3 Regulation of CTL Infiltration Within the Tumor Microenvironment

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3.1 The Predictive Capability of Tumor Infiltrating Lymphocytes

3.1.1 T-Lymphocyte Infiltration in the Tumor and Immunoscore

Classical methods for determining malignant disease prognosis are based upon the morphology and location of tumor cells at the primary sites and in lymph node tissues, and the existence of distant metastases. While this analysis provides important information about a patient's disease it fails to capture the biological complexity of the tumor microenvironment and the contribution of the anti-tumor immune response. Immunohistochemical and gene analyses of immune cells, particularly $CD3^+$ T lymphocytes in the primary tumor, provides a prognostic biomarker that is highly statistically accurate for predicting clinical outcome in the vast majority of cancer types including colorectal, lung, melanoma, ovarian, head and neck, breast, urothelial, hepatocellular, gallbladder, and esophageal (reviewed in [1–3]). Furthermore, basic histological quantification of T lymphocyte density, cytotoxicity, and memory by CD3, CD8, and CD45RO, respectively, has demonstrated that increased infiltration of T lymphocytes is associated with statistically significant improvement in patients' disease-free survival (DFS) and overall survival (OS) [2, 4, 5]. In colorectal carcinoma (CRC), further delineating the location of cytotoxic T lymphocytes (CTL--$CD3^+$, $CD8^+$) into two areas within the primary tumor, the center (CT) and the invading margin (IM), provides a statistically accurate prediction of clinical outcome [4]. Quantification of the density, phenotype, and location (CT or IM) of T lymphocytes has been termed Immunoscore [6–8]. In fact, for the first time it was shown that analysis of a marker, CD3, surpassed the gold standard of diagnostics via tumor-stage, lymph node, and metastatic invasion. Immunoscore defines patients into five categories (I0-I4) based on the distinct location (CT and IM) of $CD3^+$ and $CD8^+$ T lymphocytes within the primary tumor, where I0 has no $CD3^+$ or $CD8^+$ cells and I4 has high densities of both

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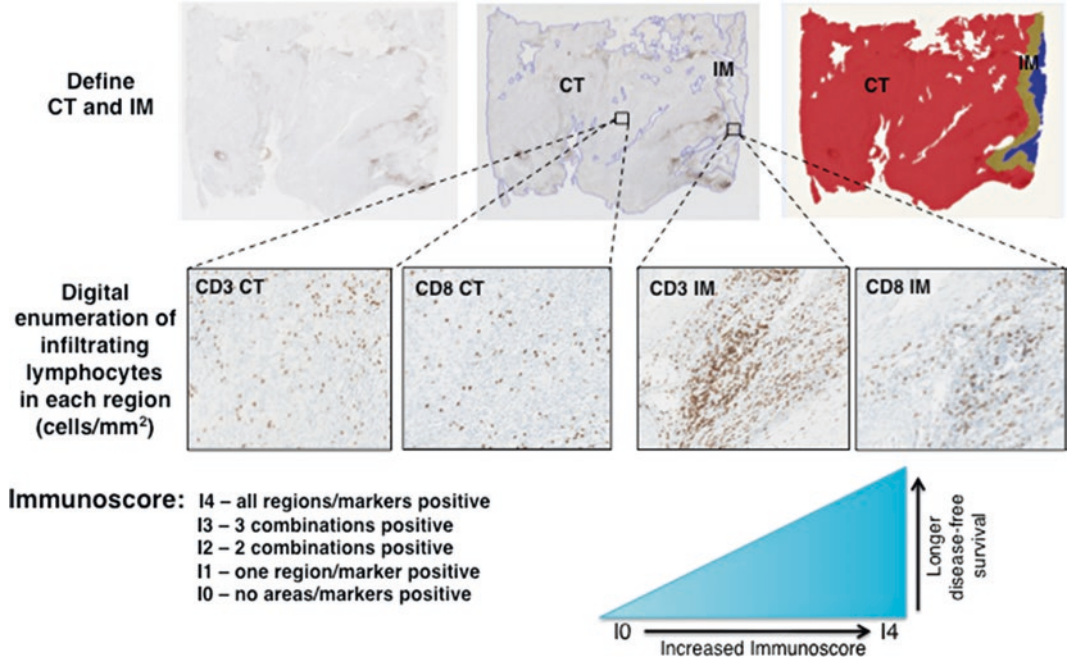

Fig. 3.1 Overview of Immunoscore. Immunoscore classifies tumors by density and location of CD3- and CD8-positive T lymphocytes. Paraffin-embedded tumor tissue is stained for CD3 or CD8. Stained tissue samples are analyzed for tumor and normal epithelium. The tumor center (CT) and invasive margin (IM) are then defined using digital software. The software then enumerates infiltrating lymphocytes in each region. Immunoscore is calculated based on the density of each marker in both regions. Patients with high Immunoscore (I4) have significantly longer disease-free survival compared to patients with low Immunoscore (I0)

$CD3^+$ and $CD8^+$ cells in the CT and IM (Fig. 3.1) [9, 10]. Immunoscore utilizes simple analysis of two markers to accurately predict a patient's clinical outcome. By combining Immunoscore with quantification of additional immune components associated with the tumor microenvironment, as a part of the immune contexture, we continue to enrich our understanding of why tumors become resistant, avoid elimination, or fail to generate a tumor-specific cytotoxic response. In particular, identifying characteristics of the tumor microenvironment that lead to low densities of immune infiltrates and consequently low Immunoscore (I0 and I1) could dramatically improve the selection of personalized treatments for cancer. In the cases with high Immunoscore, patients might be more likely to respond to immunotherapies that stimulate an existing immune response, such as checkpoint blockades, while patients with low Immunoscore would need therapy that primes a *de novo* antitumor response or facilitates trafficking of CTL to the tumor site.

3.1.2 Memory and Cytotoxic Lymphocytes Indicate Improved Prognosis

The importance of memory and CTL in the tumor microenvironment is well established. The presence of effector memory T cells in primary colorectal tumors is negatively correlated with signs of early metastatic invasion, as defined by presence of vascular emboli, lymphatic invasion, and perineural invasion [5]. This observation is supported by phenotypic analysis by flow cytometry of effector memory T lymphocytes, where patients with signs of early metastatic invasion have significantly fewer $CD3^+CD8^+CD45RO^+$

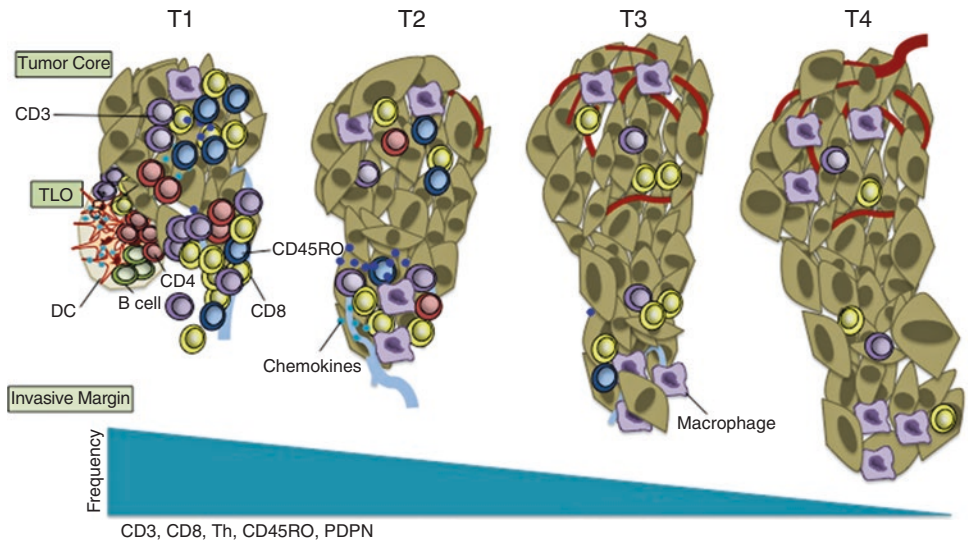

Fig. 3.2 The tumor microenvironment by progressive tumor stage. Depiction of the microenvironment of tumors from stage T1 to T4. Early stage tumors are smaller with many CD3, CD8, CD4, CD45RO, and TLO at the invasive margin and in the tumor center. T2 and T3 tumors are larger and progressively have less T lymphocytes, less lymphatic vessels (PDPN) and more angiogenesis. T4 tumors have very few T lymphocytes, increased macrophages and increased angiogenesis. The presence of CTL, memory and helper lymphocytes at the invasive margin, tumor center and in TLO predicts better clinical outcomes

and $CD3^+CD8^+CCR7^-$ cells in the tumor, as well as by immunohistochemistry measuring CD45RO, where patients with metastatic progression have significantly fewer CD45RO-positive cells in the primary tumor compared to non-invasive disease [5]. Furthermore, a high density of $CD45RO^+$ cells in the primary tumor significantly predicts better overall and disease-free survival compared to patients with low density of $CD45RO^+$ expression within their tumors [5]. High expressers had median OS of 36.5 months and DFS of 53.2 months, compared to 11.1 and 20.6 months, respectively, for low density CD45RO. This indicates that not only are CTL an excellent biomarker for determining patient disease-related survival, but also further delineates the importance of the transition of CTL into memory phenotype, which can help refining the predictive capability of intratumoral CTL on patients' clinical outcome, as illustrated in Fig. 3.2. Two important mechanisms of memory T lymphocyte development and maintenance are determined by cytokine stimulation and help by $CD4^+$ T lymphocytes [11–14]. The role of these two mechanisms in maintaining anti-tumor memory and cytotoxic lymphocytes are discussed in the following sections.

3.1.3 Th1 and Th17 Have Opposing Effects on Disease-Specific Survival

It has been previously shown that incorporating the subtype and location of T helper (Th) lymphocytes, in addition to CTL, improves the accuracy of disease-specific survival prediction [15]. Primary CRC tumors from 125 patients for the expression of 45 immune genes representing four T helper populations, Th1, Th2, Th17 and regulatory T lymphocytes (Treg) were analyzed. Hierarchical clustering revealed eight categories of Th genes as follows: Th1 cytotoxic (IRF1, GZMB, IL27, GNLY, PRF1, CCL5, CD8a, STAT1), Th1 (IL12RB1, CD28, CCR5, HLA-DMB, IL12RB2, CD38, CXCR6, TBX21), Th17 (RORC, IL-17A), Th2 (IL4, IL5, IL13) or (CCR7, CD3E, CD40LG, CCL19, CCR4,

GATA3) or (IFNGR1/2, STAT3, IL10RB, IL4R, STAT6), Th2/Treg (FOXP3, CTLA-4, CCL17, CCL22) or Tregs (IL-10, TGFB). Interestingly, when disease-free survival (DFS) was assessed based on expression of Th1 cytotoxic genes, patients with high expression had significantly increased time to relapse versus patients with low expression (78 *versus* 18 months, $p = 0.01$). Conversely, patients with low expression of Th17 genes had prolonged disease-free survival with 80% of patients not experiencing relapse after 9 years. Since the Th1 cytotoxic and Th17 gene profiles exhibited this extreme contrast in prediction of DFS, the two gene profiles were assessed for complementarity. The patients were separated into 4 groups based on high or low Th1 cytotoxic or Th17 gene expression, Th1-Hi Th17-Hi, Th1-Hi Th17-Lo, and Th1-Lo Th17-Lo. Remarkably, the few patients with Th1-Hi and Th17-Lo had no tumor recurrence at 5 years, while patients with Th1-Hi and Th17-Hi had a DFS of 65% at 5 years and patients with Th1-Lo and Th17-Hi tumors had the worst outcome with DFS of 40% at 5 years. These findings were confirmed at the protein level by immunohistochemistry (IHC) analysis of IL-17 and CD8. Density and location (CT or IM) of IL17- and CD8-positive cells were analyzed where "high" is positive in both the CT and IM, "heterologous" has high density in either the CT or IM, and "low" has low densities of cells in both regions. DFS analysis showed the vigorously augmenting effect of IL17 expression on incidence of relapse, whereas IL17-low CD8-heterologous, IL17-heterologous CD8-heterologous and IL17-high and CD8-heterologous had 8, 40, and 80% observed relapse, respectively ($p < 0.001$). These data demonstrate the benefit of complementary analysis of Th1, Th17, and CTL in the tumor microenvironment.

The finding that high density of IL-17 expressing cells in the primary CRCs is a negative prognostic biomarker is not unprecedented because IL-17 production by T lymphocytes (Th, NK, Tc, γδ), NK, neutrophil, and innate lymphoid cells has been associated with colon tumorigenesis [16]. One mechanism for this is via commensal bacteria that skew Th17-directed inflammation, leading to hyperplasia of normal colon cells and eventually colon cancer [17]. IL-17 also induces colorectal cell lines and primary cells to secrete pro-angiogenic factors, including vascular endothelial growth factor (VEGF) and can cause resistance to anti-angiogenic therapies [18, 19]. This pro-angiogenic stimulation likely also prevents trafficking of tumor-specific CTL to the tumor site, discussed in further detail below. Additional support for Th17 cells as a negative prognostic factor is that IL-17 expression defines patients with decreased disease-specific survival for pancreatic, breast, and gastric cancer [19–21], as well as increased tumor growth for intraocular lymphoma [22].

Contrary to Th17, Th1 cells are slowly becoming recognized for their important role in anti-tumor immunity for multiple types of cancer. Expression of $CD4^+$ cells within the primary tumor correlates with improved prognosis in esophageal squamous carcinoma and non-small cell lung cancer, with statistically significant additive predictability when combined with enumeration of CTL [23, 24]. Helper $CD4^+$ T lymphocytes, particularly tumor antigen-specific Th, guide CTL trafficking and maintain their cytolytic function within the tumor [25–27]. Tumor antigen-specific Th1 lymphocytes produce IFN-γ in the tumor microenvironment leading to the expression of CTL chemoattractants including CXCL9, CXCL10, CCL2, CCL3 and CCL5 [25]. Tumor-specific Th cells also produce IL-2, which is critical for CTL survival and can inhibit PD-1 mediated exhaustion of tumor-specific CTL leading to better efficacy of adoptive immunotherapy [27]. Additionally, mesenchymal stromal cells in the tumor microenvironment can directly activate CD4 T lymphocytes to become Th1 cells via IL-12 and consequently make tumor cells more vulnerable to CTL mediated destruction [28].

Regulatory T cells (Tregs) have an important role in the immune system to prevent uncontrolled immune responses to self-antigens. In cancer, this can lead to immunosuppression of the anti-tumor immune response due to self-antigens present on tumor cells. It has been shown that Treg gene signatures were not correlated to patient outcome; however, high densities

of FoxP3 protein expression was associated with increased disease-free survival of patients with colorectal cancer [15]. This is supported by other studies suggesting that in colorectal cancer, Tregs are not correlated with immunosuppression of the anti-tumor immune response and are significantly associated with high densities of CTL and Th1 T cell infiltration in the tumor [15, 29]. The effectiveness of Tregs as a prognostic maker has been variable between cancer types [30–32]. The comparisons of effector to Treg ratios, in hepatocellular and ovarian cancer showed that increased ratio of effector T cells to Tregs has positive prognostic value [33, 34]. Altogether this suggests that Tregs are a complicated biomarker for predicting patient outcome.

Finally, follicular T helper (Tfh) lymphocytes should be mentioned. Tfh cells are specialized to provide help to T and B lymphocytes, maintain memory B lymphocytes and produce IL-21 [35]. It has been previously reported that expression of the Tfh cells markers, CXCL13, CXCR5, and IL21 in the tumor were significantly correlated with prolonged disease-free survival [36]. Furthermore patients with aberration in the CXCL13 gene leading to gene deletion and dysfunction had significantly shorter disease-free survival compared to CRC patients with no aberration. High density of Tfh infiltration in the primary tumor has also been associated with prolonged disease-free survival in breast cancer [37]. The role of CXCL13 and IL-21 on CTL function will be further discussed in the following sections.

3.2 Factors Regulating Tumor Infiltration of Lymphocytes

3.2.1 T-Cell Homing Molecules Mediate Migration of CTL to Tumors

Chemokines have an important role in orchestrating both innate and adaptive immune cells chemotaxis and localization within the tumor. Chemokines can direct development and maintenance of tertiary lymphoid organs (TLO) that prime tumor-specific CTL at the tumor site, which has been described in multiple cancer types including non-small cell lung cancer, melanoma, and colorectal carcinoma [36, 38–40].

We examined the predictive capability of chemokines using data integration of gene expression in primary tumors from CRC patients [41]. We discovered a significant prolongation of DFS in patients with high expression of the chemokines CX3CL1, CXCL10, and CXCL9. CX3CL1, also known as fractalkine, mediates T lymphocyte and monocytes migration and promotes strong adhesion to endothelial cells [42]. CXCL10, also named IFN-γ protein 10, and CXCL9 are closely related cytokines in the monokine-induced by IFN-γ family. CXCL10 and CXCL9 facilitate migration of CTL, monocytes, NK and dendritic cells, inhibit angiogenesis, and have anti-tumor properties [43, 44]. CRC patients with elevated gene expression of one of these three chemokines had increased percentage and density of $CD3^+CD8^+$ T lymphocytes in the tumor as assessed by flow cytometry and immunohistochemistry [41]. Analysis by location, CT or IM, within the tumor microenvironment showed that: (I) patients with high intra-tumoral CXC3CL1 expression also had significantly increased density of effector-activated CTL ($GZMB^+$) and Th1 ($T\text{-}Bet^+$) cells; (II) tumors of patients with high CXCL9 and CXLCL10 expression levels contained a significantly increased number of memory T lymphocytes ($CD45RO^+$) and macrophage ($CD68^+$). TCR repertoire analysis of ten patients randomly selected from the same cohort showed that the TCR repertoire of patients with a high CX3CL1 level was clearly distinguishable from the repertoire of patients with low CX3CL1 expression level. One cluster with CX3CL1, CXCL9, or CXCL10 gene expression levels correlated with a specific CTL repertoire (Vb5.2L08, Vb2L03, Vb2L07), thus suggesting that these chemokines attract clonal CTL with distinct tumor-specificity. Strikingly, when CRC tumors had high expression of any of these three TCRs the patients overall 3-year survival was 100%, as opposed to 28% with low expression of these TCRs. This suggests that CX3CL1, CXCL9, and CXCL10 in the tumor

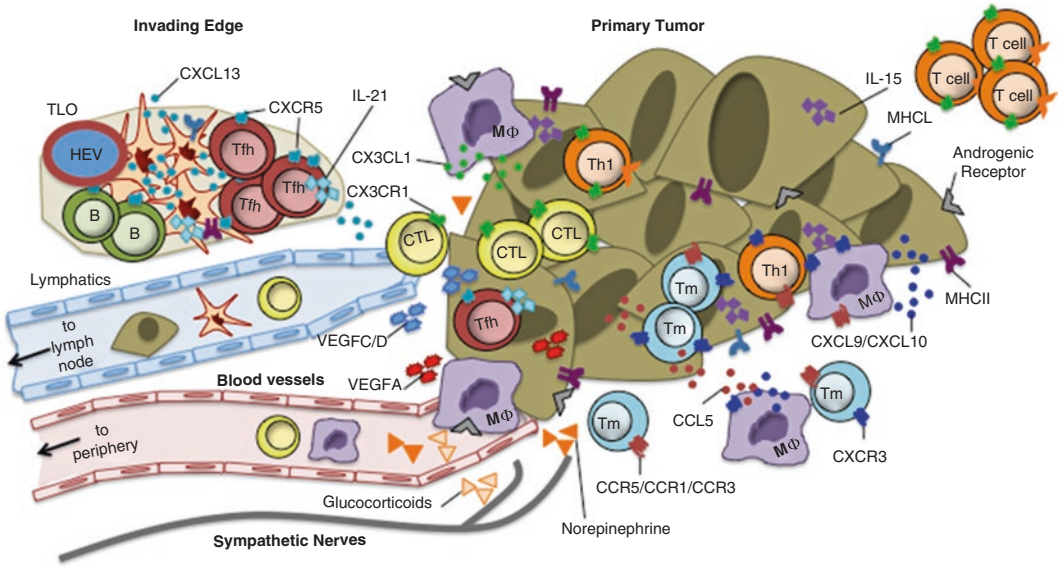

Fig. 3.3 Modulators of T lymphocytes in the tumor microenvironment. Overview of lymphatics, blood vessels, nerves, tertiary lymphoid organs (TLO), immune and tumor cells that produce cytokines, chemokines, hormones, and immunosuppressive factors that regulate function and trafficking of lymphocytes in tumors. Expression of many of these factors can predict prognosis of patients with cancer, described in Table 3.1

microenvironment recruit tumor-specific CTL to eliminate malignant cells, and tumors become resistant to CTL-mediated death when these chemokines are not present. High expression density of CXCL9 and CXCL10 also accurately predicts prolonged disease-specific survival in melanoma patients [39, 45]. Pre-clinical studies with melanoma show that blocking CXCL9 or CXCL10 substantially reduces the ability of CTL to traffic to the primary tumor and distant metastatic legions [45], which may be due to their role in directing CTL homing to the tumor by CD4 T lymphocyte help.

Another chemokine, CXCL13, was recently found to be associated with follicular helper T (Tfh) lymphocytes and also predicts patients' clinical outcome. CXCL13 is produced by and has been associated with generation of tertiary lymphoid organs (TLO) within the invasive margin of primary tumors [38, 46]. The presence of TLO in primary tumors is positively correlated to prolonged disease-free survival in multiple cancers [38, 39]. It is hypothesized that priming and activation of tumor-specific CTL is orchestrated by dendritic cells presenting tumor-antigens within these TLO. In conjunction with this observation, CXCL13 as a single biomarker can accurately predict patients' clinical outcome [36]. Earlier it was discussed that low protein expression density or chromosomal aberration of CXCL13 is associated with worse clinical prognosis in CRC [36]. Similarly, in specific subtypes of breast cancer, elevated expression of CXCL13 in the tumor is associated with increased disease-free survival compared to tumors with low expression of CXCL13 [37]. Additionally, there is evidence supporting that high or low density CXCL13 expression can accurately predict patients' response to chemotherapy [47, 48]. It seems that the CXCL13-CXCR5 axis has the highest predictive score in HER2-positive breast cancers as opposed to other breast cancer subtypes [49]. This may be due to potential immunogenicity of HER2 for generating HER2-specific T helper and CTL immune response against the tumor. Figure 3.3 shows an overview of many

Table 3.1 Factors regulating lymphocyte infiltration into tumors

	Tumor immune factor	Prognosis	Ref
Chemokines			
CX3CL1	Mediates T-lymphocyte and monocyte migration to tumors and adhesion to endothelial cells	Good	[35, 36]
CXCL9/10 CCL5	Induced on tumor cells and MΦ by IFN-γ to promote CTL, monocyte, NK, and dendritic cell migration to the tumor and anti-angiogenic properties	Good	[25, 35, 37, 38]
CXCL13	Produced by dendritic cells in the TLO and signals through the CXCR5 receptor on B cells and Tfh cells controlling the organization of TLO	Good	[30, 32, 40]
Cytokines			
IL-17	Associated with tumorigenesis. Induces tumor and primary cells to secrete pro-angiogenic factors	Poor	[15, 18, 19]
IL-15	Regulates memory T lymphocyte maintenance and homing capabilities. Shown to rescue tolerant T cells and augment tumor-reactive CTL function and survival	Good	[11, 48, 50–52]
IL-21	Produced by NKT and Th cells. Activates and prevents exhaustion of tumor-specific CTL	Good	[30, 48, 55]
Angiogensis/Lymphatics			
VEGFA	Generates leaky vasculature that prevents trafficking of leukocytes to the tumor. Stimulates suppressive Tregs and MDSC and induces immune checkpoints on endothelium (PD-L1, B7-H3, and TIM3)	Poor	[71–74]
VEGFC/D	Generates lymphatic vessels that are dysfunctional in fluid mechanics. Associated with chronic inflammation and induces secretion of immunosuppressive factors	Poor	[75, 77]
TLO/HEV	Facilitates priming, maintenance, and migration of lymphocytes in tumors. Presence in stroma correlates with high density of T and B cells	Good	[32, 78]
Neural			
Glucocorticoids	Induce expression of chemokine, cytokine, complement family members, innate immune-related genes, and TLR and repress adaptive immune-related genes. Reduce adaptive immune gene expression and skew Th1 cells to a Th2 phenotype. Upregulate IL-7Ra, enhance IL-7-mediated signaling and function, and inhibit apoptosis	Both	[82–84, 86]
Norepinephrine AR	Downregulates MHC-I, co-stimulatory molecules and increases production of IDO by tumor cells via beta-AR. AR signaling enhances Treg-mediated suppression, polarizes MΦ to a M2 phenotype and increases infiltration of MDSC	Poor	[87–92]

MΦ macrophage, *Th* T helper lymphocytes, *CTL* cytotoxic T lymphocytes, *TLO* tertiary lymphoid organ, *MDSC* myeloid-derived suppressor cells, *MHCI* MHC Class I, *AR* androgenic receptors

prognostic chemokines involved in T lymphocyte recruitment to the tumor. It is becoming increasing clear that chemokines have an essential role in trafficking CTL to the tumor site. Furthermore, the addition of chemokine expression to Immunoscore has potential to predict patient response to chemotherapy [48].

3.2.2 Cytokines Contribute to the Distribution of CTL within the Tumor

An immense number of studies have been performed to investigate the components of the cytokine milieu that regulate lymphocytes in the tumor microenvironment. Interferon-gamma (IFN-γ) is well appreciated for its capacity to

prevent tumor growth during cancer immunoediting [50]. Detection of an IFN-γ signature within the tumor has been associated with prolonged disease-specific survival in melanoma, colorectal, gastrointestinal, and ovarian cancer [51, 53]. To dissect the role of cytokines in tumor progression, a large cohort of CRC primary tumors has been analyzed for copy number variations in cytokines and cytokine receptors [54]. Fifty-nine soluble and membrane-bound proteins and their corresponding receptors from the IFN, IL, transforming growth factor (TGF), and tumor necrosis factor (TNF) families were analyzed. The majority (75%) of CRC patients displayed no difference in genomic alterations in the cytokine gene and receptor families studied. Of the remaining patients, the highest level of gain was in *IL29* and loss was in *IL15.* Furthermore, clinically advanced patients with distant metastases displayed a higher frequency of deletions in the interleukin family members *IL2, IL8, IL15,* and *IL21*. Interestingly, three of these deletions are in cytokines from the common γ-chain family, which has essential functions for maintenance, proliferation, and migration of memory, CTL, and Th lymphocytes [55]. Most strikingly, only patients with deletions in *IL2, IL15,* and *IL21* had significantly higher risk of tumor relapse [54]. On the other hand, gains or deletions of suppressive cytokines genes, *IL8, IL10,* and *TGFβ* did not correlate to tumor recurrence.

Considering the previously discussed importance of localization and density of memory $CD8^+$ T lymphocytes in the CRC tumor microenvironment, and the predominant role of IL-15 in the regulation of memory T lymphocyte homing and maintenance [56, 57], the cellular source of IL-15 within the CRC tumor microenvironment was investigated, both *in silico* and *in vitro*, by ClueGo and CluePedia, and IHC, respectively. It was discovered that tumor and myeloid cells were the source of IL-15, and increased IL-15 expression could significantly predict prolonged DFS [54]. IL-15 has been shown to rescue tolerant T lymphocytes [58] and augment the therapeutic efficacy of tumor-reactive CTL [11]. Moreover, patients with high expression of IL-15 in the tumor microenvironment have increased immune cell density, immune gene expression, and DFS compared to medium or low expressing CRC tumors [54]. These data show that deletion of the *IL15* gene and reduced production of IL-15 by tumor cells is a substantial mechanism in preventing CTL infiltration and elimination of tumor cells. Furthermore, IL-15 signaling is highly effective in augmenting the anti-tumor CTL response, both as a mechanism to enhance CD4 T cell help and to maintain adoptively-transferred CTL survival [11, 58–60].

Expression of another common receptor λ-chain cytokine family member, IL-21, also predicts clinical outcome in CRC patients. CRC patients with chromosomal aberration of the *IL21* gene leading to deletion had higher risk of relapse than those without a deletion [54]. Overrepresentation of *IL2*, *IL15*, and *IL21* deletions was seen in patients with metastases, suggesting that these cytokines may be involved in putative anti-tumor immune mechanisms. IL-21 has a broad range of therapeutic anti-cancer properties, including activating and preventing exhaustion of tumor-specific CTL [61]. IL-21 is produced by NKT cells, Th1, Th17, and Tfh cells, again suggesting a role of Tfh as a substantial player in orchestrating the CTL response in the tumor.

3.3 Global Factors that Contribute to the Immune Contexture of Tumors

3.3.1 Mutagenesis and CTL Specificity

In melanoma, historically one of the most immune responsive cancers, it is known that the most potent tumor-specific T lymphocytes are directed toward neoantigens expressed by melanoma cells [62, 63]. Similarly, in other cancer types it has been documented that CTL specific for tumor neoantigens are extremely effective at immunosurveillance, elimination of tumor cells, and predicting clinical outcome [64, 65]. Recently, Alexandrov and colleagues reported extensive somatic mutational analysis describing 30 types of human cancer, where highly immunogenic cancers including melanoma, lung and

colorectal carcinoma had the highest prevalence of somatic mutations in their genome [66], thus integrating the paradigm that increased frequency of tumorogenic mutations provides better tumor-specific CTL targets. From this, it might be hypothesized that somatic mutations could be used as predictive biomarkers of cancer patient survival and response to therapy; however, the data up to this point has been inconsistent. Enumeration of total numbers of somatic mutations does not always predict prolonged disease-free survival, however presence of selected immunogenic mutations can distinguish patients with better clinical outcome [65, 67–69]. On the other hand, low numbers of genomic mutations can predict the presence of immunosuppressive mechanisms within the tumor [70]; furthermore, immunogenic mutational gene signatures have been shown to accurately predict benefit from CTLA-4 and PD-1 blocking immunotherapies [68, 69].

A few studies have reported that cancer-driver mutations are associated with immune gene signatures in the microenvironment, most notably in the RAS and EGFR genes. Interestingly, both of these genes have been linked to immune regulatory pathways. The presence of RAS mutations in colorectal carcinoma has been associated with decreased immunogenicity of tumors [71, 72]. Evidence also suggests that mutations in KRAS correlate with downregulation of MHC Class I molecules on tumor cells [71]. Additionally, it has been found that 20 immune genes encompassing checkpoint (CTLA-4, PD-1/L1/L2, TIM3 and LAG3), MHC class II, and Th1 genes were significantly under expressed in patients with KRAS mutations, independently of microsatellite stable or unstable disease [72]. EGFR mutations in NSCLC have been linked with upregulation of PD-L1 on tumor cells leading to inhibition of T lymphocyte response [73, 74].

This suggests that mutations in cancer driver genes themselves are not the most accurate measurement of patient prognosis, but the enumeration of immune-related gene mutations, particularly those involved in MHC-processing [70]. The predictive capability of expression of genes regulating immune cells and MHC-processing has been reported in colorectal, lung, ovary, breast, brain, and renal cancers [67, 75, 76].

3.3.2 Intratumoral Blood and Lymphatic Vessels Modulate CTL Trafficking

Tumor-stimulated angiogenesis is a well-established target for anti-cancer therapies because of the necessity for tumors to obtain a sufficient supply of nutrients. However, because blood vessels generated by tumor-induced angiogenesis lack structure causing blood flow to be leaky, leukocytes are unable to traffic properly [77]. Furthermore, many of the angiogenic tumor-derived factors have promiscuous functions in stimulating suppressive immune mechanisms, such as chemotaxis of Tregs and myeloid-derived suppressor cells to the tumor [78]. Angiogenic promoters also reduce endothelial adhesion molecules, preventing CTL from attaching to the vascular walls and migrating into the tumor [79]. The early inflammatory response driven by TNF-α induces adhesion molecules such as VCAM-1 in normal endothelium, however, when pro-angiogenic factors such as basic fibroblast growth factor are present, TNF-α loses the ability to invoke adhesion molecules [79]. Lastly, pro-angiogenic factors can also induce expression of immune checkpoints, including PD-L1/L2, B7-H3, galectin-1, and TIM3, on the endothelium putting the brakes on CTL activation [79, 80]. A few studies have demonstrated that combination therapy using angiogenesis inhibitors with anti-tumor immune stimulation can restore the migratory potential of CTL [81].

Tumors not only stimulate angiogenesis, but also the generation of new lymphatic vasculature by lymphangiogenesis via production of vascular endothelial growth factor C (VEGFC) and VEGFD [82]. VEGFC and VEGFD are often associated with poor clinical prognosis and increased cancer progression. Tumor-induced lymphatic vessels are important factor for dissemination of tumor cells into the lymph node and distant metastases. These tumor-induced

vessels have dysfunctional fluid mechanics that augment chronic inflammation and secrete immunosuppressive factors, such as TGF-β [83].

On the other hand, the presence of well-ordered lymphatic structures, including TLO and high endothelial venules (HEV) have been demonstrated to facilitate priming, maintenance, and migration of lymphocytes into solid tumors in melanoma, breast, ovarian, colon, and lung cancer [38, 84]. The presence of HEVs in the tumor stroma strongly correlates with increased infiltration of T and B lymphocytes. In breast cancer, high density of tumor HEVs is associated with the extravasation of Th1, CTL, and effector memory T lymphocytes into the tumor [84]. Furthermore patients with high density of tumor HEVs have longer metastasis-free survival, DFS, and OS. These observations were independent of the density of blood vessels within the tumor. In conjunction with these reports, the Authors have observed that patients with high density of lymphatic vessels, as measured by podoplanin (PDPN), in the IM of colorectal tumors are less likely to have metastatic invasion [85]. It is possible that high ordered lymphatic vessels facilitate CTL infiltration into the tumor at the edge of the invading tumor where activated CTL function to prevent metastatic dissemination.

3.3.3 Neuromodulators in Tumor Microenvironment

Chronic exposure to hormones, such as norepinephrine, progesterone, glucocorticoids, and androgenic receptor signaling have been linked to tumorgenesis and metastatic invasion of multiple cancer types (reviewed in [86, 87]). Glucocorticoids potential for immunosuppression is harnessed as an anti-inflammatory treatment for uncontrolled immune cells in patients with autoimmune disease [88]. Glucocorticoids are the major immunomodulatory agents used in clinical medicine. However, their actions as anti-inflammatory and immunosuppressive drugs are both beneficial and deleterious. Glucocorticoids induce the expression of chemokine, cytokine, complement family members, and innate immune-related genes, including scavenger and Toll-like receptors [89]. In contrast, glucocorticoids repress the expression of adaptive immune-related genes [90]. Glucocorticoids modulate T helper lymphocyte differentiation by blocking IL-12-induced Stat4 phosphorylation without altering IL-4-induced Stat6 phosphorylation, therefore leading to suppressive action on the Th1 cellular immune response and a shift toward the Th2 humoral immune response [91]. However, glucocorticoids, in addition to their immunosuppressive function, enhance T-lymphocyte responses [92]. Glucocorticoids up-regulate IL-7RA and enhance IL-7-mediated signaling and function. Moreover, IL-7-mediated inhibition of apoptosis is increased in the presence of glucocorticoids, in a concentration-dependent manner, suggesting enhanced cell sensitivity to IL-7 following glucocorticoid exposure. These observations provide a mechanism by which glucocorticoids may also have a positive influence on T lymphocyte survival and function. Norepinephrine has been shown to downregulate expression of MHC class I molecules and co-stimulatory receptors, as well as increase production of IDO by tumor cells via beta-androgenic receptors [93]. Beta-androgenic receptor signaling has also been implicated in enhanced Treg suppression, polarization of macrophages to the M2 phenotype, and increased infiltration of myeloid-derived suppressor cells [94–98]. These hormones can be produced by cells in the tumor microenvironment or enter through the tumor vasculature. Altogether this suggests tumors that develop in conditions of chronic stress leading to hormone release may be preconditioned to an immunosuppressive immune contexture, which might lead to decreased infiltration of CTL.

3.4 Predicting Patients' Response to Treatment

The ultimate goal of predicting patient survival by immune gene signature and by the presence of immune cells in the tumor microenvironment is to accurately determine which personalized treatment will result in optimal tumor regression.

More and more clinical trials are incorporating immune cell quantification by immunohistochemistry, exome sequencing, gene expression, and flow cytometry to delineate why patients respond to therapy.

In the previous section we describe the use of immunogenic mutational analysis of tumors to predict response to CTLA-4 and PD-1 blocking immunotherapy. Recently there have been a number of reports using immunohistochemical analysis to study immune cells, particularly CTL before and after therapy. A study in patients with melanoma investigated the expression of CD8 and PD-1/PD-L1 in the tumor center and invasive margin of tumor biopsies prior to and following treatment with a humanized blocking antibody to PD-1 [99]. Patients exhibiting beneficial response had significantly higher expression of CD8, PD-1, and PD-L1 in their invasive margin before treatment than patients whose tumors progressed following treatment. This data suggests that therapy blocking the PD-1/PD-L1 pathway would be most beneficial to patients that have pre-existing CTL in the tumor microenvironment. Even though one might assume that patients with high density of immune cells would respond better to immunotherapies targeting the immune system, the presence of immune genes and TILs also predicts patients' response to traditional chemotherapies [48, 100–102]. Analysis of colorectal cancer tumors from 1156 stage III patients treated with 5-fluorouracil based chemotherapy found that patients with TILs at the time of treatment had a better survival advantage after treatment than patients lacking TILs [102]. Additionally, two studies in breast cancer compare the predictive value of TILs for three types of therapy, docetaxel, doxorubicin, and trastuxumab [100, 101]. In the first study, patients with HER2-positive breast cancer were compared for high and low density of TILs. Patients with tumors containing high densities of TILs had significantly longer disease-free (5-year 78.6 *vs*. 47%) and overall (5-year 92.9 *vs*. 70.7%) survival after treatment with doxorubicin than patients with low densities of TILs. Interestingly, this was not the case when doxorubicin and docetaxel were administered in conjunction. In another study it was shown that patients with HER2 positive breast cancer and high density TILs had better response to treatment with the anti-HER2 antibody, trastuzumab. These studies portray the importance of characterizing the immune microenvironment of tumors to determine optimal personalized beneficial treatments.

3.5 Conclusions

We ascertain that the evaluation of the CTL densities in primary tumors is a superior method of predicting patient survival for the majority cancer types. Moreover, patients that lack CTL in their primary tumor have the worst clinical prognosis and have tumors that are resistant to CTL killing because CTL are not able to traffic to the tumor site. We propose this is due to the lack of T helper lymphocytes, memory promoting cytokines, and chemoattractants, as well as dysfunctional blood and lymphatic flow preventing CTL from getting into the tumor microenvironment. We also hypothesize that potential immunosuppressive factors from stress-related hormones deters CTL from the tumor. These mechanisms are illustrated in Fig. 3.3. Plainly, if CTL are not present at the tumor site they are not able to eliminate tumor cells.

The difficult question remains, how do we induce CTL trafficking to the tumor for patients with low Immunoscore (I0-I1)? Currently systemic treatment of both recombinant human IL-15 and IL-21 are being tested in clinical trials with favorable results [57, 103]. In addition, IL-15 and IL-21 are being used in combination with adoptive immunotherapy to stimulate CTL *ex vivo* or as supplemental systemic administration. Initial studies using intratumoral injection of membrane-anchored chemokine fusion proteins, including CXCL10, are being used as a method to induce CTL trafficking to the tumor site [104, 105]. Another potential target to improve CTL migration to the tumor is by combination immunotherapy with angiogenesis-inhibitors. Inhibition of angiogenesis improves the organization of the vasculature allowing for better extravasation and migration of CTL into

the tumor [78, 79, 81, 106]. Angiogenesis inhibition has improved the therapeutic efficacy of both adoptive immunotherapy and vaccine-induced anti-tumor immunity. Finally, it will be essential that clinical studies incorporate tumor immune microenvironment analysis, such as Immunoscore, to fully understand the factors managing tumor-specific CTL trafficking to the tumor and quality of response to cancer therapy.

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