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Authors

Quigley, David A
Kristensen, Vessela

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Review

Predicting prognosis and therapeutic response from interactions between lymphocytes and tumor cells☆

David A. Quigley[a,b,*], Vessela Kristensen[c,d,**]

[a]Helen Diller Family Comprehensive Cancer Center, University of California at San Francisco, San Francisco, USA
[b]Department of Epidemiology and Biostatistics, University of California at San Francisco, San Francisco, USA
[c]Department of Genetics, Institute for Cancer Research, Oslo University Hospital Radiumhospitalet, Oslo, Norway
[d]K.G. Jebsen Center for Breast Cancer Research, Institute for Clinical Medicine, Faculty of Medicine, University of Oslo, Oslo, Norway

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ABSTRACT

As epithelial tumors grow from single cells to a malignant mass of invasive tissue, they must exploit the innate inflammatory response, while evading the adaptive immune system. Prognosis of solid tumors has historically focused on macroscopic features such as size, grade, and mitotic index. It is now clear that prognosis assessment must also consider the stromal and immune cells that surround and infiltrate the tumor. Tumors promote growth, angiogenesis, and tissue remodeling by subverting the normal functions of macrophages and other cells of the innate immune system that inhabit their microenvironment. Simultaneously, tumor cells escape from and inactivate the adaptive immune system by exploiting the mechanisms preventing damaging auto-immune responses in cytotoxic T cells. The presence of $CD8^+$ T cells within epithelial tumors is now a well-supported marker of better prognosis in many tumor types. However, this benefit is counterbalanced by immune regulatory cell populations that promote tumor escape from immune surveillance and metastasis. Therapeutic approaches that kill tumor cells selectively by re-activating immune checkpoints are becoming an established therapeutic option, but it is not yet clear how to identify which patients will benefit from this treatment modality. Evidence is accumulating that identifying the presence of T cell-activating neoantigens, produced by mutated proteins in tumors, will play an important role in checkpoint inhibitor prognosis. This review provides an overview of the evidence that lymphocytic infiltration of tumors has prognostic value in many epithelial tumor types and is linked to the success of chemical and immune checkpoint therapeutic strategies.

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* *Corresponding author*. Helen Diller Family Comprehensive Cancer Center, University of California at San Francisco, San Francisco, USA.
** *Corresponding author*. Department of Genetics, Institute for Cancer Research, Oslo University Hospital Radiumhospitalet, Oslo, Norway.
E-mail addresses: David.Quigley@ucsf.edu (D.A. Quigley), v.n.kristensen@medisin.uio.no (V. Kristensen).
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1. Background

In the classic model of tumorigenesis, an initiated cell accumulates a series of somatic changes that promote tumor growth and allow the cell to escape from signals that would normally force it into permanent growth arrest or self-destruction. A relationship between chronic inflammation and tumor growth has been long-established in some solid tumors such as colorectal cancer, for which the chronic inflammatory condition, Crohn's disease, is a predisposing risk factor (Ekbom et al., 1990). While the classic Hallmarks of Cancer review published in 2000 acknowledged that tumors co-opt their surrounding stroma to produce growth stimulatory factors and obtain nutrition via the angiogenic switch, the initiated tumor cell was portrayed as the primary driver of altered growth signaling, and the immune response was not described as a major factor (Hanahan and Weinberg, 2000). Many targeted therapeutic approaches to cancer therapy following this model have focused on blocking oncogenic mutations that activate kinases such as BRAF and KRAS, whose cell-intrinsic aberrant growth signaling drives tumor growth (Chapman et al., 2011; Druker et al., 2001; Zhang et al., 2009). This targeted approach seeks to identify and quash the activity of the protein bearing the oncogenic mutation to which the tumor is addicted. There have been dramatic successes with this approach, but in almost all applications the evolutionary pressure upon the evolving tumor cell population exerted by targeted therapy results in the emergence of disease resistant to the targeted therapy. It is only relatively recently that the role of the adaptive immune system in opposing tumor growth has been widely appreciated. After years of controversy, it is now established that reactivating the immune system is a potent tool to eradicate some forms of cancer. In Hanahan and Weinberg's follow-up to their original Hallmarks review, evasion of the immune system and the role of the tumor microenvironment were acknowledged to play prominent roles (Hanahan and Weinberg, 2011). Three factors support this change: 1) improved understanding of the relationship between the innate and adaptive immune systems and tumorigenesis; 2) consistent evidence that lymphocytic infiltration into solid tumors is a beneficial prognostic marker; and 3) the recent successes and FDA approval of immune checkpoint therapies such as pembrolizumab and ipilimumab. This review will describe how assessment of the infiltration of sub-populations of lymphocytes into tumors, as well as tumor cell-intrinsic factors that interact with the immune system, are becoming relevant for patient prognosis and treatment response.

2. Tumors co-opt the inflammatory mechanism

It has been clear for decades that chronic inflammation creates a microenvironment that favors tumor growth and metastatic spread of tumor cells (Coussens and Werb, 2002; Coussens et al., 2013). Normal local inflammatory response to either wounding or pathogenic invasion results in the production of growth factors and chemokines that attract infiltrating monocytes from the circulatory system. These monocytes differentiate into macrophages that promote temporary tissue remodeling, activation of fibroblasts, and angiogenesis as a part of the wound healing process. Macrophages are phagocytic cells of the innate immune system that digest foreign invaders, present antigens to the adaptive immune system, and produce pro-inflammatory cytokines and growth factors (Wynn et al., 2013).

Long term tissue inflammation in response to a stimulus such as a viral infection produces conditions that encourage tumor formation by overstimulating the mechanisms of growth signaling and remodeling. Although most viral infections are acute, the persistence of certain viral infections has been causally linked to tumor development. Human Papilloma Virus (HPV)-associated cervical cancer and HPV-associated Head and Neck Squamous Cell Carcinoma are caused by HPV-infected cells that evade the immune system and produce a persistent inflammatory response by pro-inflammatory cytokines (Boccardo et al., 2010; Gillison et al., 2000; Moscicki et al., 2012). Patients chronically infected with hepatitis B are at elevated risk of developing hepatocellular carcinoma (Beasley et al., 1981; Chisari and Ferrari, 1995). During persistent tumor-associated inflammation, ordinary macrophages can become co-opted by the tumor to promote abnormal growth signaling, tissue remodeling, and angiogenesis. These cells polarize into type II (M2) Tumor Associated Macrophages (TAMs). M2 macrophages have greatly reduced ability to present antigen to T cells and are immunosuppressive, while retaining their tissue remodeling and pro-angiogenic abilities (Mantovani et al., 2002; Noy and Pollard, 2014; Mantovani et al., 2002; Wynn et al., 2013). The presence of TAMs in tumor stroma is generally a negative prognostic indicator (Lewis and Pollard, 2006; Noy and Pollard, 2014; Pollard, 2004). Proteases produced by M2 macrophages play a key role in growth stimulation and metastatic spread. Reducing signaling through the macrophage recruitment factor colony stimulating factor (*CSF1*) by blockade of the CSF1 receptor CSF-1R a mouse model of mammary adenocarcinoma was shown to deplete macrophages and improve chemotherapeutic response (DeNardo et al., 2011). Blocking CSF-1R activity in a mouse model of glioblastoma also resulted in improved survival without reducing the number of TAMs present in tumors (Pyonteck et al., 2013). These preclinical studies have recently been followed by applications of monoclonal antibodies blocking CSF-1R to patients with diffuse-type giant cell tumors (Ries et al., 2014). Importantly, the study by DeNardo and colleagues showed a reciprocal relationship between the reduction in TAMs and the beneficial influx of $CD8^+$ T cells, highlighting the connections between the innate and adaptive immune cell populations.

3. T cells to the rescue, if they can find the target

Epidemiological studies have long supported the role of the adaptive immune system in blocking cancer. Retrospective studies of cancer rates in organ transplant recipients demonstrated that patients taking immunosuppressive drugs were at significantly greater risk of developing malignancies

including non-melanoma skin cancer, non-Hodgkin's lymphoma, kidney cancer, and endocrine cancer (Adami et al., 2003; Birkeland et al., 1995). Increased risk is confined to certain tissue sites, and was greatest in tumors with a viral etiology. Large surveys of kidney transplant patients did not identify any increase in risk for some of the most common tumor types, including prostate and breast tumors (Vajdic et al., 2006).

The concept that T cells were the primary means by which the immune system would combat tumors is decades old [reviewed in Dunn et al. (2002)], but, until the turn of the century, we lacked appropriate tools to directly test the hypothesis that tumors must evade or disable this process. Studies elucidating which cell populations are required for tumor immune suppression began accumulating in the 1990s, after genetically engineered mouse models had become sufficiently sophisticated to allow formal testing of the mechanistic role of the immune system in tumorigenesis. In 2001, Robert Schreiber and colleagues demonstrated that mice completely lacking a functioning adaptive immune system were at elevated risk of spontaneous and carcinogen-induced sarcomas, and that the tumors that arise in immune competent mice have reduced immunogenicity compared to those arising in immune-compromised animals (Shankaran et al., 2001). These findings renewed interest in the hypothesis that evasion of the adaptive immune system is a key step in tumorigenesis. The protective role of the adaptive immune system is now termed immune surveillance, and the effort of tumors to hide from the immune system by concealing their antigens or shutting down the normal operation of immune cell effectors is called immune editing (reviewed in Schreiber et al. (2011)).

Mutant proteins produced by Dunn et al. (2002) tumors with genome sequence alterations produce neoantigens that may appear foreign to the adaptive immune system. It was recognized early on that these neoantigens in proteins such as TP53 might be interpreted by the immune system as danger signals indicating a foreign pathogen (Crawford et al., 1982; Soussi, 2000). Additionally, tumors are frequently necrotic and hypoxic, conditions that indirectly stimulate the immune system. These local danger signals can stimulate a local response that activates the immune system at a distance. Tissue-resident dendritic cells are antigen presenting cells that serve as key messengers between a local invasion and lymphocytes that dwell in lymphoid organs, lymph, and the circulatory system. After immature dendrites are activated by pattern recognition receptors and identify a dangerous antigen, they mature, phagocytose the source of that antigen, and migrate to lymph nodes, where they convey the antigen to naïve and central T cells. When activated by antigen presentation and co-stimulatory signals from the antigen-presenting cell, T cells mature into several subpopulations, including effector T cells capable of targeted killing of pathogens expressing that antigen using cytolytic enzymes such as perforin and granzyme B. Cytotoxic $CD8^+$ T cells are not effective killers until they are activated by the combination of antigen presentation to the T cell antigen receptor complex and co-stimulatory signals from the B7 proteins CD80 and CD86 through the CD28 receptor (Townsend and Allison, 1993).

$CD8^+$ T cells are assisted by helper T cells, identifiable by expression of the CD4 receptor, which stimulate cytotoxic T cells, macrophages, and B cells. Naïve post-thymic $CD4^+$ T cells differentiate into numerous lineages, including the T_H1, T_H2, T_H17, and Treg lineages (Weaver et al., 2006). The frequency of circulating CD4/CD25 + cells increases in patients with epithelial malignancies (Wolf et al., 2003), but the impact on tumor development and metastasis of this expansion is complex. T_H1 cells secrete interferon-γ, and assist cytotoxic lymphocytes and tumor-fighting M1 macrophages. These cells are broadly anti-tumorigenic. T_H2 cells produce IL-4 and IL-13, assisting antibody-producing B cells, which would seem to make them anti-tumorigenic as well. However, there is evidence that in the MMTV-PMT mouse model of breast cancer, T_H2 cells promote metastasis by activating M2 macrophages (DeNardo et al., 2009). Pro-inflammatory T_H17 cells secrete the cytokine *IL17*, which promotes the spread of tumor cells by stimulating angiogenesis and the migration of vascular endothelial cells, and by stimulating fibroblasts (Numasaki et al., 2003). However, there is also evidence that T_H17 cells play a beneficial role in halting tumor progression by stimulating the activity of T_H1 and effector cells (Benchetrit et al., 2002; Kryczek et al., 2009; Muranski et al., 2008) [reviewed in Murugaiyan and Saha (2009)]. T regulatory (T_{reg}) cells act as immune suppressors, damping the adaptive immune response to prevent destructive autoimmune responses (Van Parijs and Abbas, 1998). (Numasaki et al., 2003; Sakaguchi et al., 2008) T_{reg} cells play an important role in reducing auto-immunity, but their immune suppressive (Wolf et al., 2003) activity can be exploited by tumors to produce negative effects on the efficacy of cytotoxic T cells and immune checkpoint inhibitors (Nishikawa and Sakaguchi, 2010). A consequence of the distinct and sometimes opposing roles of T cell subtypes is that efforts to harness T cells as tumor fighters must avoid promoting the activities of detrimental cell types to whatever extent is possible.

4. Assessing the tumor microenvironment in patients

Prognostic assessment of cancer patients has been assisted by technological advances in molecular analysis, including the characterization of tumor transcriptional activity, quantitative assessment of protein expression, and evaluation of methylation status. Despite the development of these methods, the mainstay of tumor diagnosis and prognosis remains radiographic assessment and pathological assessment of cellular phenotypes and mitotic activity in histological tumor sections. Before the development of gene transcription microarrays, retrospective studies of stained tissue sections sought to identify immune-associated factors that would predict tumor survival. These studies showed significant associations between semi-quantitative assessment of tumor-infiltrating lymphocytes and improved survival in numerous epithelial malignancies, including melanoma (Clark et al., 1989; Clemente et al., 1996), bladder (Lipponen et al., 1992), ovarian (Zhang et al., 2003), and colorectal cancer (Naito et al., 1998; Pages et al., 2005). Although gene transcriptional analysis would later add new layers of information, the fundamental demonstration of the association between

lymphocytic infiltrate and improved patient outcome was made by counting cells on a slide. These studies provided strong evidence that the presence of lymphocytes in a solid tumor was a positive prognostic sign in these tumor types.

4.1. *Colorectal cancer and the immunoscore*

In 2006, Jerome Galon and his colleagues used a combination of microarray analysis and immunohistochemistry to document that the presence, type, and location of $CD8^+$ T cell infiltrates in human colon cancer were associated with longer patient survival. This provided information that was orthogonal to and more informative than standard UICC-TNM staging for tumor site, regional lymph node involvement, and metastatic spread (Galon et al., 2006). This study emphasized that it was not only the number of T cells present overall in the tumor that mattered for prognosis, but their distribution within the tumor. Another large retrospective study of colorectal cancer stained 967 stage II and III colorectal cancers for CD8, CD45RO, and the T_{reg} marker FOXP3 (Salama et al., 2009). This study confirmed earlier correlations between $CD8^+$/$CD45RO^+$ cells and improved survival. It further found that overall number of $FOXP3^+$ T_{reg} cells in tumor tissue was associated with improved survival, but when $FOXP3^+$ T_{reg} cells occupied the surrounding stroma, survival was reduced. These observations have been formalized into a test that quantifies the number and location of $CD8^+$ T cell effectors and $CD45RO^+$ memory T cells within epithelial tumors called the Immunoscore that is now being validated in a series of studies (Galon et al., 2014). The overall message from these studies was that tumors can escape immediate destruction from the immune system, but the presence of $CD8^+$ T cells within some tumors confers better prognosis. The prognostic relevance of T_{reg} cells in these assays has been more complicated. In addition, quantifying immune infiltrate is a valuable source of prognostic information, independent of other established signs.

4.2. *$CD8^+$ T cells are beneficial in certain subtypes of breast cancer*

The prognostic value of tumor infiltrating lymphocytes (TIL) in breast cancer has been repeatedly assessed. As noted above, patients undergoing long-term immune suppression are not at elevated risk of developing breast cancer. However, as in every aspect of breast cancer, the tumor's molecular subtype is extremely important. TIL are rare in the seventy percent of tumors that respond to growth stimulation through the estrogen receptor (ER) (Curtis et al., 2012). Several large retrospective studies of breast tumors have established that the presence of lymphocytic infiltration is associated with improved prognosis in tumors that do not grow in response to stimulation through the Estrogen Receptor, or that are HER2-positive. A study of two ER-negative cohorts reported that qualitatively higher levels of TIL were associated with elevated tumor sensitivity to anthracycline in HER2-positive and triple-negative (that is, tumors negative for Estrogen Receptor, Progesterone Receptor, and HER2) cohorts (West et al., 2011). Retrospective analysis of 2009 node-positive breast cancer cases from the BIG 02-98 trial demonstrated that increases in stromal and intratumoral lymphocytic infiltration were associated with reduced risk of death for patients with triple-negative tumors (Loi et al., 2013). This study also reported that HER2-positive patients with TIL present who received a larger dose of anthracycline showed improved survival. In a follow-up study of the FinHER cohort (Loi et al., 2014), TIL infiltrate was associated with reduced risk of recurrence in two groups: patients with triple-negative disease and those with HER2-positive disease who were treated with trastuzumab. This result was particularly interesting in light of data showing anti-HER2/neu antibody therapy is dependent upon the presence of T cells (Park et al., 2010b). An analysis of TIL density in 481 tumors from ECOG trials E2197 and E1199 found stromal TIL density was an independent prognostic factor for disease-free survival and overall survival (Adams et al., 2014). The largest such study to date assessed the association between lymphocytic infiltration and survival by performing immunohistochemistry for CD8 in 8978 breast tumor samples and for FOXP3 in 5239 tumor samples (Ali et al., 2014). This study reported that $CD8^+$ T cells were associated with reduced risk of death in patients with either ER-negative or ER-positive/HER2-positive disease, and that ER-negative patients with $CD8^+$ T cells in their tumors derived greater benefit from anthracycline chemotherapy. In summary, these studies provided strong evidence that the presence of TIL was associated with better survival and improved therapeutic response, except in HER2-negative, ER-positive patients.

Gene transcriptional activity has been extensively characterized in breast tumors, and provides further evidence for the link between lymphocytic infiltration and prognosis (Curtis et al., 2012; Perou et al., 2000; Sorlie et al., 2001; van 't Veer et al., 2002). The majority of these studies have been performed using bulk sampling of tumors, which contained a mixture of tumor, stroma, normal epithelium, red blood cells, and other cell types. Among the earliest uses of these data were to develop prognostic signatures for breast cancer (Chang et al., 2005; van de Vijver et al., 2002), leading to FDA-approved prognostic tests that rely on gene expression such as the Mammaprint and OncotypeDX assays. However, interpretation of prognostic signatures must account for breast tumor subtypes, as Estrogen Receptor subtype is strongly associated with both survival and expression of many genes in breast tumors (Tofigh et al., 2014). Complementing the results observed by pathological review of tumor sections, a T cell gene expression signature has repeatedly been associated with patient survival in ER-negative patients (Desmedt et al., 2008; Kristensen et al., 2012; Mahmoud et al., 2011; Rody et al., 2009; Schmidt et al., 2008; Teschendorff et al., 2010, 2007). We recently showed that breast tumors with high expression of a T cell signature were significantly more likely to retain a functioning *TP53* pathway (Quigley et al., 2015).

In addition to studies quantifying the number or presence of lymphocytes in tumors, several recent studies have proposed the ratio of stroma to non-stromal content as a prognostic factor for risk of relapse in breast tumors (de Kruijf et al., 2011; Dekker et al., 2013; Huijbers et al., 2013). A study by de Kruijf and colleagues assigned stroma-high or stroma-low categories to 574 Hematoxylin-Eosin stained histological sections and reported that higher intra-tumor stromal content

was associated with shorter relapse-free period (de Kruijf et al., 2011), a finding they later confirmed in a larger population (Dekker et al., 2013). Similar findings have been reported in a study of 710 colon cancer patients (Huijbers et al., 2013). A study that focused on patients with ER-positive breast tumors reported better outcomes in patients with higher, rather than lower, intra-tumor stromal content (Downey et al., 2014). This finding emphasized that the role of inflammation and immune response in breast cancer must be understood within the context of tumor subtype. Other groups have examined gene expression activity in breast tumor-associated stroma directly by laser capture microdissection of stromal compared to epithelial tumor compartments. The Park group demonstrated that analysis of transcriptional activity using this approach could be used to construct a prognostic classifier (Finak et al., 2008). Better outcome was associated with a stromal transcription cluster that showed elevated expression of T cell and NK cell markers, compatible with a T_H1 response. In contrast, stromal expression of genes related to hypoxia and angiogenesis was associated with worse outcome.

4.3. *Estimating the composition of infiltrating cells from whole tissue*

Estimating the presence of T cells in a mixed population of tumor, normal tissue, and stroma is made somewhat easier by the fact that T cells bear cell-type specific surface markers. To increase the precision of these estimates of cell fractions, several groups have developed methods to deconvolute the relative numbers of individual cell populations from mixtures of mRNA derived from whole tumors. The Galon group recently described the immune landscape in human colorectal cancer, identifying transcriptional signatures of 28 different immune cell types infiltrating the tumors (Bindea et al., 2013). This study demonstrated that the immune infiltrate composition changed with tumor stage. When tumor progression was present, there were increased densities of T follicular helper (T_{fh}) cells and innate cells, while most other T cell densities were decreased. The B cell population, which increased in later stage tumors, was associated with prolonged survival. Most notably, the authors demonstrated that genomic instability in the locus harboring the chemokine *CXCL13* was associated with T_{fh} and B cell infiltration. Together with the expression of *IL21*, its expression was necessary for the T_{fh}/B cell influence on survival.

The Nanodissect algorithm accomplishes a similar task by ranking each gene in the genome according to an estimated likelihood of being cell type specific (Ju et al., 2013). Nanodissect has been applied to transcriptomes from breast tumors and normal breast tissue, estimating the presence of B cells, cytotoxic T lymphocytes, T_H1 lymphocytes, and T_H2 lymphocytes (Quigley et al., 2015). Another method, Cibersort, characterizes cell composition of complex tissues using their gene expression profiles (Newman et al., 2015). Cibersort gives relative estimates based on the mixture of cell types in a sample, and the diversity of cell types possible within a sample affects the output. Nanodissect does not *a priori* ascertain how many different cell types are in a sample and operates with absolute levels which are more easily correlated to other clinical parameters.

5. Biomarkers for immune checkpoint response

The tumor genome is shaped by selective pressure to stimulate growth and deactivate cell-intrinsic checkpoints such as the *TP53* tumor suppressor pathway. Since cytotoxic T cells can eliminate tumor cells, this produces selective pressure to disable the adaptive immune response. One mechanism for this immune suppression is to exploit immune checkpoints that normally prevent aberrant T cell responses. The PD-1 receptor is expressed on activated T cells, where it functions as an antagonist to T cell receptor signaling after binding the PD-L1 or PD-L2 ligand. Activation of the PD-1 immune checkpoint limits auto-immunity during inflammatory responses (Barber et al., 2006; Park et al., 2010a; Yang et al., 2011). Evidence supporting the importance of this pathway in tumor immune editing began accumulating fifteen years ago with the observation that while normal human tissue lacks PD-L1 expression, PD-L1 is abundantly expressed in many tumors (Dong et al., 2002). This suggested PD-1 activation was preventing T cell-mediated killing of tumor cells. It was observed that expression of PD-1 was required for the growth of myeloma cells in syngeneic host mice (Iwai et al., 2002). These observations motivated an effort to unleash an adaptive immune response against tumors by blocking this immune checkpoint using monoclonal antibodies targeted against PD-1 or the PD-L1 ligand (reviewed in Topalian et al. (2012a)). Targeting this pathway has produced durable responses in patients with advanced melanoma, renal cancer, and non-small-cell lung cancer (Brahmer et al., 2012; Topalian et al., 2012b).

Tumor expression of PD-L1 is an obvious candidate biomarker for efficacy of PD-1 checkpoint inhibition. The presence of TIL in melanomas and inflamed nevi is strongly associated with expression of PD-L1 (Taube et al., 2012). A study in 46 patients with metastatic melanoma found that response to the PD-1 inhibitor pembrolizumab was associated with the pre-treatment presence of PD-L1-expressing TIL within the tumor itself, not exclusively at the tumor margin (Tumeh et al., 2014). A recent phase I trial using the anti-PD-L1 antibody MPDL3280A in 277 patients found that elevated PD-L1 expression in infiltrating cells, but not in tumor cells, was associated with elevated objective response rates (Herbst et al., 2014). Thirteen percent of the patients with no detectable PD-L1 protein expression also showed an objective response to therapy, suggesting that PD-L1 expression alone is not an adequate biomarker for the choice to use this therapy. The most consistent result so far has been that patients lacking TIL do not benefit from checkpoint inhibitor therapy, but much remains to be determined about which patients will benefit from these approaches.

An orthogonal approach to predicting the efficacy of immune checkpoint inhibition is to identify whether tumor-specific somatic mutations produce antigens likely to be targeted by T cell killing. Several recent studies have suggested there is an association between overall tumor mutational burden and treatment response. A subset of colorectal, endometrial, and ovarian, and bladder tumors have hypermutation phenotypes due to inactivation of the mismatch repair (MMR) pathway. These tumors bear 10- or 100-fold more mutations

than MMR-competent tumors with the same tissue of origin. A small study of patients with colorectal tumors who were treated with pembrolizumab reported an objective response in 62% of patients with MMR-deficient tumors (N = 25), compared to none of the patients with MMR-competent tumors (N = 13) (Le et al., 2015). In this report, neither PD-L1 nor CD8 expression was significantly associated with objective response. Whether the lack of association between response and PD-L1 expression in these patients was a distinction between melanoma and colorectal cancer, or a function of the relatively small sample sizes that have been studied to date, is currently unclear. A study of patients with non-small cell lung cancer, another disease with a very high basal mutation rate, also reported an association between higher mutational burden and more frequent durable response (Rizvi et al., 2015). The main message from these studies is that tumors with very heavy mutational burdens are likely to produce a large number of neoantigens, and are more likely to activate cytotoxic lymphocytes when immune suppression is relieved using checkpoint blockade therapies. However, hypermutated tumors are relatively rare, and not all hypermutated tumors are vulnerable to immune checkpoint inhibitors.

Several studies have taken an additional analytical step beyond calculating tumor mutational load, attempting to identify specific antigens that predict treatment efficacy (Duan et al., 2014; Gubin et al., 2014; Rajasagi et al., 2014; Segal et al., 2008; Snyder et al., 2014). A commonly employed analytical pipeline has been to identify mutant neoepitopes using exome sequencing, and then predict neoepitope binding avidity to specific HLA complexes using NetMHC (Lundegaard et al., 2008; Nielsen et al., 2003), an analytical approach that uses neural networks. This pipeline provides a score predicting the binding avidity of each epitope to a given HLA allele. Melanoma has one of the highest mutational burdens of any non-MMR-deficient tumor type (Alexandrov et al., 2013). CTLA-4 is a second immune checkpoint protein that opposes T cell CD28 co-stimulation. The anti-CTLA-4 antibody ipilimumab was approved by the FDA in 2011 for melanoma treatment. A study of the efficacy of CTLA-4-inhibition in melanoma produced suggestive, but not definitive evidence that mutational load was associated with survival after immune checkpoint blockade with ipilimumab (Snyder et al., 2014). This result motivated a search for somatic neoepitopes associated with treatment response. They reported that no single epitope was associated with response, but they identified an ensemble of epitopes that predicted response. Trajanoski's group recently performed an *in silico* analysis of a cohort of 595 colorectal cancer patients to characterize the antigenicity of tumors (Angelova et al., 2015). Colorectal tumors with microsatellite instability (MSI-H) have extremely high mutation frequency. In this report, predicted neo-antigens were generally unique to a particular patient. Tumor hypermutation was associated with higher predicted numbers of tumor-infiltrating lymphocytes, reduced number of immunosuppressive cells, and higher expression of immunoinhibitory molecules.

The *in vitro* confirmation rate for T cell activation by predicted neoepitopes is usually low, suggesting that research in this area still has considerable development ahead. Some pipelines compare neoantigens to their corresponding unmutated counterpart epitope and use the difference in predicted binding avidity as a filter (Duan et al., 2014). Other groups have used orthogonal approaches such as mass spectrometry to provide additional filtering of candidate neoepitopes (Yadav et al., 2014). It is not yet established whether ensemble approaches that employ sets of weakly predictive neoepitopes will be generalizable to new sets of patients, in the same manner that gene expression signatures from microarray studies have been used to predict outcomes from new patients. Early evidence does not support a model where a small number of neoepitopes that recur with high frequency in patient populations will be immediate prognostic indicators for immune checkpoint inhibitor response, but larger studies are required to address this important point rigorously.

6. Concluding thoughts

The clinical importance of the immune system to cancer therapy is now beyond question. The presence of TIL in colorectal, ovarian, ER-negative breast cancer, and other tumor types has prognostic value independent of many established prognostic signs. Immune checkpoint blockade therapy will continue to grow in importance and provide real benefit to cancer patients. However, these treatments will not be a panacea, and refining the predictive biomarkers of therapeutic response is an ongoing process. These therapies will be administered in combination with existing chemotherapeutic and radiation modalities. Early evidence suggests that combinations of checkpoint inhibitors will also be more effective than single agent treatments (Kalbasi et al., 2013; Wolchok et al., 2013). A major focus of translational research will be prediction of when the tumor microenvironment is primed for T cell activation, and how to manipulate that environment when conditions are not already favorable. An important consequence of studies linking mutational burden to immune checkpoint efficacy is that mutations that were previously disregarded as "passengers", not contributing to tumor progression, may be of great importance as targets for immune checkpoint blockade. Larger studies will be necessary to identify what combination of mutational burden, antibody staining, or other biomarkers will be the most effective predictor of therapeutic efficacy.

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