FOXP3, IL-35, and PD-L1 in intra- and peritumoral lymphocytic infiltrate of cutaneous melanomas as an important part of antitumor immunity

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Background. The tumor microenvironment is a significant mediator enabling tumor growth and progression. Tumor-infiltrating lymphocytes (TILs) are an important component of this but tumor cells develop mechanisms by which they can escape the action of the immune system. Immunosuppressive mechanisms cooperate with each other and involve cells of the immune system, the tumor microenvironment itself, chemokines and cytokines. In this study, we examined the FOXP3+, IL-35+, and PD-L1+ lymphocytes in tumor tissues as they are contributing to immunosuppression in some tumors, including melanoma. Such cells are also associated with tumor progression, early metastasis, and prognosis.
Methods and Results. In this study, 95 cutaneous melanomas and 25 melanocytic nevi as a control group were examined by immunohistochemistry for FOXP3+, IL-35+, and PD-L1+ lymphocytes. Melanomas were divided into four groups according to the TNM classification: pT1 (35), pT2 (21), pT3 (21), and pT4 (18). PD-L1+ lymphocytes were enriched in pT3- and pT4-stage melanomas, especially in the periphery of the lesions (P<0.001). The number of FOXP3+ lymphocytes was positively correlated with the stage of the disease, especially in the center of the tumors (P<0.001). Likewise, IL-35+ lymphocytes (P<0.001) were enriched with the stage of the tumor.
Conclusion. This article demonstrates that the immunosuppressive environment develops in proportion to the stage of the melanoma. The most significant changes are found at the tumor periphery, confirming the heterogeneity of the tumor stroma which is more pronounced in more advanced tumors and which may contribute to the greater aggressiveness in these peripheral zones.

FOXP3, IL-35, AND PD-L1 EXPRESSION IN LYMPHOCYTIC INFILTRATE OF CUTANEOUS MELANOMAS

Tumor microenvironment is an mediator of tumor growth and progression, with important part of tumor infiltrating lymphocytes.

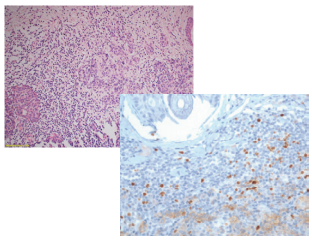
Tumor cells can develope mechanisms by which they can escape the action on immune system.

We examined the FOXP3+, IL-35+ and PD-L1+ lymphocytes in tumor tissues as they are contributing to immunosuppression in some tumors, including melanoma.

95 cutaneous melanomas and 25 melanocytic nevi as a control group were examined by immunohistochemistry for FOXP3+, IL-35+, and PD-L1+ lymphocytes.

Melanomas were divided into four groups according to the TNM classification: pT1 (35), pT2 (21), pT3 (21), and pT4 (18).

The expression of all monitored antigens was the evaluated with light microscopy over an area 1 mm2 in the places of greatest density (hot-spot) both in the center and on the periphery of tumor.



We demonstrated the association of melanoma progression with the presence of FOXP3+ T cells, which significantly increase, especially in advanced stages of melanoma (pT2 and pT3.

An interesting finding was the lower numbers of FOXP3+ in pT4 group, which could be hypothesized to be a part of tumor progression.

In comparison levels of IL-35 and PD-L1 reached their highest values in the pT4 group.

Changes in the numbers of regulatory lymphocytes and other immunosuppressive mechanisms constitute a dynamic process that reflects tumor development in the presence of the immune system.

We demonstrated a connection between the expression of the biomarkers and stage of melanoma. Mechanisms of immunosuppression are very complex. Further development of therapeutic options will focus on blocking these mechanisms.

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INTRODUCTION

Tumor growth is a complex and multi-stage process affecting locally many cell types including tumor parenchyma cells and tumor stroma cells. Tumor stroma is composed of fibroblasts, endothelium, immune cells, soluble molecules, and extracellular matrix[1].

Immune cells are a key component of the tumor microenvironment and composed of innate immunity cells represented by macrophages, dendritic cells, neutrophils, and NK cells and antigen specific immunity cells including helper and, cytotoxic T cells and B cells. Tumor-infiltrating lymphocytes (TILs), differ in their numbers significantly in different types of tumors and among individual patients[2].

The immune system provides an effective defense against tumors but during tumor development, the immune response can be diverted allowing tumor growth and uncontrolled tumor cell proliferation[3,4].

A significant association of TILs with overall survival has been reported in many malignancies, including melanoma, in which a higher representation of lymphocytes in the tumor stroma is associated with up to three times higher 5-year survival than in melanomas without TILs. The composition of TILs is not homogeneous concerning to effector/regulatory T cell subtypes in individual patients leading to high survival variability.

Because of its genetic instability, melanoma is considered a very immunogenic type of tumor, as it can produce a large number of antigens that are well recognized by the immune system[5].

T regulatory lymphocytes (Tregs) are the subtypes of $CD4^+$ and $CD8^+$ T cells that inhibit the functions of other immune cells, protecting the body from autoimmunities and excessive inflammation[6]. Forkhead box transcription factor FOXP3, specific marker of Tregs, has been proven both necessary and sufficient for Tregs suppressive activity[7].

Tregs also play an important role in antitumor immunity. They suppress the antitumor immune response and reduce damage to tumor cells caused by the inflammatory environment. Thus, they increase the tumor self-tolerance of the immune system. Distinct phenotypes and functions of Tregs within the tumor have been described including highly suppressive effector Tregs and less phenotypically stable Tregs which may also switch to regular T contributing to tumor elimination[8,9].

The prognostic significance of Tregs is still debatable, as they are associated with a worse prognosis for various types of malignancies such as melanoma, mammary gland cancer, and non-small cell lung cancer, while they indicate a better prognosis for head and neck cancers, colorectal cancer, and lymphomas. Conclusions from several studies highlight the importance of distinguishing Tregs subpopulations based on surface or intracellular expression of markers such as CD25, FoxP3, CD54RA, CCR4, CCR8, CTLA4, or PD-1 which nevertheless is not feasible for routine immunohistochemistry detection[2,8,10].

Tregs suppress effector cells through multiple mechanisms. One of these is the secretion of cytokines such as TGF-β, IL-10 or IL-35. Cytokine production in the tumor microenvironment determines how the mechanisms of innate and acquired immunity influence tumor growth and progression[11].

An important immunosuppressive cytokine involved in immune evasion IL-35 serves as a driver of tumor development. It increases angiogenesis and blocks $CD8^+$ T and $CD4^+$ T cells and NK cells[12]. IL-35 belongs to the IL-12 family, and it is mainly secreted by Tregs and induces the transformation of conventional T cells into Tregs. IL-35 secretion in the tumor microenvironment contributes to an increase in tumor growth[13,14].

A relatively new immune control mechanism involved in the regulation of T cell responses includes immune checkpoints represented by the PD-1/PD-L1/2, CTLA-4/CD80/86, LAG-3/LSECtin, TIM-3/Galectin-9/HMGB-1, and TIGIT/CD155 which has become a promising therapeutic target of immunotherapy[15-17].

PD-1 is a protein expressed on T cells in relation to their maturation and activation, with increased expression on activated T cells. PD-1 interacts with its ligands PD-L1, expressed on T cells, B cells, macrophages, DCs, bone marrow-derived mast cells, and various normal tissues as well as many tumor cells, whereas PD-L2, which is predominantly expressed on antigen-presenting cells[18,19].

The PD-1/PD-L1 interaction plays a crucial role in maintaining peripheral tolerance and is also one of the key factors contributing to tumor suppression. Inhibitors of this pathway augment the T-cell immune response directed against tumor cells and have demonstrable clinical benefit in various types of tumors, including melanoma. Detection of PD-L1 expression represents important predictive marker[20].

MATERIAL AND METHODS

We examined 95 cutaneous superficial and nodular melanomas divided according to the TNM classification into four groups: pT1 (35), pT2 (21), pT3 (21), and pT4 (18). In addition, we examined 25 benign nevi.

All examined samples were fixed with 10% formalin, embedded in paraffin blocks, and cut into 5-μm thick sections. They were then stained using the indirect immunohistochemistry method in an automated processor (Ventana Benchmark XT). We used the following primary antibodies: a monoclonal rabbit anti-FOXP3 antibody (Novus Biologicals, clone SP97, NBP2-12498, diluted 1:150, incubation time 20 min), a monoclonal rabbit anti-PD-L1 antibody (Abcam, clone 28-8, ab205921, dilution 1:50, incubation time 16 min), and a polyclonal rabbit anti-IL-35 antibody (US Biological, clone 141049, dilution 1:50, incubation time 50 min). The Ventana detection kit (Ventana iVIEW DAB Detection Kit, Catalog number 760–091) was used to detect the anti-FOXP3 and anti-IL-35 antibodies, and the Ventana detection kit (OptiView DAB IHC Detection Kit, Ventana Medical System, Catalog number 760–700) was used to detect the PD-L1 antibody.

The expression of all monitored antigens was then evaluated with light microscopy over an area of 1 mm^2 in the places of greatest density (so-called "hot spots"), both in the center of each tumor (C) and on the periphery (P). The periphery was considered to be the invasive edge of the tumor, with a total width of 1 mm, including both the peripheral area of the tumor and the adjacent, closely abutting stroma[21].

We presented patient baseline characteristics as median and range for continuous variables and the quantity with the percentage for categorical variables. Since the data were non-normally distributed according to both the Levene tests and Kolmogorov-Smirnov tests the results were statistically evaluated using the Mann-Whitney test and the Kruskal-Wallis ANOVA test with post hoc multiple comparison. The box plots were used for graphical interpretation and *P*-values of 0.05 or lower were considered statistically significant. All analysis was performed using Statistica V. 13.4.0.14 (Tibco Software Inc., VA, USA).

RESULTS

In the monitored groups pT1-pT4, patients were represented in the ratio of men and women 51:44. The age range of the patients was 23–88 years, with a median of 56 years (Table 1.).

Evaluation of FOXP3, PD-L1, and IL-35 expression

We calculated the median values of FOXP3 expression which increased together with tumor stage both in the center (C) of the lesion (P<0.001) (Fig. 1) and at the periphery P (P<0.001). The sudden drop in the pT4 group is noticeable and interesting. According to Fig. 1, the medians of pT1–3 groups are statistically significant different from control group (P<0.001). However, the median of the pT4 group cannot be considered statistically significantly different from other pT groups and the control group. The median of pT4 group is statistically significant different from pT2 and pT3 groups.

Median values of PD-L1 expression on lymphocytes in the center (C) of the tumor increased with melanoma stage (P<0.001) and, as with FOXP3, decreased with pT4 stage (Fig. 2). In peripheral (P) lymphocytes (Fig. 3), expression increased with tumor progression (P<0.001). PD-L1 expression on tumor cells increases mainly in advanced stages of melanoma compared to early stages (P<0.0065).

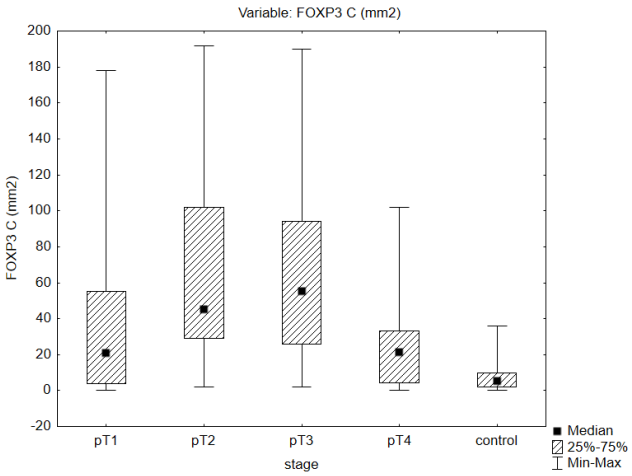

Fig. 1. Evaluation of FOXP3 expression in the center of melanomas at different tumor stages (pT1–T4) and in benign nevi.

Table 1. Characteristics of the investigated group.

Stage	n (%)	Age (median)	Age (min-max)	Men n (%)	Women n (%)
pT1	35 (36.9)	51.5	23–82	18 (35.3)	17 (38.6)
pT2	21 (22.1)	59	39–88	12 (23.5)	9 (20.5)
pT3	21 (22.1)	56.5	38–86	11 (21.6)	10 (22.7)
pT4	18 (18.9)	59	42–80	10 (19.6)	8 (18.2)
Sum	95	56	23–88	51	44

Table 2. Comparison of median cell numbers per 1 mm^2 among pT1–pT4 melanomas and benign nevi.

Markers	Staining structures	Diagnosis					*P*
		pT1	pT2	pT3	pT4	nevi	
FOXP3	lymphocytes C	22	45	52	18.5	5	< 0.001
	lymphocytes P	6	15	12	2.5	0	< 0.001
PD-L1	tumor cells	1	1	3	4.5	0	< 0.0065
	lymphocytes C	0	2	7	2	0	< 0.001
	lymphocytes P	3	6	16	48	0	< 0.001
IL-35	lymphocytes C	0	1	5	10	0	< 0.001
	lymphocytes P	3	7	32.5	37.5	0	< 0.001

C, center; P, periphery; *P*, *P*-values.

IL-35 expression levels increased continuously with increasing melanoma stage in the pT1–pT4 groups, both in the center (P<0.001) and at the periphery of the lesions (P<0.001) (Fig. 4).

DISCUSSION

In this study we evaluated the expression of FOXP3, PD-L1, and IL-35 in tumor infiltrating T lymphocytes in different stages of melanoma.

The tumor stroma is a very heterogeneous environment composed of a number of interconnected and cooperating components, including fibroblasts, epithelial cells, blood vessels, smooth muscle cells, extracellular matrix proteins, and cells of the immune system. The interaction of these components leads to a violation of normal homeostasis with the induction of angiogenesis, inflammation, changes in the extracellular matrix, and an increase in protease activity. These events then contribute to the differentiation, proliferation, migration, and invasion of tumor cells[1].

Tumor-infiltrating leukocytes have different immunophenotypes and functions (effector and regulatory T and B cells, NK cells, macrophages, and dendritic suppressor cells). They are thus able to express various immune gene products that modulate the tumor microenvironment and affect tumor development. Studies have shown that melanomas with a better prognosis have an inflammatory infiltrate composed of numerous $CD3^+$ T cells, small numbers of $CD20^+$ B cells, $CD138^+$ plasma cells, and variable numbers of $CD1a^+$ Langerhans cells. Tregs are an immunosuppressive subtype of $CD4^+$ T lymphocytes that express CD25 and FOXP3. In melanomas, their high number helps the tumor evade the antitumor immune response[10,22].

In addition, FOXP3 is expressed in various tumor and nontumor tissues outside the Treg cells, with two opposite types of expression. On the one hand, FOXP3 expression has been demonstrated in normal epithelium and in tissue of the mammary gland, prostate, ovary, and brain, with downregulation in the respective tumor cells. On the other hand, FOXP3 is overexpressed in tumor cells of the pancreas, melanoma, leukemia, hepatocellular carcinoma, bladder carcinoma, thyroid gland carcinoma, and cervical carcinoma[23]. FOXP3 expression in tumor cells has been associated with tumor proliferation, metastasis, drug resistance, and prognosis.

Studies have repeatedly demonstrated a clear association between infiltration by Tregs and poor prognosis of malignant tumors, which is consistent with their presumed immunosuppressive function that enables tumor progression. However, it remains unclear at what stage of tumor progression, from the formation of the earliest preneoplastic lesions to a fully developed invasive tumor, the induction and infiltration of Tregs is important. In pancreatic cancer, stromal infiltration of Tregs begins as early as the preneoplastic stage, and their numbers then correlate with a higher stage of the disease[24].

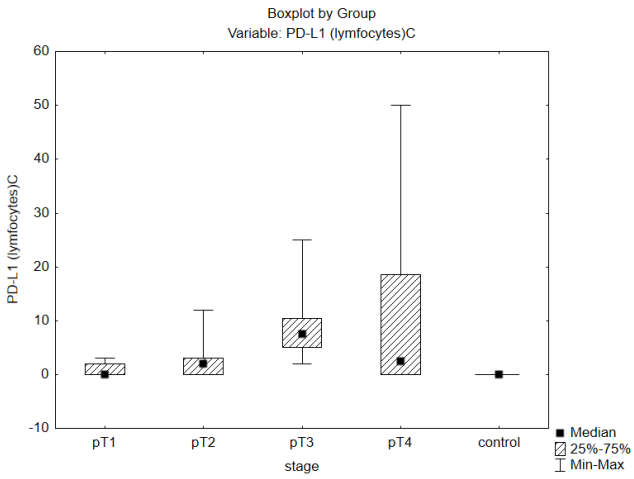

Fig. 2. Evaluation of PD-L1 expression on lymphocytes in the center of melanomas at different tumor stages (pT1–T4) and in benign nevi.

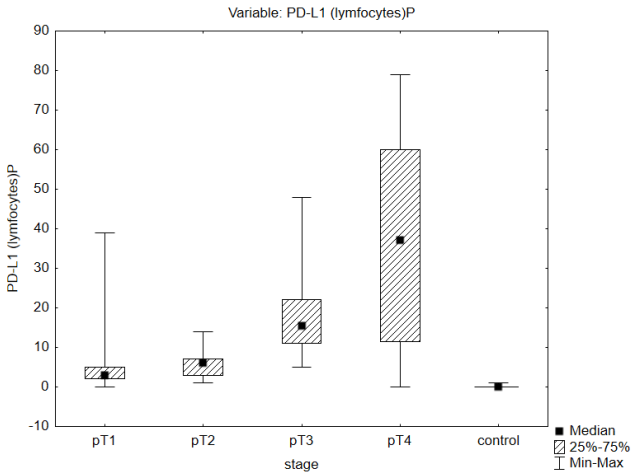

Fig. 3. Evaluation of PD-L1 expression on lymphocytes at the periphery of melanomas at different tumor stages (pT1–T4) and in benign nevi.

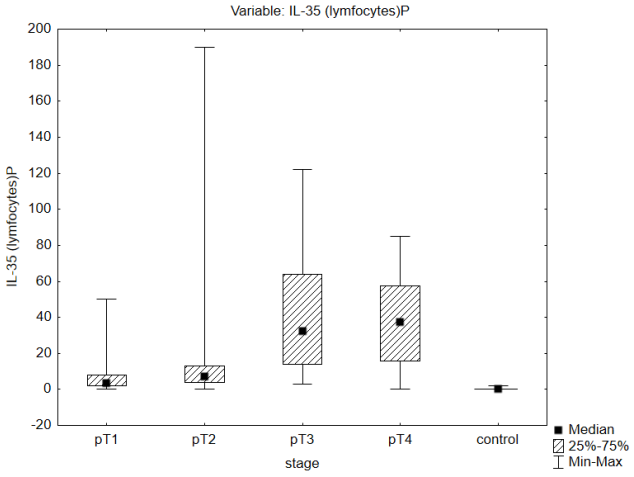

Fig. 4. Evaluation of IL-35 expression at the periphery of melanomas at different stages of disease (pT1–T4) and benign nevi.

Significantly increased numbers of Tregs are also found in colorectal cancer (CRC) in comparison with noncancerous tissue. However, the association of higher levels of Tregs with prognosis remains controversial and their presence may not always be associated with worse prognosis. The expression of FOXP3 on CRC tumor cells and Tregs was compared. High levels of FOXP3 on tumor cells were associated with worse prognosis than lower levels due to increased secretion of immunosuppressive cytokines into the tumor tissue microenvironment, which allowed tumor cells to circumvent the antitumor immune response. An interesting finding was the correlation with FOXP3 levels on Tregs showing the opposite effect[25].

This may be explained by the protective role of Tregs related to their ability to reduce the development of an aggressive, cytotoxic, and potentially proliferogenic cytokine milieu that underlies the inflammation-driven progression of malignant disease, probably due to the antiproliferative effect of TGF-β on Tregs[26].

Despite numerous studies describing the relationship of Treg levels to the prognosis of various tumors, the mechanisms by which regulatory lymphocytes induce immunosuppression are not completely known. Some data indicate the role of cell-to-cell contact and the formation of a cytokine environment that includes IL-10, TGF-β, IL-35, and many others. Furthermore, other inhibitory signals are produced directly by Tregs such as adenosine or indoleamine 2,3-dioxygenase[27].

Thus, Tregs cause immunosuppression through multiple mechanisms. The production of cytokines contributes to a long-lasting immunosuppressive state. Tregs can be divided into two groups that produce cytokines with different functions. The first are IL-35-Tregs predominantly producing IL-35 and expressing intermediate levels of CCR7 (chemokine receptor type 7), which are preferentially localized on T cells. The second are IL-10 Tregs that produce high levels of IL-10, ICOS (inducible T-cell co-stimulator), granzymes, and multiple chemokine receptors responsible for migration to peripheral nonlymphoid tissues. Unlike IL-10, which can target a variety of cell types including dendritic cells, macrophages, and T cells, IL-35 is more specific for T lymphocytes[28,29].

Overexpression of IL-35 is demonstrated in many nonneoplastic diseases including inflammatory and coronary artery diseases, and its deficiency leads to a worse course of diseases such as encephalomyelitis, liver fibrosis, and autoimmunity. However, high levels have also been demonstrated in the tumor microenvironment, where IL-35 is produced by both tumor cells and Tregs and is associated with a poor prognosis in a number of cancers (such as lung, stomach, and breast). Among the important functions of IL-35, in addition to the already mentioned immunosuppression of T cells, promotion of angiogenesis could be mentioned. Conversely, blocking of IL-35 activity leads to increased levels of $CD8^+$ T lymphocytes[30, 31].

However, different conclusions were drawn for IL-35 expression in colorectal cancer. Zhang et al.[32] correlated low levels of IL-35 in CRC with poor prognosis. Ma et al.[33], on the other hand, reported that high levels of IL-35 are associated with poor CRC progression, and especially with an increased ability to metastasize.

IL-35 is required for the maximal suppressive function of Tregs and contributes to the regulatory milieu in numerous disease states. Previous work has demonstrated an increase in IL-35 production by tumor-infiltrating Tregs in experimental mouse models (B16 tumors) and the role of this cytokine in promoting the expression of inhibitory receptors PD-1 and others[33].

Activation of the PD-1/PD-L1 pathway leads to immune escape by tumor cells, and inhibition of this pathway is the basic principle of immunotherapy. PD-L1 expression is of great importance on melanoma tumor cells, non-small cell lung cancer including adenocarcinoma and squamous cell carcinoma, and gastric cancer with microsatellite instability, where PD-L1 expression by immune cells is an important indicator of overall survival[19,20].

Likewise, PD-L1 has been shown to play a role in the induction and maintenance of Tregs, leading to their expansion in the tumor microenvironment, where these induced Tregs inhibit the T cell response. Consistent with this, inhibition of PD-L1 led to a decrease in the formation of Tregs[35].

Giavina-Bianchi, et al.[36] assumed that the expression of PD-L1 in nodular melanoma is a manifestation of an adaptive immune mechanism that is related to the presence of TILs. PD-L1 was expressed in only 10% of nodular melanomas in which TILs were evaluated (according to Clark's classification) as "absent"; while PD-L1 was expressed in 23% and 25%, respectively, of nodular melanomas in which TILs were evaluated as "non-brisk" or "brisk". However, other studies have not confirmed this connection and consider PD-L1 expression to be heterogeneous and independent of TILs.

PD-1/PD-L1 receptors are both important regulators of tumor microenvironment development, and administration of antibodies against either of these receptors has a therapeutic effect in various cancers. A number of studies have shown a relationship between IL-35 expression and the PD-1/PD-L1 pathway, both in mouse models, where elimination of IL-10 and IL-35 reduces PD-1/PD-L1 expression, and in various types of human cancers[37].

PD-L1 has become an important potentially predictive marker for a number of tumors, including melanoma. Several studies have demonstrated a better therapeutic response of anti-PD-L1 therapy in patients with PD-L1–positive tumors compared with PD-L1–negative tumors, although the limited value of a negative finding is well recognized. Depending on the study, therapy appears to be beneficial in 20–40% of cases[38,39].

Here, we demonstrated the association of melanoma progression with the presence of $FOXP3^+$ T cells which significantly increase, especially in advanced stages of melanoma (pT2 and pT3), compared with benign nevi, in which the FOXP3+ T cells expression was negligible. We detected $FOXP3^+$ cells mainly in the central area of the tumor rather than in the invasive area on its periphery. An interesting finding was the lower numbers of Tregs in the pT4 group. This reduction in Treg cell numbers can

be hypothesized to be a part of tumor progression, as the levels of the other monitored markers, IL-35 and PD-L1, reached their highest values in pT4. Thus, in contrast to other studies showing that the level of IL-35 expression usually follows the number of Tregs, especially FOXP3$^+$ Tregs, in different tumor types, the sudden reduction of Tregs in the advanced stage of melanoma that we observed indicates a new aspect of melanoma progression. It indicates that the high levels of IL-35 in pT4 melanoma are maintained by cells other than FOXP3$^+$ Tregs. Liu, et al.[38] relate the production of IL-35 to a whole range of other cells, either the tumor cells themselves or Bregs (regulatory B lymphocytes). However, we did not monitor the expression of these markers in this work.

Changes in the numbers of regulatory lymphocytes and other immunosuppressive mechanisms constitute a dynamic process that reflects tumor development in the presence of the immune system. It is logical that tumors of a higher stage are most adapted to the immune system.

CONCLUSION

This article demonstrates a connection between the expression of the biomarkers FOXP3, PD-L1, and IL-35 and the stage of cutaneous melanoma. It is evident that the mechanisms of immunosuppression are very complex and mutually influence each other. It is therefore logical that further development of therapeutic options should focus on blocking these mechanisms which are currently used to disrupt the PD-1/PD-L1 interaction, allowing reactivation of immunity against tumor cells.

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Author contribution: VZ: summarized the data and prepared the manuscript, figures and tables; PH: did the literature research and relevant studies; JS, JE: prepared all the tables; TH: prepared the immunohistochemical slides; DP: did the statistical analysis; YV, SM: clinical information.

Conflict of interest statement: All authors declare that there is no conflict of interest.

Ethical approval: The study was approved by the Institutional Ethics Committee of University Hospital Ostrava, number 305/2023.

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