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Phenotypical characterisation of immune cells associated with histological regression in cutaneous melanoma: which role in daily practice?

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Running title: IMMUNE CELLS AND REGRESSION IN CUTANEOUS MELANOMA

Summary

Histological regression and tumour infiltrating lymphocytes represent an early sign of activation of the immune system against primary melanoma. The first phenomenon has been especially discussed in the literature because of its prognostic role, but no clear agreement on its evaluation has been reached. Immunotherapy of advanced stage melanoma has recently shown promising results; an improved understanding of the initial interplay between melanoma cells and the immune system would potentially help tailor treatment for patients.

Seventy consecutive melanomas with regression were analysed to identify a prognostic cut-off value of regression extension. Then, we compared the immune infiltrate between regressed and not regressed areas of these regressed melanomas, assessing CD3, CD4, CD8, CD20, CD123, PD1 and FOXP3/CD25 expression. The immune infiltrate of these cases was further compared with 28 control melanomas without regression.

A regression extension of 10% represented a reliable cut-off to distinguish two different risk categories in regressed melanomas. Regressed areas were less infiltrated by CD4/CD25, FOXP3/CD4 or PD1/CD4 compared to not regressed areas of each sample. These lymphocyte subsets are associated with anergy and hamper the immune CD8+ response towards the cancer cells. Moreover, the relevance of these findings was further supported by the observation that not regressed controls were significantly more infiltrated by these anergic immune cell subsets compared to the regressed cases.

These results help understand the real meaning of regression in melanoma. Moreover, the association here identified between specific immunomodulatory immune cell subsets and regression could help devise new therapeutic strategies.

Key words: Tumour infiltrating lymphocytes; melanoma; histological regression; survival.

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INTRODUCTION

Despite extensive efforts aimed at identifying prognostic biomarkers in melanoma, its clinical behaviour remains often unpredictable with frequent unexpected progressions, both in terms of timing and type.¹ Additional markers are warranted to identify early stage patients at higher risk of recurrence, in order to offer patient-tailored follow up or adjuvant treatments. Currently, evidence has shown that the specific malignancy and biological features of a cancer depend not only on its genetic abnormalities, but also on the interplay between cancer cells and micro-environment. The relationship between the tumour and host's immune system is particularly intriguing, especially considering that drugs targeting these pathways have shown a significant clinical efficacy in multiple tumour types.² Histological regression in melanoma is a paradigmatic example of this phenomenon. Some authors found no association between regression and melanoma prognosis,³ while other research groups reported a significant correlation between them.⁴⁻⁶ This discordance could be due to several factors including the heterogeneity of patient populations, as well as the different research methods applied, but what matters most is probably the lack of standardised histological criteria for its assessment. In particular, there is no agreement among pathologists in defining regression or in its sub-classification.⁷

Tumour-infiltrating lymphocytes (TILs) have been identified as a favourable prognostic marker across multiple cancers including breast, ovarian, and colon cancer neoplasms and may serve as a surrogate indicator of the strength of the host anti-tumour immune response.⁸ TIL and histological regression are

two distinct, sometimes overlapping, phenomena of the melanoma biology that still deserve better explanation in order to differentiate them.

The composition of the regression infiltrate has been partially characterised. Effector and suppressor T cells, B cells, natural killer cells, macrophages, and dendritic suppressor cells, can all impart competing immuno-stimulatory or immunosuppressive effects within the tumour microenvironment.

The first aim of this study was thus to describe and histologically characterise the regression phenomenon in melanoma, assessing its impact on survival. The second aim was to evaluate the phenotype of the infiltrating lymphocytes comparing regressed and not regressed areas in order to identify cell subsets related to the phenomenon of histological regression.

METHODS

Study population

A cohort of 70 consecutive patients with primary cutaneous melanoma attending the Dermatologic Clinic in Turin between January 2003 and December 2014 were enrolled in the study. All patients signed a consent form to give permission to include their biological sample in a biobank, and the study was approved by the local ethics committee. In order to perform an optimal evaluation of the histomorphological parameters, only melanoma samples with complete removal of the lesion and in which the entire thickness of the neoplasm was submitted to histological examination were included. Therefore, shave and punch biopsies, partial excisions and samples with too little/poor material were excluded from the study. In addition, a second cohort of 28 not regressed melanomas was used to compare the immunophenotypic composition of the TIL. Patient demographics, clinical, pathological, and follow-up information were prospectively recorded and updated on an internal database. For each case, the inflammatory regression areas were assessed (TIL inside regression area, TIL-IRA). In addition, one or more areas including inflammatory cells intimately associated with the tumour cells outside the regression area (TIL-ORA) were analysed for each case if present, and in the control series (TIL-CTRL).

For full details regarding histopathological review, and immunophenotypical evaluation of TIL, ^{2,9-13} see the Supplementary Methods (Appendix A).

Morphological evaluation of histological regression^{13,14}

Histological regression: definition and phases

Regression was defined and classified as follows: (i) early phase as a dermal tumour area with a dense mononuclear infiltrate composed mainly of lymphocytes, histiocytes and melanophages that actively infiltrate the neoplastic nests and at least partially replace neoplastic melanocytes; (ii) late phase as a dermal or epidermal area with disappearance of neoplastic cells and their replacement with dense

fibrous tissue, melanophages, dilated vessels and focal or missing residual lymphocytic infiltration in the dermis.

Histological regression: ratio/percentage

Once the presence of one or more regression areas was established, the regression/tumour area ratio was calculated as the ratio between the linear extension of regression areas and tumour. If regression was present on multiple tumour samples, the mean of the measurements made on all the slides was considered. In case of multiple regression foci on the same section, the sum of the different areas was considered. If regression was present in <5%, the lesion was considered as not regressed. Extensively regressed melanoma (>75%) was not considered eligible for the study. Regression >75% in horizontal extension according to the College of American Pathologists (CAP) definitions was not considered eligible for the study because in our experience it is present in only 4% of cases;¹⁵ for this reason, we focused our analysis on the prognostic role of tumour regression <75%.

Histological regression: distribution/topography

In addition, the predominant distribution pattern of regression was evaluated (peripheral if present on the sides of the tumour, basal if distributed along the stromal-tumour interface, and intra-tumoural if central and completely surrounded by the neoplastic cells). In order to avoid an overlap with TIL, the key feature of regression was the evidence of destruction of neoplastic cells defined as ‘necrosis or apoptosis or whole disappearance of malignant tumour cells or apparent partial or total active destruction of melanocytic nests with replacement by inflammatory cells or fibrosis or melanophages’. Areas of ulceration or those intimately associated or adjacent to it were not considered.

Statistical analysis

The specific inflammatory infiltrates (TIL-IRA, TIL-ORA, TIL-CTRL) features and associations with clinicopathological characteristics were assessed using the chi-square test and the non-parametric test. Specifically, the *p* values in Table 1 were calculated as follows: if the variable had categorical data, the chi-squared test was performed; if the variable had continuous data, the Kruskal–Wallis rank sum test for median and the analysis of variance (ANOVA) for mean was performed. For more than two groups Tukey honestly significant difference (HSD) post-hoc test was performed. Logistic regression and post-estimation receiver operating characteristic (ROC) curve were used to define optimal cut-off of regression related to relapse (>10%, >20%, >30%, >40%, >50%, >60% values were tested). Disease free interval (DFS) was calculated from the date of primary lesion diagnosis to the date of tumour progression/recurrence or last follow-up. Disease specific survival (DSS) was calculated from the date of primary lesion diagnosis to the date of death or last follow-up. Survival curves were estimated with the Kaplan–Meier method and compared using the log-rank test.

RESULTS

Clinicopathological characteristics of enrolled patients

Clinicopathological characteristics are reported in Table 1. Of the 70 patients included with histological regressed melanoma, 39 (56%) were males and 31 (44%) females. Median age was 58 years (range 23–78). Primary tumours were on the trunk in 58.6% of cases (41/70), followed by lower limbs (22/70,

31.4%), upper limbs (4/70, 5.7%) and head/neck (3/70, 4.3%). The most represented histotype was superficial spreading melanoma (56/70, 80%). Mean Breslow thickness was 2.73 ± 2.10 mm. The most represented Clark level was IV (71.4%, 50/70). Ulceration was present in 33% of the lesions (23/70). Mitotic count $\geq 1/\text{mm}^2$ was present in 61% (43/70); 38.5% (27/70) showed diffuse neo-angiogenesis and 48.6% (34/70) had vascular invasion. In 20 cases (28.6%), TILs were described as 'brisk'. There was metastatic involvement of the sentinel lymph node in 37% (26/70).

According to the AJCC classification, there were eight stage I (26%), 26 stage II (37%), 26 stage III (37%) patients. After a median follow up of 4.9 years (range 0.7–8.9), 21/70 patients showed disease progression (30%), and subsequently 15 died of melanoma during follow up (21.4%). The pattern of first progression was: 12 regional metastases (12%) [four lymph node metastases in the same SLNB negative basin (false negative SLN biopsy), seven regional skin metastases, one lymph node metastasis into the parotid gland] and 13 (18%) distant metastasis. During follow-up, of 12 patients with first progression at regional site, four were free of disease after the surgical resection of the metastasis, three patients showed a *de novo* skin recurrence, one patient had lymph node metastasis and four patients progressed in a distant site (2 in the lung, 2 in the liver).

Histological regression evaluation

Percentage/ratio

Median percentage of regression was 15% (range 5–75) (Table 1). After logistic regression and post-estimation ROC analyses, a cut-off value of 10% was identified as statistically significant towards a more favourable course [odds ratio (OR) 0.35, confidence interval (CI) 0.12–0.98, $p=0.048$]. Patients with less than 10% of regression showed a higher Breslow thickness compared to the others (3.64 ± 2.32 mm vs 2.29 ± 1.85 mm, $p=0.015$), a higher prevalence of ulceration (56% vs 21%, $p=0.003$) and a higher prevalence of a mitotic count $\geq 1/\text{mm}^2$ (82% vs 51%, $p=0.011$). There were no differences concerning other variables adopting the 10% cut-off value (Table 1). We identified 46 'early' and 24 'late' regressed melanomas, respectively; no differences were observed on the basis of regression cut-off (17/23 early cases in $\leq 10\%$ vs 29/47 early cases in 10–75%, $p=0.312$).

DFS was significantly longer in patients with more than 10% of histological regression: in particular, at 1 year of follow-up, 97.8% of the patients with $>10\%$ regression were disease free compared to the 65.2% of patients with regression $<10\%$, while at 5 years the rates were 73.1% and 48.7% ($p=0.005$), respectively (Fig. 1A).

Concerning DSS, no significant difference was reached between regressed and not regressed tumours, even if the curve of the group with regression $>10\%$ looked more favourable in terms of prognosis. Percentages of survival at 1 year were 97.8% vs 82.6% and 84.6% vs 68.2% at 5 years, respectively ($p=0.07$) (Fig. 1B).

TIL immunophenotype evaluation

A total of 67 cases out of 70 histological regressed melanomas and 28 control melanomas were used for these analyses (Table 2), while three cases had insufficient material to perform all the determinations.

The control cohort of not regressed melanomas used for comparing the immunophenotype had a mean

Breslow thickness of 2.61 ± 1.29 mm, the median age at diagnosis was 53, 12/28 had ulceration of primary melanoma. There were no significant differences in the classical prognostic melanoma features between regressed and not regressed melanomas.

The distribution of immunophenotype in TILs in both regressed and not regressed lesions is reported in Table 2. No differences were observed in the immunophenotype between TIL-IRA and TIL-ORA in the 49 analysable cases when evaluating the CD3, CD4, CD8 sub-populations. Conversely, differences were detected in FOXP3/CD4 ratio in different subgroups (10.1 ± 9.28 TIL-IRA vs 23.3 ± 21.8 TIL-ORA vs 38.4 ± 25.4 TIL-CTRL; $p < 0.001$) (Fig. 2).

FOXP3/CD4 evaluation and correlation with regression

FOXP3/CD4 ratio was lower in TIL-IRA compared to TIL-ORA (10.1 ± 9.28 vs 23.3 ± 21.8 , $p = 0.0004$), but both of them were significantly lower compared to TIL-CTRL (38.4 ± 25.4 , $p = 0.0016$) (Table 2). In particular, we evaluated the differences according to the extent of the regression ($\leq 10\%$ vs $10-75\%$). FOXP3/CD4 ratio in TIL-CTRL (38.4 ± 25.4) was higher than the ratio observed in TIL-ORA of $10-75\%$ (21.8 ± 17.9 , $p = 0.0361$) (Supplementary Table 1, Appendix A). When looking at differences of immunophenotype rate between TIL-CTRL and TIL-IRA, we observed a higher ratio of FOXP3/CD4 in the TIL-CTRL subset compared to both $\leq 10\%$ and $10-75\%$ categories (38.4 ± 25.4 vs 9.7 ± 9.87 vs 10.3 ± 9.10 , $p < 0.001$) (Supplementary Table 2, Appendix A). Supplementary Table 3 (Appendix A) reports data on regressed lesions: TIL-ORA ($\leq 10\%$ and $10-75\%$ subgroups) showed a higher ratio of FOXP3/CD4 (26.0 ± 28.2 and 21.8 ± 17.9) compared to TIL-IRA ($\leq 10\%$ and $10-75\%$ subgroups) (9.7 ± 9.87 and 10.3 ± 9.10).

PD1/CD4 evaluation

PD1/CD4 ratio in TIL-IRA and TIL-ORA were significantly lower compared to TIL-CTRL (4.9 ± 5.5 and 6.9 ± 7.6 vs 12.7 ± 6.9 , $p < 0.001$) (Table 2), while no difference was detected in PD1/CD4 ratio between TIL-IRA and TIL-ORA in regressed lesions [4.9 ± 5.5 vs 6.9 ± 7.6 , $p =$ not significant (ns)].

Similarly, PD1/CD4 ratio between TIL-IRA $\leq 10\%$ VS TIL-IRA $10-75\%$ was not statistically different (5.7 ± 5.3 vs 4.5 ± 5.6 , $p =$ ns; Supplementary Table 2, Appendix A). Moreover, PD1/CD4 ratio in TIL-ORA was higher in lesions with $\leq 10\%$ of regression compared to TIL-ORA in lesions with $10-75\%$ regression (10.5 ± 8.9 vs 3.1 ± 2.9 , $p = 0.0004$) (Supplementary Table 1, Appendix A).

When looking at differences in immunophenotype rates between TIL-CTRL and TIL-IRA in $\leq 10\%$ or $10-75\%$ categories, we observed a higher ratio of PD1/CD4 in TIL-CTRL compared to both (12.7 ± 6.9 vs 5.7 ± 5.3 , $p < 0.007$; and 12.7 ± 6.9 vs 4.5 ± 5.6 , $p < 0.001$) (Supplementary Table 2, Appendix A). For TIL-CTRL and TIL-ORA in $\leq 10\%$ or $10-75\%$ categories, we observed a higher ratio of PD1/CD4 in TIL-CTRL compared to $10-75\%$ regressed melanoma (12.7 ± 6.9 vs 10.5 ± 8.9 , $p =$ ns; and 12.7 ± 6.9 vs 3.1 ± 2.9 , $p < 0.0004$) (Supplementary Table 1, Appendix A). In regressed lesions, (Supplementary Table 3, Appendix A) TIL-ORA in lesions with $\leq 10\%$ showed a higher PD1/CD4 ratio compared to TIL-IRA in both categories.

DISCUSSION

Melanoma is considered an 'immunogenic' cancer because it is often associated with lymphocytic

infiltration and is capable of spontaneous regression.^{14–17} Those phenomena are strictly related, share some similarities, but probably represent different time-points and different mechanisms during the biological history of this tumour. Although the lymphocytic infiltrate has been described as a favourable prognostic feature,¹⁸ complete agreement on the role of histological regression has not been reached to date; indeed, either a negative or protective role on prognosis has been attributed to regression.^{4–6,19} Moreover, it has been suggested that early immune activation could explain the different immunotherapy response rates in clinical practice.²⁰

After revision of slides and evaluation of the extent of regression, we identified a quantitative cut-off value (10%) of histological regression associated with a different outcome. In fact, this cut-off maintained its prognostic role in terms of DFS by survival analysis, marking the difference between the not regressed ($\leq 10\%$) and the ‘true’ regressed melanomas (10–75%). A similar regression extent cut-off in dermoscopy has been associated with a different tumour molecular landscape in terms of copy number variation, which represents a marker of tumour aggressiveness.²¹ For DSS, no significant difference was reached between regressed and not regressed tumours; this finding can be related to overlaps with the widespread availability of immunomodulatory therapy in recent years, which might differentially affect survival in melanoma patients.²²

In order to explain the different behaviour of regressed lesions, we analysed the immunophenotype of the recruited lymphocytes within and outside the ‘regression’ areas in regressed lesions compared to a control group without regression.

In our study, a lower rate of FOXP3/CD4 positive cells was observed in TIL-IRA and TIL-ORA in regressed melanoma compared to not regressed melanoma (TIL-CTRL). The FOXP3+ T-reg population has been described to decrease the innate and acquired response against the tumour through a mechanism which is physiologically involved in protecting against autoimmunity^{23,24} and has also been associated with different progression and survival rates in melanoma patients.^{18,24–26} Conversely, the absolute number of FOXP3+ cells was not significantly different between the groups in our series. When considering the FOXP3/CD4 ratio, it was inversely associated with histological regression, thus reflecting a different immunotolerance status between regressed and not regressed melanoma, potentially representing an early immunophenotypic marker of inactivation of the immune system. This finding is also supported by evidence that in metastatic melanoma FOXP3+ tumour-infiltrating immune cells are a potential predicting biomarker of response to treatment and survival. Balatoni *et al.* showed that the strongest predictor of ipilimumab treatment response was the density of FOXP3+ cells infiltrating lymph node metastases; moreover Di Gennaro *et al.* reported that electrochemotherapy induces a local response in skin melanoma metastasis by a lymphoid infiltrate characterised by a decrease in CD4+FOXP3+Treg cells.^{27–29}

Similarly, we demonstrated a different count of PD1+ out of the total CD4+ count in the control group (without regression) compared to the regressed group. The PD1/PDL1 axis maintains a balance between tolerance and autoimmunity, thus cancer cells under selective pressure can exploit this signalling pathway with lymphocytes to decrease the immune response. PD1/PDL1 interaction inhibits T lymphocyte proliferation, survival, and effector functions, induces apoptosis of antigen-specific T cells, and promotes the differentiation of CD4+ T cells into FOXP3+ regulatory T cells. Moreover new evidence is suggesting that the peritumoural and the perimetastases infiltrate can be a potential therapeutic target in melanoma.^{26,30–33} From this point of view, the histological regression depends on the immune microenvironment and can be considered an indicator of a more efficient immune system exploitable for daily clinical practice.³⁴

Limitations of the present study are due to the relatively low prevalence of regression in melanoma which, together with the need for adequate representative tissue, results in a relatively small sample size. Moreover, we have considered only lesions with extensive regression up to 75%, because melanomas with regression higher than 75% are extremely rare and harbour uncertain clinical behaviour.^{15,35} The limited number of samples could help explain why statistical significance was not achieved in survival analysis, even though a trend was observed; this finding is probably also due to the increased use of immunomodulatory therapies in recent years. Another potential limitation could be the intrinsic operator-dependency in immunophenotype assessment, but we tried to overcome it by employing multiple independent observers.

Despite these potential drawbacks, based on these results we can suggest that histological regressed melanomas represent a heterogeneous group in terms of prognosis, possibly due to different immunomodulatory mechanisms which can affect the activity of TILs. Caution should be advised when interpreting the prognostic meaning of these characteristics, but an important observation is that a regression of 10–75% should be considered a biologically and clinically significant phenomenon impacting survival. The observed heterogeneity in terms of immunophenotype between the different samples and tumour areas highlights the importance of target assessment and selection when devising future treatment strategies, and live adoptive cell therapy.³⁶ Following our results, further studies are encouraged analysing the immunophenotype of histological regression, especially evaluating its correlation with response to therapies.

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APPENDIX A: SUPPLEMENTARY DATA

Supplementary data related to this article can be found at <http://>

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FIGURE LEGENDS

Fig. 1 (A) Disease free survival and (B) disease specific survival of melanoma patients stratified for the extension of histological regression.

Fig. 2 (A) Low power H&E of a regression area (IRA): a rich inflammatory infiltrate is present with lymphocytes and melanophages replacing neoplastic cells (arrows). (B) Low power H&E of an area outside regression (ORA): an inflammatory infiltrate is present, although replacement of neoplastic cells is not (arrows). (C) Low power FOXP3 immunohistochemical staining showing few positive cells within the regression area (arrows). (D) Low power FOXP3 immunohistochemical staining showing multiple positive cells within the tumour infiltrating lymphocytes (arrows).

Table 1 Clinical characteristics in regressed and not regressed melanomas

	Histological regression stratified			Histological regression (5–75%) (n=70)	No histological regression (CTRL) (n=28)	p*
	5–10%	10–75%	p			
Age, median (interval)	55 (34–78)	59 (23–78)	0.551	58 (23–78)	53 (27–83)	0.432
Gender			0.149			0.250
Female	13	18		31	16	
Male	10	29		39	12	
Primary site			0.078			0.141
Head/neck	2	1		3	1	
Trunk	9	32		41	13	
Upper extremities	1	3		4	6	
Lower extremities	11	11		22	8	
Histotype			0.245			0.291
SSM	16	40		56	23	
ALM	5	6		11	2	
Other	2	1		3	3	
Breslow thickness, mm±SD	3.64±2.32	2.29±1.85	0.015	2.73±2.10	2.61±1.29	0.769
Breslow AJCC			0.036			0.209
≤1	3	13		16	3	
1–2	5	14		19	8	
2–4	5	14		19	13	
>4	10	6		16	4	
Clark			0.688			0.586
3	7	11		18	10	
4	15	35		50	17	
5	1	1		2	1	
Ulceration			0.003			0.351
No	10	37		47	16	
Yes	13	10		23	12	
Mitosis			0.011			0.105
<1	4	23		27	6	
≥1	19	24		43	22	
Neoangiogenesis			0.328			0.475
Absent/mild	16	27		43	15	
Present	7	20		27	13	
Inflammatory infiltrate			0.013			0.795
Scarce	14	14		28	12	
Present	9	33		42	16	
Vascular invasion			0.635			0.149
Absent	16	30		46	14	
Present	7	17		24	14	
SLN status			0.416			0.056
Negative	16	28		44	12	
Positive	7	19		26	16	
No. metastatic LN			0.615			0.185
0	16	28		44	12	
1–2	6	16		22	15	
3–4	0	2		2	1	
>4	1	1		2	0	
AJCC stage			0.181			0.182
I	4	14		18	3	
II	12	14		26	10	
III	7	19		26	15	

Brisk infiltrate			0.147		0.215
No	19	31		50	17
Yes	4	16		20	12

AJCC, American Joint Committee on Cancer; ALM: acral lentiginous melanoma; LN, lymph node; SD, standard deviation; SLN, sentinel lymph node; SSM, superficial spreading melanoma.

*Those tests are referring to the total of regressed melanoma towards the not regressed ones.

Table 2 TIL immunophenotype in regressed and not regressed lesions

Population	TIL-CTRL (A) (n=28)	TIL-ORA (B) (n=49)	TIL-IRA (C) (n=67)	Tukey HSD post-hoc test	
CD3, n° cell/mm ²	523.8±386.4	585.14±426.2	500.2±337.3	0.4874	
CD20, n° cell/mm ²	95.5±132.76	76.12±116.48	64.8±98.9	0.4757	
CD4, n° cell/mm ²	238.3±159.9	305.6±233.6	278.0±182.5	0.3570	
CD8, n° cell/mm ²	297.5±235.2	299.9±224.6	249.3±184.1	0.8332	
CD4/CD8	0.93±0.38	1.11±0.61	1.27±0.53	0.0170	A vs C 0.0148
CD25, n° cell/mm ²	38.9±37.6	35.7±46.7	27.1±27.2	0.2696	
CD123, n° cell/mm ²	7.3±11.6	5.4±10.7	3.0±5.4	0.0783	
FOXP3, n° cell/mm ²	91.2±72.5	62.8±73.9	52.7±66.3	0.0541	A vs C 0.0420
CD4/CD3, %	49.8±17.2	54.2±19.8	58.8±18.3	0.0872	
FOXP3/CD4, %	38.4±25.4	23.3±21.8	10.1±9.28	<0.001	A vs B 0.0016, A vs C <0.001, B vs C 0.0004
PD1, n° cell/mm ²	28.4±42.5	15.1±23.1	12.9±21.1	0.0380	A vs C 0.0325
PD1/CD4, %	12.7±6.9	6.9±7.6	4.9±5.5	<0.001	A vs B 0.0016, A vs C <0.001
Melanophages, n° cell/mm ²	77.2±81.8	191.8±179.8		0.0017	

HSD, honestly significant difference.