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Research Article

Characterization of the Tumor-Associated Immune Infiltrate in Acral Lentiginous Melanoma

Castaneda CA[1,2]*, Castillo M[1], Torres-Cabala C[3], Bernabe LA[1], Villegas V[1], Casavilca S[4], Sanchez J[1], Dunstan J[6], Calderon G[6], De La Cruz M[6], Cotrina JM[6], Gomez HL[5], Mejia O[1], Villa-Robles MR[4], Zevallos R[4] and Abugattas J[6]

[1]Department of Research, Instituto Nacional de Enfermedades Neoplasicas, Peru

[2]Universidad Privada San Juan Bautista, Peru

[3]Departments of Pathology and Dermatology, University of Texas MD Anderson Cancer Center, USA

[4]Department of Pathology, Instituto Nacional de Enfermedades Neoplasicas, Peru

[5]Department of Medical Oncology, Instituto Nacional de Enfermedades Neoplasicas, Peru

[6]Department of Breast Cancer Surgery, Instituto Nacional de Enfermedades Neoplasicas, Peru

***Corresponding author:** Castaneda CA, Research Department, Instituto Nacional de Enfermedades Neoplasicas, Faculty of Medicine, Universidad Privada San Juan Bautista, Peru

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Abstract

Purpose: This study aims to evaluate the composition of tumor infiltrating lymphocytes in acrallentiginous melanoma (ALM).

Materials and Methods: A cohort of 43 ALM who underwent surgery at the Instituto Nacional de Enfermedades Neoplasicas (Lima, Peru) between March 2007 and May 2012 was selected. Expression of CD3, CD4, CD8, CD20, CD68 and CD163 in the tumor-associated immune cells was evaluated by digital analysis.

Results: The median age of our patients was 66.7. The tumors had a median Breslow thickness of 5.0 mm and lymph nodes were involved in 44.2%.Most of the tumors exhibited a Grade 2 TIL (48.8%). Breslow thickness (p=0.006), stage I-II (p<0.001) and negative lymph nodes (p<0.001) were associated with longer survival. Median percentage of CD3, CD8, CD4, CD20, CD68 and CD163 immune cells in melanoma lesions were 19.3%, 8.7%, 7.8%, 3.0%, 16.6% and 19.2%, respectively. Higher density of CD4 TIL was associated to thinner Breslow's depth (p=0.033). Higher density of CD20 TIL was associated to stage I-II (p=0.013) and absence of lymph node involvement (p=0.032). Survival analysis found that longer survival was associated to high CD4 TIL (p=0.005) and had a trend to be associated to high CD8/CD3 ratio (p=0.065).

Conclusions: Infiltration of immune cell subsets in ALM lesions can be associated to tumor features and prognosis.

Keywords: Acral melanoma; Tumor infiltrating lymphocyte; Prognosis; Survival

Introduction

Tumor-associated immune cells including tumor infiltrating lymphocytes (TIL) have been associated with longer survival in patients with different malignancies [1,2]. Malignant transformation may be associated with the expression of molecules on the tumor cells, which are recognized as foreign by the specific immune system and may induce immune responses that can modulate the metastatic potential of cutaneous melanoma. The presence of inflammation and regression in primary melanomas is a relevant prognostic factor and has been considered a strong indicator for the existence of an antitumor immune response [3-5]. The assessment of TILs also appears to predict survival and lymph node involvement [6,7]. A small study also found a predictive role to IFN therapy response of TIL [8,9]. In addition, infiltration of macrophages is correlated closely with the depth of tumor and angiogenesis in melanomas [10].

CD8 cytotoxic lymphocytes are the main players in anticancer activity with some studies describing an association between higher numbers of these cells and longer survival in different malignancies, including melanoma [1,11,12]. The activity of CD8-positive lymphocytes, however, is likely depended on CD4 T helper signals. CD4-positive lymphocytes recognize tumor antigens that are expressed by activated B lymphocytes, macrophages, and dendritic cells, and can produce an activating or inhibiting effect over CD8 cytotoxic lymphocytes. FoxP3 regulatory T cells are a CD4 subpopulation that are in part responsible of the inhibiting effect [12].

Different target treatments against tumor antigens like BRAF mutations as well as immune checkpoints are currently being used in the daily treatment of cutaneous melanoma, creating an urgent need to better select patients who will benefit from specific treatment modalities. TIL are emerging as promising molecular markers that may help in this decision [12-15].

Herein we report a comprehensive analysis of CD3, CD4, CD8, CD20, CD68, and CD163 in the inflammatory cells associated with acral lentiginous melanoma samples and correlate these results with patient survival.

Materials and Methods

Patients

43 cases of Acral Lentiginous Melanoma (ALM) who underwent surgery at the Instituto Nacional de Enfermedades Neoplasicas (Lima, Peru) between March 2007 and May 2012 were selected for the research project. The Research Ethics Committee at our institutions approved the study protocol, and all the participants were well informed.

Histopathological assessment of tumor and TIL infiltrate was performed. The TIL infiltrate was prospectively classified by a pathologist following Azimi, et al. published methodology [6] who describe 4 TIL grades by combining density and distribution: grade 0

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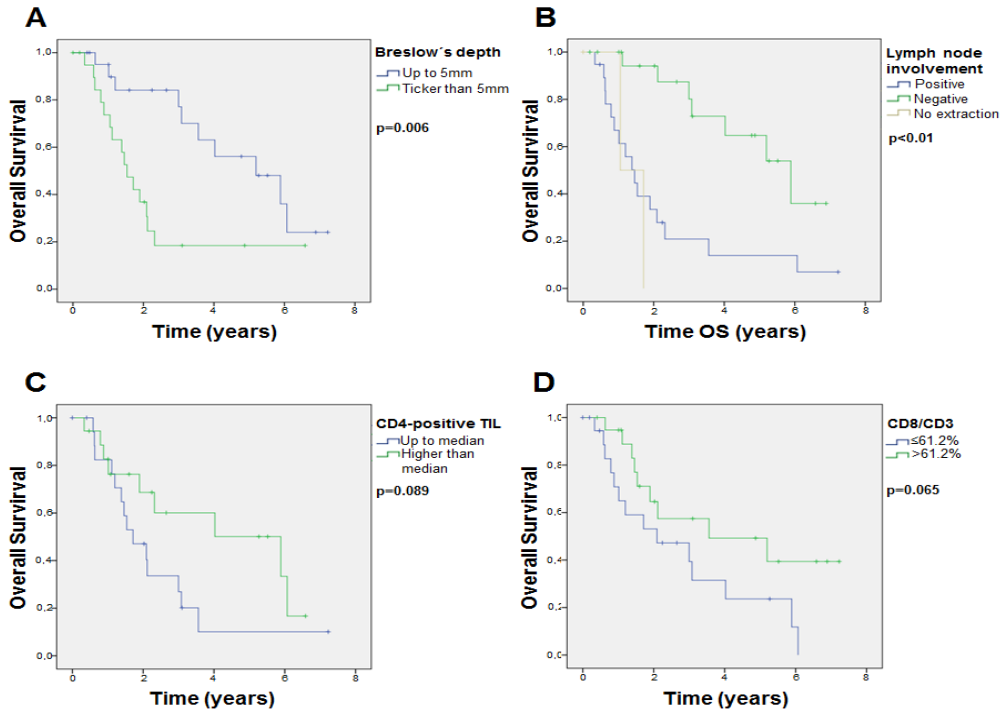

Figure 1: Correlation between overall survival and melanoma Breslow thicknesses, lymph node involvement, CD4-positive TILs and CD8/CD3 ratio in the TIL infiltrate.

(TILs absent), grade 1 (mild or moderate density and focal distribution or a mild multifocal TIL infiltrate), grade 2 (intense focal, either a moderate or intense multifocal, or a mild diffuse TIL infiltrate) and grade 3 (a moderate or intense diffuse TIL infiltrate). Areas with high mononuclear density were selected and tissue microarrays (TMA) with 8 cylinders of 0.6 cm in diameter were prepared.

The histopathological and clinical findings were utilized for staging of the cases according to the 7th edition of the AJCC Melanoma Staging and Classification [16]. Two independent pathologists (S.C. and C.T.) examined the cases on separate occasions.

Immunohistochemistry

Histological sections, 5 μm thick, obtained from formalin-fixed paraffin embedded TMA were used for the immunohistochemical demonstration of CD3, CD4, CD8, CD20, CD68 and CD163, using the alkaline phosphatase-streptavidin method, as per manufacturers protocol. The sections were rehydrated in phosphate-buffered saline (PBS) and antigen retrieval was performed by immersion in 0.1% trypsin solution in PBS at 37 °C for 5-10 minutes or by microwave heating for 5 minutes × 4 (total, 20 min) in 10 mM citrate buffer solution (pH 6.0). The sections were treated for 45 minutes with 10% normal goat serum or normal horse serum in PBS.

The following antibodies were used: mouse monoclonal antibody to human CD3 (IS503, Dako), mouse monoclonal antibody to human CD4 (IS649, Dako), mouse monoclonal antibody to human CD8 (IS623, Dako), mouse monoclonal antibody to human CD20 (IS604, Dako), mouse monoclonal antibody to human macrophages CD68 (IS613, Dako) and mouse monoclonal antibody to human CD163 (BSB6304, Bio SB). The sections were incubated further in alkaline phosphatase-streptavidin (Vector Laboratories, Burlingame, CA; 1:1000 dilution) for 30 minutes at room temperature, reacted with Fast Red Substrate System (Dakopatts) or with Dako® Fuchsin + Substrate-Chromogen, and then counterstained with Mayer hematoxylin; the sections were rinsed thoroughly in PBS between each step and finally mounted in glycerol gelatin (Sigma Chemical Company, St. Louis, MO). In negative control sections, the specificity of the antisera was tested by replacing the primary antibodies with normal serum, and human lymph node sections were used as positive controls for CD3, CD4, CD8, CD20, CD68, and CD163 antigens (Figure 1).

Statistical analysis

The median of the TIL and macrophage subpopulation counts was used for statistical analysis. An additional analysis was performed by evaluating the impact of CD8/ CD3 proportion over survival. Differences in median count for every clinicopathological features were evaluated by Chi-square test of independence or Fisher's exact test. Furthermore, we correlated these data with the survival rate by using the Kaplan–Meier method; differences between categories were tested by the log-rank or Breslow (Generalized Wilcoxon) test according to the case. Analyses were performed using the SPSS statistical package (IBM SPSS Statistical 19).

Results

Clinicopathological features

Our cohort of 43 ALM cases included 29 men (67%) and 13 women (33%). The median age was 66.7 yrs. The most common anatomic locations were: sole (16 cases, 38.1%), ankle (11 cases, 26.2%) and subungual nail bed (9 cases, 21.4%). Median Breslow thickness of the tumors was 5 mm and lymph nodes were involved in

Table 1: Correlation between clinico-pathological features of ALM and survival.

Features	n=43 (%)	OS at 5 years= 36.7% (IC95%=21-53)	p
Age			0.975
Median (range y)	66.7 (27-92)		
<66y	18 (41.9)	37.5% (13-62)	
≥66y	25 (58.1)	35.7% (16-57)	
Gender			0.329
Male	29 (67.4)	28.6% (12-47)	
Female	14 (32.6)	61.1% (26-83)	
LVI			0.162
No	25 (58.1)	39.4% (17-61)	
Yes	16 (37.2)	33.5% (12-57)	
NR	2 (4.7)	50% (1-91)	
Breslow's depth (mm)			**0.006**
Median (range)	5 (0.73-55)		
≤5 mm	22 (51.2)	56.1% (29-77)	
>5 mm	21 (48.8)	18.4% (5-39)	
Ulceration			0.149
No	11 (25.6)	70.7% (34-90)	
Yes	32 (74.4)	22.2% (8-41)	
Mitoticrate per mm²			0.49
Median (range)	3 (0-38)		
≤3	21 (48.8)	42.6% (19-64)	
>3	19 (44.2)	26.4% (7-51)	
NR	3 (7.0)	-	
Microsatelitosis			0.224
No	34 (79.1)	40.1 (21-58)	
Yes	6 (14)	33.3 (4.6-68)	
NR	3 7.0)	-	
Lymph node			0.001
Negative	21 (48.8)	64.7 (34-84)	
Positive	19 (44.2)	13.9 (3-35)	
No resection	3 (7.0)	-	
Stage			<0.002
I-II	22 (51.2)	64.7 (34-84)	
III	21 (48.8)	12.5 (2-32)	

44.2% of the cases. The cases were classified as stage I (2 cases), II (20 cases), and III (21 cases).

Most tumors had either Grade 2 (48.8%) or Grade 3 (32.6%) TIL. TIL grade was inversely associated with Breslow thickness (p=0.030) and Clark level (p=0.016). Higher TIL levels (grade 2 and 3) had a trend to be associated with absence of ulceration (p=0.069).

Eighteen patients (42%) had died at the median follow-up of 73 months. Survival analysis found that thinner Breslow thickness (p=0.006), stages I-II (p<0.001) and negative lymph nodes (p<0.001) were associated with longer survival. There was a trend for Grade 3 TIL to be associated with longer survival (p=0.34) (Table 1).

Table 2: Correlation between Tumor Infiltrating Immune cells and survival.

Features	n=43 (%)	OS at 5 years= 36.7% (IC95%=21-53)	p
TIL density			0.659
Negative- Mild	9 (20.9)	28.6 (4-61)	
Moderate	26 (60.5)	35.3 (16-56)	
Intense	8 (18.6)	52.5 (12-82)	
TIL distribution			0.166
Diffuse	15 (34.9)	52.6 (23-76)	
Focal	2 (4.7)	-	
Multifocal	25 (58.1)	23.1 (7-45)	
Negative	1 (2.3)	-	
TIL Grade			0.34
0-I	8 (18.6)	33.3 (5-68)	
II	21 (48.8)	19.5 (4-44)	
III	14 (32.6)	58 (26-80)	
CD3			0.827
Median (range)	19.3 (0.4-60)%		
<19.3%	21 (48.8)	31.4 (11-55)%	
≥19.3%	22 (51.2)	42.4 (20-63)%	
CD4			0.005
Median (range)	7.8 (0.1-28)%		
<7.8%	18 (41.9)	14.6 (2-37)%*	
≥7.8%	19 (44.2)	52.8 (25-75)%	
NR	6 (14)	-	
CD8			0.647
Median (range)	8.7 (0.6-50)%		
<8.7%	20 (46.5)	38.5 (14-63)%	
≥8.7%	20 (46.5)	33.7 (13-56)%	
NR	3 (7)	-	
CD20			0.157
Median (range)	3 (0.2-20)%		
<3%	18 (41.9)	20.8 (5-43)%	
≥3%	19 (44.2)	48 (19-73)%	
NR	6 (14)	-	
CD68			0.742
Median (range)	16.6 (4-46)%		
<16.6%	19 (44.2)	26.5 (8-49)%	
≥16.6%	19 (44.2)	41.7 (18-65)%	
NR	5 (11.6)	-	
CD163			0.863
Median (range)	19.2 (5-45)%		
<19.2%	18 (41.9)	21.2 (5-44)%	
≥19.2%	19 (44.2)	49.4 (23-71)%	
NR	6 (14)	-	

Table 3: Association between tumor infiltrating immune cells and clinico-pathological features.

Features	CD3	CD4	CD8	CD20	CD68	CD163
Age (p value)	0.897	0.385	1	0.898	0.097	0.385
<66y	18%	13.70%	9.10%	3.60%	20%	21.50%
≥66y	21.30%	6.80%	8.70%	3%	12.50%	17.60%
Gender (p value)	0.916	0.331	0.507	0.909	1	0.909
Male	21.30%	7.30%	9.60%	3.50%	16.70%	19.20%
Female	18%	15.60%	6.90%	2.90%	16.50%	19.20%
LVI (p value)	0.121	0.877	0.511	0.251	0.502	0.118
No	15.50%	8.70%	7.70%	5.70%	11.90%	17.60%
Yes	27.90%	7.80%	13.60%	2.90%	19.30%	22.30%
NR	-	-	-	-	-	-
Breslow's depth (mm) (p value)	0.65	0.033	0.752	0.402	1	0.858
≤5mm	19.50%	13.70%	8.40%	2.70%	15.40%	18.60%
>5mm	15.50%	5.70%	10.20%	3.70%	16.60%	19.70%
Ulceration (p value)	0.337	0.124	0.723	1	0.124	0.124
No	26.20%	14.80%	9%	3%	26.20%	25.60%
Yes	16.10%	6.80%	8.40%	3.20%	12.50%	17.60%
Mitotic rate per mm^2(p value)	0.752	0.73	0.9	0.515	0.6	1
≤3	19.60%	9.40%	8.40%	4%	22.90%	19.20%
>3	15.50%	7.30%	7.60%	2.70%	14.80%	18%
NR	-	-	-	-	-	-
Microsatelitosis (p value)	0.397	0.164	0.644	1	1	0.601
No	15.80%	6.30%	7.70%	3%	15.40%	18.20%
Yes	24.40%	11.10%	17%	2.70%	14.90%	23.50%
NR	-	-	-	-	-	-
Lymph node (p value)	0.987	0.6	0.516	0.011	0.516	0.187
Negative	19.60%	15.20%	8.40%	5.70%	14.70%	17.20%
Positive	19.30%	7.30%	11.80%	2.70%	20%	22.30%
No resection	-	-	-	-	-	-
Stage (p value)	0.876	0.858	0.342	0.013	0.516	0.14
I-II	18.10%	14.80%	8.40%	5.90%	13.10%	16%
III	19.30%	7.60%	13.30%	2.70%	19.30%	22.30%
TIL density (p value)	0.006	0.037	0.021	0.037	0.067	0.059
Negative- Mild	9.20%	6.10%	2%	0.70%	11.50%	20.70%
Moderate	20.60%	7.30%	8.40%	3%	11.80%	14.60%
Intense	26.80%	15.70%	15.30%	9.70%	27.80%	21.80%
TIL distribution (p value)	0.008	0.056	0.069	0.387	0.509	0.28
Diffuse	26.20%	15.20%	13.30%	5.50%	18.60%	21.30%
Focal	16.40%	11.80%	13.40%	0.40%	35.40%	36.90%
Multifocal	11.90%	5.70%	7.30%	3%	13.70%	18%
Negative	-	-	-	-	-	-
TIL Grade (p value)	0.002	0.066	0.069	0.261	0.82	0.364
0-I	7.80%	5.90%	2%	0.70%	12.50%	26.70%
II	15.50%	5.70%	8%	3%	14.90%	17.60%
III	26.20%	15.60%	13.60%	5.80%	18.90%	21.40%

CD3 (p value)	-	<0.001	<0.001	0.005	0.003	0.014
<19.3%	6.10%	4.90%	4.30%	1%	10.60%	11.90%
≥19.3%	31.80%	14.20%	21%	5.50%	22.90%	21.90%
CD4 (p value)	<0.001	-	<0.001	0.019	0.14	0.019
<7.8%	9.10%	4.30%	6.50%	1.30%	11.50%	16%
≥7.8%	27.50%	15.80%	16.60%	5.30%	24.20%	21.90%
NR	-	-	-	-	-	-
CD8 (p value)	<0.001	<0.001	-	0.072	0.002	0.008
<8.7%	6.20%	4.90%	4.20%	2.40%	10.70%	12.70%
≥8.7%	34.20%	12.70%	21.30%	4.40%	23.10%	22.30%
NR	-	-	-	-	-	-
CD20 (p value)	0.005	0.019	0.072	-	0.413	0.317
<3%	10.50%	5.40%	6.50%	1%	12.30%	17.60%
≥3%	26.30%	14.80%	13.80%	7.70%	19.30%	20.50%
NR	-	-	-	-	-	-
CD68 (p value)	0.003	0.14	0.002	0.413	-	<0.001
<16.6%	9%	5.80%	6.10%	2.70%	10.50%	11.60%
≥16.6%	27.50%	9.40%	16.10%	4%	26.20%	25.60%
NR	-	-	-	-	-	-
CD163 (p value)	0.014	0.019	0.008	0.317	<0.001	-
<19.2%	9%	5.30%	6.10%	2.40%	10.20%	11.60%
≥19.2%	26.20%	11.10%	13.80%	4%	26.20%	26%
NR	-	-	-	-	-	-

Infiltrating immune cells evaluated through immunohistochemistry

Evaluation of the composition of the immune infiltrate revealed that CD3-positive T cells (19.3%) were more frequent than CD20-positive B lymphocytes (3.0%). Density of CD4-positive and CD8-positive cells was 7.8% and 8.7%, respectively. Density of infiltrating macrophages (CD68-positive) was 16.6%, similar to that of activated macrophages (CD163-positive, 19.2%).

CD4-positive TIL were associated to thinner Breslow depth (p=0.033), Clark-level II and III (p=0.02), intense TIL density (p=0.037), high density of CD3-positive TIL (p<0.001), high density of CD8-positive TIL (p<0.001), high density of CD20-positive TIL (p=0.019) and high density of CD163-positive cells (p=0.019).

CD8-positive TIL were associated to intense TIL density (p=0.021), high density of CD3-positive TIL (p<0.001), high density of CD4-positive TIL (p<0.001), high density of CD68-positive cells (p=0.002) and high density of CD163-positive cells (p=0.008).

CD20-positive TIL were associated to stage I-II (p=0.013), absence of lymph node involvement (p=0.032), intense TIL density (p=0.037), high density of CD3 TIL (p=0.005) and high density of CD4 TIL (p=0.019).

CD68-positive cells were associated to no recurrence (p=0.02), high density of CD3-positive TIL (p=0.003), high density of CD8-positive TIL (p=0.002) and high density of CD163-positive TIL (p<0.001). CD163 TIL were associated to high density of CD3-positive TIL (p=0.014), high density of CD4-positive TIL (p=0.019), high density of CD8-positive TIL (p=0.008), and high density of CD163 TIL (p<0.001) (Table 2 and 3).

Survival analysis found that longer survival was associated to high CD4 TIL (p=0.005) and had a trend to be associated with high CD8/CD3 ratio (p=0.065). Other ratios like CD8/CD68 were tested for association with survival but no association was found (p=0.890).

Discussion

In our series of ALM we found that higher TIL counts were associated to factors predictive of good prognosis, such as low Breslow depth, and to a trend for longer survival. These findings are in keeping with our previously published results [7] as well as to the work of Clark, et al., who identified the significance of TILs in the prognosis of patients with cutaneous melanoma for the first time in 1989,evidence confirmed by subsequent reports [4,5].

The central role of T cells and antigen presenting cells in antitumor immunity has been extensively described [12]. Increased numbers of T cells, specifically activated CD8-positive cytotoxic T cells, have been found to correlate with longer survival in invasive colon, ovarian and cervical cancers [1,11,17]. In our cohort of ALM, lymphocytes in the TIL infiltrate were mainly CD3-positive T cells admixed with a minor population of CD20-positiveB cells (19.3 versus 3.0%, respectively). Densities of CD3-positive TIL and CD68-positive macrophages were very similar (18.13% *vs.* 16.8%). There were more CD8-positive (8.7%) than helper CD4-positive (7.8%) T

cells. Most infiltrating macrophages were activated CD163-positive cells (19.2%). Interestingly, higher density of CD4-positive TIL was associated to thinner Breslow depth and Clark-level II-III. Higher density of CD20 TIL was associated to stage I-II and absence of lymph node involvement.

Similarly, Erdag, et al. report that T cells (53%) are the dominant infiltrating immune cells and CD8 T cell (35%) were more frequent than CD4 T cell (19%) in a series of 183 samples of metastatic melanoma. Contrary to our findings, B lineage cells were not infrequent (B cells (25%) and plasma cells (8%)). CD163 macrophages accounted for 13% of the infiltrates. They also found that CD3, CD8 and B lymphocytes ($p<0.001$) positively correlated with overall survival. Interestingly, correlations with survival were not observed for CD4 cells, which may reflect the substantial fraction of FoxP3 regulatory T cells found in their series (7%) [12] on the opposite side, Jensen, et al. did not identify any association between CD8-positive TIL and neither aggressiveness features nor survival, in 185 cases analyzed [18].

Piras, et al. evaluated CD8 and CD4-positive lymphocytes, and CD68-positive macrophages in 47 cutaneous melanoma samples. A significant difference in 5-year survival was found between patients in groups with high (78.8%), moderate (44.4%), and low (25%) CD8-positive T lymphocytes density ($p=0.01$). No other associations with clinical variables were found. Density of CD68-positive cells failed to show any correlation with survival [19].

Capone, et al. evaluated the combination of CD3, CD8, CD20 and FOXP3 staining in 34 melanoma cases and found that CD3 and CD8 and its ratio could identify patients who relapsed [20]. Similarly, different studies have demonstrated that a higher proportion of CD8/CD3 is a strong biomarker associated to longer survival in early and advanced colorectal tumors [1].

Plasma cells in primary melanomas were correlated with Breslow thickness and ulceration, and carry negative prognostic significance in a few case series [21,22].

Storr, et al. evaluated CD68-positive tumor infiltrating macrophages in 202 cutaneous melanoma cases, identifying a high count in 51.2% of the cases, and an association with markers of aggressive disease, such as Breslow thickness ($p<0.001$), ulceration ($p<0.001$), mitotic rate ($p=0.005$), lymphatic vessel invasion ($p=0.002$), high micro vessel density ($p=0.003$), and the presence of non-brisk or brisk TILS ($p=0.005$). However, it has not association with relapse-free or overall survival [23].

Jensen, et al. evaluated 190 stage I/II melanoma cases, and found that CD163-positive macrophages were more frequent than CD68-positive macrophages and higher density of these cells in specific regions was associated to thicker and ulcerated lesions, and had a trend to be associated to poor survival [24].

It is interesting to note that there was a significant longer median survival among patients with higher CD4-positive cells but not association with CD8 or CD3 in our ALM series. This favorable prognostic effect of CD4 TIL sis contrary to the previously mentioned results described by other authors and could be a finding associated toacral location or to host immune features of our South-American population [18,19]. It is also remarkable that the CD8/CD3 proportion had a strong trend to longer survival in our series. We did not find any association between CD68 and CD163 macrophages and survival.

We can conclude that T lymphocytes constitute the most frequent immune cell in ALM TILs. Most of these lymphocytes are CD8-positive T lymphocytes. CD4 T lymphocytes were associated to thinner Breslow and longer survival. Higher CD8/ CD3 ratio had a trend to be associated with longer survival. More studies using digital analysis of infiltrating immune cell subsets are need to increase understanding of their role in melanoma lesions.

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