	12 (63.2)	14 (63.6)	26 (63.4)	0.975	****
	Yes	7 (36.8)	8 (36.4)	15 (36.6)		
	Total	19 (100)	22 (100)	41 (100)		
Ever used alcohol	No	13 (68.4)	18 (85.7)	31 (77.5)	0.265	***
	Yes	6 (31.6)	3 (14.3)	9 (22.5)		
	Total	19 (100)	21 (100)	40 (100)		
Disease stage	Early (FIGO IA-IIA)	0 (0)	14 (41.2)	14 (25)	0.000	****
	Advanced (FIGO IIB-IVA)	0 (0)	20 (58.8)	20 (35.7)		
	Normal	22 (100)	0 (0)	22 (39.3)		
	Total	22 (100)	34 (100)	56 (100)		

*Mean (SD)=Mean (Standard Deviation). **Kruskal-Wallis test. ***Fisher's exact test. ****Chi-square's test.

reactions with a negative result or samples unable to be sequenced where checked for HPV DNA by means of the INNO-LiPA HPV Genotyping v2 (Innogenetics, Gent, Belgium) or by the PapilloCheck Kit (Greiner Bio-One, Frickenhausen, Germany).

Quantification of immunohistochemistry results

The number of PD-L1, PDL2, CD1a$^+$ and CD8$^+$ cells in the stained section were counted by visual inspection. Staining was evaluated by counting the total number of immunoreactive cells (stained by DAB-brown color) per 1 mm (which is the size of each core).

Statistical analysis

Scoring of PDL-1, PDL-2, CD1a and CD8 expression in cervical samples was analyzed by the non-parametric Kruskal-Wallis test. The correlations between clinicopathological parameters and continuous data were done using the Chi-square test or Fisher's exact test. Mean values are reported as ± 1 standard deviation values. All statistical analyses were performed with IBM SPSS 20.0 and statistical significance was set at alpha ≤0.05.

Results

Study population, Socio-demographic, clinical and behavioral variables

The present study consisted of 61 cervical specimens. A total of 39 women were diagnosed with invasive cervical cancer with a mean age of 54.5 ± 13.9 years. This data was compared to 22 benign cervical epithelial samples from women who had hysterectomies for leiomyomata with a mean age of 47.5 ± SD 8.6 years. We found a statistically significant difference analyzing the number of previous pregnancies (P=0.001) and number of deaths (P=0.020) in the invasive cervical cancer group compared to the controls. For the other variables analyzed, no statistical difference was found between the two groups (**Table 1**). A compilation of the HPV DNA detection data is provided in **Table 2**.

Statistical analysis was performed to determine a correlation between the HPV type and the expression of PD-L1, PD-L2, CD8 and CD1a in ICC and controls. No correlation was found (**Table 2**).

Table 2. Immune markers analyzed across early and late stage cervical cancers compared to controls

Characteristics		PDL-1			PDL-2			CD8			CD1a		
		Mean (SD)*	N	*P***	Mean (SD)*	N	*P***	Mean (SD)*	N	*P***	Mean (SD)*	N	*P***
Patients	Controls	0 (0)	22	<0.0001	5 (11)	22	<0.0001	15.5 (10.6)	22	<0.0001	11.6 (7.1)	22	0.040
	ICC	18.2 (19.8)	39		37.8 (23.2)	39		57.4 (33.2)	39		8 (5.7)	39	
Diseasestage	Normal	0 (0)	22	<0.0001	5 (11)	22	<0.0001	15.5 (10.6)	22	<0.0001	11.6 (7.1)	22	0.037
	Early (FIGO IA-IIA)	19 (19.1)	14		37.1 (26.4)	14		582 (32)	14		8.7 (6.1)	14	
	Advanced (FIGO IIB-IVA)	17.5 (22.6)	20		35.8 (22.6)	20		57.8 (34.4)	20		6.5 (4.3)	20	
HPV 16	No	13.9 (17.8)	11	0.497	38.2 (24.7)	11	0.935	55.5 (31.1)	11	0910	8.4 (6.9)	11	0.866
	Yes	17.3 (15.7)	27		38 (23.5)	27		56.7 (34.2)	27		8.1 (5.3)	27	
HPV 30	No	16.3 (16.2)	38	-	38 (23.5)	38	-	56.3 (32.9)	38	-	8.2 (5.7)	38	-
	Yes	-	-		-	-		-	-		-	-	
HPV 33	No	15.2 (15.5)	32	0.375	39.7 (23.2)	32	0.240	56.9 (32.9)	32	0.778	8.6 (6)	32	0.354
	Yes	22.2 (19.7)	6		29.2 (25.4)	6		53.3 (36.1)	6		6 (3.5)	6	
HPV 35	No	16.9 (16.2)	35	0.475	36.7 (23.8)	35	0.163	54.9 (32.7)	35	0.341	8.3 (5.7)	35	0.649
	Yes	10 (17.3)	3		53.3 (15.3)	3		73.3 (37.9)	3		7 (7.2)	3	
HPV 52	No	15.8 (16.4)	36	0.512	38.8 (23.9)	36	0.447	54.7 (33.1)	36	0.250	8.3 (5.8)	36	0.371
	Yes	25 (7.1)	2		25 (7.1)	2		85 (7.1)	2		5 (0)	2	
HPV 54	No	15.9 (16.2)	37	0.413	38.5 (23.6)	37	0.381	55.4 (32.9)	37	0.336	8.2 (5.8)	37	0.533
	Yes	30 (0)	1		20 (0)	1		90 (0)	1		5 (0)	1	
HPV 58	No	16.2 (16.4)	37	0.923	38 (23.8)	37	0.747	56.8 (33.3)	37	0.647	7.7 (5)	37	0.084
	Yes	20 (0)	1		40 (0)	1		40 (0)	1		25 (0)	1	
HPV 67	No	16.3 (16.2)	38	-	38 (23.5)	38	-	56.3 (32.9)	38	-	8.2 (5.7)	38	-
	Yes	-	-		-	-		-	-		-	-	
HPV70	No	16.8 (16.1)	37	0.312	38.9 (23.2)	37	0.107	55.9 (33.3)	37	0748	8.2 (5.8)	37	0.533
	Yes	0 (0)	1		5 (0)	1		70 (0)	1		5 (0)	1	

*Mean (SD)=Mean (Standard Deviation). **Kruskal-Wallis test. ***Fisher' sex acttest. ****Chi-square's test.

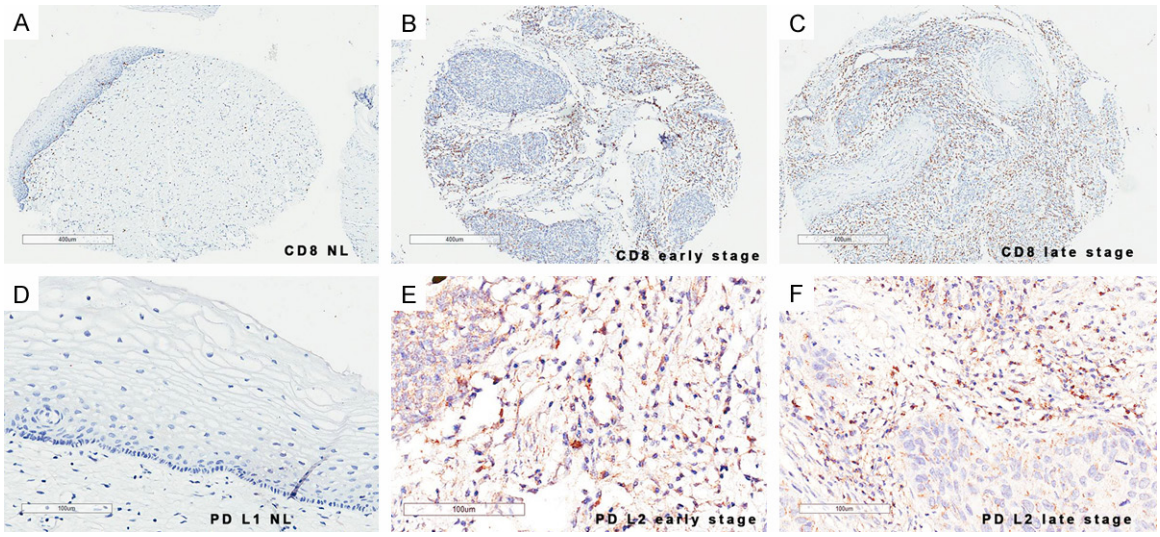

Figure 1. Detection of CD8$^+$ cells and PD-L1 in cervical cancers. (A) shows the rarity of CD8$^+$ cells in the normal cervix from a woman with no cervical disease. (B and C) show the strong infiltration of CD8$^+$ cells in both an early stage and late stage cervical cancer, respectively. PD-L1 expression was non-existent to minimal in the normal cervix (D). PD-L2 was present in both mononuclear cells and cancer cells in early and late stage cervical cancers (E and F).

PD-L1, PD-L2, CD8 and CD1a expression in ICC and controls

The expression of PD-L1, PD-L2, CD8 and CD1a were compared between the histologically normal cervical control specimens and the invasive cervical cancer cases (ICC). Early stage cervical cancer was defined as FIGO IA-IIA and advanced stage included FIGO IIB-IVA. This data is presented in **Table 2**.

PD-L1 and PD-L2 staining was evaluated by counting the number of reactive positive lymphocytes per 1 mm, which was the size of each core. The appearance of the signal for both PD-L1 and PD-L2 was cytoplasmic and membranous. We focused on the lymphocyte expression of PD-L1 and PD-L2 because lymphocytes were the major source of PD-L1 and PD-L2 in the cervical cancers. Note the very low baseline expression of PD-L1 and PD-L2 in the control tissues.

We next focused on the number of CD8 cells in the tissues since these were the primary source of PD L1 and PD L2. Scattered CD8$^+$ cells were evident in the normal cervical epithelia, typically at the epithelial-stromal interface. Also, CD1a$^+$ cells were evident in the normal cervix and were often in the epithelia. These CD1a$^+$ cells often showed fine branching processes consistent with Langerhans cells.

Also notable from **Table 2** is the dramatic increase in the number of cells expressing CD8 in the invasive cancers (57.4/core) versus the control tissues (15.5/core); this was statistically significant (P<0.001). Using a threshold of greater than 20 CD8$^+$ cells per core as a designation for a strong infiltration, 84% (33/39) of the invasive cervical cancer tissues showed a strong presence for CD8$^+$ cells. There was also a concomitant increase in the number of cells expressing PD-L1 and PD-L2 in the cancers (each again highly significant). The cells expressing PD-L1 and PD-L2 were primarily mononuclear cells with the same distribution as the CD8$^+$ cells (**Figure 1**); scattered cells with the morphology of cancer cells and macrophages also expressed these checkpoint proteins. Also note that the number of CD1a$^+$ cells decreased between the control samples (11.6) to the invasive cervical cancers (8.0) that barely reached statistical significance at P=0.040 (**Figure 2**).

Next, we analyzed whether there is some difference between the expression of PD-L1, PD-L2, or the numbers of CD8$^+$ and CD1a$^+$ cells in the early stage versus advanced FIGO stage cervical cancers. As compiled in **Table 2**, there was no stastically significant differences for PD-L1, PD-L2, CD8 or CD1a when comparing early and late stage cervical cancer (**Figure 3**).

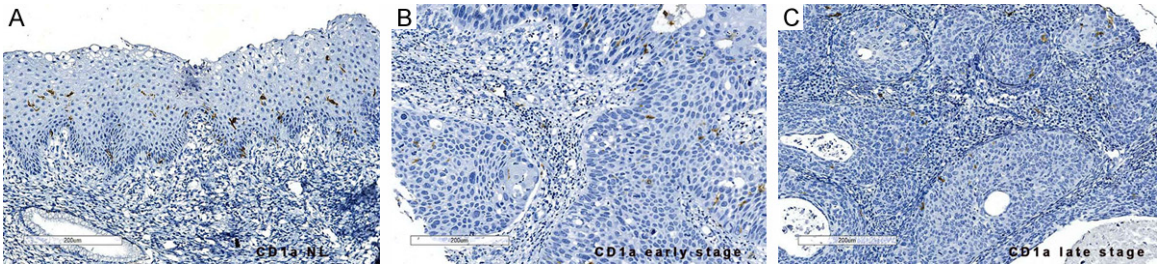

Figure 2. Detection of CD1a in controls compared to cervical cancers. (A) shows the paucity of dentritic cells around the basal epithelium on normal tissue. (B and C) show several cells positively stained for CD1a in a sample of an invasive cercal cancer.

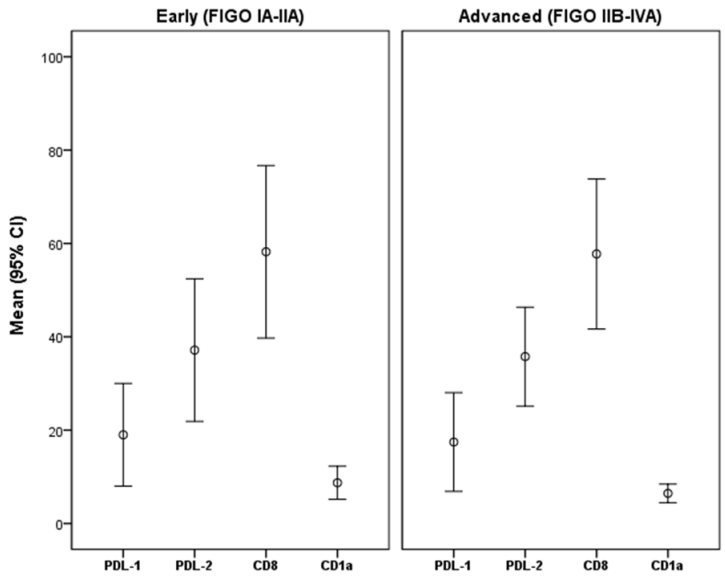

Figure 3. Comparison between the early and Advanced FIGO staged samples and the immune markers analyzed.

Discussion

A central question in the prognosis of carcinomas is whether a decreased immune response may be responsible in part for the worsening prognosis associated with advanced stages of the disease. Herbst et al [6] in a study of 175 late stage cancers including lung cancer, renal cancer, and melanoma, noted that only 22% of patients had a strong CD8 response in the tumoral tissue [6]. With regards to cervical cancer, it has been reported that CD4 and natural killer cell density decreased with worsening prognosis and stage advancement, which suggests that a weakened immune response may be in part responsible for the progression of cervical cancer to stages III and IV [3, 5]. Several groups have indicated that increased CD8 infiltration can be a good prognostic sign in colon cancer [13] and in a wide variety of other cancers such as lung, melanoma, renal cell cancer and colon cancer [6, 14]. However, the more recent studies by Herbst et al and Tumeh et al [6, 14] suggested that increased CD8 infiltration was only a good prognostic feature in stage IV cancers as an indicator for the response to anti PD-1 or anti PD-L1 therapy. Finally other groups [15] have suggested that early stage and late stage cancers are equivalent in their immune responses as measured by CD8 numbers and activation. However, their role in different types of cancers has been controversial. The main finding of this study was that there indeed was an increased CD8 infiltration in cervical cancer tissue was equivalent between early stage and late stage disease. Importantly, we also documented that two of the key checkpoint proteins of CD8 cells, PD-L1 and PD-L2, were dramatically up regulated in the invasive cervical cancer tissues and that this increased expression was equivalent in early and late stage disease.

Interestingly, we did note a slight, but significant decrease in the $CD1a^+$ cell numbers in the cancers (P=0.040) compared to the normal cervical tissues. This data, together with other studies that have shown decreased $CD4^+$ and NK cell numbers in invasive cervical cancer, do raise the question whether other arms of the immune system, relatively distinct from the cytotoxic $CD8^+$ cells, may indeed be reduced in cervical cancers. Nonetheless, since $CD8^+$ cells are considered the key anti-tumor cell in cervi-

cal carcinomas, our data indicates that, at least as measured by the numbers of infiltrating cells, early and late stage cervical cancer each show a robust and equivalent CD8 infiltration in the tumor.

The present study also found that PD-L1 and PD-L2 were highly expressed in invasive cervical cancer (ICC) compared to controls. A recent report suggested that PD-L1 is a potential biomarker of productive HPV infection of the cervix and that it is up-regulated in cervical cancer when compared with other gynecologic malignancies [8]. Further, tumor cells may release other immunosuppressive cytokines such as IFN-γ, CTLA4 and IL-10 that may provide protection from cytolysis by activated T cells [16]. Overall, this data suggests that there indeed is a strong tumor-related effort to render tumor-infiltrating $CD8^+$ cells quiescent and the presence of PD-L1 and PD-L2 are consistent with their participation in this process within cervical cancers.

A noteworthy finding in our study was that even stage III and IV tumors maintained a strong degree of $CD8^+$ cell tumoral infiltration. Indeed, 33/39 of the invasive cervical carcinomas showed a strong CD8 infiltration that was much greater than reported in other studies [6, 14]. Herbst et al [6] noted that only 21% of the stage IV tumors studied showed a strong intra-tumoral $CD8^+$ cell infiltration. Importantly, these tumors also showed the strongest PD-1 and PD-L1 expression levels that together with the number of $CD8^+$ cells appeared to be highly predictive for the response to anti PD-1 or anti PD-L1 therapy. Neither study [6, 14] studied cancers associated with a viral infection. Numerous groups have shown that viral infections increase the number of infiltrating $CD8^+$ cells. Thus, one possible explanation for our observation for a much greater degree of $CD8^+$ cell infiltration, even in the late stage cervical cancers, is that cervical cancer is invariably associated with a productive viral infection. This would suggest that cervical cancer, both early and late stage, may be especially sensitive to anti PD-1/PD-L1 and/or anti PD-L2 therapy, which will require additional research.

The present study had some limitations, such as the relatively small sample size among the different invasive cervical cancer stages that prevented additional subgroup analyses. However, our study highlighted important and new data regarding the potential application of PD-L1 and PD-L2 immunostaining as biomarkers for the presence of HPV infections that could direct further DNA analyses to test for the presence of viral genomes. Moreover, a robust immune response was still evident in the advanced tumor stages. Further studies should be done to explore the use of PD-L1 and/or PD-L2 as potential therapeutic targets in early and late stage cervical cancer treatment.

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Disclosure of conflict of interest

None.

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