A PERSONALIZED VIEW ON CANCER IMMUNOTHERAPY

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1. Introduction

Cancer therapy is currently evolving from relatively non-specific standard treatment strategies, such as surgery, radiotherapy and chemotherapy, to a more personalized targeted approach. Although surgical resection and radiotherapy are often successful for eradication of primary tumors, a commonly encountered problem is disease relapse due to residual tumor cells and/or metastases. Therefore, these therapeutic strategies are often followed by chemotherapy. Standard chemotherapy acts by killing rapidly dividing cells, although healthy cells that undergo fast cell division can be damaged by this therapy as well [1]. The identification of cancer genes and tumor-related signaling pathways that are essential for the tumor cell growth and survival has provided researchers with a better insight in tumor pathogenesis. This boosted the development of tumor-targeted therapies.

Targeted therapies focus on the inhibition of tumor-specific proteins or biological pathways involved in tumor growth and progression. These therapies are mostly based on the blockade of growth factor receptors, the inhibition of angiogenesis and the induction of apoptosis in tumor cells by the use of monoclonal antibodies or small-molecule inhibitors [2]. Examples of monoclonal antibodies that are approved by the US Food and Drug Administration (FDA) for the treatment of various cancer types are cetuximab, trastuzumab and bevacizumab [3, 4]. Cetuximab and trastuzumab are both monoclonal antibodies that bind respectively epidermal growth factor receptor (EGFR) and HER2/neu proteins, which are both members of the ErbB tyrosine kinase family [5]. The binding of the antibodies to EGFR that is overexpressed in a broad range of epithelial cancers results in the inhibition of cancer cell proliferation [6]. Trastuzumab is approved for the treatment of invasive HER2/neu positive breast cancer from which the mode of action is similar to cetuximab [7]. The monoclonal antibody bevacizumab inhibits angiogenesis by binding to vascular endothelial growth factor A (VEGF-A) and is approved for a variety of tumor types [8]. Besides monoclonal antibodies, small-molecule inhibitors have also been developed to inhibit growth factor receptors such as EGFR [9]. Furthermore, small-molecule inhibitors such as vemurafenib can interfere with oncoproteins. Vemurafenib is a specific kinase inhibitor of the mutated protein kinase B-RAF V600 (BRAF-V600) and has been proven successful to enhance the survival rates of melanoma patients [10, 11]. Although mutations in the gene encoding BRAF are present in a wide range of cancers, the highest frequency of BRAF mutations is found in melanoma [12]. In 40 to 60% of melanomas the specific BRAF V600-mutant is present. This mutation promotes tumor cell proliferation and prevents apoptosis of melanoma cells [13]. Generally, targeted therapies are not only less toxic compared to conventional chemotherapy, some targeted therapies are moreover of clinical benefit in tumor types that were resistant to conventional chemotherapy [14].

Besides inhibition of growth factor actions, oncoproteins and neovascularization, another targeted therapy is aimed at exploiting the destructive properties of the immune system to eradicate cancer cells. The presence of endogenous anti-tumor immune responses forms the basis of immunotherapy.

Immune-based therapies are developed to induce anti-tumor responses by the host immune system and became an important and well-investigated treatment option for cancer during the last three decades [15]. However, the strategy to manipulate immunity for treating cancer encounters problems in terms of individual variations in immune responses and differences in tumor types. Each patient's immune system is unique due to variability in genetics, epigenetics, nutrition, lifestyle, and environment [16]. Furthermore, due to tumor heterogeneity, certain cancer treatments are only beneficial in small subsets of cancer patients. Therefore, efficacious cancer immunotherapy requires personalization of the treatment. A first screening of patients to more precisely diagnose disease can lead to the development of more safe and effective cancer therapy. In this review, we will present an overview of the current state-of-the art in cancer immunotherapy with the focus on the implementation of personalized strategies to reduce toxic side effects and costs while improving efficacy.

2. Tumor antigens are the key to effective immunotherapy

Early attempts to detect components or functions unique to cancer cells were made over a century ago, leading to the conviction in the 1950s that 'tumor-specific materials' probably did not exist, since over 50 years of work delivered little to no evidence [17]. However, studies performed by Gross, Foley and Prehn produced already evidence in the 1940s and 1950s that tumors were antigenic after transplantation in inbred mouse lines [18-20]. These studies together with many others in the 1960s, showing the rejection of transplantable tumors in syngeneic mice, reinstated the idea of 'tumor-specific materials', at that time referred to as 'tumor-specific transplantation antigens' [21, 22]. In this regard, it is important to mention that the first evidence for the existence of such antigens was delivered by Gold and colleagues who demonstrated humoral responses to an antigen specific for colonic cancer, in particular for the carcinoembryonic antigen (CEA) [23, 24]. The existence of antigens, which can elicit specific humoral immune responses and are expressed by tumor cells was confirmed in the late 1970s by Steven Rosenberg and colleagues [25]. A decade later, it was shown by Thierry Boon and colleagues that these antigens could moreover elicit potent T cell responses [26]. These antigens are today known as tumor antigens, *i.e.* antigens that are uniquely, preferentially or in excess expressed by tumor cells. Importantly, the description of immune responses against these tumor antigens has rationalized the so-called tumor surveillance concept that was formally introduced in the early 1970s by Burnet and Thomas, which confirmed previous observations by Ehrlich [27, 28]. Thus, a model was proposed in which tumor cells are recognized as foreign and subsequently specifically eliminated without damaging healthy cells, much in the same way as for virus-infected cells. Without any doubt, the description of tumor antigens ushered in the era of immunotherapy. Generally, tumor antigens are divided into five classes, including tissue-differentiation antigens (*e.g.* Melan-A/MART-1, TRP-2, *etc.*), antigens that are overexpressed (*e.g.* survivin, telomerase, *etc.*), antigens derived from epigenetic changes or the so-called cancer-testis antigens (*e.g.* MAGE-A3, NY-ESO-1, *etc.*), antigens derived from mutated genes (*e.g.* p53, RAS, *etc.*), and viral antigens (*e.g.* derived from human papillomaviruses, Ebstein-Barr viruses, *etc.*). Alternatively, these tumor antigens can be subdivided in two main classes: self-antigens and tumor-specific antigens (Figure 1). Self-antigens are present on both tumor cells and normal tissues in contrast to the tumor-specific antigens, of which the expression is restricted to tumor cells. In 1991, van der

Bruggen and colleagues cloned the first human tumor antigen that is recognized by T cells [29]. This melanoma-associated antigen MAGE-A1 was identified by a genetic approach, which paved the way for the discovery of multiple antigens on a broad range of tumor types [30]. Most of these antigens can be classified in the group of self-antigens. A characteristic of these self-antigens is that they are usually expressed on a wide variety of tumors of different histological origins. Therefore, these antigens can also be classified as so-called shared antigens. Of note, some tumor-specific antigens can also be shared between patients, for example virus-derived antigens and BRAF-V600. In the past, researchers have focused on the identification of shared antigens for the production of off-the-shelf immunotherapeutic drugs that could be used in a broad range of patients [31].

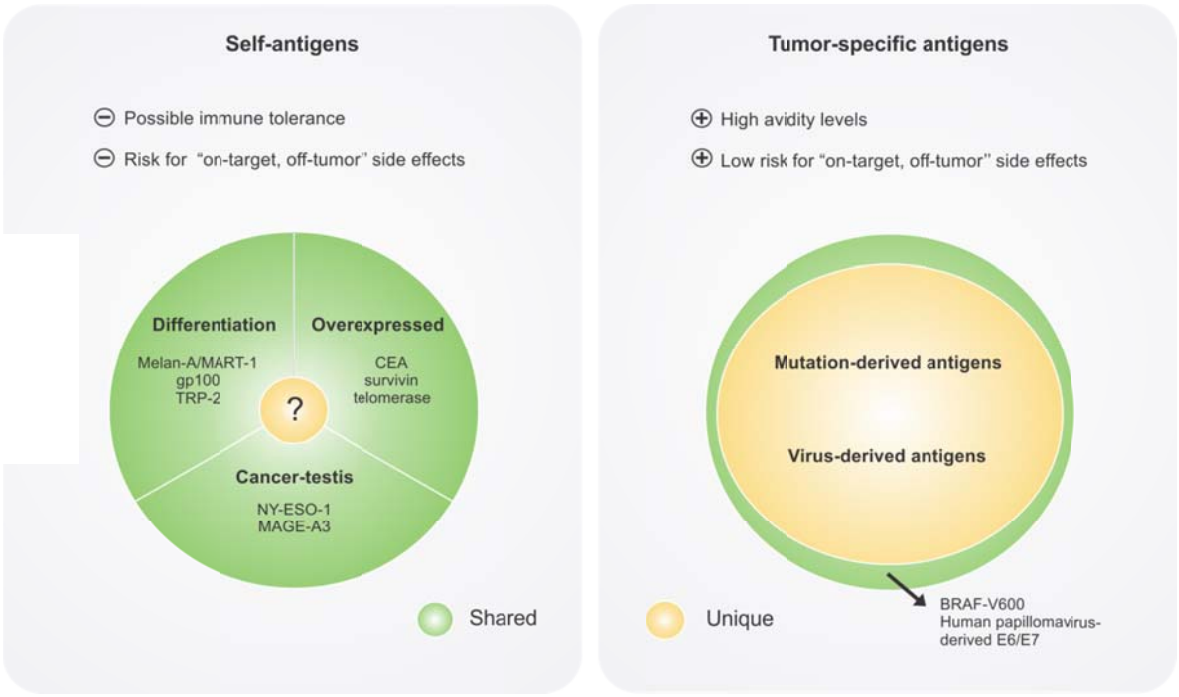

Fig. 1. Tumor antigens can be classified in two main classes: self-antigens and tumor-specific antigens. The class of self-antigens encompasses differentiation, overexpressed and cancer-testis antigens. Self-antigens are expressed on both tumor cells and normal tissues. Consequently, cancer immunotherapies that target these antigens can encounter problems due to immune tolerance and 'on-target, off-tumor' side effects. The class of tumor-specific antigens includes mutation-derived and virus-derived antigens. Cancer immunotherapies targeting these antigens have advantages in terms of high avidity levels and low risk for toxic side effects. Generally, self-antigens are shared between patients while the majority of tumor-specific antigens are unique, although virus-derived antigens and some mutation-derived antigens can also be shared between patients. Examples of tumor antigens are illustrated in the figure adapted from Ref. [52].

2.1. Cancer-testis antigens: exploiting the tumor epigenome for cancer therapy

The class of self-antigens encompasses cancer-testis antigens (CTA). These were suggested as ideal targets for immunotherapy because they are expressed in a wide range of histological different cancers while their expression on normal cells is restricted to germ line cells in the testis that are thought to be guarded against immune responses [32, 33]. Aberrant CTA expression was first described in melanoma [29]. Today, it is known that CTA are moreover abundantly expressed in hematological malignancies (*e.g.* multiple myeloma) and a wide variety of solid tumors, including breast, lung, prostate, ovarian, bladder, colorectal and hepatocellular carcinoma [34, 35]. Importantly, CTA are considered "foreign-like" antigens to which T cell tolerance is limited [36]. Therefore, it is not surprising that these antigens have been found to elicit spontaneous humoral and cell-mediated immune responses in cancer patients, highlighting their value as cancer immunotherapy targets [37]. However, caution is called for, because some CTA were observed to be also expressed on healthy tissues that are not part of an immunoprivileged site, resulting in toxicity to healthy cells [38, 39]. A recent clinical study by Rosenberg and colleagues reported on the immunotherapeutic targeting of CTA MAGE-A3/12 in 9 patients with metastatic disease. Although 5 patients were reported with cancer regression including 2 with ongoing response, 3 patients experienced mental status changes from which 2 patients died. Further investigation identified the expression of the target CTA in the human brain, which was thought to be the reason for the unexpected toxicity of this immunotherapeutic approach [40].

Although CTA can be found in a broad range of different tumors, it is possible that only a small subset of a certain tumor type expresses the CTA [41]. Therefore, it is interesting to further investigate in which tumor subtypes these antigens are expressed and first screen a patient's tumor before starting with antigen-reactive immunotherapies. Interestingly, expression of CTA is controlled by epigenetic events. In particular, expression of CTA is switched on by demethylation of the promotor region of the CTA encoding gene [42]. The latter is of importance, as several drugs have been shown to modulate the tumor epigenome, in particular to initiate the CTA transcriptional program. Examples hereof are azacitidine and decitabine, two DNA methyl transferase inhibitors as well as the immunomodulatory drug lenalidomide [35, 43-45]. These drugs enhance the expression of CTA and as such impart a more immunogenic tumor cell phenotype.

2.2. Differentiation antigens and widely overexpressed antigens as targets in immunotherapy

Besides CTA, self-antigens include differentiation and overexpressed antigens. Differentiation antigens are expressed on both tumor cells and normal cells from the same tissue. Typical examples of these antigens are Melan-A/MART-1 and gp100, which are expressed in malignant melanoma cells as well as healthy melanocytes in the skin, eye and ear [46]. A major limitation of immunotherapy towards differentiation antigens is the toxicity by virtue of immune responses directed against normal cells, as evidenced in the study by Johnson and co-workers, who observed anterior uveitis and ototoxicity upon immunotherapy targeting the differentiation antigens MART-1 or gp100 [47].

Overexpressed antigens can be classified as a third class of self-antigens. This subclass consists of antigens that are overexpressed on tumor cells, although low levels of expression were also observed on different types of normal cells. Due to their expression on a number of different tumor types, these antigens were extensively investigated for off-the-shelf immunotherapies. Carcinoembryonic antigen (CEA) is an example of an antigen that is overexpressed in a variety of epithelial cancers, even though it is also found in many normal gastrointestinal epithelial cells [48]. Clinical trials exploiting CEA as a target illustrated the objective regression of metastatic colorectal cancer in one out of three patients. However, severe transient colitis was reported for all three patients indicating the toxicity of the immunotherapy to CEA-expressing normal cells [49]. A similar toxicity problem was observed in a clinical study that targets CAIX, a frequently overexpressed antigen on renal cell carcinoma. The concurrent CAIX expression on the bile duct epithelial cells was possibly responsible for the detected liver toxicity [50]. The reported 'on-target, off-tumor' side effects of immunotherapies against self-antigens highlight the need for novel approaches against more tumor-specific antigens. Furthermore, data by Lennerz et al. demonstrated the superior reactivity of tumor infiltrating T cells (TILs) towards tumor-specific antigens compared to the already widely discovered self-antigens [51]. This can be explained by the expression of self-antigens on both tumor cells and normal cells, resulting in peripheral immune tolerance of T cells towards these antigens.

2.3. Tumor-specific antigens: exploiting the tumor mutanome for personalized cancer therapy

Most tumor-specific antigens, also called neo-antigens, arise from somatic mutations in the tumor cells and are patient-specific [51, 52]. This type of antigen is an ideal target for immunotherapy because of their expression restricted to tumor cells, which limits toxicity towards normal cells. Moreover, as these are neo-antigens, there is no tolerance to the presented mutated epitopes. Furthermore, a recent study reported on the poor reactivity of isolated TILs towards self-antigens, even though a clinical response was observed in patients after re-infusion of *ex vivo* expanded TILs. This finding may indicate the presence of TILs reactive towards neo-antigens [53]. Unfortunately, neo-antigen-based immunotherapies are not yet successfully realized due to a lack of in-depth research. In the past, the development of immunotherapies against tumor-specific antigens was hindered by the uniqueness of neo-antigens in a patient's tumor together with a lack of techniques able to systematically test T cell responses against neo-antigens. The development of the next generation sequencing (NGS) technology enables the systematic sequencing of whole cancer genomes, providing insights into the mutational landscape of various human cancers [54, 55]. Whole-genome sequencing was already employed to characterize the genome of cancers such as acute myelogenous leukemia, breast cancer and melanoma [56-58]. By comparing the genome of cancer cells with the one of the original cells, cancer-specific mutations can be identified [59]. A second strategy is whole-exome sequencing (WES) in which only 1% of the genome needs to be sequenced, making sequencing more affordable for larger sample sizes. This method elucidated multiple mutations in various cancer types such as breast cancer, ovarian, renal and head and neck carcinoma [60]. Decreasing costs of NGS should facilitate detection of patient-specific tumor mutations and identification of neo-antigens attractive for personalized immune-based cancer therapy.

Recently, a new screening approach for the identification of mutated antigens recognized by anti-tumor T cells was presented by Robbins et al. [61]. Exceptionally, some neo-antigens recognized by autologous T cells are known to be shared between patients such as BRAF-V600 and p53. In addition, a recent study reported on shared frame shift-derived neo-antigens that were recognized by antigen-specific T cells in different microsatellite-instable leukemia and lymphoma cell lines [62].

2.4. Viral antigens: exploiting the vironome for cancer therapy

Virus-derived antigens can also be classified as tumor-specific antigens. Immunotherapy against tumor cells that arise as a consequence of an infection with an oncovirus has the advantage that viral antigens are shared between patients infected with the same virus without toxicity to normal cells and that it is relatively easy to prime T cells directed against viral antigens. The human papillomaviruses (HPV) are able to induce several types of cancer such as cervical, penile, anal, and head and neck cancers [63, 64]. The HPV-derived oncoproteins E6 and E7 are currently investigated as targets for cancer immunotherapy [65, 66]. In fact, the two prophylactic HPV vaccines that are now available, a bivalent vaccine (types 16 and 18), and a quadrivalent vaccine (types 6, 11, 16, and 18), have provided powerful tools for primary prevention of cervical cancer and other HPV-associated diseases. These vaccines have been incorporated into national immunization programs in over 22 European countries. It is estimated that these prevent up to 70% of cervical cancers [67]. All these findings highlight that anti-cancer immunotherapy based on eliciting immune responses against tumor antigens is no longer science fiction but has become a reality.

3. The promise of cancer immunotherapy

With the description of tumor antigens and the immune responses they elicit, came the promise of novel immunotherapeutics that will increase our ability to fight human cancer in years to come. Several strategies have been devised so far ranging from strategies aimed at increasing tumor antigen-specific T cells *in vivo* through cancer vaccination or by transferring tumor antigen-specific T cells that have been stimulated or engineered in the laboratory to strategies aimed at supporting tumor antigen-specific T cell responses amongst which cytokine-based therapy (*e.g.* IL-2 and IFN-α2b) and antibody therapy aimed at blocking critical immune checkpoints (*e.g.* anti-CTLA-4 antibodies and anti-PD-(L)1 antibodies) [68-71]. Although immunotherapy in general and the subdomains in particular are complex and challenging fields, there have been major advances in basic and translational research in each of the approaches resulting in clinical trial activity that is now beginning to confirm this promise.

3.1. Active immunotherapy: stimulating tumor antigen-specific T cell responses in vivo

Cancer immunotherapy can be roughly divided into two classes: active and passive immunotherapy. Active immunotherapy can be generally described as the induction of a tumor-directed immune response by the vaccination of patients with tumor antigens. Vaccines are generally considered as a

preventive measure against microbial infection, although cancer vaccines are mostly used as a treatment strategy. The adaptive immune system can fight against cancer via the presentation of tumor antigens by antigen-presenting cells (APCs), notably dendritic cells, to activate $CD4^+$ and $CD8^+$ T lymphocytes. Many different cancer vaccination strategies have been proposed including the immunization with whole tumor cells, tumor lysates, peptides, proteins, recombinant viruses, and nucleic acids, such as DNA and mRNA encoding for tumor antigens [72-78]. In the past, the inclusion of immune adjuvants in the vaccines has appeared to be crucial for the induction of an efficacious immune response [79]. Another approach in active immunization is the *ex vivo* pulsing of dendritic cells with tumor-derived peptides, proteins and tumor cells [80]. In 2010, the first cell-based cancer therapy, sipuleucel-T, was approved by the US FDA. Sipuleucel-T is a patient-specific treatment for men with metastatic castration-resistant prostate cancer. In this therapy, peripheral blood mononuclear cells are isolated from the patient and *ex vivo* activated with a recombinant fusion protein consisting of a prostate antigen prostatic acid phosphatase (PAP) that is fused to granulocyte-macrophage colony-stimulating factor (GM-CSF), which is an immune stimulating growth factor. Subsequently, the cells are re-infused into the patient to activate PAP-specific T cells [81]. In a randomized placebo-controlled clinical trial in 512 patients, an improved survival of 4.1 months was observed [82].

Furthermore, dendritic cells can also be loaded *ex vivo* with tumor-associated antigens (TAAs) by genetic engineering using viral vectors, such as lentiviruses and adenoviruses, or with mRNA encoding for TAAs [83-85]. The transfer of *ex vivo* mRNA-modified dendritic cells has proven to be an elegant method for cancer vaccination. In 1997, the FDA approved the first clinical trials with *ex vivo* mRNA-transfected dendritic cells [86, 87]. Over the past decades, clinical trials have shown that vaccines consisting of autologous dendritic cells loaded with tumor antigen mRNA are safe, well tolerated and capable of inducing T cell-mediated immune responses in a significant number of cancer patients, including patients with colorectal, lung, breast, prostate cancer and melanoma [88-91]. These clinical trials, their clinical as well as immunological outcome, have been described in detail by Van Lint et al [76].

Since vaccination with dendritic cells is patient-specific and associated with a high production cost, several researchers have evaluated the *in vivo* modification of dendritic cells. In this regard, it has been shown in preclinical and clinical studies that direct administration of mRNA as an anti-cancer vaccination strategy is feasible, safe and elicits potent T cell responses with therapeutic efficacy [92-95]. It is fair to state that mRNA became the focus of research in molecular medicine at the beginning of the millennium. Since mRNA is easy and cost efficient to produce, has a favorable safety profile and enables induction of combined immune responses, it is regarded as an efficient alternative to proteins, recombinant viruses or DNA in the field of vaccination [96]. Moreover, the efficacy of mRNA vaccination was recently highlighted in a clinical study in renal cell carcinoma, in which advanced stage patients were pre-treated with GM-CSF and vaccinated by intradermal injection of tumor antigen mRNA. This vaccination regimen resulted in priming of $CD4^+$ and $CD8^+$ T cells and was shown to enhance the overall survival of patients [97]. This demonstrates that the use of mRNA as an off-the-shelf vaccine represents an important step in the development of future anti-cancer immunotherapeutic strategies.

3.2. Passive immunotherapy: providing or supporting tumor antigen-specific T cells

Passive immunotherapy can be further divided into three main approaches: administration of immune stimulating cytokines, monoclonal antibodies and/or adoptive T cells. One example of an immunostimulating cytokine is IL-2, a T cell growth factor that promotes activation, proliferation, survival, and effector functions of anti-tumor T cells. IL-2 is FDA approved due to its demonstrated complete cancer regression in 8% of the patients with metastatic renal cancer or melanoma [98]. The treatment of cancer with monoclonal antibodies such as cetuximab and trastuzumab, previously described as targeted therapies, can also be classified as passive immunotherapy, based on the immunological character of the antibodies. However, these types of antibodies do not aim to improve immunity against cancer. In contrast, monoclonal antibodies against the immunosuppressive molecules CTLA-4 and PD-1 block immunoinhibitory pathways, which may enhance endogenous anti-tumor immune responses. CTLA-4 is expressed on activated T cells including regulatory T cells (T_{regs}) and down-regulates T cell activation [99]. Ipilimumab is a human monoclonal antibody that blocks CTLA-4, which results in the enhanced proliferation and effector activity of T cells. The FDA approved this antibody in 2011 for the treatment of patients with metastatic melanoma [100]. Two other promising targets for antibody therapy are PD-1 and its ligand PD-L1 [101, 102]. Binding of PD-L1 to its PD-1 receptor on T cells inhibits T cell activation and can induce apoptosis of T cells. PD-L1 expression has been identified on APCs and several solid tumors such as renal cell, colon, breast, ovarian and hematological cancers [103, 104]. Monoclonal antibodies blocking the PD-1 pathway have been widely investigated. Topalian and colleagues reported on a phase I clinical trial with the anti-PD-1 monoclonal antibody nivolumab in which objective responses were observed in patients with metastatic renal cell carcinoma, melanoma and non-small cell lung carcinoma [101].

Despite the objective clinical responses of these strategies, it is important to mention that their use is restricted to tumors that are able to generate endogenous anti-tumor T cells such as melanoma. Melanoma and also small lung cancer carcinomas are known to have the highest mutation rates due to the exposure to respectively ultraviolet radiation and tobacco smoke, leading to more tumor antigen recognition by endogenous T cells [105, 106]. Since not all cancer types are characterized by induction of endogenous tumor-specific T cells, these strategies are often envisaged as a supporting therapy next to vaccination or adoptive T cell transfer.

The discovery of antigen-specific and tumor-reactive T lymphocytes in the tumors of patients paved the way for adoptive T cell therapy (ACT). Adoptive T cell therapy can be described as the treatment of patients with *ex vivo* selected and expanded tumor-reactive T cells. Both immediate anti-tumor responses by effector T cells and long-term effects by memory T cells can induce complete remission in patients that are incurable by other anti-cancer treatments. Two major sources of T lymphocytes for ACT are the tumor itself and the peripheral blood of the patient (Figure 2). In case the tumor is infiltrated by anti-tumor T cells, these cells can be isolated from the resected tumor mass. Rosenberg and colleagues reported on a study with 93 metastatic melanoma patients from whom TILs were isolated. These patients had failed chemotherapy and other immunotherapies such as IL-2 and anti-CTLA-4 treatments. The T cells isolated from the resected tumor were first selected for anti-tumor reactivity and expanded *in*

vitro. Twenty patients (22%) achieved a complete regression with ongoing complete responses in 19 patients after 3 years [107].

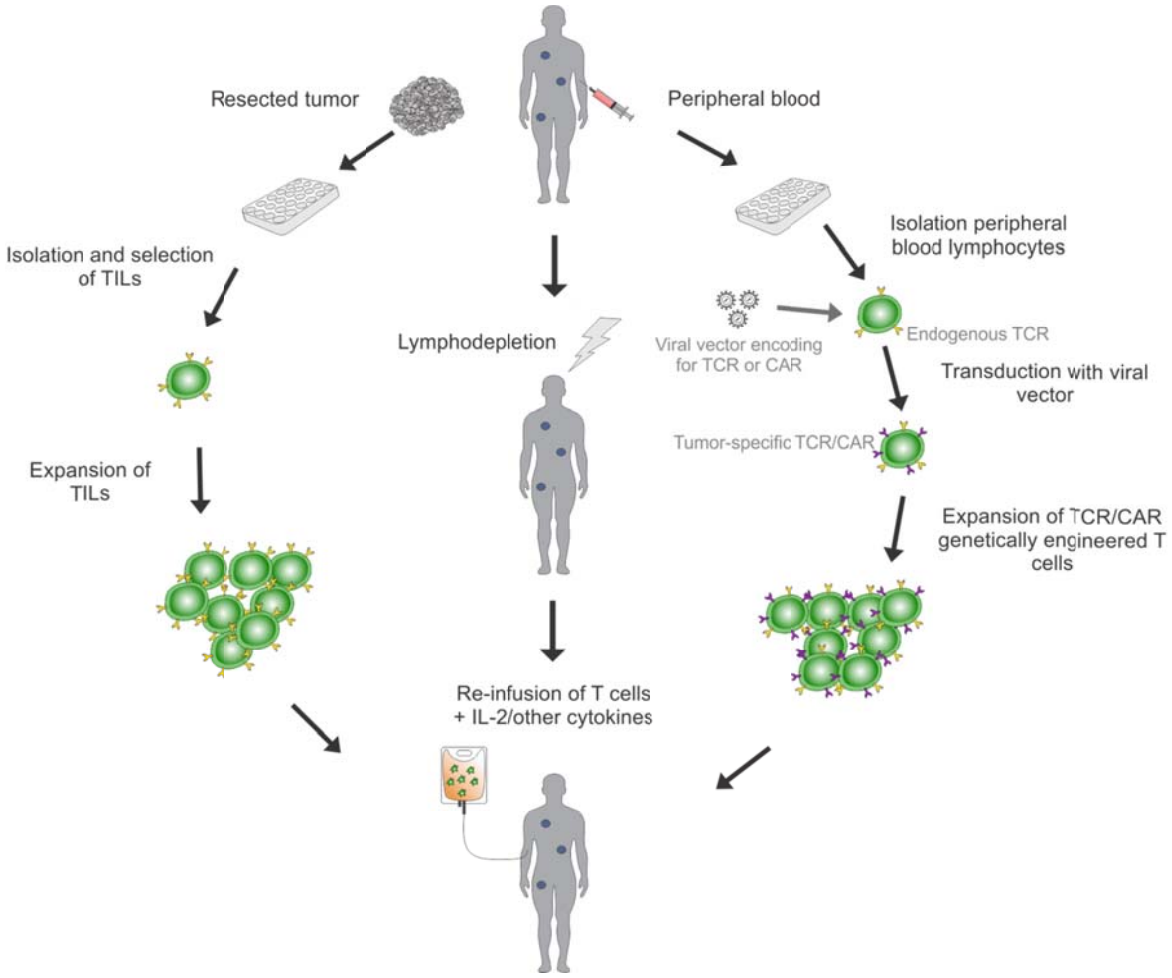

Fig. 2. Two major sources of T lymphocytes for adoptive T cell therapy are the tumor itself and the peripheral blood. In case the tumor is infiltrated by anti-tumor T lymphocytes, tumor-infiltrating lymphocytes (TILs) can be isolated and selected for tumor reactivity followed by *ex vivo* expansion. Alternatively, peripheral blood lymphocytes can be isolated from the cancer patient followed by transduction with retroviral or lentiviral vectors encoding for a T cell receptor (TCR) or chimeric antigen receptor (CAR) and *ex vivo* expansion. During the engineering and expansion process of the anti-tumor T lymphocytes, the patient is treated with lymphodepleting chemotherapy, often in combination with total body irradiation. Subsequently, the expanded anti-tumor T cells are re-infused into the patient in combination with interleukins such as IL-2.

In spite of the successful outcomes in metastatic melanoma patients, a major limitation of TIL therapy is the absence of sufficient numbers of TILs in other types of cancer than melanoma. To extend the treatment of cancers with autologous T cells to a wide variety of cancer types, an alternative approach is the transfer of antigen-specific T cell receptor (TCR) genes into lymphocytes isolated from the patient's peripheral blood [108, 109]. Via transduction of T cells with retroviruses or lentiviruses, T cells can be

engineered to recognize tumor antigens and eradicate tumor cells that express these antigens in large numbers of patients [106]. A study by Robbins and colleagues demonstrated objective clinical responses in patients with metastatic synovial cell sarcoma and melanoma treated with TCRs towards NY-ESO-1 antigens. Two melanoma patients out of 11 were observed with complete regression that persisted for more than 1 year [110]. Although most clinical trials show objective responses in melanoma patients, the challenge is now to expand this TCR engineering approach to a broader range of cancers.

Besides TCR engineered T cells, chimeric antigen receptor (CAR) modified T cells are a second class of engineered T lymphocytes. In contrast to TCRs that are specific for a certain HLA-peptide complex, CARs can recognize antigens in a non HLA-dependent way. This can be advantageous for the broad application of CARs in patients with different HLA haplotypes [111]. Furthermore, CARs increase the number of potential targets due to their recognition of not only proteins but also carbohydrates and glycolipids [112]. The broad range of tumor antigens targeted by CARs was recently reviewed elsewhere [113]. Although one of the first successful CAR therapies with complete remissions was reported on a solid neuroblastoma tumor, the most investigated tumors in CAR therapies are hematological malignancies [114]. To date, the most investigated CAR target is CD19, which is expressed on normal B cells as well as on the majority of B cell leukemia's and lymphomas. Several clinical studies on patients with B cell malignancies such as chronic lymphocytic leukemia resulted in partial and complete responses in a subset of patients [115-117]. A recent report on the CD19-targeted CAR treatment of 2 children with relapsed and refractory acute lymphoblastic leukemia described the complete regression in both children [118]. One child was reported with an ongoing complete regression while the other child relapsed due to the growth of blast cells that no longer expressed CD19. These observations demonstrated that CD19 CARs are able to kill aggressive leukemia's. Nevertheless, the development of T cells with CARs against other molecules in combination with CD19 CARs is recommended in case the tumor can escape immune responses by down-regulation of the CD19 CAR target. Based on the success of CAR therapies in hematological malignancies, the expansion of this therapy to numerous solid tumors is one of the major future goals of ACT. Of note however, the eradication of solid tumors appears to be more challenging by virtue of an immunosuppressive tumor microenvironment that may hinder cell-based immunotherapy.

4. The tumor microenvironment as a major hurdle in cancer immunotherapy

4.1. Infiltration of the tumor by cancer-specific T cells

The success of cancer vaccination and adoptive T cell therapies largely depends on the capacity to increase the amount of effector T cells in the tumor. However, it was observed that subsets of patients show no benefit from these therapies because of numerous barriers built up by the tumor microenvironment, for example the impediment of T cell infiltration and the inhibition of T cell stimulation [119]. Several studies demonstrated improved clinical benefit of immunotherapies in patients with a T cell inflamed tumor [120-122]. Therefore, the development of strategies to promote T

cell infiltration in the tumor site would be of interest. The lack of tumor-reactive T cells in the tumor microenvironment can be attributed to several features such as a dense stroma that can sterically impede the infiltration of immune cells as well as low levels of inflammatory chemokines that recruit anti-tumor T cells into the tumor. A more detailed insight in the natural process of spontaneous T cell responses in subsets of patients may educate researchers on how to improve cytotoxic T cell infiltration [123].

4.2. Immunosuppressive pathways exerted by the tumor and its environment

In case of TIL therapy, tumor-reactive T cells can infiltrate into the tumor, although clinical responses were again only seen in a subset of patients. This implies the presence of other potential tumor microenvironmental hurdles such as up-regulated suppressive pathways including the presence of immunosuppressive cells, for example myeloid derived suppressor cells (MDSCs) and T_{regs} (Figure 3). These suppressive immune cells and the mechanisms they employ to aid tumor progression and dissemination as well as inhibition of anti-tumor immune responses has been extensively reviewed by Emeagi et al [124]. MDSCs represent a heterogeneous population of immune cells that promote tumor growth by its immunosuppressive role, which includes the suppression of the activation of anti-tumor T cells [125, 126]. Therapeutic strategies for targeting MDSCs were recently reviewed by Suzanne Ostrand-Rosenberg and colleagues [127]. T_{regs} are known to inhibit T cell anti-tumor functions and are therefore eradicated by lymphodepleting regimens prior to adoptive T cell transfer of either TIL or TCR/CAR engineered T cells [128]. The clinical benefit of lymphodepletion was demonstrated by various preclinical and clinical trials [129-132]. Patients were mostly treated with the lymphoablative chemotherapeutics cyclophosphamide and fludarabine, often in combination with total body irradiation [133]. In addition, due to destructive effects of total body irradiation and lymphoablative chemotherapy on tumor cells, released tumor antigens can be taken up by APCs, resulting in the enhanced activation of adoptively transferred T cells [134, 135]. However, the toxicity associated with lymphodepleting conditioning precludes a number of patients from adoptive T cell therapy and warrants the investigation of other strategies that are better tolerated. One example of an alternative treatment to avoid pre-conditioning regimens that was tested pre-clinically depends on the genetically engineering of the adoptive anti-tumor T cells to secrete IL-12 [136]. IL-12 is an immune-stimulating cytokine that mediates both innate and adoptive immune responses against tumors, including the stimulation and homeostatic expansion of T cells [137]. Those preclinical data demonstrated that the adoptive therapy of IL-12 secreting T cells reduced the need for toxic lymphodepleting regimens.

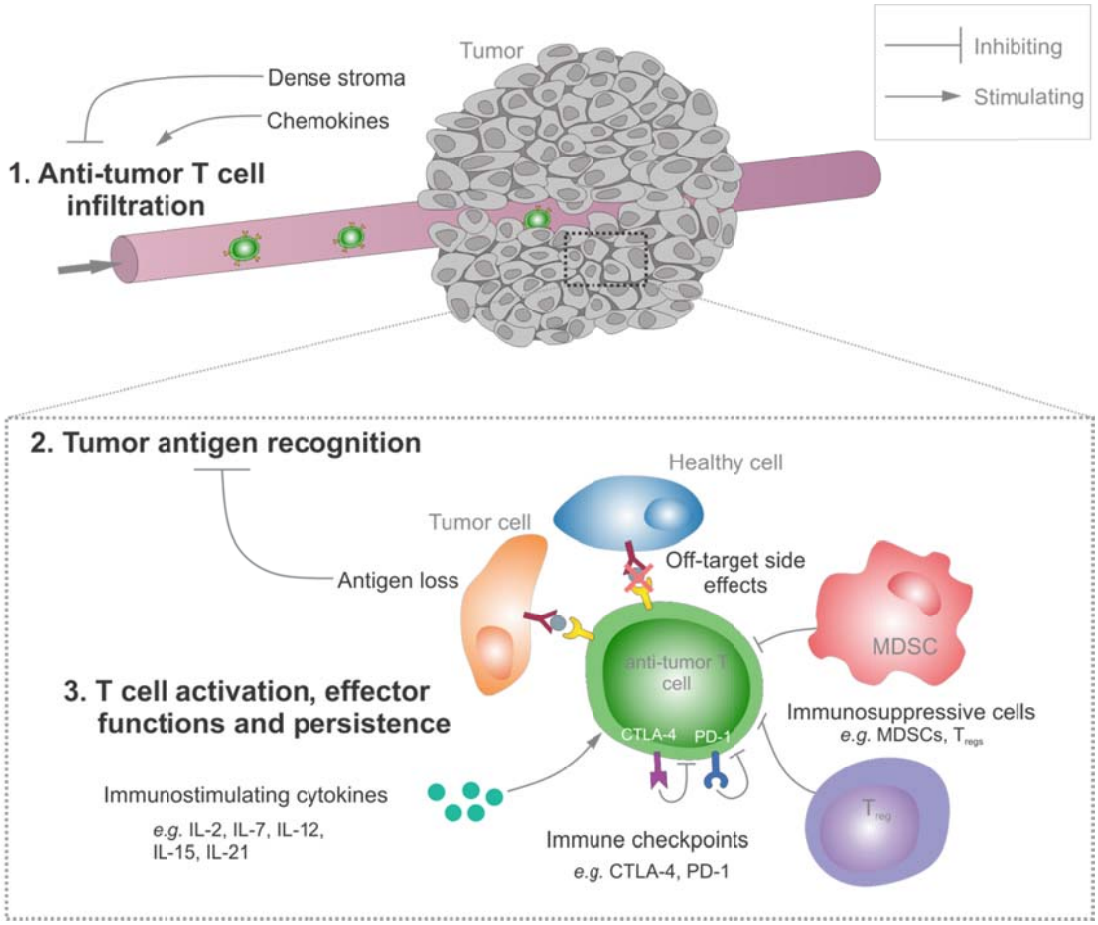

Fig. 3. Successful cancer immunotherapy requires infiltration of anti-tumor T cells, tumor antigen recognition, and T cell activation, effector actions and persistence. All of these different steps can be stimulated or inhibited by different molecules or pathways. T cell infiltration can be stimulated by the presence of chemokines in the tumor site or inhibited by a dense stroma. Tumor antigen recognition can encounter problems due to antigen loss or off-target side effects can occur by interaction of the anti-tumor T cells with healthy cells. T cell activation, effector actions and persistence can be stimulated by cytokines such as interleukins or inhibited by immunosuppressive pathways and cells such as myeloid derived suppressor cells (MDSCs) and regulatory T cells (T_{regs}).

Other interleukins that are currently investigated for the stimulation of anti-tumor T cells are IL-2, IL-7, IL-15, and IL-21. IL-2, a T cell activator, was already described as FDA approved monotherapy for patients with metastatic melanoma and renal cell carcinoma. The combination of IL-2 infusion and adoptive T cell transfer after lymphodepletion improved the clinical outcome in melanoma patients [107]. Furthermore, IL-2 is also used to expand anti-tumor T cells *ex vivo*. However, two major disadvantages of this interleukin are the concurrent stimulation of T_{regs} and the induction of high differentiation of T cells. This differentiation may reduce the generation of an anti-tumor immunological memory [138]. In adoptive T cell therapy not only the direct cytotoxic killing effect by effector $CD8^+$ T cells is important but also the persistence of T cells generating an anti-tumor memory. The combination of the homeostatic

interleukins IL-7 and IL-15 were suggested to support the survival and proliferation of adoptive transferred T cells while also enhancing the persistence *in vivo* to obtain durable cancer regression [139]. Recent clinical data demonstrated that, compared to IL-2, IL-21 promotes the expansion and function of effector $CD8^+$ T cells with a less differentiated phenotype and without co-expansion of T_{regs} [140]. These currently investigated T cell stimulating cytokines can be used to both expand anti-tumor T cells *ex vivo* and provide the transferred T cells with co-stimulatory agents after infusion into the patient [141]. However, the systemic delivery of cytokines, such as the interleukins can be limited due to toxic side effects. One strategy to overcome this toxicity is genetically engineering of isolated anti-tumor T cells to produce their own pro-inflammatory or pro-proliferative cytokines [142, 143]. Another approach to provide T cells with interleukins while minimizing systemic side effects is the chemical conjugation of interleukin-loaded nanoparticles onto the cell membrane of adoptively transferred T cells. The sustained release of the interleukins from the stably attached particles provides the cells directly with immunostimulatory agents *in vivo*. A preclinical study by Stephan and coworkers demonstrated the complete tumor regression of mice treated with anti-tumor T cells decorated with cytokine-loaded nanoparticles on the surface, in contrast to only a modest survival improvement in mice treated with anti-tumor T cells with or without the systemic infusion of the same doses of cytokines [144].

Although immunostimulating cytokines support expansion and activation of anti-tumor T cells and lymphodepletion is able to eliminate T_{regs}, several other immunosuppressive pathways may further impede cancer immunotherapy. Blocking these pathways with immunomodulating antibodies can enhance T cell immune responses against cancer cells. These inhibitory pathways, also known as immune checkpoints, are important for the maintenance of self-tolerance and regulate amplitude and duration of immune responses [145]. Tumor cells may induce some of these inhibitory pathways to escape immunity. For example the expression of the previously described PD-L1 ligand on tumor cells indicates an escape strategy of certain tumors by the induction of the PD-1 pathway. Therefore, interfering with this PD-1 and also CTLA-4 pathway by treatment with monoclonal antibodies may further enhance the anti-tumor immune responses of cancer immunotherapies such as cancer vaccination and ACT. In addition to the well-studied CTLA-4 and PD-1 targets, other co-inhibitory pathways in activated T cells such as T cell immunoglobulin and mucin-domain-containing molecule 3 (Tim-3) and lymphocyte activation gene-3 (LAG-3) have been identified. Studies about monoclonal antibodies against these two pathways reported on the improved expansion and function of antigen-specific $CD8^+$ T cells. Moreover, the combination of either Tim-3 or LAG-3 with PD-1 antibodies resulted in synergistic clinical outcomes [146, 147]. Besides antibody treatment against co-inhibitory receptors and ligands, a number of new antibodies against co-stimulatory receptors have been developed such as anti-CD137 (4-1BB), anti-OX-40 (CD134), anti-CD40, and anti-GITR [148, 149]. These agonist antibodies enhance the persistence and anti-tumor activity of the T cells (Figure 4).

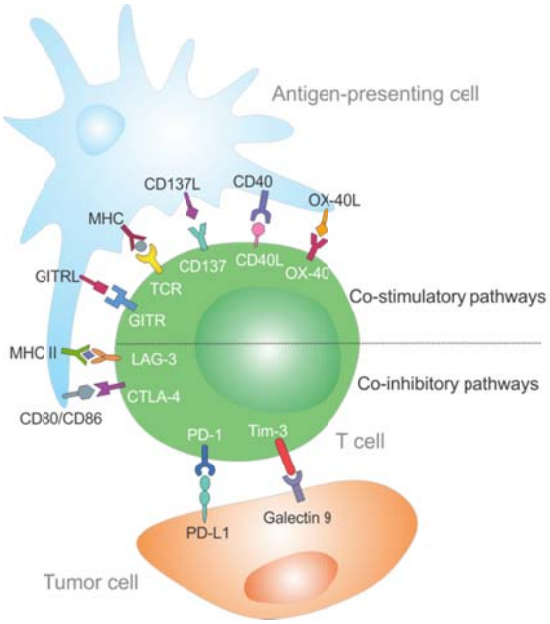

Fig. 4. Schematic overview of immune co-stimulatory and co-inhibitory receptor ligand interactions of anti-tumor T cells with antigen-presenting cells and tumor cells. Interfering with these interactions by monoclonal antibodies that either promote immune stimulating pathways or block immunosuppressive pathways enhances the anti-tumor responses and persistence of T cells. Adapted from Ref. [148].

4.3. Novel combinatorial strategies and the implementation of predictive biomarkers

Because multiple immunoinhibitory pathways are involved in the tumor development, the combination of different antibodies may enhance and prolong the anti-tumor effects. For example, in a recent preclinical study, a combination of CTLA-4 and PD-1 pathway inhibition resulted in a synergistic effect that further enhanced the activation of anti-tumor T cells and improved the long-term survival rates compared to the inhibition of both pathways alone. In addition, the tumor rejection was further improved by the combination with a cancer vaccine [150]. Numerous immunotherapy combinations are currently (pre-)clinically investigated. One example is the combination of ACT with PD-1 antibody therapy that was tested in a mouse model. This resulted in an increase in the amount of transferred T cells in the tumor site and an improved tumor regression compared with either immunotherapy alone [151]. Furthermore, combinations between immunotherapy and small-molecule inhibitors were already performed in mouse models and are currently clinically investigated (see ClinicalTrials.gov number, NCT01659151) [152].

Current progress in the elucidation of new tumor-promoting pathways has led to the development of novel immunotherapy targets and therapeutic strategies to improve immunity against cancer. However, the rising number of treatment options and combinations will further increase the complexity in treatment decision-making for clinical oncologists. Additionally, a next stumbling block is patient

heterogeneity. Patients differ in the immune response they develop against cancer based on differences that can be related to mutations or polymorphisms in immune regulatory genes or various environmental influences. Therefore, the interest in the use of biomarkers including both prognostic biomarkers and predictive biomarkers increases. Prognostic biomarkers are related to the clinical outcome independent of the treatment while predictive biomarkers assess the likely benefit of a specific treatment. Consequently, predictive biomarkers can be used to facilitate therapeutic decision-making. This means that patients who might clinically benefit from a particular treatment can be identified while patients with poor probability on clinical response may be excluded from treatment to protect them from severe drug-related side effects [153]. Furthermore, the opportunity to exclude non-responders from the therapy by screening procedures may have a high impact on treatment cost. In general, the screening for predictive biomarkers will be indispensable in a personalized immune-based cancer strategy.

For example, two suggested biomarkers in the treatment of patients with ipilimumab are indoleamine 2,3-dioxygenase (IDO) and FoxP3. IDO is an immunosuppressive enzyme that is expressed by immune cells as well as cancer cells and FoxP3 is a marker of T_{regs} [154]. The increased expression of both IDO and FoxP3 was positively associated with the clinical response to ipilimumab and therefore these factors were suggested as predictive biomarkers [155]. In another study, the pre-existence of antibody responses and the presence of tumor antigen-specific T cells isolated from the peripheral blood of a patient were suggested as predictive biomarkers for treatment with ipilimumab because the presence of these biomarkers were highly correlated with the clinical benefit of the anti-CTLA-4 treatment [156]. Furthermore, the PD-L1 ligand, expressed on various tumor cells, was suggested as a potential predictive biomarker for the antibody therapy against the PD-1 pathway [101, 157]. In general, a T cell inflamed microenvironment can be a predictive biomarker for all cancer immunotherapies because patients with the presence of T cells in the tumor site are more likely to benefit also from immunotherapies such as PD-1 pathway blockade [123, 158]. Therefore, the presence of TILs combined with the expression of PD-L1 on the tumor cells is a better predictive biomarker in the treatment of patients with PD-1 immune checkpoint blockers than PD-L1 expression alone [104]. This indicates that a combination of predictive biomarkers is more favorable for clinical decision-making.

5. Intra-tumor heterogeneity

Until now, the importance of inter-tumoral heterogeneity was illustrated. Unfortunately, personalized treatments can be hindered by intra-tumoral heterogeneity, which can be described as the presence of genetically distinct subpopulations within a tumor. Due to the ease and decreasing cost of next generation sequencing, it becomes currently more feasible to compare the genome of distinct tumor sites within 1 patient [159, 160]. Several studies demonstrated different genotypes and phenotypes within separated regions of hematological and solid cancers due to diverse somatic mutational events and DNA copy number aberrations [161]. Consequently, the identification of target neo-antigens by a single tumor biopsy sample might often not be representative for the total tumor mass, leading to recurrent disease. In general, this tumor heterogeneity may also contribute to the failure of predictive

biomarkers in the clinic. Besides somatic mutations and DNA copy number aberrations in neo-antigens leading to a decrease in antigen recognition, several studies demonstrated antigen loss or a decrease in antigen expression in tumors as a consequence of the pressure of antigen-specific T cells [129, 162-164]. In the case of a loss in HLA expression, CAR engineered T cells can be superior to TCR engineered T cells because CARs can still target tumor cells that have down-regulated HLA expression [165]. In summary, a better understanding of the clonal evolution of tumors is needed to elucidate the genetic aberrations between separated sites during tumor development and also during treatment.

6. Conclusions

In recent years, immunotherapeutics such as monoclonal antibodies and cancer vaccines have become part of a standard cancer therapy. Compared to conventional chemotherapeutics, immunotherapy has advantages in terms of reducing off-target side effects and inducing an anti-tumor memory that may prevent patients from relapse. The successful clinical data of TIL therapy in metastatic melanoma patients paved the way for the use of adoptive T cell therapy in a broader range of tumors by engineering T cells with a TCR or CAR.

However, the tumor microenvironment is one of the main challenges in the development of cancer immunotherapies. The tumor microenvironment can impede anti-tumor T cell infiltration while a lack of immunostimulatory cytokines and the up-regulation of immunosuppressive pathways inhibit T cell stimulation and persistence. Therefore, the identification and therapeutic targeting of these pathways will become increasingly important. Moreover, combining various immunotherapies has been demonstrated to result in synergistic effects. These observations currently encourage researchers to further investigate in combination therapies. However, the increasing number of immunotherapeutics, the differences in immune reactions between patients, and the tumor heterogeneity augment the need for predictive biomarkers to identify patients that may benefit from a selected therapeutic strategy. Screening for predictive biomarkers, which is part of a personalized approach, may help clinicians in their treatment decision-making. In addition, this personalized strategy may offer advantages in terms of reducing side effects and costs for patients that might not show therapeutic response.

The implementation of a personalized approach in cancer immunotherapy can be further extended to the rational selection of suitable tumor antigens as targets in cancer vaccination and adoptive cell therapy with TCR and CAR engineered T cells. Self-antigens can be used in a broad range of patients, although their clinical use is severely hampered by off-target toxicity and low avidity levels previously observed in clinical trials. Therefore, the identification of tumor-specific antigens as targets for these therapies will become increasingly important. Decreasing cost of next generation sequencing platforms will make this identification in a patient-specific manner more feasible in the future.

On the other hand, the development of immunotherapeutic personalized and cell-based therapies for cancer may give rise to a number of additional regulatory challenges. In these therapies, not all of the general guidelines in current drug development can be applied. For example assessment of the stability

and shelf life of autologous cell-based products cannot be established for every batch due to the 'on-demand' production and short time until administration. Regulatory agencies that control the pre-market testing and marketing approval currently put a lot of effort in the facilitation of the development of new cancer immunotherapeutics. A recent review on the FDA's approaches to evaluate the safety and efficacy of these products has recently been published by Vatsan et al. [166].

Although patient-specific strategies pose regulatory challenges, a personalized approach is already embedded in anti-cancer targeted therapies such as cetuximab, trastuzumab and vemurafenib. These treatments are designed for people whose cancer cells harbor a specific mutation or overexpress certain genes. Companion diagnostics that detect whether a patient's tumor has the target gene mutation or overexpression are performed prior to initiating treatment with a particular drug. Recently, the FDA approved two new companion diagnostics, one for the screening of the BRAF mutation in patients with advanced skin cancer in order to justify treatment with a BRAF inhibitor and the first companion diagnostic to detect EGFR gene mutations in non-small cell lung cancer patients who are candidate for treatment with erlotinib [167, 168]. In summary, due to advances in genetic sequencing and identification of predictive biomarkers, a personalized treatment will be implemented in the field of cancer immunotherapy to improve safety and clinical outcome in patients that are refractory to current standard cancer therapies.

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