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Review

Reprogramming the tumor microenvironment to enhance adoptive cellular therapy[☆]

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ABSTRACT

The frontiers of cancer immunotherapy are extending in terms of both the range of cancer types that can potentially be targeted and the types of therapeutics that are in clinical development. The use of adoptive cellular therapy (ACT) and its derivative, chimeric antigen receptor (CAR) T cells, is currently limited to hematological malignancies and immunogenic cancers such as melanoma and renal cell carcinoma. Although ACT utilizing *ex vivo* expanded tumor-infiltrating lymphocytes (TIL) or engineered CAR/TCR T cells have undergone clinical trials for other solid cancers, their efficacy to date has been limited. This may be due, in part, to the immunosuppressive nature of the tumor microenvironment. The development of novel combination approaches which target the immunosuppressive network engineered by tumors has raised the possibility of using ACT for a broader range of cancers. This review summarizes the potential of such strategies and outlines the clinical relevance of these observations.

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1. Introduction

The clinical success of adoptive cellular therapy (ACT) in targeting cancers such as Acute Lymphoblastic Leukemia (ALL), Chronic Lymphoblastic Leukemia (CLL), B cell lymphoma and melanoma has shown the potential for this approach [1,2]. However, attempts to recapitulate this success in other cancer types have been disappointing for a number of reasons including (i) a lack of a known tumor antigen, or one that can be suitably targeted by chimeric antigen receptor (CAR) transduced T cells, perhaps related to inherent differences in antigenicity and/or mutational load in different cancer types [3]; (ii) limited trafficking of adoptively transferred

cells to the tumor site [4] and (iii) the highly immunosuppressive tumor microenvironment. It is now known that tumors utilize multiple mechanisms to engineer this immunosuppressive environment and this has revealed a multitude of novel therapeutic targets. Given the ability of immunotherapeutic drugs targeting checkpoints such as anti-PD-1 (anti-programmed cell death protein 1) to harness endogenous anti-tumor immune responses, many preclinical studies have evaluated the potential of these approaches to enhance the efficacy of ACT.

In this review, we give a brief overview of the development of ACT technology and specifically discuss some promising approaches to enhance ACT efficacy by targeting tumor-induced immunosuppression and how these methods can be applied in the clinic.

2. Control of T cell activation in the tumor microenvironment

The ability of T cells to evoke an anti-tumor immune response within the tumor microenvironment is restrained by a multitude of immunosuppressive mechanisms, the physiological function of which is to prevent excessive inflammatory mechanisms. The best

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characterized of these in the context of tumor immunology is the ligand–ligand interactions between T cells and tumor cells or T cells and antigen presenting cells (APCs) [5]. Whilst a number of these interactions enhance T cell effector functions such as CD28, OX-40 and 4-1BB, in cancer these are often outnumbered by negative signaling moieties, the so called ‘checkpoint inhibitors’ such as PD-1, CTLA-4 (Cytotoxic T lymphocyte-associated protein 4) and TIM-3 (T cell immunoglobulin and mucin domain-3) [6]. PD-1 and CTLA-4 inhibit T cell responses through binding to their ligands PD-L1/PD-L2 (Programmed death-ligand 1/2) and CD80/CD86 respectively. Blocking these interactions has been shown to significantly enhance anti-tumor immune responses both in preclinical models [7–10], and in patients [11–17]. PD-1 blockade appears to work by blocking the interaction between PD-1 expressed on CD8⁺ T cells [18,19] and PD-L1 expressed on either tumor cells [11] or host immunosuppressive subsets such as myeloid cells [20].

In order to escape immune control, tumors also promote the differentiation/activation of immunosuppressive subsets such as regulatory T cells (Tregs) [21], myeloid-derived suppressor cells [22], alternatively activated macrophages (tumor-associated macrophages; TAMs) [23,24] and mesenchymal stem cells [25,26]. Tumor cells (and host immunosuppressive cells) also secrete chemokines such as CCL22 and CCL2 which attracts CCR4⁺ Tregs [27,28] and CCR2⁺ MDSCs (Myeloid-derived suppressor cells)/TAMs respectively [24,29]. This leads to an enrichment of these cells at the tumor site and results in the prevention of a robust anti-tumor T cell response. Tregs, TAMs and MDSCs cells suppress T cell responses through a multitude of mechanisms (reviewed by [21,22]) and the relative proportion of either Tregs or MDSCs cells to CD8⁺ T effector cells is a strong prognostic indicator for responses to chemotherapy/immunotherapy and patient outcomes [24,30,31]. The high numbers of immunosuppressive myeloid cells such as MDSCs and TAMs macrophages coupled with the heterogeneous nature of the myeloid compartment has made it difficult to identify myeloid cells capable of effectively presenting tumor antigens within the tumor microenvironment. However, recent eloquent work dissecting the myeloid subsets has identified that rare tissue resident CD103⁺ dendritic cells may be effective APCs within this context [32,33]. Further work is required to investigate the importance of these cells in the context of ACT.

3. Adoptive cellular therapy

Adoptive cellular therapy was originally devised as expanding tumor-infiltrating lymphocytes *ex vivo* and reinfusing these tumor-specific T cells back into the patient. Although this approach has yielded great success in melanoma patients [2] and to a lesser extent against other cancers such as renal cell carcinoma [34] and glioma [35], its widespread application is limited by the relatively low frequency of tumor-antigen specific lymphocytes that can be isolated from most other cancers. Generating a sufficient number of tumor-reactive T cells *ex vivo* is challenging especially as evidence suggests that prolonged culture time of tumor-infiltrating lymphocytes (TILs) can result in inferior anti-tumor responses when transferred back to the patient [36]. To tackle this problem, strategies have been designed to manipulate T cells derived from peripheral blood to express specific TCR- $\alpha\beta$ transgenes to generate a TCR capable of targeting a known tumor antigen [37]. More recently, it has been shown that by engineering T cells to express a single chain Fv region fused to the T Cell Receptor signaling domain (a so called chimeric antigen receptor; CAR), the cells can induce potent anti-tumor immune responses. Notably, this approach does not require the tumor antigens being targeted to be presented in the context of MHC and so bypasses one potential mechanism of tumor cell evasion given MHC expression is often downregulated.

The ‘first generation’ of CAR linked either the CD3 ζ or Fc γ R signaling domains to the scFv recognizing tumor antigen [38]. Although these cells were able to induce anti-tumor immune responses, their efficacy was somewhat limited by the lack of costimulatory pathway activation, and thus subsequent CARs have been designed to incorporate the CD28 or 4-1BB signaling domains (second generation CAR) [39–42]. These second generation CARs have had unprecedented success in ALL [43–46], CLL [45,47,48] and B cell lymphoma [49,50] through targeting the CD19 antigen [1]. Subsequently, additional signaling domains (4-1BB, CD28 and/or OX-40) have been added to the intracellular component of the CAR to make third generation CAR T cells. These adaptations enhance T cell proliferation and persistence *in vivo* in response to recognition of tumor antigens, and also enhance cytokine production/cytolytic function [39–41]. Moreover, by providing costimulatory signals, these latter generations of CAR T cells are thus better equipped to overcome the immunosuppressive tumor microenvironment, where costimulatory ligands are often expressed at low levels [5]. These second and third generation CARs therefore represent the first adaptations of CAR T cells to overcome tumor-induced immunosuppression. Nevertheless, the efficacy of these second and third generation CARs in solid tumors has largely been disappointing to date [51]. Preclinical evidence suggests that this is due, in part, due to immunosuppression in the tumor microenvironment. Thus, a large amount of preclinical work has now investigated the potential of other strategies to enhance the efficacy of ACT by reprogramming the tumor microenvironment (Fig. 1).

4. Targeting Immunosuppressive populations to enhance ACT

Work from preclinical mouse models has established that depleting immunosuppressive populations such as Tregs [52,53] and MDSCs [54] can enhance endogenous anti-tumor immune responses. Consequently, treatment strategies that deplete these cells would be expected to enhance the efficacy of ACT. Interestingly, the myeloablative preconditioning of patients with chemotherapy and/or radiotherapy which enhances the engraftment of T cells and consequently the efficacy of ACT [55–58], is now known to promote anti-tumor immune responses. There are a multitude of mechanisms that have been reported to underpin this (reviewed by [59]) including the induction of ‘immunogenic cell death’ [60–64] and selective depletion of Tregs [65–69] and/or MDSCs [70–78]. For example low dose cyclophosphamide has been shown to selectively deplete Tregs [65,66,79] and reprogram MDSCs into a more anti-tumor phenotype [80,81], consequently enhancing the efficacy of ACT [82,83].

Treg depletion with more specific reagents such as an IL-2 toxin conjugate [84] or anti-CD25 [85] has also been shown to increase the efficacy of ACT. However, these reagents also deplete activated effector T cells which express the IL-2R and so further work is needed to develop reagents capable of specific Treg depletion in patients. Similarly, depletion of MDSCs/TAMs using either a CSF-1R inhibitor [86], a CCR2-directed toxin [87] or splenectomy [78] has been shown to enhance the efficacy of ACT. Alternatively, disruption of MDSC function with pharmacological tools such as all-trans retinoic acid or inhibitors of ROS generation has the potential to enhance ACT [88,89]. One major mechanism by which MDSCs suppress anti-tumor T cell responses is *via* the metabolism of tryptophan, a key amino acid required for T cell proliferation, due to their high expression of indoleamine dioxygenase (IDO) [22]. A number of preclinical studies have shown the benefits of IDO inhibitors in the context of immunotherapy and these inhibitors are now entering clinical trials [63]. Interestingly, it has been shown that fludarabine and cyclophosphamide decrease

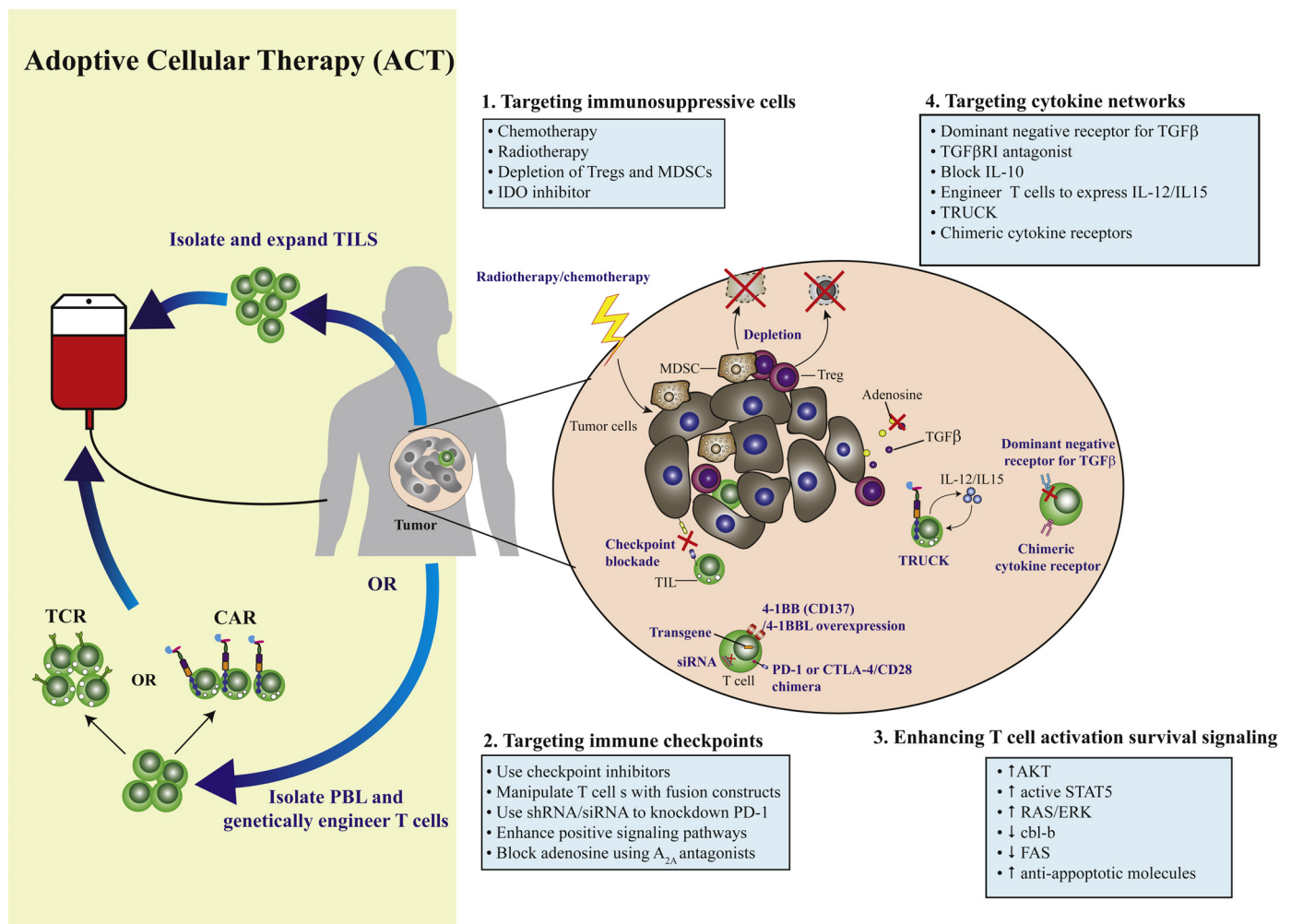


Fig. 1. Manipulating the tumor microenvironment to enhance the efficacy of adoptive cellular therapy.

the expression of IDO, leading to enhanced efficacy of CD19-CAR T cells [90]. A number of strategies to reprogram TAMs to engender a more anti-tumor immune phenotype have been developed including anti-CD40 [91,92] (reviewed by [24]). Indeed the constitutive expression of CD40L by CAR T cells was recently shown to improve therapeutic efficacy, in part through the maturation of myeloid-derived cells [93]. Thus, the combination of TAM-targeting therapies and adoptive cellular therapy warrants further investigation.

5. Targeting immune checkpoints to enhance adoptive cellular therapy

Given the success of checkpoint inhibitors as single agent therapies, particularly in melanoma, there has been great interest in pursuing these agents as adjuvant therapy to ACT. It is known that adoptively transferred T cells express high levels of PD-1, LAG-3 (Lymphocyte-activation gene 3) and TIM-3 when they are reinfused back to the patient suggesting they may be good therapeutic targets [48,94,95]. Blocking PD-1 has been shown to enhance ACT both in the context of transgenic TCR T cells targeting tumors expressing foreign/self-antigens [18,19] and CAR T cells targeting syngeneic [96] or xenograft [97] tumors. In these studies PD-1 blockade was shown to enhance IFN γ production, consequently inducing a chemokine response favourable for further T cell trafficking. Similarly, targeting CTLA-4 and LAG-3 in combination with ACT has been shown to enhance its efficacy [98,99]. A number of other checkpoint

inhibitors have been identified that warrant investigation in combination with ACT, these targets include TIM-3 [100], CEACAM1 [101], BTLA [102], B7-H3 [103], B7-H4 [104,105] and VISTA [106]. Given the success of checkpoint inhibitors in the clinic, these are likely to be among the first immunotherapies tested in combination with ACT.

An alternative approach to antibody-mediated blockade of these inhibitory pathways is to genetically engineer the T cells to modify their response to checkpoint inhibitors by fusing their extracellular domains to the signaling domains of costimulatory receptors. The proof-of-concept of this approach was elegantly shown by Shin *et al.* who showed that CTLA-4:CD28 chimeric receptors significantly enhanced the efficacy of ACT [107]. Subsequently it has been shown that a PD-1:CD28 fusion receptor enhances the activity of anti-tumor T cells both *in vitro* [108] and *in vivo* [109]. T cells manipulated with these fusion constructs are highly appealing as the activity of the T cells is amplified by ligands overexpressed by the tumor/in the tumor microenvironment thus increasing the specificity and safety of treatment. Alternatively, the effect of these pathways can be reduced by knocking down checkpoint inhibitor expression through the use of shRNA/siRNA technology. Knockdown of PD-1 using siRNA has been shown to enhance the activity of anti-tumor T cells *in vitro* [110]. Progressing this strategy into the clinic is likely to require the development of shRNA protocols in order to ensure that knockdown is stable and thus maintained after T cell proliferation *in vivo*. It is also possible to modulate the PD-1:PD-L1 interaction by targeting PD-L1 either with a targeted

antibody [19,20] or by inhibiting the expression of PD-L1 using siRNA [111].

Another immunosuppressive moiety that is highly abundant within the tumor microenvironment is adenosine, generated from ATP by the ectoenzymes CD39 and CD73 [112,113]. Adenosine suppresses T cells and NK cells through activation of A_{2A} receptors and T cells deficient for A_{2A} receptors or treated with A_{2A} antagonists show enhanced anti-tumor responses [114–120]. Indeed targeting A_{2A} or CD73 has shown to enhance the activity of adoptively transferred OT-I T cells against ova expressing tumors [121].

A complementary strategy for blocking negative checkpoint inhibitors is to enhance positive signaling pathways such as 4-1BB. For example the overexpression of either 4-1BB [122] or 4-1BBL [123] in adoptively transferred T cells has been shown to enhance the efficacy of ACT. The latter approach holds the advantage that 4-1BBL expressed on T cells can activate other tumor-infiltrating T cell *in trans*. Whilst these genetic-based approaches manipulate costimulatory/inhibitory pathways specifically in adoptively transferred cells, potentially enhancing their specificity, they may lack the full efficacy of antibody treatments since they lack the ability to target these interactions on host cells which may also be important. For example, PD-1 is also expressed on Tregs and MDSCs and may contribute in part to the efficacy of anti-PD-1 whilst 4-1BB activation can potentially activate host immune subsets such as Natural Killer (NK) cells and endothelial cells to enhance T cell trafficking [124,125] as well as by directly activating the adoptively transferred CD8⁺ CTLs [126]. Thus, comparisons of these approaches in a head to head manner is warranted and the relative efficacy maybe dependent on local tumor microenvironment factors.

6. Enhancing T cell activation/survival signaling

A number of studies have shown that genetically engineering T cell signaling pathways to protect T lymphocytes from tumor-induced immunosuppression can enhance their effectiveness in ACT [127–129]. The overexpression of the signaling molecule AKT was shown to enhance the efficacy of GD2 specific CAR T cells *in vitro*, making them less susceptible to TGFβ (Transforming Growth Factor-β) mediated suppression [127]. More recently, the overexpression of AKT in OT-I cells was shown to enhance their *in vivo* efficacy against ova-expressing tumors [130]. Similarly, CD8⁺ T cells engineered to have constitutively active STAT5 showed enhanced efficacy and resistance to PD-1/TGFβ mediated suppression [131]. Moreover, overexpression of miR-155 in adoptively transferred lymphocytes was recently shown to enhance AKT/STAT5 responses, thereby promoting T cell persistence and function [132]. Interestingly, AKT/STAT5 activation mimics the signaling induced by γ-signaling cytokines IL-2/IL-15 [133] and dephosphorylation of this pathway is the target for PD-1 signaling. Thus, targeting these signaling pathways has the potential to neutralize many immunosuppressive mechanisms simultaneously. Similarly, enhancing the RAS/ERK pathway by deactivating diacylglycerol (DAG) kinases [128] and/or knockdown of cbl-b to reduce the costimulatory requirements for adoptively transferred lymphocytes [129], represent other potential avenues for enhancing CAR T cell function. A recent screen of shRNA hairpins in combination with ACT by Zhou *et al.* revealed a number of novel intracellular targets including Ppp2r2d (Protein Phosphatase 2, Regulatory Subunit B, Delta) and smad2 (SMAD family member 2), a signaling pathway induced by TGFβ signaling [134]. Other potential intracellular targets recently identified that warrant further investigation include A20, ASAP and AKAP55 [135,136].

An alternative to manipulating T cell signaling to promote T cell proliferation is to enhance the persistence of adoptively transferred

T cells by protecting them from apoptosis. For example, it has been shown that T cell survival can be enhanced through the knockdown of FAS expression using siRNA [137] or the overexpression of anti-apoptotic molecules such as Bcl-2 [138]. However, given the safety concerns of treating patients with T cells resistant to apoptosis mechanisms, it may be necessary to utilize these types of approaches in combination with 'suicide genes' to allow for elimination of T cells following therapeutic responses [139].

7. Targeting cytokine networks to improve ACT

A major component of the immunosuppressive tumor microenvironment is the predominance of immunosuppressive cytokines such as TGFβ and IL-10 which potentially inhibit T cell responses and consequently suppress the efficacy of adoptive cellular therapy. TGFβ suppresses anti-tumor immune responses by modulating the function of a range of immune cells including effector T cells, Tregs and APCs [140]. Adoptive cellular therapy utilizing T cells deficient for TGFβRII was shown to enhance the effectiveness of therapy and correlated with increased T cell proliferation and cytokine production *in vivo* [141]. A similar approach with increased translational relevance is the overexpression of a dominant negative receptor for TGFβ, a strategy which has been shown to enhance the effectiveness of adoptively transferred T cells [142–144] and NK Cells [145,146]. Similarly, treatment of mice with a TGFβRI antagonist also enhanced the efficacy of ACT [147]. Furthermore, it has been shown that vaccinia virus-mediated production of a soluble TGFβ receptor enhanced tumor antigen specific CD8⁺ T cell responses *in vivo* [148]. These approaches also have the advantage of inhibiting the plethora of immunosuppressive effects of TGFβ on host cells as well as the adoptively transferred lymphocytes [140]. IL-10 is another cytokine that has potent immunosuppressive activity within the tumor microenvironment [149]. Whilst strategies have been developed to target IL-10 in the context of cancer immunotherapy [150,151] this has yet to be tested in the context of ACT.

The alternative to blocking the effects of immunosuppressive cytokines is to enhance cytokine networks that promote anti-tumor immune responses. Indeed IL-2 is routinely given in combination with ACT and is required for maximum therapeutic efficacy [152,153]. IL-12 is important for the effective generation of T_H1 type immune responses and effective anti-tumor immunity [154]. However, the provision of exogenous IL-2/IL-12 can result in toxicity [155,156] and so more subtle approaches have consequently been devised. CAR T cells engineered to express IL-12 specifically upon activation within the tumor microenvironment show significantly enhanced efficacy *in vivo* [157–161]. These so called 'TRUCK' cells (T cells redirected for universal cytokine-mediated killing) have been described as the fourth generation of CAR T cells and provide an additional advantage in that they allow for the activation of innate cells that may recognize tumor cells negative for the CAR target antigen [162]. A similar strategy has been devised for IL-15 [163], which may promote the generation of T_{CM} cells that are associated with enhanced T cell persistence and efficacy [164–166]. There may also be value in using this technology to program T cells to secrete other cytokines such as GM-CSF known to modulate myeloid cell differentiation to reshape the tumor microenvironment [167]. Given the role for FLT3L in the differentiation of anti-tumor CD103⁺ DCs [32,33], combining this cytokine with ACT is an attractive proposition. Indeed, GM-CSF and FLT3L were originally shown by Allison and colleagues to enhance the effectiveness of anti-CTLA-4 when given in context of a tumor cell vaccine [7]. Finally, it may also be possible to combine reducing the effects of immunosuppressive cytokines with promoting anti-tumor cytokine networks through the use of chimeric cytokine receptors such as the IL-4/IL-7

chimeric receptor which was recently shown to enhance CD8⁺ CTL responses [168].

8. Clinical perspectives

The last two decades have seen significant advances in the use of ACT in clinical practice particularly in the treatment of patients with advanced melanoma [169]. Greater understanding of the benefits of preconditioning chemotherapy/radiotherapy and 'young TIL', with shorter periods in culture and increased telomere length, has seen response rates of over 50% which are comparable with the practice-changing results seen with checkpoint blocking immunotherapy agents. Furthermore, for many patients responses are durable achieving prolonged disease control even in the setting of widespread metastatic melanoma [2,170]. Despite this, the use of ACT remains limited to a number of specialist centers in North America, Europe and Israel.

The major limitations to more widespread uptake of ACT include the labor intensive and expensive protocols involved, the absence of high-level data to demonstrate efficacy and the toxicity of the treatment in the absence of good predictors of response. Whilst ACT is highly technical and labor intensive, the costs are comparable with those of novel systemic agents in immunotherapy and numerous joint ventures between academic institutions and industry have recently been announced in attempts to commercialise and thereby increase access to these technologies [171]. Furthermore, in an attempt to demonstrate efficacy, a randomized controlled trial has recently opened in Europe comparing ACT with the CTLA-4 inhibitor, ipilimumab, for patients with metastatic melanoma which will provide high level evidence regarding the impact of treatment on patient outcomes.

Finally, in order to improve efficacy, ACT has moved beyond the use of TIL to explore many of the technologies discussed in this review. The use of CAR-T cells for haematological malignancy has demonstrated efficacy in a number of small clinical studies [43,46]. In addition, targeting of cancer testis antigens such as NYESO1 [172] or MART-1 [37,173,174] utilizing TCR engineered T cells has shown efficacy in patients. Along with this improved efficacy has come significant toxicity, the most significant of which is cytokine release syndrome [43,46], improved understanding of the mechanisms underlying this response have led to successful management strategies, specifically IL-6 blockade [175]. A better understanding of T cell engineering in the clinical setting as well as the identification of novel CAR targets, or personalized mutated neo-antigens [176] should lead to major advances in overcoming immune tolerance within the tumor microenvironment in a variety of tumor types over the next decade.

9. Future directions

The success of adoptive cellular therapy in melanoma and, in particular, CAR T cell technology in hematological malignancies has provided the framework to improve this technology further to allow for the successful targeting of highly immunosuppressive solid tumors. It is currently unknown which of the approaches discussed previously (or combinations of these approaches) provide the greatest enhancement of ACT efficacy. Further work is required to identify biomarkers that predict which strategies are likely to be favorable, such as by analyzing PD-L1 expression to determine which patients are likely to respond to PD-1 blockade. Amongst these factors, consideration of the microbiota of patients should also be included, since it is increasingly recognized that the microbiota can have a significant impact on anti-tumor immune responses and therefore may modulate the effectiveness of immunotherapy [177].

Furthermore, although ACT in this context is often considered as the infusion of CD8⁺ cytotoxic lymphocytes, the importance of CD4⁺ lymphocytes in the transferred cellular product is increasingly being recognized [178–180] and the potential of adoptively transferred allogeneic NK cells to mediate anti-tumor effects is well known [181–183]. Therefore identifying the best combination of immune cells to mediate anti-tumor effects in ACT is an important question. As technology develops to allow for increased proliferation/maintenance of other immune subsets such as NKT cells, $\gamma\delta$ T cells and MAIT (Mucosal associated invariant T cells) cells, the feasibility of using these cells as cellular therapeutics will increase. Whilst the anti-tumor efficacy of NKT cells [184] and $\gamma\delta$ T cells [185] transduced with a CAR has already been shown in proof-of-principle studies, further investigations are needed to establish their ability to enhance the efficacy of conventional ACT and importantly, their ability to evade the tumor immunosuppressive mechanisms that suppress conventional CD8⁺ T cell responses.

Tumor-infiltrating lymphocytes can be manipulated *ex vivo* and reinfused back into the patient to induce anti-tumor immune responses. The efficacy of this therapy is limited by the tumor microenvironment which suppresses anti-tumor immune responses in a number of ways. Strategies have been designed to modify the tumor microenvironment and so complement adoptive cellular therapy. This includes targeting immunosuppressive cells (1), immune checkpoints (2), manipulating T cell signaling pathways to promote proliferation/survival (3) and targeting cytokine networks (4). These approaches have all been shown to enhance the efficacy of adoptive cellular therapy in preclinical studies.

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