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ORIGINAL PAPER

Location and Density of Immune Cells in Precursor Lesions and Cervical Cancer

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Abstract Only a small proportion of women infected with *Human Papillomavirus* (HPV) develop cervical cancer. Host immune response seems to play a role eliminating the viral infection and preventing progression to cancer. Characterization of tumor infiltrating lymphocytes (TILs) in cervical pre-neoplastic lesions and cervical cancer may be helpful to understand the mechanisms that mediate this protection. The aim of this study was to determine if there are differences in the localization and density (cells/mm^2) of CD8+ T-cells, CD4+ T-cells and Tregs (CD25 + Foxp3+) in cervical pre-neoplastic lesions and cervical cancer. Immunohistochemical analysis of sections of 96 (26 CIN1, 21 CIN2, 25 CIN3, and 24 SCC) samples revealed that regardless of CIN grades, CD8+ T-cells are more abundant than CD4+, CD25+ and Foxp3+ cells in both the stroma and epithelium. There was a higher density of CD8+ cells in the stroma of cervical cancer compared to CIN3 (OR = 4.20, 95% CI 1.2-15), CIN2 (OR = 7.86, 95% CI 1.7-36.4) and CIN1 (OR = 4.25, 95% CI 1.1-17). Studies evaluating whether these cells are recruited before or after cancer progression will be helpful to understand the role of these cells in the natural history of HPV-induced lesions.

Keywords Tumor infiltrating cells · Cervical cancer · HPV infection · Immunohistochemistry

Abbreviations

HPV	Human Papillomavirus
SCC	Squamous cell carcinomas
IC	Invasive Carcinoma
CIS	Carcinoma in situ
CIN1	Cervical Intraepithelial Neoplasia 1
CIN2	Cervical Intraepithelial Neoplasia 2
CIN3	Cervical Intraepithelial Neoplasia 3

Introduction

Cervical cancer is the third most common malignancy in women worldwide. In 2008, 530,000 new cases were diagnosed and 275,000 deaths due to this disease were estimated to occur worldwide. Approximately, 85% of cases and 88% of deaths occur in women living in developing countries [1]. High-Risk Human Papillomavirus (HR-HPV) infection is the

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necessary but not sufficient cause for cervical carcinoma [2, 3]. In sexually active women, the lifetime incidence of cervical HPV infection is estimated to be as high as 80% [4]. Approximately 90% of these infections clear spontaneously in about 2 years [5–7]. In contrast, when the infection persist (about 10% of infected women) the risk for progression to high grade lesions and cervical cancer increases [8–10], especially in HPV 16 and 18 positive women [11]. In the natural history of HPV infection, Cervical intraepithelial Neoplasia (CIN) 1 lesions have higher rates of regression than CIN3, which are mostly associated with persistent HR-HPV infection [12]. Likewise, progression rates to invasive cancer for CIN1 are lower (1%) than CIN3 (>12%) [13]. Immune response has been considered a cofactor that may play a role in the different stages of the natural history of cervical cancer [13]. Although tumor infiltrates lymphocytes (TILs) have been observed in pre neoplastic and tumor tissues [14–17], it has been difficult to delineate the role that these immune cells may play in affording the regression of different grades of CIN. Studies have reported an equal proportion of CD4+ and CD8+ cells in the stroma of pre-neoplastic lesions and a low density of CD4+ cells in the epithelium of pre-neoplastic lesions when compared to normal tissues [18]. Even further, in cervical cancer, high number of CD8+ TILs are associated with absence of metastasis [19] and low numbers of immune cell types with relapse [20]. Others have reported that a high number of CD8+ cells are present in invasive cervical cancers [15]. In this study we conducted a thorough statistical analysis of the reproducibility of the counts of three important types of immune cells (CD8+, CD4+) and regulatory T cells (CD25+ Foxp3+) in premalignant and malignant lesions of cervical cancer. We also conducted a descriptive analysis of the density (number of cells per mm^2), in the infiltrates of these lesions and determined if the density of cell subtypes in the stroma or the epithelium was associated with any of the histological grades.

Material and Methods

Samples and Patients

In this retrospective study, 120 histological specimens were selected from the archives of the Department of Pathology at the School of Medicine of the University of Antioquia, Medellin, Colombia. We included samples of cases diagnosed between 2000 and 2007 histologically confirmed with CIN1 (30), CIN2 (30), CIN3 (30) and SCC (30). Paraffin blocks were recut for uniform histopathology review and micro dissection, with the first and last slides of a series of 5 reviewed by two pathologists to confirm the original diagnosis. There was 78% (93/120) agreement between pathologists and 27 cases were again read by an external pathologist. A consensus diagnosis of these cases was blindly reached by the statistician. Final group included in the analysis was conformed of 96 cases. This study was approved by the Research Ethics Committee at the University of Antioquia

Immunohistochemistry

After histopathology confirmation of the lesion, the second and third section were used for Immunohistochemical double staining which was performed on 4 μm tissue sections with the EnVision™ G|2 Doublestain System, Rabbit/Mouse kit (K5361, Dako. Denmark), using the following monoclonal antibodies: anti-CD4 (VP-C318, Vector Laboratories, CA, USA), anti-CD8 (N1592, Dako cytomation, CA, USA), anti-CD25 (sc-57297, Santa Cruz Biotech, CA, USA) and anti-Foxp3 (sc-80792, Santa Cruz Biotech, CA, USA). Tissue sections were dewaxed in xylene and rehydrated through graded ethanol. After deparaffinization, sections were immersed into preheated antigen retrieval solution (50 mM Tris –HCl at pH 9.9) at 100°C for 30 min. Endogenous peroxidase activity was blocked for 5 min using dual endogenous enzyme blocking solution. Each primary antibody was diluted as follows: 1/50 (anti-CD8, for slide 1) and 1/20 (anti–Foxp3, for slide 2) and was incubated at room temperature. Washing of sections 3 times was followed by incubation with Polymer/HRP secondary antibody. Reaction was developed with DAB + working solution and incubation for 5–15 min. Then, sections were incubated with double staining blocking solution for 3 min. Primary antibodies were diluted in antibody diluent as follows: 1/10 (anti-CD4, for slide 1) and 1/800 (anti-CD25, for slide 2) and incubated at room temperature. Washing of sections 3 times was followed by incubations with a Rabbit/Mouse (LINK) by 10 min and incubations with Polymer/AP for an additional 10 min. Finally, slides were incubated with permanent red working solution for 5–20 min and counterstained with hematoxylin.

Quantification of Labeled Cells

The number of positive cells per mm^2 was independently assessed by 2 pathologists (RJ and NO) from 10 independent areas of each slide (5 epithelial and 5 stromal areas) within the areas with most abundant infiltrates using an eyepiece graticule and 400× magnification. The mean number of cells counted per mm^2 in 5 areas of epithelium and 5 of stroma, was obtained for each case.

DNA Recovery and HPV Genotyping

Areas with lesions were circled during histopathology confirmation of the diagnosis and corresponding tumor areas micro dissected from unstained slides using a sterile surgical scalpel in each case. The dissected material was transferred to non-silicone tubes and xylene (350 ul) was added to each

sample to dissolve the paraffin. Paraffin-free tissue was precipitated with 150 ul of cold 100% ethanol and centrifuged and pellet was allowed to dry at room temperature overnight. Dry button was re-suspended in 100 ul of proteinase K buffer (10 mg/ml proteinase K in 50 mM Tris, pH 8.3) and incubated overnight at 37°C. Finally the samples were incubated at 95°C for 8 min to inactivate proteinase K and stored at–20°C until use. Paraffin blocks sectioning and DNA extraction was conducted under strict conditions to avoid contamination. Blank paraffin blocks with normal tissue were included among every 21 samples processed, and positive controls were paraffin-embedded cervical cancer tissues positive for HPV. DNA quality was evaluated by amplifying a 209 bp segment of the human β-globin gene. Initially, HPV DNA was detected by the general primer GP5+/6 + -mediated PCR, as described previously [21] and followed by genotyping of PCR products with reverse-line blot hybridization with specific probes for 37 different HPV genotypes (HPV types 6, 11, 16, 18, 26, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 57, 58, 59, 61, 66, 68, 70, 71, 72, 73, 81, 82/IS39, 82/MM4, 83, 84, CP6108). All samples negative for hybridization after the GP5+/GP6 + -mediated PCR, were subjected to HPV 16 and HPV 18 genotype specific PCR as described [22]. The primers used in this procedure amplify a fragment of 96pb (HPV-16) and 115pb (HPV-18) E6 gene of HPV, which were visualized in 2% agarose gels stained with ethidium bromide.

Statistical Analysis

The percentage agreement, the Kappa index (κ) and McNemar test, were used to assess the inter-observer agreement for identification of lesions with presence or absence of cells. The agreement was considered poor if the Kappa index was less than 0.4, good or adequate if it was between 0.4 and 0.75, and excellent if it was greater or equal than 0.75 [23]. In lesions with counts/mm^2 equal or higher to one, the cell counts obtained by each observer were compared using the Bland-Altman method. We depicted the Bland-Altman plot (data not shown) with the average of the number of cells/mm^2 counted by the two observers versus the differences in the number of cells/mm^2 counted by them. Furthermore, following the enhanced Bland-Altman analysis recently described by Muñoz [24], we conducted a lineal regression analysis of the difference of RJ and NO on the averages of RJ and NO centered around their overall average. In such regression, the intercept corresponds to the mean of the differences between the two observers (i.e., bias) and the slope corresponds to the ratio of the standard deviations. Full agreement is achieved when both the intercept and the slope are equal to zero and the correlation coefficient is near one. This analysis was conducted for CD4, CD8 and Foxp3 cells, because only in these cases the number of observations afforded an adequate statistical power to determine the association between high number of cells/mm^2 (upper tertile) and different grades of CIN lesions/invasive cancer. Then, we conducted a descriptive analysis on the amount of cells/mm^2 counted in the stroma and epithelium using the median and mean as measures of central tendency and the interquartile range and coefficient of variation as measures of dispersion. Also, the skewness was calculated. The Mann–Whitney and Kruskal-Wallis tests were used to determine if there were statistically significant differences between the number of cells/mm^2 counted in the stroma and epithelium and among CIN1, CIN2, CIN3 and invasive cancer respectively. Post-hoc tests were conducted using Benherens-Fisher test. Logistic regression ORs adjusted by age, to determine the association between high number of cells/mm^2 (upper tertile) and different grades of CIN lesions/invasive cancer, were obtained with those cells types on which was possible to conduct the Bland-Altman analysis. A significance level of 0.05 was used in all tests. The statistical program R version 2.9 (R Development Core Team (2009): A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0) was used for all analysis.

Results

Characteristics of the Study Subjects

After final review by the third pathologist, 24 cases were excluded because there was no agreement in the histological diagnosis or there was poor representation of the lesion. The final group included 96 cases. Twenty-six were CIN1, 21 CIN 2, 25 CIN3 and 24 SCC (Table 1). The mean (standard deviation) age of CIN1 cases was 33.7 (8.4) years, of CIN2 33.6 (12.2), of CIN3, 47 (13.5) and SCC 47.2 (15.3). As expected, a statistically significant difference was observed on the age of cases among the histological grades (Kruskal-Wallis p-value=0.0002).

HPV DNA Detection in Premalignant Lesions and Cervical Cancer Tissues

HPV positivity was estimated after exclusion of paraffin blocks from which it was not possible to obtain DNA for PCR (HPV and β-globin negative samples). Fifty percent (13/26) of CIN1 cases, 52.3% (11/21) of CIN2, 72% (18/25) of CIN3 and 75% (15/20) of SCC cases were HR-HPV positive. In SCC cases we found that 87% of the HPV positives (13/15) had HPV-16, 6.6% (1/15) HPV-18 and 6.6% (1/15) HPV-31. Also we found 5% (1/20) of SCC

Table 1 Immune cells density in cervical intraepithelial neoplasia and squamous cervical carcinoma

		Cell densities (cells/mm^2)					
		$CD4^+$	$CD8^+$	DP $CD4^+/CD8^+$	$CD25^+$	$Foxp3^+$	DP $CD25^+/Foxp3^+$
Intraepithelial compartment							
CIN 1	Mean	6.04	19.71	0.35	0.18	0.38	0.04
(n=26)	Median	1.00	13.25	0.00	0.00	0.00	0.00
	CV* (%)	57.0	69.8	51.3	22.4	36.1	20.0
	IQ Range	8.75	14.38	0.50	0.00	0.00	0.00
	Skewness	2.49	3.11	2.42	4.31	3.43	4.42
CIN 2	Mean	1.20	15.95	0.19	0.05	2.02	0.07
(n=21)	Median	0.5[a]	13.00	0.00	0.00	0.00	0.00
	CV* (%)	55.7	122.7	64.6	31.7	43.2	29.9
	IQ Range	1.00	1.00	0.50	0.00	2.00	0.00
	Skewness	2.15	1.89	1.14	2.56	3.17	3.04
CIN 3	Mean	5.80	17.17	0.83	0.00	2.06	0.00
(n=25)	Median	2[a]	13.00	0.00	0.00	0.00	0.00
	CV* (%)	59.5	125.7	32.1	0.0	26.9	0.0
	IQ Range	5.50	16.50	0.50	0.00	0.00	0.00
	Skewness	2.54	0.88	3.75		3.95	
SCC	Mean	5.52	15.42	0.42	0.17	1.71	0.40
(n=24)	Median	1.25	10.50	0.00	0.00	0.25	0.00
	CV* (%)	45.6	78.4	54.7	26.9	42.7	38.4
	IQ Range	4.13	17.75	0.50	0.00	1.25	0.00
	Skewness	3.10	1.87	1.76	3.98	3.39	2.46
† P value		0.06	0.67	0.97	0.41	0.06	0.13
Stromal compartment							
CIN 1	Mean	30.52	52.65	0.53	0.80	6.76	0.30
(n=26)	Median	7.75	35.75	0[c]	0.00	0.00	0.00
	CV* (%)	62.3	101.0	60.1	32.1	44.2	38.6
	IQ Range	22.50	46.63	0.88	0.50	4.00	0.00
	Skewness	1.55	1.68	1.32	4.10	2.57	2.57
CIN 2	Mean	9.45	42.52	0.62	0.81	3.06	0.28
(n=21)	Median	5.25	34.5[b]	0[d]	0.00	1.75	0.00
	CV* (%)	68.2	132.8	47.1	39.7	87.2	70.9
	IQ Range	7.63	30.88	0.50	0.00	3.25	0.50
	Skewness	2.06	1.65	2.12	2.22	1.12	0.87
CIN 3	Mean	20.62	59.93	3.59	0.08	10.04	0.10
(n=25)	Median	7.50	42.00	1[c,d]	0.00	0.00	0.00
	CV* (%)	82.9	106.1	33.7	34.6	29.7	35.4
	IQ Range	28.00	55.50	2.00	0.00	2.88	0.00
	Skewness	1.43	1.26	3.68	2.69	4.07	2.42
SCC	Mean	29.71	71.58	1.50	1.71	5.48	0.33
(n=24)	Median	12.75	76[b]	0.25	0.00	1.75	0.00
	CV* (%)	82.6	145.3	58.8	22.1	41.0	44.6
	IQ Range	48.50	73.50	2.00	0.00	4.13	0.13
	Skewness	1.20	0.28	1.78	4.29	3.76	2.35
† P value		0.22	0.28	0.13	0.22	0.32	0.32

*Coefficient of variation

†Kruskal-Wallis test: comparing the counts among the histological grades in stroma and epithelium

[a] p=0.0003. Benhrens–Fisher test. CD4+ in CIN 2 vs. CIN 3 in epithelium

[b] p=0.06. Benhrens–Fisher test. CD8+ in CIN 2 vs. SCC in stroma

[c] p=0.03. Benhrens–Fisher test. DP CD4+/CD8+ in CIN 1 vs. CIN 3 in stroma

[d] p=0.06. Benhrens–Fisher test. DP CD4+/CD8+ in CIN 2 vs. CIN 3 in stroma

DP double positive staining

cases had low risk-HPV positive. Although the HPV prevalence was low, these results are within the range of estimates obtained and explained by the low sensitivity of the Gp5+/Gp6+ PCR in paraffin-embedded tissues.

Inter-Observer Agreement in the Counting of Cell Types

There was very good agreement in the proportion of cases identified with or without CD8+ cells in the epithelium (90.7%, MacNemar test p value=0.077, κ=0.59); in the stroma, the agreement was adequate (94.1%, MacNemar test p-value=0.371) but reproducibility was poor (κ= 0.26). Likewise, for Foxp3+ cells in the epithelium there was good agreement (73.2%, McNemar test p-value=0.210) but poor reproducibility (κ=0.27) but in the stroma the agreement was good (74.7%, McNemar test p-value= 0.662, κ=0.49). For CD4+ cells, agreements were low (72% in the epithelium and 69.7% in the stroma) and there was a statistically significant difference between observers for this type of cell in both tissue compartments (McNemar test p-values<0.001). Although the agreement was good for CD25+ cells, in the epithelium (93%, McNemar test p value=0.68) and stroma (81%, McNemar test p value= 0.45), the κ index (0.22 and 0.17 respectively) was poor (Table 2). In the enhanced Bland-Altman analysis (Table 3), the comparison of counts of CD8+ cells in the epithelium and stroma and of Foxp3+ cells in the epithelium, showed that the differences between the two observers were not statistically significant (intercept p-values>0.05) but deviation standards were different (slope p-values<0.05) whereby suggesting that one of the pathologist dispersed the counts more than the other.

There was good agreement between the two observers for counts of CD4+ cells/mm^2 in stroma (intercept p-value= 0.34, slope p-value=0.10, r=0.71) but not for counts of CD4+ cells/mm^2 in epithelium (intercept p-value<0.001, slope p-value=0.23). There was very poor agreement between the two observers for counts of Foxp3+ cells obtained in the stroma (intercept p-value=0.04 and slope p-value=0.08).

Table 2 Agreement between readers for the identification of immune cells in precursor lesions and cervical cancer

Cell type	Localization	% agreement	κ index	Mc Nemar test
CD4	Epithelium	72	0.47	0.0005
	Stroma	69.7	0.17	0.0000
CD8	Epithelium	90.7	0.59	0.077
	Stroma	94.1	0.26	0.371
Foxp3	Epithelium	73.2	0.27	0.210
	Stroma	74.7	0.49	0.662
CD25	Epithelium	93	0.22	0.68
	Stroma	81	0.17	0.45
DP CD4+ CD8+	Epithelium	67	0.03	0.0001
	Stroma	57	0.06	0.0000
DP CD25+ Foxp3	Epithelium	93	0.23	0.04
	Stroma	77	0.14	0.00

Table 3 Comparison for reproducibility of cells/mm^2 counts between two observers by enhanced Bland-Altman analysis

Cell type	Localization	Intercept p value	Slope p value	Spearman correlation coefficient
CD4+	Epithelium	0.0007	0.02	0.73
	Stroma	0.34	0.10	0.72
CD8+	Epithelium	0.14	0.0004	0.62
	Stroma	0.08	0.0002	0.73
Foxp3+	Epithelium	0.1379	0.03	0.80
	Stroma	0.04	0.08	0.74

Infiltrating Immune Cells in Pre-Neoplastic Lesion and Invasive Cancer

We observed a significant predominance of CD4+, CD8+ and Foxp3+ cells/mm^2 in the infiltrates at the stromal compared to the epithelial compartment in all histological grades (Table 1). In addition, the average number of CD8+ cells/ mm^2 was higher than the average number of CD4+ and Foxp3+ cells, in all histological grades as well as in both stroma and epithelia but this difference was not statistically significant. The average number of intraepithelial CD4+ cells/mm^2 in CIN3 was higher than in CIN2, (Benhrens–Fisher p-value=0.0003) and the average number of stromal CD8+ cells/mm^2 was higher in invasive cancer than in CIN2, but this difference did not reach statistical significance (Benhrens–Fisher p-value=0.06). Double-positive CD4+CD8+ cells were observed in some pre-neoplastic lesions. When present, the average number of double-positive CD4$^+$CD8$^+$ cells/mm^2 was higher in the stroma of CIN3 than in stroma of CIN1 (Benhrens–Fisher p-value= 0.03) and CIN2 lesions (Benhrens–Fisher p-value=0.06) (Table 1). The descriptive analysis of these cell populations shows that infiltrate pre-neoplastic lesions and cancer of the cervix have a heterogeneous immune response in terms of numbers of cells/mm^2 and their relation to different histological grades (Table 1).

Association between Cellular Densities and Different Degrees of Neoplasia

In the logistic regression models the main outcome variable corresponded to different combinations of low grade and high grade disease as follows: a) SCC vs. CIN1, b) SCC vs. CIN2 and c) SCC vs. CIN3. The distribution of the frequencies of the counts of CD4+, CD8+ and Foxp3+ cell/mm^2 were positively skewed i.e. did not show a normal distribution curve, (Table 1) and showed a heavy right tail. Therefore, we used the classification by tertile (66 percentile) of cell counts as independent variable, comparing cells counts

Table 4 Analysis of association between cellular densities (cells/mm^2) of CD8+ cells in the stroma and different degrees of neoplasia

Cells density by tertiles	SCC vs. CIN1			SCC vs. CIN2			SCC vs. CIN3		
	n	SCS%	OR[a] (95%CI)	n	SCC%	OR[a] (95%CI)	n	SCC%	OR[a] (95%CI)
All others	29	31	1	26	34.6	1	26	37.5	1
Upper tertil	21	71.4	4.25 (1.1–17.0)	18	83.3	7.86 (1.7–36.4)	21	71.4	4.20 (1.2–15.0)

The reference group was all others tertiles (lowest and medium tertile). [a] Cell densities are adjusted by age in all models

in the upper tertile subgroup with all other subgroups (reference category) to test the probabilities of a relationship between counts of cells/mm^2 and different outcomes (different grades of CIN). Table 4 shows the logistic regression models comparing SCC with each of the other types of lesions. Cases with counts of CD8+ cells/mm^2 in the stroma above the upper tertile had higher risk of SCC compared to CIN1 (OR = 4.25, 95% CI 1.1-17), CIN2 (OR = 7.86, 95% CI 1.7-36.4) and CIN3 (OR = 4.20, 95% CI 1.2-15).

Discussion

Cervical intraepithelial lesions, the histologic manifestation of HPV infection are very frequent and occur almost immediately after sexual debut. Almost 90% of HPV infection and related lesions clear spontaneously within 2 years, but in a small proportion of women, persistent infection is established. The risk of progression to high grade lesions is very high in women with persistent infection; however, these high grade lesions can also eventually disappear. It has been suggested that elimination of the infection and therefore, disappearance of the lesions, may be in part afforded by an adequate immune response [25]. Until today, the roles of effectors and regulatory immune mechanisms that participate in affording elimination of viral infection have not been completely delineated. Specifically, it is known that in the tumor microenvironment, there are different cell types, including macrophages, dendritic cells, NK, B, T cells and regulatory T-cells that produce soluble factors, such as cytokines and chemokines, that can also induce the expression of surface receptors and adhesion molecules. The balance and regulation of immune responses may determine whether there is tumor cell growth promotion or inhibition [26].

Based on the assumption that the activation of an specific immune response may be reflected in the increase or decrease in the number of cell types that infiltrate the site of the precursor lesions caused by the HPV infection, we focused in accurately determining the density (cells/mm^2) of CD4+, CD8+, CD25+ and Foxp3+ cells in CIN1, CIN2, CIN3 and SCC. Histopathological diagnosis [27] and the counting of stained inflammatory cells have a very low reproducibility, the variation of estimates is wide and both variables depend heavily on the reader's experience [28]. Because there is no gold standard to obtain accurate measurements of cell counts by IHC, it is not possible to define the most accurate observer. We focused our efforts to overcome these limitations by performing a complete analysis of reproducibility and determining the level of intra- and inter-variability in the number of cells counted by the pathologists. Carreon et al. 2007 found that CIN3 histological classification is more reproducible than CIN2 [29]. In agreement with previous reports, we found that inter-observer reproducibility is very good for the classification of invasive lesions, moderate for CIN3 and very poor for CIN1 and CIN2. Therefore, only cases with full agreement on the diagnosis were included in the analysis for identification and counting of immune cells. In spite of the wide intra-variability for the identification and the counting of stained immune cells, there was good reproducibility for the proportion of cases identified with CD8+ cells (Mc Nemar test p value>0.05). The reproducibility for the proportion of cases identified with CD4+ (Mc Nemar test p value<0.05) and DP CD4+ CD8+ cells (Mc Nemar test p value<0.0001) was poor. The comparison of the counting between pathologists showed that there was acceptable correlation in the number of cells counted (Spearman correlation coefficient above 0.6 for all cell phenotypes) and intercept p values shows that the means of the differences between the 2 pathologists (bias) were not significantly different from zero for counting CD8+ cells in both tissue compartments, for CD4+ cells in the stroma and for Foxp3+ in the epithelium. However, considerable lack of agreement between pathologists was observed when the dispersion of the measurements were evaluated (slope p value<0.05), especially in the case of CD8+ cells, where one of the pathologist tended to count more cells than the other. It is not expected that both pathologists agree exactly but we saw the need to estimate how much they differ and if this difference was enough to cause problems in the interpretation of the logistic regression analysis.

We observed that CD8+ cells localized in the stroma and ephitelium of pre-neoplastic lesions and cervical cancer were more abundant than CD4+, CD25+ and FoxP3+ cells but the difference was not statistically significant. We also observed that high counts of CD8+ cells in the stroma were associated with cervical cancer as seen in Table 4. It is of interest that elevated ORs were present when comparing SCC with lesions of all grades, suggesting that this is a phenomenon associated with invasion and not with persistent infection or development of precursor lesions. Given the fact that CD8+ cells presented the highest coefficient variation, and that there was lack of agreement when the dispersion of the measurements was compared, we also estimated the ORs using the counting conducted by each pathologist to estimate the average number of cells/mm^2 in each sample (data not shown). In this analysis there were increased risks for cervical cancer associated to the upper tertile of CD8+ cells in the stroma for both reader 1 [(OR: 1.59, 95% CI:0.4-6.4 SCC vs. CIN1), (OR: 3.2, 95% CI:0.8-13.8 SCC vs. CIN2) and (OR: 3.3, 95% CI:0.9-12.2 SCC vs. CIN3)] and reader 2 [(OR: 2.69, 95% CI:0.7-10.7 SCC vs. CIN1), (OR: 5.3, 95% CI: 1.2-24.4 SCC vs. CIN2) and (OR: 1.88, 95% CI:0.6-6.4 SCC vs. CIN3)], although this relationship was not statistically significant. These data are on agreement with previous reports of a higher number of CD8+ cells in the stroma of invasive carcinoma as compared to low grade lesions [14, 15, 30, 31]. However since the confidence intervals were wide, studies with bigger sample size may help to confirm the accuracy of our observation.

Regarding of the meaning of higher number of CD8+ cells in cervical cancer, the cross-sectional nature of this study does not permit to suggest whether the presence of these cells predispose to cancer or whether the increase in the number of cells is a consequence of the invasion. A higher density of CD8+ cells in cervical cancer may be the response to more antigenic stimulation that may occur during the invasive process as tumor cells migrate from cervix to draining lymph nodes. Several authors have identified HPV antigen-specific CD8+ T cells in the cervix [32]. Zehbe et al. 2007 showed that CD8+ cells specific for the HPV 16 E6 protein fail to control tumor growth despite interferon gamma production [33] suggesting that successful immune surveillance of HPV16+ tumor cells in cervical cancer patients is impaired. There are observations that those cells may be rendered tolerant by molecules present in tumor microenviroment [34, 35]. Cervical tumors are marked by the presence of an immune regulatory microenvironment characterized by increased production of IL10 and TGFβ and decreased production of IL2 [31]. In addition, as we observed in this study, the presence of regulatory T cells may suggest a role of these cells in the induction of anergy of CD8+ cells. In our study there was no difference in CD4/CD8 ratio (data not shown) and this was below 1 in all lesions. This may suggest that in addition to CD4+ T cells other soluble mechanisms may play role in impairing the effector mechanisms of CD8+ T cells in cervical cancer. Compared to cervical carcinoma without lymph node metastasis, those lesions with lymph node metastasis have a higher proportion of CD4+ CD25+ Foxp3+ cells [36], which suggest that these cells are important in the late stages of the disease. We also found that some of the cervical cancer tissues have DP (doble positive) CD4+/CD8+ cells. This population has been observed in chronic and malignant conditions such as chronic lymphoid leukemia [37] and shown to be memory T cells with effectors capacity in animal models.

Our results are in agreement with previous reports that a higher density of CD8+ cells are observed in the stroma but not in the epithelium of SCC compared to precancerous lesions. This data strengths previous observation about the important role for CD8+ T cells in cervical cancer and warrants future studies to dilucidate if this immune response is an important cofactor in the natural history of lesions associated with HPV infection. Although the purpose of this study was not to correlate cell number or cell types with cancer prognosis, the presence of these cell types in all grades of cervical pre-neoplasic lesions and cervical cancer, suggest that immune response develops very shortly after HPV infection, since they are observed in CIN1 lesions, which are the consequence of acute HPV infection. Our results suggest that the magnitude of this response remains similar in CIN2 and CIN3, as we did not observe any difference in the density of cells. Therefore, we may suggest that differences in the quality of the immune response may be more related to cancer prognosis. However, our results have to be interpreted in the context of the limitations. Wide confidence intervals denote the lack of precision of our estimates. This may due in part to the sample size or because the wide inter-observer variability. A high variability on the average numbers as well as on the range is observed when counting histologically stained cells. Although the use of other techniques such as flow cytometry may overcome this limitation, immunohistochemistry is required in order to locate the cells in tissue compartment.

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Conflict of Interest The authors declare that they have no conflict of interest.

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