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Review

Preclinical scenario of targeting myocardial fibrosis with chimeric antigen receptor (CAR) immunotherapy

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ABSTRACT

Fibrosis is present in an important proportion of myocardial disorders. Injury activates cardiac fibroblasts, which deposit excess extracellular matrix, increasing tissue stiffness, impairing cardiac function, and leading to heart failure. Clinical therapies that directly target excessive fibrosis are limited, and more effective treatments are needed. Immunotherapy based on chimeric antigen receptor (CAR) T cells is a novel technique that redirects T lymphocytes toward specific antigens to eliminate the target cells. It is currently used in haematological cancers but has demonstrated efficacy in mouse models of hypertensive cardiac fibrosis, with activated fibroblasts as the target cells. CAR T cell therapy is associated with significant toxicities, but CAR natural killer cells can overcome efficacy and safety limitations. The use of CAR immunotherapy offers a potential alternative to current therapies for fibrosis reduction and restoration of cardiac function in patients with myocardial fibrosis.

1. Cardiac fibrosis

Fibrosis is a fundamental process in cardiac remodelling and a crucial contributor to heart failure (HF) development and progression. The presence and extent of myocardial fibrosis has prognostic value because it causes contractile dysfunction and arrhythmias in structural heart disease of various aetiologies [1–6]. Moreover, myocardial fibrosis increases the risk of rehospitalization [7] and mortality in HF [8].

The pathobiology of cardiac fibrosis has been studied extensively [2–6,9]. Fibrosis derives from excessive accumulation of extracellular matrix (ECM) and can be categorized as reparative or reactive [10,11]. The formation of an organized scar after myocardial infarction (MI) is best described as reparative or replacement fibrosis, which is needed to mechanically stabilize the necrotic tissue defect. Conversely, the fine interstitial 'reactive fibrosis' seen in non-ischemic cardiomyopathies or surviving myocardium after MI appears to result from different pathological processes that give it a characteristic structural quality, ECM composition, and metabolic properties. Additionally, scar development involves a time component that must be considered. For example, reactive fibrosis in the setting of pressure overload is initially characterized by perivascular fibrosis that progresses to interstitial fibrosis [10]. Fibrosis is also highly dynamic and typically entails recruitment of

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fibroblasts that are converted into myofibroblasts, excessive synthesis and secretion of ECM and ECM-associated modulatory glycoproteins, posttranslational modification and cross-linking of ECM proteins, and dysregulation of ECM breakdown by matrix metalloproteinases (MMPs) and their endogenous inhibitors.

In HF with reduced ejection fraction (HFrEF), specifically in ischemic events, scar tissue replaces the healthy myocardium after MI. In this scenario, reparative fibrosis replaces a massive number of cells, and reversing this process to achieve cardiac rejuvenation requires recellularization and fibrosis reversion. In contrast, in HF with preserved ejection fraction (HFpEF), compliance reduction and increased stiffness due to reactive myocardial fibrosis accumulation are the main factors responsible for ventricular dysfunction. As suggested by clinical and experimental evidence, cardiac fibrosis can be reversible [12], so that reactive fibrosis has become an important target in HFpEF treatment [13]. In fact, fibrosis is broadly a direct and indirect target of HF treatment, either by established drug therapies or specific antifibrotic drugs. However, the resilience of this tissue and the repair process require additional efforts to control, prevent or even reverse fibrotic remodelling.

In this context, Chimeric antigen receptor (CAR) T cell therapy offers a new avenue. This method was originally developed to kill cancer cells, and consist in modifying T cells from patient to express a chimeric receptor that recognizes malignant cells. Interestingly, few preclinical studies have shown promise results for specifically elimination of activated fibroblasts through fibroblast activated protein (FAP) as a therapy for HF [14–16]. This review summarizes the antifibrotic effects of current drugs and explains the rationale for the use of CAR T/NK cells, presenting the results of related preclinical studies (**Graphical abstract**).

2. HF therapies with antifibrotic effects

Renin–angiotensin–aldosterone system (RAAS) inhibitors represent the mainstay of treatment for HF. Several classes of RAAS inhibitors have been developed to control the blood pressure and sodium homeostasis avoiding their profibrotic effects: Angiotensin-Converting Enzyme inhibitors (ACEi) and Angiotensin Receptor Blockers (ARB) act specifically inhibiting Angiotensin II action as a fibrogenic stimuli, finally Mineralocorticoid Receptor Agonists (MRA) act blocking aldosterone to prevent fibrogenic response [17]. Other relevant antifibrotic drugs are Angiotensin Receptor Neprilysin inhibitor (ARNI) which are composed by the combination of two anti-hypertensive drugs: sacubitril and valsartan. Sacubitril inhibit neprilysin action avoiding the breakdown of natriuretic peptides and increasing their level whereas Valsartan is an ARB drug [18].

Here, we recapitulate the main relevant evidences for these approaches. In patients with hypertensive heart disease, 6 months of lisinopril therapy (ACEi) lead to a significant reduction in myocardial fibrosis and improvement of left ventricular diastolic dysfunction [19]. A sub-study of the PARADIGM-HF trial (Prospective Comparison of ARNI with ACEI to Determine Impact on Global Mortality and Morbidity in Heart Failure) analysed the effect of sacubitril/valsartan (ARNI) on biomarkers of ECM homoeostasis and collagen synthesis and their relationship with clinical events in patients with HFrEF. The results showed that sacubitril/valsartan decreased many of these biomarkers [20], with aldosterone, sST2, TIMP-1, MMP-9, PINP, and PIIINP having decreased more with sacubitril/valsartan than with enalapril (ACEi) at 8 months after randomization. In a *post hoc* analysis of the PARAGON-HF trial (Prospective Comparison of ARNI With ARB Global Outcomes in HF With Preserved Ejection Fraction), biomarkers reflecting ECM homeostasis were elevated in patients with HFpEF and were reduced by sacubitril/valsartan treatment [21]. As for MRA, the RALES trial (Randomized Aldactone Evaluation Study) found a reduction in circulating fibrotic markers after 6 months of treatment with spironolactone in patients with congestive HF [22]. The Aldo-DHF trial (Aldosterone Receptor Blockade in Diastolic Heart Failure), however, showed that spironolactone is less effective in HFpEF with the biochemical fingerprints of high cross-linking of myocardial collagen [23].

Cardiac fibroblasts become activated in response to various myocardial injuries through well- studied mechanisms that include transforming growth factor beta (TGF-β)-SMAD2/3, among others. TGF-β is produced within injured tissues, inhibits immune cell proliferation, and stimulates fibroblasts to synthesize ECM proteins [24]. Because of the important role of TGF-β in conversion of fibroblasts into myofibroblasts, this protein has been considered a promising therapeutic target for specific fibrotic pathway inhibition. In a preclinical study, treatment with the TGF-β inhibitor pirfenidone prevented diastolic dysfunction and left ventricular fibrosis in mice exposed to pressure overload by transaortic constriction [25]. In a model of MI, pirfenidone decreased the infarct scar, improved left ventricular function, and decreased ventricular tachycardias [26]. The phase 2 PIROUETTE trial (Pirfenidone in Heart Failure With Preserved Ejection Fraction) showed a small reduction in extracellular volume (a surrogate for myocardial fibrosis) after 52 weeks of treatment with pirfenidone in patients with HFpEF [27].

3. The CAR T cell strategy

CAR T cells are traditionally produced by transducing a genetically engineered CAR fusion protein into autologous T cells. Once modified, the T cells are expanded and infused into the patient with prior lymphodepletion [28]. After infusion, T cells proliferate and recognize their target antigen through the single chain variable fragment (scFv) binding domain, resulting in endogenous T-cell activation for target cell elimination through the cytokine release. This, together with the tumour antigen release activates the immune system to recruit and activate endogenous T cells amplifying the cytosolic effect [29,30] (Fig. 1). The revolution of immunotherapy also brought its share of challenges like the use of allogeneic T cells, sorely associated with Graft *versus* Host Disease (GvHD), to reduce the production costs and length [31].

CAR T cell therapy first appeared as a promising alternative for paediatric and adult patients with highly refractory and relapsing haematological malignancies, such as large B-cell lymphomas and lymphoblastic leukaemia. This therapy was first approved to target the CD19 protein, which is expressed in most B-cells and shows limited expression in other cells [32]. Other T-cell therapies have proved effective, such as B-cell maturation antigen targeting in multiple myeloma (BCMA) [33], B-cell precursor targeting CD22 in acute lymphoblastic leukaemia [34] or CD30 in relapsed and refractory Hodgkin lymphoma [35,36]. Also, it has been considered as a therapeutic option for other pathologies such as solid tumours [30], and new indications continue to emerge as reflected by the numerous clinical trials that are ongoing worldwide with official agencies support [37].

New advances show that cardiac fibrosis is a dynamic and reversible process that can revert the fibrotic lesion during early stages or when the underlying causes are eradicated, restoring appropriate tissue architecture and function [38,39]. To achieve cardiac fibrosis regression, several strategies are possible, such as ECM degradation or elimination of fibrogenic myofibroblasts. Considering their abundance, myofibroblasts may be a suitable target to combat fibrosis [40], an idea first introduced in a preclinical study by Aghajanian et al. [15]. In a mouse model of hypertensive cardiac injury and fibrosis, these authors engineered a CAR T cell specific for fibroblast activated protein (FAP) to specifically eliminate myofibroblasts. Adoptive transfer of FAP CAR T cells resulted in significantly reduced cardiac fibrosis and restored cardiac function (Fig. 2). Of note, although FAP also is expressed in pancreas and skin fibroblasts after wounding, no pathological responses were observed after FAP CAR T cell administration [15]. Some drawbacks of this approach are the complex process needed to obtain T cells *ex vivo* and the permanent activation of these cells in targeting myofibroblasts. Permanent activation also might be a problem because of important adverse effects observed in oncological CAR T cell clinical trials. The

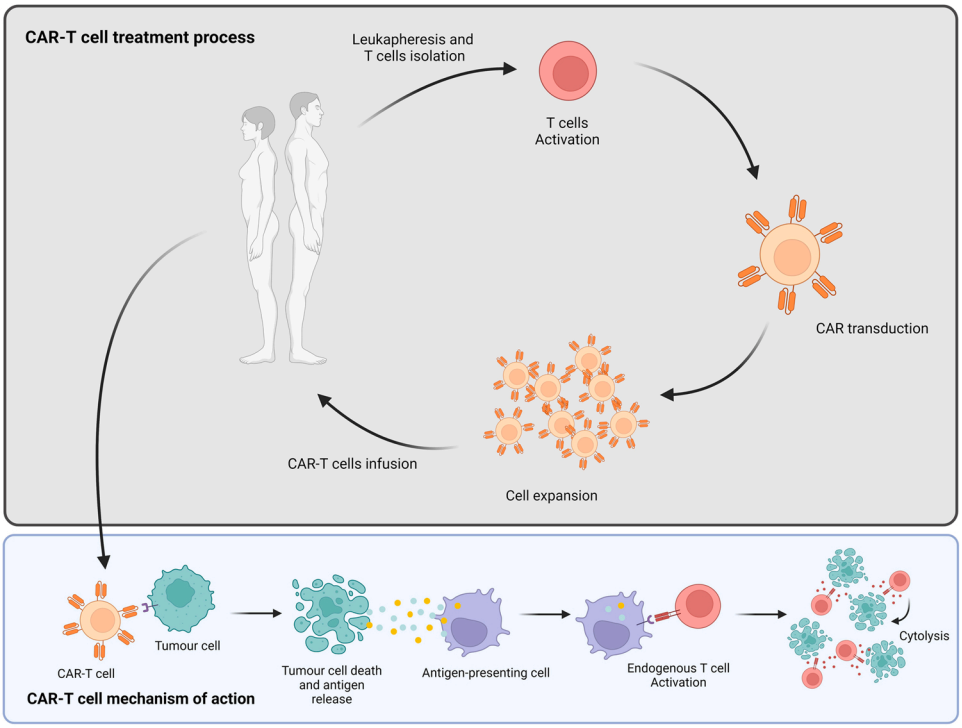

Fig. 1. Overview of the CAR T cell treatment process and mechanism of action. In CAR T cell therapies, T cells are extracted from the patient's blood and transduced *ex vivo* with a gene through viral transduction, RNA electroporation or RNA lipid nanoparticles (RNA LNP) for a chimeric antigen receptor (CAR) specific to a cancer cell antigen. CAR T cells are then expanded and infused into the patient (grey panel). After infusion (blue panel), CAR T cells travel to the tumour, identify the cancer cells through the CAR, activate and kill them *via* the perforin and granzyme axis, as well as the release of cytokines. These, together with the tumour antigen release activates the immune system to recruit and activate endogenous T cells to amplify the tumour-directed cytolysis. Created with BioRender.com

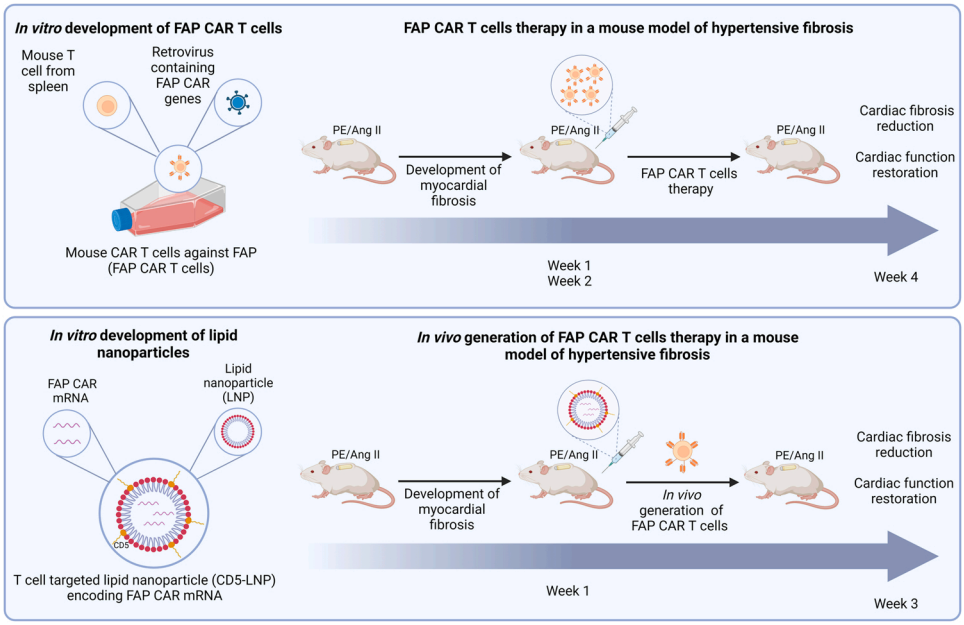

Fig. 2. *In vitro* and *in vivo* FAP CAR T cell generation strategies for hypertensive fibrosis treatment in a mouse model. In the *in vitro* model described by Aghajanian et al., FAP CAR T cells are generated and expanded *in vitro* and then administered to hypertensive mice [15]. In contrast, Rurick et al. designed lipid nanoparticles (LNPs) containing the mRNA encoding the FAP CAR and then administered them to mice with experimental cardiac fibrosis [16]. In this case, FAP CAR T cells were generated *in vivo*. Both approaches resulted in the death of FAP-expressing cells *in vivo*, reducing fibrosis and improving heart function. *PE/Ang II:phenylephrine / angiotensin II*. Created with BioRender.com

most common adverse effect is cytokine release syndrome (CRS), caused by the massive release of proinflammatory cytokines by activated T cells and other immune cells [41–43]. The incidence of CRS is 70–90% and usually with mild complications, although around 20–50% of these suffer severe complications such as vascular leak syndrome, circulatory collapse, and multiorgan failure [44]. The severity of CRS correlates with tumour burden and the CAR T cell dose injected. Off-target cross-reactivity of CAR T cells with unrelated proteins expressed by healthy tissue also has been reported [45,46].

The latest development of CAR T generation exploits the technology of lipid nanoparticles (LNPs) containing messenger RNA (mRNA) sequences. The safety and scalability of this technology has been established through its use in mRNA vaccines against Covid- 19, and it can be used to simplify the method of generating CAR T cells for the treatment of fibrosis. Rurik et al. [16] generated specific LNPs directed to T cells *in vivo* through the expression of anti-CD5 on their surface, and containing mRNA encoding a CAR against FAP (FAP CAR). After the T cell interaction with the nanoparticles, the mRNA is released to the cytoplasm, where it is translated into a functional FAP CAR. FAP CAR T cells will be able to recognize and bind to activated cardiac fibroblasts, which express FAP on their surfaces (Fig. 3). When administered to mice with induced cardiac fibrosis, the lipid nanoparticles containing the mRNA encoding the FAP CAR behaved similarly to conventionally generated (*i.e.*, "*ex vivo*") FAP-directed CAR T cells [16] (Fig. 2). Both

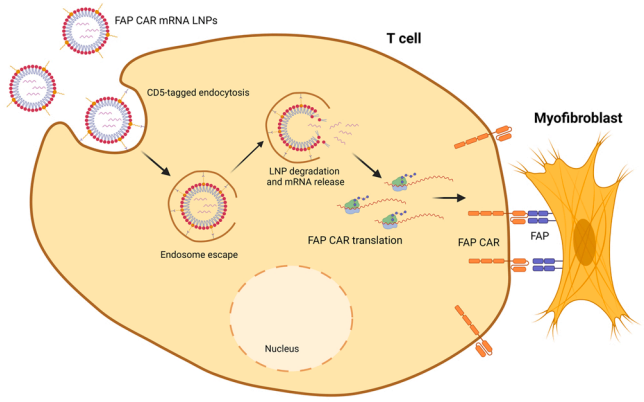

Fig. 3. CAR T cell production *in vivo*. The lipid nanoparticles (LNPs) containing mRNA encoding a CAR designed to bind FAP (FAP CAR) are tagged with an anti-CD5 that will recognize T cells. Then, LNP fuses with the T cell, and the mRNA is released to the cytoplasm, where it is translated into the FAP CAR, equipping T cells with the ability to recognize and bind to the myofibroblast [16]. Created with BioRender.com

approaches resulted in the death of FAP-expressing cells *in vivo*, reducing fibrosis and improving heart function. The findings of Rurik et al. represent a great advance for several reasons. First, rather than relying on an *ex vivo* method of preparing CAR T cells, their approach would enable the treatment to be distributed as an "off-the-shelf" quality-controlled therapeutic LNP–mRNA that generates the CAR T cell therapy *in vivo*, much like the current Covid-19 mRNA vaccines. Second, the expression of the mRNA is transient (it does not require integration of nucleic acid into the host genome), which reduces the risk of long-term genetic alterations and allows for dose adjustment and repeated administration as needed. Finally, the remarkable safety of LNP–mRNA technology has been established through its use in millions of patients worldwide. Even so, the CAR T cells complications and safety risks mentioned above, have led to consider CAR natural killer (NK) cells as a possible alternative in immunotherapy.

4. The CAR NK cell strategy

NK cells represent 5–20% of all circulating lymphocytes and are involved in the innate immune response. They originate from $CD34^+$ hematopoietic progenitor cells and are further matured in the bone marrow and secondary lymphoid tissues [47]. NK cells provide the first line of defence against viral and bacterial infections and respond quickly to tumour formation. They can detect "missing-self" markers of major histocompatibility complex (MHC) class I and immediately elicit a cytotoxic immune response [48]. Many inhibitory and activating receptors are involved in balancing NK cell functions, reducing the risk of GvHD in allogeneic transplantation [49,50]. These receptors can be grouped into two classes of glycoproteins: the killer cell immunoglobulin-like receptors (KIR) and the killer cell C-type lectin receptors (KLR). KIRs are type I transmembrane glycoproteins that specifically recognize class-I MHC or HLA in humans and are involved in self and non-self recognition. Various combinations of inhibitory and activating KIRs are expressed on NK cells, with each of them recognizing a particular MHC molecule [51]. While inhibitory KIRs are more subject to recognizing self-MHC, activating KIRs may be triggered by foreign MHC, leading to an immune response. On the other hand, KLRs are type II glycoproteins encompassing the NKG2 subfamily (or KLRC1, CD159). These receptors can also be inhibitory or activating and bind to non-classical class-I MHC molecules, such as HLA-E [52], MIC-A, or MIC-B [53]. They form heterodimers with CD94, with the exception of NKG2D, which dimerizes [54]. The expression balance of inhibitory and activating receptors is not constant and depends on the maturation stage of the NK cells [55]. When triggered, NK cells release lytic granules containing perforin, which can form pores in the target cell, and granzymes from the serine protease family, which can induce apoptosis [56].

NK cell activation also can be mediated by antibody-dependent cell-mediated cytotoxicity through the FcγRIII receptor (CD16). By binding to the constant domain of the antibody heavy chain, NK cells can detect opsonized antigens and induce a cellular response against the target [57]. In immunotherapy, this antibody-dependent activation of NK cells has been broadly exploited with the use of monoclonal antibodies specifically engineered to tackle cancers that overexpress particular antigens [58].

The unique biological characteristics of NK cells get around the major limitations of CAR T cell therapy in cancer (Fig. 4). Tumour cells tend to downregulate MHC-I, leading to the escape of T cell antitumor actions [59]. In contrast to T cells, NK cells can exert a direct cytotoxic effect against tumour tissues lacking MHC-I expression [60]. The NK cell cytotoxic response is governed by different activating and inhibitory receptors that, upon binding specific ligands, trigger the immune response. Furthermore, because of a limited lifespan of CAR NK cells, about 2 weeks in the circulation, the risk of on-target/off-tumour toxicity is low [61]. Finally, the probability of CRS in CAR NK immunotherapy is lower in part because of the secretion of different cytokines: activated CAR NKs mainly produce IFN-γ and GM-CSF [62], whereas activated CAR T cells mostly induce cytokines highly related to CRS, such as IL-2, IL-2Rα, IL-1α, IL-1RA, IL-6, TNF-α, MCP-1, IL-8, and IL-10 [63]. The superior safety of the CAR NK cells compared with CAR T cell immunotherapy has been shown in several clinical settings such as efficiency and safety as mentioned before. Allogeneic CAR NK cells provide an "off-the-shelf" therapy that eliminates the need for a patient-specific product, making feasible their large-scale production and implementation in clinical practice to reduce the enormous medical burden of cardiac fibrotic disorders [64].

As in CAR T cells, the first studies with CAR NK cells were performed in haematological cancers. In a phase I/II trial, anti-CD19 CAR NK was administered to 11 patients with non-Hodgkin's lymphoma or chronic lymphocytic leukaemia and led to complete tumour remission in seven patients without toxic effects [65]. More CAR NKs targeting other markers involved in blood malignancies have been studied, such as CD123 [66] or CD33 [67]. A vast number of *in vitro* and *in vivo* studies have tested the efficacy of CAR NK cells against several solid tumours such as brain, breast, ovarian, pancreatic, colorectal, and prostate [68], with successful results for tumour remission, in contrast to CAR T. For

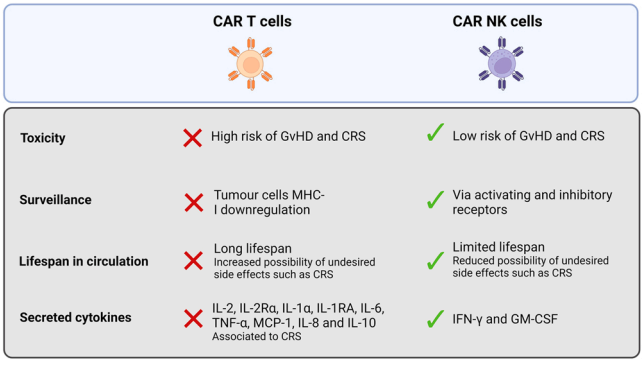

Fig. 4. Comparison of CAR T cells with CAR NK cells therapies. Toxicity: The use of allogeneic CAR T cells is associated with graft-*versus*-host disease (GvHD) and cytokine release syndrome (CRS) caused by overactivated T cells. Surveillance: Tumour cells can escape CAR T cell surveillance by major histocompatibility complex class I (MHC-I) downregulation, reducing the CAR T cell antitumor effect. In contrast, NK cells exert their cytotoxic effects *via* a combination of activating and inhibitory receptors that will trigger the immune response anyway. Lifespan in circulation: The limited lifespan of CAR NK cells in circulation (about 2 weeks) compared to CAR-T cells (years) lowers the risk of on-target/off-tumour toxicity. Secreted cytokines: Activated CAR T cells secrete cytokines that can cause CRS. Created with BioRender.com

instance, NKG2D directed CAR NK reduced the size of colorectal tumours in mice. More important, tumour regression has been observed in three patients with metastatic colorectal cancer [69]. Clinical results obtained from small trials have suggested that these cells represent a promising therapeutic tool in cancer, and several clinical trials are ongoing.

5. Conclusions and perspectives

Cardiac fibrosis represents an important clinical burden, and new therapeutic approaches are needed. CAR T cell immunotherapy has shown antifibrotic efficacy in mouse models of cardiac fibrosis. Although CRS has not been reported in the murine preclinical model, clinical data of CAR T cell immunotherapy to treat cancer have shown cytotoxicity associated to CRS as one of the major side effects of this therapy in humans, which could be circumvent with the use of CAR NK cells. Notwithstanding the above, CAR therapies still raises questions and point to challenges including in which pathologies the immunotherapy could work, cell target selection and dosage strategy. To date, it has been demonstrated their application to combat diffuse fibrosis in a hypertensive preclinical model, opening the opportunity to treat interstitial fibrosis with other origins such as diabetes or obesity. Further studies are needed to unravel if the immunotherapy would work in ischemic models, where the access to fibrogenic cells might be reduced due to scarring and tissue distortion. Interestingly, the CAR T cells target to treat myocardial fibrosis in the mouse model was FAP, being also upregulated in other pathological fibrotic tissues such as lungs, liver and submucous. This opens new opportunities for CAR immunotherapy as an antifibrotic treatment for a broader range of pathologies where fibrosis is established. The choice of cell-surface target for the CAR T cells is also critical and may vary among different organs and species. To minimize 'off-target' effects on healthy tissues, the ideal target should be restricted to the injured tissue, where fibrogenesis is concentrated. Simultaneous recognition of two fibrosis specific antigens could help to constrain the area of interest, but identifying the ideal cell-surface targets in specific contexts could prove challenging. Fibrogenic cells may support organ function in ischemic pathologies, and their elimination could disrupt tissue regeneration and repair, especially in the heart, where complete loss of the scar could lead to cardiac rupture. Many efforts are needed to identify the dosage strategy to eradicate cardiac fibrosis due to the different characteristics between cancer and cardiovascular diseases. The distinct tissue environments, including immunosuppressor stimuli or grade of inflammation, force us to adapt to the new scenario. Several questions and concerns raise including the safety dose, the number of infusions or whether may work as a unique treatment or in combination with the standard drug therapies. Rigorous tests of replications by independent laboratories are mandatory. Nonetheless, these questions provide the basis for further investigations in CAR therapies with the aim of reducing myocardial fibrosis.

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CRediT authorship contribution statement

Gemma Ferrer-Curriu: Investigation, Writing – original draft, Writing – review & editing. **Carol Soler-Botija**: Investigation, Writing – original draft, Writing – review & editing; **Sandra Charvatova**: Investigation, Writing – original draft**; Benjamin Motais**: Investigation, Writing – original draft **Santiago Roura:** Writing – review & editing, Funding acquisition; **Carolina Galvez-Monton**: Writing – review & editing; **Marta Monguió-Tortajada**: Writing – review & editing. **Oriol Iborra-Egea**: Writing – review & editing; **Michele Emdin**: Writing – review & editing; **Josep Lupón**: Writing – review & editing, Funding acquisition. **Alberto Aimo:** Writing – original draft, Writing – review & editing; **Juli R Bagó**: Investigation, Writing – original draft, Writing – review & editing. **Antoni Bayés Genís**: Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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