Expert Opinion on Biological Therapy

ISSN: 1471-2598 (Print) 1744-7682 (Online) Journal homepage: https://www.tandfonline.com/loi/iebt20

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To cite this article: Isabelle Magalhaes, Claudia Carvalho-Queiroz, Ciputra Adijaya Hartana, Andreas Kaiser, Ana Lukic, Michael Mints, Ola Nilsson, Hans Grönlund, Jonas Mattsson & Sofia Berglund (2019) Facing the future: challenges and opportunities in adoptive T cell therapy in cancer, Expert Opinion on Biological Therapy, 19:8, 811-827, DOI: 10.1080/14712598.2019.1608179

To link to this article: https://doi.org/10.1080/14712598.2019.1608179

Published online: 30 Apr 2019.

EXPERT OPINION ON BIOLOGICAL THERAPY
2019, VOL. 19, NO. 8, 811–827
https://doi.org/10.1080/14712598.2019.1608179

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REVIEW

Facing the future: challenges and opportunities in adoptive T cell therapy in cancer

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ABSTRACT

Introduction: In recent years, immunotherapy for the treatment of solid cancer has emerged as a promising therapeutic alternative. Adoptive cell therapy (ACT), especially T cell-based, has been found to cause tumor regression and even cure in a percentage of treated patients. Checkpoint inhibitors further underscore the potential of the T cell compartment in the treatment of cancer. Not all patients respond to these treatments; however, many challenges remain.

Areas covered: This review covers the challenges and progress in tumor antigen target identification and selection, and cell product manufacturing for T cell ACT. Tumor immune escape mechanisms and strategies to overcome those in the context of T cell ACT are also discussed.

Expert opinion: The immunotherapy toolbox is rapidly expanding and improving, and the future promises further breakthroughs in the T cell ACT field. The heterogeneity of the tumor microenvironment and the multiplicity of tumor immune escape mechanisms pose formidable challenges to successful T cell immunotherapy in solid tumors, however. Individualized approaches and strategies combining treatments targeting different immunotherapeutic aspects will be needed in order to expand the applicability and improve the response rates in future.

ARTICLE HISTORY
Received 7 January 2019
Accepted 12 April 2019

KEYWORDS
T cells; T cell immunotherapy; adoptive cell therapy; solid tumors; cancer

1. Introduction

Therapies harnessing the antitumor potential of the immune system have lately come to play an important role in the treatment of solid tumors, and treatments targeting the T cell compartment have been accorded a large share of the interest. Groundbreaking T cell-based adoptive cell therapies (ACTs) for cancer include tumor-infiltrating lymphocyte (TIL) treatment [1–6], and therapy with chimeric antigen receptor (CAR)- and transgenic T cell receptor (TCR)-modified T cells [7–12], all of which have been shown to induce tumor regression. However, CAR T cell therapy has so far reached the greatest success in hematological malignancies.

The impact of the immune checkpoint inhibitor therapies, releasing the 'breaks' on T cells, further demonstrates the anticancer potential and power of the T-cell compartment. The success of these therapies is reflected in their worldwide use in several different groups of malignancies [13–16] – the immune checkpoint inhibitor market during 2017 was valued at $10,566 million, and is expected to reach $56,530 million by 2025 (alliedmarketresearch.com), and in the awarding of the Nobel prize in Physiology or Medicine 2018 to two of the key scientists behind the checkpoint concept.

However, the abovementioned therapies achieve durable responses in only a fraction of treated patients – a significant number of patients do not benefit. Obstacles at several different levels contribute to preventing the success of T cell ACT in solid tumors. One major difficulty is target selection. The target antigen chosen for T cell immunotherapy can be known tumor antigens, or neoantigens formed by unique mutations in one specific tumor, or even unknown, as in the case of TIL therapy. Aspects such as levels of antigen expression, specificity to cancer tissue, and whether efficient presentation of the antigen is possible on a specific human leukocyte antigen (HLA)-molecule complicate target selection and confer risks such as insufficient targeting efficacy or off-target effects. The *in vitro* production of the T cell product adds another layer of difficulties. Factors such as the choice of cytokines during T cell culture are of great importance, as different T cell phenotypes are known to have different potency *in vivo*. The tumor itself can introduce several types of obstacles that are jointly termed immune escape mechanisms. These mechanisms vary between cancer types and also between patients with the same type of malignancy, and include aberrant blood vessels, the expression of checkpoint receptor ligands, and many other mechanisms. Certain types of suppressive immune cells in the tumor microenvironment also form a significant barrier against T cell-mediated tumor cell eradication, such as regulatory T cells (Tregs) and tumor-associated macrophages (TAMs).

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Article highlights

- An overview of the T cell ACT field is here presented from a development, strategy, and production perspective. Antigen targeting strategies, key aspects and choices in the manufacturing process, and tumor evasion strategies are explored.
- Different targeting strategies and well-known therapies adopting these strategies are presented and contrasted against each other, including TIL therapy, CAR T cell therapy, transgenic TCR therapy, and neoantigen-based therapy.
- Important aspects of T cell ACT production, such as cytokine use in the culturing protocols, and the choice of culture system are explored.
- An overview of relevant tumor evasion mechanisms is presented in order to visualize the obstacles to successful T cell ACT.
- The conclusions drawn from the knowledge summarized in this review point toward the need of multi-pronged approaches to optimize the therapeutic success of T cell ACT. The choice of target and production methods are central to the patient outcome, but additional therapies targeting tumor evasion mechanisms and the microenvironment, including suppressive immune cells, might be necessary to extend the efficacy of this type of treatment.

This box summarizes key points contained in the article.

The development of ACT strategies that combine efficient tumor cell targeting, manufacturing of potent T cell products, and methods for contravening immune escape mechanism is essential to expand the applicability and potency of T cell immunotherapy in solid cancers (Table 2). The purpose of this review is to overview the T cell ACT field from a development, strategy, and production perspective, in order to describe the known obstacles to success and explore the alternatives available for overcoming them. We hope to contribute to the ongoing discussion on how to reach the goal of making effective T cell immunotherapy available to all patients with cancer by providing a basic 'recipe' for the strategic considerations of T cell ACT development.

2. Target antigens for T cell immunotherapy

The choice of target antigen or antigens for T cell ACT is central for treatment success. There are, roughly, three main target categories: (1) Therapies with an unknown target, (2) Therapies that target known tumor-associated antigen (TAAs), and (3) Therapies that target neoantigens specific to one individual tumor. An overview of the different target types is displayed in Figure 1.

2.1. Therapies against unknown antigens

2.1.1. Tumor-infiltrating lymphocytes

Therapies targeting unknown antigens are commonly based on the sourcing of T cell populations enriched for antitumor specificity. The most well-known and extensively studied is the TIL methodology, where isolation of T cells infiltrating the

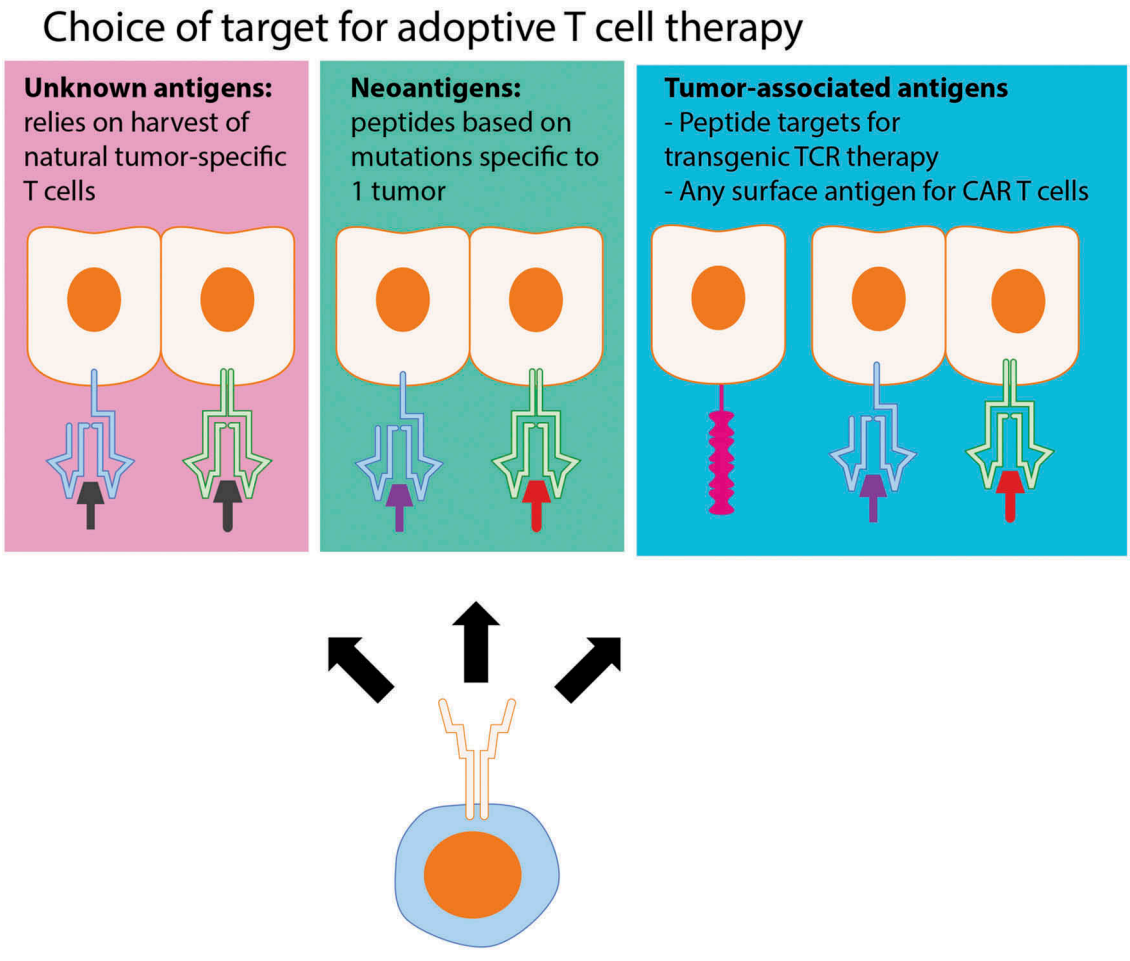

Figure 1. An overview of the different classes of T cell targets used for ACT in solid cancer.

tumor tissue is performed. The basis for harvesting this specific T cell population is the enrichment of tumor-specific T cells in the tumor tissue [2]. The proposed cause for their lack of efficiency *in vivo* is the immune-suppressive properties of the tumor microenvironment [17]. The stimulation of TILs *in vitro*, in the absence of this suppressive milieu, is believed to reverse their anergic state [2]. This hypothesis is supported by the significant response rates seen to TIL [2–5,18]. The culture process used in many of the published studies follows the 'rapid expansion protocol' (REP), which includes a first step, sometimes called the pre-REP, where the TILs are isolated and expanded from digested tumor tissue and cultured in interleukin 2 (IL-2) to obtain a starting batch of TILs. Subsequently, in the REP culture step, the TILs are re-stimulated via TCR stimulation (commonly monoclonal anti-CD3) together with irradiated allogeneic peripheral blood mononuclear cells used as feeder cells [3,4,19,20]. The treatment protocols also include lymphodepletion with chemotherapy and in some cases radiotherapy before T cell infusion and *in vivo* IL-2 administration after infusion. The majority of trials have used a high dose of IL-2, associated with extensive side effect, and a reduced dose-protocol has been introduced lately [18]. Lymphodepletion is performed to improve the survival and localization of infused TILs [21], through ensured access to homeostatic cytokines for the infused TILs, reduction of Tregs, and stimulatory effects on antigen-presenting cells (APCs) [22].

The initial indication for TIL therapy was malignant melanoma, where a number of trials have been completed and published. The response rate noted for patient with melanoma has been approximately 40–50% [2–5,18], with some reports showing rates of over 70% [6], with up to 24% reaching complete responses. The complete responders have been reported to have high rates of relapse-free survival, indicating that some of these patients are cured [3,4,6]. The localization of metastases might affect the response rate: e. g. the rate of durable responses was lower in patients with brain metastases [5]. The applicability of TIL therapy in other tumor types is being explored: a large number of trials are being conducted in patients with a wide range of cancer types, such as squamous cell carcinoma of the head and neck, non-small cell lung cancer, biliary tract cancers, ovarian cancer, pancreas cancer, cervical cancer, renal cell cancer, human papilloma virus-associated cancer types, and even T cell lymphoma (source: clinicaltrials.gov). The results are awaited with great interest.

2.1.2. Other therapies targeting unknown antigens

Another approach targeting unknown antigens is the Sentoclone® method, where the source of autologous T cells used is tumor-draining sentinel lymph nodes. Sentinel nodes, like tumor tissue, have T cell populations enriched for tumor-specific T cells [23]. The isolated T cells are stimulated with autologous tumor homogenate, expanded *in vitro* and re-infused without previous lymphodepletion or adjuvant IL-2. Objective responses have been described in patients with advanced colon cancer and urinary bladder cancer [24–26].

These results indicate that knowledge of the target of the T cell therapy is not essential for the treatment's success if a sufficiently enriched tumor-specific T cell population can be sourced.

2.2. Therapies targeting TAAs: CAR - cell therapy and other methods

The identification and targeting of TAAs is another strategy for cancer immunotherapy. Ideally, TAAs should be homogeneously, highly, stably, and specifically (i.e. not found in healthy tissues) expressed by tumors cells, present in many patients, recognized by T cells, and subsequently able to elicit T cell cytotoxicity.

Other requirements vary based on whether the T cells used for ACT rely on 'classical' T cell activation via the TCR or are based on introducing a CAR into the T cells. When naturally occurring tumor-specific T cells cannot be used (e.g. not found in patients or too few to expand), T cells' specificity can be redirected by genetic modification by introducing a TCR or a CAR. In the former case, TCR α and β genes are cloned from a T cell and transferred to new T cells, while in the latter case, CARs are synthetic receptors composed of an antibody single chain fragment variable (scFv), the CD3ζ chain, and in the case of second generation CARs a co-stimulatory domain (e.g. CD28 or 4–1BB). As described below, TCRs recognize peptides presented by HLA molecules. A large library of tumor-derived antigens can be targeted but HLA restriction (i. e. the peptides possible to present is dependent on the specific HLA type) implies that for patients with different HLA haplotypes, different TCRs are required. CARs specificity derives from the scFv; therefore, CARs can be used in any patient expressing the cognate antigen.

ACTs based on T cell recognition via the TCR require presentation of the targeted TAA in the form of peptides on HLA molecules. TCR activation of $CD4^+$ and $CD8^+$ T cells is triggered via HLA class II and HLA class I, respectively, which means that cells expressing both the HLA types are required in the tumor immune environment for activation of both T cell subsets. Also, TCRs of $CD4^+$ and $CD8^+$ T cells recognize different types of epitopes. CAR T cells are activated in an HLA-TCR independent fashion and can recognize via the scFv domain of the CAR construct virtually any kind of antigens: surface antigens such as proteins, carbohydrates, or lipids, but also peptide/MHC complexes [27] via the antibody scFv domain of the CAR construct. Consequently, the only requirement for the antigen is to be present at the cell surface. Also, the same cognate antigen activates both $CD4^+$ and $CD8^+$ CAR T cells. The amount of antigen plays also a role, as TCRs can trigger T cell activation at low antigen density [28], whereas high antigen density appears to be needed to fully activate CAR T cells [29].

Tumor antigens can be classified into different groups: tissue differentiation antigens, normal proteins overexpressed by tumor cells, tumor germline antigens, and mutated antigens [30]. In 1991, the first human tumor-specific antigen *MAGE-A1* was identified [31]. Several tumor antigens have since been reported, such as the differentiation antigens MART-1 and gp100, and the germline antigens in the MAGE family and NY-ESO-1.

The proof of concept of T cell specificity redirection by TCR engineering was first performed using murine cells in 1986 [32]. In 2006, two clinical trials using TCR engineered T cells targeting the HLA-A2 restricted MART-1 antigen demonstrated safety and therapeutic potential of the transgenic TCR technique [10,33]. Clinical trials targeting other antigens (e.g. NY-ESO -1, MAGE, gp100) have been or are being performed with variable objective clinical responses [34,35].

Most TCR (genetically engineered or native)-based ACTs rely on the infusion of antitumor $CD8^+$ T cells; however, $CD4^+$ T cells are capable of mediating potent antitumor responses as well. $CD4^+$ TILs [36] and $CD4^+$ TCR-engineered T cells [12] have been used to treat patients with cancer who subsequently experienced tumor regression. Interestingly, in a murine preclinical model mesothelin-directed $CD4^+$ CAR T cells demonstrated enhanced efficacy as compared to mesothelin-directed $CD8^+$ CAR T cells, with $CD4^+$ T cells displaying both helper and cytotoxic functions [37] highlighting the antitumor potential of $CD4^+$ T cells.

One advantage of transgenic methods, such as CAR T cell therapy and transgenic TCR T cell therapy, is that the original specificity of the T cells is of little importance, and thus, T cells from peripheral blood can be used.

Since the first report in 2010 of a patient with advanced follicular lymphoma treated with CD19-directed CAR T cells [38], CAR T cell therapy has emerged as one of the most promising ACTs. CD19 expression is restricted to the B cell lineage and is expressed by most B cell lymphomas and leukemia, consequently representing an excellent target. CD19 CAR T cells target malignant cells and normal B cells which results in B-cell aplasia. This is an unavoidable side effect, but can be mitigated by immunoglobulin replacement therapy. Impressive results have been achieved for patients with lymphomas and acute lymphoblastic leukemia (ALL) treated with CD19 CAR T cells [7–9,39], but tumor escape due to the loss of CD19 expression in patients with ALL has been reported [40]. Strategies that target dual antigens for instance CD19 and CD22 [41] may allow to circumvent antigen loss. Overall, this underlines the importance to identify additional tumor antigens, which still remains a daunting task. CAR T cell therapies for the treatment of solid tumors are now being developed, targeting for instance GD2 (neuroblastoma), and mesothelin (pancreatic, cervical, breast, ovarian cancer). However, the immunosuppressive environment in solid tumors represents a formidable challenge for T cells and to date CAR T cell treatments in solid tumors have been less successful than in hematopoietic malignancies [42].

Note that gene editing using targeted nucleases such as transcription activator-like effector nucleases and CRISP/Cas9 opens new possibilities to modify (knock-out and knock-in) engineered TCR and CAR T cells [43], which will hopefully endow engineered T cells with improved functions and efficacy particularly against solid tumors.

2.3. Therapies targeting neoantigens

Tumor-specific antigens, also called neoantigens, arise from nonsynonymous mutations and other abnormal genetic modifications [44,45]. The major benefit of targeting neoantigens over TAAs is that they are highly specific for the cancer cells of one individual tumor. They are not found in normal tissues, as the mutations do not occur in germline DNA. They can thus be differentiated from normal self-antigens and recognized as 'non-self' by T cells. However, they are specific to a single individual tumor, which requires a personalized approach.

A number of studies have demonstrated the relationship between neoantigen load (the number of individual neoantigens in one tumor) and the efficacy of checkpoint inhibitor therapy, where cancers with a high neoantigen load such as cutaneous malignant melanoma or non-small cell lung cancer generally respond well [13,14,16,46], while cancers with a low neoantigen load generally show poor responses [47,48]. Another example demonstrating the association between neoantigen load and checkpoint inhibitor response is found in colon cancer, where the tumors are either DNA repair proficient or deficient. DNA repair deficient tumors (referred to as microsatellite instability) accumulate more mutations, resulting in a higher neoantigen load. Programmed cell death protein 1 (PD-1) inhibitor treatment in an unbiased selection of colon cancer patients failed to exhibit significant clinical response [49,50], but high response rates were observed when selecting for DNA repair deficient tumors [50]. It is also clear that the characteristics of the mutations influence therapy outcome [51], as vaccination with a neoantigen based on a single mutation has been found sufficient to elicit positive clinical responses [36,52,53].

Several methods have been developed to predict which neoantigens have the potential to elicit a positive immune response. Until recently, most studies have been focused on common mutations discovered using major databases such as COSMIC or The Cancer Genome Atlas, many of which are cancer driver mutations. However, it is disputable whether driver mutations hold any advantage for neoantigen-based immunotherapy. Recent computational analyses of the mutational landscape for the binding to both HLA class I and class II suggest that driver mutations might carry an overall poorer affinity than random mutations [54,55]. Instead, the recent substantial cost reduction for next-generation sequencing (NGS) has opened up for systematic personalized neoantigen prediction for each individual patient. NGS for neoantigen prediction is based on Massive parallel sequencing of either all known DNA sequences in the genome coding for proteins (Whole Exome Sequencing, WES) or presently available RNA Transcripts (Transcriptome sequencing, RNA-seq) of a chosen tissue [56]. There are several publicly available neoantigen prediction pipelines available, including pVAC-Seq [57], MuPeXI [58], TIMiner [59], and OpenVax (https://www.openvax.org/). Most neoantigen prediction pipelines rely on WES for normal and tumor DNA combined with RNA-seq for tumor RNA, where WES traditionally is used to identify ('call') mutations only found in tumor DNA, and RNA-seq is used to verify the expression of the corresponding mRNA transcript [60]. In addition to expression, RNA-seq also reveals additional information that is not visualized by WES, such as alternate splicing variants or transcriptional errors. Also, a major effort has been put into predicting the binding of putative epitope candidates to the corresponding HLA molecules from the individual patient. The NetMHC series, currently at version 4.0 [61], is a popular tool for

prediction of peptide binding across class I HLA alleles. However, several studies have indicated that the actual number of predicted peptides to be presented on HLA molecules might be lower than 5% [62–65]. Thus, improvements are necessary. Recently, the importance for neoantigen therapy of antigen-presentation on HLA class II has been demonstrated. Neoantigens presented on HLA class II was found to promote recognition of tumor epitopes and antitumor activity as well as presentation on HLA class I [36,66]. This calls for inclusion of class II molecules in neoantigen prediction pipelines. A recent paper also highlighted the value of using proteasome-generated HLA class I peptides, i.e. spliced peptides that do not align to the original peptide chain, as a source of peptides that might be targeted as neoantigens [67]. Such peptides represent a separate fraction of antigens that may complement the non-spliced peptide pool for neoantigen targeting, particularly for self-antigens without conventionally identified neoantigens. The efficacy of using such peptides for cancer immunotherapy remains to be evaluated. Even with accurate predictions (i.e. the selected peptides are properly displayed on HLA molecules), it may prove difficult to foresee whether a neoantigen will elicit a favorable immunogenic response due to the complexity of the interactions between APCs and T cells. The presence of non-mutated wild-type peptides with the potential to cross-react with the neoantigen peptide could result in negative selection of effector T cells, or, cross-reactive, T cell responses [68].

To overcome the hurdles associated with conventional prediction pipelines, machine-learning algorithms that correlate results concerning neoantigen peptide fitness and clinical response from several datasets simultaneously might be able to improve neoantigen prediction. In a recent report, the Neopepsee machine-learning platform was able to significantly improve sensitivity and specificity [69]. Several commercial platforms based on machine-learning techniques are currently available.

Neoantigens can be used in T cell ACT strategies either to stimulate and/or select autologous tumor-specific clones or as a template for the production of a tumor-specific TCR. A venture exploring the former strategy is under development by some of the authors of this review, where the neoantigen concept is applied to a sentinel node-based T cell ACT. Neoantigen peptides are coupled to paramagnetic beads that are added to a lymph node cell culture, and the APCs from the lymph node process the neoantigens and present them to the T cells, causing specific expansion (unpublished). Several preclinical studies have demonstrated different ways to identify neoantigen-reactive T cell clones and sequencing their TCR for use in TCR-transgenic therapies. In one approach, neoantigens identified from patient tumor tissue were used to screen healthy donor T cells for reactive clones, as autologous TILs are often few and functionally suppressed. The identified neoantigen-specific TCR can then be used for transgenic transfer into the desired T cell population [70]. Another fascinating approach is a development from the TIL therapy, where TILs expressing PD-1 and/or activation markers OX40 and 4–1BB (and thus likely to be tumor-reactive) are sorted by flow cytometry, cultured, and co-cultured with APCs pulsed with neoantigen peptides. Responding clones are analyzed by TCR sequencing to obtain a TCR template in analogy with the described method above [71]. The field is highly active and techniques are constantly evolving, promising a better outcome for neoantigen-based therapies in the future.

3. The T cell act product

The production of the T cell product is the next crucial point after target selection in T cell ACT. The conditions in which T cell stimulation and expansion is performed are crucial for the potency and safety of the products. Pre-conditioning and post-infusion therapies are also essential parts of some ACT protocols. The former will not be discussed further in this review, but cytokine treatment post T cell infusion is of interest in this context as it can be considered in many T cell ACTs to improve the efficacy of the infused cells.

3.1. Cytokines in T cell immunotherapy

The use of cytokines in cancer treatment has received considerable attention in the past decades [72]. Cytokines such as IL-2, IL-12, tumor necrosis factor (TNF), and interferon γ (IFN-γ) have been used clinically with success in some cancer settings [73]. In the context of T cell ACT, the most well-known and extensively studied cytokine for *in vivo* use is IL-2. It has been used singly to improve the anticancer activity of the body's T cells [74] and as an integral part of TIL therapy protocols. The latter exemplifies one of the two modes of use of cytokines relevant to this review, where cytokines are administered to the patient after T cell infusion to boost the infused cells. Aside from IL-2, IL-7, IL-15, and IL-21 have been discussed [75]. A TIL trial utilizing IL-15 post TIL infusion instead of IL-2 was initiated, but was subsequently closed after three patients due to autoimmune toxicity (NCT01369888). Further studies to determine the benefits of post-infusion therapy with these cytokines would be of value, especially if this type of therapy is to be used in emerging T cell ACT.

Cytokines are also essential to the *in vitro* stimulation and expansion of T cell immunotherapy products. The cytokines used during production significantly affect several vital T cell characteristics. Successful T cell immunotherapy depends on properties such as differentiation state, ability to persist *in vivo*, and capacity to exert effector functions against cancer cells in the host. The differentiation and memory phenotype of the cultured T cells is of great importance for the efficacy of the cultured T cell product [75]. Studies in animal models have indicated that naïve T cells and central memory T cells are more potent and persist longer than terminally differentiated effector cells and effector memory T cells [76,77]. The definition of memory subsets is based on the expression of certain markers, for example CD45RA (naïve T cells, stem cell memory T cells, and terminally differentiated effector cells), CD45RO (central end effector memory T cells), CD62L and CCR7 (important for entrance into secondary lymphoid organs, found in naïve, stem memory and central memory T cells), and co-stimulatory receptors CD27 and CD28 (expressed in a pattern similar to CCR7 and CD62L) [75]. In human trials, higher amounts of transduced CAR T cells could be detected *in vivo* after infusion of T cell products with a higher level of expression of CD45RA, and

of CD45RA+ CCR7+ cells, indicating a larger fraction of naïve and stem memory T cells. The persistence of the CAR T cells from such products was also longer [78]. Also, T cells with longer telomeres, which correspond with 'younger,' less extensively proliferated cells, were associated with better response in melanoma patients [79]. This is also supported by findings indicating that less *in vitro* culture time correlates with higher potency in TIL and CAR therapy [80,81]. A crucial role for the cytokines used in the culture medium is thus to promote a less differentiated phenotype.

IL-2 is the most extensively used cytokine, and well known to efficiently promote proliferation of cultured T cells. It is, however, also known to stimulate to differentiation, especially in high concentrations and with longer culture time [75], while lower concentrations and short-term culture result in maintenance of less differentiated phenotypes [20,75,82]. The high IL-2 concentrations used in the REP TIL protocol (6000 IU/ml culture medium) were found to result in downregulation of CD62L and CD27, and to a lesser extent CD28, indicating loss of less differentiated memory T cells [20] (Table 1). IL-2 has been associated with activation-induced cell death as well. This has resulted in a change of strategy toward exchanging or combining IL-2 with other cytokines, such as IL-4, IL-7, IL-15, and IL-21 [78,83–91], or toward altering the concentration and timing of IL-2 administration [82,92,93]. Different strategies are summarized in Table 1.

Protocols utilizing lower IL-2 concentrations have been found to promote a less differentiated phenotype, but findings indicate that the effect is most pronounced in shorter culture protocols, longer culture time promotes differentiation even at lower IL-2 concentrations [82,92]. The timing of administration and length of exposure has also been found to be important: supplementing the culture medium with IL-2 the first 4 days after stimulation, and thereafter culturing the T cells without extraneous cytokines was found to promote proliferation and prevent apoptosis and extensive differentiation [93].

The choice of the other cytokines with receptors including the common γ-chain is based on the characteristics they share with IL-2, most prominently their capacity for stimulating T cell proliferation. IL-15 is a good alternative, as it stimulates the proliferation of activated T cells without supporting Tregs [94]. IL-7 is central for T-cell homeostasis, and maintenance of naïve T cells. *In vivo* administration in humans leads to T cell proliferation, broadening of the circulating TCR repertoire through proliferation of mainly naïve T cells, and bcl-2 upregulation [95], indicating that IL-7 is less likely to induce activation-induced cell death in *in vitro* than IL-2. Previously published studies have shown improved *in vitro* proliferation and viability of umbilical cord blood T cells when IL-2 was combined with IL-7 [83,85]. Combination of IL-7 and IL-15 has been increasingly used instead of IL-2, as results have indicated that this combination results in comparable proliferation together with higher fractions of early memory T cells in the cell product [78,84,88,96], even though reports show data similar to the results achieved using IL-2 [86]. IL-21 is of interest as it has been found to favor proliferation of highly cytotoxic $CD8^+$ T cells specifically, and to prolong their longevity and reduce the signs of replicative senescence [75,89]. The timing of administration of other cytokines as well as IL-2 has been found to be of importance: for example, intermittent administration of IL-7 has been found to promote homeostatic cycling in $CD8^+$ T cells, while prolonged signaling induced cell death [97].

The use of cytokines administered sequentially instead of single cytokines or multiple cytokines used simultaneously has also been presented as a viable alternative: IL-2 for 4 days followed by IL-12 combined with IL-7 or IL-21 for 3 days and IL-7 or IL-21 only for 5 days yielded a 'young' phenotype expressing CD62L, CCR7, CD27, and CD28 to a high extent [91].

Last, IL-4 has been entered into the T cell ACT production field. IL-4, a T helper 2-associated cytokine, is known to play a complicated role in several tumor types [90]. IL-4 in combination with IL-7 has been found to be able to induce *in vitro* proliferation in the absence of TCR stimulation, and to promote low expression of co-inhibitory receptors [87]. A novel approach introducing a fusion protein with the IL-4 receptor α chain fused with the IL-2/IL-15 receptor β chain into CAR T cells has demonstrated a way to use IL-4 to promote effects associated with IL-2 and IL-15, such as proliferation and induction of effector function in cultured T cells [90].

3.2. Cell culturing systems and culturing conditions

Other factors than the cytokine concentration in the culture medium are of importance to the quality and characteristics of the product. One interesting method for promoting a less differentiated phenotype and reducing terminal differentiation, especially for $CD8^+$ T cells, is inhibition of the P13-Akt-mTOR pathway. Akt inhibition has been shown to promote production of $CD8^+$ T cell cultures with a less differentiated phenotype [98]. Another approach worthy of consideration is culture in hypoxic conditions, which has been found to substantially increase the yield [99]. Last, in TIL therapy, 'REPs' using irradiated allogeneic feeder cells from healthy donor peripheral blood in combination with high-dose IL-2 have generated positive results [100].

Then, the choice of culturing system is another key factor to successful T cell immunotherapy. For therapeutic cell products, a closed system with a high degree of automation is preferable, as it limits the risk of contamination and human error. Other important factors include whether the cells are adherent or grow in suspension. For adherent cells, options include more traditional systems like T-flasks and cell culture bags (permeable or non-permeable to gas), and innovations like hollow fiber bioreactor systems (e.g. Quantum® from Terumo BCT and G-Rex® from Wilson Wolf). All these can be set up as closed systems, but cell culture bags and hollow fiber bioreactor systems are more suitable given the availability of sterile connectors and sterile tube welding. For suspension cell cultures, rocking motion bag systems (e.g. Xuri™/Wave from General Electric and Allegro™ from the Pall Corporation), spinner flasks, and Erlenmeyer/Fernbach flasks for shaker cultures are among the available choices. Bioreactors with an impeller can generally be used for both suspension cell cultures and adherent cell cultures. Bioreactors like iCELLis® from the Pall Corporation and rocking motion bag systems commonly offer the possibility to monitor and control parameters such as pH, dissolved oxygen, and temperature.

Table 1. Cytokines in T cell ACT production.

Strategy	Result	Interpretation	Type of therapy	Reference
- IL-2 (6000 IU/ml) - REP	Downregulation of CD62L, CD27, and CD28	This protocol favors short-lived effector cells	TILs (preclinical)	Zhou et al. [20]
- IL-2 (0, 5, 20, 100, 300 IU/ml) - (10 and 20 days of culture)	- Higher IL-2 -> increased proliferation, cytokine production, and tumor cell killing, but less early memory T cells. - Longer culture time also increased differentiation	The IL-2 concentration is a very important factor for differentiation, but the length of culture has significant impact as well.	CD19 CAR (preclinical)	Kaartinen et al. [92]
- IL-2 short term vs. long term (100 IU/ml, day 0–3 vs. entire culture) - 10 days of culture	Short-term IL-2 -> less apoptosis, better proliferation, less differentiated phenotype, higher cytotoxicity	Using IL-2 for 3 days only could reduce negative effects of IL-2	CD19 CAR (preclinical)	Zhang et al. [93]
IL-2 (100 IU/ml) ± IL-7 (10 ng/ml)	IL-2 combined with IL-7 -> less apoptosis, higher fold induction, and less differentiation than IL-2.	The negative effects of IL-2 can to some extent be counteracted by IL-7	UCB DLI	Davis et al. [85]
- IL-2 (100 IU/ml) vs. IL-7 (10 ng/ml) + IL-15 (5 ng/ml) - 10–14 days of culture	More proliferation, more early memory T cells, better response to serial stimulation with IL-7 + IL-15. *In vivo* tumor mouse model indicated better survival with IL-7 + IL-15 cultured CAR T cells.	The results described here indicate a clear advantage of the combination of IL-7 and IL-15 compared to IL-2	CD19 CAR clinical (IL-2) Preclinical (IL-2 vs. IL-7 + IL-15)	Xu et al. [78]
- IL-2 (100 IU/ml) vs. IL-7 (10 ng/ml) + IL-15 (5 ng/ml), - 10–14 days of culture	Comparable proliferation and memory phenotype	In some settings, the advantage of using IL-7 + IL-15 instead of IL-2 is not clear	GD2 CAR (preclinical)	Gargett et al. [86]
- IL-2 (100 IU/ml) - IL-7 (10 ng/ml) + IL-15 (5 ng/ml)	Clinical study, IL-2 was switched to IL-7 + IL-15 because this -> increased CM T cell % (5% -> 10%)	The benefits of using IL-7 + IL-15 instead of IL-2 were judged to motivate a switch in a clinical trial	Kappa light chain CAR (clinical)	Ramos et al. [88]
- IL-7 (10 ng) + IL-15 (5 ng), -21 days of culture - CAR T cells modified to produced IL-15 on ligation of CAR	IL-7 + IL-15 culture for 21 days -> only 5–10% CM T cells. The CAR T cells producing IL-15 upon CAR ligation had more CM T cells+, less PD-1 expression	Additional IL-15 produced by T cell at CAR ligation could reduce differentiation.	Kappa light chain CAR (preclinical)	Chen et al. [84]
- IL-2 (300 IU/ml), days 0–4 - IL-12 + IL-7 or IL-21, days 4–7 - IL-7 or IL-21, days 7–12	The protocol was used with selected CD8+ T cells. -> High expression of CD62L, CD28, CD27, and CCR7.	A sequential combination of cytokines could be a valid strategy to reduce differentiation	TCR transgenic T cells (preclinical)	Yang et al. [91]
TILs + artificial APCsS (K562 cells expressing 4–1BBL and producing IL-2, IL-15, or IL-21)	IL-21 culture -> lower proliferation, more CD8+ T cells, more CD27+ CD28+ T cells and less Tregs, better tumor cell line killing.	IL-21 -> little additional more expansion compared to only TCR + 4–1BB stimulation, but favored cytotoxic CD8 T cells	TILs (preclinical)	Santegoets et al. [89]
IL-2 (100 IU/ml) vs. IL-7 (10 ng/ml) + IL-4 (20 ng/ml) ± IL-21 (20 ng/ml)	- IL-4 + IL-7 -> expansion without TCR stimulation. - IL-7 + IL-4 ± IL-21 -> very low levels of TIM-3, LAG-3, and PD-1. - IL-4 + IL-7 + IL-21 culture -> more CM T cells.	The combination of IL-4 and IL-7 seems interesting as an alternative to IL-2. However, no T cell lineage information, such as Th/Tc identity, was provided.	CD19 CAR (preclinical)	Ptackova et al. [87]
- IL-4 receptor α chain – IL-2/IL-15 receptor β chain fusion protein - Culture with IL-4	- T cells with fusion receptor cultured with IL-4 proliferated and destroyed tumor cells repeatedly - Mainly produced IFN-γ	This technique enables using IL-4 for IL-2/IL-15-like induction of effector function and proliferation without Th2 polarization	MUC1 CAR (preclinical)	Wilkie et al. [90]

IL-2: interleukin 2; TILs: tumor-infiltrating lymphocytes; CAR: chimeric antigen receptor; IL-7: interleukin 7; UCB: umbilical cord blood; DLI: donor lymphocyte infusion; IL-15: interleukin15; GD2: ganglioside 2; CM T cell: central memory T cell; IL-21: interleukin 21; IL-4: interleukin 4; IFN-γ: interferon γ; Th: T helper; Tc: the counterpart of the Th subsets for cytotoxic $CD8^+$ T cells; MUC1: mucin 1; REP: rapid expansion protocol; TCR: T cell receptor; PD-1: programmed cell death protein 1; TIM-3: T cell immunoglobulin and mucin-domain containing 3; LAG-3: Lymphocyte-activation gene 3.

Another interesting closed system with a partly automated culture process is Zellwerk's Z®RP system. There are also highly automated systems that cover the whole range of tasks from fractionation of starting material to final product such as the CliniMACS Prodigy® from Miltenyi Biotec.

4. Tumor defense mechanisms

Another challenge to successful T cell therapy is the tumor's capacity to escape immune-mediated eradication through tumor immune escape mechanisms. Multiple mechanisms have been identified, and they are widely heterogeneous among tumor types and patients [101]. Their potency and diversity provides a formidable barrier against successful T cell immunotherapy, and strategies to target and overcome the right escape mechanism in each case are necessary [102]. In this section, we discuss the immune escape mechanisms and possible solutions to overcome them. An overview is found in Figure 2.

4.1. Escape from immune recognition

As mentioned earlier, due to transcriptional infidelity resulting in somatic mutations, neoantigens are continuously generated in tumor cells [103], and promote T cell immunity. However,

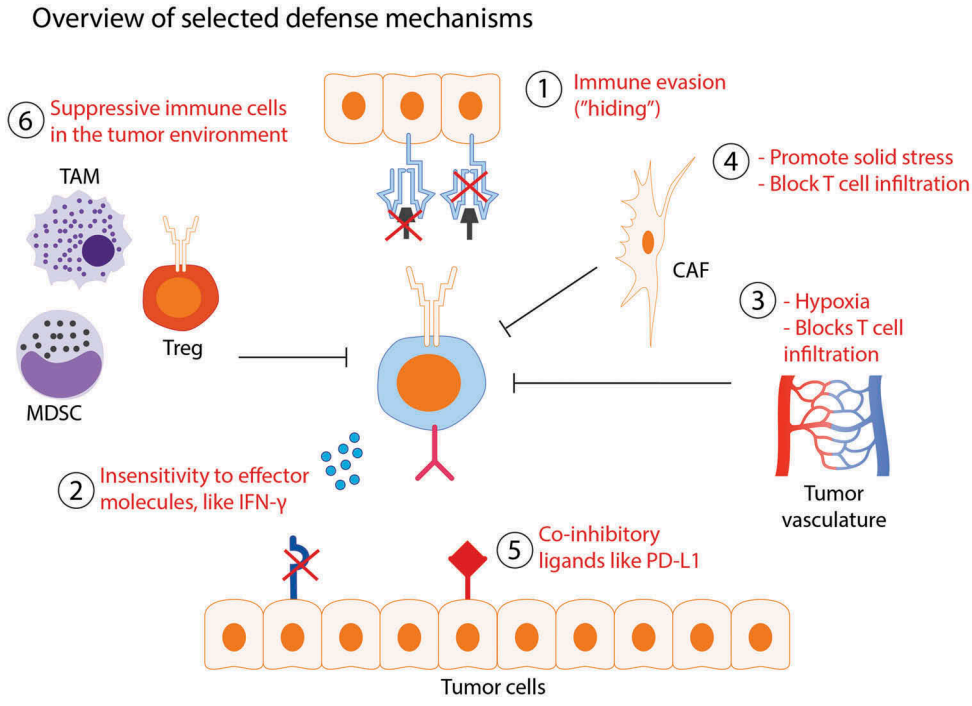

Figure 2. Schematic overview of the immune escape mechanisms described in this review.

Table 2. Immune escape mechanisms

Mechanism	Strategy/ies to counteract mechanism
(1) Immune evasion	
Lost expression of neoantigens	Targeting TAAs
Downregulation of HLA class I and defects in proteasome function	Targeting antigens independent of proteasome: HLA machinery (only possible in certain cancer types)
(2) Insensitivity to effector molecules	
Abnormalities in IFN-gamma receptor signaling	
(3) and (4) Microenvironment	
Defects/leakiness in tumor blood vessels[113,114]	Anti-VEGF [124]. This property can also be exploited for targeting drug delivery [126,127].
Hypoxia	Angiotensin inhibition and other strategies targeting the stroma [115]. This, however, is not uncontroversial [116,117].
Solid stress	
TGF-β production	
Endothelial FasL expression (apoptosis at intravasation)	FasL inhibition [121]
Endothelial endothelin B expression (limits intravasation)	Endothelin B inhibition [122]
Eicosanoids (PGE2) [132–134]	COX-2 inhibitors [136]
(5) Co-inhibitory ligands	Checkpoint blockade [13–15,46]
(6) Suppressive immune cells	
Tregs	Anti-CTLA-4 [151], -CD25, -GITR antibody therapy [152]. CCR4 blockade [153]. Cyclophosphamide [152].
TAMs	Blocking of CSF1R, and CCL2 [161]

TAA: tumor-associated antigen; VEGF: vascular endothelial growth factor; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; GITR: glucocorticoid-induced TNFR-related protein; CCR4: C-C motif chemokine receptor type 4; CCL2: C-C Motif Chemokine Ligand 2; HLA: human leukocyte antigen; IFN-gamma: interferon gamma; TGF-beta: transforming growth factor beta; FasL: Fas ligand; PGE2: prostaglandin E2; Tregs: regulatory T cells; COX-2: cyclooxygenase 2.

tumors with a high expression of neoantigen do progress and metastasize, even in the presence of infiltration of neoantigen-specific CD8 T cells after vaccination [104]. This suggests that several mechanisms, not only immunoediting but also the tumor microenvironment itself, protect cancer cells from T cell-mediated eradication.

Tumor cells have been found to evolve toward reducing or even losing the expression of neoantigens [105], which is a central issue in the T cell ACT. The targeting of normal tissue-restricted antigens found in certain tumors, such as mesothelin in for example pancreatic and ovarian cancer, can be a strategy to circumvent this problem, but this is possible in very few cancer types. Tumor cells have also been described to downregulate HLA class I expression, which complicates antigen recognition by $CD8^+$ T cells [106]. Defects in the proteasome and peptide transporters (TAP-1 and TAP-2) which are responsible for intracellular antigenic processing may contribute to reduced antigen expression on cancer cells [107]. Like in the case of reduced neoantigen expression, certain therapies targeting antigens independent of the proteasome-HLA machinery are not affected by this mechanism, but it is a significant obstacle in many T cell ACTs. Also, the loss of HLA class I expression should enable NK cell-mediated tumor eradication, but the expression of surrogate HLA class I molecules, such as HLA-G, can rescue the cancer cells from detection by NK cells [108]. Furthermore, tumor cells can demonstrate abnormalities in the IFN-γ receptor signaling pathway, leading to the development of IFN-γ insensitivity [109]. Recent evidence also demonstrates that tumors are capable of suppressing TNF signaling within $CD8^+$ T cells, which counteracts T cell-mediated immunity [110].

4.2. Escape by immunosuppressive activity

4.2.1. The tumor microenvironment

The tumor microenvironment is well studied, and its features are reviewed elsewhere. In short, it is characterized by an aberrant vasculature with large gaps between endothelial cells, increased interstitial pressure, an acidic extracellular environment [111], cancer-associated fibroblasts (CAFs) [112], and infiltration of immunosuppressive immune cells.

The leakiness of the tumor vessels increases the interstitial pressure, which limits perfusion of the tumor [113]. The high-pressure environment also promotes transforming growth factor β (TGF-β) production through mechanical stress, which leads to recruitment and expansion of immunosuppressive cells [114], and together with the accumulation of interstitial matrix factors excreted by the CAFs causes solid stress. Solid stress results in an environment hostile and inaccessible to T cells [115]. Angiotensin inhibition using the blood pressure drug losartan has been found to reduce the solid stress, and to reduce the CAF density in the tumor, and the collagen I production by CAFs through TGF-β inhibition [115]. The lowered solid stress decompresses tumor blood vessels and increases oxygen and drug delivery into the tumor [115]. Retrospective studies of the outcome of cancer patient on this type of drug for hypertension showed an association between this therapy and longer survival in pancreatic cancer, and reduced recurrence risk in breast cancer [115]. However, some strategies targeting the stromal component in pancreatic cancer have yielded disappointing results, e. g. hedgehog signaling inhibitors [116], and PEGylated recombinant human hyaluronidase [117]. This has led to discussions regarding whether the stroma has a protective role in impeding the progression of the cancer cell component in the tumor. In the latter case, the risk of increased toxicity when novel agents are included into already toxic standard regimens was raised. This indicates that this type of therapy can be a double-edged sword.

The hypoxia resulting from vessel compression and high interstitial pressure in itself suppresses the cytotoxic effects of T and NK cells; recruits myeloid-derived suppressor cells (MDSCs) [118], Tregs, and TAMs [119]; and stimulates programmed death-ligand 1(PD-L1) expression [120]. An important feature of the tumor endothelial cells (TECs) in the tumor blood vessels is FasL expression, which induces apoptosis in cells intravasating through the vessel walls. Since Tregs are somewhat protected from this effect by a high expression of c-FLIP, FasL expression by TEC selectively depletes conventional T cells, especially $CD8^+$ T cells, while promoting Tregs in the tumor microenvironment [121]. The endothelin B receptor, that is also upregulated on TECs, further limits intravasation of TILs [122].

Normalizing the tumor vasculature through knockout of the regulator of G-protein signaling 5 (RGS5), a master gene responsible for abnormal tumor vascular morphology in mice [123] or anti-vascular endothelial growth factor (VEGF) antibodies [124], improves T cell infiltration. Additionally, inhibition of FasL [121] and the endothelin B receptor [122] increases $CD8^+$ TIL numbers and suppresses tumor growth. It has been also been shown that tumor necrosis, through excretion of potassium ions into the extracellular fluid, suppresses the function of infiltrating T-lymphocytes through downregulating Akt-mTor signaling, and that this can be avoided by increasing the potassium efflux of T cells by overexpressing Kcna3 potassium channels [125].

The defective tumor vasculature could also be exploited for drug delivery. Tumor tissues are subject to the enhanced permeability and retention effect, where the leaky vessels allow accumulation of nanoparticles into the tumor, while decreased lymphatic drainage inhibits their removal. Through engineering nanoparticles of a size suited to pass through the gaps in the leaky tumor vasculature, but too large to enter through normal endothelial gaps, preferential tumor accumulation could be achieved [126]. An example of how this principle can be used is found in a study where alendronic acid in a liposomal formulation was used to selectively sensitize tumor tissue to eradication by adoptively infused γδ T cells in a mouse model [127].

Another immune escape system mechanism is posed by the tumor stroma, and especially the CAFs. In both colorectal and ovarian cancer, the prognosis is worse in tumors where TILs are physically blocked from direct access to the cancer cells by the tumor stroma [128,129]. As mentioned above, the dense extracellular matrix created by the CAFs blocks the access of T cells into the tumor. Targeting CAFs and the chemokine CXCL12, which is released by the CAFs, has been found to improve the impact of anti-PD-L1 checkpoint blockade in a pancreatic cancer model, further pointing to the role of the stroma in blocking antitumor immune responses [130].

The tumor microenvironment is immunosuppressive and yet highly inflammatory. One important class of mediators of inflammation is eicosanoids. The role of eicosanoids has been extensively studied in cancer, and prostaglandin E_2 (PGE_2) appears to be important in this context. PGE_2 is formed via the activity of cyclooxygenase-2 (COX-2) [131]. The presence of COX-2 and the M2 phenotype TAM marker CD163 is associated with poor prognosis in breast cancer, while perioperative therapy with COX-2 and beta-adrenergic blockers results in lower metastatic spread [132,133]. Similarly, PGE_2 and CD163 correlate to poor prognosis in pancreatic cancer [134]. The enzymatic activity of COX-2 can be disrupted by non-steroid anti-inflammatory drugs (NSAIDs). NSAIDs are extensively used to treat inflammation, and have been studied in the cancer context previously. They are now investigated once again as a possible alternative in cancer therapy [135] (18). Interestingly, the use of COX-2 inhibitors in combination with checkpoint inhibitors has been found to synergistically limit tumor growth [136]. The COX-2/PGE_2 cascade enhances PD-L1 expression on TAMs and contributes to immune tolerance in lung cancer by increasing PD-1 expression on T cells [137,138]. However, the best survival benefits of aspirin (an NSAID) treatment occur in colon cancer patients with low PD-L1 expression, suggesting that patients should be selected carefully [139].

4.2.2. Checkpoint receptors and their ligands, and T cell exhaustion

The regulation of T cell function is a complex subject and the details are outside the scope of this review, as is a detailed discussion about checkpoint blockade. It is, however, pertinent to mention the interplay between co-regulatory receptors on the T cells and their cognate ligands expressed on cancer cells, as this may influence the fate and efficiency of infused T cells in the immunotherapy setting.

Co-inhibitory receptors, such as PD-1, Lymphocyte-activation gene 3 (LAG3), T cell immunoglobulin and mucin-domain containing-3 (TIM-3), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), 2B4 and CD160, mediate negative regulation of T cells, and their expression increases at activation of T cells and memory T cell development [140,141], but also in exhaustion. Exhaustion is a state characterized by a lack of effector functions, such as poor capacity for proliferation, cytotoxicity, and cytokine production [142,143]. Increasing co-expression of co-inhibitory receptors correlates with a more severely exhausted phenotype [144].

Co-inhibitory receptors are associated with two negative aspects in the context of T-cell-mediated anticancer immunity: their expression makes the T cells vulnerable to negative signals from any cell expressing their corresponding ligands, and cancer cells and APCs in the tumor microenvironment are well known to be able to upregulate co-inhibitory ligands, such as PD-L1 [145,146]. Also, the chronic inflammation and continuous exposure of antigens inside the tumor microenvironment contributes to induce T cell exhaustion [142], rendering the T cells incapable of response. Further, hypoxia and IFN-γ production by T cells also contribute to inducing PD-L1 expression [146]. PD-L1 expression on tumor cells can be persistently maintained by CKLF-like MARVEL trans-membrane domain-containing protein 6 (CMTM6), which inhibits PD-L1 degradation in a lysosome-mediated fashion [147,148].

The success of the blocking antibodies directed against CTLA-4, PD-1, and PD-L1 have demonstrated how powerful the negative regulation of T cell anticancer immunity through these systems is, and how potent T cell anticancer effects are when unleashed [13–15,46]. With regard to the PD-1/PD-L1 axis, it is interesting that though treatment with PD-1 inhibition resulted in better response in patients with PD-L1 positive tumors in a trial including several different solid cancers [49], treatment in melanoma patients resulted in good responses even in patients with PD-L1 negative tumors [149].

4.2.3. Immune cells inhibiting T cell immunity

Besides lymphocytes, the tumor microenvironment is infiltrated by other immune cells, such as MDSCs and Tregs. Tregs are a suppressive subset of $CD4^+$ T cells characterized by high CD25 and FOXP3 and low CD127 expression, which limit inflammatory responses through CTLA-4 binding, PD-1 and PD-L1 expression, IL-2 consumption, and secretion of immunosuppressive cytokines like IL-10 and TGF-β [150]. High Treg/$CD8^+$ cytotoxic T cell ratios have been associated with poor prognosis. The administration of CTLA-4 blockade has been demonstrated to reduce the number of Tregs in tumor tissue, besides their effects on conventional T cells [151]. Other strategies include cyclophosphamide treatment and antibodies directed against CD25 and GITR [152]. Also, targeting C-C motif receptor 4 (CCR4), which facilitates Treg migration to the tumors, could prove successful. CCR4 blockade has been previously reported to deplete Tregs *in vivo* [153].

MDSCs are a heterogeneous population of immunoregulatory myeloid cells, including macrophages and dendritic cells, attracted to inflamed tissues such as tumors, where they hamper immune responses through stimulating Tregs by secreting TGF-β and IL-10 [154], while suppressing conventional T cell proliferation through reducing amino acids required by T cells, and by production of NO synthase [155]. Another mechanism is nitration of CCL2, which traps cytotoxic T cells in the tumor stroma and limits tumor access [156].

The TAMs are the most important APC in the tumor microenvironment. They promote an immunosuppressive microenvironment, and TAM infiltration has been linked to poor prognosis in several cancer types. This can in part be explained by production of suppressive cytokines and PD-L1 expression [157]. In the tumor-induced chronic inflammation, macrophages are polarized toward a M2 macrophage phenotype which displays anti-inflammatory and pro-tumorigenic properties [102]. M2 macrophages are characterized by poor antigen presenting capacity [158], and secrete IL-10 and TGF-β [159]. It has been shown that blocking of CSF1R, a myeloid growth factor, shifts the macrophage phenotype to facilitate antigen presentation and T cell proliferation [160], and selectively deplete TAMs [161]. Currently, inhibitors targeting the CSF1-CSF1R axis are being investigated in clinical trials [161]. It is also attractive to target the TAM-recruitment signaling pathway, often mediated by CCL2 produced by the tumor cells [162]. Several antibodies to block CCL2 are currently tested in

clinical trials [161]. TAMs may also contribute to the failure of checkpoint blockade. TAMs can inhibit activated $CD8^+$ T cells and limit their infiltration into the tumor; additionally, TAMs contribute to dissociate checkpoint inhibitors from T cells [163]. Checkpoint inhibitors can be efficiently combined with inhibitors targeting the CSF1-CSF1R axis for a better response [160]. A synergistic antitumor effect was also reported when anti-PD-1 therapy was combined with a treatment inducing a TAM phenotype shift, from M2 to M1 [164].

5. Conclusion

There are several strategies in T cell ACT that can be used to produce successful therapies. Different types of antigens can be targeted, including known TAAs, personalized neoantigens, and unknown targets, with a certain degree of success. Therapies with unknown target antigens, like the TIL approach, have resulted in tumor reduction and cure. The neoantigen promises further future developments. Last, positive responses have been observed with TAA-targeting therapies, such as transgenic TCR T cell and CAR T cell ACTs in solid cancers.

There are many variables of T cell ACT outside cancer cell targeting that are under investigation, such as the production conditions. Many variables can in all probability be significantly improved in the coming years based on emerging knowledge. The use of automated culture methods and closed systems will further revolutionize the outlook in this field.

The tumor escape mechanism adds another level of obstacles that will have to be overcome in order to expand the applicability and efficacy of T cell immunotherapy in cancer. These include mechanisms that 'hide' the tumors from detection, such as loss of HLA class I expression and defects in antigen processing and presentation. Another important factor is the aberrant tumor vasculature that creates a hostile environment and bar the egress of T cells into the tumor tissue, and thus access to the cancer cells. Expression of co-inhibitory ligands is another important factor that protects cancer cells from T cell-mediated eradication. Cells in the tumor environment such as the CAFs and the suppressive immune cells that are often present also dampen T cell-mediated antitumor immunity. There are ways of targeting these mechanisms, and several, furthermost checkpoint inhibitor therapy, have been implemented successfully. Their role in T cell ACT is currently being explored.

6. Expert opinion

The field of T cell immunotherapy in cancer is evolving rapidly and the future is expected to further establish this type of therapy as a part the standard therapy arsenal in solid cancers. The potency of T cell ACT in cancer was demonstrated with the TIL therapies, and developments in the CAR T cell field and in neoantigen-based T cell ACTs are expected to expand their applicability in future.

Target antigen selection is still a central question in the development of new T cell immunotherapy strategies. The treatments targeting unknown antigens, such as TIL therapy and the Sentoclone® treatment, have been found to be effective in a significant fraction of patients, but having an unknown target makes understanding what caused the treatment to fail in the specific case difficult. The many factors other than targeting that could influence the results, such as tumor escape mechanisms, cloud the picture, and further investigations into which antigens are targeted in the responders, could increase our understanding of the dynamics behind the treatment outcome.

The discovery of new TAAs in the solid tumor context could further increase the benefits of CAR T cell therapy and transgenic TCR T cell ACTs by expanding their applicability to more cancer types and increase efficacy. One great advantage to CAR T cell therapy is that tumor defense mechanisms affecting antigen processing and presentation by the proteasome-HLA machinery do not affect its efficacy, as the targets are expressed, not presented. The problem with off-target toxicity remains significant, and examples such as the neurotoxicity and cardiac toxicity with lethal outcome seen in trials with TCR transgenic T cells directed against the TAA MAGE-A3 are an important reminder about the dangers and difficulties inherent in targeting TAAs [165,166]. The issues with unknown targets and TAAs might indicate that the identification and selection of neoantigens to target for each individual patient may be preferable as the risk for off-target toxicity is reduced due to the selection of targets based on mutations only found in tumor tissue. There is, of course, still a low but potential risk for cross-reactivity, and consequently off-target toxicity. The identification of potential cross-reactivity is pivotal in order to maximize the safety of ACT; however, it is still difficult to predict off-target effects. Interspecies differences in MHC, antigen repertoire, and processing limit the usefulness of animal models. Different *in vitro* methods can be used to identify $CD8^+$ or $CD4^+$ cross-reactive T cells, but as mentioned above in the case of a MAGE-3 TCR, preclinical screening did not detect cross-reactivity that then has proven to be lethal. New testing methods are therefore needed, and hopefully new platforms such as the X-scan that generates mutated peptides [167], or the pMHC II-TCR (MCR) hybrid molecules that carry cDNA-derived peptides [168], will improve cross-reactivity detection.

To conclude this part of the discussion, different targeting strategies have different advantages, as well as different disadvantages and limitation. The advantages of TIL therapy and the Sentoclone approach, including relatively easy and affordable production, and not being limited to a single target antigen that can be downregulated by the tumor, are balanced by the issues associated with not knowing the target the therapy is directed against. The advantages of the CAR T cell and TCR transgenic therapies against TAAs, including powerful responses and the possibility of targeting antigens independent of HLA presentation in the case of CAR T cells, have limitations with regard to loss of the target antigen(s) and the risk for toxicity. The neoantigen discovery technology is still relatively new, and further improvement is necessary to achieve results reliable enough for clinical standards, but the field is evolving very quickly, and the possibilities are great. Possibly, the personalized character of this strategy could make it superior to the other approaches. It is being explored as a means of improving such therapies as TIL treatment and TCR transgenic T cell therapy, which could result in significant improvements in T cell ACT in the future.

The manufacturing of the T cell products for ACT is another area that is constantly improving. The choice of which cytokines and other factors to use in order to promote proliferation and a desirable T cell phenotype and other physical factors such as which oxygen pressure to apply are still subject to debate. With regard to cytokines, the current favorite in many T cell ACTs seem to be a combination of IL-7 and IL-15, which has been implemented in for example CAR T therapies. The results obtained using this cytokine combination mainly indicate significant advantages compared to using IL-2 [78,84,88]. Other promising strategies include using cytokines intermittently or for a shorter duration. The emerging new culture systems with increased automation and improved monitoring of factors such as oxygen pressure, pH, and lactate will contribute with further opportunities for improvement of the cell product.

In the opinion of the authors, the greatest issue in this research field is how to overcome the obstacles posed by the tumor itself. Optimal targeting and manufacturing of the T cell products will not suffice if the infused cells are unable to reach the tumor tissue or exert their effector functions. Given the heterogeneity and complexity of the tumor microenvironment, and the multiplicity of pathways that protect and maintain this niche, it is evident that these factors will have to be addressed in order to improve the response rates of T cell ACT in solid cancer. In this review, we have mentioned several strategies targeting different escape mechanisms. The optimal application of these should, in our opinion, be based on the specific characteristics of each tumor. In the perfect scenario, analysis of neoantigens and the immunosuppressive properties in the tumor microenvironment could be performed upfront, and a tailor-made combination of different treatments be applied. In tumors where abnormalities of the blood vessels and endothelial cells could be predicted to bar T cell infiltration into the tumor tissue, anti-VEGF treatment, and FasL and endothelin B receptor inhibition could be considered to improve the efficacy of the infused T cells. Angiotensin inhibition could be applied in order to reduce the solid stress in tumors with high interstitial pressure and dense extracellular matrix. This latter treatment would in many ways be an easy option, as it is a common and well-known drug with relatively little side effects. Targeting CAFs and the CAF chemokine CXCL12 is another option, as is focusing on the inflammatory component by, for example, targeting COX-2/PGE2.

Several other strategies that could form part of the arsenal of possible additions in T-cell ACT are treatments that target the immune suppressor cells present in the tumor microenvironment, such as Tregs and TAMs. The depletion of Tregs by one of the strategies discussed and TAMs by CSF1-CSF1R inhibition or targeting of the signaling recruiting suppressive immune cells to the tumor tissue could further pave the way for antitumor immunity by infused T cells.

All these strategies could also be combined with checkpoint blockade, which will be offered to a large percentage of eligible patients regardless of if they are scheduled to receive T cell immunotherapy as well. Checkpoint inhibition could, if administered before T cell harvest, boost the T cells to be used for ACT product manufacture. Thus, besides the original purpose, this type of drug could potentially be used to improve the quality of *ex vivo* expanded T cell immunotherapy.

We believe that the combinatorial approach is the best way to increase response rates in this type of treatment, and have noticed that several trials published during later years have combined several checkpoint blockades, or checkpoint blockade and other therapies. There is, however, still a long way to go before algorithms on how to best combine available therapies can be established. We look forward with great interest to see how the field of T cell ACT in solid cancer will evolve.

Acknowledgments

The figures were prepared using elements from the Motifolio collection.

Funding

This paper was not funded.

Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Reviewers' disclosure

Peer reviewers on this manuscript have no relevant financial relationships or otherwise to disclose.

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