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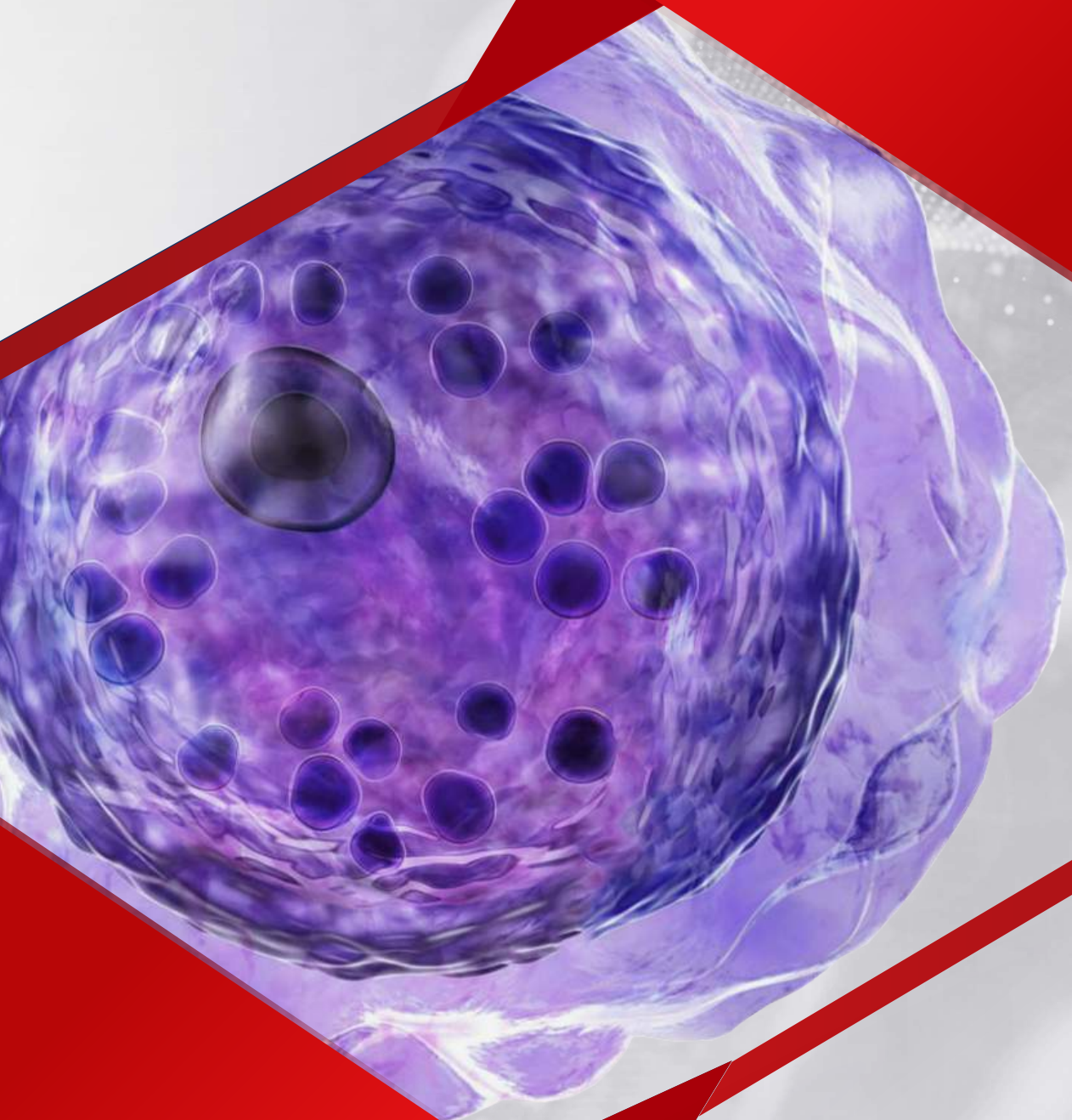

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FOREWORD

Cell and gene therapies offer hope for many as they are enhancing and saving the lives of people who often have no other treatment option. Cell therapy utilizes living cells from the patient or a donor to replace damaged or diseased cells, or to stimulate the body's immune response or regeneration.

Usually stem cells and immune cells are used, and a typical method involves removing the cells from the body, repairing or editing the error in the gene, and then returning the cells to the body. Natural killer (NK) cells are a type of immune cell that kill virally infected or malignant cells. Unlike T cells, NK cells function in an antigen independent manner, responding to anything they perceive as "non-self", including malignant cells. In addition, NK cells have a well-known safety profile. This makes them an attractive cell type for development of off-the-shelf immunotherapies.

For the clinical and commercial manufacture of cell therapies, closed systems are particularly important, as they provide the highest containment and product safety protection. In addition, using the same equipment from research through process development and manufacturing can cut process development times and delays by eliminating the need to learn and optimize new systems, while also permitting multiple batches to be processed in a shared clean room.

In this eBook, discover the latest updates in the field of cell therapies, including:

- An overview of current cell therapies, practices and future prospects
- Challenges and solutions in scaling up and manufacturing
- The additional difficulties in developing NK cell therapies and how these can be overcome

CONTENTS

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Facilitating the accessibility of lifesaving 'living drugs' for cancer patients: 60s with Yongchang Ji

Process Development Manager in the Cell and Gene Therapy team at Thermo Fisher Scientific (CA, USA), Yongchang Ji, speaks to BioTechniques about the current challenges for cell therapy manufacturing processes, how these can be overcome, as well as the additional unique challenges and solutions when developing natural killer (NK) cell therapies.

Ji is currently working on the development of closed, modular and semi-automatic workflow solutions for viral and non-viral cell therapy. Ji received his PhD in Cell Biology and Anatomy from SUNY Upstate Medical University (NY, USA) and his undergraduate degree from Xiamen University (China).

Yongchang Ji

Q) What are the challenges in current cell therapy manufacturing processes?

Most cell therapy manufacturing processes are manual and open, which means there is a risk of introducing contamination and creating batch-to-batch product variability. In addition, it is very hard to scale up from manual to larger processes.

Q) How might automated closed systems overcome these challenges?

Closed systems can significantly reduce the risk of contamination by viral, bacterial or other adventitious agents. Closed systems may be placed in a controlled but non-classified environment, improving manufacturing flexibility. Automation can improve operator safety, reduce human errors and enable processing robustness and reproducibility. Automated systems can simplify the operations overall.

Q) How can developers ensure system flexibility to allow for iterative process optimization?

One way is to create modularity in the system, where you can utilize some of the tools more efficiently. It could be within a process or across processes. Moreover, if a specific tool can be used in multiple processing steps, it will reduce personal training and equipment qualification efforts. On the scalability side, it will bring a lot of value if you can use one tool at an early stage of development for small scale processes, and later the same tool during the large-scale process.

Q) For allogeneic NK cell therapy, what are the challenges associated with commercial-scale production for therapeutic use?

There are many challenges for NK cell therapy.

Scalability is one of the main challenges facing the industry. Manufacturing high doses means significant expansion during the in vitro culture. Currently, most expansion cultures use feeder cell lines. The residuals from these feeder cells are major concerns for clinical applications. There is a need to develop a feeder-free NK culture media. Moreover, there is a regulatory incentive to replace human and animal components to generate human and animal-free media. Other challenges for NK cell therapy also include selecting the right cell source to get enough starting materials, designing culture containers for large-scale production, and so on.

Q) What unique requirements for culture and characterization do NK cells have?

In addition to the above, NK cells need to be expanded for an extended time period (usually >2 weeks) for sufficient clinical doses, which means ex vivo culture conditions need to be carefully controlled over time. That would include an optimal media formulation, the right amount and combination of certain cytokines (IL-2, IL-15, IL-21, etc.), as well as culture vessel control. Some cytokines, such as IL-15 or IL-21, can produce better expansion results if they are in a membrane-bound state. The membrane-bound state can be achieved by expressing cytokines on the feeder cells, or feeder-free NK cells themselves.

Q) How are you and your organization contributing in this area?

We have developed a closed, modular, semi-automated system for CAR-T cell therapy manufacturing. We have also developed a special medium to proliferate a high yield of functional human NK cells with or without the use of feeder cells. We hope these cell therapy solutions can facilitate the accessibility of lifesaving 'living drugs' for cancer and other disease patients.

Review

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Immunotherapy

Chimeric antigen receptor T-cell therapy beyond cancer: current practice and future prospects

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Adoptive cell therapy with chimeric antigen receptor T (CAR-T) cells has achieved remarkable efficacy in the treatment of hematological malignancies, which has inspired researchers to expand the application of CAR-T-cell therapy to other medical conditions. Here, we review the current understanding and development of CAR-T-cell therapy for infectious diseases, autoimmune diseases and allotransplantation. The limitations and challenges of CAR-T-cell therapy in the treatment of these diseases and potential solutions to overcome these shortcomings are also discussed. With the development of novel designs of CARs and preclinical/clinical investigations, CAR-T-cell therapy is expected to be a potential cure option in a wide array of disease settings in the future.

First draft submitted: 14 January 2020; Accepted for publication: 22 July 2020; Published online: 30 July 2020

Keywords: adoptive cell therapy • allotransplantation • autoantibody • autoimmune diseases • chimeric antigen receptor • infection • regulatory T cells

Adoptive T-cell immunotherapy, especially chimeric antigen receptor T (CAR-T)-cell therapy, has attracted increasing attention worldwide and emerged as a promising therapeutic approach for cancers [1–4]. CARs are versatile synthetic receptors, which comprise binding variable regions of a monoclonal antibody targeting the antigen, a hinge, a transmembrane region and costimulatory/activation domains of the T cell (such as signaling domains of CD3ζ, CD28 and/or CD137). CARs could be transduced into T cells via viral or nonviral methods (Figure 1). T cells genetically modified to express CAR activate upon binding to the target antigen without MHC restriction and specifically lyse tumor cells expressing target antigen [5]. Based on the remarkable success of CAR-T cells in the past decade, two CD19 CAR-T-cell products, Kymriah and Yescarta, were approved by the US FDA for the treatment of refractory or recurrent B-cell leukemia or lymphoma in 2017 [6–14]. In addition to hematological malignancies, CAR-T cells have also been applied to solid tumors and accumulating preclinical studies and clinical trials have demonstrated that CAR-T-cell therapy is likely to be a novel and promising treatment for solid tumors [15–19].

The success of CAR-T-cell therapy in cancers has sparked interest in broadening the spectrum of CAR-T-cell therapy beyond malignancies and applying this effective treatment to diverse medical conditions that profoundly decrease life expectancy, the quality of life and lack effective treatments [20–22]. In this review, we focus on the development, challenges and prospects of CAR-T-cell therapy in the treatment of a wide array of diseases beyond cancers.

CAR-T-cell therapy for infectious diseases

Infectious disease still represents a major menace worldwide. Despite the availability of an array of treatment options, the incidence rates and mortality rates of several infectious diseases remain high [23,24]. Moreover, the occurrence of drug resistance is another significant concern. T cells play a key role in the process and control of infectious diseases,

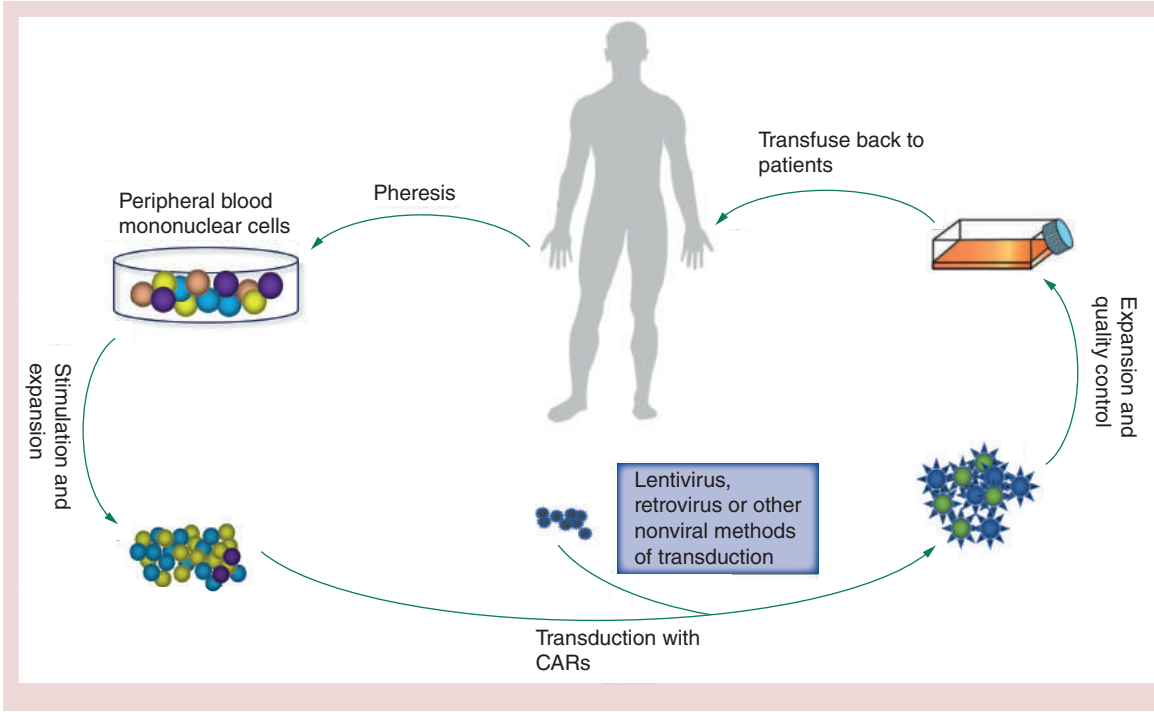

Figure 1. The process of CAR-T cell therapy.

which indicate the potential application of T-cell-based therapeutic approaches, such as CAR-T-cell therapy, in the prophylaxis and treatment of infectious diseases. In the past few years, a series of preclinical/clinical studies have been implemented to investigate the efficacy and safety of CAR-T cells in the treatment of infectious diseases.

CAR-T-cell therapy for HIV infection

AIDS represents a medical condition caused by infection with HIV. Since AIDS was identified in the early 1980s, the terrible disease has caused approximately 35 million deaths around the world [25]. HIV attacks the human immune system, including $CD4^+$ helper T cells, macrophages and dendritic cells [26–28]. If untreated, HIV leads to a low number of $CD4^+$ T cells in the body, which could make the body more susceptible to opportunistic infections or infection-related cancers [29–34]. Currently, antiretroviral therapy (ART) represents the standard treatment practice for AIDS [35]. If treated adequately, the plasma HIV viral load could be potently reduced or even could become undetectable [36,37]. Despite success, emerging evidence shows that cryptic viral replication still exists, immune dysfunction often persists during treatment and HIV could emerge after years of undetectable viremia [38,39]. In addition, the toxic effects of ART are also regarded to increase the risk of non-AIDS deaths [40,41]. Thus, the effect of the current therapeutic approaches is far from satisfactory and potent curative treatment is in pressing need.

In the past few years, the efficacy and safety of CAR-T cells in the treatment of HIV infection have been investigated and the most popular design is fused CD4 to the CD3ζ chain, which is also called CD4ζCAR [42]. Consistent with studies for solid tumors, first-generation CD4ζCAR-T cells failed to durably reduce the viral burden despite their potent antiviral effect [43–45]. Thus, in an effort to improve the persistence and enhance the anti-HIV activity of CAR-T cells, the design of CARs has been re-engineered and costimulatory signaling domains, such as those from CD28 and 4-1BB (CD137), have been added to the structure of CARs, which are called second- or third-generation CARs [46–51]. Compared with first-generation CAR-T cells, the addition of costimulatory signaling domains could enhance T-cell function, persistence and susceptibility to exhaustion [52–54]. Recently, novel designs of CARs, such as bispecific or multispecific CARs, were also developed. The bispecific CARs fused a CD4 segment to an single-chain variable fragment (scFv) or the carbohydrate recognition domain of a human C-type lectin, which could bind the CD4 fragment to the gp120 and bind to conserved glycans on Env in the case of the carbohydrate recognition domain, which could significantly improve the specificity and antiviral effect of CAR-T cells [55,56]. In a recent study, trispecific CAR targeting three putative sites on the Env trimer, including the gp120 CD4-binding

site, gp120 coreceptor-binding site and gp41, was also demonstrated to be an effective approach for the treatment of patients with HIV-1 infection [57]. Despite the great success achieved in the treatment of HIV infection by CAR-T-cell therapy, several challenges still exist, which could weaken the benefits of CAR-T-cell therapy and limit its wide application. One challenge is that in some HIV-infected patients, the quantity of virus-infected cells is substantially lower after ART. Due to the lack of antigen, CAR-T cells should be infused several times to augment the persistence of CAR-T cells after infusion, which could ensure sufficient reaction when the virus reappears from latently infected cells. Thus, in addition to constructing new designs of CARs, another approach to enhance CAR-T-cell function, persistence, is the design of hematopoietic stem and/or progenitor cell (HSPC)-based CAR-T cells. Modified with HIV-specific CARs, HSPCs could provide consistent production and long-term maintenance of CAR-T cells due to their inherent ability to self-renew and produce mature, multilineage immune cells [58–60]. After HIV challenge, CD4ζ CAR-T cells in the HSPC group retained antiviral function and proliferation. In addition, these cells could suppress HIV replication to a great extent [61,62]. Recently, the antiviral function of HSPC-based CAR-T cells was also evaluated in a nonhuman primate model of HIV infection [63]. Consistent with the results of the mouse models, CD4ζ CAR-modified HSPCs could differentiate into a series of hematopoietic lineages [64]. Although HSPC-based CAR-T cells did not achieve durable virus control, the method was regarded a safe gene therapy in a preclinical animal model. In addition, the therapy resulted in a stable production of antiviral cells for nearly 2 years, which indicated that HSPC-based CAR therapy might be a potential method to overcome the poor persistence associated with peripheral-based effector CAR-T-cell products. The second challenge is how to make CAR-T cells resistant to HIV infection. Although CAR-T cells mediate functional HIV control, they are themselves targets of HIV and the persistence and function of CAR-T cells will be destroyed if infected by HIV. To protect the persistence and function of CAR-T cells, it is crucial to protect CAR-T cells resistant to HIV infection [65]. Several approaches, including direct genetic manipulation of CAR-T cells, the redirection of HIV-1-specific immune responses or a combination of the two strategies, could prevent viral entry into CAR-T cells. Preventing viral entry could block virus propagation and the integration of the virus genes into the host genome, which could significantly reduce the latent cells. Recently, several studies have been carried out to abrogate HIV entry, of which targeting CCR5 is of great value. In a study, Hale *et al.* blocked CCR5 with a site-directed mega TAL nuclease and drove homology-directed recombination (HDR) using an adeno-associated virus donor template encoding an scFv-based CAR. HIV-specific CAR-T cells lacking CCR5 expression had better antiviral effects than CAR-T cells without protection from HIV infection [54]. In other studies, targeting CCR5 with shRNAs also downregulated the expression of CCR5 in CAR-T cells, which could also prevent HIV entry [66,67]. Furthermore, other novel strategies in efforts to inhibit HIV-1 replication and highlight the requirements for CAR-T-cell therapy have also been developed [63,68].

CAR-T-cell therapy for other infectious diseases

In addition to HIV infection, CAR-T-cell therapy has also achieved potent efficacy in its application to other types of infectious diseases. Human cytomegalovirus (CMV) is a DNA virus that can be transmitted through contact with CMV-infected fluids. Currently, the main treatments for CMV infection are ganciclovir, foscarnet and cidofovir; however, these drugs always have severe side effects and fungal infections [69]. In a study, Full *et al.* engineered T cells with a CAR targeting CMV glycoprotein B (gB), which is expressed by infected cells early in the replication cycle. These CAR-T cells were demonstrated to mediate antiviral effector functions such as cytokine production and cytolysis in an HLA-independent manner, indicating that CAR-T-cell therapy might be a potential alternative therapeutic option for CMV infection [70]. In another study, the long spacer (CH_2–CH_3 Fc domain from IgG1) was demonstrated to act as a receptor for CMV-infected cells, which indicated human CMV-encoded FcRs as an attractive target for CMV immunotherapy by CARs and bispecific antibodies [71].

Current treatments for hepatitis B virus (HBV) infection target the virus reverse transcriptase; however, these treatments rarely inhibit HBV replication completely as the HBV replication template, covalently closed circular DNA (cccDNA), persists in the host cell nucleus [72]. Recently, some preclinical studies have investigated the efficacy of CAR-T cells in the treatment of chronic hepatitis B. Bohne *et al.* designed CAR-T cells targeting HBV surface proteins S or L on HBV-infected cells. The results showed that these CAR-T cells could specifically recognize HBsAg-positive hepatocytes, release IFN-γ and IL-2 and lyse HBV-replicating cells [73]. In another study, Krebs *et al.* constructed CAR-T cells that target HBV envelope proteins (S-CAR); these CAR-T cells could recognize different HBV subtypes and localize to the liver in mice to reduce HBV replication, causing only transient liver damage, indicating that CAR-T-cell therapy is an effective and safe treatment for HBV infection [74]. Most recently,

novel CARs targeting HBsAg were engineered. HBsAg CAR-modified T cells could release significant amounts of IFN-γ, IL-2 and TNF-α when activated by HBV-positive cell lines and HBsAg particles. In a humanized HBV-infected mouse model, HBsAg CAR-T cells accumulated in the liver and significantly reduced HBsAg and HBV-DNA levels in plasma [75].

Fungal opportunistic infection is currently one of the most severe issues. The currently available antifungal drugs are usually associated with severe toxicity; thus, novel strategies should be proposed [76]. Dectin-1 is a type II transmembrane protein expressed on macrophages, neutrophils, which mediates recognition of *Aspergillus fumigatus*. Kumaresan *et al.* developed an approach to redirect the specificity of T cells by the modification of a novel CAR that incorporates the pattern-recognition ability of Dectin-1 using the nonviral *Sleeping Beauty* gene-transfer system. These genetically modified T cells express CARs with specificity for carbohydrates and effectively inhibit their hyphal growth, suggesting the potential application of CAR-T cells in the treatment of fungal infectious diseases [77].

In addition to viral infections and opportunistic fungal infections, bacterial infections represent another key field. In recent decades, the incidence and mortality of bacterial infections have been well controlled due to the emergence of various new antibiotics. However, there has been a dramatic rise in the numbers of patients with multidrug-resistant infections, which brings great challenge to the treatment of bacterial infections. In this setting, CAR-T-cell therapy may provide an attractive strategy to solve the current predicament of these medical conditions, especially for chronic infections, such as drug-resistant tuberculosis. Recently, adoptive transfer of T cells has been developed for the treatment of a series of bacterial infections; however, there is no research on CAR-T-cell therapy in bacterial infection. As distinct target antigens could be identified, it is possible that CAR-T cells for bacterial infection may also be developed, which could solve the dilemma of multidrug-resistant bacterial infection.

CAR-T cells for autoimmune diseases

Autoimmune diseases are conditions arising from abnormal immune attack to the body, and they substantially increase the morbidity, mortality and healthcare costs worldwide. It is estimated that more than 50 million people are living with autoimmune diseases [78]. To date, at least 80 types of autoimmune diseases are recognized, and almost all parts of our body could be involved. Despite various manifestations, all autoimmune diseases are thought to result from an imbalance in the immune system [79,80]. Normally, the function of the immune system is to recognize and react to foreign pathogens but not host tissues, which is known as immunological tolerance. In the process of lymphocyte development, most lymphocytes specific to self-antigens are eliminated before maturation. The remaining self-reactive T cells in healthy individuals could be counterbalanced by regulatory mechanisms (such as Tregs) [81]. Thus, autoimmune diseases occur not only by aberrant activation of self-reactive cells but also by defects in regulatory mechanisms.

Traditional therapies for autoimmune diseases are immunosuppressive agents, which could broadly inhibit the immune response. Although it has high efficacy and is regarded as the 'gold standard' of treatment for autoimmune diseases, several problems arise in long-term treatments. First, doses of these agents need to be elevated to maintain disease control. Second, patients receiving long-term treatment with these agents are often prone to life-threatening opportunistic infections and have a high risk of malignancies. Third, these agents always have severe toxicity and serious side effects, which counteract their benefits [82,83]. Recently, several specific strategies that could lower the risk of systemic immune suppression and improve tolerability have been developed [84–86]. As T cells play a key role in the process of autoimmune diseases, engineered T-cell therapy has emerged and is also regarded as a potential approach to overcome current roadblocks in the treatment of autoimmune diseases (Figure 2).

Chimeric autoantibody receptor T cells

Either self-reactive or autoantibodies play a key role in the process of autoimmune diseases [87,88]. Thus, engineering T cells to express a chimeric autoantibody receptor (CAAR) is probably a promising approach for the treatment of autoimmune diseases. Pemphigus vulgaris (PV) represents a life-threatening autoimmune disease caused by autoantibodies targeting the keratinocyte adhesion protein Dsg3 [89]. CD20-targeted B-cell depletion can induce short-term remission, however, most patients will relapse, and Dsg3-reactive B-cell clones play a key role in the relapse of the disease [90,91]. In a recent study, Ellebrecht *et al.* engineered T cells to express the PV autoantigen Dsg3, which fused to second-generation 4-1-BB-CD3ζ signaling domains. Dsg3 CAAR-T cells exhibited specific cytotoxicity against cells expressing anti-Dsg3 BCRs *in vitro*, decreasing Dsg3 autoantibodies and inducing the lysis of pathogenic B cells [92]. In addition, these cells could expand, persist and specifically eliminate Dsg3-specific B

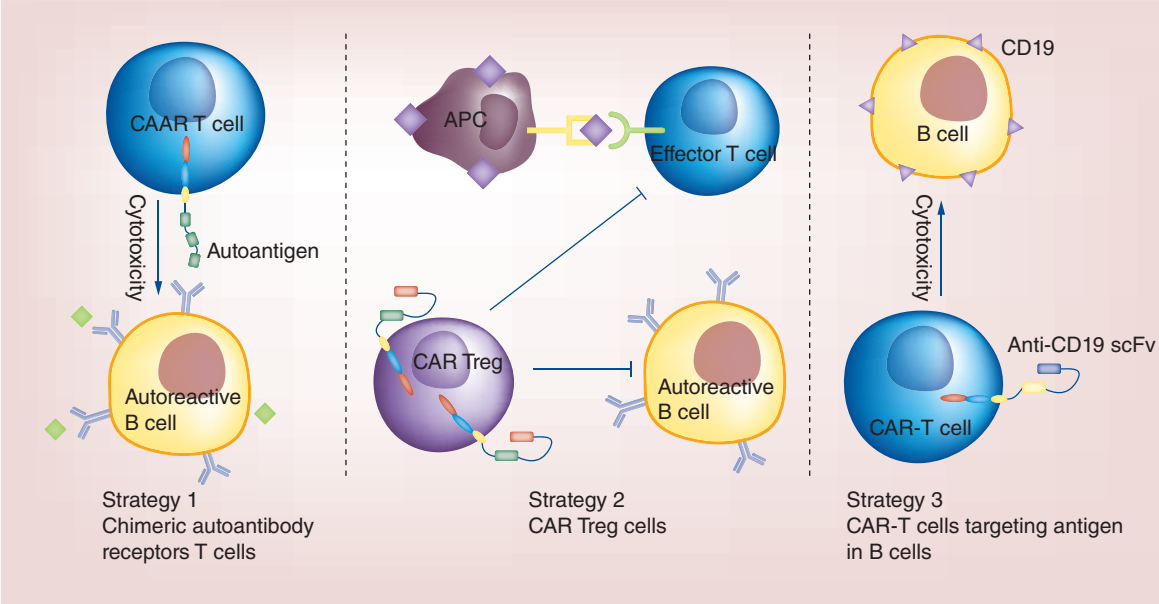

Figure 2. Strategies for CAR-T cell therapy in the treatment of autoimmune diseases.

cells *in vivo*, indicating that CAAR-T cells might be an effective strategy for autoantibody-mediated autoimmune diseases.

Although CAAR-T cells are likely to be a promising method and can be translated to several human autoimmune diseases, several key issues should be addressed. First, what is the effect of soluble antibodies on the function of CAAR-T cells? As the effect of soluble antibodies on CAAR-T cells is affected by the relative position of the epitope in the CAAR molecule, as well as the affinity and binding kinetics of the antibody, we should test different lengths for the extracellular portion to gain the optimal length to function as effective bait and could function even in the presence of soluble antibodies. Second, although CAAR-T cells have potent effect in the treatment for PV, the application of the therapy is limited. CAAR-T cells could be applied to autoimmune diseases with at least two essential prerequisites: autoantibodies play a key role in the development of the disease and the pathogenicity of autoantibodies have been clearly established; and the sequence and molecular structure of the autoantigens must be clearly identified. Currently, only a few autoimmune diseases could fulfill these prerequisites. Thus, it is essential to investigate the mechanisms of autoimmune diseases in which B cells contribute substantially to promote the development of new therapies based on CAARs. Third, the toxicity of CAAR-T cells should also be concerned about, especially when infusing activated T cells from a patient with autoimmunity back into the patients. Thus, for the sake of safety, the CAAR T cells should probably be equipped with an inducible suicide gene.

CAR Tregs

As mentioned above, Tregs also play a critical role in the maintenance of immune balance, and defects in immune tolerance are another key mechanism of autoimmune diseases. Thus, adoptive cell therapy with Tregs for the treatment of autoimmune diseases has emerged. In 1995, Sakaguchi *et al.* initiated the first Treg therapy in nude mice, and the onset of autoimmune diseases was hindered by the infusion of Treg cells [93]. Since then, a series of studies and clinical trials have been implemented to investigate the potential effect of Treg therapy in a variety of diseases, such as rheumatoid arthritis (RA) [94], multiple sclerosis (MS) [95], colitis [96], lupus erythematosus [97] and Type 1 diabetes (T1D) [98]. Despite being potentially efficacious in some conditions, including T1D, in the context of several diseases, such as graft-versus-host disease (GVHD) after bone marrow transplantation or lupus erythematosus, the efficacy of these cells is questioned, which highlights the need for potent Tregs to yield a substantial clinical advantage [99–102]. One promising way to achieve this is to use antigen-specific Tregs, and the introduction of CARs to Tregs might be a potential option to improve the efficacy of Treg therapy in autoimmune diseases. In recent years, CAR-Treg cells have been developed rapidly and become one of the most useful methods in the treatment of autoimmune diseases. In a pioneering study, Elinav *et al.* developed CAR Treg cells-expressing

chimeric receptors specific for a predetermined model antigen, 2,4,6-trinitrophenol [103]. The results of the study showed that Tregs expressing CARs could proliferate in an antigen-specific manner and accumulate at the target organ. These cells could effectively prevent or ameliorate 2,4,6-trinitrobenzenesulphonic acid (TNBS)-induced colitis, while polyclonal Treg cells had no effect. In addition to colitis, CAR Treg cells have also been demonstrated to be effective in experimental autoimmune encephalomyelitis (EAE). EAE represents an animal model of MS, which is mediated by the activation of myelin-specific T cells that infiltrate the CNS and initiate an inflammatory process. In a recent study, myelin oligodendrocyte glycoprotein (MOG)-specific CAR Treg cells were developed to treat EAE [104]. Specifically, murine FoxP3 was coexpressed with CAR, which could drive naive $CD4^+$ T-cell differentiation to Tregs. After infusion, MOG-CAR Tregs efficiently homed to the brain and suppressed ongoing encephalomyelitis. On the other hand, MOG-CAR Tregs sustained their effects, as they could protect against a second EAE challenge. Encouraged by the success of the study, several studies have also investigated the ability of CAR Treg cells in the treatment of a series of other autoimmune diseases, and they produced remarkable effects [105,106].

Despite the success achieved, disadvantages or limitations also exist in the wide application of CAR-Tregs. 'Cytokine storm' and neuronal cytotoxicity are key side effects in the treatments with antitumor CAR-T cells; however, it is not clear whether CAR-Tregs would also induce these adverse events. Furthermore, to develop effective CAR Tregs, selecting the ideal antigen target for CAR is critical. Similar to effector CAR-T cells, the ideal antigen targets are those antigens highly expressed on the target cell surface but not on the other cells or tissues. However, selecting the ideal targets for CAR Tregs is difficult, and off-target effects are one of the most noticeable problems in Tregs applications. Fortunately, the effect of off-target Treg cells seems to be less severe based on the results of several studies. Finally, the exhaustion of CAR-Tregs is another limitation in the development of CAR-Tregs. To overcome this challenge, second-generation CARs with CD28 or CD137 costimulatory domains were fused to CARs to expand the effect of CAR Tregs [48,107,108].

Novel strategies

As mentioned above, B cells are the major effector cells in autoimmunity. B cells can produce autoantibodies, contribute to antigen presentation to self-reactive T cells and secrete proinflammatory cytokines [87,88]. Thus, the inactivation or depletion of B cells might be an attractive and promising option for the treatment of autoimmune diseases [109–111]. Previously, B-cell depletion with rituximab (a monoclonal antibody against the B-cell surface marker CD20) has shown therapeutic promise in several autoimmune diseases, such as RA and MS [112,113]. However, a series of issues arise during treatment with rituximab. First, monoclonal antibodies follow drug-like pharmacokinetics with exponential decay; thus, repeated administrations are required to maintain their therapeutic dose [114]. Second, resistance to rituximab arises in the process of administration. Thus, the depletion of B cells with cell-based therapies might provide further advantages over antibodies [115]. In a recent study, Kansal *et al.* erected CAR-T cells targeting CD19 to deplete B cells in lupus models. $CD8^+$ T cells expressing CD19-targeted CARs could persistently deplete $CD19^+$ B cells completely, which significantly ameliorated the disease manifestations in target organs and extended life spans. Moreover, CAR-T cells could be detected and active for 1 year *in vivo*, indicating that the depletion of B cells with CD19-targeted CAR T cells is a stable and effective strategy for the treatment of lupus [116].

Although the depletion of B cells showed attractive effects for the treatment of autoimmune diseases, a series of challenges remain. During the process of the differentiation of B cells to plasma cells, CD19 expression is thought to decline. Several evidences have suggested that bone marrow-derived plasma cells include $CD19^+$ and CD19 subsets and that long-lived humoral immunity depends on CD19-plasma cells. Recently, Bhoj *et al.* investigated the mechanisms underlying the maintenance of long-lasting humoral immunity following CD19 CAR-T-cell treatment [117]. These results show that CD19 CAR-T cells could effectively eliminate both B cells and $CD19^+$ plasma cells, but they spare a CD19 population of plasma cells, are likely long lived given the absence of B cells to replenish this pool and most likely contribute to the stable serum antibody titers observed in patients with CD19 CAR-T-cell-induced B-cell aplasia. Thus, plasma cell targeting is likely to be another potential method to completely eliminate the source of autoantibodies in the future.

CAR-T cells for allotransplantation

Cell and organ transplantations are currently used in several disease conditions; however, immune rejection is a formidable barrier to the success of transplantation [118–122]. Thus, immunosuppressive medications should be

used after transplantation to protect transplanted cells and organs from graft rejection. Although these reagents could significantly reduce the incidence of graft rejection, they could also increase nonspecific immunosuppression, which related morbidity and mortality in the long term [123]. Previous studies have demonstrated that Tregs play a critical role in suppressing autoimmunity and alloimmunity, which inspired researchers to explore new thinking in suppressing immune rejection [124]. However, *in vitro* enrichment of alloreactive T cells by stimulation with allogeneic antigen-presenting cells (APCs) has several limitations. The frequency of alloantigen-specific cells decreases with increasing MHC matching, and cells generated by this method may suffer from limitations of cell numbers. Moreover, this method requires sufficient APCs from the donor or recipient in the case of hematopoietic stem cell transplantation (HSCT), which are not always available. Recently, emerging evidence has suggested that CAR-T-cell therapy based on Tregs could overcome all of these limitations and might be a promising approach to induce graft tolerance and improve long-term graft outcomes. MHC class I molecules are constitutively expressed on almost all transplanted cells and human leukocyte antigen (HLA)-A mismatching is often associated with poor outcomes after transplantation, which indicates that HLA-A might be a potential target antigen for designing CAR Tregs [125]. In a study, MacDonald *et al.* described the design and function of an HLA-A2-specific CAR (also called 'A2-CAR'). Both *in vitro* and *in vivo*, A2-CAR Tregs could maintain phenotype and show superior suppressive function to Tregs expressing an irrelevant CAR, which suggested that use of HLA-A2 CAR-modified Tregs might be a potent therapeutic option for graft rejection [126]. In another similar study, two HLA-A2-specific CARs, one comprising a CD28-CD3ζ signaling domain (CAR) and the other lacking an intracellular signaling domain (ΔCAR), were engineered. Compared with polyclonal Tregs, CAR Tregs could be specifically activated and showed significantly suppressive function than polyclonal or ΔCAR Tregs in the presence of HLA-A2 [127]. In general, CAR Tregs targeting HLA-A2 can provide several advantages: first, CAR Tregs could suppress alloimmune responses more effectively than polyclonal Tregs; second, CAR Tregs were activated in an MHC class II-independent manner; third, CAR Tregs targeting HLA-A2 could avoid the risk of panimmunosuppression and provide a tailored therapy. In addition to CAR Tregs targeting HLA-A2, other novel designs of CAR Treg cells have been developed and applied to the treatment for graft rejection. Recently, Treg cells modified with fluorescein isothiocyanate (FITC)-targeted CAR, which could be activated by monoclonal antibodies (mAbs) covalently conjugated to FITC, were designed. Adoptive transfer of MAdCAM1-mAbCAR permitted Treg targeting to specific tissue sites and mitigated allograft responses, which effectively prevented GVHD. In addition, mAbCAR Tregs targeted to MHC class I proteins on allografts, also significantly prolonged islet allograft survival [128]. All these studies suggested that CAR-T-cell therapy might be a promising approach for allotransplantation rejection.

Conclusion & future perspective

In recent years, CAR-T-cell therapy has emerged as a promising approach for the treatment of a series of malignant tumors. The tremendous success of CAR-T cells in malignancies inspired interest in applying CAR-T cells to other disease conditions beyond cancer. As mentioned above, CAR-T-cell therapy has been developed rapidly for the treatment of infectious diseases, autoimmune diseases and allograft rejection. Despite the success achieved by CAR-T-cell therapy, this promising therapy still faces several issues. First, more specific antigens should be evaluated in future studies. Second, to overcome drug resistance, novel designs of CARs and combinations with other drugs should also be explored. Third, animal models currently used are not suitable enough to judge the efficacy of CAR-T-cell therapy, and more suitable models, which could reflect the whole course of these diseases and the effect of CAR-T cells, are in pressing need. Thus, more work to improve the efficacy and safety of CAR-T-cell therapy is required.

Currently, most of the CAR-T cells are individualized and produced from autologous T cells. Although these CAR-T cells have achieved great success, there are several disadvantages or limitations. First, the custom pattern of CAR-T-cell production is a significant limiting factor for the large-scale clinical application as the CAR-T-cell product is individualized and therefore varies from patient to patient. Second, current individualized CAR-T-cell therapy is not a ready-to-use therapeutic agents and the manufacturing process is costly and time-consuming. Third, it is not always feasible to generate sufficient number of autologous T cells in all cases, particularly for infants or highly treated patients who are profoundly lymphopenic. Recently, several novel types of CAR-T cells, especially universal allogeneic T cells, have been developed. Universal allogeneic T cells could be produced by genetically disrupting the *TCR* gene and/or HLA class I loci of the allogeneic T cells using genome-editing technologies including ZFN (zinc finger nuclease), TALEN (transcription activator-like effector nuclease) and CRISPR-Cas9 [129–131]. These T cells could effectively abolish GVHD and can be widely used to generate 'off-the-

Table 1. Currently registered clinical trials using chimeric antigen receptor T cells for the treatment of infection, autoimmune diseases and allograft rejection.

Disease	Center	Study title	Phase	Clinicaltrials.gov identifier
HIV/AIDS	Guangzhou 8th People's Hospital, Guangzhou, Guangdong, China	The effect of CAR-T-cell therapy on the reconstitution of HIV-specific immune function	I	NCT03240328
SLE	Shanghai Jiaotong University School of Medicine, Renji Hospital Shanghai, China	A study of CD19 redirected autologous T cells for CD19-positive SLE	I	NCT03030976
HIV/AIDS	University of Pennsylvania Philadelphia, Pennsylvania, USA	CD4 CAR+ ZFN-modified T cells in HIV therapy	I	NCT03617198
POMES syndrome	Shanghai Changzheng Hospital, Shanghai, China	Anti-CD19/BCMA bispecific CAR-T-cell therapy for R/R POMES	I	NCT03879382
HIV/AIDS	Guangzhou 8th People's Hospital, Guangzhou, Guangdong, China	Effect of chidamide combined with CAT-T or TCR-T-cell therapy on HIV-1 latent reservoir	I	NCT03980691
MG	University of California Irvine, Irvine, California, United States University of Miami, Miami, Florida, USA	Descartes-08 CAR-T cells in generalized MG	Ib/IIa	NCT04146051

CAR: Chimeric antigen receptor; MG: Myasthenia gravis; SLE: Systemic lupus erythematosus.

shelf' CAR T cells. With the development of universal allogeneic CAR-T cells, and genome-editing technologies to eliminate the endogenous immunogenicity, CAR-T cells could be generated using donor-derived or third-party T cells, which can expand the clinical application and improve the safety of CAR-T-cell therapy.

So far, a series of CAR-T-cell products, including Kymriah and Yescarta, have been approved for the treatment of refractory or recurrent hematological malignancies. Meanwhile, hundreds of clinical trials using CAR-T cells to treat tumors are also underway. However, for diseases beyond cancers, CAR-T cells are still in the preclinical and early clinical stages, and there are only a few registered clinical studies at present (Table 1). Thus, there is still a long way before we can apply CAR-T cells to clinical treatment in diseases beyond cancer. Fortunately, with the development of CAR-T technology and the acceleration of clinical research approval, more and more clinical trials using CAR-T cells to treat infectious diseases, autoimmune diseases and immune rejection will be conducted, thus promoting the rapid application of the promising therapy to the clinical settings.

The current review focuses on the application of CAR-T-cell therapy to the treatment of infection, autoimmune diseases and allograft rejection. In addition to these life-threatening diseases, chronic, nonlife-threatening diseases represent another key field that affect the health and quality of life, which inspires us to consider the application of CAR-T-cell therapy to these diseases. To identify suitable diseases, several factors should be considered: Although CAR-T-cell therapy represents a medical and scientific breakthrough, the price of CAR-T-cell therapy is very high [132]. If one disease is considered as candidate for CAR-T-cell therapy, the disease must cause great harm to human health and society, and lack of effective treatment. Distinct target antigens should be identified, which indicate that it is possible to develop CAR-T cells direct to the target antigens. T cells should play a key role in the process of the disease, suggesting the potential application of T-cell-based therapeutic approaches in the treatment of the disease.

In summary, CAR-T-cell therapy has shown potential efficacy in the treatment of diverse medical conditions beyond cancer, including infectious diseases, autoimmune diseases and allograft rejection. Although a series of important issues remain to be answered before clinical application of this technique, CAR-T-cell therapy is poised to provide an alternative treatment paradigm for a variety of diseases still awaiting effective treatment options.

Author contributions

PF Zhang and Q Li were responsible for the conception, literature collection, draft writing and editing. D Xie was responsible for literature collection, draft writing and editing.

Financial & competing interests disclosure

This work was supported by the National Natural and Scientific Foundation of China (no. 81572988) and Science & Technology Department of Sichuan Province Funding Project (nos. 2016FZ0108, 2018SZ0117). The authors have no other 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 apart from those disclosed.

English editing services were provided by American Journal Experts (AJE).

Executive summary

CAR-T-cell therapy for infectious diseases

- T cells play a key role in the process and control of infectious diseases, which indicate the potential application of CAR-T-cell therapy in the prophylaxis and treatment of infectious diseases.
- In the past few years, a series of the design of CAR-T cells were developed and demonstrated to be an effective approach for the treatment of patients with HIV-1 infection.
- Besides HIV infection, CAR-T-cell therapy also achieved potent efficacy in the application to other types of infectious diseases, including human cytomegalovirus infection, hepatitis B virus infection and fungal opportunistic infection.

CAR-T cells for autoimmune diseases

- Autoimmune diseases are thought to be results from imbalance of the immune system and engineered T-cell therapies might be a potential approach to restore the homeostasis of the immune system.
- Chimeric autoantibody receptor T cell is probably a promising approach for the treatment of autoimmune diseases, in which self-reactive or autoantibodies play a key role.
- CAR-Tregs cell therapy has developed more rapidly and was applied to a wide array of autoimmune diseases for their potential of restoration of self-tolerance.
- Inactivation or depletion of B cells might be another attractive and promising option for the treatment of autoimmune diseases.

CAR-T cells for allotransplantation

- Tregs play a critical role in suppressing autoimmunity and alloimmunity, which indicates Tregs as a novel therapy to overcome the immune rejection after transplantation.
- Emerging evidences suggested that CAR-T-cell therapy based on Tregs might be a promising approach to induce graft tolerance and improve long-term graft outcomes.

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Immunotherapy

Engineering chimeric antigen receptor-natural killer cells for cancer immunotherapy

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Adoptive cell transfer has attracted considerable attention as a treatment for cancer. The success of chimeric antigen receptor (CAR)-engineered T (CAR-T) cells for the treatment of haematologic tumors has demonstrated the potential of CAR. In this review, we describe the current CAR-engineered natural killer (CAR-NK) cell construction strategies, including the design principles and structural characteristics of the extracellular, transmembrane and intracellular regions of the CAR structure. In addition, we review different cellular carriers used to develop CAR-NK cells, highlighting existing problems and challenges. We further discuss possible ways to optimize CAR from the perspective of the tumor microenvironment to harness the strength of CAR-NK cells and provided rationales to combine CAR-NK cells with other treatment regimens to enhance antitumor effects.

First draft submitted: 9 August 2019; Accepted for publication: 27 April 2020; Published online: 21 May 2020

Keywords: adoptive cell therapy • CAR-NK cells • chimeric antigen receptor (CAR) • natural killer cells • tumor immunotherapy

A chimeric antigen receptor (CAR), originally proposed by Gross *et al.* [1] is a structure that is used to reconstruct autogeneic T cells, generating CAR-T cells that can be successfully used for the treatment of patients with hematological tumors, such as acute lymphoblastic leukemia (ALL) [2,3], chronic lymphocytic leukemia (CLL) [4,5], Hodgkin's lymphoma and non-Hodgkin's lymphoma, and other B lymphomas [6,7]. Although CAR-T have demonstrated great efficacy against these hematological tumors, they also have many limitations. For example, CAR-T therapy requires pretreatment of the patient, primarily through chemotherapy and irradiation [8], and the quality of the pretreatment directly affects the efficacy. After infusion of CAR-T, patients are prone to adverse reactions, such as the cytokine storm and graft-versus-host disease (GVHD), both of which can be life threatening in severe cases [9,10]. In addition, the construction of CAR-T requires the collection of autologous T cells from the patient's own peripheral blood (PB), thus limiting their broader clinical application. Some heavily treated patients are not even suitable for this produce because of immune toxicity caused by intensive chemotherapy or radiation. Above all, the major challenge is that the outcome of CAR-T treatment of solid tumors is not satisfactory.

Therefore, researchers have been motivated to modify innate immune cells, especially natural killer (NK) cells, to develop CAR-engineered NK cells (CAR-NK) for cancer immunotherapy. The most significant promise of CAR-NK cells that is not provided by CAR-T is that they might provide an over-the-counter cellular immunotherapy product and improve the survival rate of patients with refractory solid tumors [11]. Because the killing of NK cells is not dependent on matching human leukocyte antigen (HLA), allogeneic CAR-NK cells can be generated from different sources, such as PB, umbilical cord blood cells (CB), induced pluripotent stem cells (iPSCs) and even NK cell lines, thus having broad applications for different malignancies. The primary mechanism by which tumor cells escape CAR-T-cell therapy is the loss of the targeted antigen. Although the CAR-restricted killing is shared by both CAR-T and CAR-NK cells, allogeneic CAR-NK cells may be less susceptible to this inhibitory mechanism because they can kill target cells through mechanisms other than CAR [12–14], such as various specific natural cytotoxicity receptors (NCRs), including NKG2D, NKP30, NKP44 and NKP46 [15], as well as through antibody-dependent

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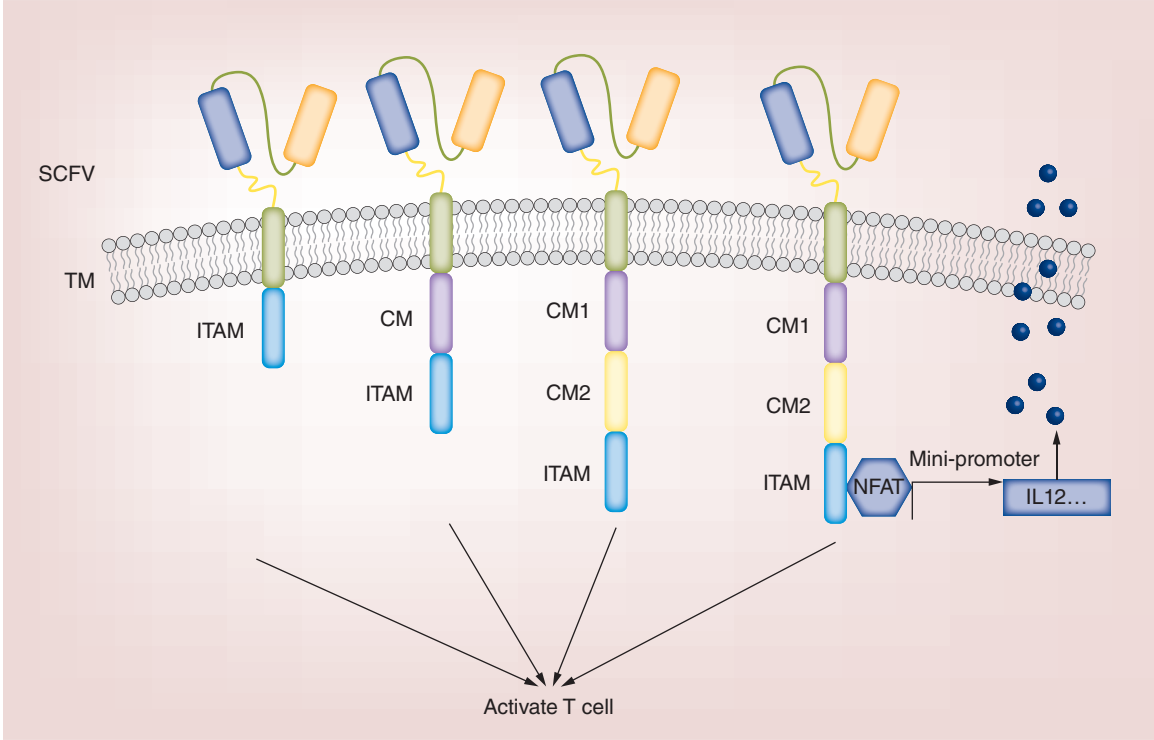

Figure 1. Structures of first- to fourth-generation chimeric antigen receptors.
CM: Costimulatory molecule; ITAM: Immunoreceptor tyrosine-based activation motif; SCFV: Single-chain variable fragment; TM: Transmembrane.

cellular cytotoxicity (ADCC). Notably, allogeneic CAR-NK cells do not match the patient's *HLA*, and thus, they can escape interference by inhibitory signals mediated by the 'self' HLA molecules expressed on tumor cells.

Moreover, CAR-NK cells are safer for clinical use compared with CAR-T cells. CAR-NK cells have a shorter lifespan after infusion and, therefore, do not persist in the circulation for long periods of time [12], reducing on-target/off-tumor toxicity, which is a significant concern for the use of CAR-T cells. For instance, CD19-targeting CAR-T cells can cause profound and long-lasting B-cell deficiencies due to persistent CAR-T activity against normal mature and progenitor B cells [16]. On the other hand, the shorter lifespan of NK cells may limit their efficacy in vivo. Recent studies have demonstrated that cytokines such as interleukin IL-12, IL-15 and IL-18 can induce the differentiation of memory-like NK cells, with enhanced persistent proliferation and antitumor capacity [17]. Consistently, human cord blood NK cells, expressing the CD19-specific CAR gene and IL-15 gene, were able to survive for several months and effectively kill tumor cells in a lymphoma mouse model [18]. Compared with CAR-T cells, which can function for up to several years after treatment [19], these memory-like CAR-NK cells, which live for several months, will be unlikely to cause significant on-target/off-tumor toxicity. In addition, activated NK cells predominately release IFN-γ and GM-CSF, which can promote continuous killing of one target to another without producing excessive pro-inflammatory cytokines, such as TNF-α, IL-1 and IL-6 [20]. These pro-inflammatory cytokines are implicated in the cytokine storm observed with the use of CAR-T cells [9].

In addition to NK cells, other innate immune cells, such as NKT and γδT cells, which possess unique features, can be engineered to express CAR. Preclinical trials have also demonstrated the effectiveness and feasibility of using CAR-NKT and CAR-γδT cells [21–23]. In this review, we mainly outline the recent advances in the development of CAR-NK cells, focusing on CAR constructs, cellular carriers and strategies to enhance CAR-NK-mediated immunotherapy.

Structure & construction of CAR

The structure of CARs used in CAR-T cells has evolved for four generations. As shown in Figure 1, the CAR structure is mainly composed of an extracellular single-chain variable fragment (scFv) derived from an antibody against a tumor antigen, a transmembrane domain (TM), and an intracellular signaling domain (ICD). The

intracellular structure of the first generation contains only a CD3ζ chain. That of the second generation contains CD3ζ chains and one costimulatory molecule, such as CD28, 4-1BB, CD134 (OX40), or an inducible T-cell costimulator (ICOS), to enhance cytotoxicity and proliferative activity. That of the third generation contains a CD3ζ chain and two or more different costimulatory molecules. The fourth generation of CAR refer to those designs that allow the expression of cytokines or costimulatory ligands upon T-cell activation mediated by the CAR [24–27]. For example, by using a nuclear factor of the activated T-cell (NFAT)-responsive promoter to drive an *IL-12* gene in CAR-T cells (Figure 1), CAR engagement and subsequent T-cell activation induced promoter activity and finally IL-12 production and release [28]. IL-12 improved CAR-T activation, modified the tumor microenvironment, and augmented the antitumor effects of CAR-T therapy in orthotopic human ovarian mouse models [29]. In view of the cytokine storm that occurs during the application of CAR-T, a suicide gene can be inserted into the new generation of CAR to add a controllable switch to the cells, so that when the risk of death occurs, a relevant drug can be used to induce apoptosis in time to terminate treatment [30,31]. Although CAR-NK cells share the same construction strategy as CAR-T, certain aspects have been modified to better suit the NK cells. The CAR-NK cell designs in recent years are described below.

Extracellular domain

The extracellular region mainly refers to the scFv for the tumor-specific antigen, that is, the VH and LH of the antibody, which are connected by a linker. Currently, many CAR-NK cell assays have been developed against different tumor antigens, such as the B-cell acute and chronic leukemia targets CD19 and CD20 [32–35], the breast cancer target HER2 [36], the neuroblastoma and melanoma target GD2 [37], the multiple myeloma target CD138 [38], the T-cell lymphoma target CD4 [39], and EGFR, which is frequently overexpressed in various solid tumors [40–42]. In addition, some researchers have focused on certain immunosuppressive cells of the human body, such as M2 macrophages and myeloid-derived suppressor cells (MDSCs), to design CAR-NK cells targets for these cells, which enhances the efficacy of other tumor killing strategies by eliminating inhibitory cells [43,44]. Moreover, researchers have also attempted to construct a bispecific CAR structure, that is, an extracellular scFv that can recognize two antigens.

The structures of the bispecific CARs shown in Figure 2A, 2B and 2C have the advantage of high sensitivity. Many tumor cells express a variety of tumor-associated antigens (TAA). Such CARs can effectively kill target cells, and to a certain extent, avoid immune escape induced by tumor tolerance. However, the disadvantages of such CARs are reduced specificity and damage to certain normal tissues that express one of the antigens. On this basis, researchers developed a fourth structure shown in Figure 2D. Since the activation of T cells requires two signals, only when both scFvs bind to the corresponding antigens can the CAR structure be fully activated to kill target cells. However, this design may not apply to NK cells. Studies have shown that when this type of CAR is engrafted on the NK92 cells, the association of the antigen with the one containing a costimulatory domain alone can activate the CAR structure *in vitro*, without requiring an association of the antigen with the one containing the CD3ζ domain [45]. The costimulatory domain in this construct consists of CD28 and CD244, and CD244 may be the molecule that plays a key role [45], which will be discussed later. In addition to using scFv as the extracellular region of CAR-NK cells, some different design ideas have been proposed, such as the use of NKG2D as an extracellular region [46]. This design has been applied in both CAR-T and CAR-NK cells and is based on the knowledge that NKG2D ligands are upregulated in a variety of tumor cells as well as on the surface of MDSCs, which are readily recruited by solid tumors [44]. Since NKG2D activation of NK cells requires interaction with DAP10 protein, researchers have constructed the NKG2D-DAP10-CD3ζ structure in human peripheral blood NK cells and T cells, which demonstrated good efficacy in mouse models of osteosarcoma [46–49].

Transmembrane domain

Most of the CAR-NK structures contain a transmembrane domain derived from CD8a, CD28 or CD3; some special ones are from NKG2D [33,50,55]. It is currently believed that the transmembrane domain has little impact on the overall structure. Alabanza *et al.* compared different transmembrane domains in CAR-T cells. They used two different scFvs targeting CD19 and two different transmembrane domains from either CD8a or CD28, and they designed four CAR structures in human peripheral blood T cells [60]. The results showed that although the two transmembrane domains were not significantly different in their ability to eliminate human primary chronic lymphocytic leukemia cells in mice, in both $CD4^+$ and $CD8^+$ T cells, degranulation and release of cytokines by CAR-T using CD28 as the transmembrane domain were higher than in those using CD8a as the transmembrane

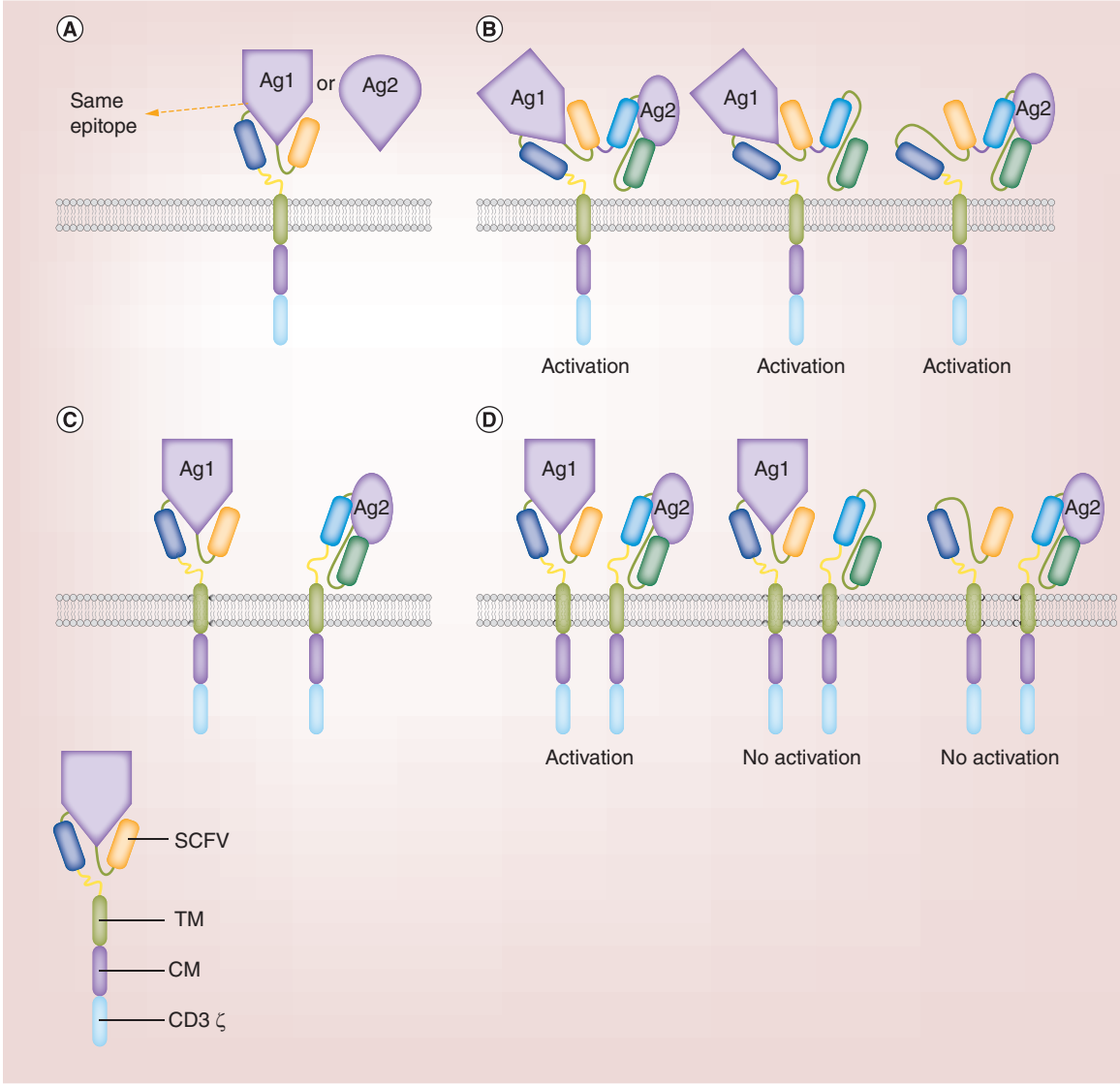

Figure 2. Bispecific CARs employed in CAR-T and CAR-NK cells. (A) Extracellular scFv is designed to target a common epitope of two different antigens, which allows CAR-modified cells to kill target cells expressing both antigens [50,51]. **(B)** Two scFvs are linked together and can simultaneously target two different antigens. The binding of either antigen activates this CAR structure [52–54]. **(C)** Two complete CAR structures are placed in parallel [55,56]. **(D)** Two different scFvs are each connected to the costimulatory molecule and to CD3ζ [57–59].

domain [60]. Guedan *et al.* demonstrated that the effect of the ICOS intracellular signaling domain was better than that of CD28 in CAR-T cells, as indicated by enhanced CAR-T persistence and tumor killing in mice [61], and additional findings suggested that the unique transmembrane region of ICOS was the key, rather than the cytoplasmic domains [62]. Furthermore, combining ICOS and 4-1BB intracellular signaling domains in a third-generation CAR-T targeting mesothelin-expressing human cancer cell lines displayed superior antitumor effects in xenograft models [63]. Interestingly, the ICOS intracellular signaling domain must be positioned proximal to the cell membrane and linked to the ICOS transmembrane domain [63]. Therefore, this information suggests that the transmembrane region of CAR-NK cells may contain unrecognized features that await further exploration.

Intracellular signaling domain

As the key component of the functional structure, the importance of the intracellular signaling region is self-evident. Most CAR-NK cells use CD3ζ as a signaling domain to conduct signal transduction and use the ITAM of CD3ζ to initiate NK cell killing [64–66]. The CD3ζ dimer can transmit signals from CD16 and can thus contribute to

Table 1. Comparison of peripheral blood NK cells and NK-92 cells as cell carriers.

	Peripheral blood NK cells	NK-92 cells
Source	Extracted from human peripheral blood, which requires the removal of white blood cells through multiple rounds of leukapheresis, a cumbersome and time-consuming process	Can be directly amplified from easily accessible commercial cell lines, which is more efficient for experiments or clinical treatments
Cell surface receptors	Include all stimulatory and inhibitory receptors	Partially lack stimulatory and inhibitory receptors. The surface is CD16-negative. Unable to kill target cells through ADCC
Cell culture	Require feeder cancer cells that express specific stimulating factors, such as IL-15 and 4-1BB	Depend on IL-2 and require specific IL-2-containing medium
Cell treatment before experiments	No irradiation is required	Irradiation (1000 cGy) is required before use to prevent Epstein–Barr virus infection, without compromising cytotoxicity
Cytotoxicity	Multiple rounds of leukapheresis may have a negative impact on NK cytotoxicity, to a certain extent	Cytotoxicity is not affected

ADCC [67,68]. Moreover, similar to experiments with CAR-T, in the construction of CAR-NK cells, Schonfeld *et al.* also compared a variety of different intracellular signaling regions in NK-92 cells [69]. The results showed that with only a single domain of CD3ζ, the CAR-NK92 cells could not effectively lyse ErbB2-expressing human tumor cells *in vitro* due to insufficient stimulatory signals [69]. Therefore, more costimulatory domains are required, including the commonly used CD28, CD137 (4-1BB), or CD244 (2B4) [70,71]. Interestingly, both CD28 and CD137 are important costimulatory molecules that function during human T-cell activation even though neither CD28 nor CD137 is expressed in human NK cells, and the classical activation pathway of NK cells is not dependent on a costimulatory signal like they are in T cells. Therefore, why is the effect of a single CD3ζ not ideal, and what roles do CD28 and CD137 play in the activation of CAR-NK cells? These questions deserve an in-depth discussion for the construction of effective CAR.

Studies have found that the stimulatory effect of the CD244 cytoplasmic domain in CAR-expressing NK92 cells is better than that of CD137 *in vitro* and in acute lymphoblastic leukemia (T-ALL) xenograft mice [72]. CD244 is a costimulatory molecule of NK cell activation and belongs to a receptor family related to lymphocyte activation signaling molecules. The cytoplasmic domain of CD244 contains an immunoreceptor tyrosine-based switch motif (ITSM), which can bind to lymphocyte activation signaling molecule (SLAM)-associated protein (SAP) and recruit and activate tyrosine kinases to initiate activation signals, which suggests that CD244 may be more suitable as an intracellular signaling domain in CAR-NK cells [73,74]. Using an ovarian cancer xenograft model, Li *et al.* found that when CD244 and CD3ζ were simultaneously used as signaling domains, mutation of key amino acids of CD3ζ did not significantly affect the function of CAR; however, mutations in key amino acids of CD244 resulted in a tremendous impact on the killing effect of human iPSC-derived CAR-NK cells [40]. These results evoke questions of whether CD244 can replace CD3ζ as the intracellular region of CAR-NK cells, which awaits confirmation in future experiments. In addition to the use of CD244, Muller *et al.* have attempted to replace CD3ζ with DAP12 in CAR-expressing YTS-NK cells and evaluated their killing effect in glioblastoma xenografts [75]. Compared with CD3ζ, however, the intracellular region of DAP12 contains only one ITAM structure [75]. The results showed that DAP12 demonstrated a better effect than CD3ζ as a signaling domain, which can recruit and phosphorylate ZAP70, and then further phosphorylate downstream signals to mediate the CAR-NK cells killing effects [75,76].

Cell carriers used for CAR-NK cells

Currently, commonly used cell carriers for CAR-NK development are human peripheral blood NK cells and the NK92 cell line. The two types of cells are compared below (Table 1). From the perspective of timeliness and practicality of treatment, the NK-92 cell line is undoubtedly superior to human peripheral blood NK cells, and studies have shown that the levels of perforin, granzyme and a series of cytokines with killing effects produced by NK-92 cells are higher than those produced by peripheral blood NK cells [77–82]. In addition to NK-92 cells, other NK cell lines were also used to express CAR. For example, Murakami *et al.* first attempted to construct a CAR structure targeting EGFRvIII using another human NK cell line, KHYG. The results showed that KHYG cells demonstrated similar cytotoxic effects as NK-92 cells in human glioblastoma xenografts [42]. However, which of the two cell lines exhibits better and safer treatment effects awaits further verification. In recent years, stem cell-derived NK cells, including embryonic stem cells (ESCs), CB stem cells, and induced pluripotent stem cells (iPSCs), have also attracted substantial attention. Since embryonic stem cells are difficult to obtain and their applications have ethical concerns, they are used less frequently. CB can be stored frozen and its application is more

Table 2. Clinical trials using CAR-NK cells.

NTC number	Target	Disease	Status	CAR design	Cell source
NCT03941457	ROBO1	Pancreatic cancer	Recruiting	Unknown	Unknown
NCT03940833	BCMA	Multiple myeloma	Recruiting	Unknown	NK-92
NCT03824964	CD19/CD22	Refractory B-cell lymphoma	Not yet recruiting	Unknown	Unknown
NCT03692663	PSMA	Castration-resistant prostate cancer	Not yet recruiting	Unknown	Unknown
NCT03692637	Mesothelin	Epithelial ovarian cancer	Not yet recruiting	Unknown	PB NK
NCT03656705	Unknown	Non-small-cell lung cancer	Enrolling by invitation	Unknown	NK-92
NCT03415100	NKG2D ligands	Unknown	Recruiting	Unknown	Unknown
NCT03383978	HER2	Glioblastoma	Recruiting	CD8a-CD28-CD3ζ	NK-92
NCT03056339	CD19	B-lymphoid malignancies, etc.	Recruiting	CD19-CD28-CD3ζ-2A-iCasp9-IL15	CB NK
NCT02944162	CD33	Acute myelogenous leukemia, etc.	Unknown	CD28-CD137-CD3ζ	NK-92
NCT02892695	CD19	Acute lymphocytic leukemia, etc.	Unknown	CD28-CD137-CD3ζ	NK-92
NCT02839954	MUC1	Hepatocellular carcinoma, etc.	Unknown	Unknown	Unknown
NCT02742727	CD7	Acute myeloid leukemia, etc.	Unknown	CD28-CD137-CD3ζ	NK-92

convenient than that of embryonic stem cells. At present, some techniques have been developed that can greatly amplify CB-derived NK cells in a short period of time, and these cells showed significant *in vivo* activity against multiple myeloma in a xenogenic mouse model [83]. iPSCs are a type of pluripotent stem cell that can be generated directly from adult cells, such as skin cells, and then induced to differentiate into NK cells. The iPSC-derived NK cells have advantages of both human peripheral blood NK cells and NK-92 cells, and standardized methods have been established for large-scale induction and amplification of NK-like cells in a short period of time [84–87]. Moreover, these NK-like cells can express biological levels of killer activation receptors (KARs), and they express a low level of killer-cell immunoglobulin-like receptor (KIR) compared with peripheral blood NK cells [88]. Li *et al.* have successfully constructed a CAR structure in iPSC-derived NK cells. Compared with traditional CAR-T, ovarian cancer mouse models transplanted with the iPSC-derived CAR-NK cells showed a higher survival rate and fewer toxic side effects [40]. However, it is worth noting that the same CAR structure may have different effects in differenT-cell carriers [71], which indicates that we cannot directly apply the CAR that is suitable for one type of carrier to another and must experimentally determine the most suitable CAR structures for different applications.

Clinical trials using CAR-NK cells against various tumors

Here, we list some ongoing clinical studies on CAR-NK cells (Table 2). In these trials, the primary cell carrier is the NK-92 cell line. CARs were designed to target antigens that are known to be specific for various tumors, and the designs mainly adopted the first- or second-generation structure. In addition, more studies used CD137 rather than CD244 as the costimulatory molecule, which we previously discussed. It is worth mentioning that in clinical study NCT03056339, Rezvani *et al.* adopted a TRUCKS-like strategy to design the CAR, adding the IL-15 gene to enhance the antitumor activity of CB derived CAR-NK cells. To improve the safety, they took advantage of an inducible caspase-9 suicide gene, whose expression can be turned on using a specific chemical inducer of dimerization.

Future perspective

In tumor immunotherapy, the tumor microenvironment is an essential factor that must be taken into account. Although NK cells might be more effective in attracting solid tumors than T cells, their infiltration can be blocked by a tumor microenvironment that is composed of various inhibitory factors. Therefore, enabling better infiltration of NK cells into the solid tumor and overcoming the inhibition of the tumor microenvironment are crucial for their antitumor activity. By inducing a permissive tumor microenvironment for immune cell infiltration, we could greatly improve the efficacy of CAR-NK treatment for solid tumors. For example, CXCL12 has been proven to be abundant in the tumor microenvironment, and it can recruit $CXCR4^+$ regulatory T cells and MDSCs into solid tumors, promoting immune suppression and tumor progression [89,90]. CXCL1 and CXCL2 are highly expressed by breast cancer cells, leading to increase infiltration of $CXCR2^+$ pro-tumorigenic myeloid cells [91]. Thus, we may

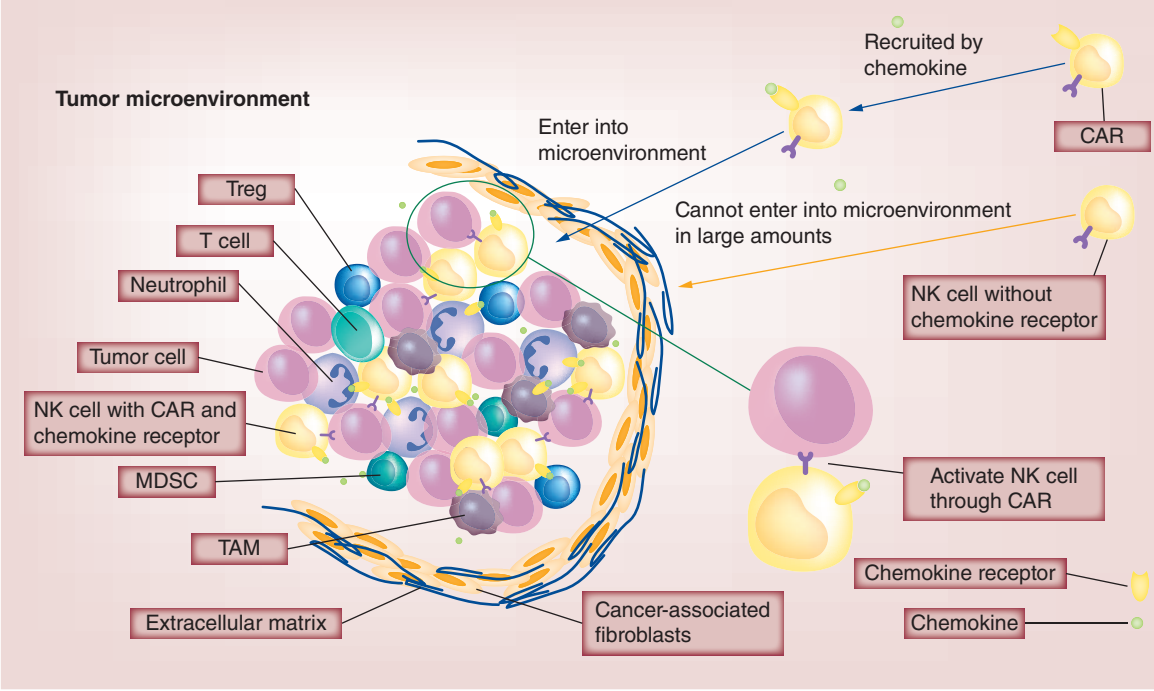

Figure 3. Chimeric antigen receptors modulate immune cell infiltration. It is difficult for NK cells or T cells to infiltrate the solid tumor microenvironment because of the presence of a large number of immunosuppressive cells. When NK cells or T cells are modified to express CAR and chemokine receptor genes, such as CXCR2 or CXCR4, which activates CXCR2/CXCL1/2 or CXCR4/CXCL12 axis, respectively, these cells may be able to enter tumor tissues in large numbers and exert a killing effect.
MDSC: Myeloid-derived suppressor cell; NK: Natural killer; TAM: Tumor-associated macrophage.

Table 3. CAR-NK cells combined with other treatments.

CAR-NK cell target	Combined treatment	Mechanism and rationale	Ref.
CD20	Romidepsin	Romidepsin induces tumor cells to express the NKG2D ligand MICA/B and enhances the cytotoxicity of CAR-NK cells	[93,95]
CD133	Cisplatinum	Cisplatinum can enhance CAR-NK cell cytotoxicity	[96]
EGFR	Cabozantinib	Cabozantinib can increase the expression of EGFR and decrease that of PD-L1 in renal cell carcinoma cells, enhancing the killing ability of CAR-NK cells, and may also activate autogeneic T cells to a certain extent because of the decrease in PD-L1	[97]
EpCAM	Regorafenib	Regorafenib enhances T- and NK-cell infiltration by upregulating the expression of T/NK cell-associated adhesion molecules and chemokines and also improves the proliferation and function of T/NK cells by downregulating immunosuppressive cell subpopulations (MDSCs, Tregs, and TAMs) and cytokines (IL-10 and TGF-β)	[94]
CD19	Nanodrugs	Owing to the repellent nature of the human body, nanodrugs cannot reach their targets efficiently. However, modified CAR-NK cells can precisely locate lesions through scFv. Therefore, NK-92 cells engineered with CAR can be used as a carrier to guide drug-loaded nanoparticles to the target, which can enhance the efficacy of *in vitro* and *in vivo* chemotherapy and simultaneously reduce off-target toxicity	[98]
CD16	CD133 antibody	Surface expression of CD16 confers to NK-92 cells a target cell-killing ability, which relies on the ADCC pathway	[99]

be able to improve the infiltration of CAR-NK cells by modifying NK cells to express CAR and the receptor of chemokine that are elevated in the tumor microenvironment (Figure 3).

Moreover, in the tumor microenvironment, the inhibitory effect of cytokines such as TGF-β on immune cells cannot be ignored. It plays important roles in the differentiation of protumorigenic immune cells, such as regulatory T cells (Treg) and M2-like TAM. If CAR-NK cells are loaded with structures that enable secretion of the soluble receptors of these cytokines, which can competitively bind to TGF-β and then inhibit TGF-β activity, the suppression of various immune cells by the tumor microenvironment might be reinvigorated [92]. In addition, the

combination of CAR and other treatments is worth exploring. Here, we present some experiments using CAR-NK in combination with chemotherapeutic drugs, antibodies and nanomolecules (Table 3). These studies mainly sought to increase the expression of the targeted antigen or to enhance the infiltration and function of CAR-T or CAR-NK cells. For instance, Chu *et al.* found that romidepsin significantly increased the expression of the NKG2D ligand MICA/B in Burkitt lymphoma cells and enhanced CAR-NK cytotoxic activity through the interaction between MICA/B and NKG2D *in vitro* and *in vivo* [93]. Zhang *et al.* demonstrated that EpCAM-specific CAR-NK-92 cells combined with regorafenib, which enhanced the infiltration of CAR-NK-92 cells, could effectively kill tumor cells in a colorectal cancer murine model. Therefore, a rational design of the combination therapy strategy may be an important direction for future CAR-NK research [94].

Conclusion

It is undeniable that CAR-NK cell therapy has tremendous potential and feasibility as a new means of adoptive immunotherapy. However, many issues still need to be addressed, such as how to improve the transfection efficiency of CAR-NK cells, exploration of the optimal CAR structures, selection of the appropriate cell carriers, the best introduction point for combined treatments, and how to eliminate possible adverse reactions after application in humans. These are all concerns that need to be resolved before CAR-NK cells can be used in clinical practice. If these problems are resolved, it is believed that CAR-NK cells will become a powerful weapon against tumors.

Executive summary

Chimeric antigen receptor

- Chimeric antigen receptor (CAR) structure was first used in T cells, and the constructed CAR-T have demonstrated a good effect in clinical treatment of hematological tumors. To further explore the antitumor potential of CAR, CAR-NK cells have been developed.

CAR-NK cell structure

- The extracellular domain is mainly composed of scFv that can bind to the target antigen and a hinge domain. As the starting point of signal transmission, the specificity and sensitivity of scFv directly determine the effects of the entire structure. Currently, there are two designs for scFv: mono-specificity and bi-specificity.
- CD8a, CD28 and CD3ζ are commonly used for constructing the transmembrane domain, and it is generally believed that different transmembrane regions have little effect on the killing effect of CAR.
- The intracellular region consists of costimulatory molecules and a signaling domain containing ITAM. The CAR-NK cells with costimulatory molecules demonstrate significantly higher cytotoxicity than CAR-NK cells without costimulatory molecules. This is an interesting phenomenon due to the finding that, unlike T cells, the activation of NK cells does not require two signals. The ITAM-containing signaling domain used by CAR-NK cells are mainly CD3ζ and DAP12.

Cell carriers

- Peripheral blood NK cells and the NK-92 cell line are the most commonly used cell carriers. In addition, NK cells derived from IPSCs also demonstrate great potential. It is worth noting that the same CAR is not necessarily suitable for all cell carriers. Determining the most suitable CAR according to the characteristics of different carriers is also a problem that needs to be resolved.

Combined treatments

- In existing studies, the combination of CAR-NK cells with other treatments shows a synergistic effect ($1 + 1 > 2$). For example, some drugs can enhance the activity of NK cells or can specifically upregulate specific antigens on the surface of target cells, thereby promoting a stronger killing effect of CARs against this antigen.

Future perspective

- The tumor microenvironment is a forbidden area for human immune cells such as T cells and NK cells. The various inhibitory factors located there prevent the massive infiltration of NK cells. We believe that loading CAR-NK cells with "navigation" that directs them to the interior of the tumor will maximize the therapeutic effect of CAR-NK cells.

Author contributions

Y Zhao and X Zhou wrote the manuscript.

Financial & competing interests disclosure

This work was supported by the National Natural Science Foundation of China, grant numbers 81571527 and 81771681. The authors have no other 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 apart from those disclosed.

The manuscript has been checked by the American Journal Experts (AJE) language editing company.

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Closed, automated wash and concentration of expanded human natural killer (NK) cells

Improve the efficiency of NK cell processing with the CTS Rotea Counterflow Centrifugation System and CTS NK-Xpander Medium

Introduction

One of the key challenges faced by the cell and gene therapy industry is poor efficiency in cell processing. A key improvement being made is the shift toward closed and automated systems, which can help reduce the risk of contamination and error, as well as provide the ability to process multiple products in parallel in less controlled spaces.

The Gibco™ CTS™ Rotea™ Counterflow Centrifugation System is a closed cell processing system developed specifically for small-batch cell therapy manufacturing. It provides output volumes as low as 5 mL with high cell recovery and viability, and is controlled via user-programmable software to enable creation of protocols for many different processes. The CTS Rotea system has been successfully used in various steps of the CAR T cell processing workflow, including isolation of peripheral blood mononuclear cells (PBMCs), cell washing, and concentration of engineered CAR T cells.

Here we demonstrate use of the CTS Rotea Counterflow Centrifugation System for automated washing and concentration of human NK cells expanded in Gibco™ CTS™ NK-Xpander™ Medium (Figure 1).

Materials and methods

Enriched NK cells were expanded in CTS NK-Xpander Medium, harvested on day 17, washed, and concentrated using the CTS Rotea Counterflow Centrifugation System. Subsequent to this, phenotypic and functional characterization was performed.

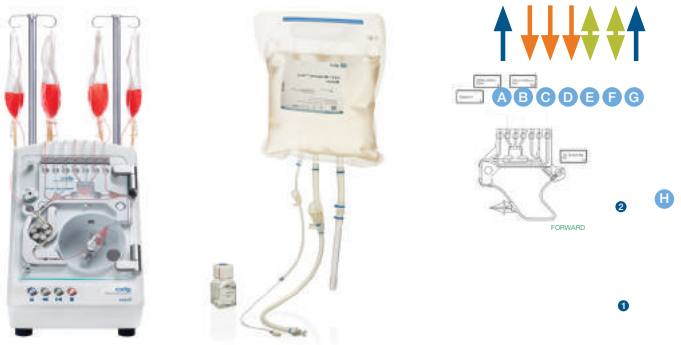

Figure 1. CTS Rotea Counterflow Centrifugation System and CTS NK-Xpander Medium.

Expand

- CTS NK-Xpander Medium
- IL-2 recombinant human protein
- Human AB serum

Wash and concentrate

- CTS Rotea Counterflow Centrifugation System (instrument, software, and single-use kits)
- CTS DPBS, bag format

Analyze

- Countess Automated Cell Counter
- Staining solutions
- Attune NxT Acoustic Focusing Cytometer
- Monoclonal antibodies

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Feeder-free NK cell expansion and activation

Enriched CD56+ NK cells from PBMCs were cultured per the CTS NK-Xpander Medium **protocol** and scaled up to a final volume of 1.5 L. Briefly, NK cells were plated at 1.25 x 10^5 cells/mL at 200 µL per well in Thermo Scientific™ Nunc™ non-treated 96-well plates and cultured for 17 days in CTS NK-Xpander Medium (Cat. No. A5019001) containing 500 U/mL recombinant human IL-2 (Cat. No. PHC0023) and 5% human AB serum (Fisher Scientific Cat. No. BP2525100). The cells were fed every 2–3 days beginning on day 5 to maintain an optimal cell density of 4–5 x 10^5 cells/mL. As cells grew, they were transferred from initial 48-well plates and split into multiple 6-well plates, then T-75 flasks, and finally to multiple T-175 non–tissue culture treated flasks to a final volume of 1.5 L. Cells were stained with trypan blue and counted using the Invitrogen™ Countess™ II FL Automated Cell Counter.

Closed, automated washing and concentration of expanded NK cells

Following expansion, NK cell washing and concentration were performed using the CTS Rotea Counterflow Centrifugation System. Prior to loading onto the CTS Rotea system, a single-use kit was constructed using bag connections made via standard welding techniques (Figure 2). The CTS Rotea System was primed by replacing air in the system with buffer. The cells were loaded into the chamber to form a fluidized bed. Fresh wash buffer, consisting of Gibco™ CTS™ DPBS (without $CaCl_2$ and $MgCl_2$) and 2% human serum albumin (Nova Biologics, Cat. No. 68982-0643-02), was allowed to flow through the bed to wash the cells. The cells were then concentrated and harvested from the system for further downstream processing.

All CTS Rotea System protocols were written using the Gibco™ CTS™ Rotea™ Protocol Builder desktop application. Table 1 lists the steps of the NK cell washing and concentration protocol, and Figure 2 illustrates the configuration of the Gibco™ CTS™ Rotea™ Single-Use Kit.

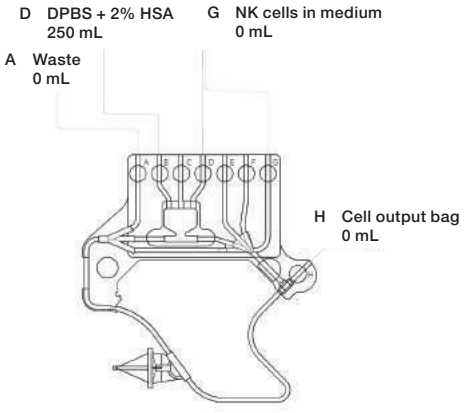


Figure 2. CTS Rotea Single-Use Kit configuration for NK cell washing and concentration.

Table 1. Sequence of NK cell washing and concentration protocol on the Rotea system, including initial priming steps.

Step	Description	Flow path	Speed	Flow rate	Step type	Trigger
Priming sequence						
1	Pre-prime	B to A	0 x *g*	100 mL/min	Normal	Input bubble sensor
2	Lubricate rotary coupling	B to A	0 x *g*	100 mL/min	Normal	Volume: 15 mL
3	Prime chamber and line A	B to A	10 x *g*	100 mL/min	Normal	Volume: 15 mL
4	Add priming volume	B to A	10 x *g*	100 mL/min	Normal	Volume: 50 mL
5	Prime bubble trap and line B	A to B	10 x *g*	100 mL/min	Normal	Volume: 15 mL
6	Prime line D	A to D	10 x *g*	50 mL/min	Normal	Volume: 5 mL
7	Pressure prime	A to EF	10 x *g*	0 mL/min	Pressure prime	
8	Prime pause	J to K	10 x *g*	25 mL/min	Pause	Volume: 3 mL
9	Ramp speed to initiate bed	J to K	2,200 x *g*	50 mL/min	Pause	Time: 10 sec
Loading and washing the NK cells						
10	Initiate bed	D to G	2,200 x *g*	50 mL/min	Normal	Time: 4 min
11	Load input material	D to A	2,250 x *g*	35 mL/min	Normal	Volume: 1 x input aliquot (mL) Input bubble sensor, pause
12	Adjust speed for wash	J to K	2,400 x *g*	25 mL/min	Pause	Time: 15 sec
13	Wash	B to A	2,400 x *g*	25 mL/min	Normal	Volume: 30 mL
14	Concentrate bed for harvest	J to K	2,500 x *g*	15 mL/min	Pause	Time: 10 sec
15	Harvest	B to H	2,500 x *g*	50 mL/min	Harvest	Volume: 1 x harvest volume (mL)
16	Ramp to stop	K to J	500 x *g*	50 mL/min	Pause	Time: 5 sec

NK cell phenotypic characterization

Expanded NK cells were gated for live cells using Invitrogen™ LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit. Their CD56, CD3, and CD16 levels were then measured using appropriate antibodies and the Invitrogen™ Attune™ NxT Acoustic Focusing Cytometer.

NK cell functionality

NK effector cells expanded in CTS NK-Xpander Medium were coincubated with K562 target cells labeled with the Invitrogen™ CellTrace™ CFSE Cell Proliferation Kit at NK:K562 cell ratios of 0.625:1, 1.25:1, 2.5:1, and 5:1 for 2 hours. Following incubation, degranulation was assessed based on the expression of CD107a by $CD56^+$ NK cells, measured on the Attune NxT Acoustic Focusing Cytometer. NK cell cytotoxicity was assessed by measuring K562 cell death on the Attune NxT system by gating for CFSE-labeled K562 cells and measuring the percentage of dead cells using the LIVE/DEAD stain kit.

Results

NK cells were expanded to 1.83 x 10^9 cells in a final volume of 1.62 L using CTS NK-Xpander Medium. Cells washed and concentrated using the CTS Rotea Counterflow Centrifugation System showed high recovery and viability post wash and maintained their phenotype and functionality.

Feeder-free NK cell expansion and activation

PBMC-derived NK cells cultured in CTS NK-Xpander Medium expanded by 1,700-fold on average after 17 days (Figure 3). The cultures started at 1.13 x 10^6 cells in 9 mL and increased to 1.83 x 10^9 cells in 1.62 L.

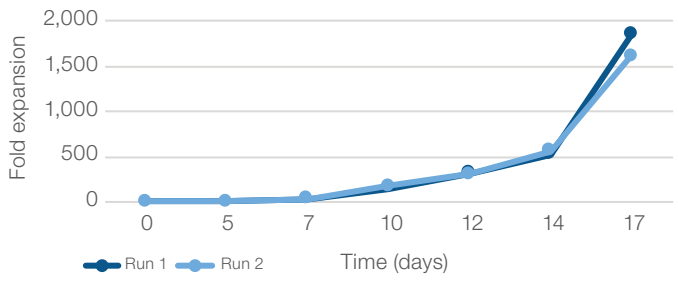
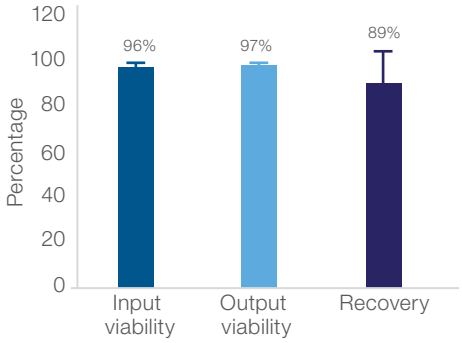


Figure 3. Fold expansion of NK cells cultured in CTS NK-Xpander Medium for 17 days.

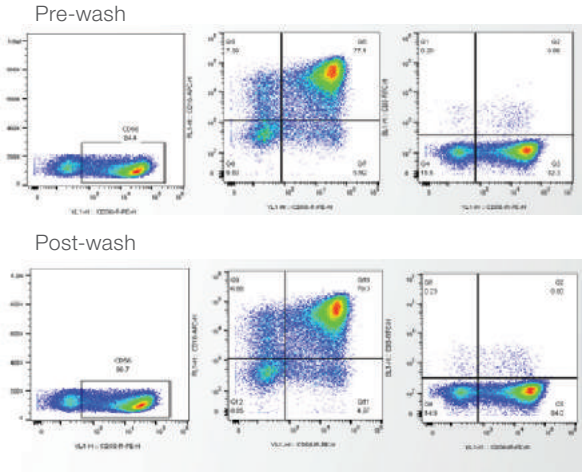
Closed, automated washing and concentration of expanded NK cells

Expanded cells were loaded into the CTS Rotea system to form a stabilized bed for subsequent washing in CTS DPBS. Recovery was ~90% with high viability and maintenance of cellular phenotype (Figure 4).

A



B



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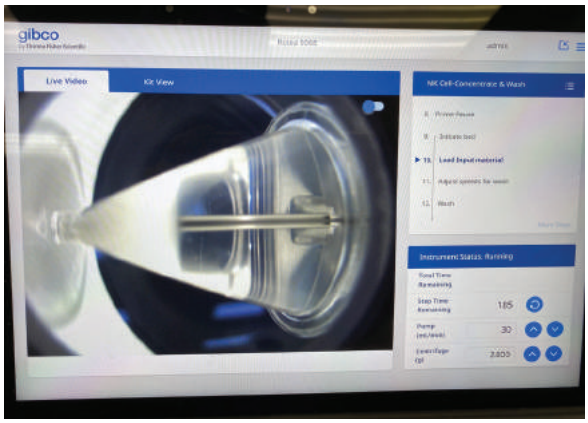

Figure 4. NK cell washing and concentration. (A) NK cell viability and recovery averaged over four washing and concentration runs. **(B)** Flow cytometry staining for CD56, CD16, and CD3 before and after washing with the CTS Rotea System NK cell wash and concentration protocol. **(C)** CTS Rotea System software image with a chamber of NK cells in a fluidized bed.

NK cell functionality

Cells washed and concentrated using the CTS Rotea system maintained cytolytic function and were able to degranulate (Figure 5) and kill K562 target cells (Figure 6) in a dose-dependent manner.

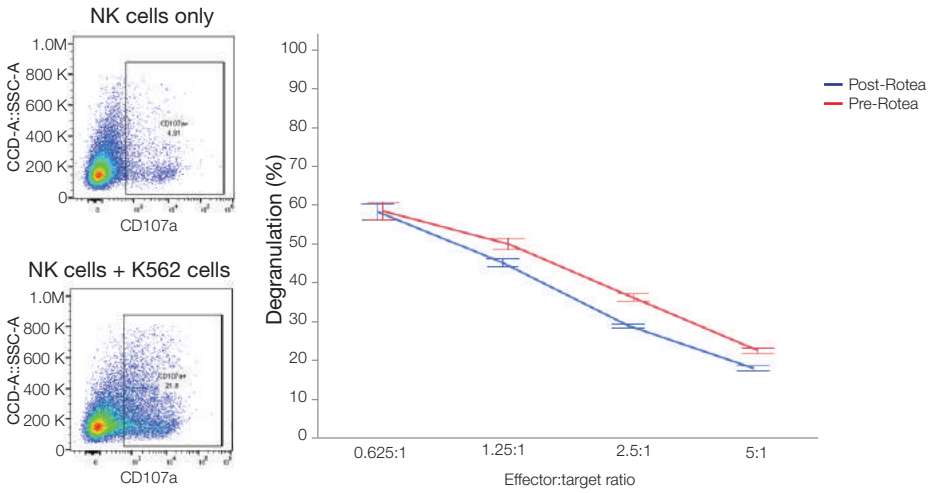

Figure 5. Maintenance of NK cell degranulation capability after washing and concentration.

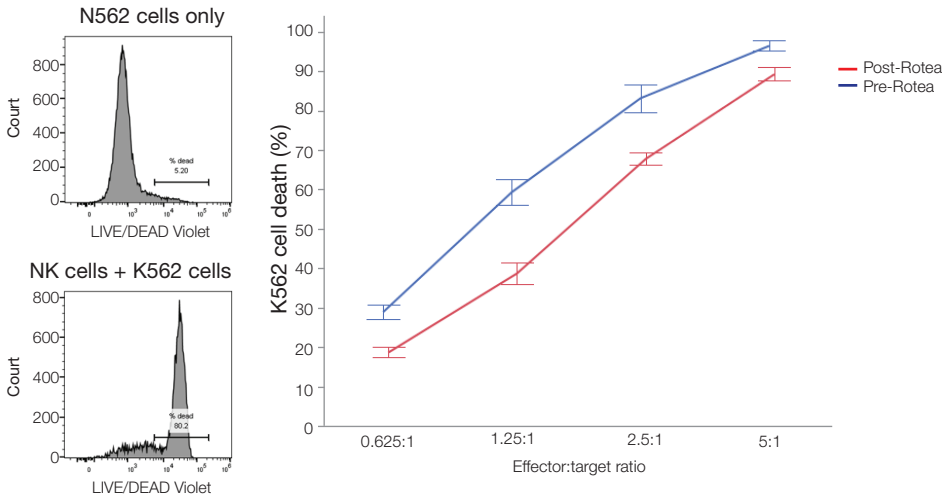

Figure 6. Maintenance of NK cell cytotoxicity after washing and concentration.

Conclusions

Critical improvements to cell and gene therapy manufacturing can be achieved by reducing risk and hands-on time using regulation-compliant reagents and closed manufacturing systems. We have demonstrated efficient NK cell expansion in a feeder-free culture system as well as high recovery after washing and concentrating the cells using a closed, automated counterflow centrifugation system. Here we have demonstrated efficient expansion of NK cells in a feeder-free culture system using CTS NK-Xpander Medium, and high recovery of cells during wash and concentration using the CTS Rotea Counterflow Centrifugation System.

gibco

Ordering information

Product	Quantity	Cat. No.
Expansion		
CTS NK-Xpander Medium	500 mL bottle	A5019001
	5 L bag	A5019002
Human IL-2 Recombinant Protein	1 mg	PHC0023
Human AB Serum	100 mL	Fisher Scientific, BP2525100
Nunc non-treated 96-well plates	Case of 160	268200
Nunc non-treated 48-well plates	Case of 75	150787
Analysis		
Countess 3 FL Automated Cell Counter	1 instrument	AMQAF2000
Trypan Blue Solution, 0.4%	100 mL	15250061
CellTrace CFSE Cell Proliferation Kit	1 kit	C34570
eBioscience Flow Cytometry Staining Buffer	600 mL	004222-26
Fc Receptor Binding Inhibitor Polyclonal Antibody	100 tests	14-9161-73
UltraComp eBeads Compensation Beads	100 tests	01-2222-42
ArC Amine Reactive Compensation Bead Kit	1 kit	A10346
Attune NxT Acoustic Focusing Cytometer	1 instrument	A24858
CD56 Monoclonal Antibody (CMSSB)	100 tests	120567-42
CD3 Monoclonal Antibody (OKT3)	100 tests	11-0037-42
CD16 Monoclonal Antibody (CB16)	100 tests	17-0168-42
CD107a (LAMP-1) Monoclonal Antibody (eBioH4A3)	100 tests	25-1079-42
LIVE/DEAD Fixable Violet Dead Cell Stain Kit, for 405 nm excitation	400 assays	L34964
Wash and concentration		
CTS Rotea Counterflow Centrifugation System with 2-year warranty, including OQ after PM plus IQOQ	1 instrument	A50757*
		A47695**
CTS Rotea Counterflow Centrifugation System with 2-year warranty, including PM	1 instrument	A50760*
		A47679**
CTS Rotea Single-Use Kit	10 pack	A49585
	5 pack	A49313
CTS Rotea Hi-Flow Single-Use Kit	10 pack	A46575
	5 pack	A49239
CTS DPBS	2 L bag	A1285602
CTS Rotea Kit Tube Clamps	100 pack	A49127
CTS Rotea Kit Sterile Luer Connectors	10 pack	A50110
CTS Rotea Kit Sterile Sample Ports	10 pack	A50111

* North America and Europe.

** Rest of the world.

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WHITE PAPER

CTS reagents and products

Autologous CAR T cell manufacturing using a semiautomatic, closed, modular workflow

Seamless transition from discovery to clinical manufacturing

Background

Cell-based chimeric antigen receptor (CAR) T cell therapies have rapidly advanced from preclinical research—with a variety of targets in clinical research and several FDA-approved products currently on the market [1]. This success has driven an influx of companies to further develop CAR T cell constructs to make them more effective, safe, and persistent. On the manufacturing side, however, errors, lot-to-lot variation, and contamination can be associated with open processing and manual handling of CAR T products. Overcoming the bioprocessing bottleneck remains a critical challenge in CAR T cell therapy scalability, which can potentially hinder both product development and patient access. It has been reported that about 7–9% of patients have been unable to receive one of the FDA-approved CAR T cell therapies because of manufacturing failures [2].

Autologous CAR T cell therapies are donor-specific, where a donor's own immune cells are used to create therapeutic CAR T (Figure 1A). During the manufacturing process, a Leukopak™ bag from the donor is received by a GMP facility, where the T cells are isolated from peripheral blood mononuclear cells (PBMCs), activated, and genetically engineered by viral transduction to express a CAR. The activated T cells are expanded in a T cell–specific cell culture medium, typically for 7–10 days to reach a therapeutically relevant number, and then they are cryopreserved. The cryopreserved CAR T cell product is then characterized and analyzed before being shipped to the treatment center, where it will be thawed and administered to the donor via infusion. This complicated, labor-intensive process usually involves many open manipulations and manual procedures, potentially

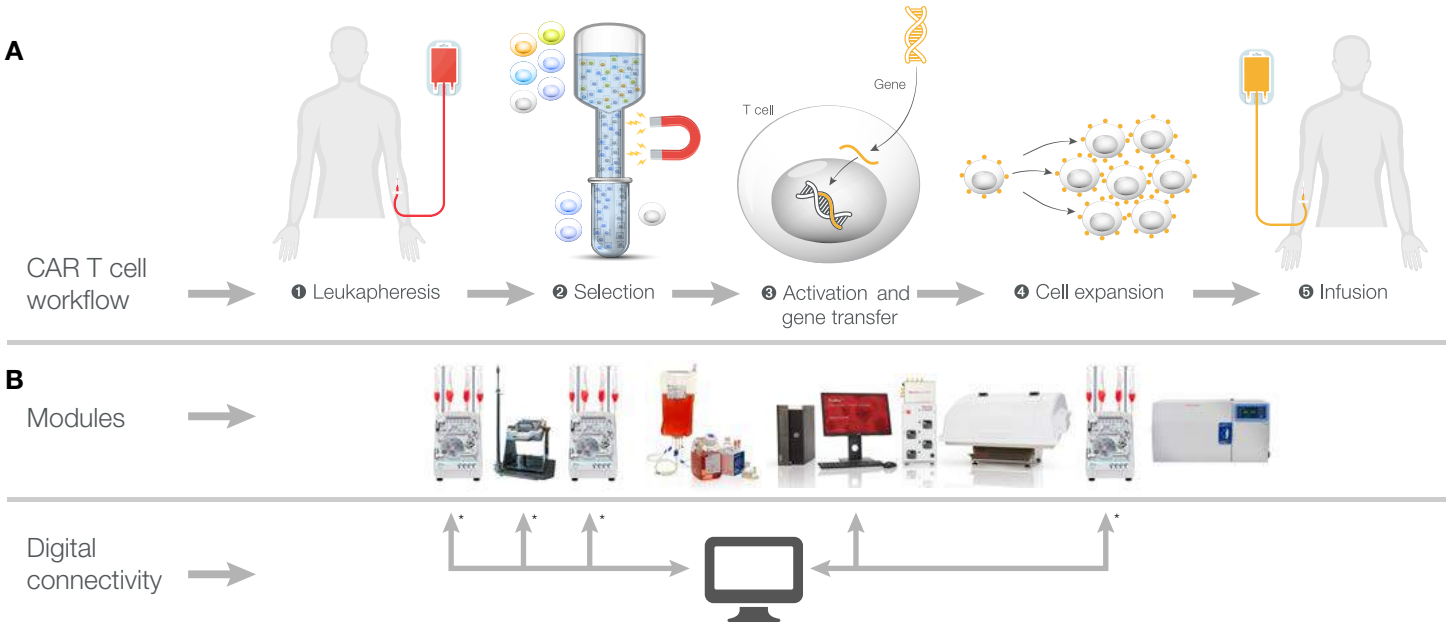

Figure 1. A typical autologous CAR T cell therapy workflow. (A) The typical CAR T cell therapy workflow. **(B)** Workflow solutions from Thermo Fisher Scientific.
* Proof-of-principle prototype solution.

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introducing inconsistencies, errors, and contamination at various steps of the workflow. Currently, no uniform, automated manufacturing process exists to accommodate the wide variety of complex workflows needed—from isolation to cryopreservation—to produce therapeutic CAR T cells. Re-engineering the CAR T cell therapy manufacturing process by integrating the complicated multistep workflow into a closed, modular, benchtop system could enable a smoother transition from the laboratory to clinical research application while improving consistency, purity, and safety of the product. Additionally, as early as possible in the discovery and development phases, the generation of CAR T cells should include safe and effective biomanufacturing processes and trackable workflows, and have the potential for cGMP compatibility.

The closed modular system developed by Thermo Fisher Scientific is a digitally compatible, GMP-compliant, semi-automated manufacturing platform, which when used in combination with Gibco™ Cell Therapy System™ (CTS™) reagents, protocols, and analytics can result in consistent, efficacious CAR T cell production. One important benefit of the closed modular system is the digital connectivity. Here we demonstrate a proof-of-principle digital integration using the DeltaV™ Distributed Control System from Emerson to control and manage the instruments in the workflow, as shown in Figure 1. Taken together with the modularity, digital connectivity, and cGMP compatibility of the various components, the manufacturing of therapeutic CAR T cells with the closed modular system can reduce contamination and production failure, and improve lot-to-lot consistency of products.

Materials and methods

Step 1. PBMC isolation

Starting material was derived from fresh or frozen quarter-size Leukopak bags (n = 7). Peripheral blood mononuclear cells (PBMCs) were isolated using the Gibco™ CTS™ Rotea™ Counterflow Centrifugation System following the predefined isolation protocol (see "Automated PBMC isolation and T cell wash and concentration by the CTS Rotea system"). During the process, red blood cells (RBCs) were eliminated with Gibco™ ACK Lysing Buffer and the PBMCs were further washed with Gibco™ DPBS with 1% human serum albumin (HSA) and 2 mM EDTA. Cells were counted using the Via1-Cassette™ system and NucleoCounter™ measuring chamber (ChemoMetec). Flow cytometry acquisition and analysis were performed using the Invitrogen™ Attune™ NxT Flow Cytometer and FCS Express™ 7 software, respectively.

Step 2. Selection—T cell isolation and activation

PBMCs were incubated with Gibco™ CTS™ Dynabeads™ CD3/CD28 at a 3:1 ratio of beads to T cells for 30 minutes at room temperature. Bound cells were captured with the Gibco™ CTS™ DynaMag™ Magnet, and unbound (nontarget) cells were removed with the supernatant. The bound cells were then washed with 1% HSA, 2 mM EDTA in DPBS, followed by resuspension in complete medium (Gibco™ CTS™ OpTmizer™ T Cell Expansion Serum Free Medium (SFM)), supplemented with Gibco™ CTS™ Immune Cell Serum Replacement (SR), 200 mM L-glutamine, and 100 U/mL IL-2. The cells were then transferred to either a Thermo Scientific™ HyPerforma™ Rocker Bioreactor with Thermo Scientific™ HyPerforma™ G3Lab™ Controller or a G-Rex™ 500M bioreactor (Wilson Wolf) at a seeding density of 1 x 10^6 cells/mL. Cells were cultured overnight and transduced with lentivirus the next day.

Step 3. Gene transfer—lentivirus-CAR transduction

To achieve gene transfer of CD19-targeted CAR into T cells, anti-CD19 CAR lentivirus (CD19 SCFv-CD3z-41BB) was generated using the Gibco™ CTS™ LV-MAX™ Lentiviral Production System (see "Integrated generation and characterization of CAR T cells" [3]). T cells that were isolated and activated for 24 hours were then transduced with CD19 CAR lentivirus at a multiplicity of infection (MOI) of 10. Cell characterization including CAR expression, CD4/CD8 ratio, cell number, and viability was performed on day 6 posttransduction.

Step 4. Expansion—CAR T cells

CAR T cells that were transduced with CD19 lentivirus vectors were then expanded in a HyPerforma Rocker Bioreactor or G-Rex bioreactor in complete medium. Feeding and monitoring was accomplished in a closed automated process controlled by the DeltaV platform in conjuction with Thermo Scientific™ TruBio™ Bioprocess Control Software.

Step 5. Cryopreservation

The CTS Rotea system was used to prepare the CAR T cells for cryopreservation by concentrating, washing, and placing the cells into a cryopreservation medium. Next, the cells were cryopreserved using the Thermo Scientific™ CryoMed™ Controlled-Rate Freezer.

Results

This section focuses on the robustness of the closed modular system in each step of the workflow as presented in Figure 1. In the manufacturing of autologous CD19 CAR T cells for these studies, the lentivirus encodes a second-generation CAR construct.

Step 1. Processing of leukapheresis product for PBMCs with the CTS Rotea system, controlled by the DeltaV platform

The generation of autologous CAR T cells for therapeutic use began by taking blood from a donor's vein through a process called leukapheresis. The white blood cells were then separated from the rest of the blood products, e.g., RBCs, plasma, and platelets. The CTS Rotea system was used to perform this step in a single-use consumable where the RBCs were lysed and eliminated, the platelets were removed in the washing steps of the process, and the PBMCs were optimally concentrated for isolation of T cells. To test the robustness of this critical first step, seven fresh or frozen quarter-size Leukopak bags were used to isolate PBMCs from seven donors. Across seven different Leukopak bags processed in the CTS Rotea closed system, RBCs were lysed efficiently and PBMCs were recovered consistently (Figure 2A). Importantly, the viability (Figure 2B) was only minimally affected by the CTS Rotea system.

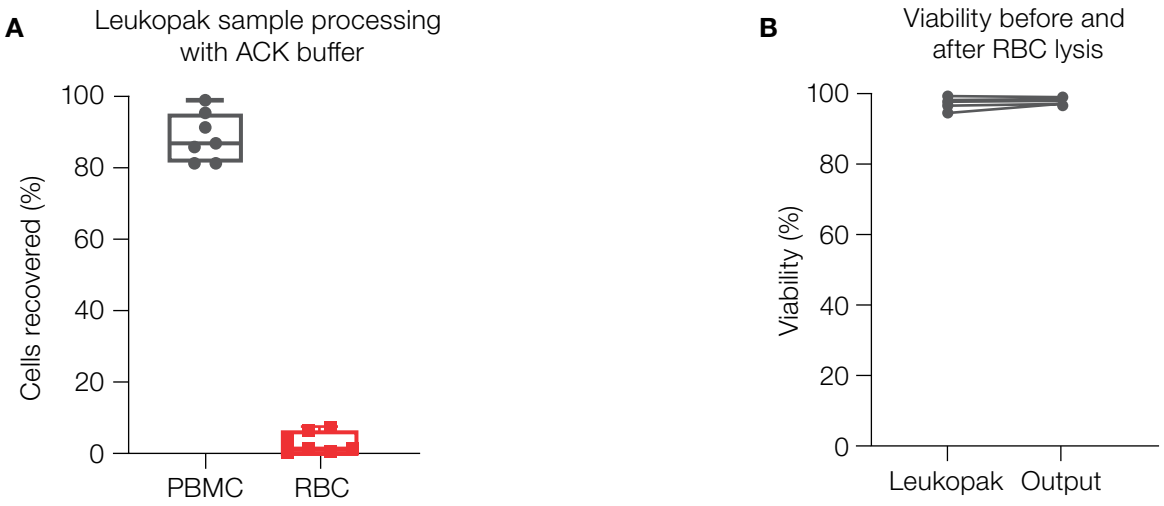

Figure 2. PBMC isolation using the Rotea system. PBMCs are efficiently recovered with no change in viability. Cells from Leukopak quarter packs were collected in the Rotea system and lysed with ACK buffer (n = 7). **(A)** 90% of PBMCs were recovered, versus 3% of red blood cells (CD235z$^+$). **(B)** No significant effect was observed on the viability before and after ACK lysis (P = 0.5).

Step 2. T cell isolation using the CTS DynaMag Magnet

Isolation of T cells from the processed PBMCs, as detailed in step 1, was carried out using CTS Dynabeads CD3/CD28. This is a one-step process to simultaneously isolate and activate T cells, which has been optimized to be used in numerous clinical research settings. As shown in Figure 3, a high percentage of the T cells were initially enriched during the PBMC isolation (step 1) using the CTS Rotea system. The cells were further enriched to 93% after step 2, using CTS Dynabeads CD3/CD28 and the CTS DynaMag Magnet.

Step 3. The generation of CD19 CAR T cells

In the context of manufacturing autologous CAR T cells, the gene transfer step used here is a well-published procedure and involves transduction of the isolated and activated T cells with a lentivirus vector made using the Gibco LV-MAX Lentiviral Production System [4,5]. T cells isolated from 3 of the 7 donors were used for the generation of CD19 CAR T cells. Transduction was performed with a lentivirus-encoded CD19 CAR at an MOI of 10, one day after T cell isolation and activation. CD19 CAR expression was assessed on day 3 or 4 and again on day 6. As shown in Table 1, CD19 expression on CAR T cells ranged from 20% to 60% among the three independent runs.

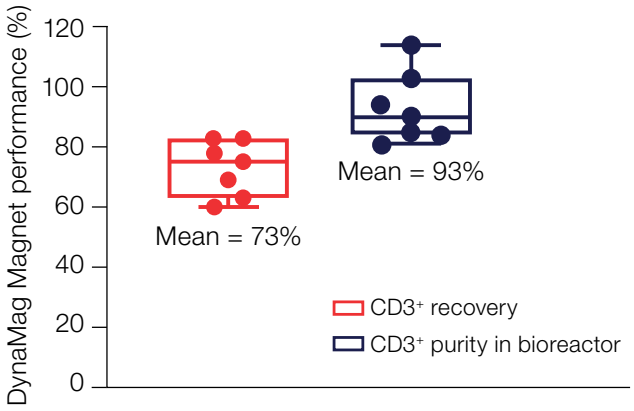

Figure 3. T cell isolation using CTS Dynabeads magnetic beads and CTS DynaMag Magnet controlled by DeltaV platform. T cells ($CD3^+$ $CD56^-$) were enriched to 73% post PBMC isolation using the Rotea system, and then were enriched to 93% post T cell isolation using CTS Dynabeads CD3/CD28.

Step 4. Expansion of CD19 CAR T cells

Culturing and expanding the CAR T cell product in CTS OpTmizer T Cell Expansion SFM is one of the most crucial steps in the manufacture of autologous CAR T cells. All aspects of this process, including every component in the autologous CAR T cell infusion bag for each donor, can be subject to evaluation by the regulatory agency for continuous compliance with quality control. The chemistry, manufacturing, and controls (CMC) process has to be tightly controlled and consistent.

The closed, semiautomated CAR T cell expansion step was carried out using the 10 L Thermo Scientific™ HyPerforma™ Rocker Bioreactor or G-Rex system, either of which can be controlled by the HyPerforma G3Lab platform. The scale can be expanded to 50 L bags if needed. If the HyPerforma Rocker Bioreactor is chosen, this process can be controlled and automated by TruBio software powered by the DeltaV system, which conforms to regulatory requirements for use in cGMP-compliant processes. As shown in Figure 4A, in 6 days there was a 20-fold expansion of T cells grown in either type of bioreactor. These CAR T cells showed significant potency in killing cancer cells, when challenged with a $CD19^+$ leukemia cell line such as Nalm6 (Figure 4B).

As summarized in Table 1, in 6 days, consistent generation of high-quality autologous CD19 CAR T cells for research or clinical use was seen in all three runs. In addition, these CD19 CAR T cells were especially enriched for cell naive/central memory–like phenotypes; these cells are known to show better persistence and function in *in vivo* assays (Figure 5).

Table 1. Characterization of CD19 CAR T cultures on day 6 of the autologous CD19 CAR T cell manufacturing process.

	Day 6 analysis of CD19 CAR T cultures from 3 runs (donors)			
	% CD19 CAR T cells	CD4/CD8 ratio	Total cell number	Viability
Range	20–60%	1.4–3.5	5,230–16,950 x 10^6	91–96%

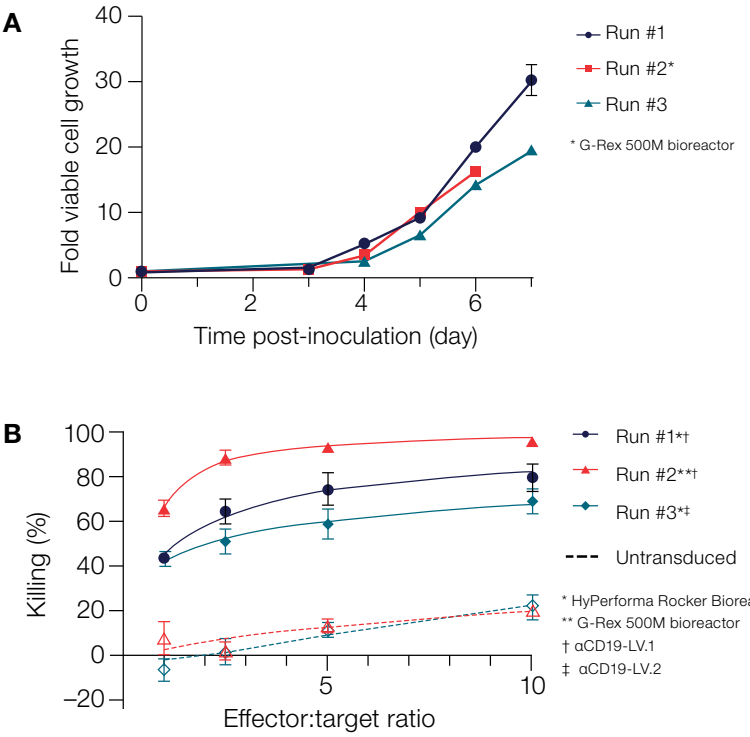

Figure 4. CD19 CAR T cell culture expansion and function. CD19 CAR T cells expanded robustly and were found to have high potency in killing cancer cells. **(A)** CD19 CAR T cells expanded in various bioreactor formats over 6–7 days—runs #1 and #3 in the 10 L HyPerforma Rocker BPC, and run #2 in the G-Rex 500M system. **(B)** On day 6, effector CD19 CAR T cells were challenged with Nalm 6 target cells (CD19⁺ leukemia cell line) at various effector:target ratios.

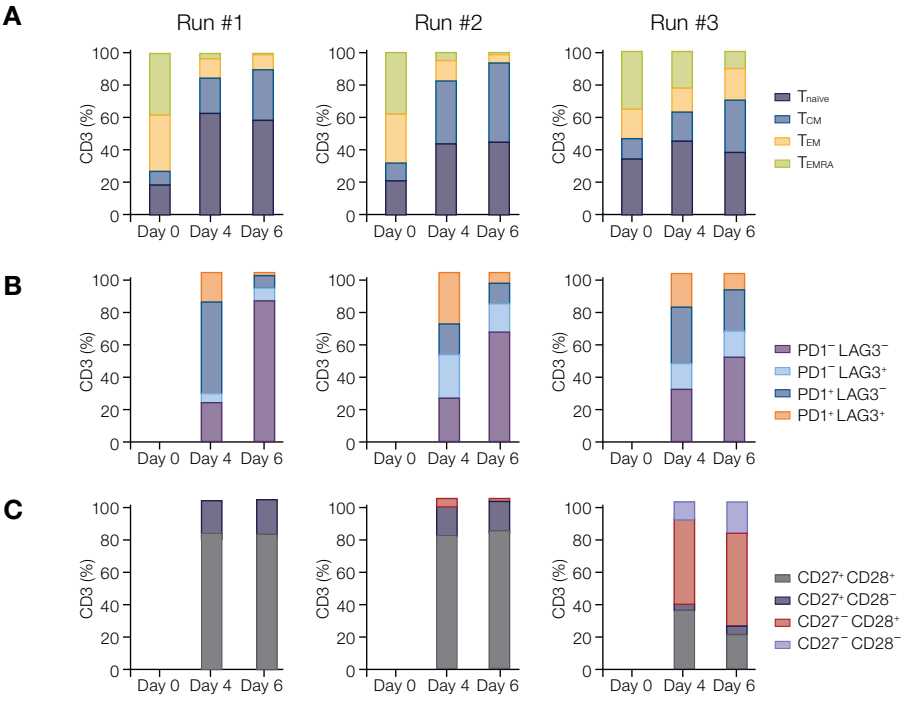

Figure 5. Analysis of CD19 CAR T cell cultures. Cell expansion condition favored both naive and central memory cell subsets over 6 days of expansion. Bioreactors used: HyPerforma Rocker Bioreactor 10 L for runs #1 and #3, and G-Rex 500M bioreactor for run #2. **(A)** T cell subsets markers: T_{naive} CD45RA⁺ CD62L⁺, T_{CM} CD45RA⁻ CD62L⁺, T_{EM} CD45RA⁻ CD62L⁻, T_{EMRA} CD45RA⁺ CD62L⁻. **(B)** Exhaustion T cell markers: PD-1, LAG3. **(C)** Naive-like markers shown to correlate with *in vivo* potency: CD27, CD28. CM: central memory; EM: effector memory; EMRA: effector memory cells re-expressing CD45RA.

Step 5. Cryopreservation of autologous CAR T cells.

The existing complex process for manufacturing autologous CAR T cells makes it challenging for the donors in clinical trials to receive freshly manufactured CAR T cells for therapy. Therefore, currently, they generally receive frozen CAR T cells transported from the manufacturing sites to the clinics, where the cells are thawed before infusion. For this reason, the cryopreservation of the CAR T cells needs to be as stringent and consistent as the manufacturing process, and requires strict regulatory compliance for clinical application if needed. Following preparation for cryopreservation with the CTS Rotea system, the cells were cryopreserved using the CryoMed Controlled-Rate Freezer, which has been routinely used in the cell therapy industry [6]. The controlled-rate freezing process maintained the integrity of the CAR T cells—the T cells were frozen, thawed, and monitored for 2 days for cell recovery, growth, and viability (Figure 6). The CD19 CAR T cells recovered and expanded well after they were placed in the CTS Rotea system and frozen using the CryoMed freezer.

Conclusions

Every component of the modular cell therapy manufacturing system described here is a GMP-compliant solution for manufacturing of CAR T cells. The "fit-for-purpose", semiautomated manufacturing platform for autologous CAR T cells includes modular instruments, proven CTS reagents, and a digital control system for the generation of a consistent CAR T cell product. The system described here is flexible and can deliver standardization, compatibility, and scalability in CAR T cell manufacturing. The modules in this system are compatible with most laboratory benchtops. The modular, GMP-compliant system allows laboratories to eliminate manual cell processing in manufacturing workflows. It is compatible with single-use consumables requiring only a class C laboratory environment, and it can be used to produce consistent CAR T cell therapy products.

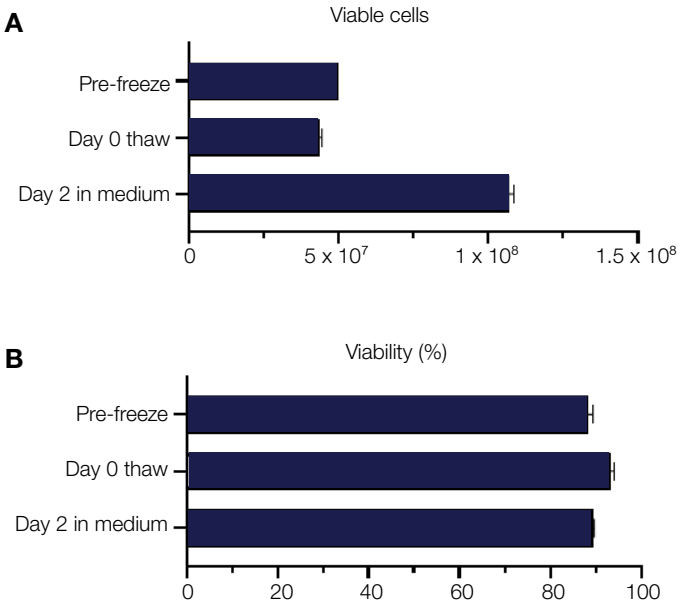

Figure 6. Characterization of cryopreserved CD19 CAR T cells. CD19 CAR T cell cultures showed consistent recovery after they were placed in the Rotea system and frozen in the CryoMed freezer. **(A)** Cell counts and **(B)** cell viability of cryopreserved and thawed CAR T cells.

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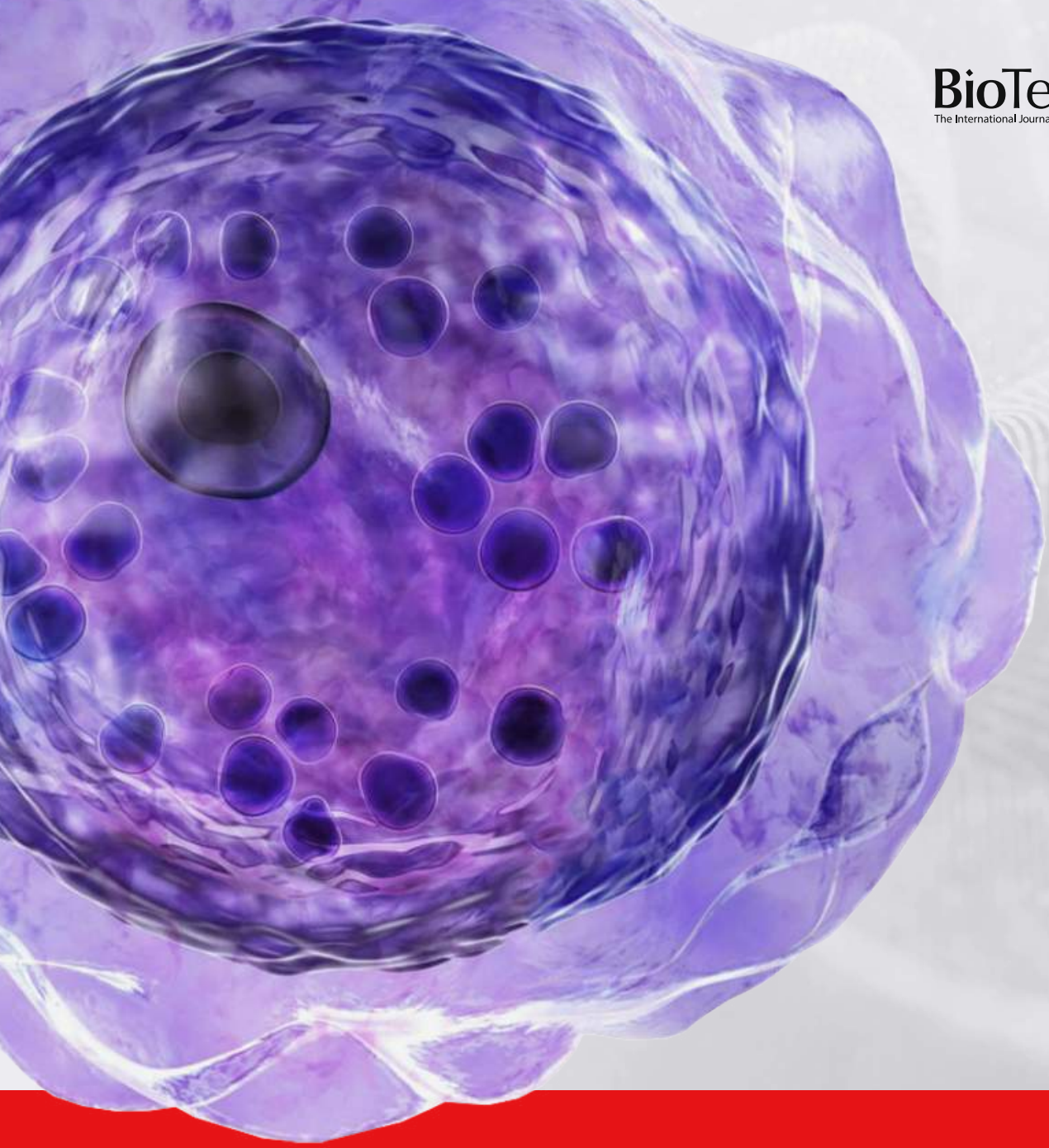

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