Chapter 9
Adoptive T-Cell Immunotherapy: Perfecting Self-Defenses

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Abstract The unrivaled potential of T cells for targeted immune function is central to the eradication of cancer. While their natural anti-tumor response might sometimes be insufficient, several studies and importantly, multiple clinical trials in terminally-ill cancer patients have demonstrated that it is possible to design novel and efficient immunotherapeutic approaches based on the adoptive transfer of autologous tumor-specific T lymphocytes. Herein, we will expand on the development and the use of such strategies using tumor-infiltrating lymphocytes or genetically-engineered T cells. We will also comment on the requirements and potential hurdles encountered when elaborating and implementing such treatments as well as the exciting prospects for this kind of emerging personalized medicine therapy.

List of Abbreviations

ACT	Adoptive cell transfer
AICD	Activation-induced T-cell death
CAIX	Carboxy-anhydrase-IX
CAR	Chimeric antigen receptor
CEA	Carcino embryonic antigen
CT	Cancer/testis
EBV	Epstein–Barr virus
HLA	Human leukocyte antigen
hTERT	Human telomerase reverse transcriptase
HTLV-1	Human T-cell lymphotrophic virus type I
IL	Interleukin

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ITAM	Immunoreceptor tyrosine-based activation motif
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
PBL	Peripheral blood lymphocyte
RCC	Renal cell carcinoma
scFv	Single-chain variable fragment
TA	Tumor antigen
TCR	T-cell receptor
TGF-β	Transforming growth factor-β
TIL	Tumor-infiltrating lymphocyte
Tregs	Regulatory T-cells

Contents

9.1 Tumor Antigens: Defining the Target

T cells play a central role in the immune response against cancer. Their activation is initiated by the interaction of the T-cell receptor (TCR) with its cognate MHC-peptide complex presented on the surface of the target cell, which activates them specifically [1]. Whether T cells could recognize endogenous tissues was a matter of debate during several decades, especially as T cells are supposed to be tolerant to self-antigens. Nevertheless, molecular and immunological advances in the 1990s led to the discovery of self-originated proteins that could be recognized

by T lymphocytes [2]. Accordingly, tumor-specific T cells have been shown to be activated through the binding of their TCR to specific epitopes derived from tumor antigens (TA) presented by a major histocompatibility complex (MHC) molecule [3]. TA are present on some tumor cells but also on normal tissues (in this case, they are termed tumor-associated antigens—TAA), and were shown to represent effective targets for T-cell-based cancer immunotherapy. They can be classified into several categories; this division pertains to the pattern of expression of these antigens (e.g., over-expressed, oncofetal, ...) and whether these antigens are "self" or mutated [4]. Several sources indicate different classifications, but five known classes of TA can be broadly described:

Cancer/testis antigens (*C/T*)—they are expressed in various human cancers, but also in normal testis tissues. Some evidences suggest that there may be some level of T-cell tolerance toward these antigens [5].

Tissue-specific differentiation antigens—these antigens are typically expressed only by the tumor and its tissue of origin. Known examples of tissue-specific differentiation antigens include the MART-1/Melan-A and gp100, which are expressed in both melanocytes and melanoma cells. These antigens have emerged as very promising target antigens for T-cell-based adoptive immunotherapy, but their presence on normal tissues can be the source of auto-immune manifestations.

Mutated self-proteins—usually when mutations occur in the initial cancerous cell (or one of its early daughter cells), this class of tumor antigens can potentially provide targets for T-cell-based immunotherapy of cancer, as they are to be expressed in most of the tumor tissues.

Over-expressed antigens—this type of antigens constitutes also an important TA class, which is relevant in both T-cell therapy and antibody-based treatments. Based on clinical data, it seems that their over-expression in several tumor tissues (e.g., Her2/neu) but then again their reduced levels in healthy cells may limit the potential for deleterious autoimmune side-effects [4].

Viral antigens—as it is believed that around 20 % of all cancer cases are linked to infectious agents [6], antigens derived from oncogenic viruses would provide a source of "non-self" targets, which would be recognized more efficiently than TAA due to a potential lack of tolerance against the viral epitopes.

9.2 Tumor-Infiltrating Lymphocytes

9.2.1 Presence of Intra-tumoral T Lymphocytes

For several decades, it has been demonstrated that tumor-specific T cells can massively migrate into tumor sites. Some of these tumor-infiltrating lymphocytes (TILs) have thus the ability to specifically recognize tumor antigens expressed on the surface of tumor cells, and may greatly influence directly or indirectly the anti-tumor immune responses and the progression of a variety of solid tumors [7]. The

presence of TILs in the tumor vicinity, and the nature of their interactions with target cells, contribute to determine whether a tumor is destroyed or grows unimpeded. It may also correlate with responses to chemotherapy/radiotherapy and disease prognosis. Indeed, high densities of $CD3^+$ T cells, $CD8^+$ cytotoxic T cells, and memory T cells into tumor sites could represent a reliable prognostic factor for the disease-free and overall survival of patients with different tumor types, such as melanoma, and head and neck, breast, bladder, urothelial, ovarian, colorectal, renal, prostatic, and lung cancer [8]. In contrast to the effects of cytotoxic T cells and memory T cells that are associated with a positive clinical outcome, the impact of $CD4^+$ T cell infiltration on survival and prognosis is unclear; for example, there are conflicting data about the role of regulatory T-cells (Tregs) in this context, and their effects on tumor progression have been a matter of debate for the past decade [7, 9]. Moreover, there is a great variability in the density and location of these infiltrating T cells between different patients bearing the same type of cancer [7].

9.2.2 Adoptive TIL Immunotherapy

Nonetheless, to harness the potential benefit of tumor-specific T cells in cancer treatment settings, pioneering therapeutic approaches (Fig. 9.1) were developed in the last three decades [10]. Adoptive immunotherapy using autologous TILs has become an appealing strategy for the treatment of mainly melanoma and renal cell carcinoma. This necessitated the development of techniques and systems to grow large numbers of anti-tumor lymphocytes. An important milestone in the development of this kind of immunotherapy occurred in 1987 when tumor-infiltrating lymphocytes from patients with metastatic malignant melanoma were successfully cultured and expanded using the T-cell growth factor interleukin 2 (IL-2) [11]. During this expansion process performed ex vivo, fragments from resected tumors were grown in culture vessels in conditions that favor T-cell growth (using for example high concentrations of IL-2). Tumor-specific T-cell populations can be identified on the basis of their reactivity with MHC-matched tumor cell lines or the autologous tumor. Reactive cultures can be then selected and expanded, and adoptively infused back into cancer patients. Furthermore, to facilitate the engraftment of this autologous T-cell transplant, patients receive high-dose intravenous bolus IL-2 [12, 13]. As exemplified in several studies, the transfer of these cells back into the patient led to dramatic partial or complete clinical responses and durable regression [14, 15].

The adoptive transfer of TILs is one of the most effective treatments for patients with stage IV melanoma. The first study aimed at directly targeting human tumor using autologous TILs to treat patients with metastatic melanoma was reported in 1988 by Rosenberg et al. at the National Cancer Institute [16], and a significant improvement in the response rate and durability of response was steadily reported in subsequent studies [15]. This improvement occurred when bulk cultures ($CD8^+$ and $CD4^+$) were transferred and more importantly, when a non-myeloablative

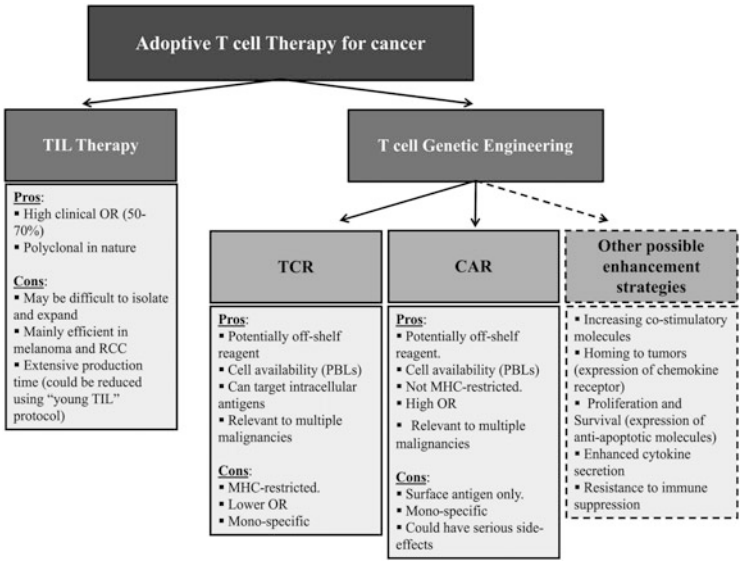

Fig. 9.1 A summary of different adoptive T-cell therapy approaches. *OR* objective response, *PBLs* peripheral blood lymphocytes

conditioning regimen (depleting chemotherapy or whole-body irradiation) was administered to the patient prior to T-cell transfer [12]. In that regard, studies reported a significant correlation between the intensity of lymphodepletion and the in-vivo anti-tumor effect of the infused cells [17]. It has been suggested that the positive impact of lymphodepletion prior to TIL transfer is based in part on the elimination of suppressive $CD4^+CD25^+$ Tregs as well as of normal endogenous lymphocytes that could compete with the transferred cells for homeostatic cytokines such as IL-7 and IL-15 [18, 19].

Recent results indicate that the objective clinical response observed in patients with metastatic melanoma that were treated with adoptively transferred autologous TILs ranges between 49 % and 72 % [15]. Importantly, objective response was highly associated with the persistence of the transferred cells [20]. Indeed, many patients in the recently reported trials display high levels of persistence, sometimes reaching up to 75 % of all of the circulating $CD8^+$ T cells. Still, it appears that persistence alone was not a sufficient requirement for an effective response [20, 21]. Studies have also shown that the state of differentiation of the transferred cells may be inversely correlated to the effectiveness of these cells in adoptive cell therapy (ACT) settings, and to their capacity to proliferate and persist [12, 22]. In other words, early effector T cells seem to mediate better anti-tumor response than intermediate and late effector T cells.

9.2.3 Tumor Microenvironment and Potential Hurdles

Solid tumors contain many other cell types, including cells derived from the innate and adoptive immune system, stromal cells, and myeloid-derived suppressor cells (MDSCs) [23, 24]. The latter are endowed with potent immunosuppressive properties, and their intratumoral presence at a high frequency correlates with a poor prognosis in patients with different tumor types. Recent findings indicate that targeting these cells, and the supportive environment (for the tumor) they promote, might represent an effective approach to promoting the destruction of cancer cells, leading to tumor elimination [25].

Despite its aforementioned success (especially in melanoma), adoptive cell transfer (ACT) therapy with autologous TILs bears some limitations which include, for example, the requirement to isolate and expand T cells with anti-tumor activity. Even if such cells are generated, adoptive T-cell therapy for some tumors will not necessarily be effective, as these may be poorly antigenic. Other tumors, such as colon and breast tumors, are infiltrated by T cells, but the specificities and functions of the latter are unclear [26, 27]. In this regard, a potential explanation as to why melanoma has been widely studied as a target for therapeutic TILs is that this type of cancer appears to be unique among human cancers because of its ability to promote elevated numbers of lymphocytes with anti-tumor activity. This might be due to the fact that melanoma tumors express a high number of mutated antigens that could help in breaking self-tolerance and were also shown to harbor class II-MHC molecules [10, 28]. Renal cell carcinoma (RCC) is also considered an immunogenic tumor that exhibits rich intra-tumoral lymphocytic infiltration. Still, it seems that T-cell activation is insufficient at the tumor site due to many immunosuppressive mechanisms induced in the microenvironment of RCC [29–32]. This may provide an explanation as to why previous clinical trials with TILs in RCC did not yield substantial benefit compared to melanoma. Nevertheless, current knowledge and experience with TIL generation from—and treatment of—melanoma patients could provide clues to elaborate an improved therapeutic regiment for ACT in RCC and other malignancies [33, 34].

9.2.4 TIL Treatment: Current Status and Future Promises

By utilizing current techniques today, tumor-infiltrating lymphocytes can be detected in approximately 80 % of melanoma patients [35]. However, in most cancer patients, those naturally-occurring TILs fail to destroy the tumor as they are outnumbered, subjected to constant immunosuppression, and due to other factors that are not fully understood. Additionally, the generation of a TIL culture (s) that prove reactive for each patient tumor is not always feasible and requires several weeks. The latter might be overcome, as exemplified in new clinical studies designed to improve the TIL anti-tumor activity, growth, and expansion by

generating "young TIL" cultures [36, 37]. In this method, tumor-infiltrating lymphocytes are grown and expanded briefly (around 2–3 weeks compared to 4–6 in the conventional TIL protocol) and are introduced back into patients without testing for selection. Thus, the "young TIL" protocol utilizes bulk unselected TIL which spend minimal time in culture by eliminating the individualized tumor reactivity screening step [38]. As no further selection process is required, all established "young TIL" cultures are technically eligible for treatment [37]. "Young TIL" protocols reduce labor time and can be implemented in most patients, but importantly, recent studies indicate that this approach leads to an objective response rate of 50 %, close to that observed in classical TIL protocols [36].

As immunomodulatory monoclonal antibodies show promise in the clinical trials recently conducted, the combination of T-cell transfer with antibodies blocking CTLA-4 or PD-1 function may help to overcome negative costimulatory signals, which may improve the function of the transferred T cells [39, 40]. In addition, it is possible to manipulate the T-cell differentiation state during culture/expansion to improve TIL-ACT for the treatment of human cancer, using, for example, molecules that may inhibit differentiation processes (e.g., GSK-3b [41]) or by subjecting TIL cultures to different cytokines, such as IL-7, IL-15, or IL-21 alone or in addition to IL-2 [42–47].

While TIL-based clinical trials have demonstrated impressive results in terminally-ill melanoma patients, they require dedicated facilities, and collaboration between surgical and cell therapy teams, which may have limited their implementation to a few clinical centers worldwide. Nonetheless, parallel approaches aimed at exploiting the unrivaled potential of T cells to mediate tumor regression are being developed, and are based on the genetic modification of T cells to express tumor-specific receptors.

9.3 Adoptive Immunotherapy Based on the Genetic Modification of Lymphocytes

9.3.1 TCR Gene Transfer

9.3.1.1 Development and Implementation of TCR Gene Transfer Approaches

As T-cell specificity is solely based on the nature of its TCR, TCR gene transfer therapy represents a promising approach based on the genetic modification of T cells engineered to recognize tumor antigens. A study by Steinmetz and colleagues back in 1986 demonstrated for the first time the feasibility of the TCR gene transfer approach. In this study, T cells were redirected by genetic engineering in order to study the receptor dynamics [48]. Since then, several studies have demonstrated

how human T cells can be redirected toward specific antigen by TCR gene transfer using a melanoma-specific TCR in vitro [49], followed by an in-vivo study using a mouse model [50]. In 2006, the first clinical trial involving TCR gene therapy was reported by Morgan et al. involving metastatic melanoma patients, who were treated with autologous peripheral blood lymphocytes (PBLs) retrovirally transduced with a MART-1 specific TCR following a lymphodepletion regimen. An objective clinical response was observed in two out of 17 patients treated in this trial (12 %), demonstrating dramatic tumor regression [51].

Three years later, the results of a second clinical trial were reported by the same group (led by Dr. Steven Rosenberg, NCI); in this trial, metastatic melanoma patients were treated with two high-affinity TCRs against the melanoma antigens MART-1 and gp100 [52]. The expression levels of the TCR and the persistence of modified T cells were markedly increased compared with the first trial, and an objective response rate of 30 % (six out of 20 patients) was reported. Since then, progress has been made towards the clinical testing of additional TCRs, specific to other antigens such as p53 [53], NY-ESO-1 [54], and CEA [55], in order to target cancers other than melanoma.

So far, TCR gene transfer has been proven to be an effective strategy to create specific tumor-reactive T cells, without the restrictions or the need of isolating natural tumor-reactive T cells from the patient. Factors that should be taken into account towards improving the clinical efficacy of this approach, and that will be discussed in part below are, for instance, the persistence of the TCR-modified T cells after infusion, the prolonged expression of the TCR genes, and the need to reach sufficient T-cell functional avidity.

9.3.1.2 How to Select the Appropriate (Suitable) Antigen?

As for other therapeutic treatments, two main factors should be considered to choose the proper target antigen for TCR gene therapy: safety and efficiency. By choosing a target antigen characterized by high levels of tumor-specific expression and lacking any expression levels in the normal tissue, one can limit the possibility of on/off-target effects and the possible dose-limiting toxicity which can result from the destruction of normal tissues that express the aimed target antigen [55].

Currently, over-expressed antigens, cancer-testis (CT) antigens, and differentiation antigens represent the most common target antigens for TCR-based adoptive immunotherapy. NY-ESO-1, a cancer-testis antigen (CT), is one of the most promising targets that have been the subject of a recent clinical trial for TCR gene therapy, which resulted in a 40–60 % objective response in melanoma and synovial cell sarcoma patients [54]. Many CT antigens have been identified in various human cancers is discussed above [5, 56], while they are normally expressed only in the human germ line. The restriction of CTs to cells that partially or do not express human leukocyte antigen (HLA) molecules (in healthy tissues) makes them unsusceptible to recognition by a TCR, thus preventing toxicity to normal tissues when targeting T cells to tumor-associated CT antigens. Two other

classes of tumor antigens that may be also taken into account as targets for TCR gene therapy are the mutation antigens and the neo-antigens [57, 58]. Indeed, it seems that the majority of these antigens are to be safe targets owing to their exclusive expression in tumor cells. While the first group is represented by antigens that are common not only to a variety of patients but also shared between several tumor types, the second group is constituted of patient-specific antigens that can be characterized using recent technological advancements such as individual tumor sequencing [57]. Still, as an immune selective pressure builds up, the down-regulation of target antigens could represent a concrete impediment to the therapeutic efficacy of TCR gene therapy [59, 60], especially as it is based on mono-specific T cells. Recently, the study of Kaluza and colleagues demonstrated tumor (B16/Ovalbumin) recurrence after adoptive transfer of specific (OT-1) effector cells, due to the loss of the target tumor antigen [61]. Possible solutions for the down-regulation of target antigen expression may consist in: (1) targeting of proteins that have an essential role in the survival of the tumor [4], (2) combining two (or more) different specificities expressed by the same T cell [61], or (3) using multiple populations of T cells, each expressing a different tumor-specific TCR.

9.3.1.3 Choosing the "Right" TCR for the "Right" pMHC Complex

Several approaches have been described in order to isolate the desirable TCR, which will not only recognize specifically the targeted peptide–MHC complex, but will also endow T cells with superior functional avidity. As mentioned above, the objective response rate observed in the first two clinical TCR-gene therapy trials, in which MART-1-specific TCRs were produced from a melanoma patient [51, 52], was low in comparison to that in TIL therapy trials [17, 38, 62]. This disparity could be due to: (1) low levels of TCR expression of the introduced TCR on the engineered T cells, (2) a diminished persistence of TCR-modified T cells after infusion, and/or (3) the induction of immunological self-tolerance that might hinder a proper response to target antigens with suboptimal affinity to their cognate TCR. Therefore, unmodified TCRs derived from melanoma patients may require further optimization steps to endow T cells with an improved performance.

High-affinity TCRs could be isolated from HLA-mismatched donors, since one does not expect that those TCRs would be subjected to any tolerance mechanism pertaining to the targeted MHC–peptide complex, which thus would be recognized as non-self [63–65]. Similarly, HLA-transgenic mice [66–69] and phage/yeast/T-cell display systems [70–73] also provide platforms that could be exploited to isolate "non-tolerized TCR." The TCR phage display technique, for example, yielded TCRs with high affinity specific for human telomerase reverse transcriptase (hTERT), human T-cell lymphotropic virus type 1 (HTLV-1), TAX antigen, and additional antigens [73, 74].

Additionally, a human-TCR repertoire transgenic mice system was recently established. In this system, the entire human TCR loci was cloned into HLA-A2-transgenic mice [75], and this resulted in the reconstitution of a potentially broad

human TCR repertoire in the mouse recipient which can provide a platform to isolate human high-affinity TCRs, provided the targeted epitope is not expressed by the mouse recipient.

9.3.1.4 TCR Expression Systems

In most of the clinical trials reported, TCR gene therapy made use of γ-retroviral vectors which are common viral expression systems that facilitate transgene integration into the genome of the host cells [76–78]. MFG/SFG-, MP71/SF91-, and MSGV1- are examples for such γ-retroviral vectors that in pre-clinical studies and clinical trials exhibit high transduction efficiency together with minimal vector-associated toxicity. Lentiviral vectors are another viral expression platform that, unlike γ-retroviral vectors, is largely independent from cells' dividing status and thus could successfully infect minimally activated T cells [79, 80]. Moreover, lentiviral vectors display a greater gene insertion capacity, allowing the transfer of larger and highly complex gene constructs into T cells.

There are also several non-viral alternatives for TCR-gene transfer into T-cells. One main advantage of the latter is that, unlike viral platforms, they require a minimal production and testing time from a regulatory standpoint. The *Sleeping Beauty* and the *piggyBac* are example of transposon-based systems that have been used to alternatively redirect T cells to express antigen-specific receptors [81, 82]. This approach relies on the expression of the transposase in the target cell, together with the transfer of the transposon that encodes the genes of interest [83, 84]. Transfer of mRNA molecules encoding TCR chains by electroporation may also be used as a non-viral expression system to modify T cells; it eliminates the risk of insertional mutagenesis. Still, the main downside of this approach is the short-term expression of the transgene (a few days), which necessitates repetitive injection of electroporated cells to achieve in-vivo effects [85].

9.3.1.5 Off-Target and Safety Risks Involved in TCR Gene Transfer Strategy

Off-target events following TCR gene therapy may be due to self/cross-reactivity of the transduced TCR and/or the formation of mixed dimmers between the two α and two β chains that are co-expressed in the transduced cell, which may potentially lead to new auto-immune specificity [86]. Four different TCR combinations can form when mixing the chains that originated from the exogenous α/β TCR with the two chains that originate from natural/endogenous α/β TCR. The two mispaired heterodimeric TCRs may result either in a non-functioning TCR or a receptor with a new specificity that can prove self-reactive. In this regard, a recent study demonstrated how the formation of mixed TCRs can result in self-reactive T cells that engendered autoimmune manifestations in a mouse model [87].

Several strategies have been devised to increase the expression of the introduced TCRs, which are often based on molecular approaches aiming for better pairing/association of the α/β chains of the introduced-exogenous TCR [86, 88]. For example, hybrid human TCRs that are composed of parts of/entire murine constant regions [89–93] mediated an improved expression of the transferred TCR. The inclusion of an additional disulfide bond within the constant region of the TCR [94, 95], molecular "knob into holes" inversions in the constant regions of the TCR chains [96], single-chain TCRs [97], and the use of TCR/CD3ζ fusion products [98] were also recently demonstrated as potential pairing-optimization strategies. Since α/β and γ/δ TCR chains cannot mutually pair [99], the use of γδ T cells that are transduced with an αβ TCR is also an alternative approach [100]. Silencing the endogenous TCRs is another strategy, which can be achieved by co-transferring siRNAs/shRNAs targeting the endogenous TCR [101] or by making use of zinc-finger nucleases (ZFNs) that are specific for the endogenous TCR chains [102].

9.3.1.6 How to Further Improve the Anti-tumor Efficacy of TCR Gene Transfer?

In addition to the aforementioned strategies to improve adoptive T-cell therapy (such as lymphodepletion and cytokine polarization), several approaches are being developed in order to enhance functional and durable responses by TCR gene therapy. TCR affinity enhancement, which is believed to lead to an improved functional avidity, could be achieved by introducing selective modifications in the CDR3 region of the TCR α or β chain, which has been shown to be crucial for the recognition and binding of the antigen [70, 73]. The use of pairing (see above) and codon optimization (to improve protein expression) may also contribute to enhancing antigen-specific reactivity in T cells [68, 103]. Additionally, it has been demonstrated that reduced TCR glycosylation can elevate functional avidity and prevent the internalization of the transduced TCRs [104]. Recently, we demonstrated that it is possible to greatly enhance T-cell functional avidity against tumor cells by mutating three transmembrane residues in the TCRα chain into hydrophobic amino acid, which led to increased TCR stability and expression and augmented TCR expression in the transduced T cells [105]. In addition, the design of the gene expression cassette may also influence TCR expression: the use of P2A or IRES elements, which link the α and β chains, has been shown to improve TCR expression and to reduce the risk of induced autoimmune pathology [87, 106].

Beyond the engineering of T-cell specificity using TCR transgenes, several genetic approaches to further amplify/generate important T-cell functions (such as co-stimulation, cytokine secretion, expression of chemokine receptors and homing factors) have been described (reviewed in [107]). For example, though the administration of IL-12 in tumor mouse models can improve host survival and tumor regression rate [108, 109], the associated toxicities are a major drawback. Engineering gene-modified T cells to produce IL-12 in vivo using an inducible retroviral vector demonstrated intensified anti-tumor activity against B16 murine

melanoma tumors [110]. Alternatively, the use of T cells that are conjugated to adjuvant cytokine-loaded nanoparticles is another potential way to lead to a local production/delivery of cytokines, while reducing toxicity [111]. The (sub-) type of T-cell to be transduced is also of importance; recent studies have demonstrated the superior properties of other kinds of lymphocytes, such as memory T cells, naïve T cells, memory stem cells and central-memory T cells [41, 112–114].

In addition to TCR signaling, T-cell function is controlled by both positive and negative regulation. The tumor microenvironment has been shown to greatly induce immune suppression. For example, the immunosuppressive role of transforming growth factor-β (TGF-β) involves the inhibition of proliferation and function of T cells [115, 116]. By expressing a non-functional TGF-β receptor, tumor cells may also escape the apoptotic effects of TGF-β [117, 118]. In order to diminish the inhibition induced by TGF-β, it is possible to express in the genetically engineered T cells a truncated (dominant negative) form of TGF-β receptor [119], or to use a decoy-soluble TGF-β receptor II [120]. Bollard et al. recently reported that human T cells transduced with a dominant negative form of TGF-β receptor were resistant to the anti-proliferative and anti-cytotoxic effects of exogenous TGF-β [121, 122]. More recently, several groups [120, 123] have shown that this strategy is also effective in vivo, though the sustained effects of this might not last as expected [123].

9.3.2 Chimeric Antigen Receptor Gene Transfer

In parallel to the TCR gene transfer approach, it is possible to redirect the specificity of T-cells using chimeric antigen receptors (CARs). These CARs, also known as "T-bodies" or "chimeric immune receptors" are fusion proteins that generally contain an extracellular targeting domains based on an antibody single-chain variable fragment (scFv) that is fused to intracellular signaling elements. As mentioned above for TCRs, transduction of peripheral blood T cells with CARs allows the redirection of T-cell specificity against tumor cell surface antigen.

9.3.2.1 CAR Development

The development of antibody-based chimer receptor, was first reported in 1989 in the pioneering studies by Gross and Eshhar [124]. They generated a chimeric T-cell receptor assembled from the TCR constant domains fused to the variable domains of an antibody specific for anti-2,4,6-trinitrophenyl (TNP). T cells that expressed this chimeric receptor successfully recognized TNP, which led to the production of IL-2 and cell-mediated cytotoxicity of TNP-expressing targets. Thus, the use of CARs enables the targeting of tumor in an HLA-independent manner, which

suggests the possibility, in theory, of treating a larger part of the population, compared to TCR-based therapies. Moreover, CARs allow the targeting of not only protein-based antigens but also carbohydrates and glycolipids, provided targeting moieties/monoclonal antibodies can be generated against these. Another advantage of the CAR approach, as these function in an MHC-independent way, is their ability to stimulate both $CD8^+$ and $CD4^+$ T cells, which have been shown to act synergistically in enhancing the T-cell anti-tumor effect [125]. Still, it is important to remember that technically CARs can target only surface expressed antigens (though intracellular antigens could be also detected by CARs based on antibodies that target a specific pMHC (peptide–MHC) complex, and thus can mimic the mode of action of the TCR [126, 127]).

9.3.2.2 CAR Structure

As mentioned earlier, the common design of CARs is based on a binding domain, an extracellular spacer/hinge element, a trans-membrane region, and an intracellular singling domain (Fig. 9.2). Most of the CAR targeting domains are scFv (i.e., the variable regions of heavy and light chains joined together by a short linker peptide). If the scFv is derived from a murine antibody, it is possible to "humanize" it by replacing the mouse framework regions by their human counterparts. Another possible design for the targeting moiety of CARs (instead of scFv) are protein receptor/ligands; such alternatives include, for instance, a vascular endothelial growth factor polypeptide [128], an integrin binding peptide heregulin [129], interleukin—13 mutein [130], NKp30 (NCR3/CD337) [131], and the NKG2D receptor [132].

The second component in this design is the hinge region that serves as spacer, which increases the distance of the binding domain from the transmembrane region, providing more flexibility for the binding domain. The nature of the hinge region can influence cytokine secretion and cell-mediated killing of target cells by CAR-modified T cells [133]. Some common examples for hinge region are immunoglobulin domains such as the fragment crystallizable (Fc) regions of antibodies, or immunoglobulin-like domains derived from CD8α and CD28 molecules. It has been found that the function of the hinge region in the CAR is dependent on the binding site on the antigen itself; if the binding site is a membrane-proximal epitope, the use of a hinge region will be beneficial. In contrast, when the binding site is a membrane-distal epitope, improved cytokine release and cytotoxicity will be higher in the absence of a hinge region [134].

The third component in the CARs is the transmembrane region: in most cases, it is based on transmembrane domains derived from co-receptor/costimulatory molecules such as CD8 and CD28.

The fourth module in the structure of the CARs is the intracellular signaling domain. Importantly, a lot of effort is being invested in order to develop optimal

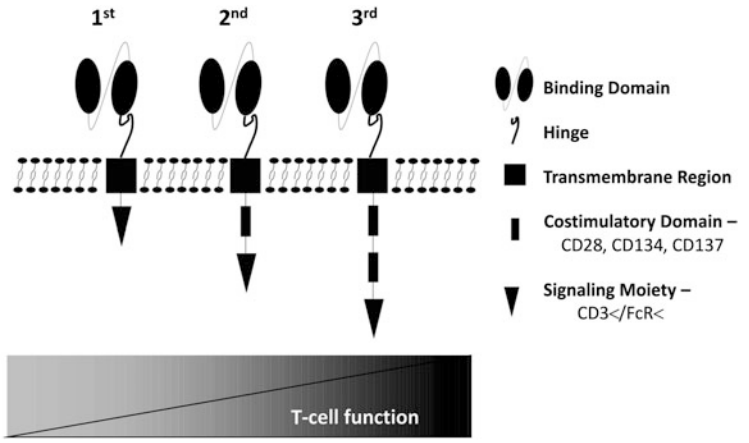

Fig. 9.2 Schematic representation of the different CAR generations

conformation of the intracellular signaling portions to achieve the best activation. The first generation of CARs included only one signaling domain (Fig. 9.2) derived either from the CD3ζ or FcRγ chains, which are the common signal-transducing subunits of the TCR or the immunoglobulin receptor respectively [135]. One main difference between these two subunits is the number of the immunoreceptor tyrosine-based activation motifs (ITAMs); while the CD3ζ chain contains three ITAMs, the FcRγ chain contains only one and this feature has been shown to impact on T-cell function and survival [136].

9.3.2.3 CAR Development and Generations

When first compared, the ζ and γ subunits were fused to single-chain variable domain chimeric receptors recognizing the carcinoembryonic antigen (CEA). Although similar levels of expression were detected after transduction, some significant functional difference was found after co-culture with target cells [137]. These assays demonstrated the superiority of the chimeric receptors that contained the CD3ζ, mainly in improved cytokine production and enhanced ability to mediate lysis of target cells. Additionally, it was revealed that CD3ζ-based chimeric receptors displayed a better ability to eradicate human tumors in vivo. While it has been postulated that the anti-tumor activity mediated by the CD3ζ moiety might result in activation-induced T-cell death (AICD) because of the numerous ITAMs (3), these claims have been refuted [138], and so far most of the CAR designs include a CD3ζ moiety as their main signaling domain.

Despite the encouraging results that were obtained in the studies with the first-generation CARs (that contained only the CD3ζ chain in the intracellular singling domain) and which demonstrated anti-tumor activity against a range of target cells

[139], the lack of co-stimulatory signals ("signal 2") led to inefficient cytokine production, reduced proliferation, and even a state of T-cell anergy [140, 141]. A second generation of CAR was designed to include a co-stimulatory portion in addition to the CD3ζ signaling domain. The most common co-stimulatory molecule that fills this role is CD28, the first isolated co-stimulatory molecule, which is essential to prevent anergy and to drive increased cytokine secretion [142]. Still, the possibility of generating two chimeras that express the ζ chain and the CD28 separately was explored, and this approach did mediate increased secretion of IL-2 in vitro [143]. More recently, a similar concept to reduce CAR side-effects made use of a first-generation CAR transduced in conjunction with a CCR (chimeric co-stimulatory receptor) specific for a second antigen, which enabled safer in-vivo targeting of tumors which expressed both cognate antigens [144]. So far, a more widespread concept is to combine both signaling moieties in the same receptor [145]. From a structural standpoint, a better surface expression of the CAR can be achieved by positioning the CD28 domain in proximity to the CD3ζ domain and immediately after the transmembrane region [146]. Several studies have demonstrated the improved function of second-generation CAR-modified T cells in mediating increased proliferation [147] and cytokine secretion (IL-2, interferon-γ, granulocyte–macrophage colony-stimulating factor) [148, 149]. Furthermore, this kind of design promoted the up-regulation of anti-apoptotic proteins such as Bcl-2 (which would contribute to reduce AICD) and better resistance to immunosuppressive conditions prevalent in the tumor microenvironment; studies have shown that second-generation CAR-modified T cells are less sensitive to TGF-β-mediated suppression [150], and could increase the expression of NFκB counteracting Tregs-induced inhibition [151].

There does not seem to be an optimal signaling moiety for CARs, and thus there is often a need to evaluate empirically several combinations for each given targeting moiety. Although most of the CARs use the CD28 signaling domain, alternative co-stimulatory molecules that were tested include the inducible T-cell costimulator (ICOS) B7 family member, and CD27, CD137 (4-1BB), and CD134 (OX-40) from the TNFR family members, which can enhance effector functions also in resting human T cells [152–154]. However, to further improve second-generation CARs, several studies have shown that it was possible to include another co-stimulatory moiety in addition to CD3ζ chain and CD28 in the signaling domain, leading to the design of third-generation CARs [155]. For example, a CAR for prostate-specific membrane antigen (PSMA), which contains $CD28^+$ $4\text{-}1BB^+$ CD3ζ signaling domain, showed an increased cytokine production and mediated an improved prostate tumor regression in vivo [154]. Furthermore, third-generation CARs can induce PI3Kinase/Akt activation and Bcl_{XL} expression and can help to reduce T-cell apoptosis. Another study showed that a CAR that contained the antigen-binding domain of the anti-GD2a fused to a CD28/OX40/ζ signaling domain endowed T-cells with improved proliferative capacity and anti-tumor function [156]. Still, the presence of the three activation/stimulation motifs in a single signaling domain may theoretically cause a lower sensitivity threshold, which should be taken into account when designing future clinical applications.

9.3.2.4 Driving the CARs into the Clinic

Results from in-vitro and in-vivo (in animal models) studies that show the potential of CARs in mediating tumor regression in several types of cancer—such as medulloblastoma, prostate [157] and colon carcinoma [158]—,facilitated their translation into the clinic. In the first clinical trial that made use of first-generation CAR-modified T cells, Lamers et al. treated three patients with metastatic renal cell carcinoma (RCC) using a CAR that recognizes carboxy-anhydrase-IX (CAIX), which is over-expressed by RCC tumors. All three patients were reported to suffer from liver toxicity, which was apparently caused by on-target effects of CAR-modified T cells against the $CAIX^+$ bile duct epithelial cells and no clinical responses were observed [159]. In another trial, 14 patients with metastatic ovarian cancer were treated with CAR-modified T cells against the ovarian cancer-associated antigen α-folate receptor (FR) [160]. Analysis of the CAR-modified T-cell presence in the circulation showed it quickly declined in the majority of the patients after 1 month, and also in this case no clinical response was observed in any of the patients treated.

Pule et al. engineered Epstein–Barr virus (EBV)-specific CTLs to express a first-generation CAR directed to the diasialoganglioside GD2 antigen, which is expressed on neuroblastoma cells. Infusion of these CAR-modified T cells seemed safe, and resulted in encouraging tumor regressions in half of the subjects tested [161]. Whereas these three clinical trials used retroviral transduction, in a clinical trial reported by Till et al., CAR-modified T cells were generated by electroporation with a vector plasmid encoding a CAR specific to CD20, to target indolent B-cell lymphoma (or mantle cell lymphoma). Out of seven patients treated, two achieved complete responses, one had a partial response, and four had stable disease [162]. Another notable clinical study was carried out recently by Kalos et al., in which three patients with advanced chronic lymphocytic leukemia (CLL) were treated with an anti-CD19 second-generation CAR that contained a CD3ζ chain coupled with CD137 domain. CAR-modified T-cells expanded over 1,000-fold in vivo, trafficked to the bone marrow and remained detectable 6 months post-infusion; a fraction of these cells even differentiated into memory T cells. Ten months after treatment, all the patients demonstrated an objective clinical response, with two of the three patients treated showing complete remission and one partial response [163]. A recent clinical trial using a third-generation CAR was conducted by Till et al. using a CAR targeting CD20 (which is expressed on indolent B-cell and mantle cell lymphomas) [164]. This third-generation CAR contained two co-stimulatory domains, CD28 and CD137, in addition to CD3ζ. CAR-modified T cells were detected for up to 1 year in patients' blood. Moreover, one out of four patients treated had an objective partial response (later relapsed a year after infusion), one patient developed transient infusional symptoms, and two patients remained progression-free for 12 and 24 months. Thus, some 20 years after they were initially developed, chimeric antigen receptors have entered the clinic and are showing promising results.

Nevertheless, one has to bear in mind that side-effects may arise, and unfortunately these may on rare occasions be lethal. In a trial that made use of a trastuzumab (Herceptin)-based third-generation CAR to target breast tumors, infusion of CAR-modified T cells led to the death of one patient. This was attributed to a "cytokine storm," possibly linked to the widespread expression of the targeted antigen, Her2/neu (ERBB2), by normal lung cells [165]. Another fatality was noted after using a second-generation CAR targeting CD19, in combination with cyclophosphamide lymphodepleting chemotherapy [166]. This treatment led to hypotension, dyspnea, and renal failure in the treated patient, and 4 days after the initial infusion the patient died. This suggests the need to include suicide genes in the CAR-bearing viral construct, or to use a dual-CAR/CCR design [144] to potentially provide another layer of safety. In addition, knocking down the expression of the endogenous TCR might prove valuable in order to prevent undesired/non-specific responses of CAR-activated T-cells [167].

9.4 Conclusions

In the past 25 years, adoptive T-cell transfer has established itself as a promising immunotherapeutic strategy for the treatment of advanced cancer. The basic idea, that the (autologous) immune system can be manipulated in order to promote tumor regression and remission, is appealing as it may provide long-lasting protection. Still, from the "bench-side" of things, additional targets/antigens have to be defined/characterized to provide safer treatments targeting a broad spectrum of tumors. From a clinical standpoint, there is a need to speed up processing times [168] and to ease regulatory requirements [169]. Improving the success rate of adoptive T-cell transfer will also require its combination with multi-modal therapies targeting, for instance, the tumor micro-environment as well as immunosuppressive agents. Much has to be done also to encourage partnership with the industry in order to commercialize this kind of immunotherapy that requires cell manipulation and conditioning [170]. Several studies also suggest that these concepts can be applied to treat other conditions than cancer [88]. Adoptive T-cell immunotherapy is certainly earning a respected place in the "Hall of Fame" of personalized medicine treatments.

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