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Significance of Tumor Microenvironment Scoring and Immune Biomarkers in Patient Stratification and Cancer Outcomes

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Abstract

Tumors appear as heterogeneous tissues that consist of tumor cells surrounding by a tumor microenvironment (TME). TME is a complex network composed of extracellular matrix (ECM), stromal cells, and immune/inflammatory cells that drive cancer cells fate from invasion to intravasation and metastasis. The stromal-inflammatory interface represents a dynamic space, in which exchange of numerous molecular information is associated with the transition into tumorigenic microenvironment. Recruitment, activation, and reprogramming of stromal and immune/inflammatory cells in the extracellular space are the consequences of a reciprocal interaction between TME and cancer cells. Recent data suggest that cancer development is influenced by TME and controlled by the host's immune system, underlying the importance of TME components and immune biomarkers in the determination of prognosis and response to therapy. The immune classification has prognostic value and may be a useful supplement to the histopathological, molecular, and TNM classifications. Nevertheless, the complexity of quantitative immunohistochemistry and the variable assay protocols, stromal and immune cell types analyzed underscore the need to harmonize the quantified methods. It is therefore important to incorporate TME and immune scoring in determinations of cancer prognosis and to make sure they become a routine part of the histopathological diagnostic and prognostic assessment of patients.

Keywords: tumor microenvironment, stromal cells, immune cells, inflammatory cells, immunoscore, immune biomarkers, PDL-1, PD-1, checkpoint inhibitors, immunoncology, patient stratification, combined immunotherapy

1. Introduction

Cancer is usually viewed as a complex process of multiple disorders that are mostly driven by somatic mutation with the involvement of several hallmarks: genomic instability, sustaining proliferative signaling, resisting cell death, enabling replicative immortality, inflammation, evading the immune system, *de novo* angiogenesis, invasion, and metastasis. The outcome prediction in cancer is usually achieved by histopathological analysis of tissue samples obtained by biopsies or surgical specimens from primary tumor or metastatic localization. However, the heterogeneity in the histological appearance of different tumors (intertumor heterogeneity) as well as of different areas in the same tumor (intratumor heterogeneity) is of uncontested relevance and can explain the histopathological classification of tumors based on the morphological patterns. In the last decade, the advent of molecular pathology has allowed the definition of molecular subtyping for several cancers, which does not completely overlap with prevailing histopathological classifications [1].

In current practice, TNM classification appears as a simple method of tumor staging used worldwide, and based on tumor burden (T), lymph nodes status (N), and presence of metastases (M). However, the TNM classification provides limited prognostic information in cancer and does not predict response to therapy. Moreover, cancer outcome can differ significantly between patients whose cancers are at the same TNM stage.

Tumor appears as heterogeneous tissues that consist of tumor cells surrounded by a tumor microenvironment (TME). TME is a complex network composed of extracellular matrix (ECM), stromal cells (fibroblasts, adipocytes, neural and neuroendocrine (NE) cells, endothelial cells (ECs), and pericytes), immune and inflammatory cells that drive cancer cells fate from invasion to intravasation and metastasis. Cancer cells need cellular, biochemical, and biophysical stimuli originating from a more adapted microenvironment by recruiting and educating various types of normal cells into their neighborhood. The stromal-inflammatory interface represents a dynamic space characterized by reversible stromal and epithelial events. Within this dynamic space, exchange of numerous molecular information is associated with the transition into tumorigenic microenvironment and includes growth factors (GFs), cytokines, chemokines, enzymes, matrix proteins, and metabolic intermediates. Recruitment, activation, reprogramming, and persistence of stromal and immune/inflammatory cells in the extracellular space are the consequences of a reciprocal interaction between TME and cancer cells [2, 3].

Recent data suggest that cancer development is influenced by TME and controlled by the host's immune system, underlying the importance of including TME components and immunological biomarkers in the determination of prognosis and response to therapy, a concept that has been termed as microenvironment score and immunoscore. Increasingly, data collected from cancer tissue samples demonstrate that immune classification has prognostic value and may be a useful supplement to the histopathological, molecular, and TNM classifications. Nevertheless, the complexity of quantitative immunohistochemistry and the variable assay protocols, stromal and immune cell types analyzed and tumor-sampling criteria underscore the need to harmonize the quantified methods. It is therefore important to incorporate TME and immune scoring in determinations of cancer prognosis and to make sure they become a routine part of the histopathological diagnostic and prognostic assessment of patients with cancer.

2. Tumor microenvironment components

2.1. Non-immune/inflammatory stromal cells

Non-immune/inflammatory stromal cells comprise fibroblasts, adipocytes, neural and neuroendocrine cells, endothelial cells, pericytes, and mesenchymal stem cells (MSCs) (**Figure 1** and **Table 1**).

2.1.1. Cancer-associated fibroblasts

Cancer-associated fibroblasts (CAFs) are a sub-population of activated fibroblasts with myofibroblastic phenotype that represent the predominant non-inflammatory stromal cell type in the TME. CAFs are heterogeneous cells of multiple origins, which are usually identified according to their different origins by expression of proteins such as mesenchymal biomarkers (vimentin and fibronectin), fibroblast-secreted protein-1 (FSP-1), α -smooth muscle actin (α SMA), tenascin-C, platelet-derived growth factor receptor (PDGFR), and fibroblast activation protein (FAP) [4, 5]. CAFs accumulation in the TME is often correlated with poor prognosis. They may promote tumor development and progression by promoting angiogenesis or by interacting with immune-inflammatory cells and neuroendocrine cells through different cell factors and cytokines [2]. CAFs may also hinder antitumor immune responses [4]. Indeed, cancer cells produce TGF- β that activates adjacent CAFs. In turn, CAFs promote tumor progression by releasing numerous interleukins (IL-1, IL-6, IL-8, and IL-22) and chemokines (CXC-chemokine ligand CXCL and CC-chemokine ligand CCL) [2]. CAFs can also secrete various chemotactic GFs (EGF, FGF, HGF PDGF, and VEGF), ECM proteins (collagens, fibronectins, tenascin C, and SPARC), enzymes such as matrix metalloproteinases (MMPs), lysyl oxidases (LOX) family, and cyclooxygenase 2 (COX2) [6].

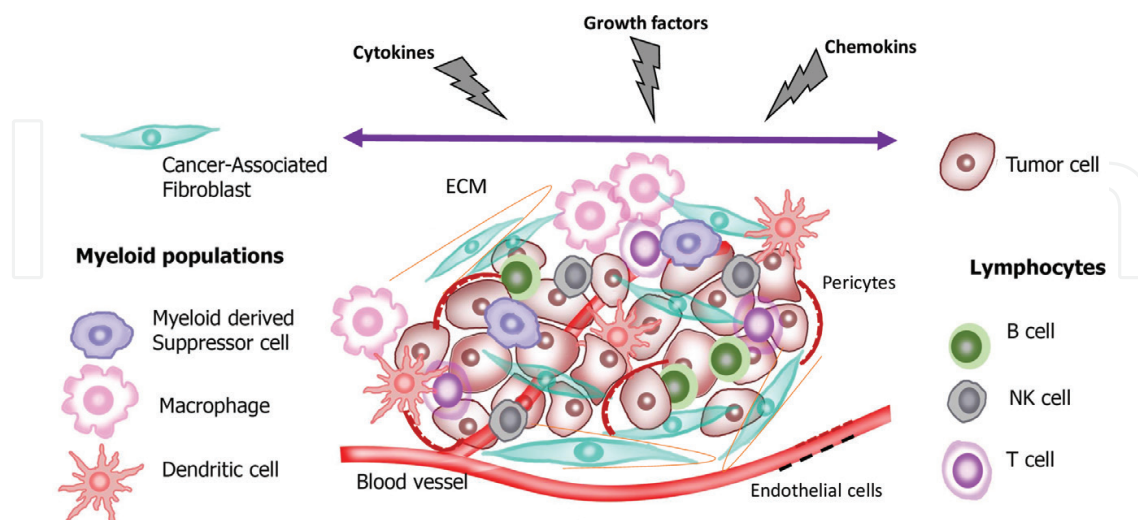


Figure 1. Tumor microenvironment is a complex network composed of extracellular matrix (ECM), stromal cells (fibroblasts, endothelial cells and pericytes) and immune and inflammatory cells (T cells, B cells, natural killer 'NK' cells, dendritic cells, macrophages and myeloid-derived suppressor cells). The stromal-inflammatory interface represents a dynamic space contains growth factors, cytokines and chemokines. Recruitment, activation, reprogramming and persistence of stromal and immune/inflammatory cells in the extracellular space are the consequences of reciprocal interactions between tumor microenvironment components and tumor cells.

Non-immune/ inflammatory stromal cell	Main markers	Main functions	Potential therapeutic targets
Cancer-associated fibroblasts (CAFs)	Vimentin, fibronectin, FSP-1, α SMA, tenascin-C, PDGFR Endosialin (CD248), and FAP	<ul style="list-style-type: none"> Tumor progression (IL-1, IL-6, IL-8, IL-22, CXCL1 to CXCL12/ SDF1, CCL2 and CCL20, VEGF, PDGF, EGF, FGF, HGF, fibronectin, collagen I and III, EDA-fibronectin, tenascin C, SPARC, MMPs, LOX family, and COX2 Promote angiogenesis Hinder antitumor immune responses 	Anti-CXCR-4 antibodies (CXCL12/SDF-1inhibition); anti-VEGF and anti-PDGF antibodies; MMP inhibitors; anti-IL6 antibodies; anti-HGF therapies; anti-FAP antibodies; Anti-TGF β inhibitors; anti-IL-11 and anti-THSB1 therapies
Cancer-associated adipocytes (CAAs)	FSP-1 expression	<ul style="list-style-type: none"> Produce adipokines (leptin, adiponectin, and apelin), angiogenic factors (VEGF), TNF-α, IL-1β, IL-6, IL-8, MCP1, CCL2 and CCL5 and HGF 	Antibodies anti-IL-6, anti-IL-8; anti-CCL2, COX2 and adiponectin inhibitors
Mesenchymal stem cells (MSCs)	Vimentin, CD29 (β 1integrin), CD44, CD73, CD90, CD105 and STRO-1	<ul style="list-style-type: none"> Stimulate tumor angiogenesis through VEGF expression Multilineage potentiate Immunoregulatory function 	Nano-engineered MSCs are used as targeted therapeutic carriers
Endothelial cells	Tip cells: VEGFR1 ^{low} , VEGFR2 ^{high} , Dll4 ^{high} , and CD34 ⁺ Stalk cells: VEGFR1 ^{high} , VEGFR2 ^{low} , Dll4 ^{low} CD34 ⁻ .	<ul style="list-style-type: none"> Implicated in metastatic niche and dormancy through TGF-β1 and POSTN 	av β 1, av β 2, a5 β 1 integrin inhibitors; anti-VEGF and VEGFR agents
Pericytes	α SMA, Desmin, NG2 (CSPG4), 3G5 antigen, PDGFR- β and Endosialin (CD248)	<ul style="list-style-type: none"> Modulate the magnitude of immune responses Prevent lymphocyte extravasation and activation in tumor tissue 	Anti-ANG2 antibody VEGFR and PDGFR- β antagonists; VEGFR, PDGFR- β , and Tie-2 agonists; anti-RSG5 and anti-PD/PD-L1 therapies
Neural cells	PGP9.5. and NGF	<ul style="list-style-type: none"> Favors tumor progression Norepinephrine, impact T-cells by inhibiting the generation of CTLs through inhibition of TNF-α synthesis 	Anti-NGF blocking antibodies, NT3 and NT4 targeted therapies; GDNF inhibitors; anti-NGF antibodies; anti-PTN antibodies and N-syndecan inhibitors; BDNF inhibitors

Abbreviations: ANG2: angiopoietin-2; *Integrins* av: av β 1, av β 2; BDNF: brain-derived neurotrophic factor; CCL: chemokine ligand; COX: cyclooxygenase; CSF: colony stimulating factor; CXCL: C-X-C chemokine ligand; CXCR: C-X-C chemokine receptor; CSPG4: Chondroitin sulfate proteoglycan 4; FAP: fibroblast activation protein; FRb: folate receptor beta; GDNF: glial cell line-derived neurotrophic factor; HRG: histadine-rich glycoprotein; IL: interleukin; MMP: matrix metalloproteinase; NGF: nerve growth factor; NT: neurotrophin; PDGF: platelet-derived growth factor; PDGFR: platelet-derived growth receptor; PD1: programmed cell death protein; PD-L1: programmed cell death ligand; PTN: pleiotropin; RSG5: regulator of protein signaling 5; SDF: stromal-derived factor; TIMP: tissue inhibitor of metalloproteinase; TGF: transforming growth factor; TLR: toll-like receptor; VEGF: vascular endothelial growth factor; VEGFR: vascular endothelial growth factor and receptor.

Table 1. Stromal cell types in tumor microenvironment: main markers and functions with potential therapeutic targets.

2.1.2. Cancer-associated adipocytes

Cancer-associated adipocytes (CAAs) possess important secretory properties that may enhance tumor aggressiveness. Compared to normal adipocytes, CAAs are characterized by the loss of adipocyte differentiation, a smaller size, and FSP-1 expression (with lack of α SMA expression). They produce adipokines (leptin, adiponectin, and apelin), angiogenic factors and GFs (VEGF and HGF), tumor necrosis factor- α (TNF- α), interleukins (IL-1 β , IL-6, and IL-8), and chemokines (MCP1, CCL2, and CCL5) [7]. They also exhibit an increased secretion of fibronectin, collagen I/VI, and MMP-11/Stromelysin-3 [2, 8]. The activation of Wnt/ β -catenin pathway in response to Wnt3a secreted by cancer cells is essential to adipocytes reprogramming. The reprogrammed CAAs located close to cancer cells can initiate protumoral heterotypic paracrine and endocrine interactions. Another type of CAAs is the adipose stem cells (ASCs). ASCs can influence the TME by worsening the tumorigenic behavior of c-Met-producing cancer cells, which in turn creates an inflammatory TME. ASCs can interact with TME through TGF- β 1-signaling pathway or promote angiogenesis by migrating toward tumor-conditioned media through the PDGF-BB/PDGF- β -signaling pathway [5].

2.1.3. Angiogenic vascular cells

Blood vessels are composed of perivascular cells termed as pericytes, endothelial cells (ECs) which form the inner lining of the vessels wall and smooth muscle cells.

Pericytes differentiate from mesenchymal precursors and are recruited to tumors by PDGF β . They possess characteristic cellular markers including 3G5 ganglioside and chondroitin sulfate proteoglycan 4 (CSPG4) also known as NG2. In tumor tissue, pericytes highly express α SMA, although it is often absent in quiescent pericytes in non-tumoral tissue. Recent experimental studies revealed that pericytes can actively modulate the magnitude of immune responses and may prevent lymphocyte extravasation and activation in tumor tissue [9].

ECs are subdivided into tip cells and stalk cells and function as active stromal regulators implicated in proliferation, invasion, secretion of inflammatory and growth mediators, and metastatic spread. Tip cell is highly migratory and polarized EC type that extends numerous filopodia and expresses low level of VEGF receptor 1 (VEGFR1^{low}), with high levels of VEGFR2 and Delta-like ligand 4 (Dll4), and in vitro CD34. The tip cell is followed by stalk cell, a proliferative and less migratory type of EC, which expresses VEGFR1^{high}, VEGFR2^{low}, Dll4^{low} and has undetectable levels of CD34 in vitro [10]. Importantly, neovascular tips are rich in active TGF- β 1 and periostin, which promote tumor growth and regulate tumor dormancy [11].

2.1.4. Neural and neuroendocrine cells

Cancer cells can support the neurogenesis by secreting several neuronal growth factors and axon guidance molecules. The majority of factors known to induce neurogenesis, such as neurotrophins, insulin-like growth factor-II (IGF-II), and fibroblast growth factor (FGF), are usually secreted by tumors with bad prognosis. These factors exert autocrine or paracrine effects in cancer cells. Norepinephrine, another neurotransmitter, has a significant impact on T-cells. It can inhibit the generation of antitumor cytotoxic T-lymphocytes (CTLs) through the inhibition of TNF- α synthesis [11]. The neural-epithelial interaction and nerve growth factor

(NGF) production by cancer cells favor tumor progression by promoting both the growth of cancer cells and neurites [12].

Neuroendocrine (NE) cells are part of the diffuse NE system and exhibit a combination of neuronal and endocrine features. NE system strongly influences the function of the immune system. It can regulate the migration and cytotoxicity in natural killer (NK) cells through neurotransmitters. Additionally, the neuroendocrine substance P (SP) blocks the β 1-integrin-mediated adhesion of T lymphocytes and increases their migratory activity [13]. SP can also induce the production of various cytokines in leukocytes. SP and the subsequent activation of the neurokinin-1 receptor (NK1R) lead to mitogen-activated protein kinase (MAPK) activation. The involvement of NK1R activation in mitogenesis, angiogenesis, cell migration, and metastasis supports the hypothesis that SP and NK1R interactions influence the TME [14].

2.1.5. Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multipotent stem cells with the capacity to differentiate into fibroblasts, adipocytes, pericytes, osteocytes, and chondrocytes. MSCs express cell surface markers CD29, CD44, CD73, CD90, CD105, and STRO-1, and lack the expression of CD14, CD34, CD45, and human leukocyte antigen HLA-DR [15]. MSCs have immunomodulatory features and secrete cytokines, VEGF, and immune receptors which regulate the microenvironment in the host tissue. Based on their multilineage potential, immunoregulatory and tissue-protective properties, MSCs are being tested for the treatment and prevention of graft-versus-host disease, chronic diseases, and certain hematologic malignancies [16].

2.2. Extracellular matrix

ECM is composed of proteins (collagens, laminins, and fibronectins), proteoglycans, and hyaluronans in a specific organization [17, 18]. CAFs are the major cell type responsible for the synthesis of ECM proteins. ECM contains all the cytokines, GFs, and hormones secreted by stromal and cancer cells. During tumor progression, ECM composition and structure change continuously. ECM heterogeneity is crucial for controlling collective cell-invasive behaviors and determining metastasis efficiency. ECM selects survival cancer cells to aid in tumor growth and invasion at the fastest rate. ECM can also affect tumor development and metastasis through extracellular secretion, or by altering the phenotype of stromal cells or cancer cells [3]. Moreover, ECM provides a hypoxic or acidic microenvironment in which cancer cells have greater survival advantages. The abundant ECM within the TME is correlated with increased tumor growth through various mechanisms, including activation of pro-survival phosphoinositide 3-kinase (PI3K)-signaling pathways and downstream of integrin receptors [2].

ECM interacts with lymphocytes and crucially influences immune cells motility and localization, which can help tumor cells to evade from immune surveillance. Increased stroma density reduces lymphocyte displacement, supporting the idea that ECM deposition can alter antitumor immune responses by limiting T-cell motility [4].

2.3. Immune and inflammatory cells

Tumor microenvironment contains numerous immune and inflammatory cells that originate from lymphoid precursors [CD8⁺ cytotoxic T-cells (CTLs), CD45⁺ memory T-cells, CD4⁺ T

helper cells (Th1, Th2 and Th17), T regulatory cells (Tregs), T follicular helper cells (TFH), NKT cells, gamma delta T ($\gamma\delta$ T) cells, B-cells, and plasmacytoid dendritic cells (pDCs)] and from myeloid precursors [tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), conventional DCs (cDCs), neutrophils, mast cells, and platelets]. The term tumor-infiltrating lymphocytes (TILs) are referred to a group of T-cells (CD3, CD4, CD8, and FoxP3) located around tumor cells [19]. In addition, the invasive margins of cancers may comprise tertiary lymphoid structures (TLSs) that exhibit strong similarities with lymph node organization.

These immune and inflammatory cells infiltrate TME via a network of inflammatory chemotactic cytokines and chemokines produced by cancer cells.

NK cells (CD56⁺/CD3⁻) belong to the innate immune system and play an important role in protecting the host from infections and cancer. NKT cells (CD56⁺/CD3⁺) share a variety of markers for both T lymphocytes and NK cells. The $\gamma\delta$ T-cells are an independent population of circulating lymphocytes that can sense pathogens. $\gamma\delta$ T-cells can also induce DC maturation, functional activation and migration, and antigen presentation. NK, NKT cells, and $\gamma\delta$ T-cells are present in TME in various cancer, and express the natural killer group 2D (NKG2D) receptor. NKG2D recognizes proteins encoded by the *MICA* and *MICB* locus, which are located within the major histocompatibility complex (MHC) on chromosome 6 near the *HLAB* locus [20].

CD4⁺ and CD8⁺ are the two main lineages of T-cells. CD4⁺ T-cells are classified into CD4⁺ Th that mediate tumor immunity and CD4⁺ CD25⁺ FoxP3⁺ Tregs that suppress antitumor immunity and promote tumor growth [21, 22].

DCs are derived from myeloid precursors (cDCs) or lymphoid precursors (pDCs) and are considered as a crucial link between innate and adaptive immunity. DCs have three maturation stages: precursor DCs, immature DCs, and mature DCs. Immature DCs interact with antigens, migrate into secondary lymphoid organs, and become antigen-presenting cells (APCs). DCs are among the first cells migrating to the tumor site by means of GFs (VEGF and HGF), chemokines (CXCL12 and CXCL8), and antimicrobial peptide (β -defensin) secreted by cancer cells and stromal cells [23–25].

MDSCs have two distinct monocytic and granulocytic subsets and can differentiate into DCs or ECs. They coordinate tumor progression and angiogenesis through the release of MMP-9 and VEGF. MDSCs can also promote immune evasion by suppressing antitumor CTLs and NK cells [26].

TAMs are multifunctional cells characterized by the expression of CD68, plasticity, and secretion of numerous immune-modulatory cytokines. Macrophages differentiation and growth are regulated by several GFs, including CSF-1 and GM-CSF. TAMs can release chemokines (CCL17, CCL18, and CCL22) and recruit non-CTLs, especially Tregs. Activated macrophages can be classified into M1 and M2 cells [27]. M1 cells are characterized by high capacity to present antigen and are involved in the response of Th1 cells to pathogens and cancer. M1 cells produce proinflammatory cytokines (TNF α and IFN- γ) and interleukins (IL-1 and IL-12) and generate reactive oxygen species (ROS) and nitric oxide (NO). By contrast, M2 cells have immunosuppressive phenotype, produce IL-10, and inhibit CTLs, which are crucial to initiate a Th2-type response. Within the TME, TAMs have generally a M2-skewed phenotype (CD163⁺, CD204⁺, and CD206⁺) that promote angiogenesis, ECM remodeling, and repair [28].

During tumor development, pre-invasive TME has antitumor property that includes predominantly M1 and Th1 with the production of IL-12, IFN γ , and inducible NO synthase (iNOS). Comparatively, the transition to invasive TME is marked by pro-tumoral properties with a shift from M1 to M2 and from Th1 to Th2 cells, a decrease of IFN γ , and an increase of IL-1, IL-6, VEGF, and indoleamine 2, 3-dioxygenase (IDO) [29].

Topographically, each type of immune and inflammatory cells has a preferred location within tumor site. CTLs and Th1 cells are located at the invasive margins and/or in the tumor core. Immature DCs are found in the tumor core, whereas mature DCs infiltrate T-cell zones in close contact with CD4⁺ and CD8⁺ T-cells. B-cells are found in TLS and at the invasive margins. TAMs and TFH are in contact or within B-cell zones, whereas NK cells are dispersed within the stroma and at the invasive margins [30].

Tumor-associated TLSs exhibit strong similarities with lymph node organization and comprise prominent B-cell follicles, T-cell marginal zones, and associated follicular DCs, very few Tregs, and high endothelial venules (HEVs). TLSs are usually located in the tumor-invasive margin and in the stroma of most cancers and their densities correlate with a favorable clinical outcome. HEVs express peripheral node addressins (PNAs) and specialized in the extravasation of circulating immune cells, and the secretion of chemokines that are crucial for lymphocyte recruitment and entry into the lymph node. Recently, a molecular signature of TLSs encoding 12 distinct chemokines (CCL2, CCL3, CCL4, CCL5, CCL8, CCL18, CCL19, CCL21, CXCL9, CXCL10, CXCL11, and CXCL13) has been identified in various tumors [31].

TLSs are associated with the generation of an adaptive immune response and represent a formidable school for T-cell priming, B-cell activation, and differentiation into plasma cells and an exquisitely located factory for antibody production [32].

3. Host immune response to cancer

3.1. Cancer immune cycle

In the early stage of carcinogenesis, cancer cells are rejected by an innate immune mechanism also referred to as immunosurveillance. The innate immune system recognizes exogenous pathogen-associated molecular patterns (PAMPs) or endogenous danger-associated molecular patterns (DAMPs). These latter ones are recognized by the host organism through various pattern recognition receptors (PRRs) that activate DNA sensors and downstream adaptors to trigger stimulation of innate immune system and to induce adaptive T-cell responses. Multiple families of PRRs, including Toll-like receptors (TLRs), have been identified within plasma membrane, intracellular vesicles, and within the cytosol of APCs [33]. Binding of ligands to PRRs activates various adaptor molecules and downstream signaling pathways, orchestrating innate immune responses and maturation of APCs (DCs), leading to production of antimicrobial peptides, cytokines, chemokines, and type I interferon (IFN) including IFN- α and IFN- β . In cancer, PRRs can also recognize various endogenous DAMPs, such as cancer-associated antigens (CAAs). Among regulators of innate immune system, recent evidence has

indicated that the major pathway involved in the induction of a spontaneous antitumor adaptive T-cell response is the stimulator of interferon genes (STING) signaling [34].

Experimental studies indicate that immune system plays a dual role in cancer, a theory known as cancer immunoediting. It can not only eliminate cancer cells or inhibit their growth but also promote tumor progression by modifying conditions within TME or by selecting more resistant cancer cells. Cancer immunoediting contains three phases: elimination, equilibrium, and escape. The immune system is directed against cancer cells through the “cancer immunity cycle” described by Chen and Mellman [35], which associates cancer antigen release by tumor cells, presentation by DCs and priming of T lymphocytes in lymph nodes, activation of peripheral immune cells, trafficking and infiltration of T-cells to the TME, cancer cells recognition, and immune-mediated cell death (T-cell-inflamed phenotype). In the elimination phase, T-cells attack tumor cells that express tumor-specific antigens in the form of complexes of tumor-derived peptides bound to MHC molecules on the cell. Naïve T-cells that differentiate in bone marrow express a unique T-cell receptor (TCR) and undergo positive and negative selection processes in thymus. T-cells become activated when tumor antigens are recognized. Then, T-cells proliferate and differentiate, leading to the T-cell’s ability to attack and destroy cells that express relevant antigens. The recognition of antigen-MHC complexes by the T-cell antigen receptor is not sufficient for the activation of naïve T-cells. However, the engagement of CD28 on T-cell surface and the expression of B7 molecules (CD80 and CD86) on APCs (DCs) provide additional costimulatory signals [36]. Cancer cells usually do not express B7 molecules (except for certain lymphomas) and hence are largely invisible to the immune system. This can be overcome by an inflammatory response, which permits APCs to take up antigen and present antigen-MHC along with B7 molecules initially in tumor-draining lymph nodes for effective activation of T-cells. After the costimulation process, tumor-specific T-cells acquire effector function, move to the tumor site, and infiltrate TME, which activates the antitumor immune response. However, the antitumor efficacy of T-cells within TME is determined by their ability to overcome barriers and counter-defenses they encounter from tumor and stromal cells, Tregs, MDSCs, and inhibitory cytokines that act to mitigate antitumor immune responses [37].

Activated T-cells express immune checkpoints such as cytotoxic T lymphocyte-associated protein 4 (CTLA-4 also known as CD152) and programmed death 1 (PD-1 also known as CD279) which act to abrogate T-cells responses. CTLA-4 competes with CD28 for binding to CD80/86, providing an inhibitory stimulus upon engagement [38].

PD-1 is a T-cell surface receptor that delivers inhibitory signals upon engagement with its ligands. PD-1 ligands (PD-L1 and PD-L2) are expressed via oncogenic expression on tumor cells or by stromal cells and may also be upregulated in the setting of high levels of IFN- γ , termed adaptive immune resistance [39].

During tumor development, a subpopulation of non-immunogenic cancer cells develops new mechanisms to evade immune surveillance and induce tumor tolerance. They include decreased expression of MHC-I and expression of immunosuppressive factors that contribute to escape from immune recognition. Consequently, tumors display a strong immunosuppressive TME and fail to elicit an appropriate adaptive immune response. This TME is associated with several molecular mechanisms in place to interfere with CTLs, resulting in poor infiltration of reactive tumor-rejecting T-cells [40].

After an efficient immune response, immune tolerance reduces ability for immune-mediated tumor eradication by associating upregulation of tumor and immune cells PD-L1, DCs and macrophages IDO expression in response to IFN γ signaling, expression of additional immunosuppressive checkpoints (LAG3), and enhanced regulatory T-cells and MDSCs activities [41].

An innate immune response leads to activation of the adaptive immune system (B- and T-cells), provided direct interactions with APCs and a proinflammatory environment. Primary adaptive responses are slower than the innate responses, as clonal expansion due to the recognition of foreign antigens is required.

The current understanding of the dichotomous nature of immune cells in tumors is that IFN γ -producing CD4⁺ Th1 and CD8⁺ CTL along with mature DCs, NK cells, M1 macrophages and type 1 NKT cells can generate antitumor responses. Conversely, CD4⁺ Th2, CD4⁺ Tregs, MDSCs, immature DCs, M2 macrophages, and type 2 NKT cells promote tumor tolerance and support tumor growth and progression [40]. Furthermore, the knowledge on the crucial antitumor activity of the immune system has generated great interest in immunotherapy of cancer, including non-immunogenic tumors.

3.2. Humoral immune response in cancer

The production of autoantibodies (AAbs) reflects the immunologic reactivity in cancer patients and enhances immune surveillance for cancer cells. AAbs level is detectable in very early cancer stages and may persist for an extended period after cancer removal, reflecting the overall host immune response toward the tumor. It is interesting to note that a repertoire of AAbs is shared by autoimmune diseases and cancer, suggesting that autoimmune conditions share many parallels with the humoral immune response to tumor-associated antigens (TAAs) [42]. Tolerance defects, inflammation, posttranslational modifications, and cell death can affect TAA immune presentation, contributing to cancer-related AAbs production. Recently, AAbs have become useful diagnostic, prognostic, and surveillance cancer biomarkers as they can be easily detected in the serum of cancer patient [43].

3.3. Genetic and germline polymorphisms of immune system

Genetic polymorphism is an alternative phenotype that appears to be widespread among the genes of the immune system and can correspond to an evolutionary adaptation of the host organism facing an environment in constant evolution. Several polymorphisms concerning genes that encode Janus kinase/signal transducer and activator of transcription (JAK/STAT), *TLR* genes, TNF- α , NF- κ B, NOD2, autophagy-related protein 16 (ATG16), and receptors for the Fc domain of immunoglobulins (FcR), are involved in the immune responses in cancer development or affect the potency of certain anticancer therapies.

JAK/STAT-signaling pathway plays a key role in the regulation of cellular responses to cytokines (IFN- α , IFN- β , IFN- γ , and IL). It has been demonstrated that genetic polymorphism involved in JAK/STAT (STAT3 and STAT4) pathway is associated with the risk of non-Hodgkin lymphoma [44]. Moreover, polymorphisms in *TLR* genes may shift balance between pro- and anti-inflammatory cytokines in the host, contributing to the onset and progression of

cancers. Recent evidence has implicated polymorphisms of FcRs in the efficacy of monoclonal antibody (mAb)-mediated therapy. Interestingly, the therapeutic effects of IgG1 mAbs (rituximab and trastuzumab) are partially mediated by the Fc γ R immune response, suggesting that polymorphisms of Fc γ R may affect the potency of the mAb treatment [45].

3.4. Microbiota

The microbiota is composed of commensal bacteria and other microorganisms that live on the epithelial barriers of the host. Microbiota influences physiological functions including the maintenance of barrier homeostasis and the regulation of metabolism, hematopoiesis, inflammation, and immunity. Recent data demonstrated the involvement of microbiota in cancer initiation, progression, and dissemination. In addition, gut microbiota can modulate the response to chemotherapy, radiotherapy and immunotherapy, and susceptibility to toxic side effects. Therefore, targeting the microbiota may improve anticancer efficacy and prevent toxicity [46].

3.5. Environmental factors

Immunity in humans can also be affected by environmental factors, including the presence of infectious agents, diet, exposure to sunlight (photoimmunity), and the intake of pharmaceuticals. Interestingly, during periods of decreased exposure to sunlight the human immune responses are associated with enhanced levels of IL-6 and C-reactive protein, which are linked to an increased propensity for autoimmunity. Therefore, it is acceptable to believe that low sunlight conditions may correlate with a more inflammatory systemic environment, leading to better responses to cancer immunotherapy [47].

4. Tumor microenvironment and immune scoring

4.1. Glasgow microenvironment score

Glasgow microenvironment score (GMS) is a cumulative prognostic score that combines Klintrup-Mäkinen (K-M) grade and tumor stroma percentage (TSP) and has an independent prognostic value. K-M grade semiquantitatively evaluates the peritumoral immune cell type and density at the invasive margin of the deepest point of tumor invasion using H&E-stained FFPE tissues. K-M grade is classified into (1) low-grade K-M: no increase or mild increase in inflammatory cells, and (2) high-grade K-M: prominent inflammatory reaction that forms a band at the invasive margin, or florid cup-like infiltrate at the invasive edge with destruction of cancer cell islands [48, 49]. K-M grade could be assessed by IHC-stained sections using CD3, CD8, CD45R0, and FoxP3 antibodies to evaluate immune T-cell type [49]. TSP evaluates the percentage of stroma using complete sections of the deepest point of tumor invasion. The proportion of stroma is calculated as the visible field at 10 \times objective, excluding areas of mucin and/or necrosis [50]. TPS is subsequently graded as low TSP (\leq 50%) or high TSP (>50%) [49]. The global GMS score is subdivided into three GMS categories: (GMS 0: high-grade K-M), (GMS 1: low-grade K-M/low-grade TSP), and (GMS 2: low-grade K-M/high-grade TSP) [51].

4.2. Microenvironment cell populations-counter

Microenvironment cell populations (MCP)-counter is a transcriptome-based computational method that quantifies the abundance of 10 stromal and immune cell populations in TME using a single-gene expression experiment. MCP-counter produces an abundance score for CD3⁺ T-cells (CD3D and CD5), CD8⁺ (CD8B) and CTLs (EOMES and GNLY), B lymphocytes (CD19, CD79A, and CD79B), NK cells (NKp46 and KIR genes), monocytic lineage (CSF1R), myeloid DCs (CD1), neutrophils (FCGR3B and CD66b), as well as fibroblasts (DCN and TAGLN) and ECs (CDH5). These scores can then be used for direct comparisons of the abundance of the corresponding cell type across samples within a cohort. MCP-counter was quantitatively validated by both using mRNA mixtures and IHC in FFPE tissues. This method can reproduce immunological and stromal prognostic classifications associated with overall survival in lung adenocarcinoma and colorectal and breast cancers [52]. However, the loss of spatial cell's localization is one of limitations when using such transcriptomic technology. Thus, histological confirmation of MCP-counter seems to be necessary in cases where contamination of samples by surrounding non-tumoral tissues is unavoidable.

4.3. Cancer transcriptomic signature

A transcriptomic classification of colorectal cancer has been recently proposed that stratifies colorectal cancer into intrinsic subtypes with different prognosis. This classification is subdivided into four consensus molecular subtypes (CMS): CMS1 (MSI-like subtype) that contains most microsatellite instability (MSI) tumors and BRAF mutations, CMS2 (canonical subtype) with high chromosomal instability (CIN), CMS3 (metabolic subtype) includes tumors with KRAS mutations and shows a disruption of metabolic pathways, and CMS4 (mesenchymal subtype) that concerns tumors with frequent CpG-island methylator phenotype (CIMP) [53]. Interestingly, a recent comparative study has demonstrated three microenvironmental signatures that correspond to each molecular subtype. The CMS1 was associated with the overexpression of genes specific to cytotoxic lymphocytes, and a good prognosis. Conversely, CMS4 revealed proinflammatory, proangiogenic, and immunosuppressive signature and was associated with poor prognosis. Finally, CMS2 and CMS3 showed almost similar TME profile and were associated with low immune and inflammatory signatures, and intermediate prognosis [54] (**Table 2**).

Comparatively, in triple-negative breast cancer, three TME subtypes using IHC analyses have been identified: (1) a first subtype with TLR9^{high} expression by cancer cells, hypercellular stroma and numerous TILs overexpressing TLR9; (2) a second subtype with TLR9^{low} expression by cancer cells, a predominantly paucicellular stroma, and rare inflammatory cells expressing TLR9 without TILs; and (3) a third subtype with TLR9^{low} expression by cancer cells, a predominantly fibrotic and vascular stroma containing some immune and inflammatory cells [55].

4.4. Tumor microenvironment of metastasis score

Tumor microenvironment of metastasis (TMEM) score is an IHC-staining score assessed by three antibodies: anti-CD31, anti-CD68, and anti-panMena. The selected area should be identified by low power, focusing on representative high density and adequacy of tumor, and lack of necrosis, inflammation, and artifacts. TMEM is defined as a structure composed of the direct

Consensus molecular subtypes (CMS)	Molecular characteristics [53]	MCP-counter signature [54]	Mechanisms of action	Prognosis
CMS1 MSI-like subtype	<ul style="list-style-type: none"> MSI tumors with mutations in genes encoding DNA mismatch-repair proteins, resulting in high mutational burden Tumors with a CIMP and BRAF mutations 	Overexpression of genes specific to cytotoxic lymphocytes	High expression of genes coding for T-attracting chemokines (CXCL9, CXCL10, and CXCL16) or TLS's formation (CXCL13), Th1 cytokines IFNG and IL15	Good prognosis
CMS2 Canonical subtype	<ul style="list-style-type: none"> Tumors with high chromosomal instability Activation of the Wnt and MYC pathways 	Low immune and inflammatory signatures		Intermediate prognosis
CMS3 Metabolic subtype	<ul style="list-style-type: none"> Tumors with KRAS mutations and disruption of metabolic pathways 	Low immune and inflammatory signatures		Intermediate prognosis
CMS4 Mesenchymal subtype	<ul style="list-style-type: none"> Tumors with mesenchymal phenotype and frequent CIMP phenotype 	Expression markers of lymphocytes and of cells of monocytic origin. Proinflammatory, proangiogenic, and immunosuppressive signature	High expression of myeloid chemokine CCL2, complement components, angiogenic factors (VEGFB, VEGFC, and PDGFC), and immunosuppressive molecules (TGFB1, TGFB3, LGALS1, and CXCL12)	Poor prognosis

Table 2. Cancer transcriptomic signature: molecular subtypes versus tumor microenvironment signature.

contact between an invasive pan Mena-overexpressing carcinoma cell, an endothelial cell (CD31⁺), and a perivascular macrophage (CD68⁺), with no discernible stroma between tumor cell and perivascular macrophage. Then, the number of TMEMs per 10 high-power fields (×400) is calculated to give a final TMEM score for each patient sample [56, 57]. In breast cancer, TMEM score is positively associated with risk of distant metastasis in ER⁺/HER2⁻ patients [57].

4.5. Recommendations for assessing TILs in breast cancer

A group of experts has proposed a step-by-step recommendation of how TILs should be evaluated by pathologists in breast carcinoma tissue samples [58], whether it can be on core biopsies or full surgical sections:

- One section (4–5 μm, magnification × 200–400) per patient is considered to be sufficient.
- Full sections are preferred over biopsies whenever possible.
- TILs should be evaluated within the borders of the invasive tumor.

- TILs should be reported as percentage for the stromal compartment (percentage of stromal TILs).
- TILs should be assessed as a continuous parameter. The percentage of stromal TILs is a semiquantitative parameter for this assessment, for example, 80% stromal TILs means that 80% of the stromal area shows a dense mononuclear infiltrate.
- All mononuclear cells (including lymphocytes and plasma cells) should be scored, but polymorphonuclear leukocytes are excluded.
- Do not focus on hotspots: a full assessment of average TILs in the tumor area should be used.
- Exclude TILs outside of the tumor border and around DCIS and normal lobules.
- Exclude TILs in tumor zones with crush artifacts, necrosis, regressive hyalinization as well as in the previous core biopsy site.

4.6. PDL-1/TILs score

Tumors can be classified into four groups based on their PD-L1 expression and the presence or absence of TILs [59, 60] (**Table 3**). The type of tumors that fit into each of PD-L1/TILs status depends on the genetic aberrations and oncogene drivers of these tumors. In melanoma, a high proportion of type I (~38%) and type II (~41%) tumors is observed, with the former having considerably the best prognosis [59]. Comparatively, pancreatic cancer has a lower level of PD-L1 expressed on tumor and immune cells [61]. By contrast, in non-small-cell lung cancer (NSCLC) where the oncogenes are more important drivers of tumor PD-L1 expression, the frequency of type III may be higher. In NSCLC, PD-L1 positivity is associated to adenocarcinoma and the presence of EGFR mutations, whereas PD-1 is associated with smoking status and the presence of KRAS mutations [62]. Additionally, increased levels of CD3 and CD8⁺ are associated with better outcome in NSCLC [63].

Accumulating data suggest that two major categories of immune resistance within the TME may exist: (i) failure of T-cell trafficking due to low levels of inflammation and lack of chemokines for migration, and (ii) dominant suppression through immune-inhibitory mechanisms. The potential reasons explaining failed tumor rejection in the cases of T-cell-inflamed TME include extrinsic inhibition by PD-L1/PD-1 interactions and the suppression effect of Tregs [64].

Expression groups	PDL-1/TILs status	Significations
Group I	PD-L1+, with presence of TILs	Drives adaptive immune resistance
Group II	PD-L1-, with no TIL	Indicates immune ignorance
Group III	PD-L1+, with no TIL	Indicates intrinsic induction
Group IV	PD-L1-, with presence of TILs	Indicates the role of other suppressor in promoting immune tolerance

Table 3. PDL-1/TILs score: tumors can be classified into four groups based on their PD-L1 expression and presence or absence of TILs.

4.7. PD-L1 tumor proportion score

Immunotherapies with checkpoint inhibitor PD-L1, which can inhibit T-cell function by binding PD-1 on T-cells, have shown encouraging results in patients with advanced NSCLC. Several agents such as pembrolizumab, nivolumab, atezolizumab, and durvalumab are approved or under clinical development for patients with metastatic NSCLC. Clinical trials have shown an association between the degree of clinical efficacy of these drugs and the level of PD-L1 expression by IHC. In two recent comparative trials, at least three PD-L1 IHC antibodies (22C3, 28-8, and SP263) are aligned regarding PD-L1 expression on tumor cells [65, 66]. A cancer cell is considered PD-L1 positive only when cell membrane is partially or completely stained. By contrast, an immune cell is considered PD-L1 positive if it features any PD-L1 staining: cell membrane or cytoplasm. PD-L1-positive immune cells are predominantly macrophages and lymphocytes. All assays revealed PD-L1 expression on immune cells, but with greater variance than expression on tumor cells. Alveolar macrophages are consistently stained with anti-PD-L1 antibody, serving as an internal positive control.

In NSCLC, PD-L1 tumor proportion score (TPS) is proposed to evaluate the IHC expression on tumor cells. The cutoffs of the different scoring criteria may be integrated into a six-step scoring system (Cologne Score: <1, ≥1, ≥5, ≥10, ≥25, ≥50%).

Currently, pathologists are confronted with two situations to evaluate TSP:

First-line metastatic NSCLC: Pembrolizumab is indicated in first-line setting as both monotherapy and combination therapy in metastatic NSCLC, which has TPS of ≥50%, with no EGFR or ALK genomic aberrations [67].

Second-line metastatic NSCLC: Pembrolizumab is indicated in second-line treatment of metastatic or locally advanced NSCLC, which has PD-L1 TPS of ≥1%. In this case, patients with EGFR or ALK genomic aberrations should have disease progression on therapy for these aberrations prior to receiving Pembrolizumab [61].

The above-cited data underline the importance of PD-L1 test as a biomarker in immunotherapy of NSCLC even in the first-line treatment. Nevertheless, the priority remains to harmonize the procedure of PD-1 testing and interpretation, which might require specific standardization. Therefore, pathologists have a major role to put in place the PDL-1 IHC test in routine practice and determine PDL-1 immunoscore on FFPE tissues.

5. Strategy panels in immunotherapy

Systemic anticancer therapies have evolved from chemotherapy through targeted therapies to immune agents and immunotherapy, which is now considered as the third paradigm in cancer treatment. Events from cancer immunity cycle and immune tolerance may serve as both predictive biomarkers and potential therapeutic targets. Immunotherapy is emerging as a novel therapeutic strategy promoting immune response against cancer cells and differing from traditional modalities that target tumor cells directly. Preclinical and clinical evidence provides the rationale for different promising immunotherapeutic approaches combining upregulation

of immune responses and downregulation of immune tolerance, to edify a cancer immunity cycle or to re-activate a neutralized preexisting anticancer immune response [68].

Immunotherapies are most effective in patients with a T-cell-inflamed phenotype. Initially, immunotherapy using high-dose interleukin 2 and adoptive T-cell transfer allowed durable clinical benefit in patients with advanced malignancies. Currently, immune strategies have shifted to targeted manipulation of immune checkpoints. Immune checkpoints refer to multiple inhibitory and costimulatory pathways that counteract certain crucial steps of T-cell-mediated immunity to maintain self-tolerance and modulate the duration and amplitude of immune responses. Immune checkpoints are initiated primarily through T-cell inhibiting and stimulating receptors and their ligands, including CTLA-4 (CD152), PD-1 (CD279) and PD-L1 (CD274) or PD-L2 (CD273), among many others [41]. The CTLA-4 antibody ipilimumab was the first approved checkpoint inhibitor after it improved overall survival in patients with advanced melanoma in two randomized phase III trials. However, objective responses are low with ipilimumab monotherapy and 22% of patients with advanced melanoma survived at least 3 years after therapy. Greater clinical benefit has been observed with inhibitors targeting PD-1 or PD-L1 checkpoints. The anti-PD-1 inhibitors pembrolizumab and nivolumab have been recently approved by the US Food and Drug Administration (FDA) for patients with advanced unresectable melanoma, NSCLC, and metastatic renal-cell carcinoma, with objective responses in 40–45, 20, and 25% of patients, respectively. FDA approvals have been announced for nivolumab in patients with refractory Hodgkin's lymphoma and for the anti-PD-L1 agent atezolizumab in patients with advanced bladder cancer. Furthermore, significant clinical benefit, including durable tumor responses and extension of progression-free and overall survival, has now been observed with other anti-PD-1 and anti-PD-L1 inhibitors in a wide spectrum of solid tumors and hematological malignancies [69, 70].

However, significant responses to immunotherapy only occur in a minority of patients. Attempts are being made to improve the activity of immunotherapies with novel combinatorial strategies and with biomarker optimization. Immuno-oncology drugs are thus currently evaluated and data from recent clinical phase I–III trials have highlighted the potential for combination therapies, including these immunomodulating inhibitory molecules (TIM-3, VISTA, LAG-3, IDO, and KIR) and costimulatory antibodies (CD40, GITR, OX40, CD137, ICOS) [41, 71, 72].

6. Biomarkers in immuno-oncology

Selection of patients based on validated predictive biomarkers is an important issue that needs to be addressed. Although most of immunotherapies are dedicated to T lymphocytes and cell-mediated cytotoxicity, cancer immune response is a very complex process characterized by numerous reciprocal interactions between tumor cells, multiple immune/stromal cellular subtypes, soluble mediators, ECM, and blood vessels. A wide spectrum of biomarkers is thus required to guide anticancer immune strategies. Immunotherapeutic agents function through different mechanisms of action, including modulation of T-cell receptors (CTLA-4 and PD-1) and adoptive T-cell therapies that associate TILs, chimeric antigen receptors (CARs), and TCR-modified T-cells. Furthermore, tumor spatio-temporal heterogeneity is characterized by different antigenic profiles over time (before and after treatment) and topography (primitive

and metastatic tumor) and numerous immunosuppressive mechanisms are promoted in the TME. Most importantly, discovering and optimizing immuno-oncology biomarkers could predict sensitivity or resistance to these immunomodulating molecules, identify their mechanisms of action, and define efficient combined therapies to rationally select patients. Thus, characterizing the anticancer immune response with multidisciplinary and multiparametric NGS and in situ technologies is pivotal to identify multiplex profiles that could allow patient's stratification for optimal personalized immunotherapy [73].

According to the thematic hallmarks of anticancer immune response, a large spectrum of potential biomarkers that could predict response to immunotherapy have been recently identified, including (i) tumor foreignness: tumor immunogenicity, high mutational load, gene expression profiling, epigenetic modifications of immune genes, intra-tumor heterogeneity; (ii) immunosuppressive tumor metabolism: LDH and TGF β levels; (iii) host immune status: total lymphocyte count, T-cell and B-cell repertoire, antitumor antibodies titers, preexisting autoimmunity; (iv) immune regulation: antigen presentation (CD40/CD40L), cancer cells reduced MHC expression, T-cell recognition, TCR repertoire diversity, IFN α and TNF α levels; (v) immune cells migration: T-cell trafficking chemokines (CCL5, CXCL9, and CXCL10), chemokines profile, VEGF levels, inflammatory signature; (vi) tumor immune infiltration: CD8⁺ TILs, FoxP3⁺ Tregs; (vii) T-cell cytotoxicity: granzyme A, perforin 1, and IFN γ levels; and (viii) immunosuppressive molecules: CTLA4, PDL1, PDL2, LAG3, TIM3, and IDO [73, 74].

These multiple predictive biomarkers present potential great interest in future practice to select patients for optimal immunotherapy: (i) PD-L1 expression in the TME may indicate increased sensibility to PD-1/PD-L1 checkpoint inhibitors; (ii) the presence of TILs suggests a preexisting antitumor immune response that can be reinitialized by immunotherapy; (iii) high tumor mutational load and neoantigens may be indicative of high tumor immunogenicity and sensitivity to immunotherapy; and (iv) the presence of immunosuppressive cells (immature DCs, MDSCs, TAMs, and Tregs), polarization of macrophages (anti-inflammatory M2 macrophages) and DCs (immunosuppressive/tolerogenic regulatory DCs), immunosuppressive molecules and immunoinhibitory cytokines may predict resistance to immunotherapy [72, 75].

Currently, only PD-L1 IHC assays have been validated for clinical utility, although several tumors, host, and environmental biomarkers are very promising candidate for patients' stratification. NGS and in situ technologies investigating tumor-immune interactions include multiplex immunohistochemistry (multiplexed-IHC), whole-exome sequencing (WES), transcriptome analysis, proteomics, and flow cytometry. However, before clinical application, each of these potential biomarkers requires high-quality validation process, comprising assessment of basic assay performance, characterization of the performance of the assay, and validation in clinical trials.

Recent technological advances have provided new tools that will facilitate an in-depth understanding of the interaction between the immune system and tumor cells, particularly in the TME and will help guide the development of personalized cancer immunotherapies. Data generated from these innovative technologies (i.e., gene microarray, deep-sequencing technologies, mass cytometry, and multicolor IHC staining) are classified into three categories: (i) function (to evaluate the function of different immune cells), (ii) phenotype (to provide the frequency and status of these cells), and (iii) signature/pattern (to elucidate the potential mechanisms of action) [76].

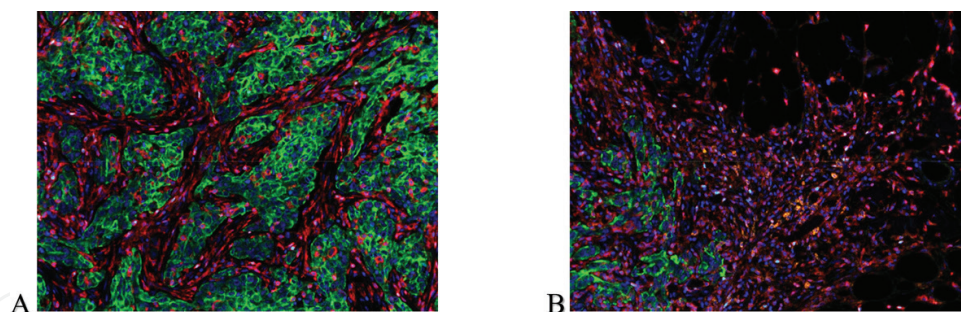


Figure 2. Fluorescent multiplex immunohistochemistry. (A) Breast cancer, section from tumor's core, and (B) section from invasive margin of the same tumor. The sections are stained with cytokeratin (cancer cells, in green), CD45RO (memory lymphocytes, in red), CD4 (T helper cells, in orange) and FoxP3 (in blue).

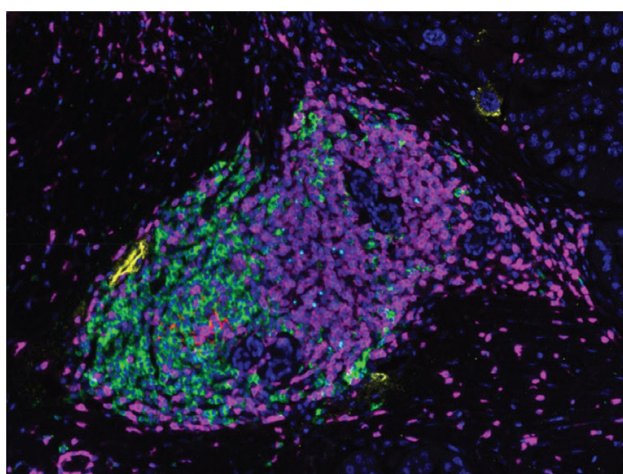


Figure 3. Fluorescent multiplex immunohistochemistry. Tertiary lymphoid structure panel stained with CD20 (B cells, in green), CD3 (CD3⁺ T lymphocytes, in purple), DC-Lamp (mature DCs, in blue), CD21 (follicular DCs) and PNAAd (high endothelial venule, in yellow).

Among these novel technologies, multiplex immunohistochemistry (multiplexed-IHC) appears as very effective and efficient method to identify on the same section and at the same time, several immune cell types, their location, and their state of activation, as well as the presence of immunoactive molecular expression. Multiplexed-IHC is a quantitative, image analysis-based method, using multicolor IHC on FFPE tissues, automated multispectral slide imaging, and advanced recognition software. When coupled with fluorophores (fluorescence multiplexed-IHC), this method takes advantage of light emission with different spectral peaks against a dark background (**Figures 2A, B** and **3**). Fluorescence multiplexed IHC provides spatial localization and distribution of phenotypic and functional biomarkers within the TME and thus is highly beneficial in experimental research for exploring immune evasion mechanisms or finding potential biomarkers [77].

7. Conclusion and perspectives

After chemotherapy and targeted therapy, immunotherapy has become the third paradigm in cancer. Immunotherapy is a key component of the therapeutic strategies to control and

potentially cure cancer. The complexity and heterogeneity of the interaction between the immune system and tumor cells, particularly in the tumor microenvironment, underlies the immune status (i.e., immunologically responsive or immunologically ignorant) of each tumor for every patient. These reciprocal interactions depend on the organ, the oncogenic processes, and their modification by treatments. Although immunomodulation by checkpoint inhibitors (targeting both CTLA-4 and the PD-1/PD-L1 axis) induced a durable tumor response in several malignancies, the use of PD-L1 immunohistochemistry alone has not been sufficient for ruling in or out the use of anti-PD-1 or anti-PD-L1 expression-based therapies. Therefore, characterization of recognized tumor antigens, effector T-cell function, and immune-suppressive mechanisms, TILs, T-cell receptor repertoire, and mutational or neoantigen burden should be aimed at creating an optimized model for predicting response to anti-PD-1 or anti-PD-L1 therapies. Furthermore, specific mechanisms of T-cell exclusion such as activation of the WNT/ β -catenin-signaling pathway, microbiota status, and genetic polymorphism should be included in future biomarker development (**Table 4**).

Accumulating evidences support that the optimal strategy for further immunotherapy development is combinatory regimens. The challenge of increasing the curative immune responses in a diverse population of patients will require multiple complementary therapeutic modalities to overcome the immunosuppressive tumor microenvironment. Thus, understanding the tumor microenvironment may offer opportunities to predict response to therapy and select the most appropriate immunotherapy for each patient. The recent availability of high-throughput next-generation sequencing and in situ technologies to quantify the different elements of the tumor microenvironment and understand their functionality opens the way for generalization of these approaches and the subsequent application of precision-personalized therapies based on these landscapes rather than on cancer subtypes only.

Stratification factors	Tumor microenvironment subtypes			Cancer immune set point
Morphology	Immune/ inflammatory	Mesenchymal	Paucicellular/ inactive	
Immunophenotype	Immune response	Immune exclusion	Immune desert	
Biological mechanisms	Tumor infiltration by cytotoxic T-cells, B-cells, MDSCs, NK cells regulatory T-cells, CAFs	Stromal-based inhibition from vessels, ECM, chemokines	Absence of preexisting antitumor immunity (ignorance, tolerance, no priming of T-cells)	
Biomarkers				
Tumor genome/epigenome				
Tumor mutation load	High	Low	Low	Positive effect
Neoantigen burden	High	Low	Low	Positive effect
Gene expression profiling	Activation signature ^{high}	Activation signature ^{low}	Activation signature ^{very low}	
Tumor cells PD-L1	High	Low	Low	Negative effect
Tumor infiltration by TILs	High	Absent	Absent	Positive effect

Stratification factors	Tumor microenvironment subtypes			Cancer immune set point
Antigen immunogenicity	High	Low	Low	Positive effect
KRAS, BRAF, B2M, MHC, IDO	High	Low	Low	Negative effect
Hypoxia	High	Low	Low	Negative effect
Tumor microenvironment				
Immune cells	High	High	Very low/absent	
Immune cells phenotypes	Effector cells ^{high}	Effector cells ^{low}	Very rare myeloid and CD8+ cells	Negative/positive effect
Spatial relationship	Immunosuppressive cells ^{low}	Immunosuppressive cells ^{high}		
Immune cells PD-L1	High	Low	Low	Positive effect
Immune gene signatures	High	Low	Low	Positive effect
Fas-L, TGF-β, LOX, VEGF, collagen, fibronectin, CXCL12	Low	High	Low	Negative effect
Host immunity/genetics				
T-cell clonal diversity	High clonality	Low clonality	Low clonality	Negative/positive effect
Priming of immune response	High	Low	Low	Positive effect
General antibody response	Robust	Weak	Weak	Positive effect
Chronic inflammation/cytokines	Proinflammatory cytokines	Immunosuppressive cytokines	Immunosuppressive cytokines	Negative/positive effect
Germline polymorphism: TLR4, TNF-α, NF-κB, NOD2, JAK-STAT, inflammasome pathway	Present	Absent	Absent	Negative effect
Environment				
Gut microbiota				Positive effect
Stress hormones				Negative effect
Glucocorticoids				
Immunotherapy	Sensitivity	Resistance	Resistance	
Therapeutic strategy	Immune checkpoint inhibitors	Surgery, radiotherapy, chemotherapy, vaccination	Surgery, radiotherapy, chemotherapy, vaccination	
	Other immunotherapies	Adaptive cellular therapy	Adaptive cellular therapy	

Table 4. A summary table describes the stratification factors implicated in the interactions between immune system, tumor microenvironment, and tumor cells, which can influence immunotherapy and therapeutic strategies (immunophenotype, tumor genome/epigenome, tumor microenvironment, Microbiota, environmental factors, host immunity and genetics). This table proposes global tumor microenvironment morphological-, immunophenotypical- and biological-based subtypes with linked immune biomarkers.

Disclosure-conflict of interest

The authors declare that they have no competing interests.

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