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CARs: a new approach for the treatment of autoimmune diseases

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The development of chimeric antigen receptor (CAR)-based therapeutic interventions represented a breakthrough in cancer treatment. Following the success of the CAR-T-cell strategy, this novel therapeutic approach has been applied to other diseases, including autoimmune diseases. Using CAR-T cells to deplete pathological immune cells (i.e., B cells, autoreactive B or T cells, and accessory antigen-presenting cells (APCs)) has resulted in favorable outcomes in diseases characterized by excessive autoantibody levels or hyperactive lymphocyte cell numbers. The importance of immunosuppressive regulatory T cells (Tregs) in restoring immune tolerance has been well established, and CAR-Tregs have shown promising therapeutic potential in treating autoimmune diseases. Moreover, prior experience from the cancer field has provided sufficient paradigms for understanding how to optimize the structure and function of CARs to improve their function, persistence, stability and safety. In this review, we describe the potential application of CAR-T cells and CAR-Tregs in the treatment of autoimmune diseases, and we summarize the currently available strategies of gene editing and synthetic biological tools that have improved the practical application of CAR-based therapies.

autoimmune diseases, chimeric antigen receptor, regulatory T cells, cellular therapy

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Introduction

Chimeric antigen receptors (CARs) are synthetic transmembrane proteins with high specificity for target antigens that can redirect lymphocytes to recognize and exert their effects under particular circumstances. To date, CD19-specific CARs have been the most successful in clinical applications, showing promise for their use as a highly effective strategy for treating $CD19^+$ B-cell haematological malignancies. In addition, the first two anti-CD19 CARs have been approved by the US Food and Drug Administration (FDA) for the treatment of relapsed or refractory diseases (Rafiq et al., 2020). These initial successes attracted substantial interest from researchers to further explore the applications of this approach in diseases other than haematological malignancies, such as solid tumours, autoimmune diseases, and infectious disorders (Hale et al., 2017; Lindo et al., 2020; Maldini et al., 2018). In recent years, rapid advances in synthetic biology have made cell re-engineering more predictable and precise, initiating a new era of using engineered CAR-T cells in personalized medicine. The expression of CARs in other cells (natural killer (NK) cells, macrophages,

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regulatory T cells (Tregs), γδ T cells, etc.) has gradually emerged, exhibiting safety and modest efficacy in both basic and clinical research (Klichinsky et al., 2020; Siegler et al., 2017).

Autoimmune diseases are characterized by a breakdown in immune tolerance that is mainly caused by interactions between genes and the environment (Jiao et al., 2020; McDevitt and Bodmer, 1974). As the third most common disease type after cancer and cardiovascular disease, autoimmune diseases currently affect nearly 10% of the world's population (Su et al., 2008). Ideal treatments for autoimmune diseases should eliminate pathological autoreactive cells while preserving protective immunity. However, traditional and non-specific treatment based on hormones and immunosuppressants partly inhibits autoantibody production by systemically suppressing the immune system; this approach may cause serious adverse effects, such as an increased risk of infection (Askling et al., 2007; Billi et al., 2019). Biologics, as exemplified by monoclonal antibodies (mAbs), including anti-TNFα and anti-IL-6R antibodies, specifically interact with their targeted antigen, resulting in more selective outcomes with fewer toxic effects than traditional treatments (Askling et al., 2007). Despite the significance of currently available therapies, achieving the effective and permanent restoration of immune homeostasis is still challenging considering that repeated antibody injection is generally needed, and an insufficient antibody dose leads to incomplete depletion and treatment failure (Baghdadi et al., 2015). In addition, the immunogenicity caused by long-term administration of antibodies remains a concern (Kuriakose et al., 2016). To this end, CAR-T-cell therapy has shown promise in the treatment of autoimmune diseases because of its specificity and the induction of durable remission of autoimmunity (Sadeqi Nezhad et al., 2020).

The number of studies on the use of CARs for autoimmune disease therapy is rapidly increasing, and these studies have mainly focused on CAR-T cells/Tregs (Chen et al., 2019). Similar to the treatment of tumours, the treatment of autoimmune diseases with designed CAR-T cells via the targeted elimination of autoreactive lymphocytes, which are defined by their reactivity to specific autoantigens, is a topic of substantial interest in recent research (Lee et al., 2021). The therapeutic potential of $FoxP3^+$ immunosuppressive Tregs and antigen-specific Tregs, as exemplified by CAR-Tregs, has been demonstrated in various autoimmune disease models (Yi et al., 2019). Numerous preclinical and clinical studies have indicated that CAR-T cells and CAR-Tregs have promising therapeutic potential in various autoimmune diseases, including multiple sclerosis (MS) (Harman et al., 2017), type 1 diabetes (T1D) (Uhlig et al., 2006), inflammatory bowel disease (IBD) (Blat et al., 2014), systemic lupus erythematosus (SLE) (Galy, 2016), and pemphigus vulgaris (PV) (Sadeqi Nezhad et al., 2020). Here, we present a brief overview of the currently available therapeutic strategies and critically summarize studies on CAR-T cells/Tregs conducted in both animal models and human clinical trials. Furthermore, we discuss the major concerns and perspectives related to CAR-based treatment of autoimmune diseases, providing a theoretical foundation for the clinical use of CARs in the future.

Car-based therapeutic strategies for autoimmune diseases

Overview of autoimmune diseases

Autoimmune diseases, such as SLE, T1D, and rheumatoid arthritis (RA), are heterogeneous disorders characterized by prominent autoimmune dysregulation; these diseases are mainly caused by self-antigens and immune complex deposition. Although the potential causative factors of autoimmune diseases remain largely unknown, there are two major causes of the failure of immune tolerance: (1) the presence of autoantibodies and (2) disease-associated autoreactive lymphocytes. Autoantibodies not only play a central role in disease diagnosis but also contribute to the pathogenesis of tissue damage mediated by a number of mechanisms, such as complement- and antibody-dependent cell-mediated cytotoxicity (CDC and ADCC, respectively) and immune complex deposition. Unlike the abundant autoantibodies in serum, autoreactive lymphocytes mainly accumulate in target organs and circulate at low levels (de la Varga-Martínez et al., 2019), providing a distinct target for disease treatment (Lee et al., 2020). The immune response is considered an orchestrated response that involves a multitude of diverse cell populations (Li et al., 2021b; Seehus et al., 2017; Wu et al., 2020b). The complexity and heterogeneity of the mechanism underlying immune dysregulation in autoimmune diseases result in many challenges associated with currently available therapies, and the development of novel therapeutic strategies that are specific and have durable treatment effects but minimal side effects remains a topic of ongoing investigation in the field of autoimmune disease treatment.

Emerging cell-based therapies for autoimmune diseases

The key to the treatment of autoimmune diseases is to restore immune tolerance and balance. Traditional treatment options and biological agents have been shown to provide effective relief of inflammation and pain within days and weeks in clinical trials as well as basic research (Chen et al., 2011; Czekalska et al., 2019; Ishihara et al., 2021; Rosenzwajg et al., 2019; Saxton et al., 2021; Zhang et al., 2021a). Nevertheless, the repeated administration of agents remains an ever-present issue. Recent progress in single-cell sorting and

cell monoculture techniques has generated increased enthusiasm for the use of adoptive cell therapy (ACT) to treat autoimmune diseases. Cell-based therapies, including therapies based on Tregs, mesenchymal stem cells and tolerogenic dendritic cells, may provide greater advantages than antibodies and cytokines in restoring immune tolerance because of the persistence and effectiveness of these therapeutic cells *in vivo* (Munir and McGettrick, 2015; Zeng et al., 2019). Given the essential role of Tregs in immunosuppression and regulation, the adoptive administration of polyclonal or antigen-specific Tregs has been explored as a potential immunosuppressive treatment option for a number of autoimmune diseases (Esensten et al., 2018). Treg-based therapy enhances immune tolerance through two distinct mechanisms: bystander suppression and infectious tolerance (Sojka et al., 2008). More than 50 ongoing or completed clinical trials are evaluating or have evaluated the safety and outcome of Treg-based therapy in diseases with potential indications, suggesting that cytotherapy is safe and that new approach may be needed to enhance the efficacy of these cells.

Design and evolution of the structure of CARs

Currently, the adoptive transfer of engineered T cells is a rapidly developing area in the field of autoimmune disease therapy, and there are two major types of engineered T cells: T-cell receptor (TCR) transgenic cells and CAR-T cells (Wu et al., 2020a; Zhao et al., 2021b). Transgenic TCR structures are designed to specifically interact with a peptide in the major histocompatibility complex (MHC), allowing T cells to respond to a specific target in a sustained manner. CAR structures provide additional advantages compared with TCRs because they are not MHC-restricted and have more flexibility for optimization (Haddadi et al., 2020; MacDonald et al., 2016). The emerging application of CARs provides a new research and development idea for the immune recovery of autoimmune diseases.

Engineered CAR molecules can be considered chimeras from antibodies and TCRs, including four essential domains: the antigen-binding motif, hinge and transmembrane domain, costimulation domain, and activating domain (CD3ζ signalling domain). The antigen-binding domain confers this receptor with antigen specificity (Figure 1). Mostly, the antigen-binding domain is derived from the variable heavy (VH) and light (VL) chains of antibodies, connected by a flexible linker to form a single-chain variable fragment (scFv). Recently, molecules other than scFv, such as nanobodies, specific antigens, cytokine-binding proteins and other domains with high affinity for targets of interest, have also been used as alternative antigen-binding domains for the extracellular part of CARs. The hinge and transmembrane domains contribute to the flexibility and provide adequate length to facilitate access to the target antigen. The intracellular domain of CARs generally comprises an activating domain and one or more costimulatory domains. First-generation CARs included only the CD3ζ chain without a costimulation domain, which was insufficient to effectively induce a T-cell response (Moritz et al., 1994). Therefore, a costimulatory signal was added to the second-generation CARs to maintain and increase the expansion, persistence, and activation of CAR-T cells. There are mainly two superfamilies of costimulatory signals, the CD28 and TNFR2 superfamilies, among which CD28 and 4-1BB (belonging to the TNFR2 superfamily) are the most frequently used (Chen and Flies, 2013). Different costimulatory domains endow T cells with a wide range of properties; therefore, third-generation CARs include two different costimulatory molecules cooperating with CD3ζ (Hombach et al., 2012). Fourth-generation CARs introduce an inducible system to make T cells secrete particular cytokines to assist in their function (Chmielewski and Abken, 2017).

In addition to the classic evolution of the CAR structure mentioned above, many basic research studies have enabled further and meticulous optimization of each component of the structure of CARs, which has been well discussed in other reviews (Rafiq et al., 2020). These varied structures give cells different functions, which are very suitable for the complex pathogenesis of autoimmune diseases. Put another way, appropriate designs are selected according to the characteristics of different diseases. A few studies have suggested that CAR-T-cell therapy could hold great promise for the treatment of many different kinds of autoimmune diseases by resulting in enhanced killing of pathogenic immune cells. Additionally, CAR-Tregs lead to improvements and the persistence of immune suppression under the activation and proliferation of Tregs under specific targets. Research on CAR-T/Treg therapy will be summarized in the next section (Figure 2).

Therapeutic potential of CAR-T cells in the treatment of autoimmune diseases

The pathological cells associated with autoimmune diseases mainly include autoantibody-producing plasma B cells, activated T cells and antigen-presenting cells (APCs). B cells play critical roles in the progression of a number of autoimmune diseases by producing autoantibodies, releasing proinflammatory cytokines, and functioning as APCs to activate autoreactive T cells; thus, recent research on CAR-based therapy has largely focused on specific or nonspecific B-cell depletion. B-cell pan marker-based cell depletion therapy (CD19, CD20, B-cell maturation antigens (BCMAs), etc.) has shown some success in human and mouse models (Furie et al., 2011; Mackay et al., 1999; Navarra et al., 2011).

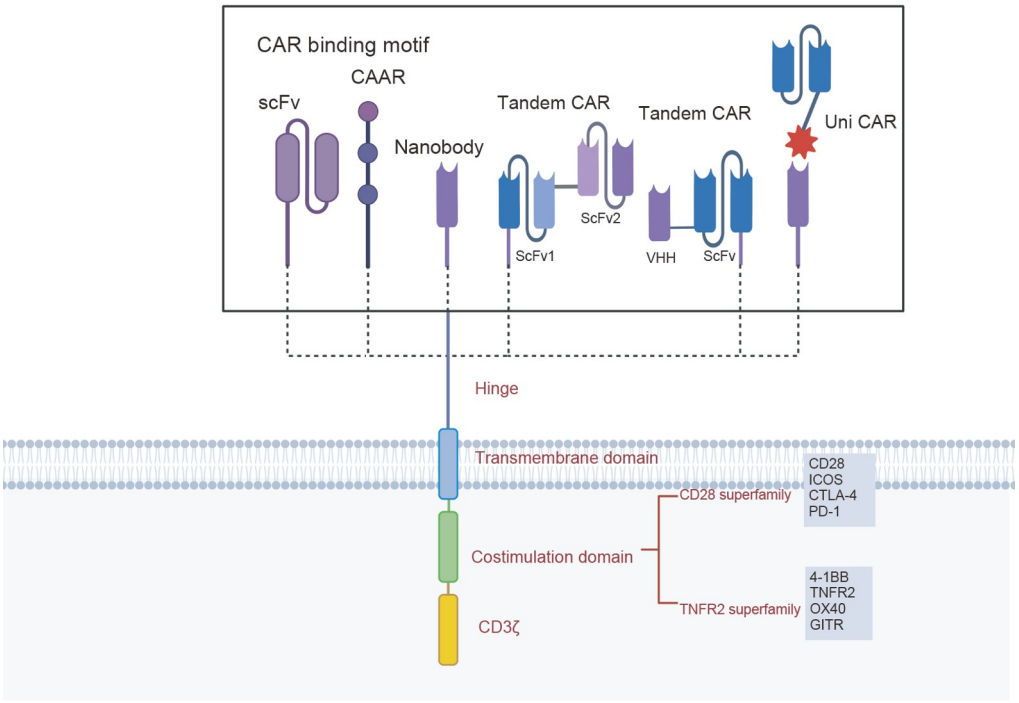

Figure 1 An overview of the design of CARs. The structure of CARs can be divided into the following parts: an antigen-binding motif, a hinge domain, a transmembrane domain, one or more costimulation domains and a constant activation domain. For the extracellular region, the binding motif can be flexibly adjusted according to different usages. Most previous work has focused on comparing coreceptor signalling domains. For CAR-T cells, 4-1BB-based CAR-T cells are more persistent and resistant than CD28-based CAR-T cells. For CAR-Tregs, the CD28-based costimulatory domain was initially used, while studies focusing on optimizing costimulatory Treg signals are always needed.

Importantly, some autoimmune disorders, such as PV, are associated with known autoantigens, which are ideal targets for chimeric autoantibody receptors (CAARs) (Ellebrecht et al., 2016). Other strategies for the specific depletion of activated T cells or accessory APCs by redirecting cytotoxic T lymphocytes (CTLs) to target clonotypic TCRs or MHC complexes have also been reported, providing promising directions in this field (Zhang et al., 2019). Nevertheless, the exact autoantibodies or molecular targets associated with particular disorders, especially in systemic autoimmune diseases, remain largely unknown, and the nonspecific clearance of B cells may still be a major direction for the treatment of these disorders (Table 1).

Nonspecific B-cell depletion by CAR-T cells

CD19 is a B-cell-receptor-associated protein that is intimately involved in B-cell signalling and contributes to marginal zone B-cell development. The preferential expression of CD19 on activated B cells makes it an ideal target for the clearance of dividing plasmablasts and early plasma cells that are directly responsible for autoantibody production (Carter et al., 1997). Studies in MRL-lpr mice have shown that the treatment of these mice with anti-CD19 CAR-T cells preserves their plasma levels of IgM and IgG to some extent (Kansal et al., 2019). Similar approaches for whole-B-cell depletion are also being used to treat disorders such as myasthenia gravis (MG) and neuromyelitis optica spectrum disorders (NMOSDs).

SLE: SLE is the prototypical systemic autoimmune disease, and it is characterized by many types of autoreactive B cells and high levels of autoantibodies (Zhao et al., 2015). Antibody therapies that target B cells are unsatisfactory, and responses vary among different patients (Vital et al., 2011). Since anti-CD19 CAR-T cells have achieved durable and long-lasting B-cell depletion in the treatment of B-cell lymphoblastic leukaemia and better therapeutic effects than antibody-based therapy (Sterner and Sterner, 2021), trials of the use of CAR-T cells to deplete B cells are urgently awaited to test their efficacy and safety in SLE patients. Kansal and colleagues evaluated the preliminary efficacy of anti-CD19 CAR-T cells in murine lupus models, and their results showed the prevention of disease progression when the treatment was administered before disease onset (Kansal et al., 2019). Jin and colleagues infused syngeneic anti-mouse CD19 CAR-T cells into MRL-lpr mice both before and after disease onset and achieved durable and considerable alleviation of disease by sustained B-cell depletion. Furthermore, these authors found that CAR-T cells equipped with costimulatory 4-1BB demonstrated more persistent effects and lower exhaustion levels than conventional CD28 T cells, emphasizing the importance of costimulatory molecules in

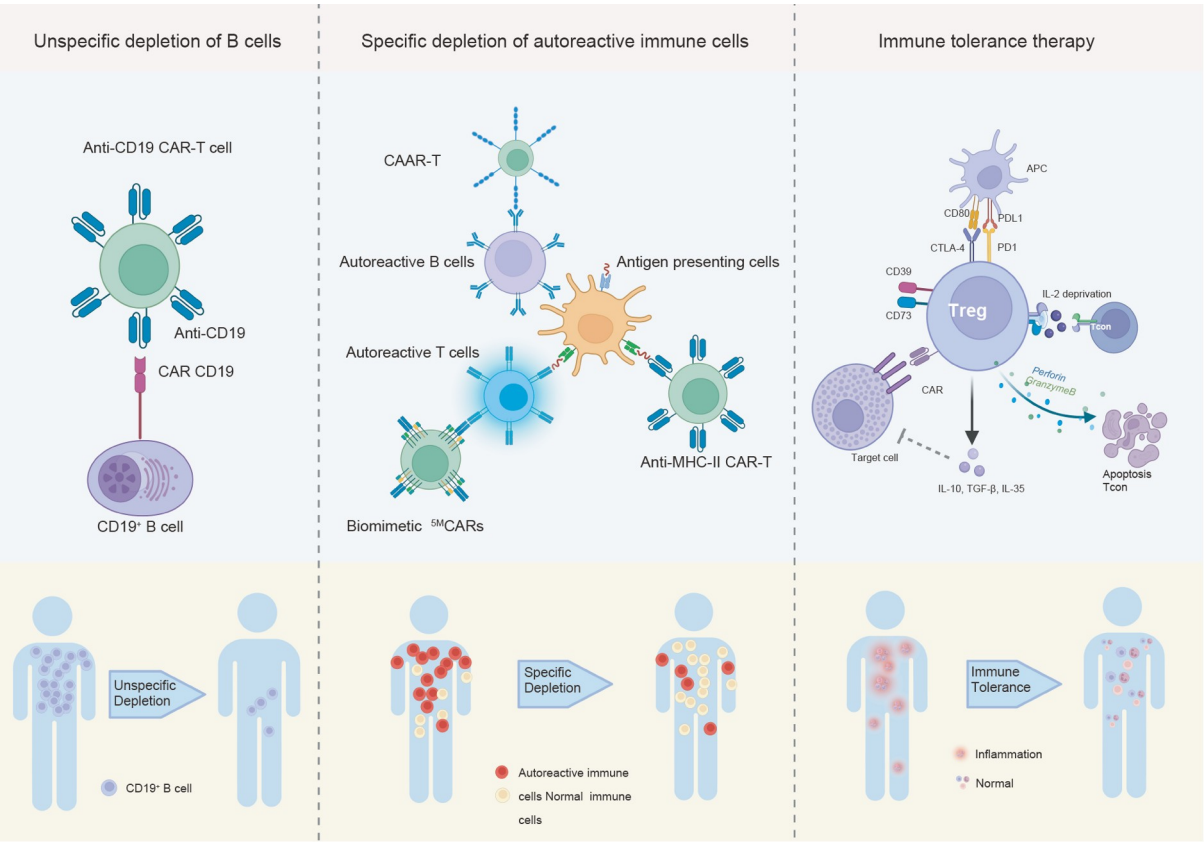

Figure 2 Mechanisms underlying the effects of CARs in the treatment of autoimmune diseases. Three different strategies for CAR-based therapy of autoimmune diseases are shown. The left panel shows anti-CD19 CAR-T cells, which can recognize and deplete $CD19^+$ autoreactive B cells as well as healthy B cells that express CD19. The middle panel shows the methods of specific autoreactive immune cell depletion. CAAR-T cells recognize autoreactive B cells with autoantigen-specific receptors as well as autoreactive APCs, thereafter exerting their cytotoxic effects. CTLs that express specific MHCII can directly kill pathological $CD4^+$ T cells in a TCR-dependent manner. The right panel shows the effect of CAR-Tregs activated by an antigen on the target cell in improving immune tolerance.

the design of CAR-T cells for SLE treatment (Jin et al., 2021). Currently, two clinical trials (NCT03030976 and NCT05030779) are underway, and the results are highly anticipated.

Notably, Mougiakakos and colleagues recently reported a clinical case in which a 20-year-old woman who presented with severe and refractory SLE with active lupus nephritis was infused with CD19 CAR-T cells after preparatory lymphodepletion and leukapheresis. This study demonstrated that CAR-T cells induced the rapid remission of refractory SLE and the disappearance of autoantibodies (Mougiakakos et al., 2021). Another clinical study reported by Zhang et al. showed that the disease activities of an SLE patient complicated with stage IV diffuse large B-cell lymphoma (DLBCL) achieved sustained remission after the infusion of CAR-T cells targeting both CD19 and BCMAs. The B cells in these patients were effectively cleared, and their disease remained in stable remission for up to 23 months during the follow-up examination (Zhang et al., 2021c). To promote the homeostasis and expansion of the infused cells, transient immunosuppression by fludarabine and glucocorticoids or other strategies is generally required in the preconditioning regimen (Jin et al., 2021). Collectively, these results are promising, and these studies provide a new approach for treating SLE patients, although the potential risk of CAR-T therapy in SLE warrants further study.

NMOSD: NMOSD is an autoimmune disorder of the central nervous system (CNS) that is characterized by the presence of anti-aquaporin 4 (AQP4) antibodies and axonal injury. The monoclonal anti-CD19 antibody was one of the first agents approved for the treatment of NMOSD, and its administration results in the lymphocytolysis of $CD19^+$ B cells and plasmablasts (Derdelinckx et al., 2021). A tandem CAR-T-cell targeting both CD19 and CD20 was being investigated in a clinical trial (NCT03605238) to examine its safety and efficacy in the treatment of NMOSD. However, this study ended due to recruitment difficulties. Another open-label phase I clinical trial (NCT04561557) using BCMA-specific CAR-T cells for the treatment of NMOSD patients with AQP4 antibodies is currently ongoing. The safety and efficacy results of this trial are expected by the end of 2023.

MG: MG is a heterogeneous neuromuscular autoimmune disease that is mainly caused by the presence of anti-acet-

Table 1 Published studies or registered clinical trials of CAR-T cell therapy for autoimmune diseases

Disease	Target	CAR-T type	Research type	Outcome/current status	Safety	Reference/NCT
SLE	CD19	Murine $CD8^+$ T cells (CD28-CD3ζ)	Basic	$CD19^+$ CAR-T cells eliminated B cells and autoantibody production, with reduced manifestations of lupus pathogenesis.	Long-term engraftment up to 1 year; continued cytotoxic efficacy; did not eliminate all IgM^+ B cells, retained pretreatment expression of plasma IgM and IgG.	Kansal et al., 2019
	CD19	Murine T cells (CD28-CD3ζ; CD137-CD3)		4-1BB CAR showed more persistent effects and lower exhaustion levels.	Milder skin ulceration and proteinuria; Effective up to 30 weeks.	Jin et al., 2021
	CD19	Human T cells (CD137-CD3ζ)	Clinical	Phase I, recruiting	Not provided.	NCT03030976
	CD19, BCMAs	Human T cells		Phase I, recruiting	Not provided.	NCT05030779
	CD19	Human T cells (ratio of $CD4^+$ to $CD8^+$ T cells, 3:1)	Case report	$CD19^+$ CAR-T-cell therapy could induce rapid remission of refractory SLE.	No evidence of CRS, neurotoxic effects, or prolonged cytopenia.	Mougiakakos et al., 2021
	CD19, BCMAs	Human $CD8^+$ T cells		Through the separate reinfusion of CD19 and BCMA dual-targeted CAR-T cells, the B cells in the patients were effectively cleared.	Absence of complement activation; limited the risk of opportunistic infection.	Zhang et al., 2021
NMOSD	CD19, CD20	Human T cells (CD137-CD3ζ)	Clinical	Phase I, withdrawn	Not provided.	NCT03605238
	BCMAs	Human T cells		Phase I, recruiting	Not provided.	NCT04561557
MG	BCMAs	Human T cells	Clinical	Phase I, II, recruiting	Not provided.	NCT04146051
	MuSK autoantibodies	Human T cells (CD137-CD3ζ)	Basic	CD137-based CAAR-T-cell therapy achieved antigen-specific B-cell depletion.	Specific cytotoxicity towards target.	Oh et al., 2020
PV	Dsg3 autoantibodies	Human T cells	Clinical	Phase I, recruiting	Not provided.	NCT04422912
	Dsg3 autoantibodies	Human T cells (CD137-CD3ζ)	Basic	Dsg3 CAAR-T cells exhibited specific cytotoxicity and suppressed Dsg3-specific hybridoma driven GvHD in NSG mice.	Engraftment and persistence for 3 weeks; Absence of off-target toxicity; Tumour lysis and CRS: uncertain.	Ellebrecht et al., 2016
	Dsg3 autoantibodies	Human T cells (CD137-CD3ζ)		DSG3 CAAR-T effectively inhibited antibodies responses accompanied by clinical and histological improvement of blisters in a rhDSG3 active immune model.	Toxicity: high dose: 20%; low dose: undetectable; CRS: uncertain; off-target effects: no evidence.	Lee et al., 2020
T1D	I-Ag7-B:9–23 (R3) complex	Murine T cells (CD28-CD3ζ; CD137-CD3)	Basic	The therapy could only delay T1D in mice; it could not prevent the development and onset of T1D.	Metabolic side effects: no evidence; engraftment for less than 25 weeks; systemic side effects: no evidence; off-target effects: uncertain.	Zhang et al., 2019
	Clonotypic TCRs	^{5M}CAR-CTLs: mouse $CD8^+$ T cells (peptide antigens (MHCII))		^{5M}CAR-CTLs not only decreased the incidence of insulitis in NOD mice but also reversed ongoing insulitis.	Off-target effects: low level; engraftment for up to a year; natural signalling helped maintain the normal function of T cells.	Kobayashi et al., 2020

ylcholine receptor (AChR) antibodies at neuromuscular junctions, resulting in poor delivery of neurotransmitters and muscle weakness in different parts of the body (Danikowski et al., 2017). A similar approach of B-cell depletion by targeting BCMAs is being investigated in an ongoing trial (NCT04146051).

In addition, RA is a common systemic autoimmune disease characterized by classic T-cell-dependent immune responses. It has been proven that the application of rituximab, a chimeric human CD20-specific antibody with the capacity to

deplete CD20-expressing B cells, is effective in the treatment of RA (Aletaha and Smolen, 2018). Other rheumatic autoimmune diseases, such as Sjogren's syndrome, systemic sclerosis and IgG4-related disease, are all characterized by acutely or chronically activated B cells (Thurlings et al., 2008), and these diseases are expected to benefit from the elimination of B cells by CAR-T cells (Barnas et al., 2019; Hofmann et al., 2018). The sustained total absence of B cells, however, increases the risk of infection and sometimes results in a failure to produce a recall response to a protective antigen (Oren et al., 2008). Therefore, a more precise therapy that targets autoantibody-producing B cells is a promising direction for further exploration.

Specific depletion of autoreactive immune cells

Autoreactive lymphocytes are defined by their reactivity against autoantigens, and these cells are an ideal target for specific depletion strategies (Ellebrecht et al., 2019). Instead of the antibody in a traditional CAR, CAARs harbour an autoantigen fragment on T cells, a transmembrane domain, and intracellular signalling abilities, and CAARs directly and specifically target autoantibody-producing B cells without inducing broad immunosuppression. The use of CAAR-T cells in the treatment of autoimmune diseases, especially diseases for which the pathological autoantibody-mediated mechanisms are clear and unequivocal, is expected to result in promising outcomes (Ellebrecht et al., 2016). In some instances, hyperactivated and self-activated T effector cells are considered to play a central role in the complex pathogenesis of autoimmune diseases, and these T cells are stimulated by professional APCs to exacerbate disease progression (Nepom and Erlich, 1991; Tsai and Santamaria, 2013). The depletion of these cells is another strategy awaiting more basic research studies to confirm its safety and effectiveness.

PV: PV is a rare life-threatening blistering disease of the skin and mucosa that is mainly caused by autoantibodies that target the keratinocyte adhesion protein Dsg3. Ellebrecht and colleagues developed CAAR-T cells that expressed the autoantigen Dsg3 (Dsg3 truncation (EC1-3/EC1-4)) as the extracellular domain, which was linked to intracellular CD137 and CD3ζ domains to target and eliminate autoreactive naïve and memory B cells that express DSG3-specific B-cell receptors. In preclinical studies, it was demonstrated that DSG3-specific CAAR-T cells induced histological and serological remission in a PV mouse model via the specific lysis of anti-DSG3 B cells both *in vitro* and *in vivo*. DSG3 CAAR-T cells did not cause cytotoxicity against keratinocytes that express the native ligands of Dsg3, emphasizing the specificity and safety of this approach. Additionally, the authors evaluated the effects of Dsg3-specific CAAR-T cells in the presence of serum anti-Dsg3 IgG antibodies, and they showed that specific antibodies may partially inhibit but not impair the effects of CAAR-T cells. This study provides a feasible and universal strategy for depleting autoantibody-producing B cells that could be applied to other antibody-mediated autoimmune diseases (Ellebrecht et al., 2016). A preclinical study using a pathological anti-Dsg3 B-cell population from PV patients was further performed to evaluate the safety and efficacy of this strategy, and the results showed that Dsg3-specific CAAR-T cells had the capacity to specifically inhibit antibody responses against the Dsg3 epitope and to clinically and histologically improve blisters (Lee et al., 2020). A clinical trial of anti-Dsg3 CAAR-T cells (NCT04422912) in patients with mucosal-dominant PV is ongoing, and the results are eagerly anticipated.

CAAR-T cells have shown promising and exciting results in preclinical experiments; however, the application of this methodology to systemic autoimmune diseases, such as RA and SLE, is limited since a CAAR (or CAR) can target only a single cell type, and this methodology is not effective against the various types of autoreactive lymphocytes present in patients with systemic autoimmune diseases. As an increasing number of autoantibodies and their antigenic epitopes have been identified (Hu et al., 2010; Juarez et al., 2016; Li et al., 2021a), it is becoming possible to individually eliminate major populations of autoreactive B cells with sets of immunodominant peptides that are customized according to the circumstances of individual patients. In a proof-of-concept study, we demonstrated a targeted and customized strategy that employed universal anti-FITC CAR-T cells combined with FITC-labelled immunodominant peptides derived from autoantigens (Zhang et al., 2021b). We preliminarily verified that such universal anti-FITC CAR-T cells could be redirected by customized FITC-labelled citrullinated autoantigen peptides to specifically eliminate autoreactive B-cell subsets that recognize these peptides and that these CAR-T cells acted in a dose-dependent manner, thus revealing the heterogeneity and safety of CAR-based approaches in the treatment of autoimmune diseases. Although further efficacy and practicality studies remain to be performed, this strategy may provide a promising direction for the development of precise and customized approaches for the treatment of systemic autoimmune diseases.

T1D: The insulin B chain (amino acids 9 to 23) is one of the main pathogenic epitopes in T1D. Zhang and colleagues redirected CTLs with mAb287, which is an mAb against the MHC class II peptide epitope complex, to kill pathological APCs both *in vitro* and *in vivo*. Although the protection was eventually lost, this study demonstrated that a single dose of 287-CAR-T cells was sufficient to delay the onset of disease in otherwise untreated animals for almost 6 weeks (Zhang et al., 2019). One point that needs to be considered is the times of infusion, as the findings from this proof-of-concept study

indicated that a single dose of CAR-T cells given early in the disease did not have a lasting protective effect due to the exhaustion of the infused cells. Additional infusions and the optimization of the administration scheme may be required in future studies (Beheshti et al., 2022; Zhang et al., 2019). Since T1D is associated with T-cell-mediated destruction of pancreatic β-cells, directly targeting pathological T cells is another strategy. Kobayashi et al. engineered a biomimetic five-module chimeric antigen consisting of the ectodomain of pMHCII assembled with CD3 signalling modules, and this module was referred to as ^{5M}CAR-CTLs; this approach redirected CTLs to specifically kill pathological CD4$^+$ T cells that express clonotypic TCRs. The authors showed that ^{5M}CAR-CTLs could reduce the incidence of diabetes in non-obese diabetic (NOD) mice and inhibit the process of insulitis (Kobayashi et al., 2020). The findings from this study support the administration of CAR-T cells in autoimmune diseases mediated by pathological T cells as well as APCs, expanding the prospect of the application of CAR-T cells to the treatment of autoimmune diseases.

MG: Given that autoantibodies against muscle-specific tyrosine kinase (MuSK) are generally observed in a substantial portion of MG patients (Stathopoulos et al., 2017), a CD137-based CAAR-T-cell that expresses the MuSK autoantigen was evaluated in a NOD-SCID-gamma (NSG) mouse model; in this model, the specific depletion of antigen-specific B cells was achieved (Oh et al., 2020).

Collectively, considering the definition of autoantigen(s) in certain patients, CAARs are an ideal strategy for eliminating autoreactive lymphocytes with minimal side effects. The CAAR strategy theoretically directly eliminates memory B cells that express immunoglobulin, thereby indirectly killing potential plasma B cells that produce disease-related autoantibodies. Similar methods, such as inserting mAb-specific domains for the MHC: peptide complex of autoreactive APCs and engineering pMHCII on CTLs to eliminate APCs or autoreactive T cells, have also been investigated. Another approach that uses CAAR-engineered NK cells, which are expected to prevent the risk of cytokine storms, was also reported (Meng et al., 2018). These studies provide promising alternative directions for the targeted treatment of autoimmune diseases and could be applied to other diseases characterized by dysfunctional immune responses.

Restoration of immune balance using Treg-based therapies

As one of the most important populations with immunosuppressive functions, Tregs exert their inhibitory effects through various mechanisms, including attenuating the activation of effector T cells; competing with effector T cells to bind to IL-2; inhibiting antigen presentation by APCs; secreting anti-inflammatory cytokines such as IL-10, TGF-β and IL-35; and directly lysing target cells through granzyme and perforin. Tregs play a predominant role in maintaining peripheral tolerance, and defects in their number or functions have been reported in a number of autoimmune diseases (Sojka et al., 2008; Xiao et al., 2020). Given the essential role of Tregs in immune regulation, the adoptive transfer of Tregs has been investigated as a promising approach to restore tolerance in a number of autoimmune diseases (Brunstein et al., 2011; Sarkar et al., 2014).

CAR-Treg-based therapy for the treatment of autoimmune diseases

To date, the majority of clinical experiments related to Tregs have used polyclonal Tregs. Although promising results have been found in these trials, the application of polyclonal Tregs is limited since a considerable number of cells are required and a general suppression of the immune system may occur (Adair et al., 2017). Moreover, given the heterogeneity of polyclonal Tregs, the infusion of a large number of cells may induce unwanted variability with limited therapeutic effects. To address these issues, antigen-specific Tregs, such as CAR-Tregs or TCR-Tregs, with a defined homogenous population would be a promising and attractive option. The therapeutic effects of antigen-specific Tregs have been demonstrated in a number of autoimmune diseases, including T1D (Radichev et al., 2020), colitis (Blat et al., 2014), graft rejection (Dawson et al., 2020), and haemophilia (Yoon et al., 2017) (Fransson et al., 2012; Skuljec et al., 2017) (Table 2).

T1D: An insulin-targeted CAR-Treg was designed, and its therapeutic efficacy was tested in a NOD/LtJ diabetic mouse model. Although insulin-specific CARs have superior specificity and minimal off-target toxicity, therapeutic effects are not observed due to the rapid metabolization of insulin (Tenspolde et al., 2019). To address this issue, Radichev et al. engineered Tregs specific for islet cells using anti-HPi2 (Radichev et al., 2020). Unfortunately, this HPi2-CAR was shown to be nonfunctional due to the unexpectedly high level of HPi2 expression by Tregs, which led to severe tonic signalling and the failure of Treg expansion and suppressive function. Glutamic acid decarboxylase 65 (GAD65) is another target in T1D. It has been reported that GAD65-specific CAR-Tregs can home to pancreatic islets and significantly lower blood glucose levels (Imam and Jaume, 2019).

Colitis: The critical role of Tregs in maintaining a healthy intestinal mucosa in patients with IBD has been widely reported (Collison et al., 2007), and carcinoembryonic antigen (CEA) is considered a major autoantigen associated with benign colon inflammation in humans (Smithson et al., 1996). Blat et al. redirected Tregs with CEA-CAR to be activated in the inflamed colon. These authors demonstrated

that these engineered CAR-Tregs suppressed the manifestations of colitis and reduced the burden of colitis-associated colorectal cancer, which is a malignant secondary change that occurs with IBD (Blat et al., 2014). Another experimental colitis study that used 2,4,6-trinitrobenzene sulfonic acid (TNBS) was also conducted to confirm the therapeutic effects of CAR-Tregs on colitis; in that study, CAR-Tregs that targeted TNP (2,4,6-trinitrophenol) exerted protective effects against TNBS-induced colitis in a mouse model (Elinav et al., 2008).

Graft-versus-host disease (GvHD): GvHD is a major complication of allogeneic bone marrow transplantation (alloBMT) and donor lymphocyte infusion, and it is mainly caused by donor T cells (Zhao et al., 2021a). MacDonald et al. evaluated the therapeutic effects of CAR-Tregs targeting human leukocyte antigen (HLA)-A2, a commonly mismatched alloantigen in transplantation, on GvHD by infusing HLA-A2-positive peripheral blood mononuclear cells (PBMCs) into NSG mice and cotransferring HLA-A2 CAR-Tregs into these mice. The authors showed that HLA-A2-specific CAR-Tregs indeed ameliorated the development of lethal GvHD in a humanized mouse model, and this effect was mediated by Treg-based inhibition of effector cells (MacDonald et al., 2016). Another study by Imura et al. demonstrated that CD19 CAR-Tregs suppressed antibody production by B cells without resulting in any GvHD symptoms (Imura et al., 2020). Similar to the findings in this study, Sicard et al. demonstrated that CAR-Tregs could suppress humoral immunity in a murine immunocompetent skin rejection model (Sicard et al., 2020). In that study, however, the authors found that sensitized recipients with a higher risk of allograft rejection could progress to skin rejection, probably due to the failure to inhibit lymphocyte responses by CAR-Tregs. This finding suggested that alternative therapies for recipients positive for donor-specific anti-HLA antibodies (DSAs) are necessary, and it is also important to optimize the infusion times and cell numbers to achieve a lasting effect (Sicard et al., 2020).

MS: MS is a chronic inflammatory and subsequently degenerative disease of the CNS. The prevailing opinion is that MS is a $CD4^+$ T-cell-mediated disease that leads to irreversible neurological deficits characterized by demyelination and axonal loss. The repressive function of Tregs has been confirmed by several studies, and antigen-specific Treg-based therapy for the improvement of MS has been investigated (Derdelinckx et al., 2021). By coexpressing FoxP3 with a CAR that targets myelin oligodendrocyte glycoprotein (MOG), which is a major target expressed in the CNS during demyelination, Fransson et al. generated sufficient numbers of Tregs with a consistent phenotype from naïve $CD4^+$ T cells (MOG-FoxP3 CAR-Tregs). These CAR-Tregs successfully suppressed ongoing inflammation and alleviated disease symptoms by preventing immune attacks against MOG^+ oligodendrocytes. Importantly, the authors provided a promising option for the production of engineered $CD4^+$ T cells with immunosuppressive abilities, addressing the issues of low frequency, slow expansion, and the phenotypic instability of Tregs (Fransson et al., 2012).

Haemophilia (HemA): HemA is an X-linked inherited disease caused by a deficiency of clotting factor VIII, which results in diminished blood clotting ability. The administration of recombinant or plasma-derived FVIII protein has generally been considered an effective treatment for HemA; however, the therapeutic effects of this approach are largely limited by the immunogenicity of FVIII under conditions of repeated and long-term injection (Iorio et al., 2010). To develop tolerogenic therapies, Scott and colleagues engineered antigen-specific Tregs, referred to as 17195TCR-Tregs, that were created by the transduction of a recombinant TCR obtained from a T-cell clone from a HemA patient; these Tregs successfully suppressed immune responses against FVIII (Yoon et al., 2017). These authors further engineered an FVIII-specific CAR, referred to as the ANS8 CAR, using an FVIII-specific scFv, and they demonstrated that ANS8 CAR-Tregs were able to suppress the proliferation of FVIII-specific effector cells via bystander suppression without MHC restriction (Yoon et al., 2017). One limitation of this study is that the effects of human Tregs on tolerance cannot be thoroughly tested in mice due to the potential immunogenicity and cross effects of species (Yoon et al., 2017). To thoroughly investigate the effects of CAR-Tregs *in vivo*, another study that used mouse Tregs was performed; in that study, engineered CAR-Tregs that targeted FVIII effectively prevented the immune response against FVIII and generated tolerance to FVIII for as long as 8 weeks (Fu et al., 2020).

Other diseases: Vitiligo is an acquired skin disorder characterized by immune dysregulation; in vitiligo, functional epidermal melanocytes are lost and degraded, mainly owing to cytotoxic $CD8^+$ T cells targeting ganglioside D3 (GD3) in the skin (Le Poole et al., 2003). CAR-Tregs that target GD3 successfully protected melanocytes from T-cell-mediated destruction in a mouse model of vitiligo, with a greater abundance of Tregs and melanocytes in the treated group (Mukhatayev et al., 2020). Skuljec et al. redirected Tregs with a CAR that recognizes CEA and demonstrated the therapeutic benefit of antigen-specific Tregs in the treatment of severe asthma (Skuljec et al., 2017).

Opportunities and challenges in the use of CAR therapy for the treatment of autoimmune diseases

Procedures for the manufacture of CAR-T cells/Tregs

The standard operating procedures according to good manufacturing practice (GMP) protocols include cell isolation, activation, genetic manipulation, and expansion and quality

Table 2 Published studies on CAR-Treg therapy for autoimmune diseases

Disease	Target	CAR-Treg type	Outcome	Safety	Reference
T1D	Insulin	Murine $CD4^+$ T cells converted with foxP3 gene (CD28-CD3ζ)	CAR-Tregs remained in spleen 17 weeks postinfusion.	Maintained FoxP3 expression; Durable for 4 months.	Tenspolde et al., 2019
	GAD65 beta cell epitopes	Human Tregs (CD28-CD3ζ)	CAR-Treg-treated mice had lower blood glucose than control mice.	Maintained FoxP3 expression; Off-target effects: no evidence.	Imam and Jaume, 2019
	HPi2	Human Tregs (CD28-CD3ζ)	The study was unsuccessful due to the unexpected high levels of HPi2 antigen present on Tregs.	Off-target effects: obvious; Tonic signalling resulted in exhaustion and potential conversion into cytotoxic T cells.	Radichev et al., 2020
Colitis	TNP	Murine $CD4^+CD25^+$Tregs (CD28-CD3ζ)	CAR-Tregs could alleviate acute TNBS colitis.	Maintained FoxP3 expression; Off-target effects: no evidence.	Elinav et al., 2008
	CEA	Murine $CD4^+CD25^+$Tregs (CD28-CD3ζ)	CAR-Tregs ameliorated colitis and prevented the development of colitis-associated colorectal cancer.	Maintain FoxP3 expression; Cytotoxicity: nonsignificant; Off-target effects: no evidence; virtually undetectable by day 9.	Blat et al., 2014
GvHD	HLA-A2	Human nTregs (CD28-CD3ζ)	Anti-HLA-A2 CAR-Tregs ameliorated the development of lethal GvHD in NSG model mice.	Maintained Treg phenotype; off-target effects: should be minimized; limited engraftment (lasted for only two weeks).	MacDonald et al., 2016
	HLA-A2	Human nTregs (CD28-CD3ζ)	CD19-targeted CAR-Tregs efficiently suppressed primary human B cells compared with polyclonal Tregs both *in vitro* and *in vivo*.	Off-target effects: no evidence; CRS: no evidence; cytotoxicity: no evidence; maintained the stability of Tregs; little risk of infection.	Imura et al., 2020
	HLA-A2	Murine $CD4^+$foxP3$^+$Tregs (CD28-CD3ζ)	Donor-specific CAR-Treg therapy could be a treatment that enables diminished immunosuppression.	Maintained FoxP3 expression; cytotoxicity: no evidence.	Sicard et al., 2020
	HLA-A2	Human nTregs (CD28-CD3ζ; ICOS-CD3ζ; CTLA-4-CD3ζ; PD-1-CD3ζ; OX40-CD3ζ; GITR-CD3ζ; 4-1BB-CD3ζ; TNFR2-CD3ζ;)	The inclusion of the CD28wt costimulatory domain was essential for the potent function of Tregs.	CD28 CAR-Tregs maintained high level of Treg phenotype.	Dawson et al., 2020
	HLA-A2	Human nTregs (CD28-CD3ζ; 4-1BB-CD3ζ)	Transient *ex vivo* mitigation of 4-1BB tonic signalling improved CAR-Treg stability and function.	Rapamycin/vitamin C rescued 4-1BB CAR-Treg function and survival for 2 months.	Lamarthée et al., 2021
MS	MOG	Murine $CD4^+$ FoxP3$^+$ T cells (CD28-CD3ζ)	CAR-Tregs localized to the brain and reduced levels of proinflammatory cytokines and disease symptoms.	Decreased suppressive capacity under the pathology of activated macrophages.	Fransson et al., 2012
Vitiligo	GD3	Murine iTregs (CD28-CD3ζ)	Antigen-specific Tregs could be prepared, used, and stored for long-term control of progressive depigmentation.	No cytotoxicity; Remained FoxP3$^+$; Long-term engraftment for 10 weeks.	Mukhatayev et al., 2020

control following reinfusion. For the isolation of T cells, $CD3^+$ T cells could be enriched *ex vivo* from autologous PBMCs, and other markers of T cells (CD4, CD8, CD25, CD62L, etc.) could also be separated for some specific purposes. For the isolation of human Tregs, there are two types of sources, namely, umbilical cord blood (UCB) and peripheral blood (PB). Previous studies demonstrated that nTregs isolated from UCB are more stable than those isolated from PB due to the relative paucity of $CD25^+$ non-Tregs in UCB (Godfrey et al., 2004). However, PB-derived Tregs are more practical for clinical settings than those derived from UCB due to the abundant number of Tregs in PB and patient compliance (Karakhanova et al., 2006). Flow cytometry-based approaches for Treg enrichment are considered better than magnetic beads in terms of isolated Treg purity and minimal percentages of $CD25^-$ effector cells, but more technical equipment is required (Hippen et al., 2011). In addition, the isolation of CD45RA-positive cells by fluorescence-activated cell sorting (FACS) to isolate Tregs allows the maintenance of all the phenotypic and functional characteristics of thymic Tregs, which is better for long-term expansion and immune regulatory function (Edinger, 2016). The activation process could be performed through the following approaches: antibodies, antibody-coated magnetic

beads, artificial antigen-presenting cells based on K562 cells, and a GMP licenced cell-based APC line that expresses the desired stimulating molecules. Anti-CD3/CD28 mAb-coated beads are generally used for cell activation and expansion, while it has been reported that the application of artificial APCs resulted in 5-fold higher proliferation of human lymphocytes (Foley et al., 2008). KT64/86 cells, a GMP licenced cell-based APC line that expresses CD86 and CD64, achieved the efficient expansion of genetically modified T cells and promoted the maintenance of CD28 expression on the surface of cultured T cells (Suhoski et al., 2007). Retroviral and lentiviral vectors are often used for CAR transgene manipulation due to their low immunogenicity and permanent delivery (Scholler et al., 2012).

A prime concern for the *ex vivo* culture of CAR-T cells is the maintenance of less differentiated phenotypes (memory stem (TSCM) and central memory (TCM) T cells). These subsets possess superior self-renewal capacity and persistence. The addition of IL-15 during culture or the incorporation of IL-15 costimulation into the CAR structure both help preserve the stem cell phenotype of T cells. The transduction of induced pluripotent stem cells (iPSCs) into naïve T cells from artificial thymic organoid systems is also a new approach needing further exploration (Montel-Hagen et al., 2019).

The phenotypic stability of Tregs has always been considered a critical concern for Treg-based therapy (Rana and Biswas, 2020). Long-term culture of Tregs may change their suppressive phenotype, which may lead to severe side effects for patients (Yu et al., 2021). A number of approaches have been reported to address this issue (Passerini et al., 2013; Segundo et al., 2006). The mechanistic target of rapamycin (mTOR) signalling pathway impacts the differentiation and functions of Tregs and conventional T cells, and rapamycin, which is an inhibitor of mTOR complex 1 (mTORC1), has been widely used to improve the stability and function of Tregs during *in vitro* culture (Battaglia et al., 2006; Strauss et al., 2007). Since FoxP3 plays a critical role in the differentiation and maintenance of Tregs, stable expression of FoxP3 by genetic engineering may block Treg conversion into effector cells and thereby improve the safety of Treg-based therapy (Fransson et al., 2012; Fu et al., 2020). The choice of costimulatory domain could also affect the stability and suppressive function of CAR-Tregs, which will be discussed in the next section (Lamarthée et al., 2021). In addition, the efficacy of other methods, including the treatment of Tregs with the vitamin A derivative all-trans retinoic acid (Zhou et al., 2010) or the administration of microbiota to a Treg-favouring environment (Atarashi et al., 2013), in maintaining the immune inhibitory phenotype of Tregs has been validated. The assessment of methylation in the Treg-specific demethylated region has been considered the gold standard to evaluate the phenotypic stability of Tregs, and this assessment has been included in the product release criteria of expanded Tregs before infusion (Floess et al., 2007; Huehn et al., 2009). To the best of our knowledge, unfortunately, a standardized quality control procedure for CAR-Tregs has not yet been well established in the clinic, and functional assays remain unavailable. The commonly used suppressive assay of CAR-Tregs *in vitro* cannot fully predict the functional activity of infused Tregs *in vivo*, and the testing process is time consuming, expensive and impractical for clinical administration (Zenclussen et al., 2005). Under stimulation from polyclones or target antigens, testing the release of inflammatory cytokines seems to be a feasible functional evaluation to exclude contamination by assumed effector T cells (Fritsche et al., 2020). In addition, other key Treg markers and homing receptors, such as CTLA-4, GITR, CD39, CD45RA, and CCR7, can also be used to monitor Treg stability during long-term *in vitro* culture.

Safety of CAR-T cells/Tregs for the treatment of autoimmune diseases

CAR-T cells/Tregs infused *in vivo* could elicit broader immune responses and destroy antigen-expressing target cells, making these cell products a potential toxic safety hazard. In general, this safety problem can be divided into the following categories: manufacturing risks, target-related risks and off-target risks.

Risks associated with during the manufacturing process are always related to the products themselves, and these include unwanted contaminants, cancerous cell products of gene editing, allogeneic T cells with potential GvHD risk and the risk of CARs transforming target cells (Marcucci et al., 2018; Neelapu et al., 2018). Given that the engineering of CAR-T cells or Tregs is generally based on viral transduction, the safety of these engineered cells for clinical application is under scrutiny. The impacts of virus-mediated gene integration in human lymphocytes, such as integration-related insertional mutagenesis, have not been fully investigated, and the incidence can be less than one event per 1,000 patients after ACT (June et al., 2015). This issue may be partly addressed by site-directed integration approaches, such as CRISPR/Cas9 and AAV-based incorporation, which may reduce the potential effect of nonspecific integration on cell survival (Papapetrou and Schambach, 2016). In some cases, allogeneic T cells are the only option, but these T cells could potentially mediate serious GvHD. From a safety perspective, the multiple possible solutions may include endogenous TCR deletion (Torikai et al., 2012), full or partial HLA matching (Brudno et al., 2016), or cell isolation from UCB (Eapen et al., 2010), which is the major challenge for the development of off-the-shelf cell therapies.

One on-target risk, cytokine release syndrome (CRS), often occurs after CAR-T therapy and the excessive immune

suppression mediated by CAR-Treg therapy. Although CAR-T cells eliminate pathological lymphocytes and have been shown to be therapeutically beneficial in autoimmune diseases, the activation of CAR-T cells and excessive production of cytokines paradoxically activate cytotoxic lymphocytes, which may exacerbate autoimmune diseases. The occurrence of CRS has long been considered the major safety concern for the treatment of cancer with CAR-T cells, and such effects in the treatment of autoimmune diseases require further clinical exploration. Tregs may be a safer option than cytotoxic T cells because of this concern (Wang et al., 2021). Inducing CAR-T-cell death by incorporating suicide genes is one of the first clinical applications of gene transfer technology in humans, and several suicide switches have been described (Zakrzewski et al., 2008). More promising switches could be envisioned in the future, such as those that autonomously induce cell death when FoxP3 expression is lost by CAR-Tregs or when the expression of IL-17 and/or another proinflammatory cytokine is acquired (Maldini et al., 2018). Additionally, CAR-Treg-mediated bystander suppression of antitumor or anti-infectious effects is another theoretical risk, and this is an important issue that must be investigated in preclinical models (Rosado-Sánchez and Levings, 2020).

Off-target risks may occur owing to intrinsic tonic CAR signalling caused by the self-aggregation of an unstable scFv. CAR-mediated tonic signalling is always independent of specific target engagement, and it is accompanied by spontaneous cytokine release and T-cell exhaustion. The modification of the CAR structure and costimulatory domain as well as amino acid substitution or humanization could likely help abrogate tonic signalling (Landoni et al., 2021).

Signalling—Distinct costimulatory requirements for cytotoxic T cells and Tregs

In second-generation CARs, costimulatory molecules are divided into CD28 and tumour necrosis factor receptor (TNFR) family proteins. It has been found that 4-1BB is an effective costimulatory domain in conventional CAR-T cells, and it confers T cells with a memory T-cell-like pattern of gene expression (Pillai et al., 2019); however, CD28-based stimulation causes the acute release of effectors (Long et al., 2015). Since Tcons and Tregs are two distinct T-cell subsets with opposite effects that undergo strikingly different metabolic patterns, the translation of CAR-T cell findings to the engineering of Tregs remains a topic of ongoing investigation in the immunotherapeutic field. Numerous approaches have been implemented to explore intracellular signalling pathways in Tregs (Spence et al., 2015). Dawson et al. compared various costimulatory signals on Tregs and found that CD28 was superior to 4-1BB in terms of maintaining the stability and immunosuppressive function of Tregs (Dawson et al., 2020). Consistent with previous findings, CD28 signalling is essential for Treg development and homeostasis, while 4-1BB-specific CARs often cause rapid proliferation and loss of function (Zheng et al., 2004). Nevertheless, the appropriate costimulatory domain for other forms of CARs, such as CARs with self-aggregating properties, still requires further investigation. Self-clustering anti-GD2 CAR-Tregs exhibit deleterious effects of CD28 signalling and lose suppressive function *in vivo* (Lamarche et al., 2020). In the absence of this tonic CAR signalling, repeated exposure of Tregs to an autoantigen or alloantigen would affect Treg stability, and persistence would be an important consideration. In addition, for peripheral Tregs isolated from PB, deficient CD28 signalling might be related to Treg-type DNA hypomethylation (Mikami et al., 2020; Wakamatsu et al., 2018). Costimulation mediated by CD28 biases the metabolic environment towards glycolytic ATP production and thus promotes T-cell terminal differentiation, which may not benefit the long-lasting function of cell products (Zappasodi et al., 2021). In addition to modulating costimulatory domains in the second-generation CARs to achieve better function, persistence and expansion of CAR-T cells/Tregs, an inducible expression cassette, such as IL-2 receptor β-chain, mRNA encoding telomerase reverse transcriptase, or PI3K inhibitor, should be considered for the optimization of the CAR structure (Bai et al., 2015; Kagoya et al., 2018; Zheng et al., 2018).

Principles for target selection in autoimmune diseases

CAR-mediated target recognition is not limited to only cell-surface proteins; natural soluble protein ligands, post-translational modifications of proteins and glycosphingolipids can also be recognized (Posey et al., 2016). The mechanisms of CAR-T therapy for disease treatment rely on the depletion of autoreactive immune cells. Therefore, an ideal target should have wide coverage and high specificity for safety and effectiveness. Systemic diseases such as lupus, affecting multiple tissues, might make it difficult to identify specific targets and measure therapeutic effectiveness. Nevertheless, nonspecific depletion of B cells by targeting CD19 has shown outstanding preclinical and clinical outcomes due to its high coverage, and potential B-cell aplasia could be rescued by effective clinical management (Wei et al., 2019).

For some diseases, such as RA and T1D, possible appropriate antigen targets have been evaluated for diagnosis and treatment. RA patients can display various synovial histological findings with different patterns of infiltration of inflammatory cells, implying different subsets of disease. The targeting of tissue pathogenic cells with specific antigens by CAR-Tregs is a feasible and challenging strategy. Treg-mediated bystander suppression and infectious tolerance are

useful tools for diseases in which antigens are various and complex. Just activated by one antigen in the location which even need not be the actual driving of diseases, Tregs could be activated and execute the immunomodulating function in the environment (Cabello-Kindelan et al., 2020).

Targeting proinflammatory cytokines is another strategy awaiting preclinical evaluation. A series of cytokines, such as TNF-α, IL-6 and IL-17A, are upregulated in autoimmune diseases (Evans et al., 2009) and play definite pathogenic roles in those diseases. The construction of a CAR with one cytokine receptor as the extracellular domain could theoretically invert proinflammatory signalling into CAR costimulation signalling. This strategy has been successfully applied in CAR-T cells for the tumour environment, tuning TGF-β signalling into CD28 costimulation (Chang et al., 2020). Engineering immune cell responses to soluble cues is a more suitable design for CAR-Tregs due to their immunomodulatory functions, which are activated in an inflammatory environment and release their tolerant cytokines.

Lessons to be learned from CAR-T cells in the treatment of tumours

The application of CAR-T cells in the field of haematological malignancies and solid tumours provides a reference and foundation for the development of CARs in autoimmune diseases. Some barriers, such as antigen escape and heterogeneity in tumours, have been addressed to a large extent by using strategies such as bispecific CAR-T cells and universal CARs. These strategies could also be applied in autoimmune diseases due to their complexity and similar heterogeneity in terms of autoantigens. Other engineering synthetic strategies, such as the emergence of nanobodies, have been well applied to replace the extracellular domain of scFv.

The clinical efficacy of using different CAR-T-cell products sequentially targeting alternative antigens has been validated (NCT03019055). Making CAR-T products target more than one antigen could be achieved by the following 3 strategies: a mixture of different CAR-T products, the transduction of different CARs into T cells, and tandem CAR-T. A tandem CAR-T-cell expresses a CAR harbouring two distinct ligand-binding domains. A tandem CD19- and CD20-targeted CAR-T product was well tolerated and showed promising response rates in patients with B-cell malignancies (Tong et al., 2020).

Developing universal CARs (UniCARs) is an important direction for CAR-based therapy, and numerous studies have been conducted (Cho et al., 2018). For example, a Zip system composed of a leucine zipper-containing universal receptor and a cognate leucine zipper linked to an antigen-specific scFv has been reported to target multiple antigens without the re-engineering of CAR-T cells (Cho et al., 2018). Other CAR constructs that include orthogonal molecules, including biotin-binding immunoreceptor, anti-FITC, and anti-PNE peptide, have also been well studied (Liu et al., 2019). Based on this methodology, the aforementioned proof-of-concept study by our group using universal anti-FITC CAR-T cells combined with FITC-labelled immunodominant peptides was conducted (Zhang et al., 2021b), and the results provided a promising direction for the precise and customized treatment of autoimmune diseases by CAR-T cells.

The scFv composed of the variable region of heavy-chain VH and light-chain VL of the antibody linked by a (GGGGS) 3 sequence is generally used as an antigen-binding domain for CAR construction. The high affinity of scFv, which lacks a constant antibody domain, exposes hydrophobic patches of the variable domain to the outside, making scFv prone to self-aggregation and the overactivation of T cells (Nieba et al., 1997; Shah and Fry, 2019). Nanobodies (VHH antibodies) are one of the smallest known antigen-binding antibody fragments, and they are derived from the variable domain of camelid antibodies (Hamers-Casterman et al., 1993). VHH demonstrated a number of advantages over scFv in terms of low immunogenicity, high solubility and good stability (Jin et al., 2022). Nanobody-based CARs have been explored over the years, and the findings of several studies have demonstrated their potential as an ideal antigen recognition domain for CAR structural optimization (Bao et al., 2021).

Other manufacturing innovations

The major hurdles associated with the development of CAR-based products are the low yield and high cost, which restrict their large-scale preparation. The *in situ* reprogramming of CARs in the body is an attractive method to reduce the cost and simplify the process of CAR-T-cell preparation (Nawaz et al., 2021). A few studies have demonstrated that nanoparticle-mediated delivery of mRNA encoding CAR or TCR complexes could routinely transfect >70% of cultured T cells, with an average of 7 days of transient expression. Parayath et al. reported that CD3-targeted nanoparticles preferentially transfected T cells with anti-CD19-specific CAR mRNA, and this *in vivo* reprogramming of CAR-T cells resulted in an antileukaemia response in immunocompetent mice (Parayath et al., 2020). Several ongoing clinical trials are examining repeated infusions of *ex vivo*-engineered mRNA-transfected CAR-T cells (NCT01355965, NCT01897415, NCT02277522 and NCT02624258), and the initial data suggested promising clinical effects. In addition, an AAV vector encoding a CD4-specific CAR (as an HIV-targeting receptor that targets gp120) was used to deliver genes *in vivo*; this approach generated CAR-T cells that have strong functions as *ex vivo*-generated CAR-T cells (Nawaz et al., 2021). Even though those *in vivo* CAR-T-cell strategies still have some limitations, such as dependence on sufficient

functional T cells in patients as well as blunted efficacy due to elicited immune responses, these technologies substantially streamline the drug-like delivery of CAR-T-cell therapy, providing the promise of translation for the treatment of autoimmune diseases.

The development of other tolerogenic immune cell subsets from innate immunity armed with CAR components is a promising direction that warrants further investigation. Immunoregulatory NK subsets characterized by high expression of CD56 are endowed with increased production of cytokines such as IL-10 and reduced cytotoxicity (Fu et al., 2014; Kucuksezer et al., 2021). The regulatory function of $CD56^{bright}$ NK cells has been validated in an experimental autoimmune encephalitis (EAE) model. Macrophages are another type of multifunctional immune cell that may either activate or inhibit immunity depending on their phenotype. Adoptive cell transfer of M2 macrophages with immunosuppressive functions has been demonstrated to be a promising immunotherapy for the treatment of autoimmune diseases such as IBD and T1D (Arranz et al., 2012; Parsa et al., 2012), and engineering M2 macrophages with CARs may be another novel immunotherapy candidate for the treatment of chronic autoimmune diseases. Further clarifying the multifaceted roles of these cells in autoimmune diseases, identifying their phenotype-associated specific markers, and addressing the issues in maintaining their regulatory function are the basis for treatment.

Conclusion

Current management strategies for autoimmune diseases that involve immunosuppressive medications and engineered biological materials have failed to achieve satisfactory clinical results. The rapid development of engineered cell-based therapies and synthetic immunological approaches has broadened their potential for the treatment of human diseases (Figure 1). CARs are specific, which is in line with the complexity of autoimmune disorders. More basic research and preclinical studies are needed to evaluate CAR-based therapy before initiating multicentre clinical trials, and further optimization to continuously increase the safety and precision of cell-based therapies is needed for the widespread use of engineered T-cell/Treg therapy in autoimmune diseases.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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