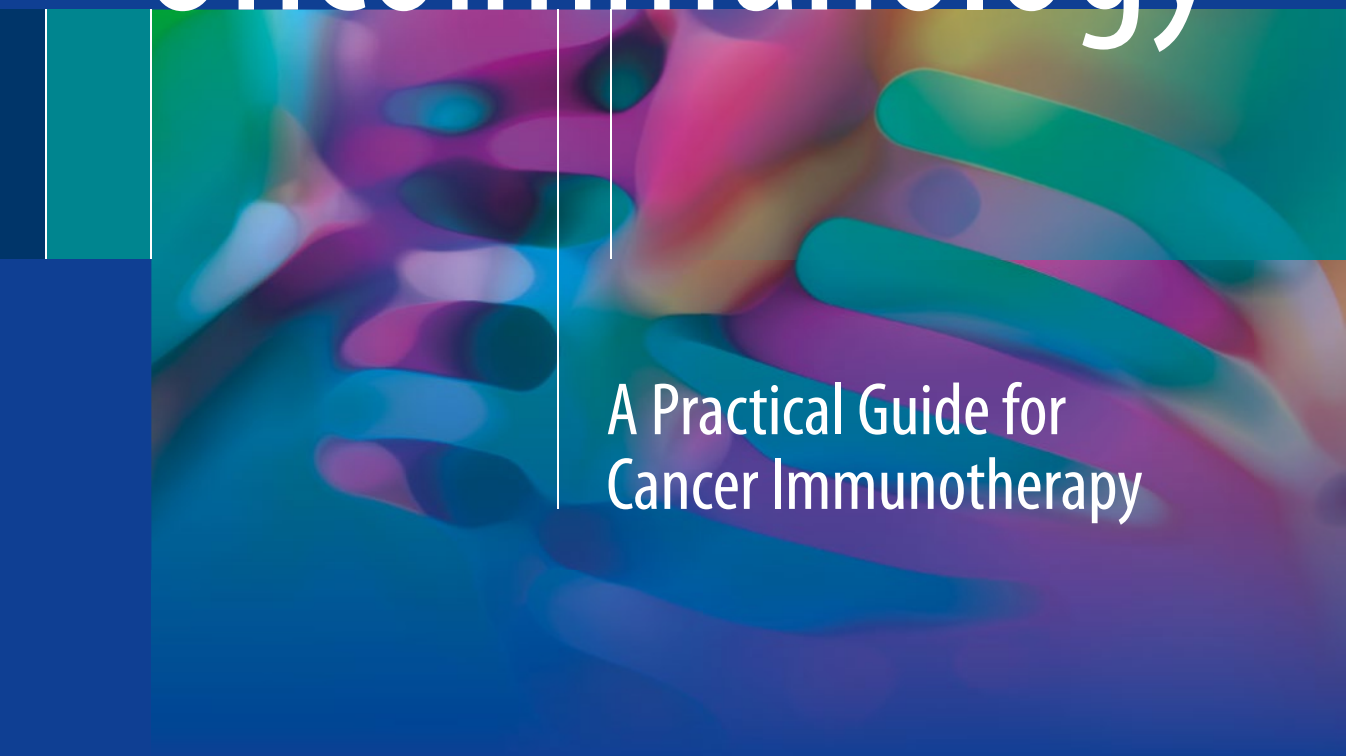
Laurence Zitvogel
Guido Kroemer
Editors

Oncoimmunology



A Practical Guide for Cancer Immunotherapy

Springer

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Our Reviewers

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And finally, we are, of course, grateful to all of the chapter contributors to this comprehensive, groundbreaking textbook in the important emerging field of immuno-oncology.

Laurence Zitvogel
Guido Kroemer

Contents

Principles of Oncoimmunology

1

Laurence Zitvogel and Guido Kroemer

The history of cancer research is marked by at least three phases that each are based on different methodologies and therapeutic strategies.

During the first phase that lasts from antiquity to the eighties of the twentieth century, cancer was considered as a cellular disease resulting from the invasion of tissues by abnormal cells. Hence, the main challenge consisted in excising the tumor with its margins to make sure that all cancer cells had been removed. In addition to mutilating surgical techniques, clinical oncologists have been applying cytotoxic agents to their patients, based on the consideration that proliferating cells had to be purged from the organism. Cancer drugs were identified by their capacity to kill cultured tumor cells in vitro and then administered to patients as "chemotherapies" at the maximum tolerated doses to obtain similar effects in vivo.

The second phase of cancer research is marked by the idea that malignant disease results from genetic and epigenetic aberrations affecting the cancer cell. This phase of research has been marked by the successful identification of tumor suppressor genes and oncogenes, the development of ever-refined tools to measure gene expression and to identify mutations in the cancer genome, to classify malignancies into different molecular subcategories, and to follow the clonal evolution of cancers as they form, progress, and escape from therapy. Driven by the identification of druggable oncogene products, a myriad of 'targeted' anticancer agents

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has been developed, heralding the era of "personalized" medicine. In this yet unattained utopia, identification of driver mutations in each patient's cancer would allow a tailor-made "precision" treatment.

The third phase of cancer research is based on the discovery that cancer is not just a genetic and epigenetic disease of aberrant cells, but that it also involves a constant struggle between malignant cells (and their precursors) with the immune system. The complex relationship between cancer and the immune system has been schematically condensed to the 3E hypothesis: initial *e*limination of malignant cells by innate or acquired immune effectors, later *e*quilibrium between cancer cells and the local immune response within an often indolent neoplastic lesion, and the final and fatal *e*scape of cancer cells from immune control. This latter event, which entails the clinical manifestation of the tumor involves the selection of non-immunogenic cancer cells (a process called "immunoselection" or "immunoediting") or active inhibition of the local immune response (a process called "immunosuppression" or "immunosubversion"). In this paradigm, it appears logical that anticancer treatments should be designed in a way that they reset the relationship between cancer and the immune system from escape to equilibrium or—ideally—elimination. Several events have lent support to this idea over the last decade. Thus, it has been discovered that the density, composition, architecture, and functional state of the immune infiltrate has a major prognostic and predictive impact on cancer. Multiple studies came to the conclusion that the relative success of chemotherapy and targeted therapy was based on the reinstatement of anticancer immunosurveillance, especially if the effects of therapy lasted beyond its discontinuation. Finally, a large panel of immunotherapies have been successfully developed and applied to patients, providing proof-of-concept that reinstating immune control leads to tangible and often spectacular clinical benefits.

Of course, it is too early to proclaim that cancer research has become victorious due to its recent paradigm change. Future will tell whether the actual triumph of immunotherapies will allow us to win the war against cancer or whether we will simply obtain a pyrrhic victory. The Editors and the authors of this textbook are optimistic about the final issue of our collective adventure.

We take this opportunity to thank Professors Pierre Galanaud and François Martin for their invaluable help in editing this book. Without their patient and constant support, this textbook would not have been printed.

Part I

Fundamentals in I-O

The Human Tumor Microenvironment

2

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2.1 Introduction

For a long time, cancer therapy has had as its sole objective the direct elimination of tumor cells. In case of nonmetastatic disease, this is accomplished by surgery, which removes the primary tumor. Radiotherapy and conventional chemotherapies also aimed at targeting tumor cells preferentially. The high capacity of tumor cells to divide as compared to the normal cells makes them more sensitive to agents that physically, in the case of radiotherapy, or chemically, in the case of chemotherapy, attack DNA and lead to cell death. Targeted therapies targeting mutations in tumor cells such as BRAF have been developed as well. However, these approaches also destroy the nonmalignant cells and/or have systemic consequences. To increase specificity toward the tumor cells, cytotoxic agents have been coupled to antibodies that bind to the tumor cells in order to allow their specific targeting to the tumor and not to the normal cells. However, the entry of such constructs into tumors still remains a major issue.

The progresses that have been accomplished in the field of tumor immunology in these last 20 years have led to a drastic change in the representation of primary tumors and metastases and to cancer treatments. Tumors are not anymore represented as a simple accumulation of cells that have undergone oncogenic processes but as a complex and dynamic structure made of tumor cells and inflamed tissue. Tumors are infiltrated with blood vessels that bring nutriments and all kinds of leukocytes inside the tumor and at its periphery, in the so-called tumor stroma that also contains matrix proteins such as collagen fibers. The transformation of a normal cell into a clinically detectable tumor can last for decades such as in the case of breast or colon cancers. Thus, tumors are dynamic structures that derive from this long process of carcinogenesis occurring in an inflamed and reactive tissue microenvironment.

Importantly, the last 20 years of intense research in the tumor immunology field unraveled the proof of concept of the immunosurveillance theory that was brought by McFarlane and Lewis Thomas in the 1950s (reviewed in [1]). These two scientists anticipated that immunosurveillance is a physiological mechanism that protects against nascent tumors. The description of immune cells with effector and memory functions within primary tumors and their metastases and the discovery of the correlation between their density at the site of the primary tumor and patient's survival more than 10 years ago unambiguously demonstrated that the immune system is capable of recognizing and eliminating tumor cells. The immune system uses the same basic mechanisms to fight against cancer as those used to eliminate viruses such as the influenza virus. Both the innate and adaptive arms of the immune system cooperate to mount an antitumor response leading to the development of effector $CD4^+$ T cells that produce cytokines, of effector $CD8^+$ T cells that kill the tumor cells and produce cytokines, and of B cells that differentiate into plasma cells that produce antibodies. Most importantly, so-called memory lymphocytes develop in parallel. All these cell types accumulate into tumors, and the memory lymphocytes circulate for a long time, with the possibility of transforming into effector lymphocytes very rapidly. They protect locally against tumor cells and systemically against metastatic cells that may escape from the primary tumor and circulate before nidation in distant organs, where they proliferate and become metastatic. An immune response is raised directed against tumor antigens. More than 15 years ago, it was proposed that tumors grow until an equilibrium is reached between tumor cells and the immune system. Only tumors, in which the tumor cell growth potential overcomes the pressure exerted by the adaptive immune response, can subsequently grow and metastasize into distant tissues. Indeed, tumor cells develop a series of mechanisms to evade the immune defenses including the downregulation of tumor antigens or the production of molecules that suppress immune functions. Therefore, tumor cells have long standing interactions with the immune system, especially in the microenvironment in the primary tumor and later in the metastases.

Finally, studies on the tumor microenvironment brought another major issue regarding the mounting and the regulation of the antitumor defenses. Immune cells were found to form aggregates at the tumor sites, mimicking those

found in inflamed tissues that reflect local consequences of a chronic antigenic challenge. A large body of evidences suggests that these so-called tertiary lymphoid structures play an important role to mount, maintain, and control the local and systemic immune defenses.

This deep knowledge of the antitumor defenses and of the composition of the tumor microenvironment brought a new paradigm for cancer treatment. Instead of targeting the tumor cells by using radiotherapy or chemotherapy, drugs targeting the tumor microenvironment have been developed. This major step in cancer therapy has been accomplished these last years. Drugs aiming to alleviate the immune defenses by unlocking the effector functions of the T cells, such as anti-CTLA4 or anti-PD-1 antibodies, have been developed. Other drugs targeting the tumor vasculature such as antibodies against factors favoring the growth of cells lining the blood vessels (vascular endothelial growth factor, VEGF) or molecules inhibiting the signaling pathways in the endothelial cells downstream VEGF (sunitinib) have been approved by the FDA for some cancers. Indeed the tumor microenvironment offers an array of potential new targets that can be used alone or in combination with the classical approaches preferentially targeting the tumor cells such as chemotherapy or radiotherapy which may also in some cases increase immune reactions to the tumors.

In this chapter, we will first describe the tumor natural history, how tumor cells progressively grow in a tissue that becomes inflamed, and how the tissue both facilitate the development of tumors and participate to their elimination. We will then describe the different cell types that are found in the tumor microenvironment, their function, their location, and their organization in human tumors. The prognostic impact of the different cell types of the tumor microenvironment will then be compared, and the immunotherapy approaches targeting the tumor microenvironment will be described.

Regarded for a long time as a genetic and cellular disease, cancer is now considered as a tissular and systemic disease whose outcome depends largely on interactions with the host, especially within the tumor microenvironment. The tumor microenvironment can promote or inhibit tumor invasion and metastasis. It changes during the course of the disease, and the understanding of this dynamic interaction makes it possible to identify new therapeutic prognostic factors and new therapeutic targets at all stages of the disease.

2.2 Cancer's Natural History

More than 40 year ago, Peter Nowell proposed that genetic alterations—induced by diverse mutagenic stimuli—could be responsible for the transformation of normal cells toward neoplastic states [2]. According to his theory, these random mutations confer cells with autonomous proliferative capacity and immortality. This concept has barely changed, and today we know that genetic instability is the hallmark initiating event of cancer cells. In fact, tumor cells acquire a series of mutations over time, and it is believed that the stepwise accumulation of genetic abnormalities eventually generate their malignant transformation. In average, a tumor cells exhibit 120 non-synonymous mutations [3] that not only confer them autonomous and uncontrolled proliferative capacities but also several other characteristics that allow them to survive in the hostile human body environment.

In 2011, Hanahan and Weinberg proposed the main hallmarks or essential characteristics that a cancer cells exhibit and allow them to self-support the development of a tumor mass [4]. With genetic instability and increased proliferative capacity leading the list, it is currently recognized that tumor cells also need to actively interact with surrounding endothelial, stromal, and immune cells, to guarantee their own survival. Thus, human cancers often promote angiogenesis and inflammation and commonly develop mechanisms to evade the immune system. While the stepwise acquisition of new mutations allows the development of these pro-tumoral functions, the pressure of the hostile environment leads to the selection of the more malignant and aggressive cell clones [5].

The cornerstone of tumor cell emergence and development is then genetic mutations, which can

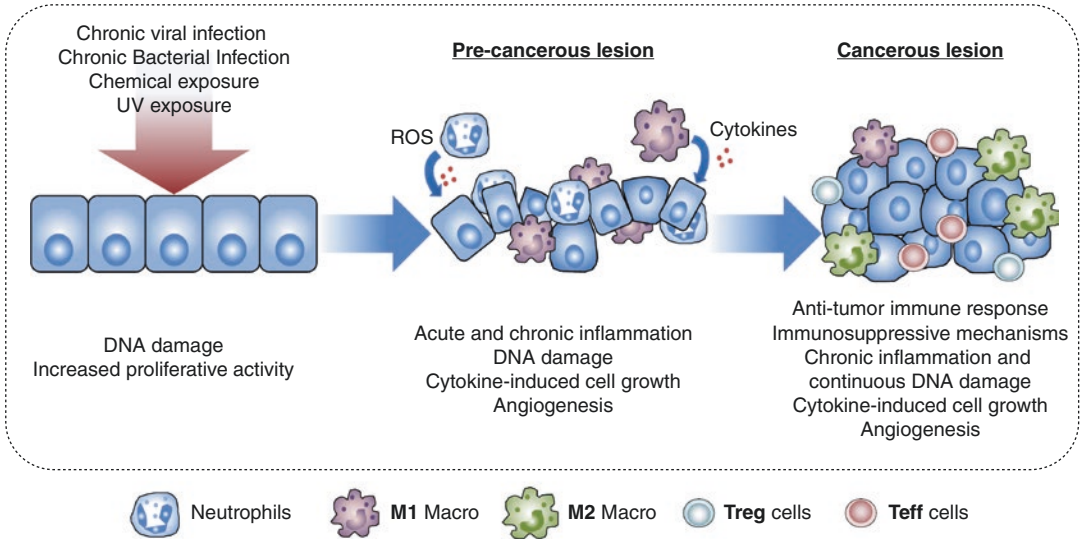

Fig. 2.1 Major immunopathological and genetic events occurring during carcinogenesis. Upon chronic inflammatory stimuli exposure, normal cells undergo transformation into precancerous cells. Local inflammation induces recruitment of myeloid-derived cells that fuel carcinogenesis via production of oxygen derivatives or cytokines. Later on, tumor growth and invasion into tissues are controlled by a balance between antitumor and immune escape mechanisms

be induced by diverse factors (Fig. 2.1). We are continuously exposed to mutagenic agent, such as UV light, pollution, or even viruses. Normal cells often possess efficient machineries that repair mutated DNA or intracellular cascades that promote cell death when the damages are irreparable [6]. Some hereditary diseases, such as xeroderma pigmentosum (associated with an extremely high risk of skin cancer at early ages due to defect in the DNA-repairing machinery), are examples of how important these proofreading systems are to prevent cancer development and how often we are exposed to mutagenic stimuli.

Inflammatory mediators are other well-known promoters of genetic alterations. In fact, many of the substances produced by the inflammatory immune cells (such as macrophages and neutrophils) can induce the direct damage of DNA in nonimmune cells. In the presence of noxious stimuli, chronic inflammation can both induce the development of driver tumorigenic mutations and promote the necessary genetic instability to allow other alterations to develop [7]. This process of cancer induced by chronic inflammation (Fig. 2.1) has been described in several pathologies, including gastric cancer in association with *Helicobacter pylori* infection, asbestos or cigarette smoke exposure and lung cancer, arsenic exposure and skin cancer, gastroesophageal reflux for cancer of the esophagus, inflammatory bowel disease for colorectal cancer, chronic pancreatitis for pancreatic cancer, and pelvic inflammatory disease for ovarian cancer [8].

Examples of inflammatory carcinogenic mediators include reactive oxygen species and matrix metalloproteinases, which can induce DNA damage and extracellular matrix disruption, respectively [9]. In addition, some cytokines can induce the growth of abnormal or preneoplastic cells, such as IL-1β for gastric carcinoma and IL-8 for melanoma. The preneoplastic potential of many other cytokines has also been described (e.g., IL-1β, IL-6, IL-23, and TNF-α).

In virus-related cancers, aside from the inflammation induced by the infection itself, the virus genetic material can integrate into the host genome and induce cell transformation by altering diverse oncogenic pathways [10]. Virus-associated cancers represent roughly 20% of all cancer types and include cervical cancer (induced by HPV), B cell

lymphoma (induced by EBV), Merkel cell carcinoma (induced by Merkel cell polyomavirus), hepatocellular carcinoma (induced by hepatitis B and C viruses), and some gastric cancer and H&N cancer (induced by EBV).

2.3 The Tumor Immune Microenvironment

As mentioned above, the tumor microenvironment is a very complex and dynamic ecosystem, where different cellular populations coexist. The major players include tumor, immune, and supporting cells (e.g., fibroblasts, stromal, and endothelial cells) [11]. Immune cells that circulate in the blood enter into tumors via transendothelial migration and are attracted by chemokines produced by tumor cells, fibroblasts, or inflammatory cells. Within the tumor mass, the immune cells locally proliferate, differentiate, exert their functions, and die, and some migrate back to the circulation. Within this population, one often can find cells related to acute inflammation (including neutrophils, basophils, and eosinophils), cells of the innate immune response (including macrophages, NK cells, and DC), and cells from the adaptive immune response (including cytotoxic $CD8^+$ T cells, Th1-/Th2-skewed T cells and B cells). We focused this subchapter in the last two populations.

2.3.1 Tumor-Associated Macrophages

Tumor-associated macrophages (TAM) represent an abundant population, and in many tumors they outnumber other immune cells [12]. Although the majority of TAM are found in the invasive margin of the tumor, we can often find also elevated densities within the tumor core [13]. TAMs exhibit an extremely plastic phenotype and function, and two main subtypes have been described: M1 TAM (induced by Toll-like receptor ligands [e.g., lipopolysaccharide and IFN-γ]) which preferentially express pro-inflammatory cytokines and inducible nitric oxide synthase and M2 TAM (induced by IL-4 or IL-13) which express arginase 1, CD206, CD163, IL-4R, TGF-β1, and PDGF [12]. Some works suggest that while M1 TAM potentiate the antitumoral Th1 response and antagonize the suppressive activities of regulatory immune cells, M2 promote angiogenesis, tumor growth, and metastasis [13].

2.3.2 NK Cells

Natural killer cells are cytotoxic effector lymphocytes of the innate immune system whose primary function is to help control infections and tumors [14]. Two major mechanisms of recognition of tumor cells by this population have been described: they can recognize cells which have downregulated major histocompatibility complex class I expression (an immunotolerance phenomenon widely described in many cancer types), or they can bind to stress-induced ligands expressed on tumor cells (e.g., MICA or MICB, which bind to NKG2D expressed on the NK cell) [14].

2.3.3 Dendritic Cells

The main function of dendritic cells (DC) is to establish a bridge between the innate and adaptive immune response. Under physiological circumstances, DC engulf and process nonself-antigens, and when they are exposed to danger or activation signals, they become activated and travel to secondary lymphoid structures in lymph nodes where they prime naïve B or T cells [15]. The DC phenotype is rather plastic, and they can produce a wide range of pro-inflammatory or immunosuppressive cytokines, as well as expressing a large series of activating or inhibition receptors, depending of the environment where they are embedded. The secondary lymphoid organs are protected environments and often provide an ideal milieu to promote a DC phenotype that effectively activates the adaptive immune response [16].

In many cancer types, tumor cells produce molecules that induce pro-inflammatory or tolerogenic DC and block their maturation at different stages.

Often, intratumor DCs exhibit an immature and inhibitory phenotype [17]. Interestingly, in recent years, several works have described the presence of tertiary lymphoid structures (TLS) in the invasive margin of many cancer types [18], where in theory the DCs are protected from tumor-produced inhibitory substances and from where they can effectively prime the antitumor immune response [19].

2.3.4 Tertiary Lymphoid Structures

TLS are highly organized lymphoid aggregates that develop in inflammatory pathologies. In cancer, TLS often develop in the invasive margin of the tumors and/or in the stroma and resemble those arising in other chronic infectious or autoimmune diseases [19]. Figure 2.2A illustrates TLS found in clear cell renal cell cancer (ccRCC). Characteristically, TLS exhibit an organization similar to secondary lymphoid organs, including a T cell zone (Fig. 2.2Aa) and a B cell follicular zone (Fig. 2.2Ab), and are often surrounded by high endothelial venules [20]. B cells in TLS form germinal centers; they undergo active proliferative machinery and somatic hypermutation [19]. T cells have a CD62L$^+$/CD45RO$^+$ central memory or a naïve phenotype, and some can be found in contact with mature DC which expresses the DC-Lamp marker (Fig. 2.2Aa) or at the periphery of B cell follicles (Fig. 2.2Ac) [20]. Follicular dendritic cells are also detected

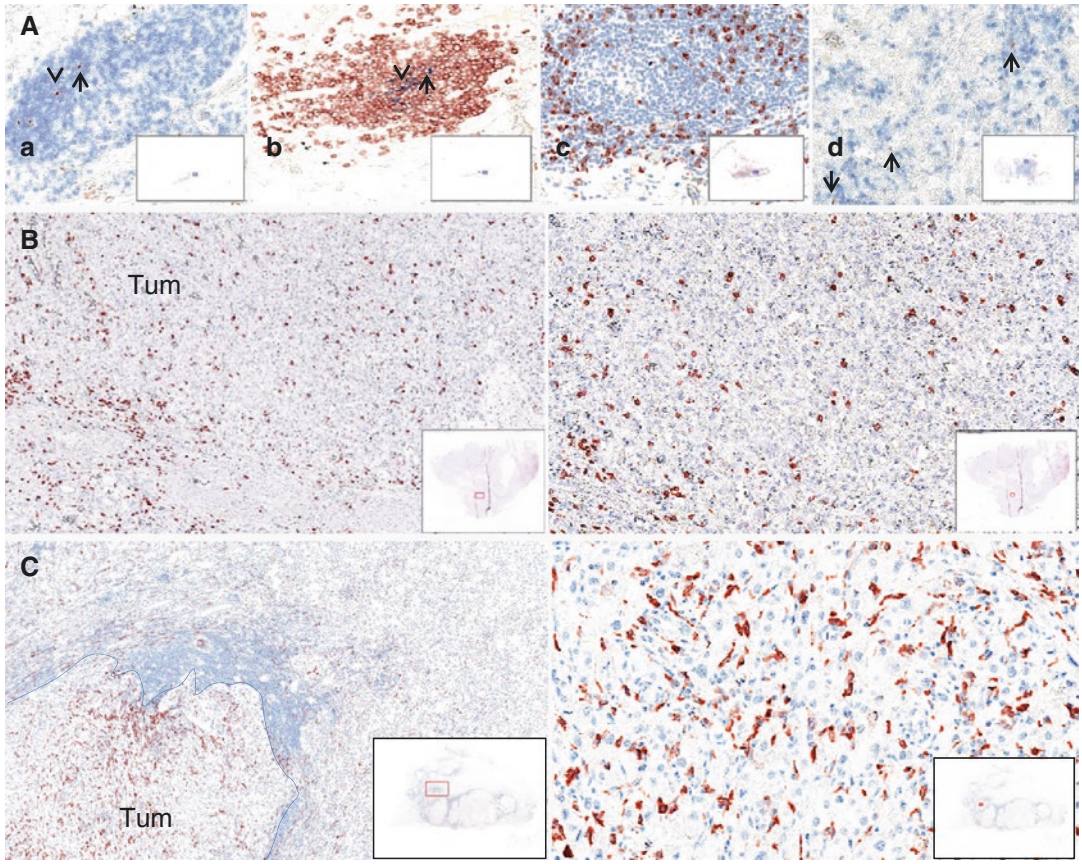

Fig. 2.2 The tumor microenvironment in human clear cell renal cell cancers as detected by IHC on paraffin sections. (**A**) Tertiary lymphoid structures: (*a*) DC-Lamp$^+$mature DC (*brown*) in the CD3$^+$T cell zone (*blue*); (*b*) CD20$^+$ B cells (*brown*) and CD21$^+$ follicular dendritic cells (*blue*) delineate the germinal center; (*c*) CD8$^+$ T cells (*brown*) are distributed around the germinal center; (*d*) non-TLS-DC-Lamp + DC (*brown*). (**B**) CD8 + T cells (*brown*) (*left* 5×, *right* 20×). (**C**) CD163+ macrophages (*red*) (*left* 5×, *right* 20×), Tum = tumor area

forming a network where immune complexes can form and be presented for selection of the high affinity B cells. Plasma cells that produce antibodies are located at the vicinity of TLS [21].

Primary tumors and metastases contain TLS at variable densities, depending on the tumor type and on the patient. As discussed below, it is assumed that TLS reflect the ongoing immune reaction within tumors. They allow the presentation of tumor antigens by mature dendritic cells to T cells leading to the differentiation of $CD4^+$ Th1 cells as reflected by the expression of the T-bet marker and the T-B cell cooperation for B cell differentiation into plasma cells. All of these events can thus occur locally, within the tumor bed. To what extent TLS bypass the need of secondary lymphoid organs to mount or control the antitumor immune reaction remains an open issue.

2.3.5 $CD4^+$ and $CD8^+$ T Cells

CD4+ T-helper cells are divided into different subtypes, including Th1, Th2, Th17, Tfh, and Treg; each subpopulation accomplishes specific roles in the antitumor immune response. Overall, a Th1-oriented response antagonizes the tumor growth and is often associated with good clinical outcome [22]. In fact, Th1-oriented cells potentiate in situ the antitumor function of cytotoxic T cells, through the production of several cytokines including IL-2 and IFN-γ. Tfh cells interact with B cells in TLS, helping antibody production.

The role of other subpopulations of tumor-infiltrating $CD4^+$ T cells (Th2, Th17, and Treg) is less well understood but is often associated with poor prognosis in different tumors [22]. Many studies suggest that Treg in cancer can dampen the antitumor immune response by two main mechanisms: (1) production of inhibitory cytokines (e.g., IL-10, TGF-β, and IL-35) and (2) suppression of DC development and maturation [23].

$CD8^+$ T cells exert a very import function in the antitumor immune response, as they are responsible of tumor cell recognition and elimination. Due to their genome instability, tumor cells often express mutant proteins at their surface. Many of these are neoantigens that can induce a tumor-specific immune response. The primed $CD8^+$ T cells are in charge of the tumor cells recognition and lysis, by mechanisms well described in the literature including the release of cytotoxic granules [24]. Interestingly, in the majority of tumors, infiltrating cytotoxic T cells express inhibitory receptors (e.g., PD-1, Tim-3, and Lag-3), whose function under physiological situations is to contract the immune response upon biding to their ligands. Many tumor cells in fact can take advantage of this inhibitory mechanism and in fact express a wide arrange of ligands (e.g., PD-L1, PD-L2) that help them escape for the T cell attack [25].

2.3.6 B Lymphocytes

In inflammatory settings other than cancer, B cells enhance T cell responses by producing antibodies and stimulatory cytokines and chemokines, serving as local antigen presenting cells and organizing the formation of TLS that sustain the immune response. In cancer, B cell can exert all of these functions and overall have an antitumor effect. In addition, recent evidence suggests they can also play an immunomodulatory role through the production of IL-10, among other cytokines [26].

2.3.7 Spatiotemporal Dynamics of the Tumor Immune Microenvironment

Chemokines ensure the local migration of these different cell types and cytokines allow their cooperation. In addition, many tumors are surrounded by a stroma containing an extracellular matrix composed of fibroblasts that form collagen fibers and produce enzymes—such as metalloproteases—that facilitate local invasion within tissues and ultimately the release of tumor cells that egress to the circulation and migrate in other tissues.

A direct consequence of these processes is that the tumor microenvironment is a tissue-dependent organized structure in which immune cells are common denominators. Figure 2.2B illustrates the presence of $CD8^+$ T cells in the

tumoral zone of clear cell renal cell cancer. A closer look into the organization of the immune microenvironment reveals that cells are not evenly distributed in the tumor area. Lymphocytes (T and B cells) are more abundant in the tissue border area called the invasive margin than in the center of the tumor [13]. They can be found dispersed or within aggregates, forming TLS in the invasive margin and/or in the stroma [18, 27]. Most of the T and B cells have a memory phenotype, $CD8^+$ T, $CD4^+$ Treg, Th1, Th2, Th17, and B cells being detected at variable densities, whereas naïve T cells and $CD4^+$ Tfh are exclusively present within TLS. NK cells are detected in the tumor stroma. Some T cells are found in close contact with tumor cells in the center of the tumor. Myeloid cells such as macrophages, myeloid-derived suppressor cells, mast cells, and neutrophils are present at high densities, both in the invasive margin and the center of the tumor. Figure 2.2C illustrates the high density of $CD163^+$ M2-oriented macrophages near the invasive margin of renal cell cancer. Immature dendritic cells are present at low densities, dispersed in the whole tumor area whereas mature dendritic cells are usually found within the TLS, in close contact with T cells (Fig. 2.2Aa). Importantly the immune composition of the tumor microenvironment evolves with the stages of tumor progression in a tumor-dependent manner. Thus, T cells are more numerous at the early stages of the disease in colorectal cancers and at their late stages in renal cell cancers [17, 28]. The density of B cells increases with tumor stage in colorectal cancers, as does that of the myeloid cells such as neutrophils, mast cells, immature dendritic cells, and macrophages. Thus, the tumor microenvironment is a complex structure, forming a tumor-dependent "immune landscape" that evolves during tumor progression.

2.4 The TME Dictates Clinical Outcome for the Patients

Quantification of immune infiltrates and its relationship with prognosis has been studied for more than 20 years. Following the observation that high T cell densities correlate with longer survival in ovarian cancer [29], the Galon, Pagès, and Fridman studies demonstrating for the first time in large cohorts of patients with colorectal cancers (CRC) the association between densities of memory T cells, early signs of metastasis, and patient's survival made a significant breakthrough in this field [28, 30]. Since then, important progresses in immunohistochemistry (IHC) with the multiplication of robust antibodies, the development of high through put technologies and of automated quantitative imaging has led to numerous studies on immune cell composition of the TME. This real enthusiasm was even more pronounced during the last 5 years with the emergence of checkpoint blockade therapy (CBT), which aims at reversing T cell exhaustion. Thus, T cell abundance in the TME and its link with outcomes and/or response to CBT is under intensive work by many teams worldwide.

2.4.1 T Cells

2.4.1.1 $CD8^+$ T Cells

T cell abundance within the TME has been extensively studied across the majority of tumor types. Our group published in 2012 a comprehensive review of the number of original articles linking immune cell populations infiltrating the tumor and prognosis [11]. We reported that high densities of $CD3^+$ T cells, $CD8^+$ cytotoxic T cells, and $CD45RO^+$ memory T cells were associated with a longer disease-free survival (DFS) and/or overall survival (OS) in most tumors (including melanoma, head and neck, breast, bladder, urothelial, ovarian, colorectal, and lung cancer) [1]. We noted at that time that clear cell renal cell carcinoma (ccRCC) was one of the rare exceptions to the rule. We updated these data last year and found similar results. In addition, we reported new tumor types such as GIST, biliary tract, thyroid, or oropharyngeal cancers where $CD8^+$ cell infiltration was associated with a good prognosis [22].

The poor prognostic value associated with $CD8^+$ T cells in ccRCC was confirmed by our group, both in kidney primary tumors [17] and in ccRCC lung metastases [31]. Besides ccRCC, studies in lung adenocarcinoma [32] and in HCC [33] also reported a poor prognostic value

associated with increased CD8+ T cell infiltration, in contradiction with other published studies. In prostatic adenocarcinoma as well, CD8+ T cell densities correlate with poor outcome [34], consistent with our own data [35].

The "Classical" Case of CRC

Colorectal cancer is the archetype of tumors where high CD8+ T cell densities are associated with good prognosis. Indeed a high infiltration of CD8+ T cells, particularly effector memory subtypes (TEM), is correlated with a low probability of metastatic spread and prolonged PFS and OS [28], suggesting T cells may control local invasion in primary tumors and confer a long-term systemic protection against metastasis. Moreover, IHC studies showed that compartmentalization of T cells in the center and the invasive margin of the tumors does matter. An immunoscore (IS) measures the density of CD3+ and CD8+ T cells in the center, and the invasive margin of the tumors has been developed by Jerome Galon's team and has been validated in a worldwide collaboration approximately 4000 CRC patients [36, 37]. Even if a high T cell density was more frequent in smaller tumors and MSI-positive tumors, the prognostic value of IS was independent from TNM stages and MSI status. Moreover IS was more accurate to predict the prognosis of patients with early stage CRC [37, 38].

The Discordant Case of ccRCC

We recently reported a clear negative association between CD8+ T cell infiltration and outcomes in ccRCC [17]. Within a cohort of 135 patients with available primary RCC tumors, we found that a high density of CD8+ cells, as assessed by IHC, was associated with a shorter disease-free survival and OS. These results were validated for OS in an independent cohort of 51 patients with (resected) lung metastases of ccRCC. The underlying mechanism for this poor prognosis value of CD8+ T cells is not fully understood. We showed that most of the intratumoral T cells have an exhausted phenotype, which may reflect impaired antigen presentation due to the presence of dysfunctional DCs with an immature phenotype (Fig. 2.2Ad). They express the DC-Lamp marker of mature DC but lack the high levels of MHC class II molecules and CD83 expressed by mature DC. They may be involved in the impairment of T cell antitumor response [17]. Consistently, in patients who have a higher density of DC within TLS, a high density of CD8+ was associated with good prognosis. Thus, antigen presentation by mature DC in the TLS seems to be a crucial event to drive antitumor response in ccRCC, in accordance with our previous observations in lung cancers [39]. Moreover, we showed by immunofluorescence (IF) that CD8+ T cells express immunoregulatory receptors such as PD-1 and/or LAG-3, suggesting a highly exhausted phenotype and both associated with poor outcomes [17].

2.4.1.2 CD4+-, Th2-, and Th17-Oriented T Cells

Consistent with CD8+ T cell infiltration, an increased in Th1-oriented CD4 T cell infiltration has been associated with favorable prognosis in almost all tumor types studied including breast cancer [40] or CRC [41].

Prognostic value of other T cell subsets (Th2, Th17) has been far less investigated first because of a low frequency in the majority of the tumors and second because of technical challenges to specifically identify these subsets.

2.4.1.3 Regulatory T Cells (Tregs)

The example of Tregs is eloquent. A high Treg density has been first associated with poor prognosis in ovarian cancer, which has been then confirmed in a variety of tumors such as in breast, lung, melanoma, or colorectal cancers (reviewed in [42]). Nevertheless, other studies reported longer survival associated with high densities of Tregs in colorectal, bladder, head and neck, or ovarian cancers. One of the reasons for these opposite results is the difficulty to identify the Treg population. Tregs are a heterogeneous population that should be ideally identified by a combination of markers (CD4+, CD25+, Foxp3+, T cells). The development of multicolor fluorescence imaging allows to increase the number of cell surface markers for their detection. Beyond the technical challenges, these results highlight that the prognostic impact of

immune cell populations depend on the tumor type and on the TME.

2.4.2 B Cells

The positive or negative role of B cells in antitumor immunity has been discussed for many years, mainly supported by mice studies. As compared to T cells, few clinical studies reported the prognostic role of intratumoral B cells. The majority of clinical studies have demonstrated that a high density of B cells within TME is associated with better prognosis including breast cancer [43], NSCLC [21], head and neck cancer [44], ovarian cancer [45], metastatic colorectal cancer [46], biliary tract cancer [47], and primary cutaneous melanoma [48]. Several nonexclusive mechanisms could explain the positive role of B cells in the antitumor immune response, some being antibody dependent by their capacity to trigger complement and antibody-dependent cell cytotoxicity (CDC and ADCC) or to form immune complexes able to activate DCs and others by acting as APC for CD4 [49] and $CD8^+$ T cell immune responses [50]. Indeed, it has been shown that B cells play a major role during initial priming and expansion of $CD4^+$ T cells [51], are able to cross-present antigens to $CD8^+$ T cells [52], and can promote cytotoxic T lymphocyte survival and proliferation [53].

On the opposite, few clinical studies reported a pro-tumoral role of B cells within the TME [54, 55]. B cells may play a pro-tumor function by the maintenance of a chronic inflammation [56], by the promotion of neoangiogenesis [57], and/or by the direct inhibition of cytotoxic T cell responses [55]. Moreover, a subpopulation of immunoregulatory B cells called "Bregs" has been described and has been shown to favor the differentiation and the recruitment of Tregs, thus amplifying the immunosuppressive environment [58].

Beyond the density of B cells, an increasing number of studies reported that the spatial localization of these cells have an impact on patient's outcome. In particular the density of B cell follicles characteristic of TLS is positively associated with outcomes. M.C. Dieu-Nojean and col. showed that an increase in B cell density within the TLS is associated with prolonged survival in NSCLC patients [21]. Similar results were reported in CRC [59] and oral squamous carcinoma [60].

2.4.3 Macrophages

Tumor-associated macrophages (TAM) are a major component of the TME, found both at the tumor core and the invasive margin. The prognostic value of TAM seems to be dependent of the tumor type. Increased density of TAMs is associated with a good prognosis in CRC [61], HCC [62], prostate [63], and cervical cancer [64]. At the opposite an increased TAM density is associated with poor prognosis in endometrial [65], gastric [66], urothelial [67], HCC [68], melanoma [69], breast [70], ovarian [71], bladder [67], NSCLC [72], and primary CRC tumors [13]. These discrepancies might be explained by the plasticity of these cells since we know that they can switch from a pro-tumoral function (M2) to an antitumoral function (M1) and vice versa [12]. M2 TAMs are associated with a shorter survival and M1 TAMs with a longer survival [22]. Unfortunately, there are no specific or consensual markers to define M1/M2 TAMs. Most of the studies used CD11c or NOS2 for M1 TAMs and CD163, CD204, or CD206 for M2 TAMs, but the use of these markers is still debated.

Tumors contain another heterogeneous subset of cells of myeloid origin, the myeloid-derived suppressor cells (MDSC). Such cells have an immature phenotype and exert profound immunosuppressive activities. Specific and robust tools are still needed for their identification in the human TME.

2.4.4 New Techniques to Estimate the Immune Cell Populations in Tumors

The most broadly used way to quantify tumor-infiltrating immune cells is to detect the protein expression of specific markers either by IHC or

IF. These techniques have been improved in the last decade, allowing to detect multiple proteins (multiplex IHC or IF) and to quantify cells automatically. Nevertheless, they remain expensive and difficult to standardize across laboratories, and available antibodies could lack sensitivity or specificity to accurately detect some of immune cell populations.

Efforts have been made to use transcriptome to estimate the composition of the TME. Nevertheless, variability in the signal has limited its applicability until recently. New methods such as CIBERSORT [13] or MCP-counter [73] aim at providing very precise quantitative information about the cell content of heterogeneous samples. Using MCP-counter, we estimated the abundance of immune cells, fibroblasts, and endothelial cell infiltrates, in transcriptomes of 25 different cancers (n = 19,000). The results showed the relative heterogeneity of the cellular composition of the tumor microenvironment in different cancers and confirmed that the inferred density of $CD8^+$ or cytotoxic T cells correlated with favorable prognosis in most cancer types [73] (Fig. 2.3).

Fig. 2.3 Estimation of the abundance of infiltrating immune and stromal cells and their prognostic significance across human solid tumors. *Left*, means of MCP-counter scores across malignant tissues (more than 19,000 tumors) in three transcriptomic platforms. *Right*, univariate prognostic values (overall survival) associated with MCP-counter scores in human solid tumors. *Green* represents significant favorable prognostic impact and *purple* significant poor prognostic impact. *Gray* represents no significant prognostic impact. Adapted from Becht E et al., Genome Biol. (2016) [73]

2.5 TME as Predictors of Response to Therapy

After decades of having targeted on tumor cells and their molecular alterations, new immuno-oncology (IO) agents such as CBT have shed a light on the crucial role of the TME. The currently approved CBT targets are CTLA-4 (ipilimumab) or the PD-1/PD-L1 axis (nivolumab, pembrolizumab, atezolizumab avelumab) [74]. These mAb block the negative signal received by T cells after their interactions with APCs or with tumor cells, thus being able to reverse T cell exhaustion.

As the main target of these agents are T cell infiltrating the tumor, efforts to predict CBT efficacy have been focusing on their characterization in terms of density, localization, phenotype and functionality, before and/or during treatment.

Other well-known and debatable candidates are still investigated as a "biomarker of efficacy" such as PD-L1 expression by IHC or the neoantigen/mutational burden, but are outside the scope of this chapter [75].

2.5.1 First Emerging Data from Checkpoint Blockade Treated Patients

2.5.1.1 Tumor-Infiltrating Lymphocytes

With the growing number of patients treated with anti-PD-1/PD-L1, translational data on the pharmacodynamics effect of these therapies on the TME are emerging. Tumeh et al. reported in patients with melanoma a higher density of CD8 TILs at baseline in responding patient to pembrolizumab (anti-PD-1) [76]. As with ipilimumab, serial biopsies on treatment showed an increased density of $CD8^+$ TILs in the responding group. In another exploratory study 53 melanoma patients who first received ipilimumab and then anti-PD-1 (pembrolizumab) at progression were serially biopsied before and on treatment. IHC analyses of the TME revealed that the increase of $CD8^+$ TIL density early on treatment was associated with response to ipilimumab, whereas baseline TIL density was not [77]. For the 46 patients who subsequently received anti-PD-1 after progression on ipilimumab, there was a statistically significant difference in the density of $CD8^+$, $CD3^+$, and $CD45RO^+$ T cells in pre-treatment samples of responders compared to nonresponders. In addition a very highly statistically significant difference in the expression of markers for T cell subsets—CD8, CD4, and CD3—and immunomodulatory molecules PD-1 and LAG3 was observed in early on-treatment tumor samples of responders versus nonresponders to therapy. Altogether these results highlight the unlocking effects of CBT on T cell response. In addition, the authors reported an increase in the ratio of $CD8^+$ TIL in the tumor center (TC) vs the IM in early on-treatment biopsies within responders compared to nonresponders suggesting an infiltration of the TILs from the IM to TC as a consequence to therapy [77]. Finally, IHC results were confirmed by gene expression analyses.

Another group performed the phenotypic analyses of TILs (flow cytometry) at baseline from 40 patients (discovery cohort and validation of 20 patients each) with metastatic melanoma treated with an anti-PD-1 [78]. CTLA4 expression by TILs was the only parameter significantly associated with a clinical response in multivariate analysis. The response rate (RR) and PFS were significantly correlated with the relative abundance of CTLA-4^{hi}PD-1^{hi} $CD8^+$ TILs.

In a multi-cohort phase I study of patients treated with atezolizumab (anti-PD-L1), both increased density of CD8 by IHC and high Teff signatures (genes regulated by interferon gamma (IFNg), including IFNg, CD8A, granzyme A, granzyme B, EOMES, and perforin) correlated with response in melanoma, but no association with clinical benefit was observed in RCC [79]. However, a higher ratio of Teff to Treg as revealed by gene expression was associated with atezolizumab response in RCC.

A translational study dedicated to investigate how VEGF blockade with bevacizumab could potentiate PD-L1 checkpoint inhibition with atezolizumab in mRCC was recently reported [80]. The authors showed that bevacizumab alone tends to increase the gene signatures associated with T-helper 1 (Th1) chemokines and CD8 T effectors, and the combination with atezolizumab further increases expression of these signatures. IHC showed similar results with an increase of $CD8^+$ density following bevacizumab, which was more pronounced with the combination. Interestingly the increased density of $CD8^+$ TILs seemed to reflect an increased trafficking into the tumor rather than

an in situ increased proliferation (unchanged ratio of Ki67+/Ki67- among CD8⁺ TIL) [80].

2.5.2 From the Molecular to the Immune Signatures

Escape to the immune surveillance has been proposed as an important mechanism of resistance to a number of systemic therapies including targeted therapies such as antiangiogenic agents [81]. Indeed, immune escape is one of the main mechanisms of resistance to VEGFR-TKI in ccRCC [82]. It was recently reported that metastatic ccRCC treated with sunitinib (VEGFR-TKI) could be classified into four distinct molecular groups (ccrcc1 to 4) using transcriptomic analysis [83]. The four groups had significantly distinct prognosis with ccrrcc1 and 4 having the poorest survival and response to sunitinib. Interestingly we found that immune cell infiltrates were different according to molecular groups [84]. For instance ccrcc4 tumors were the most highly infiltrated in T cells and had the highest expression of immunosuppressive markers such as PD-L1, PD-1, LAG-3, TIM-3, suggesting exhaustion of T cells within these tumors. Conversely, ccrcc1 tumors, which were also associated with poor prognosis, had the poorest T cell infiltration and a low expression of T CB markers. As the density of CD8⁺ infiltrating the tumor has been associated with CBT efficacy, we made the hypothesis that ccrcc4 could respond to PD-1/PD-L1 blockade alone. In contrast an anti-PD-1/PD-L1 alone might not be fully efficient in ccrcc1 due to the lack of CD8 T cells in the tumor. Another therapy able to attract T cells in tumors such as an angiogenesis inhibitor (VEGFR-TKI or anti-VEGF mAb) or CTLA4 blockade could sensitize tumors to anti-PD-1/PD-L1 therapy.

We therefore hypothesize that combination of molecular and immune signatures might be a better predictor of CBT efficacy than each signature alone. Figure 2.4 shows an example of an integrated view

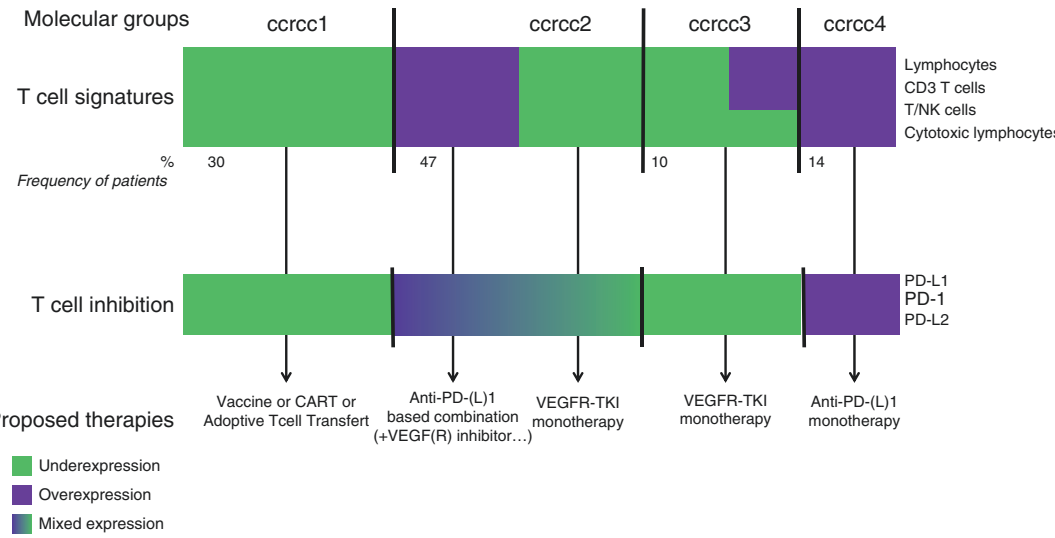

Fig. 2.4 Integrative view of biomarker-driven treatment: example of ccRCC. Using a 35-gene classifier, molecular grouping according to Beuselinck et al. [83] identified four groups of patients (ccrcc1 to 4) with distinct response to sunitinib, ccrcc3 having the best response to sunitinib. The ccrcc molecular groups have different gene expression immune profiles: immune-desert (enriched in ccrcc1), immune-competent (enriched in ccrcc3), immune-high (enriched in ccrcc4), and mixed (enriched in ccrcc2) tumors. CD8⁺ T cell infiltration evaluated by immunohistochemistry confirmed these four phenotypes [83]. T cell inhibition signatures based on the gene expression of immunoregulatory checkpoints and their ligands refine the four immunophenotypes and provide additional information to drive patient and treatment selection. ccrcc1 tumors are immune-desert and patients may benefit from a T cell attractant-based therapy such as vaccine or CAR-T cell or adoptive T cell transfer; ccrcc4 tumors are immune-high with a high density of T cells and high expression of immunoregulatory checkpoints; ccrcc4 patients may benefit from anti-PD-(L)1 alone. ccrcc3 tumors are immune-competent with a high infiltration of T cells but low expression of immunoregulatory checkpoints; VEGFR-TKI alone provides excellent results in this ccrcc3 group of patients [83]. ccrcc2 tumors are mixed in terms of T cell infiltration as well as expression of immunoregulatory checkpoints; ccrcc2 patients may be treated according to T cell infiltration and expression of immunoregulatory checkpoints

of how to combine multiple biomarkers to drive patient selection in ccRCC.

To confirm these hypotheses, we launched in March 2017 the first biomarker-driven trial to date in ccRCC called BIONIKK (BIOmarker-driven trial with Nivolumab and Ipilimumab or VEGFR tKi in naïve metastatic Kidney cancer, NCT02960906) [85]. This trial randomizes mRCC patients to receive a first line of systemic therapy with nivolumab (anti-PD-1), ipilimumab (anti-CTLA4), the combination, or a TKI according to their molecular subgroup. The primary endpoint is the objective response rate according to therapy and molecular groups. Immune infiltrates and their correlation with outcome and molecular groups will be evaluated using IHC and gene expression analyses (MCP-counter).

Conclusion

The findings of complex interactions between tumor cells and the host has led to define the concept of the immune contexture which include organization, location, density, and functional orientation of immune cells in the TME. This immune contexture helps to understand pathophysiological mechanisms that support the clinical impact of various cells of the immune response [86].

The growing approval rate of CBT targeting the PD-1/PD-L1 axis through many tumor types stimulates research teams worldwide to go deeper in the comprehension of the immune contexture to better optimize the efficacy of these agents. In addition, the high number of IO agents currently evaluated in clinical trials provides a huge competition between companies which in turn force them to understand the importance of selecting patients and to make financial efforts to support translational studies.

Many efforts are currently done to find a way to select patients who will have a durable benefit from CBT. Characterization of the tumor-infiltrating immune cells may provide one of the most promising biomarkers of efficacy. Nevertheless, some technical challenges explain why such promising biomarkers are not reproducible or difficult to assess. One of these challenges is inherent to the technique of IHC or IF. Even if major advances have been made on this field, we have to deal with high intratumor heterogeneity and lack of specific markers and to interpret a static evaluation of a dynamic process. The first two points could be partially resolved by the progress in transcriptomic analyses and particularly in the immune signatures that were recently developed such as in MCP-counter. It provides a high accuracy in defining the proportion of immune cells, is reproducible, is less dependent to tumor heterogeneity, and finally allows to compare between tumor types.

Characterization of the immune TME together with the deep characterization of malignant cells using next-generation sequencing (NGS), RNA sequencing, as well as multiplex IF will allow to treat patients with the most appropriate precision medicine and to closely monitor the dynamic changes during CBT.

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CD8+ T Cells in Immunotherapy, Radiotherapy, and Chemotherapy

3

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Contents

3.1 Introduction

Extensive studies have revealed that the infiltration of T cells, especially CD8+ T cells, into the tumor microenvironment is a favorable prognostic feature for numerous malignancies, including melanoma and head and neck, breast, ovarian, renal, bladder, urothelial, colorectal, prostatic, pancreatic, and lung cancers [1]. A high density of intratumor CD8+ T cells is associated with longer disease-free survival and overall survival. Interestingly, direct tumor contact by CD8+ T cells may not be required, as both intraepithelial and intrastromal CD8+ T cells are associated with a favorable prognosis in human breast and ovarian cancer [2–4].

CD8+ T cells are often referred to as cytotoxic T lymphocytes (CTLs) because of their

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ability to directly kill target cells. Upon antigenic stimulation, CD8+ T cells will progressively differentiate from naïve T cells into central memory T cells (T_{CM}) and effector memory T cells (T_{EM}). The effector function increases upon CD8+ T-cell differentiation, while memory function and proliferation decrease. Effector CD8+ T cells are characterized as CCR7− CD62L−CD45RO+CD95+IL-2Rb+, in addition to expressing killer cell lectin-like receptor G1 (KLRG-1) and programmed death 1 receptor (PD-1). They secrete high concentrations of IFNγ and TNFα but secrete minimal IL2. Trafficking of CD8+ T cells is mediated through chemokine-chemokine receptor interaction, which, among others, includes the ligands CXCL9 and CXCL10 with their receptor CXCR3. Increased expression of CXCL9/10 is associated with increased number of CD8 T cells in tumor microenvironment [5, 6].

CD8+ T cells are a key component of antitumor immunity and execute tumor clearance by several mechanisms. First, CD8+ T cells recognize the specific tumor-associated antigen [7] expressed on tumor cells, release cytotoxic molecular granzyme B and perforin, which are delivered into tumor cells and induce caspase activation and ultimately apoptosis [8]. Secondly, CD8+ T cells can also induce cancer cell death through the Fas/Fas ligand pathway. It has been demonstrated that the Fas ligand is essential for tumor regression mediated by CD8+ T cells in murine models of lung cancer and B-cell lymphoma [9, 10]. Finally, IFNγ and TNFα secreted by CD8+ T cells can have antitumor activity and control tumor growth. The combination of IFNγ and TNFα can drive cancer cell into senescence [11]. IFNγ is also known to be critical for cancer immunosurveillance by enhancing antigen presentation and limiting tumor angiogenesis [12].

Although tumor-reactive CD8 T cells are often found in the tumor biopsies, cancer can still progress. It has been revealed that the immunosuppressive tumor microenvironment may drive CD8 T cells into senescence or exhaustion [13]. Senescent CD8+ T cells are characterized by short telomeres, activation of DNA damage response genes, and secretion of senescence-associated secretory phenotype (SASP) factors [14]. These cells phenotypically show downregulation of the co-stimulatory molecules CD27 and CD28 and high expression of CD57 and KLRG1. Although senescent T cells are irreversibly cell-cycle arrested, they may still retain their cytotoxic capacity [15]. Exhausted CD8+ T cells are described as cells that exhibit defects in proliferation and decreased cytokine production and cytotoxic functions, as well as display higher expression of co-inhibitory molecules, such as PD-1, CD244, CD160, CTLA-4, Lag-3, and Tim-3 [16, 17]. However, it is notable that CD8+ T cell exhaustion is reversible to some extent [18]. Blockade of CTLA-4 or PD-1 has been shown to improve CD8+ T-cell effector function, resulting in improved clinical response. In addition, adoptive transfusion of ex vivo-expanded tumor-specific T cells, especially CD8+ T cells, has achieved durable tumor remission and even cure of malignant disease. Interestingly, recent studies have revealed that antibody-based targeted therapy, radiotherapy, and chemotherapy may synergistically initiate or augment antitumor immune response. The antitumor efficacies of these therapies are at least partially dependent on CD8+ T-cell immunity.

This review focuses on the convergence of adoptive T-cell transfer, checkpoint blockade, antibody-based targeted therapy, radiotherapy, and cytotoxic chemotherapy on effector CD8+ T cells. We summarize the state of knowledge regarding how these therapies increase intratumor CD8+ T-cell infiltration, induce tumor antigen-specific CD8+ T-cell response, unleash CD8+ T-cell effector function, and sensitize tumor to CD8+ T cells. Finally, we discuss how to rationally combine immunotherapy with radiotherapy or/and chemotherapy to improve cancer patient outcomes.

3.2 CD8+ T Cells in Immunotherapy

3.2.1 Adoptive T-Cell Transfer

Adoptive T-cell therapy (ACT) for cancer is a form of transfusion therapy consisting of the infusion of various ex vivo-expanded T-cell populations. The first strategy of ACT, which has been the most extensively studied in clinical trials, is the adoptive transfer of autologous ex vivo-expanded tumor-infiltrating lymphocytes (TILs). More recently, transfer of genetically modified T cells is being developed and clinically utilized. This approach includes the utilization of peripheral blood lymphocytes (PBLs)-derived T cells expressing TAA-specific T-cell receptor (TCR) or a so-called "chimeric antigen receptor" (CAR) T cells [19, 20].

CD8+ T in Tumor-Infiltrating Lymphocytes Therapy The general protocol of ACT includes (1) collection of circulating or tumor-infiltrating lymphocytes, (2) selection and expansion of tumor-specific T-cell populations ex vivo, and (3) re-administration of T cells to patients with a conditioning regimen of lymphodepletion and IL-2 administration. Thus far, ACT of ex vivo-expanded TILs is considered to be the best available treatment for patients with chemorefractory metastatic melanoma [21, 22]. Following the harvesting of TILs from the patient, long-term ex vivo IL-2 and CD3 stimulation are used to expand CD4+ and CD8+ αβ TCR+ T cells [21]. As CD8 interacts with MHC class I expressed on tumor cells, CD8+ T cells are thought to effectuate the antitumor activity of ACT, although indirect CD4+ T-cell interaction with the tumor cannot be dismissed. A recent clinical study described three sequential trials on metastatic melanoma treated with the ACT of autologous TILs combined with lymphodepletion and IL-2. Objective response rates in the three trials using different lymphodepleting preparative regimens ranged from 47% to 72% [22]. Furthermore, the number of infused CD27+ CD8+ cells was found to correlate with objective response [22]. This corroborates other melanoma ACT trials that have also found a positive correlation between a higher number of infused CD8+ T cells and clinical response [21, 23].

Nonselective expansion of polyclonal tumor-infiltrating T cells results in a population that recognizes multiple tumor-associated antigens. These antigens include cancer testis antigens that are expressed during development and reactivated in tumors, such as NY-ESO-1 and MAGE; melanocyte lineage antigens, such as gp100, MART-1, and tyrosinase; and mutational antigens generated from the low-fidelity replication present in cancer cells. A recent study analyzed the antigens recognized by clinically effective TILs from melanoma patients that experienced durable complete regressions beyond 5 years after ACT of TILs and identified both nonmutated and mutated antigens that could be recognized by autologous TILs [24]. More recently, neoantigen-specific T cells including CD8+ T cells were successfully isolated from the blood and primary tumor in patients with melanoma [25]. A recent case report demonstrated that ACT from TILs in a patient with metastatic KRAS mutant colorectal cancer could result in durable regression of all metastatic deposits. Correlative studies revealed that four different CD8+ T-cell clones that were specifically reactive to mutant KRAS G12D mediated this response [26]. This highlights an emerging strategy where cellular immunity can be harnessed to target conserved oncogenetic mutations, which have not been conducive to pharmacologic inhibition.

In addition to TAA specificity, emerging findings indicate that the differentiation state of T-cell populations is crucial to the antitumor efficacy of ACT [20, 27]. CD8+ T cells in ex vivo-expanded TILs are a mixture of mostly T_{EM} (less-differentiated effector memory T cells), T_{EFF} (more-differentiated effector T cells), and T_{TDE} (terminally differentiated effector T cells). Relatively, very few T_{CM} (central memory) are found in the ex vivo-cultured TILs, although in preclinical and clinical models T_{CM} cells have shown increased antitumor activity compared with effector T cells in mouse melanoma models [28, 29]. Currently, little is known on which state of differentiated CD8+ T cells is optimal for ACT of TILs in human.

Genetically Engineered T Cells T cells can be genetically engineered to express a T-cell receptor (TCR) with a high affinity and specificity to target antigens. Introductions of such TCR genes are accomplished by retrovirus or lentivirus-mediated transduction. Such TCR-modified T cells have specificity for tumor-associated antigens and can be rapidly expanded ex vivo and reinfused into patients for ACT. For example, TCR transduction has been used to target MART-1 and NY-ESO-1 in clinical trials for patients with melanoma. Tumor regression and durable objective responses were observed in a subset of patients [30, 31].

Chimeric antigen receptors (CARs) are another means for providing specificity to transduced T cells. CAR molecule is an artificial receptor composed of a single-chain variable fragment (scFv) derived from antibody, fused to transmembrane and cytoplasmic domains. The scFv fragment recognizes specific surface tumor antigens in an MHC-independent fashion. The cytoplasmic domains consist of a CD3 zeta activation domain and two co-stimulatory domains, CD28 and CD137/4-1BB. Upon antigen encounter, the CAR transduces the activation signals to T cells, resulting in T-cell proliferation and expansion with cytotoxic functions [32]. Clinical trials have shown excellent outcomes for CAR-T-cell adoptive transfer therapy in patients with hematologic malignancies [33]. Almost all B-cell malignancies, as well as normal B cells, express the CD19, which is absent in other cell types. Thus, anti-CD19-redirected CAR-T cells were designed to target CD19+ B cells and have achieved impressive response rates in 60–90% of patients with relapsed or refractory lymphoblastic leukemia [34–36]. CAR-T cell therapies are also being developed to target solid tumors in a number of disease sites [37, 38]; however, these efforts have been historically hindered by off-target toxicity [39, 40].

In a manner similar to TILs, genetically modified T cells for ACT also contains both CD4+ and CD8+ T-cell populations, which both confer an antitumor response. In a recent clinical trial on patients with B-cell malignances, CD19-CAR-T cells were generated from CD8+ and CD4 T-cell subsets that were separately ex vivo expanded and infused at a 1:1 ratio. This defined composition product showed remarkable antitumor activity as 93% patient achieved bone marrow remission [41]. In contrast to polyclonal TILs, genetically modified T cells have monoclonal specificity to a single target antigen, which may facilitate tumor immunoediting and allow the development and outgrowth of antigen escape tumor subclones.

3.2.2 Checkpoint Blockade

The immune system is characterized by compensatory inhibitory mechanisms to prevent the inflammatory response from precipitating autoimmunity. Tumor-infiltrating T cells that recognize and are poised to eliminate tumor cells are held in check by negative signals that reduce their activation and effector functions. Several molecules have been identified as negative regulators or checkpoints of T-cell activation, including cytotoxic T-lymphocyte-associated antigen 4 (CTLA4), PD-1, and PD-L1. Drugs interrupting these checkpoints can unleash the antitumor activity of T cells and mediate durable cancer regressions. Multiple therapeutic antibodies that block CTLA4, PD-1, or PD-L1 have been approved and have shown clinical benefits in a wide range of solid and liquid tumor types, including melanoma, non-small cell lung cancer, kidney cancer, and Hodgkin's lymphoma [42].

The Biology of the CTLA-4 Pathway CTLA-4 is a receptor that is expressed exclusively on T cells and primarily regulates the amplitude of the early stages of T-cell activation. The engagement of CTLA-4 downregulates the T-cell function, largely by counteracting the activity of the T-cell co-stimulatory receptor, CD28. The recognition of peptide-major histocompatibility complex (MHC) by the T-cell receptor (TCR) is insufficient for T-cell activation and must be amplified by the ligation of CD28 to its ligands, CD80 and CD86. CTLA-4 shares the same set of ligands with CD28 but with a much higher affinity; therefore, its expression on the surface of T cells damp-

ens the activation of T cells by outcompeting CD28 with regard to binding CD80 and CD86, as well as actively delivering inhibitory signals to the T cell [43]. CTLA-4 also confers T-cell inhibition via depletion of CD80 and CD86 from the antigen-presenting cell (APC) surface [44]. The essential role of CTLA-4 for maintaining normal immunologic homeostasis is demonstrated by the lethal systemic immune hyperactivation phenotype in CTLA-4-deficient mice [45, 46].

On the basis of CTLA-4 biology, ipilimumab, a therapeutic antibody against CTLA-4, has been developed and approved for the treatment of patients with advanced melanoma. Ipilimumab binds to CTLA-4 and blocks ligation with CD80 and CD86, which prevents inhibitory signal transduction and results in increased CD28-mediated co-stimulation. CTLA-4 is predominantly expressed on CD4+ T cells, and CTLA-4 blockade has been demonstrated to mediate antitumor immune response through enhancement of effector CD4+ T-cell activity, as well as inhibition of regulatory T (Treg)-cell-dependent immunosuppressive activity. In Treg cells, CTLA-4 is regulated by the forkhead transcription factor FOXP3 and therefore constitutively expressed. It has been demonstrated that anti-CTLA-4 antibody can deplete Treg population in the tumor microenvironment in a Fc-mediated manner through antibody-dependent cellular cytotoxicity (ADCC) [47].

CD8+ T Cells in CTLA-4 Blockade In addition to CD4+ T cells, CTLA-4 blockade enhances CD8+ T-cell response in the tumor microenvironment. Because CTLA-4 is also expressed on activated CD8+ effector T cells, CTLA-4 blockade is considered to directly regulate CD8+ T-cell activity. CTLA-4 regulates effector functions of CD8+ T cells through repressing the production of IFNγ and eomesodermin in individual CD8+ T cells [48, 49]. CTLA-4 blockade was shown to directly enhance the proliferation and activation of specific CD8+ T cells in vitro and in vivo, in a manner independent of CD4+ T-cell help [50]. However, studies using different mouse tumor models demonstrated that CTLA-4 blockade could also reverse CD8+ T-cell tolerance and mediated antitumor immune response by a CD4+ T cell-dependent mechanism [51, 52]. Regardless of which cell types are targeted by CTLA-4 blockade, the functional result of CTLA-4 blockade therapy is enhancement of tumor-specific CD8+ T cells and tumor regression. Ipilimumab treatment in melanoma patient results in clonal expansion of tumor-specific CD8+ T cells in the tumor microenvironment and systemic circulation, although it is related with ipilimumab-induced toxicities [53]. Ipilimumab also increases the absolute number of circulating CD8+ T cells, which correlated with improved clinical outcomes [54].

The Biology of PD-L1/PD-1 Pathway PD-1 is a cell surface receptor of the same immunoglobulin family as CD28 and CTLA-4. Similar to CTLA-4, PD-1 is absent on resting naive and memory T cells and is induced after T-cell activation. However, in contrast to CTLA-4, PD-1 expression on the surface of activated T cells is initiated at a transcriptional level and is therefore delayed [55]. Unlike CTLA-4, which primarily regulates T-cell activation at the earlier stage, PD-1 is believed to inhibit effector T-cell activity in the effector phase within peripheral tissue and tumors [56]. Ligand engagement of PD-1 results in activation of the inhibitory phosphatases SHP-2 and PP2A, which suppress the kinase signaling required for T-cell activation [55, 57].

The ligands for PD-1 are PD ligand 1 (PD-L1, B7-H1, CD274) and PD ligand 2 (PD-L2, B7-DC, CD273) [58, 59]. PD-L1 has immunomodulatory functions independent of PD-1 and can also bind CD80 on activated T cells and APCs to deliver inhibitory signals [60, 61]. The relevance of this interaction in antitumor immune resistance has yet to be determined. Additionally, PD-L1 engagement results in bidirectional signaling that "back" transmits signals into T cells and tumor cells to regulate their survival [62, 63]. Thus, PD-L1 could regulate tumor immunity by functioning as both a ligand and receptor. Similarly, PD-L2 can deliver suppressive signals through PD-1 and can also signal via repulsive guidance molecule b (RGMb) to promote respiratory tolerance [64]. The relevance of PD-L2 signaling to cancer immunity is unknown as it is not widely expressed by tumor or immune cells.

Clinically, the PD pathway blockade, including anti-PD-1 and anti-PD-L1 antibodies, has demonstrated highly durable response rates with minimal toxicity across a spectrum of different tumor types, spanning both solid tumors and hematologic malignancies [65]. In theory, targeting PD-1 may result in different biologic effects than targeting PD-L1 because of the different cellular populations that express these two molecules. In addition to activated T cells, PD-1 expression was found on B cells and natural killer (NK) cells, and, therefore, PD-1 blockade may influence the function of these cells as well [66, 67]. PD-L1 was highly expressed on tumor cells and tumor-associated APCs, including dendritic cells (DCs), macrophages, fibroblasts, and T cells [68–72]. PD-L1 on different types of cells may mediate immunoregulation through unique mechanisms. The comparative effectiveness between anti-PD-1 and anti-PD-L1 antibodies cannot yet be performed because of clinical data that has not yet matured, and biological inferences from the clinical studies may be limited by the differing degrees of chimerism and different isotype subgroups of the antibodies.

CD8+ T Cells in PD Blockade Although the cellular and molecular mechanisms are not completely defined, translational and clinical studies suggest that both PD-1 and PD-L1 blockades converge on tumor-infiltrating CD8+ T cells. In the tumor microenvironment, PD-1 is highly expressed on infiltrating lymphocytes, including tumor-specific CD8+ T cells, and engagement by PD-L1 on tumor cells or APCs results in CD8+ T-cell dysfunction. Analysis of melanoma patients treated with anti-PD-1 antibody (pembrolizumab) showed that the expansion of intratumoral CD8+ memory T cells was marked in those patients who responded to therapy [73, 74]. PD-1 blockade could enhance the proliferation of the effector memory CD8+ T cells with senescent phenotype [75]. Additionally, PD-L1 blockade was shown to reverse exhausted CD8+ T-cell function, and this could be synergized by anti-CD27 [76]. These studies suggest that both anti-PD-1 and anti-PD-L1 antibodies can enhance CD8+ T-cell proliferation and improve effector cytokine production to promote antitumor activity. Recent clinical studies on melanoma have further demonstrated that “inflamed” or “hot” tumors are highly responsive to PD pathway blockade [77]. An “inflamed” tumor is characterized by a Th1-type immune signature that includes Th1-type chemokines, CD8+ T cells, and a high level of PD-L1 expression [6, 65]. Tumor regression mediated by therapeutic PD blockade requires preexisting CD8+ T cells in the tumor microenvironment [73].

3.2.3 Antibody-Based Targeted Therapy

Antibody-based therapy for cancer has been established for more than a decade. The fundamental basis for this therapy is the differential upregulation or mutation of cell surface antigens on cancer cells, compared to normal tissues. Receptor tyrosine kinases, such as EGFR and HER2 (ERBB2), have been found to be overexpressed or mutated in various cancer types, including breast, lung, brain, head and neck, and colon tumors. Aberrant tyrosine kinase activity of EGFR and HER2 can promote cancer cell proliferation and tumorigenesis [78, 79]. Monoclonal antibodies targeting HER2 and EGFR have been approved by the FDA and are currently being utilized in a variety of disease sites [80]. These antibodies antagonize these oncogenic receptors, leading to reduced proliferation and increased apoptosis [78]. Additionally, the antitumoral effect of these antibodies is also mediated by the Fc region of antibody, which can bind to Fc receptors (FcRs) on macrophages, neutrophils, and natural killer (NK) cells and induce cell death through activation of complement-dependent cytotoxicity (CDC) and ADCC [80, 81].

Interestingly, recent studies suggested that adaptive immunity, including CD8+ T cells response, contributes to the efficacy of anti-HER2 and anti-EGFR antibodies. A murine HER2-overexpressing breast cancer model demonstrated that anti-HER2/neu antibody therapy required CD8+ T cells. Anti-HER2/neu antibody treatment increased CD8+ T-cell infiltration into

tumor and induced memory T-cell responses [82]. This result was corroborated by another immunocompetent murine HER2 breast cancer model, which demonstrated that IFNγ-producing CD8+ T cells are required for efficacy of the antibody therapy [83]. Similarly, a study using a murine EGFR+ lung cancer model showed that anti-EGFR antibody cetuximab induced a tumor-specific CD8+ T-cell response, which is required for efficacy of antibody [84]. Additionally, cetuximab was shown to promote dendritic cell maturation and CD8+ T-cell priming, leading to the activation of tumor-specific T cells in patients with head and neck cancer [84, 85].

Bevacizumab, a humanized monoclonal antibody targeting vascular endothelial growth factor A (VEGF-A), is used in the treatment of many malignancies, including colon cancer, lung cancer, glioblastoma multiforme, and renal cell carcinoma. VEGF-A is a secreted factor that is critical for tumor angiogenesis through binding to the VEGFR1 and VEGFR2 receptors. Bevacizumab binds to and neutralizes all human VEGF-A isoforms and thereby block angiogenesis [86]. Aside from its direct action on tumor vascularization, anti-VEGF-A antibody has been shown to modulate immune cells in the tumor microenvironment. Blockade of VEGF-A increased DCs maturation and inhibited infiltration of immunosuppressive cells, such as regulatory T cells and MDSCs [87]. In a mouse model of colorectal cancer, VEGF-A was reported to regulate CD8+ T-cell exhaustion by enhancing expression of PD-1 and other inhibitory checkpoints, and this phenotype could be abrogated by an anti-VEGF-A antibody treatment [88]. VEGF-A can also inhibit the infiltration of T cells by reducing adhesion molecule expression in endothelial cells. Modulation or normalization of tumor vasculature by anti-VEGF-A antibody can result in increased T-cell recruitment and infiltration into tumors [89, 90]. In patients with metastatic renal cell carcinoma, bevacizumab therapy increased intratumoral CD4+ and CD8+ T-cell infiltration [91]. Increased intratumoral T cells were also observed in a combination therapy of bevacizumab with anti-PD-L1 antibody in renal cell carcinoma [92].

3.3 CD8+ T Cells in Radiotherapy

3.3.1 Radiation Therapy Induces Immune Responses

Radiotherapy is a highly effective treatment modality used for the curative and palliative management of almost all cancer histologies. It is frequently combined with other treatment modalities, including surgery, chemotherapy, and more recently immunotherapy, to maximize the chance of disease control [93]. Radiotherapy is a noninvasive localized therapy that applies ionizing radiation (IR) to a tumor. This induces single- and double-stranded DNA breaks in the irradiated tissue. As cancer cells are more sensitive to DNA damage-induced cell death than normal cells because of deficiencies in DNA repair pathways, ionizing radiotherapy can selectively damage cancerous cells [94].

Consistent with this, classical radiobiologic models have shown that radiotherapy induces tumor cell intrinsic mitotic catastrophe and cell death [95]. However, recent studies have highlighted the cell extrinsic mechanisms through which radiation modulates local or systemic immune responses and highlight the challenges and promise of combining radiotherapy with immunotherapy. Low-dose total body radiotherapy was used prior to hematopoietic stem cell transplant to create an immunosuppressed host with stem cell niche availability [96]. The hematopoietic compartment, which is comprised of hematopoietic stem cells, progenitor cells, and the vast majority of innate and adaptive immune cells, is vulnerable to radiation due to a rapid cycling time. Thus, even low doses of radiation are sufficient to induce cell death and damage in mature NK cells, T and B cells, as well as bone marrow stem cell precursors of monocytes and granulocytes. Low-dose radiotherapy was also historically used for the management of benign inflammatory conditions with moderate efficacy [97]. Finally, fractionated courses of radiotherapy had often been delivered in which small doses of radiotherapy are delivered on consecutive days for a duration up to 7 weeks to allow normal tissue healing and minimize treatment-

associated toxicity. When fractionated radiotherapy is given to a large area with concurrent chemotherapy, incidental lymphopenia can result for several months, which can compromise ongoing efforts to promote tumor immunity [98].

In contrast to low-dose total body irradiation, emerging evidence demonstrates that high-dose localized radiation often initiates or enhances antitumor immune response, and the efficacy of radiotherapy even relies, in part, on the host innate and adaptive immunity [99, 100]. Over the last decade, advances in diagnostic imaging and radiotherapy delivery allow for more conformal treatments to a smaller volume without compromising local control. Radiotherapy techniques, including intensity-modulated radiotherapy, have been shown to decrease the toxicity of treatment at many disease sites, including the risk of lymphopenia [95]. Further, hypofractionated approaches, including stereotactic body radiotherapy and stereotactic radiosurgery, which provide equivalent or superior outcomes in one to five total treatments, are increasingly utilized in a variety of disease sites. Biologically, radiation induces immunogenic cell death by causing the release of tumor antigens and danger-associated molecular patterns (DAMPs), such as calreticulin, ATP, and high-mobility group protein B1 (HMGB1). DAMPs are endogenous molecules that induce immunostimulatory effects upon release or exposure during cell death and act by binding to pattern recognition receptors (PRR) expressed on innate immune cells. Simultaneously, radiation can create an inflammatory microenvironment by the induction of cytokine and chemokine production, which leads to infiltration of DCs, macrophages, cytotoxic T cells, and some immunosuppressive cells. Released DAMPs work on APCs through TLR4 signaling to promote efficient processing and cross-presentation of tumor antigens [101]. Mature APCs can migrate to the draining lymph node, where T-cell priming is augmented to initiate a systemic antitumor immune response.

3.3.2 CD8+ T Cells in Radiotherapy

Emerging evidences have demonstrated that radiotherapy can induce tumor-specific CD8+ T cell responses that are critical for radiation-mediated tumor reduction. Using a mouse B16 melanoma model, Lee et al. showed that ablative hypofractionated radiation induces significant tumor regression dependent on CD8+ T-cell activation and recruitment [102]. Radiation has also been shown to induce activation of tumor-associated DCs that support tumor-specific effector CD8+ T cells. The efficacy of radiotherapy depends on DCs and CD8+ T cells, whereas CD4+ T cells or macrophages are dispensable [103, 104]. More recent study suggested that CD8+ T cells and IFNγ contributed to radiation-induced tumor equilibrium in two animal models. Depletion of CD8+ T cells or neutralization of IFNγ leads to tumor regrowth, and blockade of PD-L1 augments CD8+ T-cell response and leads to tumor rejection [105].

Concomitant with increased T-cell activation, radiotherapy can diversify the TCR repertoire of tumor-infiltrating CD8+ T cells. Radiation increases the expression of MHC class I and the production of novel proteins to favor neoantigen presentation [106]. Another study has shown that radiation increases the expression of cancer testis antigens, which promotes the immunological recognition of cancer cells by T cells [107]. In a more recent study involving melanoma patients and a mouse melanoma model, TCR sequencing revealed that high-dose radiation increased diversity of TCR clonotypes of CD8+ TILs. The optimal antitumor response was achieved by the combination of the three treatment modalities: high-dose radiation, CTLA-4 blockade, and PD-L1 blockade [108].

Radiation could also promote tumor infiltration of CD8+ T cells through alterations in tumor vascularity and improved T-cell homing. Radiation induces a pro-inflammatory milieu including inductions of IFNγ as well as many other cytokines and chemokines. This leads to

the infiltration of different immune cell subsets, including CD8+ T cells. Radiation-induced chemokines include CXC-motif chemokine 9 (CXCL9), CXCL10, CXCL11, and CXCL16, which binds to corresponding receptors on CD8+ effector T cells, resulting in migration of T cells into the tumor microenvironment. Moreover, type I IFNs were demonstrated to be required for the CXCL10 production within tumor after radiation treatment. Radiation-induced CXCL10 expression correlated with intratumor CD8+ T-cell numbers [109].

3.4 CD8+ T in Cytotoxic Chemotherapy

3.4.1 Chemotherapeutic Agents Activate Immune Responses

Cytotoxic chemotherapy is another efficacious treatment modality used for the management of many advanced cancers. Cytotoxic chemotherapy functions by inducing tumor cell death or inhibiting tumor cell reproduction. Based on their principal mechanism of action, conventional chemotherapeutic agents can be organized as several categories:

1. Alkylating agents or DNA-damage agents, which cause DNA strand cross-link by adding alkyl groups to the electronegative groups and result in DNA-damage-induced cell death (e.g., cyclophosphamide and cisplatin)
2. Antimetabolites, which function as the building blocks by imitating purine or pyrimidine to inhibit the synthesis of DNA and RNA (e.g., 5-fluorouracil)
3. Spindle poisons, which interfere microtubule function and mitotic spindle assembly, resulting in cell-cycle arrest (e.g., paclitaxel and taxanes)
4. Topoisomerase inhibitors, which prevent the correct unwinding of DNA during replication, transcription, and repair (e.g., irinotecan and etoposide)
5. Antitumor antibiotics, which are made from natural products of soil fungus *Streptomyces* and exert antineoplastic effects by various mechanisms, including DNA intercalation, altering membrane fluidity, and generation of oxygen radicals (e.g., doxorubicin and bleomycin) [95]

The integration of cytotoxic chemotherapy with immunotherapy is the subject of several ongoing clinical trials.

Similar to radiotherapy, cytotoxic chemotherapy has historically been considered immunosuppressive because most chemotherapeutic agents indiscriminately impair cellular division and thus impact tumor cells, effector lymphocytes, and homeostasis of innate leukocytes [110, 111]. Cytotoxic chemotherapy is now the main backbone for conditioning regiments to generate lymphodepletion prior to HSCT and ACT [20]. However, recent studies demonstrated that select chemotherapy agents might also augment tumor immunity [112, 113]. Chemotherapy can initiate or promote antitumor immune response through two major mechanisms. First, chemotherapy induces immunogenic cell death on tumor cells [114]. Similar to radiotherapy, chemotherapy-induced ICD involves the release of tumor antigens and the emission of DAMPs in the tumor microenvironment. Immunogenic chemotherapy-associated DAMPs include calreticulin (CRT), heat shock protein HSP70 and HSP90, ATP, annexin A1, and HMGB1, although different drugs may correlate with different DAMPs [115–117]. For example, Obeid et al. reported that during anthracycline-induced cell death, CRT was exposed to the cellular surface and facilitates their engulfment by DCs, which leads to tumor antigen presentation and tumor-specific CTL response [115, 118]. The antitumor efficacy of many chemotherapy drugs has been demonstrated to partially rely on the induction of ICD. Secondly, chemotherapy agents could activate systemic immunity. Some chemotherapy drugs could directly stimulate the effector activity

of myeloid or lymphoid cells. Paclitaxel was shown to promote DC maturation and cross-priming in mouse breast cancer model [119] and enhance the infiltration of NK cells in a cohort of breast cancer patients [120]. Cyclophosphamide has been shown to favor Th17 and Th1 memory response through altering the composition of microbiota in the small intestine [121]. Gemcitabine resorted defective cross-presentation of tumor antigen in DCs [122]. Finally, cytotoxic chemotherapy may also preferentially target immunosuppressive cells to indirectly enhance antitumor immune response. Gemcitabine [123], 5-FU [124], docetaxel [125], oxaliplatin [126], and paclitaxel [127] have all been shown to deplete blood-borne or tumor-infiltrating Treg cells or MDSCs.

3.4.2 Chemotherapy Enhances the Antitumor Function of CD8+ T Cells

Among innate or adoptive immune cells associated with chemotherapy, cytotoxic CD8+ T lymphocytes are considered a crucial mediator for tumor regression. The antitumor efficacies of these agents even rely in part on CD8+ T cells. In a mouse sarcoma model, the depletion of CD8+ T cells using anti-CD8+ antibody abolished anthracycline-mediated tumor regression, suggesting CD8+ T cells are indispensable for the anticancer efficacy of anthracyclines [128]. Using lung adenocarcinoma mouse models, the chemotherapy of oxaliplatin combined with cyclophosphamide was shown to induce antitumor response relied on innate immune sensing through TLR4 signaling and ultimately depended on CD8+ T-cell immunity [129]. Tumor regression induced by paclitaxel combined with blockade of IL-10 receptor was dependent on CD8+ T cells in a mouse model of orthotopic PyMT-derived tumors. Correlative studies in human breast cancer have found expression of CD8A to be predictive of pathological complete response [130] to neoadjuvant paclitaxel, and patients who achieve a pathological complete response (pCR) have improved clinical outcomes [131].

Additionally, chemotherapy induces tumor antigen-specific CTL response. When chemotherapy induces tumor cell death, tumor-associated antigens are released by dying cells and are taken up by APCs and presented to T cells, resulting in increased T-cell responsiveness and expansion of tumor-specific CD8+ T cells. In an ovalbumin-expressed murine mesothelioma model, cisplatin and gemcitabine have been shown to enhance the presentation of specific epitopes and amplify the CTL response [132]. In a breast cancer patient treated with gemcitabine and radiotherapy, ex vivo analysis of the TCR-Vβ repertoire of TAA-specific T cells in blood and TILs revealed the expansion of TAA-specific CD8+ T [133]. Dacarbazine combined with peptide vaccination in melanoma patients increased the antigenic repertoire of T cells and induced greater tumor reactivity compared to the vaccine alone [134].

Chemotherapy can also increase the infiltration of CD8+ T cells. Increased tumor-infiltrating lymphocytes have been also observed after certain chemotherapy regimens, and this is explained by the induction of chemokine expression in cancer cells. Dacarbazine, temozolomide, and cisplatin were all able to induce expression of T-cell-attracting chemokines, including CCL5, CXCL9, and CXCL10 in human melanoma cell lines in vitro. Using a genetically modified mouse model of melanoma, the authors demonstrated that chemotherapy-induced intratumoral expression of these chemokines increased T-cell infiltration into cutaneous tumors. In patients with melanoma, these chemokines were also increased in chemotherapy-sensitive lesions and correlated with T-cell infiltration and patient survival [135]. The antitumor effects of anthracyclines were known to partially rely on T-cell immune response. Anthracyclines rapidly stimulate the production of type I IFNs by malignant cells. Type I IFNs then trigger autocrine and paracrine signaling on cancer cells resulting in the release of CXCL10, a potent chemotactic factor for CD8+ T cells [136].

Finally, chemotherapy sensitizes tumor cells to the killing effect of CD8+ T cells. In an earlier study that combines vaccinia viral vaccine with conventional chemotherapy, the treatment with

cisplatin or cyclophosphamide after vaccination led to complete regression of the established tumors. These chemotherapy drugs augment the antitumor effect of the tumor-specific CD8+ T cells that were induced by vaccinia virus [137]. Chemotherapy with cyclophosphamide was also shown to sensitize tumor cells to TRAIL-dependent CD8+ T-cell-mediated apoptosis in a mouse model of malignant mesothelioma [138]. Moreover, doxorubicin, cisplatin, and paclitaxel sensitized tumor cells to the cytotoxic effect of CD8+ T cells through increasing the permeability of tumor cells to granzyme B. This effect was mediated by chemotherapy-induced upregulation of mannose-6-phosphate receptors on the surface of tumor cells [139].

3.4.3 CD8+ T Cells Sensitize Tumor Cells to Chemotherapy

While most studies have focused on the effects of chemotherapy on TILs, the reciprocal relationship may also be important. A recent study demonstrated that effector CD8+ T cells could abrogate fibroblast-mediated chemoresistance in ovarian cancer. Fibroblasts in tumor microenvironment inhibit the therapeutic efficacy of cisplatin by release of cysteine and glutathione, which are both utilized by tumor cells to protect them from cisplatin-induced apoptosis. CD8+ T cells restore the cisplatin sensitivity by IFNγ-mediated alterations of glutathione and cystine metabolism in fibroblasts. The presence of CD8+ T cells is positively associated with chemotherapy response and patient survival with ovarian cancer [4]. Also in ovarian cancer model, miR-424 was shown to directly regulate PD-L1 and CD80 expression in tumor cells and enhance the efficacy of chemotherapy by activating CD8+ T cells and reducing regulatory cytokine secretions [140].

3.5 Future Directions

Immunotherapies including adoptive T-cell transfer and checkpoint blockade are efficacious in a broad spectrum of cancers and can induce durable clinical responses. Unfortunately, this result is achieved in a minority of patients. No benefit has been seen in certain cancer histologies, including ovarian, mismatch intact colorectal cancer, and pancreatic cancer. Emerging evidence suggests that immunotherapy most benefits patients with preexisting tumor-infiltrating CD8+ T cells. A current challenge is to turn "non-inflamed" tumor to "inflamed" tumor to increase CD8 T-cell infiltration in hopes that this will augment antitumor efficacy. Scientists and clinicians are now looking for the optimal strategy to achieve this goal.

One promising strategy is to enhance effector T-cell trafficking through epigenetic reprogramming. Effector T-cell tumor infiltration correlates with the level of intratumoral Th1-type chemokines, CXCL9 and CXCL10, which are frequently epigenetically repressed by histone modification and DNA methylation in tumor cells. Treatment with epigenetic modulators can enhance tumor Th1-chemokine production, increasing CD8+ T-cell tumor infiltration and augmenting antitumor efficacy of PD-L1 blockade and adoptive T-cell transfer in preclinical models [7]. Moreover, DNA methyltransferase inhibitor 5-azacitidine was shown to increase immunostimulatory genes including interferon signaling, antigen presentation, and cytokines/chemokines in several human epithelial cancers [141]. Another DNA methyltransferase inhibitor decitabine could also increase chemokine production and CD8+ T-cell infiltration in a murine ovarian cancer model [142]. Thus, epigenetic therapy may be able to increase Th-1-type chemokines, IFN signature genes, and CD8+ T-cell immunity and ultimately sensitize to checkpoint blockade therapy.

Another promising strategy is the merging immunotherapy with radiotherapy or chemotherapy. The basic scientific rationales of combining traditional cancer treatment modalities with immunotherapy have been demonstrated in many preclinical studies. Unfortunately, much of this work relies on immunocompetent murine models, and there are important distinctions between rodent and human immunology and cancer biology [143]. The successful combination of

immunotherapy with traditional cancer modalities will require both empiric discernment and rational mechanistic administration. To thoroughly assess the combination with chemotherapy, optimization of the sequencing, timing, and chemotherapy agent selection will be required. From a radiotherapy standpoint, the dosage, target, and timing will need to be assessed and optimized. Additionally, rigorous preclinical models and clinical trials will be required to realize the hope of combining the efficacious therapies already in the clinic with ground-breaking immunotherapy to unleash CD8+ T cells and achieve the best disease control and cure for cancer patients.

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Mutant Epitopes in Cancer

4

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4.1 Introduction

Targeted immunotherapy in cancer is a rapidly expanding and evolving field with a developmental history spanning at least three decades. Beginning with the identification and characterisation of tumor-specific antigens (TSA)—protein molecules which are exclusively associated with transformed cells—and very recently the dawn of neoantigen-specific immune-cell reactivity—championed by immune checkpoint blockade therapy—demonstrates that immune-based interventions will substantially shape the future of cancer therapy. Neoantigens arise from naturally processed mutated host protein molecules—eventually presented as immunogenic peptides to the immune system. However, a deeper understanding concerning the generation and recognition of neoantigens is indispensable in order to better understand the immunological and biological underpinnings in diagnostics and therapeutic applications to enhance healthcare for patients with cancer. We briefly introduce the reader to the antigen processing and presenting machinery in cancer, and provide a condensed history of cancer antigen discovery, touching upon seminal findings. Last but not least, we discuss the latest

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development in cancer immunotherapy—with a strong focus on neoantigen-directed strategies, which may be improved for the time to come in the context of clinical translation and therapy. We limit the focus in this chapter to active cellular therapy (ACT) for patients with cancer and the potential of using mutant epitopes in combination witih cellular therapy.

Harnessing the potential of neoepitope-specific T-cell subsets is highly attractive, due to their ability to recognize and respond to tumor cells with limited off-target toxicity, superior efficiency, and with the capacity to provide durable and clinically meaningful outcome in patients with cancer [1]. This anti-cancer reactivity directed against transformed cells is in essence a targeted but productive autoimmune response and dependent on the presence of a T-cell receptor T-cell receptor (TCR) repertoire capable of recognising mutant targets. Some cancer antigens have been identified as 'cancer antigens' due to their selective tissue expression or overexpression in malignant/transformed cells, i.e. mesothelin, or cancer testis antigens (discussed later in this chapter). In other cases, mutations that occur in otherwise normally expressed and functional proteins may cause them to become cancer-inducing agents. These mutated host molecules may be involved in cancer initiation (oncogenesis), disease maintenance, or in metastasis. Since some mutations are crucial for malignant transformation and for tumor cell survival, they may also be instrumental in immune escape mechanisms, either by selecting tumor-promoting T-cell responses, or - not mutually exclusive, 'blinding' anti-cancer immune responses by inducing loss of immune - 'fitness'.

Recent findings in cancer research show that the success of immune - based therapies requires a T-cell receptor repertoire capable of recognizing mutant targets along with anti-cancer directed cellular immune responses (e.g. cytotoxicity, Th1 - type immune responses, see Fig. 4.1). In line with this, T-cell-based cancer immunotherapy is gaining momentum since the most successful novel interventions against solid tumors rely on cancer-specific T-cell activity and their mobilisation to sites of disease, i.e. immune checkpoint inhibitors, chimeric antigen receptors (CARs) and T-cell receptor (TCR)-modified T-cell products [2, 3]. Local activation of antigen-specific tumour-infiltrating T lymphocytes, known as TILs, allows for recirculation of cells, robust killing of tumour cells, reduction in tumour mass and orchestration of anti-tumour responses in tissue. Monoclonal antibodies targeting PD-1 and CTLA-4 have thus revolutionised cancer therapy, with signs of potential use in treating chronic infectious diseases such as viral hepatitis, human immunodeficiency virus (HIV) infection, malaria and tuberculosis [4–7]. In particular, anti-PD-1 therapy has been shown to activate CD8 T cells specific for mutated antigens (neoantigens) associated with cancer progression in metastatic melanoma [3]. Patients showing durable responses following immunotherapy had increased numbers of neoantigen-specific T-cells in their blood, signifying the underlying mechanism of anti-PD-1 therapy.

4.2 Antigen Processing and Presentation in Cancer

In order to gain an understanding of the dynamics driving the generation and 'visibility' of antigens to the immune system, it is advantageous to provide an overview about antigen processing and presentation to immune effector cells. Antigens can be generally viewed as being either intrinsic or extrinsic in nature; they are biochemically processed within cells and presented to various T-cell subsets, B cells as well as natural killer (NK) cells [8]. The essential molecule associated with presenting antigens to the immune system is termed as the major histocompatibility complex (MHC), or specifically in humans, the human leukocyte antigen (HLA) [8]. The function of MHC/HLA system was discovered and first described by Rolf Zinkernagel and Peter Doherty in the early 1970s, the seminal work for which they were awarded the Nobel prize in Medicine and Physiology in 1996 [9–12].

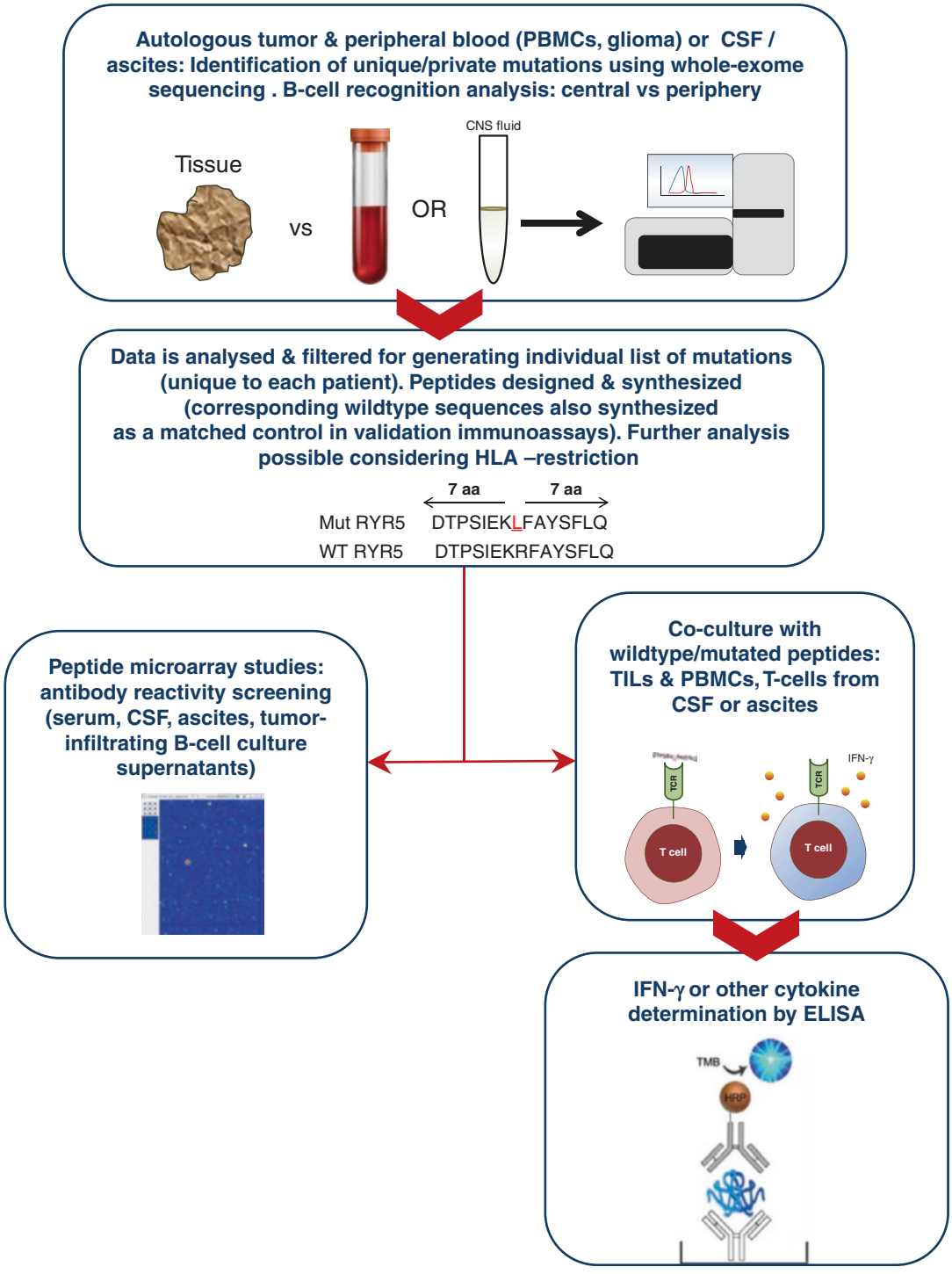

Fig. 4.1 Strategies to identify mutant epitopes from transformed cells, CSF: cerebrospinal fluid

Antigens can derive from whole pathogens, i.e. bacteria, viruses and parasitic organisms, or by non-mutant, or mutant proteins associated by transformed cells. Intrinsic antigens, also called 'endogenous' antigens, are processed and presented to the immune system in the form of specific peptides called epitopes. This pathway is termed the MHC/HLA class I pathway (hereafter referred to as the 'HLA class I pathway'), and plays a crucial role in eliciting immune responses to viruses (viral components synthesised within the host cell), intracellular bacteria as well as to cancer - associated antigens - which relies on the immune system's capacity to recognized 'self' or 'mutant self' antigens [8]. All cells of the body (with the exception of erythrocytes) are capable of processing and presenting antigens via the HLA class I pathway. The processing of antigens in this pathway involves a crucial step, where the immunoproteasome (occurring in the cytosol) cuts up denatured (unfolded) protein structures into small peptide sequences between 8 and 10 amino acids long. The amino acid junctions at which the proteasome enzymatically cuts a protein decides on which peptide or epitopes are naturally presented to immune cells. Epitopes presented by HLA class I molecules are recognised by CD8+ T-cells, which can respond by i) proliferation, ii) cytokine production and / or iii) production of cytotoxic molecules, capable of killing transformed cells [8]. CD8+ T-cells may produce perforin, granzymes, and granulolysin (that can be easily measured using an CD107a induction assay), or - not mutually exclusive - IFN-gamma in response to transformed cells [13]. If (cancer) target epitopes are identified using the 'reverse immunology strategy', i.e. that epitopes are selected based on their predicted capacity to bind to MHC class I or class II molecules, it cannot be assumed with a very high degree of certainty that T-cells will also recognise the naturally processed and presented epitopes on tumour cells—a scenario which was described more than two decades ago [14]: T-cells that were shown to be peptide specific were not able to react against naturally processed and presented peptides on tumor cells. One of the reasons driving this phenomenon is that the specialised, or 'skewed' antigen processing and presentation machinery in transformed cells may be different compared to professional and non-professional antigen presenting cells [15] that are responsible for activating and expanding antigen-reactive T-cells. Alternatively, epitopes may have been created via post-translational modifications (such as phosphorylation) that could not be predicted from the primary structure of the wildtype and/or the mutant protein [16].

Antigens that are taken up from the external environment by professional antigen presenting cells or APCs (i.e. dendritic cells, macrophages), including B-cells, that have also professional APC functions, are usually processed and presented to the immune system via the HLA class II pathway. Whole pathogens, as well as proteins, e.g. generated via destruction of cancer cells by antibody-mediated mechanism, NK or CD8 T-cells, are actively taken up by APCs in endocytic vesicles called phagosomes, after which proteolytic enzymes contained within lysosomal compartments fuse with the phagosome to digest the antigen to yield smaller peptide sequences, usually 13–17 amino acids in length. These epitopes are then presented to CD4+ T-cells, which are also termed as helper T-cells (Th), and have an indispensable role in orchestrating immune responses mainly by producing effector cytokines, i.e. IFN-γ, tumor necrosis factor alpha (TNF-α), interleukin (IL)-2 (Th1 cells), IL-4, IL-10 (Th2 cells) and in some cases, IL-17 (Th17 cells). Cytotoxic activity is not exclusively attributed to CD8+ CTLs; cytotoxic CD4+ T-cells have also been reported to mediate biologically relevant immune responses in cancer as well as in viral infections [17–19].

The T-cell receptor (TCR) on the surface of T-cells binds to the HLA-epitope complex, along with co-receptors CD8 or CD4, to initiate an immune synapse. Interactions between T-cells and tumor cells are governed by HLA-restriction—the alleles encoding a person's HLA repertoire and matching TCRs available in the tissue microenvironment and/or in blood, which dictates the nature and strength of the immune response. HLA allele-restriction of epitopes and immune cross-reactivity thereof plays an indispensable role in dictating

the nature of immune responses. For example, HLA-DQ variants have been associated with increased susceptibility to certain infectious diseases; mutations in the β57 subunit of HLA-DQ may perpetrate progression to pulmonary disease [20]. Interestingly, mutations in HLA-DQ alleles have been attributed to susceptibility to contract type 1 diabetes mellitus (T1DM). While HLA-DQ is highly prevalent among Caucasians in the Americas as well as Europe, East Asians and Africans are much less likely to express these alleles [21]. Indeed, individual HLA alleles may also favour certain immune-recognition profiles, independent of the peptide repertoire displayed by the nominal restricting MHC element, i.e. HLA-DQ0602 favours IL-17 production independent of binding peptides, as shown in the transgenic murine model of multiple sclerosis [22]. This IL-17-centric reactivity represents a double-edged sword; it may more effectively contain certain bacterial infections [23, 24] and IL-17 may be beneficial to attract immune cells to the tumor site [25] while the chronic exposure to IL-17 may rather promote malignant transformation [26–28]. Therefore, the nature, quality and quantity of immune responses following vaccination appear to greatly depend on an individual's HLA profile, which shapes the quality and quantity of ensuing cellular immune responses, including increased or decreased risk for infections, autoimmune responses or the ability to present (neo) epitopes to T-cells dependent on the restrictions imposed by the MHC–peptide complex and the responding TCR repertoire. For instance, even if neoepitopes are generated during malignant transformation, they may not be visible to the cellular immune system, if they are not processed and ultimately complexed to the respective HLA molecule and presented to responding T-cells.

Th1–Th2 Responses and MHC Restriction Most studies use IFN-gamma as the readout of T-cells responding to wildtype and mutant epitopes provided from cancer cells, yet Th2 responses, with the signature cytokines IL-4, IL-5 and IL-13 may also be present, either as an 'original' Th2 response or as a result of partial agonist peptides, imposed by the mutational event (see below) that may turn Th1 T-cells into Th2 cytokine-producing T-cells [29]. Th2-type T-cell responses may not *per se* signify an unproductive and potentially 'tolerizing' immune response; more recent reports indicate that Th2-type immune responses may also be able to mediate clinically relevant anti-cancer immune reactivities [30]. In a preclinical model, antigen-specific Th2 cells eradicated myelomas without the help of CD8 T-cells, leading to massive inflammation at the tumor site [30]. Th2-mediated tumour destruction has been shown to be associated with IL-1, TNF-alpha (Th1) and Th2 cytokine (IL-4, IL-5, IL-13) production in situ, while passively transferred Th2 cells were able to confer long-lasting cellular anti-cancer directed immune responses. CD8-independent and antigen-specific T-cells in Th2-mediated immune responses were shown to be eotaxin- and STAT6-dependent [31–36]. In general, Th2 infiltrates in human cancers have not been studied extensively and some studies even suggested a better outcome with Th2-type cytokines [36]. The nature of Th2 responses in recognising mutant epitopes is not well explored at this time. The more detailed association of CD4 Th2 responses may also benefit from closer association of T-cells with the restricting MHC class II elements. For instance, previous studies reported Th1/Th2 CD4+ T-cell responses against NY-ESO-1 in DPB1*0401/0402-positive patients with ovarian cancer [37]. Much more information is available concerning the nature of the cellular immune response directed against peptides presented by the rather less variant (as compared to HLA-DR) HLA-DP molecules from infectious pathogens, e.g. Hepatitis B or MHC class II molecules that pre-dispose humans to certain autoimmune diseases (e.g. gluten-associated colitis) [37–45]. The impact of variant epitopes in association with certain MHC alleles that are associated with certain cytokine production patterns (IL-17, Th1, Th2) is unexplored up to now. Table 4.1 provides an overview of wildtype and mutant target epitopes recognised in TIL from patients with glioma, demonstrating that Th2 responses exist in the TCR repertoire from individual patients directed against mutant epitopes.

Table 4.1 TIL reacting against wildtype and mutant epitopes—Th1 and Th2 patterns

ID	Patient code	Wildtype sequence	IFN-γ wildtype	IFN-γ mutant	TNF-α wildtype	TNF-α mutant	IL-17A wildtype	IL-17A mutant	IL-4 wildtype	IL-4 mutant	IL-5 wildtype	IL-5 mutant	IL-13 wildtype	IL-13 mutant	Mutated sequence	Gene ID
1	GBM-1	ALYDICSKTLKLPTP	193.09	570.95	64.68			22.69			11.75	36.71	45.35	33.71	ALYDICSRTLKLPTP	TUBB8
2	GBM-1	SSGGCCGSSSGGCCS		63.38							4.92	9.07		3.96	SSGGCCGSSSGGCCS	LCE1F
3	GBM-1	AKQTSNCVLEICAEQ									17.37	4.74		2.31	AKQTSNCILEICAEQ	ESPNP
4	GBM-1	REQEEKMWRQEEKIR					126.84	85.39			3.94	30.39	4.95	11.85	REQEEKMRRQEEKIR	NCKAP1L
5	GBM-1	REDAGAGGEDVGAGG		88.74								37.28		1.98	REDAGAGEEDVGAGG	GOLGA6L1
6	GBM-1	IREQEEMLREQEAQR	105.48	754.23				35.73			18.34	53.61			IREQEEMiREQEAQR	GOLGA6L2
7	GBM-1	PPTWSGRRAPGDRDN		270.84						69.22				19.06	PPTWSGRHAPGDRDN	LOC645752
8	GBM-1	QFLIPTSLSVSSNSV		30.48											QFLIPTSFSVSSNSV	DSPP
9	GBM-2	WPSFSEAHGTSGSDE	428.21	747.66							76.27	86.03		28.36	WPSFSEARGTSGSDE	MSRB2
10	GBM-2	TATASSTQATAGTPH				196.06									TATASSTRATAGTPH	MUC5B
11	GBM-2	TATATTTGATGSVAT				31.21									TATATTTRATGSVAT	MUC5B
12	GBM-2	NLKEKCFLTQLAGFL					9.24								NLKEKCFVTQLAGFL	NBPF8
13	GBM-2	LLTPDEPDKSQGQDL					18.28	3.1	116.92						LLTPDEPEKSQGQDL	NBPF12
14	GBM-2	PDAVGKCRSAGIKVI	353.94	865.82							15.74	60.5		36.45	PDAVGKCGSAGIKVI	ATP1A2
15	GBM-2	ARCSSEDDDSKESTC		412.16			47.28				7.53	147.1		26.11	ARCSSEDSKESTCSP	PHOX2A
16	GBM-2	RWEEWNKRLEEVKRE					30.69								RWEEWNK	AK7
17	GBM-2	PGEGHGGEHLDSEGE					7.41								PGEGHGGDHLDSEGE	GOLGA8DP
18	GBM-2	PSDLRRHVRTHTGEK					81.47	24.61			42.07	30.96			PSDLRRHMRTHTGEK	ZNF764
19	GBM-2	EGGPAAPRLGSRTAP		288.05											EGGPAAPHLGSRTAP	LINC00273
20	GBM-2	NRPTSGPWQRHTRRS		495.14						19.38					NRPTSGPRQRHTRRS	LINC00273
21	GBM-2	ADPIPSGLSPGPCGA				31.21									ADPIPSGRSPGPCGA	LINC00273
22	GBM-2	MKDCQLRKQQNENVS					41.12								MKDCQLRRQQNENVS	SLFN12L
23	GBM-2	VKRNPPKTAKVSEPG	246.07	1275.82			9.24		60.58		84.52	169.86		49.05	VKRNPPKKAKVSEPG	HOXB1
24	GBM-2	SAFEPEGVLANVLGL								65.93				25.85	SAFEPEGGLANVLGL	CYB561
25	GBM-2	GSGPSCRRWEKKLAS	376.6								25.49				GSGPSCRQWEKKLAS	PIK3R5
26	GBM-2	DMYGTGQESLYS		472.45								69.15		31.08	DMYGTGQQSLYS	CDH7
27	GBM-2	QSYKNDFNAEYSEYR		385.13							37.04				QSYKNDFSAEYSEYR	ELL
28	GBM-2	ARKAKYNVHATVRYQ		376.14							3.01				ARKAKYNLHATVRYQ	NCAN
29	GBM-2	MRVMKFSVSPVVRVA			258.99										MRVMKFSISPVVRVA	EEF2
30	GBM-2	YAPCGDLSGMLQERG						23.08							YAPCGDLNGMLQERG	SBK3
31	GBM-2	GQLAVSKRLALEVTV			304.12		39.39								GQLAVSKCLALEVTV	SIRPG
32	GBM-2	QRAAAIARQKAEIAA		889.28			112.28								QRAAAIACQKAEIAA	JPH2

33	GBM-2	ISPSRAARQLMERTQ	606.87								120.34				ISPSRAACQLMERTQ	ELMO2
34	GBM-2	FARKLKDVHETLGFP		410.66											FARKLKDIHETLGFP	TTN
35	GBM-2	EPDNIKYVISEEKGS		361.16											EPDNIKYMISEEKGS	TTN
36	GBM-2	DNHCEQLRVKIRKLK	>1864.49	>1864.49							184.5				DNHCEQLGVKIRKLK	ANKRD36C
37	GBM-2	LTELKDNHCEQLRVK	495.5	561.95	1759.72							80.89		34.27	LTELKDNLCEQLRVK	ANKRD36C
38	GBM-2	DFSVIIMAYVSENIK	669.42												DFSVIIMVYVSENIK	SCN5A
39	GBM-2	GKGVMLAVSQGRVQT	328.34	676.71		702.12						69.23		53.48	GKGVMLAISQGRVQT	TENM3
40	GBM-2	ESRGLLQRRAQAAQE									214.08				ESRGLLQHRAQAAQE	CFAP99
41	GBM-2	SRELCPGRWRAGPWS	467.9		1023.03						52.66				SRELCPGHWRAGPWS	ADAMTS2
42	GBM-2	WDLTDALRLAALSIE			423.55										WDLTDALWLAALSIE	LAMA4
43	GBM-2	SHLIAASNCHSLELQ	993.08								146.98	33.34			SHLIAASSCHSLELQ	CAPN11
44	GBM-2	EDVKWPPTLQPPTLR			682.55										EDVKWPPPTLQPPTL	IRF5
45	GBM-2	NGMEWNGMEWNRIES	343.39												NGMEWNGIEWNRIES	PCSK5
46	GBM-2	AATSHPKPTTGHIKP	307.31	1000.95					14.03		134.72	109.23		33.29	AATSHPKHIKPATSH	GPR50

TIL were harvested from 2 patients with glioma (GBM1 and GBM2) using IL-2, IL-15 and IL-21 without restimulation with autologous tumor cells. TIL were tested against individual mutant epitopes and the corresponding wildtype sequences, followed by detection of Th1 and Th2 cytokines by ELISA. Note a focused T-cell recognition pattern against a low number of mutations in TIL from GBM1 versus a diverse spectrum of recognition in TIL from GBM2 exhibiting as well Th2-cytokine production against mutation epitopes. This test was performed with synthetic epitopes and does not necessarily imply that the wildtype or the mutant target is naturally processed and presented by tumor cells. Nevertheless, TIL reactivity is detectable that falls into the categories that either the mutant epitope is exclusively recognized, or both the wildtype and the mutant epitope sequence are recognised. Numbers are picogram (pg) cytokine production 1×10^5 TIL.

Processing and presentation of neoantigens may yield mutant epitopes (neoepitopes) that are shared as well as patient-specific ('private'). This of course depends on the location of the mutation, i.e. point mutation which might disrupt the naturally occurring cleavage site and the nature of the mutation itself i.e. point mutation vs. chromosomal deletion vs. premature stop codons. A comprehensive analysis of somatic mutations in the HLA class I pathway, using DNA isolated from tumour and non-tumour tissue from patients representing 20 different cancer types, revealed a high likelihood for loss-of-function mutations occurring in the N-terminus of the HLA class I molecule, which abrogates transport of the peptide-HLA complex to the cell surface [46]. Furthermore, in all cancers tested, the most frequent mutations were found to occur in the α3 region of the HLA class I molecule, which is required for binding of the CD8 co-receptor on T-cells during an immune synapse for subsequent activation of the CD8- TCR complex [8].

4.3 Cancer Antigens and Epitopes: From Discovery to Therapeutic Application

Preclinical studies in the mouse model of human cancer, in particular melanoma, provided the first insights into cancer antigen discovery and functional characterisation, in the context of tumour rejection. Thierry Boon and colleagues had shown in the late 1980s that the tumor antigen P19A, heterologously expressed in mouse P815 tumour cells (isolated from DBA/2 mice bearing methylcholanthrene-induced sarcoma), contains an HLA class I epitope (within a 13-mer sequence harbouring a point mutation) capable of eliciting potent CTL responses and lysis of target cells [47].

Epitope mining in the human cancer setting was first performed using tumour tissue derived from human melanoma lesions, spearheaded by groups in Europe and the United States. Thierry Boon, Pierre Coulie and colleagues at the Ludwig Institute in Brussels, Belgium discovered the first tumour-associated antigen (TAA) in 1991, after in vitro characterisation of CTL responses using melanoma cell lines derived from an anonymous patient MZ2 who had metastatic disease [48]. This TAA, first annotated as MZ2-E and later renamed as melanoma-associated antigen 1 (MAGE-1, cancer testis antigen), was recognised by an autologous CTL line and induced lysis of the tumour cell line expressing the MAGE-1 DNA and restricted by HLA-A1 [48]. Further work with a cell line from the same patient led to the discovery of MZ2-F, or as it is known today, G antigen 1 (GAGE-1) [49]. Much of the ongoing work at the time focussed on discovering novel immunogenic HLA class I-restricted antigens that mediated CTL reactivity and lysis of melanoma cells from patients, with a strong interest to first understand and then to develop immune-based interventions; Melan-A (HLA-A2+ epitope) [50]; MAGE-3 (HLA-A1+ epitope)-specific CTL response in a patient vaccinated with MAGE-3.A1 peptide [51].

Simultaneous efforts by researchers in Europe and the United States revealed another important cancer antigen, the cancer testis antigen NY-ESO-1, which was discovered by serological analysis of expression cDNA libraries (SEREX) (indicating the presence of antibody responses), using cDNA prepared from human oesophageal squamous carcinoma cells [52]. NY-ESO-1 was later shown by Elke Jäger and co-workers (Frankfurt) to contain biologically functional CD8+ (HLA-A2/B51) and CD4+ (HLA-DRB*1) T-cell epitopes, based on seminal studies performed on human melanoma cells as well as transfected T2 cells as a model [53–56]. The afore-mentioned T2 cells harbour a defect in the transporter associated with antigen processing (TAP), which in turn inhibits them to present endogenous cytosolic cytosolic peptides (except for some leader peptide sequences loaded onto HLA-A2 molecules), but accommodates the introduction of exogenously added HLA class I epitopes for CTL recognition assays [57].

Steven Rosenberg and colleagues at the Surgery Branch, National Cancer Institute (NCI, National Institutes of Health (NIH), Bethesda, MD) made pivotal contributions to antigen discovery in human melanoma, in particular those that induce reactivity among TILs: the tyrosine related protein 1 (TRP-1) or gp75 restricted by the HLA-A31 molecule in 1995 [58]; HLA-A31-restricted

TRP-2 peptide LLPGGRPYR, which was a major target of TILs infused into a patient with metastatic melanoma who thereafter showed disease regression [58]; epitopes from TRP-1 and TRP-2 ($TRP_{197–205}$) restricted by HLA-A31 as well as HLA-A33 [59]; a mutated epitope derived from triosephosphate isomerase restricted by HLA-DR1 and recognised by CD4+ TIL and cell division cycle protein 27 homolog (CDC27) epitope restricted by HLA-DR4 [60, 61]. Collectively, these early efforts (over a span of 15 years, from the late 1980s to early 2000s) provided an excellent foundation which lead to the expansion of the field of targeted cancer immunotherapy.

A whole series of other molecules were identified to be associated with transformed cells. For instance, mesothelin was discovered as a marker of several important solid cancers, i.e. mesothelioma, ovarian cancer, pancreatic ductal adenocarcinoma based on serological (a murine 'Ki antibody' recognising human mesothelin) and genetic analyses [62–64]. Further exploration of the clinical significance of this molecule in ovarian cancer, mesothelioma and squamous cell carcinomas, and in conjunction with measurable mesothelin as well as antibody responses in sera of patients, indicated the immunogenic potential of mesothelin and its designation as a legitimate cancer antigen [65, 66]. An experimental immunotoxin developed based on the mesothelin-binding region of the K1 antibody was among the earliest attempted targeted immune-based interventions, with preclinical studies performed in a murine model of human carcinoma xenografts [67].

Work implemented in the later part of the 1990s placed a greater focus on studying mutated proteins in human cancer cells, and the possibility of discovering mutated antigenic determinants (neoepitopes) presented by HLA restricting elements, with biological and clinical relevance in therapy. An early example is a neoepitope derived from melanoma ubiquitous mutated 1 protein (MUM-1, initially named LB33-B, after the patient from whom the melanoma tumour was obtained, LB33 [68]), which is restricted by the HLA-B*44*02 allele. This 9-mer neoepitope was identified following in vitro cytotoxicity studies directed against the autologous melanoma cell line LB33-MEL.A-1; the same cytolytic activity was not seen with the wildtype peptide sequence [69]. A 10-mer neoepitope (amino acids 23–32) from mutated cyclin-dependent kinase 4 ($CDK4_{R24C}$) protein, restricted by HLA-A*0201, was also shown to mediate cytolytic activity by autologous CTLs in a dose-dependent manner, when exposed to T2 cells transfected with the $CDK4_{R24C}$ cDNA [70]. A caspase 8-derived mutated peptide restricted by HLA-B*3503, which showed potent cytolytic activity against the autologous head and neck cancer cells as well as tumour cDNA-transfected B-cell lines [71] further strengthened the field of neoepitope mining from human cancer cells.

A high-throughput analysis of whole genomic as well as exomic DNA from clinical tumor samples representing thirty different human cancers revealed the unique mutational burden in each cancer type, in addition to specific mutational signatures characterising these cancers [72]. Although this provides an elegant view of the general landscape of mutational burden in human cancers, the mutational signature in each patient varies—thus giving rise to a 'compendium' of private mutational signatures involved not only in driving and maintaining malignant transformation, but also in the activation and expansion of immune effector cells.

The mutated form of the V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog, or known as KRAS in short, is a well-established neoantigen implicated in the pathogenesis of pancreatic, colorectal and lung cancers [73–76]. Native KRAS was discovered in 1982 following gene sequencing of human lung adenocarcinomas, and is a guanine triphosphatase involved in cellular signal transduction [77]; however, mutations at positions 12, 13 and 16 are associated with oncogenesis, thus making it a proto-oncogene in humans.

Steven Rosenberg and colleagues at the Surgery Branch, National Institutes of Health (Bethesda, MD) recently developed a cutting-edge approach to screen for neoepitope-specific T-cell responses for individual patients. This method has been termed the 'tandem minigene (TMG)' approach, which first requires whole-exome sequencing data of genomic DNA isolated from patients' tumor tissue samples. The sequencing data then yield all non-somatic mutations contained within gene-coding DNA of the patient. This allows for