Journal of High School Science

The potential of immunotherapy treatments to facilitate post-cardiac injury recovery

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Submitted: August 25, 2022, revised: version 1, October 2, 2022
Accepted: October 8, 2022

Abstract
As the prevalence of cardiac disease and injury increases across the world, there is a greater need to understand its pathology and how to effectively combat it. An avenue of treatment, known as immunotherapy, has gained traction in recent years due to studies suggesting its potential to favorably treat cardiac disease. The four immunotherapies that this paper explores are: immunoadsorption and immunoglobulin treatment, CAR T-cell therapy, macrophage-derived cardiac regeneration, and regulatory T-cell cardioprotective function. Experimental data suggest that all four of these treatments result in improvement in cardiac function in either animal or human models. A meta-analysis of studies involving human models indicated that immunoadsorption treatment and its combination with immunoglobulin treatment can improve the left ventricular ejection fraction, reduce left ventricular end diastolic diameter, and thus improve clinical outcomes in dilated cardiomyopathy patients. Additionally, expression analysis of cardiac fibroblast gene signatures from healthy versus diseased human hearts identified an endogenous target, the fibroblast activation protein (FAP). The adoptive transfer of T-cells expressing a chimeric antigen receptor (CAR) against FAP in a murine model resulted in a significant reduction in cardiac fibrosis and restoration of function post injury. Using animal models of mice and Zebrafish, macrophage-induced cardiac regeneration was shown to contribute to collagen deposition in post-injury scars. This contradicts the previous scar formation model, which asserted that myofibroblasts were the only cell type that performed this function. Finally, regulatory T-cells found in the myocardium have been shown to express Sparc, a gene critical for cardioprotection, by increasing collagen content and maturation after myocardial infarction. However, although these immunotherapies have shown unprecedented success in relieving cardiac disease and regaining normal cardiac function, further research is necessary to validate the safety and efficacy of these therapies for treating this disease in humans.

Keywords
Immunotherapy, Cardiac injury, Cardioprotection, CAR T-cells, Macrophages, Regulatory T-cells, Immunoadsorption treatment, Myocardial infarction, Cardiac fibrosis

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Introduction

Cardiac injury is the leading cause of disease and death across the world. Even though significant advances have been made in cardiology, scientists still do not completely understand why the heart does not fully heal after an injury or why progressive heart failure persists and worsens over time. Currently, no therapeutics/treatments, other than heart transplantation, are effective to stop or revert the evident progression of heart disease in the majority of patients. Effective treatments for heart failure have focused on modifying the blood flow in the body by stimulating compensatory mechanisms. Compensatory mechanisms exist to manage cardiac failure, and common examples of such mechanisms are arterial and vein vasoconstriction and vasodilation to control blood pressure or blood volume, or changes in the function of the cardiomyocytes to modify the cardiac muscle contractility.

The human body responds to cardiac injuries through acute and chronic adaptive processes to maintain the heart's ability to pump blood. This response is only possible through inflammation and immune cell signaling. For example, after an acute ischemic injury, immune response activated cardiac fibrosis is critical to avoid myocardial rupture (1). Cardiac fibrosis is one of the main compensatory mechanisms that is triggered following a cardiac injury. When looking at the development of myocardial fibrosis, multiple pathways exist for fibroblast activation. RNA sequencing of fibroblasts has shown several fibroblast populations before and after injury with distinct transcriptome signatures. Though researchers do not yet completely understand the relationship between the various fibroblast subsets and their physiology, they do understand that the immune response's regulation of cardiac fibrosis impacts cardiac function in both the short-term and long-term (2). Fibroblasts within the tissue differentiate in the early stages of cardiogenesis, and during homeostasis, these cardiac fibroblasts secrete and maintain the extracellular matrix (ECM). However, after an acute cardiac injury, these resident fibroblasts are activated, and as a result, their gene expression is altered. The alteration of the gene leads to the acceleration of the production of ECM. During a myocardial infarction, these activated fibroblasts are recruited through the process of epithelial-mesenchymal transformation, and travel to the regions of damaged cardiac muscle.

Fibroblasts are activated in response to the presence of multiple cytokines, specifically transforming growth factor beta (TGFb), which is produced by immune cells and activated after leaving the ECM. The ECM produced by activated fibroblasts allows for the cardiac tissue to remain structured and healthy while also limiting the occurrences of cardiac dilation and rupture (2). This is a positive response for acute cardiac injury in the short term however, in the long term, the occurring fibrosis has a negative impact on myocardial health. Myocardial fibrosis has repeatedly had poor clinical outcomes as it adversely alters the heart physiology, directly and indirectly. Excess ECM directly transforms the compliance and malleability of the heart muscle, causing it to become stiffer and unmalleable. This stiffness causes the cardiomyocytes to work harder than they should, thereby intensifying their dysfunction and decreasing the diastolic and systolic cardiac function (3). Furthermore,

excess collagen deposits are associated with defects in electrical conduction and arrhythmias. Activated fibroblasts and the ECM components signal infiltrating immune cells to the cardiac microenvironment. For instance, the ECM protein tenascin-c accelerates macrophage recruitment to the myocardium and inflammatory environment.

Besides the recruitment of fibroblasts from the epicardium, activating interstitial fibroblasts result in the formation of scars that maintain the structure, strength, and consistency of the cardiac chambers. However, even though the fibroblast response is initially beneficial in preventing myocardial rupture, the response, in many cases, becomes flawed and, overtime, results in the development of excess ECM (4). This buildup of ECM not only stiffens and hardens the cardiac muscle, but also negatively impacts the cardiac muscle integrity, leading to deterioration of cardiac function over time. In every myocardial disease, cardiac fibrosis is present. After an acute cardiac injury, cardiac fibroblasts begin to deposit ECM in the myocardium, causing increased stiffness of cardiac tissue and reduced compliance of the myocardium (5). The excessive buildup of ECM and fibrosis is a major factor in the rapid progression of cardiac disease and heart failure.

There is evidence to suggest that fibrosis causes diastolic heart failure, which decreases the elasticity of the cardiac heart muscle. As a result of this loss of elasticity, the heart fails to fill up with a sufficient amount of blood. Another type of heart failure is systolic heart failure, where the heart becomes less efficient in pumping blood out to the rest of the body. Systolic heart failure is attributed to dilated cardiomyopathy (DCM), which is characterized by the impairment of the left ventricle as well as the dilation and weakening of ventricular walls (6). As one of the most common ways of developing end-stage heart failure, DCM frequently requires heart transplants. The onset of DCM is associated with multiple factors; inherited DCM is caused by genetic variations, while whole acquired DCM is a result of toxins, viruses, immune disorders, and arrhythmias (7). In terms of prevalence, DCM is more common in men than women, but is also relatively uncommon within the general population. Out of 100,000, only 40 people will be affected by DCM with a yearly DCM incidence of 7 out of 100,000. Even though progress in combating DCM has been made, the mortality rate of DCM patients still remains significantly high (8).

Besides the compensatory mechanisms the human body has, such as cardiac contractility and blood vessel biology, there is a significant role that the immune system plays in modifying and potentially preventing the future/downstream problems that are associated with cardiac injury [7]. Immune response to cardiac injury is only partially understood but is known to be influenced by the differences in injury type, in addition to host and environmental factors. The factors responsible for initiating the pro-inflammatory cardiac environment during a myocardial injury are damage associated molecular patterns (DAMPs), which are released by the dying cells as a result of the myocardial injury, the cytosolic DNA sensing pathway (cGAS-STING), and cardiomyocyte NOD-like receptor protein 3 (NLRP3) inflammasome (9). Without an inflammatory response to cardiac injury, scar tissue will not form on the

myocardium, thereby making the heart more prone to cardiac rupture and death.

Cardiac resident macrophages are the first type of immune cell that respond to injury. These cells are self-renewing, created in the embryonic myocardium, and are critical for myocardial development, specifically for the structure of the cardiac lymphatic system and the proper conduction of electrical impulses. Macrophages are also involved in the maintenance of cardiac energy homeostasis, which is enhanced in times of myocardial stress.

The significance of macrophages is demonstrated by several studies that have concluded that cardiac dysfunction is the result of the depletion of cardiac macrophages. During an injury, activated fibroblasts, cardiomyocytes, and other immune cells release cytokines, which cause the nearby macrophages and chemokines to recruit more monocytes from the circulation to migrate to the injury site. As a result, the tissue resident CCR2 macrophages are activated and multiply (9). Scientists have discovered that these cells play an important role in protecting and healing the heart post-injury, especially by facilitating neo-vascularization. Also, during an injury, the inflammatory signaling environment created by immune cells recruits more "infiltrating" immune cells from the bloodstream (10). For example, infiltrating CCR2+ monocytes mature into macrophages, and these pro-inflammatory Ly6c macrophages release a mixture of different cytokines including interleukin-1B (IL-1B) and Tumor Necrosis Factor alpha (TNFa).

The timing and ratio of the two macrophage phenotypes, M1-like (detrimental) and M2-like(reparative), into the injury chronology, are also critical. CCR2+ macrophages are thought to mature into the M1-like phenotype during the early phase of injury response, resulting in the secretion of cytokines. The development into the M2-like phenotype has been found to stimulate heart repair and improve heart function in animal models (11).

In addition to macrophages, neutrophils also infiltrate the inflammatory cardiac environment. Neutrophils work to amplify proinflammatory signals, produce oxygen species, and secrete proteolytic enzymes that reduce the ECM. Neutrophils bolster cardiac recovery by stimulating macrophages to develop into one of the two phenotypes (either M1-like or M2-like phenotypes) (12). Additionally, natural killer cells are involved in the cardiac microenvironment by restraining chemokine production and secreting interferon gamma (INF-y), perforin, and various anti-inflammatory chemokines to limit the infiltration of damaging immune cells. These cells prevent activated fibroblasts from over-producing collagen while also limiting apoptosis of cardiomyocytes (13).

The adaptive immune system is critical in maintaining the inflammatory environment when the heart is injured. B- and T-lymphocytes play an important role in the maintenance of cardiac homeostasis and response to injury. B-cells produce potent apoptotic signals and activate auto-antibodies when the heart experiences acute decompensation or ischemia-reperfusion to combat the cardiac injury (11). Some of the T-cells that infiltrate the injured myocardium

have been evidenced to demonstrate protective behavior. There is evidence to suggest that CD4+ specific effector T-cells (Teff) protect the heart against post-inflammatory fibrosis in experimental models. Furthermore, there are multiple possible mechanisms for protective effects of the non-specific Teff cells (14). The main idea is that if there is an increasingly greater quantity and concentration of heart non-specific Teff cells than antigen-specific T-eff cells, the levels of pro-inflammatory cytokines will be reduced. This, therefore, results in the limitation of cardiac fibrosis. While Teff cells demonstrate protective behavior in the cardiac environment, pro-inflammatory T-cells are stimulated to infiltrate the injured myocardium and cardiac environment by dendritic cell activation and chemotactic signals. The pro-inflammatory response of CD4+ and CD8+ T-cells benefits cardiac function and health in acute phases of cardiac injury, as they increase scar formation and prevent myocardial rupture (11). However, the pro-inflammatory response has the opposite effect in the long term as the increased quantity and prevalence of non-malleable scars result in decreased elasticity and integrity of the myocardium, which thereby, decreases cardiac function.

Understanding the role of the immune system in maintaining normal cardiac function and its response to cardiac injuries is an increasingly exciting area of research. Therapeutic interventions that employ the power of immune cells, known as immunotherapies, have been recognized by the scientific community as new avenues for cardiac research. Currently, scientists look toward providing patients with a more personalized and effective treatment to manage cardiac injury and myocardial disease using targeted immunotherapy and immunomodulation. This review paper will describe the following immunotherapies - immunoadsorption and immunoglobulin treatment, CAR T-cell therapy, macrophage-induced cardiac regeneration, and regulatory T-cell's cardioprotective function - to determine whether these tools can facilitate the treatment of cardiac injury and disease.

Immunoadsorption therapy

One of the novel immunotherapies researchers are currently studying is immunoadsorption (IA) therapy. IA therapy is a selective method used for the removal of specific antibodies and immune complexes from the bloodstream, sparing other plasma components and preventing the need for plasma replacements. Various adsorbents are now available and allow for both a nonselective removal of all immunoglobulins, like Immunoglobulin G (IgG), and a selective removal of disease specific molecules (15). The selectivity of IA therapy, paired with its efficient removal of molecules and favorable side effects has made IA an increasingly capable therapeutic modality to combat a range of autoimmune diseases, specifically in cardiac injury.

Programmed cell death protein 1 is a negative immunoregulatory receptor that is expressed on the surface of T lymphocytes. In a study involving programmed cell death protein 1-deficient mice, researchers found that IgG was accumulated on the surface of cardiomyocytes. This suggests that the immune system plays a role in the progression of DCM in patients. Since antibodies contribute to DCM progression, removing these antibodies through the process of immunosuppression can improve blood flow. IA and subsequent intravenous immunoglobulin (IVIG) substitution (IA/IgG)

were effective to relieve DCM symptoms by improving left ventricular ejection fraction (LVEF) and increasing cardiac index (16). Several studies have been performed to study the safety and efficacy of IA in treating DCM; however, these studies suffer from small sample sizes. Therefore, a meta-analysis of twelve existing and viable IA treatment studies was performed to better understand IA's efficacy and side effects when treating DCM patients.

The meta-analysis investigated 395 DCM patients. Of the 395 patients, 201 of them received IA therapy, and the other 194 received medical treatments other than IA. The studies in the meta-analysis were between 2000 and 2013, and the age range of patients was approximately 43 to 60.5 years. Furthermore, 5 of the studies assessed IA therapy, 4 assessed IA/IgG polytherapy, 3 studies assessed IVIG, and 2 studies used placebo treatment in the control group (17).

The results of the meta-analysis showed that IA therapy significantly improved LVEF as compared with that of placebo control group, and a comparison of the IA subgroup test subjects to the other treatment group's test subjects showed that only IA therapy significantly improved the LVEF. On the other hand, IVIG showed no significant effect on improving LVEF (18). Four studies involving 145 patients presented the left ventricular end diastolic diameter (LVEDD) data, and the result of the meta-analysis indicated that IA therapy was associated with significantly improved LVEDD while IVIG was not. However, there were a handful of test subjects who despite receiving the IA therapy treatment, showed no signs of improvement in LVEF or LVEDD (17).

There is emerging evidence that the immune system plays a major role in the progression of cardiac diseases. Activation of the humoral immunity results in the circulation of cardiac autoantibodies, and in patients with myocarditis and DCM, cardiac autoantibodies have been detected circulating in the bloodstream. The main pathological character of DCM is the adverse remodeling of cardiac chambers, and during ventricular remodeling, gene expression and protein composition are changed in response to IA therapy (6). The data from the meta-analysis highlighted the efficacy of IA therapy in DCM patients and found that IA therapy improved LVEF, reduced LVEDD, and relieved DCM symptoms, using the New York Heart Association (NYHA) functional classification, in patients with DCM (17). IA followed by IVIG substitution treatment also significantly improved LVEF as compared with that of the placebo control group, whereas IVIG by itself did not produce the same level of improved results (Figure 1). Additionally, there were significant subgroup interactions in the IA and IA/IgG groups but not in the IVIG subgroup. Scientists also believe that IVIG supplements were not as effective in DCM patients because certain cardiac antibodies, inflammatory markers, and activated T-cells might also be involved in cardiac dysfunction (19). Overall, the meta-analysis revealed the positive effects of IA therapy in improving cardiac function and clinical outcomes in DCM patients, and recent studies suggest that IA therapy facilitates the positive remodeling of the left ventricle.

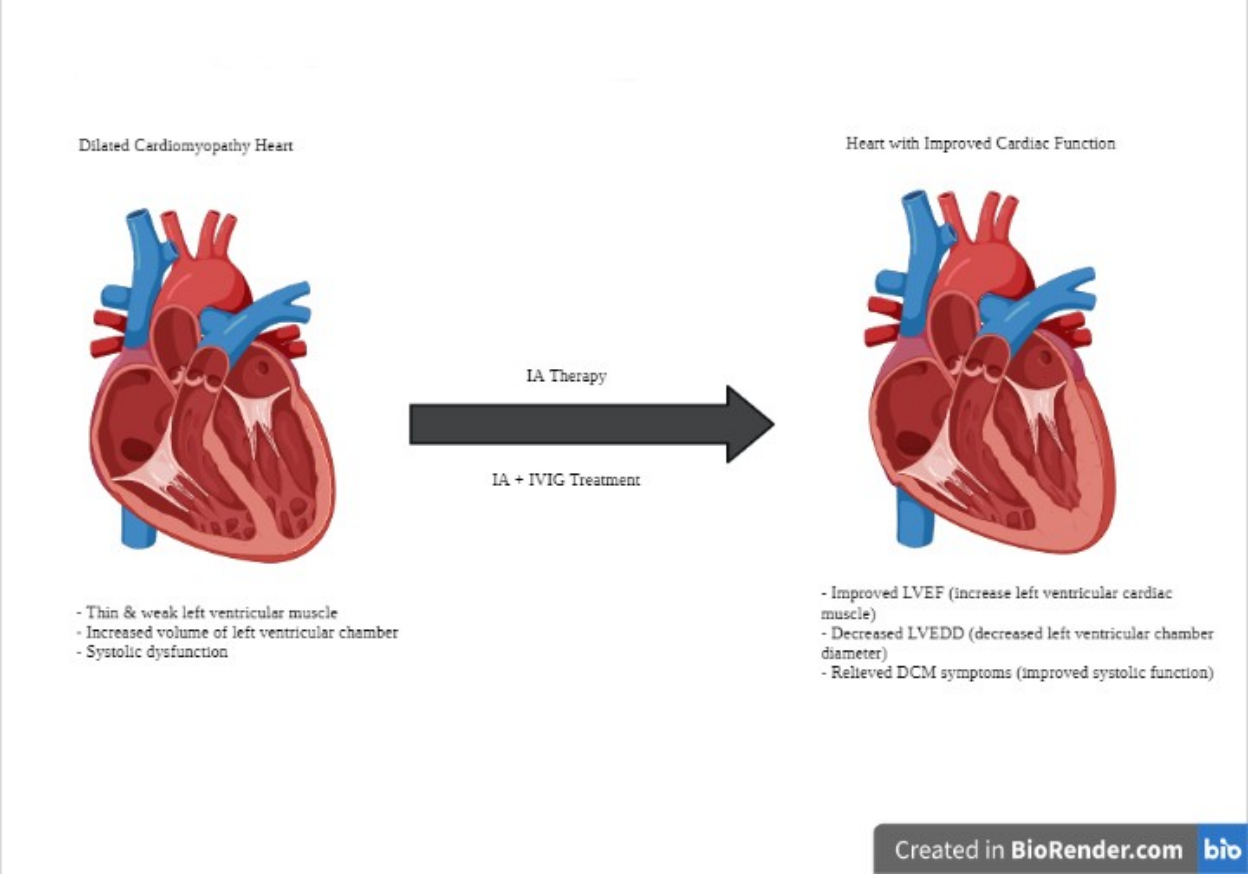


Figure 1: Effects of IA and IA + IVIG treatments in meta-analysis patients.

Since the immune activation and heart failure markers were increased in DCM patients, scientists believe that an increased immune response accelerates the onset of DCM. In this study, IA therapy was associated with improved LVEF and other measurements of improved cardiac function, but further research is still needed to confirm the efficacy and safety of IA therapy. Clinical studies suggest that the development of effective IA is essential to protect the body against cardiac autoantibodies and reduce myocardial inflammation (15). Consistent with clinical studies, the examined meta-analysis indicates that IA followed IgG substitution may be an additional therapeutic tool for stabilization of the cardiovascular function of patients with severe heart failure due to DCM.

Immunoadsorption discussion

Immunoadsorption is a valuable tool that does relieve myocarditis and DCM, but may be cardiotoxic. An increased immune response has been known to accelerate the onset of DCM, and for IA therapy to be effective, it must protect the body against cardiac autoantibodies and reduce myocardial inflammation. However, IA therapy is not a universally effective treatment since patients face the risk of cardiotoxicity following IA and IA/IVIG

therapy. Further research is necessary to determine how the treatment can be modified to successfully treat the majority of patients suffering from DCM without the added cardiotoxicity. In addition, in the meta-analysis, there was a group of patients who received IA Therapy or IA/IVIG therapy but showed no signs of improvement in cardiac function. It would be interesting to follow up on these patients and determine their susceptibility to treatment to understand the lack of response to this therapy. By identifying why these patients failed to respond to the therapy, patient eligibility and inclusion criteria can be better defined. To improve the efficacy of Immunoadsorption therapy, future studies must explore specific cardiac antibodies' adsorption columns as potential treatment to gauge whether the results show a greater percentage of improvement than conventional IA therapy. Researching the blood signatures of responders and non-responders to IA therapy can allow for modifications and the creation of a more effective and universal treatment for DCM in the future.

CAR T-cell therapy

Revolutionary breakthroughs have been made in cancer treatment by redirecting cytotoxic t-cells to target specific antigens on cancer cells and successfully eliminating the cancer. The most common example of this technique is CAR T-cells (20). Using this same idea, engineering T-cells to target activated cardiac fibroblasts in the heart could become a revolutionary treatment and prevention method for all cardiac diseases.

Inactive fibroblasts are critical to the normal structure and function of the myocardium. However, fibroblasts activated by acute cardiac injury increase the stiffness and lack of compliance of the cardiac muscle. These fibroblasts signal more cardiomyocytes to stiffen, resulting in the maladaptive function and formation of the heart, with significant problems in the diastolic period (21). The genetic ablation of cardiac fibroblasts following a hypertensive or ischemic injury has been tested in mice and has been shown to reduce fibrosis and, therefore, to increase cardiac function.

Genetically engineered cytotoxic T-cells have been engineered to recognize a specific antigen by expressing a chimeric antigen receptor (CAR) on the activated CD8+ T-cells. The chimeric antigen receptor is a combination of a single-chain variable fragment (scFv) against a specific antigen. This protein design results in one-step cytotoxicity induced through antigen binding alone, as opposed to T-cell receptor (TCR) mediated cytotoxicity, which requires several more complex components (22). In the setting of heart failure, scientists are targeting a xenogeneic antigen that activated cardiac fibroblasts exhibit through the adoptive transfer of engineered CD8+ T cells, since activated cardiac fibroblasts are genetically different from homeostatic fibroblasts as they express distinct cell surface markers. This means that cardiac fibroblasts can be specifically targeted, and that adverse fibrosis can be mitigated (23).

An early indication that this approach might be effective in reversing fibrosis and improving function after injury came from mouse studies in which activated fibroblasts were eliminated by genetic ablation using diphtheria toxin in animals that were engineered to express the diphtheria toxin receptor on activated fibroblasts (24). In order to replicate these

findings in humans, researchers turned to target activated fibroblasts with an engineered T-cell.

In the first series of experiments, researchers stimulated mice to present the xenogeneic antigen ovalbumin peptide (OVA), thereby using OVA as the target of CAR T-cells. OVA is not normally found in mice but can be conditionally presented using tamoxifen induced expression on the surface of cardiac fibroblasts when they are activated (25). Within the first week of angiotensin II and phenylephrine (Angll/PE) treatment to induce cardiac injury, a significant degree of fibrosis was observed throughout the myocardium, and OVA expressing cardiac fibroblasts were targeted by the adoptive transfer of CD8+ cells that expressed a cognate T-cell receptor against the OVA peptide (CD8+ OT-I T cells). At week 4, there was significantly less cardiac fibrosis in the mice treated with OT-I T-cells as compared to the controls (Figure 2) (26).

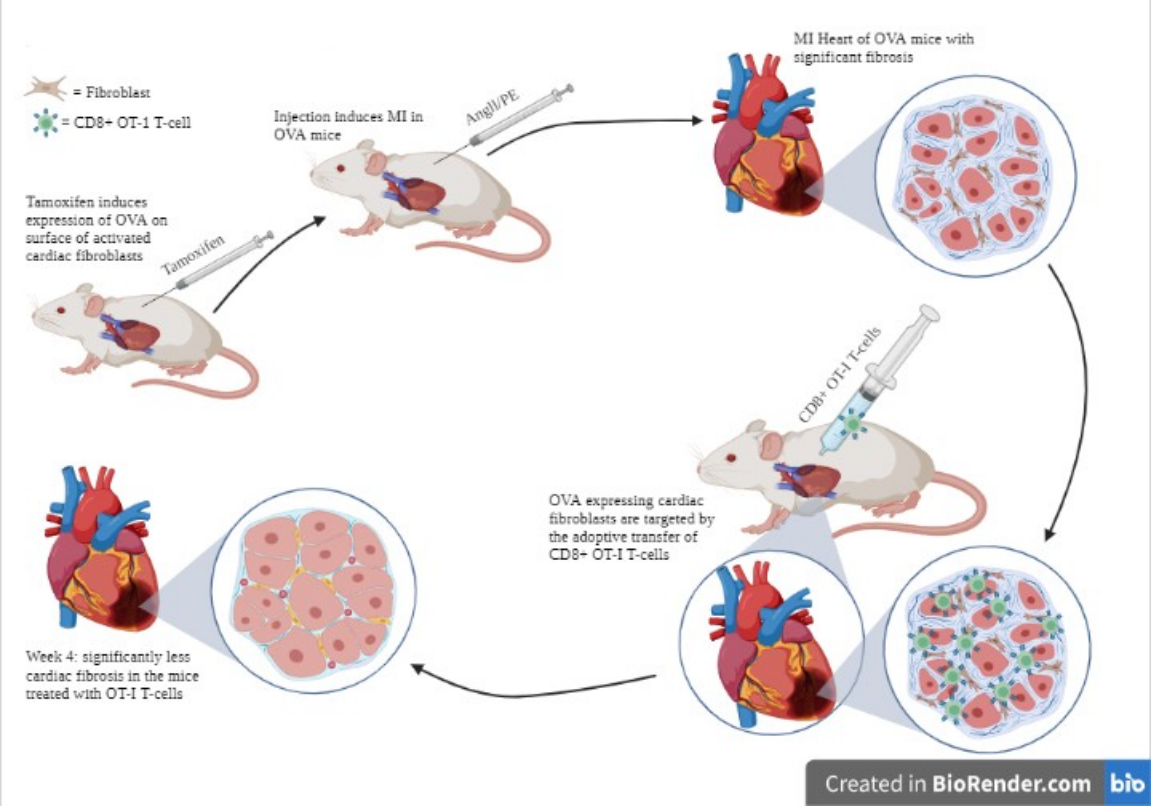


Figure 2: OVA as target for CAR T-cell therapy.

In addition to OVA, a unique molecular marker of activated fibroblasts was identified using transcriptomics when comparing healthy human hearts to dilated and hypertrophic hearts. The experimental data showed that the fibroblast activation protein alpha (FAP) was highly upregulated in both disease states when compared to healthy hearts. FAP is a cell surface glycoprotein that is expressed during embryonic development and where tissue is actively being remodeled, such as areas of wound repair or tissue fibrosis (27). In adults,

FAP protein expression is restricted to activated fibroblasts in various disease states and after injury. On the other hand, this protein is absent in healthy adult tissues of both mice and humans.

Using a mouse model of cardiac fibrosis and heart failure produced by the constant infusion of angiotensin II and phenylephrine, scientists discovered that FAP CAR T-cells were effective at restoring cardiac function and significantly lowering the burden of fibrosis even when the CAR T-cells were delivered after fibrosis had already accumulated (Figure 3) (28). The resorption of previously accumulated ECM is thought to be the result of macrophage, non-targeted fibroblasts, and neutrophil activity, which is increased once the cells producing the excessive matrix are destroyed by the CAR T-cell therapy.

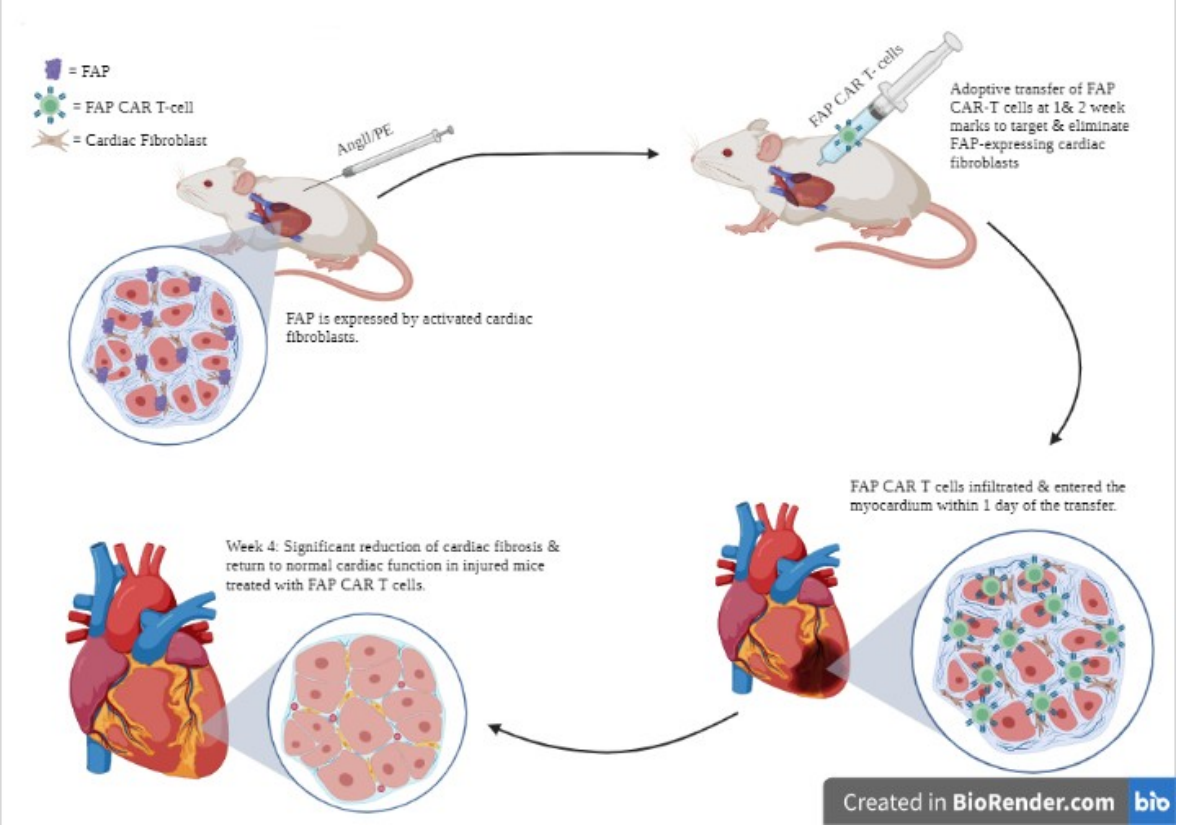


Figure 3: FAP as target for CAR T-cell therapy.

One advantage of FAP as the antigen target is the ability to visualize expression in vivo using imaging probes. Unlike in cancer where curative therapy requires the total elimination of every cancer cell, CAR T-cell therapy for fibrosis may be effective at restoring function even if only a fraction of the disease-causing fibroblasts are destroyed (26). It is critical to modulate the quantity, potency, and longevity of the CAR T-cells to ensure that the beneficial, homeostatic fibroblasts remain within the myocardium to maintain a healthy ECM after the excessive fibrosis is reduced. To produce temporary anti-fibrotic CAR T-cells, scientists suggest the usage of kill switches

incorporated into the engineered CAR T-cells (29).

This finding is also consistent in human patients as expression analysis of fibroblast gene signatures from a healthy human heart and a diseased human heart identified the FAP of cardiac fibroblasts as the target of CD8+ T-cells. The adoptive transfer of T-cells genetically modified to express the chimeric antigen receptor (CAR) against FAP, resulted in a significant reduction of cardiac fibrosis and the restoration of cardiac function in mice (30). These findings are consistent in both human and mice models of cardiac injury.

To identify endogenous proteins/antigens that were solely expressed by activated cardiac fibroblasts, a RNAseq database of 238 human heart transplant donor and recipient left ventricular (LV) tissue was analyzed. This analysis identified a number of fibroblast-specific genes that were upregulated in the myocardium in patients with either hypertrophic cardiomyopathy (HCM) or DCM but regulated at controlled levels in healthy and non-failing donor hearts. FAP showed the greatest structural/folding change and upregulation after comparing the DCM and HCM samples to the control (healthy hearts) (24). In regard to cardiac injury, FAP has been expressed in human hearts following acute myocardial infarction injury, and researchers have shown that in the failing DCM and HCM human hearts, FAP is expressed solely by cardiac fibroblasts in the left ventricular tissue. On the other hand, FAP is minimally expressed in normal human hearts (26). Since FAP is only expressed by activated cardiac fibroblasts in hearts with DCM, it is a logical candidate to target the cardiac fibroblasts and prevent/reverse fibrosis.

To test FAP as a target for cardiac-based immunotherapy, researchers utilized the AngII/PE model of hypertensive cardiac injury and fibrosis in mice and confirmed the expression of FAP by activated cardiac fibroblasts present. Immunohistochemistry showed that FAP is only expressed on activated fibroblasts following acute myocardial injury, such as after 1 and 2 weeks of AngII/PE exposure or other models of cardiac injury, such as myocardial infarction, transverse aortic constriction, and muscular dystrophy (31). To target and eliminate the FAP-expressing cardiac fibroblasts in the AngII/PE model, researchers adoptively transferred FAP CAR T-cells at 1- and 2- weeks post AngII/PE initiation. A second injection of the FAP CAR T-cells was administered in the experiment due to the short half-life of mouse FAP CAR T-cells (24). After the injection, researchers detected FAP CAR T-cells infiltrating the myocardium within 1 day of the transfer. By 4 weeks, researchers observed a significant reduction of cardiac fibrosis, and a return to normal cardiac function in injured mice treated with FAP CAR T cells when compared to the controls.

Next, to determine if FAP CAR T cell treatment was cardiotoxic, researchers utilized a cardiotoxicity array to test 84 genes associated with myocardial toxicity. At 4 weeks, there were gene expression differences consistent with cardiotoxicity between AngII/PE and AngII/PE + FAP CAR T cell-treated animals (32). Immunohistologic analysis for immune cell infiltration revealed variations in CD3+, CD4+, CD8+, or MPO+

cell counts, a reduction in CD19+ cells, and an increase in F4/80+ macrophages in AngII/PE +FAP CAR T-cell compared to AngII/PE-treated samples at 4 weeks. All FAP CAR T-cell treated animals survived to 12 weeks with no evidence of severe toxicity. The results of this experiment provide evidence for the possibility of targeting cardiac fibrosis in mice with engineered T cells and immunotherapy (33), however, it is necessary to conduct further testing to determine whether the presented cardiotoxicity will progress as the number of days after CAR T-cell treatment increase, and if it has the potential endanger the health and safety of patients.

CAR T-cell therapy discussion

For CAR T-cell therapy, specific unplanned errors are common, such as non-specific targeting, and can lead to cardiotoxicity and additional cardiac damage. With any T-cell therapy, the effects of an off-target treatment can be very severe, so it may be necessary to engineer modified T cells with a "kill-switch" to limit time of survival so as to minimize any off-target side-effects. More studies must be conducted if FAP is the optimal and safest target of cardiac immunotherapy before testing this approach on humans. Further investigation into activated cardiac fibroblasts may reveal additional targets, such as alternative antigens or combinations of antigens, as more effective and safer targets for cardiac immunotherapy in humans. Furthermore, fine-tuning the process of genetically modifying CAR T-cells while also taking into consideration the patient's genetic make-up and severity of cardiac disease will allow for a more effective treatment. T-cell exhaustion is an area to consider when analyzing the efficacy of CAR T-cell therapy. These T-cells are not immortal, and at some point, after a batch of CAR T-cells is administered, the population will cease to function or combat existing cardiac fibrosis. Therefore, to prevent rapid exhaustion and disappearance of the CAR T-cells from the cardiac microenvironment, future studies must address mechanisms and methods to control the exact amount of time CAR T-cells are active within the myocardium.

Macrophage-induced cardiac regeneration

Macrophages are highly adaptable and functionally diverse during steady-state conditions and following tissue injury or disease. In the heart, tissue resident macrophages play a critical role in coronary vessel development and have recently been found to contribute to cardiac conduction. Following myocardial infarction (MI), macrophages are the predominant immune cell type within the cardiac microenvironment. Macrophages arise from monocytes which infiltrate from the spleen and bone marrow and carry out their functions depending on the context of the microenvironment. For example, during the initial inflammatory phase, macrophages adopt a classically activated-like state. This state is associated with the release of pro-inflammatory cytokines, and when in this state, macrophages work to digest and remove dying neutrophils, dead tissue and necrotic debris (11). Subsequently, they transition to a reparative state and contribute to cardiac fibrosis through the production of cytokines, chemokines, and growth factors following an acute cardiac injury. These alternatively activated macrophages modify ECM turnover by regulating the balance of matrix metalloproteinases (MMPs) and their tissue inhibitors, while also activating cardiac fibroblasts to become myofibroblasts - cells

that deposit collagen to form a scar (34). Ultimately, the replacement of dead cardiomyocytes with non-contractile scar tissue stabilizes the injury site but leads to longer-term adverse ventricular remodeling and heart failure.

Unlike the adverse cardiac remodeling in humans, several species can regenerate their hearts following an injury either in the absence of scar formation or through scar resolution. For instance, in Zebrafish, following the resection of the ventricle, complete heart regeneration occurs without scar formation. The post-resection Zebrafish heart differs in many ways to the infarcted human heart. The main difference is that it lacks ischemia-induced cell death and forms a fibril layer, composed of fibrin fibers with minor collagen deposition (35). On the other hand, following cryoinjury, heart regeneration is delayed, due to widespread cell death and fibrotic scar formation. This aspect of Zebrafish heart regeneration resembles the human heart post-MI more closely. However, unlike human hearts, following cryoinjury in Zebrafish hearts, regeneration occurs even in the presence of scarring. In neonatal mice, the heart can regenerate during a limited period of time, which extends to around 7 days after MI or ventricular resection (36). The transition from a tissue repair phase to a regenerative phase following cardiac injury requires a well-balanced and organized immune response to manage both a pro-fibrotic and a pro-regenerative environment. For this balance to be perfect, macrophages are integral to both repair by scar formation and tissue regeneration, but scientists still do not understand the regenerative or pro-fibrotic phenotype factors.

To determine the temporal and dynamic response of macrophages to injury in both adult Zebrafish and neonatal versus adult mouse models, gene expression analysis was performed in Zebrafish at 11 time points following either resection or cryoinjury over the span of 14 days (Figure 4) (36). The macrophage-specific marker (mpeg1) revealed increased macrophage infiltration at 1 day-post-injury (dpi) in both injury models. This was accompanied by neutrophil infiltration, which was determined by tracking the myeloid-specific peroxidase (mpx) expression. While the neutrophil presence at the site of injury was short-lived, macrophages persisted for an extended period in both injury settings (12).
Next, the spatial distribution of macrophages was analyzed at 1 dpi when mpeg1 expression experienced a sharp increase following cardiac injury, and at 5 dpi, when mpeg1 was significantly higher following cryoinjury. The mpeg-1 section analysis revealed that macrophages were distributed in the atrium, throughout the ventricle, and near the epicardium, with increased concentrations surrounding the injury site. Additionally, by 14 dpi, inflammation of the area decreased (fewer macrophages) following ventricular resection, while mpeg1 expression was still high following cryoinjury and subsequent scarring.

Following the spatial distribution analysis, myocardial infarction (MI) was stimulated in the prenatal stage 1 (P1), the prenatal stage 7 (P7) and in the adult stages of mice. As a result, CD68+ macrophages were observed to be localized to the infarct zone at day 4. Flow cytometry of the neonatal adult mice revealed significant elevation of macrophages in adult infarcted hearts across days 1 and 4. By day 7,

the numbers of all immune cell types were reduced to the starting amount across all three stages (36). The resulting data is consistent with the significantly decreased magnitude of the macrophage response in neonates compared to adults.

Table 1: Upregulated and downregulated genes in the nuclei of macrophages in Zebrafish following cardiac injury.

Days post injury (dpi)	Ventricular resection		Cryoinjury	
	Downregulated	Upregulated	Downregulated	Upregulated
1	578	3664	704	722
5	1303	2308	348	693
14	Data not collected		58	320

To better understand the molecular mechanisms that drive macrophage response during transient scarring and regeneration as opposed to stable scar formation, scientists compared macrophage transcriptomes in adult Zebrafish following resection versus cryoinjury and in post-MI mice, at neonatal P1 and P7 stages versus the adult (Figure 4) (37). A number of genes have been identified to induce healthy proliferation, specifically the genes fgf7, involved in positive epithelial cell proliferation in the intestines, and pdgfr1, which was implicated in dilated cardiomyopathy. Both these genes are not carcinogenic and have not been found to crosstalk with carcinogenic amplifying pathways (37). The differential expression analysis of the nuclei following cryoinjury at various dpi are shown in Table 1 (35, 36). Further analysis identified factors involved in re-innervation, ECM components, ECM enzymes, resolution of inflammation and regeneration, pro-inflammatory mediators, macrophage function, and monocyte to macrophage differentiation. Macrophages following resection exhibited both acute inflammatory and regenerative responses at 1dpi. On the other hand, post-cryoinjury, macrophages revealed a coordinated response: genes associated with pro-inflammation were upregulated at 1 dpi and were followed by pro-regenerative gene signatures at 5 dpi and 14 dpi. Additionally, 6 groups of co-expressed factors were identified across different time points and in the different injury settings (36). The data suggests that a robust immune response is responsible for the heart regeneration in Zebrafish and the differential responses of macrophages, following resection or cryoinjury insults, may influence downstream wound healing.

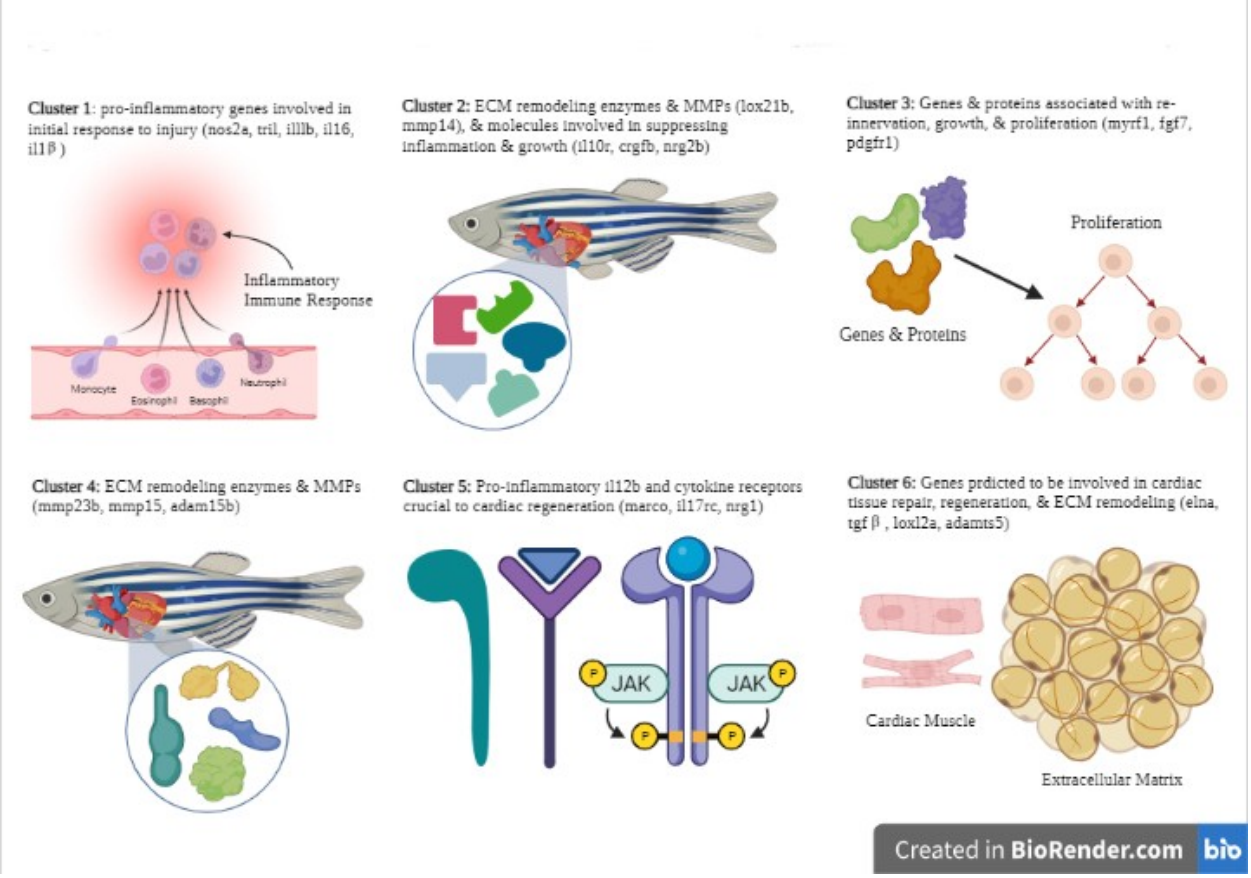


Figure 4: Unbiased clustering of RNA sequence datasets reveals six groups of co-expressed factors across different time points and injury settings in Zebrafish

RNA-seq analysis in mice of macrophages at baseline, day 4 post-MI, and between day 4 & 7 post-MI, revealed 1500 differentially expressed genes in the adult as compared to 398 and 470 genes in the P1 and P7 models, respectively. Of the genes upregulated in the adult at day 4 post-injury, only 39 were conserved in both the P1 and P7 samples at the same point in time. Additionally, analysis at day 4 and day 7 post-injury revealed that only 43 genes were shared across all samples. The data hints that the macrophage response is less complex with distinct gene-regulatory mechanisms in neonates compared to adults. Next, semi-hierarchical clustering, comparing the regenerative P1 macrophage response to that of the scar forming P7 adult response at post-injury day 4 and day 7, revealed upregulation of fibrosis mediators, including Ctgf and Serpine123 (36). In contrast, P1 macrophages uniquely upregulated a subset of genes that may be linked to heart regeneration, based on known functions in other tissue-injury settings (38). To further understand the differences in the functional responses of macrophages in the P1 and adult hearts, unbiased clustering of adult heart RNA-Seq datasets was utilized. Based on gene expression

dynamics, distinct gene clusters at different phases of myocardial injury and repair were identified (Figure 5). Further clustering of P1 RNA- seq datasets (day 4-7) identified multiple soluble factors with reported pro-survival, and angiogenic functions, including Bone Morphogenetic Protein 7 (Bmp7), which is involved in neonatal digit regeneration post-amputation. Moreover, careful analysis of genes that were differentially expressed between P1 and P7 stages suggested that genes upregulated at P7 were associated with ECM organization and genes upregulated at P1 were associated with nucleosome assembly and positive regulation of IFN-gamma production (39). These results suggest that unique transcriptomes guide macrophage function during cardiac injury and contribute to the transition from regenerative to scar-based repair for both neonatal and adult mice (36).

During transcriptional analyses, researchers previously observed differential expression of collagens and related ECM genes in macrophages derived from injured Zebrafish hearts and between neonatal P1 and P7 stages mice, which suggests a direct pro-fibrotic function. Different collagen types were expressed within the heart on day 5 post-injury as well (23). Additionally, genes involved in ECM function and fibrillar collagen mRNA and isoforms were detected at P7. Furthermore, there was a co-localization of macrophages and collagen, which suggested that macrophages contribute to P7 scar formation (36).

To address macrophage's role in collagen accumulation on hearts, a transgenic mouse line with fluorescently labelled type-1 collagen fibrils was used. Donor monocytes were injected into the peri-infarct myocardium of adult wild type mice undergoing MI surgery (30). Seven days after the onset of MI, a fluorescent signal was observed in the cardiac scar region with CD68+ macrophages and collagen-1 antibody staining (α-Col1). By day 21, the fluorescent signal had increased and was detected within multiple regions of the scar, including fiber-based structures. This provided evidence to support the idea that macrophage-derived collagen contributes to scar formation post-MI (23). Additionally, the relative proportions of macrophage-derived collagen versus a myofibroblast source were determined at day 7 post-MI. Macrophages comprised approximately 6% and fibroblasts 75% of the total fluorescent cell population. Even though macrophages made up only 0.45% of the total cells in the heart compared to fibroblasts which accounted for 6.1% of the total macrophage population, 46.2% of the macrophages were fluorescent while only 22.5% of fibroblasts were fluorescent. The data suggest that while fibroblasts are likely to account for the majority of collagen-deposition in terms of the abundance of Col1a+ cells, the macrophage contribution is not insignificant, since it amounts to 6.8% of the total (36).

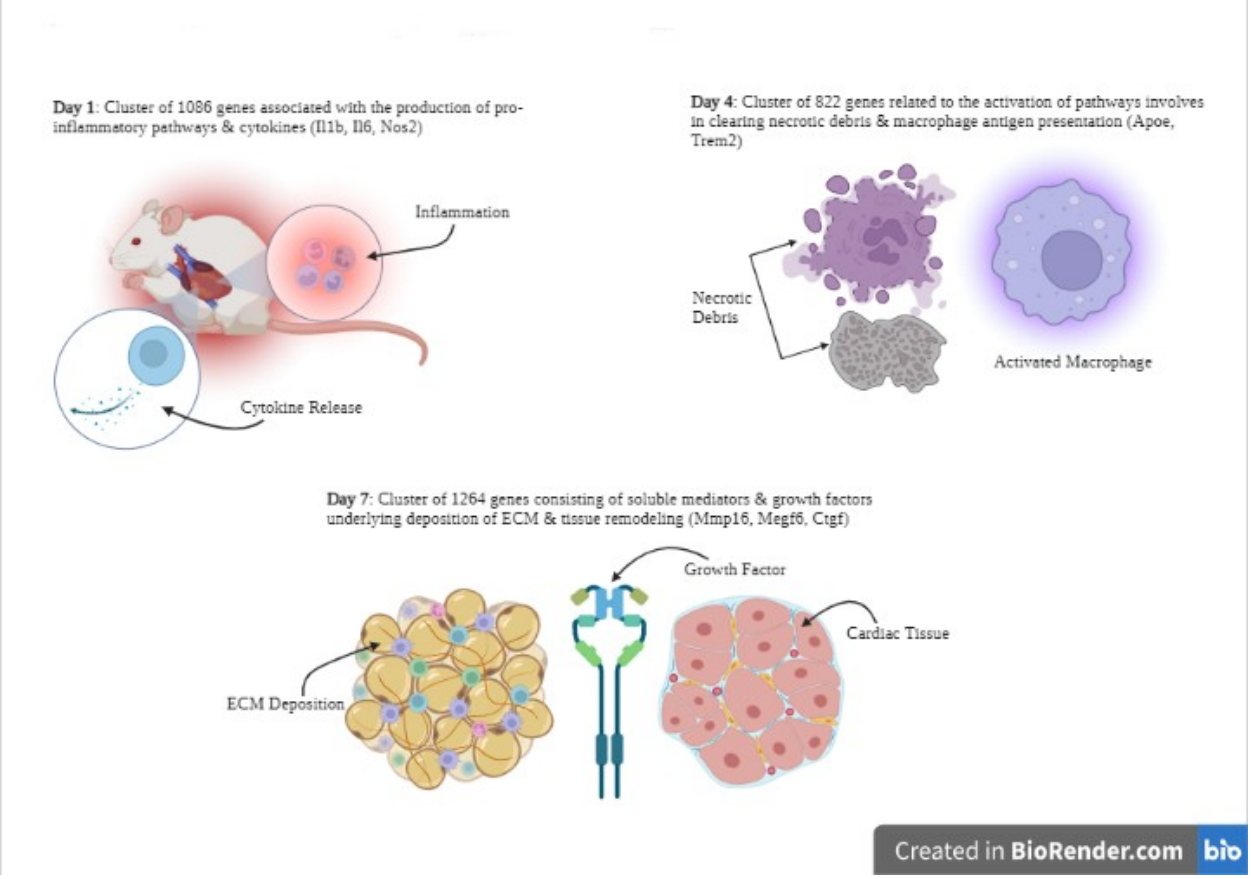


Figure 5: Unbiased clustering of RNA sequence datasets reveals distinct gene clusters at different phases of myocardial injury in adult mice.

To determine whether macrophages can directly contribute collagen to scar formation in Zebrafish, fluorescently labelled collagen was used and revealed mosaic expression in macrophages at day 6 post-injury. Additionally, immunostaining revealed that the extracellular fibrotic response surrounding the injury located on the cryoinjured heart was positive for fluorescent collagen and was fibrillar in nature at 14 dpi and 21 dpi (40). The majority of labelled collagen was deposited near the injury, within the overlying epicardial region, instead of directly within the major site of fibrosis. This difference suggests a possible distinction between macrophage-collagen deposition and collagen deposited by activated myofibroblasts (39). This reveals that macrophages not only express collagens at the intracellular level but can also contribute to the forming of the extracellular scar by direct collagen deposition. To investigate the role of macrophages in cardiac fibrosis and collagen deposition, a knockout of col4a3bpa and col4a1 was performed in Zebrafish and showed a reduction in scarring, which confirmed the role macrophages play in scar formation (40, 41).

The regenerative competence of the injured heart and the transition from the immediate fibrotic-based repair, pro-fibrotic environment, into a transient response, pro-regenerative environment, allows for effective cardiac regeneration. Recently, macrophages have been demonstrated to be critical to the regeneration of the salamander limb, adult zebrafish tail fin, and neonatal mouse heart. This concept is further supported by how Zebrafish studies have reported impaired heart regeneration following a decreased macrophage response (36). However, researchers have not completely understood the molecular mechanisms utilized by macrophages to combat the adverse effects of inflammation or directly exacerbate fibrosis during wound healing.

The transcriptional state of macrophages responding to distinct injury cues at different stages of Zebrafish heart regeneration and between neonatal and adult mouse hearts post-MI were studied and characterized. The temporal characterization of the cellular and molecular immune responses revealed exact timing for the recruitment of myeloid cells and the establishment of a regenerative immune environment from a fibrotic immune environment (37).

In adult Zebrafish, the significance of the immune response was large enough to counter the existing dogma that regeneration is incompatible following an immune response to injury. Additional transcriptional analyses identified key differences in the macrophage regulatory profiles when activated in resection versus cryoinjury conditions. In Zebrafish, the occurrence of a synchronized and simultaneous pro- and anti-inflammatory response was associated with rapid regeneration following ventricle resection (23). Conversely, in the cryoinjury setting, the replacement of the initial pro-inflammatory response with the anti-inflammatory macrophages allowed for cardiac regeneration and prevented chronic fibrosis. When comparing P1 neonates to P7 and adult mice, the global macrophage transcriptional response in P1 neonates was significantly decreased when compared to the intermediate response in P7 mice and highly active response in the adult immediately post-MI (42). Interestingly, the transcriptional analyses in Zebrafish and mice demonstrated that macrophages express collagen and collagen-associated gene isoforms following cardiac injury. Myofibroblasts, which are periostin-expressing cells that arise from Tcf21-derived tissue-resident fibroblasts, were previously known as the exclusive source of collagen deposition during scar formation. Experts previously believed that after depositions, macrophages only worked to increase the survival and activation of myofibroblasts by releasing soluble factors, like TGFβ and Fibroblast Growth Factors 50 (FGFs50), and thereby indirectly influenced scarring (41).

However, the previously discussed experimental data suggest that macrophages also directly deposit collagen and directly contribute to scar formation. In mice, adoptive transfer experiments revealed the fusion-protein within the adult and neonatal heart and fluorescent collagen-fiber deposition within forming scars. On the other hand, in Zebrafish, following macrophage adoptive transfer, collagen deposition was observed on the sides of the cryoinjury wound (5). These results indicate that macrophages directly contribute to cardiac fibrosis via autonomous collagen

deposition. However, the important aspect of this discovery was that macrophages' function of collagen deposition in the process of scar formation is conserved across species (fish and mammals) and challenges current dogma that the myofibroblast is the only cell type that forms post-cardiac injury scars.

Adoptive cell transfer of macrophages from resected zebrafish hearts to cryoinjured recipient ventricles not only revealed excessive scar formation but also indicated that macrophages exhibit a high degree of plasticity. This means that macrophages can alter their function when transferred from a regenerative environment into a more scar-inducing environment (36). When contrasting this discovery with the experiment conducted in mice where the transfer of monocytes from adult mouse spleen into P1 neonatal hearts induced scar formation, regardless of the differences in the regenerative environment between the spleen and heart, suggests that macrophages derived from adult monocytes are completely programmed to induce fibrosis. The plasticity of differentiated macrophages in adult Zebrafish allows for transplanted bone marrow precursors and differentiated macrophages to be reprogrammed when transferred into a new organ (35). Macrophages' plasticity also allows them to quickly and easily adapt and regulate opposing states, such as regeneration versus fibrotic repair. In contrast, the apparent genetic programming and set function of adult mouse macrophages following cardiac injury may inhibit default pro-fibrotic wound healing in the mammalian heart and contribute to the lack of the self-driven regeneration in all mammal hearts.

Macrophage-induced cardiac regeneration discussion

Unlike immunoadsorption treatment, macrophage-induced cardiac regeneration has not yet been tested in human clinical trials. Only animal models of mice and Zebrafish have undergone treatment with macrophages, and in these cases cardiac regeneration was successful. However, besides the successful restoration of cardiac function, the functional relevance of macrophage-derived collagen production in addition to the safety and potential side effects of macrophage-based treatment still needs to be investigated in the future. To improve the existing capabilities of macrophage regeneration as a viable treatment option for patients, more research must be conducted to gain a comprehensive understanding of the regenerative environment in which the innate immune system still exists to fight disease but also permits regeneration. Conducting an adoptive cell transfer from P1 (pro-regenerative) monocytes and macrophages into the adult mice heart, would allow for determining the variations in the extent of scarring in the adult heart. Additional research targeting macrophage-induced pro-fibrotic pathways will also greatly aid in the development of immunomodulatory therapies for patients suffering from acute MI. Furthermore, it should be noted that it is possible to intervene pharmacologically and reprogram immune cells, such as macrophages, to transition from one type to another. For example, a M2 macrophage can be stimulated to become a M1 macrophage. The idea of genetically programming M2 macrophages, which are immunosuppressive and tumor promoting, to perform anti-tumor inflammatory functions in a cancer setting by converting it into a M1 type has been explored (43). This

study discovered that utilizing a nanocarrier formulated with mRNAs encoding interferon regulatory factor 5 in combination with its activating kinase IKKβ can reverse the tumor suppressive state of M2 macrophages, and reprogram them to the M1 phenotype, which promotes tumor regression. The infusion of these nanoparticles has been shown to be successful in converting tumor-associated macrophages into M1 macrophages in models of ovarian cancer, melanoma, and glioblastoma. Such a tool is incredibly valuable since it would allow physicians to stimulate the body to create more of a specific cell type that would be more beneficial in a certain medical situation than another cell type.

Regulatory T-cell cardioprotection

Regulatory T cells (Tregs) are critical in maintaining immune homeostasis and regulating inflammatory disease progression. Tregs modulate the activation and function of a variety of immunocytes in both the innate and adaptive immune system. In addition to their immunoregulatory function, Tregs play a major role in promoting effective tissue repair in peripheral tissue and maintaining local homeostasis. Tissue-resident Tregs were first identified by the Mathis team (44, 45) when they described visceral adipose tissue Tregs and injured muscle Tregs. Since then, Tregs with unique phenotypes and functions have been reported in the pancreas, skin, brain, and lungs (31). However, scientists still do not fully understand the function and existence of Tregs in the hearts, especially in the myocardium during myocardial infarction (MI). MI is one of the most common cardiac diseases that severely damage the myocardium, and previous experiments have shown that Tregs play a protective role in the myocardium following MI by alleviating local inflammation, protecting cardiomyocytes from apoptosis, and modulating macrophage differentiation and myofibroblast activation (46). To further understand the characteristics of Tregs in MI hearts, scientists utilized multiple transcriptional and functional assays, revealing a unique population of tissue related Tregs in the hearts after MI which have been identified by their unique phenotypes, sources, and sustaining factors.

To track the infiltration of heart Tregs into the myocardium after the onset of MI, flow cytometry was performed, which revealed that Tregs in the myocardium increased at day 1 post-MI, reached the highest value at day 7, and still maintained high values at day 14. In addition to Tregs, the number of heart CD4+ T cells and heart conventional T cells (Tconvs) peaked at 7 days post-MI. The frequency of heart Tregs along with the CD4+ T cells increased 7 days after MI and maintained consistent values until around 28 days (47). A similar accumulation of Tregs was also observed in hearts after myocardial ischemia/reperfusion injury (I/Rinjury). Regarding I/Rinjury, Tregs infiltrated injured hearts within 1 day, peaked at day 3, and remained elevated until 7 days. The heart Treg count also reached a peak on day 3 after I/R injury (48). On the other hand, when the same tests were conducted on spleen Tregs, the spleen Treg population showed little variation over the course of 28 days, and the population that reached a peak on day 3 post-I/R injury was significantly lower than the heart Treg proportions. These results indicate that heart Tregs are a unique Treg population that play a role in maintaining the myocardium after cardiac injury.

To identify transcriptional phenotypes of heart Tregs 7 days post-MI, RNAseq was performed on isolated Tregs and Tconvs from the heart, spleen, and non-draining lymph nodes (NDLNs). Volcano plots suggested that the transcriptome of heart Tregs differed from that of spleen or NDLN Tregs, while the spleen and NDLN Tregs shared transcriptome similarities. Although heart Tregs exhibited a distinct transcriptome, they were clearly Tregs based on the expression of 79% of the canonical Treg signature, including elevated expression of hallmark transcripts such as scurfin (Foxp3) and Interleukin-2 Receptor Alpha Chain (CD25) (29). Furthermore, differentially expressed genes upregulated in skin and muscle Tregs were also increased in heart Tregs. Principal components analysis and transcriptomic analysis suggested transcriptional differences among the skin, muscle, and heart tissue Tregs and also between tissue Tregs and lymphoid organ Tregs. Following this realization, scientists analyzed the upregulated differentially expressed genes in heart Tregs. The first group included Cytotoxic T-Lymphocyte Associated Protein 4 (Ctla4), Amphiregulin (Areg), and Interleukin 1 receptor-like 1 (Il1rl1), which were also upregulated in muscle Tregs and skin Tregs, while the second group was composed of numerous cytokines, cytokine receptors, chemokines, and chemokine receptor–related genes (49). Gene set enrichment analysis evidenced that genes upregulated in heart Tregs displayed enrichment for Gene Ontology biological processes terms that are all related to tissue repair, such as extracellular structure and matrix organization, collagen metabolic and biosynthesis processes, wound healing, and regulation of smooth muscle cell proliferation (49).

To determine whether the altered genetic signatures in heart Tregs contribute to the cardiac injury microenvironment, the transcriptome of heart Tregs with heart-draining mediastinal lymph node (MLN) Tregs were compared 7 days after MI. Volcano plots and principal components analysis provided evidence that the transcriptome of heart Tregs is different from MLN Tregs. When compared to MLN Tregs, the aforementioned Gene Ontology biological process terms and genes of heart Tregs are significantly upregulated in heart Tregs (47). To identify the transcriptional characteristics of heart Tregs in I/R injury, RNA sequencing was performed on the isolated Tregs from hearts, spleens, and MLNs 3 days post-I/R injury. Like the previous results, volcano plots showed that the transcriptome of heart Tregs differed from spleen or MLN Tregs. Gene set enrichment analysis also showed that genes involved in ECM organization and extracellular structure organization were significantly upregulated in heart Tregs unlike spleen or MLN Tregs. Principal components and transcriptomic analysis, identified that heart Tregs from two mouse models demonstrating two different kinds of acute cardiac injury shared very similar transcriptomes (49), therefore, suggesting that heart Tregs are a novel population of tissue Tregs that may play a major role in all forms of cardiac repair.

To determine whether the circulating Treg pool contributed to the accumulation of heart Tregs after MI, mice were treated with FTY720, an S1P1 receptor agonist that secures lymphocytes in secondary lymphoid tissue. Receiving this

treatment, the number of Tregs recruited to ischemic hearts dropped significantly on days 3 and 7 following MI. Similarly, the number of CD4+T cells infiltrating infarcted hearts also showed a pronounced decrease. These results demonstrated that the accumulation of heart Tregs after an MI or acute cardiac injury depends on the circulating Treg pool (49). Following this discovery, to evaluate the ability of Tregs to infiltrate the infarcted myocardium from circulation after MI, transgenic mice were used to track the migration of stained Tregs and Tconvs from the cervical lymph nodes to the axillary lymph nodes, MLN, and heart. Four days later, the tracking analysis showed that stained Tregs and Tconvs emigrated from the cervical lymph nodes in mice, and a significant number of Tregs or Tconvs were measured in the hearts, MLNs, and axillary lymph nodes. It was determined that Tregs manifested more effective migration to ischemic hearts than Tconvs, whereas no significant difference in migration between Tregs and Tconvs was observed in MLNs (49).

Next, the parabiosis experiment, used to analyze the contribution of the circulating Treg pool to heart Tregs, showed that the recruitment of Tregs from circulation accounted for nearly 95% of the Tregs within the infarcted hearts, therefore, suggesting that the accumulation of Tregs in the post-MI hearts mainly depends on recruitment from the circulating Treg pool (48).

To test the possibility that proliferation also contributes to the rapid accumulation of heart Tregs post-MI, Tregs were stained with Ki67, and approximately 75% of heart Tregs were Ki67-positive, much higher than those in spleen or NDLN Tregs 7 days post-MI, which was consistent with the 5-ethynyl-2′-deoxyuridine (EdU) incorporation, that showed approximately 25% EdU+ cells among heart Tregs, but a lower fraction within spleen or NDLN Tregs [49]. These findings indicate a higher proliferation rate of heart Tregs than Tconvs and lymphoid organ Tregs post-MI (48). Using the TCR repertoires to determine the clonality of the heart Tregs post-MI, it was found that a significant fraction of heart Tregs clonally expanded while the spleen Tregs showed a much lower frequency of clonality, and there were only a few TCR sequences shared between spleen Tregs and heart Tregs (50). These results suggest a unique TCR repertoire of heart Tregs and their local clonal expansion in the infarcted hearts after MI. Additionally, since Foxp3 expression in the peripheral sites is important to convert Tconvs to peripheral Tregs (pTregs), the donor cells that were initially Foxp3– essentially could be detected in organs two weeks after the transfer, but more importantly, Foxp3+ cells, donor derived Tregs, were detected in the infarcted hearts post-MI (49).

To identify the mechanism of heart Treg accumulation after MI, the role of IL-33 was examined. Researchers noticed that Il1r1l, which encodes Suppression of Tumorigenicity 2 (ST2), the receptor of interleukin-33 (IL-33), was significantly upregulated in heart Tregs. Since IL-33 plays an important role in Treg homeostasis in tissue; flow cytometry measuring the ST2 protein was performed, to test whether heart Tregs would accumulate in response to IL-33, The results showed that the percentage of ST2+ Tregs within heart Tregs was much higher than spleen Tregs, and the fraction of ST2+ heart Tregs elevated at day 3 stayed elevated for 28 more days. Moreover,

both Il33 transcript and IL-33 protein levels were increased after MI, peaking at day 5. Furthermore, the cellular source of IL-33 in the infarcted hearts was investigated by sorting the CD45+ and CD45– cell fractions from the hearts 5 days post-MI and measuring their Il33 transcript levels (49). This revealed that IL-33 was mainly expressed by CD45- cells. Endothelial cells, cardiomyocytes, and cardiac fibroblasts from the post-MI hearts were sorted and their Il33 transcript levels were measured. Results showed that IL-33 was highly expressed in cardiac fibroblasts, and cardiomyocytes expressed a small amount of IL-33, but for endothelial cells IL-33 was scarcely expressed. Immunofluorescence staining confirmed the cell source of IL-33 as Vimentin+ fibroblasts, but none of them were co-stained with CD31 (49). This suggests that the main source was cardiac fibroblasts. Following this understanding, a series of loss- and gain-of-function experiments evaluating the function of the IL-33/ST2 axis in the accumulation of heart Tregs after MI showed that the number of heart Tregs was decreased in Il1rl1 -/- mice when compared to wild-type mice 7 days after MI, but there were not significant changes in spleen Tregs levels. IL-33 administration also elevated the proportions of proliferating heart Tregs (Ki67+CD4+Foxp3+) and ST2-positive heart Tregs (ST2+CD4+Foxp3+) (31). These observations combined indicate that the IL-33/ST2 axis induces the heart Treg expansion by promoting their proliferation (Figure 6).

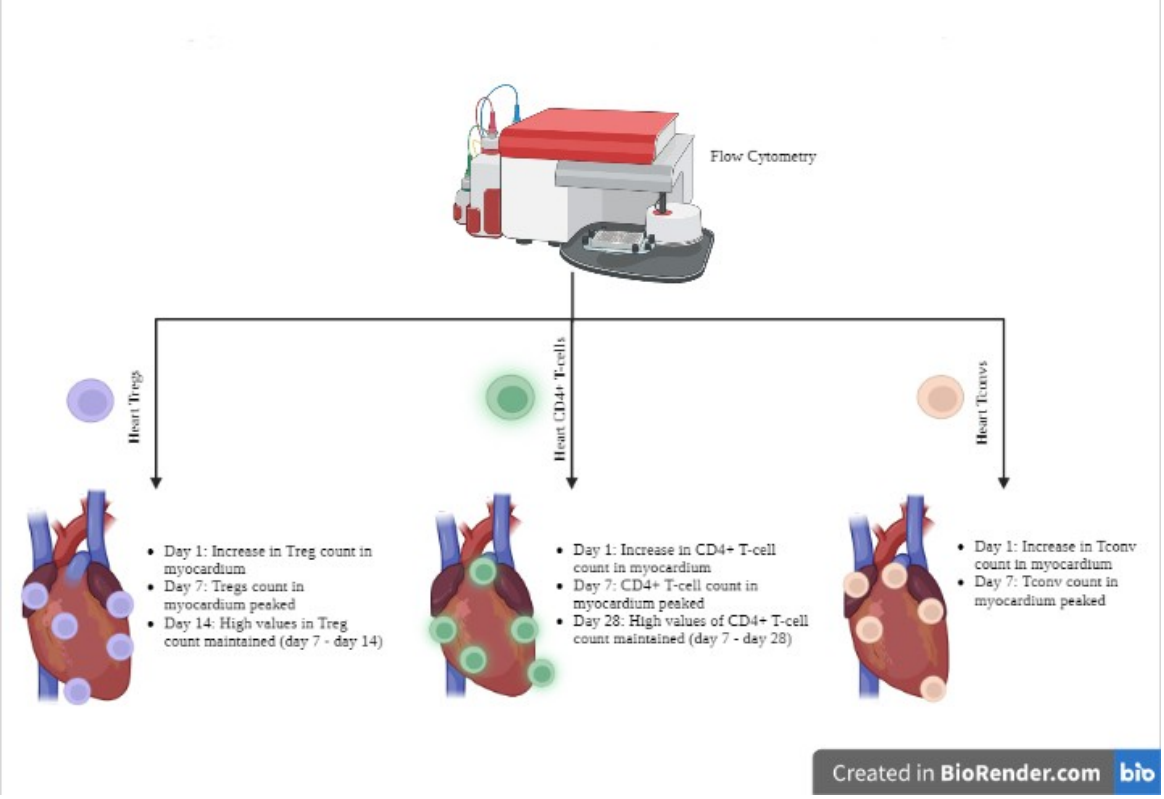
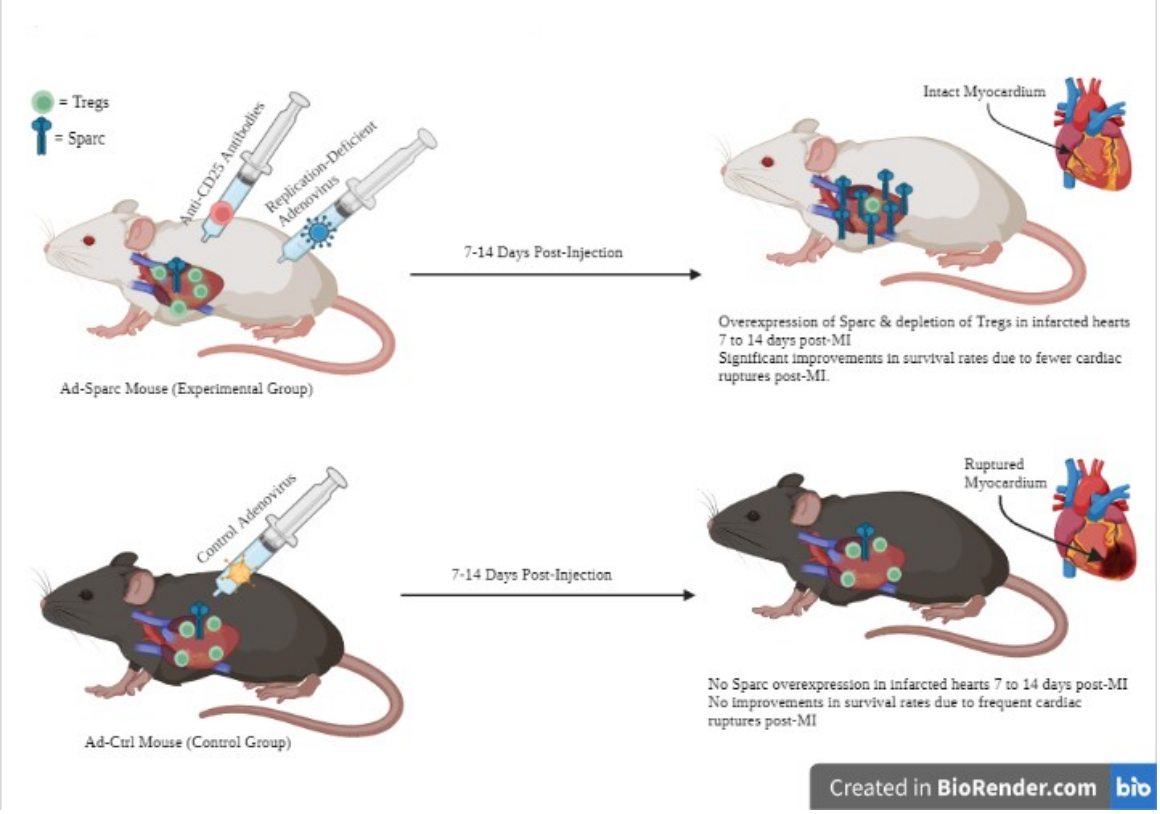


Figure 6: Accumulation of Regulatory T-cells, Conventional T-cells, and CD4+ T-cells within the myocardium following myocardial infarction.

Tregs have been known to engage in anti-inflammatory activities after MI, but their direct influence on pathological processes within the tissue of infarcted hearts is not fully understood. To better understand heart Tregs' reparative role in the myocardium post-MI, their transcriptome was analyzed and revealed that Sparc was among the highest differentially expressed genes. Sparc (secreted acidic cysteine-rich glycoprotein, also known as osteonectin), a collagen-binding matricellular protein, plays a pivotal role in collagen assembly into the ECM and the preservation of the structure and composition of the ventricles after MI (51).

To ascertain the role of Sparc in heart Treg-mediated tissue repair post-MI, Anti-CD25 antibody was administered to deplete Tregs, and an injection of replication-deficient adenovirus harboring mouse Sparc (Ad-Sparc) was administered to overexpress Sparc globally. The control group represented the Control adenovirus (Ad-Ctrl). Unlike the Ad-Ctrl–treated Treg-ablated mice, the Ad-Sparc–treated Treg-ablated mice showed significant improvements in survival rates due to fewer cardiac ruptures after MI. Echocardiography was performed 14 days post-MI in surviving mice, and no statistically significant differences were identified between the mice (49). The experiment suggested that there was improvement of cardiac function following Sparc overexpression, which may be explained by the longer survival of mice with poor cardiac function in the Ad-Sparc–treated group than in the Ad-Ctrl–treated group.

Next, researchers focused on better understanding the processes of scar formation within the infarct zone. The scar size of mice treated with Ad-Sparc and Ad-ctrl did not significantly differ, but the infarct thickness was greater in Ad-Sparc–treated Treg-ablated mice than the scar thickness of the Ad-Ctrl–treated Treg-ablated group. This was consistent with the result of lower cardiac ruptures as thick scars were less prone to ruptures. Since collagen is an abundant structural component of infarct scar tissue, the collagen deposition amount and maturation were analyzed after MI. First, researchers noticed that Treg depletion reduced the blood vessel-based collagen content of the infarct zone (52). However, Sparc overexpression reversed these effects. Furthermore, the deposition of collagen strengthens the scars, and is reparative in nature in the short term (53). However, in the long term, more scarring will inhibit cardiac function. Additionally, thicker tightly packed collagen fibers were dominant within infarct scars in Ad-Sparc–treated Treg-ablated mice than in control Ad-Ctrl–treated Treg-ablated mice, suggesting that Sparc overexpression improved college maturation after Treg depletion. Moreover, Treg depletion resulted in loose and thinner collagen fibers in the infarct scars, whereas after Sparc overexpression, collagen fiber organization was tight and parallel along with a larger diameter, indicating improved collagen maturation (54). This confirms that heart Tregs lead to increased collagen content and enhanced maturation in the infarct scars, thereby suggesting Sparc plays a critical role in this process (Figure 7).



After analyzing the unique transcriptome of Tregs, scientists identified an unrecognized tissue-Treg population in hearts. The distinctly altered transcripts of the heart Tregs in the transcriptome data from other tissue Tregs are genes encoding molecules related to Treg suppressive capacity. Ctla-4 and Klrg-1 genes were upregulated in heart Tregs, thereby supporting the evidence that heart Tregs play a critical inhibitory role in the inflammatory cardiac microenvironment (50). Scientists have also determined that heart Tregs overexpress ECM organization or collagen synthesis–related genes, which differentiates them from lymphoid organ Tregs, and contributes to maintaining cardiac function and integrity of the myocardium after MI. Even though tissue Tregs, in general, share similarities in their transcriptomes, tissue Tregs are significantly distinct from other Treg populations depending on which organ they are associated with. Due to similarities in the injury to the myocardium, heart Tregs in I/R injury models also have a pro healing phenotype similar to heart Tregs in MI models. Additionally, it has been determined that CD4+ T cells first become activated and proliferate in the heart-draining MLNs post-MI, and then infiltrate into injured hearts (47). When scientists compared transcriptomic characteristics between heart Tregs and MLN Tregs, the results showed that heart Tregs displayed a distinct signature characterized by a pro-healing phenotype, unlike the MLN Tregs. The data from the experiments conducted also revealed that T-cells infiltrating the infarcted myocardium display a unique signature and clonally expand in situ, but Treg suppressive molecular Ctla4 was upregulated in

Tregs, the results showed that heart Tregs displayed a distinct signature characterized by a pro-healing phenotype, unlike the MLN Tregs. The data from the experiments conducted also revealed that T-cells infiltrating the infarcted myocardium display a unique signature and clonally expand in situ, but Treg suppressive molecular Ctla4 was upregulated in MLN Tregs when compared to spleen and NDLN Tregs (49). Therefore, from existing data, heart Tregs follow a lymphocyte-trafficking mechanism, resulting in Tregs becoming primed and proliferative in MLNs. Furthermore, the injured myocardium promotes the development of heart Tregs to exhibit a cardioprotective phenotype during the healing stage.

Tissue Tregs have various recruitment sources, such as the peripheral Treg pool, cloning of recruited or locally colonized Tregs, and Tconvs conversion. Researchers believe that Tregs are mainly recruited from the peripheral circulation after MI, but there is a lack of evidence supporting this notion. FTY720 and stain-based tracking experiments confirmed that circulating Tregs migrate to the hearts after MI, and that 95% of the increased Treg population in the myocardium is derived from the peripheral circulation (50). When compared to lymphoid organs, the grouping of Tregs in the injured hearts depends on the upregulated expression of chemokines and chemokine receptors. Moreover, TCR repertoire data revealed that the clonal expansion of heart Tregs occurred in response to MI and was more pronounced in heart Tregs than in spleen Tregs. This hints at the possibility of specific antigen-TCR recognition by heart Tregs. Therefore, even though heart Tregs expressed high levels of biomarkers associated with thymus derived Tregs, experimental results suggest that only a minute proportion of heart Tregs was converted from Tconvs. By using the heart-specific antigen transgenic mice, it was also found that Tconvs can be transformed into Tregs in MI. However, Tconvs did not convert to visceral adipose tissue Tregs from Tconvs, suggesting that heart Tregs are distinct sources of individual tissue Tregs (49). Since ST2 is upregulated on heart Tregs, it was thought that IL-33 drove heart Tregs enrichment, and it was verified that IL-33/ST2 axis contributed to the accumulation of heart Tregs through gain- and loss-of-function experiments. Regarding the mechanism of IL-33–induced Treg expansion, experimental evidence has shown that IL-33 injection enhanced the proliferation of Tregs and has demonstrated that IL-33 attenuates cardiac remodeling after MI, which is consistent with the protective role of Tregs.

The upregulation of ECM proteins and collagen-related genes associated with heart Tregs was identified by transcriptomic data, which indicates an increased ability to promote cardiac repair after MI. Therefore, the reparative traits of tissue Tregs suggest that heart Tregs are more likely to play a cardioprotective role by directly promoting repair instead of simply suppressing inflammation. Among the various differentially expressed transcripts associated with the post-cardiac injury microenvironment, Sparc is the prime example since it is sparsely expressed in lymphoid Tregs but is highly expressed by heart Tregs (51). Sparc is a cellular matrix protein that governs cell-matrix interactions and regulates ECM production and assembly in the myocardium. The absence of Sparc in mice increases the mortality rate, which is attributed to an increased frequency of cardiac rupture

and dysfunction after MI. This effect is a result of impaired granulation tissue formation and faulty collagen maturation following the knockout of Sparc (52). The experimental data evidenced decreased mortality and cardiac rupture in Treg-ablated mice. These observations are associated with increased collagen content and enhanced maturation in the infarct scars as a result of the upregulated expression of Sparc. This revised the defects in tissue repair caused by Treg removal.

Regulatory T-cell cardioprotection discussion

Unlike all three of the aforementioned immunotherapies, the cardioprotection function of Regulatory T-cells (Tregs) has not yet been transformed into a treatment option. However, further research about Tregs wound repair ability will allow for the development of safer options for acute cardiac injury recovery. As heart Treg accumulation is dependent on the IL-33/ST2 axis, incorporating this component in the development of a Treg-based treatment will allow for preserving cardiac integrity after acute cardiac injury. Regarding the experiments about Treg clonality and infiltration, these can be expanded with a time course to see whether donor derived Tregs are detected in infarcted hearts at different time periods post-MI, such as 4 days or 2 weeks post-MI. Furthermore, as Sparc overexpression has been associated with decreased mortality and cardiac rupture in Treg-ablated mice, modulating Sparc expression in Tregs, or even other Sparc producing cells (fibroblasts and macrophages) in the cardiac microenvironment will allow for more effective and universal treatments against cardiac injury. However, there are some drawbacks to using Sparc to combat cardiac injury. As Sparc increases collagen deposition and maturation, more sturdy scars are constructed on the heart. Though, in the short term, this scarring is reparative and prevents cardiac rupture, in the long term, it progresses into adverse remodeling of the cardiac muscle, resulting in a decrease in cardiac function. Therefore, it is important for researchers to study how to modulate the expression of Sparc to control the amount and thickness of cardiac scarring so that only reparative fibrosis is presented, and it does not progress into adverse fibrosis and ventricular remodeling over time. Furthermore, in addition to Sparc, heart Tregs also highly expressed other ECM molecules, such as Areg and Ctla4. Therefore, research must be conducted to determine whether these additional molecules contribute to maintaining or modulating heart Treg function, and based on this information, a variety of therapies targeting or utilizing different molecules can be developed. RNA interference or oligonucleotide therapeutics represents a potential method to modulate presented transcriptomes at pre-defined intervals into disease progression, so as to control the switch between regenerative and scar-based repair. This method has not yet been tested with pausing Sparc overexpression specifically, but there are currently two clinical trials being conducted by the Brigham and Women's Hospital (55, 56); one of them is active (NCT03702829) and the second one has not yet started (NCT04843020). These studies explore the use of oligonucleotides in treating transthyretin (TTR) amyloid cardiomyopathy, which is another condition that also affects myocardial structure, similar to MI and fibrosis. Even though no results have been released yet, if this method is found to be effective in the trial, researchers can focus on

expanding the domain of oligonucleotide therapeutics to modulating Sparc expression to switch between regenerative and scar-based repair.

Discussion

Cardiac disease and injury is the leading cause of death worldwide. As the death rates continue to increase, the need for effective treatments and therapies to combat cardiac disease becomes even greater; scientists have developed various forms of treatment, ranging from simple lifestyle changes and medical therapies to complex cardiac surgeries. However, in the past few years, studies have revealed the potential of immunotherapies in efficiently targeting and treating cardiac disease. These immunotherapies, such as immunoadsorption and immunoglobulin treatments, CAR T-cells, macrophage cardiac regeneration, and even cardiac protection by regulatory T-cells, may be the solution to the long-standing battle against cardiac disease.

Significant progress has been made over the past two decades. Twenty years ago, CAR T-cell therapy did not exist. But today, there are several immunotherapies, ranging from macrophage regeneration to various applications of CAR T-cells, that show potential in successfully treating cardiac disease and injury. The field has progressed exponentially in the past few decades. However, there are still areas of improvement for each of the therapies discussed in this paper, specifically relating to side effects and patient safety. Ultimately, the potential benefit of each treatment may outweigh its side effects. From over-the-counter medications to complex surgeries, today, there is no single treatment without side effects. Even though receiving immunotherapy may lead to some adverse events, this might still be the best option for patients to regain cardiac function after cardiac injury.

The ideal scenario to treat cardiac injury or disease is to grow new muscle or regenerate the heart, and this method is now being researched in clinical trials. Potential ways to access this type of regenerative capability in humans is to conduct research on embryonic or induced pluripotent stem cell therapies and how we can manipulate them to develop into cardiac tissue and muscle to replace the entire heart. Understanding the key to stimulating cardiac regeneration has the potential to transform and change the way cardiac disease and injuries are treated.

Conclusion

The pathology of myocardial injury and repair is governed by complex inflammatory pathways and a variety of immune cell types. Our understanding of the beneficial and harmful contributions of specific immune cells and cytokines, in addition to targeted modulation of the immune system to boost myocardial recovery and repair is an important goal of cardio- immunology. Cell types, like M2-like macrophages, natural killer cells, and regulatory T cells as well as engineered immune cells, such as CAR T cells, are all targets and tools of novel cardiac research. In animal and human models, immunoadsorption followed by immunoglobulin therapy, CAR T-cell therapy, macrophage-derived cardiac regeneration, and regulatory T-cell cardioprotection capabilities have all improved cardiac function following acute cardiac injuries, including arrhythmias, myocardial infarctions, dilated cardiomyopathy, and

myocarditis. Further research is necessary to fine tune these existing immunotherapies as well as to explore novel ones so that they can be safely and efficiently administered in human patients. In the future, through more research, our understanding of not only the innate/adaptive immune system and existing immunotherapies but also the cardiac microenvironment will provide researchers with the ability to develop new treatment strategies for cardiac disease and injury.

References

1. Doran, A.C., Inflammation Resolution: Implications for Atherosclerosis. Circ Res, 2022. 130(1): p. 130-148.

2. Khawaja, A. and D.I. Bromage, The innate immune response in myocarditis. Int J Biochem Cell Biol, 2021. 134: p. 105973.

3. Kurose, H., Cardiac Fibrosis and Fibroblasts. Cells, 2021. 10(7).

4. Okamoto, R., Cardiac Fibrosis: Chronic Inflammatory Disease and Promising Therapeutic Target. Int J Mol Sci, 2022. 23(15).

5. Rizzoni, D., et al., Immune System and Microvascular Remodeling in Humans. Hypertension, 2022. 79(4): p. 691-705.

6. Rosch, S., et al., Characteristics of Heart Failure With Preserved Ejection Fraction Across the Range of Left Ventricular Ejection Fraction. Circulation, 2022. 146:p. 506-518

7. Kadhi, A., F. Mohammed, and G. Nemer, The Genetic Pathways Underlying Immunotherapy in Dilated Cardiomyopathy. Front Cardiovasc Med, 2021. 8: p. 613295.

8. Yoshikawa, T., A. Baba, and Y. Nagatomo, Autoimmune mechanisms underlying dilated cardiomyopathy. Circ J, 2009. 73(4): p. 602-7.

9. Anzai, A., S. Ko, and K. Fukuda, Immune and Inflammatory Networks in Myocardial Infarction: Current Research and Its Potential Implications for the Clinic. Int J Mol Sci, 2022. 23(9).

10. Jui, E., et al., The Immune and Inflammatory Basis of Acquired Pediatric Cardiac Disease. Front Cardiovasc Med, 2021. 8: p. 701224.

11. Rurik, J.G., H. Aghajanian, and J.A. Epstein, Immune Cells and Immunotherapy for Cardiac Injury and Repair. Circ Res, 2021. 128(11): p. 1766-1779.

12. Ma, Y., Role of Neutrophils in Cardiac Injury and Repair Following Myocardial Infarction. Cells, 2021. 10(7).

13. Feng, L., et al., Identification of Genes Linking Natural Killer Cells to Apoptosis in Acute Myocardial Infarction and Ischemic Stroke. Front Immunol, 2022. 13: p. 817377.

14. Vakrakou, A.G., et al., Peripheral blood natural killer cells in sarcoidosis are associated with early cardiac involvement. Eur J Clin Invest, 2022. 52(5): p. e13742.

15. Huang, J., et al., Vibrational spectroscopy as a powerful tool for follow-up immunoadsorption therapy treatment of dilated cardiomyopathy - a case report. Analyst, 2020. 145(2): p. 486-496.

16. Fuchs, K., et al., Performance, clinical effectiveness, and safety of immunoadsorption in a wide range of indications. Ther Apher Dial, 2022. 26(1): p. 229-241.

17. Bian, R.T., Z.T. Wang, and W.Y. Li, Immunoadsorption treatment for dilated cardiomyopathy: A PRISMA-compliant systematic review and meta-analysis. Medicine (Baltimore), 2021. 100(26): p. e26475.

18. Weinmann, K., et al., Use of Cardiac Biomarkers for Monitoring Improvement of Left Ventricular Function by Immunoadsorption Treatment in Dilated Cardiomyopathy. Biomolecules, 2019. 9(11).

19. Weinmann, K., et al., Add-on Immunoadsorption Shortly-after Optimal Medical Treatment Further Significantly and Persistently Improves Cardiac Function and Symptoms in Recent-Onset Heart Failure-A Single Center Experience. Biomolecules, 2018. 8(4).

20. Simbaqueba, C.C., et al., Cardiovascular Complications of Chimeric Antigen Receptor T-Cell Therapy: The Cytokine Release Syndrome and Associated Arrhythmias. J Immunother Precis Oncol, 2020. 3(3): p. 113-120.

21. Stein-Merlob, A.F., et al., Immunotherapy-Associated Cardiotoxicity of Immune Checkpoint Inhibitors and Chimeric Antigen Receptor T Cell Therapy: Diagnostic and Management Challenges and Strategies. Curr Cardiol Rep, 2021. 23(3): p. 11.

22. Nishi, M., P.Y. Wang, and P.M. Hwang, Cardiotoxicity of Cancer Treatments: Focus on Anthracycline Cardiomyopathy. Arterioscler Thromb Vasc Biol, 2021. 41(11): p. 2648-2660.

23. Barbosa, C.D., et al., Cytotoxic CD4. Elife, 2022. 11.

24. June, C.H. and M. Sadelain, Chimeric Antigen Receptor Therapy. N Engl J Med, 2018. 379(1): p. 64-73.

25. Miyagawa, F., et al., The use of mouse models to better understand mechanisms of autoimmunity and tolerance. J Autoimmun, 2010. 35(3): p. 192-8.

26. Aghajanian, H., et al., Targeting cardiac fibrosis with engineered T cells. Nature, 2019. 573(7774): p. 430-433.

27. Stein-Merlob, A.F., S. Ganatra, and E.H. Yang, T-cell Immunotherapy and Cardiovascular Disease: Chimeric Antigen Receptor T-cell and Bispecific T-cell Engager Therapies. Heart Fail Clin, 2022. 18(3): p. 443-454.

28. Mladosievičová, B., et al., Challenges and solutions in management of cardiotoxicity induced by checkpoint inhibitors. Klin Onkol, 2020. 33(5): p. 350-355.

29. Patel, N.P., et al., Cardiovascular Toxicities of CAR T-cell Therapy. Curr Oncol Rep, 2021. 23(7): p. 78.

30. June, C.H., S.R. Riddell, and T.N. Schumacher, Adoptive cellular therapy: a race to the finish line. Sci Transl Med, 2015. 7(280): p. 280ps7.

31. Vagnozzi, R.J. and T.A. McKinsey, T cell immunotherapy for cardiac fibrosis: mRNA starts the CAR. Cell Stem Cell, 2022. 29(3): p. 352-354.

32. Nenna, A., et al., Cardiotoxicity of Chimeric Antigen Receptor T-Cell (CAR-T) Therapy: Pathophysiology, Clinical Implications, and Echocardiographic Assessment. Int J Mol Sci, 2022. 23(15).

33. Guha, A., et al., Cardiovascular Events Associated with Chimeric Antigen Receptor T Cell Therapy: Cross-Sectional FDA Adverse Events Reporting System Analysis. Biol Blood Marrow Transplant, 2020. 26(12): p. 2211-2216.

34. Li, Z., et al., Effects of IL-38 on Macrophages and Myocardial Ischemic Injury. Front Immunol, 2022. 13: p. 894002.

35. Bruton, F.A., et al., Macrophages trigger cardiomyocyte proliferation by increasing epicardial vegfaa expression during larval zebrafish heart regeneration. Dev Cell, 2022. 57(12): p. 1512-1528.e5.

36. Simões, F.C., et al., Macrophages directly contribute collagen to scar formation during zebrafish heart regeneration and mouse heart repair. Nat Commun, 2020. 11(1): p. 600.

37. Wong, E.V. 13.1: Introduction to Extracellular Matrix and Cell Adhesion. Biology LibreTexts 2021 [cited 2022 8/2/2022].

38. Chen, B., et al., Identification of macrophages in normal and injured mouse tissues using reporter lines and antibodies. Sci Rep, 2022. 12(1): p. 4542.

39. Xu, X., et al., The future Landscape of macrophage research in cardiovascular disease: a bibliometric analysis. Curr Probl Cardiol, 2022: p. 101311.

40. Wang, Y., et al., Macrophage-targeting gene silencing orchestrates myocardial microenvironment remodeling toward the anti-inflammatory treatment of ischemia-reperfusion (IR) injury. Bioact Mater, 2022. 17: p. 320-333.

41. Booz, G.W., R. Altara, and F.A. Zouein, Editorial: Immunomodulatory Approaches in Cardiovascular Diseases. Front Cardiovasc Med, 2022. 9: p. 873452.

42. Rizzo, G., et al., Dynamics of monocyte-derived macrophage diversity in experimental myocardial infarction. Cardiovasc Res, 2022.

43. Stephan, M.T., et al., Genetic programming of macrophages to perform anti-tumor functions using targeted mRNA nanocarriers. 2019.

44. Mathis, D., et al., Fat Treg cells: a liaison between the immune and metabolic systems. 2009, National Library of Medicine.

45. Mathis, D., M. Panduro, and C. Benoist, TISSUE-Tregs. 2016.

46. Grabie, N., A.H. Lichtman, and R. Padera, T cell checkpoint regulators in the heart. Cardiovasc Res, 2019. 115(5): p. 869-877.

47. Vdovenko, D. and U. Eriksson, Regulatory Role of CD4. J Immunol Res, 2018. 2018: p. 4396351.

48. Kino, T., M. Khan, and S. Mohsin, The Regulatory Role of T Cell Responses in Cardiac Remodeling Following Myocardial Infarction. Int J Mol Sci, 2020. 21(14).

49. Xia, N., et al., A Unique Population of Regulatory T Cells in Heart Potentiates Cardiac Protection From Myocardial Infarction. Circulation, 2020. 142(20): p. 1956-1973.

50. Lu, Y., N. Xia, and X. Cheng, Regulatory T Cells in Chronic Heart Failure. Front Immunol, 2021. 12: p. 732794.

51. Deng, S.K., Y. Jin, and J.F. Wang, SPARC induces M2 polarization of macrophages to promote proliferation, migration, and angiogenesis of cholangiocarcinoma cells. Neoplasma, 2022.

52. Nakamura, T., et al., SPARC promotes production of type IV and VII collagen and their skin basement membrane accumulation. J Dermatol Sci, 2022.

53. Leancă, S.A., et al., Left Ventricular Remodeling after Myocardial Infarction: From Physiopathology to Treatment. Life (Basel), 2022. 12(8).

54. Veith, C., H.A. Ghofrani, and N. Weissmann, Response by Veith et al to Letter Regarding Article, "SPARC, A Novel Regulator of Vascular Cell Function in Pulmonary Hypertension". Circulation, 2022. 146(5): p. e16-e17.

55. Hospital, B.a.W.s., 24 Month Open Label Study of the Tolerability and Efficacy of Inotersen in TTR Amyloid Cardiomyopathy Patients. 2018.

56. Hospital, B.a.W.s., ION-682884 in Patients With TTR Amyloid Cardiomyopathy. 2021.