

Tumor Immunology and Tumor Evolution: Intertwined Histories

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Cancer is a complex disease whose outcome depends largely on the cross-talk between the tumor and its microenvironment. Here, we review the evolution of the field of tumor immunology and the advances, in lock-step, of our understanding of cancer as a disease. We discuss the involvement of different immune cells at distinct stages of tumor progression and how immune contexture determinants shaping tumor development are being exploited therapeutically. Current clinical stratification schemes focus on the tumor histopathology and the molecular characteristics of the tumor cell. We argue for the importance of revising these stratification systems to include immune parameters so as to address the immediate need for improved prognostic and/or predictive information to guide clinical decisions.

Introduction

Although the first indications of the involvement of the immune system in cancer control can be traced back to over a century ago, tumor immunology can be regarded as an emerging field, rightly complementing and completing that of oncology. Indeed, the renaissance of tumor immunology came in the past two decades, with the demonstration of two key concepts that bring to fore the major role of the pre-existing adaptive immunity within tumors: immuno-surveillance and immunoediting (Schreiber et al., 2011; Shankaran et al., 2001) and the importance of immune contexture (Galon et al., 2006; Mascaux et al., 2019). The encouraging results obtained with the employment of novel cancer immunotherapies such as immune checkpoint inhibitors (ICIs) revealed the power of the immune system to counteract and possibly defeat the disease. The recognition of the tumor immunology field was conclusively sealed by the Nobel Prize in Physiology or Medicine 2018, awarded for the discovery that inhibiting the negative immune regulation of T cells could be exploited as a powerful anti-cancer strategy. These groundbreaking findings were accompanied by numerous studies elucidating how various components of the immune system control or contribute to disease progression, thus revealing their part in the natural history of the tumor, as well as their prognostic value. Indeed, it is now appreciated that innate and adaptive immune cells influence cancer evolution directly and indirectly, including at the pre-cancerous stages.

Here, we discuss the current understanding of how components of the immune system shape the progression of cancer. The fact that ICIs yield clinical benefit in a limited percentage of patients indicates the need for a deeper knowledge of the different actors playing a role in cancer development. Nonetheless, readily available powerful evidence makes it already plausible to encourage the adoption of immune-based parameters to improve patient stratification and guide treatment decisions. Thus, we argue for the importance of bringing the current cancer classification system up-to-date to take into account recent advances in tumor immunology.

The Dawn of Tumor Immunology

The field of tumor immunology has deep roots, dating back to the nineteenth century (Figure 1). Rudolf Virchow reported a link between inflammation and cancer in 1863. Virchow hypothesized that cancer is caused by severe irritation in the tissues; it was not until the 1990s that his theory—known as “chronic irritation theory”—was substantiated by solid evidence, at least in certain cancer types (Balkwill and Mantovani, 2001). A few decades later, in 1891, the bone sarcoma surgeon William Bradley Coley started the first systematic study of immunotherapy (Coley, 1893). Inspired by tumor regression in a *Streptococcus*-infected (and seemingly doomed) patient, he first formulated and tested the hypothesis that cancer could be defeated by stimulating the patient’s immune system. Hence, the so-called Coley’s Toxins (a mix of bacteria and bacterial products) were injected in over 1,000 patients over the subsequent 40 years, yielding remarkable results. Despite the evidence, many clinicians didn’t trust his results, and his methods were gradually forgotten, replaced by the more promising novel radiotherapeutic (1896) (Holsti, 1995) and chemotherapeutic (1942) (Fenn and Udelsman, 2011) approaches. What couldn’t be suspected at the time is that the efficacy of these seemingly unrelated therapeutic modalities might be due at least in part to their modulatory and potentially activating effects on the immune system (Formenti and Demaria, 2013; Galluzzi et al., 2015).

Around Coley’s time, another important milestone was provided by George Beatson (Beatson, 1896), who exploited ovarian ablation in an attempt to cure unresectable breast cancer. In doing so, Beatson leveraged on the connection between sex steroid hormones and cancer (Beatson, 1896), thereby paving the way to hormonal therapy in hormone-sensitive cancers (such as breast, ovarian, endometrial, and prostate cancers). This treatment modality became later a widely deployed strategy, still in use today (Shevach et al., 2019; Zang et al., 2019). Nonetheless, the underlying link between hormones, inflammation, and cancer began to unravel only more recently (Gharwan et al., 2015; Key, 1995; Mantovani et al., 2008; Özdemir and Dotto, 2019).



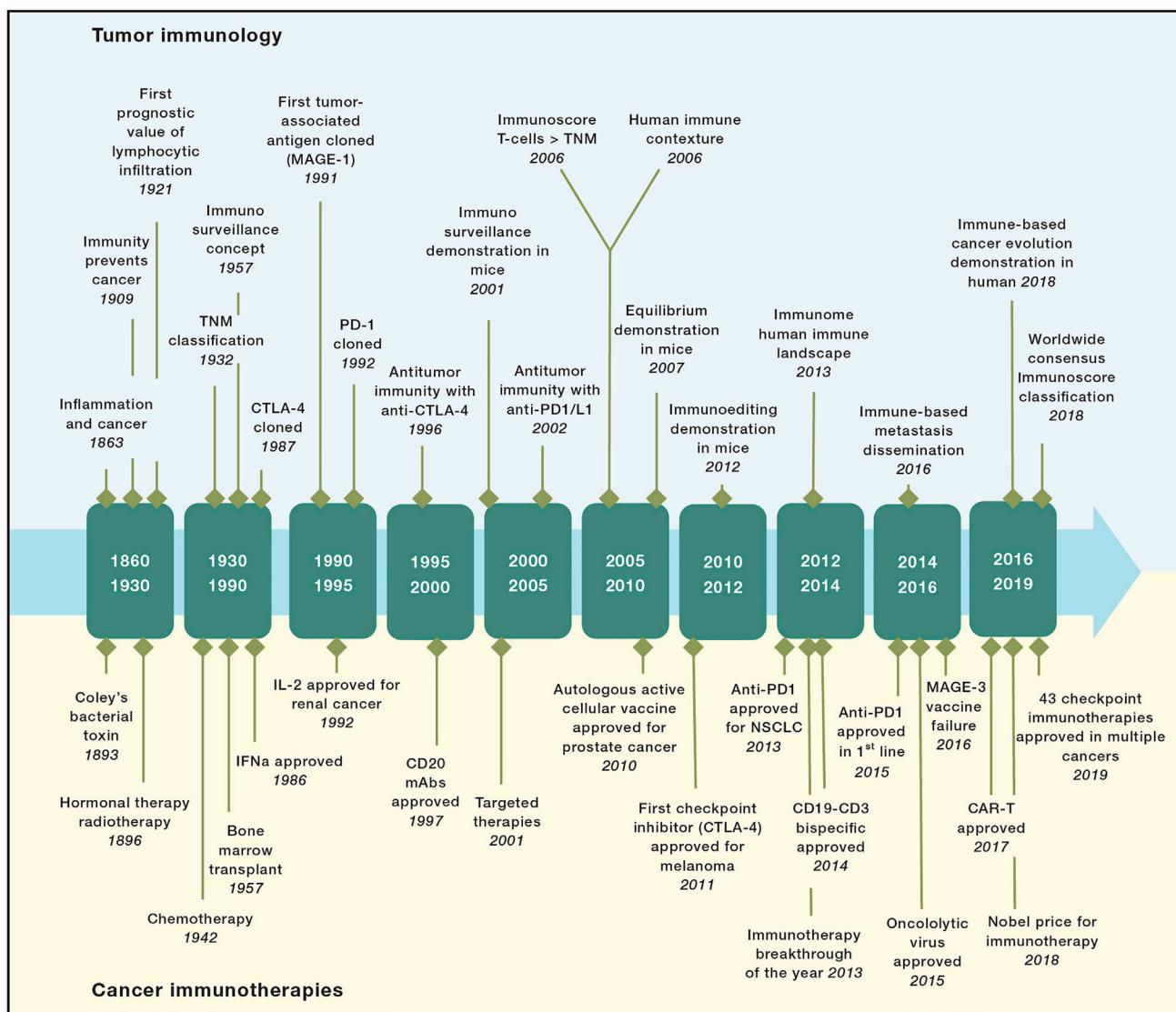


Figure 1. History of Tumor-Immunology and Cancer (Immuno)therapy

The main milestones, by year, of tumor immunology are highlighted (top), including the first proposed link between inflammation and cancer by Rudolf Virchow ([Balkwill and Mantovani, 2001](#)); the hypothesis that the immune system prevents neoplastic development ([Ehrlich, 1909](#)); the prognostic value of immune infiltration ([MacCarty and Mahle, 1921](#)); the concept of immunosurveillance ([Burnet, 1957](#)); the cloning of CTLA-4 and PD-1 ([Brunet et al., 1987; Ishida et al., 1992](#)) and their anti-tumor immunity role ([Dong et al., 2002; Leach et al., 1996](#)); the first cloning of a tumor antigen ([van der Bruggen et al., 1991](#)); the demonstration of immunosurveillance ([Shankaran et al., 2001](#)), equilibrium ([Koebel et al., 2007](#)), and immunoediting ([Matsushita et al., 2012](#)) in mouse models; the concept of immune contexture in humans ([Galon et al., 2006](#)); the Immunoscore and demonstration that TNM-staging is dependent on T cells ([Galon et al., 2006](#)); the concept of immunome and the immune landscape in humans ([Bindea et al., 2013](#)); the demonstration of immune-based metastasis dissemination in humans ([Mlecnik et al., 2016b](#)); the demonstration of an immune-based theory of cancer evolution in humans ([Angelova et al., 2018](#)); and the worldwide consensus Immunoscore validation ([Pagès et al., 2018](#)). The main milestones of cancer therapy and immunotherapy are highlighted (bottom), with the Coley's bacterial toxin ([Coley, 1893](#)); radiotherapy ([Holsti, 1995](#)); hormonal therapy ([Beatson, 1896](#)); chemotherapy ([Fenn and Udelsman, 2011](#)); IFN- α and IL-2 FDA approval in 1986 and 1992, respectively; the first targeted therapy (the tyrosine kinase inhibitor Imatinib) approved by the FDA against chronic myeloid leukemia (2001); the first FDA-approved therapeutic vaccine against prostate cancer (2010); the first FDA-approved checkpoint inhibitor, anti-CTLA-4, for melanoma (2011); FDA-approved anti-PD-1 in lung cancer (2013); Immunotherapy defined as “Breakthrough of the Year” by Science ([Couzin-Frankel, 2013](#)); first FDA-approved bi-specific CD19-CD3 antibody (Blinatumomab) for the treatment of acute lymphoblastic leukemia (2014); anti-PD-1 approved by the FDA as first-line treatment in lung cancer (2015); FDA approval of an oncolytic virus (talimogene laherparepvec [T-VEC]) for use in melanoma patients (2015); the MAGE-3 vaccine failure in a large Phase III study ([Vansteenkiste et al., 2016](#)); the first FDA-approved CAR T cell therapy (tisagenlecleucel) for pediatric and young adult patients with relapsed and/or refractory B cell precursor acute lymphoblastic leukemia (2017); and the current (as of February 2019) 43 checkpoint cancer immunotherapies overall approved by the FDA.

A similarly hypothesis-driven path was followed by use of the bacille Calmette-Guérin (BCG) in bladder cancer settings. Originally developed by Calmette and Guérin in the early 20th century

as a vaccine for tuberculosis (TB) ([Calmette, 1927; Luca and Mihaescu, 2013](#)), the BCG remains to date the only available vaccine against TB. TB was first linked to low rates of cancer

in 1929 by Raymond Pearl (Pearl, 1929), while he was carrying out a series of autopsies at Johns Hopkins Hospital. A few decades later, also on the basis of this evidence, Morales et al. (1976) performed the first intravesical administration of BCG to patients with recurrent superficial bladder tumors, which showed indeed promising results. Fibronectin was shown to mediate the attachment of BCG to tumor cells (Ratliff et al., 1988), and fibronectin-mediated internalization of BCG was a necessary prerequisite for its anti-tumor activity (Kavoussi et al., 1990). The consequently initiated functional immune response includes an enhancement of antigen presentation and cytotoxic T-cell-mediated immunity, as well as an increase in pro-inflammatory mediators occurring after successive treatments (Fuge et al., 2015). Despite a lack of understanding of its mode of action at the time, Morales et al. (1976) initiated a therapeutic modality that is still used today for treating early-stage bladder cancer, representing the only remnant of the microbial era of immunotherapy efforts.

The Advent of Cellular and Antibody Therapies

Apart from these exceptions, the fields of oncology and immunology developed relatively independently since the late 19th century, and only truly merged in the past two decades. This is despite some pioneering hypotheses such as that the host defense might prevent neoplastic cells from developing into tumors, by Paul Ehrlich in 1909 (Ehrlich, 1909); that the humoral immune system might recognize newly arising tumors antigens, by Lewis Thomas in 1959 (Thomas, 1959); the theory of cancer immuno-surveillance, by Frank MacFarlane Burnet in 1957 (Burnet, 1957); and related studies (Foley, 1953; Graham and Graham, 1959; Gross, 1943).

In 1957, Edward Donnall Thomas treated a leukemia patient, after total-body high irradiation therapy, with bone marrow infusion from his identical twin (Thomas et al., 1957). This represents *de facto* the first form of clinically useful cellular therapy, or hematopoietic stem cell transplantation (HSCT). Thomas was awarded the 1990 Nobel Prize in Physiology or Medicine for fathering this groundbreaking approach, which significantly boosted survival rates for blood malignancies. Therefore, John Kersey performed the world's first bone marrow transplant for lymphoma in 1975 (Kersey, 2007); although the technique was admittedly crude and risky, his first patient is still alive. The success of this technique and other allogeneic HSCT is because of the ability of donor-derived stem cells to provide allo-immunity, thus enabling a graft-versus-tumor effect to eradicate residual disease and prevent relapse. Indeed, graft versus leukemia reaction (GvLR) remains an important therapy against hematological malignancies (Dickinson et al., 2017; Yeshurun et al., 2019).

Instrumental for paving the way from a tumor-centric to a tumor *plus* immune vision of cancer were the advances in basic immunology: the discovery of the interferons (IFN) by Isaacs and Lindenmann in 1957 (Isaacs and Lindenmann, 1957; Isaacs et al., 1957) and that of T cells and their critical role in the adaptive immune responses (Miller et al., 1967) were only the beginning of an avalanche of crucial findings (Kiessling et al., 1975; Steinman and Cohn, 1973; Zinkernagel and Doherty, 1974). Subsequently, the first steps into more modern immunotherapies were taken. The use of purified, large-scale-produced IFN- α

against chronic myeloid leukemia yielded promising results (Talpaz et al., 1983) but was still insufficient to convince the clinical oncologists of the utility of immunotherapy. Again, a deeper basic knowledge was needed, as well as translational research to bring it from bench to bedside.

First discovered in 1976 as a "T cell growth factor" (Morgan et al., 1976), interleukin-2 (IL-2) represents the first soluble immune mediator administered to cancer patients (Lotze et al., 1985). It subsequently received U.S. Food and Drug Administration (FDA) approval for renal cell cancer in 1992. In 1986, the adoptive transfer of IL-2-expanded tumor-infiltrating lymphocytes (TILs) efficiently cured tumor-bearing mice, providing a rationale for the use of TILs in the treatment of humans with advanced cancer (Rosenberg et al., 1986). First clinical trials employing the adoptive transfer of expanded TILs to patients with metastatic disease were reported in 1987 (Topalian et al., 1987). Follow-up studies demonstrated the feasibility and safety of using retroviral gene transduction for human gene therapy, which had implications for the design of TILs with improved antitumor potency (Rosenberg et al., 1990). The origin of one of the most recently developed immunotherapies, chimeric antigen receptor (CAR) T cells, can be traced back to the same time period. In fact, the generation and expression of functional CAR-expressing T cells were shown in 1989 (Gross et al., 1989), thereby initiating a process ultimately leading to FDA approval of CAR-T-cell-based therapies for hematologic malignancies in 2017. The potency of CAR T cell therapy was shown by several clinical trials, in which even end-stage patients experienced a full recovery of up to 92% in acute lymphocytic leukemia (Abramson et al., 2018; Miliotou and Papadopoulou, 2018) and 82% objective response in refractory large B cell lymphoma (Neelapu et al., 2017). Fratricide-resistant and allo-tolerant "off-the-shelf" CAR T cells have facilitated clinical adoption of this costly therapeutic modality (Cooper et al., 2018; Qasim et al., 2017; Sommer et al., 2019). Nevertheless, disease relapse after CAR T cell therapy is not uncommon (Abbasi, 2018; Kim et al., 2017; Ruella et al., 2018), indicating the need to further refine this type of therapy, or to combine it with further treatments (Galon and Bruni, 2019; Liu et al., 2018), depending on the specific case. Mostly successful against hematological malignancies, the use of CAR T cells in solid tumor settings is hampered by their limited ability to find, enter, and survive in the tumor (Martinez and Moon, 2019). These limitations are being gradually overcome by the use of bispecific CAR designs or local administration, and that of combinatorial strategies, such as the combination with checkpoint inhibitors to mitigate T cell exhaustion (DeRenzo and Gottschalk, 2019; Gargett et al., 2016).

Apart from T cells, other types of immune cells have been employed for adoptive cellular immunotherapies over the years, including dendritic cells (DCs), natural killer (NK) cells, lymphokine-activated killer (LAK) cells, and cytokine-induced killer (CIK) cells. As for T cells, cellular immunotherapies relying on DCs require a first antigenic characterization and, most commonly, the use of autologous cells, thereby slowing clinical development. By virtue of DCs' ability to most efficiently prime T cell responses (Banchereau and Steinman, 1998), and considering that vaccine adjuvants are known to act via the induction of DC maturation, it's not surprising that DCs are being studied as tools to effective therapeutic vaccination against cancer

(Palucka and Banchereau, 2013; Lee et al., 2018) and approved Sipuleucel-T (Cheever and Higano, 2011) in prostate cancer.

Conversely, LAK cells, CIK cells, and NK cells display antigen-independent cytolytic activity against cancer cells, and represent attractive, off-the-shelf immunotherapy options. Since their discovery by Kiessling et al. (1975) and, independently, by Herberman et al. (1975) in 1975, NK cells have been implicated in anti-cancer surveillance and anti-tumor immunity (Chiossone et al., 2018) and have been exploited for the design of anti-cancer therapies (Cheng et al., 2011; Souza-Fonseca-Guimaraes et al., 2019). Instrumental to these developments was the pioneering concept of missing-self recognition, whereby the absence or incomplete expression of host major histocompatibility complex (MHC) class I molecules in a normal cell would make it susceptible to NK-cell-mediated killing. This hypothesis was formulated by Klaus Kärre in his PhD thesis (Kärre, 1981), then in subsequent proceedings and articles (Kärre, 1985; Kärre et al., 1986). Hence, the groups of Yokoyama (Karlhofer et al., 1992) and Moretta (Moretta et al., 1993) discovered the first NK inhibitory receptors (killer-cell immunoglobulin-like receptors [KIRs]) restraining NK cells cytolytic activity. Since, several KIRs as well as functionally opposite killer activation receptors (KARs) have been described (Molgora et al., 2017; Sivori et al., 2019; Vély et al., 1996). Anti-KIR antibodies recently entered clinical trials because of their ability to unleash NK-cell-mediated antitumor responses (Benson et al., 2015; Vey et al., 2018). The first successful use of allogenic mismatched NK cells was reported in 2002 by Velardi's group in acute myeloid leukemia (AML) patients (Ruggeri et al., 2002).

First described in 1994, the generation of LAK cells (a mixture of NK and NKT cells) involved culturing of peripheral blood mononuclear cells (PBMCs) with IL-2 and then cluster of differentiation 3 (CD3) antibody (OKT3 [Van Wauwe et al., 1980]) (Escudier et al., 1994). LAK-cell-based immunotherapy didn't elicit satisfactory anti-tumor efficiency in patients, especially as a monotherapy. Novel combinatorial approaches might yield clinical benefit (Saito et al., 2014), which nonetheless remains to be proven in human settings. CIK cells were described over two decades ago (Lu and Negrin, 1994). CIK cells share features of both NK and T cells, such as functional (T cell receptor) TCR and NK cell molecules. They are expanded from circulating precursors by treatment with interferon- γ (IFN- γ), OKT3, and IL-2 (Introna and Correnti, 2018), after which they acquire a potent, MHC-unrestricted cytotoxicity against (mostly) hematological malignancies in a natural killer group 2 member D (NKG2D)-receptor-dependent manner. CIK cells' anti-tumor efficacy has been demonstrated in various hematopoietic neoplasms with varying yet encouraging results (Introna et al., 2007; Introna et al., 2017; Zhou et al., 2013).

First discovered in 1883 by Élie Metchnikoff (Metchnikoff, 1883), a Russian zoologist, macrophages are innate immune cells critically involved in a multitude of steps of tumor initiation and progression. Notably, Metchnikoff and Paul Ehrlich (the latter for his work on mast cells, described below) were jointly awarded the 1908 Nobel Prize in Physiology or Medicine "in recognition of their work on immunity." According to a current paradigm, rooted in pioneering hypotheses formulated since the late 1970s (Balkwill and Mantovani, 2001; Bottazzi et al., 1983; Mantovani et al., 2017; Mills et al., 2000; North, 1978),

classically activated M1 macrophages accompany the early stages of cancer initiation and development, where they play an inflammatory, anti-cancer role. With disease progression, a shift toward pro-tumor M2 or M2-like macrophages occurs: these cells are responsible for the tissue remodeling, angiogenesis, and adaptive immune suppression typically contributing to tumor growth and dissemination (Mantovani et al., 2017). Recent reports indicate the possibility to leverage on the presence of checkpoint molecules (such as programmed cell death-1 [PD-1] [Gordon et al., 2017] or the newly discovered Clever-1 [Viitala et al., 2019]) impairing the sought phagocytic ability and anti-cancer functional properties of macrophages. Indeed, blocking PD-1 on tumor-associated macrophages (TAMs) resulted in increased phagocytic properties, reduced tumor growth and increased survival *in vivo*. Similarly, blocking Clever-1 could switch TAMs toward an M1-like phenotype, resulting in the activation of a protective cytotoxic T cell response. Clever-1 targeting also decreased PD-L1 expression both on TAMs and on cancer cells, providing a rationale for dual (Clever-1 and PD-L1) blockade (Viitala et al., 2019). The exploitation of macrophage-targeting strategies in humans, if successful, could provide a further powerful anti-tumor weapon.

Another milestone in the field of immunotherapy was reached with the use of the anti-CD20 monoclonal antibody (mAb) rituximab in patients with recurrent B cell lymphoma (Maloney et al., 1994). Indeed, anti-CD20 mAb became the first mAb approved by the FDA for cancer treatment in 1997.

The first human tumor-associated antigens (TAA) reported was the epithelial mucin MUC-1, recognized by cytotoxic T cells (CTLs) isolated and grown from a pancreatic cancer patient (Barnd et al., 1989). Since, other TAAs were discovered such as MZ2-E (a product of the MAGE-1 gene) (van der Bruggen et al., 1991) and MZ2-E (coded by the MAGE-3 gene) (Gaugler et al., 1994), both on human melanoma. Subsequently, the antigen encoded by the BAGE gene was found expressed in varying percentages in several cancer types, including melanoma, infiltrating bladder carcinoma, mammary carcinoma, head and neck squamous cell carcinoma, and non-small-cell lung carcinoma (NSCLC) (Boël et al., 1995). Like the MAGE genes (Gaugler et al., 1994; van der Bruggen et al., 1991), BAGE is not expressed in healthy tissues, except for the testis (Boël et al., 1995). The existence of TAAs generated tremendous enthusiasm in the field for their possible exploitation as therapeutic targets. However, recent findings indicate no increased benefit associated with tumor-antigen-specific vaccines, compared with placebo, in the randomized phase III clinical trials in NSCLC (Vansteenkiste et al., 2016) and melanoma (Dreno et al., 2018). Despite this setback, it is possible that targeting TAAs could be effective in combinatorial strategies. Indeed, neoantigen vaccines generated intra-tumoral T cell responses and demonstrated clinical responses in recent studies (Hilf et al., 2019; Keskin et al., 2019; Ott et al., 2017; Sahin et al., 2017).

The Renaissance: Immunosurveillance and Immune Contexture

The term "immunosurveillance" refers to the physiological process by which the immune system recognizes and destroys transformed cells. Preliminary experimental evidence for tumor immunosurveillance was provided over 60 years ago (Klein

et al., 1960; Prehn and Main, 1957) and further supported by evidence on the role of IFN- γ and perforin in tumor surveillance and rejection (Dighe et al., 1994; Kaplan et al., 1998; Smyth et al., 2000; van den Broek et al., 1996). Such was the scenario that welcomed the concept of “tumor immunoediting” formulated by Schreiber and colleagues (Shankaran et al., 2001), based on a study in mouse models, which presented a renewed and improved immunosurveillance hypothesis (Dunn et al., 2002). Using WT and Rag2-deficient mice, they found three outcomes resulting from the continuous cross-talk between tumor cell and immune system: tumor elimination (Shankaran et al., 2001), equilibrium (Koebel et al., 2007), or tumor escape from immune control (Matsushita et al., 2012). The equilibrium phase is characterized not by tumor dormancy, as previously postulated (Weinhold et al., 1979; Matsuzawa et al., 1991), but by a continuous proliferation and mutation—the phenomenon of immunoediting—in an attempt to attenuate tumor antigenicity and ultimately escape the immune response (Dunn et al., 2002). The ability of tumor cells to survive immune attack is now a recognized hallmark of cancer (Hanahan and Weinberg, 2011). In the escape phase, when the tumor versus immunity battle is seemingly won by the former, the immune system can still provide useful information on patients’ prognosis and disease outcome.

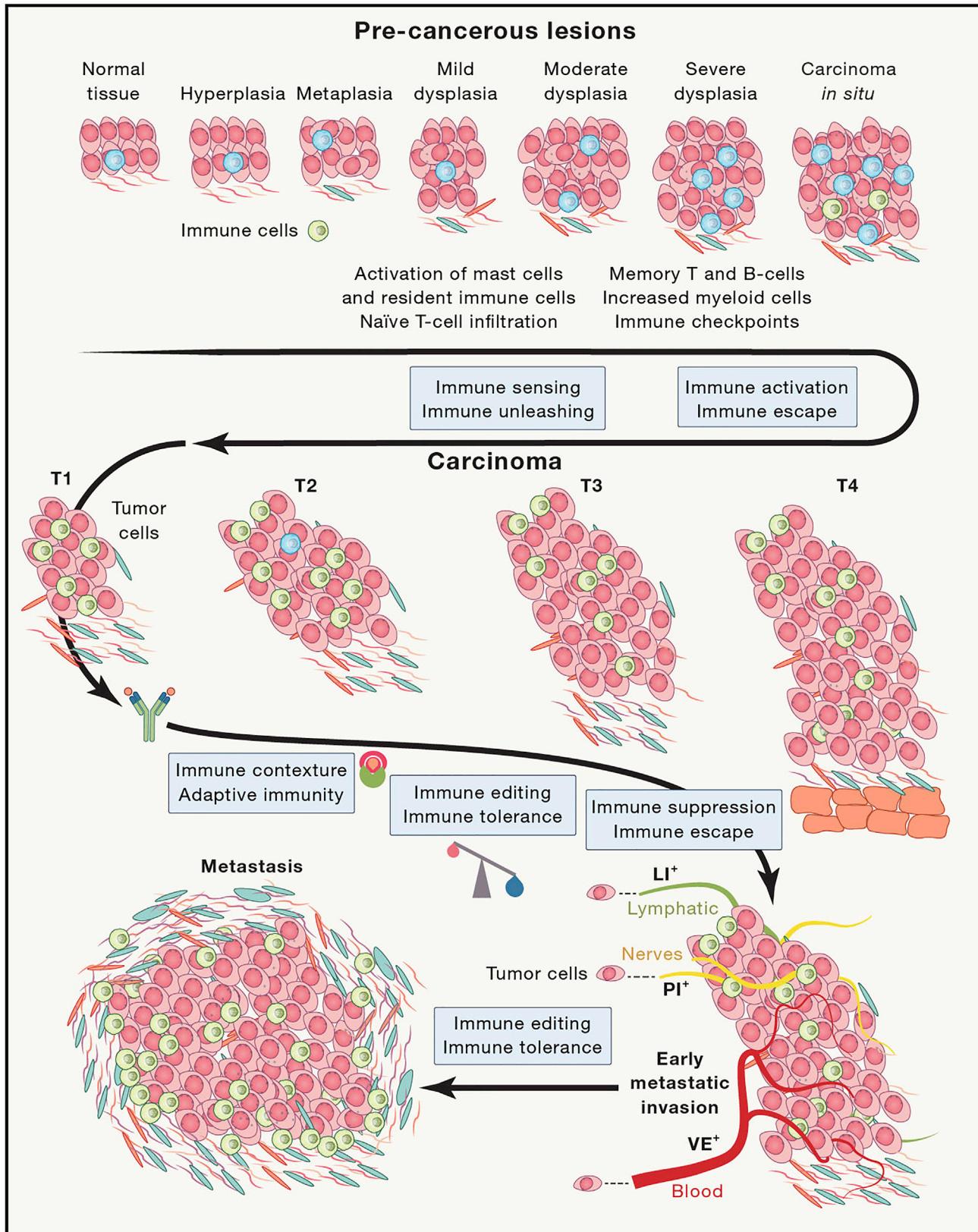
The association of prolonged survival of patients with cancer with intratumoral lymphocytic infiltration was noted in the 1920s (MacCarty and Mahle, 1921). This association became clearer in the late 1990s and the early 2000s; the first reports showed how specific tumor-infiltrating immune cells types can influence cancer progression and clinical outcome, and there was a prominent role for cytotoxic and effector memory T cells: the presence of these cells correlated with increased survival of patients with cancer (Clemente et al., 1996; Naito et al., 1998; Pagès et al., 2005; Zhang et al., 2003). Since the original classification of rectal cancer (Dukes, 1932), now expanded to all solid tumors, cancer staging relies on the Tumor (T), Node (N), Metastasis (M) AJCC/UICC-TNM cancer classification. The importance of the density and location of cytotoxic and memory T cells within the tumor was demonstrated by studies showing that these parameters were superior predictors of outcome to cancer staging using the AJCC/UICC-TNM classification and that, in fact, tumor progression and invasion were statistically dependent upon the pre-existing T cells (Galon et al., 2006). This evidence constituted the cradle of the concept of “immune contexture” (Galon et al., 2007), highlighting the importance of the quantity and the quality of the immune infiltrates for the survival of patients with cancer. The four major immune parameters of the “immune contexture” are defined by the nature, density, immune functional orientation, and location of tumor-associated immune infiltrates. Importantly, tumor progression (T-stage), tumor invasion (N-stage), tumor differentiation (grade), and early-metastatic invasion (emboli and vessel invasion) are dependent upon pre-existing anti-tumor immunity (Galon et al., 2013; Galon et al., 2006; Galon et al., 2014; Mlecnik et al., 2011; Pagès et al., 2009). Originally applied to colorectal cancer (CRC), the immune contexture concept is nowadays widely applied to most solid cancers (Fridman et al., 2012; Remark et al., 2015; Tazzari et al., 2018; Zhao et al., 2018). Molecular profiling of melanoma metastases revealed the importance of T helper 1 (Th1) signature and suggested classifiers of immune

responsiveness (Wang et al., 2002). We previously proposed a continuum of cancer immuno-surveillance in human and a broader immunological interpretation of three concepts: immune contexture, Immunoscore, and immunologic constant of rejection, which segregated oncogenic processes independently of their tissue origin (Galon et al., 2013). The strength of the immune contexture proved instrumental for the development of the consensus Immunoscore, an immunohistochemistry (IHC)-based assay, defining hot (inflamed) and cold (non-inflamed) tumors and providing prognostic information based on the presence of CD3⁺ and CD8⁺ in specific regions (center and invasive margin) of the tumor (Galon and Bruni, 2019). A recent worldwide validation confirmed that the consensus Immunoscore holds prognostic value superior to that of the AJCC/UICC-TNM staging system (Pagès et al., 2018).

Tumor Immunology Coming-of-Age: Anti-CTLA-4 and Anti-PD-1 as Prototype Checkpoint Inhibitors

Advances in molecular biology between the 1980s and the 1990s enabled the elucidation of the mechanisms underlying T cell cytotoxicity, such as the perforin-granzyme and the FAS-FASL pathway (Golstein and Griffiths, 2018). These advances were also instrumental to the discovery of key molecular players in immunity. Brunet and colleagues reported the identification of the immunoglobulin superfamily member CTLA-4 in mice and revealed its inducible nature upon T cell activation (Brunet et al., 1987). These findings were soon extended to humans (Dariavach et al., 1988). These discoveries were contemporary to the identification of the co-stimulatory receptor CD28 (Aruffo and Seed, 1987). Although the ability of CTLA-4 to bind the CD28 ligand B7-1 with high affinity was demonstrated in 1991 (Linsley et al., 1991), the role of CTLA-4 remained controversial until 1995, when an antibody blocking the interaction of CTLA-4 with antigen-presenting cell (APC)-derived B7-2 (functionally equivalent to B7-1 [Pentcheva-Hoang et al., 2004; Sigal et al., 1998]) was shown to inhibit T cell proliferation (Krummel and Allison, 1995). Of note, at the time, the role of CD28 in preventing T cell anergy had just been revealed (Harding et al., 1992). Shortly after, the analysis of a CTLA-4-deficient mouse model revealed its crucial role as negative regulator of T lymphocyte activation, therefore contributing to T cell homeostasis and protecting from lethal tissue damage (Waterhouse et al., 1995; Tivol et al., 1995). Subsequent studies revealed that CTLA-4 ligation inhibits CD28-dependent IL-2 production (Walunas et al., 1996). Importantly, mAbs targeting CTLA-4 reversed its inhibitory effect on T cells (Walunas et al., 1994). A big breakthrough came with the discovery by Allison and colleagues that administration of anti-CTLA-4 antibodies could mediate tumor rejection in mouse models *in vivo* and confer long-lasting immune protection (Leach et al., 1996).

A similar pre-clinical path was followed by the other prototype checkpoint inhibitor PD-1 (also known as CD279). Also belonging to the immunoglobulin gene superfamily, PD-1 emerged during a screen for genes involved in apoptosis carried out by Ishida et al. (1992). PD-1 is expressed by activated T cells, and also by B cells and myeloid cells. It took 8 more years to conclusively demonstrate the immunoinhibitory role of PD-1 via ligation of the B7 family member PD-L1 on antigen-presenting cells (APCs), as well as non-lymphoid tissue (Freeman et al., 2000).



(legend on next page)

Altogether, these studies demonstrated the existence of fine-tuning mechanisms regulating tolerance and controlling autoimmunity, and conversely (as we know now) contributing to tumor immune escape. Tumor cells express PD-L1 (also known as CD274 or B7 homolog 1-B7-H1), and an anti-PD-L1 antibody reduced tumorigenesis and tumor invasiveness and enhanced cytotoxic T-cell-mediated tumor cell lysis (Iwai et al., 2002). PD-L1 is not normally expressed by healthy human cells (with the exception of macrophages) but is highly expressed by tumor cells in response to IFN- γ (Dong et al., 2002). PD-1-PD-L1 binding, with concomitant TCR engagement, results in antigen-specific T cell apoptosis, thus promoting tumor growth *in vivo* (Dong et al., 2002). PD-L2, another ligand for PD-1, appears to be functionally redundant to PD-L1 and is expressed by tumor cells as well as by some normal tissues and by APCs upon activation (Latchman et al., 2001).

It was already clear in the early 2000s that CTLA4 and PD-1 were profoundly different checkpoints. One of the most striking observations was the different phenotypes of the CTLA-4- or PD-1-deficient mouse models, the former lethal within a few weeks (Tivol et al., 1995; Waterhouse et al., 1995), the latter developing lupus-like disease with age (Nishimura et al., 1999). Furthermore, the relevant ligands display distinct expression patterns: B7-1 and B7-2 are found mainly on professional APCs (most typically residing in lymph nodes or spleen), whereas PD-L1 and PD-L2 are widely expressed, most notably in peripheral tissues (Buchbinder and Desai, 2016; Latchman et al., 2001).

At this point in time, three key concepts came together: (1) the demonstration of the phenomenon of immunosurveillance in mice; (2) the demonstration of the importance of a pre-existing favorable immune contexture, and (3) the possibility to unleash these tumor-infiltrating T cells induced by these immune checkpoint antibodies. These major milestones generated great enthusiasm and brought forth the renewal of the field of tumor immunology. Indeed, cancer immunotherapy was named “Breakthrough of the Year” by Science in 2013 (Couzin-Frankel, 2013). The subsequent clinical application of monoclonal antibodies against CTLA-4 and PD-1 ultimately resulted in regulatory approval in a growing number of indications (particularly for PD-1) since 2011. The milestones in tumor immunology discussed here are summarized in a timeline (Figure 1). As of February 2019, these agents have been approved for 43 different cancer indications. The award of the 2018 Nobel Prize in Physiology or Medicine to immunologists James Allison and Tasuku Honjo should be seen not only as their recognition, but also as a global acknowledgment of the field and of the underlying effort of all contributing parties.

Pre-cancerous Lesions: A Cradle for Cancer

It is assumed that most solid cancers develop through a multi-step physiopathological process: the normal mucosa-to-

adenoma-to-carcinoma sequence, or pre-malignant-to-low-grade-to-high-grade lesions (Figure 2). This sequence was thought to be characterized by pre-defined steps of sequential genetic mutations (Fearon and Vogelstein, 1990; Kinzler and Vogelstein, 1996). More recently, the notion that a branched, rather than linear acquisition of specific mutations applied to the growth and dissemination of primary tumors (Gerlinger et al., 2012; Kim et al., 2015; Sottoriva et al., 2015; Thirlwell et al., 2010) has been widely accepted. This notion, together with the observation that specific, supposedly required key genetic mutations are not systematically found in established tumors suggest further revisions to the classical model. Such revision is even more strongly encouraged in the light of the most recent findings on the role of the immune system in shaping later stages of cancer evolution (Angelova et al., 2018). Whether a similarly branched, immune-influenced modality applies to the stages of pre-cancer development constitutes a plausible hypothesis, which nonetheless remains to be established.

Mutations enhancing proliferative signaling by determining the activation of oncogenes and/or the negative regulation of tumor suppression genes, as well as those conferring resistance to cell death and evasion of transforming growth factor- β (TGF- β) signaling are among the most critically involved in the carcinogenic process (Hanahan and Weinberg, 2011). These mutations can result from genomic instability, defined as the loss or rearrangement of the chromosomes during cell division, but also be initiated and/or potentiated by factors directly or indirectly causing DNA disruptions and damage. Smoking (Murphy et al., 2019; Siegel et al., 2015), exposure to radiation (Ozasa et al., 2012; Preston et al., 2007; Williams et al., 2004), and oncogenic viruses (Boda et al., 2018; Koeppel et al., 2015; McBride, 2017; Parfenov et al., 2014; Slebos et al., 2013) are examples of established carcinogens directly causing DNA damage, although indirect effects also contribute to the carcinogenic process. Other factors also play a pro-tumorigenic role, including obesity (Calle et al., 2003; Wade et al., 2019) and lack of physical exercise (Dietz et al., 2016; Stenholm et al., 2016; Zauber et al., 2012). Linked to most, if not all the above-mentioned factors is chronic inflammation, which in fact provides a fertile niche promoting carcinogenesis, and is indeed a recognized hallmark of cancer (Colotta et al., 2009; Hanahan and Weinberg, 2011). Apart from setting up a tumorigenic microenvironment, several lines of evidence indicate that chronic inflammation is associated with chromosomal instability (Colotta et al., 2009; Kitamura et al., 2015; Lin et al., 2016), thereby highlighting the crosstalk among different tumorigenic components. It should be noted that not all chromosomal abnormalities seem to be linked to carcinogenesis (Vitre et al., 2015), further reiterating its complexity and multifactorial nature.

Whereas the mechanism underlying the connections between inflammation and carcinogenesis remain to be elucidated, a

Figure 2. History of Cancer: from Pre-cancerous Lesions, to Primary Tumors, to Metastases

The main pathological stages characterizing the pre-malignant lesions are shown, followed by the development of the primary tumor. The main parameters regulating the progression of primary tumors along the classical, TNM-based tumor stages, are displayed: the immune contexture and adaptive immunity counteracting tumor growth yet shaping tumor progression via the immunoediting. The ability of the tumor to evade the immune recognition, with the phenomena of immune tolerance, immune suppression, and immune escape, favor tumor progression. Therefore, these factors contribute to the early metastatic invasion, characterized by vascular emboli, lymphatic invasion and perineural invasion (VELPI) as well as sustaining distant metastatic spreading.

growing body of evidence implicates several immune and immune-derived components in the onset and the progression of cancer. Malignant cells release pro-inflammatory mediators and chemoattractants that promote immune cell infiltration (Gao et al., 2017; Johnson et al., 2014; Xia et al., 2012). Furthermore, malignant cells are able to reprogram resident fibroblasts turning them into cancer-associated fibroblasts (CAFs); in turn, CAFs play a pro-tumorigenic role, both directly (by stimulating the growth of tumor cells and angiogenesis) and indirectly, via the recruitment of immune cells to the tumor microenvironment (TME) (Ao et al., 2007; Erez et al., 2010; Vicent et al., 2012). Of note, CAFs in mouse pancreatic ductal adenocarcinoma (PDAC) and mammary tumors, but not cervical tumors, are pro-inflammatory (Erez et al., 2010). The use of a Kras-driven mouse model of PDAC revealed the critical role for the inflammatory mediator signal transducer and activator of transcription 3 (STAT3) in supporting cell proliferation, metaplasia associated inflammation and matrix metalloproteinase 7 (MMP7) expression during tumorigenesis (Fukuda et al., 2011). Of note, MMP7 and other metalloproteinases are involved in extracellular matrix (ECM) remodeling, which typically accompanies the malignant transformation (Das et al., 2017; Heslin et al., 2001; Page-McCaw et al., 2007).

Neutrophils, macrophages, and lymphocytes infiltrate the inflammatory site, release a plethora of soluble mediators including pro-inflammatory cytokines and growth factors, and determine the production of genotoxic species, such as reactive oxygen species (ROS) and nitrogen oxide (NO), inducing DNA damage and genetic mutations (Kanda et al., 2017; Okada, 2014). Langerhans cells can create a mutagenic environment by metabolically converting chemical carcinogens into active mutagens, thus inducing DNA-damage-induced squamous cell carcinoma (Modi et al., 2012). Lesion-infiltrating bone-marrow-derived myeloid cells not only drive the inflammatory process, but also produce ECM remodeling factors, pro-angiogenic growth factors and vascular-modulating enzymes (Jiang and Lim, 2016), thereby setting up the stage for the developing malignancy. Neutrophils are generally pro-tumorigenic during the initiation and progression of tumors (Tazawa et al., 2003), and often a source of ROS (Canli et al., 2017; Nicolás-Ávila et al., 2017). Other neutrophil-derived genotoxic substances, such as defensins, were shown to induce DNA strand breaks in target cells (Gera and Lichtenstein, 1991). *In vivo* studies implicated neutrophils in the neoangiogenetic process in early preneoplastic hepatocellular (Huo et al., 2019; Kuang et al., 2011) and pancreatic (Nozawa et al., 2006) lesions, thus favoring malignant progression. The observation that systemic depletion of neutrophils inhibits bacteria-triggered mammary tumor development in mice further demonstrates the key role of these cells in tumorigenesis (Lakritz et al., 2015).

In addition to neutrophils, other ROS-producing myeloid cells, i.e., macrophages, can contribute to carcinogen-independent intestinal tumor initiation and progression (Canli et al., 2017). *In vivo* evidence suggests a prominent role for macrophage-derived inducible nitric oxide synthase (NOS2) in promoting lung squamous cell carcinoma (SCC) (Wang et al., 2018), confirming previous observations (Liu et al., 1998; Okayama et al., 2013). Pro-inflammatory macrophages are among the responsible for the low-grade inflammation observed in obese individ-

uals, involving the production of IL-6 and tumor necrosis factor- α (TNF- α) and the derived hepatic carcinogenesis (Park et al., 2010). However, pro-inflammatory M1-like macrophages seem to play a less prominent role in tumor progression than their anti-inflammatory counterparts, often referred to as M2 macrophages (Wang et al., 2015). The expression pattern of inflammatory macrophages is unchanged along the pre-cancerous stages preceding CRC, whereas the frequency of M2-like macrophages markedly increased with the pathological stages of progression (Wang et al., 2015). M2-like macrophages can promote carcinogenesis by driving an oncogenic program in epithelial cells (Morales et al., 2014). The cytokine IL-4, in combination with macrophage colony-stimulating factor induces macrophage polarization to an anti-inflammatory phenotype (Yang and Zhang, 2017). The increased expression of the oncogenic (Nussinov et al., 2016; Tao et al., 2014) protein Yes-associated protein 1 (YAP1) found in colorectal adenocarcinoma, compared with that in normal tissue, can drive IL-4- and IL-13-induced macrophage polarization (Huang et al., 2017). It should be noted that a high degree of plasticity exists among the different macrophage populations, and that the M1 versus M2 distinction represents an oversimplification of a wide phenotypical spectrum. The significance of the heterogeneity within TAMs (Aras and Zaidi, 2017) in different cancer contexts is an important area of investigation.

Eosinophils and mast cells can also contribute to malignant transformation. The number of tissue-infiltrating eosinophils decreases during the progression of colorectal adenoma-carcinoma (Cho et al., 2016; Kızılış et al., 2008; Moezzi et al., 2000), suggesting a protective role for these cells and/or the establishment of mechanisms of escape. The association of mast cells with tumorigenesis was first reported by Paul Ehrlich (Ehrlich, 1879a, 1879b). Pioneering work in a model of skin carcinogenesis showed that the number of mast cells increased throughout the pre-cancerous stages and then decreased at the onset of cancer (Cramer and Simpson, 1944). Mast cell activation, presumably degranulation, occurred at the most advanced pre-malignant phases, and high activation intensity was proposed to act as a functional defensive process against the development of skin cancer (Cramer and Simpson, 1944). Further studies in different contexts have reported both pro- and anti-tumorigenic roles for mast cells. For instance, mast cells promoted pre-malignant angiogenesis and pro-tumoral stromal remodeling in squamous epithelial carcinogenesis in mice (Coussens et al., 1999), and degranulation-dependent (pre)tumor cell proliferation in mammary carcinogenesis in rats (Faustino-Rocha et al., 2017). On the other hand, mast cells promoted eosinophil infiltration and tumor cell apoptosis in a model of early-stage intestinal tumorigenesis (Sinnamon et al., 2008). These differences might reflect a cancer- and/or localization-specific role for these cells, as recently suggested (Varicchi et al., 2017).

It is estimated that chronic inflammation increases the risk of human cancers involving almost all organs or tissues (Kanda et al., 2017). In support of this observation, very recently, a tumor-promoting inflammatory microenvironment was found to promote skin carcinogenesis in discoid lupus erythematosus (DLE) patients. Reduced CD8 $^{+}$ T cells, combined with enhanced lesion-infiltrating regulatory T (T_{REG}) CD4 $^{+}$ T cells, M2 macrophages, mast cells, as well as upregulated STAT3 signaling in

keratinocytes were found in cancer-prone DLE patients (Zaalberg et al., 2019). Therefore, it is not surprising that strategies and candidate agents aiming at preventing inflammation-related carcinogenesis have been proposed (Kanda et al., 2017), including aspirin for CRC prevention (Coyle et al., 2016; Garcia-Albeniz and Chan, 2011). Of note, not all chronic inflammations lead to carcinogenesis; for instance, some of chronic inflammatory diseases, such as rheumatoid arthritis, are not linked to cancer risk, whereas others are even leading to tumor regression (Okada, 2014). The crucial genetic and/or immune determinants leading from inflammation to cancer haven't been fully elucidated yet. Notwithstanding, the notion that immune-based mechanisms control the transformation of benign to malignant lesions (or fail to do so) has been advocated (Cui et al., 2012). Recently, the examination of the nine morphological stages of lung squamous carcinogenesis identified specific modules of co-expressed genes and evolutionary trajectories of cancer and immune pathways (Mascaux et al., 2019). A continuous increase of proliferation and DNA repair linearly form normal tissue to cancer along the pre-cancerous stages was found. This was accompanied by a transitory increase of metabolism and early immune sensing through activation of resident immune cells, including mast cells, in low-grade pre-invasive lesions. Subsequently, the activation of the innate and adaptive immune response and the expression of immune checkpoints and suppressive interleukins were detected from high-grade pre-invasive lesions, indicating the establishment of an immune escape phase. The invasive, more advanced stage was characterized by the activation of the epithelial-mesenchymal transition (EMT). High-grade lesions were characterized by the highest percentage of immune-related gene expression, including that of activated T cells, neutrophils, and M1 macrophages. The immune composition within lesions from the same patients varied along the stages, and there was a shift from naive to memory T and B cell and a gradual increase in mast cell activation. The abundance of naive CD4⁺ T cells peaked at the early stage of mild dysplasia then rapidly declined concomitantly with the increase of activated CD4⁺ memory T cells in the successive stages. It would be of crucial interest to pinpoint key determinants involved in the failure of the immune system in controlling and possibly removing (pre)malignant clones. Nevertheless, this study represents a first comprehensive immune assessment at the precancerous stages, a first step toward a desired identification of biomarkers of (pre)disease progression or recurrence, and the possibility to perform immunotherapy at a pre-cancer stage.

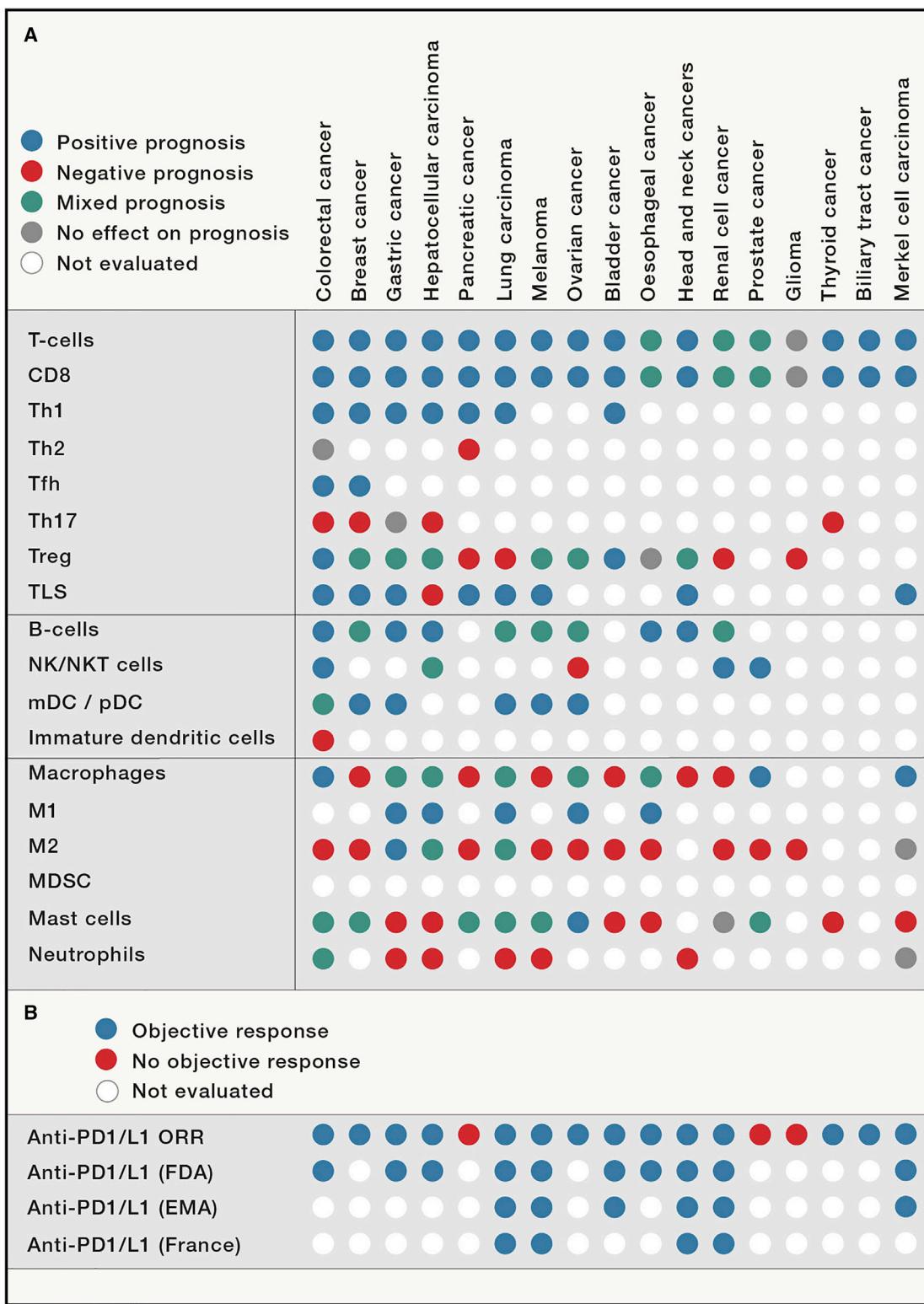
Immunity Versus (or Pro) Cancer in Primary Tumors

Primary tumors are characterized by a wide array of immunological scenarios (Galon et al., 2013), partly depending on the tissue of origin (Oliver et al., 2018; Santegoets et al., 2019), tumor location (Zhang et al., 2018), the underlying spectrum of genetic mutations (Giannakis et al., 2016) and genetic background (Lim et al., 2018), epigenetic features (Jones et al., 2019), the composition of the host gut microbiome (Cremonesi et al., 2018; Gopalakrishnan et al., 2018), and environmental conditions (Yi et al., 2015). All these factors contribute to shape the tumor-associated immune landscape and consequently both the natural progression of the disease, as well as the response to (im-

muno)therapeutic agents (Bindea et al., 2014; Galon and Bruni, 2019). The analysis of the prognostic value of individual cell types enabled their labeling as friends or foes, although this clear-cut distinction is not applicable to all cell subtypes and/or to all cancer types (Fridman et al., 2012) (Figure 3). Such prognostic variability might reflect local or tissual differences, thereby affecting the milieu of the TME, but it might also arise from: a lack of harmonization in marker selection; technical differences; the exclusion of spatial parameters (localization of specific immune cells in specific tumor regions); the need for combinatorial assessments, whereby combinations of immune cells and/or factors (and their topography within the tumor), rather than individual ones, hold superior prognostic power.

In mechanistic terms, what has been clearly established is that CD8⁺ cytotoxic T cells are the ultimate effectors of tumor rejection because of their cytolytic capabilities and of course their specificity against tumor cells, conferring long-lasting protection, thus protecting against cancer recurrence. It should be noted that also innate immune cells can control cancer directly by interacting with tumor cells, and/or indirectly by favoring the anti-tumor activities of CD8⁺ T cells. NK cells, $\gamma\delta$ T cells and macrophages are among the documented mediators of direct tumor cell lysis (Chan et al., 2009; Hayakawa et al., 2002; Jadus et al., 1996; Kägi et al., 1994; Uno et al., 2007; Wrobel et al., 2007; Wu et al., 2015). Activated NK cells and $\gamma\delta$ T cells are also potent IFN- γ producers. IFN- γ increases MHC and immunoproteasome expression by tumor cells (Cheon et al., 2014; Rouette et al., 2016), thereby sensitizing tumor cells to CD8⁺ T cell killing. More complex is the role of macrophages, which are mostly considered as pro-, rather than anti-tumor, as detailed below. Nonetheless, the expression of a fixed set of germline-encoded receptors, characteristic of innate immune cells, shows that innate cells mediated cancer surveillance, which is fundamentally different from that by the adaptive immune system, which instead relies on a unique specificity for tumor antigens. Therefore, CD8⁺ cytotoxic T-cell-mediated tumor rejection represents the last step of a complex dynamic process, nicely exemplified with the proposed cancer-immunity cycle (Chen and Mellman, 2013). As a result of the oncogenic transformation, cancer cells express modified antigens (neoantigens) which are released upon tumor cell death. Neoantigens are then captured by APCs, most typically in humans by conventional type 1 dendritic cells (cDC1s) (Sánchez-Paulete et al., 2017), which then drive the priming and activation of T cell responses, possibly within tertiary lymphoid structures (TLSs) (Zhu et al., 2015), in the presence of the right set of co-stimulatory signals and cytokine milieu (Gardner and Ruffell, 2016). This represents a critical step, given that distinct parameters and factors can tip the balance in favor of either anti-tumor effector T cells, or pro-tumor T_{REG} cells (Chen and Mellman, 2013). Of note, the neoantigen specificity of both tumor-associated and circulating T_{REG} cells has been demonstrated only very recently (Ahmadzadeh et al., 2019).

T_{REG} cells are not the only immune cells shown to favor cancer progression: monocytic myeloid-derived suppressor cells (MDSCs) and M2-like TAMs (both falling within the broad category of monocyte/macrophage lineage cells [MMLCs]) are the most notable examples of pro-tumor immune cells by acting at multiple levels (reviewed in Chew et al., 2012; Lindau et al., 2013; Takeuchi and Nishikawa, 2016) (Aras and Zaidi, 2017). The degree of CD8⁺

**Figure 3. Prognostic Effect of Immune Cells in Solid Cancer**

An overview of the main innate and adaptive immune cell types and their prognostic effect in distinct solid cancers in humans, as determined by IHC staining of tumors. Adapted from Aponte-López et al., (2018), Delahaye et al., (2011), Dong et al., (2006), Dundar et al., (2008), Dydych et al., (2012), Galon and Bruni, (2019), Gu-Trantien et al., (2017), Mussai et al., (2012), Pasero et al., (2015), Powell and Huttenlocher, (2016), Rajput et al., (2008), Sautès-Fridman et al., (2016), Sun et al., (2018), Truxova et al., (2018), Wouters and Nelson, (2018), Xu et al., (2016), and Yano et al., (1999).

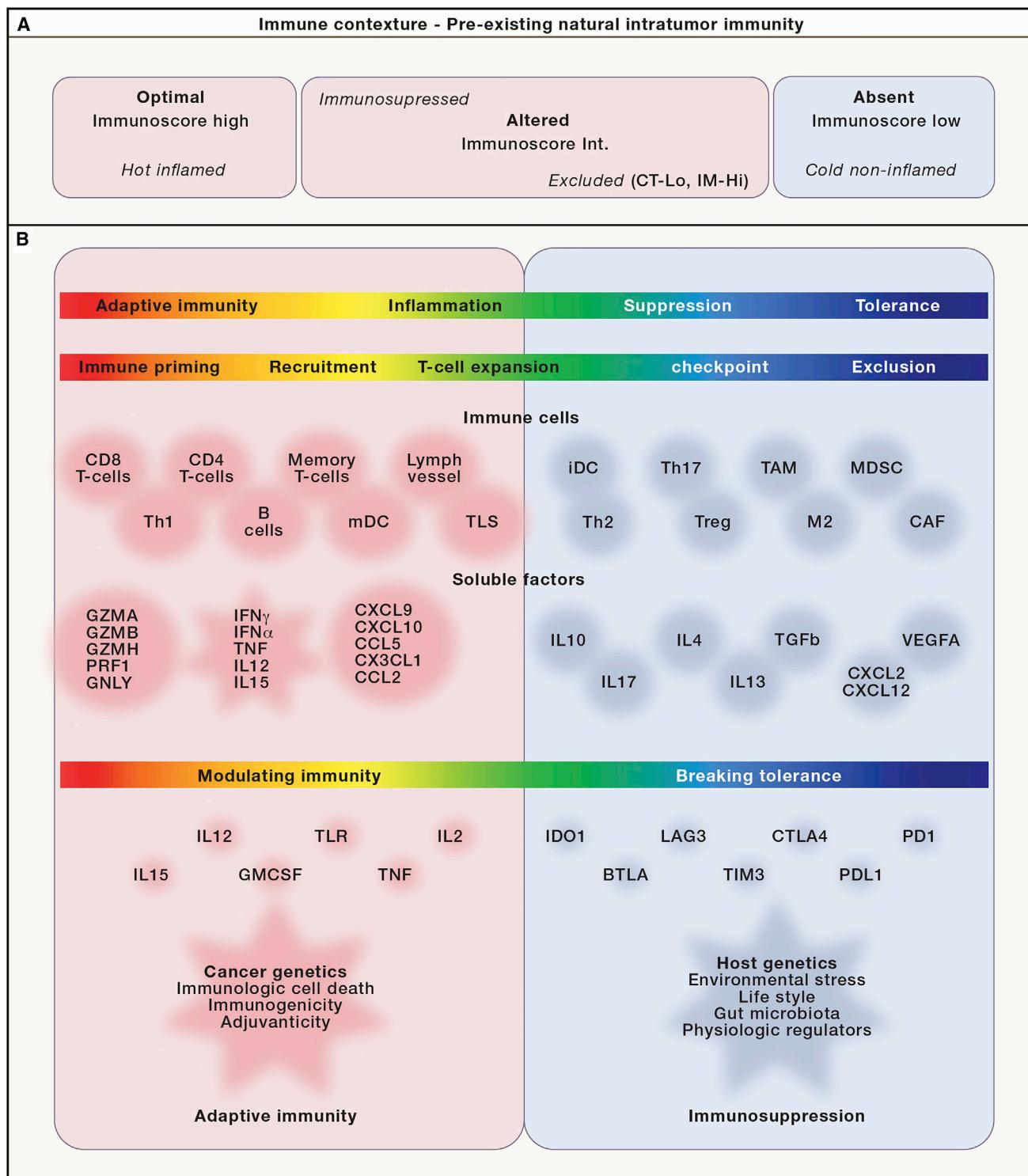


Figure 4. Immune Contexture: Good and Bad Immunity

(A) The pre-existing natural intra-tumor immunity can classify cancer patients into four main categories based on Immunoscore: the optimal immunity (high Immunoscore and hot, inflamed tumors), the altered immunity (intermediate Immunoscore and with either an immunosuppressed phenotype or an exclusion phenotype [high density of T cells only at the tumor margin]), and the absent immunity (low Immunoscore and cold, non-inflamed tumors).

(B) The immune microenvironment comes in different flavors including adaptive immunity, inflamed, immunosuppressed, or with a tolerogenic phenotype. The immune contexture, defined as the type, density, immune functional orientation, and location of immune cells within distinct tumor regions shapes the proper

(legend continued on next page)

T cell infiltration is affected by both TAMs and T_{REG} cells. Tumor cells are a source of colony stimulating factor 1 (CSF-1), which recruits CSF-1 receptor (CSF-1R)-expressing TAMs (Dwyer et al., 2017), thereby providing at once a survival, proliferation, and differentiation stimulus. The *in vivo* pharmacological inhibition of the CSF-1R signaling pathway (Strachan et al., 2013), or the genetic ablation of CSF-1 in colorectal cancer cells (Abbasi, 2018) promoted CD8⁺ T cell infiltration, which was counteracted by an enhanced influx of T_{REG} cells (Gyori et al., 2018). Co-depletion of both TAMs and T_{REG} cells increased of CD8⁺ T cell density and activity and reduced tumor growth (Gyori et al., 2018). Hepatocellular carcinoma cells can activate tumor-associated monocytes, showing increased HLA-DR, CD80, and CD86 amounts with concomitant PD-L1 expression, thereby suppressing tumor-specific T-cell-mediated immunity (Kuang et al., 2009). Components of the TME such as mucins promote an immune suppressive phenotype (increased production of IL-10, decreased Th1-recruiting CCL3, and suppressed IL-12) in TAMs by binding the mannose receptor on these cells and determining its internalization (Allavena et al., 2010). Indeed, tumors would seem to distinctively reprogram TAMs, as shown by the transcriptional differences observed between monocytes, tissue resident macrophages, and TAMs from endometrial and breast cancers (Cassetta et al., 2019). Tumor cells (as well as TAMs themselves) are a source of TNF- α , which supports CCL8 production by TAMs; in turn, CCL8 promotes CSF-1 production by cancer cells, thereby supporting TAM survival and proliferation (Cassetta et al., 2019). Furthermore, TAMs can promote angiogenesis (the “angiogenic switch”) by producing a plethora of factors (vascular endothelial growth factor A [VEGFA], epidermal growth factor [EGF], basic fibroblast growth factor 2 [FGF2], chemokines CXCL8 and CXCL12, TNF- α , semaphorin 4D, adrenomedullin, and thymidine phosphorylase) and activating and recruiting endothelial cells (Leek et al., 1998; Riabov et al., 2014; Sierra et al., 2008; Zhou et al., 2012).

Although a general consensus emerged on the pro-tumoral role of T_{REG} cells, MMLCs display a more complex profile. A recent study in early-stage human lung tumors showed that PD-L1-expressing TAMs did not generally suppress tumor-specific effector T cell responses, as opposed to PD-L1-expressing tumor cells; on the other hand, tumor monocytes tended to inhibit more the T cell responses (Singhal et al., 2019). Apart from potentially expressing PD-L1, TAMs express increasing amounts of checkpoint molecules (PD-1 [Gordon

et al., 2017] and Clever-1 [Viitala et al., 2019]) over the course of primary human cancers, as discussed above. This evidence indirectly shows the good, anti-tumor side of TAMs.

Chemokines and factors keeping the “good” immune cells within the TME are essential to enable an efficient anti-tumor response. The rarity of cDC1s in the TME, and their most frequent absence from early tumor stages was suggested to contribute to cancer progression (Bottcher et al., 2018). NK cells were recently shown to be important producers of the cDC1 chemoattractants CCL5 and XCL1 in melanoma (Bottcher et al., 2018); in turn, cDC1s can recruit effector T cells by releasing CXCL9 and CXCL10, which of note can also attract NK cells in a positive feedback loop (Fessenden et al., 2018; Spranger et al., 2017; Zelenay et al., 2015). Th1 cells also significantly contribute to the production of CXCL9 and CXCL10; their epigenetic silencing in these cells was associated with decreased intratumoral CD8⁺ T cells and reduced survival in human ovarian cancer, thus representing an immune-evasion mechanism of tumors (Peng et al., 2015). The critical role played by chemokines in creating an immune optimal or immune hot tumor (highly infiltrated by CD8⁺ T cells) was shown by using multiple approaches (Mlecnik et al., 2010) and very recently confirmed by the George Coukos group (Dangaj et al., 2019). These studies showed an association between CCL5 and CXCL9 co-expression and CD8⁺ T cell infiltration across human solid tumors. Tumor cells were identified as a constitutive source of CCL5, where it could be epigenetically repressed, whereas CXCL9 was induced in tumor-associated myeloid cells (TAMs and DCs) by IFN- γ , independently on tumor antigen recognition by TILs (Dangaj et al., 2019).

Novel proteogenomic approaches enabled the identification of further mechanisms regulating T cell infiltration into microsatellite instability-high (MSI-H) colon tumors (Vasaikar et al., 2019). MSI-H tumors are characterized by an increased mutational and neoantigen load compared with the microsatellite stable (MSS) phenotype, which is typically accompanied by an increase in TILs (Smyrk et al., 2001). While no noticeable differences were found at the messenger RNA (mRNA) level, a marked increase in the protein amounts of glycolytic enzymes was found in a subgroup of MSI-H tumors, and then linked to impaired T cell function and trafficking to the TME (Vasaikar et al., 2019).

Once recruited to the TME, CD8⁺ effector T cells further propagate this loop by producing, among others, CCL5 and XCL1 (Fessenden et al., 2018). Of note, a lack of recruitment of immune

pre-existing adaptive immunity, in contrast to immunosuppressed microenvironment. The main anti-cancer (left) and pro-cancer (right) immune cells and soluble factors are illustrated. To shape a proper immune contexture, immune priming, recruitment of T cells and other immune cells, and T cell expansion are necessary (right), whereas immune negative feedback-loop mechanism (checkpoint) could lead to immunosuppression, and intrinsic tumor mechanisms could lead to immune exclusion (left). Immunotherapy could be performed by breaking tolerance through immune checkpoints such as FDA approved PD1, PDL1, and CTLA4 or checkpoints undergoing clinical studies TIM3, LAG3, BTLA, and IDO1 and by modulating immunity with soluble molecules such as IL-2, TNF, TLR agonists, GMCSF, IL-12, and IL-15. Cancer genetic parameters such as the immunogenicity, adjuvanticity, and immunologic cell death of tumor cells could modulate the adaptive immunity. Host genetic parameters, environmental stress, life style, and gut microbiota could further modulate the immune contexture.

Abbreviations are as follows: ThX, Type “X” T helper cell; TLS, tertiary lymphoid structure; cDC, conventional dendritic cell; iDC, immature dendritic cell; T_{REG}, regulatory T cell; TAM, tumor-associated macrophage; M2, alternatively activated macrophages; MDSC, myeloid-derived suppressor cell; CAF, cancer-associated fibroblast; GZMA, Granzyme A; GZMB, Granzyme B; GZMH, Granzyme H; PRF1, perforin 1; GNLY, Granulysin; IFN- γ , interferon gamma; IFN- α , interferon α ; TNF, tumor necrosis factor; IL, interleukin; CXCL, C-X-C motif chemokine ligand; CCL, C-C motif chemokine ligand; CX3CL1, C-X3-C motif chemokine ligand 1; TGF- β , transforming growth factor beta; VEGFA, vascular endothelial growth factor A; GM-CSF, granulocyte-macrophage colony-stimulating factor; TLR, toll-like receptor; IDO, indoleamine-pyrole 2,3-dioxygenase; BTLA, B- and T-lymphocyte-associated or cluster of differentiation 272 (CD272); TIM-3, T cell immunoglobulin and mucin-domain containing-3; LAG-3, lymphocyte-activation gene 3; CTLA-4, cytotoxic T-lymphocyte-associated antigen; PD-1, programmed death-ligand 1; TIGIT, T cell immunoreceptor with Ig and ITIM domains.

cells was proposed to be one possible cause for the existence of so-called excluded tumors, characterized by the presence of effector T cells at the tumor borders, but not at its core (Figure 4) (Galon and Bruni, 2019). It remains to be established how this chain of events starts, i.e., how NK cells (which are lowly, if at all present, within the TME) are recruited in the first place; nonetheless, it is clear that it ends, when successful, with the CD8⁺ cytotoxic T-cell-mediated killing of cancer cells.

The epigenetic modulation of chemokine expression shown above are only some of the numerous immune escape mechanisms developed by cancers to avoid immune recognition, activation, and effector functions. The expression of immune checkpoints, which occurs already at the pre-cancerous stages (Mascaux et al., 2019) and the development of tolerance are the most notable examples of well-established mechanisms of immune control. Apart from the immune-mediated mechanisms of escape, the tumor itself can directly or indirectly evade the anti-tumor immune response. A comprehensive analysis of tumor-cell-intrinsic mechanisms of primary and secondary immune evasion have been extensively provided elsewhere (Wellenstein and de Visser, 2018). More recently, an additional mechanism of tumor-mediated immune escape has been provided by Bottcher and colleagues. Tumor cells were previously found to be a source of prostaglandin E2 (PGE₂) (Zelenay et al., 2015) (Jung et al., 2003). Novel evidence showed how tumor-derived PGE₂ reduced the secretion of CCL5 and XCL1 by NK cells, impaired the expression of chemokine receptors on cDC1s, and increased NK cell death (Bottcher et al., 2018). These novel mechanisms could contribute to explain the previously known pro-tumorigenic role of PGE₂ and other eicosanoids (Wang and Dubois, 2010), as well as further demonstrate the complexity of the interplay between tumor and tumor-associated immune components. It is the combination and simultaneous crosstalk among all positive and negative factors that ultimately shapes cancer progression (Figures 3 and 4).

The TME is a highly dynamic entity in terms of both cellular immune components and soluble mediators. The great majority of the studies describing the TME composition reflect a specific point in space and time, which is an evident limitation of studies in humans. Nonetheless, some studies assessed the changes accompanying tumor progression, offering precious insights into the associated everchanging immune landscape. To our knowledge, we were the first proposing an integrative study of the spatio-temporal dynamics of tumor-associated immune cell types with immune-cell-specific gene modules (immunome) showing how the immune infiltrate composition changes at each tumor stage (Bindea et al., 2013). Densities of the TLS-associated T follicular helper (T_{FH}) cells and innate cells (including macrophages, mast cells, neutrophils, and plasmacytoid DCs) increased, whereas most T cell densities (activated T cells, memory T cells, effector T cells, CD4⁺ helper, and CD8⁺ cytotoxic T cells) decreased along with tumor progression through T stages and AJCC/UICC-TNM classification (Bindea et al., 2013), as also found in a previous study (Mlecnik et al., 2011). B cells increased at a later stage, showing a dual effect on recurrence and tumor progression. CXCL13 and IL-21 were pivotal factors for the T_{FH}-B cell axis correlating with prolonged survival and supporting a good immune contexture (Th1, cytotoxic, and memory T cells). These findings, first observed with gene expres-

sion and immunome analyses, were confirmed by multiple approaches, including quantitative IHC and mouse models (Bindea et al., 2013). Recently, T_{FH} cells were found to enhance CD8⁺ T cells effector function in an IL-21-dependent manner in CRC patients (Shi et al., 2018). This potent T_{FH} cell function was impaired in the presence of PD-1/PD-L1-mediated suppression (Shi et al., 2018).

The Immune Landscape of Primary Tumors: Clinical Implications

The study of the immune landscapes associated with cancers has deep clinical implications. One clear example is constituted by primary CRCs, which are surgically removed in the majority of cases. To date, there is no parameter in clinical use that can predict CRC recurrence, which indeed happens in 40% of surgically removed CRC cases within 5 years (Augestad et al., 2017). The analysis of the immune infiltrate and Immunoscore of the resected primary tumors were demonstrated to predict tumor recurrence: indeed, a low adaptive immune reaction in the center and invasive margin of the primary tumor was found in recurrent patients. This Immunoscore feature was observed in early-stage tumor (stage I) as well as in late tumor stages (stages III or IV) (Mlecnik et al., 2011; Pagès et al., 2009; Pagès et al., 2018). Thus, immune features of the TME associated with CRC progression and recurrence could prove instrumental to guiding the choice for additional (neo)adjuvant therapy.

The immune landscape of cancer across 33 different cancer types was recently defined, identifying six immune subtypes on the basis of differences in both immune (macrophage or lymphocyte signatures, Th1:Th2 cell ratio, and expression of immunomodulatory genes) and seemingly non-immune parameters (intratumoral heterogeneity, aneuploidy, neoantigen load, overall cell proliferation, and patients' prognosis) (Thorsson et al., 2018). This genomic approach linked the presence of specific driver mutations with lower (CTNNB1, NRAS, or IDH1) or higher (BRAF, TP53, or CASP8) leukocytes frequencies across cancer types. Whether the occurrence of these mutations at different stages of the disease has a differential effect on the amount and/or composition of immune tumor infiltrates (and therefore on patients' prognosis) remains to be established. A limitation of the study is that it only relied on RNA sequencing (RNA-seq) and exome sequencing (Exome-Seq) data. Nonetheless, this work has the undoubted merit of having highlighted features of the TME that are shared among histologically distinct tumors, suggesting that the choice of therapeutic strategies should rely on immune parameters rather than cancer type. A first regulatory opening in this sense came with the approval by the FDA in May 2017 of the anti-PD-1 pembrolizumab for the treatment of adult and pediatric patients with unresectable or metastatic solid tumors based on a tumor biomarker (MSI-H or mismatch repair deficient [dMMR]). Immune biomarkers constitute likely candidates for future regulatory approvals.

Another important consideration having likely repercussions on prognosis and clinical management of cancer patients concerns the spatial localization and relative distribution of immune cells within the TME. The co-localization of TAMs or B cells with cytotoxic T cells was associated with complete pathological response after neo-adjuvant chemotherapy in inflammatory breast cancer patients (Van Berckelaer et al., 2018). Moreover,

although the number of PDL1⁺ or CD8⁺ cells was not prognostic per se, the presence of more PDL1⁺ cells around CD8⁺ cells ($r = 30 \mu\text{m}$) correlated with worse prognosis (Van Berckelaer et al., 2018) or with absence of immunoediting (Angelova et al., 2018).

Multiplex immunohistochemistry (mIHC) enables the simultaneous detection and spatial resolution of multiple immune determinants at once, and its adoption in clinical routine has been advocated (Hofman et al., 2019). A multiplex analysis of PDAC patients who received neoadjuvant a granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting pancreatic tumor vaccine (GVAX) (Lutz et al., 2014) showed how response to therapy correlated with degree of myeloid cell density and percentages of exhausted CD8⁺ T cells. Alternative approaches to mIHC have been proposed. For instance, deep learning mapping of TILs from hematoxylin and eosin (H&E) stain images from 13 The Cancer Genome Atlas (TCGA) tumor types revealed local spatial structures in TIL patterns and their correlation with overall survival (Saltz et al., 2018). Tumor, immune, and molecular subtypes were characterized by differential TIL densities and spatial structures enrichment, possibly reflecting specific tumor cell aberrations. Local clustering of TILs was a more distinctive (and better prognostic) feature than overall TIL infiltration in pancreatic adenocarcinoma and prostate adenocarcinoma as opposed to other tumor types (Saltz et al., 2018), indicating the importance of the spatial immune patterns in affecting survival in specific cancer settings. The reason for these tissue-specific differences are not clear; also unclear is the mechanism regulating the formation and evolution of these patterns overtime. Future investigation on these aspects is urgently needed, especially in view of the adoption of evermore biomarker-based therapeutic approaches.

Early Cancer Dissemination and Distant Metastases

Tumor progression comes most often with the spreading of the primary tumor to other anatomical locations throughout the body. The metastatic cascade is a multi-step process involving incompletely defined pathological changes of the tumor cell, which has to be able to leave the primary tumor, enter in systemic circulation, and penetrate and colonize distant sites, often after a phase of dormancy (Kienast et al., 2010; Luzzi et al., 1998). The epithelial-mesenchymal transition (EMT) is a widely recognized process leading to tumor cell invasion and metastatic spreading, and distinct EMT programs were recently associated with different invasion modalities (Aiello et al., 2018). The hypothesis that the both innate and adaptive arms of the immune system play a role in modulating (positively or negatively) one or more steps in the metastatic cascade is being ever-growingly appreciated and substantiated by compelling evidence.

Macrophages and MMLCs in general are among the most prominent innate immune cells promoting all steps characterizing metastatic spreading (Lewis et al., 2016; Nielsen and Schmid, 2017; Qian and Pollard, 2010). Such prominence was shown, for instance, by the observation that the genetic ablation of CSF-1 completely abolished the metastatic process in a mouse model of breast cancer (Lin et al., 2001). In fact, macrophages were directly linked to metastatic dissemination in multiple pre-clinical models (Griesmann et al., 2017; Headley et al., 2016; Linde et al., 2018).

Several mechanisms underpinning macrophages' ability to favor the metastatic process have been shown, involving in many cases their angiogenic and ECM remodeling properties, and cytokine and/or chemokine production. MMLC-driven remodeling mechanisms facilitate the migration of malignant cells from the primary tumor to nearby or distant sites. Indeed, the presence of venous invasion and liver metastasis were linked to increased expression TAM-derived MMP-2 and MMP-9 in pancreatic cancer patients (Nagakawa et al., 2002). TAMs, together with tumor cells, are a source of cathepsin S, which is able to proteolytically process the blood-brain barrier junctional adhesion molecule JAM-B, thereby promoting transmigration of breast-derived tumor cells into the brain (Sevenich et al., 2014). Another pro-metastatic mechanism involves the typically M2 (Kodelja et al., 1998) chemokine CCL18, produced by TAMs and acting on PITPNM3-expressing cancer cells, where it triggers integrin clustering, thus enhancing their adherence to the ECM (Chen et al., 2011). This mechanism was shown to promote breast cancer metastasis formation *in vivo* (Chen et al., 2011). MMLCs seem also to set up a favorable stage for metastatic seeding, by remodeling the pre-metastatic niche and secreting tumor-cell-attracting factors. Monocytic MDSCs (mo-MDSCs), a subtype of MDSCs, are recruited in a CCL12-dependent manner to the pre-metastatic lungs, where they constitute a major source of IL-1 β . This cytokine increases E-selectin expression on endothelial cells, thus promoting tumor cell arrest (Shi et al., 2017). Within the metastatic site, macrophages and MMLCs in general display most commonly an immunosuppressive, M2-like phenotype (Biswas and Mantovani, 2010).

Neutrophils represent another crucial innate immune cell type favoring the metastatic process, as shown in several pre-clinical models (Tütting and de Visser, 2016). In patients, a relationship between increased circulating neutrophils and metastases has been established for several solid tumors including melanoma (Bald et al., 2014) and PDAC (Tao et al., 2016). The proposed hypothesis that neutrophils might interact with circulating tumor cells (CTCs) in the blood and assist these potentially metastatic cells (Tao et al., 2016) has been validated recently by Nicola Aceoto's team (Szczera et al., 2019). The same group revealed how the association with neutrophils promotes cell cycle progression in CTCs, increasing their metastatic potential, compared with that of unescorted CTCs (Szczera et al., 2019). Further evidence showed how neutrophils can also shape a favorable pre-metastatic niche in distant organs. This has been first shown in pre-clinical models of breast cancer (Wculek and Malanchi, 2015) and ovarian cancer (Lee et al., 2019). Neutrophils undergo a specific type of cell death termed NETosis involving the extrusion of chromatin and the generation of neutrophil extracellular traps (NETs) (Singel and Segal, 2016). NETs were previously shown to facilitate tumor progression through promotion of thrombosis and angiogenesis (Singel and Segal, 2016). NETs were also required for awakening dormant metastatic cancer cells in inflamed murine lungs, via the cleavage of laminin mediated by NET-associated proteases. Proteolytically remodeled laminin activated integrin $\alpha 3\beta 1$ signaling in the dormant cancer cells, thus inducing their proliferation (Albrengues et al., 2018). Finally, recent evidence showed how omentum-migrating neutrophils at the early ovarian cancer stages promoted metastatic seeding in this organ in a NET-dependent manner.

(Lee et al., 2019). Altogether, the presented evidence highlights the multifaceted role of neutrophils in favoring tumor progression, although mechanisms showing anti-tumor roles for these innate immune cells also exist (Bindea et al., 2013; Singel and Segal, 2016), possibly suggesting an incomplete understanding of such complex system.

A recently developed stable intravital two-photon imaging model in mice enabled the direct observation of the arrival of CTCs in the lung, and the subsequent immune dynamics (Headley et al., 2016). Upon arrival in the lung capillaries, CTCs shed microparticles that either remained attached to the lung vasculature or migrated through it. These particles were then ingested by distinct temporal waves of myeloid cells: neutrophil first, then conventional monocytes, non-alveolar macrophages, patrolling monocytes, and cDCs. Successful metastatic development occurred after accumulation of these interstitial myeloid cells, which was favored by the CCR2-CCL2 axis, previously shown to promote myeloid recruitment in the lung (Qian et al., 2011). This study also implied a host-protective role for cDCs, competing with the pro-tumor macrophages by engulfing tumor-derived material in the lung and subsequently migrate to the mesenteric lymph nodes to engage with cognate T cells (Headley et al., 2016).

NK cells represent yet another innate immune cell type linked to the modulation of metastatic spreading. NK cells are more prominently known to exert direct cytotoxicity against tumor cells, and they appear to kill tumor cells in circulation more efficiently than those within the TME (Larsen et al., 2014). NK cells are scarcely infiltrating solid tumors but can be found at the tumor borders, where EMT takes place (Cantoni et al., 2016). Their ability to kill tumor cells undergoing EMT has been demonstrated in lung cancer (Chockley et al., 2018), although mechanisms of resistance to NK-mediated cytotoxicity have been also described (Terry et al., 2017). Therefore, by potentially acting on both the putative metastatic precursors (the EMT-undergone malignant cells) and the CTCs, NK cells appear to limit tumor colonization of distant sites.

NK cells inhibited lung colonization by tumor cells after administration of a phospholipid-conjugated Toll-like receptor 7 (TLR7) agonist in murine models of breast cancer, melanoma, and Lewis lung carcinoma (Hosoya et al., 2018). Whether this effect is due to an NK-mediated direct effect on metastatic precursors or CTCs remains to be elucidated. The same study showed the temporal sequence of immune-mediated anti-dissemination effects, whereby NK cells constrain early tumor colonization, followed by a cytotoxic CD8⁺ T cell anti-metastatic response at the later phases (Hosoya et al., 2018). Indeed, cytotoxic immune cells and other adaptive immune cells were also found to affect metastatic spreading. For instance, the presence of high amounts of infiltrating memory CD45RO⁺ T cells correlated with the absence of early signs of invasiveness around the tumor (such as venous emboli, lymphatic invasion, and perineural invasion [VELIPI]) (Figure 2), as well as increased CRC patient's survival (Pagès et al., 2005). This also highlighted that a weak pre-existing adaptive intra-tumor immunity gave the license to the tumor to invade vessels, even at very early stage of disease. A subsequent study on NSCLC correlated the presence of high levels of intraepithelial CD45RO⁺ cells in lymph node metastases with increased patients' survival (Kilvaer et al., 2016). Of note,

this held true in squamous cell carcinoma, but not in adenocarcinoma patients (Kilvaer et al., 2016). The long-lasting anti-tumor capacity of memory T cells (CD45RO⁺) was shown two decades ago in mouse models of colon carcinoma metastases (Xiang et al., 1999). The presence of enduring human memory T cells (Sallusto et al., 2004) might be a key immune hallmark of tumor control, explaining long-term survival in some cancer patients. Recent evidence shows how the interplay between circulating memory T cells and resident memory CD8⁺ T cells (T_{RM}) is needed to ensure optimal anti-tumor immunity (Enamorado et al., 2017).

Apart from the demonstration of anti-metastatic effect in synergy with NK cells described above (Hosoya et al., 2018), further *in vivo* studies demonstrated the ability of CD8⁺ cytotoxic T cells to protect against metastatic lesion formation. CD8⁺ T cells (and NK cells) protected against metastatic spreading in a type-I-IFN-dependent manner, as demonstrated by the *in vivo* analysis of bone metastasis from breast carcinoma (Bidwell et al., 2012). Furthermore, depletion of CD8⁺ T cells in a spontaneous melanoma mouse model resulted in an increased formation of lung and reproductive tract metastases (Eyles et al., 2010; Lengagne et al., 2008). In patients with inflammatory breast cancer, a correlation between CTCs (mediators of metastatic dissemination) and defects in adaptive immunity has been demonstrated (Mego et al., 2016). Similarly, CTC amounts negatively correlated with circulating T cell amounts in lung cancer patients (Sun et al., 2017).

Increased levels of immune cytotoxicity and lymphatic vessel density were shown to hinder the generation of distant metastases from CRC, whereas tumor cell genomic alterations didn't seem to affect this process (Mlecnik et al., 2016b). Indeed, a sole immune-related gene expression profiling could distinguish patients with or without distant metastases, indicating the strength of the immune parameters (Mlecnik et al., 2016b). TILs have been shown to directly affect the metastatic landscape in human CRC (Halama et al., 2011; Kwak et al., 2016; Mlecnik et al., 2018; Van den Eynde et al., 2018). We provided *ex vivo* evidence of the protective role of CD8⁺ T cells against metastatic spreading, with killing of tumor cells by anti-frameshift mutations neoepitope-targeting CD8⁺ T cells (Mlecnik et al., 2016b). We also showed the non-dissemination of immunoedited tumor clones in the presence of CD8⁺ cytotoxic T cells and proliferating T cells in CRC (Angelova et al., 2018). Elevated TIL densities, as determined by Immunoscore, measured on the least infiltrated metastases correlated with longer patients' survival (Mlecnik et al., 2018); indeed, the phenotypes of such metastases were the better predictor patients' outcome (Van den Eynde et al., 2018).

The brain is typically considered as an immune-privileged site. Whether T cells exert an anti-metastatic effector response within the brain parenchyma remained a matter of debate until recently (Gonzalez et al., 2018). An analysis of the immune microenvironment across brain metastases from any type of human primary tumor revealed that Immunoscore was profoundly associated with long-term patients' survival (Berghoff et al., 2015). This work would support the existence of a T-cell-mediated anti-tumor immune responses within brain metastases.

A recent analysis comparing the TCR-β repertoire of NSCLC primary lesions and paired brain metastases showed a minimal

overlap in T cell clones between paired lesions (Mansfield et al., 2018), showing clonal heterogeneity, as well as the occurrence of effector responses in the brain. Brain metastases displayed higher mutational burden, yet a significant reduction in the number of unique T cell clones, than did matched primary lesions (Mansfield et al., 2018). This might reflect a more immunosuppressive nature of the TME within the brain than the lung and/or the presence of less immunogenic clones in metastases, compared with primary sites. Further evidence of the direct role of TILs in the control of tumor dissemination was recently provided by Angelova et al. (2018) by following the metastatic progression of CRC patients, as well as their associated immune landscape, over an 11-year follow-up period. In addition, this study proved the validity of the concept of immunoediting in humans and, for the first time, its effect on progression and evolution of human cancers (Angelova et al., 2018). Immunoediting was previously defined as the concept describing the change of tumor immunogenicity over time under the selective pressure of the immune system (Dunn et al., 2002; Mittal et al., 2014). The immune system contribution to the steps defining tumor evolution has been elegantly shown in mouse models (Matsushita et al., 2012). The genetic evidence of immunoediting was then shown in humans for missense (Rooney et al., 2015), then missense and frameshift mutations (Mlecnik et al., 2016a). Angelova et al. (2018) first demonstrated in humans that tumor clones for which immunoediting was more prominent tend to disappear, whereas, conversely, the least immunogenic ones (therefore the most resistant to immune attack) were persisting in subsequent metastatic sites. Accordingly, the immunoediting and Immunoscore were found to be the two best predictors of favorable clinical outcome (Angelova et al., 2018). Thus, a novel “parallel immune selection model” of tumor evolution in humans was proposed, incorporating the effects of the immune system (Angelova et al., 2018). The most currently accepted “branched evolution model” by Gerlinger et al. (2012) describe a landscape of multiple co-evolving and co-existing malignant clones featuring distinct mutations and deriving from a common mutated ancestor; similarly to previously proposed models (Fearon and Vogelstein, 1990; Sottoriva et al., 2015), it is solely focused and based on tumor cell features. Although agreeing with the underlying concept of parallel co-evolution and co-existence, the parallel immune selection model first assigns a central role to the immune system in driving and directing this process.

In vivo evidence shows how other types of adaptive immune cells (CD4⁺ T helper cells and T_{REG} cells) effect metastatic spreading. CD4⁺ T cells can have both pro- and anti-metastatic roles via their effects on other immune cells. By acting on TAMs in an IL-4-dependent manner, CD4⁺ T cells promote invasion and pulmonary metastasis of mammary carcinomas. The consequent phenotypic shift from inflammatory M1-like TAMs to EGF-expressing M2-like TAMs activated EGFR signaling programs in malignant epithelial cells and resulted in tumor cell dissemination and outgrowth in the lung (DeNardo et al., 2009). Of note, this phenotypic switch in TAMs is well in line with the previously proposed concept of “smoldering” inflammation that characterizes tumor progression (Mantovani et al., 2008). On the other hand, a mouse model of permanent immunomediated metastatic dormancy showed a critical role for CD4⁺ T cells (but more prom-

inently for CD8⁺ T cells) in restraining the awakening of dormant tumor cells and the development of pulmonary metastases (Romero et al., 2014). Perhaps a better phenotypic characterization of these cells could clarify these apparently contrasting results. For instance, subgroups of CD4⁺FOXP3⁻ T cells are known to exhibit (as does their Foxp3⁺ counterpart) potent suppressor functions (Han et al., 2009), and facilitated melanoma metastasis *in vivo*, possibly via their inhibitory effect on NK cells (Wang et al., 2012).

A more general, although not complete (Wang et al., 2014), consensus exists on the pro-metastatic effect of T_{REG} cells, and an association between T_{REG} cells and incidence of metastasis has been reported in several cancer types, including NSCLC (Erfani et al., 2012), breast cancer (Metelli et al., 2016), and hepatocellular carcinoma (Ye et al., 2016). Inhibition of T_{REG} cells is likely to prevent metastasis, given the ability of T_{REG} cells to counteract cancer-killing immune cells from both the adaptive and innate immune systems (Chen et al., 2005; Ghiringhelli et al., 2005; McNally et al., 2011). Furthermore, T_{REG} cells supported directly mammary cancer metastasis by producing receptor activator of nuclear factor kappa-B ligand (RANKL). RANKL-stimulated pulmonary metastasis of RANK⁺ human breast carcinoma cells *in vivo* (Tan et al., 2011). A mechanism promoting metastatic development in the lung, involving the induction of T_{REG} cells and the concomitant limitation of CD4⁺-T-helper-cell- and CD8⁺-T-cell-mediated responses has been recently proposed (Clever et al., 2016). The oxygen-sensing prolyl-hydroxylase (PHD) proteins limit hypoxia-inducible factors (HIF)-driven glycolytic programs within T cells, thus inhibiting spontaneous CD4⁺ T helper differentiation while promoting T_{REG} cell commitment and restraining local CD8⁺ T cell responses, thereby establishing the lung as an immunologically permissive metastatic site (Clever et al., 2016).

Finally, it should also be noted that immune and non-immune (stroma and tumor cell)-derived chemokines and cytokines, apart from orchestrating the infiltration and function of the immune cells, also modulate the colonization capabilities of tumor cells, as broadly discussed elsewhere (Mantovani et al., 2008; Yao et al., 2016).

Concluding Remarks

The realization that the concerted action and interaction among all immune and non-immune components in space and time ultimately determine cancer initiation, development, and dissemination and bring along the need to reconsider the way cancers are classified. Conventional classification strategies are based on histopathological features of tumor cells, tumor morphology, tumor cell of origin, deregulation of tumor-cell-associated molecular pathways, mutational status, and tumor gene expression signatures, as well as on cancer progression (tumor [T]) and invasion of nearby tissues, lymph nodes (node [N]) and distant organs (metastasis [M]). The currently used TNM tumor staging is indeed based on these cancer-cell-focused characteristics. No immune parameter is yet used in clinical practice, even if their inclusion in this context has been advocated to better stratify cancer patients. This is despite evidence that the histopathologic parameters of tumor cells, tumor progression, and tumor invasion might be

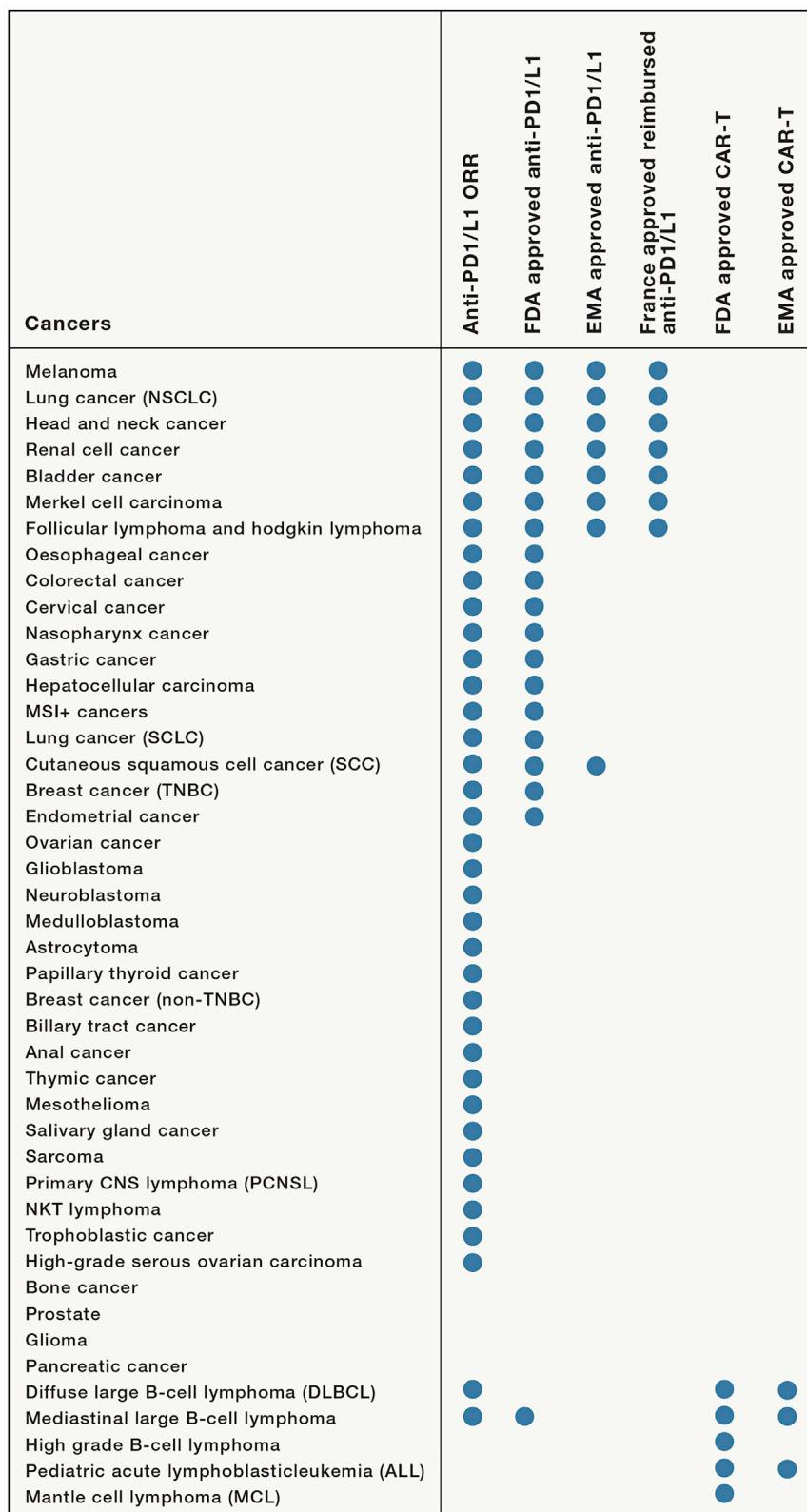


Figure 5. Cancer Types Responding to Anti-PD-1/PD-L1 Immunotherapy and to CAR T Cell Therapy

Abbreviations are as follows: ORR, overall response rate; FDA, food and drug administration; EMA, european medical association.

because of underlying differences in the nature and density of tumor-infiltrating adaptive immune cells (Galon et al., 2014; Pagès et al., 2018). Other immune-based prognostic signatures based on machine learning, which are improving the TNM staging, have also been recently proposed (Brieu et al., 2018). A revised, immune-including classification should take into account a multimodal combination of several parameters and biomarkers, possibly including, but not limited to: parameters of cytotoxic T cell response and T cell exhaustion, tumor mutational burden, immune gene expression signatures, and parameters of immune suppression (presence of immunosuppressive cells, such as T_{REG} cells and MDSCs). Fundamentally, the proposed models to guide tailored treatment options, such as the “cancer immunogram” (Blank et al., 2016), rely on the same valuable parameters and biomarkers to be adopted, in our opinion, to upgrade the current cancer classification to the era of cancer immunotherapy. An opening toward the introduction of immune parameters in clinical settings has been achieved after the ever-growing use of immunotherapy (Figure 5) with the adoption of immune-based response parameters, including immune-related adverse effects (irAEs) (Kottschade, 2018), immune-related response criteria (irRC) (Wolchok et al., 2009), irRECIST (immune-related response evaluation criteria in solid tumor) (Seymour et al., 2017). As the field progresses, it is likely that more powerful stratification systems will be implemented. Nonetheless, it would be reasonable to apply our current knowledge to better satisfy the immediate need for an improved prognostic and/or predictive information and ultimately guide clinical decisions. What these past two decades have established is not only the rebirth of tumor

determined by the immune components of the TME, and that the lack of accuracy of the TNM-staging-based prognosis among patients within the same tumor stage might be

immunology, and its rightful position as an academic entity, but more importantly as a gold mine from which to draw rational approaches to potentially defeat cancer.

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