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Adoptive T cell therapy: Boosting the immune system to fight cancer

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Abstract

Cellular therapies have shown increasing promise as a cancer treatment. Encouraging results against hematologic malignancies are paving the way to move into solid tumors. In this review, we will focus on T-cell therapies starting from tumor infiltrating lymphocytes (TILs) to optimized T-cell receptor-modified (TCR) cells and chimeric antigen receptor-modified T cells (CAR-Ts). We will discuss the positive preclinical and clinical findings of these approaches, along with some of the persisting barriers that need to be overcome to improve outcomes.

Keywords

CAR-T cells; T-cell receptor-modified (TCR) cells; Cytolytic T cells (CTLs); Tumor infiltrating T cells (TILs); Adoptive cell therapy; hematologic malignancies

1. Introduction

In recent years, the use of cellular therapies has energized the field of oncologic immunotherapy. While immune cells like Natural Killer (NK) and NKT cells are also increasingly utilized for cancer therapy, we will here focus on the use of T lymphocytes. T cells are uniquely positioned for fighting cancer because of their direct effector activity and helper function through the recruitment of other components of the immune response. Furthermore, T lymphocytes can expand ex-vivo and establish a memory compartment, a major property for antitumor surveillance.

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Starting with tumor infiltrating lymphocytes (TILs) and ex-vivo expanded cytotoxic T cells (CTLs), and moving to optimized T-cell receptor-modified T cells (TCR-Ts) and chimeric antigen receptor-modified T cells (CAR-Ts), each of these technologies displayed a gradual improvement in our understanding of harnessing the power of immune cellular elements to eradicate cancer. There have been promising results with clinical trials, especially with CAR-Ts, demonstrating long-term efficacy and remission potentials with hematologic malignancies. However, some hurdles hamper T-cell therapies from achieving higher levels of success and require further research. In this review, we will discuss the positive preclinical and clinical findings, along with some of the current barriers of T-cell-based therapies.

2. TILs and ex-vivo expanded CTLs: Dawn of Adoptive Cell Therapy (ACT)

Seminal studies in humans have shown that the presence of cytotoxic (CD8+) and helper (CD4+) T lymphocytes infiltrating tumors and the surrounding stroma is not only indicative of an ongoing antitumor response by the host, but also correlates with clinical outcomes in several tumor types, especially melanoma [1–2]. Tumor-specific CTLs could also be found in the circulation, although at very low frequencies [2]. These initial observations propelled improvements in culture conditions for the ex-vivo expansion of these cells, with the goal of returning them in large numbers to patients to eliminate tumors (Figure 1).

2.1 Rules of engagement

TILs and CTLs target tumors via their native TCR, that recognize tumor-associated antigens (TAAs), presented as epitopes via the major histocompatibility complex (MHC) on the surface of antigen-presenting cells, an interaction that is highly specific and results in T-cell activation [1,4]. MHC proteins are encoded by the human leukocyte antigen (HLA-A, B, and C) family of genes, which are highly polymorphic, resulting in these T cells being MHC-restricted [3]. TAAs susceptible for TIL- and CTL-targeting comprise of epitopes derived from viral open reading frames (for virus-induced tumors), non-mutated proteins overexpressed in cancer cells, to which T-cell tolerance is incomplete (like the cancer-testis antigens), or peptides that are absent in the normal human genome, so-called neo-antigens. These epitopes can be derived from any antigen, whether it is an intracellular or a cell surface protein.

2.2 Clinical relevance

Because of the high mutational load and accompanying high neo-antigen rates of melanoma [4–7], the adoptive transfer of autologous TILs represents one of the more effective approaches to try and induce complete regression in patients with metastatic melanoma [8–13]. For similar reasons, durable tumor regression have been observed for metastatic melanoma, and partial responses in patients with pulmonary or hepatic metastasis from melanoma, colon cancer, and renal cancer [14]. TILs have also proven effective when targeting immunogenic viral proteins, like in Human Papilloma Virus (HPV)-associated cancers, which overexpress the HPV-E6 and -E7 oncoproteins [17,18]. Equally, CTLs have been found effective against HPV-associated cancers and in eradicating other virus-associated tumors like Hodgkin and Non-Hodgkin lymphomas, and nasopharyngeal

carcinoma when they express Epstein Barr Virus-associated antigens [17–19]. They have also proven successful for treatment of hematological cancers, because of their expression of tumor restricted antigens, like Wilms tumor gene product 1 (WT1), preferentially expressed antigen in melanoma (PRAME), and melanoma-associated antigen (MAGE) [20,21].

2.3 Generation of TAA-specific T cells

Traditionally, TILs are isolated from resected tumor specimens, ex-vivo expanded with OKT3, irradiated feeder cells, and IL-2 [1,22,23] and thus retain the targeted antigen specificity to the tumor from which they were isolated. Approximately 6–8 weeks after tumor resection, up to 10^{11} lymphocytes can be prepared for infusion into patients (Figure 1A) [1,22,23]. Although the expansion can be reduced to 5 weeks, the procedure remains highly specialized and labor intensive, often producing “aged” cells. Additional concerns include the possibility that TILs may be rendered metabolically unfit by the chronic exposure to antigen before their isolation [24,25], and the potential expansion of less effective clones.

CTLs are generated *ex-vivo* over the course of 2–3 weeks, by repeated stimulations with cytokines and professional antigen presenting cells presenting the specific antigen (Figure 1B) [26]. T-cell clones have also been used, but like TILs they require extensive cultures and may generate exhausted cells. Unless targeting viral-associated antigen, the production of CTLs and clones for clinical purposes remain suboptimal, due to the low frequency of CTLs with adequate TCR affinity in the circulation as well as central tolerance.

To overcome the shortcomings of TIL and CTL therapy, including access to tumors, and expansion to sufficient cell numbers for therapy, research has concentrated on redirecting the antigen specificity to T cells through genetic engineering.

3. TCR-T cells: Genetic modifications make a debut in ACT

The ability to confer antigen specific recognition through the direct expression of ectopic TCRs allows to produce large quantities of TAA-specific T cells from the peripheral blood. In addition, TCR transgenic expression allows the recruitment of the auxiliary molecules of the signal transduction pathway, resulting in T cells being fully activated by a small amount of antigen (Figure 1C) [27].

3.1 Generating effective TCRs

Similarly to TILs and CTLs, candidates for TCR-based T-cell therapy are antigens either over-expressed or associated with tumor differentiation, like MAGE-A3, MAGE-A4 [28], melanoma antigen recognized by T cells (MART)-1 [29], gp100 [30], New York esophageal squamous cell carcinoma-1 (NY-ESO-1) [29], and carcinoembryonic antigens (CEA) [31], or viral antigens like the HPV-E6 and E7 proteins [32,33]. TCR sequences were traditionally identified by isolating single cells and expanding each clone, but major advances in engineering technologies have facilitated the identification and optimization of TCR specificity and affinity. Such approaches rely on using structure-guided modulation of the TCR-peptide-MHC interface, on selecting targeted mutations using computational design to characterize binding parameters and structure, or on rationally designing sequence

substitutions in contact areas using known TCR-pMHC structures. In some instances, deep mutational scanning of TCR-pMHC interactions are used to test the effects of amino acid alterations at multiple positions. These techniques are coupled with in-silico binding analyses, to provide a more informed high-throughput screening of engineered TCRs than traditional yeast and phage display technologies [34–41].

3.2 Limitations of TCR-based therapies

TCR-based T cells have produced successful responses in melanoma, myeloma, and HPV-associated cancers [16,28,32,42–47] While these successes have highlighted the feasibility and clinical potency of this strategy, limitations have also progressively emerged.

First, despite having the advantage of targeting any peptide, including those derived from intracellular protein degradation, this strategy remains MHC-restricted, making the TCR sequence only useful for individuals that share the same identical HLA. The majority of TCR-based trials are HLA-A2-restricted because of the prevalence of this haplotype in most human populations [48]. Expanding the targeting of TCR-gene therapies beyond HLA-A2 to a more diverse panel of targetable MHC alleles would make these therapies accessible to diverse populations.

Second, because antigen recognition by TCRs is regulated by MHC-presentation, there is the possibility of tumor escape due to MHC downregulation, or dysfunction of the antigen processing machinery [49]. Identifying TCR restricted for various HLA alleles may not only extend the strategy to a greater subset of patients, but also broaden the repertoire of TCRs to recognize other epitopes from the same antigen, ultimately overcoming the issue of heterogenous MHC expression [50].

Finally, on-target/off-tumor toxicities can occur if TCR-Ts attack antigens expressed on healthy tissues. Two separate clinical trials reported fatal neurotoxicity and cardiotoxicity events [51–53], caused by a MAGE-A3-TCR which was affinity enhanced in the α-chain of the complementarity-determining region (CDR)2 region, generating unforeseen cross-reactions with healthy tissues. An additional safety factor is the potential occurrence of TCR mispairing. Hybrid TCRs, formed from the combination of endogenous and exogenous chains, have the potential to generate unknown reactivity. Additionally, hybrid TCRs, even if not toxic per se, may cause T-cell dysfunction by competing with the intended α/β-pairs for surface and, thus, functional expression [54]. Mitigation strategies are being explored, like downregulating the endogenous TCR by small-interfering RNA (siRNA) and Dicer-substrate small interfering RNA (DsiRNA), which result in high transgene TCR expression with a reduced degree of TCR mispairing [55,56].

4. CAR-T cells: An evolution of ACT

Since their initial development, CAR-Ts have conquered some inherent weaknesses of the former two methodologies. First, CAR-Ts can be rapidly generated with known tumor specificities within weeks. Second, their tumor recognition is MHC-unrestricted, making them broadly applicable to multiple individuals. Third, unlike TCRs, CARs can bind not only to extracellular proteins but also to surface carbohydrate and glycolipid structures,

resulting in an expanded range of targets. However, CAR signaling cannot fully recapitulate the physiologic events that arise from the native TCR engagement and further engineering had to be developed. Regardless, their use has significantly accelerated the development of personalized medicine.

4.1 CAR Structure

The structure of the CAR construct consists of primarily three sections: an extracellular ectodomain, responsible for binding to the targeted antigen, a transmembrane domain, and an endodomain responsible for signal transduction that carries out the effector component of the CAR-T-cell apparatus (Figure 1D).

4.1.1. Extracellular domain.—This portion comprises of a single-chain variable fragment (scFv), traditionally derived from a monoclonal antibody, which is the tumor antigen-binding moiety of the CAR construct. The scFv is usually composed of a variable light (V_L) and a variable heavy (V_H) region, joined by a linker segment. Work is on-going for deriving these moieties from alternative antigen-binding proteins that are smaller in size, more stable, and less likely to aggregate, which should facilitate the construction of multi-specific recognition motifs. Examples of single domain antibody mimetic proteins are: monobodies, based on the type III domain of fibronectin [57]; affibodies, based on a three-helix bundle Z domain [58]; and DARPins, based on the designed ankyrin repeat protein [59]. A hinge segment flexibly anchors the scFv to the transmembrane domain. Some of the hinges used contributed to activation-induced death, while others led to off-target activation, or played a role in tonic signaling [60–62], suggesting that this region can have significant impact on the overall CAR structure and functionality.

4.1.2. Transmembrane Domain.—The transmembrane domain connects, via the hinge region, the scFv to the signaling endodomain. Initially, transmembrane domains were derived from CD3ζ, CD4, CD8, or CD28 molecules, but more recent second-generation CAR transmembrane domains are derived solely from CD8 and CD28 molecules. Though difficult to compare across each of these subtypes, the transmembrane domains acts as a dynamic part of the CAR construct [63]. Together with the hinge, the transmembrane domain participates in modulating CAR function, which may vary depending on the type or the location of the tumor antigen epitope [61,64].

4.1.3. Endodomains.—This segment is tasked with coordinating and recruiting signaling elements through its immunoreceptor tyrosine-based activation motifs (ITAMs) [65,66]. Initially, CARs only contained a signaling domain, commonly derived from the CD3-ζ chain (1st generation CAR). This iteration of CAR, while showing preclinical cytotoxicity, exhibited only limited persistence, expansion, and antitumor efficacy [67]. Incorporation of co-stimulatory domains from either the CD28 or 4–1BB molecules in tandem to the ζ-chain promoted superior expansion, longevity, and tumor cytotoxicity preclinically and clinically (2nd generation CARs) [68,69]. While there is no clear consensus on which co-stimulatory domain is superior, key intrinsic differences are emerging. CD28-based CAR-Ts appear to proliferate and expand more rapidly and secrete larger amounts of Th1 cytokines like IFN-γ and IL-2; 4–1BB-based CAR-Ts display a more gradual response,

but with less exhausted phenotypes and superior persistence in-vivo [70]. These distinctions also reflect specific metabolic pathways. CD28-CAR-Ts express a genetic signature pattern, which coopts an enhanced aerobic glycolytic pathway, resulting in effector memory differentiation and phenotype with less potential for extended survival and persistence [71]. In contrast, 4–1BB-CAR-Ts show increased oxidative phosphorylation capabilities, with enhanced fatty acid oxidation and mitochondrial biogenesis, which leads to more central memory phenotype. Intrinsic cellular kinases like lymphocyte-specific protein tyrosine kinase (LCK) in CD28-CARs and phosphatases such as SHP1 in 4–1BB-CARs also work differentially in each respective construct to further generate distinct modulation of cellular kinetics, metabolism, and persistence of each of these CAR-T types [71,72]. Additional co-stimulatory domains, such as OX40, CD27, and ICOS, have shown promise in preclinical CAR-T cell studies. Combination of multiple co-stimulatory molecules (3rd generation CARs) are also being studied [73,74], but more clinical experience is necessary to prove superiority [75].

4.2 Lessons learned from CAR-Ts in Hematologic Malignancies

The largest clinical experience with CAR-Ts is in hematologic malignancies, especially in those expressing the CD19 molecule, solely found on B-cell lineage cells. Over the course of the past 10 years multiple trials have shown unprecedented high and durable response rates in adults and pediatric patients with acute lymphoblastic leukemia (ALL) and aggressive B-cell non-Hodgkin lymphomas (B-NHL). Overall, these trials have resulted in the approval of a few CD19.CAR-T cell products for ALL, aggressive B-NHL, and mantle cell lymphoma [84–86]. CARs are also in clinical trials for non-B-cell hematological malignancies. For multiple myeloma, CAR-Ts targeting the B-cell maturation antigen (BCMA) are the most advanced in the clinic [76–78]. For Hodgkin Lymphoma, phase I-II trials with CAR-Ts targeting the CD30 antigen have shown safety and encouraging efficacy [79–81].

Despite the successes, these trials are previewing upcoming issues that CAR-based therapy is likely to have when applied to solid tumors. For example, the on-target/off-tumor toxicity of CD19.CAR-Ts produce B-cell aplasia and hypogammaglobulinemia with increased susceptibility to infections that can be long-lasting [82–84]. Supportive care with intravenous immunoglobulin infusions and prophylactic antibiotics help lessen the impact, but approaches are being actively explored to mitigate these on-target/off-tumor toxicities while retaining high clinical efficacy. Examples for B-cell malignancies are CARs targeting the surface immunoglobulin light-chains [85] (kappa or lambda), which are clonally restricted in expression and would spare the reciprocal light chain-expressing, non-malignant B cells. On-target/off-tumor toxicities have also delayed the application of CAR-Ts for the treatment of T-cell leukemia and lymphoma and for acute myeloid leukemia (AML), due to failures in identifying targets not shared by the leukemic and healthy T and myeloid precursor cells [86,87].

Another example is the loss or downregulation of the targeted antigen. Despite CD19 being highly expressed by blasts, antigen escape can still occur because of either selection pressure due to therapy, presence of clone containing a preexisting alternatively spliced CD19

isoform, a mutated CD19 protein, or occurrence of a lineage switch from lymphoid to myeloid [89–91].

Finally, epitope masking has been observed after the accidental transduction of B blasts by the CD19.CAR [90]. Although this is a rare phenomenon, the risk that this may occur in malignancies with circulating tumors, especially T-cell leukemia, should not be underestimated.

4.3 CAR-Ts Take the Stage in Solid Tumors

The application of CAR-Ts in solid tumors remains a work in progress. Solid malignancies exhibit unique impediments to CAR-T-cell therapies and clinical trials have so far displayed only modest activity. The reasons for these tempered results are multifactorial. Some are intrinsic to the CAR strategy and will be described in this section; others are common to the TIL/TCR-Ts approach and will be described in the next section.

4.3.1. Shared expression of TAA—One problem with the physiology of solid tumors is that cell surface targetable antigens on cancer cells are almost invariably also expressed on healthy cells. Unlike blood cancers where tumors usually express a cell-lineage specific marker, solid tumors more commonly express TAA, which while enriched in expression levels on tumor surfaces, tend to exist at low levels on normal tissues. For example, a trial with carboxyanhydrase-IX (CAIX).CAR-Ts in patients with renal cell carcinoma had to be stopped due to dose-limiting hepatotoxicity stemming from low-level expression of the CAIX antigen in normal bile duct epithelial cells [91]. In another patient with metastatic colon cancer, Her2.CAR-Ts caused severe respiratory failure, leading to the patient's death. Low levels of Her2 on the lung epithelial cells were likely the culprit [92]. To circumvent this problem, researchers are exploring ways to modulate the affinity of the scFv towards its target. The critical question is how one can tune CARs to sense antigen density without impairing efficacy.

One way to modulate CAR signaling is by modifying the scFv affinity for the tumor antigen. However, challenges lie in balancing the efficiency of the CAR construct-mediated activation: if affinities are too weak, the recognition of TAAs can be ineffective; if too augmented, on-target/off-tumor toxicity could prevail (Figure 2A). An alternative approach uses the tyrosine kinase inhibitor dasatinib, which reduces LCK phosphorylation of CARs. This reversible inhibition curtails CAR-T-cell proliferation and cytokine production. Although potentially useful for abrogating toxicities like cytokine release syndrome (CRS) and neurotoxicity [93,94], side effects are expected to reoccur when the drug is withdrawn (Figure 2A). Different strategies looked at tightly regulating the expression of a cross-reactive CAR. One of these methods incorporates combinatorial antigen recognition with split signaling, as well as with balanced T-cell activation and costimulation. In this model, T cells co-express a CAR construct targeting the TAA of interest lacking costimulatory endodomains, and a chimeric costimulatory receptor (CCR) motif, which recognizes a second TAA and provides the costimulatory signal [95] (Figure 2B). Alternatively, adapter-mediated CARs separate the tumor antigen recognition motif and the CAR signaling, which allows for titratable tumor killing and reversible control of the CAR-Ts. Affinity between the

adaptor and the CAR (zipper pair), and the affinity between tumor antigen and scFv can also be modified to provide additional tuning of CAR activation [96,97] (Figure 2B). To minimize off-tumor activation, CAR expression can be restricted to the tumor microenvironment (TME). This can be accomplished by incorporating a hypoxia inducible factor (HIF) within the construct. Such CAR molecules are continuously degraded when transduced cells are present in a normoxic environment, but expressed when cells are in hypoxic conditions like upon entering the TME [98] (Figure 2B). Finally, combinatorial logic for the precise recognition of tumors have been explored. For example, T cells have been engineered to express combinatorial circuits, in which a synthetic receptor (Notch) for one antigen induces the expression of a CAR for a second antigen. This increases the threshold of activation of CAR-Ts, so that they are armed and activated in the presence of dual antigen tumor cells (AND-gate strategy) [99] (Figure 2C).

Suicide genes represent alternative methods to remove CAR-Ts in the event of a life-threatening on-target/off-tumor toxicity, although with the downside of also halting efficacy. Titratable safety switches, such as the inducible caspase 9 (iCasp9), may be more useful to not jeopardize clinical responses, by potentially allowing a small remnant population of functional CAR-Ts behind (Figure 2B) [100,101].

4.3.2. Antigen Expression Levels.—Surface antigen expression presents inter- and intra-heterogeneity, with proportions of cancer cells expressing no or low levels of antigen. This variation affects the binding capacities of CARs, as, in contrast to TCR, they require a higher threshold of antigen expression for activation [102,103]. While an advantage when targeting shared-TAA (see above), a low-activation threshold can be a hinderance in case of tumor heterogeneity, with cells expressing antigens at low levels escaping killing. Enhancing the strength of CAR-signaling should increase activity against low-antigen density tumors [104], but it an undesirable trade-off if resulting in off-tumor toxicities, over-activation, and premature exhaustion. This may imply fine tuning and balancing, and the requirement for tailoring CARs to each tumor model.

An alternative compromise may rely on the use of CAR-Ts that recognize multiple targets to prevent the upfront tumor antigen remodeling driven by the treatment pressure. For tumors with intra-tumor variability, a hierarchy of two or three non-overlapping antigens could be sufficient [105]. While a combination of “monovalent” CAR-Ts, each targeting a highly expressed antigen, to be infused simultaneously would allow flexibility to mix and match, the approach comes with regulatory hurdles and costs, and complex logistics [106]. Alternatively, multiple CAR constructs could be engineered in the same T-cell, although their expression seems overall reduced, and there are concerns about their functionality. Recently bi- or trivalent CAR constructs that recognize 2 or 3 antigens have been developed and tested preclinically with impressive results. To overcome some of the challenges of assembling several scFv’s in one single CAR, such as maintaining optimal protein folding, researchers are sourcing to antibody mimics [57,59].

5. Roadblocks to Effective ACT

Whether ACT occurs in the form of TILs, CTLs, TCR-Ts or CAR-Ts, there are several common barriers when targeting solid tumors. Because CAR-Ts are broadly applicable (as MHC-unrestricted) and readily available (quick turn-around of product through genetic engineering), the obstacles for targeting solid tumors have become better appreciated in the context of this modality.

5.1. Trafficking and infiltration.

For productive homing after infusion, there must be a match between the chemokine released by the tumor and/or its environment (TME) and the receptors that a T-cell expresses. T cells are expected to traffic to lymphoid tissues and bone marrow, but this homing can be suboptimal for certain locations (i.e. brain, pleural or peritoneum spaces). To overcome this obstacle, cells can be delivered locally, with successful results [107,108]. However, not every tumor is accessible for local delivery, and metastases to multiple sites render this approach unpractical. Leveraging on specific chemokine gradients/chemokine receptor pairs represents a possible solution. If the chemokine gradient is unique to the tumor, then the strategy is straightforward, and the match can be artificially created by forcing the expression of homing receptors and/or chemokines. Examples can be found both for TILs [109] and CAR-Ts [110–114], and some of these strategies are reaching clinical application. Making the tumor and/or its TME release chemokines that attract T cells may produce the same results [110,115].

In addition to homing, physical barriers also impede T-cell infiltration into cancer sites, especially in solid malignancies, where cancer-associated stromal cells and extracellular matrix (ECM) remodeling enzymes create highly desmoplastic TMEs [116]. Reducing or degrading the ECM components could assist in tumor eradication. For example, CAR-Ts targeting fibroblast activating protein on stromal cells augmented endogenous antitumor responses in mice [117]. Coexpression by CAR-Ts of enzymes capable of degrading the ECM has also demonstrated enhanced tumoricidal efficacy in preclinical models of stromal rich tumors [118].

5.2. Nutrient Starvation and Hypoxia

The lack of nutrients constitutes a major barrier for T cells in the TME. Indoleamine 2,3 dioxygenase (IDO) is an intracellular enzyme involved in the oxidative catalysis of tryptophan into kynurenine, with a vital regulatory role in mediating effects on innate and adaptive immunity in response to inflammation[119]. In the context of tumor anti-immunity, the IDO pathway is a potential tumor escape mechanism, through induction of cell-cycle arrest and CD8+ T-cell anergy, and by blocking the differentiation of CD4+ T cells to T helper (TH)17, while promoting differentiation, activation, and tolerogenicity of regulatory T cells (Treg) [120,121]. Additionally, kyneurine and its catabolites can inhibit T-cell proliferation, cytokine secretion, and cytotoxicity [122,123]. Tumor-IDO expression can finally orchestrate the recruitment of myeloid derived suppressor cells (MDSCs), which leads to further amino acid depletion through the secretion of arginase-1 [124,125]. By encouraging the differentiation and activation of immunosuppressive Tregs, depleting the

local tryptophan supply, and recruiting MDSCs, IDO expression in tumors creates an oppressive and toxic environment for T cells. Engineering of CAR-Ts to become “IDO-insensitive” have been developed [123].

Hypoxia, along with nutrient depletion, is a characteristic trait of the TME. Deficient perfusion due to aberrant angiogenesis and ineffective delivery of oxygen are causative factors for this phenomenon. Hypoxia can negatively affect T-cell metabolism and effector function, by inducing inefficient cellular metabolism and enhancing glycolysis, instead of the tricarboxylic acid cycle [126]. Upregulation of HIF1-alpha further promotes MDSC recruitment, enhancing the immune suppressive environment [127]. Lessening the deleterious effects of hypoxia could help boost T-cell function. Recently, a CAR engineered to intrinsically activate the IL-23 pathway, via expression of p40, resulted in enriched hypoxia-related genes, ultimately leading to enhanced antitumor activity in solid tumor models [128].

5.3. Inhibitory Cytokines and Soluble Factors

Inhibitory cytokines and soluble factors are other prominent hallmarks of the TME. They can directly encumber T-cell cytotoxicity, proliferation and survival, and indirectly hamper them by recruiting immunosuppressive cells such as MDSCs and Tregs.

Chemotherapy regimens or irradiation prior to ATC infusions have proven helpful in the removal of suppressive cells and competing endogenous lymphoid sinks for the homeostatic cytokines, thus creating a favorable milieu supportive for T-cell persistence and function [129]. However, the effects of lymphodepleting conditioning are transient, and neither is poised to reshape inhibitory TMEs.

For example, adenosine and prostaglandin E2 (PGE2) are two of the molecules over-expressed by hypoxic TMEs. Both can subvert executive function of T cells through intracellular activation of protein kinase A (PKA) in a cyclic AMP (cAMP)-dependent fashion [130]. Specifically, the regulatory subunit I-anchoring disruptor (RIAD) is a protein that interrupts the PKA anchorage close to adenylyl cyclase and its expression in mesothelin-directed CAR-Ts has resulted in superior tumor infiltration and cytotoxicity [131].

Transforming growth factor beta (TGFβ), IL-4, and IL-10 are among the more predominant immunosuppressive cytokines secreted into the TME milieu by tumors, Tregs, and tumor-associated macrophages [132–134]. In advanced phases of tumor invasion and metastasis, TGFβ helps to stimulate the epithelial-to-mesenchymal transition of malignant cells, and assists in immune evasion by skewing T cells towards a Th2 phenotype [135]. IL-10 stifles antigen presentation and T-cell differentiation through suppression of antigen presenting cells [136]. Blocking the effects of these cytokines poses a chance to enhance the efficacy of T cells. Augmented tumoricidal effects, both in-vitro and in-vivo, were demonstrated by incorporating a dominant negative TGFβ receptor into antigen-specific CTLs and CAR-Ts [137,138]. The strategy is now in the clinic for tumor-specific CTLs, with very encouraging results [139]. To combat IL-4-mediated immunosuppression in the TME, a chimeric cytokine receptor comprising of an extracellular IL-4 receptor domain fused to an activating

IL-7 cytoplasmic domain has been used to enhance persistence and effector function of CAR-Ts [140].

6. Immune Checkpoints to synergize with ACT

CTLA-4 and PD-1 are inhibitory protein receptors expressed by activated T cells, including CAR-Ts. They are categorized as negative regulators, or checkpoints, of T-cell activation, as they blunt T-cell-mediated anti-tumor activity. CTLA-4, a homolog of the T-cell co-stimulatory receptor CD28, dampens T-cell responses by out-competing CD28 for CD80 and CD86 ligands, and disrupting intracellular stimulatory signaling processes [141]. Most of the success with ipilimumab, a CTLA-4 antibody, has been with tumors carrying a high mutational and neo-antigen burden, such as melanoma [142]. PD-1 is also a homolog of both CTLA-4 and CD28; its binding to the B7 ligands PD-L1 and PD-L2, which many tumors constitutively express, causes reduction in T-cell metabolism and proliferation [143]. Analyses with melanoma in a murine model showed that production of proinflammatory cytokines from activated CD8+ T cells correlated with induced upregulation of PD-L1 [144]. There have been encouraging results with blockade of PD-L1 in patients with advanced stage cancers including in combination with TILs [145].

Preventing CAR-T-cell tolerance from such negative regulatory mechanisms has become a major driving point to boost effector function. There is substantial data demonstrating how these surface inhibitory markers play a critical role in modulating CAR-T-cell efficacy. In a mesothelioma mouse model, mesothelin.CAR-Ts showed an augmented expression of PD-1 at the tumor site, with resultant hypofunction. Their cytotoxicity was restored with the addition of a PD-L1 antibody in the presence of tumor cells ex vivo [117]. In mice bearing Her2+ tumors, there was a distinct advantage in tumor regression when combining Her2-CAR-Ts with PD-1 antibody [146]. However, clinical studies of combination therapy with CAR-Ts and immune checkpoint inhibitors have failed to recapitulate these promising preclinical results [147]. While combinations may prove effective only in certain tumor models, strategies to overcome the inhibitory pathway directly in T cells are being explored. An example is engineering CAR-Ts to secrete a PD-1-blocking scFv [148]. The secreted scFv improved anti-tumor activity of both CAR-Ts and bystander TILs. A more structural way of safeguarding CAR-Ts against the negative impact of immune checkpoint inhibition is the incorporation of a chimeric switch-receptor, containing the extracellular domain of PD-1 fused to the transmembrane and cytoplasmic domain of the costimulatory molecule CD28. This approach was incorporated into mesothelin.CAR-Ts and resulted in amplified tumor cytotoxicity, producing tumor regression in preclinical models of mesothelioma-bearing mice [149].

7. Conclusions and perspective

The first observation that the immune system plays a role in antitumor activity is attributed to Dr. Coley, who in 1890 linked immune responses to bacterial infections and subsequent remission of cancer in the same patients. It took almost a century to make ACT important tools in the therapy for cancer. TILs and CTLs first demonstrated the curative potential of ACT. The introduction of genetic engineering offered the opportunity to overcome the

shortcomings of TIL and CTL therapy, by conferring direct antigen specificity to T cells. The creation of CAR molecules marked a new beginning for innovative advances within ACTs, with CAR-Ts addressing some inherent weaknesses in the former two methodologies.

Although cellular therapy remains in relatively nascent stages of development as a therapeutic modality, we should be encouraged by the exponential advances the field of ACT has reached in the past two decades (Figure 3). Despite the prodigious amount of emerging and encouraging data from clinical studies with TILs, CTLs, TCR-T cells, and CAR-T-cells, the field of cellular therapy still has ample room for growth and improvement. As our understanding of cellular physiology, mechanisms, and processes advances, our ability to tackle roadblocks to ACT and augment their clinical efficacy with innovative approaches will also improve.

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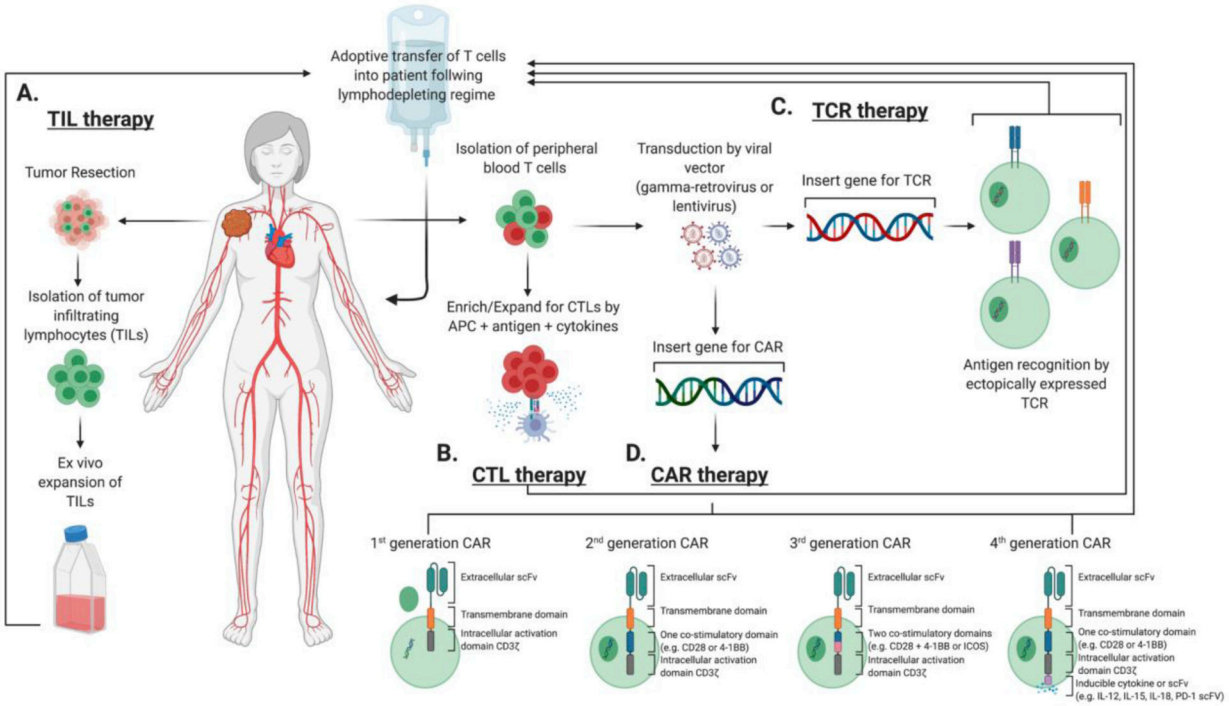

Figure 1.

Overview of the adoptive cell therapy (ACT) process for cancer treatment using tumor-infiltrating lymphocytes (TILs), cytotoxic T lymphocytes (CTL), or genetically modified TCR-T or CAR-T cells. **A.** For TIL ACT, tumors are resected, and tumor-reactive T cells are isolated and expanded ex vivo. **B.** CTL therapy relies on enriching and expanding peripheral blood T cells with known TAA specificity. **C & D.** ACT with TCR-T or CAR-T cells relies on the genetic modification of peripheral blood T cells by viral vectors to express a specific TCR or CAR. Patients are pretreated with a lymphodepletion regime before adoptive transfer of TILs, TCR-T, or CAR-T cells to create space and availability of homeostatic and activating cytokines. Figure created with Biorender.

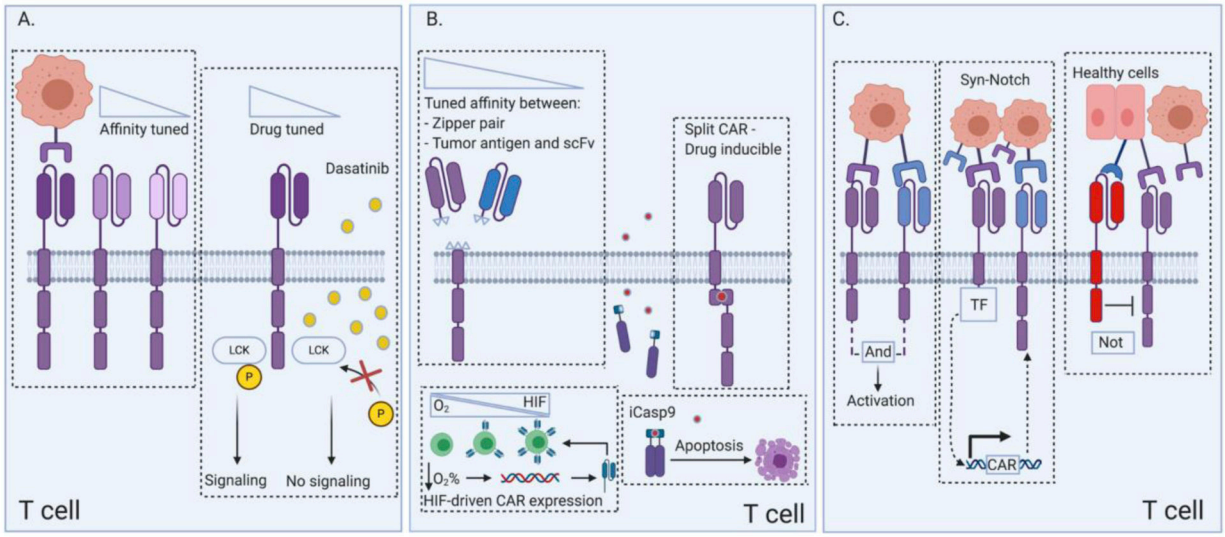

Figure 2.

Schematic representation of CAR T cell control mechanisms. **A**. Tuning the affinity of the scFv to tumor antigen and drug-induced inhibition of CAR T cell signaling by dasatinib. **B**. Split-CAR signaling by adaptors or drug-induced dimerization. HIF-driven CAR expression is regulated by hypoxic environment i.e. tumor microenvironment. The suicide gene iCasp9 can be activated by a dimerizable drug to kill CAR T cells. **C**. CAR T cells can have autonomous regulation in the killing of targets based on cell surface protein expression by cancerous and healthy cells. Figure created with Biorender.

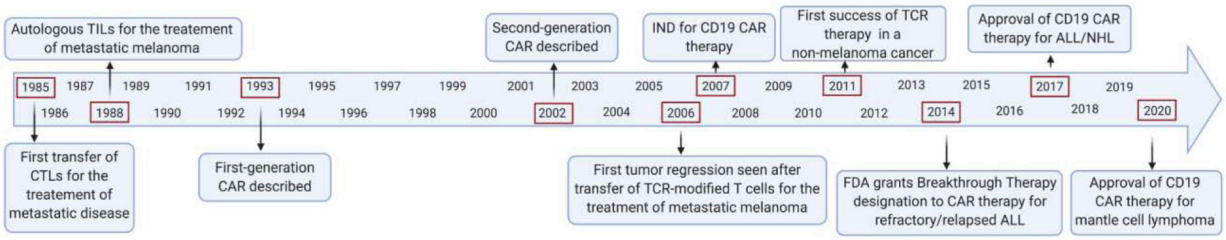

Figure 3.

History of adoptive cell therapy (ACT) in cancer. This timeline highlights important developments in the field of adoptive T-cell therapy. Figure created with Biorender.