Tumor infiltrating lymphocytes in malignant melanoma – allies or foes?

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This is an overview of current problematics regarding the role of tumor infiltrating lymphocytes (TILs) in malignant melanomas. Various and often conflicting data have been published, correlating tumor type, stage, prognosis, as well as sex and age of patients. This is partly due to heterogeneity in scaling systems and unstandardized TILs grading but also due to changes of tumor-host interactions. Melanomas are an immunologically heterogeneous group with variability of TILs, where distinct gene expression patterns were found in tumors with absent, and/or non-brisk TIL grade versus brisk TIL grade. However, the presence of TILs alone appears to be inadequate for implicating them as immunologically functional. Further characterisation of TIL phenotype and function is warranted. This especially concerns, evaluation of TILs of the suppressor phenotype but rather than as a prognostic factor, more for prediction of targeted immunotherapy.

Key words: melanoma, tumor infiltrating lymphocytes, regulatory lymphocytes, immune check point

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INTRODUCTION

Tumor growth is a complex process that involves numbers of interactions between tumor cells and immune host system through multiple cellular and molecular factors in the tumor specific microenvironment. Many genetic and epigenetic alterations in cancer cells provide a diverse set of antigens¹, which are recognized by the immune system. An effective cancer immune surveillance system is able to destroy premalignant and malignant cells, but its avoidance can be considered as a hallmark of cancer. The term “immunoediting” has been proposed to describe the events when the immune system interacts with malignant cells during the course of cancer². Interaction between tumor and immune cells can crucially determine the outcome of the disease. Anticancer immune response is a very complex process, where T lymphocytes which are able to recognize specific neoantigens, play a key role. Tumor infiltrating lymphocytes (TILs) are lymphocytes in close association with cancer cells and have infiltrated and disrupted nests of malignant cells. They infiltrate the central areas of tumors as well as their edges in a neighbourhood with healthy non-tumorous tissue. As they interact most closely with the cancer cells, they are likely to more accurately reflect tumor-host interactions. Increased density of TILs is recognized as a sign of efficient anti-tumor immunity and generally associated with a better outcome. There are still many obscurities and questions deserving clarification.

Melanoma is considered an extremely immunogenic neoplasm. This is partly due to its higher genomic instability leading to formation of many neoantigens which are

recognized by immune cells. Activation of the immune system is observed even in dysplastic nevi, which are taken as melanoma precursors. We know that the immune response cannot directly prevent melanoma formation; however, it can make a difference to the disease outcome³. The melanoma immunogenicity is reflected by the density of lymphocytic infiltrate surrounding the malignant cells, as well as by the relatively high grade of melanoma spontaneous regressions. What is interesting is that despite this regression, which is an obvious result of local elimination of malignant cells by immune cytotoxicity, in many cases there was metastatic spread of tumor cell, which is a prognostically negative feature⁴. Better understanding of an effective antitumor immune response in patients with melanoma is desirable for assessment of a disease prognosis and prediction, as improved survival has been shown by blocking mechanisms that are responsible for immune down regulation^{5,6}.

Quantification of TILs in melanoma

One of the most frequently used scoring system is Clark’s method grading TILs as absent, non-brisk, or brisk^{7,8}. Other studies grades TILs infiltrate as a range of 0 to 3 or even only as absent or present^{9,10}. Heterogeneity in scaling system and a lack of standardization of TILs grading method contributes to limitation and diversity of results in the current literature on the prognostic value of TILs, along with the heterogeneity of patients’ selection criteria and often small sample sizes. This may explain the often controversial data regarding variability in numbers of TILs according a melanoma type and its outcome.

TILs and the age and sex of patients

Age is an independent negative predictor of melanoma survival. Multiple factors may contribute to this observation including decline in host antitumor immune response with aging. It is not so surprising that a higher TIL grade has been found to be more common in younger patients¹¹. On the other hand, once brisk TIL grade in old patients was found, it was associated with prolonged melanoma-specific survival¹². Evaluation of TILs may so have greater prognostic value in patients above 45 years¹².

It has been documented that sex also affects clinical outcomes of melanomas, as women tend to exhibit a more favourable melanoma-specific prognosis in comparison to men. Women have also been reported to have better outcomes in overall survival (OS) and a lower tendency for metastasis. A relationship between sex and sentinel lymph node (SNL) positivity has been inconsistently reported. The precise mechanism for this sex-specific prognosis still remains unclear, it is probable that varying patterns of immunity might be involved. SNL metastases were associated with brisk TILs among men but not in women. Also among men prolonged OS was associated with presence of brisk TILs. However, no association between TIL status and OS among women has been found¹³.

However, there are also opposite data, where age difference and male to female ratio did not differ between different grades of TILs (ref.³).

TILs and melanoma type

Concerning melanoma type, there is not full consensus regarding relation between histological type and intensity of TILs. Whereas some authors found no differences, especially between more frequent melanoma types as acral lentiginous, lentigo maligna, nodular, superficial spreading³, others documented their significant decrease in some types as are desmoplastic and nodular melanomas¹². These melanomas have a less favourable prognosis, based on their apparently aggressive clinical behaviour.

TILs and worse pathologic characteristic

Various, often conflicting data have been published regarding worse melanoma histopathologic features such as ulceration, increased mitotic activity or formation of satellite foci. Generally, absence of infiltrates was found in tumors with increased mitotic activity and ulceration¹⁴. Significant correlation also exists with diminished rate of microsatellitosis and increased numbers of TILs (ref.³).

TILs and melanoma stage and prognosis

Melanomas with brisk density are more frequently associated with better prognosis than tumors in which lymphocytic infiltrate was absent or low. But only brisk TILs have been shown to improve recurrence-free survival and overall survival compared to non-brisk or absent metastatic melanomas, where TILs are low or absent^{12,15}. Several studies found that TILs response as a major predictor of sentinel lymph node metastasis, whereas metastasis decreased with increasing incidence of TILs (ref.^{3,16}). However, others published that TILs did not predict sur-

vival¹⁷ and there is no association of their presence and improved melanoma prognosis^{11,17-19}.

It is assumed that one of major determinants of immune cell infiltration is the stage of disease where host immune answer decreases with increasing tumor thickness²⁰. Consistent results are published for advanced T4 melanoma. In T1-T3 stages, however, there are diverse data where even T3 tumors may have higher TILs, while in T1 or T2 melanomas their density may be low²¹. It also has been described that the distribution of TILs is heterogenous, and in more advanced stages they become more accumulated at the peripheral areas associated with invasiveness. Heterogeneity of TILs distribution should be taken in consideration as it seems to support tumor progression²¹. The paradox that presence of TILs does not always correlate with improved prognosis may be explained by the presence of different lymphocyte subtypes²². Hence, the role of TIL has been studied with an increased interest.

The role of CD8+T lymphocytes

CD8+T cells belong to main subsets of T cells that constitutively mediate an effective antitumor response. Their transfer to patients with melanomas has been shown to be associated with beneficent therapeutic effect. While CD8+T cells can produce Th1 (T helper type 1) cytokines when activated, their primary functions is production of cytotoxic proteins, mainly perforin and granzyme, which are secreted at the point of the immunologic synapse – the place of contact with the target cell. Then specific killing without bystander cellular damage is a result of the process. Perforin is a protein disrupting cellular membrane and it also facilitates the ability of granzymes to induce apoptosis in the target cell²³.

Antigens that are expressed on cancer cells and recognized by T lymphocytes are used in tumor-specific immunotherapy. However, the effectiveness of currently available therapeutic strategies is limited, as only about 5% of vaccinated patients with metastatic melanoma exhibited a complete or partial clinical response, whereas additional 10% showed limited evidence of melanoma regression without clear clinical benefit²⁴.

The role of CD4+ T lymphocytes

Important role for activation and maintenance of immune response against tumor cells also belongs to CD4+ T lymphocytes. Although these cells have ability to eliminate tumor cells in the absence of cytotoxic lymphocytes, more often both cell types are required for tumor rejection²⁵. As the main effector mechanism of antitumor immune response is direct cells lysis through the major histocompatibility complex (MHC) class I recognizing CD8+ CTL (cytotoxic lymphocytes), the key role of CD4+ T cells is in the activation of CTL. Based on their cytokine profile, CD4+ T-cell response can be sub-classified into different types. Th1 cells, which typically produce interleukin-2 (IL-2), lymphotoxin α (LT α) and interferon- γ (IFN γ) are able to induce macrophage activation, and also proliferation and activation of CD8+ T cells²⁶. Th1

response is generally correlated with a better cellular immune activation²⁷. In contrast, Th2 (T helper type 2) cells produce IL-4, IL-5, IL-10 and IL-13 and tend to elicit humoral immunity. In some reports, Th2 and their cytokines have been shown to down regulate antitumor activity²⁸. A shift from a Th1 to a Th2 cytokine profile is considered to be a major contributor to the failure of T cell mediated immunity. On the contrary, there are many opposite data that Th2 specific clones have been demonstrated to strong anti-tumor activity. The mechanisms how Th2 cells destroy tumors are not fully understood, but it may be through the activation of innate immune response, such as eosinophils and macrophages which in turn secrete superoxide and nitric oxide. Effective anti-tumor immunity seems to be a result of balance cooperation between Th1 and Th2 cell types.

Melanomas with present TILs represent an immunologically heterogeneous group

A gene expression analysis identified immunologic heterogeneity among melanomas depending on the intensity of lymphocytic infiltrate. Melanomas with absent and non-brisk TIL grades had the most similar immunoregulatory gene expression patterns and were immunologically distinguishable from tumors with brisk TIL grade. This group of melanomas had gene expression profile associated with T lymphocyte activation which encompass T helper cell differentiation, T cell receptor, dendritic cell maturation, T cell co-stimulation (CD28, CD5), cytokine signalling (IL-2), and other signalling pathways like interferon, Toll-like receptor and JAK/STAT (Janus kinase/Signal Transducers and Activators of Transcription) signalling. Moreover, immune checkpoint regulators such as PD-1 (Programmed cell death 1) and CTLA4 (Cytotoxic T-Lymphocyte Associated Protein 4) were more inhibited. It could be emphasized that melanoma with brisk TILs represent a distinct immunologic entity¹².

Adverse effect of TILs

Despite the well established immunogenicity of many melanomas, it is obvious that immune answer often fails to regulate and/or inhibit tumor progression. Several local factors have been described to be responsible for attenuation of effective immune response, and an immunosuppressive microenvironment is created. In this way, immune cells may not only help cancer cells to escape immune surveillance, they are able even to support tumor progression.

T regulatory lymphocytes (Tregs)

Tregs are CD25⁺ CD4⁺ T lymphocytes, they are considered to be one of the main regulatory cell types which inhibit and effector functions by dampening the T-cell-mediated immune response against the tumor cells^{5,29-31}. The transcription factor FOXP3 (forkhead box P3) plays a key role in CD4⁺ CD25⁺ regulatory T cell function and differentiation and represents a specific marker for these cells³². FOXP3 is a member of the forkhead or winged helix family of transcription factors^{33,34}. Tregs can be recruited in the periphery from conventional CD4⁺ helpers

(adaptive/induced cells) where T lymphocytes infiltrating the area of a tumor may be compromised or may adversely adapt to the suppressive environment to promote growth instead of regression³¹. Their majority is generated during normal process of maturation in the thymus. Recent reports suggest a role for TGF- β (transforming growth factor β) in generation of Tregs from CD4⁺ CD25⁻ precursors³⁵.

Tregs account for only 5-10% of CD4⁺ cells, their activation is associated with inhibition of cytotoxic lymphocytes and NK (Natural Killer) cells³⁶. However, the precise role of Tregs in cancer development and progression is not clear. In many studies Tregs were proved to promote growth of many cancers. They are responsible for immune tolerance against tumor antigens by dampening the T cell mediated immune response, which enable malignant cells to evade the immune destruction. Mechanism of their immunosuppression is dependent on a mutual intercellular contact which leads via granzyme and/or perforin dependent pathway to inhibition of T cell proliferation. Also in malignant melanoma recent studies have documented that the presence of Foxp3⁺ Tregs in the microenvironment may significantly contribute to the immune resistance. This is supported by results of both experimental animal melanoma models as well as patient studies where high numbers of circulating Tregs were associated with rapid progression of the disease. The presence of FOXP3 lymphocytes in primary cutaneous melanomas were associated with a higher frequency of metastases in the sentinel lymph node^{37,38}. FOXP3 expression in melanomas so seems to be associated with worse overall survival and FOXP3⁺ Tregs are thought to be predictive of patient survival as a marker of early metastatic spread or recurrence of the disease^{22,39,42}. Moreover, therapeutic inhibition of Tregs has been proved to impair their immunosuppressive effect, which is associated with better outcome of the disease⁴³.

Higher densities of Treg were observed in more advanced stages of melanoma in a vertical growth phase and in metastatic melanomas^{22,39,42}. In more advanced stages of melanomas the most pronounced changes in CD3⁺/Treg ratio in behalf of Tregs have been also found. What is interesting that higher accumulation of Tregs was predominantly at the periphery of tumors, at the front line in the neighbourhood of benign tissue. This finding, indicating that advanced tumors create an immunosuppressive microenvironment, may explain their resistance against immune destruction^{21,44}. Moreover, heterogeneity of lymphocytic infiltrate should be taken into consideration, where immunosuppression in the front line may promote melanoma progression. Surprisingly, some T4 stages of melanoma exhibited low Treg density which may be a feature of a severe failure of immune system, and be another feature of higher aggressiveness of the tumor as a presence of Tregs reflects at least partially preserved immune functionality. Similar changes were also for example described in colorectal carcinomas⁴⁵.

Immune Checkpoints

Immune checkpoint molecules refer to a group of immune receptors that upon engagement with their ligand

transmit inhibitory signals suppressing effector function. In this way cancer may evade anti-tumor immunity⁴⁶. Programmed cell death signalling pathway (PD-1/PD-L1) (Programmed death-ligand 1) has become one of the most discussed inhibition pathways. PD-1 is expressed on many immune cells; among them T lymphocytes belong to the most important. PD-1 is activated by its ligands PD-L1, also known as B7-H1 protein and PD-L2 (Programmed death-ligand 2) (ref.^{47,48}).

The engagement of PD-1 by its ligands induces apoptosis or exhaustion in activated T cells and some cytokines production, such as IFN- γ and IL-2. The result is lymphocyte deletion and establishment of immunological tolerance⁴⁹. Compared to PD-L2 that can be found only in activated dendritic cells and macrophages, PD-L1 is expressed by T and B cells, macrophages and often on many tumors including melanoma^{50,51}.

Once TILs recognize tumors they start to produce IFN- γ and other pro-inflammatory cytokines up-regulating PD-L1 expression. PD-L1, in turn, may ligate PD-1 antigen on lymphocytes inducing their down regulation. This adaptive PD-L1 expression in the melanoma microenvironment has been associated with an improved prognosis. It also may be a predictor of anti-PD1/PD-L1 targeted therapy response.

On the other hand, constitutive PD-1 expression by tumor specific T cells was associated with the induction of inhibitory receptors expression followed by an impairment of T cell functions, tumor cells may then escape upon ligation to PD-L1. This is for example accompanied by loss of PTEN (Phosphatase and tensin homologue) with consequent activation of PI3K (Phosphoinositide 3-kinase) pathway. Constitutive PD-1 expression so constitutes a form of immune adaptation and tumor tolerance.

Considering up-to now published data, there is an ambiguous and not fully understood role of PD-1 in creation of efficient or ineffective T lymphocyte responses⁵². In spite of an indisputable inhibitory signalling upon ligation with its ligands, it is now clear that PD-1 expression is a first marker of T cell activation allowing the identification of the tumor reactive CD8⁺ T cell population in melanoma tumors^{53,54}. Moreover, it is a sign of high avidity of cytotoxic lymphocytes to specific neoantigens, the level of PD-1 expression so reflects functionality of specific T cells.

The complex regulation of PD-1 expression unequivocally demonstrates that PD-1 expression status alone cannot distinguish between exhausted and activated T cells that are the result of distinct genetic and epigenetic programs dictated by T-cell receptor signalling strength and microenvironment. It also explains the fact that, despite PD-1 undisputed clinical effectiveness compared to chemo- or radiotherapy, anti-PD-1 monotherapy remains inefficient in more than 60% of cancer patients⁵⁵.

There are studies highlighting that PD-L1 expression may depend on melanoma subtype. Melanomas with high UVC radiation exposure (high cumulative sun damage) had the highest expression of PD-L1; it reflects high mutational load in these tumors and provide a possible ex-

planation that these patients had high rates of responses to anti-PD-1 monotherapy. By contrast, uveal, acral and mucosal melanomas demonstrate the lowest TIL densities and PD-L1 positivity which may be due to the fact they have the least genomic instability. These melanoma subtypes also exhibit low response rates to anti-PD-1 monotherapy.

Furthermore, PD-1 expression seems to be dependent on tumor thickness with higher density in T3 and T4 melanomas. Also distribution of these lymphocytes is not homogenous, they tend to be increased at the tumor edges²¹. Peripheral areas, where malignant cells meet neighbour microenvironment, seem to be more critical for the fate of the lesion.

CONCLUSION

Melanoma represents immunologically heterogeneous group with variability of TILs predominantly in dependence on the type and stage of the disease with inconsistent results regarding outcome of the disease. The presence of TILs alone is not enough to implicate them as immunologically functional. In light of recent studies interested in the possible benefit of TILs to determine therapeutic outcome, further characterisation of TIL phenotype and their function seems to be important. Especially, evaluation of TILs focusing on suppressor phenotype is essential, but rather than as a prognostic factor, more for prediction of targeted immunotherapy. However, still some degree of ambiguity exists, which will require further investigation.

ABBREVIATIONS

TILs, Tumor infiltrating lymphocytes; OS, Overall survival; SNL, Sentinel lymph node; Th1, T helper type 1; MHC, Major histocompatibility complex; CTL, Cytotoxic lymphocytes; IL-2, Interleukin-2; LT α , Lymphotoxin α ; IFN γ , Interferon- γ ; Th2, T helper type 2; JAK/STAT, Janus kinase/Signal Transducers and Activators of Transcription; PD-1, Programmed cell death 1; CTLA4, Cytotoxic T-Lymphocyte Associated Protein 4; Tregs, T regulatory lymphocytes; FOXP3, Forkhead box P3; TGF- β , Transforming growth factor β ; NK, Natural Killer; PD-L1, Programmed death-ligand 1; PD-L2, Programmed death-ligand 2; PTEN Phosphatase and tensin homologue; PI3K, Phosphoinositide 3-kinase.

Search strategy and selection criteria

Our research strategy was focused on evaluating of studies on the role of tumor infiltrating T lymphocytes in cutaneous malignant melanoma progression. In particular, we aimed at T regulatory lymphocytes and immune checkpoint molecules PD-1/PD-L1. Scientific articles from 1989 to 2019 were searched using the PubMed, Web of Science databases, and Researchgate. All searches were up to date as of March 2019. The searched terms

included melanoma, tumor infiltrating lymphocytes, T regulatory lymphocytes, immune checkpoints, stage, prognosis. Czech and English language papers were reviewed.

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