Inflammation in Heart Failure

Is our immune response failing our heart?

Patricia van den Hoogen

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Laat onze immuunrespons ons hart falen?
(met een samenvatting in het Nederlands)

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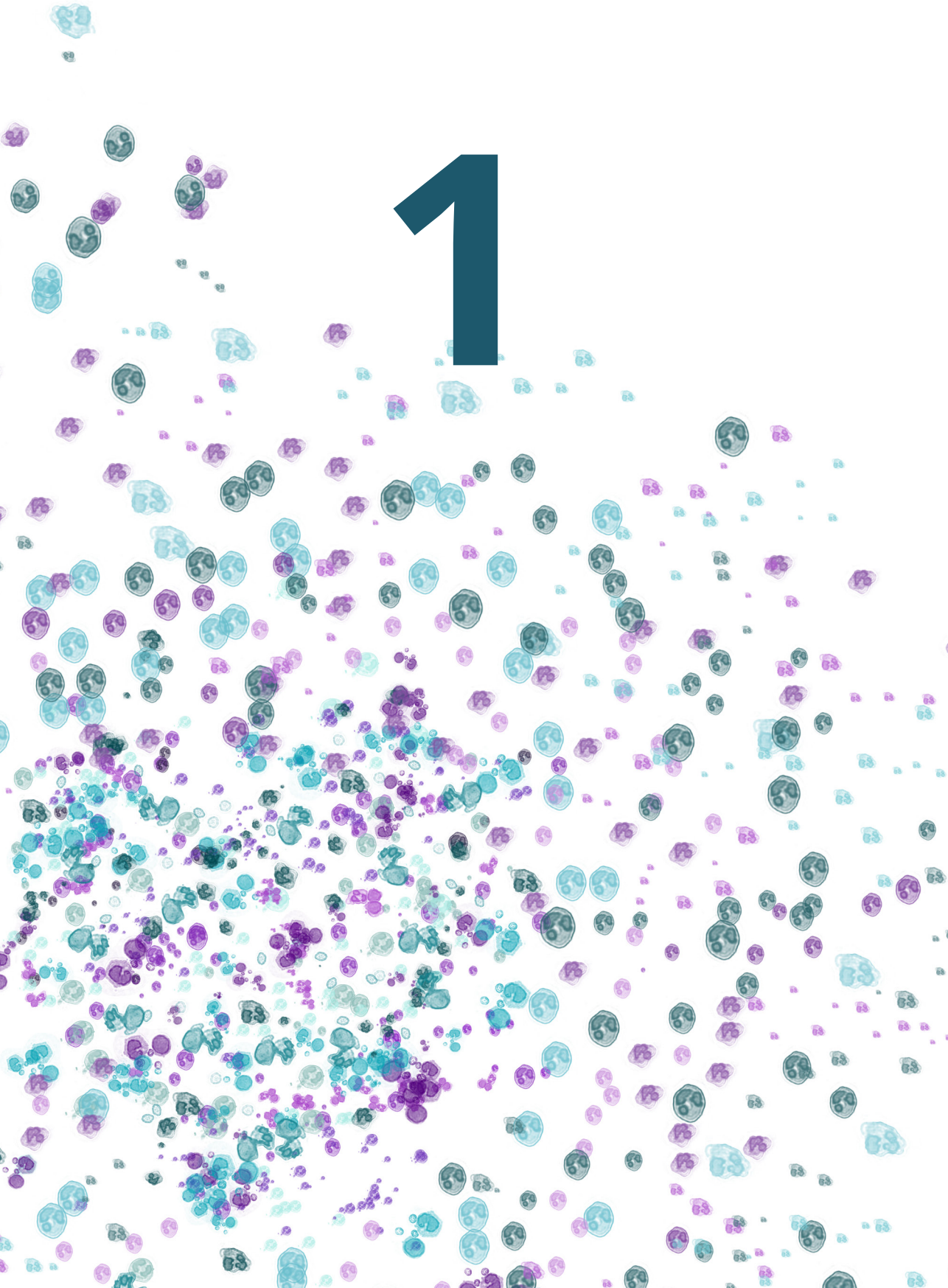

General introduction and thesis outline

Heart failure

Heart failure (HF) is one of the most common diseases globally, affecting approximately 26 million people worldwide[1]. The prevalence of HF is still rising, which leads to enormous health expenditures for cardiovascular diseases[2]. Survival rates for HF are poor, where 65% of the patients die within 5 years after diagnosis[1]. HF is characterized by adverse cardiac remodeling and a reduced ability of the heart to maintain cardiac output[3]. Different causes leading to HF have been described, such as ischemic myocardial injury, cardiac autoimmune responses, and genetic mutations in e.g. contractile proteins[4–7]. HF has been classified into different subtypes, including HF with reduced ejection fraction (HFrEF), HF with mid-range ejection fraction (HFmrEF), and HF with preserved ejection fraction (HFpEF)[8,9]. HFrEF is most often the consequence of contractile cell loss due to cell death in e.g. myocardial infarction (MI), whereas HFmREF and HFpEF are more associated with hypertension, diabetes, overweight, and previous ischemic heart disease (IHD)[3,8]. Diagnosis of HF is still difficult and mainly based on clinical symptoms, including edema, fatigue, and shortness of breath[3]. The severity of HF is classified by symptoms and physical activity, which is represented in class I-IV by the New York Heart Association (NYHA)[10]. Currently available therapies include the prescription of medications, including statins, aspirin, β-blockers, AT1R antagonists, and P2Y12-inhibitors[11,12]. These drugs have shown to reduce symptoms of HF, yet do not repair the injured heart[13–15]. The only treatment option for patients with end-stage HF is mechanical support of the heart using a left ventricular assist device (LVAD) or ultimately heart transplantation (HTx)[16]. Unfortunately, there is still a shortage of donor hearts, thereby limiting the number of transplantations[17,18]. Moreover, long-term survival rates after HTx are relatively low due post-transplant morbidities, such as cardiac allograft vasculopathy (CAV) and antibody-mediated rejection[19,20].Therefore, there is still a need to find new and better strategies for the treatment of HF. A strong common denominator, both in the progression of HF and post-HTx is inflammation[21,22], which makes the immune response an attractive area in the search for new interventions.

(Auto) immune responses in heart failure

After ischemic cardiac damage, the immune response is characterized by different phases, including a pro-inflammatory phase and a reparative phase[23]. In the acute phase, necrotic cardiomyocytes release their intracellular contents, which induce an pro-inflammatory immune response[24]. Complement activation and the release of reactive oxygen species (ROS) attract neutrophils and inflammatory monocytes, which clear the myocardium from dead cells and debris[25]. The release of pro-inflammatory cytokines and chemokines subsequently attracts lymphocytes, which in their turn recruit more inflammatory monocytes to the myocardium[26].

In the second phase, a switch to an anti-inflammatory immune response is initiated, which leads to resolution of the initial pro-inflammatory response[27]. The secretion of anti-inflammatory and pro-fibrotic cytokines induces a shift towards reparative monocytes, which create a reparative environment allowing wound healing[25,28]. The transition between these phases is a complex interaction between many players and components of the immune system. Small imbalances in the transition of the pro- and anti-inflammatory phases can

result in persistent pro-inflammatory responses and chronic inflammation[29]. Chronic inflammation is characterized by increased abundance of lymphocytes in the myocardium, secreting pro-inflammatory cytokines and antibodies, thereby attracting more inflammatory cells which induce additional cardiac damage[30]. This vicious cycle supports ongoing chronic immune responses in the heart and contributes to adverse cardiac remodeling and HF progression [23], as represented in *Figure 1.*

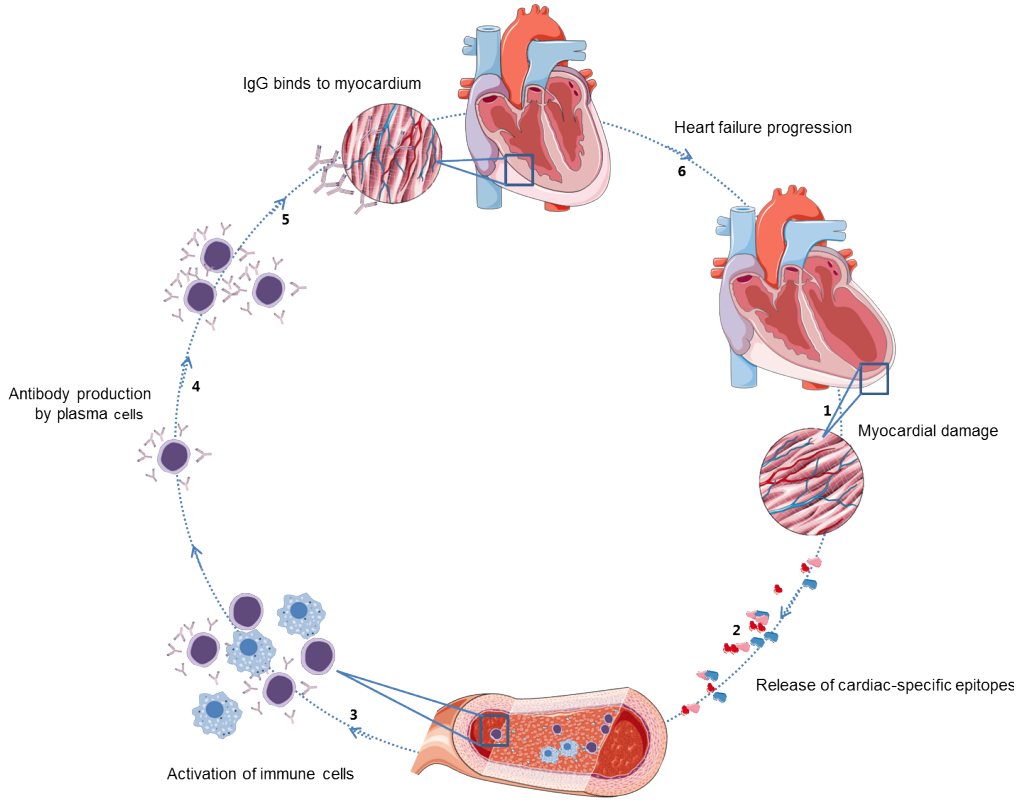

Figure 1. Potential role of (auto) immune responses in the progression of HF
1) HF is caused by structural or functional cardiac abnormalities. 2) As a consequence of cardiac stress, damaged cardiomyocytes release intracellular proteins, which can act as cardiac-specific immune antigens. 3) Exposure of these cardiac antigens in a chronic inflammatory environment subsequently leads to the activation of autoreactive T and B lymphocytes. 4) B cells differentiate into plasma cells, which enter the circulation and start producing cardiac-specific antibodies. 5-6) These antibodies will bind to the myocardium, thereby inducing more cardiac damage and HF progression.

The adaptive immune response is a key mediator of adverse cardiac remodeling[31,32]. In the context of a highly inflammatory environment the release of cardiac proteins by necrotic cardiomyocytes may break immune tolerance to self-proteins and activate autoreactive T and B cells[33]. Activated B cells generate cardiac-specific antibodies and pro-inflammatory cytokines[34]. It has been suggested that binding of these autoantibodies to cardiac-specific

antigens on cardiomyocytes can induce cardiac dysfunction, for instance by affecting contractility or by inducing cellular toxicity[35,36]. Binding of antibodies activates the process of complement activation, thereby inducing complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC), resulting in lysis of cardiomyocytes[37,38]. Cardiac-specific antibodies have already been detected in plasma of end-stage HF patients[39,40]. For example, anti-myosin antibodies are associated with impaired cardiac function in patients with DCM[37]. In addition, IHD patients show high levels of antibodies targeting cardiomyocyte surface receptors, including β1-adrenergic receptors [41]. The most abundant immunoglobulin subtype observed in patients with HF is IgG, including IgG1 and IgG3 subclasses[42]. IgG1 and IgG3 show high reactivity against protein epitopes and are strongly associated with complement activation[43,44]. These findings indicate that immune activation by the release of cardiac antigens by necrotic cardiomyocytes in different etiologies of HF results in increased cardiac damage and might accelerate the progression of HF.
The presence of cardiac-specific (auto) antibodies in end-stage HF closely resembles autoimmune features as observed in non-ischemic cardiomyopathies, such as myocarditis[45,46]. Myocarditis is defined as an inflammatory disease of the heart, which is often caused by viral infections or post-viral immune-mediated responses[47,48]. After internalization of the coxsackie B3 virus into cardiomyocytes, acute injury is induced by viral replication, leading to cardiomyocyte necrosis and exposure of intracellular proteins that can function as antigens[49]. The release of intracellular proteins in a highly inflammatory environment will activate T cells, which can also attack healthy cardiomyocytes via mimicry between viral epitopes and cardiac epitopes, leading to autoimmune responses[45,50]. In chronic myocarditis patients, heart-reactive autoantibodies, against cardiac myosin heavy chain (MHC), cardiac troponin I and mitochondrial proteins have been found, which further aggravate cardiac damage[45,46,51]. Ultimately, chronic inflammation and autoimmune responses in myocarditis can induce severe myocardial remodeling, which leads to end-stage HF.

(Auto) immune responses in heart transplantation and rejection

Antibody-mediated immune responses are not limited to the progression of HF, but are also implicated after HTx[52,53]. HTx is considered as the last treatment option for patients with end-stage HF[16], however, challenges exist for improvement of post-HTx outcome[54]. Despite a decreasing incidence of acute rejection of the transplanted heart due to immunosuppressive interventions, chronic rejection is still the primary cause of allograft failure, consequently limiting long-term survival[55,56]. One of the hallmarks of chronic rejection is cardiac allograft vasculopathy (CAV), which is characterized by diffuse concentric intimal thickening of the coronary arteries, leading to ischemic damage to the transplanted heart or sudden cardiac death[57,58]. The exact cause of CAV is not fully elucidated yet, but an antibody-mediated immune response against the allograft is suggested to be of major importance[59]. Antibody production can take place in the lymphoreticular system of the recipient, but also in ectopic lymphoid structures (ELS) in the donor heart[59]. ELS consist of clusters of inflammatory cells in the adventitia and adipose tissue surrounding epicardial coronary arteries with CAV[60]. These clusters contain B and T lymphocytes, macrophages and active plasma cells, which produce immunoglobulins, including donor-specific antibodies

(DSA), against donor human leukocyte antigens (HLA)[60]. However, also antibodies against non-HLA, including myosin, vimentin and anti–angiotensin II type 1 receptor, can be found in patients with CAV and contribute to transplant rejection[21,61]. These findings suggest that already prior to HTx, the immune system of the recipient is activated and high levels of antibodies might already be present that potentially reduce post-HTx survival. Whether pre-HTx autoimmune responses are associated with post-HTx rejection, survival, and CAV development is currently unknown.

Cellular therapeutics against cardiac inflammation

Although inflammation is a strong common denominator in different etiologies of HF, none of the currently used HF medications are aimed at specifically modulating the immune response. Several attempts to prevent excessive immune responses upon cardiac damage, for example by the use of broad-spectrum immunosuppression with corticosteroids, have demonstrated controversial effects. The use of broad immunosuppression showed detrimental effects on the clearance of necrotic cardiomyocytes, impaired collagen deposition, disrupted post-MI wound healing and resulted in cardiac rupture[35]. These findings indicate that the immune response is essential in the acute phase of myocardial injury. Since adaptive immunity is more active in the chronic phase of adverse cardiac remodeling and the development of HF[34,62], strategies aiming at B cells and antibody-mediated responses may be more promising.

Currently, B-cell depleting strategies, using monoclonal antibodies, such as Rituximab (anti-CD20), are becoming of more interest. B cell depletion significantly improved cardiac remodeling after MI or transverse aortic constriction (TAC) induced pressure overload in small animals, thereby suggesting that B cell depletion might be useful in the acute setting of cardiac damage[63–65]. The use of B cell depletion in patients after MI is currently being investigated in the first clinical trials. However, potential side effects of the long-term use of B cell depletion in patients with cardiovascular disease are still unknown and remain to be elucidated.

The clearance of circulating antibodies in HF patients using intravenous immunoglobulins (IVIG) showed limited and contradictory results[66,67]. IVIG improved cardiac function in patients with end-stage DCM[68], however, the use of IVIG in an early phase of cardiomyopathy did not show any improvement[69]. B cell depletion and clearing circulating antibodies might be interfering with several other innate and adaptive immune pathways and does not restore the balance between pro- and anti-inflammatory immune cell subpopulations. Therefore, restoring immune tolerance against cardiac antigens and the balance between pro-inflammatory and anti-inflammatory responses by immunomodulation would be a better strategy.

A promising strategy to repair the damaged heart and to modulate the activated immune response is the use of cellular therapeutics, using progenitor cells[70]. These progenitor cells are defined as cells with the ability to differentiate into other cell types and have multiple molecular and functional properties[71]. For example, mesenchymal stromal cells (MSC) and cardiac progenitor cells (CPC) are known to improve cardiac function, despite poor engraftment in the heart[72,73]. In addition, MSC and CPC are able to suppress inflammatory

responses, mostly in a paracrine manner[74,75]. Important paracrine mediators are extracellular vesicles (EVs), small lipid bi-layered vesicles containing lipids, small RNAs and proteins, which are able to influence many processes, including inflammation[76,77]. Progenitor cells and their EVs also showed immunosuppressive effects on the adaptive immune response[78–80]. MSC and MSC-derived EVs have been shown to modulate B cells, inhibit the formation of plasma cells, and lower antibody production *in vitro*[74,81–83]. Moreover, MSC are used as potential immunosuppressive agents for multiple autoimmune disorders and graft versus host diseases (GVHD)[84,85], and could therefore also be of major importance to suppress antibody-mediated immune responses, as observed in pre- and end-stage HF. For CPC, it is known that they are able to inhibit T-cell proliferation *in vitro*[86]. Interestingly, less is known about their immunomodulating effects on B cells and antibody-mediated immune responses.
Despite the beneficial effects of progenitor cells and cellular therapeutics on cardiac function and cardiac inflammation, as observed *in vitro* and in small and large animal models of MI, the efficacy of cellular therapeutics on top of standard prescribed medications in the clinical setting is disappointing. Several randomized clinical trials, using cell therapeutics to improve cardiac function in cardiovascular disease, showed inconsistent results and modest efficacy of progenitor cells[71,87–89]. More often, promising new therapies which are successful in preclinical phases fail to be translated into actual beneficial interventions that reach the clinical arena, which is also referred to as 'translational failure'. Surprisingly, standard medications, which are clinically prescribed after MI, such as aspirin, P2Y12-inhibitors, β-blockers, AT1R antagonists and statins, are barely incorporated into preclinical studies when testing a new therapy, such as cellular therapeutics. These medications have made substantial impact on cardiovascular disease burden by improving outcome and preventing cardiovascular events[15,17]. However, it might be possible that the (non-) administration of regularly prescribed MI drugs explains part of the reduction in efficacy when translating any new therapy from the laboratory and animal models to the clinical setting.

THESIS OUTLINE

Part one: Antibody-mediated immune responses in pre- and end-stage heart failure
Chronic inflammation has gained much interest as being a key mediator in the progression of HF. In the first part of this thesis, we describe the role of antibody-mediated immune responses in different etiologies of HF. In **chapter 2,** we demonstrated that patients with end-stage HF have high levels of circulating immunoglobulins compared to healthy controls. Hence, high levels of immunoglobulins could already be detected in men with left ventricular diastolic dysfunction, a pre-stage of HF. Moreover, we demonstrated that in the myocardium of end-stage HF patients, many infiltrating immune cells and IgG deposits are present. In **chapter 3**, the role of antibody-mediated immune responses was studied in patients with a genetic cardiomyopathy, caused by a mutation in the phospholamban (PLN) gene. These PLN patients also demonstrate high levels of circulating immunoglobulins and IgG deposits in the myocardium upon end-stage HF. An epitope discovery screen, including 26,000 known cardiovascular peptides, revealed that these antibodies are present in the circulation of PLN

patients and are potentially directed against cell adhesion molecules and structural components of cardiomyocytes.
Since antibody-mediated immune responses play a key role in end-stage HF, we hypothesized that this also has an effect on HF and antibody-mediated rejection post-HTx. In **chapter 4**, we give an overview of the post-HTx immune response, including antibody-mediated rejection and the development of CAV. Also, the contribution of donor and recipient cells is discussed. To investigate whether high immunoglobulins prior to HTx are affecting post-HTx survival, we measured pre-HTx immunoglobulin levels in cardiac recipients and correlated these levels to years of survival in **chapter 5**. We demonstrated a strong correlation between high pre-HTx antibody levels and survival, where patients with high levels pre-HTx had a significant lower survival compared to patients with low antibody titers pre-HTx. In addition, we show that these patients more often develop an inflammatory CAV phenotype.

Part two: Antibody-mediated immune responses in myocarditis
The presence of cardiac-related antibodies in end-stage HF closely resembles autoimmune responses as observed in autoimmune-induced cardiomyopathies, such as myocarditis. Myocarditis is an inflammatory disease of the heart, which leads to severe cardiac damage and ultimately HF progression. In **chapter 6**, we outline the pathogenesis of myocarditis and the development of HF. Moreover, the role of miRNAs in the progression towards HF is described, with some suggestions for future interventions. In **chapter 7**, we set out to reproduce a described model for experimental autoimmune myocarditis (EAM) in mice, as a representative model of autoimmune mediated HF, with the aim to adapt this model to study the role of cardiac-specific antigens in HF progression. Therefore, we immunized mice with α-myosin heavy chain (MHC) emulsified in Complete Freund Adjuvant (CFA). However, in contrast with the literature, we failed to induce myocarditis in these mice. Despite a functional immunization protocol, marked by increased levels of IgG1 and IgG3, we did not observe any signs of cardiac inflammation or deterioration of cardiac function up to 6-weeks after immunization. This underscored the need for a relevant and translational small animal model to study autoimmune responses in the development of HF. Creating a reproducible and translational EAM model would provide the basis to investigate the role of other cardiac-specific antigens involved in autoimmune responses in HF.

Part three: Therapeutic potential of progenitor cells in heart failure
Cellular therapeutics might be a promising strategy in the treatment of HF. These progenitor cells do not only show beneficial effects on cardiac function, but they also exert strong immunosuppressive properties. For MSC, it has already been established that they can modulate the adaptive immune response by suppressing plasma cell formation and antibody production. In **chapter 8**, we investigate the immunosuppressive effect of CPC and CPC-derived EVs on antibody production *in vitro*. We showed that CPC also inhibit antibody production by immune cells-derived of healthy controls or end-stage HF patients, however, the strongest suppressive effects were observed using MSC.
Since there is a huge translational gap between the *in vitro* effects of progenitor cells and the effects observed when reaching the phase of clinical trials, we created an *in vivo* model

in **chapter 9**, where we combined the use of MSC and clinically prescribed medications in a mouse model of ischemia/reperfusion injury. We demonstrated that only using clinically prescribed medications after MI already improved cardiac function in mice, with no further additional effects of MSC.
In **chapter 10**, we summarize the conclusions and discussions of all preceding chapters and put these into context of current literature.

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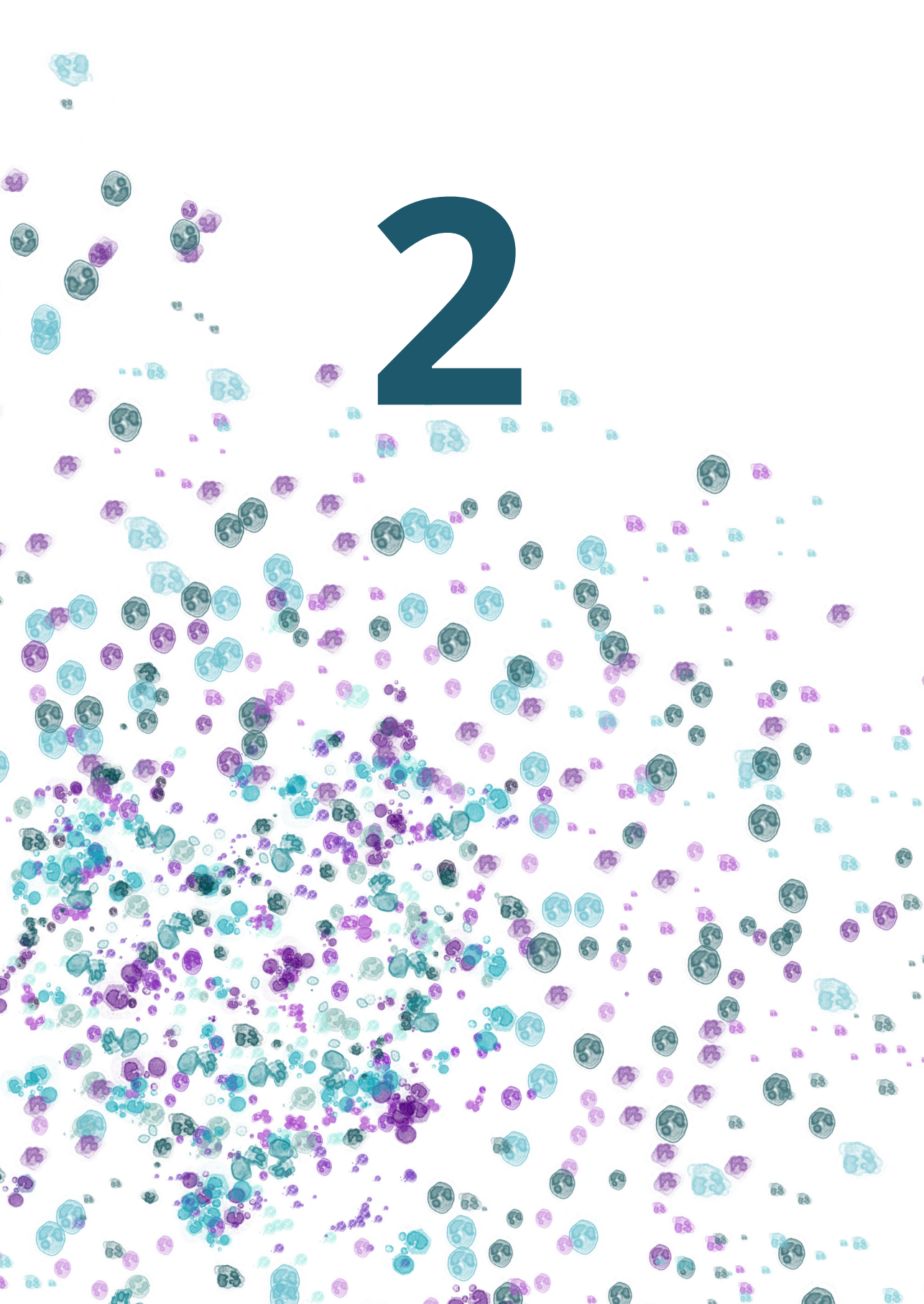
Part one

Antibody-mediated immune responses in pre- and end-stage heart failure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Increased circulating IgG levels, myocardial immune cells and IgG deposits support a role for an immune response in pre- and end-stage heart failure

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ABSTRACT

Background

The chronic inflammatory response plays an important role in adverse cardiac remodeling and the development of heart failure (HF). There is also evidence that in the pathogenesis of several cardiovascular diseases, chronic inflammation is accompanied by antibody and complement deposits in the heart, suggestive of a true autoimmune response. However, the role of antibody-mediated immune responses in HF progression is less clear. We assessed whether immune cell infiltration and immunoglobulin levels are associated with HF type and disease stage, taking sex differences into account.

Methods and results

We found IgG deposits and increased infiltration of immune cells in the affected myocardium of patients with end-stage HF with reduced ejection fraction (HFrEF, n=20). Circulating levels of IgG1 and IgG3 were elevated in these patients. Furthermore, the percentage of transitional/regulatory B cells was decreased (from 6.9% to 2.4%) compared to healthy controls (n=5). Similarly, increased levels of circulating IgG1 and IgG3 were observed in men with left ventricular diastolic dysfunction (LVDD, n=5), possibly an early stage of HF with preserved EF (HFpEF).

Conclusion

IgG deposits and infiltrates of immune cells are present in end-stage HFrEF. In addition, both LVDD patients and end-stage HFrEF patients show elevated levels of circulating IgG1 and IgG3, suggesting an antibody-mediated immune response upon cardiac remodeling, which in the early phase of remodeling appear to differ between men and women. These immunoglobulin subclasses might be used as marker for pre-stage HF and its progression. Future identification of auto-antigens might open possibilities for new therapeutic interventions.

INTRODUCTION

Heart failure (HF) is a clinical syndrome that affects approximately 1-2% of people in the western world[1]. HF is caused by structural or functional cardiac abnormalities, resulting in a reduced cardiac output or increased filling pressures[2]. This can be caused by systolic dysfunction leading to HF with reduced ejection fraction (HFrEF), or by diastolic dysfunction leading to HF with preserved EF (HFpEF)[2]. A large proportion of patients with HFrEF are diagnosed with ischemic heart disease (IHD), due to the consequences of an acute myocardial infarction[3,4]. Another cause of systolic HF is dilated cardiomyopathy (DCM), in which the heart is functionally decompensating as a result of genetic pathogenic mutations or other causes, such as viral myocarditis[2,5,6].

Regardless of HF etiology, cardiac remodeling is one of the hallmarks, in which HF progressively worsens by adverse remodeling of the heart to compensate for losses in contractility or impaired relaxation with increased filling pressures[7,8]. One of the main players in adverse cardiac remodeling is the inflammatory response[8–11]. In the acute phase upon a myocardial infarction, mainly neutrophils and monocytes are important for the clearance of necrotic cells and debris[10]. The chronic phase of remodeling is hallmarked by a prolonged inflammatory response. It has been suggested that this chronic inflammatory response has detrimental effects on the heart due to chronic activation of macrophages and B and T lymphocytes[11]. These leukocytes secrete pro-inflammatory cytokines, growth factors, and immunoglobulins, thereby inducing adverse cardiac remodeling[11–13]. Clinical trials targeting the chronic pro-inflammatory response by immuno-adsorption showed beneficial effects on mortality in patients with DCM as clearance of all four immunoglobulin G (IgG) subclasses improved cardiac performance[14–18]. Conversely, general inhibition of the immune response by corticosteroids or intravenous immunoglobulin (IVIG) administration showed no effect on mortality in HF patients compared to conventional treatment[19–23]. These previous observations suggest a pathogenic role of a specific antibody-mediated immune response. In line with this hypothesis, deposits of antibodies (mainly IgG1 and IgG3) in the myocardium of HF patients have been observed. These antibodies, reacting to cardiac tissue, are bound to the failing myocardium where they induce complement activation and most likely, play an important role in HF progression[24–29]. In previous studies, circumstantial evidence has been provided for an antibody-mediated immune response in end-stage HF. However, the question remains whether this antibody-mediated immune response is associated with the severity of HF. In addition it remains to be elucidated whether this immune response is a generalized phenomenon, or that differences between sex or HF etiology exist.

Therefore, we investigated the presence of a potential antibody-mediated immune response in patients with end-stage HFrEF, including IHD and DCM. We investigated the presence and localization of different immunoglobulin subclasses and immune cells locally in the myocardium as well as in the circulation. In order to establish whether immunoglobulins are also detectable in the earliest phase of HFpEF, we measured immunoglobulin levels in patients with different stages of left ventricle diastolic dysfunction (LVDD).

MATERIAL AND METHODS

Patient population with end-stage heart failure

Patient's myocardial tissue was stored in the cardiac tissue biobank of the University Medical Centre Utrecht in compliance with the *Declaration of Helsinki*[30]. The study was approved by the local medical ethics committee (METC, reference number 12/387). Written informed consent for collection and biobanking of tissue samples and blood was obtained prior to transplantation or, in certain cases, approved by the ethics committee when obtaining informed consent was not possible due to death of the patient. Myocardial tissue from 10 IHD patients and 10 DCM patients was obtained from the explanted heart during heart transplantation (HTx; *Supplementary Table 1*). Patients carrying a left ventricular assist device (LVAD) prior to transplantation were not included. Three control hearts, two donor hearts not-used for transplantation and one heart obtained at autopsy, were used as reference. Fresh plasma samples of age and sex matched IHD (n=9) and DCM (n=7) patients prior to HTx (2018-2019) were collected and compared to fresh plasma samples of healthy controls without cardiovascular disease history (n=21).

Patient population with early left ventricular diastolic dysfunction

Plasma of 260 patients with different stages of diastolic dysfunction was collected in the HELPFul study. HELPFul is an ongoing single center, prospective observational study conducted at a cardiac diagnostic outpatient center in The Netherlands[31] (*Supplementary Table 2*). Eligible patients were persons aged 45 years or older referred by their general practitioner for evaluation of a cardiac cause of symptoms, e.g. chest discomfort, shortness of breath, palpitations. Patients who had a history of coronary intervention, cardiac (bypass) surgery, or with congenital heart disease were excluded. Written informed consent was obtained from all participants. The ethics committee approved the study (reference number NTR6016). Patients were categorized using the diagnostic algorithm presented at the ESC congress in Munich by the Heart Failure Association (2018). The algorithm is a scoring system to estimate the likelihood of HFpEF, which ranges from zero to six points based on minor or major abnormalities of echocardiographic diastolic function parameters (i.e. septal and lateral early diastolic mitral annular recoil velocity (e'), ratio of peak early (E) diastolic filling velocity to average e'(E/e' ratio), left atrial volume index (LAVI), left ventricular mass index (LVMI), tricuspid regurgitation velocity, relative wall thickness (RWT) and left ventricular wall thickness) and levels of natriuretic peptides. The algorithm is proposed to categorize patients into three groups: no HFpEF (HFpEF likelihood score 0-1), indeterminate for HFpEF (HFpEF likelihood score 2-4), and definite HFpEF (HFpEF likelihood score 5-6). However, since the algorithm score uses diastolic function criteria and natriuretic peptides, we interpreted it as a diastolic function score with no LVDD, indeterminate LVDD and definite LVDD respectively.

Myocardial tissue selection

Myocardial tissues were transversally sliced, thereby obtaining cross-sectional overviews of the diseased heart. Non-ischemic regions of a mid-ventricular heart slice of the left

ventricle were selected using Hematoxylin and Eosin (H&E) and Masson trichrome stained sections using light microscopy. These remote sections were defined as regions with little fibrosis and the absence of necrotic tissue. The epicardial layer and any adipose tissue, if present, were removed from the tissue in further analyses. Both paraffin embedded and cryo-sections were obtained from the myocardium.

Immunohistochemistry (IHC) for the detection of inflammatory cells

Tissue sections (4 µm) of formalin fixed-and paraffin embedded (FFPE) myocardium were stained with H&E and consecutive sections with markers for different immune cell types using immunohistochemistry. Sections were stained for T cells (CD3 DAKO, A0452, 1:100), B cells (CD20 Roche, 790-2531, undiluted), macrophages (CD68 Novocastra, NCL-CD68-KP1, 1:1600), and plasma cells (CD138 Serotec, MCA681A647, 1:500) using the Ventana automatic slide staining system. Detection of enzymatic activity was performed using diaminobenzidine (DAB). Histological sections were analyzed using semi-quantitative analysis. Immune cell infiltration was manually scored and classified into five phenotypes per immune cell type, ranging from 0-4 (0=no inflammation, complete absence of infiltrating cells, 1= mild inflammation, 0-5 immune cells present per field, 2=moderate inflammation, >5 immune cells diffusely present per field, 3= moderate/severe inflammation, clusters of immune cells present, 4=severe inflammation, excessive amount of infiltrating immune cells and clusters). Each histological section was assessed by randomly scoring five high power fields (magnification 400x) throughout the tissue section, which were averaged as a mean score per section. The scoring and classifications were determined by a certified pathologist and two independent observers blinded to section origin.

Immunofluorescence immunohistochemistry (IF) for the detection of immunoglobulins

Cryo-sections (8 µm) of human myocardium were incubated with FITC-labeled anti-IgG1 (Sigma, F0767, 1:30), FITC-labeled anti-IgG3 (Sigma, F4641, 1:15), and FITC-labeled anti-complement component 3 (C3c) (DAKO, F0201, 1:10). Sections of diseased kidney tissue (8 µm) served as positive control. Negative control stainings were included in which an antibody without fluorescent label was used. Slides were incubated with Sudan black (0.1%, Sigma-Aldrich, 4197-25-5) for 20 min to limit background lipofuscin fluorescence. To visualize the localization of the antibodies in the myocardium, images were taken using a Zeiss Axiovert 200M microscope.

Tissue lysates of myocardium

Cryopreserved myocardium was cut into 10 sections of 10 µm and collected in tubes containing micro-beads. Tissue extraction buffer (100mM Tris (pH 7.4); Roche, 10708976001), 150mM NaCl (Sigma Aldrich, S7653), 1mM EGTA (Sigma Aldrich, 03777), 1mM EDTA (Sigma Aldrich, E4884), 1% Triton X-100 (Sigma, T8787), and 0.5% sodium deoxycholate (Sigma, 30970) dissolved miliQ water) was added and the tissue was homogenized for 3x 35 sec using a bead shaker (Biospec). Constant agitation was maintained by rotating the lysates for 2 h at 4 ˚C. The samples were then centrifuged for 20 min (13,000 rpm at 4 ˚C). Next, the supernatant was collected, aliquoted, and stored at -80˚C.

Multiplex immunoassay

Levels of IgM and IgG subclasses (IgG1, IgG2, IgG3, IgG4) were measured in tissue lysates and fresh plasma samples using a Bio-Plex Pro™ Human Isotyping immunoassay 6-plex (Bio-Rad, 171A3100M) according to manufacturer's instructions. Plasma and tissue lysate immunoglobulin levels were calculated using internal standards.

IgG immunoprecipitation, gel electrophoresis and Western blot

Immunoprecipitation (IP) of IgG was performed according to manufacturer's protocol (Bio-rad, USA). In brief, protein G coated magnetic beads (SureBeads™ Protein G Magnetic Beads, Bio-Rad 161-4023, USA) were washed with PBS-T (PBS pH 7.4 and 0.1% Tween 20; EMD Millipore, 9005-64-5) and incubated with 1 µg of goat anti-human IgG antibody (EMD Millipore, AP112, 1:400) for 1h. IgG coupled beads were incubated o/n with 15 µg protein from tissue lysates diluted in PBS. Magnetic beads were washed with PBS and dissolved in 40 µL Laemmli Buffer and 1% Nu-Page sample reducing agent (Invitrogen, NP0004) and incubated for 10 min at 70 ˚C. The precipitate was collected and used for gel electrophoresis and Western blotting (WB). Total of 15 µg protein per sample was loaded on pre-casted Bolt 4-12% Tris-Plus Gels (Invitrogen, NW04120BOX) for 1h at 160V in MOPS SDS running buffer (Invitrogen, NP0001-02). Proteins were transferred to PVDF membranes (Millipore, IPVH00010) and incubated o/n with a primary antibody (mouse anti-human IgG; Novus, IG226, 1:400) and 1 h with a secondary HRPO polyclonal Rabbit anti-mouse IgG (Dako, P0260, 1:2000). For visualization, a chemiluminescent peroxidase substrate (Sigma, CPS1120) was used and images were quantified using Image Lab Software (Bio-Rad, Hercules, CA, USA, 5.1V).

Flow cytometry

Cryopreserved peripheral blood-derived mononuclear cells (PBMC), derived from five age and sex matched end-stage IHD patients and five matched end-stage DCM patients, were collected. PBMC were thawed and washed with RPMI (61870010, Gibco) supplemented with GlutaMax (room temperature) containing 25nM HEPES, 1% penicillin/streptomycin and 2% Fetal Bovine Serum (FBS) (10270-106, Gibco). PBMC were filtered over a 40 µm cell strainer (542040, Greiner bio-one). The single cell suspension was added to an antibody mixture containing different cell surface markers to identify B-cell subtypes as described before[32]. Cells were stained with a fixable viability dye (eBioscience, eFluor-506, 65-0866-14). Viable $CD19^{+}CD3^{-}$ B lymphocytes were selected for further gating of $C24^{-}CD38^{+}$ plasmablasts and $CD27^{-}$, IgG^{+},$CD24^{+}$,$CD38^{+}$ transitional/regulatory B cells using gating strategy as described by Meeuwsen *et al*, 2017[32]. All appropriate controls were included in the experiments, including isotype/subclass-matched primary antibody of irrelevant specificity. After flow cytometry, data was analyzed using Kaluza 1.5a software (Beckman Coulter).

Statistical analysis

Statistical analysis and data representation were performed using IBM SPSS statistics 21 and GraphPad Prism© (GraphPad Software Inc. version 7.02, CA, USA). Normal data distribution was tested and normally distributed data were analyzed using an unpaired t-test. Non-normally distributed data were compared using a Mann-Whitney test. Group comparison was performed by a one-way ANOVA or Kruskal-Wallis test, corrected for

multiple comparison testing. An UNIANOVA was used with age as covariate for the immunoglobulin analyses of the HELPFul cohort Data are presented as mean ± SEM, unless stated otherwise. Values of $p<0.05$ were considered significant.

RESULTS

High levels of IgG deposits in the myocardium of IHD patients with end-stage HF

To investigate the presence of IgG deposits in the HFrEF patient cohort, myocardial lysates of the LV were used for immunoprecipitation. IgG precipitation followed by WB analysis showed high levels of IgG in myocardial lysates of IHD patients as compared to controls and DCM patients (*Figure 1A*). On average, myocardial IHD IgG levels were 2.7-fold higher compared to DCM (p=0.01) and 1.9-fold higher compared to controls (*Figure 1B*, not significant).

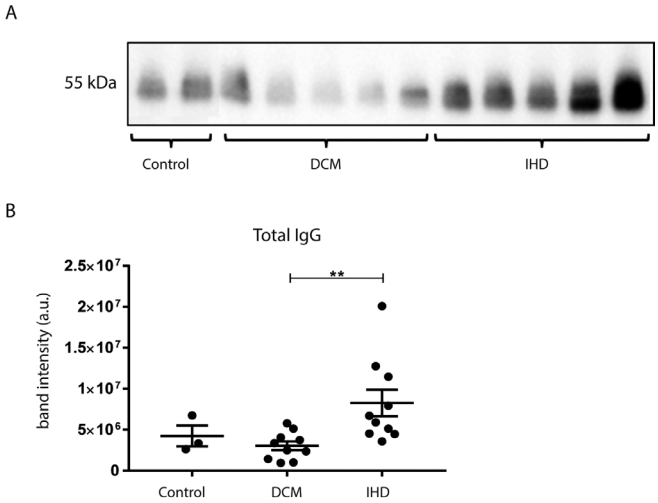

Figure 1. Deposits of IgG in myocardial lysates
Total IgG levels in myocardial lysates were determined using IP and subsequent WB (A). Quantification of band intensity is shown in (B), where total IgG levels were increased in IHD patients compared to DCM patients. *IP: immunoprecipitation; WB: Western Blot; DCM: dilated cardiomyopathy; IHD: ischemic heart disease. Myocardial lysates: control n=3, DCM n=10, IHD n=10. ** p<0.01*

Increased myocardial IgG3 and C3c deposits in end-stage HF

To visualize the localization of IgG deposits in the myocardium and to investigate IgG subclasses and potential co-binding with complement factor C3c, myocardial sections were fluorescently stained for IgG1, IgG3, and C3c (*Figure 2*). IgG1 showed no clear staining in both control and IHD/ DCM patients. IgG3 was visible in IHD and DCM patients, but barely in controls. C3c was clearly elevated in end-stage HF myocardium in both DCM and IHD patients, as compared to control myocardium. These findings indicate that IgG3 and C3c form a network of deposit throughout the myocardium of HF patients.

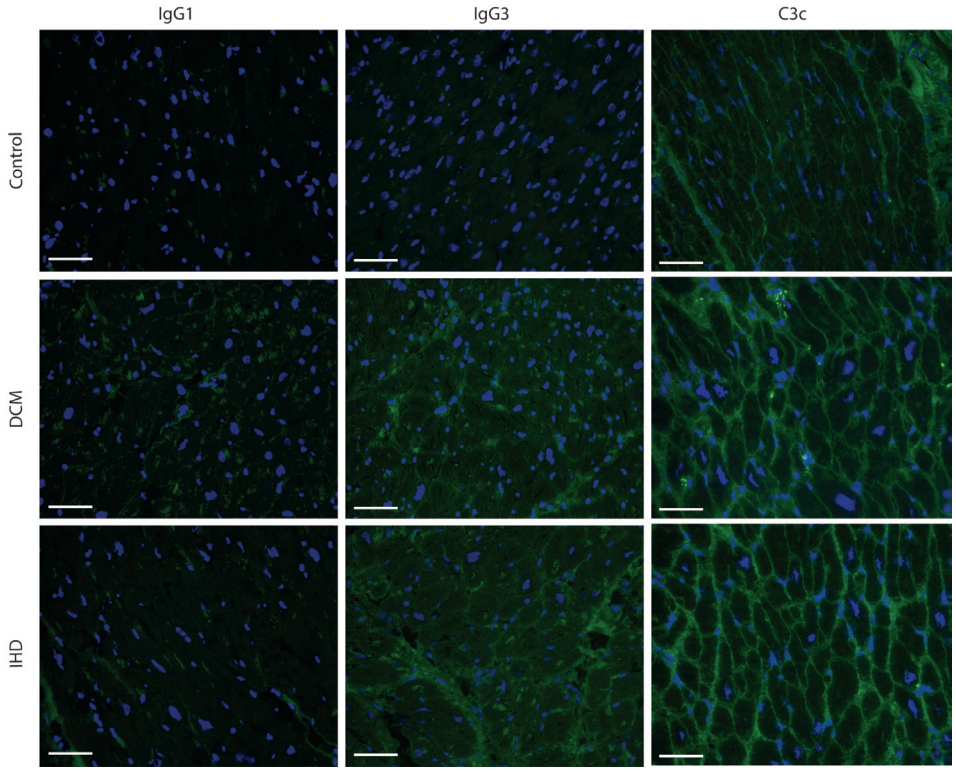

Figure 2. Deposition of IgG1, IgG3 and C3c in healthy and diseased myocardium
Cardiac tissue was fluorescently stained for IgG1, IgG3, and C3c (reflecting complement activation). DCM and IHD patients showed more deposits of IgG3 and C3c compared to control myocardium. IgG3 and C3c form an extensive network throughout the myocardium. *Line bar indicates 50 µm, magnification 20x, C3c: complement factor 3c; DCM: dilated cardiomyopathy; IHD: ischemic heart disease.*

Increased numbers of myocardial immune cells in end-stage HF

Next, we explored if immunoglobulin deposits are accompanied with increased numbers of immune cells in the myocardium, i.e. T cells (CD3), macrophages (CD68), B cells (CD20), and plasma cells (CD138) (*Figure 3*). Although traditionally thought to be linked only to acute MI, more infiltrating immune cells were observed in both the myocardium of DCM and IHD patients as compared to controls (*Figure 3A*). Semi-quantitative analysis (*Figure 3B-D*) showed a significantly higher number of CD3+ T cells, CD68+ macrophages, and CD20+ B cells in both IHD and DCM patients, as compared to controls (T cells; DCM p=0.02, IHD p=0.02, macrophages; DCM p=0.002, IHD p=0.014, and B cells; DCM p=0.02, IHD p=0.04). Only a few CD138+ plasma cells were observed in the myocardium and no difference in plasma cell numbers were observed between HF patients and controls (*Figure 3E*).

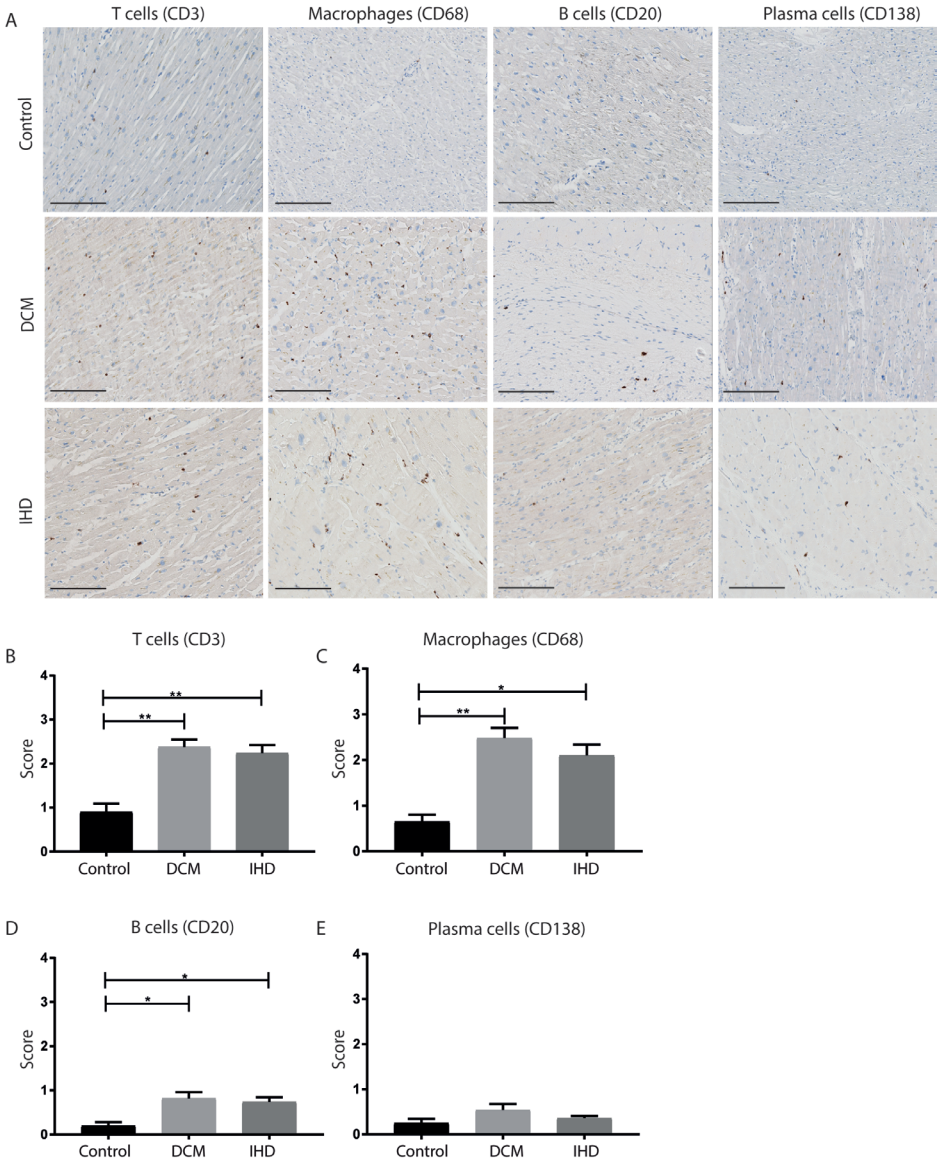

Figure 3. Infiltration of different immune cell types in the myocardium upon HF
Staining for T cells (CD3), macrophages (CD68), B cells (CD20), and plasma cells (CD138) in the myocardium of controls, DCM patients and IHD patients (A). The number of infiltrating immune cells was scored by semi-quantitative analysis. End-stage HF patients, both DCM and IHD, showed increased numbers of T cells (B), macrophages (C), and B cells (D). The number of plasma cells did not differ between the patient groups (E). *Line bar indicates 200 µm, magnification 10x*. *HF: heart failure*; *DCM: dilated cardiomyopathy; IHD: ischemic heart disease. Control n=3, DCM n=10, IHD n=10.* * p<0.05, ** p<0.01.

Increased IgG1 and IgG3 levels and decreased circulating regulatory B cells in HF patients

Flow cytometry was performed on PBMCs derived from 10 end-stage HFrEF patients (5 DCM and 5 IHD) and stained for different B-cell markers to identify B-cell subsets[32] (*Figure 4A*). Data is presented as the percentage of total B-cell count. The percentage of plasmablasts (CD24-CD38+) in HF patients did not differ significantly from controls (p=0.12) (*Figure 4B*). The percentage of anti-inflammatory transitional/regulatory B cells ($CD27^-$, IgG^+,$CD24^+$,$CD38^+$) was decreased in HF patients as compared to controls (p=0.03; *Figure 4C*). Next, levels of IgM, IgG1, IgG3, and IgG4 were measured in freshly collected plasma samples of HF patients (n=16) and compared to healthy controls (n=21) (*Figure 4D-G*). IgG1 and IgG3 levels were significantly increased in patients with end-stage HF compared to healthy controls (IgG1 p=0.0003, IgG3 p=0.0003) (*Figure 4E-F*). IgM and IgG4 levels did not differ significantly between healthy controls and HF patients (*Figure 4D-G,* IgM p=0.11, IgG4 p=0.56).

Circulating pro-inflammatory markers mostly pronounced in IHD patients

When the complete HF cohort was divided into DCM and IHD, the increase in percentage of plasmablasts was most pronounced in IHD. Furthermore, IHD patients showed significantly fewer transitional/regulatory B cells (CD38+CD24+) (p=0.04) as compared to healthy controls (*Figure 5A-B*). In addition, IgG1 and IgG3 levels were significantly increased in IHD patients (IgG1= $1.1{\cdot}10^7$ vs $4.5{\cdot}10^6$ ng/ml, p<0.0001, IgG3= $1.3{\cdot}10^6$ vs $6.0{\cdot}10^5$ ng/ml, p=0.002; *Figure 5D-E*). IgG3 levels were also increased in DCM patients compared to healthy controls (IgG3= $1.1{\cdot}10^6$ vs $6.0{\cdot}10^5$ ng/ml, p=0.02). IgG1 levels showed a trend towards an increase, but did not reach statistical significance (IgG1 p=0.09). IgM and IgG4 levels did not differ significantly between the groups (*Figure 5C, 5F*).

IgG1 and IgG3 levels as possible early markers of diastolic dysfunction in men

To assess if IgGs are possible markers for pre-stage heart failure, we measured IgG1 and IgG3 levels in a cohort of patients, ranging from only slightly elevated filling pressures to more severe LVDD (*Figure 6*). Another advantage of this unique cohort is that it was specifically designed to assess sex differences in the progression of LVDD to HFpEF. Interestingly, men with LVDD show significantly increased levels of IgG1 ($8.2{\cdot}10^6$ vs $5.2{\cdot}10^6$ ng/ml, p=0.05) and IgG3 ($1.0{\cdot}10^6$ vs $5.5{\cdot}10^5$ ng/ml, p<0.0001) in the circulation as compared to men without LVDD (*Figure 6A*), when corrected for age. In addition, men with increasing HFpEF likelihood score showed an increasing trend in IgG1 levels (p=0.084) and a significant increase in IgG3 (p=0.003) (*Figure 6B*). We did not find a correlation between IgG1 levels and C-reactive protein (CRP) or brain natriuretic peptide (BNP) levels, confirming that these increased antibody levels are not part of a general inflammatory reaction (*Supplementary Table 3-4*). Strikingly, women with LVDD or increasing HFpEF likelihood score showed no difference in IgG1 and IgG3 levels as compared to women without LVDD (*Supplementary Figure 1*), suggesting a male-specific effect of the immunoglobulin levels in diastolic function. The levels of IgG4 and IgM did not differ between the different groups or men and women (data not shown).

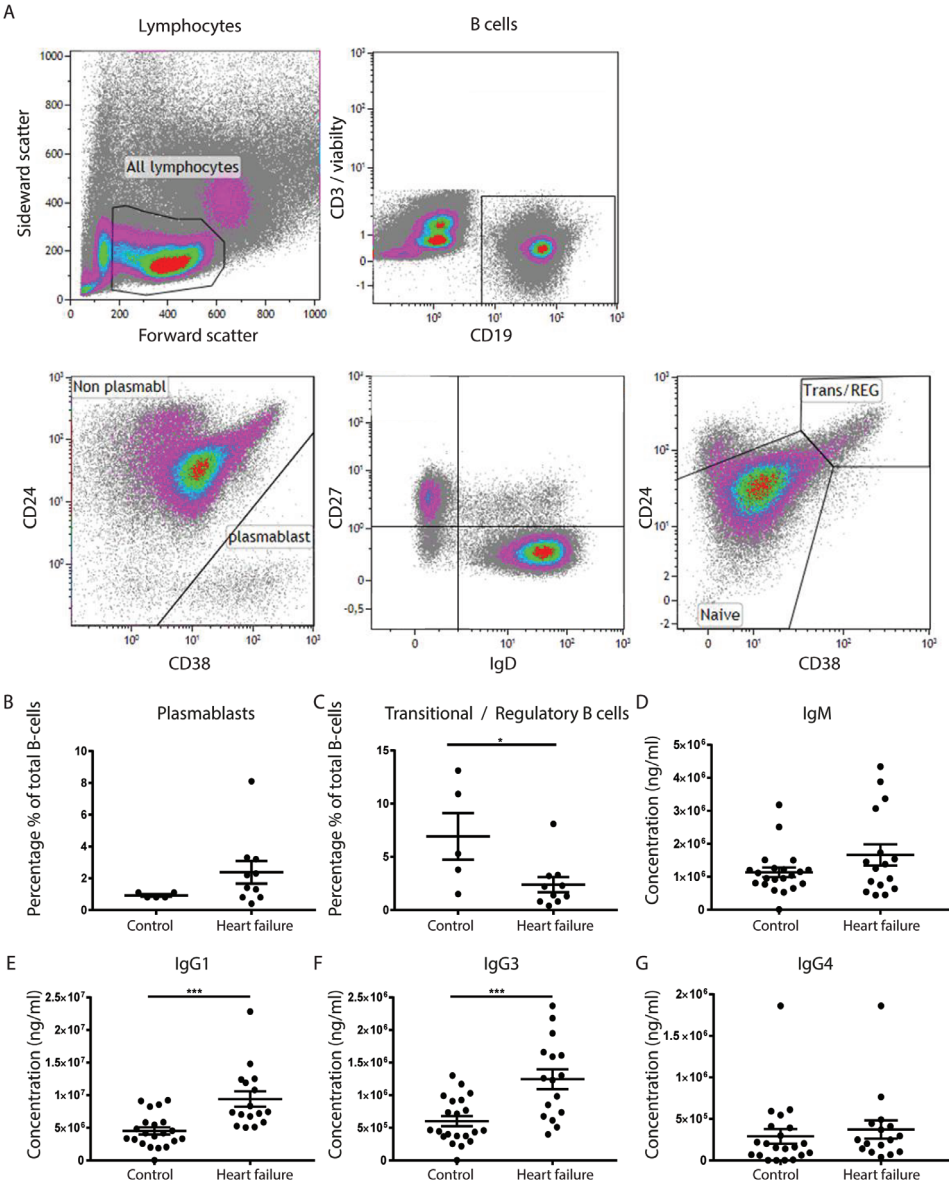

Figure 4. B-cell subsets and immunoglobulin levels in HF patients
Flow cytometry on cryopreserved PBMC was used to identify different B-cell subsets. (A) Gating strategy for selecting different B-cell subsets. HF patients showed an increase in the percentage of plasmablasts (CD24-CD38+), albeit not significant (B). Transitional/regulatory B cells, defined as CD38+CD24+, were decreased upon HF as compared to healthy controls (C). IgG1 and IgG3 levels were increased in HF patients (E-F). IgM and IgG4 levels did not differ between the groups (D-G). *PBMC: peripheral blood mononuclear cells; HF: heart failure. For PBMCs: control n=5, HF n=10. For plasma samples: control n=21, HF n=16. * p<0.05, *** p<0.001.*

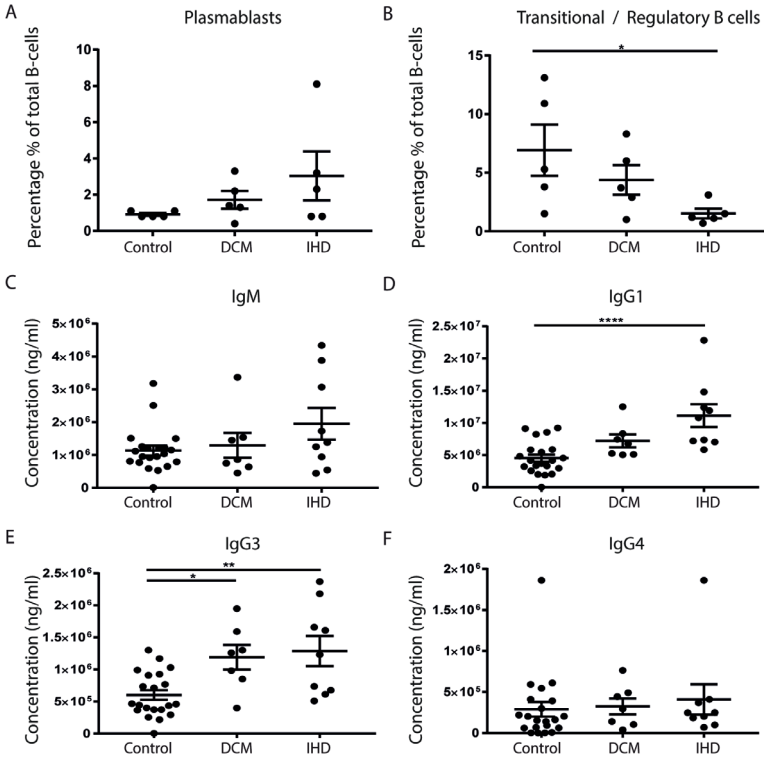

Figure 5. B-cell subsets and immunoglobulins levels in IHD and DCM patients
When HF patients were divided into subgroups of DCM and IHD etiology, IHD patients showed no significant increase in the percentage of plasmablasts (A) but did have a significantly lower number of transitional/ regulatory B cells (B). There were no differences in IgM and IgG4 levels (C-F). However, IgG1 and IgG3 levels were both significantly increased in IHD patients compared to healthy controls (D-E). *HF: heart failure; DCM: dilated cardiomyopathy; IHD: ischemic heart disease. For PBMCs: control n=5, DCM n=5, IHD n=5. For plasma samples: control n=21, DCM n=7, IHD n=9. * p<0.05, ** p<0.01, **** p<0.0001.*

DISCUSSION

The inflammatory response, in particular of T and B lymphocytes, plays an important role in adverse cardiac remodeling in HF[28]. Furthermore, deposits of IgG can be found in end-stage HFrEF myocardium[27,33,34], thereby suggesting the potential role of an (auto)-immune response. However, the role of an antibody-mediated immune response in HF progression is less clear and might differ between HF etiologies. To investigate whether immune cell infiltration and immunoglobulin levels are associated with HF type and disease stage, we studied the immune response in patient cohorts with LVDD and late stage HFrEF, in which we also took sex differences into account.

In line with previous studies, we found an increased amount of IgG deposits in the myocardium of end-stage HFrEF patients[25]. This increase was significant in IHD patients as compared to DCM. Upon MI, secreted immunoglobulins are able to recognize cardiac specific

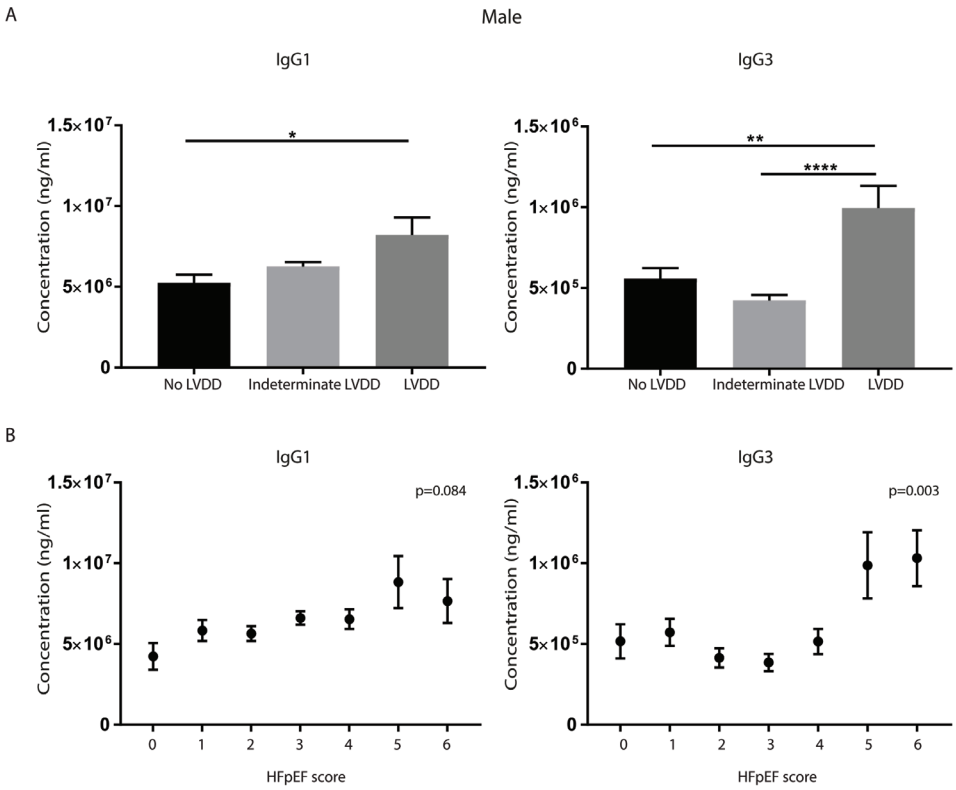

Figure 6. IgG1 and IgG3 in male patients with diastolic dysfunction
IgG1 and IgG3 levels measured in men with different stages of LVDD and levels (corrected for age). Men with LVDD showed significantly increased levels of IgG1 and IgG3 in plasma compared to those without LVDD (A). Increase in the HFpEF likelihood scores in men showed a significant trend towards higher IgG1 levels. Men with a HFpEF likelihood score of 5-6 had significantly higher levels of IgG3 as compared to lower HFpEF likelihood scores (B). *LVDD: left ventricular diastolic dysfunction; HF: heart failure. No LVDD (score 0-1) n=20, indeterminate LVDD (score 2-4) n=64, LVDD (score 5-6) n=5. * p<0.05, ** p<0.01, **** p<0.0001.*

antigens, which are suddenly exposed in great amounts upon ischemia-reperfusion injury. In this way, the immune system possibly becomes sensitized for these cardiac specific antigens. In DCM the myocardial damage is less massive and therefore the immune system might be exposed to less cardiac specific epitopes possibly resulting in a less severe antibody-mediated immune response. Binding of immunoglobulins to cardiac-specific antigens on cardiomyocytes can initiate the process of complement activation and antibody-dependent cellular cytotoxicity (ADCC), resulting in lysis of cardiomyocytes[29,35–37]. In DCM, it has also been described that immunoglobulins can directly bind to antigens expressed by the cardiomyocytes, and then crosslink to the Fc gamma receptor IIa on the cardiomyocyte, resulting in reduced calcium transients, cell shortening, and initiating cellular apoptosis[37–39]. The direct binding of immunoglobulins can also lead to complement-dependent cytotoxicity (CDC). Using immunofluorescence, we observed an increased deposition of both IgG3 and

C3c in the myocardial tissue of DCM and IHD patients. IgG3 has a high capability to activate components of the complement system[40], which probably explains the deposits of C3c in the failing hearts. Which specific epitopes are leading to this cascade is still under debate and is currently being investigated.

We also observed increased numbers of macrophages, T cells, and B cells in the myocardium of HF patients compared to control myocardium. Long-lived plasma cells migrate to the bone marrow and this perhaps explains why we did not find increased numbers of plasma cells in the myocardium[41]. The increased numbers of different inflammatory cell types in the chronically failing heart suggest a persistent low grade inflammatory response in these patients, which until now has mainly been described for the acute phases post-MI and in myocarditis patients[42,43].

Despite low numbers of plasma cells in the myocardium of HF patients, we did find an increase in the number of plasmablasts in the circulation, albeit fell short of being significant. Plasmablasts develop upon antigen-stimulation and secrete immunoglobulins[44]. The percentage of transitional/ regulatory B cells was decreased in IHD patients compared to healthy controls. This population is characterized by its immunosuppressive capacity, often mediated by IL-10[45]. A potential increase in the numbers of plasmablasts and a parallel/ concomitant decrease in the numbers of immunosuppressive transitional/regulatory B cells, as observed in our IHD patients, might promote the putative autoimmune response in HF.

Consistent with our observations in the myocardium, we observed increased levels of IgG1 and IgG3 in fresh plasma samples of patients with end-stage HF, most pronounced in IHD patients. Antibody responses to protein antigens are initiated via B cells and lead to the production of IgG1 and IgG3 specifically[46]. Therefore, increased levels of IgG1 and IgG3 in end-stage HF patients support the hypothesis of an immune response against cardiac proteins upon HF. Moreover, we showed that elevated levels of IgG1 and IgG3 are not limited to the end-stage HFrEF population only, but can also already be found in patients with LVDD, the potential early phase of HFpEF. We demonstrated a significant correlation between the severity of LVDD and circulating levels of IgG1 and IgG3 in men. Possibly already in this early stage, small myocardial damage has occurred that sensitizes the immune system, e.g. due to microvascular dysfunction or increased wall pressure. In patients with indeterminate LVDD, IgG1 levels were starting to increase, whereas IgG3 levels were increasing at a later stage. This might be the initial phase of myocardial damage, leading to the production of IgG1 primarily, which is later accompanied by IgG3. Interestingly, the levels of IgG1 and IgG3 did not differ in women with LVDD, despite the fact that in general women are more prone to develop autoimmune diseases as compared to men[47,48]. In addition, women are more prone to develop HFpEF, whereas men more often develop HFrEF[31]. What is causing this sex difference in the progression of LVDD to HFpEF is still poorly understood. Our findings suggest a possible difference in immune responses in early HF stages between men and women, thereby probably affecting HF progression.

Possible limitations of this study are the low number of control hearts and the low number of plasma samples used for the end-stage HFrEF patients. Generally, the numbers of HTx are very limited due to the lack of donor hearts. In case a donor heart is not used for transplantation, and a consent is available to donate the heart to science, the heart can be used as a control heart. Therefore, the availability of control hearts for research purposes

is extremely low. In addition, since only fresh plasma samples were collected upon HTx during the study, the numbers of plasma samples were limited to the numbers of HTx performed during the study. Another limitation is the low number of HFpEF patients in our cohort. HFpEF is a disorder with non-specific HF-like symptoms, which makes the diagnosis of the disease challenging. Diagnosis guidelines provided by the ESC are evolving over time, which leads to variation in the different classifications of HFpEF, and often also LVDD, upon each published guideline consensus paper. Nevertheless, the strong association of the immunoglobulins with the different groups of diastolic function in this heterogeneous population of patients at risk for LVDD underscores their potential pathogenic importance. In conclusion, our study demonstrates an increased inflammatory state in end-stage HFrEF, mostly pronounced in IHD, which includes increased amounts of IgG deposits, increased numbers of macrophages and lymphocytes in the myocardium, fewer transitional/regulatory B cells, and increased levels of circulating IgG1 and IgG3. In addition, increased IgG1 and IgG3 levels already occurs in patients with more severe LVDD, possibly before development of clinical symptoms and signs, i.e. HFpEF, as shown in men with definite diastolic dysfunction. These findings support a role of ongoing sex-dependent autoimmune responses, starting in an pre-clinical phase of LVDD, which has been shown to confer a higher risk of eventually developing HF. Therefore this chronic immune response most likely influences progression of the disease. Our data suggest, that increased levels of IgG1 and IgG3 may be useful biomarkers for early detection of HF progression, before clinical symptoms are present, which contribute to lifestyle and/or therapeutic intervention in an early stage of disease. Future studies should focus on the identification and validation of the epitopes recognized by autoantibodies. In addition, the role of IgG1 and IgG3 as potential markers for early HF recognition, screening, and progression should be explored.

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Conflicts of interest
None declared.

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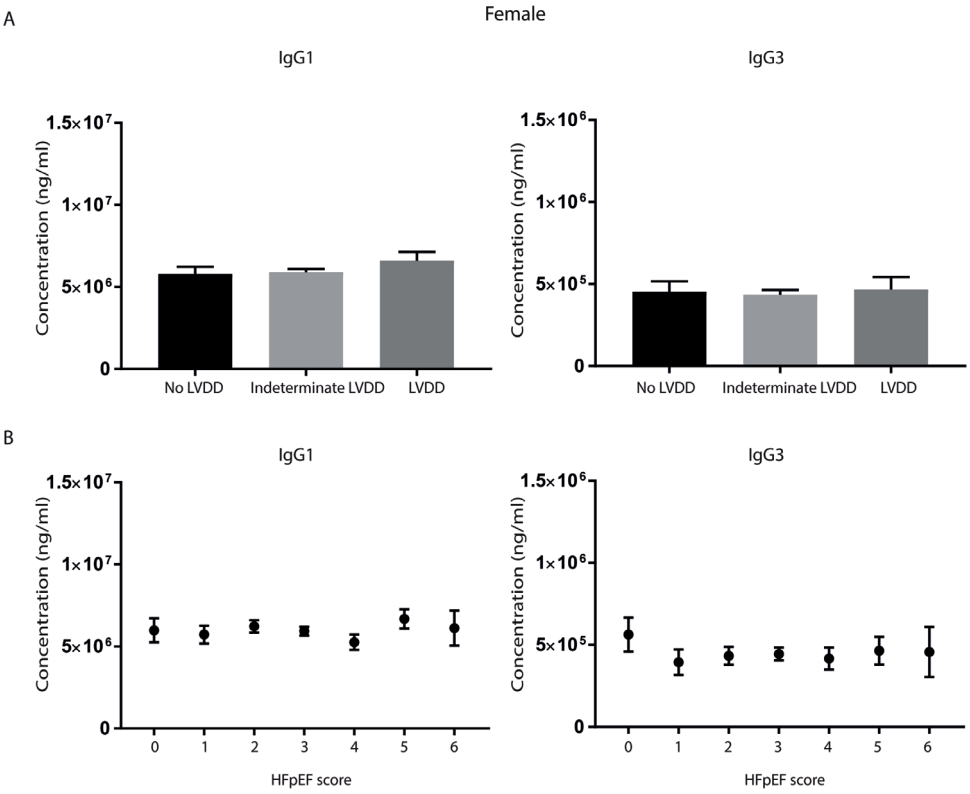
SUPPLEMENTARY FIGURES



Supplementary Figure 1. IgG1 and IgG3 in in female patients with diastolic dysfunction
IgG1 and IgG3 levels measured in women with different stages of LVDD (corrected for age). Women at different stages of LVDD showed no difference in IgG1 or IgG3 levels (A). There was no difference in IgG1 and IgG3 levels upon increasing HFpEF score (B). *LVDD: left ventricular diastolic dysfunction*; *HF: heart failure. No LVDD (score 0-1) n=27, indeterminate LVDD (score 2-4) n=124, LVDD (score 5-6) n=20*.

SUPPLEMENTARY TABLES

Supplementary Baseline Table 1. Clinical characteristics of end-stage HFrEF patients at time of heart transplantation

Clinical characteristics of end-stage HF patients at time of transplantation	
Overall n=20, mean ± SD	
Patient age (years)	57 ± 11.6
Sex (% men)	12 (60)
(% women)	8 (40)
Primary cardiac diagnosis	
Ischemic heart disease (IHD)	
Number	10
BMI	26.2 ± 2.18
BNP (pmol/L)	122.5 ± 111
CRP (mg/L)	7.76 ± 7.62
eGFRcys (ml/min/1.73 m^2)	53.7 ± 14.7
EF (%)	20.0 ± 5.27
LVMi (g/m^2)	109.7 ± 19.5
Dilated cardiomyopathy (DCM)	
Number	10
BMI	20.61 ± 3.56
BNP (pmol/L)	394.7 ± 311
CRP (mg/L)	9.71 ± 6.49
eGFRcys (ml/min/1.73 m^2)	78.0 ± 35.4
EF (%)	17.2 ± 6.18
LVMi (g/m^2)	132.5 ± 38.5

Supplementary Baseline Table 2. Clinical characteristics of patients with left ventricular diastolic dysfunction

Clinical characteristics of left ventricular diastolic dysfunction patients		
Overall n=260, mean ± SD		
	Men	**Women**
Number (%)	89 (34.2)	171 (65.8)
Patient age (years)	62.9 ± 9.40	63.5 ± 10.0
BMI	25.0 ± 14.3	27.4 ± 4.99
HsTnI (pg/ml)	4.00 ± 19.0	2.50 ± 10.5
BNP (pg/ml)	19.3 ± 66.9	21.9 ± 52.5
CRP (mg/L)	1.47 ± 14.9	1.74 ± 4.80
eGFRcys (ml/min/1.73 m^2)	80.1 ± 12.0	76.8 ± 14.9
EF (%)	67.7 ± 8.57	66.8 ± 8.99
LVMi (g/m^2)	80.2 ± 19.3	74.0 ± 18.5
E/e' ratio	8.80 ± 2.79	9.49 ± 2.99
E' septal (cm/s)	0.07 ± 0.02	0.07 ± 0.02
E' lateral (cm/s)	0.09 ± 0.02	0.08 ± 0.02
RWT	0.42 ± 0.09	0.42 ± 0.10
LAVi (ml/m^2)	24.2 ± 9.98	26.4 ± 13.3

Supplementary Baseline Table 2. Continued

Clinical characteristics of left ventricular diastolic dysfunction patients		
Primary cardiac diagnosis		
No LVDD *HFpEF likelihood score 0-1*	**Men**	**Women**
Number	20	27
Patient age (years)	57.2 ± 6.80	57.3 ± 7.30
BMI	27.0 ± 3.09	27.0 ± 4.24
HsTnI (pg/ml)	3.60 ± 26.3	2.10 ± 3.50
BNP (pg/ml)	12.8 ± 30.1	17.2 ± 13.6
CRP (mg/L)	1.57 ± 16.7	3.10 ± 4.81
eGFRcys (ml/min/1.73 m^2)	84.3 ± 140	80.0 ± 13.4
EF (%)	68.9 ± 7.99	65.5 ± 8.37
LVMi (g/m^2)	77.5 ± 170	61.8 ± 190
E/e' ratio	6.70 ± 2.02	8.00 ± 1.39
E' septal (cm/s)	0.08 ± 0.01	0.09 ± 0.01
E' lateral (cm/s)	0.11 ± 0.01	0.11 ± 0.02
RWT	0.38 ± 0.08	0.37 ± 0.06
LAVi (ml/m^2)	22.7 ± 4.55	21.5 ± 5.70
Indeterminate for LVDD *HFpEF likelihood score 2-4*	**Men**	**Women**
Number	64	124
Patient age (years)	63.4 ± 9.50	63.1 ± 8.50
BMI	24.9 ± 16.5	27.6 ± 5.31
HsTnI (pg/ml)	3.90 ± 16.5	2.50 ± 10.1
BNP (pg/ml)	20.3 ± 27.8	21.3 ± 22.5
CRP (mg/L)	1.47 ± 11.0	1.65 ± 4.81
eGFRcys (ml/min/1.73 m^2)	79.9 ± 10.7	77.0 ± 14.4
EF (%)	67.7 ± 7.82	67.2 ± 7.58
LVMi (g/m^2)	79.1 ± 19.2	74.5 ± 15.0
E/e' ratio	9.10 ± 1.89	9.75 ± 2.56
E' septal (cm/s)	0.07 ± 0.02	0.06 ± 0.02
E' lateral (cm/s)	0.08 ± 0.02	0.08 ± 0.02
RWT	0.44 ± 0.10	0.42 ± 0.10
LAVi (ml/m^2)	23.7 ± 7.04	25.3 ± 8.53
LVDD *HFpEF likelihood score 5-6*	**Men**	**Women**
Number	5	20
Patient age (years)	79.8 ± 5.40	73.9 ± 8.80
BMI	19.0 ± 10.6	26.2 ± 3.72
HsTnI (pg/ml)	15.5 ± 9.50	3.30 ± 17.2
BNP (pg/ml)	161 ± 185	82.3 ± 117
CRP (mg/L)	1.41 ± 37.2	1.36 ± 4.23
eGFRcys (ml/min/1.73 m^2)	65.1 ± 9.17	72.0 ± 16.9
EF (%)	60.1 ± 16.4	66.1 ± 14.9
LVMi (g/m^2)	104 ± 12.8	87.9 ± 27.8
E/e' ratio	11.1 ± 6.44	12.4 ± 4.36
E' septal (cm/s)	0.06 ± 0.01	0.05 ± 0.01
E' lateral (cm/s)	0.09 ± 0.02	0.07 ± 0.02
RWT	0.42 ± 0.06	0.49 ± 0.08
LAVi (ml/m^2)	51.0 ± 16.4	36.8 ± 26.0

Supplementary Table 3. Correlation of IgG1 and IgG3 with BNP level in men with LVDD

Coefficients[a,b]						
		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
Model		B	Std. Error	Beta		
1	(Constant)	-150,119	45,836		-3,275	0,002
	Age	2,528	0,663	0,376	3,811	0,000
	IgG1ng/ml	4,110E-06	0,000	0,138	1,393	0,167

a. Gender_E1_C2 = men
b. Dependent Variable: BNP (pg/mL)

Coefficients[a,b]						
		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
Model		B	Std. Error	Beta		
1	(Constant)	-148,641	39,785		-3,736	0,000
	IgG3ng/ml	8,028E-05	0,000	0,363	3,927	0,000
	Age	2,286	0,621	0,340	3,682	0,000

a. Gender_E1_C2 = men
b. Dependent Variable: BNP (pg/mL)

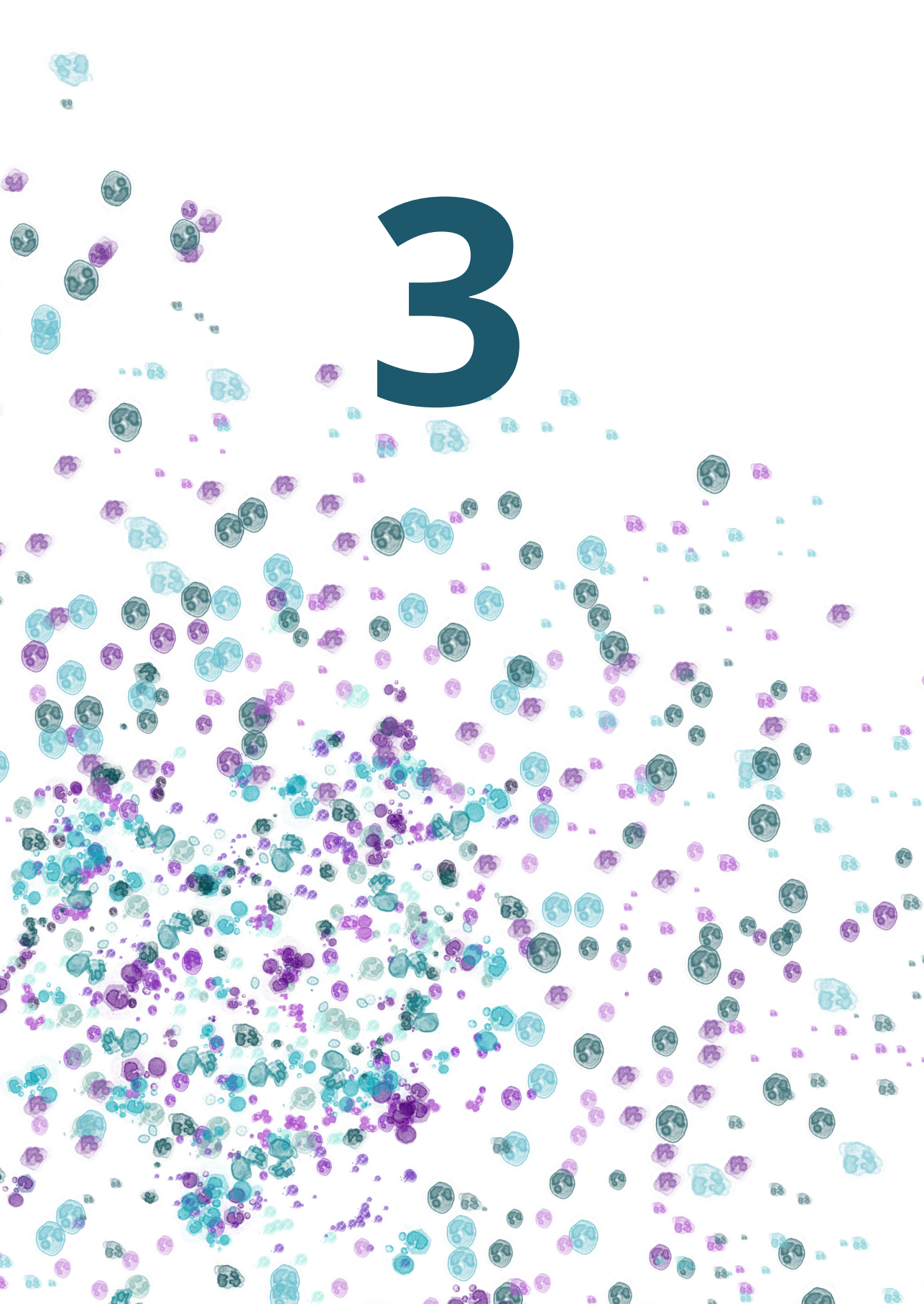
Supplementary Table 4. Correlation of IgG1 and IgG3 with CRP level in men with LVDD

Coefficients[a,b]						
		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
Model		B	Std. Error	Beta		
1	(Constant)	1,946	11,038		,176	,860
	Age	-,021	,160	-,014	-,130	,897
	IgG1ng/ml	8,757E-7	0,000	,132	1,232	,221

a. Gender_E1_C2 = men
b. Dependent Variable: CRP (mg/L)

Coefficients[a,b]						
		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
Model		B	Std. Error	Beta		
1	(Constant)	2,676	9,845		0,272	0,786
	Age	-0,068	0,154	-0,045	-0,442	0,660
	IgG3ng/ml	1,567E-05	0,000	0,319	3,098	0,003

a. Gender_E1_C2 = men
b. Dependent Variable: CRP (mg/L)


3

Myocardial immune cells and high levels of cardiac-related immunoglobulins potentially targeting cell-cell adhesion proteins in phospholamban p.Arg14del cardiomyopathy

In preparation

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ABSTRACT

Background

Dilated cardiomyopathy (DCM) is characterized by dilation and impaired contraction of the ventricles, leading to severe heart failure (HF). In 15% of DCM patients, a mutation in the phospholamban (PLN) protein has been identified, which can lead to severe myocardial damage and ultimately HF. However, it remains unclear how the mutation is leading to the progression of HF, since it can also be found in asymptomatic carriers. These findings indicate that next to the p.Arg14del mutation, an additional second 'hit' might be needed. The inflammatory response is an important component in the progression of many cardiomyopathies, including DCM. The role of inflammation in PLN carriers and the development of HF have, until now, not been studied before. We investigated whether myocardial immune cell infiltration and systemic immune activation could be observed in PLN patients with end-stage HF.

Methods and results

In PLN patients with end-stage HF (n=10), increased numbers of infiltrating myocardial immune cells and IgG deposits were found. The infiltration of T lymphocytes was already observed in the myocardium of PLN carriers without HF (n=4), who died from sudden cardiac death. Moreover, high levels of circulating IgG1 and IgG4 were found in PLN patients with end-stage HF, which upon isolation were shown to bind to induced pluripotent stem cell-derived cardiomyocytes. In order to establish which cardiac proteins are targeted by the produced IgGs, an epitope discovery screen was performed, which revealed that cell adhesion proteins may be potential targets.

Conclusion

High numbers of myocardial immune cells, IgG deposits and circulating IgG1 and IgG4 in PLN patients, suggest a role for inflammation including an antibody-mediated immune response in disease progression. Potential targets of this autoimmune response are cell adhesion proteins, which might contribute to the development of arrhythmias. Further identification of the target epitopes might lead to new diagnostic tools or therapeutic strategies for PLN-induced cardiomyopathy.

INTRODUCTION

Cardiomyopathy is a myocardial disorder in which the heart muscle is structurally and functionally abnormal, including a wide variety of different functional phenotypes[1–3]. A large proportion of cardiomyopathies are caused by mutations in genes coding for cardiac proteins, also referred as inherited cardiomyopathies[4]. These mutations are associated with the development of arrhythmogenic cardiomyopathy (ACM), hypertrophic cardiomyopathy (HCM), and dilated cardiomyopathy (DCM)[1,4]. One of the genes implicated in DCM and ACM encodes phospholamban (PLN)[5,6]. PLN is an important regulator of calcium (Ca^{2+}) homeostasis by regulating Ca^{2+} re-uptake to the sarcoplasmic reticulum (SR) by SR Ca^{2+}-ATPase (SERCA) in cardiomyocytes[7]. The pathogenic p.Arg14del mutation in the PLN gene is identified in 10-15% of the DCM patients and in 12% of ACM patients in The Netherlands[8]. These PLN patients show signs of ventricular arrhythmias, PLN protein aggregates, aggresomes, myocardial fibrosis, fibro-fatty replacement and ultimately heart failure (HF)[7,9]. However, it remains unclear how exactly the PLN mutation leads to HF, since the p.Arg14del mutation can also be found in asymptomatic carriers[7]. A potential second hit might be needed in PLN carriers to induce HF progression.

One of the hallmarks of cardiomyopathies, like DCM and ACM, is the inflammatory response[10–12]. Inflammation is an important component in the progression of many cardiovascular diseases because it is a natural response to injury[13]. However, it is also often the key driver of adverse cardiac remodeling, leading to increased cardiac damage[14]. There is growing evidence for potential autoimmune responses in multiple cardiomyopathies, such as DCM[12,15]. B cells and the production of immunoglobulins have already been proposed as crucial players in the progression of HF[12,16,17]. Cardiac-specific antibodies can be found in a large proportion of HF patients and most likely form deposits in the failing myocardium, where they might add to further deterioration of myocardial function[18,19].

Surprisingly, the role of inflammation in PLN carriers and the development of HF have, until now, not been studied before. To investigate whether inflammation is present in p.Arg14del carriers with HF, we studied the levels of circulating immunoglobulins and the presence of immune cell infiltrates in the myocardium. Moreover, we aimed to get some insights in the binding of immunoglobulins to the heart, by creating an *in vitro* cell model using iPS-derived cardiomyocytes and isolated PLN-derived IgG. Finally, we performed an epitope discovery screen, including 26,000 cardiovascular peptides, in order to identify potential cardiovascular target epitopes.

MATERIAL AND METHODS

Myocardial tissues of patients with phospholamban p.Arg14del mutation

Patient-derived myocardial tissue from explanted hearts was obtained from the cardiac tissue biobank of the University Medical Centre Utrecht. The collection of the material was approved by the local scientific advisory board of the biobank (protocol no. 12/387) and was in compliance with the *Declaration of Helsinki*[20]. Written informed consent for biobanking of

tissue samples was obtained prior to cardiac transplantation. Autopsy material was obtained from the archives of the Pathology departments of the University Medical Center Groningen and the Erasmus University Medical Center in Rotterdam. Myocardial tissue was included of patients who carried the PLN p.Arg14del mutation and either underwent heart transplantation (HTx, n=10) or died from sudden cardiac death without any history of HF (n=4). Additionally, three control hearts were included, two donor hearts that were not used for transplantation, and one heart that was obtained at autopsy.

Plasma samples of patients with phospholamban p.Arg14del mutation

Plasma of patients carrying the p.Arg14del (n=23) was collected and approved by the local medical ethics committee (METC, reference number 12/387). Written informed consent for collecting and biobanking of the samples was obtained. Additionally, fresh plasma samples of healthy controls (n=21) without cardiovascular disease history were used as controls.

Myocardial tissue selection

Myocardial tissue was transversally sliced, thereby obtaining cross sectional overview of the diseased heart. For the end-stage PLN hearts, four different regions of the heart were selected (*Figure 1*), including left ventricle (LV) with fibro-fatty replacement, least affected remote part of the LV, septum and the right ventricle (RV). Regions were selected using hematoxylin and eosin (H&E), and Masson Trichrome stained sections. Both paraffin embedded and cryo-sections were obtained from the myocardium. For the sudden death PLN hearts, paraffin embedded sections of the LV were used.

Immunohistochemistry (IHC) for the detection of inflammatory cells, autophagy and complement deposition

Tissue sections (4 µm) of formalin fixed-and paraffin embedded myocardium were stained with H&E and consecutive sections with markers for different immune cell types, an autophagy marker (P62, Santa Cruz, sc-28359, 1:100), and a complement activation marker (C4d Cellmarque, 404A-16, 1:80). Sections were stained for T cells (CD3 DAKO, A0452, 1:100), B cells (CD20 Roche, 790-2531, undiluted), macrophages (CD68 Novocastra, NCL-CD68-KP1, 1:1600), and plasma cells (CD138 Serotec, MCA681A647, 1:500) using the Ventana automatic slide staining system. Detection of enzymatic activity was performed using diaminobenzidine (DAB). Histological sections were analyzed using semi-quantitative analysis, as described previously[21]. Immune cell infiltration was manually scored and classified into five categories per immune cell type, ranging from 0-4 (0=no inflammation, complete absence of infiltrating cells, 1= mild inflammation, 0-5 immune cells present per high power field, 2=moderate inflammation, >5 immune cells diffusely present per field, 3= moderate/severe inflammation, clusters of immune cells present, 4=severe inflammation, excessive amount of infiltrating immune cells including clusters). Each histological section was assessed by randomly scoring five high power fields (magnification 400x) throughout the tissue section, which were averaged as a mean score per section. The scoring and classifications were determined by a certified pathologist and two independent observers blinded to section origin.

Tissue lysates of myocardium

Cryopreserved myocardium of the four selected regions was cut into 10 sections of 10 µm and collected in Eppendorf tubes containing micro-beads, as described before[21]. Tissue extraction buffer (100 mM Tris (pH 7.4); Roche, 10708976001), 150 mM NaCl (Sigma Aldrich, S7653), 1mM EGTA (Sigma Aldrich, 03777), 1 mM EDTA (Sigma Aldrich, E4884), 1% Triton X-100 (Sigma, T8787), and 0.5% sodium deoxycholate (Sigma, 30970) dissolved miliQ water) was added and the tissue was homogenized for 3x 35 sec using a bead shaker (Biospec). Constant agitation was maintained by rotating the lysates for 2 h at 4 ˚C. The samples were centrifuged for 20 min (13,000 rpm at 4 ˚C). Next, the supernatant was collected, aliquoted, and stored at -80˚C. Protein levels in tissue lysates were measured using micro bicinchoninic acid assay (BCA) (ThermoFisher, Waltham, MA), according to manufacturer's instructions using a Microplate Reader Benchmark (Bio-Rad, Hercules, CA, measured at 560 nm).

Multiplex immunoassay

Levels of IgM and IgG subclasses (IgG1, IgG2, IgG3, IgG4) were measured in tissue lysates (1:100) and plasma (1:40.000) using a Bio-Plex Pro™ Human Isotyping immunoassay 6-plex (Bio-Rad, 171A3100M) according to manufacturer's instructions. Plasma and tissue lysate immunoglobulin levels were calculated using internal standards, included in the immunoassay.

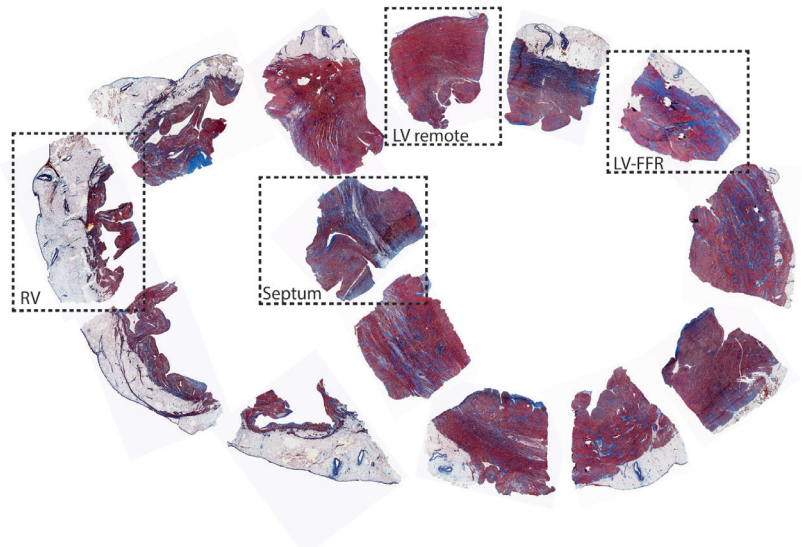

Figure 1. Explanted end-stage PLN heart and selection of regions
Different regions of the explanted PLN heart were selected for IHC and used for making tissue lysates. Four regions were selected, marked by the black squares, including 1) LV with typical fibro-fatty replacement, 2) least affected part of the LV, 3) part of the septum, 4) part of the RV. A total of 10 end-stage PLN hearts were included.
PLN: phospholamban, LV: left ventricle, RV: right ventricle, FFR: fibro-fatty replacement.

IgG immunoprecipitation, gel electrophoresis and Western blotting

Immunoprecipitation (IP) of IgG was performed according to manufacturer's protocol (Bio-Rad). In brief, protein G coated magnetic beads (SureBeads™ Protein G Magnetic Beads, Bio-Rad 161-4023) were washed with PBS-T (PBS pH 7.4 and 0.1% Tween 20; EMD Millipore, 9005-64-5) and incubated for 1 h with 1 µg of goat anti-human IgG antibody (EMD Millipore, AP112, 1:400). IgG coupled beads were incubated o/n with 15 µg protein from tissue lysates diluted in PBS. Magnetic beads were washed with PBS and dissolved in 40 µL Laemmli Buffer and 1% Nu-Page sample reducing agent (Invitrogen, NP0004) and incubated for 10 min at 70 ˚C. The precipitate was collected and used for gel electrophoresis and Western blotting (WB). A total of 15 µg protein per sample was loaded on pre-casted Bolt 4-12% Tris-Plus Gels (Invitrogen, NW04120BOX) for 1 h at 160V in MOPS SDS running buffer (Invitrogen, NP0001-02). Proteins were transferred to PVDF membranes (Millipore, IPVH00010), which were incubated o/n with a primary antibody (mouse anti-human IgG; Novus, IG226, 1:400) and 1h with a HRPO polyclonal rabbit anti-mouse IgG antibody (Dako, P0260, 1:2000). For visualization, a chemiluminescent peroxidase substrate (Sigma, CPS1120) was used and images were quantified using Image Lab Software (Bio-Rad, Hercules, CA, USA, 5.1V).

IgG isolation from plasma samples

To obtain purified IgG from patient and healthy control plasma samples, affinity chromatography was performed using HiTrap Protein G High Performance (Sigma Aldrich, GE17-0405-01). A protein G column was attached to the ÄKTA Fast Protein Liquid Chromatography (GE Healthcare) and isolation was performed utilizing 0.1 M glycine buffer (pH 2.7; Sigma Aldrich, G8898) for elution, 0.1 M Tris buffer (pH 9.0, Sigma-Aldrich, T6687) for neutralization, and 1 M HBS buffer (pH 7.4) for equilibration. After isolation, fractions containing IgG were pooled and dialyzed for 4 h at room temp. Dialysis was performed, using Slide-A-Lyzer Dialysis Cassettes (10K MWCO, 3 ml, ThermoFisher Scientific, 66380), according to the manufacturer's protocol, changing PBS buffer (1 M, 2 liters) each hour. Purity of isolated IgG was assessed by gel electrophoresis, followed by Coomassie Blue staining (SimplyBlue Safestain, ThermoFisher Scientific, LC6060) according to manufacturer's protocol.

IgG Biotin labeling

The total amount of IgG protein after isolation was determined by using micro-BCA protein assay according to manufacturer's protocol (sample dilution 1:50). Biotin labeling was performed according to the included protocol of EZ-Link NHS-PEG4-Biotin (No-Weigh Format, 8x2 mg, 21329). After labelling at room temp for 30 min, Zeba Spin Desalting Columns (7K MWCO, 5 mL, ThermoFisher Scientific, 89891) were used according to the manufacturer's protocol to remove excess biotin labelling. Samples were stored at 4°C until further use.

Culturing of iPSC-derived cardiomyocytes

Human fibroblast-derived pluripotent stem cells (iPSC) were derived from a skin biopsy of a PLN patient carrying the p.Arg14del mutation, provided by the department of Cell Biology of the University Medical Center Utrecht. iPSC were cultured in E8 medium (GibcoTM,

A1517001) on 1:400 growth-factor-reduced Matrigel. Cells were passaged when reaching 80% of confluency using EDTA (Invitrogen, 15575020) every 4–5 days, and medium was replaced every day. To obtain iPSC-derived cardiomyocytes (iPSC-CM), cells were grown until they reached 80% of confluency, after which medium was replaced by heparin medium (DMEM/F12 (Gibco, 31331028) supplemented with chemically defined lipid concentrate (Gibco, 11905031), 213 µg/ml L-ascorbic acid 2-phosphate (Sigma Aldrich, A8960), 1.5 IU/ml heparin (LEO pharma BV) and 1% penicillin/streptomycin (GibcoTM, 15150122). At day 0, medium was supplemented with 3-6 µM CHIR99021 (Selleckchem, S2924), which was replaced by medium containing 2 µM Wnt-C59 (Selleckchem, S7037) after 48 h. Medium was changed every other day until spontaneously contracting cells were observed around day 7. To purify iPSC-CM, medium was replaced with RPMI 1640 L-glutamine without glucose (Gibco, 11879020) and with sodium dl-lactate (Sigma Alderich, L1375) for 4 days. On day 25, cells were re-plated into 24-wells plates with coverslips (200,000 cells per well) and cultured for 5 days, after which cells were fixed using 4% paraformaldehyde (PFA).

IgG binding assay with iPSC-derived cardiomyocytes

To assess binding of IgG to cardiomyocytes, biotinylated IgG samples (200 µg IgG per 24-well) were incubated o/n with fixated iPSC-derived cardiomyocytes. After o/n incubation, washing with 1 M PBS was performed 3x 5 min to remove unbound IgG. Cells were incubated with Streptavidin Alexa Fluor 488 (Life Technologies, S32354, 1:1,000) for 1 h at room temp. Cell nuclei were stained using Hoechst (Thermo, Scientific, 33342, 1:10,000). All appropriate staining controls were included. Coverslips were mounted with Fluoromount (SouthernBiotech, 0100-01) and microscopic images were taken using confocal microscopy.

Epitope discovery screen

To identify the target epitopes to which the bound IgG is directed, an epitope discovery screen was performed. The microarray (PEPperPRINT) was coated with 176 antigens associated with cardiovascular diseases (*Supplementary Table 1*). Linked antigen sequences were translated into 26,364 15-mer peptides in duplicates, with a peptide-peptide overlap of 10 amino acids. Control peptides included were Polio (KEVPALTAVETGAT), Flag (DYKDDDDKGG), and human influenza hemagglutinin (HA) (YPYDVPDYAG). After 15 min incubation with washing buffer (PBS, pH 7.4 with 0.05% Tween 20) and 30 min in blocking buffer (Rockland blocking buffer MB-070), pooled human plasma samples (1:250), including healthy controls and end-stage HF patients, were incubated for 16 h at 4ºC. After washing, the microarray was incubated with a goat anti-human IgG (Fc) DyLight680 (1:5,000) antibody. As internal control, mouse monoclonal anti-HA (12CA5) DyLight800 (1:2,000) was used to stain HA control peptides. Spot intensity was measured using Odyssey Imaging System (LI-COR Bio-sciences) and quantified with a PepSlide® Analyzer. After the initial discovery screen, the selection of epitopes was narrowed down to 290 peptides. These peptides showed the highest mean fluorescent intensity (MFI) after subtraction using MFI values of healthy controls and were included in the next screening. In this next screening, individual patient plasma samples were used, including PLN carriers with and without HF and unaffected family members of PLN carriers.

Statistical analysis

Statistical analysis and data representation were performed using IBM SPSS statistics 21 and GraphPad Prism© (GraphPad Software Inc. version 7.02, San Diego, CA). Normal data distribution was tested using the Kolmogorov-Smirnov test. Group comparison was performed by a one-way ANOVA or Kruskal-Wallis test, corrected for multiple comparison testing. Correlation was assessed using Pearson correlation coefficient. Gene ontology enrichment analysis was performed and visualized using clusterProfiler (v3.10.0) in R-studio (v3.5.1). Data is presented as mean ± SEM, unless stated otherwise. Values of $p<0.05$ were considered significant.

RESULTS

High numbers of infiltrating immune cells are present in the myocardium of end-stage PLN hearts

To explore whether immune cells are present in the failing PLN heart, cardiac tissue was stained for immune cell types, including T cells (CD3), macrophages (CD68), B cells (CD20), and plasma cells (CD138). Four different regions of the myocardium were selected including LV fibro-fatty replacement, LV remote (with the least fibrosis), septum and RV. These regions of the heart showed distinct features of fibro-fatty replacement, hypertrophic cardiomyocytes, and moderate to severe interstitial fibrosis (*Figure 1*). Microscopic images of the myocardium showed increased numbers of immune cells in the end-stage PLN heart as compared to control myocardium (*Figure 2A*). Semi-quantitative analysis showed a significant increase in the numbers of T cells, macrophages, B cells, and plasma cells compared to control myocardium (*Figure 2B*, for T cells p=0.01; macrophages p=0.0004; B cells p=0.0005; and plasma cells p=0.01). The increased number of infiltrating immune cells was visible in each region of the PLN heart (*Supplementary Figure 1*), thus not limited to most affected myocardial tissue. Moreover, significantly higher numbers of myocardial T cells were present in an earlier phase of the disease, as shown in PLN hearts of patients who died of sudden cardiac arrest without pre-existing HF compared to healthy controls (p=0.01) (*Supplementary Figure 2*). The numbers of macrophages, B cells, and plasma cells were not statically different compared to controls.

One of the characteristics observed in the myocardium of PLN patients is the presence of PLN protein aggregates that form aggresomes in the sarcolemma of cardiomyocytes[9]. To assess whether immune cells colocalize with these aggresomes, a P62 staining was performed (*Figure 2C*). Despite the fact that some T cells were in close proximity to P62-positive cells, T cells were in general diffusely present throughout the myocardium and not limited to areas with P62 positive aggresomes in cardiomyocytes. Finally, a C4d staining was performed to assess whether end-stage PLN hearts show signs of complement activation in the myocardium, which can also induce immune-mediated damage and cell death[22] (*Figure 2D*). End-stage PLN hearts revealed some C4d deposits, thereby suggesting that complement activation is involved.

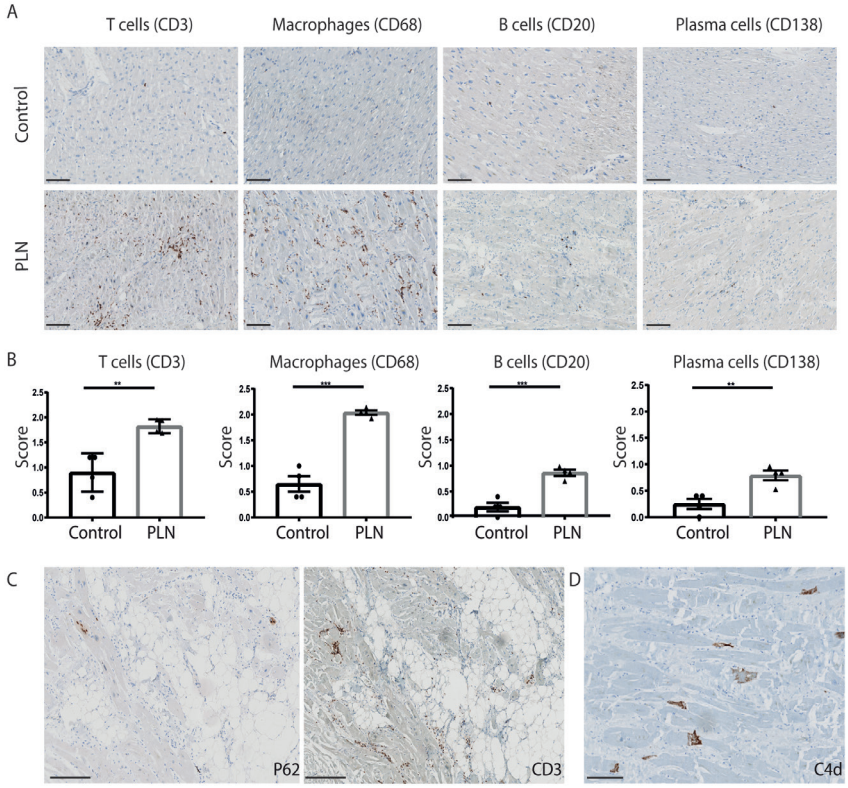

Figure 2. Immune cell infiltration, autophagy, and complement activation in end-stage PLN hearts
T cells (CD3), macrophages (CD68), B cells (CD20) and plasma cells (CD138) in the myocardium of controls, and PLN patients (A). The number of infiltrating immune cells was scored by semi-quantitative analysis. End-stage PLN patients showed significantly increased numbers of myocardial T cells, macrophages, B cells, and plasma cells (B). To assess if immune cells colocalize with PLN protein aggresomes, a P62 staining was performed (C). Immune cells were diffusely present throughout the myocardium and not limited to regions with P62 positive cells. A C4d staining showed signs of complement activation in end-stage PLN hearts (D). *Line bar indicates 200 µm for immune cells and C4d staining or 300 µm for P62 and CD3 staining, magnification 10x. PLN: phospholamban, C4d: complement component 4d. Control n=3, PLN n=10. ** p<0.01, *** p<0.001.*

IgG deposits are present in end-stage PLN hearts and are correlated with B and T cell infiltration in the myocardium

To investigate whether antibody deposits are also present in the failing heart, myocardial lysates were used for IgG immunoprecipitation. WB analysis of the IgG precipitate showed increased levels of IgG deposits in all four regions of the end-stage PLN heart compared to control myocardium (*Figure 3A*). Quantification of IgG band intensities showed a significantly increased level of IgG deposits in PLN hearts compared to controls (*Figure 3B*, p=0.002). No differences were observed in IgG deposits between the four different regions of the PLN heart (*Figure 3C*).

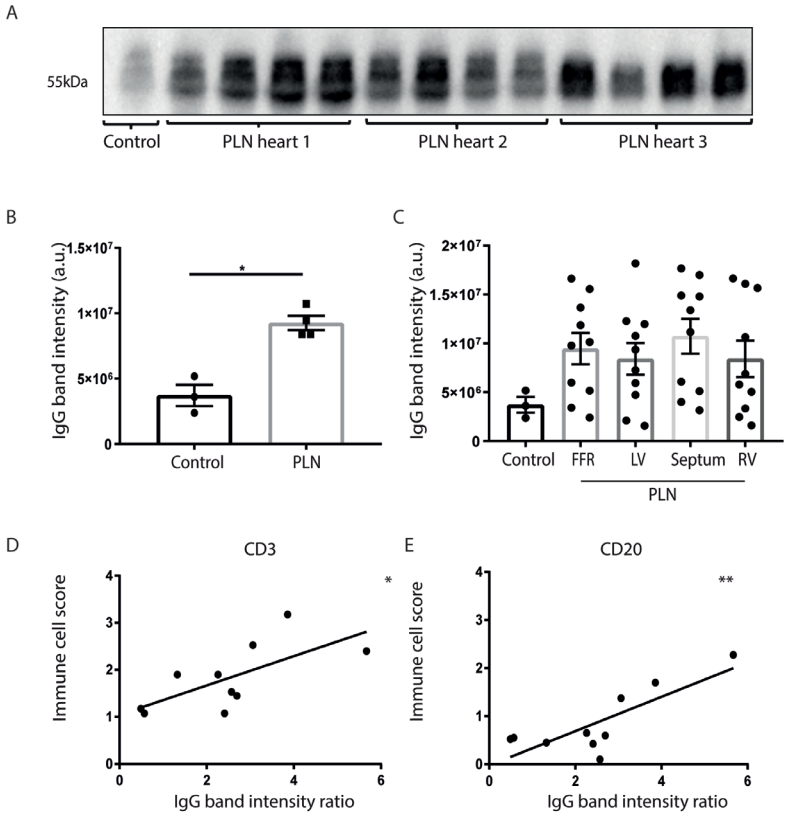

Figure 3. IgG deposits in the myocardium and correlation with infiltrating immune cell subsets
Total IgG levels in myocardial lysates of the four selected regions of the PLN heart were determined using IP and subsequent WB (A). Quantification of band intensity is shown in (B), where end-stage PLN hearts showed a significant increase in the levels of IgG deposits in the myocardium compared to controls. No differences in IgG deposits were observed in the four regions within the PLN heart (C). IgG deposits were significantly correlated with the number of infiltrating T or B cells in the myocardium (D-E). *IP: immunoprecipitation, WB: Western Blot, PLN: phospholamban. Control n=3, PLN n=10 per region. * p<0.05, ** p<0.01.*

To assess if there is a correlation between the number of immune cells and IgG deposition in the myocardium, a Pearson correlation coefficient analysis was performed (*Figure 3D-E*). End-stage PLN hearts showed a significant correlation between the number of infiltrating B or T cells and IgG deposits in the myocardium (CD3+T cells; r=0.68 p=0.03, CD20+B cells; r=0.80 p=0.005). No significant correlation was found for the number of infiltrating macrophages or plasma cells and IgG deposits (data not shown).

Circulating IgG1 and IgG4 levels are increased in end-stage PLN patients
In addition to IgG deposits in the heart, the levels of circulating immunoglobulins were measured in plasma of PLN patients and healthy controls (*Figure 4*). PLN patients showed significantly increased levels of IgG1 ($4.5x10^6$ ng/ml vs $8.3x10^6$ ng/ml, p=0.0007) and IgG4

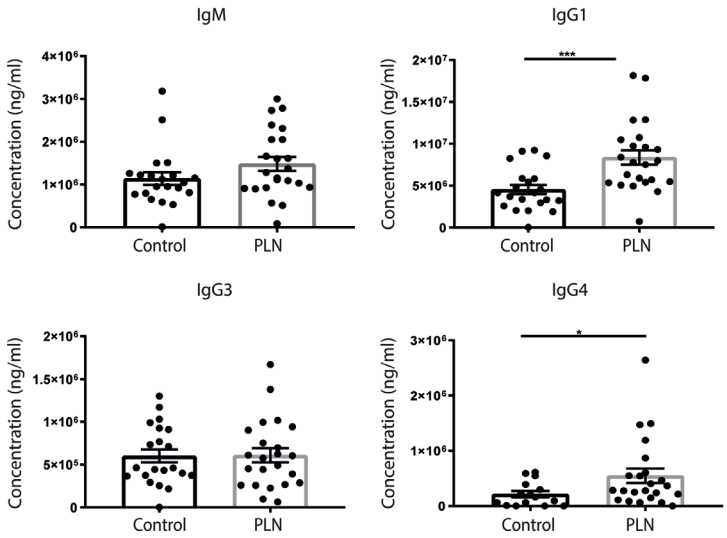

Figure 4. Circulating IgG1 and IgG4 levels are increased in PLN patients
Immunoglobulin levels were measured in plasma of PLN patients and compared to healthy controls. The levels of IgG1 and Ig4 were significantly increased in PLN patients compared to healthy controls. The levels of IgM and IgG3 did not differ between the groups. *PLN: phospholamban. Control n=21, PLN patients n=23. * p<0.05, **** p<0.0001.*

($1.6x10^6$ ng/ml vs $2.8x10^6$ ng/ml, p=0.04) compared to healthy controls. Levels of IgM and IgG3 did not differ significantly between PLN patients and healthy controls (p=0.14 and p=0.96 respectively).

PLN patient-derived IgG specifically binds to iPSC-derived cardiomyocytes with the p.Arg14del mutation

To assess whether PLN-derived IgG is binding to cardiomyocytes, patient-derived IgG was isolated, fluorescently labeled and incubated with iPSC-derived cardiomyocytes with the p.Arg14del mutation (*Figure 5*). IgG-derived from patients carrying the PLN mutation showed binding to iPSC-cardiomyocytes (*Figure 5A*). IgG derived from healthy controls demonstrated less binding to cardiomyocytes as compared to PLN-derived IgG (*Figure 5B*). These findings would suggest that IgG derived from patients with the p.Arg14del mutation is directed against cardiac epitopes.

Epitope mapping of PLN patient-derived IgG reveals a distinct pattern of epitopes involved in cell adhesion processes

To identify target epitopes and affected proteins of antibodies bound to cardiomyocytes, an epitope discovery screen was performed including PLN carriers with and without cardiomyopathy, healthy controls, and family members of PLN patients. A Venn diagram visualized shared and group-specific epitopes which are targeted by IgG (*Figure 6A*). PLN carriers with and without phenotype showed a specific set of cardiac epitopes (PLN

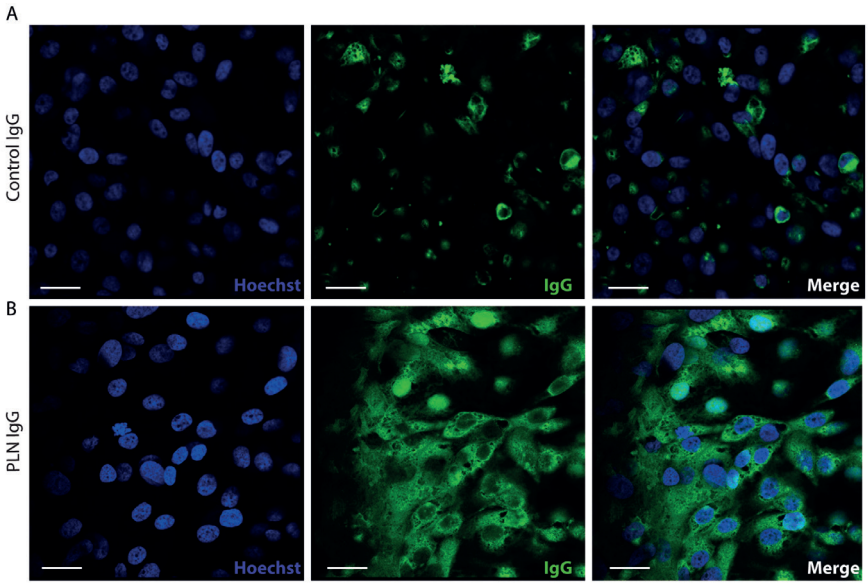

Figure 5. PLN patient-derived IgG binds to p.Arg14del iPSC-derived cardiomyocytes
Total IgG was isolated from plasma of healthy controls or PLN patients, fluorescently labeled, and added to iPSC-derived cardiomyocytes with the p.Arg14del Cell nuclei of cardiomyocytes were stained with Hoechst. IgG derived from PLN patients showed clear binding to iPSC-derived cardiomyocytes, whereas IgG derived from healthy controls hardly showed any binding. *Line bar indicates 75 µm, magnification 20x. PLN: phospholamban, iPSC: induced pluripotent stem cell.*

disease=12 and PLN non-disease=19). Next, we explored the molecular function of these group-specific proteins in PLN carriers with and without cardiomyopathy and family members (*Figure 6B*). PLN patients with cardiomyopathy showed an IgG response against proteins involved in cell adhesion and structural components of cardiac muscle as compared to non-carriers. PLN carriers without cardiomyopathy also showed a response against cell-adhesion proteins, however, did not show a response against structural components of the cardiac muscle. These findings indicate that antibodies potentially bind to cellular adhesion molecules, thereby affecting cell-cell interactions of cardiomyocytes.

DISCUSSION

The p.Arg14del mutation in the PLN gene can lead to the development of cardiomyopathy, which is characterized by increased risk of ventricular arrhythmias, ventricular dilatation, and severe HF[23,24]. However, which PLN carriers will develop HF is still unknown and a potential second hit still needs to be identified. The inflammatory response plays a critical role in cardiac remodeling and the development of HF[14,25,26]. Deposits of cardiac antibodies can be observed in the myocardium of end-stage HF patients and accumulating evidence revealed a role for inflammation in the pathogenesis of DCM and ARVC[13,18,27]. Current studies

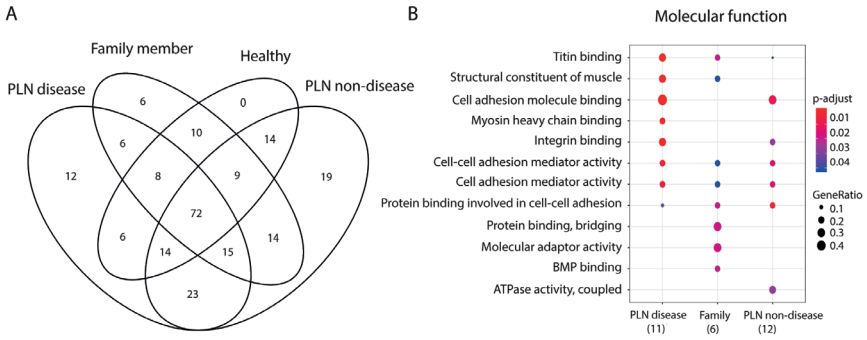

Figure 6. Epitope mapping of plasma-derived IgG in PLN patients, family-members and healthy controls
Epitope screening was performed to identify target proteins of the bound IgG. Venn diagram showing common and group-specific epitopes (A). PLN carriers with and without phenotype showed a specific set of cardiac epitopes which are targeted by PLN-derived IgG. GO-analysis showed the molecular functions of the proteins which are targeted by IgG binding (B). A dot plot is shown for GO-enrichments in the gene sets of PLN carriers with and without phenotype and non-carriers. Terms are shown on the rows, the color indicates significance, and size of the dot indicates the ratio of genes that are present from the gene sets. The number indicates the number of genes found in any of the sets tested. In PLN patients with cardiomyopathy, structural components of cardiomyocytes and cell adhesion proteins seem to be affected. *PLN: phospholamban, GO: gene ontology analysis. PLN with cardiomyopathy n=11, PLN without cardiomyopathy n=12, PLN family members n=6.*

are mainly focusing on strategies to correct the mutated PLN protein and are not focusing on the influence of inflammation[28]. In this study, we investigated the role of the inflammatory response in PLN-induced cardiomyopathy and we aimed to identify potential pathways which may contribute to HF progression in these patients.

The presence of inflammatory cells in the myocardium of end-stage HF was recently described for ischemic heart disease (IHD) and DCM[21]. In line with these findings, we observed significantly increased numbers of macrophages, T cells, B cells and also plasma cells in the myocardium of end-stage PLN patients. It is known that PLN patients have P62-positive aggregates of the mutated PLN protein in dense perinuclear aggresomes in cardiomyocytes[9,23,29]. To investigate whether myocardial immune cells colocalize with these aggresomes, a P62 staining was performed. We observed some infiltrating T cells surrounding P62-positive aggresomes, but generally, T cells were diffusely present throughout the myocardium. This suggests that the persistent low-grade inflammatory response in the myocardium of these PLN patients with end-stage HF is not limited to intra-myocardial PLN aggregates. It is known that chronic inflammation in the myocardium can also activate the complement system, thereby inducing cellular toxicity[22,30]. To investigate whether complement activation is also visible in end-stage PLN hearts, a C4d staining was performed. C4d is a marker of recent activation of the classical pathway of compliment activation and is often used in the field of antibody-mediated rejection (AMR), where C4 deposits can be found in the microvasculature of transplanted hearts[31]. Moreover, C4d can also bind to necrotic cardiomyocytes after myocardial injury in the non-transplant setting[32]. In end-stage PLN hearts, the presence of C4d deposits in the myocardium was observed. No signs of AMR were detected, since no C4d deposits were found in endothelial cells of capillaries. We

might only speculate that C4d deposits are be indicative for necrotic cardiomyocytes, which suggest that the complement system is activated upon cardiac damage and might also contribute to the increased inflammatory state in end-stage PLN hearts.

In order to compare PLN patients with end-stage HF to a more acute phase of PLN-induced cardiomyopathy without HF, 4 PLN patients who died of sudden cardiac death were included. Despite the fact that these patients had no prior symptoms of cardiomyopathy, already significantly higher numbers of myocardial T cells were found in these hearts. Currently, the association between inflammation and sudden cardiac death has been controversial[33,34]. Our findings suggest that in an early phase of the disease, an inflammatory response in the myocardium is ongoing, which might influence disease progression.

In addition to high numbers of inflammatory cells, increased levels of IgG deposits were observed in the myocardium of PLN patients with end-stage HF compared to controls. Moreover, we demonstrated that the amount of these IgG deposits is correlated with the number of myocardial T- and B lymphocytes. Antibody deposition has also been observed in other HF etiologies, including IHD[21]. Our hypothesis is that upon myocardial damage, cardiac antigens are being released, thereby activating an autoimmune response against the heart. In PLN induced cardiomyopathy, the loss of cardiomyocytes is accompanied by fibro-fatty replacement, which might expose desmosomes and gap-junctions functionally bridging cardiomyocytes[35]. The exposure of these cell adhesion proteins in combination with an active inflammatory response might induce an antibody-mediated immune response, which is characterized by IgG deposition in the myocardium.

In addition to IgG deposits in the myocardium, PLN patients also showed significantly increased levels of circulating IgG1 and IgG4. Elevated levels of IgG1 were also described in other types of cardiomyopathies and are produced upon antibody responses to cardiac proteins[21,36]. The role of IgG4 in cardiovascular disease is less well established. Increased levels of IgG4 are described in chronic, fibrotic inflammatory diseases and organ dysfunction, including the heart[37,38]. High levels of IgG4 in PLN patients could therefore be indicative of cardiac dysfunction.

To investigate whether these circulating immunoglobulins are directed against cardiomyocytes, we created an *in vitro* cell model, using iPSC-derived cardiomyocytes with the p.Arg14del and fluorescently labeled IgG. We demonstrated that IgG from PLN patients binds to cardiomyocytes, whereas IgG derived from healthy controls did not show clear binding. Binding of IgG to cardiomyocytes might lead to complement activation and cellular apoptosis, thereby leading to more cardiac damage in these patients. However, these findings still have to be replicated and validated using more patients in additional experiments.

To get more insights into the cardiac proteins that are being recognized by these circulating antibodies, we performed an epitope discovery screen including a broad spectrum of 176 known cardiovascular proteins. We found group-specific epitopes in PLN patients with/without cardiomyopathy and family members. GO-enrichment analysis showed a clear IgG response against structural components of cardiomyocytes and cell adhesion proteins in PLN carriers with cardiomyopathy. This response might be caused by the release of intracellular proteins by necrotic cardiomyocytes in a highly inflammatory environment,

which can lead to the development of cardiac antibodies against structural components of cardiomyocytes. In addition, proper mechanical and electrical coupling between cardiomyocytes is crucial for conducting the electrical impulse of cardiomyocytes and to preserve cardiac function[39,40]. Our findings suggest that cardiac-related antibodies in PLN patients might affect cardiomyocyte coupling and could therefore be of great importance in the pathogenesis of arrhythmias. This could be incorporated in future assays using iPSC-derived cardiomyocytes and isolated IgG to investigate functional effects on contractility by measuring action potentials and calcium fluxes of cardiomyocytes upon IgG binding. Moreover, the difference between PLN carriers with and without a phenotype could potentially be used in diagnostic approaches to predict early manifestation of the disease or for new therapeutic interventions in PLN carriers.

In conclusion, our study supports a potential role of an antibody-mediated immune response in PLN-induced cardiomyopathy. End-stage PLN hearts show infiltrating immune cells, myocardial IgG deposits, and high levels of circulating IgG1 and IgG4. These antibodies bind to iPSC-derived cardiomyocytes and might be directed to cell-cell adhesion proteins, thereby affecting electronical coupling of cardiomyocytes and the progression of HF. Future studies should focus on the identification of target epitopes to create new possibilities of diagnostic tools and interventions for PLN-induced cardiomyopathy.

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Conflicts of interest

None declared.

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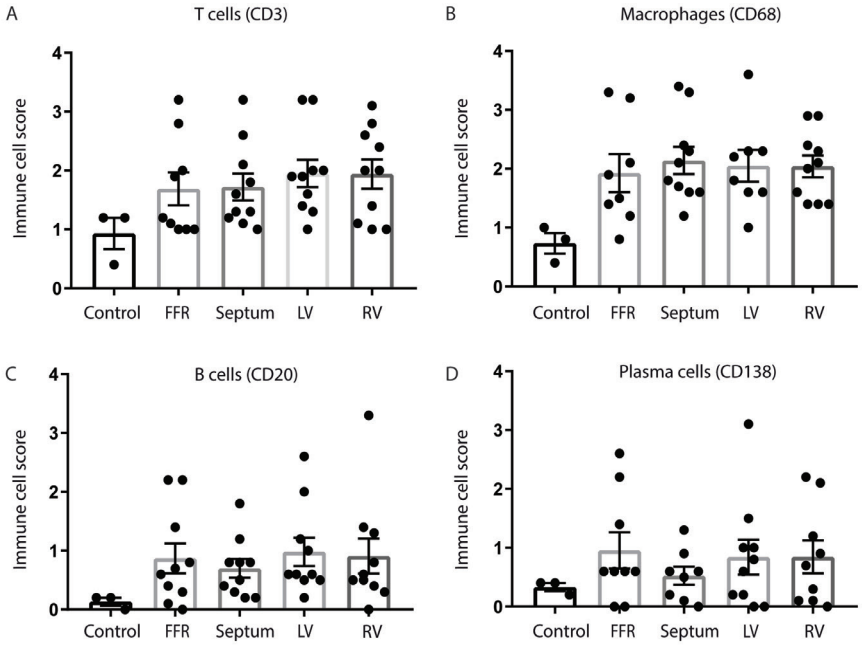
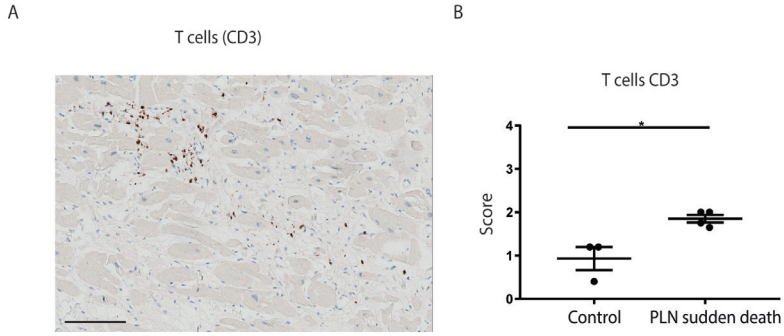
SUPPLEMENTARY FIGURES



Supplementary Figure 1. Myocardial immune cell infiltration is not limited to a specific region of the PLN heart

T cells (CD3), macrophages (CD68), B cells (CD20), and plasma cells (CD138) in the different regions of the PLN heart. The number of infiltrating immune cells was scored by semi-quantitative analysis (A-D). More infiltrating immune cells can be found in every region of the PLN heart compared to control myocardium, albeit not significant. *PLN: phospholamban, FFR: fibro-fatty replacement, LV: left ventricle, RV: right ventricle. Control n=3, PLN n=10.*



Supplementary Figure 2. Infiltrating myocardial T cells in PLN patients who died from sudden cardiac death without history of HF

T cells (CD3) were visualized in the myocardium of PLN patients who died from sudden cardiac death without any history of HF (A). Semi-quantitative scoring showed a significant increase in the number of infiltrating T cells in PLN hearts without any history of cardiomyopathy prior to death (B). *Line bar indicates 200 µm, magnification 10x. PLN: phospholamban, Control n=3, PLN n=4. * p<0.05.*

SUPPLEMENTARY TABLES

Supplementary Table 1. Cardiovascular antigens included in the epitope discovery screen

UniProt ID	Protein Name
P01185	Vasopressin-neurophysin 2-copeptin
Q96L12	Calreticulin-3
O95817	BAG family molecular chaperone regulator 3
Q5T481	RNA-binding protein 20
O95677	Eyes absent homolog 4
P42167	Thymopoietin, isoforms beta/gamma
O76041	Nebulette
P35318	ADM
P05305	Endothelin-1
Q14896	Myosin-binding protein C, cardiac-type
P12883	Myosin-7
P45379	Troponin T, cardiac muscle
P19429	Troponin I, cardiac muscle
P09493	Tropomyosin alpha-1 chain
P08590	Myosin light chain 3
P10916	Myosin regulatory light chain 2 ventricular/cardiac muscle isoform
Q59H18	Serine/threonine-protein kinase TNNI3K
P68032	Actin, alpha cardiac muscle 1
Q8WZ42	Titin
P63316	Troponin C, slow skeletal and cardiac muscles
P13533	Myosin-6
P50461	Cysteine and glycine-rich protein 3
P56539	Caveolin-3
O15273	Telethonin
P18206	Vinculin
Q9BR39	Junctophilin-2
Q5VST9	Obscurin
Q9NPC6	Myozenin-2
Q15327	Ankyrin repeat domain-containing protein 1
P17661	Desmin
P02545	Prelamin-A/C
Q92629	Delta-sarcoglycan
P35609	Alpha-actinin-2
O75112	LIM domain-binding protein 3
P26678	Cardiac phospholamban
O60706	ATP-binding cassette sub-family C member 9
Q14524	Sodium channel protein type 5 subunit alpha
P02511	Alpha-crystallin B chain
P49768	Presenilin-1
P49810	Presenilin-2
Q14192	Four and a half LIM domains protein 2
Q13418	Integrin-linked protein kinase
Q86TC9	Myopalladin
P08172	Muscarinic acetylcholine receptor M2
P11532	Dystrophin
P50402	Emerin
Q16635	Tafazzin
O75072	Fukutin
P15924	Desmoplakin
P14923	Junction plakoglobin

Supplementary Table 1. Continued

UniProt ID	Protein Name
Q99959	Plakophilin-2
P10600	Transforming growth factor beta-3
Q92736	Ryanodine receptor 2
P32926	Desmoglein-3
Q9Y4J8	Dystrobrevin alpha
P49840	Glycogen synthase kinase-3 alpha
P49841	Glycogen synthase kinase-3 beta
P09429	High mobility group protein B1
Q13627	Dual specificity tyrosine-phosphorylation-regulated kinase 1A
P41159	Leptin
O43665	Regulator of G-protein signaling 10
P41220	Regulator of G-protein signaling 2
P51636	Caveolin-2
P07355	Annexin A2
P07858	Cathepsin B
Q9BTV4	Transmembrane protein 43
P13473	Lysosome-associated membrane glycoprotein 2
P06280	Alpha-galactosidase A
P54646	5′-AMP-activated protein kinase catalytic subunit alpha-2
P19474	E3 ubiquitin-protein ligase TRIM21
Q9BQ13	BTB/POZ domain-containing protein KCTD14
P23297	Protein S100-A1
Q8IUC6	TIR domain-containing adapter molecule 1
P19320	Vascular cell adhesion protein 1
P05362	Intercellular adhesion molecule 1
P16581	E-selectin
P05121	Plasminogen activator inhibitor 1
Q0ZGT2	Nexilin
P08588	Beta-1 adrenergic receptor
P62158	Calmodulin
Q969Q1	E3 ubiquitin-protein ligase TRIM63
Q9NPC6	Myozenin-2
Q9UGJ0	5′-AMP-activated protein kinase subunit gamma-2
Q01484	Ankyrin-2
Q8IWT1	Sodium channel subunit beta-4
Q13936	Voltage-dependent L-type calcium channel subunit alpha-1C
P63252	Inward rectifier potassium channel 2
Q9Y6J6	Potassium voltage-gated channel subfamily E member 2
P51787	Potassium voltage-gated channel subfamily KQT member 1
P21796	Voltage-dependent anion-selective channel protein 1
P06733	Alpha-enolase
P50454	Serpin H1
P11086	Phenylethanolamine N-methyltransferase
Q01860	POU domain, class 5 transcription factor 1
P52952	Homeobox protein Nkx-2.5
Q06413	Myocyte-specific enhancer factor 2C
P43694	Transcription factor GATA-4
P04792	Heat shock protein beta-1
Q12988	Heat shock protein beta-3
P10809	60 kDa heat shock protein, mitochondrial
P07900	Heat shock protein HSP 90-alpha
P08238	Heat shock protein HSP 90-beta
Q13639	5-hydroxytryptamine receptor 4

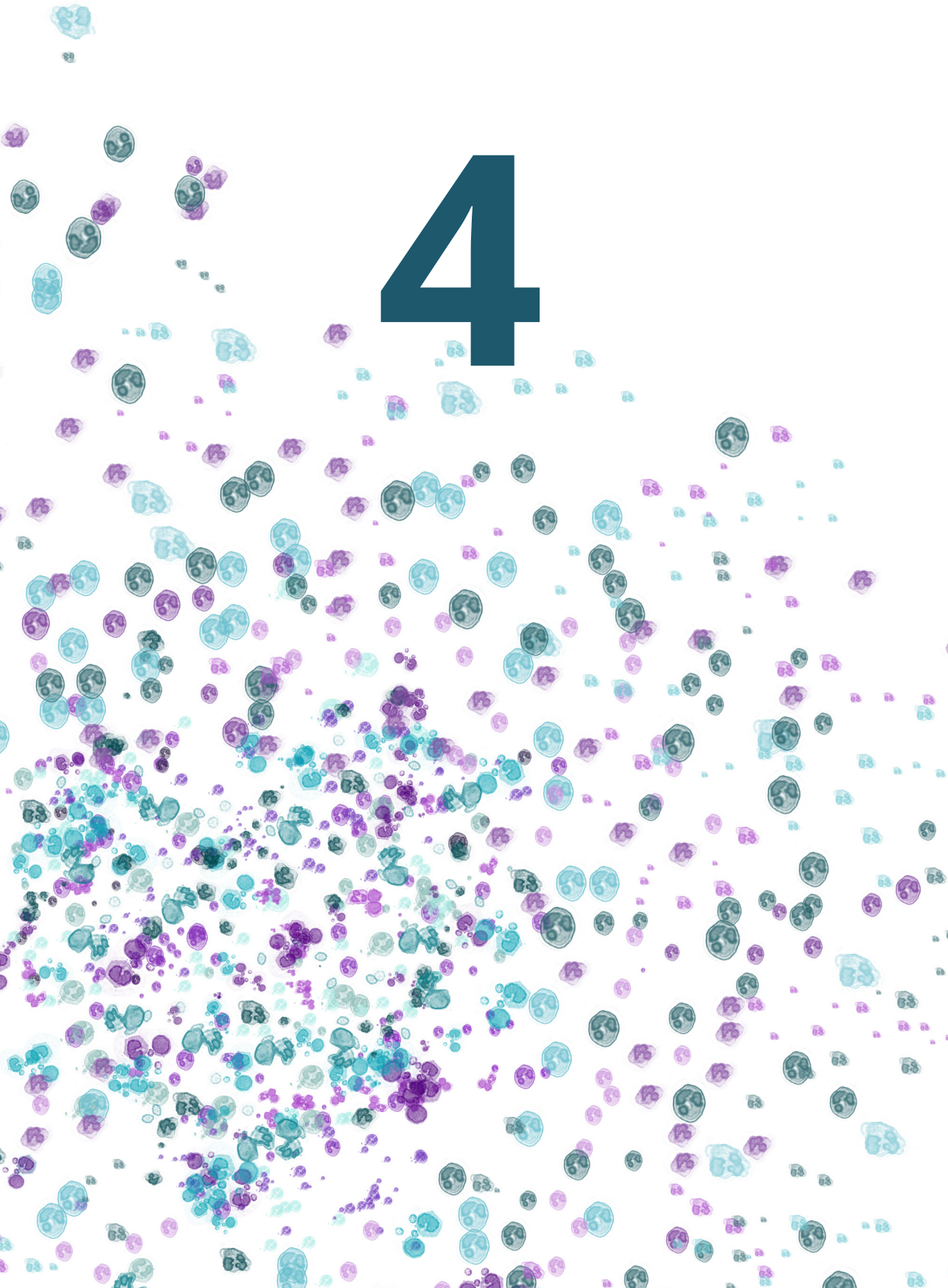
Supplementary Table 1. Continued

UniProt ID	Protein Name
O14983	Sarcoplasmic/endoplasmic reticulum calcium ATPase 1
P16615	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2
Q93084	Sarcoplasmic/endoplasmic reticulum calcium ATPase 3
P08758	Annexin A5
P08133	Annexin A6
P01160	Natriuretic peptides A
P09038	Heparin-binding growth factor 2
P16860	Natriuretic peptides B
P09543	2',3'-cyclic-nucleotide 3'-phosphodiesterase
P17302	Gap junction alpha-1 protein
P61296	Heart- and neural crest derivatives-expressed protein 2
O96004	Heart- and neural crest derivatives-expressed protein 1
Q92908	Transcription factor GATA-6
P05413	Fatty acid-binding protein, heart
P05019	Insulin-like growth factor I
P11047	Laminin subunit gamma-1
O15230	Laminin subunit alpha-5
Q16363	Laminin subunit alpha-4
P24043	Laminin subunit alpha-2
P07942	Laminin subunit beta-1
P55268	Laminin subunit beta-2
P25391	Laminin subunit alpha-1
Q9Y6N6	Laminin subunit gamma-3
A4D0S4	Laminin subunit beta-4
Q13751	Laminin subunit beta-3
Q16787	Laminin subunit alpha-3
Q13753	Laminin subunit gamma-2
P50993	Sodium/potassium-transporting ATPase subunit alpha-2
P13637	Sodium/potassium-transporting ATPase subunit alpha-3
O00755	Protein Wnt-7a
O00744	Protein Wnt-10b
Q9GZT5	Protein Wnt-10a
Q93098	Protein Wnt-8b
P56704	Protein Wnt-3a
O14905	Protein Wnt-9b
Q93097	Protein Wnt-2b
Q9UBV4	Protein Wnt-16
O96014	Protein Wnt-11
O14904	Protein Wnt-9a
P41221	Protein Wnt-5a
P56705	Protein Wnt-4
Q9H1J7	Protein Wnt-5b
P56706	Protein Wnt-7b
Q9Y6F9	Protein Wnt-6
Q9H1J5	Protein Wnt-8a
P09544	Protein Wnt-2
P56703	Proto-oncogene Wnt-3
P04628	Proto-oncogene Wnt-1
Q13835	Plakophilin-1
Q99959	Plakophilin-2
Q9Y446	Plakophilin-3
Q99569	Plakophilin-4
P49841	Glycogen synthase kinase-3 beta

Supplementary Table 1. Continued

UniProt ID	Protein Name
P04085	Platelet-derived growth factor subunit A
P01127	Platelet-derived growth factor subunit B
Q9NRA1	Platelet-derived growth factor C
Q9GZP0	Platelet-derived growth factor D
P19022	Cadherin-2
P55289	Cadherin-12
P01033	Metalloproteinase inhibitor 1
P35625	Metalloproteinase inhibitor 3
P16035	Metalloproteinase inhibitor 2
Q99727	Metalloproteinase inhibitor 4
P12532	Creatine kinase U-type, mitochondrial
Q01449	Myosin regulatory light chain 2 atrial isoform
Q15746	Myosin light chain kinase, smooth muscle
9651188	Cardiac myosin light chain-1
1220301	Cardiac myosin light chain 2
P05305	Endothelin-1
P35318	ADM
O95817	BAG family molecular chaperone regulator 3
O95677	Eyes absent homolog 4
O76041	Nebulette
Q96L12	Calreticulin-3

4



Cardiac allograft vasculopathy: a donor or recipient induced pathology?

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ABSTRACT

Cardiac allograft vasculopathy (CAV) is one of the main causes of late stage heart failure after heart transplantation. CAV is characterized by concentric luminal narrowing of the coronary arteries, but the exact pathogenesis of CAV is still not unraveled. Many researchers show evidence of an allogeneic immune response of the recipient, whereas others show contrasting results in which donor-derived cells induce an immune response against the graft. In addition, fibrosis of the neo-intima can be induced by recipient-derived circulating cells or donor-derived cells.

In this review, both donor and recipient sides of the story are described to obtain better insights in the pathogenesis of CAV. Dual outcomes were found regarding the contribution of donor and recipient cells in the initiation of the immune response and the development of fibrosis during CAV. Future research could focus more on the potential synergistic interaction of donor and recipient cells leading to CAV.

Clinical relevance

Cardiac allograft vasculopathy (CAV) is one of the main causes of late stage heart failure after heart transplantation. The exact mechanism in which CAV can be induced is not fully elucidated, however, it is known that both donor and recipient are involved. By clarifying the role of donor and recipient cells, better treatment options could be considered.

INTRODUCTION

Cardiac transplantation is often successfully applied in the treatment of end-stage heart failure[1]. Since 1982, more than 110,000 heart transplantations have been performed globally and these numbers are still rising[2]. Over the years, early survival rates of recipients, which received a heart transplantation, have significantly improved[2]. In the first months after transplantation, acute rejection of the transplanted heart can occur[3]. Much progress has been made in controlling this acute rejection phase, resulting in increased early survival rates[2]. However, chronic rejection is one of the major issues that affects long-term survival of heart transplant recipients[4].

One of the main causes of chronic rejection is cardiac allograft vasculopathy (CAV)[1–5]. CAV is an accelerated form of coronary artery disease[6] and affects both males and females[3]. The mechanism by which CAV develops is not fully elucidated, but it is estimated that 50% of heart transplantation recipients are developing CAV within five years after transplantation[7]. Hence, CAV is responsible for 10-15% of cardiac deaths after transplantation[8]. CAV affects the vasculature of the transplanted heart, resulting in congestive heart failure, arrhythmias, myocardial infarction or sudden cardiac death[1,9]. Both immunologic factors and non-immunologic factors, such as age, gender, and brain injury, are involved in the development of CAV, although immunologic factors have shown to be the most important players[5,10].

CAV is characterized by diffuse intimal thickening leading to progressive narrowing of the coronary arteries[5,11]. There are different types of lesions in CAV patients, including intimal hyperplasia, atherosclerotic lesions and vasculitis[3]. Within the lesions of intimal hyperplasia, three histopathological phenotypes of CAV can be observed: 1) loose connective tissue with inflammatory cells, 2) lesions with smooth muscle cells and 3) fibrotic lesions (*Figure 1*)[12]. Most commonly seen characteristic in CAV is fibromuscular hyperplasia of the intima, which also distinguishes CAV from atherosclerosis[8]. Ultimately, progressive narrowing of the coronary artery results in critical stenosis and ischemia of the graft[3].

The exact mechanism in which CAV is induced after heart transplantation is not elucidated, but it is known that both donor- and recipient cells are involved[13]. The question remains whether cells of the recipient react on cells of the donor heart or vice versa. Multiple researchers have studied the mechanism of CAV and the results were often contradictory. For example, one study revealed that donor dendritic cells (DCs) transmigrate through host secondary lymphoid organs, thereby promoting T lymphocytes of the recipient, which may promote graft rejection [14]. However, others propose that allo-recognition of donor major histocompatibility complexes (MHC) by recipient immune cells leads to graft rejection[15]. The same is true for the development of fibrosis; are recipient-derived endothelial progenitor cells or endothelial-mesenchymal transition of donor cells responsible for the progressive lesion formation[16,17]? The immune response could be the initial trigger for fibrosis, however, other mechanisms of fibrosis may be involved as well. In this overview, recipient and donor sides of the stories (immune response and fibrosis) are highlighted to obtain better insights in the pathogenesis of CAV.

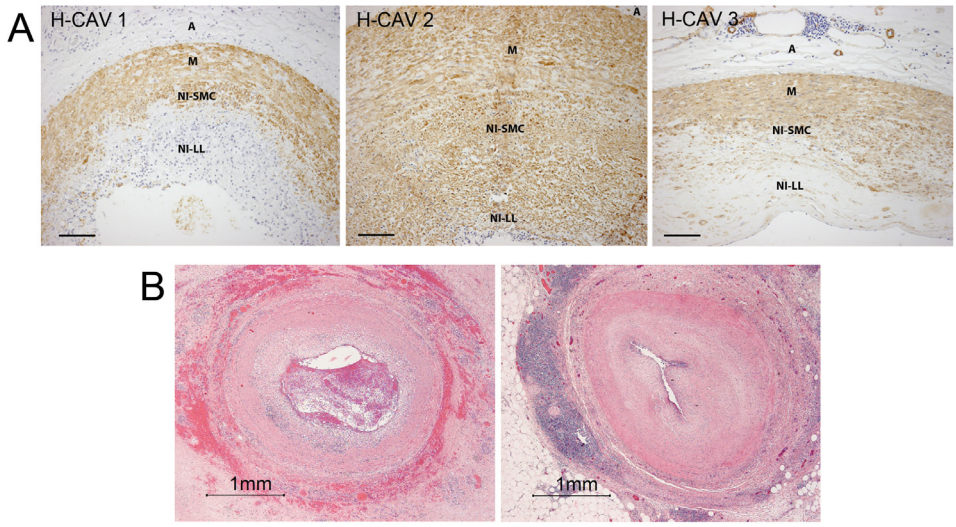

Figure 1. Microscopic images of the three histopathological phenotypes of CAV in the coronary artery of heart transplantation recipients
H-CAV 1 lesion, which shows infiltration of lymphocytes in the neo-intima layer (A). H-CAV 2 lesion, showing infiltration of lymphocytes together with infiltration of smooth muscle cells and formation of connective tissue. H-CAV 3 lesion, which shows a large fibrotic intimal lesion without inflammatory infiltrate (*αSMA staining, magnification 100x, line indicates 100μm*). Microscopic pictures of occluded coronary arteries by a thrombus or fibrotic tissue respectively (B). *(HE staining, magnification 20x, line bar indicates 1 mm).*

Cardiac allograft vasculopathy: Immune response

Recipient-derived immune response

According to multiple research groups, the onset of CAV is caused by an immune response of the recipient against the donor[3,5,9,18]. The hypothesis is that after the heart is transplanted, both cellular and humoral immune responses of the recipient are generated against the graft[3]. The immune response of the recipient can be triggered via a (1) direct- , an (2) indirect- or a (3) semi-direct pathway (*Figure 2*)[18–21] . Although all three pathways can be involved, the semi-direct and direct pathways are less well described in the process of CAV. (1) In the direct pathway recipient T lymphocytes are activated after recognition of allogeneic MHCs (with a foreign antigen) of donor antigen presenting cells (APCs)[18]. (2) The indirect pathway is activated by allo-recognition of processed foreign antigens by APCs of the recipient itself[19]. The recognition of donor antigens on recipient APCs leads to the activation and proliferation of T lymphocytes. (3) The semi-direct pathway, a new pathway which may be involved, is activated by recipient APCs, presenting donor MHC molecules on their surface[20]. The theory is that recipient APCs acquire donor MHC via cell-cell interaction (intercellular exchange) with donor cells or via the uptake of donor-derived exosomes[21]. The subsequent presentation of donor antigens by donor MHC molecules on recipient APCs will mount a host T-lymphocyte response, leading to the development of chronic rejection.
In all three pathways the activation of T lymphocytes will lead to secretion of cytokines such

as interleukin-2 (IL-2) and interferon-γ (IFN-γ)[22]. Cytotoxic T lymphocytes, B lymphocytes and macrophages are activated by these cytokines. In addition, endothelial cells are activated and start expressing vascular cell adhesion molecules, which leads to the recruitment of more immune cells[7]. The pro-inflammatory cytokines also enhance the proliferation of smooth muscle cells (SMCs)[15]. Activated B lymphocytes start to secrete donor-specific HLA antibodies. These antibodies are important mediators in the development of CAV[23,24]. They are able to bind the allo-antigens to enable complement factor binding, leading to the activation of the complement system. Furthermore, immune cells, such as macrophages, can bind the donor specific antibodies, which activates antibody-mediated lysis[18]. All of the indicated pathways will ultimately result in vascular injury, ischemia and damage to the allograft[9].

In addition, there is also evidence that "autoimmunity" plays an important role in the development of CAV[25]. For example, in lung transplant recipients, chronic allograft rejection developed even in the absence of human leukocyte antigen (HLA) antibodies[26]. An explanation for this phenomenon might be the presence of antibodies against non-HLA antigens[25], but the question remains which non-HLA antigens are involved. Recent studies showed that "auto-antibodies" against cardiac myosin and vimentin can be detected after heart transplantation[27,28]. These "auto-antibodies" are probably induced via antigen mimicry between the donor MHC peptides and auto-antigen peptides of the recipient[28]. T lymphocytes, which are activated by an indirect allo-immune response, are able to induce chronic rejection by recognition of these auto-antigens[29]. It has been shown in mouse models that induction of tolerance to cardiac myosin leads to a decrease in chronic rejection and an increase in long term survival after heart transplantation[30]. Therefore, reactivity of the T lymphocytes towards auto-antigens is likely involved in the development of CAV[28].
Furthermore, the development of anti MHC-class-1-chain-related-A (MICA) antibodies may play a role in the development of CAV[23,25]. Normally, these antigens are expressed in fibroblasts, monocytes and endothelial cells[24]. In CAV there is an increase in MICA expression on endothelial cells[31]. In addition, allo-antibodies against MICA are detected, which actively induce an immune response and cause damage to the endothelium[23].
Cytomegalovirus (CMV) infection has also been known to affect transplantation outcome and CAV. Systemic replication of CMV is associated with increased risk of rejection of the graft and the development of CAV[32]. The theory is that CMV infection induces inflammatory responses of the recipient, thereby contributing to vascular damage and accelerating the pathogenesis of CAV [33].
The trigger of these responses (direct-, indirect-, and semi-direct pathways, "autoimmunity" and CMV infection) ultimately leads to the proliferation of smooth muscle cells (SMCs), accumulation of extracellular matrix and hyperplasia of the intima of the vessel wall (*Figure 2*)[15,22,23].

Donor-derived immune response
Next to the recipient-derived immune response, there is also evidence that donor factors are involved in the immune-pathogenesis of chronic rejection and CAV[34,35]. Donor factors contributing to CAV include the status of the donor heart and donor-derived cells

transplanted during the procedure. The brain death status of the donor greatly influences CAV development[36,37]. The release of catecholamines during brain death induces endothelial injury leading to cytokine release and MHC up-regulation on donor endothelium[38]. This pathway is mainly investigated in kidney transplant models[37,39]. However, endothelial dysfunction is generally accepted as one of the strongest predictors of CAV[40]. This process in the donor heart accelerates the early allo-immune response leading to CAV initiation.

Donor-derived cells also play an important role in the pathogenesis of CAV. The current theory is that remaining donor cells within the transplanted heart are able to actively induce an immune response of recipient immune cells[41,42]. It has been shown that donor-derived immune cells are able to migrate to lymph nodes of the recipient and locally present allo-antigens[43]. Heart-derived donor dendritic cells (DCs) can already be found three hours after transplantation in secondary lymphoid organs and are not as short-lived in recipients as previously thought[41]. A rodent animal study demonstrated that donor DCs can be found in T lymphocyte areas of the host spleen and hepatic lymphnodes[35]. Hereby, cluster formation of donor DCs and host T lymphocytes was initiated that activated T-lymphocyte proliferation[35]. These results suggest a donor-derived immune response, initiated by donor DCs. In addition to donor DCs, the functional activity of donor CD4 T lymphocytes was studied. In a mouse model the development of autoimmune reactions after heart transplantation and the contribution to CAV was analyzed[42]. Donor CD4 T lymphocyte allo-recognition of MHC-II on recipient B lymphocytes enhanced the production of auto-antibodies, thereby contributing to the development of CAV[42]. When donor CD4 T lymphocytes were depleted, a significant decrease in both antibody and complement deposition was observed in the allograft[42]. Furthermore, transplant studies showed a mixture (chimerism) of donor and recipient leucocytes, including T lymphocytes, in heart transplant recipients[44]. However, to what extent donor T lymphocytes are contributing to CAV after heart transplantation is still unknown.

The expression of donor programmed death–ligand1 (PD-L1) is also involved in the development of CAV[45]. This ligand plays an important role in the regulation of an allo-immune response by regulating activation of CD4 and CD8 T lymphocytes[46]. Donor deficiency of PD-L1 accelerates allograft rejection and the development of CAV compared to PD-L1 deficient recipients[45]. Deficiency of donor PD-L1 leads to the secretion of IFN-γ and proliferation of allo-reactive T lymphocytes of the recipient, thereby promoting a recipient allo-immune response[46]. These findings show that PD-L1 expression on cardiac tissue or leukocytes of the donor is critical in the regulation of an allograft immune response in heart transplant recipients[45,46].

In addition, it has been shown that donor-derived selectins play an important role in the development of CAV[34]. Selectins are involved in adhesion of leukocytes to the endothelium of the vessel wall[34]. Donor-derived E and P-selectin, located on the endothelium of the graft, interact with L-selectin on recipient-derived leukocytes, thereby enhancing the attraction of immune cells[34]. In rats, there is a significant correlation between the amount of P-selectin expression and intimal thickening of the vessel wall[47]. Corresponding results were found in human recipients with a lung allograft[48]. Furthermore, an increased long-term graft survival with minimal vasculopathy was seen in recipients lacking donor-expressed selectins. This indicates the importance of donor-derived selectins in the development of CAV[49].

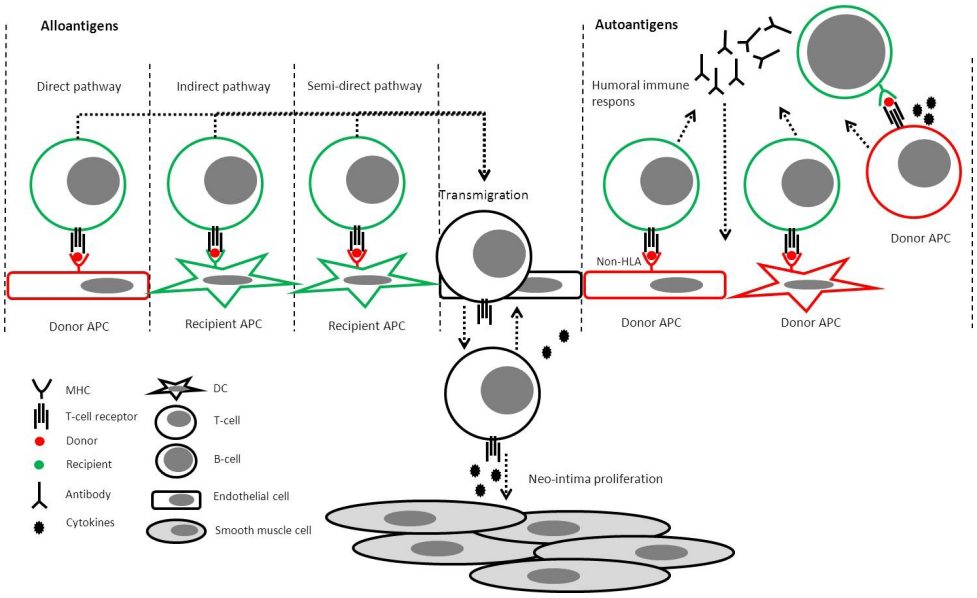

Figure 2. Pathways in recipient-derived immune response
Primary event is the recognition of allo-antigens by T lymphocytes via one of the indicated pathways. The direct pathway is activated by the recognition of MHC complexes with a foreign HLA-antigen (red) presented by donor APCs (red). The indirect pathway is activated when T lymphocytes recognize processed allo-antigens presented by recipient APCs (green). The semi-direct pathway is activated when T lymphocytes recognize allo-antigens presented on donor MHC on recipient APCs. In addition, non-HLA antigens might be involved, which are bound by "auto-antibodies". This will lead to complement activation and T lymphocyte activation. Activation of B cells can be initiated by donor DCs and donor T lymphocytes. All of the indicated pathways lead to activation of T lymphocytes, which start to secrete pro-inflammatory cytokines such as IFN-γ. The secretion of IFN-γ recruits more immune cells, such as NK-cells and macrophages, and acts on SMCs. The proliferation of SMCs will ultimately result in proliferation of the intima and occlusion of the artery, which are the characteristics of CAV.

According to this accumulating evidence, there is a donor-derived immune response causing the development of allograft vasculopathy and donor cells are involved in regulating the allo-immune response of the recipient. Since this is a relatively new insight, more focus on these aspects is needed to reveal the exact mechanism and to define all of the donor and recipient cells involved.

Differences in immune response in gender mismatch transplantations

Interesting differences in transplantation outcome have been reported between males receiving a female heart or females receiving a male heart, the so called donor-recipient gender mismatch transplantations[50]. Donor-recipient gender mismatch has been shown to influence the early pathogenesis of CAV[51]. At the vascular level, male recipients with a female allograft developed significantly higher amounts of intimal thickening within one year of transplantation[52]. Females receiving a male allograft only developed non-severe thickening of the intima[52]. The combination of male recipients receiving a female heart have been correlated with worse outcomes at several levels besides CAV[53]. Which factors are involved

is still under investigation, but there is evidence that smaller heart size, shear stress, and loss of the estrogen-protected environment of the female heart are important factors[1,52]. Additionally, these factors contribute to initial endothelial damage of the coronary arteries, thereby initiating CAV development. Furthermore, the vasculature of the female heart is thought to be immunologically more susceptible compared to male hearts[52]: a possible explanation is that the vasculature of the female heart expresses more HLA and non-HLA endothelial antigens than their male counterparts[54], which leads to triggering of the male immune system and thereby an earlier development of CAV[52].

At the organ level, contrasting studies showed higher incidents of rejection of female recipients receiving a male heart[55]. These high rejection rates might be explained by greater immuno-competence of the female by developing HLA antibodies against H-Y antigens, presented by cells of the male heart[55]. Presentation of these antigens can lead to an immune response followed by the formation of allogeneic antibodies[25]. The allogeneic immune response against H-Y antigens can lead to graft destruction and ultimately results in rejection of the male heart[56].

Based on these findings, donor-recipient gender mismatch in heart transplantation is followed by dual outcomes. Transplantation of male hearts into females is characterized by higher rejection rates, but in the end a higher long-term survival[55]. Transplantation of female hearts into males is characterized by an earlier development of CAV[55]. However, some of the studies were limited by the small numbers of gender mismatch transplantations available[51].

Cardiac allograft vasculopathy: Fibrosis

The before-mentioned immune reactions could be followed by fibrosis, which plays an important role in the progressive thickening of the neo-intima and subsequently in the development of CAV[57]. In some recipients, the neo-intima of the coronary arteries almost completely exists of fibrotic tissue[58]. It is known that some inflammatory cells of the recipient, such as T lymphocytes and macrophages, are involved in the initiation of the fibrotic process[57]. The secretion of cytokines like IFN-γ and transforming growth factor-β (TGF-β) by T lymphocytes leads to the activation of macrophages and fibroblasts respectively[25,59]. It has been shown that especially recipient-derived macrophages type 2 (M2) are increased in the neo-intima of CAV arteries[60,61]. These macrophages are involved in tissue remodeling and matrix deposition and play an important role in the development of fibrotic lesions[60,62]. They are known to infiltrate the allograft and produce growth factors, such as TGF-β, which increases neo-intimal proliferation[61]. However, more cell types are involved. Next to identified circulating cells of the recipient, there is also evidence of the involvement of donor-derived cells[13,63].

Recipient-derived circulating cells

There is evidence emerging for the role of recipient endothelial progenitor cells (EPCs) in CAV[29]. EPCs ($CD133^+CD34^+Flk1$ [$VEGF\text{-}R1]^+$ in bone marrow, CD31 [$PECAM\text{-}1]^+CD146^+vWF^+NOS^+$ in circulation) are bone marrow-derived cells, which have endothelial regenerative properties[64]. Healthy endothelium of the vessel wall normally undergoes degeneration and

regeneration[65]. By an imbalance in these processes, endothelial dysfunction occurs that can lead to injury of the vessel wall[65]. EPCs are able to adhere to the sides of injury and promote healing and repair[66]. Increased numbers of circulating EPCs have been shown to prevent cardiovascular diseases and to reduce neo-intimal hyperplasia in men[67]. However, upon heart transplantation, the protective role of EPCs changes and EPCs of the recipient may participate in the pathogenesis of CAV (*Figure 3A*) [16,65,68]. Circulating EPCs attach to the vessel wall and start to proliferate as a result of a persistent allograft immune response[68]. The EPCs become uncontrolled, thereby contributing to chronic allograft rejection via accumulation of endothelial cells and SMCs, which in turn leads to occlusive narrowing of the coronary vessels[65]. Upon culturing mononuclear cells from blood, fewer colonies of circulating EPCs were found in heart transplantation recipients with vasculopathy during chronic rejection, compared to transplantation recipients without evidence of vasculopathy[16]. Interestingly, there was also an increase of attached recipient EPCs in the coronary arties of the donor heart, where eventually CAV developed[16]. The hypothesis is that excessive numbers of recipient EPCs differentiate into endothelial cells and SMCs, and that an overload of these cells leads to hyperplasia of the neo-intima and fibrosis[16,68]. The number of circulating EPCs becomes depleted and thereby the protective effects, as mentioned before, are lost[16].

In addition to EPCs, there is also evidence for a role of recipient-derived extra-cardiac progenitor cells[69]. These extra-cardiac progenitor cells are thought to be derived from mesenchymal precursor cells and migrate towards to allograft where they differentiate into multiple cell lineages[70]. Engraftment of recipient-derived cells in the allograft, such as cardiac progenitor cells, resulted in chimerism of the transplanted heart, which can be beneficial by repopulating the niches of rejected donor cells of the graft[66,71,72] However, chimerism of the transplanted heart by extra-cardiac progenitor cells has been shown to be linked to CAV and intimal fibrosis[70]. A possible explanation is that, during cell death of donor cells, an immune response is locally triggered leading to vascular damage of the coronary arteries[64]. In response to tissue injury, recipient-derived mesenchymal precursor cells are attracted and migrate towards the allograft where they differentiate into fibroblasts[73]. In rats suffering from chronic allograft rejection, it has been shown that more than 65% of fibroblasts in the allograft are of recipient origin[73]. When these fibroblast are activated upon inflammation, they start to proliferate and produce extracellular matrix (ECM)[74]. Ongoing inflammation in chronic rejection leads to a continuous fibrogenic environment, which ultimately leads to fibrosis of the neo-intima in CAV[57,71,73,74].

Although chimerism of the transplanted heart provides evidence for the involvement of recipient-derived circulating cells, the contribution of these cells in the pathogenesis of fibrosis in human CAV is still conflicting and remains to be resolved.

Donor-derived cells

Besides the contribution of recipient-derived cells, there is also evidence that donor cells are involved in the formation of fibrosis[13,64] It is thought that especially donor-derived SMCs produce extracellular matrix and contribute to the formation of fibrotic lesions in the neo-intima[65]. In CAV, most of the cells in the neo-intima of coronary vessels express markers of

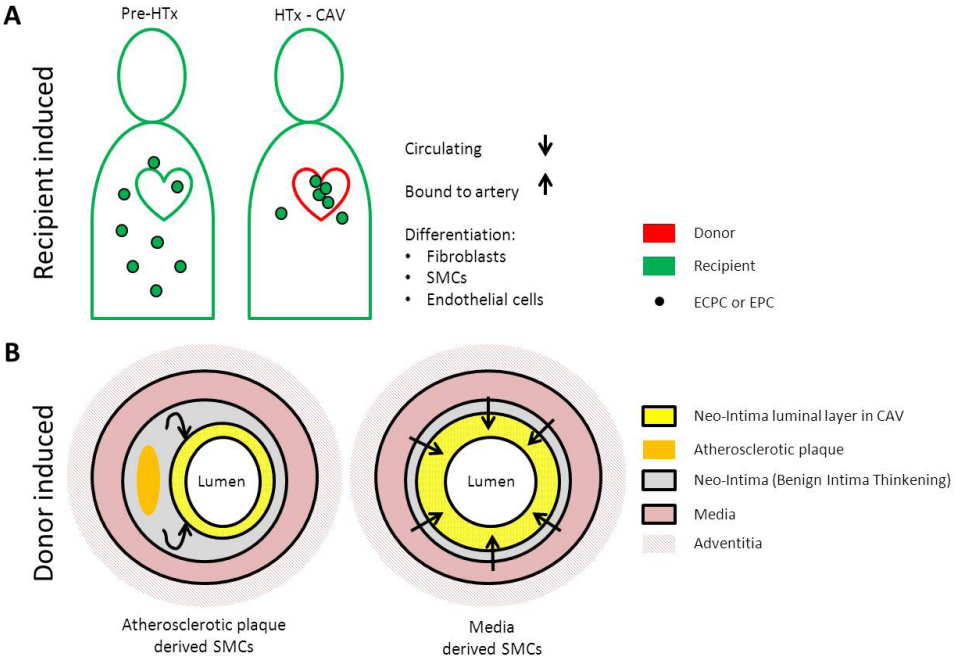

Figure 3. Role of recipient and donor-derived cells in concentric narrowing and fibrosis of the coronary artery
Recipient-derived circulating cells, such as EPCs and ECPCs, contribute to concentric narrowing and fibrosis of the coronary arteries. Increased accumulation of circulating cells bound to the vessel wall induces differentiation of these cells towards fibroblasts, SMCs and endothelial cells, which enhances concentric narrowing of the coronary arteries (A). Donor-derived cells, such as SMCs, are migrating from atherosclerotic lesions or the media layer towards the neo-intima. Accumulation of donor-derived SMCs and production of ECM will lead to the expansion of the neo-intima resulting in narrowing and fibrosis of the coronary artery (B).

SMCs[75]. In human CAV, the majority of these cells are derived from the graft and not from the host[13]. It is not known where these SMCs are originating from. It is possible that resident intimal SMCs expand in number upon inflammation or that they derive from the media and migrate towards the intima to sites with vascular damage (*Figure 3B*) [9]. There is also evidence that these donor-derived SMCs originate from endothelial mesenchymal transitions of donor cells[17]. However, the exact role of these donor-derived SMCs needs to be clarified.
Endothelial-to-mesenchymal transition (Endo-MT) and epithelial-to-mesenchymal transition (EMT) have shown to be potential contributors to neo-intima formation[76–78]. Endo-MT is the trans-differentiation of endothelial cells into mesenchymal cells, such as SMCs and fibroblasts, whereas EMT represents the trans-differentiation of epithelial cells into mesenchymal cells[17,76]. This process normally occurs during certain stages of embryonic development of the heart under influence of TGF-β signaling and is implicated in fibrosis formation[76,79]. For example, biopsies of human kidney transplants with allograft vasculopathy showed a loss of epithelial markers and an increase in mesenchymal markers[80]. The same trend was observed in studies with cardiac fibrosis, where endo-MT significantly contributed

to the development of fibrosis in chronic cardiac disease[81]. It is thought that both endothelial and epicardial-derived cells of the donor, located on the transplanted heart, use this mechanism to differentiate into SMCs and myofibroblasts, thereby contributing to the development of neo-intima fibrosis in CAV[76,81–83]. Although this hypothesis is gaining attention, it still needs to be unraveled where these donor-derived cells come from.

Donor-derived atherosclerotic plaques

Next to individual donor-derived cells, it has been suggested that atherosclerotic plaques in coronary vessels of the donor, pre-existing in the transplanted heart, influence the outcome of CAV in the recipient[84]. The atherosclerotic lesions make the intima and the endothelium of the donor coronary arteries more vulnerable for the development of fibrotic lesions during CAV[12]. In these arteries, the fibrotic process and proliferation of immune cells and SMCs was already ongoing in the plaque and could further develop in the transplanted heart, thereby causing neo-intima formation (*Figure 3B*). It is also possible that these atherosclerotic lesions develop after transplantation, but there seems to be a correlation between pre-existing atherosclerotic lesions and a more fibrotic CAV outcome[8].

In conclusion, it appears that not only inflammatory cells of the donor are involved in the pathogenesis of CAV, but also donor-derived cells, such as endothelial cells, epicardial cells and smooth muscle cells. In addition, presence of atherosclerotic plaques might be correlated to fibrotic lesions in CAV. These findings provide new insights in a possible role for the donor in the development of neo-intima fibrosis in CAV, however, this is still under debate.

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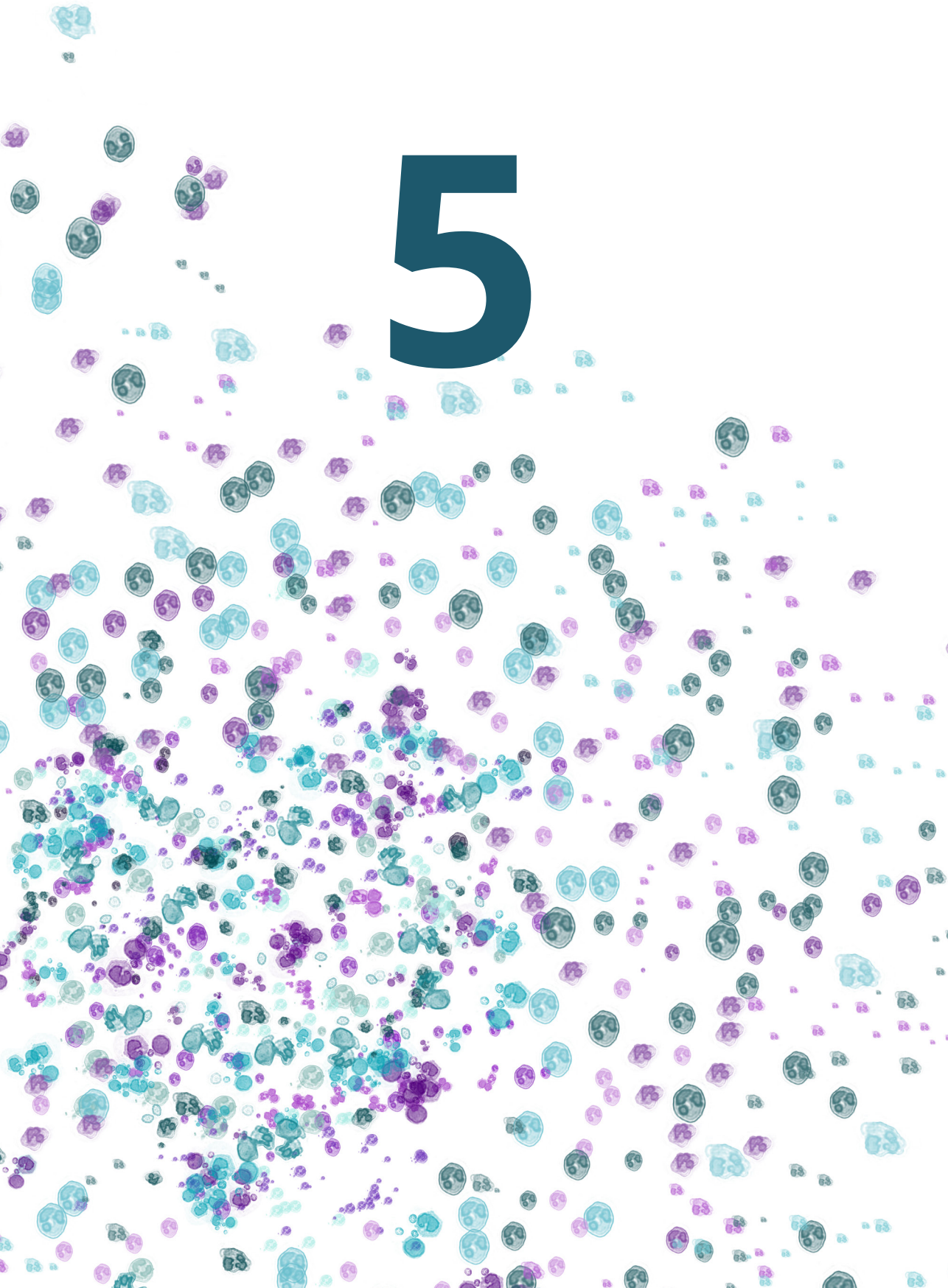

Plasma immunoglobulin levels prior to heart transplantation are associated with post-transplantation survival

Submitted

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ABSTRACT

Background

It is difficult to predict which patients will have a long term survival after heart transplantation (HTx). Cardiac allograft vasculopathy (CAV) and antibody mediated rejection are long-term complications after HTx, limiting survival. Both are caused by an immune response against the donor heart. Interestingly, already prior to HTx, increased plasma levels of immunoglobulins have been found in end-stage heart failure (HF) patients. The objective of this study was to determine whether circulating immunoglobulins pre-HTx are associated with post-HTx survival.

Methods

Epicardial tissue was collected from 61 HTx recipients at autopsy. In addition, of 36 patients, pre-HTx until 6 months post-HTx plasma samples were collected. Immunoglobulin levels were measured in plasma samples and tissue lysates and correlated to survival-time post-HTx and CAV phenotype.

Results

High pre-HTx plasma levels of IgG1 and IgG2 were associated with a shorter survival-time post-HTx. Patients with high immunoglobulin levels pre-HTx also revealed high levels in the first 6 months after HTx, whereas patients with low pre-HTx levels remained low. Similarly, in the cardiac tissue, immunoglobulin deposition was significantly elevated in patients with a survival of less than 3 years compared to patients with a long survival. Both in pre-HTx plasma and post-HTx cardiac tissue, IgG1 and IgG2 levels were significantly increased in patients with an inflammatory CAV type.

Conclusion

High pre-HTx immunoglobulin levels correlate with shorter post-HTx survival, thereby suggesting that an activated immune response before the transplantation might have an adverse effect on graft survival.

INTRODUCTION

Heart failure (HF) is a complex clinical syndrome in which the heart is functionally and structurally abnormal, which results in reduced cardiac output or increased filling pressures[1]. Approximately 1-2% of people in the Western world is affected by HF and these numbers are expected to increase[2]. Over the past four decades, heart transplantation (HTx) has evolved as a the ultimate treatment for end-stage HF[3,4]. Currently, the 1-year survival rate after HTx is 82%, however, long-term survival rates are relatively low (69% after 5 years and 25% after 20 years)[5,6].

One of the main risk factors influencing patient survival after transplantation is cardiac allograft vasculopathy (CAV)[7,8]. CAV is characterized by diffuse concentric intimal thickening of the coronary arteries, leading to ischemic damage to the transplanted heart or sudden cardiac death[7]. CAV can be classified into different histopathological stages: 1) intima consisting of loose connective tissue with inflammatory cells, 2) lesions with smooth muscle cells or myofibroblasts, and 3) fibrotic lesions[9]. These different CAV stages at autopsy are linked to survival time after transplantation[9]. The exact cause of CAV is not fully elucidated yet, but an immune response against the allograft is suggested to be of major importance[10]. Although the production of most cytokines is hampered due to immunosuppressive therapies, the production of antibodies in CAV patients has been described in multiple studies[11,12].

Antibody production can take place in the lymphoreticular system of the recipient, but also in ectopic lymphoid structures (ELS) in the donor heart[10]. ELS consist of clusters of inflammatory cells in the adventitia and adipose tissue surrounding epicardial coronary arteries with CAV[13]. These clusters contain B and T lymphocytes, macrophages and active plasma cells, which produce immunoglobulins including donor-specific antibodies (DSA) against donor HLA[13]. Interestingly, the presence of these antibody-producing ELS at autopsy also correlates to survival of the recipient[10].

Antibodies against the donor HLA are not the only antibodies observed after HTx. Also antibodies against non-HLA antigens, like angiotensin II type-1 receptor, endothelin receptor type A, and vimentin have been described to be associated with the development of CAV and transplant rejection[14–17]. This suggests that not only a specific immune response against HLA of the transplanted heart, but also against other non-HLA epitopes might contribute to antibody-mediated rejection and CAV. Recently, it has been shown that end-stage HF patients show substantial amounts of myocardial IgG deposits and increased circulating IgG1 and IgG3 levels[18,19]. These findings suggest that already prior to HTx, the adaptive immune system is activated and the presence of antibodies targeting the myocardium can potentially influence post-HTx survival.

In this study we assessed whether pre-HTx antibody levels are associated with post-HTx outcome. We measured immunoglobulin levels at different time points after HTx, in plasma of the recipient and in post-mortal tissue lysates of the donor heart. We investigated whether there is a correlation between pre-HTx antibody levels and (a) survival post-HTx, (b) histological CAV stage and (c) presence of ELS.

METHODS

Patient population

Collection of myocardial tissue and blood was approved by the local medical ethics committee (12/387 UNRAVEL) and was in compliance with the *Declaration of Helsinki*[20]. Written informed consent for biobanking of tissue samples and blood was obtained prior to transplantation or, in certain cases from before 2010, waived by the ethics committee when informed consent was not possible due to death of the patient. Patients were included who underwent autopsy after HTx in the period from 1985 until 2018 (n=61). Fresh frozen material of the transplanted heart was collected at the time of autopsy. Plasma samples were collected prior to transplantation, and at different time points post-HTx (n=36 patients). Plasma samples stored after the year 2000 were included for immunoglobulin measurements. Patients were categorized into four quartiles based on post-HTx survival, including Q1 (0-1 month post-HTx), Q2 (1 month-3 years post-HTx), Q3 (3 years-10.5 years post-HTx), and Q4 (>10.5 years post-HTx. Clinical characteristics of patients are listed in *Supplementary Table 1* (tissue lysates) and *Supplementary Table 2* (plasma).

(Immuno)histochemistry

Consecutive sections of formalin fixed paraffin embedded tissue were stained with haematoxylin and eosin (H&E) and Elastic van Gieson (EvG) for general morphology. To study vessel wall composition, an immunostain for smooth muscle cells (aSMA; Sigma-Aldrich, 1:32,000) was used. To study the cellular composition of ELS immunostains for T lymphocytes (CD3; DAKO, Santa Clare, 1:100), B lymphocytes (CD20; Roche, 1:200), macrophages (CD68; Novocastra, 1:1000) and plasma cells (CD138; Serotec, 1:250) were used. The histological stages of CAV (H-CAV 0-3) were categorized by cellular density and composition of the intimal layer, as described previously[9]. In addition, patients were categorized in ELS stage (ELS-0-2) using the median size and number of ELS, as described before[10].

Tissue lysates

All patients of which fresh frozen epicardial tissue was available, including at least 1 of the major coronary arteries were used for tissue lysate antibody analyses (n=61). Tissue lysates of epicardial tissue were made as described previously[13]. Tissue lysates were aliquoted and stored at -80˚C. In the presented data, antibody concentrations were corrected for input of tissue weight and displayed as µg/ml/mg tissue.

Multiplex immunoassay for immunoglobulin detection

IgM levels and IgG subclasses (IgG1, IgG2, and IgG3) in tissue lysates and plasma were measured using a Bio-Plex Pro™ Human Isotyping immunoassay 6-plex (Bio-Rad, 171A3100M) according to manufacturer's instructions. Plasma and tissue lysate immunoglobulin levels were calculated using internal standards, included in the immunoassay. Tissue lysates were 100x diluted and plasma samples were 40,000x diluted. Immunoglobulin levels were measured using the Luminex LabScan200 with xPONENT software (Luminex, Austin, TX).

Statistical analysis

Normal distribution of the data was tested using the Kolmogorov-Smirnov test. A one-way ANOVA, two-way ANOVA or Kruskal-Wallis test, corrected for multiple testing, was used to compare different groups. Correlation was assessed using Pearson correlation coefficient. All statistics were performed using SPSS (IBM SPSS Statistics 25; IBMCorp, Armonk, NY) and GraphPad Prism (version 7.04). Two-sided p-values of $p<0.05$ were considered significant.

RESULTS

High levels of pre-HTx circulating immunoglobulins are associated with a shorter survival time

To investigate whether pre-HTx immunoglobulin levels are associated with post-HTx overall survival, we measured the levels of IgM and IgG1, IgG2, and IgG3 prior to HTx and compared these levels with survival time post-HTx**.** High levels of IgG1 and IgG2 correlated significantly with shorter survival post-HTx (*Figure 1*, for IgG1 r=-0.42, p=0.01; for IgG2 r=-0.43, p<0.01). Levels of IgM and IgG3 showed the same correlation, although did not reach statistical significance (IgM r= -0.33, p=0.052; for IgG3 r= -0.29, p=0.1). Since a relatively large part of the patients had a survival of less than one month, we performed subgroup analysis in those patients that survived at least one month and investigated whether immunoglobulins levels 1 month post-HTx were also associated with post-HTx survival. High levels of IgM, IgG2 and IgG3 1 month post-HTx were also associated with a limited post-HTx survival (*Supplementary Figure 1*, for IgM r=-0.46, p=0.04; IgG2 r=-0.60, p=0.005; IgG3 r=-0.47, p=0.03).

Patients with high plasma immunoglobulin levels pre-HTx also have relatively high plasma immunoglobulin levels after HTx

To get more insight in the alterations in levels of immunoglobulins after transplantation, we measured the concentration of immunoglobulin at different time points after HTx in plasma of the recipient. Levels of circulating IgG1, IgG2, and IgG3 in plasma of patients after HTx were significantly decreased after 1 month of transplantation compared to pre-HTx levels (*Supplementary Figure 2*, for IgG1 p=0.004; IgG2 p=0.003; IgG3 p=0.006). Immunoglobulin levels seem to rise 6 months after transplantation, albeit not statistical significant. To determine whether pre-HTx immunoglobulin levels might influence the level of immunoglobulins post-HTx, patients were divided into two groups, including high pre-HTx immunoglobulin levels (above the mean concentration) and low pre-HTx levels (below the mean concentration). Interestingly, patients with high antibody titers prior to transplantation also showed significantly higher immunoglobulin levels in the first month post-HTx compared to patients with lower pre-HTx levels (*Figure 2A***,** for IgM p<0.0001; IgG1 p=0.01; IgG2 p=0.02). In addition, the levels of IgG2 and IgG3 remained elevated 6 months after HTx in patients with high pre-HTx levels compared to low pre-HTx levels (IgG2 p=0.003 and IgG3 p=0.0007). This suggests that the immune system of patients with high immunoglobulin levels prior to transplantation remains relatively active after transplantation despite the use of immunosuppression.

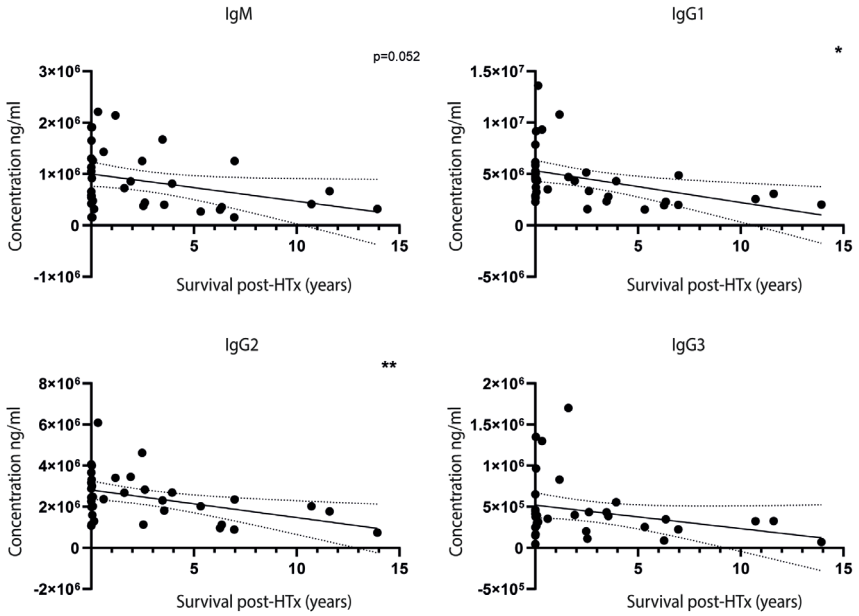

Figure 1. Pre-HTx circulating immunoglobulin levels are associated with survival time post-HTx
Pre-HTx plasma levels of different immunoglobulin subtypes were measured using multiplex immunoassay and correlated with survival post-HTx. Levels of IgG1 and IgG2 were significantly correlated with post-HTx survival, where high immunoglobulin levels resulted in a lower survival post-HTx. *HTx: heart transplantation. n=36. * p<0.05, ** p<0.01.*

Immunoglobulin tissue levels in the donor heart are higher in patients with short-term survival compared to patients with long-term survival

To study whether immunoglobulin levels in the heart are also associated with survival, we measured immunoglobulin levels in the epicardial tissue of the transplanted donor heart upon autopsy. Interestingly, in patients that died within the first 3 years after HTx, immunoglobulin levels in epicardial tissue of the donor heart were significantly increased compared to patients that died more than 10.5 years post-HTx *(Figure 2B*, for IgM p=0.004; IgG1 p=0.004; IgG2 p=0.03; IgG3 p=0.0002). To investigate whether the inflammatory state of the donor heart post-HTx correlates to high levels of pre-HTx immunoglobulins in the recipient, plasma levels of immunoglobulins prior to HTx were compared with immunoglobulin levels in the transplanted heart upon autopsy. A correlation was found between pre-HTx IgG1 plasma levels and post-HTx tissue levels (*Supplementary Figure 3*, r=0.55, p=0.0005).

High levels of IgG in plasma and epicardial tissue are related to the inflammatory CAV phenotype

Since we observed a significant correlation between the levels of IgG1 and IgG2 pre-HTx and survival post-HTx, we hypothesized that elevated immunoglobulin levels in either plasma or tissue lysates might be related to CAV. As already described in previous observations[9],

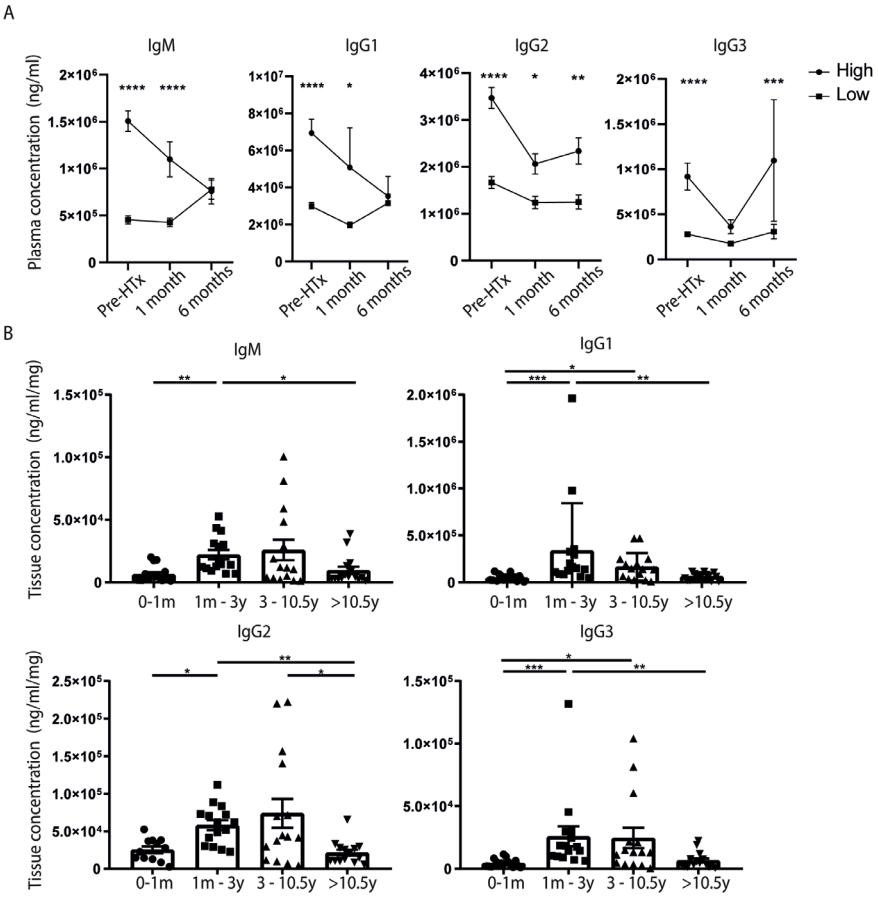

Figure 2. Immunoglobulin levels in the plasma and tissue pre- and post-HTx
Plasma immunoglobulin concentrations were measured prior to HTx until 6 months post transplantation. Patients with high antibody titers (above the mean concentration) prior to HTx also remained high in the first 1-6 months compared to patients with low levels (below the mean concentration) before HTx (A). In addition, immunoglobulin levels were significantly increased in cardiac tissue of patients who died within the first 3-years post-HTx (B). *HTx: heart transplantation. For plasma samples: n=36, for tissue lysates n=61 (0-1 month n=16, 1 month-3 years n=15, 3-10.5 years n=15, more than 10.5 years n=15). * p<0.05, ** p<0.01, **** p<0.0001.*

the H-CAV 1 phenotype is characterized by an intense inflammatory reaction with many lymphocytes infiltrating the intima (*Figure 3A-B*). Interestingly, patients who revealed H-CAV 1 at autopsy also showed higher levels of IgG1 or IgG2 in plasma already prior to HTx compared to patients with H-CAV 2 (*Figure 3C*, for IgG1 p=0.03; IgG2 p=0.03). Consistent with high plasma levels, also locally in the epicardial tissue of the donor heart, immunoglobulin levels were significantly increased in patients with H-CAV 1 compared to patients without H-CAV (*Figure 3D*, for IgG1 p=0.03, IgG2 p=0.03, and IgG3 p=0.03).

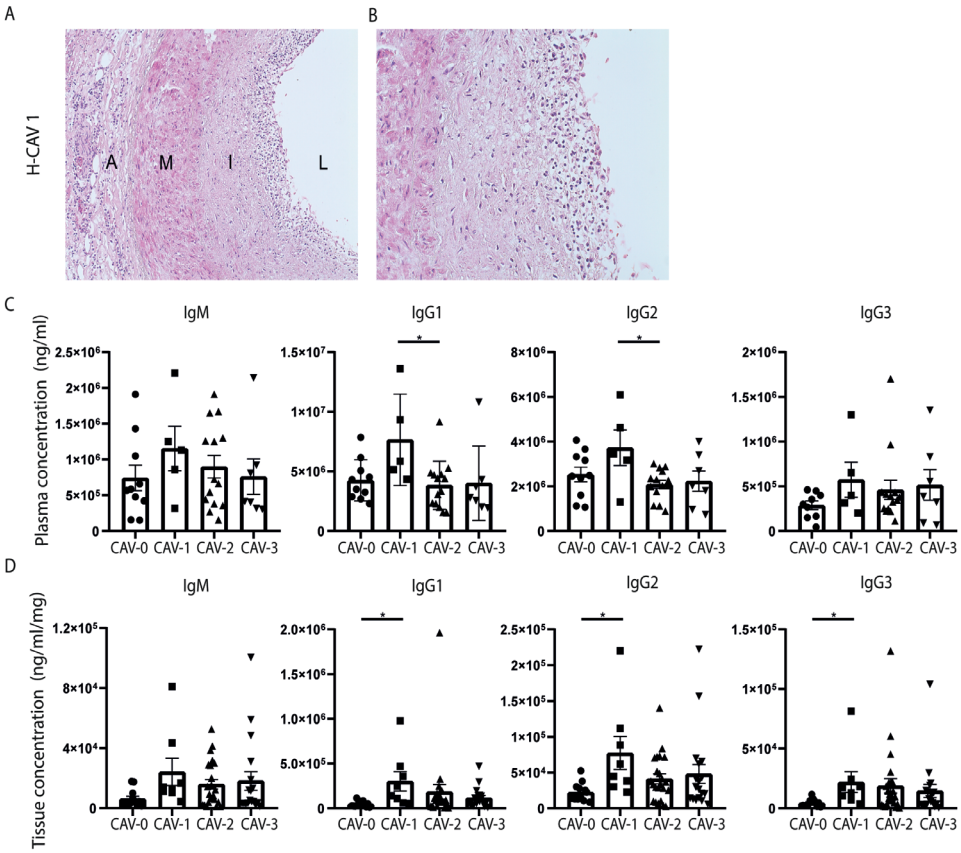

Figure 3. High pre-HTx plasma levels and post-HTx tissue levels of IgG1 and IgG2 in patients with inflammatory H-CAV 1

Histology of a coronary artery with H-CAV 1 (A), showing the adventitia (A), media (M) and neointima (I) surrounding the lumen (L). High magnification images showing infiltrating lymphocytes in the neointima (B). Patients with H-CAV 1 showed a significant increase in IgG1 and IgG2 plasma levels already prior to HTx compared to patients with H-CAV 2 (C). The levels of IgM and IgG3 showed the same trend, although did not reach statistical significance between H-CAV phenotypes. Consistent with plasma levels pre-HTx, the levels of IgG1 and IgG2 were also elevated in cardiac tissue of the transplanted heart with H-CAV 1 (D). *Magnification 200x and 400x. H-CAV: histological cardiac allograft vasculopathy phenotype, HTx: heart transplantation. For plasma samples H-CAV 0 n=10, H-CAV 1 n=5, H-CAV 2 n=14, H-CAV 3 n=7, for tissue lysates H-CAV 0 n=12, H-CAV 1 n=8, H-CAV 2 n=23, H-CAV 3 n=18. * p<0.0.5*

High levels of pre-HTx immunoglobulins are not associated with the presence of ectopic lymphoid structures in the donor heart

ELS surrounding the coronary arteries contain IgM and IgG-producing plasma cells, which may contribute to antibody mediated rejection[13]. To examine whether patients with high immunoglobulin titers pre-HTx are prone to develop ELS, we studied the correlation between immunoglobulins levels in the plasma prior to HTx and the development of ELS in the epicardial tissue of the donor heart at time of autopsy. However, high antibody levels prior to HTx were not associated with the presence of ELS (*Figure 4*).

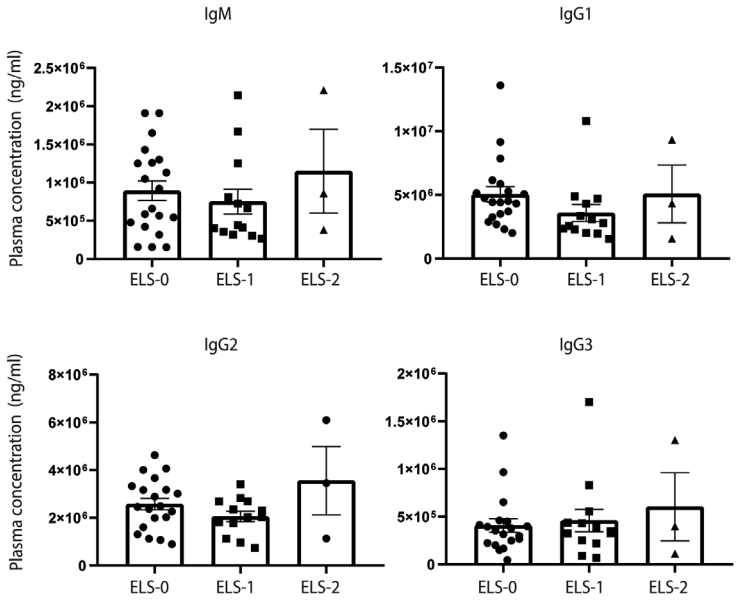

Figure 4. High levels of pre-HTx immunoglobulins are not associated with the development of ectopic lymphoid structures in epicardial tissue of the donor heart
Pre-HTx plasma levels of different immunoglobulin subtypes were measured and correlated with the presence of ELS in the transplanted heart at autopsy. No significant differences were observed between the levels of pre-HTx immunoglobulins and ELS stage. *ELS: ectopic lymphoid structures. For plasma samples ELS-0 n=20, ELS-1 n=13, ELS-2 n=3, for tissue lysates ELS-0 n=31, ELS-1 n=25, ELS-2 n=5. * p<0.05, ** p<0.01, *** p<0.001.*

DISCUSSION

To improve survival of patients after cardiac transplantation, it is important to understand the pathophysiological mechanism of post-HTx morbidities that affect long-term survival, like CAV and antibody-mediated rejection. Local production of immunoglobulins in the donor heart, possibly produced by ELS, is a local rejection process within the transplanted heart[10]. Also systemically, high serum levels of immunoglobulins have been described in CAV patients, despite immunosuppressive therapy[21]. Hence, it is known that cardiac antibodies exist in patients with end-stage HF, already prior to transplantation[22], thereby indicating that in some patients high levels of immunoglobulins already exist before transplantation that might affect post-HTx outcome. Therefore, in this study, we investigated whether antibody levels prior to HTx are associated with post-HTx outcome.
We demonstrated a significant correlation between pre-HTx immunoglobulin levels and survival post-HTx, as high levels of IgG1 or IgG2 were associated with a shorter survival time after HTx. Because the patient cohort includes a relatively large group of patients who died within a few days after transplantation the observed association might be influenced by these short survival patients. Consistently, also in subgroup analysis of patients that survived at least 1 month, this correlation was present, as 1 month post-HTx immunoglobulin levels

also correlated with survival. This might indicate that high immunoglobulin levels before and after HTx might affect survival of the cardiac graft. In patients with an activated adaptive immune response before transplantation, immune reactions against the donor heart might be more severe, leading to a more rapid dysfunction of the graft.

Next, we studied levels of circulating immunoglobulins from the time of transplantation until 6 months post-HTx. One month after HTx, antibody levels were significantly lower compared to pre-HTx levels. This effect is probably explained by the high dose of immunosuppressive therapy given early after transplantation[23]. This is in line with other studies, demonstrating decreased levels of IgG early after transplantation, and thereby, increasing the susceptibility for severe infections[24,25]. Hence, patients with high immunoglobulin levels prior to HTx maintained relatively high levels after transplantation in comparison to patients with low pre-HTx immunoglobulin levels. This observation is in line with the results of a previous study in lung transplant patients[26]. This suggests that once the adaptive immune system is activated pre-HTx, it remains relatively active after HTx despite immunosuppressive therapies.

In addition to plasma, the levels of immunoglobulins were also measured in epicardial tissue of the donor heart upon autopsy post HTx. Interestingly, patients who died in the first 3 years post-HTx showed significantly increased levels of immunoglobulins in the transplanted heart compared to patients with a survival of more than 10 years. This observation is in line with the observations in plasma and suggests that an activated adaptive immune system has an adverse effect on graft survival.

To assess whether high levels of immunoglobulins pre-HTx may be associated with a certain phenotype of CAV after transplantation, patients were divided into the different H-CAV categories. Increased pre-HTx plasma levels of IgG1 and IgG2 were found in patients with H-CAV 1, the inflammatory phenotype of CAV. In addition, epicardial tissue of the donor heart showed an increase in IgG1, IgG2, and also IgM upon autopsy in this CAV 1 type. Previous studies already showed an association between circulating inflammatory markers, such as C-reactive protein (CRP), and CAV development[21]. Our results suggest that an activated immune system pre-HTx may influence inflammation in the CAV lesion and thereby the more rapid development of these lesions.

In contrast with CAV phenotype, no clear association was found between circulating pre-HTx immunoglobulin levels and ELS stage. ELS mostly develop due to chronic inflammation and are effective sites of local production of auto-antibodies[27]. Our findings suggest that the formation of ELS in the donor heart is not clearly influenced by the degree of activation of the immune system before the transplantation, but are more likely to be formed by the recipient as a tertiary lymphoid organ to eliminate the persistent antigenic stimulation[27].

The results of this study may have possible consequences for a more personalized approach in immunosuppressive therapy. Possibly patients with an active immune system with high pre-transplantation immunoglobulin levels need a more aggressive immunosuppressive approach than patients with a relatively inactive immune system. This might be an interesting topic for future patient studies.

Our study is limited by the relative small patient group. Therefore our results need to be confirmed in future studies with larger patient groups. Because our cohort was based on

the availability of autopsy, our cohort contained a relatively large percentage of patients with a short survival, thereby not reflecting the average survival after transplantation.
In conclusion, increased immunoglobulin levels prior to HTx are associated with worse survival post-HTx. Also immunoglobulin levels in cardiac tissue after transplantation correlate with outcome. IgG1 and IgG2 levels in pre-HTx plasma and in post-HTx cardiac tissue were significantly increased in patients with an inflammatory H-CAV 1 type. This suggests that an activated immune system before transplantation probably has an adverse effect on graft survival after transplantation and that patients might benefit from a more personalized approach in immunosuppressive therapy.

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Conflicts of interest
None declared.

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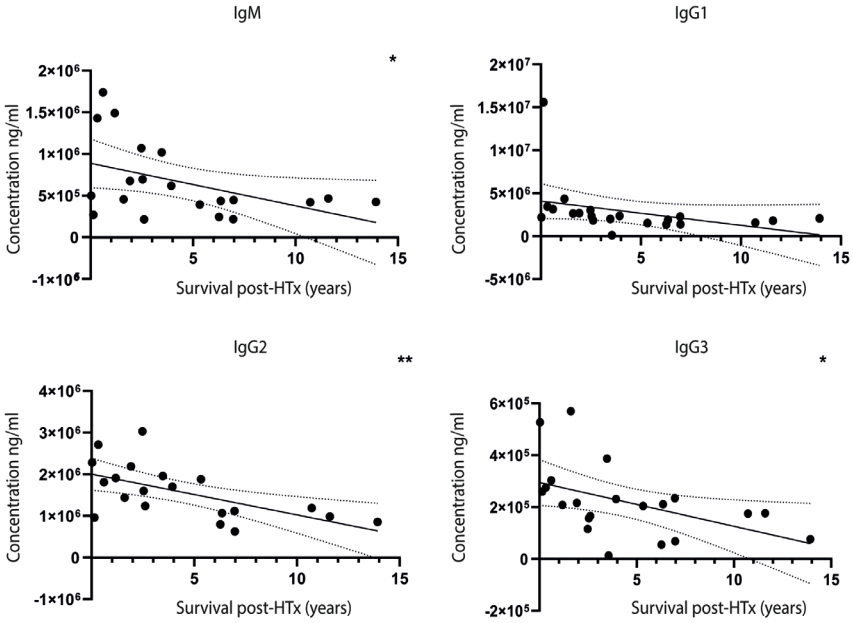
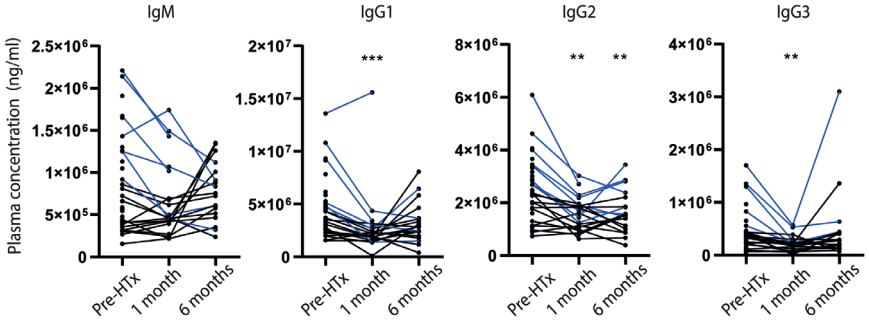
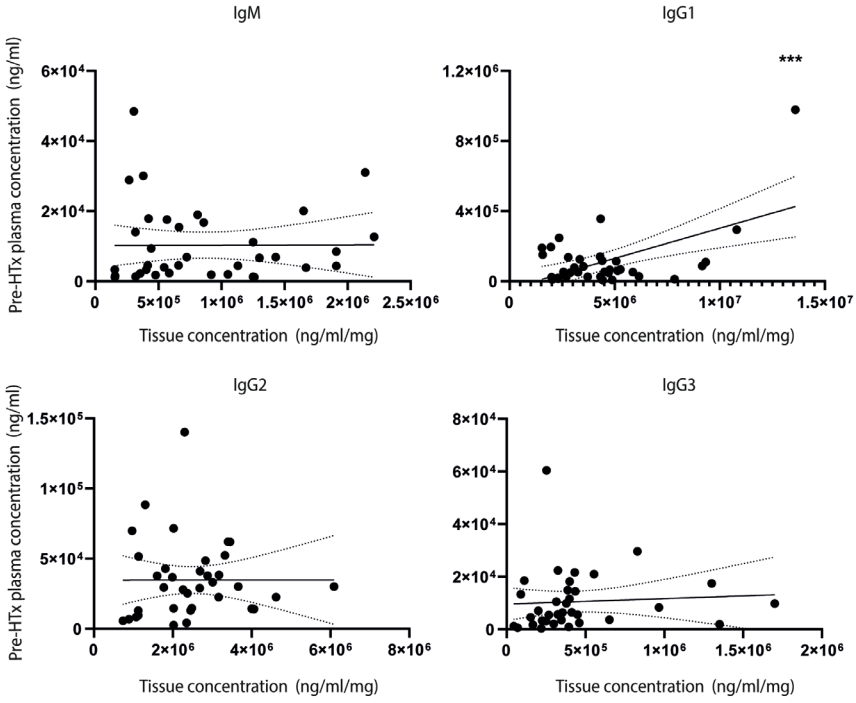
SUPPLEMENTARY FIGURES



Supplementary Figure 1. 1 month post-HTx circulating immunoglobulin levels are associated with survival post-HTx
In the subgroup of patients with survival of ≥1 month, plasma levels of different immunoglobulin subtypes were measured 1 month post-HTx using multiplex immunoassay and correlated with survival post-HTx. Levels of IgM, IgG2, and IgG3 were significantly correlated with post-HTx survival, where high immunoglobulin levels resulted in a lower survival post-HTx. *HTx: heart transplantation. n=21. * p<0.05, ** p<0.01.*



Supplementary Figure 2. Plasma levels of immunoglobulins pre- and post-HTx
The levels of immunoglobulins in plasma were measured prior to HTx until 6 months post transplantation. The blue lines represent patients with high pre-HTx levels (above the mean concentration) and the black lines represent patients with low pre-HTx levels (below the mean concentration). Levels of IgG1, IgG2, and IgG3 were significantly decreased after 1 month of HTx compared to pre-HTx levels. After 6 months, immunoglobulin levels seem to increase again, albeit not statistical significant. *HTx: heart transplantation plasma samples pre-HTx n=36, 1 month n=21, 6 months n=19. ** p<0.01, ** p<0.01.*



Supplementary Figure 3. Correlation of pre-HTx immunoglobulin levels and post-HTx immunoglobulins in epicardial tissue of the donor heart

Immunoglobulin levels in plasma pre-HTx were measured and correlated with immunoglobulin levels in the epicardium of the transplanted heart at autopsy. IgG1 showed a significant correlation between pre-HTx plasma levels and post-HTx tissue levels, where a high level of IgG1 pre-HTx was associated with high IgG1 levels in the donor heart. No significant correlation was found for IgM, IgG2 and IgG3. *HTx: heart transplantation. For plasma samples and tissue lysates n=36. ** p<0.01.*

SUPPLEMENTARY TABLES

Supplementary Table 1. Baseline patient characteristics of patients with myocardial tissue lysates (all patients)

Characteristics	value	Percentage / STDEV
Number of patients	61	
Gender		
Male	47	78%
Female	14	23%
Patient age (years)	46	±13
LVAD pre-HTx	19	31%
Survival post-HTx (years)	6.2	±7.1
Primary cardiac diagnosis		
ICM	28	46%
DCM	23	38%
HCM	3	5%
RCM	2	3%
Other	4	7%
H-CAV		
H-CAV 0	12	20%
H-CAV 1	8	13%
H-CAV 2	23	37%
H-CAV 3	18	30%
ELS		
ELS 0	31	51%
ELS 1	25	41%
ELS 2	5	8%

LVAD: left ventricular assist device, HTx: heart transplantation, ICM: idiopathic cardiomyopathy, DCM: dilated cardiomyopathy, HCM: hypertrophic cardiomyopathy, RCM: restrictive cardiomyopathy, H-CAV: histological cardiac allograft vasculopathy phenotype, ELS: ectopic lymphoid structures

Supplementary Table 2. Baseline patient characteristics of subgroup of patients with available plasma samples

Characteristics	**value**	**Percentage / STDEV**
Number of patients	36	
Gender		
Male	29	81%
Female	7	19%
Patient age	46	±14
LVAD pre-HTx	14	39%
Survival post-HTx (years)	2.6	±3.7
Primary cardiac diagnosis		
ICM	15	41%
DCM	13	36%
HCM	2	6%
RCM	2	6%
Other	4	11%
H-CAV		
H-CAV 0	10	28%
H-CAV 1	5	14%
H-CAV 2	14	39%
H-CAV 3	7	19%
ELS		
ELS 0	20	56%
ELS 1	13	36%
ELS 2	3	8%

LVAD: left ventricular assist device, HTx: heart transplantation, ICM: idiopathic cardiomyopathy, DCM: dilated cardiomyopathy, HCM: hypertrophic cardiomyopathy, RCM: restrictive cardiomyopathy, H-CAV: histological cardiac allograft vasculopathy phenotype, ELS: ectopic lymphoid structures

Part two

Antibody-mediated immune responses in myocarditis

6

Heart failure in chronic myocarditis: a role for microRNAs?

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ABSTRACT

Myocarditis is an inflammatory disease of the heart, which can persist over a long time. During this time, known as the chronic phase of myocarditis, ongoing inflammation damages the cardiomyocytes. The loss of cardiac cells culminates in the development of dilated cardiomyopathy, often followed by non-ischemic heart failure due to diminished cardiac function. During the course of the disease, expression levels of non-coding small RNAs, called microRNAs (miRNAs), change. Although mainly studied in the acute setting, some of these changes in expression level appear to persist in the chronic phase. In addition to being a much-needed diagnostic tool, these miRNA could provide new treatment options. miRNA-based intervention strategies already showed promising results in the treatment of ischemic cardiovascular diseases in preclinical animal models. By implementing more knowledge on the role of miRNAs in the progression towards heart failure, this can potentially be used in the development of miRNA-based therapeutic interventions in the treatment of myocarditis and thereby preventing the progression towards heart failure. The first part of this review will focus on the natural course of myocarditis and the progression towards heart failure. Secondly, we will discuss the current knowledge on alterations of miRNA expression patterns, and suggest some possible future interventions.

Myocarditis

Myocarditis is an inflammatory disease of the heart, which often results in heart failure or sudden cardiac death[1]. The disease mostly occurs in young, healthy people in the age of 20-51 years[2]. Estimations of the incidence of myocarditis are variable due to the non-specific symptoms, leading to underestimation of the disease. The incidence of myocarditis in cases with unexplained heart failure is estimated at 9,6%[2]. Bacterial infections, viruses, autoimmune diseases and other factors are able to induce myocarditis, with viruses being the most common cause[3,4]. Structural and functional damage of the myocardium, caused by these factors, activates the innate and adaptive immune response, which can lead to severe inflammation[5]. The immune response is eventually downregulated, however, myocardial inflammation can also persist. Persistent inflammation is characterized by an ongoing damage to the cardiomyocytes and ultimately results in non-ischemic heart failure[6]. In 30% of the cases, dilated cardiomyopathy (DCM) occurs, which is a major cause of heart failure and an important indication for cardiac transplantation[7]. Hence, the treatment of myocarditis is difficult due to late diagnosis and irreversible damage that has occurred[8]. Final clinical outcome of the disease is depending on the host response, the amount of irreversible damage, and the use of therapeutic interventions[9,10]. Patients with severe acute myocarditis have a better prognosis, while patients with moderate chronic myocarditis are more prone to develop heart failure[1,2].

Phases

Myocarditis is a three-phase process, consisting of (1) an acute phase, (2) a sub-acute phase and (3) a chronic phase. In the (1) acute phase (first 3-4 days), infection is inducing cardiomyocyte damage via the induction of apoptotic signaling pathways and the release of proteolytic enzymes[3,11]. This leads to the activation of the immune system and the production of pro-inflammatory cytokines, such as interferon-γ (IFN-γ), interleukins-1,-6 and -12 (IL-1, IL-6, IL-12) and tumor necrosis factor-α (TNF-α)[1,12]. Classical activated macrophages type 1 (M1) become activated, which have pro-inflammatory properties and further enhance the immune response[1]. In addition, up-regulation of adhesion molecules on endothelial cells leads to the recruitment of more immune cells, thereby activating the innate immune response[11,12].

In the (2) sub-acute phase (day 4-5), the innate immune response remains activated and immune cells are infiltrating into the heart[13]. Phagocytosis of dead cells and debris is initiated by monocytes, which augment the expression of pro-inflammatory cytokines[5]. Immune cells of the adaptive immune system, such as T cells and B cells, also accumulate in the infected heart[14]. Licensed cytotoxic T cells (CD8+) recognize virus-infected cardiomyocytes via the interaction and presentation of antigens loaded on major histocompatibility complex type 1 (MHC-I)[15]. The cytotoxic T cell directly kills the infected cardiomyocyte by releasing perforin and granzymes, triggering the caspase cascade and inducing apoptosis. Antigen presenting cells (APCs), like dendritic cells (DCs), take up the debris of dead cardiomyocytes and present the ingested antigens on MHC-2[16]. T-helper cells (CD4+) are able to recognize these presented antigens via the interaction of the T-cell receptor and the presented antigen loaded on MHC-II. This subsequently leads to the activation of T-helper cells, which can license cytotoxic

T cells to kill infected cells or activate B cells, which produce neutralizing antibodies[16]. During the sub-acute phase, the immune response not only eliminates infected and dead cells, but also significantly contributes to irreversible damage of the myocardium by damaging of healthy cardiomyocytes[1].
In the (3) chronic phase (day 14), repair and remodeling of the myocardium is initiated[1,12]. Regulatory T cells (CD4+CD25+FoxP3+) respond to the production of IL-2 by T-helper cells and start proliferating rapidly[1]. Anti-inflammatory cytokines are being produced, such as transforming growth factor-beta (TGF-β) and IL-10, to down-regulate the immune response and to reduce cardiac damage[11,12]. Secondly, an alternative type of macrophages becomes activated, macrophage type 2 (M2). This type of macrophage produces more TGF-β and IL-10 to reduce inflammation and to stimulate repair mechanisms[17]. Fibroblasts start to proliferate and differentiate into myofibroblasts, which contain contractile properties like smooth muscle cells, to replace the lost cardiomyocytes[18]. Fibroblasts and M2-macrophages produce collagen to restore the extracellular matrix and form a permanent scar[1,12]. In some cases, the immune system fails to completely clear all the infected cells or auto-reactive immune cells persist in the myocardium, resulting in chronic inflammation[2,9]. The chronically activated immune system produces cytokines, which activates matrix metalloproteases (MMPs) that can digest interstitial collagen and elastin. Additionally, pro-fibrotic factors are produced and thereby facilitate the dilatation of the heart[11], which can lead to dilated cardiomyopathy (DCM), an irreversible disease with systolic and diastolic dysfunction[7,19].

Diagnosis

Clinical manifestation of myocarditis includes a broad spectrum of non-specific flu-like symptoms and signs of myocardial infarction[20]. Clinical presentation is usually not sufficient for the diagnosis of myocarditis. Myocarditis is considered in young patients with rapidly progressive cardiomyopathy, arrhythmias and acute myocardial-infarction-like symptoms in combination with normal healthy coronary arteries[20]. In these cases, symptoms often mimic myocardial infarction due to segmental wall abnormalities and elevated levels of troponin and creatine kinases found in blood samples[8]. In patients with acute myocarditis, concentrations of troponin I and T are more elevated than creatine kinase[21]. In addition, inflammatory markers such as C-reactive protein and the number of circulating leukocytes can be increased[5]. In sub-acute and chronic myocarditis, anti-cardiac auto-antibodies and immunoglobulins can be detected in blood samples[19]. High levels of IgM are an indication for the presence of an active virus and can be used to determine the possible cause of myocarditis[11].
Electrocardiogram (ECG) is widely used as one of the diagnostic tools for the diagnosis of myocarditis, although it has a low sensitivity of only 47%[22]. Most of the patients with (acute) myocarditis show abnormalities in their ECG[6,23], which often mimics the ECG of a patient with a myocardial infarction[20]. Although ECGs can vary between patients, they often show non-specific T-waves, ventricular arrhythmias, PQ-segment depressions, and ST-segment changes by which high levels of Q waves are associated with higher rates of deaths and transplantations[5].
When myocarditis is suspected, an endomyocardial biopsy (EMB) is taken and analyzed to

detect presence of a virus via immunohistological techniques. Diagnosis of these histopathological analysis is based on the Dallas-criteria, which indicates myocarditis if inflammatory cellular infiltrates with or without associated cardiomyocyte necrosis is present[24]. However, the interpretation of different clinicians and the lack of prognostic values make these criteria not very sensitive or conclusive[19].

The most effective tool in the diagnosis of myocarditis is cardiac magnetic resonance imaging (CMR), which is able to distinguish ischemic and non-ischemic cardiomyopathy[21]. It makes use of different parameters such as gadolinium late enhancement (LGE) and transmural enhancement (TE). High levels of LGE are an indication of myocardial injury and can be used to distinguish between myocardial infarction and myocarditis[21]. In myocarditis, the elevated LGE levels are more diffuse and nodular, whereas myocardial infarction shows a smaller distribution[3]. T1-weighted-CMR marks capillary leakage and T2-weighted-CMR images are able to mark interstitial and extracellular edema[9,21]. Interstitial edema occurs during the inflammatory response and can be predictive for myocarditis. A combined approach, using T2-weighted-CMR images and LGE parameters, increases the accuracy of the diagnosis and assessment of myocarditis patients[25].

Non-ischemic heart failure

Heart failure is a complex clinical syndrome that can result from an abnormal cardiac structure or function leading to failure of the heart to deliver oxygen and nutrients to metabolizing tissues[26]. According to the etiology and pathophysiology, heart failure can be categorized in ischemic or non-ischemic and in anatomical designations as hypertrophic and dilated. Of these, dilated cardiomyopathy (DCM) is the most prevalent form of non-ischemic cardiomyopathy with a relative poor overall prognosis of 25% mortality in 5-years[27]. DCM is predominantly defined by left ventricular dilatation and reduced cardiac contraction in the absence of coronary artery disease and abnormal loading conditions. Amongst other secondary causes, e.g. chemotherapy, neuromuscular disease and alcohol abuse, myocarditis can result in DCM and eventually heart failure[7,28].

Early diagnosis of DCM is hindered by the fact that the majority of patients are asymptomatic in early stages of the disease, therefore patients often present themselves in the late stage of the disease with symptoms of exercise intolerance, breathlessness and edema[26]. Additionally, in some of these patients the conduction system is affected that lead to arrhythmias and an increased risk for sudden cardiac death. For diagnosis of DCM, laboratory test and imaging techniques are important to define disease severity. Imaging often show dilated cardiomyopathy, with general wall motion abnormalities and MRI using gallium-late-enhancement could show diffuse damage throughout the heart[21]. Since fast initiation of treatment is beneficial for the prognosis of heart failure, a clear diagnosis of DCM and also the contributing etiology should be known to give the best available therapy[27]. Accordingly, taking cardiac biopsies is indicated in patients with DCM from unknown etiology, and surprisingly, in 9-16% of patients presenting with DCM, traces of prior myocarditis are observed[7].

After diagnosis of heart failure, all patient with DCM are treated with standard heart failure therapy such as ACE-inhibitors, mineralocorticoid receptor antagonist, diuretics and

β-blockers[10,26,29]. The primary aim of this treatment is to relieve symptoms of heart failure, to improve survival and to prevent ongoing remodeling and worsening of heart function[26,29]. For myocarditis induced DCM, additional immunosuppressive drugs are being used[3], however this is not beneficial for viral myocarditis and can even cause harm when used for this purpose[12].
Despite poor overall prognosis and lack of specific treatments, 25% of DCM patients with recent onset heart failure will undergo reverse remodeling and demonstrate a (partially) improved cardiac function[30]. The ability to recover is observed in the majority of dilated cardiomyopathy etiologies, including myocarditis. The fact that there is a certain degree of recovery and reversibility in cardiac function, attention should be focused on inducing this process to guide improvement.
Since prominent roles have been uncovered for miRNAs in the treatment of cardiovascular disorders[31], miRNAs might also have a potential role in treatment of myocarditis and reversibility of myocarditis induced-DCM.

The role of miRNAs

microRNAs (miRNAs, miR) are small non-coding RNAs with a length of approximately 20-24 nucleotides, which are involved in the post-transcriptional regulation of protein expression by binding messenger RNAs (mRNAs)[32]. miRNAs are able to use two silencing mechanisms to down-regulate specific target genes: degradation or translational repression of the mRNA, depending on the complementarity with the target gene. If the miRNA and their target gene are complementary, the mRNA will be cleaved and degraded. If the miRNA and the target gene are not perfectly complementary, miRNAs suppress translation of the target gene, however without affecting the stability of the mRNA. miRNAs can be directly produced by cells themselves or secreted via e.g. microvesicles, such as exosomes[33]. miRNAs are involved in many processes, such as cell proliferation and apoptosis but also in the regulation of the immune response. Emerging evidence is found for the contribution of miRNAs in myocardial pathological processes by regulating angiogenesis, apoptosis and differentiation of cardiomyocytes[34,35]. The expression of certain miRNAs changes during cardiac disease and heart failure, which makes them interesting targets in the potential treatment of cardiovascular diseases, such as myocarditis[32,36].

Role of miRNAs in myocarditis

The role of miRNAs in human myocarditis is not fully elucidated. Currently, only a couple of miRNAs have been identified, which might be correlated with viral myocarditis, especially during the acute phase of myocarditis. Two identified microRNAs, miR-208b and miR-499, are elevated in viral myocarditis and can be detected in plasma of myocarditis patients[37]. miR-208b is expressed by cardiomyocytes and is involved in pathological processes, such as cardiac growth, fibrosis and inflammation by increasing MHC-expression[35]. Upregulation of miR-208b induces adverse cardiac remodeling. miR-499 is also expressed by cardiomyocytes and upregulated during cardiac disease[38]. Both of these microRNAs are released upon myocardial damage and can potentially be used in the diagnosis of myocarditis to determine the severity of the disease[37]. Elevated levels of miR-499 can be

detected in all patients, whereas the expression of both miR-208b and miR-499 was only found in fulminant virus-induced myocarditis[37]. These miRNAs, however, are not specific for myocarditis but probably reflect myocardial injury in general, as was observed for ACS patients[39].

miRNAs involved in acute myocarditis

Current research is focusing on miRNA screenings in the different stages of myocarditis to identify additional microRNAs involved in the pathogenesis of the disease. In myocarditis patients, a cardiac miRNA profile was identified involved in the acute viral phase and the inflammatory phase of acute myocarditis. The study of Corsten *et al.* showed that, for example, miRNA-155 is highly expressed in cardiac tissue of myocarditis patients[40]. miR-155 was found to be up-regulated in cardiac tissue in both human and mice with viral-induced myocarditis[40]. miR-155 is known to be pro-inflammatory and involved in multiple processes, such as immune cell functioning, and is expressed by inflammatory cells especially in the acute inflammatory phase of myocarditis[41]. In virus-induced myocarditis mice models, blockage of miR-155 by antagonists showed attenuated cardiac inflammation and less necrosis[41]. These results indicate that miR-155 plays an important role in the inflammatory response of viral-induced myocarditis. Next to miR-155, miR-21 and miR-146b were found to be upregulated in myocarditis patients, which also have a central role in immune activation and inflammation[40]. Silencing of these miRNAs by specific inhibitors showed a strong attenuation of myocarditis in viral-induced myocarditis mice[42]. The exact role of these miRNAs in the pathology of myocarditis is not elucidated, however, it is shown that the expression of miR-21 and miR-146b is correlated with IL-17 expression[42]. These findings suggest that miR-21 and miR-146b are involved in the regulation of Th17 differentiation and thereby control autoimmunity. The function of RORct, a transcription factor of Th17 differentiation, is enhanced by increased expression of these miRNAs, which leads to the differentiation of mature T cells towards Th17 cells[42]. Furthermore, miR-21 is also involved in interstitial fibrosis and cardiac hypertrophy[43]. Interestingly, other studies showed contradictory results as significantly decreased levels of miR-21 expression were found. The expression of miR-21 in the myocardium in coxsackievirus B3 (CVB3)-myocarditis mice was significantly reduced and a negative correlation was found between the severity of myocarditis and the expression of miR-21[44]. When mice were treated with miR-21 precursors, the myocarditis was alleviated and apoptosis was reduced, indicating that miR-21 also has a protective role by regulating programmed cell death 4 (PDC4)-mediated apoptosis[44]. Based on the different outcomes of these studies, it might be that the role of miR-21 in myocarditis depends on the temporal and spatial expression of its targets.

In one study, additional elevated miRNAs, including miR-511 and miR-212[40], were found in patients with acute viral myocarditis. miR-511 functions as a positive regulator of Toll Like Receptor-4 (TLR) signaling, whereas miR-212 is involved in the hypertrophic responses of cardiomyocytes[40,45].

Next to upregulated miRNAs there is also evidence that miRNAs are involved by their down-regulation. Anti-inflammatory miR-106a and miR-93 were downregulated in the acute phase

of human myocarditis[40]; both are involved in anti-inflammatory responses by inhibiting the production of inflammatory cytokines[43,46].

miRNAs involved in chronic myocarditis

Most research thus far was focused on miRNAs involved in the acute phase of myocarditis, however, less is known about miRNAs involved in the chronic phase of the disease. Inflammatory miRNAs and miRNAs associated with cardiac damage, involved in the acute phase of myocarditis, might remain affected and involved in the chronic phase, stimulating the progression of the disease[7]. Recently, miR-21 was found to be involved in the progression of viral myocarditis towards dilated cardiomyopathy[47]. These findings indicate that changes in miR-21 expression might contribute to the progression of myocarditis to dilated cardiomyopathy. In addition, elevated levels of miR-208b and miR-499 are also found in later phases after viral-induced myocarditis in patients and in patients with dilated cardiomyopathy[48]. Besides miR-21, miR-208b and miR-499 no specific miRNAs involved the chronic phase of myocarditis are identified, however, it might be that inflammatory and damage-associated miRNAs in the acute phase are also persistently expressed in the chronic phase of myocarditis.

Overall, miRNAs involved in inflammatory responses are dysregulated and muscle specific miRNAs (myomiRs) are mostly upregulated during human myocarditis (*Table 1*). Since the chronic phase of myocarditis can progress into heart failure, it is also hypothesized that miRNAs involved in the development of (non-ischemic) heart failure might also be involved.

Role of miRNAs in (non-ischemic) heart failure

microRNAs are involved in the pathogenesis and progression of heart failure[49]. It is known that the heart responds to cardiac injury by activating signaling pathways, which leads to remodeling and hypertrophy of cardiomyocytes[50]. Eventually, myocardial fibrosis and dilation of the left ventricle can result in heart failure. Multiple patterns of miRNAs, which are consistently aberrantly expressed, are identified in ischemic heart failure patients[51–54]. For example miR-21, -22, -23, -146, -195, -199 and 499 are found to be upregulated in heart failure patients, whereas miR-1, -29, -133 and -150 are found to be downregulated[55,56]. Clinical studies showed that reactivation of fetal gene expression patterns are induced in failing hearts, which results in an altered contractile function of the heart[51].

miRNAs involved in inflammatory responses, leading to non-ischemic heart failure, are still subject of investigation. Currently, some potential miRNAs have been identified in patients with non-ischemic heart failure, such as miR-21, miR-146a/b, -155, -423-5p, and the miR-17-92 cluster[57–59]. Furthermore, miR-200b, -519, -520d, -558, and -622 are identified as biomarkers for non-ischemic heart failure with reduced ejection fraction (HF-REF), however, these miRNAs still have to be validated in other studies[58]. Some of the miRNAs that have been identified in heart failure patients are also aberrantly expressed in myocarditis patients *(Table 1*), thereby pointing to a potential role of these miRNAs in the progression of myocarditis towards heart failure. However, additional research has to be performed to verify these miRNAs and to identify whether additional myocarditis-specific miRNAs can be identified and are specifically involved in the progression towards heart failure (*Figure 1*).

Therapeutic options using miRNA-based interventions

In addition to current used therapeutics, miRNA-based interventions can potentially be used for the treatment of cardiovascular diseases, thereby including myocarditis. miRNA expression can therapeutically be manipulated via different mechanisms, having promising results in animal models[31] but also already in phase I clinical trials for hepatitis C virus (HCV) infection[60]. One approach that can be used is the inhibition of specific miRNAs involved in disease progression by using modified antisense oligonucleotides (antimiRs)[61,62]. antimiRs are able to inhibit miRNA function through complementary base pairing with their corresponding miRNA[31] and, as a result, the miRNA is inhibited and the target mRNA expression is restored.

In addition, miRNA mimics (pre-miRs) can be used to elevate the expression of certain miRNAs[50]. miRNA mimics consist of a synthetic double stranded structure of oligonucleotides, which is complementary to the miRNA sequence[63]. In situations in which decreased levels of miRNAs are causing a disease, miRNA mimics can be used to restore miRNA expression. Although these approaches are promising, their organ selectivity is still limited and improved targeting or local delivery is essential[64].

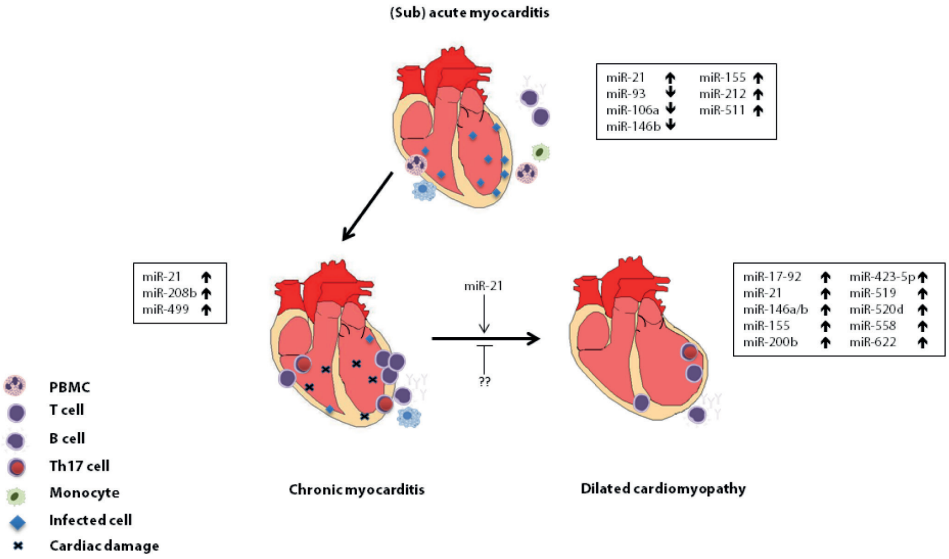

Figure 1. Different stages of myocarditis and the progression towards dilated cardiomyopathy
In the (sub)-acute phase of myocarditis, infected cells are recognized by immune cells and inflammation is initiated. The immune response can either be downregulated or infected cells persist, which leads to chronic myocarditis. Chronic inflammatory reactions are causing damage to the myocardium, which is further enhanced by autoimmune responses. Ultimately, chronic myocarditis can progress towards dilated cardiomyopathy (DCM). Multiple miRNAs have shown to be involved in the different phases of myocarditis and DCM, however, miRNAs involved in the progression of myocarditis towards heart failure are not identified yet. These miRNAs can be of great importance for developing potential therapeutic interventions.

Table 1. microRNAs involved in myocarditis and (non-ischemic) heart failure

	microRNA	Expression	Function	Reference
Acute myocarditis	miR-21	↑	Interstitial fibrosis, cardiac hypertrophy, immune activation	40–43
	miR-21	↓	PDC4-mediated apoptosis	43,44
	miR-93	↓	Inhibition pro-inflammatory cytokine production	40,43,46
	miR-106a	↓	Inhibition pro-inflammatory cytokine production	40,43,46
	miR146b	↑	Immune activation TH-17 differentiation	40–42
	miR-155	↑	Pro-inflammatory immune cell functioning	40,41
	miR-212	↑	Cardiomyocyte hypertrophy	38,40
	miR-511	↑	TLR signaling	38,40
Chronic myocarditis	miR-21	↑	Interstitial fibrosis, cardiac hypertrophy, immune activation, progression DCM	47
	miR-208b	↑	Myocardial damage, fibrosis and dilatation	35,37
	miR-499	↑	Myocardial damage	37, 38
(Non-ischemic) heart failure	miR-21	↑	Interstitial fibrosis, cardiac hypertrophy, immune activation, progression DCM	50
	miR-17-92	↑	Immune cell proliferation, cardiac development	43,52
	miR-146a/b	↑	Immune activation TH-17 differentiation	52
	miR-155	↑	Pro-inflammatory immune cell functioning	41,52
	miR-423-5p	↑	Biomarker of heart failure	37,58,59
	miR-200b,-519,-520d, -558, -622	↑	Biomarkers non-ischemic heart failure	58

CONCLUSION

Many studies demonstrated the role of miRNAs in the development and progression of cardiac diseases, thereby recently also including myocarditis. Aberrantly expressed miRNAs are mostly examined in the acute phase of myocarditis, whereas only a few miRNAs are studied in the chronic phase of myocarditis. Since the chronic phase often progress into heart failure, the question remains whether myocarditis specific miRNAs are involved. By studying miRNAs involved in the progression of chronic myocarditis towards heart failure, potential miRNA-based therapeutic approaches can be developed. Modulation of miRNA expression can be a promising strategy in the treatment of chronic myocarditis by limiting inflammation and cardiac damage and thereby preventing non-ischemic heart failure.

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Conflict of Interest
The authors declare that there is no conflict of interest.

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7

Short communication

Experimental autoimmune myocarditis in mice: Limitations and future perspectives

In preparation

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ABSTRACT

Background

Chronic inflammation is a key driver of adverse cardiac remodeling in heart failure (HF). In the context of a highly inflammatory environment, the release of cardiac proteins by damaged cardiomyocytes may break immune tolerance, which leads to the production of cardiac autoantibodies, as observed in the circulation and myocardium of end-stage HF patients. These autoimmune responses closely resemble immune responses as observed in inflammatory cardiomyopathy, such as myocarditis. Different mouse models have been developed to induce myocarditis to study disease progression. However, suitable *in vivo* mouse models to study the role of cardiac-specific epitopes and autoimmune responses in the development of HF are still lacking. Therefore, we set out to reproduce the classical experimental model of autoimmune myocarditis (EAM) as a representative model of autoimmune-mediated HF with the aim to adapt this model to study the role of cardiac-specific antigens in HF progression.

Methods and results

BALB/c mice were subcutaneously injected with an α-myosin heavy chain (α-MHC) peptide, emulsified with Complete Freund Adjuvant (CFA) to induce EAM. Interestingly, similar to what we observe in HF patients, mice immunized with the cardiac peptide specifically showed increased levels of IgG1 and IgG3. However, despite increased IgG titers, we did not observe any signs of adverse cardiac remodeling, cell death, increased influx of inflammatory cells, or a decrease in cardiac function.

Conclusion

Increased antibody titers after a successful immunization with α-MHC do not necessarily lead to the development of EAM. The development of EAM is a multifactorial process and additional stimuli might be needed to promote antigen release and to induce autoimmune-mediated responses and HF progression.

INTRODUCTION

HF is still the leading cause of mortality worldwide, affecting approximately 26 million people[1]. An important hallmark of HF progression is adverse cardiac remodeling, which is characterized by a chronic inflammatory response[2,3]. The adaptive immune response is a key mediator in adverse cardiac remodeling and HF progression[4,5]. High levels of circulating immunoglobulins. including IgG1 and IgG3, cardiac IgG deposits and signs of complement activation have been found in patients with different etiologies of end-stage HF[6,7]. In the context of a highly inflammatory environment, the exposure of cardiac proteins by damaged cardiomyocytes may break immune tolerance to self-proteins and activates auto-reactive T and B cells, subsequently leading to the production of cardiac-specific antibodies. It has been suggested that binding of these (auto) antibodies to myocardial tissue can induce cardiac dysfunction for instance by affecting contractility or by inducing cellular toxicity in cardiomyocytes, thereby accelerating HF progression[4,8].

The presence of cardiac-specific antibodies in end-stage HF closely resembles autoimmune features as observed in non-ischemic cardiomyopathies, such as myocarditis[9–12]. Myocarditis is an inflammatory condition of the cardiac muscle and is an important cause of dilated cardiomyopathy (DCM) or sudden cardiac death[13–15]. A persistent and self-sustained autoimmune response is thought to be the major cause of chronic damage, loss of cardiomyocytes, and the development of HF in myocarditis[4,10]. The exposure of intracellular proteins upon cardiomyocyte stress or death can activate autoreactive T and B cells[16].

Significant effort has been made to understand the immunological processes underlying the pathogenesis and importantly, the progression of HF. However, suitable and robust *in vivo* mouse models to study the role of cardiac-specific epitopes and autoimmune responses in the development of HF are still lacking. Different mouse models have been developed to induce myocarditis in order to study disease progression and to test potential therapeutic strategies, albeit with variations in the severity and reproducibility of the disease[17–19]. The classical model to study the effects of a pure autoimmune response in the development of myocarditis, without the contribution of a viral component, is the experimental autoimmune myocarditis (EAM) model. Therefore, in this study, we aimed to reproduce the classical EAM model to ultimately use this model as a basis to study autoimmune responses in HF progression, using other cardiac-related antigens that we have identified in patient material (*chapter 2 and 3*).

EXPERIMENTAL PROCEDURE

To induce EAM, male BALB/c mice (8-14 weeks old, weight 20-30 g) were randomized into treatment groups (*Figure 1*) and subcutaneously injected with 100 µg of α-MHC peptide (Pepscan, The Netherlands, sequence peptide: Ac-SLKLMATLFSTYASAD-OH) emulsified with 100 µg Complete Freund Adjuvant (CFA, Sigma, F5881) (*Figure 2A*). After 7 days, 100 µl of booster emulsion (100 µg α-MHC/100 µg CFA) was injected subcutaneously in the other flank of the mice. Control animals received PBS emulsified with CFA. Mice were fed with

standard chow *ad libitum* for 6 weeks. Animals were housed under standard conditions, with a 12 h light/dark cycle. The study was approved by the Ethical Committee on Animal Experimentation of the University Medical Center Utrecht and conformed to the 'Guide for the care and use of laboratory animals'. Myocardial deformation was measured by global longitudinal strain (GLS) using 3D-echocardiography (Vevo 2100 System, VisualSonics Inc, Toronto, Canada) at baseline, 14, 21, 35 and 42 days after immunization by a researcher blinded to treatment groups. Mice were euthanized after 6 weeks of follow-up using i.p. injected sodium pentobarbital (60.0 g/kg). Blood was collected using an orbital puncture in EDTA-coated tubes and centrifuged at 1,850 g for 10 min. Plasma was aliquoted and stored at -80 ºC for immunoglobulin measurements. The heart was flushed with phosphate-buffered saline (PBS), weighed, and fixed in 4% paraformaldehyde. Heart weight normalized to body weight or tibia length was used as a measure for cardiac hypertrophy. Sections of 3 µm were made with an electronic rotary microtome (Thermo Scientific, HM 340E) and general morphology of the cardiac tissue was visualized using hematoxylin and eosin (H&E) staining. Microscopic images were captured using light microscopy (Olympus Corporation). Plasma IgG1, IgG2a, IgG2b, and IgG3 levels were measured using a mouse antibody isotyping procartaplex assay (Thermofisher Scientific, EPX070-20815-901), according to manufacturer's protocol.

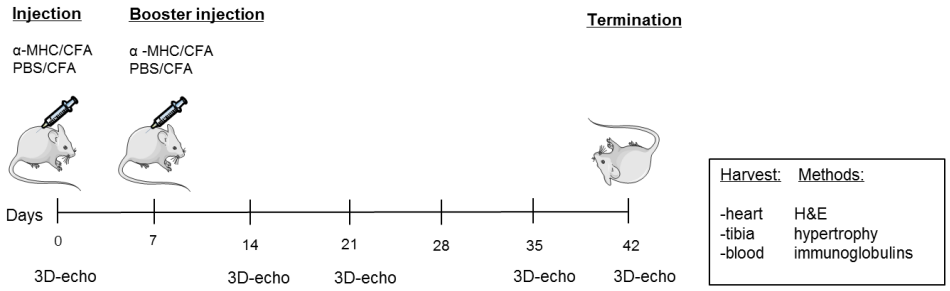

Figure 1. Experimental design
Overview of the EAM model. Mice were subcutaneously injected with 100 µg α-MHC/100 µg CFA emulsion or vehicle. After 7 days, 100 µl of booster emulsion was injected. 3D-echocardiography was performed at baseline, 14, 21, 35, and 42 days after the first immunization. After 6 weeks, mice were sacrificed and tissue and blood was collected for immunohistochemistry and immunoglobulin measurements.

RESULTS AND DISCUSSION

Upon injection of the stable α-MHC/CFA emulsion in the flank of the mice, a clear subcutaneous deposit was visible (*Figure 2B*). Six-weeks after primary immunization, plasma immunoglobulin levels were measured to ensure that autoantibodies were induced. In mice, IgG-switching reflects the polarization of the T-helper cell response, where class switching towards IgG1 and IgG3 upon immunization is specific for a Th2 response[20–22]. Indeed, we observed an increasing trend in IgG1 and IgG3 levels in mice immunized with α-MHC, as

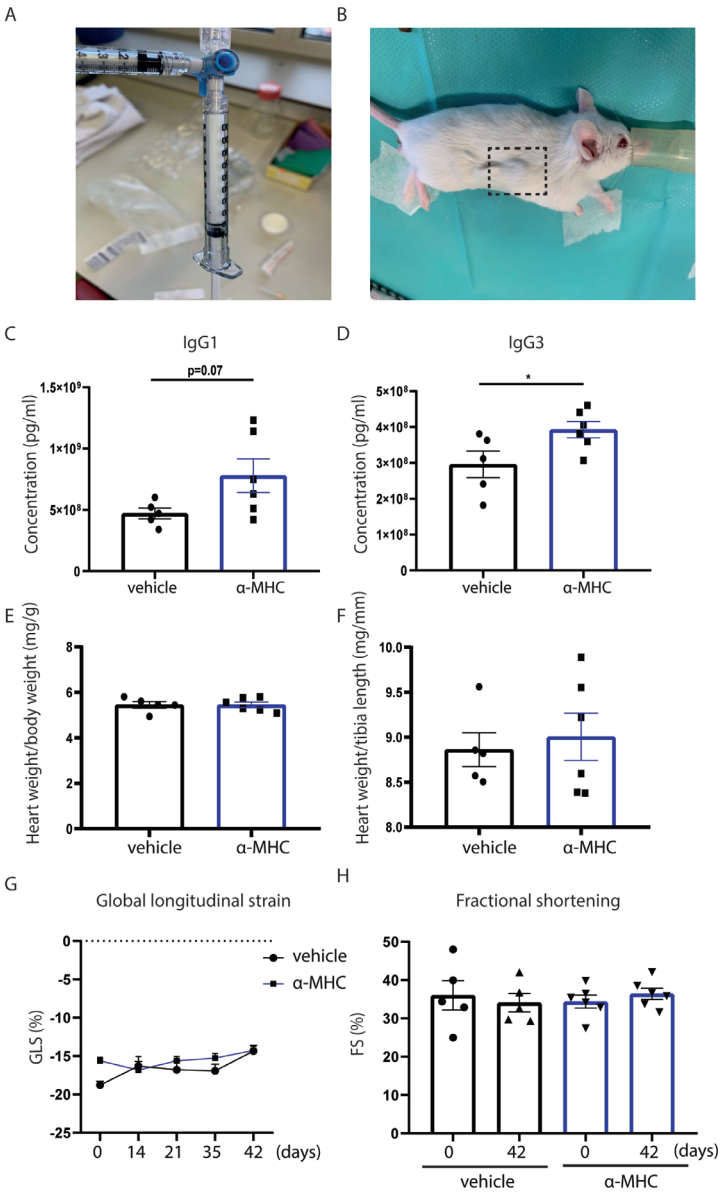

Figure 2. Increased IgG1 and IgG3 titers upon immunization with α-MHC
To induce EAM, mice were subcutaneously injected with α-MHC emulsified in CFA (A). After injection, a small depot of the emulsion was visible in the flank of the mice (B). 6 weeks after immunization, plasma immunoglobulin levels were significantly increased in mice injected with α-MHC/CFA as compared to PBC control animals (C-D). No differences were observed in heart weight/body weight or heart weight/tibia length ratio between de different groups (E-F). In addition, global longitudinal strain or fractional shortening did not differ significantly between EAM-induced mice or PBS treated animals (G-H). *EAM: experimental autoimmune myocarditis, CFA: Complete Freund's adjuvant, MHC: myosin heavy chain. PBS group n=5, α-MHC group n=6. * p<0.05*

compared to vehicle control animals (*Figure 2C-D*, for IgG1; $7.8x10^8$ vs $4.7x10^8$ pg/ml p=0.07, and IgG3; $3.0x10^8$ vs $3.9x10^8$ pg/ml p=0.05). These findings indicated that our immunization protocol was functional and suggests that an antibody-response specifically against α-MHC was induced compared to vehicle treated animals.

Following the acute phase of myocarditis, mice are expected to progress to chronic myocarditis, which is characterized by left ventricular dilation and impaired cardiac function from day 28[18,23]. To investigate dilation of the heart and signs of cardiomyopathy, we assessed heart weight/body weight ratio and heart weigh/tibia length ratio as a measure for cardiac hypertrophy (*Figure 2E-F*). However, despite the increase in IgG1 and IgG3, no differences were observed in heart weight/body weight ratio (5.44±0.14 vs 5.45±0.12mg/g) or heart weight/tibia length ratio (8.86±0.19 vs 9.0±0.26 mg/mm) 6 weeks after immunization with α-MHC. In addition, we also did not detect any effect on functional parameters, like global longitudinal strain (GLS, -14.35±0.78 vs -14.25±0.53%) and fractional shortening (FS, 34.09±2.39 vs 36.41±1.49%), which were similar between the α-MHC and vehicle treated groups (*Figure 2G-H*). Based on these findings, we can conclude that immunization with α-MHC/CFA did induce the production of IgG1 and IgG3, but did not induce cardiac remodeling or myocardial deformation up to 6 weeks after immunization. This is in sharp contrast with other published studies, where severe deterioration of systolic and diastolic

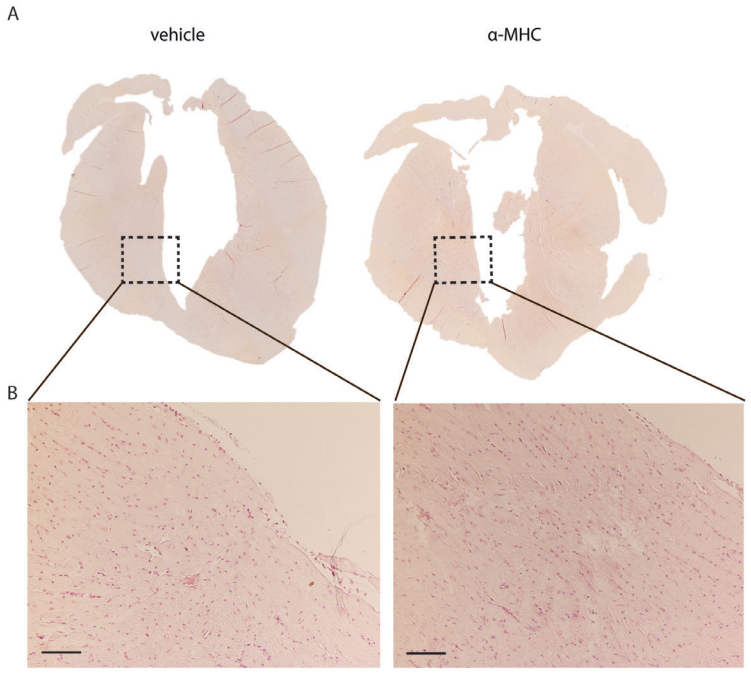

Figure 3. No signs of cardiac inflammation, fibrosis or left ventricular dilation in EAM-induced mice
General morphology was assessed by H&E staining, 6-weeks after the first immunization (A). EAM-induced mice did not show dilation of the LV compared to PBS controls. Moreover, no signs of infiltrating immune cells or cardiac fibrosis were observed in EAM-induced animals (B). *H&E: hematoxylin and eosin, MHC: myosin heavy chain. Magnification 10x, line bar indicates 200 µm.*

parameters, lower cardiac output, myocardial stiffness, and the progression to DCM at day 40-60 was observed[19,24].

Important characteristics of EAM are massive infiltration of immune cells in the myocardium between 14-21 days after first immunization, which is followed by progressive accumulation of fibrotic tissue and ventricular dilation[10,25]. To assess general morphology, as well as immune cell infiltration into the hearts, H&E staining was performed (*Figure 3A*). Similar to earlier observations, the left ventricle did not show any signs of dilation in α-MHC immunized mice. Moreover, we did not observe differences in immune cell infiltration or myocardial fibrosis in α-MHC immunized mice compared to vehicle controls (*Figure 3B*). It is known that inflammation of the cardiac tissue and massive infiltration of immune cells typically occurs 14-21 days after the first immunization, after which resolution of cardiac inflammation is initiated[18]. Therefore, it may be possible that immune cells are already cleared from the myocardium 6 weeks after immunization and are not visible anymore in our model. However, as inflammatory cells are major contributors to fibrosis formation and no signs of enhanced cardiac fibrosis were observed, we suspect no differences in inflammatory cell recruitment at earlier time points as well. In line with observations on cardiac hypertrophy and fibrosis, no left ventricular dilation was observed. These findings indicate that increased antibody titers after immunization do not always lead to the development of myocarditis and DCM, which questions the translational value and reproducibility of this EAM protocol.

LIMITATIONS AND FUTURE PERSPECTIVES

Since we demonstrated increased antibody titers upon immunization, we can assume that our immunization protocol was successful. The observed concentrations of IgG1 and IgG3 are also in line with other comparable immunization protocols in mice, using CFA as an adjuvant[26,27]. These findings suggest that despite sufficiently increased antibody titers, the autoimmune response in our model was not strong enough to induce cardiac remodeling or fibrosis. We suspect that the antigen release in the heart is insufficient and other stimuli are needed to promote antigen exposure in order to induce cardiac damage.

An important factor to boost the immune response in EAM is the use of adjuvants, typically involving TLR ligands[28]. These adjuvants have to be provided simultaneously with the cardiac self-antigen in order to generate a cardiac-specific autoimmune response[25,29]. In our study, we made use of CFA, which is widely used in EAM models. Other combinations of adjuvants, including Incomplete Freund adjuvant (IFA), Titermax or alum, were not sufficient to induce myocarditis and were shown to induce a non-antigen specific antibody response compared to CFA[28]. However, EAM models using only CFA as an adjuvant also vary greatly in severity of myocarditis[17,29].Therefore, some research groups also included a second adjuvant, including pertussis toxin (PT) or lipopolysaccharide (LPS) to increase the severity of EAM[17,19,29]. The additional dose of PT was shown to promote inflammatory cell infiltration and induced severe additional damage to the myocardium compared to studies using only CFA[17]. However, the question is whether this model is still translational to the clinical setting of autoimmune-induced myocarditis and HF. Another potential 'stressor', used to promote

antigen exposure to induce myocarditis after immunization, might be exercise. In virus-induced myocarditis, increased exercise by swimming resulted in increased influx of cytotoxic CD8+ T cells and larger necrotic lesions[30]. Whether exercise also has an effect in EAM is unknown and remains to be elucidated in future studies.

In addition to antigen release, the development of EAM also strongly depends on other factors, including genetic predisposition[31]. It was shown that immunization with α-MHC/CFA only induces EAM in susceptible mice strains[32,33]. Mice on BALB/c, A/J or A.SW background are the only strains susceptible to immunization protocols, whereas mice on the C57BL/6 background are resistant due to different immunological responses (Th1-mediated) to antigen immunization[34–36]. Susceptible strains, including BALB/c mice, respond with a Th2-mediated immune response upon immunization[37], which is essential to induce an autoimmune response and was therefore used in our study. Interestingly, mice lacking PD-1, an important negative regulator of T-cell responses to (self)-antigens, spontaneously develop organ-specific inflammation, progressive myocarditis, and HF[38]. Moreover, the hearts of PD-1 deficient mice showed diffuse deposition of IgG on the surface of cardiomyocytes[39], thereby suggesting that these mice might be a better model to mimic autoimmune responses as observed in HF patients. However, the spontaneous development of severe inflammation, also in other organs of these mice, might not be favorable and PD-1 inhibitors might preferably be used as additional stimulus in EAM models to boost a cardiac-specific autoimmune response as observed in humans[40,41].

Another important feature of EAM models is a sex bias, since male mice are more susceptible to the induction of heart-specific autoimmunity compared to female mice[10]. In mice, males produce higher amounts of pro-inflammatory cytokines during myocarditis, toll-like receptor 4 (TLR-4) is more expressed by male antigen-presenting cells, and females show a stronger anti-inflammatory regulatory T cell response[10,42,43]. In humans, inflammatory responses in myocarditis and subsequent progression towards HF is also more severe in men than in women, and therefore the reason we only included male mice in our mice study. However, in general in humans, autoimmune diseases, such as rheumatic arthritis, are more common in women[43,44]. Moreover, the development of HF is different in men than in women, where HF with reduced ventricular function is more common in men, and HF with preserved left ventricular function is more common in women[45,46]. Therefore, using the current EAM mouse model as it is now, with only using males, as a basis for a translational *in vivo* model to study autoimmune-induced HF might not be optimal.

In conclusion, increased antibody titers after immunization do not necessarily lead to adverse cardiac remodeling and disease manifestation and additional stimuli might be needed. This underscores the need for a relevant and translational small animal model to study autoimmune responses in the development of HF. Creating a reproducible and translational EAM model would provide the basis to investigate the role of other cardiac-specific antigens involved in autoimmune responses in HF.

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Conflicts of interest
The authors declare no conflicts of interest.

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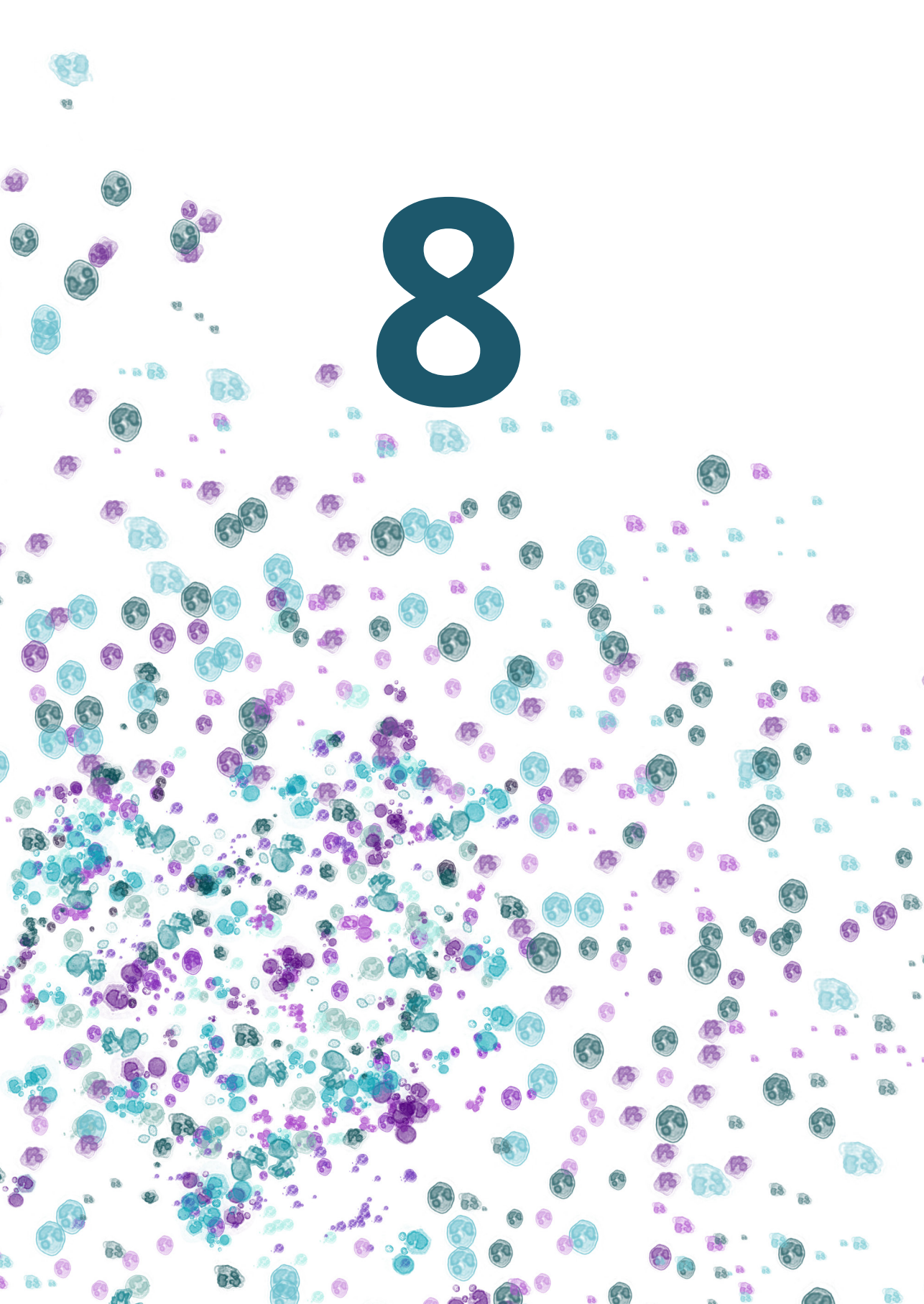
Part three

Therapeutic potential of progenitor cells in heart failure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Potential of mesenchymal- and cardiac progenitor cells for therapeutic targeting of B cells and antibody responses in end-stage heart failure

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ABSTRACT

Background

Upon myocardial damage, the release of cardiac proteins induces a strong antibody-mediated immune response, which can lead to adverse cardiac remodeling and eventually heart failure (HF). Stem cell therapy using mesenchymal stromal cells (MSC) or cardiomyocyte progenitor cells (CPC) previously showed beneficial effects on cardiac function despite low engraftment in the heart. Paracrine mediators are likely of great importance, where, for example, MSC-derived extracellular vesicles (EVs) also show immunosuppressive properties *in vitro*. However, the limited capacity of MSC to differentiate into cardiac cells and the sufficient scaling of MSC-derived EVs remain a challenge to clinical translation. Therefore, we investigated the immunosuppressive actions of endogenous CPC and CPC-derived EVs on antibody production *in vitro*, using both healthy controls and end-stage HF patients.

Methods and results

Both MSC and CPC strongly inhibit lymphocyte proliferation and antibody production *in vitro*. Furthermore, CPC-derived EVs significantly lowered the levels of IgG1, IgG4, and IgM, especially when administered for longer duration. In line with previous findings, plasma cells of end-stage HF patients showed high production of IgG3, which can be inhibited by MSC *in vitro*.

Conclusion

MSC and CPC inhibit *in vitro* antibody production of both healthy and end-stage HF-derived immune cells. CPC-derived paracrine factors, such as EVs, show similar effects, but do not provide the complete immunosuppressive capacity of CPC. The strongest immunosuppressive effects were observed using MSC, suggesting that MSC might be the best candidates for therapeutic targeting of B-cell responses in HF.

INTRODUCTION

Cardiovascular disease (CVD) is the most common cause of death globally with almost 18 million deaths per year[1]. A prominent CVD-subtype is ischemic heart disease (IHD), which is characterized by myocardial cell death due to prolonged ischemia[2]. After subsequent reperfusion strategies, further myocardial damage is initiated by the release of cardiac proteins, which can induce an inflammatory response[3,4]. Activated T and B lymphocytes significantly contribute to adverse cardiac remodeling via the production of pro-inflammatory cytokines and antibodies[5–7], which can progress to severe heart failure (HF)[6,8–10].
Currently, progenitor cell therapy is gaining a lot of interest in order to regenerate the damaged heart due to their regenerative properties and the ability to differentiate into other cell types[11–13]. Mesenchymal stromal cells (MSC) improve cardiac function by reducing scar size and increasing left ventricular ejection fraction (LVEF) with 2-4%[14,15]. However, engraftment of these cells in the heart is relatively poor, where less than 10% of the injected cells remain at the site of injection[16,17]. In addition, the few remaining cells rarely differentiate into cardiac cells[18]. In addition to their regenerative capacity, MSC have also been shown to suppress inflammatory responses, antibody production, and fibrosis, mostly in a paracrine manner[19,20]. Important paracrine mediators are extracellular vesicles (EVs), small lipid bi-layered vesicles containing lipids, small RNAs and proteins, which are able to influence many processes including inflammation[21,22]. Multiple studies investigated the therapeutic potency of MSC and MSC derived EVs in CVD[13,23,24]. MSC-derived EVs were found to reduce infarct size and infiltration of immune cells into the affected myocardium after myocardial infarction (MI) in animal models[25]. These findings suggest that the use of MSC-derived EVs might be a promising strategy to restore cardiac function, however, technical difficulties in large scale production and purification of MSC-EV are still limiting the translation to the clinic[19,26].
Considering the developmental origin of endogenous cardiac-derived progenitor cells (CPC), these cells might prove better candidates for cell therapy for cardiac repair. Endogenous CPC were previously tested in several clinical trials where they improved cardiac function[12,27], especially when combined with MSC[28,29]. CPC also have immunosuppressive properties, for example by inhibiting T-cell proliferation, which is partly mediated by paracrine factors[30]. CPC-derived EVs are proposed to be of great importance as paracrine mediators of these cells[31–33]. However, the immunosuppressive capacity of CPC or CPC-derived EVs on B cells and antibody-mediated immune responses has not been elucidated yet. Therefore, we investigated the *in vitro* inhibitory actions of CPC and CPC-derived EVs on lymphocyte proliferation and the production of immunoglobulin subclasses, using immune cells from healthy controls and end-stage HF patients.

MATERIAL AND METHODS

Culture of human-derived progenitor cells
Human bone marrow-derived mesenchymal stromal cells (MSC) and cardiomyocyte progenitor cells (CPC) were obtained and isolated as described before[34,35]. MSC were cultured in MEM-alpha (Gibco, 32561-037) supplemented with 10% fetal bovine serum (Gibco, 10270-

106) + 1% PenStrep (Lonza, 17-602E) + 0.2 mM L-ascorbic acid-2-phospate (Sigma A4034) + 1 ng/ml bFGF (Sigma F0291). CPC were cultured in SP++ (25% EGM-2 (Lonza, CC-3156) + 75% M199 (Gibco, 31150-022) supplemented with 10% fetal bovine serum + 1% PenStrep + 1% non-essential amino acids (Lonza, 13-114). Cultures were incubated at 37°C (5% CO2 and 20% O2) and adherent cells were passaged when reaching 80-90% of confluency using trypsin digestion (0.25%, Lonza, CC-5012). MSC and CPC from fetal or adult donors were used in the co-cultures between passage 6-17.

Isolation of CPC-derived extracellular vesicles and Western blotting

CPC-derived EVs were isolated using size-exclusion chromatography (SEC), as previously described[36]. In brief, fetal-derived CPC were cultured until they reached a confluency of 80-90%, after which the medium was replaced with serum-free medium (M-199, Gibco 31150-022). After 24 h, conditioned medium (CM), containing the EVs, was collected, centrifuged at 2000g for 15 min, and filtered (0.45 µm) to remove dead cells and debris. Next, CM was concentrated using 100-kDA molecular weight cut-off Amicon spin filters (Merck Milipore) and loaded onto a S400 highprep column (GE healthcare, Uppsala, Sweden) using an AKTA start (GE Healthcare) containing an UV 280 nm flow cell. Fractions containing EVs were pooled and filtered (0.45 µm) before further concentration procedures. The number of particles and mean size distribution were measured using Nanoparticle Tracking Analysis (Nanosight NS500, Malvern) as described before[36]. Protein concentration was measured using microBCA protein assay kit (Thermo Scientific). Vesicle markers were assessed by Western blotting (WB) as previously described[36]. EV protein fractions were loaded on pre-casted Bis-Tris protein gels (ThermoFischer, NW04125BOX) and run for 1 h at 160V. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, IPVH00010) and stained for general EV markers[37] Alix (1:1000, Abcam, 177840), CD63 (1:1000, Abcam, 8219), CD81 (1:1000, Santa Cruz, Sc-166029), or Calnexin (1:1000, Tebu-bio, GTX101676). Proteins were detected using chemiluminescent peroxidase substrate (Sigma, CPS1120).

Isolation of peripheral mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from fresh whole blood samples of healthy controls or end-stage HF patients, in compliance with the *declaration of Helsinki* and under approval of the Medical Ethics Committee Utrecht (METC, reference number 12/387). Written informed consent for collection and biobanking of blood samples was obtained. End-stage HF derived PBMC were obtained from blood samples prior to heart transplantation. PBMC were isolated using Ficoll-plaque PLUS gradient (GE life sciences, 17-1440-03) according to the manufacturers protocol. A total of $2{,}5x10^5$ PBMC were added per well (48-wells plate) in RPMI-1640 medium (Lonza, BE12-702F) supplemented with 10% fetal bovine serum and 1% PenStrep.

PBMC stimulation and co-culture with progenitor cells or purified EVs

For the stimulation of PBMC and subsequent antibody production, a combination of IL-2 (120 IU/ml, BD Pharmingen, 554563) and PMA (0.123 ng/ml, Sigma, P8139) was used as

previously described[33]. PBMC were co-cultured for 10 days, without medium change, in 48-well plates ($2.5x10^5$ PBMC/well) with MSC, CPC ($5.0x10^4$ cells/well), or CPC-derived EVs (1x 10 µg= $6.3x10^{10}$ particles), immediately upon co-culture or 3x 10 µg ($6.3x10^{10}$ particles) added every 3 days of co-culture. After 10 days of co-culture, light-microscopic images were taken using an Olympus CKX41 microscope in combination with CellSense software. Non-adherent cells, containing the lymphocytes, were collected and processed for further analysis using flow cytometry (Gallios, Beckmann Coulter). Co-culture supernatant was collected, centrifuged at 500g for 10 min, aliquoted and stored at -80°C for immunoglobulin measurements.

Lymphocyte proliferation

Flow cytometry (Gallios, Beckmann Coulter) was used to assess lymphocyte proliferation. Prior to co-culture, PBMC were labeled with 1.5mM carboxyfluorescein succinimidyl ester (CFSE, Sigma, 21888) as described previously[33]. In brief, PBMC were incubated with CFSE for 10 min at 37 °C in a dark shaking bath. After 10 minutes, 5% of FBS was used to prevent further uptake. After two washing steps with PBS, PBMC were incubated for 30 min with fluorescent antibodies, including CD3 for T cells (Brilliant Violet 510, Biolegend, 317332) and CD19 for B cells (PE/Cy5, Biolegend 302210). After washing with PBS, PBMC were incubated for 30 min with a fixable viability dye (eFluor506, Bioscience, 65-0866-14) to exclude dead cells. Prior to culture, general cell composition per donor was assessed by measuring the percentage of CD3+ T cells and CD19+ B cells, to ensure that the cell populations were similar between the different donors at baseline. Lymphocyte proliferation was calculated by measuring CFSE intensity and the number of cells present in each division as described before[33]. Since we encountered some donor variations in the absolute number of proliferating cells in the stimulation assays, the stimulated PBMC condition was considered as maximum response and defined as 100% proliferation (ratio=1) and used for normalization of the data per donor and per experiment. Data was analyzed using Kaluza Analysis Software (Beckman Coulter, version 1.3).

Immunoglobulin multiplex

The levels of IgM and IgG subclasses (IgG1, IgG2, IgG3, IgG4) in the co-cultures (5x diluted) were measured using a Bio-Plex Pro™ human isotyping immunoassay 6-plex (Bio-Rad, 171A3100M) according to manufacturer's instructions and were all within the detection limit of the assay. Immunoglobulin levels in the supernatant after co-culture with MSC/CPC or CPC-derived EVs were calculated using internal standards included in the assay. Immunoglobulin levels are represented as relative production, with the stimulated PBMC condition defined as 100% antibody production (ratio=1) and used for normalization of the data per donor and experiment.

Statistics

Statistical analysis and data representation were performed using IBM SPSS Statistics 21 and Graphpad Prism© (GraphPad Software Inc. version 8.01, San Diego CA, USA). Normal data distribution was tested using the Kolmogorov-Smirnov test. Group comparison was

performed by a one-way ANOVA or Kruskal-Wallis test, corrected for multiple comparison testing. Each individual PBMC donor is considered as an independent individual experimental number (n), ranging from 2-8 donors per experiment. Data was considered significant with two-tailed p-values <0.05 and is presented as mean ± SEM.

RESULTS

Progenitor cells suppress lymphocyte proliferation upon cell-cell contact

To investigate the immunosuppressive effects of progenitor cells on the proliferation of lymphocytes, a co-culture using MSC or CPC was performed (*Figure 1*). To represent normal lymphocyte activation by antigen-presenting cells, the total PBMC population was used. After 10 days of co-culture, large clusters of proliferating T cells were visible upon stimulation with IL-2 and PMA. These large clusters were smaller or even absent when PBMC were cultured in the presence of MSC or CPC (*Figure 1A*). Flow cytometry was used to measure CFSE intensity and to assess lymphocyte proliferation (*Figure 1B-C*). FACS plots clearly showed active cell proliferation upon stimulation with IL-2 and PMA and suppression of proliferation when PBMC were cultured with MSC or CPC. Quantification showed that both MSC and CPC significantly decreased proliferation of lymphocytes by 64±18.6% and 19±12.5% respectively (MSC $p<0.0001$, CPC $p<0.05$).

Production of IgM and different IgG subclasses is suppressed by cardiac-derived progenitor cells

Next to reduced cell proliferation, MSC are also able to inhibit several immune cell functions, such as antibody secretion[38]. To examine whether this also holds true for CPC, we collected the supernatant after 10 days of co-culture and measured the levels of different immunoglobulin subclasses (*Figure 2A*). Since it is known that the age of the donor can affect their inhibitory potency[39,40], both fetal and adult MSC and CPC were included. Adult and fetal-derived MSC significantly inhibited antibody production from stimulated PBMC (*Figure 2B-F*). Fetal and adult MSC significantly reduced the production of IgM (aMSC=0.005±0.0 fMSC=0.02±0.0; $p<0.0001$), IgG1 (aMSC=0.24±0.06, fMSC=0.28±0.06; $p<0.0001$), IgG3 (aMSC=0.19±0.06, fMSC=0.25±0.09; $p<0.0001$) and IgG4 (aMSC=0.29±0.07; $p<0.01$, fMSC=0.43±0.1; $p<0.05$). In addition, also CPCs showed strong suppressive effects on the production of mainly IgM (aCPC=0.02±0.0; $p<0.0001$, fCPC=0.38±0.16; $p<0.001$), IgG1 (aCPC=0.12±0.02; $p<0.001$, fCPC=0.55±0.18; $p<0.05$) and IgG3 (aCPC=0.06±0.0; $p<0.0001$, fCPC=0.44±0.14; $p<0.001$). For CPCs, the strongest immunosuppression was observed using adult CPCs.

CPC-derived extracellular vesicles suppress antibody production, but are not as effective as direct cell-cell interaction when using CPC

To explore whether the suppressive capacity of CPC on antibody production is mediated by paracrine factors, we assessed the potential of CPC-derived EVs (*Figure 3*). We experienced that it is technically challenging to obtain sufficient MSC-derived EVs using SEC. Therefore, we only included CPC-derived EVs in our co-cultures. Prior to co-culture, EVs were

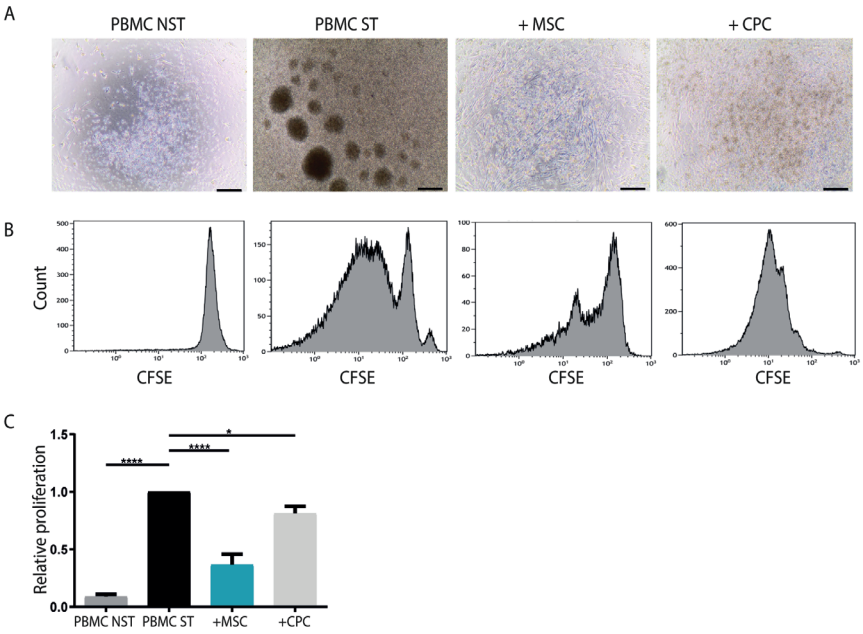

Figure 1. Progenitor cells suppress lymphocyte proliferation
Lymphocyte proliferation was measured after 10 days of co-culture of PBMC with MSC or CPC. Representative microscopic images after 10 days of co-culture (A). Upon PBMC stimulation, large clusters of proliferating cells were observed. These large clusters were absent in the presence of MSC or CPC. PBMC were labeled with CFSE and lymphocyte proliferation was assessed by measuring CFSE intensity using flow cytometry (B). FACS plots of non-stimulated lymphocytes show one peak of undivided cells, whereas upon stimulation, lymphocytes start to divide. Quantification of lymphocyte proliferation, where stimulated lymphocytes were used as normalization (C). Both MSC and CPC show a significant decrease of lymphocyte proliferation upon co-culture. Strongest effects were observed using MSC, where proliferation was inhibited towards 36% compared to CPC (81%). *PBMC: pheripheral blood mononuclear cells, MSC: mesenchymal stromal cell, CPC: cardiac progenitor cell. Per condition n=4. Line bar indicates 200µm, magnification 4x. * p<0.05, **** p<0.0001.*

characterized based on size distribution and the presence or absence of protein markers[37]. Isolated EVs showed a representative size distribution profile with the highest peak at approximately 90 nm (*Figure 3A*). In line with previous findings[36], WB analysis showed that CPC-derived EVs were enriched for the typical EV proteins Alix, CD81, and CD63. Calnexin was only detectable in the cell lysate, thereby confirming the absence of contaminations with other membrane compartments (*Figure 3B*). An amount of 1x10 µg or 3x10 µg (every 3 days of co-culture) was added to the PBMC cultures (*Figure 3C*). After 10 days of co-culture, antibody secretion was significantly suppressed by EVs (*Figure 3D*). The production of IgM, IgG1, and IgG4 was significantly decreased using the 3x dose of CPC-derived EVs (IgM=0.35±0.05; p<0.05, IgG1=0.57±0.03; p<0.05, and IgG4=0.66±0.0; p=0.03), thereby indicating that long term suppression is more effective than a single dose of EVs. However, the inhibitory effect was most robust when adding CPC and not CPC-derived EVs, with strongest suppressive effects on the release of IgG1 (0.59±0.1; p<0.05), IgG2 (0.23±0.06; p=0.02), IgG4 (0.53±0.03; p=0.01) and IgM (0.17±0.03; p<0.01).

MSC show the strongest immunosuppressive effects and are more likely to be used as cell therapy in end-stage HF patients

Since we observed that CPC-derived EVs do not give the same degree of immunosuppression as CPC, we decided to continue with CPC to examine their potential suppressive effect on antibody-mediated immune responses in end-stage HF patients. PBMC were isolated from end-stage HF patients and cultured with or without MSC/CPC (*Figure 4*). At baseline culture,

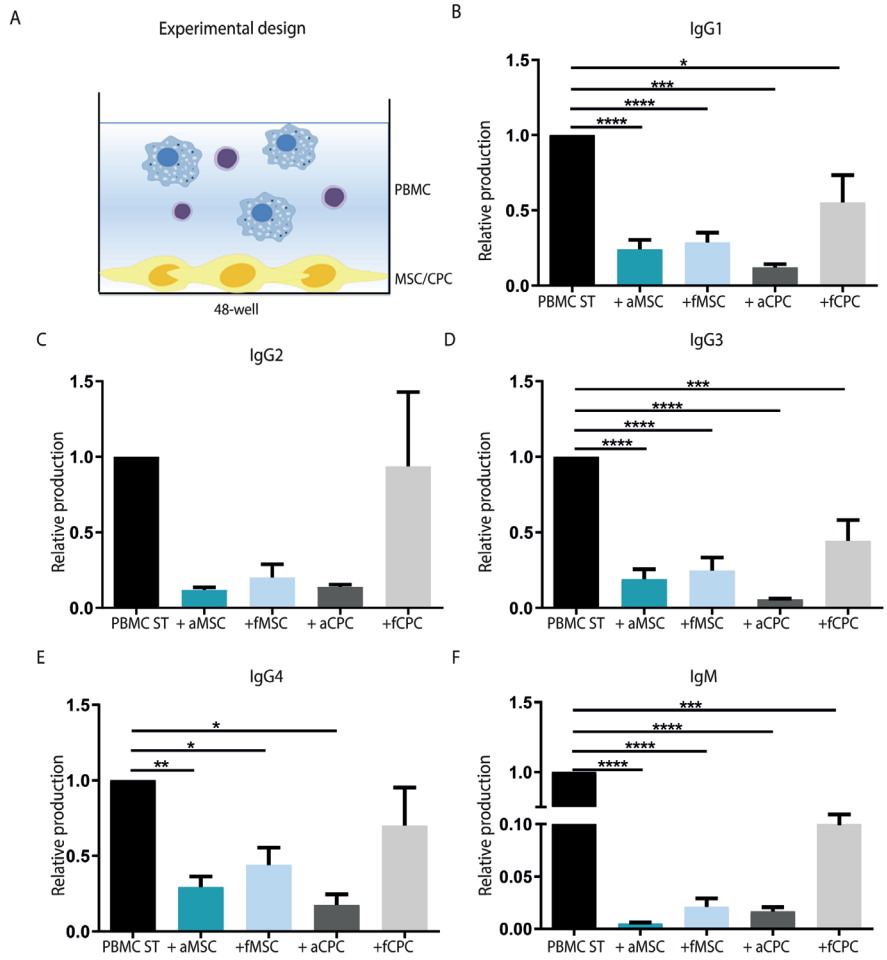

Figure 2. Progenitor cells suppress the production of immunoglobulins

Antibody production was measured after 10 days of co-culture with fetal or adult-derived MSC or CPC. Experimental design of the co-culture (A). Both fetal and adult MSC showed strong immunosuppressive effects on the production of different immunoglobulin isotypes and subclasses (B-F). IgM, IgG1, IgG3, and IgG4 levels were significantly decreased upon co-culture with MSC. For CPC, strongest effects were observed in cultures using adult-derived CPC, where the production of IgM, IgG1, IgG3, and IgG4 was significantly suppressed. *aMSC: adult-derived mesenchymal stromal cell, fMSC: fetal-derived mesenchymal stromal cell, aCPC: adult-derived cardiac progenitor cell, fCPC: fetal-derived cardiac progenitor cell. For aMSC, fMSC and fCPC n=7, for aCPC n=3. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.*

non-stimulated PBMC derived from end-stage HF patients produced similar amounts of IgGs with the exception of IgG3, which is, slightly but not significantly, increased compared to PBMC derived from healthy controls (*Figure 4A*). Upon co-culture of patient-derived PBMC with MSC or CPC, antibody production was significantly suppressed (*Figure 4B-C*). Mainly MSC showed strong suppressive effects, as they significantly decreased the production of IgM (0.02±0.0; p<0.0001), as well as all IgG subclasses (IgG1=0.25±0.08; p=0.001, IgG2=0.03±0.02; p<0.0001, IgG3=0.25±0.08; p=0.009, IgG4=0.19±0.07; p=0.0006). Co-cultures using CPC showed similar suppressions, albeit at a lower level and the differences were only statistically significant for IgM (0.20±0.06; p<0.0001) and IgG2 (0.31±0.14; p=0.0003).

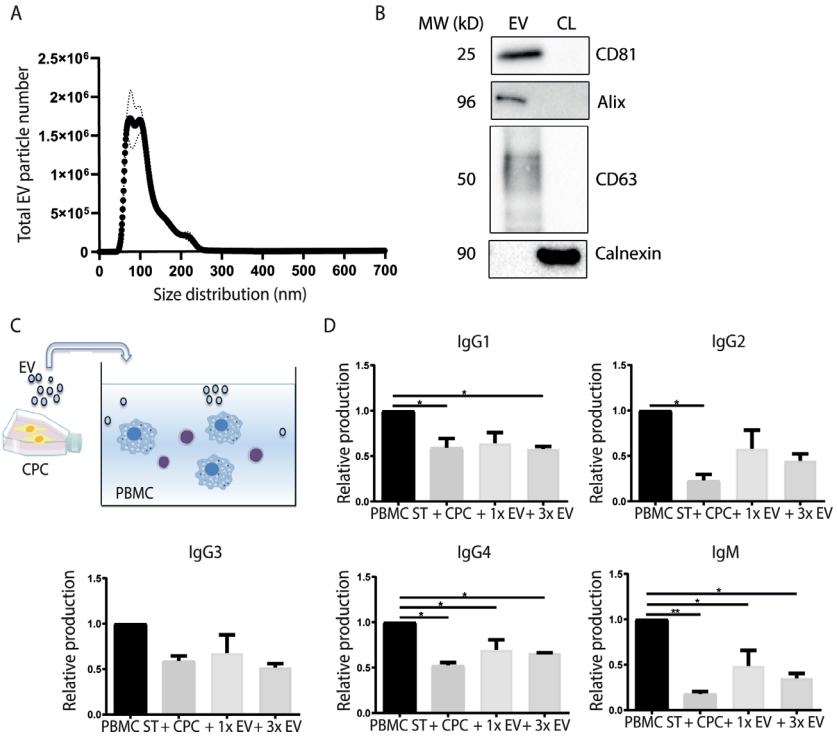

Figure 3. Immunosuppressive capacity of CPC-derived EVs on immunoglobulin production
To assess whether CPC-derived paracrine factors can be used, EVs were isolated and used in the PBMC co-cultures. EVs with a size of approximately 90 nm were isolated using SEC (A). WB of EVs and CL with general EV markers and calnexin (B). Experimental setup of the co-culture model, where either CPC or CPC-derived EVs with a total of 1x 10 µg or 3x 10 µg (every 3 days of co-culture) was added to PBMC (C). CPC-derived EVs showed a significant decrease of immunoglobulin production, especially when administered for a longer period of time (D). Levels of IgM, IgG1, and IgG4 were significantly decreased when, every 3 days of co-culture, 10 µg of EVs were added to stimulated PBMC. However, the strongest inhibition of antibody production was observed when CPC were used. *CPC: cardiac progenitor cell, EV: extracellular vesicles, CL: cell lysate, SEC: size-exclusion chromatography, PBMC: pheripheral blood mononuclear cells. For each condition n=2. * p<0.05, ** p<0.01.*

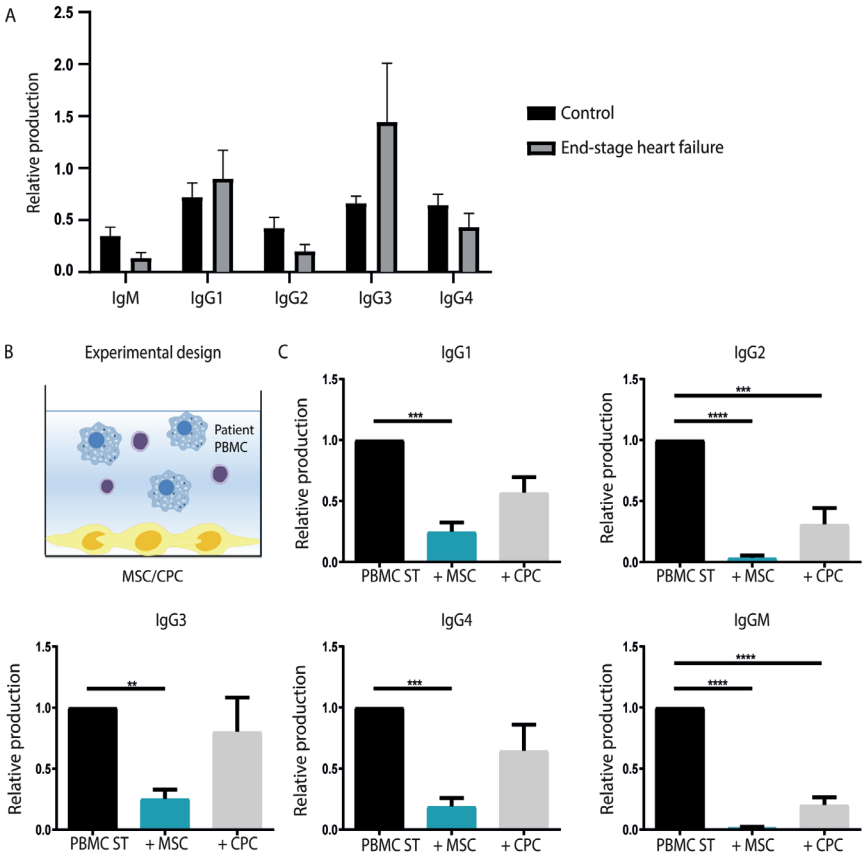

Figure 4. Inhibition of immunoglobulin production by progenitor cells in end-stage HF
The immunosuppressive actions of CPC on antibody production in end-stage HF was investigated using patient-derived PBMC. Baseline antibody levels of unstimulated PBMC in culture were measured and compared to end-stage HF-derived PBMC (A). Before co-culture with MSC/CPC, HF patients showed high levels of IgG1 and IgG3 compared to healthy controls. Experimental set-up of the co-culture (B). Levels of IgM and IgG1-IgG4 were significantly decreased upon co-culture of patient-derived PBMC with MSC (C). CPC were able to significantly suppress IgM and IgG2, however, were not as potent as MSC. *MSC: mesenchymal stromal cell, CPC: cardiac progenitor cell, HF: heart failure, PBMC: pheripheral blood mononuclear cells. Per condition n=8. ** p<0.01, *** p<0.001, **** p<0.0001.*

DISCUSSION

The post-MI immune response is an important contributor to adverse cardiac remodeling and the development of HF[41–44]. The release of cardiac proteins upon MI can trigger antibody-mediated immune responses, which further induce cardiac damage and HF[45–47]. Stem cell therapy using progenitor cells, such as MSC or CPC, showed promising reparative effects on cardiac function despite poor engraftment in the myocardium[17,48]. This indicates that paracrine mediators, secreted by progenitor cells, can be of great importance. MSC and

MSC-derived EVs also have immunosuppressive properties, for example by lowering antibody production *in vitro*[49,50]. However, the immunosuppressive capacity of endogenous CPC and CPC-derived EVs on B cells and antibody production has not been elucidated yet. Consequently in this study, we investigated the immunosuppressive effects of CPC and CPC-derived paracrine mediators on antibody production using immune cells of both healthy controls and end-stage HF patients.

In line with previous findings, we showed that both MSC and CPC significantly suppressed proliferation of lymphocytes[30,32,38]. The suppressive effects of MSC were more effective than CPC. The suppressive effects of MSC and CPC on effector and regulatory T cells have been described before, where several studies show T cell inhibition via PDL-1/PD1 in a direct cell communication manner[32,51]. Moreover, both MSCs and CPCs are also able to suppress CD4+ T helper cell-mediated immune responses[52]. However, the interaction of progenitor cells with B cells is still controversial and this issue has recently gained more interest[53–55]. MSC can inhibit plasma cell formation and subsequent IgG production in a cell-cell contact dependent as well as in an independent manner[38,55]. It is not known whether CPC are also able to suppress antibody production *in vitro*. We demonstrated that, similar to MSC, CPC effectively suppress antibody production *in vitro*. We showed that both adult- and fetal-derived CPC significantly inhibit the levels of IgM, IgG1, and IgG3, of which IgM was most efficiently suppressed, despite variation between different donors. These findings are in line with the effects of MSC, where MSC are known to exert an inhibitory effect on T helper cells, B-cell differentiation and class switching into IgG-producing cells[56,57]. Therefore, we could speculate that CPC might use a similar mechanism, in which IgG production might be suppressed either by inhibiting T-helper cell responses, thereby influencing B-cell activation and antibody production, or by directly influencing B-cell differentiation and subsequent class-switching.

To facilitate clinical translation, we examined if the strong immunosuppressive effects of CPC and MSC on antibody production using healthy donors, can be confirmed for IgG production using HF patient-derived PBMC. MSC were able to significantly inhibit the production of IgM and all IgG subclasses. For CPC, the immunosuppressive effects were not as potent compared to MSC, where CPC only significantly lowered the production of IgM and IgG2. In end-stage HF, chronically activated immune cells progressively worsen cardiac function, for example by the production of cardiac antibodies[58,59]. Our findings indicate that progenitor cells, preferably MSC, might be used as therapeutic agents to suppress antibody-mediated immune responses as observed in end-stage HF. However, mimicking the physiological immune response *in vitro,* as observed in end-stage HF patients, is still complicated. Therefore, these findings still have to be validated *in vivo*.

Part of the immunosuppressive properties of MSC is mediated by paracrine factors, such as EVs[19,21]. The advantage of using EVs is that they can be used as a cell-free approach, thereby increasing safety, and allowing a longer duration of the treatment[19,26]. However, high variability in quantity and quality in the scaling and production process of MSC-derived EVs has been a limitation[26]. CPC-derived EVs might provide a promising alternative, not only due to their regenerative and immune modulating capacities[60], but also for their culture scalability. CPC-derived EVs have immunosuppressive effects on T cells[30,60], however, the

effects on B cells and antibody-mediated responses is not clear. Our findings showed that CPC-derived EVs lower the different immunoglobulin isotypes and subclasses, such as IgM, IgG1, and IgG4. However, the number of EVs needed to reach similar suppressive effects compared to CPC, remains challenging. In this study, we were only able to test EVs produced by fetal CPC due to technical difficulties in obtaining sufficient numbers of EVs from adult CPC. Fetal-derived progenitor cells might exert different effects than adult-derived cells, where, for example, adult-derived MSC show stronger immunosuppressive capacities relative to fetal-derived MSC[61]. For CPC, it has been described that fetal- and adult-derived CPC have different developmental potentials, and adult CPC may be more effective in cardiac repair[62,63]. In addition, fetal-derived CPCs are highly proliferative as compared to adult-derived cells. Due to this proliferative state CPCs may secrete a different palette of paracrine factors that are more associated to cell cycle rather than immunomodulation. Therefore, the effects of EVs from adult CPC may differ from fetal–derived CPC and have to be investigated in future studies. Nonetheless, from our data, it is clear that EVs can be used as immunosuppressive mediators, but do completely cover the strong immunosuppressive effect of CPC.

In conclusion, we demonstrated immunosuppressive actions of both MSC and CPC on lymphocyte proliferation and antibody production, with strongest effects observed when using MSC. These are partly mediated by EVs, in a time-dependent matter. Lastly, we showed that CPC and especially MSC were able to suppress antibody production by patient-derived cells, thereby indicating the therapeutic potential of progenitor cells in HF. Currently, cell therapy using MSC is no longer the holy grail for true cardiac regeneration and cell replacement therapy, however, MSC might be promising candidates for targeting the post-MI immune response and HF progression. Future studies should focus on the identification of the cardiac antigens which are targeted by the produced IgGs and the potential of combination therapies, using both MSC and CPC, to simultaneously target cardiac regeneration and antibody-mediated immune responses.

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Conflicts of interest

None declared.

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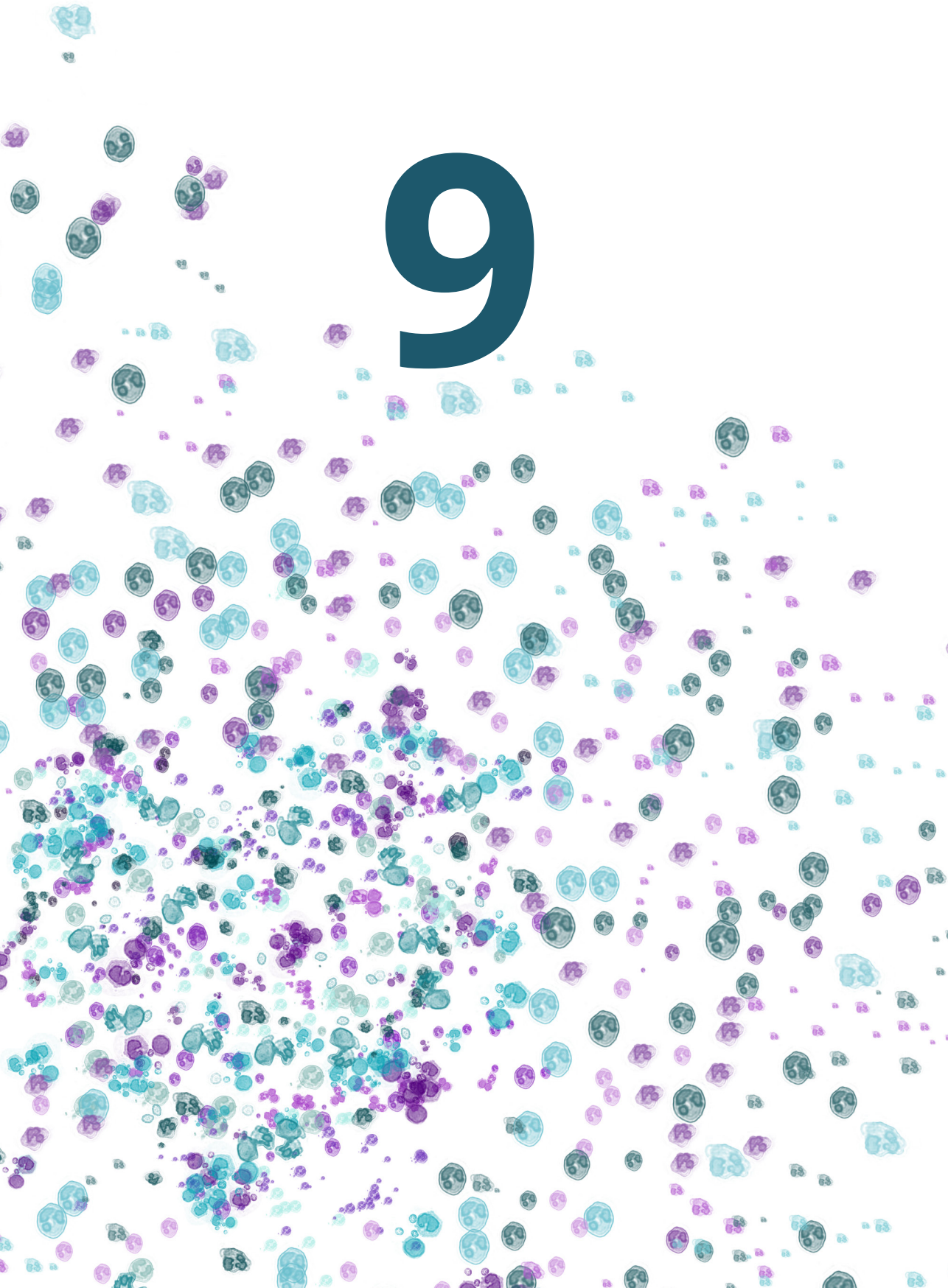

Clinically used cardiovascular medication in preclinical models improves functional outcome upon myocardial infarction, the new standard in pre-clinical research?

In preparation

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ABSTRACT

Background

Many therapeutic strategies in the treatment of myocardial infarction (MI) have been tested *in vitro* and in small animal models. The use of progenitor cells post-MI showed promising effects on cardiac function in these preclinical phases, however, effect sizes declined while moving up the translational axis with only very limited effects observed when testing these approaches in clinical trials. Currently, there is a translational gap, also referred to as the translational valley of death, between preclinical findings and therapies that actually reach the clinical arena. Surprisingly, the combination of medications which are clinically prescribed as standard care after MI, being aspirin, P2Y12-inhibitors, β-blockers, AT1R antagonists and statins, is barely incorporated in preclinical studies when testing a new therapy. This might explain part of the reduced efficacy of new therapeutics, when translating a new therapy from bench to bedside.

Methods

Using a mouse-model of ischemia and reperfusion (I/R) injury, we investigated the effect of these five clinically prescribed medications on adverse cardiac remodeling, cardiac function and fibrosis. Moreover, we tested if treatment with these clinically prescribed medications in mice influences efficacy of cell therapeutics, by intra-myocardial injection of mouse bone marrow-derived mesenchymal stromal cells (MSC), with or without the medication.

Results

We observed a significant decrease of adverse cardiac remodeling upon treatment with all five medication after I/R injury. Heart weight/body weight ratio and cardiac volumes were reduced in mice who received medication compared to mice with regular food. When medication was combined with MSC therapies, no beneficial effects of MSC were observed on cardiac function or fibrosis compared to the use of medication alone.

Conclusion

The administration of five clinically prescribed standard medications reduced adverse cardiac remodeling and improved cardiac function in a mouse model of MI. No additional beneficial effects of MSC were observed. Clinically prescribed medications should be added to confirmatory *in vitro* and *in vivo* studies when testing new therapies to optimize translational success.

INTRODUCTION

The past decades have given us many therapeutic and interventional strategies to treat patients after myocardial infarction (MI)[1,2]. The introduction of percutaneous interventions and the prescription of medications, including aspirin, P2Y12-inhibitors, β-blockers, AT1R antagonists and statins, have made a substantial impact on cardiovascular disease burden[2,3]. These medications reduce symptoms, the chance of secondary events and progression towards HF, but yet do not repair the injured heart[3,4]. Therefore, additional strategies are needed, which are focusing on reducing the loss of myocardial tissue and stimulating cardiac repair[5,6].

The use of progenitor cells has been a promising strategy to improve cardiac function after MI[7]. Multiple preclinical studies using progenitor cells, such as mesenchymal stromal cells (MSC), in small animal models of MI showed improved cardiac function, by increasing ejection fraction and reducing cardiac fibrosis[8–10]. When these cells were used in large animal models of MI, progenitor cells were still able to increase cardiac function, albeit already less pronounced than in small animals[11,12]. Once translating preclinical findings into actual clinical interventions on top of standard prescribed medications, mixed outcomes were reported. Some studies showed no significant improvement of cardiac function, whereas others did show an increased left ventricular ejection fraction after cell therapy, albeit with a lower effect size than observed in large animal studies[13–15]. It seems that we are less able to translate promising therapies, such as cellular therapeutics, to clinical interventions which show an additional benefit on top of standard care, an issue referred to as 'translational failure'[16].

Surprisingly, these clinically standard prescribed medications, also called the golden five, are barely incorporated in preclinical studies when testing new therapies. The use of β-blockers in a mouse model of dilated cardiomyopathy (DCM) prevented cardiac dysfunction and reduced adverse cardiac remodeling[17]. Hence, the administration of statins in mouse models of atherosclerosis, using LDL-receptor deficient mice or Apo-E knockout mice, resulted in reduced vascular inflammation and plaque instability[18,19]. Despite clear beneficial effects of a single clinically prescribed medication in small animal models of cardiovascular disease, not a single study reported the use of all five medications simultaneously or in combination with an additional therapy, such as cellular therapeutics.

Our hypothesis is that the (non-) administration of regularly prescribed MI drugs might explain part of the reduction in therapeutic efficacy when moving up the translational ladder from bench to bedside. Therefore, in this study, we investigated whether the five clinically prescribed standard medications affect cardiac outcome in a mouse model of I/R injury. In addition, we assessed whether these medications influenced the therapeutic potential of progenitor cells post-MI.

MATERIAL AND METHODS

Clinically prescribed MI drugs

As a reflection of standard cardiovascular care the following medications were used in the experiments: aspirin (Selleckchem, S3017), ticagrelor (Selleckchem, S4079), metoprolol (AstraZeneca, 1 mg/ml), captopril (Selleckchem, S2051) and atorvastatin (Selleckchem, S2077). The combination of all 5 will be referred to as 'comedication'. All compounds were selected for their common use in daily clinical practice and being an active metabolite in dilution. For *in vitro* assays, pure compounds in powder form were dissolved in DMSO in a 10 mM stock concentration. All DMSO-dissolved drugs were stored at -80 ^{0}C until further use. All drugs were subsequently dissolved in corresponding culture media (final concentration of 1 µM) at the start of the *in vitro* experiments. As a control, similar concentrations of DMSO were used.

Bone-marrow-derived mouse MSC culture

Bone marrow-derived mesenchymal stromal cells (MSC), isolated from BALB/c mice, were isolated and characterized as described previously[20]. MSC were cultured in 0.1% gelatin coated flaks in DMEM medium (Gibco, Life Technologies, 41965-039) supplemented with 15% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin (P/S, Lonza, 17-602E). MSCs were passaged when reaching 80-90% confluency using trypsin digestion (0.25%, Lonza, CC-5012) at 37 ^{o}C for 2 min. For conditioned medium, MSC were cultured for at least 24 h in plain DMEM medium, after which conditioned medium was collected, centrifuged at 2000g and stored at -20 ^{0}C. Microscopic images were made using CellSens (Olympus Corporation, Tokyo, Japan). Cells were used in *in vitro* and *in vivo* experiments between passages 8-10.

Endothelial cell migration assay (scratch assay)

To test for cell migration, human microvascular endothelial cells (HMEC) were cultured in MCDB-131 medium (Invitrogen, 10372019), supplemented with hEGF (Peprotech/Invitrogen, 016100-15A), L-glutamine (Invitrogen, 25030024), hydrocortisone (Sigma, H6909-10), and FBS. Cells were seeded in 48-wells plates o/n until they reached 90-100% confluency. To assess migration, the cell monolayer was scraped in a straight line using a sterile 200 µl pipet tip to induce a scratch. Remaining medium was removed and cells were washed with plain MCDB-131 medium before adding the appropriate treatment: 1) positive control (20% FBS in plain MCDB-131 medium), 2) negative control (plain MCDB-131 medium), 3) conditioned medium of MSC, 4) comedication (dissolved in plain MCDB-131 medium). Two distinct microscopic images were made at 0 h and 6 h (magnification 4x). Images were analyzed using Adobe Photoshop CS6 to quantify closure of the scratch by measuring the inner area of the scratch, as previously described[21].

Medication-food preparation for small animal experiments

Comedication was orally administered via powdered food. Optimal dosing of medications was established in previous *in vivo* experiments based on plasma concentrations (data not shown). Mice received aspirin (200 mg/kg), ticagrelor (30 mg/kg), metroprolol (10 mg/kg),

atorvastatin (10 mg/kg) and captopril (25 mg/kg), dissolved in powder food. In brief, concentrations of the medications were based on the average food intake per mouse (4-5 g/day). Medications, as purified powder, were dissolved in absolute ethanol and the mixture was then sprayed on the powder food while constantly mixing. Powder food sprayed with ethanol only was used for the control groups. Food was kept in a closed flow cabinet at room temp for at least 72 h, to ensure complete evaporation of ethanol. Researchers were blinded for the type of food and the food was stored at -20 ^{0}C until use. During the animal experiments, the food was stored at 4 ºC with a maximum storage of 2 weeks.

Animal experiments

All animal experiments were approved by the Ethical Committee on Animal Experimentation of the University Medical Center Utrecht and conform to the 'Guide for the care and use of laboratory animals'. Healthy male and female BALB/c mice (age 10-14 weeks, weight 20-30 g) received standard food and drinking water *ad libitum* until experimental procedures. Animals were housed under standard conditions, with a 12 h light/dark cycle. Mice were randomized into different groups to MI or sham surgery (*Supplementary Figure 1)*. All mice were operated by a bio-technician blinded to the treatment. In brief, mice were anesthetized by intraperitoneal injection (i.p) of medetomidinehydrochloride (1.0 g/kg body weight), midazolam (10.0 mg/kg) and fentanyl (0.1 mg/kg). Mice were intubated and ventilated using a rodent respirator (Minivent, Hugo Sachs Electronics, Germany). A core body temperature of 37 ºC was maintained during the surgery using a rectal probe and heat blanket. A left lateral thoracotomy was performed and the left ascending coronary artery (LAD) was ligated for 60 min with an 8.0 Ethilon suture. Ischemia was confirmed by bleaching of the myocardium. For the cell therapy experiment, reperfusion was immediately followed by two intra-myocardial injections (29G), containing 5 µl PBS or 5 µl MSC (2x 5.0x10^{6} cells). Surgical wounds were closed and antagonist was subcutaneously injected containing atipamezole hydrochloride (3.3 mg/kg), flumazenil (0.5 mg/kg) and buprenorphin (0.15 mg/kg) as analgesia. After 24 h and 48 h, mice received another dose of analgesia (buprenorphin (0.15mg/kg) subcutaneously. After the operation, the mice were fed with either regular powder food or powder food supplemented with comedication for the rest of the experiment.

Echocardiography

Cardiac function was measured using transthoracic 3D-echocardiography (Vevo 2100 System, VisualSonics Inc., Toronto, Canada), at baseline, 1 week, and 4 weeks after the operation. Anaesthesia was induced by inhalation of 2% isoflurane in a mixture of oxygen/air (1:1). Heart rate, core body temperature and respiration were carefully monitored. Echocardiography and functional analyses were performed by researchers that were blinded for treatment groups using VevoLab software (Fujifilm VisualSonics Inc., Toronto, Canada).

Tissue processing and histology

Mice were euthanized after 4 weeks of follow up using i.p. injected sodium pentobarbital (60.0 g/kg). Blood was collected using an orbital puncture in EDTA-coated tubes and

centrifuged at 1,850 g for 10 min. Serum was aliquoted and stored at -80 ºC until further use. The heart was flushed with phosphate-buffered saline (PBS), weighed, and fixed in 4% paraformaldehyde. Heart weight, normalized to body weight or tibia length, was used as a measure for cardiac hypertrophy. After 24-48 h of fixation, tissue was embedded in paraffin. Sections of 3 µm were made with an electronic rotary microtome (Thermo Scientific, HM 340E) and used for further stainings. Morphology of cardiac tissue was visualized using hematoxylin and eosin (H&E) staining. Collagen density was analyzed using picrosirius red staining. Microscopic images were taken under polarized light, which were then converted to gray scale images. Collagen density was represented as a percentage of the left ventricle. Images were captured and analyzed using CellSens (Olympus Corporation, Tokyo, Japan).

Statistical analysis

Normal data distribution was tested using the Kolmorov-Smirnof test. A one-way ANOVA or Kruskal-Wallis test was performed to analyze the different groups, with correction for multiple comparison testing. All statistical analyses were performed using Graphpad Prism© (GraphPad Software Inc. version 8.01, San Diego CA). A two-sided p-value of 0.05 was considered as statistically significant. Data are expressed as mean ± SEM.

RESULTS

Administration of comedication reduces adverse cardiac remodeling

To determine the impact of comedication on cardiac remodeling, mice were subjected to I/R injury for 60 min and received regular powdered food or powdered food supplemented with comedication for 4 weeks (*Figure 1*). As depicted by HE staining, left ventricular wall thinning as a consequence of MI was clearly visible, which was most pronounced in mice with regular food and reduced upon comedication treatment (*Figure 1A*). Accordingly, heart weight to body weight and heart weight to tibia length ratios were significantly lower in mice who received comedication after MI compared to mice with regular food (*Figure 1B-C*, 5.04±0.16 vs 6.02±0.15 mg/g; p=0.001 and 6.98±0.49 vs 9.30±0.61 mg/mm; p=0.02 respectively). Cardiac function was measured using 3D-echocardiography at baseline, and 1 and 4 weeks post-MI (*Figure 1D-F*). Mice with comedication showed no significant changes in EDV, ESV or EF compared to sham operated animals, while mice receiving regular food demonstrated a significant increase in both EDV and ESV (92.27±8.44 vs 68.74±5.02 µl; p=0.02 and 60.08±8.08 vs 31.61±3.57 µl; p=0.004, respectively) and a reduced EF (37.85±3.56 vs 53.49±4.56%; p=0.02) compared to sham-operated animals. These findings indicate that comedication reduces adverse cardiac remodeling after I/R injury, thereby limiting further deterioration of cardiac function.

Cardiac fibrosis is increased upon I/R injury, however is not influenced by comedication

To assess the effect of comedication on cardiac fibrosis, collagen was stained using picrosirius red (*Figure 2*). As expected, after 4 weeks of I/R injury, a substantial amount of cardiac fibrosis was observed in the left ventricle of MI mice (*Figure 2A-B*). The total amount of collagen was

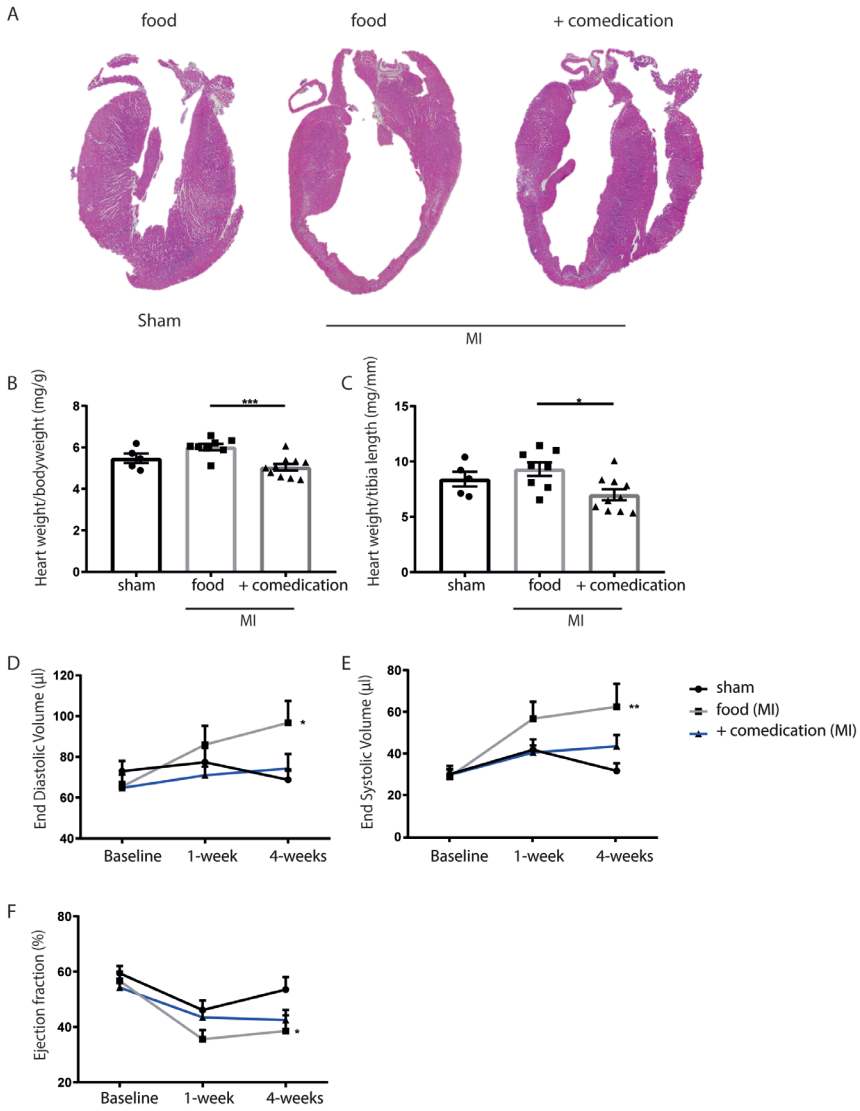

Figure 1. Comedication reduces adverse cardiac remodeling after I/R injury
General morphology and cardiac function were assessed 4 weeks after MI. Representative H&E images of the heart after 4 weeks of I/R injury (A). Heart weight/ body weight ratio and heart weight/tibia length were decreased in mice treated with comedication directly after MI (B-C). Echocardiography showed significantly increased EDV and ESV in mice without comedication after 4 weeks of I/R injury compared to sham control animals (D-E). In addition, EF was decreased in mice without comedication compared to sham animals (F). *I/R: ischemia reperfusion, H&E: hematoxylin and eosin, MI: myocardial infarction, EDV: end diastolic volume, ESV: end systolic volume, EF: ejection fraction. Sham n=5, regular food n=8, comedication n=10. * p<0.05, ** p<0.01, *** p<0.001.*

increased upon MI compared to sham operated animals, although this did not reach statistical significance (regular food p=0.1 and comedication p=0.06). Furthermore, no difference in the total amount of cardiac fibrosis was visible between MI mice with or without comedication, thereby indicating that comedication alone does not influence the total amount of cardiac fibrosis. Interestingly, in mice treated with comedication, thick collagen fibers (depicted as orange) are located near the luminal side of the LV, whereas mice with regular food showed thin collagen fibers (depicted as green) throughout the entire LV wall.

MSC and their paracrine function are not affected by comedication *in vitro*

After we observed that the administration of comedication affects cardiac remodeling upon I/R injury in mice, we subsequently investigated whether comedication might influence the efficacy of cell therapeutics. Before MSC were administered intra-myocardial in mice, their morphology and functional paracrine effects were first tested *in vitro (Figure 3*). MSC were cultured with or without the presence of comedication for 24 h (*Figure 3A*), which resulted

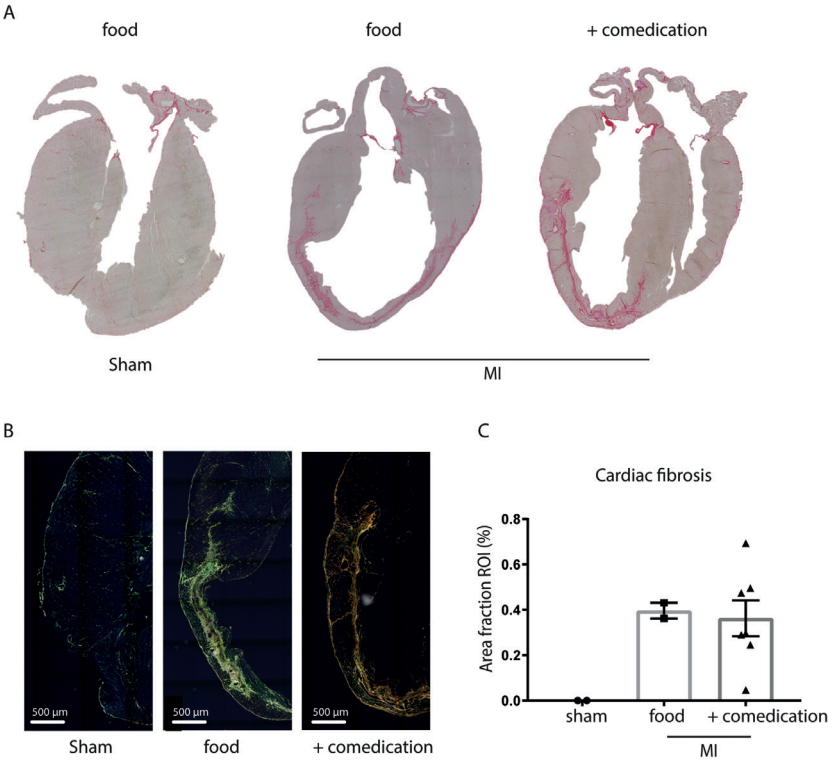

Figure 2. Cardiac fibrosis is not reduced by comedication

Collagen deposition was assessed using picrosirius red staining. Representative images of picrosirius stained hearts, showing a substantial amount of collagen deposition in the LV (A-B). Quantification of the amount of cardiac fibrosis, depicted as percentage of the LV (C). Upon I/R injury, cardiac fibrosis is increased, however, not altered by the administration of comedication. *Magnification 10x, line bar indicates 500 µm. LV: left ventricle, I/R: ischemia reperfusion. Sham n=2, regular food n=2, comedication n=7.*

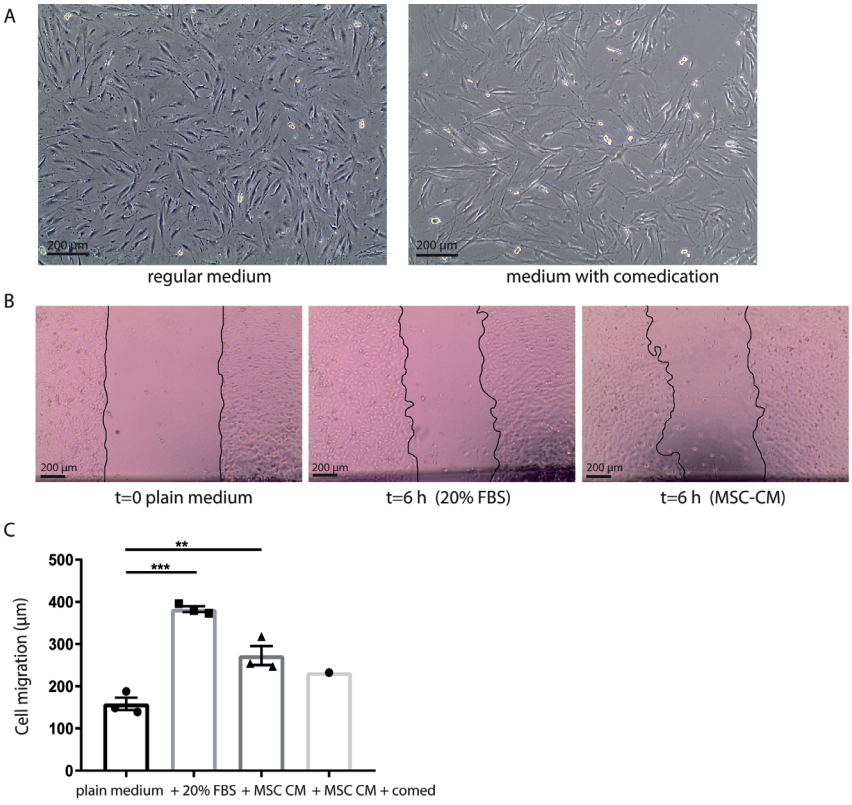

Figure 3. Independent of comedication, MSC secrete paracrine factors which stimulate cell migration *in vitro*

MSC were cultured and cellular morphology and paracrine effects were assessed *in vitro*. Microscopic images of cultured MSC in the absence or presence of comedication in the medium (A). MSC morphology slightly changed upon exposure to comedication, with more elongated cells present in the culture. Scratch assay was performed to investigate the paracrine actions of MSC on endothelial cell migration (B). MSC-derived conditioned medium induced closure of the scratch after 6 hours of culture. Further quantification showed a significant increase in endothelial cell migration when MSC-derived conditioned medium was added compared to plain medium (C). When MSC were cultured with comedication before collecting conditioned medium, the paracrine effect on endothelial cell migration was not affected compared to MSC cultured without comedication. *Scale bar indicates 200 µm. MSC: bone marrow-derived mesenchymal stromal cells, CM: conditioned medium. Plain medium n=3, 20% FBS n=3, MSC-CM n=3, MSC-Cm + comedication n=1. ** p<0.01,*** p<0.001.*

in a slightly affected, more elongated, cell morphology after comedication treatment. However, no limitations on cell proliferation or cell viability were observed. Previous studies already demonstrated the capacity of human-derived MSC to induce cell migration, of for example endothelial cells, by secreting paracrine factors[21,22]. To assess whether comedication influences the paracrine function of MSC on cell migration *in vitro*, a scratch assay was performed (*Figure 3B*). Microscopic images showed closure of the scratch by MSC-CM compared to t=0, albeit less pronounced than the positive control consisting of 20% FBS. Next, cell migration was further quantified by measuring the distance of scratch closure

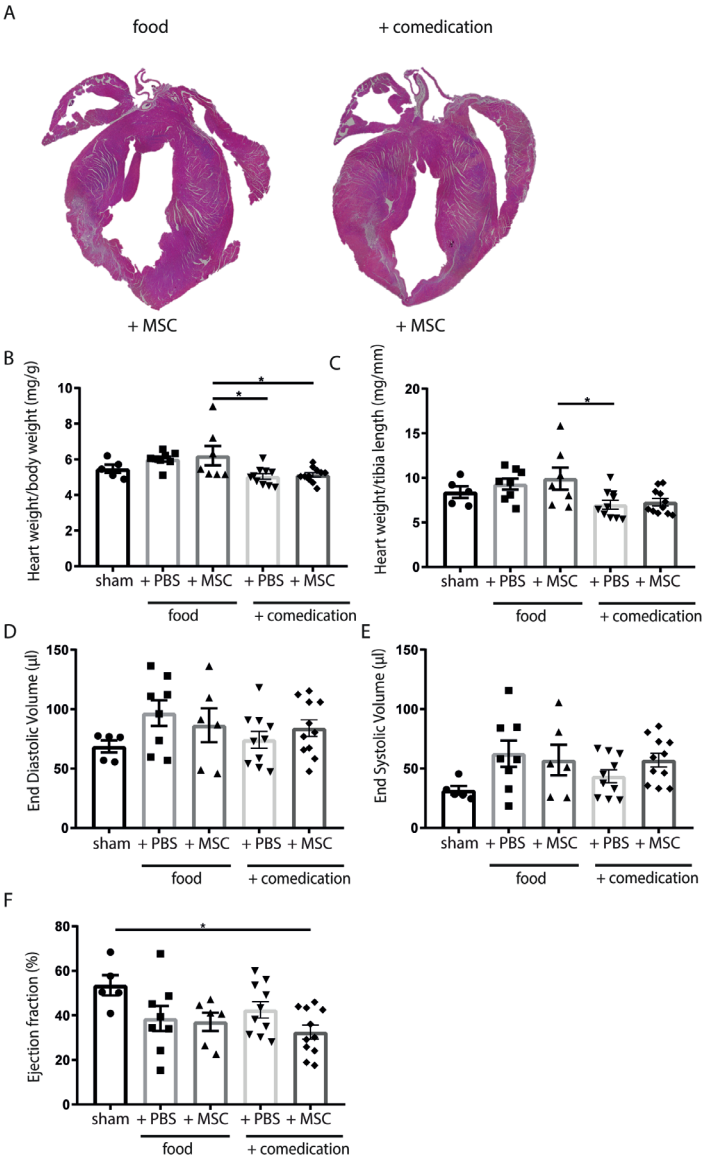

Figure 4. MSC in addition to comedication do not further reduce cardiac remodeling after I/R injury
General morphology and cardiac function were assessed 4 weeks after I/R and MSC injection. Representative H&E images of the heart after 4 weeks of I/R injury with MSC treatment (A). Infarction was clearly visible in the LV of both MSC treated mice with or without comedication. Heart weight/body weight ratio and heart weight/tibia length ratio were significantly lower in mice treated with MSC and comedication compared to mice who only received MSC (B-C). No significant differences in EDV or ESV were found between mice treated with PBS or MSC in both groups (D-E). No additional reduction in volumes was observed when MSC and comedication were used simultaneously. EF was significantly reduced in mice with MSC and comedication compared to sham control animals (F). *Sham n=5, regular food+ PBS n=8, regular food+ MSC n=6, comedication+ PBS n=10, comedication+ MSC n=11. * p<0.05.*

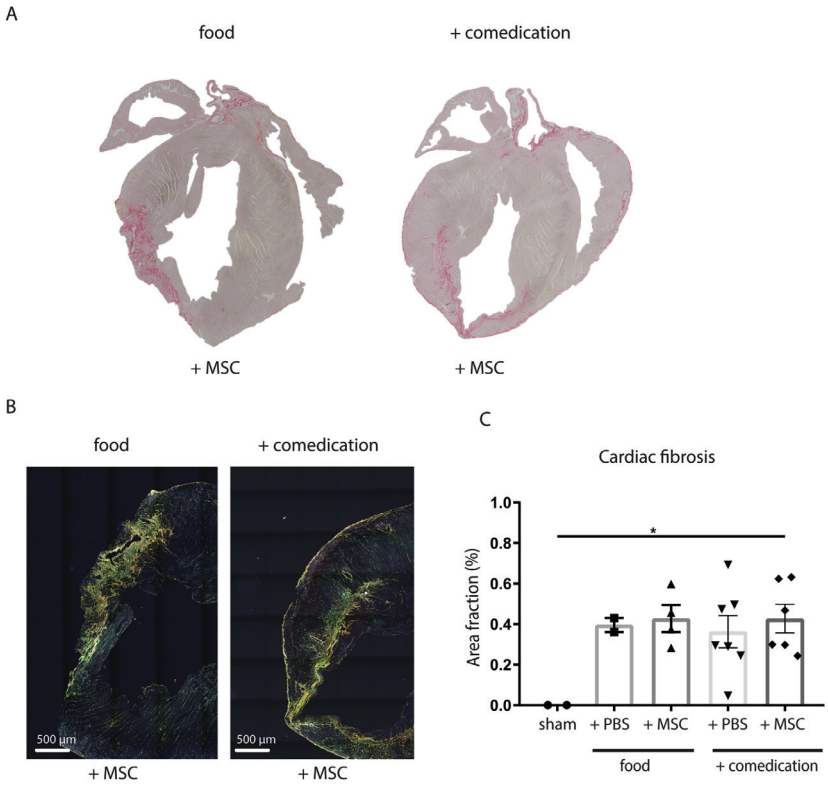

Figure 5. Cardiac fibrosis is not influenced by MSC therapy as compared to comedication
Collagen deposition was assessed using picrosirius red staining 4 weeks after I/R injury and MSC injection. Representative images showing cardiac fibrosis and collagen deposition in MSC-treated mice (A-B). Quantification of the total amount of collagen deposition in the LV showed no differences between PBS- and MSC-treated animals in both groups (C). *Scale bar indicates 500 µm. Sham n=2, regular food+ PBS n=2, regular food+ MSC n=4, comedication+ PBS n=7, comedication+ MSC n=6. * p<0.05.*

(*Figure 3C*). MSC-CM significantly increased cell migration of endothelial cells compared to plain medium (p=0.006), which was not affected by pre-treatment with comedication.

Intra-myocardial injection of MSC does not increase the beneficial effect of comedication on cardiac remodeling or cardiac fibrosis

Next, we studied whether comedication might influence the efficacy of MSC on cardiac function. Therefore, we subjected mice to I/R injury for 60 min, after which MSC or PBS was injected intra-myocardial (*Figure 4*). General morphology of hearts of mice treated with comedication and MSC demonstrated a smaller infarction compared to mice with only MSC (*Figure 4A*). Accordingly, heart weight/body weight (5.13±0.12 vs 6.21±0.55 mg/g; p=0.03) and heart weight/tibia length (7.27±0.41 vs 9.92±1.25 mg/mm; p=0.056) were lower in comedication and MSC-treated mice compared to MSC-treated mice with regular food (*Figure 4B-C*). Despite of what has been published before and the functional paracrine effects of

MSC observed *in vitro*, no beneficial effects of MSC on cardiac function were observed (*Figure 4D-F*, EF; PBS 38.58±5.64%, PBS+MSC 37.07±4.13%; comed 42.50±3.70%, comed+ MSC 32.51±3.14%).
To investigate whether MSC reduce cardiac fibrosis after I/R injury, the amount of collagen deposition was measured (*Figure 5*). The addition of MSC did not induce differences in the amount of collagen deposition between mice with regular food or comedication (*Figure 5A-B*). Additional quantification showed no significant differences between MSC or PBS treated mice in both the regular food group and mice with comedication (*Figure 5C*). These data suggest that MSC does not improve cardiac function or decrease cardiac fibrosis by itself or when combined with comedication.

DISCUSSION

Currently, there is a translational failure between preclinical findings and therapies that actually reach the clinical arena, such as cellular therapeutics. Progenitor cells significantly improve cardiac function in preclinical studies[8,11,23], however, limited effects were observed in clinical trials[13,14]. Surprisingly, medications which are prescribed as standard care for patients after MI, are barely incorporated into preclinical studies. This might explain part of the reduced efficacy of new therapeutics, when translating from the lab towards the clinic. Therefore, in this study, we investigated whether clinically prescribed medications affect cardiac outcome in a small animal model of I/R injury and whether these medications might also influence the efficacy of cellular therapeutics using BM-derived MSC.
We demonstrated that the administration of comedication after I/R significantly reduced adverse cardiac remodeling. Mice that received comedication showed less cardiac hypertrophy upon I/R injury, as represented by a decreased heart weight to body weight and heart weight to tibia length ratio compared to mice with regular food. In addition, mice with comedication showed no significant changes in EDV, ESV or EF compared to sham control animals, whereas cardiac function was significantly deteriorated in mice with regular food. These findings indicate that the use of comedication significantly reduced adverse cardiac remodeling in preclinical studies using small animals. The beneficial effect of comedication is in line with the clinical situation, as patients treated with clinically prescribed medications show an increased function capacity next to an improved clinical status and quality of life[3].
Next, the influence of comedication on the formation of cardiac fibrosis was investigated. However, no difference in the total amount of collagen deposition between mice treated with comedication or mice receiving regular food was found. Previous studies already showed that the use of statins after MI reduced myocardial fibrosis and infarct size in small animals[24,25]. In contrast, long term administration of β-blockers in healthy mice induced cardiac fibrosis by inhibiting activation of the β1-adrenergic receptor[26]. Moreover, the beneficial effects of statins on infarct size were blunted when aspirin was added in small animals[27]. These findings suggests that the combination of all 5 medications in a mouse model of I/R injury interferes with the actions of a single compound, and this could explain

why we did not observe a decrease in cardiac fibrosis in our model. We did observe a difference in localization and type of collagen fibers, where mice treated with comedication showed thicker collagen fibers, presumably collagen type I, mainly at the luminal side of the LV wall. Mice with regular food demonstrated thinner interstitial collagen fibers, presumably collagen type III, throughout the entire LV wall. It is known that the use of atorvastatin in rats after LAD ligation affects collagen metabolism, which might lead to different collagen types[28]. In addition, increased deposition of collagen type III in the myocardium of ischemic cardiomyopathy patients was shown to alter myocardial compliance[29]. The use of captopril in these patients resulted in a lower concentration of collagen type III compared to patients without treatment, which is in line with our observations. Moreover, interstitial fibrosis leads to a stiffer myocardium, thereby inducing diastolic and systolic dysfunction[30], which is also in line with the increased EDV and ESV we observed in mice with regular food. However, since no specific staining for collagen subtypes were performed in our study, at this point we could only speculate that mice with regular food might have more interstitial fibrosis throughout the LV wall, which might lead to a stiffer myocardium compared to mice with comedication.

Since we show that the use of comedication in small animal models of I/R already greatly influenced the process of cardiac remodeling, it is likely that comedication also influences the efficacy of other therapeutics, like cell therapy. In both small and large animal studies, the beneficial added effect of cell therapy compared to a control group decreased when medication was incorporated[23,31]. The main question is whether there still is an additive beneficial effect of cell therapy when all five of the clinically prescribed medications are incorporated in preclinical studies. Therefore, we investigated whether the use of comedication affected the efficacy of bone marrow-derived MSC on cardiac remodeling and fibrosis, by intra-myocardial injections of MSC directly after I/R injury.

Despite confirmed functional paracrine actions of the MSCs *in vitro*, we did not observe a beneficial effect of MSC on cardiac function or cardiac fibrosis. Hence, in animals with regular food, MSC did not improve cardiac function and were not able to decrease cardiac fibrosis. This is in contrast with other findings, where MSC improved EF with approximately 8% and were able to decrease infarct size and adverse cardiac remodeling after MI[8,10,23,32]. Intra-myocardial injections are the most favorable route of delivery in both preclinical and clinical trials, because of the reduction in infarct size and improvement in LVEF[33]. Therefore, considering the translational value of our model, we also made use of intra-myocardial injections of MSC. We might only speculate that our time window of the cell injections was not optimal, since recently, it has been shown that the optimal time window for MSC therapy pre-treated with statins is during the mid-term stage (second week) after MI[34]. Another important aspect of MSC is their cellular heterogeneity, which can lead to different phenotypes and functional characteristics[35]. Therefore, additional experiments are needed, using different cell batches, to evaluate the effect of comedication on the efficacy of cell therapeutics *in vitro* and *in vivo*. A limitation of our study is the relatively low number of animals which could be used for IHC. Due to reduced quality of the myocardial tissue as a consequence of technical issues with tissue embedding and making paraffin sections, many mice had to be excluded based on the lack of an intact LV wall. Therefore, this study has to

be considered as a pilot study and additional experiments are needed.
In conclusion, it is clear that cardiovascular research models need to incorporate clinically prescribed drugs when testing the potential of new therapeutic approaches, since also in clinical trials, new therapeutics have to show an additional effect on top of standard or even optimal care. Our data show that, when using preclinical models, it is crucial to mimic the clinical situation in the best way possible in order to expedite proper translation of new therapeutics. This will not only lead to a more efficient translation of new therapeutics to the clinical situation, but will also save resources as it allows to terminate the development on non-effective therapeutics in an earlier stage.

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Conflicts of interest
The authors declare no conflicts of interest.

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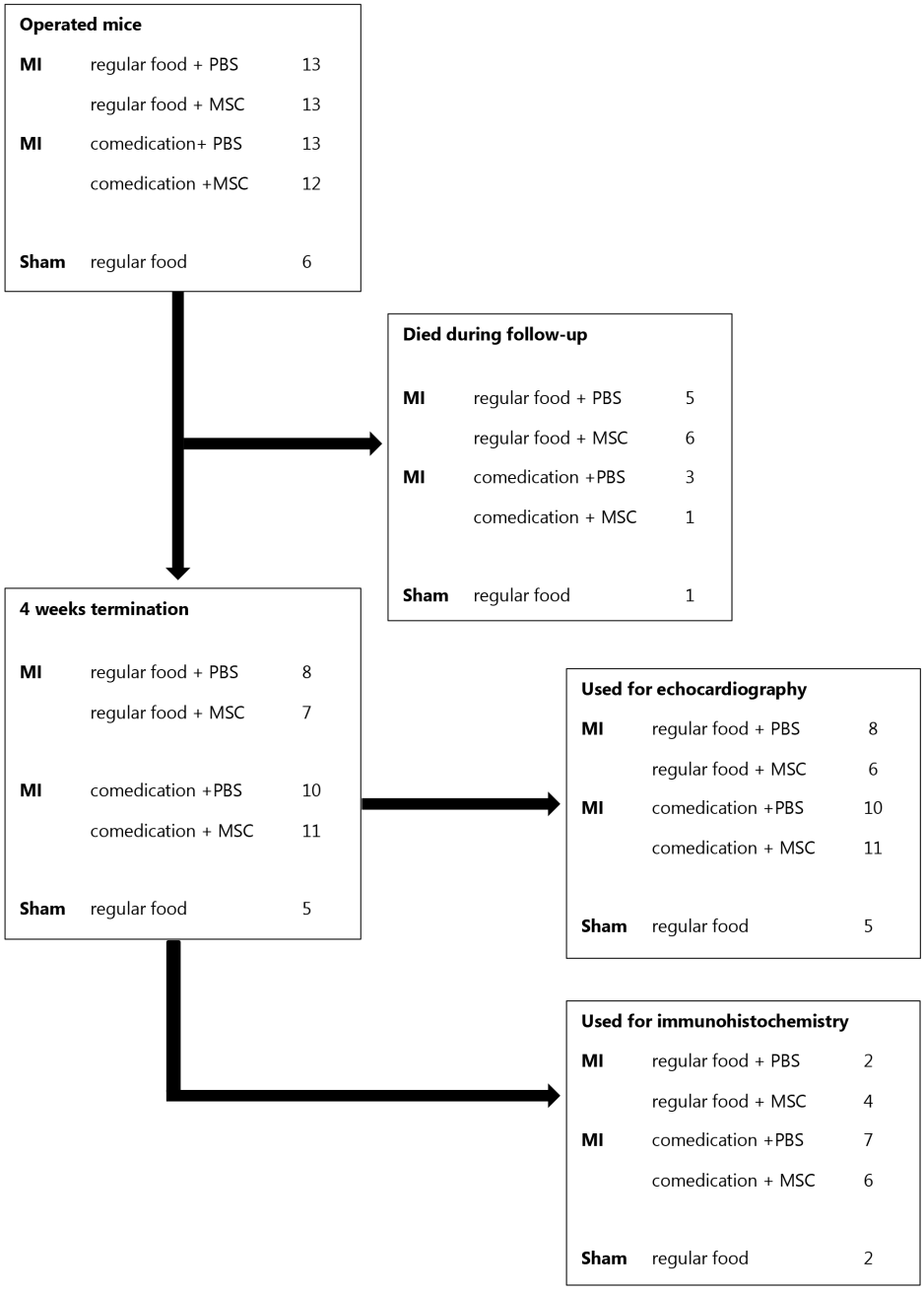
SUPPLEMENTARY FIGURES



Supplementary Figure 1. Flow chart of mice used in the study

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Summary and general discussion

Despite improved therapeutic strategies, cardiovascular disease (CVD) is still the number one cause of death. Heart failure (HF) is one of the most common cardiovascular diseases, affecting approximately 26 million people worldwide and is characterized by adverse cardiac remodeling and a reduced ability of the heart to maintain cardiac output[1,2]. HF has been classified into different subtypes, including HF with reduced ejection fraction (HFrEF), HF with mid-range ejection fraction (HFmrEF), and HF with preserved ejection fraction (HFpEF)[3,4]. In contrast to acute myocardial infarction (AMI), where the use of reperfusion strategies significantly improved survival rates by decreasing the amount of injury to the heart, patients are still at increased risk of developing HF[5]. However, limited treatment options are available for patients with HF[5–7]. To date, heart transplantation (HTx) is the only treatment modality for patients with end-stage HF, nonetheless long-term survival rates after transplantation are relatively poor[8,9]. In addition, the main limitation of HTx is the lack of sufficient and suitable donor hearts worldwide, which results in long waiting times[9,10]. Therefore, new treatment strategies aiming to limit adverse cardiac remodeling after cardiac damage and upon HF progression are of great importance. One of the key players in the chronic setting of adverse cardiac remodeling is the inflammatory response[11–13]. Increasing evidence supports a potential role for an autoimmune response in HF, which is directed against the myocardium itself after initial cardiac damage, and might accelerate disease progression[14,15]. In this thesis, we aimed to explore the role of antibody-mediated immune responses in different etiologies and stages of HF, and to extend the conceptual basis for novel therapeutic interventions.

Cardiac inflammation, a double-edged sword

The immune response is essential to clear the myocardium of dead cells and debris and contributes to scar formation after initial cardiac damage. However, it is considered more-and-more detrimental in the chronic phases of cardiac remodeling[11]. The inflammatory phase after cardiac injury is often not properly balanced, leading to a chronic immune response and additional damage to the myocardium, perpetuating the process of immune activation. This vicious cycle supports the presence of a chronic immune response in the heart, leading to increased cardiac damage and thereby contributes to adverse cardiac remodeling and HF progression. It is therefore clear that cardiac inflammation plays a dual role, which is both essential in the initial phase upon cardiac damage, but seems detrimental for the heart on the long run. The challenge is to explore these detrimental pathways of chronic inflammation, which contribute to HF progression in order to develop new diagnostic and therapeutic interventions. Potential key players in chronic inflammation are both T and B lymphocytes, which upon over-activation can induce an exacerbated immune response and additional damage to the myocardium via the production of pro-inflammatory cytokines, cytotoxicity, and antibodies[12,16]. In patients with a history of myocardial infarction (MI), autoantibodies of various specificities have been found, including anti-troponin, anti-myosin and anti-actin antibodies. These autoantibodies were shown to affect cardiac function and may even contribute to disease progression[16]. Therefore, B cells and antibody-mediated immune responses might be one of the detrimental pathways of chronic inflammation.

Antibody-mediated (auto) immune responses in heart failure

The contribution of B cells and antibody-mediated immune responses to the progression of HF has been the subject of only a limited number of studies. It has been postulated that B cells play an important role through antibody-dependent mechanisms, in which myocardial-derived autoantigens are involved[17]. The exposure of cardiac proteins, released by damaged or necrotic cells in a highly inflammatory environment after initial cardiac damage, may disrupt the immune balance and elicits an immune response towards self-proteins by activating autoreactive T and B cells. Plasma cells can subsequently generate cardiac-specific antibodies and pro-inflammatory cytokines[12]. It has been suggested that binding of these autoantibodies to cardiac-specific antigens expressed on the myocardium can induce cardiac dysfunction, for instance by affecting contractility or by inducing complement-dependent toxicity (CDC)[18–20]. Moreover, autoantibodies directed against endothelial cells might induce additional vascular injury[21]. Therefore, in **chapter 2**, we investigated the presence of an antibody-mediated immune response in patients with left ventricular diastolic dysfunction (LVDD), a potential early phase of HFpEF, and in patients with ischemic heart disease (IHD) and dilated cardiomyopathy (DCM), the end-stage of HFrEF. We found infiltrating immune cells (macrophages, T cells and B cells), myocardial IgG deposits and high levels of circulating immunoglobulins in patients with end-stage HFrEF compared to healthy controls. This antibody-mediated immune response was more severe in patients with IHD compared to patients with DCM. This might be explained by differences in quantity and identity of antigens released. In DCM, myocardial damage might be less pronounced as compared to IHD and consequently the immune system may be exposed to less cardiac antigens, possibly resulting in a less severe antibody-mediated immune response. However, additional experiments are needed to define to what extent B-cell activation and subsequent differentiation into other B-cell subsets including plasma cells has occurred, for example by using flow cytometry. Next to increased antibody levels, deposits of complement factor 3c (C3c) were observed in the myocardium of end-stage HFrEF patients, which also indicates activation of the complement system. This is in line with the fact that IgG1 and IgG3 have a high capability to activate components of the complement system, which further promotes the inflammatory response in the myocardium[15,22]. In addition, in men with LVDD, elevated levels of IgG1 and IgG3 were observed, which were also correlated to the severity of LVDD. Possibly in this early stage, myocardial damage has occurred that sensitizes the immune system, e.g. due to microvascular dysfunction or increased wall pressure. Interestingly, we did not observe increased antibody titers in women with LVDD. It has already been described that the prevalence of LVDD is similar in men and women[3,23], but women with LVDD are more prone to develop HFpEF, whereas men more often develop HFrEF[24,25]. This sex difference is still poorly understood, but might be explained by a differential antibody-mediated response upon cardiac damage, as suggested by our data. Due to the limited numbers of LVDD patients, we can only speculate that upon myocardial damage, men develop a stronger antibody-mediated immune response, characterized by increased levels of IgG1 and IgG3, leading to additional cardiac damage and the progression to HFrEF. However, additional studies are necessary to completely uncover the role of potential sex-dependent autoimmune responses in the development of HF.

In **chapter 3**, we evaluated the contribution of antibody-mediated immune responses in patients with a genetic cardiomyopathy. Patients with a mutation in the phospholamban (PLN) gene demonstrate severe myocardial damage and have a high risk of developing HF[26,27]. However, there is still considerable debate how the mutation is leading to the development and progression of HF, since the mutation can also be found in asymptomatic carriers[28]. One of the possibilities might be the contribution of a 'second hit', potentially caused by a chronic inflammatory response that will lead to additional damage upon auto-reactivity to myocardial tissue and subsequent HF progression. In line with other HF etiologies, as shown in **chapter 2**, we found increased numbers of myocardial immune cells (macrophages, T cells, B cells and plasma cells), IgG deposits, signs of complement activation, and circulating immunoglobulins in PLN carriers with end-stage HF. These findings underscored the importance of an antibody-mediated immune response in PLN-mediated HF. To get more insight into potential targets of this immune response, we performed an epitope discovery screen, including 26,000 linear epitopes from cardiovascular proteins, to identify the type of proteins targeted by autoantibodies. We demonstrated that PLN carriers with HF show high levels of antibodies against cell adhesion proteins and structural components of cardiomyocytes compared to PLN carriers without HF, and unaffected family members. Our findings suggest that the binding of antibodies to cell adhesion proteins interferes with mechanical and electrical coupling between cardiomyocytes, which could be of great importance in the pathogenesis of arrhythmias and HF. Therefore, we set out to create an *in vitro* model, using induced pluripotent stem cell-derived cardiomyocytes (iPSC-CM) carrying the p.Arg14del mutation and patient-derived IgG, to reproduce the process of antibody binding as observed in PLN carriers. Using this model, we were able to show specific binding of PLN patient-derived IgG to iPSC-CM *in vitro*. However, the question remains how antibody binding is leading to impaired functional properties of PLN-derived cardiomyocytes. The next step would be to use this model to investigate the functional effects of antibody binding, for example by looking at cardiomyocyte contractility, beating rates, and action potentials. If we are able to unravel the exact mechanisms of antibody-mediated immune responses in the progression of HF, we hopefully further confirm the inflammatory hypothesis of HF progression, which paves the way for new therapeutic approaches.

Currently, the only treatment option for end-stage HF is cardiac transplantation, however, even after HTx, chronic inflammation is still limiting survival of patients[29,30]. One of the primary causes is cardiac allograft vasculopathy (CAV), which is characterized by concentric luminal narrowing of the coronary arteries of the graft, leading to severe ischemic damage and ultimately HF[31]. There is considerable controversy on the possible pathologies leading to CAV, where some state that an allogeneic immune response of the recipient is involved, whereas others argue that a donor cell-derived immune response against the graft plays a role[32,33]. In **chapter 4**, we reviewed the pathology of CAV by highlighting both the donor and recipient side of the disease. We postulated that both donor and recipient cells are involved. The immune system of the recipient might the most important player in the development of CAV, since immune activation of the recipient initiates allograft immune responses. Donor-

derived immune cells might enhance the immune response of the recipient, but they are not capable to induce CAV independently[34]. Interestingly, despite immunosuppressive therapies to limit post-HTx inflammation in the recipient, high levels of circulating antibodies were still found in CAV patients[35,36]. We hypothesized that a pro-inflammatory state of the end-stage HF recipient prior to HTx might actively contribute to CAV development and graft failure. Therefore, in **chapter 5**, we assessed whether pre-HTx antibody levels were associated with post-HTx survival. We demonstrated a significant correlation between pre-HTx immunoglobulin levels and survival post-HTx, as high levels of IgG1 and IgG2 were associated with a shorter survival time after HTx. Hence, patients with high immunoglobulin levels prior to HTx maintained relatively high levels after transplantation in comparison to patients with low pre-HTx immunoglobulin levels. This suggests that once the adaptive immunity is activated pre-HTx, it remains relatively active post-HTx despite immunosuppressive therapies, which questions current immunosuppression approaches. Instead of general immunosuppression, for example using corticosteroids, strategies specifically aiming at B cells and antibody production prior and post-HTx might be considered. The use of B-cell depleting strategies in combination with intravenous immunoglobulins have proven to reduce antibody-mediated rejection in kidney transplants[37] and might therefore also be effective in HTx recipients.
Moreover, we found an association between increased antibody titers pre-HTx and an inflammatory CAV phenotype, which suggests that an activated immune system pre-HTx also influences inflammation in the CAV lesion and thereby initiates a more rapid development of these lesions and limits survival. These findings have to be validated in larger HTx cohorts, but if indeed confirmed, screening of HTx recipients prior to transplantation for their immunological state and levels of circulating antibodies could be considered. This might pave the way for future transplantation protocols with anti-inflammatory therapies, aiming at B-cell responses and depleting circulating antibodies, implemented already prior to transplantation.

Animal models to study antibody-mediated immune responses in heart failure

Animal research in the field of HF often makes use of mouse models of MI or transverse-aortic constriction (TAC) models, to represent HF induced by ischemia and pressure overload[38]. However, suitable *in vivo* mouse models to study the role of cardiac-specific autoimmune responses in the development of HF are still lacking. Since the presence of cardiac-related antibodies in end-stage HF closely resembles autoimmune responses as observed in myocarditis, we hypothesized that myocarditis models can be the basis for future disease models to elucidate autoimmune-induced HF. Myocarditis is an inflammatory cardiomyopathy, caused by a persistent and self-sustained autoimmune response against cardiac myosin[39,40], as highlighted in **chapter 6**. Different mouse models have been developed to induce myocarditis to study the role of chronic inflammation in HF progression[41–43]. In **chapter 7**, we set out to reproduce the classical experimental model of autoimmune myocarditis (EAM) as a representative model of autoimmune mediated HF, with the aim to adapt this model to study the role of cardiac-specific antigens in HF progression. However, in contrast to other studies, we failed to induce EAM in our mice.

Despite increased antibody titers upon immunization, we did not observe any signs of adverse cardiac remodeling, influx of immune cells, or cardiac fibrosis six weeks after immunization. Perhaps, the degree of antigen release in our model was not sufficient to induce a robust cardiac-specific immune response and additional stimuli may be needed to boost antigen release and the immune response. It has already been shown that the use of Complete Freund adjuvant (CFA) results in varied outcome of myocarditis severity[44,45]. Currently, there is discussion which type of adjuvant might be the best to induce autoimmune myocarditis. Therefore, some research groups also include a second booster adjuvant including pertussis toxin (PT) or lipopolysaccharide (LPS) to increase the severity of EAM[42,44,45]. However, it can be questioned whether these approaches are still translatable to the clinical setting of autoimmune-induced myocarditis and HF progression, when administration of multiple adjuvants and toxins is needed to induce a sufficiently strong immune response. One of the drawbacks of using small animals to study autoimmune responses is that the mouse immune system might not be fully representative for immune-pathological processes as observed in humans[46]. For example, not all strains are susceptible for immunization protocols. Certain strains, including BALB/c mice, are known to respond with a Th2-mediated immune responses upon immunization[47], whereas e.g. C57BL/6 are less susceptible due to different immunological responses (Th1-mediated) to antigen immunization[48–50]. Another important feature of EAM models is a sex bias, since male mice are more susceptible to induction of heart-specific autoimmunity compared to female mice, which corresponds to the prevalence of human myocarditis[40] and our findings in **chapter 2**. However, in general, autoimmune diseases, such as RA or multiple sclerosis (MS), which are also often associated with the progression of HF, are more common in women[51–53]. Therefore, much effort must be put into creating a reproducible and translational experimental autoimmune-induced HF mouse model, which closely resembles immune responses as observed in humans. A potential model to closely resemble human immune responses might be the humanized mouse model. The engraftment of human hematopoietic stem cells into immune-deficient mice is used to create a mouse with a human-like immune system to study the pathogenesis of several autoimmune diseases and the identify new therapeutic targets[54]. Whether the humanized mouse model might also be suitable to study cardiac-specific autoimmune responses in the development of HF remains to be elucidated in future studies.

Translational failure in the development of therapeutic interventions

As touched upon in **chapter 7**, we encounter translational difficulties in creating small animal models to study mechanisms of disease as observed in humans. The same holds true for testing new therapeutic interventions and the translation of new therapeutics to the clinic. One example is the use of cellular therapeutics, for example progenitor cells, which has been a promising strategy to improve cardiac function after MI[55]. These progenitor cells, including mesenchymal stromal cells (MSC) and cardiac-derived progenitor cells (CPC), are defined as cells with the ability to differentiate into other cell types and have multiple molecular and functional properties[56]. In small animal models, the use of MSC and CPC might also be a promising strategy in the treatment of HF, since these cells do not only show beneficial effects on cardiac function but also exert strong immunosuppressive properties,

mainly in a paracrine matter[56,57]. Important paracrine mediators are extracellular vesicles (EVs), small lipid bi-layered vesicles containing lipids, small RNAs and proteins, which are able to influence many processes, including chronic inflammation[58,59]. Therefore, the use of EVs has become a hot topic currently not only as new therapeutics in CVD, but also as potential immunosuppressive agents for multiple autoimmune disorders[60]. For MSC and MSC-derived EVs, it has already been established that they can modulate the adaptive immune response by suppressing plasma cell formation and antibody production[60,61].
Interestingly, less is known about the therapeutic immunosuppressive capacity of CPC or CPC-derived EVs on B cells and antibody-mediated immune responses as observed in HF. Therefore, in **chapter 8**, we sought to evaluate the *in vitro* inhibitory actions of different progenitor cells, including MSC, CPC, and CPC-derived EVs on lymphocyte proliferation and the production of immunoglobulin subclasses. We demonstrated immunosuppressive actions of both MSC and CPC on lymphocyte proliferation and antibody secretion, with the strongest effects observed when using MSC. Moreover, we showed that CPC and especially MSC were able to suppress antibody secretion by patient-derived cells, thereby indicating the *in vitro* potency of progenitor cells to dampen antibody-mediated immune responses in HF.
After validating these results in additional *in vitro* experiments, the next step would be to test the use of progenitor cells in preclinical animal models. However, the further cellular therapeutics move along the translational axis, the lower their efficacy[62]. Despite beneficial effects of progenitor cells and cellular therapeutics on cardiac function and cardiac inflammation, as observed *in vitro* and in small and large animal models[63–65], the efficacy of cellular therapeutics on top of standard prescribed medications in the clinical setting is disappointing. Several randomized clinical trials, using cell therapeutics to improve cardiac function in cardiovascular disease, showed inconsistent results and modest efficacy of progenitor cells[56,66–68]. Currently, there is a translational gap, also referred to as the translational valley of death, between preclinical findings and therapies that actually show an additional benefit on top of standard care. Surprisingly, the combination of all medications which are clinically prescribed as standard care after MI, being aspirin, P2Y12-inhibitors, β-blockers, AT1R antagonists and statins, is barely incorporated into preclinical studies when testing a new therapy. Despite clear beneficial effects of a single medication in small animal models of MI, such as the use of statins or P2Y12-inhibitors[69,70], not a single study reported the use of all five medications simultaneously or in combination with an additional therapy, such as cellular therapeutics. This might explain part of the reduced efficacy of new therapeutics, when translating from the lab towards the clinic. Therefore, in **chapter 9**, we assessed the effects of all five clinically prescribed medications on adverse cardiac remodeling, cardiac function and fibrosis in a small animal model of ischemia and reperfusion (I/R) injury. Hence, we tested whether or not treatment with these clinically prescribed medications influences the efficacy of cell therapeutics. We showed that the administration of five clinically prescribed standard medications significantly reduced adverse cardiac remodeling and improved cardiac function in small animal models of MI. Mice that received comedication showed less cardiac hypertrophy upon I/R injury and no significant changes in EDV, ESV or EF compared to sham control animals, whereas cardiac

function was significantly deteriorated in mice without comedication. No additional beneficial effects of MSC were observed. From our data is it clear that cardiovascular research models need to incorporate clinically prescribed drugs when testing the potential of new therapeutic approaches, since also in clinical trials, new therapeutics have to show an additional effect on top of standard care or even optimal care. Despite the promising effects observed *in vitro*, it must be kept in mind that it is crucial to mimic the clinical situation in the best way possible to expedite proper translation of new therapeutics and to increase translational success.

FUTURE PERSPECTIVES

The studies described in this thesis provided new insights into the contribution of antibody-mediated immune responses in different etiologies and stages of HF. This chronic inflammatory response is a key player in end-stage HF and is also limiting survival after HTx. These findings indicate that HF should also be considered as an autoimmune disorder rather than only being a heart disease. For this, we need to identify the detrimental pathways of antibody-mediated immune responses, which contribute to HF progression in order to find new diagnostic and therapeutic interventions, as suggested in *Figure 1*. The first step would be to explore the functional effects of antibody binding to target cells *in vitro,* as briefly touched upon in **chapter 3**. If we are able to identify the pathological role of autoantibodies *in vitro* and possible target peptides, these findings can be validated in small animals, by immunizing mice with the antigen of interest and subsequently study disease progression and to test therapeutic interventions. However, the first obstacle is to find an appropriate animal model, which closely resembles autoimmune-induced HF as observed in humans and is also suitable to incorporate sex differences. Therefore, much effort must be put into elucidating the potential of humanized mouse models to study autoimmunity in HF progression.

Moreover, an important aspect is the identification of the myocardial autoantigens. One of the possibilities is to use epitope discovery screens in a large cohort of patients with different etiologies of HF, to develop potential diagnostic and prognostic screening platforms. As suggested by our epitope screening data, different etiologies of HF may result in different myocardial antigens which are being exposed, which also leads to different autoantibody subtypes. If we are able to find a specific epitope-based fingerprint for each type of HF, we could create screening arrays to diagnose patients at risk based on a single blood sample. This would also provide insights into new therapeutic targets, which may differ per HF subtype and stage of the disease. However, these epitope screens have to be performed in large numbers of patients, using different patient cohorts worldwide, which would be extremely costly. The future will teach us whether epitope screenings will be implemented as potential diagnostic or prognostic tool for HF.

The important role of chronic inflammation in adverse cardiac remodeling and HF progression also indicates that current HF therapeutic approaches may have to be reevaluated and possibly adapted. We might have to incorporate immunosuppressive

therapies, which are modulating inflammation and specifically targeting B cells and autoantibody production, for example using B-cell depleting strategies and immunoadsorption. Therapies that specifically deplete pathogenic antibody-producing plasma cells using proteasome inhibitors (bortezomib) are effective in autoimmune disorders, such as systemic lupus erythematosus (SLE)[71], and might also be effective to target antibody-mediated immune responses in HF. However, safety and efficacy of bortezomib in HF patients should first be evaluated in clinical trials. Moreover, using immunoadsorption in DCM patients to eliminate circulating IgG already demonstrated a decrease in the number of myocardial T and B lymphocytes and HLA-II expression[71]. However, currently it is not clear whether antibody depletion alone might be sufficient to modulate humoral immunity in the long run, or whether it might be necessary to incorporate suppressive therapies for cellular-mediated immunity as well. Therefore, future studies will have to establish if B-cell depleting strategies and immunoadsorption might be safe and effective in HF patients.

The same holds true for the only treatment option for end-stage HF patients, being HTx, in its current form. As demonstrated in **chapter 5**, the inflammatory state of the recipient greatly influences patient outcome and survival after HTx. Does this mean that we have to be selective which patients that are eligible for transplantation based on their immune state? Or do we have to use a more personalized therapeutic approach with immunosuppressive therapies, where patients with high antibody titers pre-HTx have to be treated with more aggressive or more specific immunosuppressive agents? These important questions all have to be addressed in future studies, using larger numbers of patients.

Another important aspect that must be kept in mind is the difference between men and women. With regards to HF pathology and different immunological responses, researchers should thoroughly evaluate the influence of sex differences in HF progression. As demonstrated in **chapter 2**, men already showed high levels of IgG1 and IgG3 in a pre-stage of HFpEF, whereas elevated antibody titers were absent in women. This is line with the prevalence of myocarditis in humans, however, in contrast to other autoimmune disorders, including RA and MS, which are far more prevalent in women and can also induce HF progression[53,72]. The exact cause of sex differences in immune responses are not fully elucidated yet. The majority of research on sex differences focused on the role of sex hormones, however, increasing evidence suggests that also genes on the Y-chromosome contribute to immune susceptibility[73]. For example, non-pseudoautosomal gens on the Y chromosomes might have a protective role in autoimmune diseases, which could explain why women in general are more affected by autoimmune diseases as compared to men. However, the exact role of sex differences in antibody-mediated immune responses in HF progression has yet to be determined.

Our immune response is failing our heart

In conclusion, this thesis underscored the potential roles of antibody-mediated immune responses in different etiologies and stages of HF and highlighted some future directions. It is clear that chronic inflammation is failing our heart even more after initial cardiac damage and might therefore be an essential target in the treatment of HF. The next step is to use

the identified cardiac-specific epitopes to our advantage as diagnostic screening tool to not only monitor HF progression in patients, but also to create new patient-specific immunosuppressive therapies.

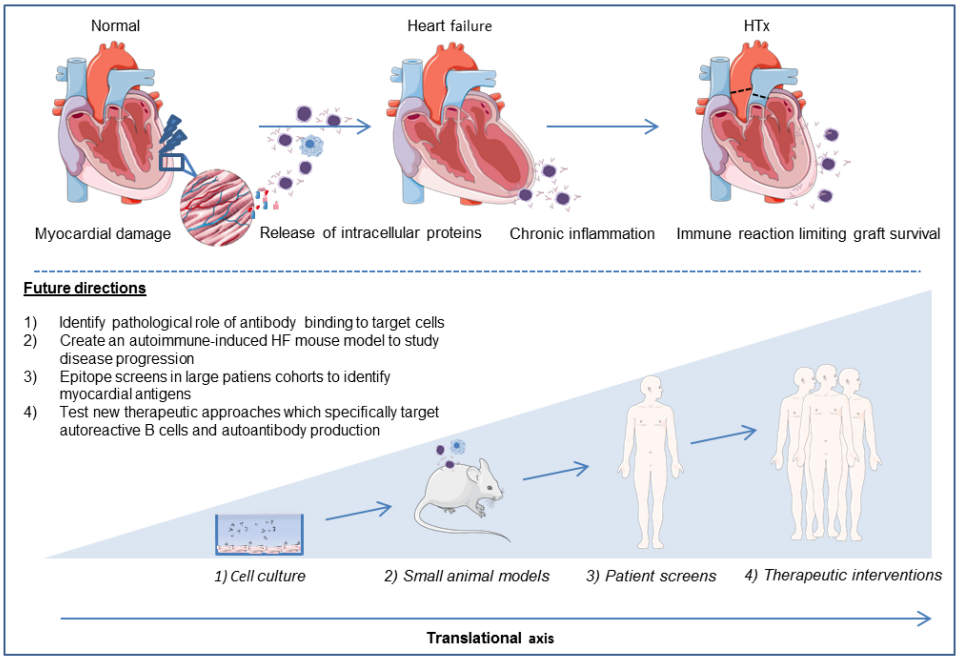

Figure 1. Overview of future perspectives

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Appendix

NEDERLANDSE SAMENVATTING

Hart- en vaatziekten zijn nog steeds de meest voorkomende doodsoorzaak wereldwijd ondanks dat er grote vooruitgang op het gebied van behandelingen is geboekt. De komst van dotterbehandelingen voor patiënten met een acuut hartinfarct zorgt in eerste instantie voor een aanzienlijke vermindering van de schade aan het hart. Op lange termijn blijven deze patiënten echter een verhoogd risico houden op het ontwikkelen van hartfalen.
Hartfalen is een veel voorkomende vorm van hart- en vaatziekte, waarbij het hart minder goed functioneert en hierdoor niet goed in staat is om het lichaam van voldoende bloed te voorzien. Er zijn verschillende vormen van hartfalen die variëren van hartfalen met een verminderde pompfunctie tot hartfalen met een behouden pompfunctie. In tegenstelling tot patiënten met een acuut hartinfarct, zijn beperkte behandelingen beschikbaar voor patiënten met hartfalen. Tot op de dag van vandaag is harttransplantatie de enige echte behandelingsoptie voor patiënten met hartfalen. De levensverwachting van mensen met een donorhart is echter maar beperkt. Daarnaast is er wereldwijd een tekort aan geschikte donorharten wat leidt tot lange wachtlijsten. Om deze redenen is onderzoek naar factoren die een rol spelen in de progressie van hartfalen en daarnaast het vinden van nieuwe behandelingsmethoden van groot belang.
In dit proefschrift hebben wij onderzoek gedaan naar een van de mogelijke processen die een belangrijke rol speelt in de ontwikkeling van hartfalen, namelijk de immuunrespons. Deze ontstekingsreactie is in eerste instantie essentieel in de vroege fase na schade aan het hart om ervoor te zorgen dat dode cellen worden opgeruimd en een litteken wordt gevormd. Echter, deze ontstekingsreactie blijkt vaak te heftig en te lang aanwezig in het hart, waardoor uiteindelijk juist meer schade wordt veroorzaakt. Dit is een belangrijk onderdeel van het ontwikkelen van hartfalen. Het is duidelijk dat de ontstekingsreactie een dubbelrol speelt, welke enerzijds essentieel is in de vroege fase van hartschade maar anderzijds op lange termijn juist nadelig kan zijn. De uitdaging is om de cellen te onderscheiden die betrokken zijn bij de chronische ontstekingsreactie en mogelijk dus ook betrokken zijn bij de progressie van hartfalen, met als doel hier nieuwe aangrijpingspunten voor therapie voor te ontwikkelen.

Belangrijke ontstekingscellen die een rol spelen bij chronische ontsteking zijn lymfocyten, welke bestaan uit B-cellen en T-cellen. Deze cellen zijn betrokken bij de normale afweer tegen bacteriën en virussen en spelen daarnaast ook een belangrijke rol in de ontstekingsreactie in het hart. Normaal gesproken markeren antilichamen lichaamsvreemde indringers en zorgen ervoor dat deze worden opgeruimd door het immuunsysteem. Steeds meer onderzoekers beweren dat over-activatie van lymfocyten kan leiden tot schade aan het hart door de productie van antilichamen die gericht zijn tegen het eigen hart. Grote hoeveelheden antilichamen lijken ook aanwezig te zijn in het bloed van patiënten met hartschade en steeds meer onderzoek laat zien dat deze mogelijk zijn gericht tegen hart-specifieke eiwitten. Deze antilichamen zouden dus een auto-immuunreactie tegen het eigen hart kunnen opwekken, wat zorgt voor meer hartschade en wat uiteindelijk kan bijdragen aan een verminderde hartfunctie en de progressie naar hartfalen. Echter, tot op heden is

er nog maar weinig bekend over de specifieke rol van B-cellen en hart-specifieke antilichamen in de ontwikkeling van hartfalen.
Het doel van dit proefschrift was om de rol van een antilichaam-gemedieerde ontstekingsreactie te bestuderen in patiënten met verschillende types en gradaties van hartfalen om zo mogelijk nieuwe aangrijpingspunten voor therapie te kunnen vinden. Hiervoor hebben wij in **deel 1** van dit proefschrift gekeken naar de aanwezigheid van antilichaam-gemedieerde ontsteking in patiënten met verschillende types en gradaties van hartfalen, zowel voor als na harttransplantatie. In **deel 2** van dit proefschrift hebben wij gebruik gemaakt van een muizenmodel om meer inzicht te krijgen in het onderliggende ziekteproces van het ontwikkelen van hartfalen door chronische ontsteking. In **deel 3** hebben wij geprobeerd om de mogelijkheden van stamcellen te onderzoeken om de chronische ontsteking te remmen en mogelijk de vertaalslag te maken naar nieuwe behandelingen.

Deel 1- De rol van een antilichaam-gemedieerde ontstekingsreactie in hartfalen
Om te onderzoeken of B-cellen en antilichamen een rol spelen in de ontwikkeling van hartfalen hebben wij in **hoofdstuk 2** gekeken naar de aanwezigheid van ontstekingscellen en antilichamen in patiënten met verschillende types en gradaties van hartfalen. Patiënten met ernstig hartfalen lieten opvallend hoge aantallen ontstekingscellen zien in het hartweefsel ten opzichte van 'gezond' hartweefsel. Daarnaast vonden wij verhoogde hoeveelheden antilichamen in het bloed als ook antilichamen gebonden aan het hartweefsel in deze patiënten. Bovendien zagen we in patiënten met ernstig hartfalen die in het verleden een hartinfarct hebben doorgemaakt de hoogste ontstekingswaarden, wat zou kunnen betekenen dat de mate van ontsteking kan variëren tussen patiënten met verschillende oorzaken van hartfalen. Om te onderzoeken of verhoogde hoeveelheden antilichamen al in een vroege fase aanwezig zijn in de ontwikkeling van hartfalen, hebben we gekeken in patiënten met een voorstadium van hartfalen. Ook in deze patiënten vonden we verhoogde ontstekingswaardes, echter opvallend genoeg alleen in mannen. We weten inmiddels dat de ontwikkeling van hartfalen kan verschillen tussen mannen en vrouwen en ook onze bevindingen wijzen hierop. Hoe de aanwezigheid van grote hoeveelheden antilichamen en ontstekingscellen precies bijdraagt aan de ontwikkeling van hartfalen in mannen en vrouwen is nog niet precies bekend en dient dan ook verder onderzocht worden.
Tevens hebben wij in **hoofdstuk 3** onderzocht of een antilichaam-gemedieerde ontstekingsreactie ook betrokken is bij het ontstaan van hartfalen in patiënten met een erfelijke vorm van hartfalen. Dit type hartfalen ontstaat door een 'foutje', ook wel mutatie genoemd, in het DNA waardoor de functie van een eiwit verstoord raakt. Een genetische vorm van hartfalen in Nederland wordt veroorzaakt door een mutatie in het phospholamban eiwit (PLN), waardoor patiënten al op jonge leeftijd hartfalen kunnen ontwikkelen. Echter, niet alle patiënten met de PLN-mutatie ontwikkelen uiteindelijk hartfalen, wat erop wijst dat ook andere factoren betrokken zouden kunnen zijn, waaronder bijvoorbeeld ontsteking. Ook in PLN-patiënten met ernstig hartfalen vonden wij verhoogde aantallen ontstekingscellen in het hartweefsel en hogere hoeveelheden antilichamen in zowel het bloed als ook in het hartweefsel. Tevens vonden wij in PLN-patiënten zonder symptomen van hartproblemen,

wie in een vroege fase onverwachts overleden zijn aan de gevolgen van een hartstilstand tijdens het sporten, al verhoogde aantallen ontstekingscellen in het hart. Dit zou erop kunnen wijzen dat reeds in een vroege fase van hartfalen de ontstekingsreactie een rol speelt. Om meer inzicht te krijgen waar de antilichamen tegen gericht kunnen zijn hebben we een screening uitgevoerd met daarin alle eiwitten die bekend zijn bij hart- en vaatziekten. Hieruit bleek dat de vele antilichamen in PLN-patiënten mogelijk gericht zijn tegen eiwitten die betrokken zijn bij de verbindingen tussen de hartcellen. Dit suggereert dat de ontstekingsreactie in PLN-patiënten de koppeling tussen hartcellen mogelijk aantast, wat hartritmestoornissen zou kunnen veroorzaken en uiteindelijk ook hartfalen. Echter, meer onderzoek is nodig om het precieze mechanisme hiervan te ontrafelen om zo de weg vrij te maken voor nieuwe behandelingen.

De rol van een antilichaam-gemedieerde ontstekingsreactie na harttransplantatie

Niet alleen in de ontwikkeling van hartfalen speelt ontsteking een belangrijke rol, maar ook na een harttransplantatie, waarbij afstoting van het donorhart door de ontvanger nog steeds een groot probleem vormt. Een van de grootste oorzaken van afstoting is de ontwikkeling van vernauwingen in de coronaire vaten (CAV) van het donorhart, wat kan leiden tot zuurstofgebrek naar het hart en uiteindelijk hartfalen. Het is nog onduidelijk hoe deze vernauwingen precies worden veroorzaakt. Hiervoor zijn verschillende theorieën, waarbij het onderzoeksveld is verdeeld in groepen die beweren dat een ontstekingsreactie van de ontvanger tegen het 'vreemde' donorhart bijdraagt aan de ontwikkeling van CAV en groepen die beweren dat resterende ontstekingscellen in het donorhart zijn betrokken. Beide kanten van het verhaal worden uitgelicht in **hoofdstuk 4**. Hierin beschrijven we dat het immuunsysteem van de ontvanger zeer waarschijnlijk de belangrijkste speler is voor het ontwikkelen van een ontstekingsreactie tegen het 'vreemde' donorhart en daarbij de ontwikkeling van CAV. Resterende donorcellen in het donorhart kunnen de ontstekingsreactie verergeren, echter niet onafhankelijk van de ontvanger CAV veroorzaken.

Opvallend is dat ondanks immunosuppressiva wat standaard wordt gegeven na harttransplantatie, CAV-patiënten nog steeds hoge levels van antilichamen laten zien in het bloed. Omdat we in voorafgaande hoofdstukken van dit proefschrift hebben laten zien dat deze verhoogde hoeveelheden al voor transplantatie aanwezig zijn tijdens hartfalen, hebben wij in **hoofdstuk 5** onderzocht of dit mogelijk de overleving van de patiënt na transplantatie zou kunnen beïnvloeden. In dit hoofdstuk laten we zien dat er een verband is tussen hoge antilichaam levels voor transplantatie en kortere overleving na transplantatie. Bovendien lijkt immunosuppressiva minder effectief in deze patiënten. Dit zou kunnen betekenen dat we misschien patiënten anders zouden moeten behandelen met specifieke immunosuppressiva gericht tegen B-cellen en de productie van antilichamen, zowel voor als na transplantatie. Daarnaast vonden we ook een verband tussen verhoogde antilichamen levels voor transplantatie en de ontwikkeling van een meer ontstekingachtig type CAV. Deze bevindingen moeten echter nog gevalideerd worden in grotere aantallen patiënten, maar het zou erop kunnen wijzen dat een geactiveerd immuunsysteem voor transplantatie kan leiden tot een snellere ontwikkeling van CAV en daardoor een kortere overleving na transplantatie.

Deel 2- Diermodellen om antilichaam-gemedieerde hartfalen te onderzoeken

Diermodellen worden veelal gebruikt om de ontwikkeling van hartfalen te begrijpen en te bestuderen. Er zijn verschillende muis modellen beschikbaar om zowel drukoverbelasting-geïnduceerde hartfalen te bestuderen als ook hartfalen door de gevolgen van een acuut hartinfarct. Echter, geschikte muismodellen om het ontstaan van hartfalen door een hart-specifieke ontstekingsreactie te bestuderen ontbreken nog. Omdat de ontstekingsreactie in het hart bij ernstig hartfalen vergelijkbaar is met de ontstekingsreactie bij myocarditis, hebben we ervoor gekozen dit muizenmodel te gebruiken als startpunt om vervolgens verder te kunnen ontwikkelen. In **hoofdstuk 6** van dit proefschrift beschrijven we het proces van myocarditis, wat wordt veroorzaakt door een ontsteking van het hart, in de meeste gevallen na een virusinfectie. Deze ontsteking kan zo heftig zijn dat er een langdurige en blijvende ontstekingsreactie kan ontstaan wat leidt tot extra schade aan het hart en sterke auto-immuun reacties, welke niet meer alleen gericht zijn tegen het virus maar ook tegen het eigen hart.

Er zijn verschillende myocarditis muis modellen ontwikkeld om het proces van myocarditis te besturen. In **hoofdstuk 7** hebben we geprobeerd het experimentele auto-immuun myocarditis (EAM) model na te bootsen, met als doel dit model aan te passen en hart-specifieke eiwitten te gebruiken die we hopelijk in de toekomst kunnen identificeren die belangrijk zijn in de progressie van hartfalen. Om EAM te induceren hebben we stukjes myosine, een harteiwit betrokken bij het ontstaan van myocarditis, opgelost in een emulsie met inactieve bacteriën en onderhuids ingespoten. Het doel is dat de muizen een ontstekingsreactie gaan ontwikkelen, opgewekt door de bacteriën, gericht tegen myosine en dus uiteindelijk het hart zullen gaan aanvallen. Ondanks dat we verhoogde levels van antilichamen in het bloed vonden na 6 weken, zagen we helaas geen verhoogde aantallen ontstekingscellen in het hart en geen tekenen van hartschade of hartfalen in de muizen. Toekomstig onderzoek zal moeten uitwijzen welke factoren in dit model zouden kunnen leiden tot een andere uitkomst dan in andere onderzoeksgroepen en of dit model representatief genoeg is voor de klinische setting om antilichaam-gemedieerde hartfalen te bestuderen.

Deel 3- Het maken van de vertaalslag naar therapeutische interventies

In het laatste deel van dit proefschrift hebben we onderzoek gedaan naar de potentie van stamcellen, ook wel progenitor cellen, om de ontstekingsreactie na hartschade mogelijk te kunnen remmen. Progenitor cellen zijn voorlopercellen met de mogelijkheid om onder de juiste omstandigheden te veranderen in andere celtypes. Deze cellen kunnen geïsoleerd worden uit verschillende soorten weefsels, waaronder beenmerg (mesenchymale stromale cellen, MSC) of het hart (cardiale progenitor cellen, CPC). Behalve dat progenitor cellen veelbelovend zijn om de hartfunctie te verbeteren in kleine proefdieren na een acuut hartinfarct, bevatten deze cellen ook sterke immunosuppressieve eigenschappen. Voornamelijk MSC en hun paracriene factoren, zoals kleine blaasjes (EV) die ze uitscheiden, zijn veelvuldig onderzocht als nieuwe behandeling om antilichaam productie te remmen in zowel hart en vaatziekten als ook auto-immuunziekten. Echter, minder is bekend over de potentie van progenitor cellen van het hart zelf, zoals CPC, om antilichaam-gemedieerde

ontstekingsreacties te remmen. Daarom hebben we in **hoofdstuk 8** naast MSC, de remmende werking onderzocht van CPC en hun EV op de activatie van B-cellen en de productie van antilichamen. Hiervoor hebben we zowel ontstekingscellen van gezonde personen als ook van patiënten met hartfalen gebruikt in een celkweek model. De aanwezigheid van CPC of MSC zorgde voor een sterke verlaging van B-cel activatie en antilichaam productie door ontstekingscellen van zowel gezonde personen als ook van patiënten met hartfalen. De sterkste effecten waren zichtbaar wanneer MSC werden gebruikt. Deze eerste resultaten zouden erop kunnen wijzen dat progenitor cellen mogelijk gebruikt zouden kunnen worden om antilichaam productie te remmen.
Na validatie van deze bevindingen in vervolg experimenten zou de volgende stap zijn om de potentie van progenitor cellen te testen in diermodellen. Echter, hoe verder we de vertaalslag maken naar de mens, hoe minder zichtbaar de effecten van celtherapie blijken te zijn. Ondanks veelbelovende effecten van celtherapie op hartfunctie in kleine proefdieren vallen de uiteindelijke effecten in klinische studies helaas tegen. Tot op de dag vandaag zit er een groot gat tussen preklinische bevindingen en de uiteindelijke effecten in de mens bovenop de huidige medicatie. Verrassend genoeg wordt het gebruik van de huidige medicatie, zoals aspirine, statines en β-blokkers, die voorgeschreven worden aan patiënten na een hartinfarct bijna nooit toegepast of gecombineerd met nieuwe therapieën in experimentele en preklinische studies. Onze hypothese is dat het effect van celtherapie misschien wel vergelijkbaar is met de effecten van de huidige medicatie en daarom ook geen toegevoegd effect laten zien zodra ze gecombineerd worden met medicatie in klinische studies. In **hoofdstuk 9** hebben we de klinisch voorgeschreven medicaties voor een hartinfarct toegediend middels het voer in een muismodel van het hartinfarct. De dieren die medicatie in het voer kregen lieten minder negatieve remodelering van het hart zien en een verbeterde hartfunctie in vergelijking met dieren zonder medicatie. Daarnaast hebben we onderzocht of klinische medicatie de effectiviteit van celtherapie eventueel beïnvloedt. Helaas zagen we in onze studie geen effect van celtherapie op hartfunctie of de grootte van het litteken, ook niet in dieren die alleen celtherapie kregen. Echter, onze resultaten wijzen erop dat het essentieel is om in diermodellen van hart- en vaatziekten klinische medicatie toe te passen, wanneer een potentieel nieuwe therapie wordt getest. Onze studie laat duidelijk zien dat het gebruik van klinisch voorgeschreven medicatie in kleine proefdieren een groot effect kan hebben op de uitkomst van een hartinfarct. Gezien het feit dat ook in klinische studies een nieuwe therapie een toegevoegde waarde moet hebben bovenop de huidige medicatie, is het van groot belang deze medicaties al in een preklinisch stadium toe te passen om ervoor te zorgen dat vertaalslag van nieuwe therapeutische interventies een groter succes wordt.

Laat onze immuunrespons ons hart falen?
Concluderend onderstreept dit proefschrift het belang van een antilichaam-gemedieerde ontstekingsreactie in verschillende type en gradaties van hartfalen, zowel voor als na harttransplantatie. Het is duidelijk dat langdurige ontsteking een belangrijke rol speelt in de ontwikkeling van hartfalen en na initiële schade het hart uiteindelijk alleen maar meer laat falen op de lange termijn. Deze bevindingen wijzen erop dat we hartfalen misschien

wel kunnen beschouwen als een auto-immuunziekte. Het remmen van de ontstekingsreactie is hoogst waarschijnlijk een essentiële stap in de behandeling van hartfalen, waarbij de identificatie van de hart-specifieke eiwitten, die worden aangetast in de verschillende type en gradaties van hartfalen, gebruikt kan worden voor diagnostische doeleinden en nieuwe patiënt-specifieke behandelingen.

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onze tijd samen, onze eindeloze telefoon / Facetime gesprekken, onze shop dagen en wanneer we samen gaan lunchen en wijntjes drinken. Ik ben stapelgek op je! Lieve pap en mam, dit proefschrift is dan ook voor jullie, ik hou van jullie!

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Liefs Patricia

LIST OF PUBLICATIONS

Published

Patricia van den Hoogen, Saskia C.A. de Jager, Emma A. Mol, Arjan H. Schoneveld, Manon M.H. Huibers, Aryan Vink, Pieter A. Doevendans, Jon D. Laman, Joost P.G. Sluijter. *Potential of mesenchymal- and cardiac progenitor cells for therapeutic targeting of B cells and antibody responses in end-stage heart failure.* PLoS One, 2019.

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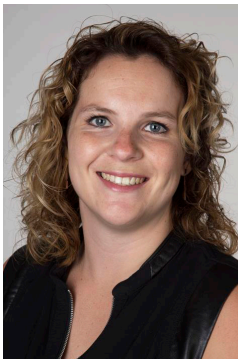
In preparation

Patricia van den Hoogen, Saskia C.A. de Jager, Noor A.J. van den Bosch, Robin J.G. Hartman, Arjan H. Schoneveld, Jon D. Laman, Pieter A. Doevendans, Aryan Vink, Joost P.G. Sluijter. *Myocardial immune cells and high levels of cardiac-related immunoglobulins potentially targeting cell-cell adhesion proteins in phospholamban p.Arg14del cardiomyopathy.*

Patricia van den Hoogen, Maike A.D. Brans, Frederieke van den Akker, Joost P.G. Sluijter, Saskia C.A. de Jager. *Experimental autoimmune myocarditis in mice: Limitations and future perspectives.*

Patricia van den Hoogen*, Peter-Paul M. Zwetsloot*, Emma A. Mol, Ellen P.J. Graumans, Cleo Arkenaar, Maike A.D. Brans, Steven A.J. Chamuleau, Pieter A. Doevendans, Saskia C.A. de Jager, Joost P.G. Sluijter. *Clinically used cardiovascular medication in preclinical models improves functional outcome upon myocardial infarction, the new standard in pre-clinical research?*

CURRICULUM VITAE



Patricia van den Hoogen was born on 18 December 1991 in Utrecht, The Netherlands. She grew up with her parents and her sister in Bilthoven. After finishing high school at Het Nieuwe Lyceum in Bilthoven, she continued with her studies at Utrecht University. There, she completed her bachelor Biomedical Sciences, followed by the master Biology of Disease. During her master internship at the Experimental Cardiology department, her interest for cardiovascular immunology was triggered. During her second master internship, she started with her PhD at the Experimental Cardiology under the supervision of Prof. Joost Sluijter, Prof. Pieter Doevendans, and Prof. Jon Laman. Her research focused on the role of chronic inflammation in heart failure, with a great interest in antibody-mediated immune responses. In 2017, she was awarded with a grant of the PLN foundation to start her own research project, where she studied the role in inflammation in PLN-induced cardiomyopathy. The results she obtained during her four-year PhD trajectory are summarized in this thesis. She is currently working as a project manager at Netherlands Heart Institute in Utrecht.