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# Macrophages, inflammation and risk of cervical intraepithelial neoplasia (CIN) progression—Clinicopathological correlation

Luciano S. Hammes [a,b,*], Rajeshwar Rao Tekmal [b], Paulo Naud [a], Maria Isabel Edelweiss [c], Nameer Kirma [b], Philip T. Valente [d], Kari J. Syrjänen [e], João Sabino Cunha-Filho [a]

[a] Department of Obstetrics and Gynecology, Hospital de Clinicas de Porto Alegre, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil
[b] Department of Obstetrics and Gynecology, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229-3900, USA
[c] Department of Pathology, Hospital de Clínicas de Porto Alegre, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil
[d] Department of Pathology, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229-3900, USA
[e] Department of Oncology and Radiotherapy, Turku University Hospital, Turku, Finland

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**Abstract**

*Objective.* To evaluate the population of macrophages during the cervical malignant transformation and its influence in CIN outcome.

*Methods.* Biopsies from 26 normal cervix, 28 low-grade (LSIL), 30 high grade squamous intraepithelial lesions (HSIL) and 28 squamous cell carcinomas (SCC) were stained by H&E to assess inflammation and by immunohistochemistry with anti-CD68 to detect macrophages. The macrophage count was corrected for the epithelial and stromal compartments using appropriate software. Clinical and prospective follow-up data were also available.

*Results.* We identified that macrophage count increased linearly with disease progression (median count per case at ×200 magnification: normal, 5.1; LSIL, 5.5; HSIL, 9.9; SCC, 14.5; $P<0.001$), that inflammation also increased (moderate–intense inflammation present in 25%, 46.1%, 58.4% and 89.3% of normal, LSIL, HSIL and SCC, respectively; $P<0.001$) and that macrophage count was independently associated with the lesion grade ($P<0.001$). Moreover, macrophages showed an increasing migration into the epithelium along with the progression of CIN to invasive cancer. Of the 24 LSIL cases with information available, followed-up for 805±140 days, 16 regressed, 6 persisted and 2 progressed. Age, high-risk HPV or inflammation were not risk factors for persistent/progressed LSIL in our cohort. However, LSIL that persisted or progressed showed a higher macrophage count (median of 10.8) than lesions that regressed (7; $P=0.031$).

*Conclusions.* The study on macrophages offers a potential approach for cervical cancer treatment, since macrophages are closely related to progression of CIN, and can be used as an applicable marker of such a risk.


*Keywords:* Macrophages; Monocytes; CD68 protien; Immunohistochemistry; Inflammation; Cervical intraepithelial neoplasia; Cervical cancer; Disease progression

## Introduction

Malignant tumors are complex structures that for purposes of growth and invasiveness must interact with the surrounding environment. The ultimate goal of this interaction is to promote blood supply, block negative growth signals, increase positive growth signals, create resistance to apoptosis and promote unlimited cell replication [1–3].

Convincing data has emerged in recent years that inflammation plays an important role in this process. Epidemiological studies have demonstrated that the use of anti-inflammatory drugs is associated with a reduced risk of cancer as well as with a decrease of precancer lesions. Among the inflammatory cells, tumor-associated macrophages (TAM) have been identified as important components not only in terms of their number but also with regard to their function [2,4,5].

Macrophages are derived from bone marrow monocytes that adopt different phenotypes when entering the circulatory

* Corresponding author. Department of Obstetrics and Gynecology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900, USA. Fax: +1 210 567 4958.
*E-mail address:* hammes@cpovo.net (L.S. Hammes).

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system. While in tissues, monocytes give rise to a variety of tissue dendritic cells (called Langerhans cells in the epidermis), macrophages and osteoclasts. Langerhans cells (LC) are "resident" monocytes that constitute the first immunological barrier against pathogens and environmental insults. Macrophages, stimulated by inflammation, migrate later, and because of this, these cells are also referred to as "inflammatory" monocytes [6–8].

In benign conditions, macrophages protect tissues from bacterial infections, regulate tissue remodeling and repair injuries, thereby functioning as an essential cellular component of the innate immune system. Macrophages are able to secrete a wide array of cytokines, growth factors, lysozymes, proteases, complement components, coagulation factors and prostaglandins [1,3]. However, in tumors, macrophages can exhibit a different phenotype and thus contribute to tumor growth, invasiveness, metastasis, local immunoregulation and angiogenesis [5]. In several different tumors, the accumulation of macrophages, which sometimes is the main component of the inflammatory infiltrate, is associated with worst prognosis as, for example, breast and ovary carcinoma [5].

It has been described that cervical cancer cells also express macrophage attractants, including monocyte chemoattractant protein-1 (CCL2) , macrophage colony stimulating factor-1 (CSF-1) and vascular endothelial growth facto (VEGF), not only locally but also identifiable in the peripheral blood [9–17]. For example, CSF-1 serum levels are elevated in cases with cervical human papillomavirus (HPV) infection and cervical intraepithelial neoplasia (CIN) [12]. However, data are conflicting on the role of macrophages in cervical carcinogenesis. Some authors have demonstrated that macrophages increase along with the progression of CIN to invasive cancer, but analyses regarding prognosis and correlation with HPV infection are rarely discussed [18–24]. Furthermore, other authors have described that the macrophage population decreases in CIN as compared to normal cervix, attributing this reduction to the presence of HPV E6 and E7 oncogenes [25–29].

To elucidate this issue, association of macrophages with malignant transformation of the cervix and risk of lesion progression, we conducted a careful analysis of inflammatory reaction and macrophages in samples of the normal cervix, all grades of CIN and squamous cell carcinoma (SCC), using H&E staining and immunohistochemistry (IHC) with anti-CD68 antibody.

## Materials and methods

### *Design and subjects*

Routine cervical biopsies from women participated in cervical cancer screening between 2000 and 2003 at Hospital de Clínicas de Porto Alegre, Brazil, were selected for analysis. Lesions were categorized as normal cervix, low grade squamous intraepithelial lesions (LSIL, equivalent to CIN 1), high grade squamous intraepithelial lesions (HSIL, which comprises CIN 2 and CIN 3) and SCC.

Normal cervix, LSIL and HSIL samples were selected from a cohort of patients screened for cervical cancer by conventional Pap smear, naked eye visual inspection with acetic acid (VIA) and Lugol's iodine (VILI), HPV testing for high-risk HPV and, when necessary, colposcopy and biopsy. The cohort is part of the Latin America Screening Study (LAMS Study).

Biopsies were performed with intention of diagnosis and not excision, preserving the lesions at the cervix. Patients with HSIL or higher were promptly submitted for appropriate treatment. LSIL cases were followed by visits every 6 months, when Pap smear, VIA, VILI, HPV testing (this exam every 12 months) and colposcopy were performed. For the analysis of LSIL outcome, we considered: (a) progression, when LSIL region became subsequently CIN 2, 3 or SCC at biopsy; (b) persistence, when LSIL lesion remained with the same histological diagnosis during follow-up for more than 330 days (second biopsy was necessary to confirm diagnosis); and (c) regression, when the lesion previously diagnosed was not visible any more at colposcopic examination or, even with colposcopic changes, the subsequent biopsy at the same place did not show lesion. To assure that benign histological samples were, in fact, normal, only samples that were negative for high-risk HPV were included in the normal category.

Squamous cell carcinoma samples were selected from archival paraffin blocks, derived from women who were treated during the same period in this same hospital, but not included in the LAMS cohort. All biopsies were fixed in formaldehyde and embedded in paraffin according to routine procedures. Local ethics committee approval was obtained prior to any procedure and informed consent was obtained from each subject.

### *Selection of regions for analysis*

Samples were cut at 4 μm, with the first section stained with H&E to confirm the histological diagnosis accessed by two independent pathologists, and subsequent section was stained for CD68 by IHC at the University of Texas Health Science Center at San Antonio. For each H&E slide, a mean of three representative areas of the lesion were selected at ×200 magnification (blind to IHC results), and marked for subsequent analysis on the IHC slides.

### *HPV testing*

HPV testing was carried out by the Hybrid Capture 2 assay (HCII-Digene Corporation; Gaithersburg, MD) using cervical swabs collected before biopsies. Samples were analyzed by automated HCII test system for the presence of high-risk HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68, according to manufacturer's protocol. For each specimen, RLU/CO values were calculated as the ratio of the specimen luminescence (RLU) relative to the luminescence of the 1.0 pg/ml HPV-16 cutoff standard (CO, 100,000 HPV-16 genomes/ml) and values equal to or higher than 1 RLU/CO were considered positive.

### *Immunohistochemistry*

Slides were dried at 60 °C for 20 min, de-paraffinized in xylene, re-hydrated through a graded ethanol series and then washed with distilled water. Antigen retrieval was achieved first by boiling the tissues in citrate buffer, pH 6.0, for 20 min and then by cooling the slides for 20 min. Slides were then placed in a semi-automated immunostainer (Sequenza, Thermo Electron Corporation; Waltham, MA).

IHC was carried out with the avidin–biotin complex (ABC), using LV's Ultravision® Kit (Lab Vision Corporation; Fremont, CA), at room temperature (RT), according to the manufacturer's instructions. Hydrogen Peroxide Block was applied for 12 min to quench endogenous peroxidase activity followed by Ultra V Block for 5 min to block avidin/biotin. Slides were incubated with primary antibody mouse monoclonal anti-human CD68 (Clone PG-M1; Lab Vision Corporation; Fremont, CA) diluted at 1:100 for 30 min at RT. CD68 is a trans-membrane glycoprotein that is highly expressed in human macrophages and its precursor, monocytes, and rarely by LC. For purposes of this study, macrophages and its precursors CD68 positive (CD68+) will be referred as macrophages.

Subsequently, biotinylated secondary antibody, goat anti-mouse, was applied for 30 min followed by streptavidin–peroxidase for 10 min. Immunoreactive

complexes were detected using diaminobenzidine chromogen exposure for 5 min. Finally, slides were counterstained with methyl green for 5 min, washed in distilled water, dehydrated in graded ethanol, cleared with xylene and mounted.

Negative controls were concomitantly processed by omitting the primary antibody. Because it was technically not possible to manage all slides at the same time, each IHC batch contained exactly the same proportion of normal, LSIL, HSIL, SCC, negative and positive controls slides.

To validate the CD68 staining, a subset of same samples (3 normal cervices, 3 LSIL, 3 HSIL and 3 cervical carcinoma samples) were also stained for CD14 (Clone 7; Lab Vision Corporation; Fremont, CA), which is considered a marker for inflammatory monocytes/macrophages, as well [7]. This antibody was expected to be expressed strongly on monocytes and macrophage and weakly on the surface of neutrophils. IHC procedure for CD 14 was similar to CD68, with the primary antibody diluted at 1:20, applied for 60 min at RT.

Benign hyperplastic adult tonsil were also evaluated morphologically as a positive control to determine if CD68 and CD14 stain macrophages at the germinal center of lymphoid follicle and crypts, where macrophages have been reported to be localized [30–32]. Additionally, lymphoid follicles from cervix, a known source of macrophages, were also selected for analysis to confirm CD68 antibody staining [33].

### *Interpretation*

H&E and IHC images were captured according to the areas pre-selected on H&E slides using a Nikon DXM 1200F digital camera microscope, with ×200 objective lens (Nikon; Melville, NY) and were stored and processed in a personal Windows-based PC. Each acquired image represents an area of 0.28 $mm^2$.

Intensity of stromal inflammatory reaction was scored semi-quantitatively by the density (nuclear counting) of lymphocytes and monocytes/macrophages adjacent to lesions, that comprised chronic inflammation, and were categorized as previously reported in: 0—no inflammation; 1—weak inflammation; 2—moderate inflammation; and 3—intense inflammation [34]. Granulocytes from acute inflammation, that were rarely identified in our cases, were not included in the inflammatory score.

For IHC interpretation, images were then opened in Corel Photo Paint X3 (Corel Corporation; Eden Prairie, MN), and manual marking of each CD68+ cell was performed. The use of images allowed us to zoom in specific regions and analyze details of the ×200 microscopic fields. CD68+ cells with a completely or partially visible nucleus were counted, while CD68+ debris, for example cell membrane fragments, were not counted.

Additionally, the area of the epithelial and the stromal compartment were separately measured with Corel Draw X3 (Corel Corporation; Eden Prairie, MN) and the number of macrophages was corrected proportionate to these areas, using the following formulas: (a) final number of macrophages in the stroma = (number of macrophages in the stroma × 100)/percentage of stroma in the image; and (b) final number macrophages in the epithelium = (number of macrophages in the epithelium × 100)/percentage of epithelium in the image. For the total count of macrophages, we summed up the epithelial and stromal macrophage counts and divided that by two.

### *Statistical analysis*

IHC measurements were accessed in two different ways. To demonstrate the presence of CD68+ macrophages in normal, LSIL, HSIL and SCC stages, each case was considered as a mean of all regions counted. To show the association between inflammation and macrophages and to evaluate the risk of progression, each region was considered as an individual value in the analysis.

The correlation between CD68 and CD14 staining were evaluated based on the Spearman Correlation Coefficient. Comparisons of macrophage populations in each CIN grade were evaluated by non-parametric Kruskal–Wallis test and proportions by Pearson Chi-Square. Means of variables with normal distribution were compared by Student *t*-test. In all tests, $P<0.05$ was considered statistically significant. Macrophage counts that presented non-normal distribution were shown in median (25th–75th percentiles). Values with normal distribution are presented as mean ± standard deviation (SD).

## Results

Because an association of macrophages with malignant transformation of the cervix has been suggested, we have studied macrophage infiltration by examining their number with CD68 marker in precancerous cervical lesions (LSIL and HSIL) and carcinomas as compared to normal cervical tissue. The present series comprises 112 patients, including 26 normal, 28 LSIL, 30 HSIL and 28 SCC cases. In total, 324 different regions were analyzed.

A direct relationship was found between the increasing grade of the lesion and the number of macrophages in the epithelium, in the stroma and the total macrophage counts analyzed together (Figs. 1–3).

The stromal compartment constantly showed higher macrophage counts than the epithelium. This difference decreased as the lesions progressed, indicating a macrophage switch from the stroma to the epithelium. The epithelial infiltrating macrophages represented (median[25th–75th percentile]) 0% (0–26.7%), 13.5% (0–45%), 25% (7.1%–48.3%) and 31.3% (18.3%–47.1%) of all macrophages in normal, LSIL, HSIL and SCC samples, respectively ($P<0.001$).

Intensity of the inflammatory reaction was also closely associated with the lesion grade (Table 1). For example, using the negative–weak and moderate–intense inflammation as cut-off, normal, LSIL, HSIL, and SCC regions were positive for inflammation in 25%, 46.1%, 58.4% and 89.3% of the regions analyzed, respectively ($P<0.001$).

Macrophages were consistently more frequent in moderate–intense than in negative–weak inflammatory regions for normal, LSIL, HSIL and SCC cases (Table 2). Macrophage

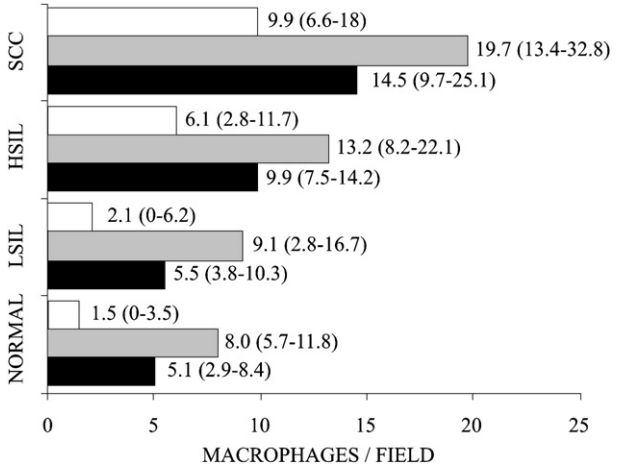


Fig. 1. Macrophage counts (per ×200 field) in epithelium (white bars), stroma (gray bars) and both compartments combined (black bars) in different lesions. The macrophage counts were not different between normal cervix and LSIL (Mann–Whitney *U* test) in the epithelium ($P=0.653$), stroma ($P=0.119$) and combined ($P=0.328$). However, in all other comparisons, macrophages counting was statistically different (Kruskal–Wallis test), both in the epithelium ($P<0.001$), stroma ($P<0.001$) and combined ($P<0.001$). Values presented in median (25th–75th percentile). LSIL—low grade squamous intraepithelial lesion; HSIL—high grade squamous intraepithelial lesion; SCC—squamous cell carcinoma.

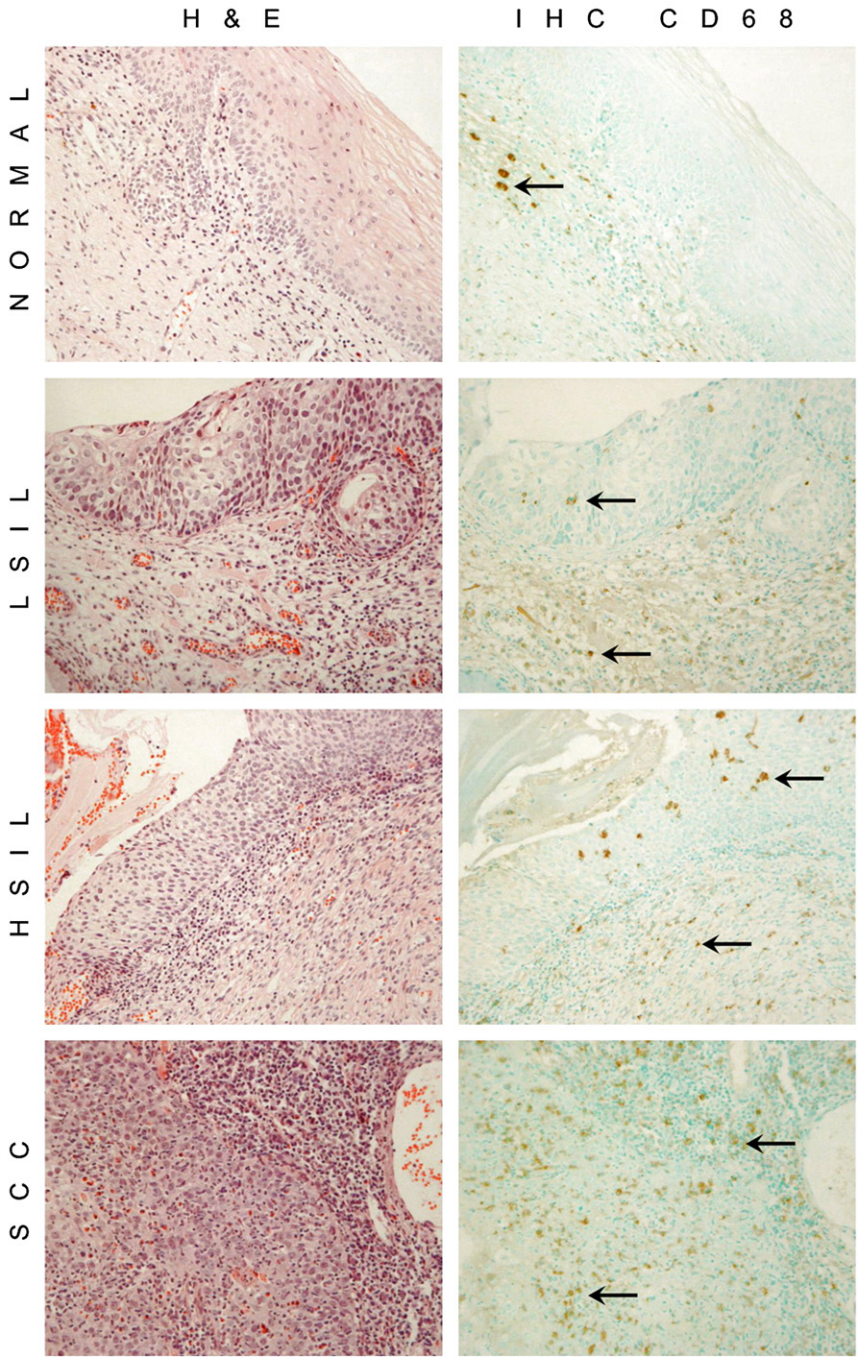


Fig. 2. Panel of H&E slides and respective immunohistochemistry staining for CD68 in normal cervix, low grade squamous intraepithelial lesion (LSIL), high grade squamous intraepithelial lesion (HSIL) and squamous cell carcinoma. Note that the presence of both macrophages (black arrows) and inflammation increase as the lesions progressed. Normal case—weak inflammatory stroma with few macrophages and absence of macrophages in the epithelium. LSIL—weak inflammation with few macrophages in the stroma and scattered macrophages in the epithelium. HSIL—moderate inflammation with the increase of macrophage population and various infiltrating the epithelium. SCC—intense inflammation and high count of macrophages in the epithelium and in the stroma (magnification ×200).

counts among negative-weak and moderate-intense inflammatory reactions were also related to lesion grade (Table 2). This finding indicates that a significant relationship between the macrophage count and the lesion grade still existed, irrespective of the density of the inflammatory reaction.

Results of high-risk HPV testing were available for 26 normal, 23 LSIL and 30 HSIL cases with 0%, 78.3% and 93.3% of positive results (Pearson Chi-Square, $p<0.001$). Interestingly, macrophage counts were not different between high-risk HPV positive and negative cases, as stratified by the lesion grade.

Even though CD68 is a well established marker of macrophages, we wanted to verify that our staining with CD68 identified macrophages. Therefore, we also examined the expression of CD14 macrophage marker in a subset of 12 cervical samples and found that both markers presented a strong

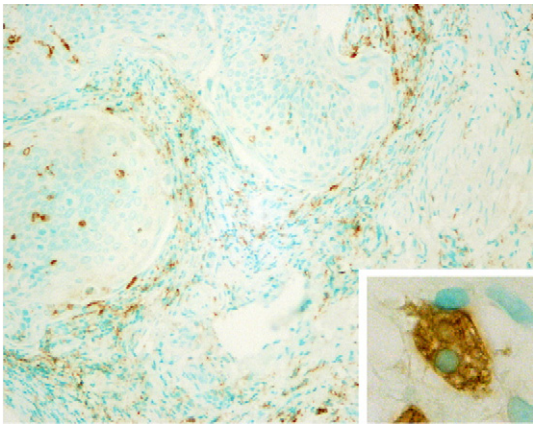

Fig. 3. Immunohistochemistry staining for CD68 in invasive squamous cell carcinoma (SCC). Intense macrophage infiltration is evidenced in stroma surrounding epithelium of SCC (magnification ×200). Inset: macrophage morphology, with intense cytoplasmatic staining, microvacuolization and small cytoplasmatic projection.

correlation (Spearman Correlation Coefficient = 0.84; $P < 0.001$). CD68 antibody clearly identified macrophages in our study, with a intense cytoplasm staining, whereas CD14 presented a surface and cytoplasmatic moderate staining. Considering all cases together, the number of cells identified by CD68 per ×200 field in epithelium, (median [25th–75th percentile]), 6.5 (0.25–15.75), and stroma, 19.5 (8.00–27.75), was slightly lower than the number marked by CD14, 7.5 (1.25–25) and 20 (13.00–39.00), respectively, but this difference was not statistically significant (Mann–Whitney *U* test; $P = 0.381$ and $P = 0.335$) (Fig. 4).

The morphological analysis of tonsil (positive control) identified that macrophages CD68+ were inhomogeneously distributed within the tonsilar tissue, with a predominance in the germinal center of lymphoid follicle and crypts, similar to CD14 marker (Fig. 4). Additionally, macrophages morphologically identified by H&E in cervical lymphoid follicle were also stained by CD68 antibody, indicating that the marker correctly identified the cells (Fig. 5).

Finally, we examined if a correlation exists between macrophage infiltration and clinical outcome (persistence-progression) of LSIL, based on follow-up data of 24 LSIL patients (mean age 32.9 ± 7.5 years old), followed-up for 805 ± 140 days. A total of 16 patients (66.7%) had their lesions regressed during follow-up, 6 (25%) patients persisted (for a mean of 512 ± 140 days) and 2 (8.3%) progressed to HSIL/carcinoma. In a linear regression model, patient's age, HPV status or inflammation were not risk factors for persistence/progression, but the total macrophage count in LSIL lesions that regressed was lower than in persistent-progressed lesions (Table 3).

To confirm that our results, which showed an increase of macrophage population according to lesion grade, were not by chance, a power analysis was repeated indicating 93.7% of statistical power (Pβ).

Table 1
Inflammation as related to cervical lesion grade

| Lesion grade | Intensity of inflammation | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Negative | | Weak | | Moderate | | Intense | |
| | N | % | N | % | N | % | N | % |
| Normal | 18 | 23.7 | 39 | 51.3 | 14 | 18.4 | 5 | 6.6 |
| LSIL | 7 | 9.3 | 33 | 44 | 16 | 21.3 | 19 | 25.3 |
| HSIL | 8 | 9 | 29 | 32.6 | 32 | 36 | 20 | 22.5 |
| SCC | 0 | 0 | 9 | 10.7 | 27 | 32.1 | 48 | 57.1 |
| Total ($n = 324$) | 33 | 10.2 | 110 | 34.0 | 89 | 27.5 | 92 | 28.4 |

Pearson Chi-Square, $P < 0.001$; N, number of regions analyzed; LSIL, low grade squamous intraepithelial lesion; HSIL, high grade squamous intraepithelial lesion; SCC, squamous cell carcinoma.

Table 2
Inflammation and total number of macrophages in different lesions

| Lesion grade | Total number of macrophages[a] | | | | $P$[b] |
|---|---|---|---|---|---|
| | negative–weak inflammation | | Moderate-intense inflammation | | |
| Normal | 4.00 | (2.04–6.46) | 6.90 | (3–11.25) | 0.041 |
| LSIL | 4.58 | (2–7.38) | 7.78 | (5–16.25) | 0.001 |
| HSIL | 8.00 | (5–12.69) | 12.05 | (8.14–16.56) | 0.003 |
| SCC | 8.75 | (5.46–12.29) | 16.27 | (8.75–24.96) | 0.044 |
| $P$[c] | <0.001 | | <0.001 | | |

LSIL, low grade squamous intraepithelial lesion; HSIL, high grade squamous intraepithelial lesion; SCC, squamous cell carcinoma.

[a] Per field magnification ×200, values in median (25th–75th percentile).

[b] Mann–Whitney *U* test for comparison of macrophage count between negative-weak and moderate-intense inflammation.

[c] Kruskal–Wallis test for comparison of macrophage count between different lesion grades, controlling for inflammation.

## Discussion

Our study clearly demonstrates a strong association between the malignant transformation of the cervix and an increase in the number of tumor-associated macrophages in the stroma as well as in the epithelium. Besides the increase of macrophages upon lesion progression, we detected also an augment of macrophage population in the epithelium proportionally to stroma, suggesting that there is an influx of macrophages into the epithelium or local proliferation.

Additionally, the inflammatory response was more evident in severe lesions than in normal cervix. Given that macrophages and inflammation increased in parallel with the progressive lesions, we suspect that the inflammation attracted macrophages, as could be anticipated physiologically. Indeed, the overall macrophage population was positively influenced by the degree of inflammation in all lesion categories. For example, in LSIL cases, the macrophages in moderate-intense inflammatory regions were almost two times more numerous than in negative-weak reactions. However, when we analyzed macrophages according to the lesion grade and controlled for inflammation (i.e., using the negative–weak and moderate–intense cut-offs), we still found a significant positive correlation between macrophage counts and lesion grade. This argues strongly that the inflammatory environment not only attracts macrophages but that the lesion also itself attracts and influence the proliferation of these cells.

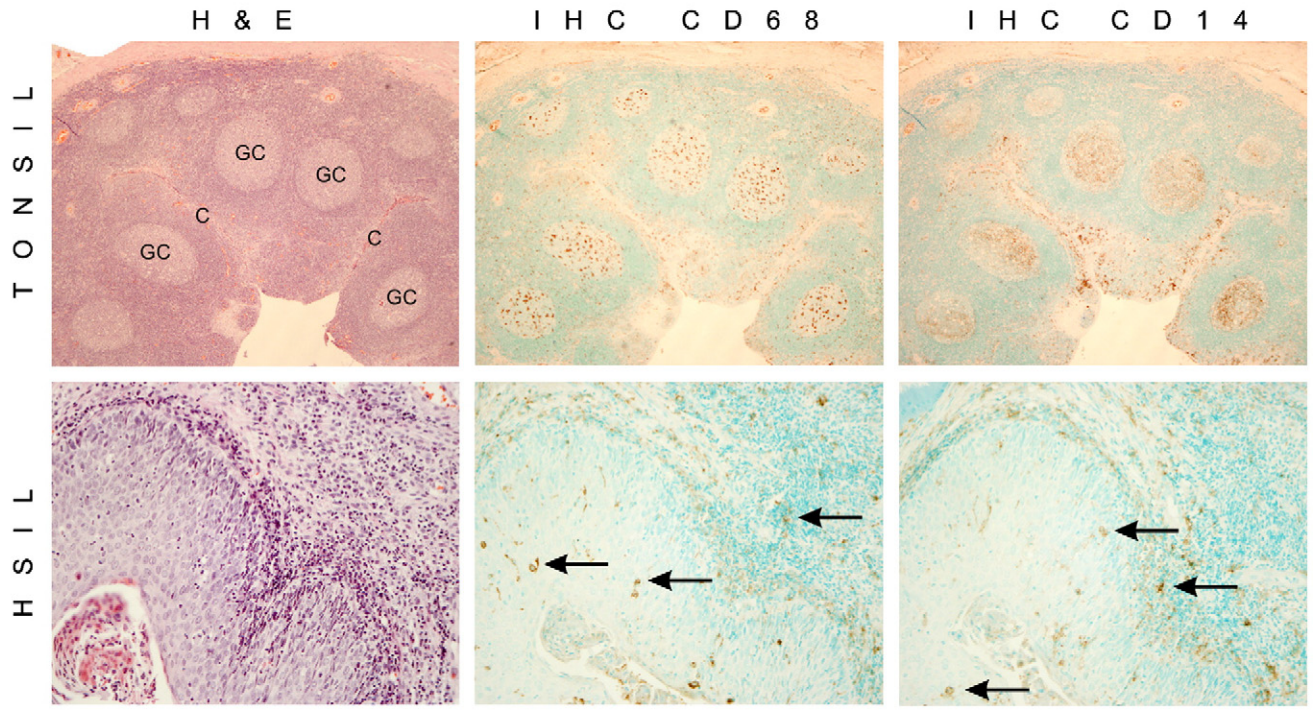


Fig. 4. Panel of H&E slides and respective immunohistochemistry staining for CD68 and CD14 in benign adult tonsil and high grade squamous intraepithelial lesion (HSIL). In tonsil, macrophages were identified predominately at germinal center of lymphoid follicle (GC) and crypts (C) similarly by CD18 and CD14 antibody (magnification ×40). Note that CD68 staining identified more clearly individual cells than CD14. In HSIL, the distribution of cells identified by CD68 and CD14 (black arrows) was similar (magnification ×200).

As related with patients' clinicopathological data, macrophage infiltration was higher in low-grade lesions which persisted-progressed than in those that did not persist-progress. Because of our limited sample size, we were not able to include a multivariate model of risk. However, individual risk factors, such as age and HPV status did not reach statistical significance in our analysis. By contrast, the macrophage count was statistically significant, thus indicating that an increased number of macrophages may be an important risk factor for lesion progression. However, in view of the small number of cases, this finding should be interpreted with caution. Further studies with larger and prospective cases are required to verify the macrophage infiltration as a prognostic marker in intraepithelial lesions.

Many studies on cervical cancer have identified a significant increase of macrophages according to the severity of cervical lesions, including cancer precursors, but few studies have addressed clinical correlations [18–24,35]. In one of the rare studies about prognosis, macrophages were a significant source of interleukin-8 that was closely associated with micro-vessel density and poor prognosis in invasive disease [20]. To our knowledge, our description, where lesions were carefully identified, epithelium and stroma areas were measured separately and data were compared to clinical outcome, is the first positive correlation of macrophage count and cervical intraepithelial lesion persistence-progression.

However, some authors have argued the exact opposite, i.e., macrophage density decreases with disease progression [25–27,36]. One explanation for this discrepancy is that most of the time, CIN lesions are restricted to a very specific area of the epithelium and a correct selection of this area is required to assure consistent results. Moreover, it is necessary to pay close attention to the markers used for macrophages. Most studies that refer to a decrease in macrophage population, actually point to a decrease in Langerhans cells that are the resident-monocytes, highly specialized in antigen-presenting and constitute the first immunological barrier against the external environment [24,37–39]. Langerhans cells are generally stained with an antibody against S-100 protein or CD1a, whereas macrophages are defined by antigens like CD68 and Ki-M1p. Indeed, the studies using the latter constantly report an increase of macrophages towards high-grade lesions and cancer [18,19,21,23,24,35]. As determined from the published literature, Langerhans cells are initially suppressed by the tumor to permit an initial growth, but, as suggested by our results, macrophages are subsequently recruited to support the progression to invasive malignancy.

In the recent literature, the majority of the studies reported an increase of macrophages in human tumors to be related to poor prognosis. Such lesions include cancer of the breast, prostate, ovary, uterine cervix, lung and bladder. Most of these studies also reported a positive correlation between macrophages and tumor angiogenesis [5].

Some of the main macrophage chemoattractants are products of dysplastic cervical cells or derived from the surrounding stroma. CSF-1, also known as macrophage-colony stimulating factor (M-CSF), which is responsible for promoting differentiation, proliferation and activation of mononuclear phagocytes,

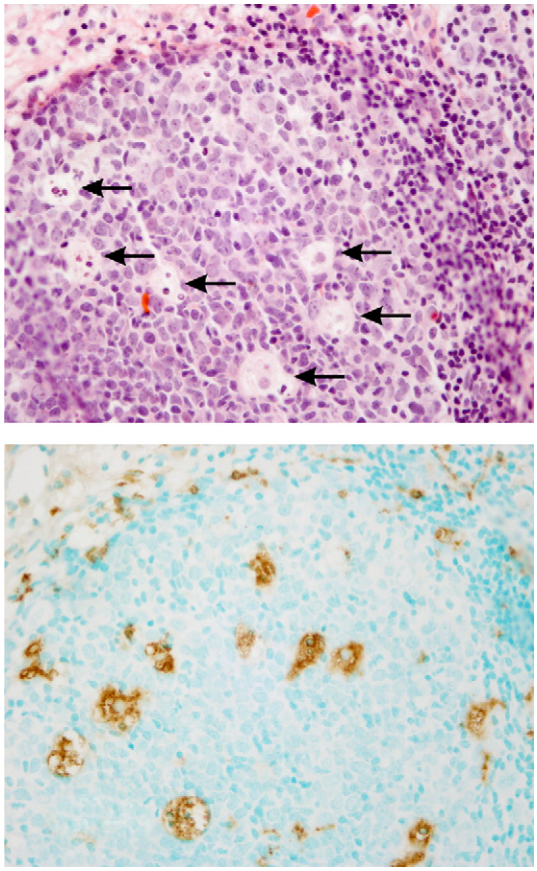

Fig. 5. H&E and respective CD68 immunohistochemistry staining of a lymphoid follicle at normal cervix. Macrophages identified morphologically at H&E slide (black arrows) stained positively for CD68. (magnification ×400).

has been identified at high concentrations in the peritoneal fluid and serum of patients with cervical cancer [15]. A single infection by HPV has been associated also with an increase of CSF-1 serum levels [12]. In vitro studies with immortalized cervico-vaginal cell lines have demonstrated that epithelial cells in the lower female genital tract also participate in the immunological function producing CSF-1 [14]. Another example of chemoattractant produced by cervical tumoral cells is VEGF, a potent pro-angiogenic cytokine, largely found in cervical cancer and its precursor lesions [10,11,16,17].

The same cytokines that attract macrophages, such as interleukin-4 (IL-4), IL-10, transforming growth factor β-1 (TGF β-1) and prostaglandin E2 (PGE2), can be responsible for changing the macrophage phenotype to become a pro-tumor agent [2,5]. Once activated by tumors, macrophages lose their natural phenotype, resulting in poor antigen presenting capability, suppressing T-cell proliferation, promoting higher angiogenesis and increasing their capability to remodel tissues, thereby allowing tumor cell invasion and metastasis [3]. As an example how the tumor might suppress macrophage activity are experiments where macrophages collected from the patients, activated ex vivo and then re-infused as autologous therapies, are not effective as anti-cancer therapy [5].

In in situ tumor stages (e.g. ductal carcinoma in situ in the breast) macrophages can be found close to the points of basal membrane disruption, suggesting that their proteases are required to facilitate the invasion of tumor cells [3]. Growth factors, such as epidermal growth factor, are also released by macrophages, acting directly upon the surrounding tumor cells. In addition, macrophages can secrete angiogenic factors, such as VEGF and angiogenin that supplement tumor production, thereby enhancing the neovascularization [40]. Finally, macrophages are capable of producing more chemoattractant proteins that, in turn, perpetuate their migration in an infinite feedback loop.

Because of the close relationship between macrophages and tumors, macrophages have been considered an attractive target for therapies. Macrophages have a stable genome, with rare mutations, and therefore with fewer chances of developing drug resistance. Blockage of some steps of macrophage–tumor interaction may promote tumor growth control. In fact, removal of macrophages in a mice breast carcinoma model, by null mutation of CSF-1, reduced markedly the rate of tumor growth and metastasis [41]. Another interesting approach in macrophage therapy is by transfecting macrophages ex vivo with genes encoding for anti-cancer agents, as anti-angiogenic factors, and reinserting them in the tumors. Initial studies have demonstrated the ability of macrophages to migrate into hypoxic areas of tumor mass and effectively deliver the manipulated gene products [2].

In conclusion, our study indicates that CD68+ macrophages are associated with cervical carcinogenesis from intraepithelial lesions to invasive stages. Macrophages increase linearly with the progression of CIN, migrating from the stroma into the epithelium, and are influenced not only by inflammation itself but also by the dysplastic (transformed) cells. Additionally, our data suggest that macrophages can be a risk factor for persistence-progression of LSIL. However, because our sample size was small, further studies with larger prospective data are necessary to demonstrate this prognostic importance. Taken together, these results suggest that macrophages might offer a novel approach for cervical cancer treatment, and in upcoming trials, macrophages should be investigated as a potential marker of disease progression.

Table 3
Risk of LSIL progression according to age, HPV status, inflammation and total number of macrophages

| Risk factor | LSIL | | *P* |
|---|---|---|---|
| | Regression (*N*=16 cases; 42 regions) | Persistence-Progression (*N*=8 cases; 22 regions) | |
| Age (years; mean±SD) | 30.5±7.9 | 34.1±7.2 | 0.599 [a] |
| High-risk HPV test (positive percentage) | 62.5% | 80% | 0.363 [b] |
| Inflammation moderate-intense | 47.6% | 40.9% | 0.609 [b] |
| Macrophages (median; 25th–75th percentile) | 7 (2.7–9.1) | 10.8 (5–16.2) | 0.031 [c] |

[a] Student *t*-test.
[b] Pearson Chi-Square test.
[c] Mann–Whitney *U* test.

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