# Characteristics of Lymphocytic Inflammatory Infiltrate in Basal Cell Carcinoma of the Oral and Maxillofacial Area

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## Abstract

**Background**. Basal cell carcinoma (BCC) is a very frequent skin malignancy, with slow evolution and sparse metastases. Host tissues react to tumor invasion through complex inflammatory response, comprising varied inflammatory cells.

**Materials and methods**. The expression of lymphocytes was assessed in 27 archived formalin-fixed paraffin-embedded tissue samples of BCC from the oral and maxillofacial areas by means of haematoxylin and eosin standard stain and immunohistochemical (IHC) method. IHC was done for UCHL1, CD4, CD8 and L26 markers, using an indirect bistadial technique with hydrosoluble polymerized dextran, according to manufacturer specifications (Dako EnVision Systems, Glostrup, Denmark). The cases with uncertain staining or scant tumor area were eliminated.

**Results**. Lymphocytes were found in great numbers in BCC stroma, displaying nodular, linear or scattered disposition within tumor stroma. UCHL1 was positive in T-lymphocytes in all cases (n = 27) 100%. The positivity for CD4 in T helper (Th) lymphocytes was 62.96%, (17/27 cases) for CD8 in cytotoxic T-lymphocytes (CTL) – 92.59% (25/27 cases) and for L26 in B-lymphocytes – 76% (19/25 cases). We found a statistically significant negative correlation between Th/CD4+ and CTL/CD8+ lymphocytes ($r = - 0.54$, $p = 0.004$).

**Conclusions**. The results of our research demonstrate the presence of abundant lymphocytic inflammatory infiltrate in BCC stroma. The predominance of T cells over B cells suggests the extensiveness of the cell-mediated immune response, rather than a humoral immune reaction. Cytotoxic T-lymphocytes were prevalent as against Th lymphocytes, which evidence that the immune mechanism prevalent in BCC is of a cytotoxic type.

**Keywords***: basal cell carcinoma, lymphocytes, UCHL1, CD4, CD8, L26.*

## Introduction

Basal cell carcinoma (BCC) is the most frequent skin cancer, arising from the basal cells of the epidermis and pilosebaceous units. It is most often located in sun-exposed skin, especially the face and neck, has slow growth rate and local destructive potential. The clinical and microscopical features are varied. The histological appearance includes undifferentiated (nodular and infiltrative subtypes) and differentiated forms. The differentiation is slight and tends towards the cutaneous appendages of hair (*keratotic* BCC), sebaceous glands (BCC with *sebaceous differentiation*), and apocrine/eccrine glands (*adenoid* BCC). Many BCCs display both undifferentiated and differentiated areas. There is no difference in the rate of growth between the two groups of BCCs. Other microscopic types are: morpheaform (sclerodermiform), fibroepithelioma, superficial, adamantinoid, granular, clear-cell and with matricial differentiation (1,2,3).

Certain anatomic locations and histopathological subtypes may trigger deep invasion of tissue structures, with progression toward vital organs. This situation is detected in BCCs in the vicinity of facial cavities (mouth, nose, ear, eyes) and in deeply penetrating BCC subtypes, such as infiltrative, morpheaform, micronodular or their combinations (4).

Host tissues react to the tumoral invasion by various mechanisms, such as an intense inflammatory reaction, comprising dense lymphocytic infiltrates, mast cells, dendritic cells, plasmocytes, neutrophils, eosinophils, etc.

**Lymphocytes** are one of the five kinds of white blood cells (or leukocytes), circulating in the blood. The most abundant lymphocytes are B-lymphocytes (often simply called **B cells**) and T-lymphocytes (**T cells**). Each B cell and T cell is *specific* for a particular *antigen*, meaning that each is able to *bind to* a particular molecular structure. The specificity of binding resides in a *receptor* for antigen: the B cell receptor (BCR) for antigen and the T cell receptor (TCR) respectively (5). T cells take part both in the cell-mediated immunity and antibody-mediated immunity, while B cells are involved in the antibody-mediated immunity (5).

**CD8** (cluster of differentiation 8) is a transmembrane glycoprotein that serves as a co-receptor for the T cell receptor (TCR). The CD8 co-receptor is predominantly expressed on the surface of cytotoxic T cells, but can also be found on natural killer cells.

**Cytotoxic T-lymphocytes (CTLs)** are defense cells that recognize and destroy infected cells and tumor cells. Most of these CTLs will die (of apoptosis) when they have done their job, but some (those that have received "help" from helper T cells) will become *memory cells*: long-lived cells poised to respond to the antigen if it should reappear.

**CD4** (cluster of differentiation 4) is a glycoprotein expressed on the surface of T helper cells, regulatory T cells, monocytes, macrophages, and dendritic cells. CD4 is a co-receptor that assists the T cell receptor (TCR) to activate its T cell following an interaction with an antigen-presenting cell.

**Helper T cells (Th)** are T lymphocytes that belong to the **$CD4^+$ subset**. When they are presented with both an antigen and appropriate cytokines, they begin to proliferate and become activated (5).

**UCHL1** (ubiquitin carboxyl-terminal esterase L1 – ubiquitin thiolesterase) is a murine monoclonal antibody that recognizes a 180-185 kDa determinant on CD4 (72%) and CD8 (36%) positive T cells. This antibody is effective in formalin fixed and paraffin embedded tissues, using the immunoperoxidase method. It marks the membrane of T-lymphocytes (6).

The **CD20** antigen is a membrane-embedded, non-glycosylated phosphoprotein involved in the regulation of B-cell activation, proliferation and differentiation. Surface expression of CD20 on activated B cells is approximately 4-fold greater than that found on resting B cells (7,8). The monoclonal antibody **L26** recognizes a membrane epitope of CD20.

**The aim** of the study was to assess the expression and distribution of lymphocytes in skin basal cell carcinoma, using the immunohistochemical markers UCHL1, CD4, CD8 and L26.

## Materials and methods

Twenty-seven archived formalin-fixed, paraffin-embedded tissue samples of BCC, belonging to the Department of Pathology of Constanta Polyclinic no. 2, were randomly selected for the histopathological analysis, using the standard haematoxylin and eosin stain. Tumor biopsies derived from the oral and maxillofacial region of 27 patients, who had undergone surgery in the Constanţa Oral and Maxillofacial Surgery Clinic for tumor excision during 5 years of practice (2000-2005). Samples were taken after informed consent, using a protocol approved by the local Bioethics Committee, in accordance to generally accepted international practice.

The BCC samples were afterwards assessed by immunohistochemical analysis (IHC) at „Victor Babeş" National Institute of Pathology, Bucharest. The immuno-detection of antigens is a multi-step process involving first, the binding of an antibody to the antigen of interest, and second, the detection and visualization of the bound antibody. This is accomplished by using an enzyme that catalyzes the deposition of a reddish-brown marker at the site of the antigen of interest.

IHC was done for UCHL1, CD4, CD8 and L26 markers, using an indirect bistadial technique with hydrosoluble polymerized dextran, according to manufacturer's specifications (Dako EnVision Systems, Glostrup, Denmark). All specimens were counterstained with Mayer's haematoxylin, examined and photographed on a Nikon Eclipse 600 microscope.

Negative controls used a primary irrelevant mouse antibody on normal skin or replacement of the secondary antibody with phosphate buffered-saline (PBS). Positive controls consisted of examining the expression of antibody in the peritumoral cutaneous tissue (positive internal control on slides). Also, to ensure immunohistochemical validity, an internal quality control was carried out, according to a quality guarantee certificate system (ISO 9001/2008). The antibodies used in the study are shown in *Table 1*.

**Table 1**. Antibodies used in the IHC study

| Antibody | Producer | Dilution | Clone | Specificity |
|---|---|---|---|---|
| **UCHL1** | Dako (Glostrup, Denmark) | 1:50 | UCHL1 | T-lymphocytes * |
| **CD4** | Neomarkers (Fremont, CA, USA) | 1:25 | OPD4 | T helper lymphocytes * |
| **CD8** | Neomarkers (Fremont, CA, USA) | 1:50 | 144B | Cytotoxic T-lymphocytes * |
| **L26/CD20** | BioGenex (San Ramon, CA, USA) | 1:50 | L26 | B-lymphocytes * |

* membrane staining

The distribution of UCHL1, CD4, CD8 and L26 positivity in BCC samples was assessed by a qualitative method, using the modified Quick score method (9), which takes into account the intensity and distribution of positivity: negative (no staining) = 0; weak (only visible at high magnification) = 1; moderate (readily visible at low magnification) = 2; strong (strikingly positive at low magnification) = 3. The cases in which the staining was uncertain or the tumor area needed for assessment was scant, were eliminated. Data were statistically analyzed using the t-Student parametrical test, "paired two sample for means, one group two-tails" from Analysis Tool Pak of Microsoft Excel 2007. A p-value $< 0.05$ was considered statistically significant.

## Results

### 1. T-lymphocytes in BCC

The assessment of T-lymphocytes showing positivity to the UCHL1 marker was made on 27 BCC cases. The inflammatory infiltrate showed varied disposition, either scattered within all tumor stroma (*Figure 1*), or follicle-like or linear (*Figure 2*), at the periphery of tumor islands (*Table 2*). Notably in the nodular and linear dispositions there was a tendecy for the lymphocytes to penetrate the tumor masses.

Regarding the intensity of the inflammatory process, all BCC cases studied were found positive to UCHL1; the majority (17 out of 27) showed an abundant (score 3) lymphocytic infiltrate, 8 cases registered a moderate score (2), only two cases showing a reduced inflammation (score 1), *Figure 3*.

**Figure 1**. IHC positive reaction to UCHL1 in scattered T-lymphocytes, 20x

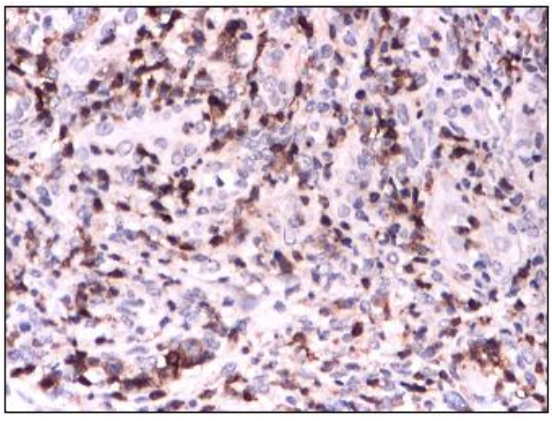


**Figure 2**. IHC positive reaction to UCHL1 in T-lymphocytes, with nodular disposition, 10x

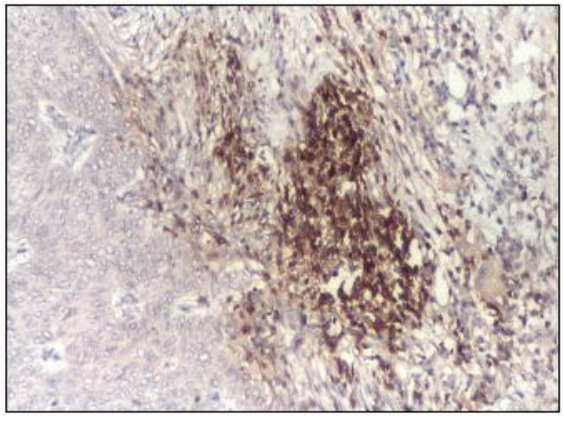


**Figure 3.**

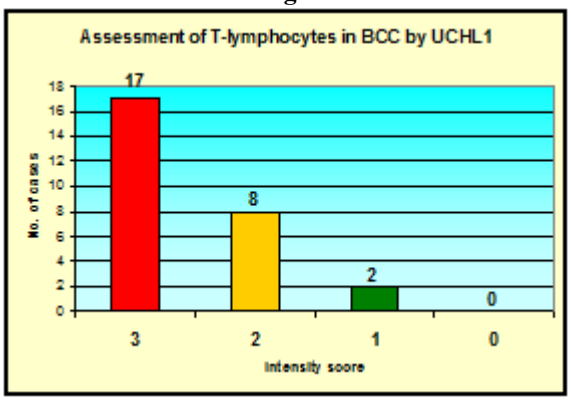



**Table 2**. Disposition of T-lymphocytes in BCC

| **Disposition** | Scattered | Nodular (follicle-like) | Linear |
|---|---|---|---|
| **Cases/Total** | 19/27 | 8/27 | 3/27 |

**Note**: the values are not cumulative. In the same case, we occasionally found two different disposition modalities of the T-lymphocytes.

## 2. T helper lymphocytes in BCC

Th lymphocytes positivity to CD4 (*Figure 4*) was low in most cases, indicating a reduced presence of these cells within the lymphocytic inflammatory infiltrate. Intensity scores were moderate (7 cases), weak (10 cases) and negative (10 cases). We did not record any strong positive reaction (*Figure 5*).

**Figure 4**. IHC positive reaction to CD4 in Th lymphocytes, 20x

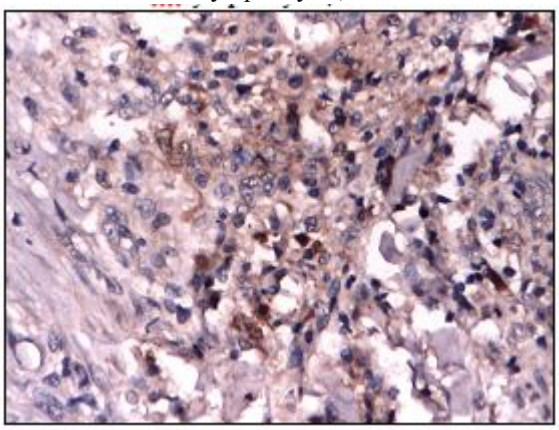
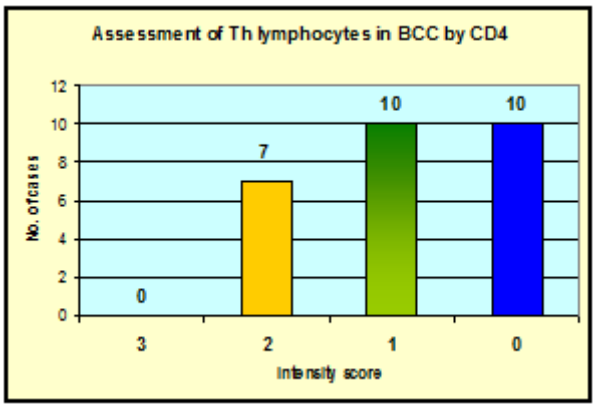


**Figure 5.**




## 3. Cytotoxic T-lymphocytes in BCC

The assessment of CTLs (*Figures 6 and 7*) shows totally different results from those displayed by Th lymphocytes. Thus, 25 cases out of 27 (92,59%) were positive to CD8. Seven cases registered high intensity scores (3), eight cases – a moderate score (2) and 10 cases – a weak positive reaction (score 1). Only two cases were negative to CD4 (score 0), *Figure 8*.

**Figure 6**. IHC positive reaction to CD8 in CTL with nodular disposition, 20x

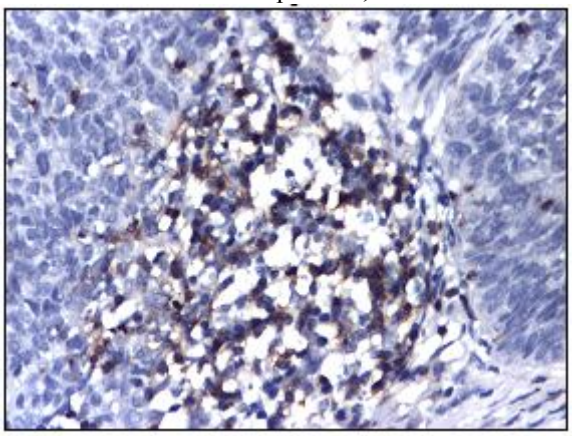


**Figure 7**. IHC positive reaction to CD8 in CTL, 60x

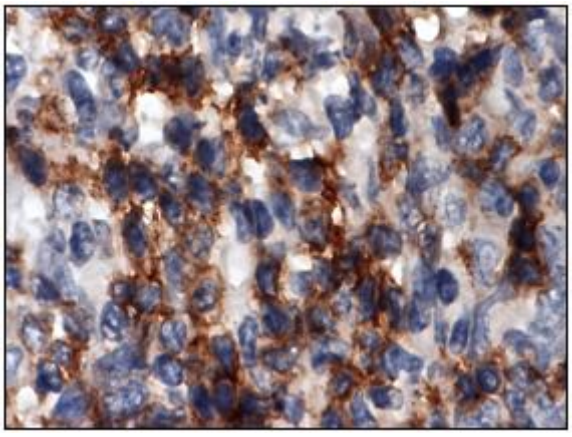
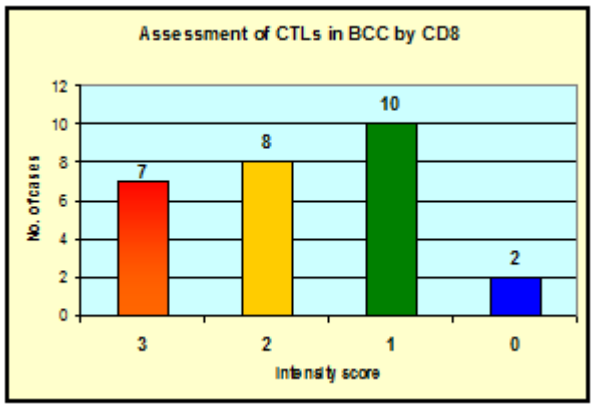


**Figure 8**.




### 4. B-lymphocytes in BCC

Immunohistochemical assessment of B-lymphocytes was carried out on 25 BCC cases. Nineteen cases (76%) were positive to L26, in seven of which the cells' disposition was nodular and in the remaining 12 was scattered (*Figures 9, 10 and 11*).

The intensity of the IHC reaction to L26 was moderate in 5 cases and weak in 12 cases. Six cases were negative and only two were strongly positive (*Figure 12*).

**Figure 9**. IHC positive reaction to L26 in B-lymphocytes, 60x

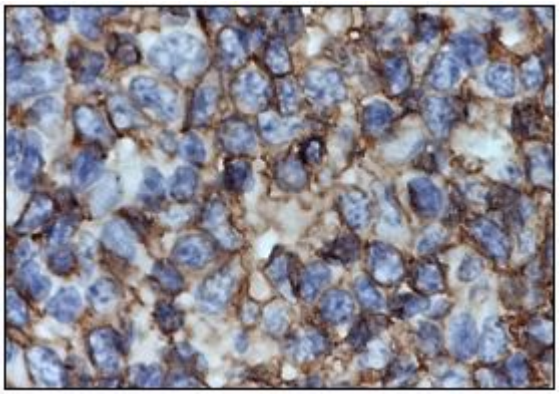


**Figure 10**. IHC positive reaction to L26 in B-lymphocytes with nodular disposition, 20x

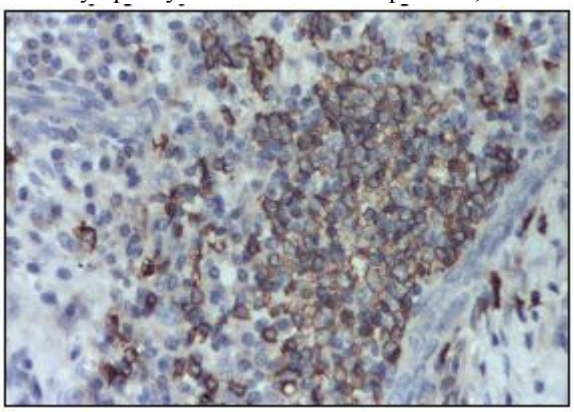


**Figure 11**. IHC positive reaction to L26 in B-lymphocytes, 20x

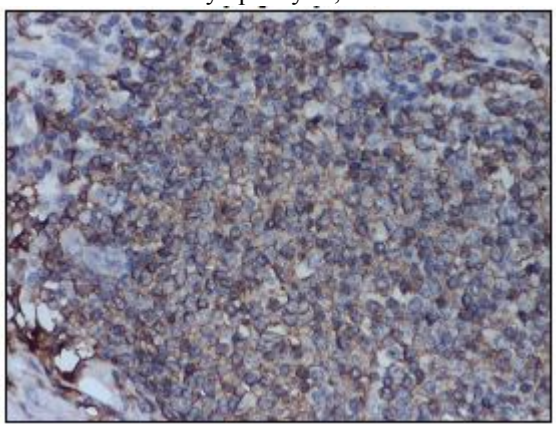
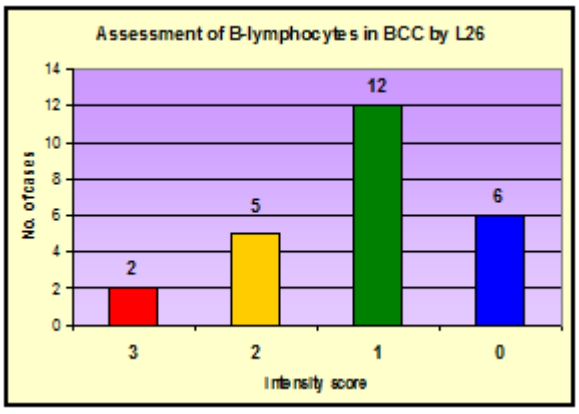


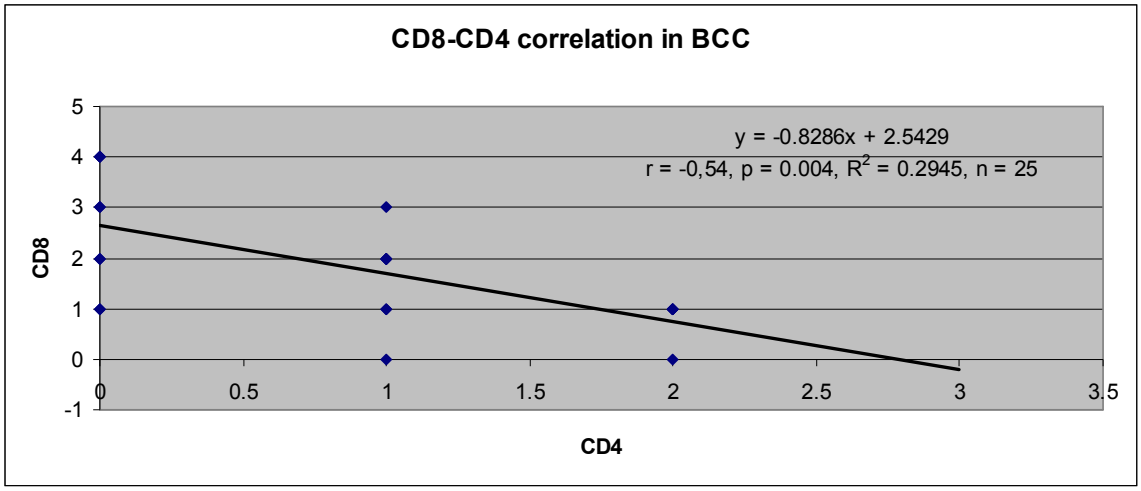
**Figure 12**.




We found a statistically significant difference ($p < 0.001$) between the population of T-lymphocytes, assessed by UCHL1 marker and that of B-lymphocytes, assessed by L26. The T-lymphocyte infiltrate was four times more abundant than that of the B-lymphocytes.

In surveying the T-lymphocytes population, we found a statistically significant (p = 0.004) and moderate negative correlation (r = -0.54) between Th/CD4+ and CTL/CD8+ lymphocytes, *Figure 13*.

**Figure 13.**




## Discussion

The results obtained from our study revealed the existence of an abundant inflammatory infiltrate, with T-lymphocytes predominating over B-lymphocytes. This finding is also supported by other research studies (10,11,12,13) and might suggest that the mononuclear infiltrate surrounding the BCC lesions is the expression of a cell-mediated response, rather than antibody-mediated immunity.

The positioning of T cells in the nearmost vicinity to neoplastic cells suggests that they might be effector cells (14,15). This supposition is based on previous studies stating that activated T-lymphocytes were frequently located nearby tumor lesions (12,14,15). Furthermore, the epithelial cells situated at the periphery of tumor islands reacted to growth-stimulating antigens secreted by T-lymphocytes. It was thus assumed that the peripherally situated intraepithelial T cells might be playing a role in regulating the proliferative activity of tumor cells.

Although B cells have been rarely observed in BCC as compared to T cells, their presence suggests a possible role in the immune response while their location in the proximity of tumor islands could be responsible for regulating the proliferation of tumor cells and might explain the localized nature and slow growth of BCC (16).

Our research showed that, in less than one third of cases, both T and B-lymphocytes had nodular distribution. The identification of lymphoid follicles, comprising B cells in the middle and T cells at the periphery might indicate an immune reaction partly organized against tumor antigens (11,17).

*Sanaa Saleh et al*. (10) have investigated the presence of T and B-lymphocytes in a batch of 20 BCCs from the head and neck areas, by immunohistochemical methods. All BCC cases displayed a positive reaction to both IHC markers, the positivity being assessed by a semiquantitative method. The authors discovered that the dense lymphocytic infiltrate around tumor cells consisted mainly of T-lymphocytes. These were prevalent in most cases (85%), while B cells were sparse in many cases (70%). T cells were mainly found as irregularly dispersed aggregates within the stroma, some being situated very close to the BCC lesion. Frequently, some T cells were even detected inside the tumor masses. B-lymphocytes displayed a similar disposition, as small aggregates within the stromal connective tissue, and even at the tumor margin or between the neoplastic cells. T cells consistently occupied the peripheral position within the inflammatory follicles, and B cells, the central position.

In our study, we found increased immunostaining for CTLs, as compared to Th lymphocytes. These findings differ from those in the literature and might signify that the destruction of BCC neoplastic cells is mostly carried out through a cytotoxic mechanism.

In contrast, *Synkowski DR et al*. (18) assessed the inflammatory infiltrate around 32 BCCs by IHC and semiquantitative assay. They found high intensity scores for T-lymphocytes (3-4) and B (over 3). The Th/CTL ratio was almost unitary. In their study, *Deng et al*. (19), noticed that the majority of T cells in the inflammatory infiltrate belong to the CD4+ subset; CD8+ cells were present in the peritumoral area but in reduced numbers. Similar findings were reported by *Aoki et al*. (20), who observed that CD4+ T cells represented the majority of BCC lymphocytic infiltrate.

## Conclusions

1. IHC markers used to assess lymphocytes showed positive reactions in the majority of cases. Thus, the positivity to UCHL1 in T-lymphocytes was 100%, for CD4 in Th lymphocytes it was 62.96%, for CD8 in CTL it was 92.59% and for L26 in B-lymphocytes it was 76%.

2. We found a statistically significant difference ($p < 0.001$) between the population of T-lymphocytes and that of B-lymphocytes. The two inflammatory infiltrates are independent statistically, the T-lymphocytes infiltrate being four times more abundant than that of B-lymphocytes. This finding might suggest the extensiveness of the cell-mediated immune response rather than a humoral immune reaction.

3. Cytotoxic T-lymphocytes were prevalent compared to Th lymphocytes, which suggest that the immune mechanism prevalent in BCC might be of the cytotoxic type.

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