Percutaneous therapy for mitral valve regurgitation modulates level and phenotype of circulating blood cells

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<tr>
<td>HF</td>
<td>heart failure</td>
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<tr>
<td>MI</td>
<td>myocardial infarction</td>
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<td>LV</td>
<td>left ventricle</td>
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<td>MR</td>
<td>mitral regurgitation</td>
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<td>AS</td>
<td>aortic stenosis</td>
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<td>DCM</td>
<td>dilated cardiomyopathy</td>
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<td>ACS</td>
<td>acute coronary syndromes</td>
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<tr>
<td>TLR4</td>
<td>toll-like receptor 4</td>
</tr>
<tr>
<td>TLRs</td>
<td>toll-like receptors</td>
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<tr>
<td>PRRs</td>
<td>pattern recognition receptors</td>
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<tr>
<td>PAMPs</td>
<td>pathogen associated molecular patterns</td>
</tr>
<tr>
<td>DAMPs</td>
<td>damage associated molecular patterns</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>IFR</td>
<td>interferon regulatory factor transcription factors</td>
</tr>
<tr>
<td>AngII</td>
<td>angiotensin II</td>
</tr>
<tr>
<td>TAC</td>
<td>transverse aortic constriction</td>
</tr>
<tr>
<td>NLRP3</td>
<td>NOD-like receptor family pyrin domain containing protein 3</td>
</tr>
<tr>
<td>TNFR1</td>
<td>TNF receptor 1</td>
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<tr>
<td>EMB</td>
<td>endomyocardial biopsy</td>
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<td>DCs</td>
<td>dendritic cells</td>
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<tr>
<td>Abbreviation</td>
<td>Full Term</td>
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<td>--------------</td>
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<tr>
<td>mDC</td>
<td>myeloid dendritic cell</td>
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<tr>
<td>pDC</td>
<td>plasmacytoid dendritic cell</td>
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<tr>
<td>EAM</td>
<td>autoimmune myocarditis</td>
</tr>
<tr>
<td>CAD</td>
<td>coronary artery disease</td>
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<tr>
<td>IVIG</td>
<td>intravenous immunoglobulin</td>
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<tr>
<td>CRT</td>
<td>cardiac resynchronization therapy</td>
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<tr>
<td>PMVR</td>
<td>percutaneous mitral valve repair</td>
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<tr>
<td>RT</td>
<td>room temperature</td>
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<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
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<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assays</td>
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<tr>
<td>6MWT</td>
<td>6-minute walk test</td>
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<tr>
<td>LVEDD</td>
<td>left ventricular end diastolic diameter</td>
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<tr>
<td>SSC</td>
<td>side scatter</td>
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<tr>
<td>FSC</td>
<td>forward scatter</td>
</tr>
<tr>
<td>TTE</td>
<td>transthoracic echocardiograph</td>
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<tr>
<td>TEE</td>
<td>transesophageal echocardiography</td>
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<tr>
<td>IL6</td>
<td>interleukin-6</td>
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<td>CRP</td>
<td>C-reactive protein</td>
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1. Introduction

1.1 Heart failure

Heart failure (HF) is considered as the inability of the heart to transport sufficient amounts of blood to meet the demands of body at normal filling pressures, finally causing a complex and severe disease syndrome. Despite remarkable progress in HF therapy regarding both treatment and prevention, the prognosis of patients after the first hospital admission is still far from favorable, as it is followed by repeated and prolonged hospitalization, and the mortality of HF within 5 years is up to 50% (1). Hence, better understanding of the pathophysiological mechanisms for HF may accelerate investigations, medical progress and therapy for acute and chronic disease.

1.1.1 Heart failure – a complex and multifaceted disease with increasing medical and socioeconomic relevance

Several underlying causes and involved mechanisms are known for the development of HF, which can be classified into categories. Among all, ischemia induced by myocardial infarction (MI) or chronic ischemic cardiomyopathy in conjunction with further risk factors such as diabetes is the most common cause for the progression of left ventricle (LV) dysfunction and HF (2). The predominant mechanism is cell death due to lack in tissue supply with oxygen, nutrients as well as the removal of noxen generated during the metabolic process. In the
similarly prevalent cause for HF in elder patients is caused by chronic overstraining of the heart muscle machinery. Most commonly, this mechanical stress is derived from arterial hypertension or heart valve dysfunction such as mitral regurgitation (MR) or aortic stenosis (AS). These processes result in alterations of the heart structure such as ventricular dilation in the case of volume overload or hypertrophy in the case of pressure overload or combination of both. Commonly such structural alterations are associated with increase in cardiac fibrosis, and eventually progress into LV dysfunction (3,4). There are also diseases resulting in heart failure, which affect more commonly younger patients including autoimmune and microbial infections inflicting damage to the heart muscle cells mediated either directly or through innate and adaptive immune responses triggered by the infectious agent (5,6). Last but not least, genetic diseases such as dilated cardiomyopathy (DCM) are a and rather heterogeneous not well understood scenario with heart failure (7).

The two main different types of HF is chronic HF and acute HF. Chronic HF is more common and symptoms appear slowly over time and worsen gradually with the underlying mechanisms mentioned above. This situation is of particular clinical and therapeutic importance as the majority of patients who are diagnosed with chronic HF are those who most likely had HF for some time, and presented to hospital only when they became symptomatic. (8) Acute HF can be determined as a new symptom or modifies the course of chronic HF and
worsens outcome via a combination of potential mechanisms, including noncompliance with medication, aggravated hypertension, acute coronary syndromes (ACS), arrhythmias and systemic inflammation, (9) which requires urgent therapy. Moreover, they are considered as chronic but no longer as acute HF once in stabilization after the initial management. (10)

Recent years, more attentions are focus on the correlation between socioeconomic status and disease prognosis. Environmental and social contexts play a vital role in health outcomes (11), moreover, in industrial countries, socioeconomic imbalance regarding cardiovascular disease present a major and ongoing challenge to public health. (12) In HF patients, socioeconomic status also displays as predictors of incident HF, for instance, higher rehospitalization, incidence and mortality rate is seen in patients under low socioeconomic situation. (13,14)

1.2 Immune response involve in the progress of heart failure

1.2.1 Relevance of immune mechanisms in heart failure – experimental evidence

Though different mechanisms of HF, there is common to each type with a correlation between elevated circulating pro-inflammatory cytokines and adverse clinical outcomes. (15) However, different to immune based, immune response acts as the secondary response in the other causes.
Immune response not only plays as a protective response to organism injuring, but also plays an important role in the progress of atherosclerosis (16,17). The complicated nature of immune response made it a double-edged sword. In ischemic based heart failure, for instance, in the event of MI, inflammatory cells infiltrate into the infarct area to initiate the reparative process triggered by ischemia and necrotic myocardial cells. In this phase, irreparably damaged or dead cells were removed, and infarct was repaired to maintain cardiac integrity by scar formation. Therefore, the infiltration of inflammatory cells to the injured tissue is considered to be an essential participator for wound healing. The sustained inflammation beyond the initial reparative process, however, may later extend into the non-infarct remote myocardium, subsequently plays a role in the long-term adverse cardiac remodeling. (18,19) In contrast, few studies focus on the role of inflammation in the chronic setting of ischemic relevant chronic HF, though it plays an important role for cardiac remodeling. To point out, the role of toll-like receptor 4 (TLR4) in the progress of chronic HF has been demonstrated by recent animal data. (20) TLR4 is a member of toll-like receptors (TLRs) which is response for activating the innate immunity. TLRs belong to pattern recognition receptors (PRRs), (21) that could identify specific ligands and involve in defense against damage associated molecular patterns (DAMPs) or pathogen associated molecular patterns (PAMPs) which derive from damaged myocardium and pathogens, respectively. (21) The activated signaling
cascades activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), interferon regulatory factor transcription factors (IFR) and activator protein 1, that lead to the expression of inflammatory genes. (22) (Figure 1) Many stressors, including ischemia, can stimulate cardiomyocytes to release DAMPs and activate the above signaling that initiate innate immunity. Furthermore, TLR4 upregulation persists in chronic HF, and LV function could be improved by the blockade of TLR4 signaling during chronic phase. (20)

LV overload by mechanical stress can result in myocardial inflammation which is triggered by leucocyte infiltration and cardiac pro-inflammatory cytokines release, that mostly indicate with two main mouse models, transverse aortic constriction (TAC) and angiotensin II (AngII) infusion. Macrophages have been shown to mediate hypertension, cardiac remodeling and fibrosis after TAC or AngII infusion, and depletion of macrophages resulted in reduced cardiac fibrosis and decreased LV hypertrophy. (23,24) The pathological effects of macrophages are in part dependent on cytokines release (25), but the role of cytokines in regulation of cardiac hypertrophy remains disputable. For example, inhibition of IL-6 has been shown to reduce fibrosis and cardiac hypertrophy after AngII infusion (26), but it has no effect after TAC (27), moreover, deletion of GP130, which is recognized as part of IL-6 receptor complex, surprisingly impairs cardiac function leading to dilated cardiomyopathy and increase mortality. (28) This indicates the importance of cause and the temporal aspect of disease
progression after the different initial insults. Similarly, in chronic HF model of mechanical stress by 6 weeks high salt diet, depletion of monocytes/macrophages for additional 4 weeks prevents LV remodeling and fibrosis, and preserves cardiac function. (24) However, due to the challenge to establish MR model with small animals, the role of inflammation for volume overload HF remains rare.

Myocarditis is interpreted as an inflammatory disease of myocardium, which can be acute, subacute or chronic, consistently, each type can be inferred as HF with LV dysfunction by means of echocardiography. (29) Different to other causes of HF, inflammation is involved in this type as the leading response. Viral infection is demonstrated to constitute the most prevalent cause of myocarditis among all the causes. (29) Onset of viral infection, the myocardial tissue triggers the activation of the host defense immune response, which is hallmarked as the infiltration of natural killer cells as well as macrophages, subsequently by virus specific T lymphocytes. (6) The initial activation of immune response is beneficial to the host by attenuating viral proliferation, however, the persistent and excessive immune response conveys harmful consequences by contributing to the progression of myocarditis and DCM. (30) Besides, special statement that damaged cardiomyocytes can lead to the release of intracellular proteins (e.g. myosin, actin, troponin, tropomyosin, β1-adrenoreceptors and muscarinic receptors) which serve as self-antigens in order to provoke humoral response.
result in the generation of auto-antibodies, and this takes an important part in the initiation and progression of DCM by aggravating myocardial contractile dysfunction. (31) On the other hand, as observed in the cardiac complications from some systemic autoimmune disorders, dysregulated humoral response itself can directly cause the production of auto-antibodies. The pathogenic potential of auto-antibodies has been proved to lead to ventricular dilation and dysfunction in animal models of antibodies transfer or by active immunization that directly against corresponding epitopes. (32–34)

Genetics also contribute to the development and progression of DCM. (7) Unfortunately, prior studies of transgenic mice model for human familial HF do not often explore the role of inflammatory cells and pathways that may potential involve in the progress of the disease. Given the association between cardiomyocytes fibrotic pathways and immune response (21), it is likely that immune response may also modulate the fibrotic progression to genetic abnormalities altered cardiomyocyte function that leading to HF development, hence, this needs further investigation.

Furthermore, in recent years, growing evidence come out to support the role of gut in the progression of HF. The theory assumed intestinal edema and ischemia induced by decreased cardiac output could lead to enhanced intestinal permeability, and lead to the entry of lipopolysaccharides into circulation that
produced by gram-negative bacteria. (35,36) Moreover, lipopolysaccharides serves as type of PAMPs could initiate innate immunity (Figure1) that may contribute to the progression of LV dysfunction and HF syndrome. Indeed, selective elimination of intestinal aerobic gram-negative bacilli resulted in decreased fecal endotoxin concentration, monocyte expression and intracellular pro-inflammatory cytokines production in patients with severe HF. (37) This provided proof for the gut hypothesis, although this was not successful to display significant changes in circulating pro-inflammatory cytokines and endotoxin levels. (37)
1.2.2 Relevance of immune mechanisms in heart failure - clinical evidence

Elevated inflammatory biomarkers, which includes TNF-α, ST2, IL-6, galectin-3 and pentraxin-3, is a hallmark characteristic for HF. Measurement of biomarkers in HF patients and animal studies has convincingly demonstrated the elevation of inflammatory cytokines during HF progression, and supporting the “cytokine
hypothesis” that inflammation contributes to HF.

The role of several members in the inflammatory cytokine family has to be highlighted. IL-1β following processing by NOD-like receptor family pyrin domain containing protein 3 (NLRP3) inflammasome, involves in bone marrow activation and leads to leucocyte production. (38) In addition, murine studies displayed that TNF-α promotes inflammation and exerts pro-apoptotic effect via the interaction of TNF receptor 1 (TNFR1). (39,40) Alternatively, it has the opposite effect via the interaction of TNFR2. (39,40) Further, ST2 belongs to IL-1 cytokine superfamily and serves as the receptor of IL-33. In response to mechanical stress, cultured myocytes contribute to secrete ST2 and thus, ST2 represent a marker for inflammation and mechanical stress. (41) Moreover, galectin-3 belongs to the lectin family. In response to damaged cells or injured tissue, macrophages release galectin-3 that involves in the activation of fibroblast and lead to the formation of fibrosis. (42) Furthermore, as mentioned above, function of IL-6 in the progress of HF is controversial. In contrast to other cytokines, IL-10 serves as a major anti-inflammatory cytokine, is proved to reduce macrophage accumulation (43) and to reduce inflammatory cytokine expression via suppression of NF-κB signaling (44), leading to attenuated LV remodeling.

In addition, elevated levels of circulating pro-inflammatory cytokines do not only associate with disease severity, but also predict the mortality of HF patients. For
example, circulating levels of IL-6, TNF, TNFR1 are demonstrated to correlate to poorer survival rate of HF patients. (45)

Clinically, diagnosis of dilated cardiomyopathy (DCM) in HF patients with endomyocardial biopsy (EMB) is recommended by present guidelines for both diagnosis and treatment of HF (10). In biopsy, myocarditis is indicated as the presence of inflammatory cells, fibrosis and tissue necrosis. During acute inflammatory disease courses, EMB often shows focal or diffuse lymphocytes and/or macrophages infiltration, but rarely eosinophils or giant cells. (46) In patients with DCM and chronic HF, up to 30% of EMB is seen with inflammation. (46) Whereas compare to acute cases, there is lower number of inflammatory cells, and they distribute in a more diffuse manner. In addition, the presence of cardiomyocytes hypertrophy, scarring and interstitial fibrosis are the features as symbol of lost myocardium. (46)

1.2.3 Antigen presenting cells and their role in heart failure

Dendritic cells (DCs) are central to immune activation as their capacity to induce naive T cells activation so that to initiate adaptive immune response, (Figure 2) as well as their critical role in innate immunity. (47) In the blood circulation, two major types of DCs with different functions have been identified, the so called myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs). In human, mDCs mostly express CD1c and CD11c, which usually are activated by
bacterial products and functional for TLR2 and TLR4; pDCs mostly express CD303 and CD123, and is respond to viral infections that major activate TLR9. (47,48)

Figure 2.

**Figure 2. Role of dendritic cells (DCs) in inflammatory disease**

DCs could recruit from circulation or proliferate from monocytes to the inflamed tissue. In the absence of inflammation, DCs may induce tolerance by presenting autoantigens and environment antigens to the naïve T cells via MHC class II molecules, leading release of IL-10. Meanwhile, DCs may progress to mature state with enhanced antigen presenting capacities, and upregulate their production of cytokines and co-stimulatory molecules. With this, mature DCs can induce the naïve T cells to differentiate into different types of CD4+ T cells, which is responsible for adaptive immune response.
In cardiovascular system, DCs do not only present in myocardium, studies by Steinman et al. firmly demonstrated that DCs localized in aortic wall and cardiac valves as well (49). The turbulent flow by aortic wall and cardiac valves may lead DCs to accumulate and capture disease-related pathogens and present to T cells (49), accordingly take part in the progress of relevant diseases. Study with acute myocardial ischemia mouse model showed that ablation of DCs enhanced monocyte/macrophage infiltration and abundance of inflammatory cytokines, led to a modulation of LV remodeling and deterioration of LV function. (50) Additionally, the role of DCs in autoimmune myocarditis has been recognized (5), and its deterioration to DCM is demonstrated in the experimental autoimmune myocarditis (EAM) animal model, however, it comes to controversial conclusions. For example, one study group demonstrated that DCs are responsible for cardiac fibrosis and involved in the progress of HF, (51) but another study group suggested that DCs transfected of herpes virus entry mediator could prevent EAM in mice. (52)

In human, significant reduction of circulating DCs precursors has been reported in HF patients due to dilated and ischemic cardiomyopathy in all stages of disease severity. (53) Study with MI patients displayed the down-regulation of circulating DCs cause by their enhanced recruitment into the inflamed myocardial tissue (54), however, this seems not to be the case in HF. Decreased numbers of DCs were found in a group of DCM patients with heart biopsies, and
this phenomenon indicates an unfavorable correlation with outcome in terms of HF and tissue fibrosis. (55) To the disappointing results, the authors assumed that possibly due to several reasons, for instance, the apoptotic of DCs, insufficient vascularization in the area of infected cardiomyocytes, and/or the immunomodulation led by regulatory T cells. (55)

In addition, immune cells take place in the progress of heart valve dysfunction. (56) In clinical study, inflammatory response firmly negative correlates with LV parameters in valve dysfunction related HF. (57,58) The complex interactions of immune cells in heart valve may involve in valvular disease related HF, and DCs might be the vital participator, however, with further studies required to clarify the crosstalk between DCs and valvular disease related HF.

### 1.3 Available treatment options

Several therapeutic manners have been proposed to test the principle to interfere with inflammatory pathway of HF patients, however, the results of large studies have produced fairly disappointing results. Stains are widely known agents for inhibiting the formation of low density lipoprotein in patients with coronary artery disease (CAD), but they also exert anti-inflammatory effects through different ways (59,60). Randomized studies with statins to alter inflammation in patients of chronic HF did not show benefit, unless in the subpopulation presence of dyslipidemia or CAD. (39–40) However, in the
retrospective analysis of CORONA study, stain therapy is benefit in patients with increased high sensitive-CRP, who showed no difference in previous CAD and cholesterol level. (63) Nevertheless, prospective randomized trials have to be done to confirm the result. Pro-inflammatory cytokine TNF-α has received much attention as a therapeutic target, in view of its central role in the pathophysiology of HF. Trails with TNF-α inhibitors infliximab and etanercept did not show benefit in HF patients. (64,65) Although trails with pentoxiylline have demonstrated improved HF symptoms and LV function, (66–68) as a nonselective phosphodiesterase inhibitor, the effects of pentoxiylline to HF maybe beyond its anti-inflammatory properties. In addition, intravenous immunoglobulin (IVIG) also serves as a potential tool for HF therapy, as its double effect on the cytokine network. For example, it is confirmed that IVIG could downregulate the pro-inflammatory mediators (such as IL-1), and upregulate the anti-inflammatory mediators (such as IL-10). (69) However, the results came conflicting as well (69–71). Hence, the overall benefit of anti-inflammatory therapy for HF is still unclear.

In addition, several therapeutic manners have been proposed to indirectly interfere the inflammatory pathway of HF patient. Recent years, cardiac resynchronization therapy (CRT) represents a valuable option in the treatment for a subgroup of HF patients, (10) with this, a biventricular pacemaker is implanted to help the heart pump blood through the ventricles to the body more
efficiently. As CRT improved cardiac contraction, increase cardiac output reduces intestine oedema and correct peripheral circulation that reduce inflammatory mediator synthesis, a reduction of inflammatory mediators in CRT treated HF patients were found in several trails, which link to reverse cardiac remodeling and improve LV function. (72–75) However, there are also several studies demonstrated no changes in circulating inflammatory markers (76–78), which might cause by statistical errors from a small study population or heterogeneity between patient groups.

Although many studies and clinical trials regarding treatment target on inflammation in HF, the results remain controversial, which might due to the complex nature of the immune cells, inflammatory cytokines as well as the mechanisms of different HF subtypes. As we gain a greater understanding of immune mediator and their role in cardiac injure and repair, but from the mentioned above, our knowledge is still superficial to date. Hence, more studies have to be carried out, especially regarding chronic HF as it represents most of the cases in clinical HF patients, and inflammation in genetic as well as valve dysfunction related HF, which is scarce studies before. Also a better understanding of the functional implication with the immune mediators in HF may help focus new therapies target in the future.

1.4 Aims of study
There is emerging evidence that immune response plays a vital role in the progress of LV remodeling, however, data for patients of volume overload HF are not available, so far. Percutaneous edge-to-edge mitral valve repair (PMVR) for patients of MR with MitraClip® system represents a good human model to study volume stress to the heart before and after treatment, because compare to the on pump surgery, it avoids the bias caused by the artificial circulation. Since DCs mediate as center of any immune response, we investigated the number and phenotype of DCs precursors in patients underwent PMVR with MR related volume overload HF.

The aims of the thesis were the following:

To show the number change of both mDCs and pDCs in circulating before PMVR and during follow up.

To show the altered phenotype of both mDCs and pDCs in circulating before PMVR and during follow up.

To assess a possible correlation between DCs and clinical parameters.

To investigate an influence of DCs numbers with systemic inflammation status.

To investigate an influence of DCs changing on functional recovery.
2. Materials and methods

2.1 Study population

The study population consisted of 125 patients with grade 3 or 4 MR, who underwent PMVR at the University Hospital, Department for Cardiology and Cardiovascular Medicine, University of Tuebingen. We included 80 patients and re-evaluated them at 6-month follow-up. 45 patients were lost to follow up, as 35 patients passed away and 10 patients opted to withdraw from the study. Venous blood samples of 11 healthy donors served as controls. All the patients were informed and agreed before the blood taken, and the consent forms were obtained from all the subjects. Heart failure patients had to be on optimal medical treatment for at least 3 months prior to MitraClip® treatment according to current guidelines.

2.2 Identification of DC precursors by flow cytometry

All blood samples were collected using Lithium-Heparin blood collection tubes (Sarstedt, Nuembrecht, Germany) and analyzed immediately after sample submission. Dates of analysis were the day of PMVR and at a median of 5.8 months (4-10.2 months) after PMVR. The used fluorescence conjugated mouse anti-human antibodies are as following: CD1c (BDCA-1)-PE, CD303 (BDCA-2)-PE (both from Miltenyi Biotec, Germany), CD14-PerCPCy5.5, CD19-PerCPCy5.5, CD11a-FITC, CD40-FITC, CD86-APC, HLA-DR-APC (all
from BioLegend, San Diego, USA). FACS analysis was performed according to
the Blood Dendritic Cell Enumeration Kit protocol from Miltenyi-Biotec. In brief,
300µl of whole blood was added to each sample and erythrocytes were lysed by
ammonium chloride (Stemcell, Cologne, Germany). Platelets were removed by
two centrifugation steps at 300g each. The samples were incubated with human
Fc-Receptor Block (Miltenyi Biotec, Germany) for 10 min on ice, then incubated
with fluorescence conjugated mAb or isotype control murine IgG (Miltenyi Biotec,
Germany) for 10 min at room temperature (RT). After staining, cells were
incubated in 1% paraformaldehyde for fixation and tested by flow cytometry
(FACS-Calibur flow cytometer Becton-Dickinson, Heidelberg, Germany) within
24 hours. Data analysis was carried out with FlowJo software (Tree Star,
Ashland, USA). As DC precursors represent a low population (about 1% of
peripheral blood mononuclear cells (PBMCs)), in order to detect the numbers of
circulating mDC and pDC precursors more accurately, a special gate strategy
was designed (Figure 3.) according to previous study (54). To avoid the
methodical bias caused by the small cell population, we analyzed the blood
sample from each patient for three times, and mDC and pDC numbers were
described as the mean value of the resulting data. The percentage of CD1c
labeled cells (gated in R3a) and CD303 labeled cells (gated in R3b) to PBMCs
(gated in R1) were determined as the relative numbers of mDC and pDC
precursors, respectively. The absolute numbers of mDC and pDC precursors
were described as cells per microliter (cells/µl), which is based on the relative DCs numbers in relation to leucocytes count, and the leucocytes number was measured with an automated cell counter (Sysmex, Norderstedt, Germany).

**Figure 3.**

![Gate strategy for discrimination of circulating mDC and pDC precursors by flow cytometry. Region R1 = acquiring of peripheral blood mononuclear cells (PBMCs) according to the forward scatter (FSC) and side scatter (SSC). Region R2 = exclusion of granulocytes by SSC, monocytes by CD14 staining, B lymphocytes by CD19 staining. Region R3a and R3b = identification of circulating mDC and pDC precursors according to their specific CD1c and CD303 staining, respectively.](image)

**2.3 Analysis of DCs surface markers with Imaging Flow Cytometer**

As the small population of DCs in circulating, it is a challenge to isolate mDCs and pDCs from peripheral blood. We determined the surface expression of
co-stimulatory molecules CD86 and HLA-DR, adhesion molecules CD11a on single mDCs or pDCs with ImageStream®X Mark II Imaging Flow Cytometer (Merck Millipore, Germany). Blood samples were collected from 5 patients using citrate blood collection tubes (Sarstedt, Nuembrecht, Germany) and after submission samples were analyzed immediately. The used fluorescence conjugated mouse anti-human antibodies are as following: CD303 (BDCA-2)-PE (Miltenyi Biotec, Germany), CD1c (BDCA-1)-PE/Cy7, CD11a-PerCP, CD86-Brilliant Violet 421, and HLA-DR-Brilliant Violet 605 (all from BioLegend, San Diego, USA). DCs purification was carried out according to the Blood Dendritic Cell Isolation Kit II of Miltenyi-Biotec (Miltenyi Biotec, Germany). In brief, PBMCs were isolated using density gradient centrifugation with Ficoll-Paque (Sigma, Germany). Platelets were removed by two centrifugation steps at 150g each. Total human DCs were purified by two steps separation using MACS system. For that, PBMCs were incubated with cocktail of biotin-conjugated monoclonal anti-human CD1c antibody, and microbeads conjugated monoclonal anti-human CD14 and CD19 antibodies. During the first negative selection, B cells and monocytes were bound by their specific antibodies coupled to beads. Therefore, DCs enriched cell population flow through the column in the magnetic field. In the subsequent step, the flow through population was incubated with microbeads conjugated DCs enrichment cocktail. In this positive selection step, the magnetic beads retain the DCs in the
column, and DCs were eluted from the column outside of the magnetic field. For the detection of surface markers, pre-purified DCs pellets containing $2 \times 10^5$ cells were resuspended in 1.5ml Eppendorf tubes with FACS buffer (PBS, 5%BSA, 0.1%NaN3 sodium azide). After washing steps, the cell pellets were incubated with human Fc-Receptor Block (Miltenyi Biotec, Germany) for 10 min on ice, then incubated with a mixture of fluorescence conjugated antibodies for 10min at RT. After staining, cells were incubated with 1% paraformaldehyde for fixation. After washing steps, cells were resuspended in PBS and analyzed with imaging flow cytometer within 24 hours.

**2.4 Multiplex Enzyme linked immunosorbent assays (ELISA)**

CRP, IL-6, ST2, TNF-α and IL-10 levels were analyzed in a subgroup of patients by commercially available ELISA kits (eBioscience, R&D) according to the manufacturers’ protocol. For analysis of blood samples, Lithium-Heparin or EDTA plasma probes were initially centrifuged for 10min at 1250g within 30 minutes of collection. Samples were directly put in -80°C refrigerator and stored until analysis.

**2.5 Clinical parameters**

To have an objective measure of functional capacity, patients underwent the 6-minute walk test (6MWT) before PMVR and at follow up, monitored by the
same investigator according to the recommendation of the American Thoracic Society. (79) Transthoracic echocardiography (TTE) and transesophageal echocardiography (TEE) were performed in all patients within 30 days prior to PMVR and at follow up using a Philips CX 50 and iE 33 machine (Philips HealthCare, Hamburg, Germany). The severity of MR at baseline was determined according to the current European Association of Echocardiography guidelines. (80)

2.6 Statistical analysis

Statistical analysis was performed with GraphPad Prism 6 software (GraphPad, San Diego, USA), including descriptive, comparative and relational analysis. Continuous variables are presented as means ± standard error of the mean (SEM), categorical variables are shown with relative or absolute numbers. For comparisons between groups, Mann-Whitney rank test was used for statistical comparison. For correlation analysis, Spearman rank test was performed to test the relativity. The 2-tailed p value< 0.05 was considered statistically significant for both tests.
3. Results

3.1 Baseline Characteristics

A total of 80 patients undergoing PMVR was finally enrolled. The mean age was 75.2 years (range, 38 to 89). Median follow up was 5.8 months (range: 4 to 10). Baseline characteristics of all patients are depicted in Table 1.
Table 1
Baseline patient characteristics (n=80)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>75.2 (38 to 89)</td>
</tr>
<tr>
<td>Male gender</td>
<td>47 (58.8%)</td>
</tr>
<tr>
<td>Coronary heart disease</td>
<td>57 (71.3%)</td>
</tr>
<tr>
<td>Atrial fibrillation</td>
<td>49 (61.3%)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>54 (67.5%)</td>
</tr>
<tr>
<td>Smoker</td>
<td>14 (17.5%)</td>
</tr>
<tr>
<td>Hyperlipoproteinemia</td>
<td>37 (46.3%)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>24 (30.0%)</td>
</tr>
<tr>
<td>NYHA-class 3-4</td>
<td>76 (95.0%)</td>
</tr>
<tr>
<td>Renal insufficiency</td>
<td>33 (41.3%)</td>
</tr>
<tr>
<td>LV Function</td>
<td></td>
</tr>
<tr>
<td>≤35%</td>
<td>39 (48.8%)</td>
</tr>
<tr>
<td>36-50%</td>
<td>20 (25.0%)</td>
</tr>
<tr>
<td>&gt;50%</td>
<td>21 (26.2%)</td>
</tr>
<tr>
<td>Regurgitation etiology</td>
<td></td>
</tr>
<tr>
<td>Functional</td>
<td>41 (51.3%)</td>
</tr>
<tr>
<td>Degenerative</td>
<td>27 (33.7%)</td>
</tr>
<tr>
<td>Mixed</td>
<td>12 (15.0%)</td>
</tr>
<tr>
<td>Betablockers</td>
<td>71 (88.8%)</td>
</tr>
<tr>
<td>Aldosteron antagonist</td>
<td>42 (52.5%)</td>
</tr>
<tr>
<td>ACE-inhibitors/Sartane</td>
<td>71 (88.8%)</td>
</tr>
<tr>
<td>Diuretics</td>
<td>74 (92.5%)</td>
</tr>
<tr>
<td>Digitalis</td>
<td>6 (7.5%)</td>
</tr>
<tr>
<td>Calcium-Antagonists</td>
<td>11 (13.8%)</td>
</tr>
<tr>
<td>Anticoagulation</td>
<td>52 (65.0%)</td>
</tr>
</tbody>
</table>
3.2 FACS analysis of circulating DCs

Circulating mDC and pDC precursors were discriminated according to their specific surface markers CD1c and CD303, respectively. B cells and monocytes which also express DC markers were prior excluded by their surface markers CD19 and CD14 (Figure 3). Circulating mDC numbers ranged from 0.01% to 0.3% of leukocyte counts and pDC numbers from 0.01% to 0.4% of leukocyte counts in our FACS analysis, respectively. These values are in the range as previous studies described. (53,54) When comparing with the patients who underwent PMVR, a reduced number of mDCs was observed in patients before PMVR as compared to controls. (0.11 vs. 0.19%, P<0.001) (Figure 4A). Such a reduction was not only seen in relative, but also in absolute numbers (6.87/µl vs. 10.38/µl, P=0.003) (Figure 4B), hence excluding a possible dilution effect. Interestingly, the level of circulating mDC precursors increased significantly 6 months after treatment and returned to values similar to the healthy control group (Figure 4B), with a similar tendency in relative numbers (Figure 4A). Similar to the findings for mDCs, relative pDCs were significantly decreased in patients with MR compared to the healthy control subjects (0.07 vs. 0.1%, P<0.05) (Figure 4C). After treatment, the number of circulating pDC precursors significantly increased (0.07% vs. 0.1%, P=0.0002 for relative number; 4.86/µl vs. 6.56/µl, P=0.0011 for absolute number) (Figure 4C, D).
Figure 4. Relative and absolute numbers of circulating mDC and pDC precursors in control group (n=11), patients with MR before PMVR (pre PMVR, n=80) and at 6 months follow up (follow up, n=80). Circulating mDC precursors are shown as a percentage of leucocytes (A) and as cells per microliter (cells/μl) (B). Circulating pDC precursors are shown in (C, D). Graphs depict median (line in the box), 25th (upper boundary of box) and 75th (lower boundary of box) percentile. *P<0.05.
3.3 Analysis for phenotype of DC precursors

To phenotype DCs, we detected the expression of adhesion molecule CD11a, co-stimulatory molecules HLA-DR (MHC class II) and CD86 with imaging flow cytometer (Figure 5). A high amount of CD11a and HLA-DR was found constitutively expressed on both mDC and pDC precursors, which is known in the prior studies (48,81). By FACS, we detected the adhesion molecule CD11a, co-stimulatory molecules CD40, CD86 and HLA-DR on DC precursors. We detected no significant change in co-stimulatory molecules for both mDC and pDC precursors before and after PMVR, although there is a significant increased expression of CD40 and decreased expression of HLA-DR on mDC precursors in patients compare to control subjects. (Figure 6) However, we observed a significantly increased expression of adhesion molecule CD11a on mDC precursors before PMVR compared to control subjects, (Figure 7) whereas no significant difference for pDC surface expression of CD11a was detected. Interestingly, at follow up of 6 months after PMVR, both mDC and pDC surface expression of CD11a were significantly decreased (MFI: 135 vs. 105, P<0.0001 for mDC; 97 vs. 72, P=0.002 for pDC). Thus, the levels and the phenotype of circulating DC precursors change after PMVR.
Figure 5. (A) Screen capture of gate strategy for analysis of DCs. Representative scatter plots of cells labeled with CD1c (gated in yellow) and CD303 (gated in blue). In the image gallery of single cells, the yellow color represents CD1c labelled cells, blue color represents CD303 labelled cells, pink represents CD11a, purple represents CD86 and orange represents HLA-DR. (B1) and (C1) represent the single staining for mDCs and pDCs, respectively. (B2) and (C2) represent the overlay of surface markers with mDCs and pDCs, respectively. Scale bar = 7 µm.
Figure 6. Circulating mDC and pDC precursors surface expression of co-stimulatory molecules. Levels of CD40 (A), CD86 (B) and HLA-DR (C) shown as mean fluorescence intensity (MFI) were analyzed by flow cytometry. control, n=11; pre PMVR, n=80; follow up, n=80. Data is shown as mean ± SEM.
Figure 7. Expression of homing and adhesion molecule CD11a on mDC (A) and pDC (B) precursors. Expression of CD11a is presented as mean fluorescence intensity (MFI). control, n=11; pre PMVR, n=80; follow up, n=80. Data is shown as mean ± SEM. *P<0.05
3.4 Correlations of circulating mDCs with CD40 and HLA-DR

As the significant changing of CD40 and HLA-DR on mDCs between control group and patients, we also examined the correlations between mDCs and CD40, HLA-DR expression in patients. We found that the up regulation of CD40 in patients was inverse correlated with circulating mDCs, and the down regulation of HLA-DR in patients was positive correlated with circulating mDCs (Figure 8).

Figure 8. Association of mDC precursors with co-stimulatory molecules. Significant inverse correlation between the relative circulating mDC precursors numbers and their surface expression of CD40 (A), n=80. Significant positive correlation between the relative circulating mDC precursors numbers and their surface expression of HLA-DR (B), n=80.
3.5 Improvement of clinical parameters

In order to identify a possible improvement of clinical parameters for HF, we measured the typical parameters of LV remodeling and HF, such as left ventricular end diastolic diameter (LVEDD), distance of 6-minute walk test (6MWT), as well as heart failure biomarkers, such as pro-inflammatory markers CRP, IL-6, ST2, TNF-α and anti-inflammatory marker IL-10, before PMVR and at 6 month follow up. There is significant changing of LVEDD, 6MWT, IL-6 and CRP, see Figure 9, which parallel with the changing of circulating DCs.
Figure 9. Left ventricular end diastolic diameter (LVEDD, A) as a parameter for volume overload was determined, n=55. Distance of 6-minute walk test (6MWT, B), n=46. Plasma levels of interleukin-6 (IL6, C), n=70, and C-reactive protein (CRP, D), n=75 were analyzed. Data is shown as mean ± SEM. *P<0.05
3.6 Correlation analysis of circulating mDCs and heart failure parameters

To confirm the relation of DCs to heart failure parameters, we further conducted correlation statistics between the changing of DCs and the changing of above clinical heart failure parameters. The recovery of mDCs correlated well with 6MWT, as well as inversely correlated with IL-6 and CRP. (Figure 10)

In contrast to the above findings for mDCs, pDC analyses did not yield any correlates among groups. (Figure 11)

Figure 10. Association of mDC precursors with markers of heart failure parameters. Significant positive correlation between the increased relative circulating mDC precursors numbers and the improvement in 6-minute walk test (6MWT) distance (A). Significant inverse correlation between the change in relative circulating mDC precursors numbers and change of plasma interleukin-6 (IL6, B) and C-reactive protein (CRP, C) levels.
Figure 11. No correlation was observed between the change in the relative number of circulating pDC precursors and the improved functional capacity of HF patients as assessed with the 6-minute walk test (6MWT) distance (A), with plasma levels of interleukin-6 (IL6, B) or with C-reactive protein (CRP, C).
4. Discussion

In our study we were able to show that i) the numbers of circulating mDC and pDC precursors are reduced in MR related HF patients, and PMVR treatment increased their levels at a follow up after 6 months, furthermore, ii) the reduction of mDCs in patients correlates with the expression of co-stimulatory molecules CD40 and HLA-DR. iii) After PMVR, the phenotype of circulating DCs changed, which showed for example decreased expression of the homing molecule CD11a and that iii) change of mDC precursors correlated with improvement of clinical parameters of HF and inversely correlated with markers of an inflammatory response.

Two major different types of DCs and their migratory route have been described in human. (48,82) Both types of DCs appear to be in transit in the peripheral circulation as precursor cells before they migrate into peripheral tissues. (83) Significant alteration of circulating DC precursors has been reported in several immune-mediate diseases, (54,84,85) and it is assumed that their reduced presence is mediated by enhanced recruitment into inflamed tissue (54). In the cardiovascular system in mice, it was recently demonstrated that DCs are present within the aortic wall and heart valves, and the localization of DCs is mainly in areas with turbulent flow. (49). In MR patients, volume overload causes disturbed flow at various areas of the heart tissue including the heart valves,
predominantly during the systolic period. (86)

In the present study, we explored whether any change of circulating DC precursors might be associated with an immune activation in advanced heart failure. We found that the number of peripheral circulating mDC precursors is significantly decreased in patients with MR related heart failure compared with healthy control subjects. Additionally, the treatment of PMVR and, thus, reduction of volume overload resulted in increased number of circulating mDC and pDC precursors at 6 month follow up, similar to levels of the healthy control group. More interestingly, we observed the decreased number of mDCs in patients before treatment is associated with the change of phenotypes of CD40 and HLA-DR.

Elevated inflammatory biomarkers, which includes TNF-α, ST2, IL-6, CRP, is a hallmark characteristic for HF. (21) Measurement of biomarkers in HF patients and animal studies have convincingly demonstrated the elevation of inflammatory cytokines during HF progression. (21) Previous clinical intervention studies have tested the principle to interfere with inflammatory pathways. (65,69,71) The achieved properties in patients with HF, however, have produced fairly disappointing results. (21,62,65,71) This outcome may reflect the complexity of HF pathophysiology with different underlying disorders from inflammatory cardiomyopathy to ischemic heart disease. We assessed instead
patients with congestive HF caused by MR featuring volume overload as a predominant mechanism of disease. Future studies will have to scrutinize the number of circulating DC precursors, their phenotype and their correlation with clinical parameters, in other settings of heart failure as well. In our patient collective, we furthermore observed a significant reduction of classical pro-inflammatory cytokines IL-6 and CRP in MR patients 6 months after PMVR treatment. The increased levels of circulating mDC precursors correlated with the change of plasma IL-6 and CRP levels, thus reflecting a potential crosstalk between the presence and phenotype of these immune cells to inflammatory processes in HF patients, rather than it being an isolated phenomenon. Further studies, however, will have to test this hypothesis more profoundly. Interestingly, mDC precursors showed a significant correlation with the functional capacity in heart failure patients. In contrast, we did not find any correlation with circulating pDC precursors suggesting cell specificity for this observation. In oder to further challenge and characterize the involvement of circulating DC precursors and their phenotype as a decisive factor or a potential biomarker in patients with HF caused by volume overload, future basic and clinical studies are warranted.

In conclusion, we demonstrate to our knowledge for the first time an effect of interventional treatment for MR featuring cardiac volume stress on levels of circulating DC precursors and their phenotype, which was associated with increase in functional capacity and reduction of inflammatory markers. Thus, our
results point to a previously unattended intersection point between volume overload and a cellular immune response.
5. Summary

**Aim:** Immune mechanisms and inflammation contribute to the pathophysiology of heart failure (HF) in different ways. As the center of immune response, dendritic cells (DCs) play an important role in LV remodeling after myocardial ischemia and myocarditis, however, the role of dendritic cells in non-ischemic HF with volume overload has not be addressed, so far. Here, we investigated the number and phenotype of circulating DC precursors in a patient collective undergoing percutaneous mitral valve repair (PMVR) of mitral regurgitation to uncover the potential crosstalk of volume overload HF with immune regulation.

**Methods:** Using flow cytometry, we determined the numbers of circulating myeloid DCs (mDCs), plasmacytoid DCs (pDCs) and their surface expression of co-stimulatory molecules CD40, CD86, HLA-DR, as well as adhesion molecule CD11a in healthy control subjects (n=11) and MR patients at the time of PMVR (n=125) and at a median follow-up (n=80) of 5.8 (4 to 10) months. Levels of plasma inflammatory cytokines and change in left ventricular end-diastolic diameter (LVEDD) were assessed, and a 6-minute walk test was carried out prior to PMVR and during follow-up. **Results:** Compared to controls (mDC 10.38/µl and pDC 5.44/µl, respectively), patients with MR had significantly decreased numbers of circulating mDC precursors (6.87/µl, P<0.01), whereas there was no significant difference regarding pDC precursors (4.86/µl). While a significant up-regulation of mDC surface expression of CD11a (mean
fluorescence intensity (MFI) 160, P<0.05) was detected compared to healthy controls (MFI: 130), no significant differences for CD40, CD86 and HLA-DR were found. During follow-up, PMVR treatment restored reduction of circulating mDC precursors (8.93/µl, P<0.001). Furthermore, we observed an increase in circulating pDC precursors (6.56/µl, P<0.01). Simultaneously, surface expression of CD11a on circulating mDC and pDC significantly decreased after PMVR. The increased numbers of circulating DC precursors after PMVR was paralleled by a reduction of CRP, IL6 levels, decreased LVEDD and an improvement of 6-minute walk test distance. Interestingly, the change in mDC levels showed a positive correlation with 6-minute walk test distance and an inverse correlation with inflammatory markers CRP and IL-6. **Conclusions:** Our findings suggest that the change of circulating DC precursors may be involved in the pathophysiology of MR relevant HF.
6. Zusammenfassung

Ziel: Immune Mechanismen und Entzündungen werden vorgeschlagen, um die Pathophysiologie der Herzinsuffizienz (HF) auf unterschiedliche Weise zu beeinflussen. Als Zentrum der Immunantwort spielen auch dendritische Zellen (DCs) eine wichtige Rolle bei der LV-Remodellierung nach Myokardischämie und Myokarditis, jedoch ist die Rolle der dendritischen Zellen bei nicht-ischämischen HF mit Volumenüberlastung bisher nicht angesprochen worden. Hier untersuchten wir die Anzahl und den Phänotyp zirkulierender DC-Vorstufen in einem Patientenkollektiv, das einer perkutanen Mitralklappenreparatur (PMVR) der Mitralinsuffizienz unterzogen wurde, um das mögliche Übersprechen der Volumenüberlastung HF mit Immunregulation aufzudecken. Methoden: Mit Hilfe der Durchflusszytometrie ermittelten wir die Anzahl der zirkulierenden myeloiden DCs (mDC), der Plasmacytoid-DCs (pDC) und ihrer Oberflächenexpression der co-stimulatorischen Moleküle CD40, CD86, HLA-DR sowie des Adhäsionsmoleküls CD11a bei gesunden Kontrollpersonen (N = 11) und MR-Patienten zum Zeitpunkt der PMVR (n = 125) und bei einem medianen Follow-up (N =80) von 5,8 (Bereich: 4 bis 10) Monate. Ebenen der Plasma-entzündlichen Zytokine, Veränderung des linksventrikulären enddiastolischen Durchmessers (LVEDD) wurden beurteilt und ein 6-Minuten-Gehetest wurde vor dem PMVR und während des Follow-up durchgeführt. Ergebnisse: Im Vergleich zu den Kontrollproben (mDC 10,38/µl
bzw. pDC 5,44/μl) zeigten die Patienten mit MR signifikant verminderte Anzahl an zirkulierenden mDC-Vorläufern (6,87/μl, P <0,01) War kein signifikanter Unterschied bezüglich der pDC-Vorläufer (4,86/μl). Während eine signifikante Erhöhung der mDC-Oberflächenexpression von CD11a (mittlere Fluoreszenzintensität (MFI) 160, P <0,05) im Vergleich zu gesunden Kontrollen (MFI: 130) festgestellt wurde, wurden keine signifikanten Unterschiede für CD40, CD86 und HLA-DR gefunden. Während der Nachuntersuchung führte die PMVR-Behandlung zu einer Reduktion von zirkulierenden mDC-Vorläufern (8,93/μl, P<0,001). Darüber hinaus beobachteten wir eine Zunahme der zirkulierenden pDC-Vorstufen (6,56/μl, P <0,01). Gleichzeitig verringerte sich die Oberflächenexpression von CD11a beim Zirkulieren von mDC und pDC nach PMVR signifikant. Die erhöhte Anzahl von zirkulierenden DC-Vorläufern nach PMVR wurde durch eine Reduktion von CRP, IL6 Niveaus, vermindertem LVEDD und einer Verbesserung der 6-minütigen Spaziergangsteststrecke verglichen. Interessanterweise zeigte die Veränderung der mDC-Spiegel eine positive Korrelation mit einer 6-minütigen Wanderteststrecke und einer inversen Korrelation mit den entzündlichen Markern CRP und IL-6. **Schlussfolgerungen:** Unsere Ergebnisse deuten darauf hin, dass die Veränderung der zirkulierenden DC Vorläufer in der Pathophysiologie der MR relevant HF beteiligt sein kann.
7. References


10. Ponikowski P, Voors AA, Anker SD, Bueno H, Cleland JGF, Coats AJS,


8. Declaration

I hereby declare that all the experiments work of this thesis were carried out by myself under the supervision of Jun. - Prof. Dr. med Harald F. Langer. I affirm that I wrote the manuscript by myself, and this thesis is not published anywhere before.

Place/Date:

Signature:
9. Acknowledgement

First, I would like to thank my supervisor, Jun. - Prof. Dr. med. Harald F. Langer, for offering me the opportunity to undertake my thesis in his group, and for his constant support, valuable advice, knowledge and understanding throughout my postgraduate research.

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Most importantly, I have to thank my parents for their support, encouragement and endless love, without them I could not become a person like today. Also, I would like to extend an expression of my heart-felt gratitude to my lover, Zaitao Sun, who sacrificed his valuable time to accompany me all this way and it is a joy to share with him the most wonderful and longest ride of our lives. I love you all.

Yingying Zhang

Nov, 2016, Tübingen