Cancer Immunol Immunother (2018) 67:141–151
https://doi.org/10.1007/s00262-017-2072-1

ORIGINAL ARTICLE

Tumor-infiltrating immune cells as potential biomarkers predicting response to treatment and survival in patients with metastatic melanoma receiving ipilimumab therapy

Tímea Balatoni[1] · Anita Mohos[2] · Eszter Papp[3] · Tímea Sebestyén[4] · Gabriella Liszkay[1] · Judit Oláh[5] · Anita Varga[5] · Zsuzsanna Lengyel[6] · Gabriella Emri[7] · István Gaudi[8] · Andrea Ladányi[3]

Received: 11 May 2017 / Accepted: 27 September 2017 / Published online: 7 October 2017

Abstract Monoclonal antibodies targeting immune checkpoints are gaining ground in the treatment of melanoma and other cancers, and considerable effort is made to identify biomarkers predicting the efficacy of these therapies. Our retrospective study was performed on surgical tissue samples (52 lymph nodes and 34 cutaneous/subcutaneous metastases) from 30 patients with metastatic melanoma treated with ipilimumab. Using a panel of 11 antibodies against different immune cell types, intratumoral immune cell densities were determined and evaluated in relation to response to ipilimumab treatment and disease outcome. For most markers studied, median immune cell densities were at least two times higher in lymph node metastases compared to skin/subcutaneous ones; therefore, the prognostic and predictive associations of immune cell infiltration were evaluated separately in the two groups of metastases as well as in all samples as a whole. Higher prevalence of several immune cell types was seen in lymph node metastases of the responders compared to non-responders, particularly FOXP3$^+$ cells and CD8$^+$ T lymphocytes. In subcutaneous or cutaneous metastases, on the other hand, significant difference could be observed only in the case of CD16 and CD68. Associations of labeled cell densities with survival were also found for most cell types studied in nodal metastases, and for CD16$^+$ and CD68$^+$ cells in skin/s.c. metastatic cases. Our results corroborate the previous findings suggesting an association between an immunologically active tumor microenvironment and response to ipilimumab treatment, and propose new potential biomarkers for predicting treatment efficacy and disease outcome.

Keywords Melanoma · Immunotherapy · Ipilimumab · Biomarker · Tumor-infiltrating immune cells

Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
AEC	3-Amino-9-ethylcarbazole
CRP	C-reactive protein

Part of this work was presented as a poster at the European Cancer Congress 2015, September 25–29, Vienna, Austria (ECC 2015) [1] and as oral presentation at Melanoma Bridge 2016, November 30–December 3, Naples, Italy [2].

Tímea Balatoni and Anita Mohos contributed equally to the work.

✉ Andrea Ladányi
ladanyi@oncol.hu

[1] Department of Oncodermatology, National Institute of Oncology, Budapest, Hungary

[2] 1st Institute of Pathology and Experimental Cancer Research, Semmelweis University, Budapest, Hungary

[3] Department of Surgical and Molecular Pathology, National Institute of Oncology, 7-9. Ráth György u., Budapest H-1122, Hungary

[4] Department of Pathology, St. John's Hospital, Budapest, Hungary

[5] Department of Dermatology and Allergology, Albert Szent-Györgyi Medical Center, University of Szeged, Szeged, Hungary

[6] Department of Dermatology, Venerology and Oncodermatology, University of Pécs, Pécs, Hungary

[7] Department of Dermatology, Faculty of Medicine, University of Debrecen, Debrecen, Hungary

[8] National Korányi Institute of TB and Pulmonology, Budapest, Hungary

ECOG	Eastern cooperative oncology group
NY-ESO-1	New York esophageal squamous cell carcinoma-1
VEGF	Vascular endothelial growth factor

Introduction

Immunotherapeutic modalities of cancer treatment have been increasingly gaining ground in the past few years. Understanding the mechanisms regulating antitumor immune response led to the development of a new class of immunotherapeutic agents targeting molecular interactions blocking T cell activation, the so called immune checkpoint inhibitors [3]. 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 [4, 5]. 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. 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. In the case of ipilimumab, long-term survival was observed in approximately 20% of patients [6]. On the other hand, serious side effects, often immune-related, were reported to occur in a higher percentage of patients [7]. 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.

Although no validated predictive biomarkers are available yet for routine clinical use of ipilimumab, several candidates have been suggested, mainly concerning peripheral blood immune cells or serum factors. The most consistent finding was the observation of association between baseline absolute lymphocyte count, or its rise during treatment, and response to therapy or survival [8–11]. In some studies, correlation of high absolute eosinophil counts, low neutrophil/lymphocyte ratio or absolute monocyte counts, as well as low number or therapy-induced decrease of circulating myeloid-derived suppressor cells, with patient survival or response to ipilimumab treatment was described [8, 10–12]. Correlations of baseline number or therapy-induced change in T cells expressing specific, proliferation- or activation-associated markers (e.g., Ki67, and ICOS) with outcome have also been observed [8, 10]. Moreover, the presence of antibodies to NY-ESO-1 (New York esophageal squamous cell carcinoma-1) was found to correlate with clinical benefit of ipilimumab treatment, and patients with simultaneous presence of anti-NY-ESO-1 $CD8^+$ (but not $CD4^+$) T cell response experienced a clinical response and a survival advantage more frequently [13]. Among serum factors, high levels of lactate dehydrogenase (LDH), inflammatory markers as C-reactive protein (CRP), or vascular endothelial factor (VEGF) were found associated with poor outcome in ipilimumab-treated patients [8, 9].

The role of tumor-infiltrating immune cells in predicting the efficacy of CTLA-4 inhibitors was less intensely studied. In melanoma patients, ipilimumab therapy enhanced the expression of many immune-related genes in metastatic tumor samples, and most genes showing higher pretreatment expression in responders were immune-related [14]. In the same trial, clinical activity correlated with baseline staining for FOXP3 and indoleamine-2,3-dioxygenase (IDO), as well as with an increase in TIL during treatment, while no association was observed for pretreatment TIL or other immune cell subsets [15]. Enhanced tumor infiltration by $CD4^+$, $CD8^+$, HLA-DR^+/$CD45RO^+$, and $FOXP3^+$ cells was reported in melanoma patients receiving tremelimumab, but no correlation with the therapeutic effect was observed [16].

A novel and intriguing field in the study of predictive biomarkers of immune checkpoint inhibitors is the analysis of somatic mutations and the resulting neoantigens in tumors [17]. Mutational burden was associated with long-term clinical benefit in ipilimumab-treated melanoma patients. Moreover, a neoepitope signature was identified that predicted the response to CTLA-4 blockade [18].

Most of the candidate biomarkers listed above were investigated only in one or a few studies, and none of them have been validated and can be used in routine clinical practice. The objective of this study was to explore tumor-infiltrating immune cell types as potential biomarkers predicting response to treatment and survival in melanoma patients receiving ipilimumab therapy.

Materials and methods

Patients and samples

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. In the study, we included only cases with available tumor samples excised within 1 year before ipilimumab therapy and the study population consisted of 30 patients (1–25 lesions per patient). 86 samples were selected for the investigations: 52 lymph node metastases, and 34 subcutaneous/cutaneous metastases. The primary site was cutaneous in 28 cases and unknown in 2 cases. Most patients ($n = 18$) received ipilimumab treatment in the Expanded Access Program and 3 patients after drug commercialization (3 mg/kg

four times every 3 weeks), while 9 patients participated in the CA-184-169 trial (receiving 3 or 10 mg/kg ipilimumab doses). Most patients ($n = 26$) had at least one prior systemic treatment; all of them received chemotherapy, while eight also had radiotherapy, one received BRAF inhibitor, and two patients were treated with electrochemotherapy. Twenty-six patients received all four doses while the remaining four patients received three cycles of ipilimumab treatment (three because of progression and one because of adverse events). Response assessment was based on immune-related response criteria (irRC) [19]. For our analysis, patients were considered "responders" if the best overall response was complete or partial response, or stable disease lasting for at least 6 months. 13 of the 30 patients belonged to this group, including 3 patients showing complete response lasting more than 24 months (27, 49, 57 + months). Patient and sample characteristics are shown in Table 1.

Immunohistochemical detection of immune cell types

Three-micrometer sections from formalin-fixed, paraffin-embedded tumor samples were used in the study. Immunohistochemistry was performed as described earlier [20–22], using monoclonal antibodies against CD8, CD20cy, CD45RO, CD68 (Dako, Glostrup, Denmark), CD16, CD137 (Santa Cruz Biotechnology, Dallas, TX), CD134 (BD Biosciences Eastern Europe, Heidelberg, Germany), FOXP3 (236A/E7; eBioscience, San Diego, CA), NKp46 (R&D Systems, Abingdon, UK), CD4, and PD-1 (Cell Marque, Rocklin, CA). For detecting staining, we used the SS™ One-Step

Table 1 Patient and sample characteristics

	Responders ($n = 13$)[a]	Non-responders ($n = 17$)[a]	p value
Age—years, median (range)	67 (51–78)	53 (30–66)	0.0000[b]
Gender—n (%)			
Female	5 (38)	8 (47)	NS[c]
Male	8 (62)	9 (63)	
Disease stage—n (%)			NS[c]
III N3	1 (8)	0 (0)	
IV M1a	4 (31)	2 (12)	
IV M1b	3 (23)	5 (29)	
IV M1c	5 (38)	10 (59)	
Number of organs involved			NS[c]
1–2	8 (62)	14 (82)	
> 2	5 (38)	3 (18)	
ECOG performance status			NS[c]
0	10 (77)	10 (59)	
1	3 (23)	5 (29)	
2	0 (0)	2 (12)	
LDH			NS[c]
Normal	10 (77)	8 (47)	
> ULN	3 (23)	9 (53)	
BRAF V600 mutation			NS[c]
Present	5 (38)	3 (18)	
Absent	4 (31)	6 (35)	
Unknown	4 (31)	8 (47)	
Progression-free survival—months, median (range)	9 (6–57)	2 (1–5)	0.0000[b]
Overall survival—months, median (range)	30 (10–61)	7 (2–30)	0.0000[b]
Samples evaluated, all	34	52	NS[c]
Lymph node metastases	20	32	
Cutaneous/s.c. metastases	14	20	

ULN upper limit of normal

[a]In the analysis, patients were considered responders if the best overall response was complete or partial response, or stable disease lasting for at least 6 months

[b]Mann–Whitney test

[c]χ^2 test

Polymer-HRP IHC Detection System (BioGenex, Fremont, CA), 3-amino-9-ethylcarbazole (AEC; Vector Laboratories, Inc., Burlingame, CA) for visualization, and hematoxylin counterstaining.

Evaluation of the immune reactions

Counting of labeled cells was performed by light microscope equipped with an eyepiece graticule, independently by two researchers who were blinded to the clinical information, and the mean value of their separate counts was used for the analysis. The number of the labeled cells within the metastases was registered in at least 10 (median: 20, range: 10–80) randomly chosen fields per section, using the graticule of 10 × 10 squares designating an area of 0.0625 mm^2 at 400 × magnification. These fields were blindly chosen from different, non-adjacent areas of the metastases, omitting necrotic areas; in the case of smaller metastases, the whole tumor area was evaluated. Inter-observer concordance in density values was excellent (correlation coefficients between 0.6814 and 0.9156 for the different immune cell markers). For patients with more than one sample available, the mean labeled cell densities of all metastases studied were also determined. Cutoff levels were set up for each marker, based on the median of the given variable in the whole patient cohort, with minor adjustments for better discriminating power in some cases (46, 215, 430, 8, 10, 70, 83, 53, 5, 450, and 450 cells/mm^2 for CD4, CD8, CD45RO, CD134, CD137, FOXP3, PD-1, CD20, NKp46, CD16, and CD68, respectively) in lymph node metastases. In cutaneous/s.c. metastases, the cutoff values used were 18, 21, 220, 4, 3, 20, 5, 4, 4, 370, and 500 cells/mm^2, while in the case of all samples evaluated together, they were 24, 155, 440, 8, 7, 60, 28, 34, 4, 440, and 400 cells/mm^2, in the same order. The proportion of patients with a mean cell density higher than the cutoff level was determined.

Statistical analysis

We used the Mann–Whitney *U* test for the statistical evaluation of differences in cell densities between different patient groups, χ^2 test for comparing the proportions of samples with high cell densities, and the Pearson test for analyzing correlation between the densities of the different cell types. The Kaplan–Meier method and Mantel-Cox test were applied for evaluating survival. Univariate and multivariate Cox regression analyses were also performed using mean immune cell densities and patients' age as continuous, while patient gender, disease stage, ECOG status, number of organs involved, LDH level, and previous treatments (chemo-, and radiotherapy) as categorical variables. Differences were considered significant in the case of p values ≤ 0.05. Statistics were calculated using the BMDP Statistical Software Pack.

Results

We determined the intratumoral density of immune cells expressing the different markers (CD4, CD8, CD45RO, CD20, CD134, CD137, FOXP3, PD-1, NKp46, CD16, and CD68) (Fig. 1) in 52 lymph node and 34 cutaneous or subcutaneous metastases. In lymph node metastases, $CD45RO^+$ T lymphocytes, $CD16^+$ cells, and $CD68^+$ macrophages were the most numerous, followed by $CD8^+$ T lymphocytes, $FOXP3^+$ cells, $CD20^+$ B lymphocytes, $CD4^+$, and $PD\text{-}1^+$ cells, while cells expressing the CD134 and CD137 activation markers, as well as $NKp46^+$ NK cells were present only in small amounts (Fig. 2). For all cell types except $CD68^+$ macrophages and $NKp46^+$ NK cells, density values were significantly lower in subcutaneous/cutaneous lesions compared to nodal metastases (Fig. 2). Because of the large difference in immune cell densities 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 ("non-responders"). In lymph node metastases, mean densities of $CD4^+$, $CD8^+$, $FOXP3^+$, $CD134^+$ lymphocytes, $CD20^+$ B cells, and $NKp46^+$ NK cells were significantly higher in the responder group compared to non-responders (Fig. 3). For each cell type, a cutoff value was introduced based on the median of the given variable in the whole patient group (see in Materials and methods), and the proportion of patients with a mean intratumoral cell density exceeding this value was calculated and analyzed according to the efficacy of ipilimumab treatment. In this comparison, the above mentioned lymphocyte markers as well as CD137 also showed higher prevalence in the responders than in non-responders (Table 2). On the other hand, in subcutaneous/cutaneous metastases significant difference between responders and non-responders was found only in the proportion of patients with high mean density of $CD68^+$ macrophages and $CD16^+$ cells (Table 2). When all samples were evaluated together, significant association with response to treatment was found in the case of NK cell density values ($p = 0.0182$; not shown) and for proportion of patients with high density of NK cells as well as that of $FOXP3^+$ cells and $CD68^+$ macrophages (Table 2).

The densities of most of the studied immune cell types strongly correlated with each other and they frequently

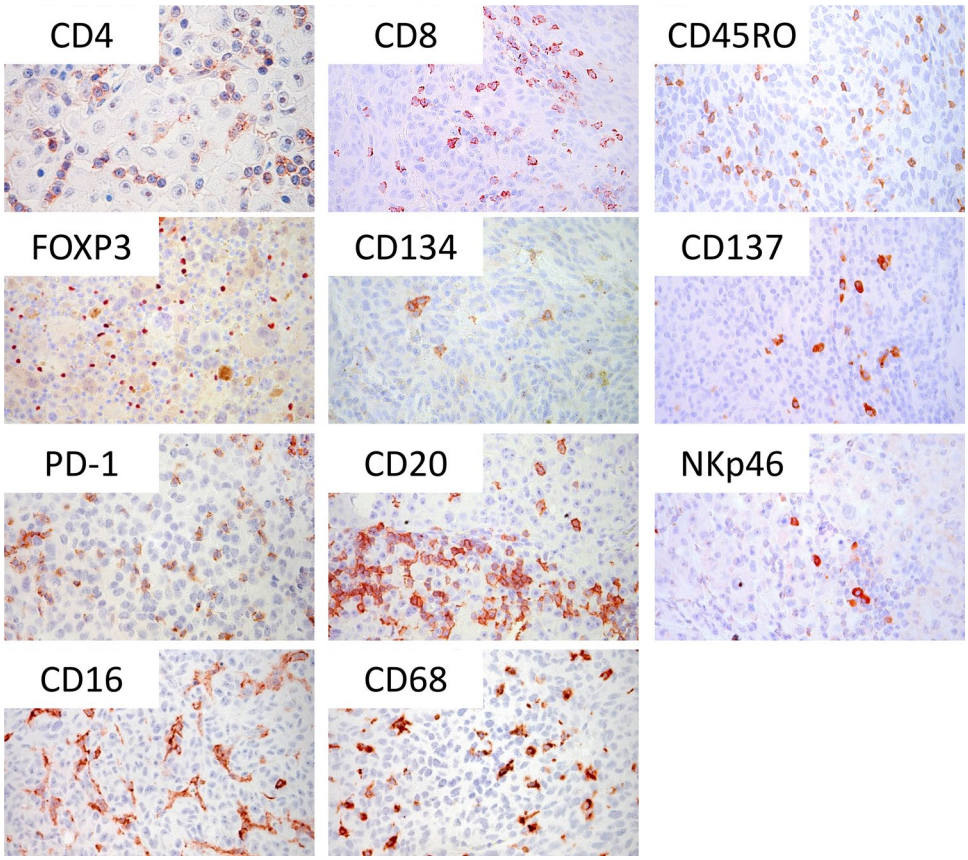

Fig. 1 Immunohistochemical labeling of $CD4^+$, $CD8^+$, $CD45RO^+$, $FOXP3^+$, $CD134^+$, $CD137^+$, $PD\text{-}1^+$, $CD20^+$, $NKp46^+$, $CD16^+$, and $CD68^+$ cells in metastatic melanoma samples (AEC, red)

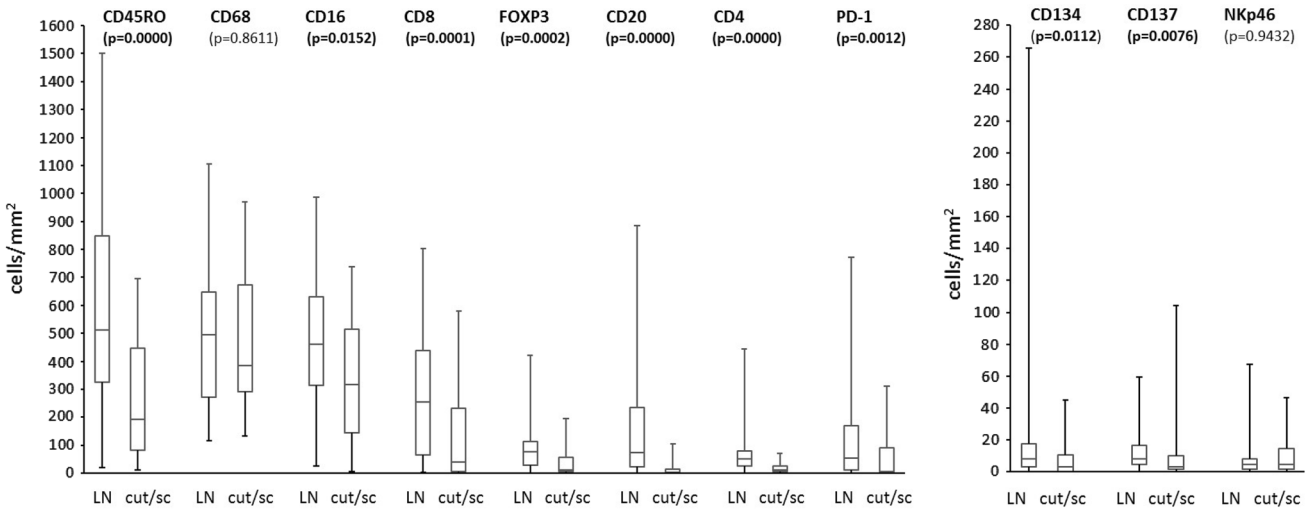

Fig. 2 Density of immune cell types in pretreatment lymph node ($n = 52$) and subcutaneous/cutaneous metastasis samples ($n = 34$) from ipilimumab-treated patients. Boxes show first and third quartiles, horizontal lines are the medians, and whiskers indicate minimum and maximum values. *LN* lymph node, *cut/sc* cutaneous/subcutaneous

showed coordinate presence. In lymph node metastases, high expression of at least 7 of the 11 markers studied was found in 6 of the 7 responders (86%), compared to only 3 of 12 non-responders (25%) ($p = 0.0106$).

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

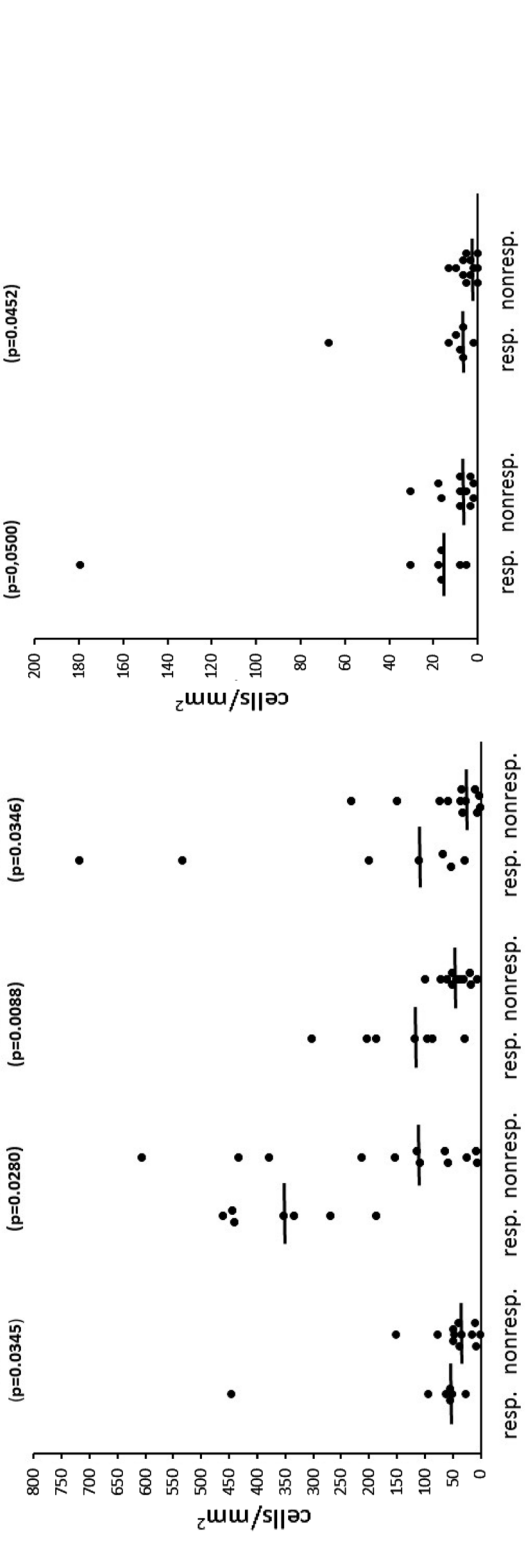

Fig. 3 Density of immune cell types in pretreatment lymph node metastasis samples from ipilimumab-treated patients responding (resp.; $n = 7$) or not responding to treatment (nonresp.; $n = 12$). Circles: mean density values of samples from individual patients; horizontal line: median

Table 2 Relationship of treatment response with proportion of patients with significant mean density of immune cells infiltrating metastases

Immune cell marker	Lymph node metastases (no. of patients: 19)			Cutaneous/s.c. metastases (no. of patients: 19)			All metastases (no. of patients: 30)		
	Resp. ($n = 7$)	Non-resp. ($n = 12$)	p value	Resp. ($n = 9$)	Non-resp. ($n = 10$)	p value	Resp. ($n = 13$)	Non-resp. ($n = 17$)	p value
CD4	**6 (86)**	**4 (33)**	**0.0274**	5 (56)	5 (50)	0.8087	8 (62)	9 (53)	0.6377
CD8	**6 (86)**	**3 (25)**	**0.0106**	4 (44)	5 (50)	0.8087	8 (62)	5 (29)	0.0785
CD45RO	6 (86)	6 (50)	0.1195	6 (67)	4 (40)	0.2451	7 (54)	5 (29)	0.1758
CD20	**6 (86)**	**4 (33)**	**0.0274**	3 (33)	5 (50)	0.4625	6 (46)	4 (24)	0.1927
CD134	**5 (71)**	**3 (25)**	**0.0480**	5 (56)	4 (40)	0.4977	8 (62)	5 (29)	0.0785
CD137	**5 (71)**	**2 (17)**	**0.0170**	4 (44)	5 (50)	0.8087	7 (54)	7 (41)	0.4906
FOXP3	**6 (86)**	**1 (8)**	**0.0009**	5 (56)	5 (50)	0.8087	**7 (54)**	**3 (18)**	**0.0371**
PD-1	5 (71)	5 (42)	0.2101	3 (33)	6 (60)	0.1775	6 (46)	8 (47)	0.9607
CD16	5 (71)	6 (50)	0.3615	**7 (78)**	**3 (30)**	**0.0373**	8 (62)	6 (35)	0.1533
CD68	4 (57)	5 (42)	0.5146	**8 (89)**	**3 (30)**	**0.0094**	**11 (85)**	**8 (47)**	**0.0344**
NKp46[b]	**6 (86)**	**4 (33)**	**0.0274**	4 (50)	3 (30)	0.1353	**10 (83)**	**7 (41)**	**0.0232**

Significant differences are shown in bold

Resp. responder, *Non-resp.* non-responder

[a]Data are presented as number of patients (%)

[b]One case with lymph node metastasis could not be evaluated

(Fig. 4a). 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 ($p = 0.0290$, $p = 0.0093$, $p = 0.0180$, $p = 0.0083$, and $p = 0.0047$, respectively), besides ECOG status ($p = 0.0009$) and LDH ($p = 0.0227$). Multivariate analysis including all immune cell density values as well as clinicopathologic parameters identified ECOG status ($p = 0.001$) and $FOXP3^+$ cell density ($p = 0.004$) as significant independent predictors of survival. Similar associations were found when all samples were evaluated together, using either Kaplan–Meier analysis (not shown) or Cox regression demonstrating significantly longer survival in case of high number of cells expressing CD4, CD8, CD45RO, FOXP3, CD16, CD68, or CD20 ($p = 0.0348$, $p = 0.0136$, $p = 0.0113$, $p = 0.0121$, $p = 0.0055$, $p = 0.0168$, or $p = 0.0372$, respectively), as well as in cases with better ECOG status ($p = 0.0026$) and normal LDH level ($p = 0.0006$). In multivariate analysis, LDH ($p = 0.001$) and the amount of $FOXP3^+$ cells ($p = 0.016$) proved as 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 (Fig. 4b) and Cox's proportional hazards model ($p = 0.0197$ and $p = 0.0175$, respectively); in this group, only LDH level proved as independent predictor of survival ($p = 0.002$). Disease stage, patients' age and gender, the number of organs involved, and previous chemo- or radiotherapy were not found as significant parameters in univariate or multivariate analyses in either group studied.

Discussion

The introduction of ipilimumab, the first representative of the new class of immunotherapy, immune checkpoint agents, has revolutionized the treatment of metastatic melanoma, and paved the way for other immunomodulatory antibodies as PD-1/PD-L1 inhibitors, expanding the range of targeted tumor types. However, even in the case of the most efficient immunotherapeutic modalities, the majority of patients does not derive clinical benefit but are still at risk of developing side effects. Furthermore, the therapeutic arsenal of several tumor types has recently been widened with the introduction of other, potentially effective treatment modalities, e.g., targeted therapies, making it even more important to identify clinically usable predictive markers which could help in choosing the optimal treatment strategy for a given patient.

In our study, the density of several immune cell types, such as $CD4^+$, $CD8^+$ 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, and cells expressing CD16 (Fcγ receptor III), was determined in pretreatment surgical tumor samples of melanoma patients receiving ipilimumab therapy.

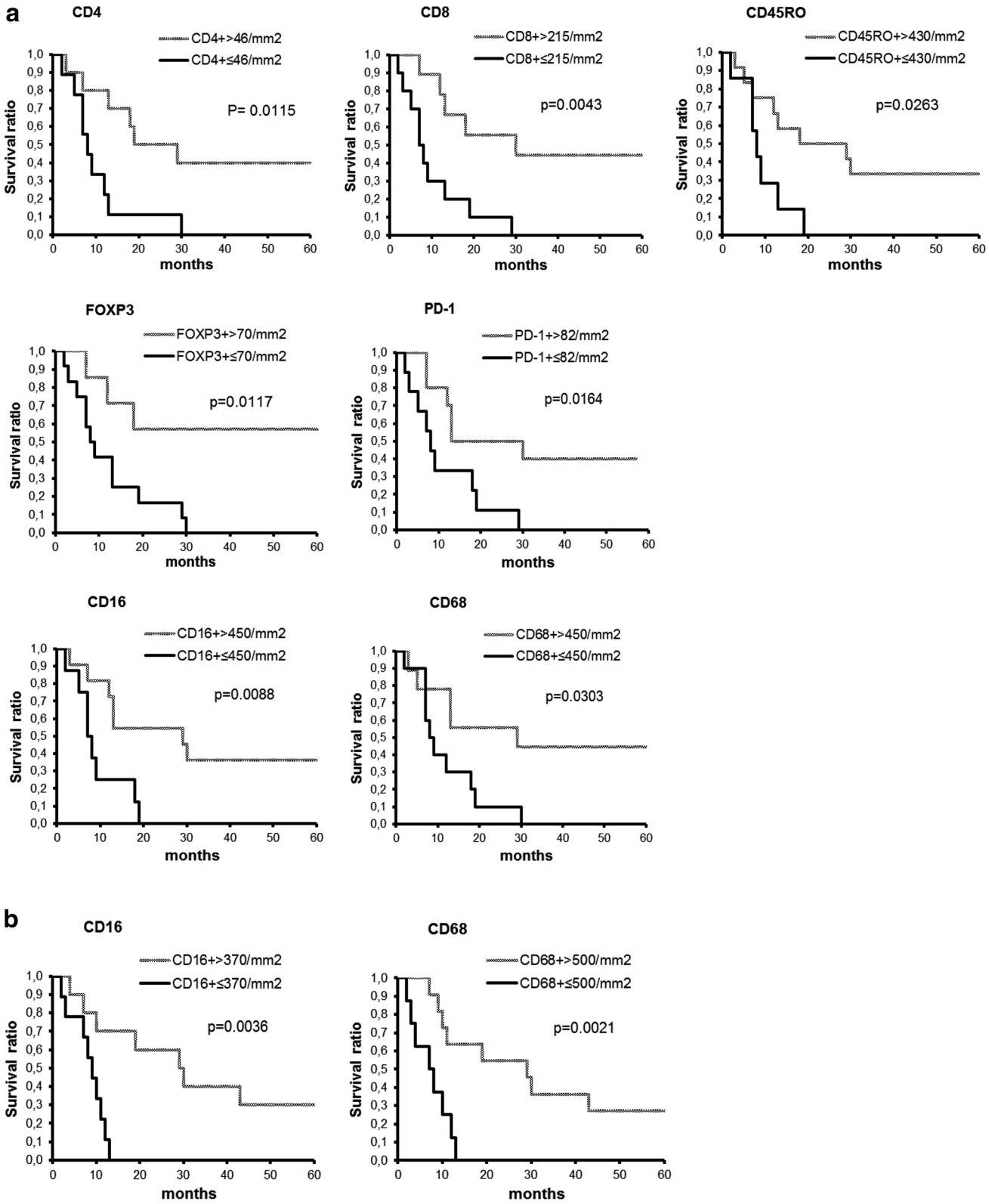

Fig. 4 Kaplan–Meier curves of overall survival for melanoma patients subdivided according to mean density of immune cells in lymph node (**a**), or cutaneous/subcutaneous metastases (**b**)

Density values of each cell type were evaluated with regard to response to treatment and the outcome of the disease.

In this study, we restricted sample collection to metastases operated within 1 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. To our knowledge, our study is the first that attempted to correlate density values of a panel of infiltrating immune cell types with response to treatment with ipilimumab in an everyday clinical setting, outside of clinical trials.

We found associations of treatment response and survival with intratumoral density of several infiltrating immune cell types. In lymph node metastases, the amount of $CD4^+$, $CD8^+$ T lymphocytes, $FOXP3^+$ lymphocytes, cells carrying the CD134 activation marker, as well as $CD20^+$ B cells and $NKp46^+$ NK cells was higher in responders; the most pronounced difference could be seen in the case of the FOXP3 marker. The densities of $CD45RO^+$, $PD\text{-}1^+$, $CD16^+$, and $CD68^+$ cells showed correlation with survival, but not with treatment response. Analyzing subcutaneous/cutaneous metastases, on the other hand, resulted in significant associations of clinical response and survival with $CD16^+$ and $CD68^+$ cell density values. The amount of some of these immune cell types as $CD8^+$ T lymphocytes and $CD20^+$ B cells was also found prognostic in an earlier study on melanoma metastases (mostly lymph node and skin or soft tissue), while no association with survival was observed in the case of $FOXP3^+$ cells [23].

Our results demonstrating association of T cell density with the efficacy of ipilimumab therapy fit well with 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 [24]. In support of this hypothesis, correlations between favorable disease outcome after checkpoint blockade and high expression of genes related to immune activity have been found in different tumor types [14, 25, 26]. Moreover, $CD8^+$ T cell density in pretreatment biopsies proved predictive of response of melanoma patients to pembrolizumab treatment [27]; 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 [15, 16, 25, 28]. 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 differences between responders and non-responders.

According to our results, the factor most strongly predicting treatment response was the density of $FOXP3^+$ cells infiltrating lymph node metastases. Similarly, strong baseline staining for FOXP3 and IDO was found to correlate with benefit from ipilimumab treatment in another study on melanoma patients [15]. The upregulation of immunosuppressive mechanisms in the tumors could also be connected to an active immune microenvironment, probably acting as counter-regulatory mechanisms. Indeed, Spranger et al. demonstrated correlation of IDO, PD-L1, and FOXP3 expression with each other and with the amount of $CD8^+$ T lymphocytes in melanoma metastases, and proved in murine models that the presence of these inhibitory mechanisms was driven by infiltrating $CD8^+$ T cells [29]. Our study also showed strong correlation between the densities of $FOXP3^+$ cells and $CD8^+$ or $CD45RO^+$ lymphocytes ($p < 0.001$). Hypothetically, in T cell-rich tumors simultaneously present suppressive mechanisms may be responsible for inhibiting the antitumor effect of T lymphocytes, and therapeutic strategies aiming at blocking these mechanisms, such as immune checkpoint inhibitors, could be expected to be beneficial in these cases.

Recent studies have raised the possibility of ADCC-mediated intratumoral regulatory T cell depletion by Fcγ receptor expressing monocytes/macrophages as a mechanism of action of anti-CTLA-4 antibody therapy [30–33]. In metastases of melanoma patients receiving ipilimumab therapy, the density of $CD16^+CD68^+$ cells was higher in pretreatment samples of responders compared to non-responders [33]. Our findings partly correspond to these results, although significant differences in the density of $CD16^+$ and $CD68^+$ cells according to clinical response was found only in the case of cutaneous/subcutaneous metastases, while correlation with survival rather than with clinical response was seen in the case of nodal metastases. Together with the association of $FOXP3^+$ cell density with treatment response [the present study and 15], these results may support a potential role of regulatory T cell killing by $CD16^+$ effector cells in the effect of ipilimumab. We also observed a higher amount of the other potential ADCC effector NK cells in responders, however, the density of these cells was very low, which may question their possible biological significance.

We recognize the inherent limitations of our study caused by its retrospective nature. Moreover, the availability of sufficient surgical samples limits the number of patients that could be included in immunohistochemistry testing. On the other hand, the strengths of our analysis are that its data

are derived mainly from a 'real world' patient cohort and that it evaluated pretreatment immune parameters, enabling the identification of predictive biomarkers that could help in making treatment decisions.

In conclusion, our data suggest that infiltration by $FOXP3^+$ cells, $CD4^+$, $CD8^+$, $CD134^+$ T lymphocytes, $CD20^+$ B cells, and $NKp46^+$ NK cells in lymph node metastases, as well as the prevalence of $CD16^+$ cells and $CD68^+$ macrophages in cutaneous/subcutaneous ones could be considered as candidate predictive markers in melanoma patients receiving ipilimumab therapy. Studies on larger patient cohorts are required to prospectively validate these and other parameters of tumor immune contexture as potential biomarkers of clinical response to ipilimumab treatment of melanoma patients. Investigation of the predictive value of these biomarkers in the case of anti-PD-1 or anti-PD-L1 agents as well as of anti-CTLA-4/anti-PD-1 combination therapies is also warranted.

Acknowledgements The authors thank Katalin Derecskei and Miklós Kónya (National Institute of Oncology, Budapest) for their excellent technical assistance.

Compliance with ethical standards

Funding The study was supported by the National Research, Development and Innovation Office Grants NKFI K105132, K116295, and by European Union GINOP_2.3.2-15-2016-00020.

Conflict of interest Tímea Balatoni has received speaker honoraria and financial support for attending symposia from Bristol-Myers Squibb, MSD Sharp and Dohme (MSD), Novartis, and Roche. Gabriella Liszkay is on the advisory board and has received honoraria for speaking at conferences as well as financial support for educational programs from Bristol-Myers Squibb, GlaxoSmithKline, MSD, Novartis, and Roche. Judit Oláh has acted as a speaker of symposia and consultant for Bristol-Myers Squibb, MSD, Novartis and Roche. Zsuzsanna Lengyel has received speaker honoraria from Bristol-Myers Squibb, MSD, Novartis, and Roche. Gabriella Emri has received speaker honoraria from Bristol-Myers Squibb, MSD, and Roche. All other authors declare that they have no conflict of interest.

Ethical approval and ethical standards The study followed the Declaration of Helsinki and was approved by the Scientific and Ethical Committee of Medical Research Council, Hungary (2506-3/2017/EKU). Informed consents from patients were not required by the board in case of retrospective studies where it is not possible to obtain consents from the majority of patients as in this case where most patients were deceased at the time of the study.

References

1. Ladányi A, Sebestyén T, Balatoni T, Varga A, Oláh J, Liszkay G (2015) Tumor-infiltrating immune cells as potential biomarkers predicting response to treatment and survival in patients with metastatic melanoma receiving ipilimumab. Eur J Cancer 51(Suppl 3):S111–112 **(Abstract)**
2. Balatoni T, Mohos A, Sebestyén T, Varga A, Oláh J, Lengyel Z, Emri G, Liszkay G, Ladányi A (2017) Tumor-infiltrating immune cells as potential predictive markers of response to treatment and survival in metastatic melanoma patients receiving ipilimumab. J Transl Med 15(Suppl 1):8 **(O7, Abstract)**
3. Pardoll DM (2012) The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer 12:252–264
4. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC et al (2010) Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 363:711–723
5. Robert C, Thomas L, Bondarenko I, O'Day S, Weber J, Garbe C, Lebbe C, Baurain JF, Testori A, Grob JJ et al (2011) Ipilimumab plus dacarbazine for previously untreated melanoma. N Engl J Med 364:2517–2526
6. Schadendorf D, Hodi FS, Robert C, Weber JS, Margolin K, Hamid O, Patt D, Chen TT, Berman DM, Wolchok JD (2015) Pooled analysis of long-term survival data from phase II and phase III trials of ipilimumab in unresectable or metastatic melanoma. J Clin Oncol 33:1889–1894
7. Feng Y, Roy A, Masson E, Chen TT, Humphrey R, Weber JS (2013) Exposure–response relationship of the efficacy and safety of ipilimumab in patients with advanced melanoma. Clin Cancer Res 19:3977–3986
8. Ascierto PA, Kalos M, Schaer DA, Callahan MK, Wolchok JD (2013) Biomarkers for immunostimulatory monoclonal antibodies in combination strategies for melanoma and other tumor types. Clin Cancer Res 19:1009–1020
9. Blank CU, Enk A (2014) Therapeutic use of anti-CTLA-4 antibodies. Int Immunol 27:3–10
10. Weide B, Di Giacomo AM, Fonsatti E, Zitvogel L (2015) Immunologic correlates in the course of treatment with immunomodulating antibodies. Semin Oncol 42:448–458
11. Friedman CF, Postow MA (2016) Emerging tissue and blood-based biomarkers that may predict response to immune checkpoint inhibition. Curr Oncol Rep 18:21
12. Martens A, Wistuba-Hamprecht K, Geukes Foppen M, Yuan J, Postow MA, Wong P, Romano E, Khammari A, Dreno B, Capone M et al (2016) Baseline peripheral blood biomarkers associated with clinical outcome of advanced melanoma patients treated with ipilimumab. Clin Cancer Res 22:2908–2918
13. Yuan J, Adamow M, Ginsberg BA, Rasalan TS, Ritter E, Gallardo HF, Xu Y, Pogoriler E, Terzulli SL, Kuk D, Panageas KS, Ritter G, Sznol M, Halaban R, Allison JP, Old LJ, Wolchok JD, Gnjatic S (2011) Integrated NY-ESO-1 antibody and $CD8^+$ T-cell responses correlate with clinical benefit in advanced melanoma patients treated with ipilimumab. Proc Natl Acad Sci USA 108:16723–16728
14. Ji RR, Chasalow SD, Wang L, Hamid O, Schmidt H, Cogswell J, Alaparthy S, Berman D, Jure-Kunkel M, Siemers NO, Jackson JR, Shahabi V (2012) An immune-active tumor microenvironment favors clinical response to ipilimumab. Cancer Immunol Immunother 61:1019–1031
15. Hamid O, Schmidt H, Nissan A, Ridolfi L, Aamdal S, Hansson J, Guida M, Hyams DM, Gómez H, Bastholt L, Chasalow SD, Berman D (2011) A prospective phase II trial exploring the association between tumor microenvironment biomarkers and clinical activity of ipilimumab in advanced melanoma. J Transl Med 9:204
16. Huang RR, Jalil J, Economou JS, Chmielowski B, Koya RC, Mok S, Sazegar H, Seja E, Villanueva A, Gomez-Navarro J, Glaspy JA, Cochran AJ, Ribas A (2011) CTLA4 blockade induces frequent tumor infiltration by activated lymphocytes regardless of clinical responses in humans. Clin Cancer Res 17:4101–4109
17. Champiat S, Ferté C, Lebel-Binay S, Eggermont A, Soria JC (2014) Exomics and immunogenics. Bridging mutational load and immune checkpoints efficacy. OncoImmunology 3:e27817

18. Snyder A, Makarov V, Merghoub T, Yuan J, Zaretsky JM, Desrichard A, Walsh LA, Postow MA, Wong P, Ho TS et al (2014) Genetic basis for clinical response to CTLA-4 blockade in melanoma. N Engl J Med 371:2189–2199
19. Wolchok JD, Hoos A, O'Day S, Weber JS, Hamid O, Lebbé C, Maio M, Binder M, Bonsack O, Nichol G, Humphrey R, Hodi FS (2009) Guidelines for the evaluation of immune therapy activity in solid tumors: immune-related response criteria. Clin Cancer Res 15:7412–7420
20. Ladányi A, Somlai B, Gilde K, Fejös Z, Gaudi I, Tímár J (2004) T-cell activation marker expression on tumor-infiltrating lymphocytes as prognostic factor in cutaneous malignant melanoma. Clin Cancer Res 10:521–530
21. Ladányi A, Mohos A, Somlai B, Liszkay G, Gilde K, Fejős Z, Gaudi I, Tímár J (2010) FOXP3$^+$ cell density in primary tumor has no prognostic impact in patients with cutaneous malignant melanoma. Pathol Oncol Res 16:303–309
22. Ladányi A, Kiss J, Mohos A, Somlai B, Liszkay G, Gilde K, Fejős Z, Gaudi I, Dobos J, Tímár J (2011) Prognostic impact of B-cell density in cutaneous melanoma. Cancer Immunol Immunother 60:1729–1738
23. Erdag G, Schaefer JT, Smolkin ME, Deacon DH, Shea SM, Dengel LT, Patterson JW, Slingluff CL Jr (2012) Immunotype and immunohistologic characteristics of tumor-infiltrating immune cells are associated with clinical outcome in metastatic melanoma. Cancer Res 72:1070–1080
24. Gajewski TF, Woo SR, Zha Y, Spaapen R, Zheng Y, Corrales L, Spranger S (2013) Cancer immunotherapy strategies based on overcoming barriers within the tumor microenvironment. Curr Opin Immunol 25:268–276
25. Herbst RS, Soria JC, Kowanetz M, Fine GD, Hamid O, Gordon MS, Sosman JA, McDermott DF, Powderly JD, Gettinger SN et al (2014) Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. Nature 515:563–567
26. Fehrenbacher L, Spira A, Ballinger M, Vansteenkiste J, Mazieres J, Park K, Smith D, Artal-Cortes A, Lewanski C, Braiteh F, Waterkamp D, He P, Zou W, Chen DS, Yi J, Sandler A, Rittmeyer A, for the POPLAR Study Group (2016) Atezolizumab versus docetaxel for patients with previously treated non-small-cell lung cancer (POPLAR): a multicenter, open-label, phase 2 randomised controlled trial. Lancet 387:1837–1846
27. Tumeh PC, Harview CL, Yearley JH, Shintaku IP, Taylor EJ, Robert L, Chmielowski B, Spasic M, Henry G, Ciobanu V et al (2014) PD-1 blockade induces responses by inhibiting adaptive immune resistance. Nature 515:568–571
28. Taube JM, Klein A, Brahmer JB, Xu H, Pan X, Kim JH, Chen L, Pardoll DM, Topalian SL, Anders RA (2014) Association of PD-1, PD-1 ligands, and other features of the tumor immune microenvironment with response to anti-PD-1 therapy. Clin Cancer Res 20:5064–5074
29. Spranger S, Spaapen RM, Zha Y, Williams J, Meng Y, Ha TT, Gajewski TF (2013) Up-regulation of PD-L1, IDO, and T_{regs} in the melanoma tumor microenvironment is driven by CD8$^+$ T cells. Sci Transl Med 5:200ra116
30. Bulliard Y, Jolicoeur R, Windman M, Rue SM, Ettenberg S, Knee DA, Wilson NS, Dranoff G, Brogdon JL (2013) Activating Fc γ receptors contribute to the antitumor activities of immunoregulatory receptor-targeting antibodies. J Exp Med 210:1685–1693
31. Selby MJ, Engelhardt JJ, Quigley M, Henning KA, Chen T, Srinivasan M, Korman AJ (2013) Anti-CTLA-4 antibodies of IgG2a isotype enhance antitumor activity through reduction of intratumoral regulatory T cells. Cancer Immunol Res 1:32–42
32. Simpson TR, Li F, Montalvo-Ortiz W, Sepulveda MA, Bergerhoff K, Arce F, Roddie C, Henry JY, Yagita H, Wolchok JD, Peggs KS, Ravetch JV, Allison JP, Quezada SA (2013) Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma. J Exp Med 210:1695–1710
33. Romano E, Kusio-Kobialka M, Foukas PG, Baumgaertner P, Meyer C, Ballabeni P, Michielin O, Weide B, Romero P, Speiser DE (2015) Ipilimumab-dependent cell-mediated cytotoxicity of regulatory T cells ex vivo by nonclassical monocytes in melanoma patients. Proc Natl Acad Sci 112:6140–6145