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Engineered T cells and their therapeutic applications in autoimmune diseases

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ABSTRACT

Chimeric antigen receptor T cells (CAR-T cells) are engineered recombinant T cells, which were initially used to treat hematopoietic malignancies and are now widely used in the treatment of various diseases. Considering their intrinsic targeting efficiency, CAR-T cells show considerable potential in the treatment of autoimmune diseases. Furthermore, regulatory T cells (Treg), a subset of CD4 T cells exhibiting immunosuppressive functions, have attracted increasing attention regarding CAR-Treg cell production. In this review, we report on recent developments in preclinical and clinical studies on CAR-T cells in autoimmune diseases and provide an outlook on opportunities and challenges of CAR-T application in such diseases.

Keywords: Chimeric antigen receptor T cells; Cell immunotherapy; Autoimmune diseases

INTRODUCTION

Systemic autoimmune diseases are characterized by the production of autoantibodies and autoreactive T lymphocytes, which attack the body's own organs (Figure 1). Autoimmune diseases suggest a breakdown of the body's immune tolerance, but the causes are unknown (Rose, 2016; Wang et al., 2015). In terms of immune mechanisms, autoimmune diseases fall into two main categories: autoantibodies that destroy cells by binding to the cell surface and autoreactive cytotoxic T lymphocytes (CTLs) that recognize target cells through the formation of T cell receptor (TCR)-major histocompatibility complex class I (MHC-I) complexes (Wahren-Herlenius & Dörner, 2013). Clinical treatments for autoimmune diseases mainly rely on the use of immunosuppressants, which not only exhibit low specificity and potentially cause long-term infection but are generally unable to eliminate the source of the disease (Durcan et al., 2019; Schmidt, 2018; Wang et al., 2015). Fortunately, chimeric antigen receptor T (CAR-T) cells have emerged as a novel therapeutic strategy for autoimmune diseases.

Chimeric antigen receptors (CARs) typically consist of a high-affinity antigen binding site on a monoclonal antibody (single chain fragment variant (scFv)) and a T cell-activating signal transduction domain, which allows them to recognize MHC-free antigens (Ramos & Dotti, 2011). Thus far, CARs have gone through four generations (Figure 2). First-generation CARs combined the scFV domains and CD4 (extracellular domain) with CD3ζ (intracellular domain), resulting in poor stability and clinical efficacy; second-generation CARs added additional co-stimulatory signaling domains, such as CD28 or 4-1BB, to CD3ζ, leading to enhanced *in vivo* expansion and persistence; and third- and

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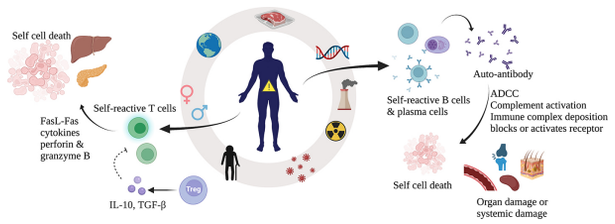

Figure 1 Mechanism of autoimmune diseases

Autoimmune diseases are characterized by an excessive host immune response, and are influenced by age, sex, region, genetics, infection, nutrition, environment, stress, and other factors (Wang et al., 2015). These diseases are also typically characterized by the presence of self-reactive B cells and T cells in the body (Wang et al., 2015). Tregs are natural immune regulatory cells that reduce the killing activity of T cells, establish immune surveillance, and prevent T cells from overreacting (Sakaguchi et al., 2008). Autoreactive CTLs recognize target cells by the binding of TCRs to a combination of MHC-I and autoantigen-derived peptides (Carreño et al., 2006). Subsequently, CTLs kill target cells by various mechanisms, leading to autoimmune disease (Halle et al., 2017). Autoantibodies can bind to cell surface markers, lyse cells, activate and block receptors, and form immune complexes with antibodies to deposit in blood vessels or tissues (Wang et al., 2015). Autoantibodies can mediate organ-specific or systemic damage through a variety of mechanisms, resulting in autoimmune disorders (Davidson & Diamond, 2001). CTL: Cytotoxic T lymphocyte; TCR: T cell receptor; FasL: Fas ligand; ADCC: Antibody-dependent cell-mediated cytotoxicity; Tregs: Regulatory T cells.

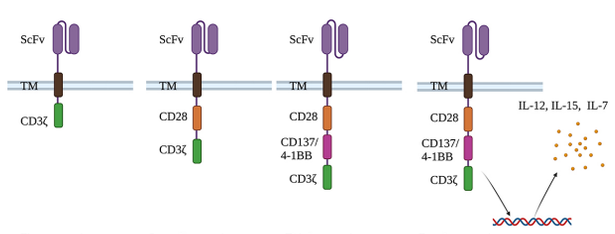

Figure 2 Four generations of CARs

First-generation CARs contain activation domain CDζ; Second-generation CARs contain an added co-stimulatory domain (CD28); Third-generation CARs contain another added co-stimulatory domain; and Fourth-generation CARs allow CAR-T cells to express certain cytokines, e.g., IL-12, IL-15, and IL-7, which significantly enhance T cell expansion. ScFv: Single chain antibody fragment; TM: Transmembrane domain; CD: Cluster of differentiation; IL: Interleukin.

fourth-generation CARs combined other co-stimulatory domains to exhibit different characteristics (June & Sadelain, 2018; Zhang et al., 2020).

Engineered CARs expressed in T cells or regulatory T (Treg) cells are called CAR-T and CAR-Treg cells, respectively. Methods for generating these kinds of T cells are similar. Briefly, the T cells or Treg cells are isolated from the blood of patients, then transduced with an overexpression vector encoding a synthetic receptor through viral or non-viral approaches (Zhang et al., 2020). Before injection into patients, CAR-Ts or CAR-Tregs need to be expanded to a specific number. To drive CAR-T cell expansion, the interleukin 2 (IL-2) cytokine and anti-CD3 antibodies are usually used by the cell processing centers (Makita et al., 2017). For Tregs, cell source is a major factor determining experimental success or failure. For example, Tregs directly induced by MHC peptides from the thymus (i.e., tTregs) are considered safer than peripheral Tregs (pTregs). Among tTregs, CD45RA$^+$ cells are considered safer than CD45RO$^+$ cells because CD45RO$^+$ cells may contain pTregs or effector T cells, which can expand quickly *in vitro*, but also exhibit high cytotoxicity rather than immunosuppression (Rosado-Sánchez & Levings, 2020).

As a highly promising approach in cancer therapy, CAR-T cells play an important role in the treatment of hematological malignancies (Yu et al., 2019) and are now being investigated in solid tumors (Martinez & Moon, 2019) and autoimmune diseases. Previous studies have mainly focused on the therapeutic efficacy of CD8$^+$ T cells (Maus et al., 2014). However, CD4$^+$ T cells, another subset of T cells in peripheral blood, have gained attention in CAR engineering (Zhang et al., 2020). After binding to the target antigen in the body, CAR-T cells undergo activation and proliferation, and then become cytotoxic and finally kill the target cells (Sadelain et al., 2013). As a living drug, CAR-T cells show good targeting, persistence, and clinical activity, and persist in the peripheral blood of patients with B cell acute lymphoblastic leukemia (B-ALL), diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), and multiple myeloma (MM) for at least 3–6 months (Abramson, 2020; Dai et al., 2020; Finney et al., 2019; Majzner & Mackall, 2019; Raje et al., 2019; Turtle et al., 2016). Furthermore, 30%–40% of patients appear to achieve long-term remission, with few relapses occurring beyond 12 months, indicating a potential cure (Kersten et al., 2020). Although cytokine release syndrome (CRS) and neurological toxicity have been observed in patients with refractory B-cell lymphoma when treated with CD19-CAR-T cells (Acharya et al., 2019; Neelapu et al., 2017), clinical trials have also shown encouraging activity and disease relief (Abramson, 2020; Brudno et al., 2020; Raje et al., 2019; Schuster et al., 2017). Furthermore, such adverse reactions can be decreased through dosage reduction, steroid therapy, and antibody introduction (especially anti-IL-6R antibodies) (Acharya et al., 2019; Hartmann et al., 2017). Currently, five CAR-T cell therapies have been approved, including anti-CD19-CAR-T cells for lymphoma treatment (Abramson, 2020; Sermer & Brentjens, 2019) and anti-B cell maturation antigen (BCMA)-CAR-T cells for MM treatment (D'Agostino & Raje, 2020). Several phase I clinical trials of BCMA/CD19-CAR-T cells have been reported in refractory autoimmune diseases (Table 1), which may become a new therapeutic option. In addition, specifically designed CAR-T cells are showing better potency in treating autoimmune diseases than traditional ones, which we discuss in this review.

CAR technology has now been extended to CAR-Treg therapy. Tregs are a subset of CD4$^+$ T cells with immunosuppressive functions and include tTregs (directly induced by MHC peptides from the thymus), pTregs (stimulated by antigens in peripheral lymphoid tissue), and iTregs (induced *in vitro*). Tregs are characterized by the co-expression of CD25 and Foxp3. Foxp3 plays an important role

Table 1 Clinical trials of CAR-T or CAR-Treg cell treatment for autoimmune diseases

Specific autoantigen	Intervention	Disease	Phase	Status	Completion date	NCT ID
BCMA(CD269)	Descartes-08 CAR-T	Myasthenia Gravis (MG)	I&II	Recruiting	1 April 2022	NCT04146051
	CT103A cell	Neuromyelitis optica spectrum disorder (NMOSD)	I	Recruiting	31 December 2023	NCT04561557
BCMA&CD19	CD19/BCMA CAR T	Sjogren's syndrome (SS)	I	Recruiting	5 November 2024	NCT05085431
	CD19/BCMA CAR T	Scleroderma	I	Recruiting	8 October 2024	NCT05085444
	CD19 / BCMA CAR T	Immune Nephritis	I	Recruiting	5 November 2024	NCT05085418
	CD19/BCMA CAR T	Systemic Lupus Erythematosus (SLE）	I	Recruiting	10 September 2022	NCT05030779
CD19	Anti-CD19 CAR-T	Systemic Lupus Erythematosus (SLE）	I	Unknown	March 2018	NCT03030976
Anti-Dsg3 surface immunoglobulin	DSG3-CAAR T	Mucosal-dominant PV (mPV)	I	Recruiting	September 2026	NCT04422912

BCMA: B cell maturation antigen; CAR-T: Chimeric antigen receptor T cell; Dsg3: Desmosomal core glycoprotein-3.

in the function of Treg cells, and its mutation can lead to serious autoimmune diseases (Göschl et al., 2019). Treg cell-based therapy has been applied in several preclinical and clinical trials, such as graft versus-host disease (GvHD) in non-cytotoxic conditions and type I diabetes mellitus (T1D) to prolong islet survival time. However, traditional methods for amplifying Treg or gene-edited TCR-Treg cells are inefficient and MHC dependent. In addition, the number of cells required for infusion is very large. Moreover, non-specific immunosuppression may occur during injection. Therefore, CAR-Treg cells are gradually being developed to play a safer and more effective therapeutic role (Zhang et al., 2018). First, CAR-Treg cells can recognize target antigen on target cells through the CAR structure and inhibit effector T cell function. For example, Fas ligands (FasL) on Treg cells can bind to Fas on effector T cells to induce apoptosis. CTLA-4, another membrane protein, can competitively interact with antigen-presenting cells (APCs) to inhibit CD28-mediated T cell activation. On the other hand, IL-2 plays an important role in the proliferation and differentiation of effector T cells. However, CAR-Treg cells can competitively bind to IL-2 and inhibit effector T cell proliferation (Yamaguchi et al., 2011). CAR-Treg cells have been successfully applied in colitis, multiple sclerosis (MS), and T1D. Carcinoembryonic antigen (CEA) CAR-Tregs (SCA431, scFv, CD28, and CD3ζ) have been used in colitis treatment, with effective results (Blat et al., 2014). Myelin oligodendrocyte glycoprotein (MOG) CAR-Tregs (MOG scFv, CD28, and CD3ζ) have been used in MS treatment, showing effective control of inflammation (Fransson et al., 2012). CAR-Treg cells (insulin scFv, CD28, and CD3ζ) have also been used in T1D, showing successful treatment with no effects on recipient immunity (Tenspolde et al., 2019). Other potential clinical applications include treatment for GVHD, hemophilia A, and asthma. For example, HLA-A2 CAR-Tregs may be effective in preventing GvHD (MacDonald et al., 2016). Compared with polyclonal Tregs or TCR-Tregs, CAR-Tregs are MHC independent, with high target site specificity, low non-specific immunosuppression, and low sensitivity to IL-2 concentration.

In this article, we analyze the use of CAR-T and CAR-Treg cells in autoimmune diseases and discuss future prospects for these diseases (Table 2).

SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)

Introduction of disease

SLE is an autoimmune disease caused by the immune system mistakenly attacking healthy tissues in many parts of the body (Poole et al., 2009) and is characterized by red facial rashes (Tebbe & Orfanos, 1997). There is currently no cure for SLE, but conventional treatments include non-steroidal anti-inflammatory drugs, corticosteroids, immunosuppressants, hydroxychloroquine, and methotrexate (Murphy & Isenberg, 2013). As SLE significantly increases the risk of cardiovascular diseases, patients often die from SLE, and their life expectancy is usually short (Murphy & Isenberg, 2013).

SLE is caused by genetic susceptibility and environmental triggers (He & Sawalha, 2018; Yang et al., 2009). From the genetic perspective, multiple genes can affect the incidence rate of SLE. Studies have shown that HLA class I, class II, and class III genes are related to SLE, and genes such as *IRF5*, *PTPN22*, and *STAT4* are also involved in disease progression (Yang et al., 2009). From an environmental perspective, lack of vitamin D as well as the use of certain drugs, such as procainamide, isoniazid, hydralazine, quinidine, and phenytoin, may induce lupus (He & Sawalha, 2018).

Application of CAR-T and CAR-Treg in SLE

Although CAR-T has been successfully applied in SLE animal models, CAR-Tregs remain relatively rare, but both show good prospects. For example, CAR-T therapy targeting $CD19^+$ B cells in MRL-lpr mice can reverse diseased organ damage and prolong survival time with reduction in autoimmune antibody production (Kansal et al., 2019). In addition, after transfusing $CD8^+$ T cells transduced with relevant A-MLV retrovirus, CD19 gene probes have proven that CAR-T can effectively treat mice with SLE, resulting in reduced *CD19* gene expression in the spleen and extended lifespan in MRL-lpr mice. Similarly, researchers have used CAR-T in New Zealand black×New Zealand white (NZB×NZW) F1 mice by isolating, purifying, and modifying $CD8^+$ T cells to eliminate potential disease enhancement of $CD4^+$ T cells (Kansal et al., 2019). Specifically, CD19-targeted CAR-T plasmids are transduced into splenic $CD8^+$ T cells through different viral transduction methods, then infused into mice with obvious SLE symptoms, resulting in $CD19^+$ B cell hypoplasia, thus

Table 2 Summary of therapeutic targets of CAR-T and CAR-Treg cells and established animal models for studies on autoimmune diseases

CAR	Disease	Target	Animal model	CAR reference
Anti-CD19 CAR-T	SLE	CD19	MRL/lpr mice NZB×NZW F1 mice	Kansal et al., 2019; Jin et al., 2021 Kansal et al., 2019
Insulin/IGPR-reactive CAR-T	T1D	Insulin/IGPR-reactive TCR	NOD mice	Fishman et al., 2017
mAb287-CAR-T	T1D	I-A^{g7}-B:9-23 (R3) antigen complex	NOD mice	Zhang et al., 2019
Insulin-specific CAR-Treg	T1D	Insulin	NOD mice	Tenspolde et al., 2019
TNP-TPCR Treg	Colitis	TNP	Hapten-mediated colitis mice	Elinav et al., 2009
CEA-CAR-Treg	Colitis and colorectal cancer	CEA	DSS-induced colitis mice, AOM and DSS-induced colorectal cancer mice	Blat et al., 2014
CARαMOG-FoxP3-Treg	MS	MOG	Chronic EAE mice	Fransson et al., 2012
MBP-CAR Treg and MOG-CAR Treg	MS	MBP and MOG	Chronic EAE mice	De Paula Pohl et al., 2020
Anti-FITC CAR-T	RA	Antigenic FITC-peptide	Collagen-induced arthritis (CIA) mice	Zhang et al., 2021
Dsg3 CAAR-T	PV	Anti-Dsg3 surface immunoglobulin	Autoantigen-knockout mice Passive transfer mice Skin graft mice	Ellebrecht et al., 2016

CAR-T: Chimeric antigen receptor T cell; CAR-Treg: Chimeric antigen receptor regulatory T cell; SLE: Systemic lupus erythematosus; T1D: Type 1 diabetes; MS: Multiple sclerosis; RA: Rheumatoid arthritis; PV: Pemphigus; TNP: 2,4,6-trinitrophenol; TPCR: Tripartite chimeric receptor; TNBS: 2,4,6-trinitrobenzenesulphonic acid; CEA: Carcinoembryonic antigen; DSS: Dextran sulfate sodium; AOM: Azoxymethane; MOG: Myelin oligodendrocyte glycoprotein; EAE: Encephalomyelitis; MBP: Myelin basic protein; CFA: Freund's adjuvant; PT: Pertussis toxin; FITC: Fluorescein isothiocyanate; Dsg3: Desmosomal core glycoprotein-3; CAAR-T: Chimeric autoantibody receptor T cells.

suggesting effective treatment (Figure 3) (Kansal et al., 2019). Jin et al. (2021) also infused MRL-lpr mice with the same anti-mouse CD19 CAR-T cells and tested different treatment strategies, e.g., CAR-T and monoclonal antibody therapies and different second-generation CAR-T cells, by adopting different co-stimulatory motifs (e.g., CD28 and 4-1BB) in the intracellular domain of CAR-T cells. For the former, they found that infusion of anti-CD19 CAR-T cells showed more sustained B cell depletion in MRL-lpr mice; for the latter, they found that CAR-T cells with 4-1BB showed better treatment effects. They also found that pretreating mice with low-dose total body irradiation (TBI) greatly improved the survival rate (Figure 3). Recently, CAR-T therapy targeting CD19 molecules on B cells was used for a female patient with severe SLE, who had failed to respond to various treatments (Mougiakakos et al., 2021). Results showed that 44 days after CAR-T cell infusion, autoimmune antibodies were undetectable and the disease condition was alleviated, with no obvious side effects (Figure 3). For CAR-Treg cells, the immune environment in SLE patients can significantly reduce the number and inhibitory function of Treg cells, and thus disrupt immune homeostasis (Kim et al., 2015). First, the large amount of IL-6 and interferon (IFN)-α secreted by dendritic cells (DCs) can hinder the inhibitory function of Treg cells, and IL-21 secreted by helper $CD4^+$ T cells can also affect the survival and function of Treg cells (Guo et al., 2019). Second, the strong pro-inflammatory environment in SLE patients can also transform Treg cells to secrete IL-17 and other inflammatory cytokines. As CAR-Treg itself has monoclonal resistance, restoring the number of Treg cells and related inhibitory functions through CAR-Treg can effectively prevent autoimmune diseases (Haddadi et al., 2020). At the same time, SLE can induce many autoantibodies (~180), suggesting many autoantigens can be targeted, including nuclear antigens, cytoplasmic antigens, cell membrane antigens, phospholipid-related antigens, blood cells, endothelial cells, and nervous system antigens (Yaniv et al., 2015). Autologous Treg infusion can activate Tregs in inflamed skin, thus weakening the IFN-γ pathway and $CD4^+$ effector cell infiltration (Dall'Era et al., 2019). Low-dose IL-2 treatment of 60 patients with active SLE was shown to be effective and

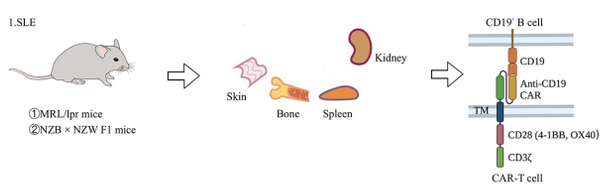

Figure 3 Application of CAR-T cells in animal model of SLE

SLE is an autoimmune disease caused by the immune system mistakenly attacking healthy tissues in many parts of the body. Currently, gene-edited T cells have been used in two mouse models: MRL/lpr mice and NZB×NZW F1 mice. MRL/lpr mice are produced by mating LG/J, AKR/J, C3H/HeDi, and C57BL/6J mouse strains, which are characterized by accumulation of double negative ($CD4^-CD8^-$) $B220^+$ T cells (Murphy & Roths, 1978). NZB×NZW F1 mice are produced by crossing NZB and NZW mice (Helyer & Howie, 1963). In this animal model, anti-RNA-related antibodies are rarely produced and SLE pathogenesis is mainly due to suppression of Treg cells through its own MHC (Helyer & Howie, 1963). Treatment is to inhibit $CD19^+$ B cells by CAR-T cells specifically targeting CD19, thereby curing SLE. SLE: Systemic lupus erythematosus; 4-1BB: Tumor necrosis factor receptor superfamily member 9; OX40: Tumor necrosis factor receptor superfamily member 4; lpr: Lymphoid proliferation gene; NZB: New Zealand black mouse; NZW: New Zealand white mouse; MHC: Major histocompatibility complex.

restored tolerance (He et al., 2020). From these findings, CAR-Treg therapy for skin and kidney treatments could restore immune tolerance and reduce inflammation within the affected tissues.

Currently, two phase I clinical studies are being carried out in China. One study at the recruitment stage (NCT05030779) aims to assess the safety and effectiveness of CD19/BCMA CAR-T cells in the treatment of refractory SLE. The second study (NCT03030976) aims to assess the safety and efficacy of second-generation CAR-T cells with an optimized hinge and transmembrane domain in the treatment of SLE patients, although the results are not yet known.

Of course, CAR-T treatment for SLE has several unique advantages and limitations. For its advantages, CAR-T can eliminate $CD19^+$ B cells in a targeted manner, reduce the production of autoimmune antibodies, and show persistence in elimination functions. However, researchers have also identified a subgroup of B cells with lower IgM levels (IgMlo) in CAR-T-treated MRL-lpr mice that do not express CD19 targets (Kansal et al., 2019), with the CAR-T-treated MRL-lpr mice still retaining antibody-producing cells and showing incomplete disease treatment. In addition, control (TBI+phosphate-buffered saline) and TBI+CAR-T-treated mice show no significant differences in their concentrations of certain antibodies, such as serum anti-dsDNA and anti-nuclear antibodies (Jin et al., 2021). The non-reduction of these antibodies is a considerable issue in the treatment of SLE.

TYPE 1 DIABETES (T1D)

Introduction of disease

Also known as juvenile diabetes, T1D is characterized by patients producing little or no insulin from the β cells of the islets of Langerhans in the pancreas (Bluestone et al., 2010). Typical symptoms include frequent urination, thirst, increased hunger, and weight loss (Torpy et al., 2007), and potentially blurred vision, fatigue, and slow wound healing (Toni et al., 2017).

T1D is caused by the destruction of β cells (the only insulin-producing cells in the body) and subsequent progressive lack of insulin (Bluestone et al., 2010). In 70%–90% of cases, β cells are destroyed by one's own immune system, while the specific reason for this is not fully understood (Katsarou et al., 2017). The remaining 10%–30% of patients show β cell destruction but no signs of autoimmune disease, and thus are diagnosed with idiopathic diabetes (Katsarou et al., 2017). At present, various theories have been espoused to explain the mechanisms of T1D, including genetic susceptibility with familial tendencies and environmental factors, e.g., maternal obesity, caesarean birth, and high dietary sugar intake (Norris et al., 2020).

Application of CAR-T and CAR-Treg in T1D

To date, CAR treatment for T1D has primarily focused on CAR-T, with some limited application of CAR-Treg. Notably, Treg cells can inhibit autoreactive T cells, which is essential for maintaining immune tolerance and homeostasis of the immune system, and even impacts the sex differences found in autoimmune diseases (Nie et al., 2015).

For CAR-T, researchers have redirected genetically modified T cells to pathogenic $CD8^+$ T cells using MHC as an activation receptor (Fishman et al., 2017). Based on the conclusion that the signaling component of MHC-I molecules is the fusion product of b2 microglobulin (b2m) and CD3-ζ chain, studies have combined H-2Kd with the insulin B chain (amino acids 15–23 (InsB15-23)) and linked it to the N-terminus of the b2m/CD3ζ daughter chain. This design forms a specific MHC activation receptor, which specifically targets $CD8^+$ T cells carrying TCRs with certain peptides and specifically inhibits insulin-responsive or pancreatic islet-specific glucose-6-phosphatase-related protein (IGRP)-responsive T cells. After using this CAR-T therapy, non-obese diabetic (NOD) mice are reported to show significant relief from insulitis and diabetes (Figure 4). Rather than targeting $CD8^+$ T cells, Zhang et al. (2019) targeted the APC of $CD4^+$ T cells in T1D NOD mice for CAR-T. This APC usually carries an I-Ag7-B:9-23 (R3) antigen complex, which consists of 9–23 residues of the insulin B chain (InsB9-23) bound to MHC class II molecules (I-Ag7), to stimulate the proliferation of $CD4^+$ T cells. Thus, Zhang et al. (2019) established a monoclonal antibody (mAb287) against this I-Ag7-B:9-23 (R3) antigen complex for use as the extracellular domain of CAR-T, resulting in cytotoxic effects on APC and delayed T1D onset in NOD mice (Figure 4).

Before CAR-Treg was developed, cell therapy for T1D mainly focused on Treg cell re-infusion (Tang et al., 2004; Bluestone et al., 2015). However, T1D patients often present

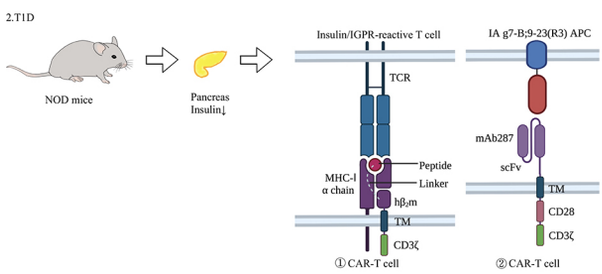

Figure 4 Application of CAR-T cells in animal model of type 1 diabetes

T1D is characterized by a progressive lack of insulin caused by the destruction of pancreatic β cells, leading to typical symptoms of diabetes. At present, the application of gene-edited T cells is limited to animal models such as NOD mice. NOD mice are established via inbreeding and selective breeding, resulting in pancreatic atrophy and fibrosis, decreased insulin secretion, and clinical symptoms resembling human diabetes (Makino et al., 1980). Various treatments have been established: e.g., construction of specific MHC activators by linking the H-2Kd and amino acid 15–23 (InsB15-23) complex of the insulin B chain peptide to the N-terminus of b2m/CD3ζ receptors, thereby targeting $CD8^+$ T cells with specific peptides to inhibit insulin-responsive or IGRP-responsive T cells (Fishman et al., 2017); and establishing monoclonal antibodies (mAb287) against the I-Ag7-B:9-23 (R3) antigen complex for use as the extracellular domain of CAR-T to target $CD4^+$T APCs (Zhang et al., 2019). TCR: T cell receptor; MHC-1α chain: Major histocompatibility complex-1α chain; mAb287: Monoclonal antibody287; scFv: Single chain Fv; NOD: Non-obese diabetic.

with a small number of $CD4^+CD25^+$ Tregs but a large expansion of autoreactive T cells. To solve this issue, researchers have long explored technologies for the rapid and massive proliferation of specific Tregs. For example, Tang et al. (2004) used a combination of anti-CD3, anti-CD28, and IL-2 antibodies to achieve 200 times purification of $CD4^+CD25^+$ Tregs in less than 2 weeks, with the amplified Tregs not only expressing classic cell surface antigens, but also inhibiting effector T cells *in vitro* and *in vivo*. They also infused related Treg cells into NOD mice, which showed effective reversal of the diabetic phenotype (Tang et al., 2004). In addition, Bluestone et al. (2015) reported on a phase I clinical trial that isolated and expanded Tregs from T1D patients and applied adoptive immunotherapy in 14 adult T1D patients. Their results showed that one year after infusion, Tregs remained at a quarter of the peak level and retained the $FOXP3^+CD4^+CD25^{hi}CD127^{lo}$ phenotype, with no obvious clinical adverse reactions. Although the targeting of the above-mentioned Treg cells to self-reactive T cells is not yet clear, CAR-Treg therapy not only reduces the number of Treg cells required for an effective response, but also shows good specificity and low off-target effects. Tenspolde et al. (2019) first redirected T effector cells to Tregs through Foxp3 transduction, and then used CAR technology to direct CAR-Tregs to the pancreatic region of NOD mice. These transformed CAR-Tregs showed continuous inhibitory properties and pancreatic region specificity, with no apparent effects on overall immunity of the recipient.

However, CAR-Treg and CAR-T application also show limitations in current T1D treatment. For example, although Zhang et al. (2019) induced a delayed in T1D onset in NOD mice, treatment did not stop disease occurrence. In addition, Tenspolde et al. (2019) only found strong immunosuppressive function of CAR-Treg *in vitro*, not in live NOD mice. Therefore, it is necessary to discover further strategies to prolong and expand metastatic CAR-T cell activity. The stability of CAR-Treg cells is also worth attention as the transformation of CAR-Treg cells into effector T cells may further aggravate the disease. For example, research on mice has shown that, when exposed to inflammatory conditions, some Tregs will lose Foxp3 expression and undergo transformation, resulting in a pro-inflammatory phenotype (Zhou et al., 2009). In addition, disturbance in the balance between Treg and Th17 can induce immune disorders (Zhang et al., 2021b). Moreover, the separation and purification of CAR-T and CAR-Treg can be difficult as current clinical GMP reagents rarely reach 100% purity. Furthermore, our understanding of how CAR-T and CAR-Treg cells differentially proliferate and transport in the body remains incomplete. Therefore, $CD8^+$ CAR-T cells that have not been isolated may expand and cause disease aggravation and possible adverse effects (Magnuson et al., 2015).

COLITIS

Introduction of disease

Colitis is a chronic inflammatory disease of the colon (Adams & Bornemann, 2013). The etiology of microscopic colitis is unknown, but is associated with autoimmune disorders, such as celiac disease, polyarthritis, and thyroid disorders (Langner et al., 2015). Exposure to medications, such as non-steroidal anti-inflammatory drugs, proton pump inhibitors, and selective serotonin reuptake inhibitors, is suspected to play a role in colitis, although their direct causal relationship has not been proven (Park et al., 2015).

Application of CAR-T and CAR-Treg in colitis

Application of 2,4,6-trinitrophenol (TNP)-tripartite chimeric receptor (TPCR) Tregs in treatment of colitis: $Foxp3^+$ Tregs express the key transcription factor FOXP3, which plays a critical role in the maintenance of immune tolerance and homeostasis (Wang et al., 2021). Insufficient accumulation or impaired function of Treg cells in inflammatory sites is the main cause of a variety of autoimmune diseases: including autoimmune thyroiditis, gastritis, insulitis, arthritis, encephalomyelitis, and colitis (Maul et al., 2005). However, as direct Treg therapies may be inadequate for controlling autoimmunity and inflammation, strategies to induce or expand endogenous Treg cells *in vivo* may be a good option (Liang et al., 2021). In animal models, adoptive transfer of Treg cells can effectively prevent and improve autoimmune diseases caused by Treg cell abnormalities. As antigen-specific Treg cells are extremely rare, studies usually use adoptive transfer of a large number of non-specific Tregs or TCR-expressing Tregs to recognize specific MHC antigen peptide complexes isolated from transgenic mice (Chen et al., 2019). Using a transgenic method, Elinav et al. (2009) developed Treg cells expressing a chimeric receptor (CR) containing an antibody variable region specific for TNP, which fuses to the extracellular and transmembrane domains of CD28 co-stimulatory molecules and intracellular domain of stimulatory Fc-γ receptor chain (Figure 5). Transgenic mice expressing this receptor on all T cells are resistant to 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis (Elinav et al., 2008). For non-transgenic mice receiving a small amount of TPCR-expressing

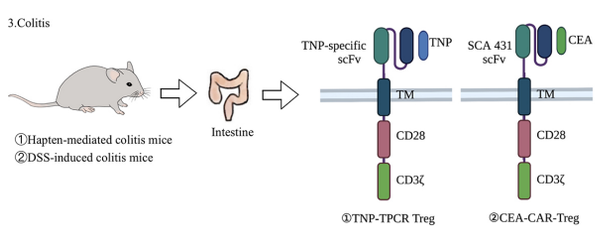

Figure 5 Application of CAR-Treg cells in animal model of colitis

Two CAR-Tregs have been applied in colitis. TNP-TPCR Tregs express a TNP-specific scFv, which is fused to the extracellular and transmembrane domain of CD28 co-stimulatory molecule and intracellular domain of stimulatory Fc-γ receptor chain. TNP-TPCR Tregs have been applied in hapten-mediated colitis mouse models, which are induced by TNBS or oxazolone (Elinav et al., 2008). CEA-CAR-Tregs express CEA-SCA431 CAR signaling domains fused with CD28-CD3ζ in $CD4^+CD25^+$ Treg cells. CEA-CAR-Tregs have been applied in dextran sulfate sodium (DSS)-induced colitis mouse models (Tanaka et al., 2003). TNP: 2,4,6-trinitrophenol; TPCR: Tripartite chimeric receptor; scFv: Single chain antibody fragment; CEA: Carcinoembryonic antigen.

Treg cells, cells accumulate and activate in the inflamed colon. TNP-TPCR Tregs can cure acute experimental colitis via their antigen specificity, no MHC restriction, and co-stimulatory signal independence. In addition, TNBS has also been used to inhibit non-TNBS-induced colitis. Although, Tregs containing TNP-specific CR did not inhibit oxazolidone-induced colitis, the addition of trace amounts of TNBS cured oxazolidone-inflamed colons, thereby proving the "bystander effect" of TNBS (Elinav et al., 2008).

To use TNP-TPCR Tregs in non-transgenic environments (such as in human diseases), Tregs must be transduced with a vector encoding CR, screened according to the expression level of TPCR, and finally amplified *in vitro* for clinical application (Elinav et al., 2009). Fontenot et al. (2003) transduced effector T cells with retrovirus or lentivirus encoding Foxp3 to partially obtain regulatory function. However, naturally occurring Tregs (nTregs) are highly resistant to retroviral and lentiviral vector transduction, which may hinder the pre-activation of Tregs, a necessary step for successful and effective retroviral transduction (Li et al., 2005) and thus clinical application. Li et al. (2005) described a pathway that can achieve efficient and reproducible retroviral transduction as well as selection and amplification of mouse nTregs, thus allowing rapid *in vivo* and *in vitro* amplification of antigen-specific nTregs that retain Foxp3 expression and inhibitory function. This enables TNP-TPCR Tregs to proliferate in an antigen-specific manner and accumulate in target organs for clinical treatment of colitis (Maldini et al., 2018).

TNP-TPCR Tregs have advantages over normal human Tregs. As mentioned above, their characteristic surface molecule (CR) is composed of an antibody variable region specific to TNP, extracellular and transmembrane domains of co-stimulatory molecule CD28, and intracellular domains of the stimulatory Fc-γ receptor chain. As such, TNP-TPCR Tregs exhibit antigen specificity, no MHC restriction, co-stimulatory signal independence, specific proliferation, good targeting, and rapid action. However, the resistance of nTregs to viral vector transduction is a challenge for TNP-TPCR Treg production, and a major problem for clinical application. In addition, adoptive transfer of engineered Treg cells contains some risks, e.g., the *in vivo* inflammatory environment may trigger the transformation of Treg cells into antigen-specific pathogenic effector T cells (McGovern et al., 2017). To solve this problem, techniques such as CRISPR can be used for gene editing. For example, Treg cells can be protected from inflammatory signals by editing cytokine receptors such as IL-6 and the tendency of Treg cells in autoimmune patients to produce inflammatory cytokines can be eliminated by removing IFN-γ or IL-17 genes from engineered Treg cells (Freen-van Heeren, 2021).

Application of CEA-CAR-Treg in treatment of colitis: Carcinoembryonic antigen (CEA)-CAR-Tregs are obtained by transducing specific CEA-SCA431 CAR signaling domains fused with CD28-CD3ζ in $CD4^+CD25^+$ Treg cells (Blat et al., 2014) (Figure 5), about 90% of which are $Foxp3^+$. CEA-CAR-Tregs can effectively alleviate T cell metastatic colitis and inhibit the occurrence of colitis-related colorectal cancer induced by azoxymethane-dextran sodium sulfate (AOM-DSS) (Elinav et al., 2008). CEA-CAR-Tregs can reside and accumulate at sites that express CEAs, and mainly accumulate in the inflamed colon, with much lower concentrations found in the small intestine and other internal organs (Blat et al., 2014). They have many advantages, such as strong antigen specificity, no MHC restriction, specific proliferation, co-stimulatory signal independence, good targeting, and rapid action. As such, CEA-CAR-Tregs show great potential for improving ameliorated colitis and preventing colitis-associated colorectal cancer. However, CEA-CAR-Tregs have a short lifespan, accumulating and expanding in the colon for only about 7 days, then diminishing rapidly to undetectable levels at 9 days after injection (Zmievskaya et al., 2021). This sharp decline in signal intensity could be attributable to an immune response against potential epitopes on the CAR or against the luciferase reporter protein (Blat et al., 2014). Activation-induced cell death may also be responsible for the rather short longevity of CAR-Tregs *in vivo*. At present, their short lifespan has limited the clinical application of CEA-CAR-Tregs in the treatment of colitis, with no clinical trials yet reported.

MULTIPLE SCLEROSIS (MS)

Introduction of disease

MS is a chronic immune-mediated demyelinating disease of the central nervous system (Compston & Coles, 2008). Although its etiology remains elusive, environmental factors and susceptibility genes are involved in disease pathogenesis (Correale et al., 2017). MS is widely considered an autoimmune demyelinating disease, with autoimmune reactions by myelin-specific $CD4^+$ T helper 1 (Th1) and Th17 cells (Sospedra & Martin, 2005). Results from immunological, genetic, and histopathological studies of MA patients further support the concept that autoimmunity plays a major role in the pathogenesis of MS (McFarland & Martin, 2007).

Application of CAR-T and CAR-Treg in MS

CAR treatments for MS have primarily focused on the transformation of CAR-Tregs. Fransson et al. (2012) used the lentiviral vector system to transduce $CD4^+$ T cells to express the CAR-targeting myelin oligodendrocyte glycoprotein (MOG), which were then induced by mouse FoxP3 for differentiation into Tregs and CD28-CD3ζ signaling domains (Figure 6). They used CAR-Tregs to conduct both *in vitro* and *in vivo* experiments and found that CARαMOG-FoxP3-Tregs can continuously inhibit T cell proliferation in the presence of MOG^+ cells and in the presence of activated macrophages. For the *in vivo* experiments, cells were administered to autoimmune encephalomyelitis (EAE) mice by intranasal (in) cell delivery. First, after 24 h of infusion of GFP and CARαMOG-FoxP3-co-expressing Tregs, immunohisto-chemical markers for myelination (myelin basic protein, MBP) and reactive astrogliosis (glial fibrillary acidic protein, GFAP) were recovered in treated mice compared to the controls, suggesting good targeting by the CAR-Tregs. Second, treatment of mice with an EAE score of 4 (hind limb paralysis) resulted in a reduction in the score in both the $CD4^+$T cell and CAR-Treg groups. At day 7, only the CAR-Treg group showed

continuous relief of symptoms and by day 25, the CAR-Treg group was asymptomatic. Symptom-free mice were rechallenged with a second EAE-inducing inoculum but remained healthy, demonstrating the sustained effect of engineered Tregs. Third, mice in the CAR-Treg group also showed decreased expression of IL-12 and IFN-γ mRNA in the brain, which may be due to the reduction in inflammation and inhibition of DC maturation (Fransson et al., 2012). In 2020, based on the bystander effect, researchers used both MBP-CAR Treg and MOG-CAR Treg therapies in an EAE model (Figure 6), and found that the combination of both significantly inhibited inflammation and myelin-specific T cell responses, thereby inhibiting EAE progress (De Paula Pohl et al., 2020).

The advantages of CAR treatment for MS are obvious. First, intranasal delivery can facilitate the accumulation of CAR-Tregs in the correct place under a small dose, avoiding the risk of systemic exposure and entry into other vital organs. Second, CAR-Tregs show good targeting and durability. For example, if EAE is again induced by antigens, CAR-Tregs can still inhibit disease development. However, CAR-Treg therapy has some safety issues. First, because the nasal mucosa is highly vascularized, CAR-Tregs may enter blood circulation throughout the body. Second, after nasal injection, cells from the nasal mucosa may migrate to the brain and cerebrospinal fluid (CSF) through the lamina along the olfactory nerve pathway (Danielyan et al., 2009).

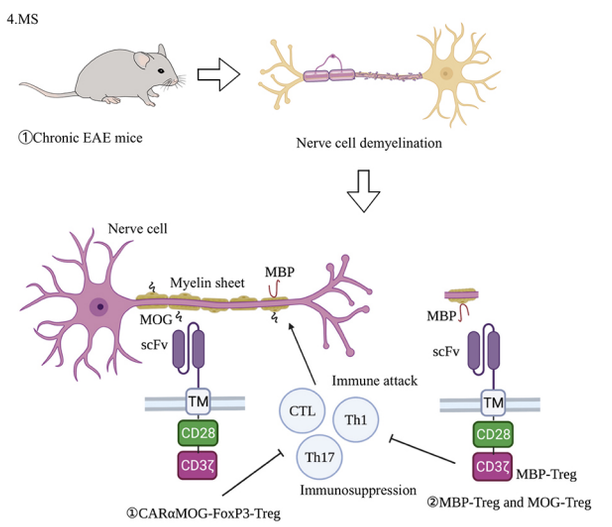

Figure 6 Application of CAR-Treg cells in animal model of MS

Chronic EAE mouse model is used in MS, induced by MOG_{35-55} and enhanced by Freund's adjuvant (CFA) or pertussis toxin (PT) (Berard et al., 2010). CARαMOG-FoxP3-Tregs express MOG-targeting CAR, FoxP3, and CD28-CD3ζ signaling domains (Fransson et al., 2012). From *in vitro* experiments, CARαMOG-FoxP3-Tregs inhibit T cell proliferation in the presence of MOG^+ cells. Based on the bystander effect, both MBP-CAR Treg and MOG-CAR Treg therapies have been used for EAE model treatment, with their combination significantly inhibiting inflammatory and myelin-specific T cell responses, and thereby EAE progress. EAE: Experimental autoimmune encephalomyelitis; MBP: Myelin basic protein; MOG: Myelin oligodendrocyte glycoprotein; scFv: Single chain antibody fragment; CTL: Cytotoxic T lymphocyte.

RHEUMATOID ARTHRITIS (RA)

Introduction of disease

RA is a common autoimmune disease characterized by an increase in rheumatoid factor (RF) and citrullinated protein antigen (ACPA) (Koivula et al., 2007), accompanied by joint swelling and systemic inflammation. In the early stage, autoantibodies and systemic inflammation are limited, but the adaptive response and tissue damage expand over time (van Venrooij et al., 2011).

Application of CAR-T and CAR-Treg in RA

For the treatment of RA, anti-fluorescein isothiocyanate (FITC) CAR-T has been applied to eliminate autoreactive B cells (Zhang et al., 2021a). Key steps include: determining autoantibody type in patients by enzyme-linked immunosorbent assay (ELISA), preparing FITC CAR-T and FITC-labeled autoantibody positive peptides; and eliminating autoreactive B cells by peptide targeting CAR-T. RA is characterized by the production of autoantibodies against citrulline antigens, so citrulline vimentin, citrulline type II collagen, citrulline fibrinogen and tenascin-C, and cyclic citrulline peptide-1 can be selected as autoantigens for targeting autoreactive B cells (Vossenaar et al., 2004). FITC can only be recognized by CAR-T and autoimmune B cells. Thus, anti-FITC CAR-T can be directed to various types of autoreactive B cells via recognition of FITC-labeled autoantigen peptides. In *in vitro* models, FITC CAR-T can recognize and kill hybridoma cells by producing antibodies against corresponding antigens, by binding with corresponding antigenic FITC-coupled peptides, and by recognizing and killing anti-COII antibody-producing B cells from collagen-induced arthritis (CIA) mice. FITC CAR-T also has cytotoxic effects on human RA autoimmune B cells *in vitro* (Figure 7) (Zhang et al., 2021a). Chimeric autoantibody receptor (CAAR) T cell (CAAR-T)-expressing citrullinated antigens have also been used to reduce the number of B cells resistant to citrullinated protein, while retaining protective B cells (Orvain et al., 2021). Anti-citrullinated vimentin CAR Tregs (CV CAR Tregs) also show potential in RA treatment but have not yet progressed beyond the concept stage (Orvain et al., 2021).

Anti-FITC CAR-T is not prone to abrogation and shows few side effects. Studies have demonstrated that in the presence of specific antibodies, anti-FITC CAR-T cells preferentially kill target antibody-secreting cells rather than FCγR-expressing cells (such as macrophages expressing CD64) (Zhang et al., 2021a). Even if the amount of specific antibody is high, CAR-T cell cytotoxicity is still far less than that of antibody-secreting target cells, and this weak off-target effect can be reduced by blocking FCγR with irrelevant antibodies (Zhang et al., 2021a). This mechanism avoids the effects of FITC CAR-T on FCγR-expressing macrophages and natural killer (NK) cells. Avoiding excessive immune cell damage prevents excessive weakening of human immunity, thus reducing the risk of infection with other diseases or cancer development due to CAR-T treatment, while enhancing the specificity and

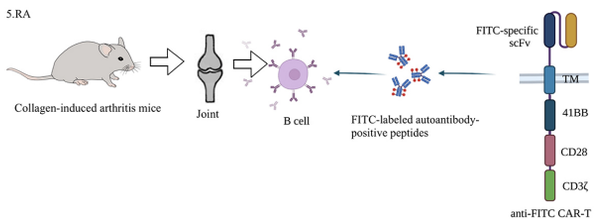

Figure 7 Application of CAR-T cells in animal model of RA

RA produces autoantibodies that cause joint swelling and systemic inflammation. Methods for eliminating autoimmune B cells by CAR-T are as follows: first, autoantibody type is determined by ELISA, and then anti-FITC CAR-T and FITC-labeled autoantibody-positive peptides are prepared to eliminate autoreactive B cells under peptide-mediated CAR-T toxicity in mice. *In vitro* experiments have shown that anti-FITC CAR-T cells recognize and kill anti-COII antibody-secreting B cells from CIA mice by binding with an antigenic FITC-conjugated peptide. CIA mice are RA mice induced by type II collagen and CFA. DBA/1 mice are commonly used (Kannan et al., 2005). RA: Rheumatoid arthritis; ELISA: Enzyme-linked immunosorbent assay; FITC: Fluorescein isothiocyanate; CIA: Collagen-induced arthritis; CFA: Complete Freund's adjuvant.

sustainability of treatment. In addition, theoretically, anti-FITC CAR-T may not only directly eliminate high-level specific B cell receptor (BCR)-expressing autoantigen B cells, but also indirectly affect the division and differentiation of memory B cells to produce autoantibody-secreting plasma cells. The main limitation of this method is that its ability to eliminate autoreactive B cells has only been proven *in vitro*, and its toxic effects on autoimmune B cells and safety *in vivo* remain to be clarified. Second, the stability of autoantigens and FITC-coupling mediators needs to be improved. Stability could potentially be enhanced by optimizing the structure and increasing the molecular weight, e.g., adding antigenic peptides and immunogenic domains of antibodies (Rodgers et al., 2016).

PEMPHIGUS VULGARIS (PV)

Introduction of disease

PV is an autoimmune vesicular disease affecting skin and mucosa (Sanders & Nelson, 1965). PV is mediated by immunoglobulin G (IgG) autoantibodies against desmoglein (Dsg). Autoantibodies bind to keratinocyte adhesion proteins Dsg1 and Dsg3 (Amagai et al., 1991), which interferes with keratinocyte adhesion (Shimizu et al., 2002), resulting in spinous layer lysis and blister formation. Dsg3 plays a major role in PV (Amagai et al., 1991).

Application of CAR-T or CAR-Treg in pemphigus

In 2016, a recombinant TCR was developed by fusing the autoantigen (Dsg3) in the CAR structure with CD137-CD3ζ (Ellebrecht et al., 2016) (Figure 8), named a chimeric autoantibody receptor (CAAR) or B cell antibody targeting receptor (BAR) (Parvathaneni & Scott, 2018). Ellebrecht et al. (2016) used several mouse models and *in vitro* experiments to evaluate the activity of the different autoantibody fragments of CAAR-T, cytotoxic potencies *in vivo* and *in vitro*, and on-target affinity and off-target conditions in the presence of circulating soluble autoantibodies. Dsg3 is composed of five extracellular cadherin (EC) domains (Ohyama et al., 2012), with CAAR expressing EC1-4 showing the highest potential (Figure 8). This CAAR-T cell directly eliminates memory B cells with anti-Dsg3 surface Ig (sIg) and indirectly eliminates sIg-Dsg3-specific short-lived pathogenic antibodies producing plasma cells. In the presence of pathogenic IgG, Dsg3-CAAR-T is not be eliminated. CAAR-T cells exhibit mature specificity and only target pathogenic B cells and Dsg3 EC1-4 CAAR-T cells exhibit no off-target toxicity. Lee et al. (2020) also evaluated the cytotoxicity, pharmacological and toxicological effects, dose-response effects, and off-target effects of CAAR-T cells in a mouse model. They found that CAAR-T cells reduce anti-Dsg3 IgG B cells in mice by 83.1%–92.3%, without decreasing total pathogenic B cells, confirming the CAAR-T cells effectively target the pathogenic anti-Dsg3 B cell population in PV. In mouse models, Dsg3-CAAR-T does not follow a strict dose-response relationship, but shows a threshold-dose relationship, suggesting that a conservative fractional initial dose should be used in subsequent clinical studies (Ellebrecht et al., 2016). In addition, screening potential desmosome ligands of Dsg3 and membrane proteins that may be targeted by CAAR verified that Dsg3 EC1-4 CAAR-T exhibits little possibility of off-target toxicity, consistent with previous research (Ellebrecht et al., 2016).

Overall, current preclinical research indicates that CAAR-T cells show high affinity and low toxicity. There are many

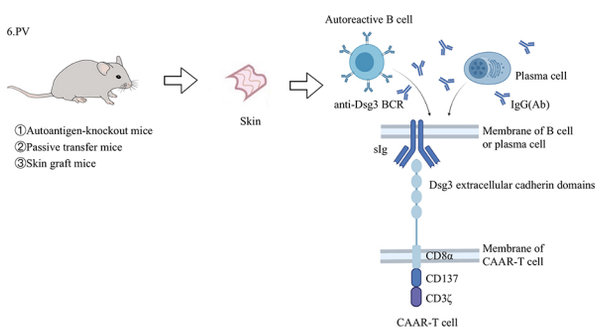

Figure 8 Application of CAAR-T cells in animal model of PV

PV develops in mucosa and skin, leading to a deadly autoimmune blistering disease. Three mouse models have been used in CAAR-T cell research. The "autoantigen-knockout mouse model" is a PV active model generated by transferring mDsg3-immunized splenocytes from Dsg3-/- mice to Rag2-/- immunodeficient mice (Amagai et al., 2000). The "passive transfer model" is established by injecting anti-mDsg3 IgG hybridoma cells into immunodeficient mice (Tsunoda et al., 2003). The "skin graft mouse model" is constructed by transplanting human skin grafts into immunodeficient mice and then transferring the hDsg3-secreting hybridoma cells of PV patients into mice (Ellebrecht et al., 2016). By fusing the EC domain (especially EC1-4) of Dsg3 into the CD137-CD3 ζ domain, CAAR-T cells can be directed to memory B cells and short-lived plasma cells, which express anti-Dsg3 sIg, thus curing the disease. PV: Pemphigus vulgaris; CAAR: Chimeric autoantibody receptor; Dsg3: Desmoglein3; mDsg3: Mouse desmoglein3; hDsg3: Human desmoglein3; EC: Extracellular cadherin; sIg: Surface immunoglobulin.

obvious advantages of CAAR-T cells, including excellent targeting properties, limited toxicity and side effects, and effective continuous effects. CAAR-T cells can eliminate the autoreactive cloning of immune cells while retaining non-pathogenic B cells and patient immunity (Zmievskaya et al., 2021), which is significantly different from the B cell-targeted killing of CAR-T19 cells. In addition, the limited toxicity/side effects could be attributed to several reasons. First, current routine clinical treatment of PV includes corticosteroids (CS) and immunosuppressive agents (ISA) (Yanovsky et al., 2019). Treatment with high-dose corticosteroids and the anti-CD20 antibody rituximab takes a relatively long time to induce remission, which can cause long-term and systemic immunosuppression, potentially leading to repeated and serious infections (Schmidt et al., 2017; Siddiqi et al., 2018). Second, no off-target effects have been observed in CAAR-T cell experiments. In addition, because anti-Dsg3 B cells account for only a small part of total B cells in patients (Lee et al., 2020; Nishifuji et al., 2000), they are unlikely to cause CRS in CAR-T therapy (Ellebrecht et al., 2016). Moreover, more than 90% of PV patients relapse without repeated infusion of rituximab (anti-CD20 antibody) (Colliou et al., 2013), whereas CAAR-T cells are a “living” drug with the potential to expand and persist *in vivo*.

However, there are some concerns about CAAR-Ts: (1) Detection methods and mouse models are limited. Most of our understanding of the persistence and effects of CAAR-T (or CAR-T) cells comes from measuring their abundance in peripheral blood (Maldini et al., 2018), which cannot reflect all effects. Furthermore, hybridoma cells injected into mice are unlikely to have the same distribution pattern as patients’ autoreactive B cells (Galy, 2016; Maldini et al., 2018). (2) Similar to CAR-T cell therapy, CAAR-T is an individualized treatment plan, which is highly time-consuming and costly (MacLeod et al., 2017; Yanovsky et al., 2019). (3) At present, CAAR-T therapy is still at the preclinical test stage, so it is hard to predict the whole picture when used on patients. A phase I clinical trial on the maximum tolerated dose of Dsg3-CAAR-T in patients with mucosal dominant PV is currently in the recruitment stage (NCT04422912). However, in the PV mouse model, CAAR-T therapy represents a highly personalized treatment method, with great research value and potential.

PERSPECTIVES

Although CAR-T and CAR-Treg cells have a series of advantages, such as good targeting, good persistence, and small side effects, they still have shortcomings in terms of safety, effectiveness, persistence, and manufacturability. We will discuss the shortcomings of CAR-T and CAR-Treg from these four aspects and explore their future developments.

Regarding safety, there are several issues with CAR-T therapy, including cell response release syndrome (Breslin, 2007), neurotoxicity (Bonifant et al., 2016), and off-target identification attacks (Makita et al., 2017). At present, researchers have added relevant mechanisms to reduce the side effects of CAR-T cells and regulate their persistence and activity in human tumor-related fields. In the future, these mechanisms could be applied to the treatment of autoimmune diseases. The first mechanism is the activation of suicide genes. Herpes simplex virus-thymidine kinase (*HSV-TK*) and inducible caspase 9 (*iCasp9*) are two suicide genes that have been integrated into CAR-T cells (Bonini et al., 1997; Quintarelli et al., 2007; Zhang & Xu, 2017). The suicide system is activated via the administration of rimiducid, which controls the intensity of side effects by the induction of rapid CAR-T cell apoptosis (Bonini et al., 1997; Quintarelli et al., 2007; Zhang & Xu, 2017). The second mechanism is the construction of switches, such as the ON-switch system applied in tumors. In this system, engineered CARs are divided into two independent proteins. One protein usually contains an extracellular antigen-binding domain, while the other protein contains downstream signaling elements and co-stimulatory molecules (such as CD3ζ and 4-1BB) (Wu et al., 2015). Only through the activation of benign foreign molecules can the two proteins dimerize, allowing CAR-T cells to attack tumors but effectively controlling their abnormal activation (Wu et al., 2015). The third mechanism is the manufacture of bispecific linkers through the small molecule drug conjugate (SMDC) platform, which could potentially be applied for autoimmune diseases in the future (Gallo et al., 2021; Zhuang et al., 2019). These unique bispecific linkers are composed of FITC molecules and tumor homing molecules, which can accurately connect universal CAR-T cells and cancer cells, thereby causing local T cell activation (Gallo et al., 2021; Zhuang et al., 2019). In addition, adjusting the dose of adaptor molecules can precisely control CAR-T and other side effects (Gallo et al., 2021; Zhuang et al., 2019). CAR-T also has safety problems in administration. As mentioned above, intranasal delivery of CAR-T for the treatment of MS may be risky, and further experiments are needed to optimize dose and reduce risk (Danielyan et al., 2009).

CAR-T effectiveness is also limited by the lack of antigens (Schultz & Mackall, 2019). As mentioned previously, SLE MRL-lpr mice treated with CAR-T still retain IgMlo subgroups of B cells, and therefore CAR-T cannot completely cure the disease as antibody-producing cells still exist after treatment (Kansal et al., 2019). In addition, because of immune escape caused by antigen deletion, the long-term survival rate of CAR-T cells in leukemia is quite low (Schultz & Mackall, 2019). As such, bispecific targeting of CAR-T to circumvent the down-regulation of the CD19 antigen is considered a good direction for research (Schultz & Mackall, 2019). Another issue is the stability of the CAR structure after infusion, e.g., *in vivo* stability of FITC in the treatment of RA (Zhang et al., 2021a). Researchers have proposed the structural optimization of bifunctional antigen-specific targeting ligands, such as improvement in the coupled immunogenic domain containing antigen peptides and antibody fragments to increase the molecular weight of the medium (Zhang et al., 2021a).

Regarding persistence, CAR-T cannot guarantee its activity, stability, and continuous proliferation under inflammatory microenvironments *in vivo*. For example, Zhang et al. (2019) showed that CAR-T can induce a delay in T1D onset in NOD mice, but cannot stop the occurrence of T1D, which may be a problem for the expansion of CAR-T *in vivo*. To search for strategies to extend the metastatic activities of CAR-T cells,

genome sequencing of CAR insertion sites in T cells has been established to better understand CAR-T cell function and persistence *in vivo* (Li et al., 2020). At the same time, modification of the CAR-T structure is also ongoing. Fourth-generation CARs have added factors (such as IL-2, IL-5, IL-12, and co-stimulatory ligands) to enhance the expansion, persistence, and anti-tumor activity of CAR-T cells (Chmielewski & Abken, 2015; Kueberuwa et al., 2018). Furthermore, CARs incorporating IL-2 receptor β-chain (Kagoya et al., 2018), telomerase reverse transcriptase co-transduction (Bai et al., 2015), or PI3K inhibitor treatment (Zheng et al., 2018) can improve *in vivo* persistence of CAR-T cells.

Concerning manufacturability, CAR-T therapy is an individualized treatment plan and complex cell manufacturing is required in a dedicated facility (Lyman et al., 2020). Furthermore, due to risks such as CRS, a high level of hospital care is required after the administration of CAR-T cells. Therefore, the price of treatment is usually high (Lyman et al., 2020). Thus, advances in CAR technology and market supervision should be strengthened to reduce the high costs of CAR-T therapy.

It is generally accepted that antigen-specific Tregs, including TCR-Tregs and CAR-Tregs, are superior to polyclonal Tregs in their suppression because they are antigen-specific and tend to migrate to a target organ harboring a specific antigen (Zheng et al., 2018). Moreover, CAR-Tregs hold advantages over TCR-Tregs as they are non-MHC-restricted and less dependent on IL-2 (Rosado-Sánchez & Levings, 2020). However, CAR-Tregs still exhibit some disadvantages and limitations.

Regarding CAR-Treg safety, the immunosuppressive phenotype of Tregs can change after losing Foxp3 expression under inflammatory microenvironments and engineered Treg cells may transform into antigen-specific pathogenic effector T cells that can aggravate disease symptoms (Juan et al., 2017). Several approaches to maintain the immune inhibitory phenotype of Tregs have been reported, e.g., treating Tregs with vitamin A derivative all-trans retinoic acid to sustain Treg stability and functionality (Zhou et al., 2010), administering a Treg-favoring microbiota to the gut (Atarashi et al., 2013), and inducing ectopic expression of the *Foxp3* gene to stabilize the regulatory Treg phenotype (Beavis et al., 2011). It is well known that CAR-T cell treatment can cause side effects related to cytokine storm and neuronal cytotoxicity (Breslin, 2007), but whether CAR-Tregs also induce these adverse reactions remains to be determined. The tendency of Treg cells to produce inflammatory cytokines can be eliminated using CRISPR to remove IFN-γ or IL-17 genes (Freen-van Heeren, 2021). Off-target effects may also occur in CAR-Treg therapy. For example, when CAR-Tregs are given via nasal injection, cells from the nasal mucosa may migrate to the brain and cause damage (Danielyan et al., 2009), which could result in pan-immunosuppression, reduced immune responses against opportunistic infections, and possibly even cancer development (Mohseni et al., 2020). One way to control this may be to integrate a suicide gene into the genetically modified therapeutic cells before injecting them into the patient.

Regarding effectiveness, it is difficult to ensure that CAR-Treg cells are effective in all diseases. It is a necessary to characterize antibodies specific for self- or allo-antigens in order to construction efficient and specific CAR-Tregs. However, both the selection of CAR-targeted antigens and development of specific antibodies can be time-consuming and may be difficult in some disease models. If antigens are not chosen properly, CAR-Tregs may be ineffective, with low specificity and off-target effects.

Regarding CAR-Treg cell persistence, the exhaustion of CAR-Tregs can limit their efficacy in suppression. For example, Blat et al. (2014) reported that CEA-CAR-Tregs diminish rapidly *in vivo*, greatly limiting their clinical application in colitis. Because CD28 plays an important role in Treg development and expansion (Bour-Jordan & Bluestone, 2009), most CAR-Treg studies use second-generation CARs with a CD28 co-stimulatory domain to expand Tregs. However, several studies have indicated that substituting CD28 with CD137 are a co-stimulatory can ameliorate T cell exhaustion, thus improving CAR-T persistence (Long et al., 2015; Quintarelli et al., 2018). However, CD28 is critical for maintaining Treg homeostasis and plays a critical role in Treg proliferation, differentiation, and survival as well as IL-2 production and Foxp3 expression (Bour-Jordan & Bluestone, 2009). Thus, the inclusion of both CD28 and CD137 co-stimulatory domains may help maintain CAR-Tregs. More studies are needed to select the best co-stimulatory signals for optimizing CAR-Treg suppression.

The manufacture of CAR-Treg cells is complex and costly, which influences their translational success. Current CAR-Treg manufacturing presents several challenges in cell purification, yield, expansion, vector production/transduction, cryopreservation, and product release testing (Fritsche et al., 2020). For example, Elinav et al. (2009) experienced significant obstacles in vector transduction when producing TNP-TPCR Tregs. Several methods can be utilized to improve CAR-Treg manufacture. For example, the development of a multi-parameter flow cytometry panel with consistent marker profiles can simplify the isolation and sorting processes (Fritsche et al., 2020). Implementing self-dissolving or easily removable beads can simplify the activation process (Bernstroem et al., 2016). In CAR design and delivery, batch production using flexible module methods, such as UniCAR, not only improve safety and cost-benefit ratios (Koristka et al., 2018; Zhang et al., 2018) but also optimize non-viral platforms (transposon or CRISPR/Cas technologies) and improve safety and efficacy. Sleeping Beauty transposon systems hold great promise for reducing costs, eliminating the need for clinical-grade viral vector manufacture (Ramanayake et al., 2015), and lowering the risk of insertional oncogenesis (Gogol-Döring et al., 2016). Using CRISPR/Cas9 with non-viral delivery of template DNA to eliminate large genes and orthotopic placement of transgenic constructs opens new delivery opportunities (Schober et al., 2019). For CAR-Treg expansion, semi- or fully automated closed-system bioreactors with integrated gas transfer and fully automated process control could pave the way toward an automated and standardized *ex vivo* expansion procedure as well as improve safety and decrease the costs of manufacturing. Furthermore,

implementation of allogeneic off-the-shelf products, elimination of endogenous TCRs and MHC molecules using genome-editing technologies, increased expression of NK cell inhibitory molecules, and application of iPSC-derived Treg technology may improve the safety of third generation CAR-Tregs at the manufacturing scale (Depil et al., 2020; MacDonald et al., 2019).

In conclusion, more studies are needed to improve the safety, effectiveness, persistence, and manufacturability of CAR-T and CAR-Treg cells. Despite their shortcomings, CAR-T and CAR-Treg cells show considerable potential for application in autoimmune diseases.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Z.W. and B.L. conceived the review. L.B. contributed to the introduction and pemphigus vulgaris section. H.W.C. contributed to the introduction and rheumatoid arthritis section. C.Q. contributed to the discussion and colitis and multiple sclerosis sections. X.C.B. contributed to the discussion and systemic lupus erythematosus, type 1 diabetes, and multiple sclerosis sections. X.C.B., L.B., H.W.C., and C.Q. contributed to the figures and tables. All authors read and approved the final version of the manuscript.

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