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REVIEW

Rational bases for the use of the Immunoscore in routine clinical settings as a prognostic and predictive biomarker in cancer patients

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Abstract

The American Joint Committee on Cancer/Union Internationale Contre le Cancer (AJCC/UICC) tumor, nodes, metastasis (TNM) classification system based on tumor features is used for prognosis estimation and treatment recommendations in most cancers. However, the clinical outcome can vary significantly among patients within the same tumor stage and TNM classification does not predict response to therapy. Therefore, many efforts have been focused on the identification of new markers. Multiple tumor cell-based approaches have been proposed but very few have been translated into the clinic. The recent demonstration of the essential role of the immune system in tumor progression has allowed great advances in the understanding of this complex disease and in the design of novel therapies. The analysis of the immune infiltrate by imaging techniques in large patient cohorts highlighted the prognostic impact of the *in situ* immune cell infiltrate in tumors. Moreover, the characterization of the immune infiltrates (e.g. type, density, distribution within the tumor, phenotype, activation status) in patients treated with checkpoint-blockade strategies could provide information to predict the disease outcome. In colorectal cancer, we have developed a prognostic score ('Immunoscore') that takes into account the distribution of the density of both $CD3^+$ lymphocytes and $CD8^+$ cytotoxic T cells in the tumor core and the invasive margin that could outperform TNM staging. Currently, an international retrospective study is under way to validate the Immunoscore prognostic performance in patients with colon cancer. The use of Immunoscore in clinical practice could improve the patients' prognostic assessment and therapeutic management.

Keywords: colorectal cancer, immune response, lymphocyte, T cells, tumor microenvironment

Introduction: from a tumor cell-oriented model to the integration of the tumor microenvironment

The many biological discoveries and the derived concepts that dominated the 20th century led to a strictly cell-based view of cancer (1). The resulting theory of cancer origin, referred to as the somatic mutation theory, defined cancer as a cell disease caused by DNA damage. In this 'cell-oriented' model, the cancer cells are autonomous and operate independently from their microenvironment. Advances in the knowledge of cancer molecular biology have gradually revealed the limits of this cell-oriented model (2). Indeed, the extreme complexity of the genome, the diversity and sheer number of genomic alterations observed in cancer cells and genomic instability prevent any structuring vision of cancer (3).

On the basis of these observations, Hanahan and Weinberg (4) proposed, at the end of the second millennium, to modify the cell-oriented model; cancer was then defined by the acquisition of six major behavioral traits (hallmarks) secondary to genomic changes. In 2011, two new features were

added to the previous major hallmarks: reprogramming of energy metabolism (the Warburg effect) and immune surveillance escape (5). Beyond the recognition of the essential role of the immune system in tumor initiation and progression, this article marked a conceptual breakthrough. The 'tumor cell-oriented' paradigm is replaced by a holistic vision including the tumor environment as a major player in the formation and development of cancer (6).

The microenvironment is composed of a set of cellular compartments comprising vascular, neuroendocrine, stromal, epithelial and immune cells. These compartments constitute a heterogeneous and dynamic set, where all the players communicate with each other directly by cell contact or through secreted molecules (5). The tumor environment definition is likely to evolve further by integrating novel components such as the microbiota (7, 8).

The natural immune response: from prognosis to therapy

Cancer natural history involves interactions between tumor and host defense mechanisms. The concept that the immune system can recognize and eliminate primary developing tumors has been postulated for nearly 100 years (9). The validity of this concept, named 'cancer immunosurveillance' (10), has been difficult to establish but is now demonstrated with a considerable amount of data from animal models and human patients (11). This was later integrated into the theory of 'cancer immunoediting' that takes into account the interactions between cancer and immune cells, each one influencing and changing the behavior of the other (12). When the tumor elimination is incomplete, a temporary state of equilibrium occurs. The selective pressure exerted by the immune cells induces a selection of tumor cell variants that leads to the escape phase. During the escape phase, the immune system is no longer able to contain tumor growth leading to clinically detectable malignant tumors (11).

Despite the immune escape, human solid tumors are commonly infiltrated by cells from the innate immune system (innate lymphoid cells, NK cells, NK-T cells, γδ T cells, macrophages, neutrophils, eosinophils and mast cells) and adaptive immunity (T lymphocytes, B lymphocytes and dendritic cells). For over 30 years, several publications have evaluated, with an increasing level of precision, the quality, the density and the functional orientation of T-cells infiltrating human tumors. There is now accumulative evidence showing a positive association between the density of intratumoral lymphocyte infiltrates in solid tumors and increased patient survival (13). The studies involved thousands of human solid tumor samples. A beneficial *in situ* immune reaction is not restricted to patients with minimal tumor invasion, indicating that the *in situ* immunologic forces may persist along with tumor progression.

This corpus of data provides strong support for the existence of a natural anti-tumor immune response in immunocompetent individuals. Strikingly, this immune response influences the course of the disease despite the apparent insensitivity of the tumor cells at the primary site to the immune attack. The search of the mechanisms involved in T-cell dysfunction has revealed that exhaustion of T cells, originally identified in $CD8^+$ T cells during chronic infection (e.g. by HIV, hepatitis C virus and hepatitis B virus), also occurred in cancer (14). Exhausted T cells overexpress multiple inhibitory receptors including programmed cell death 1 (PD-1), cytotoxic T-lymphocyte antigen 4 (CTLA-4) and others (15). Importantly, antibodies targeting and blocking these inhibitory 'checkpoint' molecules have recently been shown to be effective in the treatment of human solid tumors (16). These therapies have begun to revolutionize the current standard cancer treatment in multiple cancer types.

Effective clinical responses have revealed that it is possible to augment the function of endogenous anti-tumor T-cell responses. Thus, $CD8^+$ T cells of the tumor microenvironment might not be terminally dysfunctional and could be reinvigorated (17). Unfortunately, most patients do not experience complete responses and some do not respond at all. Thus, identifying predictive markers of the efficacy to checkpoint blockade strategies is needed. Analysis of pretreatment tumors has recently shown that the *in situ* natural immune response may be explored to predict and monitor response to checkpoint blockade (18, 19).

Hence, a biological test assessing the tumor immune infiltrate (e.g. type, density, distribution within the tumor and phenotype activation status) could become a central biomarker that is predictive for prognosis and response to (immuno) therapy. In order to satisfy this expectation, a methodology named the 'Immunoscore' has been defined to quantify the *in situ* immune infiltrate. This review aims to summarize (i) the most convincing evidence from cohort studies of the prognostic and predictive roles of the immune infiltrate in cancer patients and (ii) the performance of the Immunoscore and the state of advancement of the international Immunoscore program.

The prognostic value of tumor-infiltrating lymphocytes and the T_h1 immune orientation at the tumor site

In 1931, MacCarty observed, by histological analysis of colon cancer sections stained with hematoxylin and eosin (H&E), that a strong intratumoral immune infiltrate conferred an advantage in terms of survival (20). This pioneer observation was later confirmed in colorectal (21, 22), melanoma (23, 24), breast (25) and others cancers. In 1986, Jass (26) demonstrated for the first time that high lymphocyte density evaluated on histological sections in the invasive margin (IM) of rectal tumors was the only variable to be accepted in a multivariate prognostic model along with the tumor, nodes, metastasis (TNM) classification. This observation was of paramount importance, since it revealed that the *in situ* adaptive immune reaction was a critical variable influencing overall survival times independently of the influence of the tumor extension, challenging our understanding of the natural history of cancer.

The subsequent identification of specific markers and transcriptional profiles allowed the quantification of the immune sub-populations and determination of their functional orientation. It has been demonstrated in a large number of solid tumors (ovarian (27), head and neck (28, 29), bladder (30), breast (31), liver (32), prostate (33, 34), melanoma (35), lung (36, 37), esophagus (38) and colorectal (39–45) cancers) that the presence of T cells expressing CD3 with CD8 or CD4

and memory T cells expressing CD45RO were associated with good prognosis. However, discordant results have been reported in clear-cell renal cell carcinoma (46, 47), Hodgkin lymphoma or uveal melanoma (48).

T_h1 cells play a key role by linking innate immunity and adaptive immunity (49). T_h1 cells facilitate the development of tumor-specific $CD8^+$ cytotoxic T cells induced via cross-presentation of antigenic tumor peptides on MHC class I molecules presented by dendritic cells (50, 51). Further studies have demonstrated that the T_h1 cytokine IFN-γ has antiproliferative and/or proapoptotic effects on tumor cells (52, 53) and the capacity to enhance tumor cell immunogenicity by upregulating components of the MHC class I antigen-processing and antigen-presentation pathway (54, 55). Moreover IFN-γ can promote the production of tumoricidal molecules (e.g. nitric oxide and superoxide) by macrophages (56, 57) and NK cells activated by IFN-γ can kill tumor cells (58). In colorectal cancers, we have observed a correlation between cell densities of intratumoral T cytotoxic $CD8^+$, T memory $CD45RO^+$ and T_h1 cells that express the transcription factor T-box transcription factor (T-bet; A. Kirilovsky *et al.*, unpublished data)

Moreover, transcriptomic analyses have confirmed a strong association between the cytotoxic/memory tumor-infiltrating profile and the expression of genes involved in T_h1 orientation such as transcription factors T-bet (*TBX21*), IFN-regulatory factor 1 (*IRF1*) and signal transducer and activator of transcription 1 (*STAT1*), cytokines IFN-γ (*IFNG*) and IL-12 (*IL12*), chemokines (*CXCL9*, *CXCL10*, *CCL5*, *CX3CL1* and *CCL2*), adhesion molecules (intercellular adhesion molecule 1 (*ICAM1*), mucosal vascular addressin cell adhesion molecule 1 (*MADCAM1*) and vascular cell adhesion molecule 1 *VCAM1*) and the cytotoxic factors granzymes (*GZM*s), perforin 1 (*PRF1*) and granulysin (*GNLY*) (59, 60). Thus, the local presence of IL-12 and IFN-γ, chemoattractants and adhesion molecules could attract T cells with a T_h1, cytotoxic and memory profiles at tumor site.

Importantly, T_h1 gene signatures were correlated with good prognosis in colorectal cancer (59–63) but also in breast (64, 65), ovarian (27, 66) and melanoma (67) tumors. These observations are in accordance with many mouse models showing that deficiency in genes involved in the T_h1-oriented response (e.g. IFN-γ, IFN-γR and IL-12) increased the frequency of spontaneous or carcinogen-induced tumors (11).

Prognostic value of other immune orientations at the tumor site

Data on the impact of T_h2 immune orientation in cancers are still controversial. Components of T_h2-oriented immunity such as T_h2 cytokines (IL-4 and IL-13) and B cells might have dual effects on tumor progression (68–70). Moreover, eosinophils have been shown to decrease tumor growth and initiate antitumor activity, (71) and the immunosuppressive cytokine IL-10 is traditionally considered to favor tumor growth (68, 69). The prognosis of T_h2 immune orientation was assessed in a small number of studies and results are also contradictory. Thus, in ovarian (72), pancreatic (73) and gastric (74) cancers, T_h2 responses are associated with poor prognosis. Conversely, in breast cancer (75) and follicular or Hodgkin lymphoma, (76) there is a beneficial association between immune T_h2 infiltrate and survival. Finally, in colorectal cancer, a T_h2 gene signature did not correlate with the clinical outcome (63).

T_h17 cells are currently recognized as an independent T-cell lineage from T_h1 and T_h2 (77, 78). T_h17 cells producing IL-17 and IFN-γ induce inflammation (79) and can therefore promote inflammation-dependent tumor cell growth. T_h17 cells have been detected in several cancers, but the prognostic value associated with this infiltrate varies according to the cancer type. For instance, T_h17 cells have been associated with poor prognosis in colorectal (63), lung (80) and hepatocellular (81) carcinoma and with a good prognosis in ovarian (82) and esophageal (83) cancers. Overall, a strong intratumoral T_h17 cell infiltration is associated with a slower tumor progression of prostate cancer, (84) whereas the opposite effect is observed in hormone-refractory prostate cancer (85). These opposite results could be related to a plasticity of T_h17 cells; those cells having the ability to redifferentiate into suppressive T_{reg} cells or alternatively into T_h1-like pro-inflammatory cells capable of activating cytotoxic immune effectors (86, 87).

Finally, T_{reg} cells are the fourth major subset of $CD4^+$ T cells, characterized by the expression of the α chain of the IL-2R (CD25) and the transcription factor Forkhead box P3 (FoxP3) (88). T_{reg} cells can suppress the function of effector T cells and antigen-presenting cells (APCs) by either cell–cell interactions or by the release of TGF-β and IL-10, two suppressive cytokines (89). The prognostic value of T_{reg} cell density at the tumor site is still debated. A pioneering publication by Curiel *et al.* (90) positively correlated the presence of a high number of $FoxP3^+$ cells in ovarian carcinoma ascites with the degree of tumor extension and reduced survival. This negative prognostic value was also reported in other solid cancers such as pancreatic (91), liver (92) and breast (93) tumors.

Based on these results, it was hypothesized that the presence of natural or induced T_{reg} cells at the tumor site could be a major mechanism of tumor escape from the cytotoxic immune response. Since then, conflicting evidence has been complicating the picture. Thus, a favorable prognosis associated with a high density of $FoxP3^+$ intratumoral cells was reported in follicular and Hodgkin lymphomas (94, 95), head and neck cancers (29) and colorectal cancers (63, 96–98). Additional studies are required because the phenotypic and functional markers currently used to identify T_{reg} cells are not fully specific (99, 100).

Overall, analysis of data from the literature on the prognostic effect of different immune T populations reveals that cytotoxic $CD8^+$ T cells and memory T cells associated with a T_h1-oriented immune reaction strongly correlate with good clinical course in most studied cancer types (13, 101). On the other hand, the prognostic value of T_h2, T_h17 or T_{reg} cell populations is inconsistent and varies depending on the cancer type and stage (13).

Toward a clinical application in solid tumors: the Immunoscore

In colorectal tumors, immune cells are present within the tumor glands, in the surrounding stroma, at a distance within the IM as well as in newly formed tertiary lymphoid islets located in the tumor vicinity (102). As the immune infiltration in

tumors is heterogeneous, we hypothesized that the analysis of each tumor region could provide information on the tumor pathophysiology and possibly prognosis. Thus, we measured the density of immune cells and their distribution in the tumor core (CT) and the IM in three independent retrospectives cohort studies of colorectal cancer (n = 609 patients) (61). We performed an *in situ* quantification of T lymphocytes ($CD3^+$), memory T cells ($CD45RO^+$), cytotoxic T lymphocytes ($CD8^+$) and their cytotoxic molecules (granzyme B) using a dedicated image analysis program after immunostaining with specific antibodies.

We found a significant correlation between the density of immune cell populations in the two tumor regions (CT and IM) and the patients' clinical outcome, in terms of disease-free survival (DFS) and overall survival (OS). Moreover, when the CT and IM cell densities were considered together, the outcome differences between groups of patients with a high immune cell density in both tumor regions compared with patients with a low immune density in both areas further increased. Unexpectedly, multivariate analysis indicated that the 'weight' of the immune density parameter was independent from, and larger than, that provided by the pathology-based prognostic evaluation (i.e. the TNM staging) (61, 103).

Thus, the immune 'contexture'—defined as the type, functional orientation, density and location of adaptive immune cells within distinct tumor regions (61, 104)—appears to be the strongest prognostic factor for DFS and OS in colorectal cancer. The statistical preponderance of the immune assessment could be explained by the observation that the immune density is inversely correlated with tumor extension (T stage) but is constantly low in patients whose tumors will relapse, even at early stages of tumor progression (103). This information cannot be provided by the simple assessment of the TNM staging. Altogether, these results strongly suggest that tumor behavior should now be considered as the result of a balance between the invasive tumor process and a coordinated immune reaction of the host.

To evaluate the prognostic performance of the immune contexture, we then focused on patients with clinically localized colorectal cancers (stage I–II), among whom 25% will experience a relapse after surgery. We combined the analysis of CD8 and CD45RO staining in tumor regions (CT and IM) in a large retrospective cohort (n = 602 patients) (59). Five years after diagnosis, only 4.8% of patients with a strong infiltrate had relapsed and 13.8% had died. Conversely, 75% of patients with a low immune infiltrate had a relapse and 72.5% had died (log-rank test $P < 0.0001$).

We then derived a simple test named Immunoscore to facilitate the transfer of this discovery to the clinic (http://www.Immunoscore.org; Fig. 1). The Immunoscore is based on the numeration of two lymphocyte populations, $CD3^+$ and $CD8^+$, in the CT and in IM regions. $CD45RO^+$ memory T-cell density is highly overlapping with $CD3^+$ T-cell density and because of background staining and rapid loss of antigenicity after cutting tumor slides, CD45RO was excluded from the final test. The density of $CD3^+$ cells and $CD8^+$ cells is quantified using a dedicated image analysis workstation.

Each tumor is categorized into high or low density for each marker in each tumor region, according to an optimal cutoff value determined using the minimum P value approach (61). Patients are stratified according to a score ranging from I0 to I4 (103, 105), depending on the total number of high densities

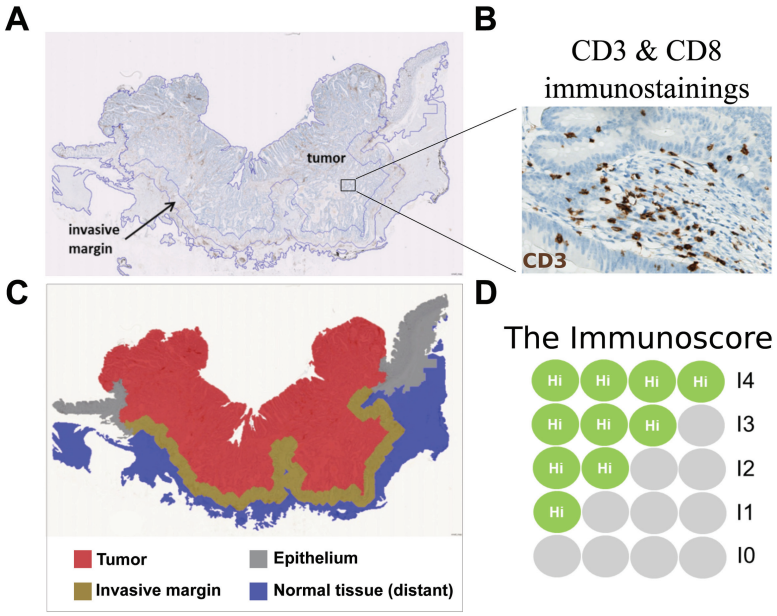

Fig. 1. (A) A section of colonic cancer immunostained for CD3, showing the regions of interest (the CT and the IM). (B) An enlargement showing $CD3^+$ cells (stained brown) in the stroma and within tumor glands (original magnification ×300). (C) The tumor (shown in red) and the IM (shown in brown) are selected to determine the Immunoscore. (D) The Immunoscore is based on the numeration of $CD3^+$ and of $CD8^+$ cells in the tumor and the IM. The densities of stained cells are determined using an image analysis workstation. The immune densities are categorized into Hi (high) or Lo (low) in each tumor region, according to a predetermined cutoff value. Patients are stratified according to a score ranging from I0 to I4, depending on the total number of high densities observed (the two markers CD3 and CD8 are assessed in the CT and the two markers are assessed in the IM).

observed (two markers assessed in CT and two markers assessed in IM). For example, I4 refers to a tumor with high densities of $CD3^+$ and $CD8^+$ cells in CT and IM regions of the tumor. I3 refers to tumors with three high densities. Patients with low densities of CD3 and CD8 in both tumor regions (0 high density) is classified I0 (Fig. 1).

We built an immunomonitoring platform at the Hospital Européen Georges Pompidou in Paris to perform the Immunoscore in routine settings on large cohorts and to achieve multiple quality controls and we coordinated an international retrospective study, under the supervision of the Society for Immunotherapy of Cancer (SITC), to validate the prognostic performance of the Immunoscore in patients with colonic cancer (105). The international retrospective study involved 23 centers in 17 countries worldwide. Thousands of colonic tumors have been evaluated on whole slide sections by international expert pathologists and immunologists of each center.

The study is now completed. Analyses are currently being performed by external statisticians according to a predetermined workplan. Results will be presented at the next American Society of Clinical Oncology (ASCO) annual meeting (June 3–7, 2016; Chicago, IL, USA). Rectal tumors were excluded from the Immunoscore SITC study because of distinct clinicopathologic features and treatment regimens. We have conducted an ancillary study to evaluate the prognostic performance of the Immunoscore on localized rectal cancers (106). The Immunoscore classified nearly 50% of the patients with very distinct behaviors: 35% with very a good outcome (score I4) as opposed to 12% with a poor outcome (score I0 or I1). Cox multivariate analysis supported the advantage of the Immunoscore compared with TNM staging in predicting recurrence and survival.

This methodology is still under investigation in several other types of cancer. Thus, in hepatocellular carcinoma, the Immunoscore has been already strongly associated with cancer outcome (107). Even in brain metastasis, one of the most common complications from cancer and which has a very poor prognosis, the Immunoscore was significantly correlated with survival prognosis and was independent from other prognostic parameters at multivariable analysis (108). Hopefully, these initiatives will result in the implementation of the Immunoscore as a new component for the classification of cancer: TNM-I (TNM-immune).

Prediction of the response to chemotherapy and radiotherapy

The innate and adaptive immune responses elicited by anthracyclines, oxaliplatin and ionizing irradiation are required for an optimal response of these anticancer treatments. The main mechanisms involve the release of tumor antigen, ATP and the purinergic receptor and the induction of an immunogenic cell death with the exposure of calreticulin, facilitating the engulfment of dying cells by APCs and the release of high mobility group box 1 (HMGB1) that binds TLR4 and stimulates antigen presentation (109).

In breast cancer, anthracycline-based neoadjuvant therapy is more effective when the tumor is infiltrated by T cells before the beginning of chemotherapy (110). It induces a significant influx of $CD8^+$ T cells into the tumor bed and a decreased density of immunosuppressive cells—T_{reg} cells and monocytic myeloid-derived suppressor cells (MDSCs) (111). A meta-analysis has recently shown that higher values of total tumor-infiltrating lymphocytes (TILs) predicted a better response to neoadjuvant chemotherapy in most breast cancers, except hormone-receptor negative ones (112). Moreover, intratumoral lymphocytes with a concomitant up-regulation of *CD3D* and *CXCL9* were independent predictors of complete response (110). In rectal cancers, we observed a significant correlation between densities of $CD3^+$ and $CD8^+$ cells and the pathological response to neoadjuvant radiochemotherapy (106). Association of $CD3^+$ and $CD8^+$ TILs with good response after neoadjuvant treatments was confirmed in two studies (113, 114).

Prediction of the response to immunotherapy

The clinical response to immunotherapies could be influenced by the quality of the natural immune response observed at the tumor site (Table 1). The data from the first immunotherapy trials with anti-CTLA-4 in melanoma have confirmed this hypothesis. Indeed, patients with tumors expressing a higher level of genes involved in T_h1-oriented (IFN-γ) and cytotoxic responses (perforin, granzyme and granulysin) or chemoattraction (*CCR5*, *CCL4*, *CCL5*, *CXCR3*, *CXCL9*, *CXCL10* and *CXCL11*) were more likely to respond favorably to ipilimumab (monoclonal anti-CTLA-4 antibody) treatment (18, 118, 119). Moreover, these immune reaction markers were significantly increased in tumors after treatment, and this increase was higher in responding tumors (18, 118). Fully activated $CD4^+$ and $CD8^+$ T cells with evidence of induction/potentiation of a memory phenotype ($CD45RO^+$) were observed following on treatment (115).

Interestingly, clinical activity was correlated with a higher baseline expression of FoxP3 and indoleamine 2,3-dioxygenase (IDO) proteins in tumors and a decreased expression after treatment in responding tumors (18). In a limited number of regionally advanced melanomas monitored by flow cytometry (115), T_{reg} cell levels tended to be higher at week 6 in the disease progression group, whereas the opposite was observed in the clinical benefit group. And a significant decrease in tumor MDSCs was associated with improved progression-free survival at 1 year.

Tumor expression of PD-1 ligand 1 (PD-L1) in melanoma is associated with the presence of TILs and a strong expression of IFN-γ transcripts, suggesting an adaptive tumor resistance mechanism (130). A recent study (120) on several types of solid cancers including melanoma and non-squamous non–small cell lung carcinoma treated by anti-PD-1 showed that PD-L1 tumoral expression was geographically associated with the presence of an immune infiltration. Furthermore, PD-L1 expression by both the tumor and the immune infiltrate was associated with PD-1 expression on lymphocytes. In this study, these parameters were associated with a good clinical response. However, in another study considering squamous non-small cell lung carcinoma, PD-L1 status was not correlated with a survival increase suggesting the importance of histological types and subtypes and should therefore be taken into account while considering predictive markers (131).

Table 1. Predictive, treatment response and surrogate markers for immune checkpoint inhibitors in cancer immunotherapies

Checkpoint inhibitor	Method	Marker	Predictive marker (baseline)	Biomarker (treatment response)	Surrogate marker (clinical response)	References
Anti-CTLA-4	Staining (H&E)	TIL	-	NT	+	(18)
	Immunohistochemistry	CD8	-	+	+	(18, 115, 116)
		FoxP3	+	NT	+ (inverse)	(18)
		IDO	+	NT	+ (inverse)	(18)
		PD-L1 (tumor cells)	NT	NT	+ (inverse)	(117)
	Cytometry	Activated CD4/CD8 T cells	NT	+	NT	(115)
		Memory CD8 T cells	NT	+	NT	(115)
		MDSCs	NT	NT	+ (inverse)	(115)
	Transcriptomics	T cell signature	+	+	+	(18, 118)
		Cytotoxicity signature	+	+	+	(18, 118, 119)
		Th1 signature	+	+	+	(18, 118)
		CXCR3/CXCL9–11 Pathway	+	+	+	(18, 118)
		CCR5/CCL3–5 Pathway	+	+	NT	(18, 118)
		MHC-II	+	+	NT	(18, 118)
		CXCR6	+	+	+	(18, 118)
		CTLA-4/PD-L2	+	NT	NT	(119)
	Whole-exome sequencing	Mutational load	+	NT	NT	(119)
		Neoantigen load	+	NT	NT	(119)
Anti-PD-1	Immunohistochemistry	PD-L1 (tumor cells)	+	NT	NT	(120–124)
		PD-L1 (immune & tumor cells)	+	NT	NT	(19, 120)
		CD8	+	NT	+	(19)
		CD8 + Ki67	NT	NT	+	(19)
		Granzyme B	NT	NT	+	(19)
		pSTAT1 (IM)	+	NT	+	(19)
		PD-1 (immune cells)	+	NT	NT	(19)
		CD4	-	NT	NT	(125)
		MHC-II (tumor cells)	+	NT	NT	(125)
	Cytometry	T cells ($CD3^+$)	NT	+	+	(126)
		B cells ($CD19^+$ or $CD20^+$)	NT	+	NT	(126)
		MDSCs	-	+	NT	(126)
		% CD8 Tem	NT	+	+	(126)
		% CD4 Tem	NT	+ (inverse)	NT	(126)
		% CD4 T effector T-cell–like	NT	+	+ (inverse)	(126)
	Next-generation sequencing	TCR clonality	+ (inverse)	NT	+	(19)
	Whole-exome sequencing	Mutational load	+	NT	NT	(127)
		Neoantigen load	+	NT	NT	(127)
Anti-PD-L1	Immunohistochemistry	PD-L1 (immune cells)	+	NT	+	(128, 129)
		PD-L1 (tumor cells)	-	NT	NT	(128, 129)
		CD8	-	NT	+	(128)
	Transcriptomics	CX3CL1 (fractalkine)	+ (inverse)	NT	NT	(128)
		IFN-γ	NT	NT	NT	(128)
		CTLA-4	+	NT	+	(128)
		IDO1	+	NT	NT	(128)
		CXCL9	+	NT	NT	(128)
		CXCL10	NT	NT	+	(128)
		Cytotoxicity signature	NT	NT	+	(128)
		EOMES	NT	NT	+	(128)

Association between markers and events are depicted as follows:
+ is a significant positive correlation.
+ (inverse) is an inverse correlation between marker presence and event.
- is an absence of significant correlation.
The abbreviations used are: TIL, Tumor infiltrating lymphocytes; FoxP3, Forkhead box P3; IDO, Indoleamine 2,3-dioxygenase; PD-1, Programmed cell death 1; PD-L, Programmed cell death ligand; MDSCs, monocytic myeloid-derived suppressor cells; Th1, T helper cell type 1; MHC-II, Major histocompatibility complex class II; pSTAT, phosphorylated signal transducers and activator of transcription; CTLA-4, Cytotoxic T lymphocyte-associated protein 4; Tem, T effector memory; IFN, interferon; TNF, Tumor necrosis factor, EOMES, eomesodermin; IM, Invasive margin; NT, not tested.

Consistent with these observations, Tumeh *et al.* (19) recently reported in patients with stage III melanoma treated with anti-PD-1 that the most strongly predictive marker of clinical response was not PD-1 or PD-L1, but the density of $CD8^+$ T cells in the IM as well as in the CT. T-cell clonality in the tumor was also more pronounced in responders. In addition, the density of T $CD8^+$ cells increased during treatment in responders whereas it remained weak in nonresponders (19).

Thus, the existence of a natural T $CD8^+$ cytotoxic immune reaction at the tumor site seems to be a prerequisite necessary to induce/reinvigorate an anti-tumor immune response with anti-PD-1. The presence of T $CD8^+$ cytotoxic cells with tumor cells expressing PD-L1 in a context of IFN-γ production could be the most favorable ground for anti-PD-1 immunotherapies. Recent reports further indicate that the presence of $CD8^+$ T cells expressing PD-L1 could predict a response to anti-PD-L1 treatment, and a global increase of the $CD8^+$ T-cell density was observed after treatment in responding tumors (128, 129).

Conclusion

Despite a partial exhaustion of the anti-tumor immune response, the intratumoral immune reaction is an important parameter influencing the natural course of the disease. The presence of cytotoxic T cells and a T_h1 immune reaction in tumor microenvironment is almost constantly associated with an increase in the patient's survival. In addition, there is increasing evidence supporting the hypothesis that an immune-active tumor microenvironment correlates with improved patient response to immune checkpoint inhibitors.

However, there are unresolved issues regarding measuring levels of the immune infiltrate. The essential parameters defining the immune populations have been grouped according to a concept of contexture that considers immune cell type, the functional orientation, density and location of adaptive immune cells in different tumor regions. For routine evaluation, a simple test called Immunoscore has been established and could improve patients' care. The result of the international validation of the Immunoscore will be presented shortly at the annual ASCO meeting. The Immunoscore could address difficulties and be sufficiently convenient to use in a clinical setting to provide an accurate prediction of a patient's prognosis and clinical response.

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