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# Langerhans Cells Ascertaining in Cervical Tissues Obtained from Women with Cervical Intraepithelial Neoplasia

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## Abstract

**Objectives:** Impairment of cell-mediated immunity in cervical cancer (CC) and intraepithelial neoplasia (CIN) has been reported. In this study, Langerhans cells (LC) subpopulations were quantitatively evaluated in cervical tissue-samples obtained from Brazilian patients exhibiting progressive grades of CIN, (CIN)I (n=3), CINII (n=3) and CINIII (n=3), in addition to three normal controls.

**Methods:** The precise number of cervical LC was determined in the entire area of lesioned epithelium from CIN samples, by performing a morphometric analysis of Langerhans cells positive for two distinct markers, S100 and Langerin (Lang) detected either by Immunofluorescence or Immunohistochemistry analysis.

**Results:** In normal cervix, a higher density of Langerin+ cells was observed whereas S100+ LC were predominant in pre-neoplastic lesions samples. Increased numbers of intraepithelial S100+ and Lang+ cells were observed in CIN samples, with an important predominance of Langerhans cell in CINII samples, despite the LC marker or the histological technique employed for the analysis. Curiously, a severe decrease in S100+, but particularly in Lang+ Langerhans cells was observed in cervical tissues exhibiting CINIII.

**Conclusions**: Our results suggest that the morphometric evaluation of Langerhans cells number is an effective approach to determine LC number in cervical tissues, and that those immune-cells are possibly involved in the surveillance against the cervical lesion development.

**Keywords:** Langerhans cell subpopulations; Immunofluorescence; Immunohistochemistry; Cervical intraepithelial neoplasia; Mucosal immune-response

## Introduction

Cervical carcinoma has been the subject of much interest due to its high incidence worldwide and its etiologic relaction with an infective cause, the HPV [1]. In Brazil, cervical cancer (CC) is the third leading cancer following breast cancer, with an overall incidence rate of 5.7% among the female population, with an estimated rate of 15.33 cases per 100.000 women [2]. More importantly, a pronounced incidence of high-grade SILs (Squamous Intraepithelial Lesions) or HSIL (9.8%) has been detected among women with cytology abnormalities submitted to the National CC screening program [2]. Cell-mediated immunity (CMI) has a main role in limiting viral infection and avoiding the progression of pre-neoplastic lesions, known as cervical intraepithelial neoplasia (CIN) grades I, II and III, to more severe stages [3]. Langerhans cells (LC) play a pivotal role in immune surveillance against cutaneous and mucosal viral infections and neoplasms due to their capacity to capture and present antigens and induce a specific T-cell activation [4]. Despite the high-prevalence of HPV infection, cervical carcinoma and HSIL lesions amongst Brazilian women, few studies were dedicated to evaluate the frequency of Langerhans cells [5] in this population, a crucial aspect for guiding immunotherapy and vaccination strategies [6]. Although there are a number of reports dedicated to ascertain the Langerhans and/or Dendritic (DC) cells in the human genital tract, especially in the cervix, these appear to be contradictory because some describe no changes [7], some have found decreased numbers of LC/DC [8-11] whereas others have seen increased LC/DC numbers [12-14]. Accordingly, most of those studies were based on the partial analysis of affected areas from the cervical epithelium and/or stroma by using the immunohistochemistry for the evaluation of LC densities [5-14]. Therefore, in this study we have employed a morphometric approach, and two distinct immunohistological methods to perform a detailed evaluation of the distribution of two Langerhans cells subpopulations infiltrating Cervical Intraepithelial Neoplasia tissues obtained from Brazilian women.

## Materials and Methods

### Tissue samples

Twelve cervical samples obtained by LEEP, surgery or colposcopy-directed biopsies were retrospectively obtained from an archive of Paraffin-Embedded Tissues (PETs) from a large histopathological diagnostic routine service Laboratory. Histopathological diagnosis

were independently reviewed and confirmed by three certified pathologists (including A.T. and P.A.F.), and corresponded to 3 CINI, 3 CINII and 3 CINIII cases. Three women who had undergone hysterectomy for benign conditions and presented negative cytology and normal colposcopic/examination before hysterectomy, were included as normal controls. This study was approved by the local Institutional Ethical Review Board.

### Indirect immunofluorescence

Serial sections (5 μM thick) were obtained from PET samples, and one was stained by H&E to confirm the histopathological diagnosis. Consecutive sections were processed for IF analysis. For antigen retrieval, sections were immersed in xylene to remove paraffin, re-hydrated and heated in water-bath for 60 minutes (min) in 10 mM citrate-buffer (pH6.0). Blocking of nonspecific binding sites was performed with 2% BSA (Bovine Serum Albumin)/1xPBS for 60 min at room temperature (RT). After washing, sections were incubated for an hour at RT with the primary antibody rabbit anti-human S100 (CodeDK-26000, Dako, USA) diluted at 1:200 in 1xPBS-2% BSA. Tissue sections were also incubated overnight at 4°C with the primary antibody mouse monoclonal anti-human Langerin (CodeAB49730, Abcam-USA) diluted at 1:400 in 1xPBS-2% BSA. Sections were exhaustively washed in 1xPBS, and incubated with the secondary antibodies for one hour at RT: anti-rabbit Cy5-conjugated/Affinity Pure (Code11-175-144), anti-mouse Biotin-SP-conjugated/Affinity Pure (Code11-065-146) and Streptavidin Cy2-conjugated (Code016-220-084) all from Molecular Probes Inc.-USA. Nuclei immuno-labeling was performed with Propidium iodide. Controls were included in every batch of tests. Negative controls consisted of the primary antibodies omission, and they were invariably negative. Positive controls were done using cervical sections obtained from histopathological negative women for Langerin labeling, and from CINII patients presenting large epithelial lesions for S100 immuno-labeling. After being washed and mounted in Hydramount (National Diagnostics-USA), sections were examined at magnification of 400X (X40 objective and X10 eyepiece) by Scanning Confocal Microscopy (Zeiss-LSM 510Meta). Cells displaying nuclear, cytoplasmic and membrane fluorescent staining, in addition to appropriate morphological features were included.

### Immunohistochemistry

For performing the IHC analysis a conventional protocol was employed based on the IF protocol above described. Serial sections (5 μM thick) were subjected to the removal paraffin, antigen retrieval and blocking of nonspecific binding sites steps. After, sections were incubated at RT with anti-human S100 and at 4°C overnight with anti-human Langerin primary antibodies diluted in 1xPBS-2% BSA. Blocking of endogenous peroxidase was performed with $H_2O_2$ at RT for 30 min. Sections were next incubated with secondary antibodies (Vectastain ABC-PK 6101 and-PK6102 kits, Vector Laboratories-USA) for 30 min, washed in 1xPBS and incubated with the ABC solution. Tissue-sections were counterstained with Harris' hematoxylin, dehydrated and mounted in Enthelan (Merck/Millipore-Germany). Appropriate negative and positive controls were included in every batch of tests. Cells displaying nuclear, cytoplasmic and membrane brown staining, in addition to appropriate morphological features were included. The number of positive Langerhans cells was ascertained in CIN tissue-samples in the whole intraepithelial-lesioned area, and in normal tissues in representative areas of intraepithelial LC.

### Morphometric analysis of langerhans cells density

For immunofluorescence analysis, LC-number was determined in each CIN sample in the area covering the whole intraepithelial lesion. Images obtained at high-magnification fields (400X) were digitalized, and the quantitative determination of positive LC-numbers was performed by using the software KS300 (Image Analyzer Carl Zeiss, Oberkochen, Germany). In normal tissues, LC number was ascertained from an extensive epithelial tissue-area which did not exhibit any cellular abnormalities. A similar approach was employed to determine the LC-number either in CIN samples or in normal tissues by the Immunohistochemistry analysis, except with respect to the LC quantification step, where the positive cells counting and the digitalization of the epithelial surface areas were performed in the Image Analyzer Carl Zeiss, by using the software KS400.

By considering the existing differences in the area sizes amongst distinct digitalized fields from the same sample, the smallest digitalized tissue-area obtained from each sample was considered as the standard area. Thus, the number of positive LC quantified from each digitalized high-power field was adjusted for this determined standard area. The median values of positive cell number were expressed per mm2 of tissue areas.

### Statistical analyses

Data were expressed as median, range, first and third quartiles. The Kruskal-Wallis (non-parametric) 1-way ANOVA test was used to determine significant differences among three and more groups. Statistical significance was considered as $P<0.05$.

## Results

### Langerhans cells evaluation in cervical tissues

In normal cervix, Lang+ (Figures 1A, 1B and 1D, Supplementary information - SI) and S100+ (Figures 1C and 1D) LC exhibiting typical dendritic morphology were visualized in the whole three layers from the epithelium. Langerhans cells positive for Langerin (Figures 1E and 1F) and S100 (Figures 1G and 1H) markers were also present in the epithelium of CINI samples, where they also showed a variable epithelial location. However, Langerhans cells present in CINII and CINIII tissues were preferentially placed at the middle epithelial layer in CINII samples (Figures 1I-1L), and at the upper epithelium stratum in CINIII tissues (Figures 1M-1P), even though Lang+ LC could be eventually observed at the entire epithelium thickness from CINIII samples (Figures 2A-2C-SI). Langerhans cells positive for both Lang and S100 markers in a representative CINIII sample is showed in Figure 2D-SI, arrows.

### Quantification of Langerhans cells subpopulations

All numerical densities (expressed as median [25%-75%] cells number per $mm^2$) of Langerhans cells positive to Langerin and S100 are shown in Table 1, as well as in Figures 2E-SI -2F-SI for Langerin and for S100 quantification, respectively. The number of Lang+ LC was higher in normal epithelium when compared with those present in CIN lesions, especially when considering the IHC results. However, elevated values in the number of LC labeled with S100 were observed in the epithelium from lesion groups when compared with normal tissues (Table 1). An important finding of our study was the predominance of positive S100 and Langerin LC in the epithelium of

patients with CINII lesions (Table 1; Figures 2E-SD-2F-SD). Concerning the lesion severity degree (CINI→II→III) a relevant reduction in the number of Lang+ and S100+ LC was observed from CIN II to III, despite the immunolabeling method employed.

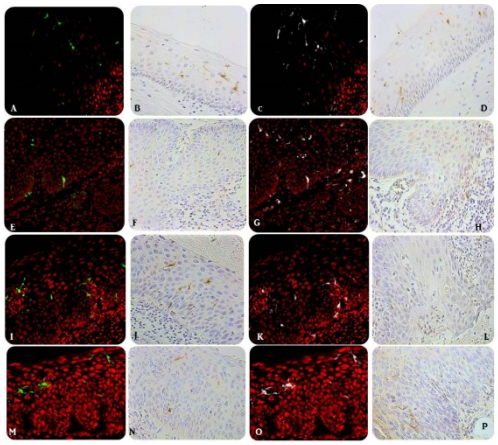

**Figure 1**: Langerhans cells subpopulations in cervical tissues. Langerhans cells detection by IF (A,E,I,M – Langerin and C,G,K,O – S100 markers) and by IHC (B,F,J,N – Langerin and D,H,L,P – S100 markers) in the cervical epithelium of CIN and normal tissues. S100+ cells show white labeling whereas Lang+ LCs exhibit green fluorescence. Nuclei from epithelial and stroma cells are stained in red by Propidium Iodide. Langerhans cells present in the epithelium without morphological alterations are shown (A, B, C, D). Representative images of LC obtained from CIN tissues analysis either by IF or IHC are shown: CINI (E, F,G,H), CINII (I,J,K,L) and CINIII (M, N, O, P). 400X magnification.

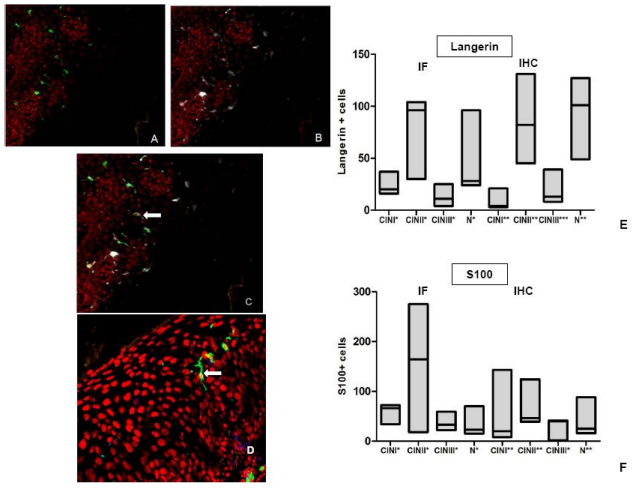


**Figure 2:** Supplementary Information. Langerhans cells subpopulations and quantification in cervical tissues Representative images of LC obtained by IF analysis of a CIN III (A-C) and normal (D) tissue samples: Lang+ LC show green fluorescence (A), S100+ show white fluorescence (B) and a merged image of LCs positive for Lang+ andS100+LC is shown in (C). Langerin positive LCs are shown in (D), exhibiting a detailed morphology (arrows). Nuclei from epithelial and stroma cells are stained in red by Propidium Iodide. 400X magnification. Box and whisker plot showing the Langerhans cells quantification from the epithelium of cervical samples by using Langerin (E) and S100 (F) markers [median, range, first and third quartiles] (*) P<0.005.

| | §S100+ | | §Lang+ | |
|---|---|---|---|---|
| **Cases (#)** | **IF** | **IHC** | **IF (*)** | **IHC (*)** |
| CINI (3) | 66 | 20 | 20 | 4 |
| CINII (3) | 164 | 46 | 96 | 82 |
| CINIII (3) | 33 | 40 | 11 | 13 |
| Normal (3) | 23 | 25 | 28 | 101 |

§ The number of positive Langerhans cells was quantified from each digitalized high-power field within the entire lesioned area. Median values of positive Langerhans cells number were expressed as median, range, first and third quartiles per mm2 of tissue areas. The Kruskal-Wallis (non-parametric) 1-way ANOVA test was used to determine significant differences among three and more groups. IF: Immunofluorescence; IHC: Immunohistochemical. (*) P=0.02 as determined by the - non-parametric statistical test.

**Table 1**: Median values of S100 and Langerin positive cells in the cervical epithelium from patients with CINI, CINII and CINIII and from patients without cervical alterations.

## Discussion

It is well established that LC play a central role in the process of antigen presentation [15] and specific T-cell activation in the afferent arm of cell-mediated immunity (CMI) [3]. Accordingly, a number of reports focused on the IHC evaluation of LC density from cervical HPV-infected and altered tissues have been developed, but conflicting results have somewhat been demonstrated. Several reasons have been suggested for these discrepancies [10], including differences in (i) the histological locations from where biopsies were taken; (ii) the disease stages of HPV-infected samples; (iii) the antibodies used to identify LC; most studies have employed S100 marker, while Langerin, the most specific Langerhans cell's marker [15] has rarely been used for LC evaluation; (iv) the methodological approach used to analyze the LC densities, since most studies were based on the partial analysis of selected tissue samples areas by IHC [5-14]. In the present study, a

morphometric analysis of the densities of two LC subpopulations, including Lang+ LC was performed in the entire injured epithelium area from cervical tissues obtained from patients with CIN lesions. This approach allowed us to precisely establish the number of these immune-competent cells in distinct CIN grades categories, and to obtain a better picture of the local immune response in the uterine cervix, which is a critical aspect for adoption of adequate clinical interventions such as protective vaccines [16,17].

Several studies reported a depletion and morphological alterations of Langerhans cells labeled with S100 [8,9], Langerin [10] and Cd1a - a marker of immature LC - [1,9] in low and high-grade SILs. These authors hypothesized that the low LC frequency observed in cervical samples could generate a more permissive microenvironment for the HPV initiate and establish infection within the regional tissues [10], in addition to probably inhibit an efficient activation and T-cell recruitment towards cervical mucosa [16,18]. Alternatively, a reduction in the production of cytokines (CK) which have a stimulatory effect on LC as TNF alfa and GM-CSF by HPV-infected KNCs [8,19,20], along with increased levels of immune-supressive CKs as Interleukin-10 (IL10) [21] in the cervical milieu could play a role in the depletion or the inhibition of maturation and function of Langerhans cell [22,23].

Nevertheless, our data are in agreement with those from other studies which found a significant increase in the densities of AuCl3+ [7], S100+ [12,14], Cd83+, a marker of mature cells [11] Cd1a+and Langerin+ [13] LC subpopulations in the epithelium of CIN lesions when compared with cervical tissues without alterations. Increased density of L1+ and CD68+ macrophages and DC was observed in the stroma underlying SILs compared with normal and metaplasic epithelium [8,24]. These influx of macrophages/dendrocytes cells may be related to specific stimulus such as the release of chemoattractants (as Monocyte Chemoattractant Protein-MCP1 and Macrophage Inflammatory Protein-MIP3α) due to the action of pro-inflammatory CKs as TNF-α and IFN-γ [19-23].

It has been hypothesized that, once the neoplastic lesion has developed, some kind of signals generated either by altered cervical cells or other immune-cells could trigger an increased frequency of LC in pre-neoplastic and neoplastic tissues in order to contain the carcinogenic process [16,20]. The LC mobilization and migration are probably orchestrated by a sequence of interdependent interactions between CK, chemokines and their respective receptors. In this way, some kinds of CK and chemokines existing or being produced in neoplastic tissues might exert an influence in attracting LC to the anomalous epithelium.

An ellegant study based on the isolation and culture of epithelial cells from normal and affected cervical tissues investigated the influence of the local microenvironment within the TZ (transformation zone) and SILs on the function and density of LC [13]. They showed that TZ was associated with a significant reduction in the density of immature LC (CD1a/LAG) compared to the exocervix, whereas the development of SILs was attributed with a relative increased density of immature LC, compared to the TZ. Additionally they showed that this variability in LC density was correlated with a differential expression of TNFα and MIP3α within the micro-environment of the TZ and SILs. Finally, the authors suggest that the immunosurveillance within the epithelium of the TZ may be intrinsically perturbed due to the altered expression of chemokines/cytokines and the concomitant diminished density of LC [13,19-23].

Therefore the local increase of Langerhans cells in CIN lesions which has been emphasized in several reports, and particularly observed in the present study in CINII lesions may be probably due to an intraepithelial proliferation of LC and/or a higher migration rate of LC precursors from the bone-marrow [4,15].

Recent therapy advances for treatment of cervical cancer and dysplasia have directed research towards the cellular and molecular components in the uterine cervix. Despite the high-prevalence of HPV infection, cervical carcinoma and HSIL lesions amongst Brazilian women, few studies were dedicated to evaluate the tissue frequency of Langerhans cells [14] and T-cell lymphocytes in this population, a crucial aspect for guiding immunotherapy and vaccination strategies [6,17].

## Conclusion

Our study suggests that specific LC populations may be involved with the establishment and maintenance of an effective, and possibly specific, immune response at the uterine cervix, which might be committed to the control of CIN progression at specific stages, and probably associated to the T-cells maturation, activation and recruitment to the cervical mucosal. Proper stimulation of the local immune response, using strategies such as prophylatic vaccines or treatments that stimulate local immunity at the onset of HPV infection, may contribute to the control of neoplastic progression.

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## Disclosures

The authors declare that there are no financial and/or commercial interest conflicts.

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