Chapter 3
Tumor-Infiltrating Lymphocytes and Their Role in Solid Tumor Progression

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Contents

Abstract Tumor-infiltrating lymphocytes (TIL) are an important component of the tumor environment. Their role in tumor growth and progression has been debated for decades. Today, emphasis has shifted to beneficial effects of TIL for the host and to therapies optimizing the benefits by reducing immune suppression in the tumor microenvironment. Evidence indicates that when TILs are present in the tumor as dense aggregates of activated immune cells, tumor prognosis and responses to therapy are favorable. Gene signatures and protein profiling of TIL at the population and single-cell levels provide clues not only about their phenotype and numbers but also about TIL potential functions in the tumor. Correlations of the TIL data with clinicopathological tumor characteristics, clinical outcome, and patients' survival indicate that TILs exert influence on the disease progression, especially in colorectal carcinomas and breast cancer. At the same time, the recognition that TIL signatures vary with time and cancer progression has initiated investigations of TIL as potential prognostic biomarkers. Multiple mechanisms are utilized by tumors to subvert the host immune system. The balance between pro- and antitumor responses of TIL largely depends on the tumor microenvironment, which is unique in each cancer patient. This balance is orchestrated by the tumor and thus is shifted toward the promotion of tumor growth. Changes occurring in TIL during tumor progression

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appear to serve as a measure of tumor aggressiveness and potentially provide a key to selecting therapeutic strategies and inform about prognosis.

Keywords Cancer · Tumor-infiltrating cells · Lymphocytes · Prognosis

Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
CTL	Cytolytic T cell
CTLA-4	Cytotoxic T lymphocyte-associated antigen-4
DC	Dendritic cells
EVs	Extracellular vesicles
ICIs	Immune checkpoint inhibitors
IFN-γ	Interferon γ
IGKC	IgG kappa chain
IL	Interleukin
MHC	Major histocompatibility complex
NK	Natural killer cells
NKG2D	nk2G gene
NSCLC	Nonsmall cell lung cancer
PD-1	Programmed cell death protein-1
TAA	Tumor-associated antigen
TCR	T-cell receptor
TGF-β	Transforming growth factor-β
Th	T helper cell
TIL	Tumor-infiltrating lymphocytes
TME	Tumor microenvironment
Treg	Regulatory T cells

3.1 Introduction

The immune cells present in the tumor microenvironment belong to both adaptive and innate arms of the immune system and are found in virtually all human solid tumors. They may be present at various densities ranging from subtle infiltration to overt inflammation. As lymphocytes usually constitute the largest component of these immune infiltrates, they are commonly referred to as "tumor-infiltrating lymphocytes" or TIL. Attention given to TIL has progressively grown in the last two decades, largely because of the perception that TIL might play a critical role in carcinogenesis and also might be therapeutically useful. In fact, inflammatory infiltrates into tumors have achieved the status of one of the "Hallmarks of Cancer" by Hanahan and Weinberg (2011) in recognition of the role they play in tumor

progression and in tumor escape from the host immune system. Recent technological advances have allowed for a better examination of tumor infiltrates and for the identification of immune-related gene signatures expressed in the tumor microenvironment (TME). Phenotypic and functional characteristics of TIL, their localization *in situ*, and their interactions with the tumor cells or nonmalignant cells residing in the tumor have become a subject of intense investigations worldwide. These studies are aimed at the confirmation and validation of prognostic and predictive significance of TIL in patients with cancer. It has also become clear that cancer cells have a complex relationship with the immune system, and that even subtle differences in immune cell infiltrates into the tumor can result in the eradication of cancer cells or in enhancement of their growth.

The dynamic relationship existing between TIL and the tumor has been extensively evaluated in mouse models of tumor growth (Allen et al. 2020) as well as human tumor tissues (Thommen and Schumacher 2018). The TME is formed as a result of prolonged and constantly changing interactions between the developing tumor and the host immune system responsible for immune surveillance (Fouad and Aanei 2017). From its inception, the tumor protects itself from elimination by immune cells and gradually develops mechanisms for suppression of their functions. As tumor progresses, TILs accumulating in the TME become dysfunctional and fail to arrest the tumor progression. The mechanisms of tumor-induced immune suppression include a variety of cellular elements, soluble factors, and subcellular components and are unique in every tumor (Whiteside 2010). The key role tumor-derived factors, including extracellular vesicles (EVs) or exosomes, play in regulating intercellular interactions in the TME has emerged as the major theme of cancer research. The results suggest that every tumor creates its own TME and establishes its own ways for disarming the immune system. While the molecular pathways leading to immune suppression in the TME might be the same, the constellation or mix of various suppressive factors seems to be distinct for each tumor. Thus, interactions between the tumor and TIL are unique for each tumor, even for the tumors of the same origin and histology. Further, the heterogeneity in immunoregulatory pathways may exist within the same tumor, depending on regional or local environmental stimuli. The term "tumor heterogeneity" implies that within the tumor mass, there are considerable differences in cellular as well as molecular and genetic characteristics.

In this brief review, I will summarize the current perception of the role TILs play in tumor progression or responses to oncologic therapies and describe immunoregulatory mechanisms that exist in the TME. I will focus on T cells, B cells, and natural killer (NK) cells. While other leukocytes, M1 and M2 macrophages, dendritic cells (DC), and neutrophils (PMN) are all important components of the TME, it is TILs that remain in the highlights. This is due to newly acquired insights into potential of TIL as potential prognostic or predictive biomarkers in cancer and also as components of a promising therapeutic strategy, in which *in vitro*-expanded TILs are adoptively transferred to patients with cancer.

3.2 Studies of the Intratumor Immune Landscape

Technological advances in cellular, molecular, and genetic evaluation of TIL populations or single infiltrating immune cells have provided a wealth of novel information about the spatial distribution of TIL in the tumor, frequency of various TIL subsets, and their functional attributes. Given the heterogeneity of human tumors and the complexity of personalized cellular and molecular interactions in the TME, it is not surprising that monitoring of the TME has been a difficult task, and that biomarkers of tumor progression or response to therapy are not readily identifiable. Dissecting the complex interplay between immune and tumor cells to identify such biomarkers requires the integration of multiple currently available approaches into a "systems biology" approach (Bracci et al. 2020). Systems biology employing multiomics technologies represents a combination of genetic, epigenetic, transcriptional, proteomic, and metabolomic methodologies with immunological insights to provide a comprehensive view of the tumor immune landscape (Bracci et al. 2020). Systems biology employing multiomics technologies is most likely to characterize mechanisms underlying cellular interactions in the TME and to define biomarkers of response to therapy (Bracci et al. 2020). Today, while various multiomics technologies are slowly being applied to studies of immune-tumor interactions, the integrative analyses of TILs *in situ* supported by bioinformatics, computational science, and clinical correlations are still not widely available and require implementation.

Despite the existing barriers, studies of TIL *in situ* have rapidly progressed from immunohistology profiling of immune phenotypes or definition of immunoregulatory cell subsets, to highly sophisticated, multiparameter genetic, and immunological analyses of the TME, where interaction of TIL with tumor cells and each other takes place. A broad variety of monitoring strategies is now available for studies of TIL and tumor cells *in situ* (Yadav et al. 2014). These include sequencing of the whole genome, defining of gene signatures, epigenetic modifications, and changes in protein expression of tumor and immune cells. Further, in TIL, we can define the immune score, T- or B-cell receptor repertoires, identify different types of immune cells by flow cytometry or CyTOFF-based mass spectrometry, and perform multispectral immunocytochemistry (Galon et al. 2012; Giraldo et al. 2019; Maby et al. 2020). Using these strategies, human tumors can be categorized into immune cell-rich ("hot") or immune cell-depleted ("cold") tumors (Giraldo et al. 2014). The former are considered to be immunologically responsive, or "hot," and the latter immunologically unresponsive ("cold") tumor types (Giraldo et al. 2014). Thus, the extent of infiltration of immune cells into the TME emerges as a general measure of the tumor response to immunotherapy. "Sterile" or poorly infiltrated tumors might not be suitable candidates for immune therapy.

The mutational tumor load might be a promising predictive measure of therapeutic response, whereby tumors with a high mutational burden, and consequently enriched in neoantigens, are viewed as immunogenic and potentially more responsive when treated with immune therapies (Snyder and Chan 2015; Strickler et al. 2021). Efforts made to correlate mutational tumor loads with immune cell landscapes

to reinforce the predictive algorithm of response to therapy are ongoing and remain inconclusive. Whole-genome sequencing and RNAseq of formalin fixed and paraffin embedded (FFPE) or fresh-frozen tumor tissues are routine procedures that are widely used to define the mutational landscape of tumors and to identify the potential driver mutations in individual tumors (Snyder and Chan 2015; Duan et al. 2014; Robins 2013). The availability of The Cancer Genome Atlas (TCGA) database with its extensive roster of gene profiles for different tumors or types has been a valuable resource for identifying mutations as well as immune subtypes and functional gene modules, including immune cell-specific genes (Thorsson et al. 2018). Next-generation sequencing (NGS) in combination with newly developed bioinformatic programs offers the means for establishing gene signatures/patterns not only for tumor cells but also for TIL. The intratumoral signatures of these T cells can be determined on a patient-specific basis (Fridman et al. 2017). Further, NGS data can be applied to the neoantigen prediction pipeline that evaluates antigen processing, binding to MHC class I and gene expression to generate a map of mutation-associated neoantigens (MANAs) specific to the patient's HLA haplotype. Neoantigen expression and immune signatures can then be further interrogated by RNAseq.

Single-cell sequencing of tumor cells as well as immune cells is readily applicable to fresh human tumor specimens. Tumor tissues are enzymatically digested and single tumor or single immune cells are isolated by flow cytometry for single-cell (sc)RNAseq (Tirosh et al. 2016). This approach provides gene profiles of both tumor and immune cell types and allows for testing of correlations between the mutational tumor landscape and immune cells in the TME. A search for T cells which are naïve, regulatory, cytotoxic, or exhausted, based on differentially expressed genes typifying these T-cell subsets, identifies distinct clusters of the T cells and allows for heat maps to be constructed and for the estimation of their abundance in the tumor tissue. Special computational algorithms are available to do so, and the immune signatures of TILs can be identified and chartered (Wang et al. 2016). Specifically, signatures of immune dysfunction-associated genes, such as, e.g., elevations in the FOXP3 gene expression characterizing Tregs or in genes for exhaustion markers in $CD8^+$ T cells, can be established. Overexpression of genes that mediate immune dysfunction in the TME (e.g., TGFβ, CTLA-4, PD-L1) is often a sign of neoplastic progression. Although these analyses performed at the RNA level may be potentially skewed because of the presence of posttranscriptional modifications in proteins that mediate cellular functions, studies of transcriptomes from tumors have been useful in defining the TME in individual tumors (i.e., personalized analysis) or in tumors with a common histologic type.

Protein-based phenotypic and functional analyses of immunoinhibitory ligands associated with immune dysfunction, such as PD-L1, CTLA4, or TGF-β, are an important tool. Based on results of these analyses, it may be possible to establish an association between the signature of immune dysfunction in the tumor, the immunomodulatory ligands expression in the TME, and the genetic alterations identified by NGS. The next critical step would be to link these findings to clinical endpoints, including a patient's response to therapy and outcome. This type of assessment, which is applicable to FFPE tissue samples and is largely based on genetic profiling

of the tumor and of immune cells found in the TME, is slowly eliminating the dependence on conventional pathological examinations. Phenotypic and functional assessments of isolated TILs without mechanistic and genetic insights that shape their physiology have become obsolete. The above-described analyses of TIL in tumor tissues have resulted in the recognition of TIL as a biomarker of prognosis and response to therapy (Fridman et al. 2017). Further, TIL and their antitumor potential are being explored in adoptive immunotherapy of cancer.

3.3 Immune Score in the TME

Favorable associations of dense T-cell infiltrates with improved prognosis of many human cancers have been reported for decades. Immunohistochemistry of fresh-frozen or FFPE tumor sections has been instrumental in establishing the grading scale for immune cell infiltrations into the tumor now referred to as "immune score" (Galon et al. 2012). In 2006, Galon and colleagues demonstrated the prognostic significance of these TILs (Galon et al. 2006). The immune score uses systems biology and an objective scoring system to measure the type, density, and localization of immune cells within the TME. In a series of studies in colorectal carcinoma (Mlecnik et al. 2011) and later in other solid tumors (Fridman et al. 2011), Fridman et al. performed immunostaining of hundreds of tumor specimens and showed that a strong local immune reaction, including $CD3^+CD8^+$ and memory $CD45RO^+$ T cells, correlated with a favorable prognosis regardless of the regional tumor involvement or the tumor stage (Fridman et al. 2011). In subsequent independent studies, the prognostic role of infiltrating T cells was confirmed and has led to the proposal for routine evaluation of the TME for density, location, phenotype, and function of immune cells as a part of the standard pathological examination (Galon et al. 2014). The globally collected data strongly support the predictive value of the immune score (Van den Eynde et al. 2018), which is currently widely employed for testing its predictive value for response to immunotherapies, including immune checkpoint inhibitors (ICIs).

3.4 Antitumor Effects of TIL

Traditionally, T lymphocytes, and especially $CD8^+$ cytolytic T cells (CTL), have been considered the major antitumor immune effector cells. They are MHC class I-restricted and when specific for cognate tumor-associated antigens (TAA) become activated, produce perforin, granzymes, and cytokines which induce death of tumor cells but spare nonmalignant cells. A subset of $CD4^+$ T helper (Th) cells is essential for providing cytokine-mediated support for CTL expansion and functions. NK cells, which are not MHC restricted and do not require prior sensitization to antigens, can also recognize and eliminate tumor cells by mechanisms that involve a release of

perforin, granzymes, and cytokines (Fregni et al. 2012). These lymphocytes are mediators of cellular antitumor immunity. B cells, which upon Ag-specific activation give rise to antibody (Ab)-producing plasma cells, mediate humoral antitumor immunity. It has been debated whether it is T or B cells that play a more important role in the control of tumor progression. Contributions of NK cells to antitumor immunity have been largely considered in the context of antibody-dependent cytotoxicity (ADCC) during cancer therapy with antibodies. Today, it is evident that cooperative interactions of these cells are critical for the development of effective antitumor responses. The presence of B cells, which often form follicular-like structures in the TME, has been recently recognized as a potential prognostic biomarker, and the involvement of infiltrating NK cells in cooperative antitumor effects has been confirmed (Freud et al. 2017). These antitumor effects of TIL are being actively explored in cancer therapy (Freud et al. 2017).

3.4.1 CD8$^+$ Cytolytic T Cells

The presence and effector functions of T cells in the tumor remain the major interest of most studies. Analyses of the diversity in cellular composition of immune infiltrates in various tumor types can define unique tumor "immune signatures" that correlate TIL with outcome, providing prognostically relevant immune classification of human cancer potentially equal to or better than the conventional tumor-node-metastasis (TNM) classification (Hendry et al. 2017). In addition to the overall TIL immune score, the presence, frequency, or *in situ* localization of CD8$^+$ T cells in immune tumor infiltrates is of critical importance as is functional evaluation of their antitumor activity. The availability of standardized single-cell assays able to detect tumor antigen-specific T cells (ELISPOT, cytokine flow cytometry, and tetramer binding) among TIL has greatly facilitated evaluations of their potential value as prognostic biomarkers in cancer (Britten et al. 2011). However, it has been also observed that tumor epitope-specific CD8$^+$ T cells present *in situ* or in the peripheral circulation of patients with cancer were often preferentially eliminated either directly via the Fas/FasL or the Trail/TrailR pathways (Whiteside 2008) or indirectly through the release of tumor-derived exosomes carrying death receptor ligands (Whiteside 2013). The propensity of TIL isolated from human solid tumors to undergo spontaneous apoptosis was measured by Annexin V binding in flow cytometry assays, and tumor-epitope reactive, activated CD8$^+$ T cells which expressed Fas were shown to be particularly sensitive to tumor-induced effects (Whiteside 2008). Specifically, FasL+ tumor-derived exosomes isolated tumor cell supernatants or plasma of cancer patients have been recently linked to tumor progression, demonstrating that the presence of membrane-tethered FasL, and potentially of other molecules such as PD-L1 or TGF-β in exosomes, could contribute to apoptosis of antitumor effector T cells among TIL and thus to tumor escape from the host immune system (Ferrone and Whiteside 2007). In aggregate, these studies suggest that the presence of death-inducing ligands on tumor cells or carried by tumor-derived exosomes contributes to

elimination of TIL responsible for antitumor effects in the TME (Mittendorf and Sharma 2010). Thus, antitumor effector $CD8^+$ T cells accumulating in the TME and expected to eliminate tumor cells become dysfunctional or "exhausted" due to immunosuppressive activities of the tumor. TIL exhaustion in the TME favors tumor progression. For this reason, the "immune score" when used as a biomarker of outcome should contain estimates of tumor-induced suppression, e.g., numbers and disposition of exhausted T cells. The exhausted T cells overexpress various inhibitory surface receptors, such as PD-L1, lymphocyte activation gene-3 (LAG-3), T-cell immunoglobulin and mucin domain-3 (TIM-3); secrete interferon (IFN) γ and low levels of the effector cytokine, tumor necrosis factor (TNF) α. In the TME, where ligands that stimulate signaling via these receptors are commonly present, suppression of antitumor responses is profound. These receptors are therapeutic targets for checkpoint inhibition aimed at restoration of antitumor activity of T cells (Pardoll 2012).

Although activated $CD8^+$ T cells are present in many human tumors, these tumors fail to undergo spontaneous regression. This is likely due to regulatory mechanisms which inhibit T-cell responses in the TME (Mittendorf and Sharma 2010). These mechanisms can operate at the level of tumor cells inducing, e.g., loss of tumor antigens or downregulation of class I MHC molecules rendering the tumor invisible to CD 8^+ effector T cells (Ferrone and Whiteside 2007). Alternatively, as suggested above, T cells upregulate immune checkpoints or inhibitory pathways that are hard-wired into all T-cell responses to prevent excessive activation and tissue damage. For example, following T-cell receptor (TCR) engagement by an antigen, T cells upregulate CTLA-4, an inhibitory receptor that counteracts the stimulatory receptor, CD28 (Pardoll 2012). Tumor cells often express PD-L1, a ligand for another inhibitory receptor, PD-1. Activation of the PD-1/PD-L1 pathway in T cells decreases their proliferation, survival, and cytokine production (Hugo et al. 2016). Still another regulatory break is the presence in the tumor microenvironment of suppressor cells, such as Treg (see below) or myeloid-derived suppressor cells. These regulatory cells produce inhibitory cytokines (e.g., IL-10, TGF-β) or suppressive factors which dampen or abrogate antitumor immunity (Groth et al. 2019; Whiteside 2012).

Today, in the checkpoint inhibitor era, much attention has been paid to T-cell activation or reinvigoration in the periphery and in the TME after immunotherapy. It appears that patients with solid tumors who respond to ICIs have greater $CD8^+$ T-cell density at the tumor margin and their numbers/phenotypes are associated with the gene inflammation signature and high tumor mutational burden (Linette and Carreno 2019). However, the specificity of $CD8^+$ TIL for tumor-associated antigens or neoantigens remains poorly defined representing a significant challenge for cancer immunologists (Linette and Carreno 2019). NGS of TCR-Vβ repertoire in TILs can reveal different levels of TCR diversity and prevalence in the tumor as compared to peripheral blood, suggesting that antigen-driven proliferation of cognate T cells occurs in the tumor (Lucca et al. 2021). In some cases, T-cell diversity appears to correlate with the mutational burden of the tumor (Van Allen et al. 2015). Newer data suggest that neoantigen-specific $CD8^+$ T cells are the major effector cells that

mediate tumor regression following checkpoint inhibition (Linette and Carreno 2019).

A subset of $CD8^+$ T cells present in tumors, and relatively recently identified using transcriptome analysis as tissue resident memory T cells (T_{RM}), is a heterogeneous T-cell population with functions of effector and memory T cells (Okla et al. 2021). T_{RM} downregulate the expression markers that regulate their exit from tissue and overexpress markers for tissue retention. This phenotype enables them to traffic to, reside in and patrol, various tissues, exercising a long-term protective role. In tumors, T_{RM} infiltration was shown to correlate with enhanced patients' responses to immunotherapy and associates with favorable prognosis. T_{RM} in the tumor undergo a unique, hybrid effector cell-memory cell differentiation program of effector cells by expression of PD-1, IFN-γ, perforin, and granzymes and of memory cells by their stem-like properties (Okla et al. 2021). Tumor-specific T_{RM} preferentially reside in the tumor milieu, where they proliferate in response to TAA and combat tumor cells or eliminate transformed cells *in situ* (Okla et al. 2021). The reportedly potent antitumor effects of T_{RM} cells suggest they represent potential therapeutic targets for enhancing responses to immunotherapy.

3.4.2 $CD4^+$ Helper T Cells

This subset of T cells is present in solid tumors with the frequency that equals or exceeds that of $CD8^+$ T cells. Several subsets of helper T cells (Th) are recognized, including Th1, Th2, Th17, and Treg. The well-known "Th1/Th2" paradigm (Romagnani 1997) refers to the balance that exists between the functionally distinct subsets of T helper cells (Th). Th1 cells produce cytokines, notably IL-2 and IFN-γ, which play a role in activating and enhancing expansion as well as effector functions of $CD8^+$ T cells and NK cells (Kalams and Walker 1998). Th1 cells also influence the antigen-presenting capacity of DC, thus shaping CTL responses (Knutson and Disis 2005). In contrast, Th2 cells secrete cytokines that are important for B-cell maturation, clonal expansion, and class switching, thus promoting humoral immune responses. The Th1/Th2 ratio is altered in cancer and other diseases, with Th2 cells often outnumbering Th1 cells in the blood and tumor tissues of patients with cancer (Zhu and Paul 2010). There are no surface markers distinguishing these two Th subsets, but cytokine production and gene expression profiles have been used to discriminate Th1 from Th2 responses (Tatsumi et al. 2002). In a study of 400 ER-negative breast tumors, the Th1 profile (IL-2, IL-12, IFN-γ) was inversely correlated with the Th2 profile (IL-13, TGF-β), and Th1 responses associated with a lower risk for distant metastases (Teschendorff et al. 2010). Th2 responses were associated with a higher risk. The combination of both pathways allowed for a better prediction of metastasis-free survival than either of the pathways alone (Teschendorff et al. 2010). This example emphasizes the potential importance of Th1 versus Th2 responses at tumor sites for disease outcome and indicates that

immune response developing in the microenvironment of tumors serves as an important prognostic factor.

A relatively recent addition of Th17 cells, characterized by the production of IL-17, to the T-cell repertoire has altered the Th1/Th2 paradigm. The Th17 cells play a major role in autoimmunity, and their involvement in cancer has been less well studied. A study of human breast tumors identified Th17 cells as a prominent component of infiltrates and established a negative association between their presence and the disease stage or number of involved lymph nodes, suggesting that Th17 are involved in antitumor responses (Yang et al. 2012). In a study of patients with ovarian carcinoma, Kryczek et al. reported that patients with higher numbers of Th17 cells had significantly improved overall survival, irrespective of the tumor stage. Further, the frequency of Th17 cells inversely correlated with that of tumor-infiltrating $FOXP3^+$ Treg (Kryczek et al. 2009). However, experiments in mouse models of cancer indicate that Th17 may also be involved in protumor functions by promoting angiogenesis (Silva-Santos 2010). IL-17 has been shown to induce expression of proangiogenic factors such as vascular endothelial growth factor, angiotensin, IL-8, and prostaglandin E_2 in stromal, endothelial, and tumor cells (Silva-Santos 2010). The exact cellular mechanisms that determine pro- vs. antitumor functions of $Th17^+$ TIL remain unclear and need further investigations. Nevertheless, given that angiogenesis remains a major feature of progressing tumors, the presence and quality of Th17 infiltrates are likely to be of considerable importance in cancer prognosis.

3.4.3 Regulatory T Cells (Treg)

This relatively minor subset of $CD4^+$ T cells (~5%) is well represented among TIL, and Treg play a major role in modulating immune responses *in situ*. Tumors appear to recruit Treg to the tumor microenvironment, where they accumulate, representing a substantial component of TIL in multiple tumor types [reviewed in 33]. The presence and functional competence of Treg inversely correlates with outcome in many, but not all, human tumors (Whiteside 2012; Lanca and Silva-Santos 2012). The existing conflicting reports in respect to the role of Treg in promoting tumor progression vs. its regression have largely originated from the lack of a definite phenotypic profile for human Treg. It appears that the $CD4^+CD25^{high}FOXP3^+$ natural (n) Treg, normally responsible for maintaining peripheral tolerance, control cancer-associated inflammation (Whiteside et al. 2012), while another subset of Treg, inducible (i) Treg which may or may not be $FOXP3^+$ but produce adenosine and TGF-β, arises by tumor-driven conversion of conventional $CD4^+$ T cells to highly suppressive, therapy-resistant cells. These iTreg appear to be responsible for downregulating antitumor immune responses *in situ* (Whiteside et al. 2012). The iTreg promote tumor growth, expand, and accumulate in cancer, and their presence in TIL predicts poor outcome. In ovarian carcinoma, melanoma, breast cancer, and glioblastoma, the frequency of Treg among TIL correlated with tumor grade and

reduced patient survival (Lanca and Silva-Santos 2012). Because Treg are heterogeneous, consisting of many subsets of functionally distinct cells, and because no universal distinguishing marker for human Treg is currently available, their use as a biomarker of prognosis is limited. On the other hand, Treg maintain a strong suppression of effector cells in the TME, and their functional attributes might serve as markers of suppression levels existing in the TME. Treg possess a metabolic profile that is distinct from that of effector T cells (Watson et al. 2021). Recent studies showed that glucose uptake by Treg correlates with their poor suppressor function and their long-term instability. In contrast, Treg upregulate lactic acid metabolism, withstand high lactate conditions, and successfully proliferate in the TME. These metabolic differences in utilization of the glycolytic pathway by Treg illustrate their flexibility for survival in the hostile TME by excluding glucose uptake in favor of lactic acid (Watson et al. 2021). Treg exploit the metabolism in the TME and, unlike effector T cells, thrive in the lactate-rich milieu and mediate high levels of immunosuppression. Additional studies evaluating the role of Treg present in the tumor microenvironment as an independent predictor of prognosis in cancer are necessary.

3.4.4 B Cells

B cells originate in the bone marrow and then migrate to secondary lymphoid organs, e.g., lymph nodes, where they interact with antigens, differentiate into plasma cells, and produce antigen-specific Abs. TIL populations in human solid tumors include variable proportions of infiltrating B cells. While a search for promising immune correlates of cancer diagnosis, prognosis, and survival has been largely limited to T-cell responses, newer reports indicate that B cells might be critically important for outcome. Two recent independent studies provide useful insights into the prognostic role of B cells in cancer. Schmidt and colleagues have reported data that validate the B-cell signature as the most robust prognostic factor in breast cancer and other human tumors (Schmidt et al. 2008, 2012). These investigators identified the immunoglobulin G kappa chain (IGKC) as an immunologic biomarker of prognosis and response to chemotherapy in hundreds of patients with breast cancer, nonsmall cell lung cancer (NSCLC), and colorectal cancer (CRC) (Schmidt et al. 2012; Whiteside and Ferrone 2012). In this multiinstitutional study, the IGKC was microscopically identified as a product of plasma cells present in the tumor stroma and was validated as a prognostic biomarker by the RNA- and protein-based expression studies independently performed in thousands of formalin-fixed, paraffin-embedded specimens at 20 different centers (Schmidt et al. 2012). Expression of the IGKC transcript was the strongest discriminator of patients with breast cancer with and without metastases among the 60 genes found in the B-cell metagene, while transcripts of the T-cell metagene had lesser prognostic significance (Schmidt et al. 2008, 2012). Infiltrates of both T and B cells were found to be associated with better prognosis. However, the most important finding was that IGKC predicted responses

to neoadjuvant therapy in breast cancer and thus qualifies it as the first immune marker of response to cancer treatment. The finding of the B-cell signature as a validated biomarker of prognosis and response to therapy provides a strong support for the role of humoral immunity in controlling cancer (Whiteside and Ferrone 2012).

In support of this key role of the B-cell signature, Nielsen et al. (2012) reported that among TIL present in high-grade serous ovarian carcinomas, $CD20^+$ B cells colocalized with activated $CD8^+$ T cells and expressed markers of antigen presentation, including MHC class I and class II antigens, CD40, CD80, and CD86. These B cells were antigen experienced. The presence among TIL of both $CD20^+$ B and $CD8^+$ T cells correlated with a better patient survival than that compared to $CD8^+$ T cells alone. Although these $CD20^+$ B cells had an atypical CD27(−) memory B-cell phenotype, together with $CD8^+$ T cells, they promoted favorable prognosis in ovarian cancer (Nielsen et al. 2012).

Recently, the role of tertiary lymphoid structures (TLS), which are ectopic cellular aggregates, resembles secondary lymphoid organs in the cellular content and structural organization (Jacquelot et al. 2021). TLS are formed in nonlymphoid tissues in response to local inflammation and are found in solid tumors (Jacquelot et al. 2021). Composed of the antigen-specific B cells and T cells as well as dendritic cells, TLS drive the antitumor immune responses and have an impact on tumor progression. Formation of TLS in the tumor and abundance of TLS associates with favorable clinical outcome (Sautes-Fridman et al. 2019).

The emerging evidence for a significant role of the B-cell signature as a biomarker of prognosis and possibly of metastasis in several human malignancies deserves careful attention particularly in view of novel insights into functional heterogeneity of this lymphocyte subset, which appears to play a pivotal role in regulating T-cell responses (Biragyn and Lee-Chang 2012). Thus, human B cells were found to express CD39 and CD73, the ectoenzymes hydrolyzing exogenous ATP to adenosine (Saze et al. 2013). The ability of activated $CD19^+$ B cells to regulate T cells via the adenosine pathway and adenosine receptor signaling places these lymphoid cells in the category of regulatory elements potentially as effective as Treg (Saze et al. 2013).

3.4.5 Natural Killer (NK) Cells

NK cells mediate innate immune responses and can mediate direct cellular cytotoxicity without a need for prior sensitization (Freud et al. 2017). NK cells play a key role in cancer immunosurveillance. In contrast to T cells, NK cells are not HLA restricted. They are regulated by a set of receptors, such as killer inhibitory receptors or KIRs, and of activating receptors, such as NKG2D and several others (Freud et al. 2017), which calibrate antitumor functions of these cells. As a result, NK cells eliminate tumors that lack MHC class I expression or that overexpress ligands for NKG2D, including MICA, MICB, and UL16-binding proteins, which are minimally

or not expressed in nonmalignant cells or tissues. These ligands are promptly and efficiently induced by stress, including malignant transformation, and their overexpression on activated NK cells is regarded as the "danger signal" marking cells for immune elimination. There is little evidence for an association of the NK-cell presence in the TME and clinical outcome in solid tumors. Nevertheless, there is evidence that NK cells, which express high levels of low-affinity Fc receptors (CD16) for IgG, are critical for ADCC. NK cells are also strong IFN-γ producers (Vivier et al. 2011). Unfortunately, NK-cell functions are often found to be downregulated in cancer, and in a study of highly aggressive NSCLC, NK cells were found to have an altered phenotype and were impaired in the ability to secrete IFN-γ (Melaiu et al. 2019). Tumor- and peripheral blood-derived NK cells in patients with cancer are frequently compromised, and in many cases, this impairment has been linked to the tumor progression and poor prognosis (Platonova et al. 2011). Recently, it has been reported that EVs produced by tumor cells play a key role in regulating of immune surveillance by NK cells, which is dependent on receptor–ligand interactions driven by MICA expression in the tumor-derived EVs (Wu et al. 2021). Thus, another mechanism of tumor-induced immune suppression is revealed, and the focus on this mechanism might provide evidence for an association of inhibitory ligand carrying EVs with cancer progression in the near future.

3.5 Summary and Conclusions

The antitumor immune response, which is mediated by subsets of lymphoid cells, can have a powerful influence on the survival of patients with cancer. In this respect, evidence is especially strong for colorectal and breast cancers, but this is now being extended to other solid tumors (Fridman et al. 2017). Patients with large infiltrates of T or B cells or increased expression of genes encoding T-cell or B-cell signatures (i.e., high immune score) tend to have better survival compared to those with few tumor-infiltrating immune cells (Fridman et al. 2017). TIL can be divided into at least three distinct cell types: effector cells, regulatory cells, and inflammatory cells, all of which can influence each other's functions through production of cytokines, soluble factors, and membrane-bound EVs. Tumor cells themselves also produce immunosuppressive cytokines, a variety of soluble and masses of EVs decorated with immunoinhibitory ligands, which have direct as well indirect effects on immune cells recruited to the TME (Marar et al. 2021). Therefore, cellular composition of the TME and interactions of cells residing within the tumor determine the outcome of antitumor immune responses. As neither the cellular composition nor the cytokine milieus in the microenvironment are constant, because they undergo changes as tumors progress from premalignant to malignant and eventually metastatic phenotype, the impact TIL may have on outcome is highly variable. Current data suggest that it may be dependent on the balance existing between inflammatory and regulatory TIL. This balance may be a critical part of the underlying molecular mechanisms that are responsible for the influence TIL exert on cancer patient

outcome. Understanding of the cellular and molecular mechanisms involved in creating and maintaining this balance is, therefore, necessary for determining of how TIL contribute to survival of patients with cancer and for the selection of therapeutic strategies that could improve patient survival.

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