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ROLE OF TUMOR-INFILTRATING IMMUNE CELLS IN INFLUENCING THE EFFECTIVENESS OF ANTICANCER TREATMENT IN PATIENTS WITH MELANOMA, HEAD AND NECK, OR RECTAL CANCER

Tumor-infiltrating immune cells as predictive markers in metastatic melanoma patients treated with ipilimumab

Immunotherapeutic modalities of cancer treatment have been increasingly gaining ground in the past few years. Understanding the mechanisms regulating antitumor immune response led to development of a new class of immunotherapeutic agents targeting molecular interactions blocking T-cell activation, the so called immune checkpoint inhibitors (ICIs) (1, **2**). The first such agent, ipilimumab which blocks CTLA-4 (cytotoxic T lymphocyte-associated antigen 4), was added to the therapeutic arsenal of advanced melanoma in 2011 (3, 4). It also paved the way for agents targeting other immune regulatory pathways, of which antibodies blocking PD-1 or its ligand, PD-L1 represent the most promising treatment modality in a widening spectrum of tumor types (**5-7**). In a significant part of responding patients, immune checkpoint agents induce durable remission, showing unprecedented clinical efficacy in this patient population with advanced stage cancers.

Nevertheless, generally only a smaller proportion of patients benefit from immune stimulating antibody therapy; the mechanisms underlying immunotherapy resistance are poorly elucidated. On the other hand, serious side effects, often immune-related, may occur in a higher percentage of patients, especially in the case of ipilimumab and combination strategies. In order to improve the benefit/risk ratio of individual patients, it is of primary importance to search for biomarkers that could predict the likelihood of therapeutic effect (**5**, 8-**10**). At present, no validated predictive biomarkers are available for routine clinical use of ICIs. For ipilimumab, several candidates have been suggested, mainly concerning peripheral blood immune cells or serum factors. Few studies analyzed the role of tumor-infiltrating immune cells in predicting the efficacy of CTLA-4 inhibitors, linking therapeutic effects with baseline staining for the immunosuppressive enzyme indoleamine-2,3-dioxygenase as well as with FOXP3 regulatory T cells but not with other immune cell types studied (11-**13**).

The aim of our study was to explore tumor-infiltrating immune cell types as potential biomarkers predicting response to treatment and survival in melanoma patients receiving ipilimumab therapy. Archived paraffin blocks of surgical tissue samples were collected from patients with metastatic melanoma who received ipilimumab treatment from 2010 to 2014 at four centers in Hungary (National Institute of Oncology, University of Szeged, University of Pécs, University of Debrecen). The necessity of inclusion of the latter three centers, in order to obtain a sufficient number of cases to study, became evident during the work. We restricted sample collection to metastases operated within one year before ipilimumab therapy, in an attempt to minimize potential changes in immune microenvironment during time elapsed between surgery and ipilimumab treatment. Furthermore, we evaluated surgical samples instead of small biopsies, and more than one metastasis per patient when possible, to reduce the confounding effect of intrapatient heterogeneity. The study population consisted of 30 patients (1-25 lesions per patient). Eighty-six samples were selected for the investigations, 52 lymph node metastases and 34 subcutaneous/cutaneous metastases.

We determined the intratumoral density of immune cells expressing different markers: CD4, CD8, CD45RO (T-cell subsets), CD20 (B lymphocytes), CD134, CD137, PD-1 (activation markers/immune checkpoints), FOXP3 (regulatory T cells), NKp46 (NK cells), CD68 (macrophages), CD16 (Fcγ receptor III). The latter three markers (not involved in the original plan) were included in the studies because of recent reports suggesting the possible involvement of regulatory T cell elimination by ADCC effectors as a mechanism of effect of ipilimumab (14-16). For all cell types except $CD68^+$ macrophages and $NKp46^+$ NK cells, density values were significantly lower in subcutaneous/cutaneous lesions compared to nodal metastases. Because of the large difference in the amount of immune cells between the two locations, the prognostic and predictive associations of immune cell infiltration were also evaluated separately in the two groups of metastases.

Intratumoral infiltration of the labeled cells was evaluated in relation to response to ipilimumab treatment and disease outcome. Patients were categorized in two groups according to clinical efficacy of ipilimumab, showing either complete or partial response, or stable disease for at least 6 months ("responders") or none of the above ("nonresponders"). In lymph node metastases, the density of $CD4^+$, $CD8^+$, $FOXP3^+$, $CD134^+$ lymphocytes, $CD20^+$ B cells and $NKp46^+$ NK cells were higher in the responder group compared to nonresponders; the most significant difference was observed in the case of the FOXP3 and CD8 markers. In subcutaneous/cutaneous metastases, on the other hand, significant difference between responders and nonresponders was found only in the proportion of patients with high mean density of $CD68^+$ macrophages and $CD16^+$ cells (**17**).

Kaplan-Meier analysis of survival according to the mean immune cell density in lymph node metastases revealed that high densities were associated with significantly longer overall survival (OS) in the case of 7 of the 11 cell types studied. The potential prognostic effect of immune cell densities evaluated as continuous variables (together with disease stage, patients' age and gender, ECOG status, number of organs involved, LDH level and previous treatments) was also analyzed using Cox's proportional hazards model. In univariate analysis $CD4^+$, $CD8^+$, $CD45RO^+$, $FOXP3^+$ and $CD16^+$ cell densities were found significantly associated with overall survival, besides ECOG status and LDH. Multivariate analysis including all immune cell density values as well as clinicopathologic parameters identified ECOG status and $FOXP3^+$ cell density as significant independent predictors of survival. Similar associations were found when all samples were evaluated together, either using Kaplan-Meier analysis or Cox regression. In multivariate analysis, LDH and the amount of $FOXP3^+$ cells proved independent predictive factors. In the s.c./cutaneous location, on the other hand, the mean density of $CD16^+$ and $CD68^+$ cells showed correlation with OS both in Kaplan-Meier analysis and Cox's proportional hazards model; in this group only LDH level proved independent predictor of survival (**17**).

Our results demonstrating association of T-cell density with the efficacy of ipilimumab therapy fit well the recent hypothesis suggesting different sensitivity of the so called "T-cell-inflamed" and "non-inflamed" tumors to the various immunotherapeutic approaches, with higher probability of T-cell-rich tumors to benefit from immunotherapies based on blocking immune suppressive mechanisms (18). In support of this hypothesis, $CD8^+$ T-cell density in pretreatment biopsies proved predictive of response of melanoma patients to pembrolizumab treatment (19), however, no such associations between the amount of infiltrating T-cell subsets and clinical activity were apparent in the case of other anti-PD-1/PD-L1 agents or CTLA-4 blocking antibodies (11, 12, 20, 21). A possible explanation for the lack of predictive power of T-cell density in some studies could be that they analyzed together metastases of different locations, from patients with different tumor types in some cases. A potentially important finding of our study is that in melanoma patients immune cell densities as well as their predictive impact were different for lymph node vs. cutaneous/subcutaneous metastases, suggesting that evaluating all metastases without distinction according to their locations may not be optimal for revealing all existing differences between responders and nonresponders (**17**).

Beside the presence of tumor antigens and the cells involved in their recognition, the expression of HLA class I antigens by the tumor cells is also essential for the development of an effective antitumor immune reaction; its importance in the efficacy of immune checkpoint inhibitors is beginning to be acknowledged (22). Therefore (although it was not included in our original plan), in a preliminary study we investigated the expression of certain HLA class I molecules in the samples used in the above mentioned studies. We found correlation with T-cell density and with patients' survival in the case of lymph node metastases. In conclusion, our results indicate different predictive impact of immune cell infiltration and HLA class I expression in lymphoid vs. non-lymphoid metastases of melanoma patients treated with ipilimumab (**23**).

In another study involving melanoma patients treated with ipilimumab at the National Institute of Oncology, we analyzed factors associated with therapeutic efficacy and survival. We investigated patient and tumor characteristics (as age, sex, performance status, site of the primary melanoma, tumor burden, metastatic stage, previous treatments, presence of brain metastasis, tumor-infiltrating lymphocyte grade in primary tumor), serum parameters (LDH level, erythrocyte sedimentation rate [ESR]), and peripheral blood cell counts (absolute lymphocyte, neutrophil and eosinophil counts [ALC, ANC, AEC], neutrophil/lymphocyte ratio [NLR], eosinophil/lymphocyte ratio [ELR]). Univariate analysis of pretreatment patient characteristics revealed that LDH>1.5×ULN, ESR>1×ULN and AEC>0.1 G/L were significantly correlated with diminished PFS. In a multivariate analysis including LDH, ESR and AEC, no variable remained

significantly associated with disease progression. In the case of overall survival, univariate analysis showed that factors significantly associated with diminished OS were LDH>1.5×ULN, ESR>1×ULN, NLR≥4, AEC>0.1 G/L, ELR>0.1, performance status >0 and multi-organ disease. High LDH level proved the only independent predictor of poor survival. In this study, no association was found between disease outcome and the presence of tumor-infiltrating lymphocytes in the primary melanoma. This finding, compared to results of the above mentioned studies on immune cells infiltrating melanoma metastases operated within one year before ipilimumab treatment (**17**) support the importance of optimal sample selection (with regard to the source and timing) for use in biomarker studies. The manuscript describing the above results is in the phase of author correction based on the reviewers' comments (Pathol. Oncol. Res., manuscript no. PORE-D-18-00013; minor revision) (**24**).

We also planned to analyze tumor-infiltrating immune cell types as potential predictive markers in melanoma patients receiving dacarbazine, which was the standard treatment of metastatic melanoma at the time of project planning. Since then, on the other hand, the therapeutic landscape of melanoma (and many other tumor types) has radically changed with the advancement of immune checkpoint inhibitors. Therefore, although we have preliminary data on the prognostic effect of several immune cell types (using the markers CD8, CD45RO, CD20, CD25, CD134, CD137, FOXP3 and DC-LAMP) evaluated with regard to dacarbazine treatment, we did not pursue these studies because of the lack of interest (and, consequently, probable refusal) of manuscripts dealing with this subject.

Ectopic lymphoid structures and MECA-79$^+$ high endothelial venules in primary melanoma

In search for easily applicable predictive markers of tumor therapies, we explored the usefulness of two markers that recently came in the forefront of tumor immunological research: the so called tertiary lymphoid structures (TLS) and high endothelial venules (HEVs) that are frequently associated with them (25-32); as a first step we tested their prognostic value in primary melanoma. TLS, composed of B lymphocytes organized in follicles, adjacent T-cell clusters associated with mature DCs and HEVs, are hypothesized to facilitate the interactions of tumor antigens, antigen presenting cells and T cells (25-30). Ectopic lymph node-like structures or B-cell follicles have been observed in a number of tumor types, and their presence at tumor sites has been correlated with favorable disease outcome in several (but not all) cancer types, while less data are available on the prognostic effect of HEVs (27, 30, 31).

The presence of TLS was documented in metastases (but not primary tumors) of melanoma patients (26). In a previous publication we noted the occurrence of lymphoid structures resembling follicles in a series of primary cutaneous melanomas (**33**). Based on this finding we now performed studies aiming at characterization of cell types involved in these cell clusters and at evaluating the association of their presence with clinicopathological parameters and the outcome of the disease. We found B-cell follicles in 39 of 147 cases (27%) in primary melanomas. B-cell clusters were associated with T lymphocytes, most of which belonging to CD45RO$^+$ memory T cells. A network of CD21$^+$ follicular dendritic cells was demonstrated in 8 of 22 cases with TLS studied (36%). The appearance of ectopic lymphoid structures did not show association with the outcome of the disease, although a trend for their higher prevalence was observed in thicker tumors (**34**).

We also investigated the potential prognostic value of MECA-79-positive HEV-like vessels in primary melanoma samples (**35**). These vessels were observed in the neighborhood of ectopic lymphoid structures in the majority of cases, however, their presence was not confined to tumors hosting ectopic lymphoid structures. Density of MECA-79$^+$ vessels as well as that of 8 immune cell types (CD8$^+$ and CD45RO$^+$ T lymphocytes, cells expressing the CD25, CD134 or CD137 activation markers, FOXP3$^+$ regulatory T cells, CD20$^+$ B cells, DC-LAMP$^+$ mature dendritic cells) was determined in 118 melanoma samples. The number of MECA-79$^+$ vessels was analyzed for potential associations with intra- and peritumoral immune cell densities, patient and tumor characteristics as tumor thickness, ulceration, histologic type, location, patient age and gender, as well as with the outcome of the disease. Statistical analyses were performed on all data as well as separately on two patient cohorts: Cohort 1 (47 patients, operated at Semmelweis University); Cohort 2 (71 patients, operated at the National Institute of Oncology). The presence of MECA-79-positive HEV-like vessels was demonstrated in the majority of primary melanoma samples (all cases: 93/118, 79%; Cohort 1: 35/47, 74%; Cohort 2: 58/71, 82%), with a median density of 2.4/mm^2 (range 0–33). These vessels were localized to lymphocyte-rich peritumoral areas and, according to Spearman's rank correlation assay,

their number correlated with peritumoral (but not with intratumoral) immune cell densities, with the strongest correlation observed in the case of $CD20^+$ B lymphocytes ($p<0.001$ in both cohorts and in all samples as well). Peritumoral density of $FOXP3^+$, $CD8^+$ and $CD45RO^+$ cells also showed correlation with MECA-79^+ vessel number in all cohorts, while correlation for the other immune cell types was weaker and observed only in one of the patient cohorts. Analysis of the connection of MECA-79^+ vessel density with clinicopathologic parameters revealed associations with tumor site and with patient gender. Strikingly higher values were found in tumors of axial location (trunk, head or neck) compared to those located to the extremities ($p=0.0000$, $p=0.0005$ and $p=0.0001$ for all patients, Cohort 1 and Cohort 2, respectively). Moreover, the number of MECA-79^+ vessels was higher in men compared to women, although the difference between genders was less prominent ($p=0.0156$, $p=0.0756$ and $p=0.0827$ for all patients and the two cohorts, respectively). The amount of MECA-79^+ vessels did not show association with metastasis formation during the 5-year follow-up period, or with survival of the patients. Our manuscript reporting these findings is under review after correction based on the reviewers' comments (Melanoma Res., manuscript no. MR-D-17-00236; minor revision) (**35**). The overall conclusion drawn from the latter two studies is that neither the presence of TLS, nor the amount of HEV at tumor sites has prognostic value in melanoma. Based on these data and controversial results on their prognostic impact in the literature (27), we did not pursue further testing of the predictive power of these two markers.

Tumor-associated immune cells as predictive markers in head and neck cancer patients treated with induction chemotherapy and cetuximab

Head and neck squamous cell carcinoma (HNSCC) represents a major health problem in Hungary, which in the past decade was leading mortality statistics of oral, pharyngeal and laryngeal cancers in Europe, in both sexes (36, 37). The most important known risk factors include tobacco use, alcohol consumption, and human papilloma virus (HPV) infection, especially high-risk subtype 16. The majority of HNSCC patients presents with stage III/IVA-B (locoregionally advanced) disease at diagnosis, with a rather dismal prognosis. Treatment of stage III/IV HNSCC requires a multimodal approach combining surgery, radio- and chemotherapy, or the anti-EGFR antibody cetuximab. An alternative approach is the use of induction chemotherapy (ICT) followed by definitive radiotherapy or chemoradiotherapy. TPF (docetaxel, cisplatin, 5-fluorouracil) ICT was proved more effective compared to the PF (cisplatin, 5-fluorouracil) regimen (38, 39). However, the value of the addition of ICT to radio- or chemoradiotherapy in terms of improving overall survival is controversial (40). Addition of cetuximab to this regimen may increase efficiency but at the cost of increased toxicity (41, 42). Hence, it is important to search for biomarkers allowing prospective identification of patients likely to derive benefit from the therapy.

Beside their role in influencing the outcome of various types of cancer immunotherapy, evidence accumulating from both experimental and clinical studies supports the importance of the host immune status in shaping the effects of other, non-immune-based treatment modalities as chemo-, radio-, or targeted therapy (43-45). These therapies can affect the immune system in many ways which, in turn, may contribute to the success of the therapeutic agents, and evaluation of the components of immune infiltrate was found useful in predicting the efficacy of the treatment and disease outcome in several cancer types (46-50). In patients receiving targeted antibody treatment (trastuzumab, rituximab or cetuximab) single nucleotide polymorphisms in Fc receptors FcγRIIa or FcγRIIIa predicted clinical response and survival, indicating that antibody-dependent cell-mediated cytotoxicity (ADCC) by host effector cells carrying Fc receptors is involved in the therapeutic effect of these agents (45, 51). Moreover, a role of T-cell mediated adaptive immune responses has also been suggested in the mechanism of action of cetuximab through dendritic cell activation by cetuximab-stimulated NK cells (51-54).

In our study we determined infiltration level of a panel of 11 immune cell types in biopsies of HNSCC patients receiving TPF induction chemotherapy and cetuximab, and evaluated in association with response to treatment and the outcome of the disease, as well as with HPV status based on p16 expression, peripheral blood neutrophil and lymphocyte counts, and other clinicopathologic parameters. Archived pretreatment biopsy samples of 50 patients enrolled in an investigator-initiated, phase II single center prospective trial performed between 2007 and 2010 at the National Institute of Oncology, Budapest (**55**) were used. Samples of 47 cases were available for p16 immunohistochemistry and 45 for immune cell analysis; because of insufficient amount of tumor tissue in certain samples, evaluation of infiltration by the different immune cell types with reliable results could be performed in 35-40 cases. Pretreatment peripheral blood neutrophil and

lymphocyte counts were also registered. Response to ICT, evaluated by CT or MRI at the end of the treatment, was available in the case of 47 patients; 33 partial responses ("responders"), 13 stable diseases and 1 progressive disease ("nonresponders").

Tumor-associated immune cell types ($CD8^+$ and $CD45RO^+$ T cells, $CD20^+$ B cells, lymphocytes expressing the activation markers CD134, CD137 or PD-1, $FOXP3^+$ regulatory T cells, $NKp46^+$ NK cells, $CD68^+$ macrophages, cells expressing CD16 (Fcγ receptor III) and $myeloperoxidase^+$ neutrophil granulocytes) were identified by immunohistochemistry. The amount of DC-$LAMP^+$ mature dendritic cells as well as that of lymphocytes carrying the PD-1 activation marker showed a tendency of being higher in the responder group compared to nonresponders ($p=0.0695$ and $p=0.0574$, respectively; Mann-Whitney U test). Moreover, the ratio of cases demonstrating high numbers of DC-$LAMP^+$ and PD-1^+ cells was found significantly associated with response to ICT. The amount of the other immune cell types studied did not show correlation with treatment response. Among blood cell parameters studied, high (>6.6 G/L) pretreatment peripheral blood neutrophil count was associated with response to ICT ($p=0.0274$). Using Cox regression analysis evaluating immune cell densities as continuous variables, high density of DC-$LAMP^+$ mature dendritic cells was associated with better progression-free survival ($p=0.0402$), while none of the parameters showed significant correlation with overall survival.

p16 positivity was observed in 12/47 (26%) samples in the whole population, 9/18 (50%) oropharyngeal, and 3/14 (21%) hypopharyngeal cancers, while all 9 oral cavity and 6 laryngeal tumors tested were p16-negative ($p=0.0121$). p16 status did not show correlation with response to ICT, survival, or with other clinicopathologic parameters studied (patient age, gender, smoking, alcohol consumption, tumor stage). Several immune cell types showed some tendency to be higher in $p16^+$ cases compared with p16-negative ones, but the difference was significant only in the case of $CD20^+$ B cells ($p=0.0158$). For B lymphocytes, as well as for $NKp46^+$ NK cells and $CD137^+$ cells, differences with regard to tumor site were also observed, with high cell densities in oropharyngeal and hypopharyngeal tumors and low densities in laryngeal ones. B cells, NK cells, and $CD8^+$ T lymphocytes were more numerous in T1-2 stage tumors compared to T3-4 ones. We also evaluated possible correlations of local immune parameters with peripheral blood neutrophil and lymphocyte counts; a strong correlation was found only in the case of CD16 immunohistochemistry results and neutrophil count ($r=0.411$, $p<0.01$).

Evaluating our results, we could not prove association between therapeutic effect or survival and the amount of known ADCC effectors (macrophages, NK cells and neutrophils) or cells expressing CD16 (the Fc receptor involved in ADCC) in our analysis of tumor-infiltrating cells. Nevertheless, high peripheral blood neutrophil count was found associated with response to ICT, and it showed correlation with the amount of tumor-associated $CD16^+$ cells (mostly neutrophils). Among the local immune parameters, only 2 of the 11 immune cell types, DC-$LAMP^+$ mature dendritic cells and PD-1^+ lymphocytes could be implicated in response to ICT and cetuximab in HNSCC patients according to our investigation, however, studies on larger patient cohorts are warranted to confirm these findings. Part of these results were presented at the Fifth World Congress of International Federation of Head and Neck Oncologic Societies (IFHNOS) (**56**) and a manuscript containing all results has been submitted to Head & Neck (manuscript no. HED-18-0256) (**57**).

Tumor-associated immune cells in rectal carcinoma treated with neoadjuvant radio- or chemoradiotherapy

In the original work plan we included investigation of immune cells in rectal cancer samples after neoadjuvant radio- or chemoradiotherapy as well, since only one report on similar studies had appeared at the time of project planning (58). However, by the time these studies were scheduled, many reports were published in the literature on this subject (59-66), therefore, we changed our plans and omitted these studies.

Because of the manuscripts presently under review, I would like to ask for a re-evaluation after the acceptance/publication of these manuscripts.

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