Inflammation in cardiac disease: towards improving diagnosis and new therapies

Linde Woudstra
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Inflammation in cardiac disease: towards improving diagnosis and new therapies

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Linde Woudstra

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copromotoren: dr. P.A.J. Krijnen
dr. L.J.M. Juffermans
“Doubt is the origin of wisdom”

René Descartes
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General introduction
INTRODUCTION

Myocarditis is defined as inflammation (*itis*) of the heart muscle (*myocardium*). The cause of myocarditis can be infectious agents; viruses, bacteria, protozoans and fungi, but also an autoimmune reaction or toxins.[1] Since there is such a variety in causes the clinical presentation of myocarditis is also highly diverse and varies from shortness of breath and flu-like symptoms to chest pain, heart failure and sometimes sudden death.[2-4] In most cases myocarditis is caused by a viral infection. In young adults myocarditis is one of the leading causes of cardiac failure and sudden death.[4, 5] Due to the large varieties in clinical presentation, viral myocarditis is a complicated clinical entity and is difficult to diagnose. Moreover, to date there is a lack of specific therapies to treat viral myocarditis. Therefore, there is a need for a better understanding of the pathogenesis of viral myocarditis to enable the development of improved diagnostic tools and potential new therapies.

Worldwide cardiovascular disease is the number one cause of death. Within Europe more than half of all deaths are caused by cardiovascular disease, especially heart attacks. A heart attack, i.e. myocardial infarction (MI), is usually an acute event as a result of an obstruction within the coronary arteries that supply the heart with oxygenized blood. Such obstructions are generally the result of erosion, inflammation and/or rupture of atherosclerotic plaques in the coronary artery that lead to blood clot formation (thrombus). The clinical presentation of MI is more clear than that of myocarditis and diagnosis is e.g. based on specific changes in the electrocardiogram. Echocardiography can even further specify regional wall motion abnormalities[6], in the blood biomarkers like troponin and creatine kinase-MB are elevated through leaking out of damaged cardiomyocytes. Furthermore coronary angiography can be performed to confirm the differential diagnosis of an MI and precisely localize obstructions in the coronary arteries.[7] Although treatment of MI has improved significantly in recent years there still is a demand for improved therapies to prevent loss of cardiomyocytes and/or regenerate myocardial tissue. Inflammation plays pivotal roles both in the pathogenesis of viral myocarditis [8] as in the induction and the outcome of MI [9, 10] and hence may be used for the development of new diagnostics and as a target for new therapies. This thesis describes inflammation both in viral myocarditis and MI towards the improvement of diagnosis and the search for new therapies.

LYMPHO CYTIC MYOCARDITIS

Pathogenesis

As mentioned above, myocarditis is most often caused by a viral infection.[1] Most of our knowledge on the pathogenesis of viral myocarditis is obtained from studies in mice with experimentally induced viral myocarditis. The pathological course of viral myocarditis can be divided in three different phases.[11, 12] The first phase occurs within the first days after virus infection, wherein the virus infects the heart. Virus replication within the heart then causes cardiac injury, which in turn leads to the activation of the innate immune response and the release of pro-inflammatory cytokines.[12] The second phase, after one week, involves both the innate and acquired immune response. Inflammatory cells namely infiltrate the heart, characterized by the invasion of macrophages and subsequently T-lymphocytes.
Increased levels of cytokines and antibodies against viral proteins but often also cardiac proteins jeopardize the heart and impair cardiac function.[13, 14] In the third phase, usually two weeks after infection, the virus is eliminated and the immune response will decline facilitating recovery of acute myocarditis. However, sometimes viral infection persists in the heart and/or autoimmunity develops, whereby the immune response attacks self-antigens in the heart. This leads to a more chronic phase characterized by extensive cardiac remodeling that can progress into a so-called dilated cardiomyopathy, in which the heart becomes enlarged and the pump function decreases.[13] Myocarditis is characterized by a diffuse inflammation of the myocardium, wherein inflammatory cells spread patchy over the myocardium (Figure 1). In viral myocarditis lymphocytes are the predominant infiltrating cells. Therefore, in pathology such a patchy lymphocytic infiltrate is often referred to as lymphocytic myocarditis (LM), which in majority has a viral etiology.

**Figure 1.** Example of cross sectional heart slice of a mouse with coxsackievirus induced acute myocarditis. The healthy myocardium is pink and the inflammatory sites are purple (arrows).

**Diagnosis**
Due to the diversity in clinical presentation the diagnosis of LM is difficult.[15] Cardiac magnetic resonance imaging (MRI) is a non-invasive technique often used to establish final diagnosis of myocarditis. It can be used to assess both the structure and function of the heart with high accuracy and demonstrates typical features of acute inflammation, such as dysfunction, edema and necrosis.[16] Nevertheless, even with cardiac MRI the diagnosis of myocarditis can still be missed and, in addition, it is not possible to determine the underlying cause of myocarditis.[17] Therefore the gold standard for diagnosing myocarditis is via endomyocardial biopsies. In the late 1980s the diagnosis of LM in endomyocardial biopsies was based on the so-called Dallas criteria, relying on histological identification of inflammatory cells within the myocardium, coinciding with cardiomyocyte necrosis of non-ischemic origin.[1] However, these criteria were found to be insufficiently specific and sensitive and quantitative immunohistochemical analyses have been added to improve the pathological diagnosis of LM in endomyocardial biopsies.[18-20] A clear cut-off value is recommended of ≥14 leukocytes per mm², including macrophages and (CD3-positive) T-
lymphocytes.[1, 21] Although for the diagnosis of myocarditis research has been performed to assess the specificity of these guidelines, limited research has been performed on the sensitivity of the markers used for particular inflammatory cells. In Chapter 2 we studied therefore whether the use of a more common leukocyte marker would increase the diagnostic sensitivity of LM. For this we compared the efficacy of the common leukocyte antigen i.e. CD45 with the often used specific T-lymphocyte marker CD3 in the diagnosis of LM on post-mortem material.

Although an endomyocardial biopsy is the gold standard to diagnose LM, these biopsies have their limitations. LM is often characterized by a patchy inflammatory infiltrate within the myocardium, which creates a significant risk of underdiagnosing LM in endomyocardial biopsies because of sampling errors.[22, 23] Moreover, with the collection of endomyocardial biopsies there is a small risk of potentially serious complications, such as cardiac perforation and valvular damage.[24] Taken together new diagnostic methods are necessary to determine LM. From patients with chronic heart failure it is known that degenerative alterations also occur in the skeletal muscle.[25] These alterations have been linked to increased levels of pro-inflammatory cytokines, indicating that the immune system has a potential role in the skeletal muscle alterations. Moreover, several case reports have demonstrated that cardiotropic viruses can infect not only cardiac but also skeletal muscle tissue.[26, 27] Since the quadriceps muscle would be a safe and practical site to obtain skeletal muscle biopsies, we wondered whether the skeletal muscle may be representative for the inflammation within the cardiac muscle. In Chapter 3 we examined whether a biopsy of the quadriceps muscle can serve as a potential new diagnostic tool for LM.

Treatment

Due to the highly variable etiology and clinical presentation in patients with LM there is no specific treatment. The treatment of LM patients with reduced cardiac function consists of supportive therapy and cardiovascular stabilization by treating the associated heart failure.[1] Standard heart failure therapy consists of beta-blockers, diuretics and angiotensin converting enzyme inhibitors.[13] In the case of a viral myocarditis antiviral drugs can be prescribed to the patient. Since the immune system plays an important role in the pathogenesis of myocarditis, immunomodulatory and immunosuppressive therapy, such as prednisone, azathioprine and cyclosporine, have also been the explored. However, the results of clinical trials are controversial since often no adequate immunohistological analysis of the endomyocardial biopsy was performed and a control group was absent.[13] Moreover, one of the largest clinical trials for myocarditis immunosuppressive therapy, consisting of prednisone with either azathioprine or cyclosporine in addition to heart failure therapy, showed no improvement in heart function nor mortality 1 year after treatment.[28] Although the standard heart failure therapy is useful in symptom repression and preventing on-going decline of cardiac function, the underlying disease then is not treated. Therefore, other treatments are needed. For pericarditis, i.e. inflammation of the pericardium, recent publications have shown effectiveness of treatment with colchicine.[29] Colchicine is an immunosuppressive drug that has been used for over 2000 years to treat gout and is rapidly becoming standard therapy for this condition.[30, 31] As pericarditis can coincide with myocarditis,[32] colchicine could be an interesting potential treatment option for myocarditis as well. In Chapter 4 we investigated the effect of colchicine treatment in a mouse model of acute coxsackievirus B3 induced myocarditis.
**Influence of myocarditis on the cardiac vasculature**

Patients with LM can present with a variety of clinical symptoms that can indicate a myocardial infarction (MI), including chest pain, electrocardiographic ST-segment elevation, wall motion abnormalities and increased blood levels of cardiac enzymes.[2, 33-37] Indeed in 50 to 78% of the patients with acute chest pain but with angiographically normal coronary arteries, the underlying cause was acute myocarditis.[38-40] However, in clinical practice, LM is only considered as potential underlying cause of infarct-like complaints when MI is ruled out, based on the absence of coronary narrowing or obstruction as detected by coronary angiography. In case of normal or non-obstructed coronary arteries, cardiac MRI is then often employed as a complementary imaging tool to differentiate between MI and LM, wherein myocardial injury is mainly located in the subendocardium with MI as opposed to a more (sub)epicardial location with LM.[41, 42] The general consensus in the literature is that although LM and MI can be similar in clinical presentation they are distinct clinical entities. However, there is accumulating evidence suggesting an interrelatedness between LM and MI.[43] Since LM is an inflammatory disease of the heart this inflammation may also affect the coronary arteries. Inflammation has been found to be an important mediator of atherosclerotic plaque destabilization that renders them more vulnerable for complication, like MI.[44] Therefore, in Chapter 5 we analyzed in post-mortem hearts of patients with LM coinciding with and without a very recent MI the inflammatory infiltrate and plaque stability in coronary atherosclerotic lesions.

Part of the clinical symptoms of patients with myocarditis can be explained by changes within epicardial and intramyocardial blood vessels. Myocarditis can influence the cardiac vasculature in different ways. The activation of the immune response and/or the presence of viruses can activate the endothelium of the cardiac vasculature and thereby increase the extravasation of inflammatory cells, most likely exacerbating the inflammation in the myocardium. Moreover, the composition of the atherosclerotic plaques within the coronary arteries can be affected by myocarditis. The increase of inflammatory cells within the atherosclerotic plaques, but also the elevated activity of the coagulation system can lead to increase chance of thrombus formation. Next to structural changes, also functional changes, such as impaired blood flow and vasospasm, can be induced by myocarditis. The importance of structural and functional changes of the cardiac vasculature in patients with infectious myocarditis is discussed in an overview in Chapter 6.

**MYOCARDIAL INFARCTION**

**Pathogenesis**

The cause of a myocardial infarction (MI) is often an obstruction of one or more of the epicardial coronary arteries that supply the heart with blood (Figure 2A). This obstruction can be caused by a complication of the atherosclerotic plaque within an epicardial coronary artery. Atherosclerotic plaques consist of cholesterol, inflammatory cells and fibrous tissue which usually take decades to develop.[44] These plaques can become so-called unstable, i.e. promoting thrombus formation that will occlude the coronary artery.[45]

Subsequent to MI an inflammatory response is induced that attracts inflammatory cells to the site of injury. This inflammatory reaction plays an important role in the healing of the infarcted heart. Within in 6-12 hours post-MI neutrophilic granulocytes infiltrate the affected
myocardium up to 5 days post-MI.[46, 47] Within the first days also macrophages and lymphocytes infiltrate the affected myocardium. Both neutrophils and macrophages are important for the clearance of the dead cardiomyocytes and cell debris.[48] Moreover, macrophages and lymphocytes release cytokines that promote inflammation and stimulate angiogenesis. Finally, from day 5-14 post-MI the inflammation subsides and extracellular matrix proteins are produced to stimulate the formation of collagen to replace cardiomyocyte loss.[49] As a result of this scar formation remodeling and thinning of the ventricular wall occurs (Figure 2B). Scar formation can be extensive and can induce a decrease in heart function, as well as arrhythmias[6].

**Treatment**

One important aspect to limit cardiomyocyte death after a MI is reperfusion therapy. As treatment to unblock the coronary artery and restore reperfusion the thrombus can be dissolved using thrombolytic enzymes or the thrombus can be surgically removed from the coronary artery by the procedure called angioplasty. If it is impossible to remove the obstruction a coronary artery bypass grafting can be performed that redirects the blood flow around the obstruction. Post-MI the treatment consists of limiting infarct size expansion and the risk of heart failure, by thinning the blood (aspirin), lowering blood pressure and relaxing the heart muscle (beta-blockers, angiotensin converting enzyme inhibitors).[50]

![Figure 2. A) Schematic drawing of myocardial infarction. Due to thrombus formation out of the atherosclerotic plaque there is an obstruction of the coronary artery causing cardiomyocyte cell death behind the obstruction. B) Example of cross sectional heart slice of a mouse with a transmural myocardial infarction. The infarct area is pink (arrows) and the healthy myocardium is purple.](image-url)
StemBells as therapy for myocardial infarction

One of the major problems after MI is the loss of cardiomyocytes. Even with reperfusion therapy and medication most ischemic jeopardized cardiomyocytes are lost and replaced with non-contractile scar tissue, while the regeneration capacity of cardiomyocytes is very low. A promising therapy for regenerative tissue repair, to prevent heart failure development after acute MI, is adipose tissue-derived stem cell (ASC) therapy. ASCs have the ability to differentiate into cardiomyocytes, but can also secrete paracrine factors that can influence cardiac inflammation and cardiomyocyte regeneration.

One of the major problems of stem cell therapy is lack of engraftment of sufficient stem cells at the site of injury. Therefore, we designed a novel targeting technique that can direct ASC specifically to the activated endothelium of blood vessels within the infarct area by coating them with dual-targeted microbubbles (Figure 3). These microbubbles are small (2-4 μm) gas-filled bubbles and originally developed as contrast agents for echocardiography. The microbubbles are coated with an antibody against ASC cell surface marker CD90 and can thereby be coupled to the ASC creating an ASC-microbubble complex, i.e. StemBells. Moreover, the microbubbles are also coated with antibodies against Intercellular Adhesion Molecule 1 (ICAM-1) to improve attachment of the StemBells in the infarct area. ICAM-1 is namely expressed on activated endothelium of blood vessels within the infarct area. As microbubbles are susceptible to the acoustic radiation force exerted by diagnostic ultrasound, in theory StemBells will also acquire this susceptibility to ultrasound. Hence StemBells can be stimulated to migrate from the center of the blood stream to the vessel wall by ultrasound, further enhancing the effect of targeting. In Chapter 7 we first studied in vitro whether ultrasound could induce migration of these StemBells to the vessel wall in a flow model and subsequently we studied in vivo the effect of StemBell therapy 7 days post-MI in a rat model.

The first week after a MI there is a strong inflammatory response in the infarct area. This inflammatory response clears the myocardium of necrotic cells and debris and provides signals to initiate reparative pathways. Nonetheless, this inflammatory response also causes further damage to cardiomyocytes, resulting in an increased infarct size and adverse ventricular remodeling. Therefore, through the immunomodulatory effects of stem cells, and StemBells, this therapy may be improved when they are administrated prior to the inflammatory response post-MI. In Chapter 8 we compared the effect of StemBell administration 1 day or 7 days post-MI in a rat model.
StemBells as therapy for atherosclerosis

It is now generally accepted that inflammation is an important mediator for atherosclerotic plaque development and destabilization.[44] After a MI the atherosclerotic plaque can grow more rapidly and there is an increase of inflammatory cell content within the plaque leading to further destabilization of the plaque.[61-63] Since MI can directly accelerate atherosclerosis and increase plaque vulnerability, the chance of re-infarction increases. Recent studies have already shown in pre-clinical models of mice, rats and rabbits that stem cells can reduce atherosclerosis.[64-66] In atherosclerotic mice and rats stem cell therapy reduced aortic thickness, intima proliferation and plaque size and importantly also reduced plaque development and macrophage content within the plaque inducing a more stable morphological structure.[64, 66] With use of StemBells the stem cells may theoretically also enter the atherosclerotic plaque more easily through the ICAM-1 expression and influence plaque composition by secreting soluble immunomodulatory factors. In Chapter 9 we studied if StemBell therapy could reduce atherosclerosis after acute MI in an atherosclerotic mouse model.

Figure 3. Schematic drawing of a StemBell: a stem cell-microbubble complex coupled via streptavidin-biotin-antibody bridging. The gas-filled microbubbles are coated with anti-CD90 antibodies, to bind them to the stem cell, and with anti-Intercellular Adhesion Molecule 1 (ICAM-1) antibodies to bind to the activated endothelial cells within the infarct myocardium.
REFERENCES


CD45 is a more sensitive marker than CD3 to diagnose lymphocytic myocarditis in the endomyocardium

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ABSTRACT

To diagnose lymphocytic myocarditis (LM) immuno-histopathological examination of endomyocardial biopsies (EMB) is used with a cut-off value of ≥14 leukocytes/mm², composed of CD3 and CD68 positive cells. We hypothesized that a more common leukocyte marker, CD45, instead of CD3 could increase the diagnostic sensitivity. In hearts of mice with acute viral myocarditis (n=9), controls (n=7) and in the EMB sampling area of the left ventricular posterior wall (LVPW) obtained from autopsy hearts of patients diagnosed with LM (n=18) and controls (n=6) were stained with anti-CD68, -CD3 and -CD45. When applying the threshold of ≥14 leukocytes/mm², 33% of the mice would be diagnosed for LM with the use of CD3+CD68 and 89% with the use of CD45+CD68. In the EMB sampling area of autopsied hearts, using the cut-off value of ≥14 leukocytes/mm², CD3+CD68 could only confirm 17% of the diagnosis of LM, while CD45+CD68 could confirm 50% of the LM cases. Moreover, we compared inflammation in the EMB sampling area of the LVPW to the remaining myocardium of the LVPW and observed a significant increase of CD45+CD68 cells/mm² in LM patients. In conclusion, the use of the common leukocyte marker CD45 increases the sensitivity of the diagnosis of LM. Furthermore, the inflammatory infiltrate in the EMB sampling area is significantly increased compared to the remaining LVPW, indicating that the sampling area constitutes the highest chance for histological diagnosis of LM.
INTRODUCTION

Lymphocytic myocarditis (LM) is an important cause of cardiac failure and death in young patients. In part of the patients LM leads to chronic myocarditis, dilated cardiomyopathy and heart failure.[1] The clinical presentation of LM is highly diverse and varies from shortness of breath and flu-like symptoms to chest pain, heart failure and sometimes sudden death.[2-4] Due to this diversity in clinical presentation the diagnosis of LM is difficult.[5] The gold standard for diagnosing myocarditis includes (immuno)histological examination of endomyocardial biopsies (EMB). However, LM is often characterized by a patchy inflammatory infiltrate, which creates a significant risk of underdiagnosing LM in EMB because of sampling errors.[6, 7] In the past the diagnosis of LM in EMB was based on the so-called Dallas criteria as histological evidence of an increase of inflammatory cells within the myocardium, coinciding with cardiomyocyte necrosis of non-ischemic origin.[1] However, these criteria were found to be insufficiently specific and sensitive for interobserver variability.[8-10] Therefore quantitative immunohistochemical analyses have been added to improve the pathological diagnosis of LM in EMB that are now recommended as guidelines by the European Society of Cardiology Working Group.[11] A clear cut-off value is recommended of ≥14 leukocytes per mm², including up to four (CD68-positive) macrophages per mm² with the presence of ≥7 (CD3-positive) T-lymphocytes per mm².[11, 12] We hypothesized that the use of a more common leukocyte marker i.e. CD45 could increase the pathological diagnostic sensitivity for LM. CD45 is a leukocyte marker expressed on hematopoietic cells, including lymphocytes, macrophages and monocytes. In this study we aimed to investigate how the ventricular endocardium, from where EMB are usually taken, relates to the rest of the myocardium in the diagnosis of LM. In addition, we aimed to investigate how the ventricular endocardium, from where EMB are usually taken, relates to the rest of the myocardium in the diagnosis of LM. This because late gadolinium enhanced cardiovascular magnetic resonance (CMR) primary detects myocardial injury related to LM in the subepicardial layer, often as a patchy distribution and almost never in the subependocardial compartment as seen with ischemia-mediated injury.[13-16] Disturbances of the distribution volume of gadolinium chelate relate to fibrosis, inflammatory processes as well as cardiomyocyte damage,[15, 17] suggesting that the endocardium, albeit the only practically available site to collect biopsies, may in fact be a poor location to diagnose LM.[18] Therefore in this study we also compared the inflammatory infiltrate in the endomyocardium of the left ventricular posterior wall (LVPW), a common EMB sampling area, to that of the remaining LVPW.

METHODS AND MATERIALS

Viral myocarditis in mice

Male C3H mice (Harlan, Horst, The Netherlands, 20-25 grams) were injected intraperitoneally with 1x10⁵ plaque forming units of coxsackievirus B3 (CVB3; Nancy strain, ATCC, Manassas, Virginia, United States) to develop myocarditis (n=9) or with phosphate buffered saline as healthy control (n=7) at day zero. Mice were euthanized 14 days after infection. Hearts were fixed in 4% formaldehyde and embedded in paraffin. All virus injected animals developed acute myocarditis as confirmed by the presence of multiple inflammatory
lesions throughout the entire heart. Inflammatory cells were investigated on cross sections in the middle of the heart. All animal procedures were approved by the animal ethics committee of the VU University Medical Center, and conforms to the Guide for care and use of laboratory animals published by the US National Institutes of Health.

**Patient material**

Transmural human heart tissue of the LVPW, the LV lateral wall (LVLW), the LV anterior wall (LVAW), the septum and the right ventricular anterior wall (RVAW) was obtained at autopsy. LM \( (n=18) \) in the LV was based on the Dallas criteria, namely the histological evidence of the aggregates of lymphocytes, adherent to cardiomyocytes and myocytolysis.\[1\] One of these patient had borderline myocarditis, i.e. myocarditis without myocytolysis. In addition, control patients \( (n=6) \) were included that died of a cause not related to cardiac disease as confirmed by (immune)histological analysis of the LVPW, LVLW, LVAW, septum and RVAW. The characteristics of patients are depicted in Table 1. There were no significant differences in sex \( (p=0.871) \) or age between the groups (mean age of control 68.17 ± 10.91 and of LM 51.83 ± 22.81; \( p=0.137 \)).

All heart tissue samples obtained from patients were fixed in 4% formaldehyde and embedded in paraffin. This study was approved by and performed according to the guidelines of the ethics committee of the VU University Medical Center, Amsterdam, and conforms to the principles of the Declaration of Helsinki. Use of the leftover material after the pathological examination has been completed is part of the patient contract in our hospital.

**Immunohistochemical analysis**

All immunohistochemical stainings were performed on paraffin sections of 4 \( \mu m \). First, sections were deparaffinized, rehydrated and blocked for endogenous peroxidases (incubation in 0.3% \( \text{H}_2\text{O}_2 \) diluted in methanol, 30 minutes). Antigen retrieval was then performed by heat inactivation in 10mM citrate buffer (pH 6.0; boiled for 10 minutes) for all stainings, except for slides stained with anti-mouse CD3 that were heated in a 10 mM Tris-EDTA buffer (pH 9.0; boiled for 10 minutes) and anti-human CD45 staining that required no antigen retrieval step. Subsequently, the sections of mouse heart tissue stained for CD3 and macrophages were pre-incubated with Normal Swine Serum \( (1:20, \text{Monosan X10964}) \) for 10 minutes. Then, the mouse sections were incubated with either CD3 \( (1:50, \text{Abcam Ab16669}) \) or Macrophage \( (1:1000, \text{Gentake A1AD31240}) \) for 60 minutes or with CD45 \( (1:50, \text{B&D 550539}) \) overnight. The human sections were incubated with CD45 \( (1:100, \text{Dako M0701}), \) CD3 \( (1:100, \text{Dako A0452}) \), CD68 \( (1:400, \text{Dako M0814}) \) or C3d \( (1:1000, \text{Dako A0063}) \) for 60 minutes. As secondary antibodies Envision \( (\text{Dako K5007, 30 min}) \) was used for the human sections and anti-rabbit/rat horseradish peroxidase \( (1:200, \text{Dako P0217/1:50,Dako P0450}) \) for the mouse sections. The staining was visualized using 3,3’-diaminobenzidine \( (0.1 \text{mg/ml, Dako K3468}) \) for 10 minutes. Finally, the slides were counterstained with haematoxylin, dehydrated and covered. With each staining slides were included incubated without a primary antibody as a negative control and all these controls showed no staining.

Moreover, of 10 randomly selected patients (LM \( n=5 \), control \( n=5 \)) the heart tissue was also stained at the Academic Medical Center (AMC) with their staining protocol. Slides were stained in an immunostainer with CD3 marker \( (1:100, \text{ThermoFisher RM-9107-S}) \) or CD45 \( (1:400, \text{Dako M0701}) \) and subsequently an optiview DAB detection kit \( (06396500001, \text{Roche}) \) was used.
CD45 is a more sensitive marker than CD3 to diagnose LM

### Table 1. Patient characteristics LM group and of control.

<table>
<thead>
<tr>
<th>Patient</th>
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<th>Primary cause of death after autopsy</th>
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<tr>
<td>1</td>
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<td>M</td>
<td>Sudden death</td>
<td>LM with pneumonia</td>
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<td>Sudden death</td>
<td>LM</td>
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<td>LM</td>
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<tr>
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Abbreviations: male (M), female (F), lymphocytic myocarditis (LM).

### Identification and quantification of inflammatory cells

Inflammatory cells on serial cross sectional slides of the heart were counted under a light microscope (Zeiss, Germany, 250x magnification) and the number of inflammatory cells was calculated per mm². Extravascular inflammatory intact cells were included. Epitopes of activated degraded cells were not included as positive cells since the original number of cells could not be extrapolated. CD45-positive mononuclear leukocytes were counted based on their morphology. Only the CD45+ cells that were round with scant cytoplasm and showed a distinct peripheral reactivity for CD45 were counted and not the elongated cells with large cytoplasmatic compartments as these are most likely macrophages.[19]

In the autopsied hearts the EMB sampling area was first defined as a 2.0 mm thick area of intact endocardium of the LVPW (representing a common biopsy area).

The slides of the human hearts were scanned using the Panoramic Desk scanner (3DHistech, Budapest, Hungary) and the surface areas of the EMB sampling area and the remaining LVPW were measured using Panoramic Viewer 1.15.2 software (3DHistech, Budapest, Hungary). The total cross-sectional surface area of the mouse hearts was determined on scanned slides using the Pathscan Enabler IV slide scanner (Meyer Instruments, Houston, Texas) and with QuickPhoto Micro analysis software (Windows version 3.0, Promicra, Prague, Czech Republic).
Statistics
Statistical analysis was performed with SPSS software (version 20). Statistical significance between groups was evaluated with a Mann-Whitney U test and if data was paired with a Wilcoxon signed rank test or with a Friedman test followed by a Wilcoxon posthoc test. Patients characteristics were analyzed with the chi-square test. A p-value <0.05 was considered statistically significant. Numeral data in the text and figures is expressed as median with interquartile range.

RESULTS
Comparing CD45 and CD3 staining in the hearts of mice with viral myocarditis
We hypothesized that use of an antibody against CD45 rather than CD3 could improve the diagnostic sensitivity of myocarditis. This was first studied in mice with viral myocarditis. Both CD3+ T-lymphocytes and CD45+ leukocytes (Figure 1A) were found in inflammatory lesions as well as solitary cells scattered in the rest of the heart. As seen clearly in mice with viral myocarditis there were more positive stained inflammatory cells as compared to control (Figure 1A).

Moreover, in the myocardium of mice with viral myocarditis the number of CD45+ leukocytes (29.73; 22.08-71.21 per mm²) was a significant 3.8-fold higher (p=0.011) than the number of CD3+ T-lymphocytes (8.02; 4.10-12.62 per mm²) (Figure 1B).

Although not designed for mice, we applied the current guidelines of LM on EMB to the whole myocardium of mice with viral myocarditis by adding the numbers of either CD45+ leukocytes or CD3+ T-lymphocytes to a maximum of four macrophages (Figure 1C).[11] The combined score of CD45+ leukocytes and macrophages (33.73; 25.83-75.21 per mm²) was significantly higher compared to the combined score of CD3+ T-lymphocytes and macrophages (12.02; 7.86-16.62 per mm²) (p=0.008). Moreover, when using CD3, in only 3 of the 9 mice (33%) the threshold of ≥14 leukocytes per mm² was reached and would be diagnosed with myocarditis, whereas with CD45 in 8 of the 9 mice (89%) the threshold was reached (Figure 1C). None of the healthy control mice reached the threshold of ≥14 leukocytes per mm² when combining the numbers of either CD45 leukocytes or CD3+ T-lymphocytes with maximum four macrophages.

These data thus indicate that CD45 is a more robust marker for the diagnosis of viral myocarditis in mice than CD3, suggesting that the use of CD45 could increase the diagnostic sensitivity of LM in humans also.

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**Figure 1. Inflammatory cells in mice with viral myocarditis. A)** Example of staining of Hematoxylin-Eosin, CD45-positive leukocytes, CD3 positive T-cells and macrophages (MØ) in the heart of a mouse with viral myocarditis (VM) and a healthy control. The pictures of the immunological stainings of the mice with VM were stained on serial section and are of the same area. A black arrow indicates an example of a positive cell. The bar indicates 50 μm. **B)** Number of extravascular CD45, CD3 positive cells and macrophages (mø) per mm² in mice with VM (n=9) or control (n=7). **C)** Number of extravascular positive CD45 or CD3 cells in combination with up to four macrophages per mm² in mice with VM (n=9) or control (n=7). Each point in the graphs represents the value of one individual sample; the dotted line represents the number of 14 cells per mm²; * p<0.05; ** p<0.01; *** P<0.001. Data is expressed as median with interquartile range.
CD45 is a more sensitive marker than CD3 to diagnose LM.
Comparing CD45 and CD3 staining in the EMB sampling area of autopsied LM patients

To compare the use of CD45+ leukocytes with CD3+ T-lymphocytes in diagnosing LM in humans, we quantified these cells and CD68+ macrophages in the EMB sampling area of the LVPW (Figure 2A) of autopsied hearts obtained from LM patients and controls (Figure 2B). The patient characteristics are summarized in Table 1. First of all we observed in LM patients stained with CD45 residual epitopes of aggregated and activated leukocytes adherent on the cell surface of cardiomyocytes, but not with CD3 staining (Figure 2B). This indicates that activated deformed leukocytes are missed with CD3 staining, but are identified with CD45 staining. Similar as in the mice, we found in the EMB sampling area of LM patients a significant 2.4-fold higher number of CD45+ leukocytes (11.19; 6.42-20.62 per mm²) compared to CD3+ T-lymphocytes (4.69; 1.72-6.70 per mm²) (p<0.001; Figure 2C). We again applied the guidelines for the diagnosis of LM on EMB by adding the numbers of either CD45+ leukocytes or CD3+ T-lymphocytes to a maximum of four macrophages. When combining the score of CD45+ leukocytes with macrophages (15.19; 10.10-38.10 per mm²) this resulted in a significantly higher number of cells than combining CD3+ T-lymphocytes with macrophages (7.47; 5.28-10.05 per mm²) in the EMB area of LM patients (p<0.001; Figure 2D). When applying the cut-off criteria only 3 out of 18 cases (17%) were diagnosed with LM for the combined score of CD3+ T-lymphocytes and CD68+ macrophages in the LM group. However, LM was diagnosed in 9 out of 18 cases (50%) when applying the combined score of CD45+ leukocytes with macrophages. Importantly, none of the control patients exceeds the 14 leucocytes per mm² cut-off value when combining the numbers of either CD45 leukocytes or CD3+ T-lymphocytes with maximum four macrophages. To determine whether the difference in cell numbers between CD45 and CD3 could also be extrapolated to other diagnostic pathology centers we compared cell numbers from a randomly selection of patients stained for CD45 and CD3 at the VU University Medical Center with slides from the same patients stained at the Academic Medical Center according to their staining protocols. No differences were found in cell numbers in the EMB sampling area of the patients for both CD45 (p=0.85, Figure 2E) and CD3 (p=0.43, Figure 2F) between sections stained at the different centers. These results indicate that the quantification of CD45+ leukocytes in the LVPW biopsy area is a more potent diagnostic marker to identify LM than quantification of CD3+ T-lymphocytes.

Increased inflammatory infiltrate in the EMB sampling area compared to LVPW

We demonstrated that use of CD45 rather than CD3 can improve the diagnosis of LM in the EMB sampling area of patients with LM. However, it is unknown how inflammation in the endocardium relates to the rest of the myocardium, especially since with CMR myocardial injury related to LM is primarily seen in the subepicardial compartment rather than the endomyocardial compartment. Therefore, we quantified the number of CD45+ leukocytes and a maximum of four macrophages of the EMB sampling area in the LVPW, a common biopsy site, and compared this with the remaining LVPW in autopsied hearts obtained from patients with LM (n=18) and controls (n=6).
Interestingly, we found a significant 2.9-fold increase in the number of CD45+ leukocytes combined to macrophages in the EMB sampling area (15.19; 10.10-24.62 per mm²) compared to the remaining LVPW (5.25; 2.69-8.04 per mm²) in patients with LM (p<0.001; Figure 3). Also in the control patients there were significant more cells in the EMB sampling area (8.58; 4.99-10.72 per mm²) than in the remaining LVPW (4.49; 2.56-5.28 per mm²; p=0.036). In addition, the number of CD45+ leukocytes combined with macrophages in the EMB sampling area of LM patients was significantly higher than that in the EMB sampling area in control patients (p=0.032). Whereas there was no significant difference between the numbers of CD45+ leukocytes combined with macrophages in the remaining LVPW of LM patients and controls.

DISCUSSION

In this study we compared the sensitivity of the recommended pan T-lymphocyte marker CD3 with the leukocyte common antigen CD45 to diagnose LM in the hearts of mice with CVB3-induced myocarditis and in patients with LM. In mice using the recommended threshold of ≥14 leukocytes per mm² we found that CD45 increased the diagnostic sensitivity to 89% from only 33% with CD3. Also in the EMB sampling area of LM patients the diagnosis of LM could be made in only 17% of cases with CD3 when the recommended threshold was applied. This increased significantly to 50% with CD45. In addition, we compared diagnostic sensitivity of a common endocardial EMB sampling area with the rest of the ventricular wall. Interestingly, the number of CD45+ leukocytes with macrophages was almost 3-fold higher in the EMB sampling area of the LVPW than in the remaining of the LVPW, indicating that despite the risk of underdiagnoses in EMB, the endocardial sampling area constitutes the highest chance for positive histological diagnosis of LM.

Especially in the hearts of mice with viral myocarditis, but also in the hearts of LM patients, we found considerably more CD45+ cells than CD3+ cells. On average there were respectively, 3.8-fold and 2.4-fold more CD45+ cells than CD3+ cells indicating that the majority of the cells identified with the CD45 antigen were not T-lymphocytes. We deliberately excluded those CD45+ cells that, based on their morphology, were macrophages. Probably the other CD45+ non T-cells are other subtypes of lymphocytes. However, it is also known that not all T-lymphocytes express CD3 and T-lymphocytes are thus automatically underscored. Even more, T-lymphocytes can lose re-expression of CD3 on their surface when activated, however this is only reported with flow cytometry.[20]

Currently, the mostly used criteria for LM in EMB is an inflammatory infiltrate of ≥14 leukocytes per mm², composed of activated T-lymphocytes and up to four macrophages per mm².[12] It could be that the clear cut off value is too strict. Related to an autopsy study with LM patients based on the Dallas criteria Nielsen et al. suggested that the cut-off value can range between 11 and 16 CD3 positive T-cells per mm² for the diagnosis of LM.[21] They stained for CD3 cells in LM patients and evaluated the accuracy by combining the sensitivity and specificity between the cut-off value from 5 to 25 T-cells per mm². The highest accuracy of was observed at a cut-off value of 13 T-cells per mm², however this combined level of sensitivity and specificity remained the same within the range between 11 and 16 T-cells per mm². [21] Moreover, Maisch et al. suggested that LM could also be diagnosed in EMB even
CD45 is a more sensitive marker than CD3 to diagnose LM

Figure 2. Inflammatory cells in the EMB sampling area of patients with lymphocytic myocarditis. A) Example of the heart tissue of the EMB sampling area of the endocardium of 2.0 mm thickness with endothelial cells at the endocardial side of the LVPW. Bar represents 5.0 mm. B) Example of CD45, CD3 and CD68 staining in the heart tissue of a post-mortem patient with LM and in controls. The pictures of heart tissue of the LM patient is of the same area and was stained on serial section. Blue arrows indicate example of positive CD45 epitopes of degraded activated cells on cardiomyocytes and black arrows indicate example of a positive cell. The bar indicates 50 μm. C) Number of extravascular positive CD45, CD3 and CD68 cells in EMB sampling area of LM patients (n=18). Data is expressed as median with interquartile range. D) The number of extravascular positive CD45 or CD3 cells in combination with maximum four CD68 cells per mm² in the EMB sampling area of LM patients (n=18) and controls (n=6). The dotted line represents the number of 14 cells per mm². Data is expressed as median with interquartile range. E-F) Number of extravascular positive CD45 (F) and CD3 (G) cells in EMB sampling area of LM (n=5) and control (n=5) patients compared between VU University Medical Center (VUmc) and Academic Medical Center (AMC). Each point in the graphs represents the value of one individual sample; * p<0.05; ** p<0.01; *** P<0.001.

when the criteria of ≥14 leukocytes per mm² is not reached, if there are extravascular foci’s of leukocytes (≥3 lymphocytes, preferably T-cells). In this study we clearly show that foci’s of leukocytes are revealed much more clearly with the CD45 staining, since CD45 is still expressed in degraded activated cells. Therefore, CD45 makes it easier to distinguish aggregates of lymphocytes and to find the distribution of the inflammatory infiltrates than with CD3. This is due to an increase of the number of positive CD45 intact cells, but also especially due to positively stained epitopes of degraded cells. Since these positively stained degraded cells were not included as lymphocytes the lymphocyte count is even underscored. Moreover, there is only a small interobserver discordance for identifying lymphocytes and diagnosing myocarditis in EMB with myocarditis stained for this common leukocyte marker.

The Dallas criteria were suggested in 1987 and have provided a pathological categorization on which the diagnosis of myocarditis can be made in EMB. However, the Dallas criteria do not take in account sampling error of EMB, variation in expert interpretation, variation in stainings for inflammatory cells or the number of inflammatory cells. Hauck et al. already demonstrated in 1989 that one EMB of the left ventricle could diagnose myocarditis only in 21% of the cases in postmortem hearts of patients who died with myocarditis. Additional EMB will increase the chance of diagnosing myocarditis, as Chow et al have shown that myocarditis could be diagnosed in 79% of the cases when on average 17,2 EMB per patient were taken. Unquestionably this is a clinically unrealistic number in living patients. In conclusion, the use of the common leukocyte marker CD45 increases the sensitivity of the diagnosis of LM. In mice with viral myocarditis the diagnosis of myocarditis was increased from 33% to 89% when using a CD45 staining compared to a CD3 staining, in combination with a macrophage staining. Also, in the EMB sampling area of autopsy hearts with LM, diagnosis of LM increased form 17% to 50% with these stainings. In addition, the EMB sampling area of the LVPW has increased amount of leukocytes with macrophages compared to the rest of the myocardium of the LVPW. Even though there is a high probability of underdiagnoses in the EMB sampling area in patients with LM, EMBs are taken at a representative area.
Figure 3. Inflammatory cells in EMB sampling area compared with the remaining LVPW. The number of extravascular CD45-positive leukocytes and up to four CD68-positive macrophages per mm$^2$ of the EMB sampling area and the remaining left ventricle posterior wall (LVPW) for LM patients (n=18) and controls (n=6) patients. The dotted line represents the number of 14 cells per mm$^2$. Each point in the graphs represents the value of one individual sample; * p<0.05; *** p<0.001. Data is expressed as median with interquartile range.
CD45 is a more sensitive marker than CD3 to diagnose LM

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Lymphocytes infiltrate the quadriceps muscle in lymphocytic myocarditis patients: A potentially new diagnostic

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Chapter 3

ABSTRACT

Background. Diagnosing lymphocytic myocarditis is challenging due to the large variety in clinical presentation and the limitations inherent to current diagnostic tools. The objective of this study was to analyze infiltration of inflammatory cells in quadriceps skeletal muscle of lymphocytic myocarditis patients and investigate the diagnostic potential of these infiltrating inflammatory cells.

Methods. Quadriceps muscle tissue, obtained at autopsy from control patients (n=9) and lymphocytic myocarditis patients (n=21), was analyzed via immunohistochemistry for infiltration of lymphocytes (CD45), macrophages (CD68), neutrophilic granulocytes (myeloperoxidase) and several lymphocyte subtypes (CD3, CD4, CD8, CD20) and via PCR for a panel of myocarditis-associated viruses. Additionally, quadriceps muscle from mice with acute coxsackievirus B3-induced myocarditis and control mice was analyzed for presence of lymphocytes and virus.

Results. In quadriceps muscle of lymphocytic myocarditis patients the numbers of infiltrating lymphocytes were significantly increased and could diagnose lymphocytic myocarditis with specificity of 100% and sensitivity of 71%. Parvovirus B19 was the primary virus found in our patient groups, found in quadriceps tissue of 3 lymphocytic myocarditis patients though also in 1 control patient. In the mice, enteroviral RNA was present in the quadriceps muscle, although enteroviral capsid proteins and lymphocyte infiltration were found in the adipose tissue within and directly adjacent to the myocyte tissue, rather than in the myocyte tissue itself.

Conclusions. Lymphocytic myocarditis is associated with lymphocyte infiltration and with viral presence in quadriceps muscle. This indicates that skeletal muscle may have potential as a diagnostic tool for lymphocytic myocarditis patients.
INTRODUCTION

Lymphocytic myocarditis (LM) is an inflammatory heart disease, mainly the result of viral infection,[1] which can cause severe cardiac dysfunction. LM is a complicated clinical entity and difficult to diagnose due to large varieties in clinical presentation.[2, 3] At present, the gold standard of diagnosing LM is evidence of cardiomyocyte myocytolysis combined with intramyocardial lymphocyte infiltration in endomyocardial biopsies (EMB), in the absence of an ischemic event.[4, 5] While valuable for the diagnosis of LM, the usefulness of EMB is limited. As LM can present with diffuse, patchy inflammation in the heart,[6] evidence of LM may not be present in EMB, even with multiple biopsies.[7] Furthermore, contraindications may exist to refrain from taking EMB and although complications are rare,[8] the collection of EMB is no sinecure for patients. As a consequence, LM is underdiagnosed and there is a clinical need for additional diagnostic methods to determine LM.

Several observations in animals and humans suggest that skeletal muscle changes can occur concomitantly with virus-induced myocarditis. In the Atlantic salmon for instance, presence of piscine reovirus in the heart coincided with heart and skeletal muscle inflammation.[9] Moreover, in several case reports on myocarditis patients, cardiotropic viruses have been shown to infect skeletal muscle,[10, 11] suggesting the possibility that skeletal muscle may reflect virus-induced myocarditis in the heart.

The inflammatory infiltrate in the heart during acute LM consists mainly of lymphocytes, although macrophages and neutrophilic granulocytes also infiltrate the heart during LM.[12] Our objectives were to quantify these cells in post-mortem material of LM patient quadriceps muscle, and to investigate whether infiltration of inflammatory cells has diagnostic potential for LM.

MATERIALS AND METHODS

Patients

Quadriceps muscle tissue was obtained at autopsy at the VU University Medical Center (VUmc, Amsterdam, the Netherlands) and used retrospectively in this study. The use of post-mortem patient material in the VUmc is conform patient contract and includes obtaining explicit written consent from relatives, in accordance with the ethical guidelines set up by the World Medical Association (The declaration of Helsinki). Details of all included patients are listed in supplementary table S1. In addition, information regarding in- and out-of-hospital deaths, as well as clinical suspicion of viral related disease in the LM patients is listed in supplementary table S2. 15 LM patients died suddenly out-of-hospital and 6 died in hospital of which 4 had a clinical suspicion of virus-related disease.

Animal procedures

All animal procedures were approved by the local institution animal ethical committee, in correspondence with the European Commission Directive 2010/63/EU. Four-week old C3H mice (Harlan, Boxmeer, The Netherlands) received 2x10^5 plaque-forming units of coxsackievirus B3 (CVB3, Nancy strain, ATCC, Manassas, VA) intraperitoneally, or a control injection. After 7 days, the mice were terminated. Of each mouse, one quadriceps muscle
was fixed in 4% formaldehyde for immunohistochemical analysis, and the other quadriceps muscle was snap-frozen and stored at -80°C for enteroviral RNA analysis.

**Immunohistochemistry**

Tissue samples were fixed with 4% formaldehyde and mounted in paraffin. Tissue slides (4μm) were deparaffinised in xylene, dehydrated in 100% ethanol and endogenous peroxidase was blocked in methanol + 0.3% H₂O₂. For immunostaining of CD8, CD20, CD68, myeloperoxidase (MPO), and mouse CD45, antigen retrieval was performed by heating the slides in boiling 0.01M sodium-citrate buffer, pH6. For CD3 and CD4 immunostaining, 0.01M Tris/EDTA buffer, pH9 was used for antigen retrieval. No antigen retrieval was used for CD45 immunostaining on human tissue. Slides were incubated with primary and secondary antibodies for 60 and 30 minutes respectively at room temperature (see supplementary table S3 for antibody details). Horseradish peroxidase (HRP) was visualized with diaminobenzidine (Dako, Glostrup, Denmark). The slides were counterstained with haematoxylin, and covered.

Enterovirus-staining was performed using the Mouse On Mouse™ Immunodetection Kit (Vector Laboratories, Burlingame, CA), following manufacturer’s instructions. Deparaffinising/hydrating, HRP visualization, endogenous peroxidase blocking, washing and antigen retrieval (0.01M sodium-citrate buffer) steps were done as described above. Avidin/Biotin blocking was done with a Biotin blocking System (Dako). Streptavidin-HRP (Dako) was used to visualize the secondary antibody.

**Quantitative analysis of immunohistochemistry**

Extravascular positive-staining cells were counted using a light microscope. Quadriceps muscle fibres and adjacent adipose tissue were analyzed separately for both human and mice. The tissue surface areas were measured with Qprodit v3.2 (Leica Microsystems, Wetzlar, Germany), using a Leica DM/LM microscope.

**Detection of viral genome**

Of the human paraffin-embedded tissues, genomic material was extracted using the ALLPrep DNA/RNA FFPE Kit (Qiagen, Venlo, The Netherlands). RNA and DNA were isolated following the manufacturer’s protocol, though DNase steps were omitted from the RNA isolation. DNA and RNA isolates from the same tissue sample were pooled. RNA of frozen (-80°C) mouse quadriceps tissues was extracted using the MagnaPureLC total nucleic acid isolation kit (Roche Diagnostics, Almere, The Netherlands) and total nucleic acid HP 200 protocol. Amplification and quality control was performed as described before.[13] Additionally, the Rodent GAPDH control reagents kit (Lifetechnologies, Nieuwerkerk a/d/ IJssel, The Netherlands) and primers/probe specific for human GAPDH were used to adjust for sampling errors. Primer and probe details can be found in supplementary table S4.

**Statistical analysis**

Statistical significance of differences between control and disease groups was determined using a Mann-Whitney U test. Sensitivity and specificity of the CD45-positive cells in quadriceps muscle was approached using a Receiver operating characteristic (ROC)-analysis. Correlations between CD45-positive cells in quadriceps muscle and CD45-positive
Lymphocytes infiltrate the quadriceps muscle in LM patients

cells in cardiac muscle, patient age or patient gender were calculated using Spearman’s rank correlation coefficient. Distributions of age and gender were compared between patient groups using the Kruskal-Wallis test. P<0.05 was considered statistically significant for all used statistical tests. Cell numbers in the text represent mean number of cells per mm² ± standard error.

RESULTS

Patient group criteria
All patients included in this study were selected based on cardiac histology at autopsy. Patients included in the LM group (n=21) were diagnosed based on the presence of multiple aggregates of extravascular lymphocytes, partly adherent to cardiomyocytes with myocytolysis, visualized with complement staining. Catecholamine-induced myocarditis (CM, n=6) was diagnosed as a mixed infiltration of lymphocytes, macrophages, and neutrophilic granulocytes, accompanied by complement positivity of cardiomyocytes. Acute myocardial infarction (AMI), n=26) were subdivided into three subgroups according to the age (phase) of the infarction based on microscopic criteria as described before[14]: phase 1 (3–12 hours after AMI, n=4), phase 2 (12 hours–5 days after AMI, n=10), and phase 3 (5–14 days after AMI, n=12). There was no significant difference in age and gender distribution between the included patient groups.

Inflammatory cell infiltration of the lymphocytic myocarditis patient’s quadriceps muscle
The number of CD45-positive (lymphocytes), CD68-positive (macrophages), and MPO-positive (neutrophilic granulocytes) cells was determined per mm² quadriceps muscle tissue of 21 LM patients and 9 control patients. The mean number of CD45-positive cells in quadriceps muscle tissue of LM patients was significantly higher compared to control patients (3.12±0.53 and 0.67±0.11 respectively, figure 1A). We also found an increased, although insignificant, number of CD68-positive cells (1.04±0.44 and 0.23±0.08 respectively, figure 1B) and MPO-positive cells (0.72±0.13 and 0.41±0.08 respectively, figure 1C) in LM patients compared to control patients.

The potential of lymphocytes in quadriceps muscle as diagnostic marker for lymphocytic myocarditis
As significantly more CD45-positive cells infiltrated the quadriceps muscle of LM patients compared to control patients, its potential as diagnostic marker for LM was further studied. First, the number of LM patients with a significant increase of CD45-positive cells in quadriceps muscle was determined. For this, the threshold value was determined (mean number of CD45-positive cells in the control group plus 2 standard deviations[15]), which was 1.35 cells per mm². In 15 of 21 LM patients the number of CD45-positive cells in the quadriceps muscle exceeded this threshold value (71%). Subsequently, the sensitivity and specificity of CD45-positive cells for diagnosing LM was determined for cell numbers ranging between 0.2 and 1.6 cells per mm² (figure 2A). The best diagnostic value was observed for 1.5 cells per mm², where CD45-positive cells had a sensitivity of 71% and a specificity of 100% in diagnosing LM. CD45-positive cell numbers in quadriceps muscle were not found to
correlate with cell numbers in cardiac muscle (figure 2B), patient age (figure 2C) or gender (figure 2D).

**Lymphocytes in the quadriceps muscle of patients with other inflammatory cardiac diseases**

To determine whether CD45-positive cells also infiltrate the quadriceps muscle in patients with other inflammatory cardiac diseases, we analyzed the quadriceps muscle of patients that were diagnosed with CM ($n=6$), phase 1 AMI ($n=4$), phase 2 AMI ($n=10$) or phase 3 AMI ($n=12$) (figure 2E). The number of CD45-positive cells in quadriceps muscle of patients with CM (1.15±0.14), phase 1 AMI (1.66±0.90), or phase 2 AMI (0.89±0.13) did not differ significantly compared to control patients (0.67±0.11). However, a limited but significant increase in CD45-positive cells was observed in patients with phase 3 AMI (1.55±0.35). The number of CD45-positive cells in quadriceps muscle of phase 3 AMI patients was lower, but not significantly, compared to LM patients.

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**Figure 1.** Quantification of infiltrated mononuclear cells per mm$^2$ in quadriceps muscle tissue of control ($n=9$) and lymphocytic myocarditis patients ($n=21$). A) CD45-positive cells (lymphocytes). B) CD68-positive cells (macrophages). C) MPO-positive cells (neutrophilic granulocytes). Each dot in the graphs represents the value of one individual patient; horizontal lines represent the mean values. NS: Not significant.
Figure 2. A) CD45-positive cells as predictive marker for LM in quadriceps muscle tissue in the range of 0.2 to 1.6 cells per mm² B) Correlation (rho-value=0.17, NS) of CD45-positive cells between quadriceps muscle and cardiac muscle in LM patients (n=21). C) Correlation (rho-value=0.18, NS) between CD45-positive cells in LM patient quadriceps muscle tissue (n=21) and patient age. D) CD45-positive cells in quadriceps muscle tissue in male (n=14) and female (n=7) LM patients. Horizontal lines: mean. E) The number of CD45-positive cells per mm² quadriceps muscle tissue of control patients (n=9), catecholamine-induced myocarditis patients (CM, n=6) and phase 1 (F1, n=4), phase 2 (F2, n=10), and phase 3 (F3, n=12) acute myocardial infarction patients. Horizontal lines: mean. Each point in graphs B, C, D and E represents the value of one individual patient. NS: Not significant.
Presence of viral genome in the heart and quadriceps muscle

As viral infection is the primary cause of LM, we analyzed both cardiac and quadriceps tissue of LM and control patients for presence of viral RNA/DNA. Quantitative PCR analysis was performed for 10 viruses that have been associated with LM (supplementary table S5). In LM patients, parvovirus B19 was found in cardiac tissue (9 of 20 patients) and in quadriceps muscle tissue (3 of 20 patients). The cardiac tissue of one of these patients was also positive for human herpesvirus 6. No other virus was detected in any sample. Notably, parvovirus B19 was also detected in cardiac tissue (3 of 9 patients) and quadriceps muscle (1 of 9 patients) of control patients. Parvovirus B19 positivity in quadriceps muscle coincided with positivity in cardiac muscle for 3 of 4 patients (2 LM patients and 1 control patient).

Figure 3. Lymphocyte subtype cell numbers per mm² quadriceps muscle tissue of control patients (n=9) and lymphocytic myocarditis patients (n=21). A) CD3-positive cells (T-lymphocytes). B) CD8-positive cells (Cytotoxic T-lymphocytes). C) CD4-positive cells (T-helper cells). D) CD20-positive cells (B-lymphocytes). Each point in the graphs represents the value of one individual patient. Horizontal lines represent the mean values. NS: Not significant.
**Lymphocyte subtypes in lymphocytic myocarditis patient’s quadriceps muscle**

To gain more insight into the nature of the lymphocyte response in the quadriceps muscle of LM patients, CD3-positive (T-lymphocytes), CD4-positive (T-helper cells), CD8-positive (cytotoxic T-lymphocytes), and CD20-positive (B-lymphocytes) cells were quantified. Both the number of CD3-positive cells (1.09±0.18) and CD8-positive cells (0.15±0.05) was significantly higher in quadriceps muscle tissue of LM patients compared to control patients (0.27±0.07 for CD3 and 0.004±0.003 for CD8, figures 3A+B). The number of CD4-positive cells (0.38±0.11) and CD20-positive cells (0.026±0.009) in quadriceps muscle tissue of LM patients were higher compared to control patients (0.13±0.04, for CD4 and 0.003±0.002 for CD20), but not significantly (figures 3C+D). Residual tissue was not sufficient to obtain data on CD3-positive cells for one control patient and CD4-positive cells for one LM patient.

**Lymphocytic infiltration in mouse quadriceps muscle**

Next, we wanted to verify in a mouse model whether acute CVB3-induced myocarditis coincides with lymphocyte infiltration of quadriceps muscle tissue. CD45-positive cells were quantified in quadriceps muscle of CVB3-mice (n=8) and control mice (n=4). In the myocyte tissue, no increase in the number of CD45-positive cells was found in CVB3-mice compared with control mice (0.93±0.09 and 0.75±0.13 respectively, figure 4A). Interestingly, in the adipose tissue within and directly adjacent to the myocyte tissue a significant increase in CD45-positive cells was found in CVB3-mice compared with control mice (186.1±59.5 and 25.8±10.2 respectively, figures 4B+C). In the patient data described above, adipose tissue was not included in the determination of the cell numbers. However, subsequent analysis of the adipose tissue in the muscle of LM patients and control patients revealed no significant differences in CD45-positive cell numbers (not shown).

**Presence of coxsackievirus B3 in mouse quadriceps muscle**

Finally, we analyzed whether CVB3 actively infects quadriceps muscle in CVB3-mice. PCR analysis showed enteroviral RNA in the quadriceps muscle in 7 out of 8 CVB3-mice, while no enteroviral RNA was detected in control mice (p=0.016, figure 4D). No correlation was found between the quantity of enteroviral RNA and the number of CD45-positive cells in either myocyte tissue or the adipose tissue of the quadriceps muscle (not shown). In addition, we investigated the location of the CVB3 infection within the quadriceps muscle tissue using an immunohistochemical staining specific for enteroviral envelope protein VP1. CVB3 was present in the adipose tissue of 6 out of 8 CVB3-mice (figure 4E), whereas no CVB3 was found in myocyte tissue. No CVB3 was found in control mice.

**DISCUSSION**

Diagnosing LM is challenging, and there is a clinical need for additional methods to diagnose LM. In this study, we for the first time show a significant increase of lymphocytes in quadriceps muscle from LM patients compared to control patients. Moreover, lymphocyte infiltration of the quadriceps muscle could predict LM with a sensitivity of 71% and specificity of 100%. These data suggest that quadriceps muscle tissue may have potential diagnostic value for LM patients.
Figure 4. A+B) Quantification of CD45-positive cells (lymphocytes) per mm² quadriceps muscle of mice with acute Coxsackievirus B3-induced myocarditis (CVB3, n=8) and uninfected control mice (n=4). A) Lymphocyte numbers in myocyte tissue. B) Lymphocyte numbers in adipose tissue surrounding muscle fibre bundles. C) Infiltrating CD45-positive cells (stained brown) in adipose tissue adjacent to quadriceps muscle fibres of a CVB3-mouse. D) Enteroviral RNA quantity in quadriceps muscle of CVB3-mice (n=8) and uninfected control mice (n=4), relative to the quantity of GAPDH RNA. E) The presence of active enterovirus (stained brown) in adipose tissue adjacent to quadriceps muscle fibres of a CVB3-mouse. Each point in graphs A, B and D represents the value of one individual mouse. Horizontal lines: mean. NS: Not significant. MT: Myocyte tissue. AT: Adipose tissue

At present, the mechanisms underlying this increase in intramuscular lymphocytes remain subject to speculation. An obvious explanation may be viral infection of both cardiac and quadriceps muscle. Indeed, coxsackievirus B1 can infect mouse skeletal muscle and cause local lymphocytic inflammation.[16] Furthermore, we found parvovirus B19 DNA in quadriceps muscle tissue of 3 LM patients, and a significant increase of (CD8-positive) cytotoxic T-lymphocytes, a cell type that typically eliminates virus-infected cells. These data support the hypothesis that lymphocytes infiltrate the quadriceps muscle in response to local viral infection.

In our study, viral genome in the quadriceps muscle and the heart was detected only in a minority of LM patients. Albeit, this lack of viral genome detection both in the heart and quadriceps muscle in the majority of our LM patients may be due to viral clearance. In
Lymphocytes infiltrate the quadriceps muscle in LM patients

addition, it is known that retrospective molecular analysis in formalin-fixed paraffin-embedded autopsy material may be less sensitive due to degradation of RNA/DNA.[17] Unfortunately, frozen tissue samples were not available for these patients.

Putative viral co-infection of the heart and skeletal muscle in LM may also offer the possibility for viral diagnostics in skeletal muscle. It is known, especially from case reports of patients with inflammatory muscle diseases, that numerous cardiotropic viruses can also infect human skeletal muscle.[10, 11, 18-23] In addition, for influenza- and coxsackievirus infection, myocarditis and myositis coincidence has been observed,[10, 11] although inflammatory cell types in skeletal muscle were not specified. These combined data suggest the possibility that in addition to inflammation, skeletal muscle may also reflect viral presence in the heart.

However, similar to viral diagnostics in the myocardium, certain important limitations exist regarding viral diagnostics in quadriceps muscle. For instance, parvovirus B19 DNA was also detected in the heart and quadriceps of 3 and 1 control patients respectively. Prevalence of parvovirus B19 in the myocardium of patients without evidence myocarditis has been shown before[24], and its role as causative agent of myocarditis remains controversial. Also, detection of viral genomic material by PCR does not necessarily constitute infection of the (cardio)myocytes. Indeed, in 7 out of 8 of our CVB3-mice enteroviral RNA was detected by PCR in quadriceps tissue, although immunohistochemistry revealed that viral capsid proteins and lymphocyte infiltration occurred in the adipose tissue within and directly adjacent to the myocyte tissue, rather than in the myocytes themselves.

Besides viral infection, autoimmunity may underlie the increased presence of lymphocytes in the quadriceps muscle in LM. Autoimmunity is a well recognized causal factor in the propagation of cardiac damage and inflammation after viral clearance.[25] This provides an additional reason why a viral agent may not be detected in the heart. Alternatively, myocarditis is also known to occur as side-effect of autoimmune disorders such as systemic lupus erythematosus, although this occurs rarely. Interestingly, increased serum autoantibodies that target skeletal muscle have been described for Chagasic cardiopathy[26] and in mouse models of CVB3- and cytomegalovirus-induced myocarditis.[27, 28] However, in these studies lymphocyte infiltration into skeletal muscle was not investigated.

Concluding remarks

The objective of this study was to assess infiltration of inflammatory cells in quadriceps muscle of LM patients and to evaluate their diagnostic potential. This study demonstrates that LM is associated with lymphocyte infiltration in the quadriceps muscle and that these lymphocytes may have diagnostic potential. In this study this concept was explored in autopsy material in relatively small patient groups. Therefore, to further investigate this concept in living patients, a prospective clinical trial has recently started in our hospital (the INFLAME-study).
REFERENCES


Lymphocytes infiltrate the quadriceps muscle in LM patients


Supplementary table S1. Patient characteristics.

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Catecholamine myocarditis patients

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<td>AMI</td>
<td>-</td>
<td>&lt;12h</td>
<td>Unknown</td>
<td>Atrial Fibrillation</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>75</td>
<td>AMI</td>
<td>Asthma Pneumonia</td>
<td>26h</td>
<td>Unknown</td>
<td>Hypotension</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>47</td>
<td>AMI</td>
<td>Sarcoidosis (lungs) DM type 2 Epicarditis</td>
<td>24h</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>F</td>
<td>82</td>
<td>AMI</td>
<td>-</td>
<td>42h</td>
<td>Unknown</td>
<td></td>
</tr>
</tbody>
</table>


PC: Possible confounders. A: Patient used prednisone, which may have negatively affected lymphocyte numbers in tissue. B: Immune cell infiltration of skeletal muscles has also been reported in literature for COPD
Supplementary table S2. In-hospital deaths of lymphocytic myocarditis patients.

<table>
<thead>
<tr>
<th>Lymphocytic myocarditis $n=21$</th>
<th>Out-of-hospital death $n=15$</th>
<th>Clinical suspicion of virus-related disease $n=4$</th>
<th>1. Pneumonia</th>
</tr>
</thead>
<tbody>
<tr>
<td>In-hospital death $n=6$</td>
<td></td>
<td></td>
<td>2. Haemophagocytic syndrome</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pneumonia</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pericarditis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3. Respiratory insufficiency</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4. Respiratory insufficiency</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Haemodynamic instability</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Leopard Syndrome</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chronic epi/pericarditis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No clinical suspicion of virus-related disease $n=2$</td>
<td>1. Cholangiocarcinoma</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Hepatitis</td>
<td></td>
</tr>
</tbody>
</table>

Right column lists secondary pathologies of patients who died in-hospital.

Supplementary table S3. Antibodies used for immunohistochemical analysis

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary antibodies</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rabbit $\alpha$-human CD3</td>
<td>1:100</td>
<td>Dako1, cat. nr. A0452</td>
</tr>
<tr>
<td>mouse $\alpha$-human CD4</td>
<td>1:50</td>
<td>Thermo Fischer Scientific2, cat. nr. MA5-12259</td>
</tr>
<tr>
<td>mouse $\alpha$-human CD8</td>
<td>1:100</td>
<td>Dako1, cat. nr. M7103</td>
</tr>
<tr>
<td>mouse $\alpha$-human CD20</td>
<td>1:200</td>
<td>Dako1, cat. nr. M0755</td>
</tr>
<tr>
<td>mouse $\alpha$-human CD45</td>
<td>1:50</td>
<td>Dako1, cat. nr. M0701</td>
</tr>
<tr>
<td>mouse $\alpha$-human CD68</td>
<td>1:400</td>
<td>Dako1, cat. nr. M0814</td>
</tr>
<tr>
<td>rabbit $\alpha$-human MPO</td>
<td>1:500</td>
<td>Dako1, cat. nr. A0398</td>
</tr>
<tr>
<td>rat $\alpha$-mouse CD45</td>
<td>1:50</td>
<td>BD Biosciences3, cat. nr. 550539</td>
</tr>
<tr>
<td>mouse-$\alpha$-enteroviral VP1 protein</td>
<td>1:1000</td>
<td>Leica Microsystems4, prod. code. NCL-ENTERO</td>
</tr>
<tr>
<td><strong>Secondary antibodies</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Real\textsuperscript{TM} EnVision\textsuperscript{TM} HRP $\alpha$-mouse/rabbit</td>
<td>undiluted</td>
<td>Dako\textsuperscript{1}, cat. nr. K5007</td>
</tr>
<tr>
<td>rabbit $\alpha$-rat-HRP</td>
<td>1:50</td>
<td>Dako\textsuperscript{1}, cat. nr. P0450</td>
</tr>
</tbody>
</table>

1: Dako, Glostrup, Denmark.
2: Thermo Fischer Scientific, Waltham, Massachusetts, United States.
3: BD Biosciences, Franklin Lakes, New Jersey, United States.
4: Leica Microsystems BV, Rijswijk, the Netherlands.
### Supplementary table S4. Primers and probes used for qPCR analysis

<table>
<thead>
<tr>
<th>Virus</th>
<th>Primer/probe sequences (5’ → 3’)</th>
<th>Concentration in PCR-mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV</td>
<td>Forward: GCGGATCGTAAAGAGATGAAGAC&lt;br&gt;Reverse: CTCGTGCCTGTGCTACGAGA&lt;br&gt;Probe: FAM-AGTGCAAGCCCCGACACATCGTTC-BHQ-1</td>
<td>200 nM&lt;br&gt;800 nM&lt;br&gt;100 nM</td>
</tr>
<tr>
<td>EBV</td>
<td>Forward: GGAACTGTGTACTCTTTGC&lt;br&gt;Reverse: ACCTGCATGGACCGGTAAAT&lt;br&gt;Probe: FAM-CGCAAGCCACTGTACTCGCTGCT-BHQ-1</td>
<td>900 nM&lt;br&gt;50 nM&lt;br&gt;100 nM</td>
</tr>
<tr>
<td>HSV-1</td>
<td>Forward: TCCTXGTTCTCTMACKGCCTCCC1&lt;br&gt;Reverse: GCAGICAXACGTAAACGCACGTCT1&lt;br&gt;Probe: FAM-CGCAAGCCACTGTACTCGCTGCT-BHQ-1</td>
<td>300 nM&lt;br&gt;900 nM&lt;br&gt;100 nM</td>
</tr>
<tr>
<td>HSV-2</td>
<td>Forward: CGCCTAATCGACTTACGA&lt;br&gt;Reverse: GAGGTTCCTCCCAGCAAT&lt;br&gt;Probe: DF-CTCGCTTAAGATGGCCGATCCCAATC-BHQ-2</td>
<td>200 nM&lt;br&gt;900 nM&lt;br&gt;100 nM</td>
</tr>
<tr>
<td>HHV-6</td>
<td>Forward: GAAGCAAGCAATCGCAACACA&lt;br&gt;Reverse: ATGTAACTCGTGTAAGGTTGA&lt;br&gt;Probe: FAM-CGCAAGCCACTGTACTCGCTGCT-BHQ-1</td>
<td>300 nM&lt;br&gt;900 nM&lt;br&gt;100 nM</td>
</tr>
<tr>
<td>Parvovirus</td>
<td>Forward: GACAGTTAATCTGACCACCCA&lt;br&gt;Reverse: GCTAATTGCCAGCATTGT&lt;br&gt;Probe: DF-CGCTAATCTGACCACCCAATC-BHQ-1</td>
<td>500 nM&lt;br&gt;600 nM&lt;br&gt;200 nM</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>Forward: GCCACCGTGTTGTTCTAATCTT&lt;br&gt;Reverse: GCCCCATCGTGCTATCGACATC&lt;br&gt;Probe: DF-TGCCACGTCAGCGCTCACTCGCA-&lt;br&gt;BHQ-2</td>
<td>200 nM&lt;br&gt;900 nM&lt;br&gt;100 nM</td>
</tr>
<tr>
<td>Enterovirus</td>
<td>Forward: GACATGTTGXXGAAGAGTCTATTGA1&lt;br&gt;Reverse: GATTGTCACCATAAGCAGCCA&lt;br&gt;Probe: FAM-CGCAAGCCACTGTACTCGCTGCT-BHQ-1</td>
<td>900 nM&lt;br&gt;50 nM&lt;br&gt;100 nM</td>
</tr>
<tr>
<td>Influenza A</td>
<td>Forward: AAGACCAATCCTGTCACCTCTGA&lt;br&gt;Reverse: CAAAGCGTCTACGCTGCAGTCC&lt;br&gt;Probe: FAM-CTGCTACGCTGAAGAGTCTATTGA1</td>
<td>800 nM&lt;br&gt;600 nM&lt;br&gt;100 nM</td>
</tr>
<tr>
<td>Influenza B</td>
<td>Forward: GXACAAATGACAACACAACAAAT1&lt;br&gt;Reverse: CACTCGCAATTCTGCTTCAA1&lt;br&gt;Probe: DF-CGCGAGCAACCACATCCATGGCCGGT-&lt;br&gt;BHQ-2</td>
<td>600 nM&lt;br&gt;900 nM&lt;br&gt;100 nM</td>
</tr>
<tr>
<td>Human</td>
<td>Forward: CAAGCAGATTTGCTCGTATGG&lt;br&gt;Reverse: GCAACAATATCCACTTACCAGAGTTAA&lt;br&gt;Probe: FAM-CGCGGCTGCGCGCCGACATGGCCGGT-BHQ-1</td>
<td>600 nM&lt;br&gt;600 nM&lt;br&gt;100 nM</td>
</tr>
</tbody>
</table>

**Supplementary table S5. PCR-analysis of patient material**

<table>
<thead>
<tr>
<th></th>
<th>Control patients (n=9)</th>
<th>LM patients (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heart</td>
<td>Quadriceps</td>
</tr>
<tr>
<td>Virus positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMV</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>EBV</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>HSV-1</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>HSV-2</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>HHV-6</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Parvovirus B19</td>
<td>3 (33%)</td>
<td>1 (11%)</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Enterovirus</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Influenza A</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Influenza B</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

Data is displayed as number of patients (percentage of patients) with positive PCR-signal for each virus. LM: Lymphocytic myocarditis. CMV: Cytomegalovirus. EBV: Epstein-Barr Virus. HSV-1/2: Herpes Simplex Virus-1/2. HHV-6: Human Herpes Virus 6.

\(^a\): Of 1 patient, left-over material was insufficient for PCR analysis.

\(^b\): In the heart of 1 patient, a co-infection of Parvovirus B19 and HHV-6 was found.
Lymphocytes infiltrate the quadriceps muscle in LM patients
Colchicine aggravates coxsackievirus B3 infection in mice

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\textsuperscript{7}Department of Virology, University Medical Center Utrecht, Utrecht, the Netherlands.
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\textsuperscript{*}Both authors contributed equally to this study
\textsuperscript{##}Both authors contributed equally to this study

ABSTRACT

Background. There is a clinical need for immunosuppressive therapy that can treat myocarditis patients in the presence of an active viral infection. In this study we therefore investigated the effects of colchicine, an immunosuppressive drug which has been used successfully as treatment for pericarditis patients, in a mouse model of coxsackievirus B3(CVB3)-induced myocarditis.

Methods. Four groups of C3H mice were included: Control mice (n=8), mice infected with CVB3 (1x10^5 PFU, n=10), mice with colchicine administration (2 mg/kg i.p, n=5) and mice with combined CVB3 infection and colchicine administration (n=10). After three days, the heart, pancreas and spleen were harvested and evaluated using (immuno)histochemical analysis and CVB3 qPCR.

Results. Mice were terminated at day 3 post-virus infection as colchicine treatment rapidly resulted in severe illness and mortality in CVB3-infected mice. Colchicine significantly decreased the number of macrophages in the heart in CVB3-infected mice (p<0.01) but significantly increased the number of neutrophils (p<0.01). In the pancreas, colchicine caused complete destruction of the acini in the CVB3-infected mice and also significantly decreased macrophage (p<0.01) and increased neutrophil numbers (p<0.01). In the spleen, colchicine treatment of CVB3-infected mice induced massive apoptosis in the white pulp and significantly inhibited the virus-induced increase of megakaryocytes in the spleen (p<0.001). Finally, we observed that colchicine significantly increased CVB3 levels in both the pancreas and the heart.

Conclusions. Colchicine treatment in CVB3-induced myocarditis has a detrimental effect as it causes complete destruction of the exocrine pancreas and enhances viral load in both heart and pancreas.
INTRODUCTION

Myocarditis is a difficult clinical entity due to its heterogeneity in etiology, clinical presentation and outcome as well as the lack of specific treatment options. Current myocarditis treatment generally consists of standard heart failure therapeutics[1]. Although these are useful in symptom repression and preventing on-going decline of cardiac function, they do not treat the underlying disease itself. As such, other treatment options are needed. Several clinical trials have investigated immunosuppressive drugs, such as prednisone, azathioprine and cyclosporine as treatment for myocarditis[2-4]. The results of several of these trials have suggested that patients with active viral infection do not respond favorably to immunosuppression[2, 3], presumably because immunosuppression hinders or prevents viral clearance. Current guidelines state that immunosuppressive therapy may be considered in myocarditis patients only after ruling out active infection on endomyocardial biopsies via PCR[1]. As viral infection is the most prevalent cause of myocarditis[5], immunosuppressive treatment that does not negatively affect viral clearance and therefore does not require virus-negativity would be of great benefit to the management of myocarditis patients.

An immunosuppressive drug that increasingly is becoming of interest for cardiovascular disease in general is colchicine[6]. Colchicine has been used for over 2000 years to treat gout[7]. Recent publications have shown effectiveness of colchicine for treating pericarditis[8] and it is rapidly becoming standard therapy for this condition[9]. As pericarditis can coincide with myocarditis[10], colchicine may be an interesting potential treatment option for myocarditis patients as well. There is some evidence that colchicine might be beneficial in patients even while active viral infection exists. In a recent case series of 5 patients with Epstein-Barr virus/Cytomegalovirus co-infection leading to viral myocarditis, the use of colchicine as adjunct to conventional therapy resulted in increased left ventricular ejection fraction[11]. Also, a large part of acute pericarditis is thought to be infectious, especially of viral etiology[12]. In several clinical trials studying the effects of colchicine treatment on acute as well as recurrent pericarditis, significant benefit over conventional treatment and no serious adverse effects of colchicine treatment were reported, while in these studies patients with viral infections were not excluded[8]. These results further suggest that colchicine may be suitable for treatment of viral inflammation. We therefore examined the effects of colchicine administration in a mouse model of acute coxsackie virus B3 (CVB3)-induced myocarditis.

METHODS AND MATERIALS

Animal procedures
This study was approved by the VUmc animal ethics and welfare committee, and conforms to the Guide for care and use of laboratory animals published by the US National Institutes of Health. Thirty-three male C3H mice (Harlan, Horst, the Netherlands) were divided into four groups: uninfected control mice (Control group; n=8, 10-12 weeks old), CVB3-infected mice (CVB3 group; n=10, 6 weeks old), colchicine-treated uninfected mice (colchicine group; n=5, 6 weeks old) and CVB3-infected colchicine-treated mice (CVB3+colchicine group; n=10, 6 weeks old). The CVB3 and CVB3+colchicine groups received an intraperitoneal (i.p.) injection containing 1x10⁵ plaque forming units of CVB3 (Nancy strain, ATCC,
Manassas, VA) on day 0, while the control and colchicine groups received an i.p. saline injection. On day 1, the colchicine and CVB3+colchicine groups received an i.p. colchicine injection (2 mg/kg, Sigma-Aldrich, St Louis, MO), while the control and CVB3 groups received an i.p. saline injection. Mice were terminated on day 3 and the heart, lungs, spleen, kidney, liver and pancreas were excised. Of the hearts, a cross-sectional ventricular tissue sample was frozen in -80°C for viral genome analysis. The remaining heart tissue and other organs were fixed in 4% formaldehyde and embedded in paraffin for histochemical and immunohistochemical analyses.

**Histological staining**
Formaldehyde-fixed paraffin-embedded tissue sections (4μm thick) were deparaffinized and rehydrated using xylene and ethanol respectively. For histological tissue analysis a hematoxylin-eosin stain was used. This stain was performed by submerging deparaffinized sections in Meyer's hematoxylin for 5 minutes followed by washing in tap water and counterstaining with eosin for 30 seconds. Afterwards, the slides were washed again in tap water, dehydrated and covered.

**Immunohistochemistry**
Deparaffinized and rehydrated formaldehyde-fixed tissue sections (4μm thick) were submerged in methanol containing 0.3% H$_2$O$_2$ to block endogenous peroxidase. Antigen retrieval was achieved by either boiling in citrate buffer (pH 6) in a microwave oven for (for CD45, macrophage, γH2AX- and VP1 staining) or by incubation with activated pepsin at 37°C (for Ly-6G and CD61 staining). Details on the used antibodies, sera and reagents can be found in *supplementary table 1*. In short: For macrophage, Ly-6G and γH2AX stainings, the sections were pre-treated with normal sera. Lymphocytes were visualized with rat-α-mouse-CD45 (BD Biosciences, Breda, the Netherlands), macrophages with rabbit-α-mouse-macrophage (Accurate Chemical & Scientific Corporation, Westbury, NY), neutrophils with rat-α-mouse-Ly-6G (BD Biosciences), double-stranded DNA breaks with rabbit-α-γH2AX (Cell Signaling Technology, Danvers, MA), megakaryocytes with rabbit-α-CD61 (GenTex Inc., Irvine, CA) and enteroviral protein with mouse-α-enterovirus VP1 (Leica Biosystems, Son, The Netherlands). Bound primary antibodies were labeled with horseradish peroxidase (HRP), which was visualized with diaminobenzidine (Dako, Glostrup, Denmark). For VP1, the Mouse-On-Mouse (M.O.M.) ImmunodetectionKit™ (Vector Laboratories, Burlingame, CA) was used. The slides were counterstained with hematoxylin, dehydrated and covered.

**Quantitative analysis of tissue slides**
All quantitative analyses were performed blinded using a light microscope at 400x magnification, apart from VP1-stained sections and the surface areas which were analyzed on scanned slides.

**Pancreas**: Morphological normal pancreatic acini and Langerhans islets were counted on HE-stained slides. The numbers of lymphocytes (CD45), macrophages, neutrophils (Ly-6G) and γH2ax-positive cells were counted. Only positive extravascular cells within the exocrine or endocrine tissue of the pancreas were counted. CD45 is also present on non-lymphocytic cells, but it is known that CD45 can be used as general lymphocyte marker based on its morphology[13].

60
**Heart**: The numbers of extravascular lymphocytes, macrophages and neutrophils within the myocardium were counted.

**Spleen**: Megakaryocytes were counted based on their characteristic morphology and CD61-positivity. Cell numbers were expressed per mm² of red and white pulp combined.

The surface areas of heart and pancreatic tissue were measured using a PathScan Enabler IV digital slide scanner (Meyer Instruments, Houston, TX). Surface measurements were performed using QuickPHOTO MICRO software v3.0 (Promicra, Prague, Czech Republic). Spleen sections and VP1-stained pancreatic sections were scanned using a Pannoramic DESK (3D histech, Budapest, Hungary). Red and white pulp surface areas were measured using Pannoramic Viewer v1.15.3 (3D histech). VP1-stained sections were analyzed with ImageJ software (Bethesda, MD) using color thresholding to determine the percentage of positively stained tissue.

**Coxsackievirus qPCR**

For detection of CVB3 in the mouse cardiac tissue samples, tissue samples were homogenized and genomic material was isolated using the Magna Pure LC Total Nucleic Acid Isolation Kit (Roche, Penzberg, Germany), following manufacturer’s instructions. Genomic DNA (gDNA) and RNA were isolated separately. RNA was converted to cDNA and pooled with the isolated genomic DNA prior to enteroviral qPCR. Conversion to cDNA and the enteroviral qPCR in the mouse hearts was performed as described previously[14]. To compensate for variations in the amount of genomic material put in the PCR-reactions, DNA concentrations in the pooled gDNA/cDNA samples were measured using a NanoDrop1000 spectrophotometer v3.7.1 (Thermo Fischer Scientific, Waltham, MA).

**Statistical Analysis**

All statistical analysis was performed using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA). As the data were not normally distributed the Mann–Whitney U-test was used to compare groups. The differences in survival were analyzed with the Mantel-Cox test. A p-value smaller than 0.05 was considered statistically significant. Values in text are given as median(25th percentile–75th percentile).

**RESULTS**

**Colchicine causes terminal illness in mice after CVB3 infection**

Three days after viral infection (two days after colchicine administration) 5 out of 10 mice in the CVB3+colchicine group were found dead (*Figure 1*), and the remaining 5 mice showed signs of severe discomfort (closed eyes, pilo-erection, arched back, lack of activity). Because of this, all the mice of the different experimental groups were terminated 3 days post-infection. Mice from the other three experimental groups showed no signs of discomfort at this point, although the mice in the colchicine group experienced a slight weight loss (9.4(7.4-9.6)% loss compared to start weight).
Colchicine increases neutrophils and decreases macrophages in the heart after CVB3 infection

The hearts were examined to determine whether colchicine had affected CVB3-induced myocarditis. In control mice a low number of lymphocytes was present in the myocardium (0.2(0.1-0.3) cells/mm²)(Figure 2A). Compared to the control group, the CVB3 (4.1(3.1-5.2) cells/mm²), colchicine (3.2(2.2-4.0) cells/mm²) and CVB3+colchicine (2.9(1.3-4.0) cells/mm²) groups showed a statistically significant increase in lymphocytes. The control and CVB3 groups had a similar low number of macrophages (0.5(0.2-0.7) and 0.6(0.4-1.0) cells/mm² respectively; Figure 2C). In the colchicine group significantly increased macrophage numbers (1.2(0.7-2.1) cells/mm²) were observed compared to the control group, while in the CVB3+colchicine group macrophage numbers (0.2(0.1-0.3) cells/mm²) were significantly decreased compared to the other three groups.

Neutrophil numbers were similar between the control (1.1(0.5-2.1) cells/mm²), CVB3 (0.9(0.5-2.1) cells/mm²) and colchicine groups (0.9(0.3-1.4) cells/mm²)(Figure 2C). However, in the CVB3+colchicine group the number of neutrophils was significantly increased (4.1(1.7-5.6) cells/mm²) compared to the other three groups.

---

**Figure 2. Inflammatory cell numbers in cardiac tissue.** A-C) Number of lymphocytes(A), macrophages(B) and neutrophils(C) per mm² ventricular myocardium of control mice, Coxsackievirus B3-infected mice, colchicine-treated mice and Coxsackievirus B3-infected mice with colchicine treatment. Horizontal lines in dot plots: median. *: p<0.05, **: p<0.01, ***: p<0.001.
Colchicine causes severe damage to pancreatic acini after CVB3 infection

We subsequently examined HE-stained slides of several organs. No morphological cell damage was seen in the heart, liver, kidney or lungs in any of the groups. However, in the pancreas we found considerable damage of the pancreatic acini (Figure 3A-D). Compared to the control group (439(392-474) acini/mm²), the number of intact pancreatic acini was significantly decreased in the CVB3 group (116(78-216) acini/mm²), but also in the colchicine group (228(208-251) acini/mm²)(Figure 3E). In the CVB3+colchicine group, intact acini were virtually absent (0.2(0.1-1.1) acini/mm²) and significantly lower compared to all other groups. Next, we stained the pancreases for γH2A (Figure 3F), which is part of the DNA-repair mechanism and used as a biomarker of double-strand DNA breaks[15]. It is known that γH2A staining can identify damaged cells in advance of morphological signs of cell damage, and remains present until cell death when DNA repair is ineffective[15]. The γH2A-positive nuclei were found diffusely in both areas of still intact acini and morphologically damaged areas. Compared to the control group (0.0(0.0-0.1) nuclei/mm²), the number of γH2A-positive nuclei in the CVB3 (80.3(58.2-122.0) nuclei/mm²), colchicine (14.7(11.1-16.0) nuclei/mm²) and CVB3+colchicine groups (17.0(15.0-20.4) nuclei/mm²) were significantly increased. A significantly decreased number of γH2A-positive nuclei were present in the pancreases of mice in the CVB3+colchicine group compared to the CVB3 group (Figure 3G), albeit in the CVB3+colchicine group morphological normal acini were almost completely diminished already what could explain the lack of γH2A staining in this particular group.

The Langerhans islets were not morphologically affected by either CVB3, colchicine or CVB3+colchicine (Figure 3A-D). In line herewith, only a few sporadic γH2A-positive nuclei were found in the CVB3 group (0 to 1 positive nuclei per islet), but none in the other groups. However, compared to the control group (1.1(0.8-1.5) islets/mm²), the CVB3 and CVB3+colchicine groups showed a significant decrease in the number of Langerhans islets (0.6(0.2-0.9) and 0.2(0.1-0.4) islets/mm² respectively; Figure 3H). The number of Langerhans islets in the colchicine group (1.1(0.7-2.7) islets/mm²) differed significantly only compared to the CVB3+colchicine group.

Colchicine increases neutrophils and decreases macrophages in the pancreas after CVB3 infection

To analyze the inflammatory response in the pancreas, extravascular lymphocytes, macrophages and neutrophils were quantified in the combined acini and Langerhans islets of the pancreas. In line with the cellular damage, inflammatory cells were only found in the acini areas, not in the Langerhans islets (not shown). Lymphocytes were virtually absent in the pancreas of control mice (0.1(0.0-0.2) cells/mm²; Figure 4A). Compared to the control group, the CVB3 (2.0(0.7-5.8) cells/mm²), colchicine (3.3(2.5-11.5) cells/mm²) and CVB3+colchicine groups (5.0(2.6-9.0) cells/mm²) all showed a significant increase in the number of lymphocytes, which did not differ significantly between each other. Macrophone numbers in the CVB3, Colchicine and CVB3+colchicine groups (10.2(8.1-15.7), 5.8(1.9-11.9) and 1.3(0.8-4.7) cells/mm² respectively) were significantly increased compared to the control group (0.1(0.0-0.3) cells/mm²)(Figure 4B), although in the CVB3+colchicine group they were significantly decreased compared to the CVB3 group. Similarly, in the CVB3, Colchicine and CVB3+colchicine groups significantly increased neutrophils numbers

Colchicine aggravates coxsackievirus B3 infection

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Figure 3. Pancreatic damage. A-D) Haematoxylin-Eosin staining of a pancreatic tissue sample from a representative control mouse (A), Coxsackievirus B3-infected mouse (B), colchicine-treated mouse (C) and Coxsackievirus B3-infected mouse with colchicine treatment (D), displaying intact pancreatic acini (examples marked with arrows) and Langerhans islets (L I). Scalebar: 100 μm. E) Number of intact pancreatic acini per mm² of pancreatic tissue of control mice, Coxsackievirus B3-infected mice, colchicine-treated mice and Coxsackievirus B3-infected mice with colchicine treatment. F) γH2AX-positive nuclei (arrows) in the pancreas of a CVB3-infected mouse. Scalebar: 50 μm. G) Number of γH2AX-positive nuclei per mm² of pancreatic tissue of control mice, Coxsackievirus B3-infected mice, colchicine-treated mice and Coxsackievirus B3-infected mice with colchicine treatment. H) Number of Langerhans islets per mm² of pancreatic tissue of control mice, Coxsackievirus B3-infected mice, colchicine-treated mice and Coxsackievirus B3-infected mice with colchicine treatment. Horizontal lines in dot plots: median. *: p<0.05, **: p<0.01, ***: p<0.001.
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were observed (7.4(6.4-13.2), 3.5(0.9-17.3) and 22.3(15.2-24.6) cells/mm² respectively; Figure 4C) compared to the control group (0.1(0.0-0.2) cells/mm²). The highest number of neutrophils was found in the CVB3+colchicine group which was significantly increased compared to the CVB3 group.

**Colchicine causes massive apoptosis in the white pulp of the spleen after CVB3 infection, and decreases the number of megakaryocytes**

Morphological changes were also observed in the spleen of CVB3+colchicine treated mice. Compared to the control group (Figure 5A), the CVB3 group (Figure 5B) and the colchicine group (Figure 5C), the white pulp of the CVB3+colchicine group displayed massive apoptosis (Figure 5D). The relative surface areas of red and white pulp did not differ between the groups (Figure 5E). We also observed changes in megakaryocyte (Figure 5F) numbers in the red pulp. In both the colchicine and CVB3+colchicine groups the megakaryocytes numbers were significantly decreased (0.3(0.2-0.7) and 2.5(2.2-4.5) cells/mm² respectively) compared to both the control group (4.0(3.3-8.7) cells/mm²) and CVB3 group (7.4(5.7-8.5 cells/mm²; Figure 5G). In addition, for the colchicine group this was also significantly decreased compared to the CVB3+colchicine group.

**Colchicine increases the virus load in the heart and pancreas after CVB3 infection**

Finally, we studied whether colchicine affected the viral load of the heart and the pancreas. In the frozen tissues samples obtained from the hearts, the levels of CVB3 RNA were determined by qPCR. Compared to the CVB3 group, the CVB3+colchicine group had a significantly increased quantity of viral RNA in the heart (159(27-2738) and 27517(22451–51889) arbitrary units respectively, Figure 6A). Heart samples from the control group and colchicine group were negative for CVB3 RNA (not shown).

![Figure 4. Inflammatory cell numbers in pancreatic tissue. A-C) Number of lymphocytes(A), macrophages(B) and neutrophils(C) per mm² pancreatic tissue of control mice, Coxsackievirus B3-infected mice, colchicine-treated mice and Coxsackievirus B3-infected mice with colchicine treatment. Horizontal lines in dot plots: median. **: p<0.01, ***: p<0.001.](image-url)
Of the pancreas, no frozen tissue was obtained. Therefore, in the pancreas the viral load was quantified using an immunohistochemical staining against the CVB3 protein VP1. VP1-stainings in the control and colchicine groups only showed minor a-specific background staining (not shown). In the CVB3 group (Figure 6B) and CVB3+colchicine group (Figure 6C) strong VP1-staining was found in the pancreatic acini. In the CVB3+colchicine group some VP1 staining was also seen in the periphery of the Langerhans islets (Figure 6D). The percentages of the pancreas positive for VP1 in both the CVB3 group (21.9(13.5-30.9)% of surface area) and CVB3+colchicine group (49.7(38.8-56.9)% of surface area) were significantly larger compared with the control and colchicine groups. In addition, the VP1-positive area in the CVB3+colchicine group was significantly larger compared to the CVB3 group (Figure 6E).

**DISCUSSION**

We have studied colchicine therapy in mice with acute CVB3-induced myocarditis. We found that colchicine treatment induced severe illness and increased mortality within three days in mice with CVB3-induced myocarditis. Colchicine also caused an increase in neutrophil infiltration but a decrease in macrophage infiltration in both pancreas and heart, coinciding with a significantly increased viral load in both organs. Additionally, CVB3 alone caused a partial degeneration of the pancreatic acini, while for CVB3 and colchicine combined these were almost completely destroyed. Finally, colchicine induced massive apoptosis in the white pulp of the spleen of CVB3-infected mice. These results indicate that colchicine treatment may cause serious adverse effects in case of an active viral infection.

In the past decade, several clinical trials have shown both in patients with acute pericarditis and recurrent pericarditis that colchicine reduced the recurrence rate of pericarditis and reduced symptom persistence (reviewed by Imazio[8]). There is evidence suggesting that recurrent pericarditis is mainly immune-mediated, whereas acute pericarditis often has an infectious cause, which is thought to be predominantly viral in developed countries[16]. Therefore it has been suggested that corticosteroids given in the index pericarditis attack can favor the occurrence of relapses because of their detrimental effect on viral replication[17]. However, in none of the colchicine trials, serious adverse effects related to the colchicine treatment were observed. In contrast, in the present study colchicine induced a large increase in viral load both in the heart and the pancreas, which seems at odds with results from the acute pericarditis trials. Although the inclusion criteria in these trials encompassed patients with a viral etiology, there was no mention of viral diagnostics having been performed[8] and consequently no proof was presented of active viral infection in these patients, nor of putative effects of colchicine thereon. Even more, if patients with pericarditis of viral etiology were included in these trials, given the high prevalence, it would have been unknown how long after putative infection colchicine therapy was started. In our study colchicine therapy was started one day after infection. It is theoretically possible that this early time point prevented the initiation of a proper immune response leading to uncontrolled viral spread and replication and high viral loads, whereas it is unlikely that colchicine therapy was started this early after infection in the patient studies.
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Figure 5. Spleen histology. A-D) Haematoxylin-Eosin staining of a splenic tissue sample from a representative control mouse (A), Coxsackievirus B3-infected mouse (B), colchicine-treated mouse (C) and Coxsackievirus B3-infected mouse with colchicine treatment (D), displaying red and white pulp. Scalebar: 100 μm. D) Apoptosis in white pulp (example 4x magnified). E) Area of white pulp as percentage of the total splenic tissue area of control mice, Coxsackievirus B3-infected mice, colchicine-treated mice and Coxsackievirus B3-infected mice with colchicine treatment. F) CD61-staining of the spleen of a CVB3-infected mouse, displaying a megakaryocyte (brown). Scalebar: 25 μm. G) Number of megakaryocytes per mm² spleen of control mice, Coxsackievirus B3-infected mice, colchicine-treated mice and Coxsackievirus B3-infected mice with colchicine treatment. For one mouse in the control group and one mouse in the Colchicine group, the remaining splenic tissue was insufficient for megakaryocyte analysis. Horizontal lines in dot plots: median. *: p<0.05, **: p<0.01, ***: p<0.001.
The primary mechanism of action of colchicine is tubulin disruption. Through this mechanism colchicine was shown to inhibit leukocyte recruitment, adhesion and transmigration\[18, 19\]. Additionally, colchicine was shown to inhibit several cellular leukocyte functions, such as activation of the inflammasome and IL1β release in macrophages\[20\] and superoxide production in neutrophils\[21\] in response to inflammatory microcrystals as well as to down-regulate tumor necrosis factor receptors on macrophages\[22\]. The results in the present study were not uniformly in line with these findings. Although the number of macrophages was indeed decreased in the affected organs of colchicine-treated CVB3-infected mice compared to untreated CVB3-infected mice, conversely the number of infiltrated neutrophils was increased. We are unsure what precisely caused this difference. Interestingly, colchicine at high doses has also been reported to have pro-inflammatory effects on neutrophils rather than anti-inflammatory effects.\[23\] A pro-inflammatory effect on neutrophils and an anti-inflammatory effect on macrophages may have contributed to the

![Figure 6. Viral load in heart and pancreas. A) Coxsackievirus B3 DNA/RNA levels in cardiac tissue of Coxsackievirus B3-infected mice and Coxsackievirus-infected mice treated with colchicine. B-C) Coxsackievirus B3 protein VP1 staining (brown) on pancreatic tissue of a representative Coxsackievirus B3-infected mouse(B), and Coxsackievirus B3-infected mouse with colchicine treatment(C). Scalebar: 200μm. D) Coxsackievirus B3 protein VP1 staining (brown) on a Langerhans islet of a Coxsackievirus B3-infected mouse with colchicine treatment. Scalebar: 100 μm. E) Percentage of spleen tissue area with positive VP1-staining of control mice, Coxsackievirus B3-infected mice, colchicine-treated mice and Coxsackievirus B3-infected mice with colchicine treatment. Horizontal lines in dot plots: median. ***: p<0.001.](image-url)
opposite effect on cell numbers we have observed in our mice. The effects of colchicine on neutrophil extravasation in the setting of an acute viral infection however were, to the best of our knowledge, never studied before. Moreover, it is unknown whether the functioning of leukocytes, those infiltrated as well as those in the blood, was affected in the colchicine-treated mice. It may well be that the dramatic increase in viral load in the affected organs of colchicine-treated CVB3-infected mice is more the result of its inhibiting effect on systemic leukocyte function than of the number of cells infiltrating the affected organs. This theory is supported by the relative low number of infiltrated leukocytes in the hearts of colchicine-treated CVB3-infected mice despite the high viral load. It is also unidentified whether the disastrous effects of colchicine in our model were virus-specific, or whether colchicine would have similar effects in infections of other common cardiotropic viruses such as parvovirus B19 and influenza viruses. Gultekin and Kucukates treated five patients with low dose colchicine (0.5 mg twice daily) for myocarditis associated with EBV/CMV co-infection (without pericarditis) and found no severe complications in these patients[11]. However, effects of colchicine treatment on virus titers in these patients were not reported.

Although it is known that myocarditis can coincide with pericarditis[10], in only two of the colchicine trials, i.e. the ICAP trial and the CORP-2 trial, patients with evidence of myocardial involvement, as indicated by elevated serum troponin levels, were excluded[8]. While in the other trials myocarditis was not listed as an exclusion criterium, nor was myocarditis depicted in the patient characteristics. Therefore it is unclear whether patients with myocarditis were included in these studies.

In the spleen, we observed that colchicine induced massive apoptosis of the white pulp in the spleen in CVB3-infected mice. Apoptosis of both B and T lymphocytes in the spleen also early after virus infection has been reported before in mice[24], the extent of which has been related to the viral dose[25]. As colchicine treatment induced such a high viral load, this may explain why the splenic apoptosis we observed in the colchicine-treated CVB3-infected mice was much more apparent than in the untreated CVB3-infected mice. Moreover, the splenic apoptosis likely was not a direct effect of colchicine as in uninfected colchicine-treated mice no apparent splenic apoptosis was noted. Noteworthy, colchicine also had an effect on megakaryocyte numbers in the spleen. Colchicine significantly reduced the number of megakaryocytes in the red pulp, both in uninfected and CVB3-infected mice, to well below their level in control mice. Whether this is a result of a toxic effect of colchicine on megakaryocytes or that colchicine prevents megakaryocytic entry into the spleen is not clear. To the best of our knowledge a reducing effect of colchicine on the number of splenic megakaryocytes in vivo has never been described before.

CVB3 infection and damage of the pancreas in mice has been described before[26]. In accordance with our study it was shown that CVB3 primarily infects the acini and not the Langerhans islets. Colchicine treatment increased the damage exclusively in the acini, not in the islets, suggesting this additional effect is likely explained by the induced increase in viral titers. The number of intact acini in uninfected colchicine-treated mice was also decreased compared to controls, suggesting a cytotoxic effect of colchicine in these areas as well. The increase of inflammatory cells in the heart due to colchicine alone further suggests cytotoxicity. Colchicine was administered before in a similar dose (2 mg/kg) in mouse models[27-29], without reported adverse effects, although whether the pancreas was
studied is unclear. In a study into the role of microtubules in acute pancreatitis in rats Ueda et al used a high dose of colchicine (10 mg/kg), without noting any histopathological damage[30]. Notwithstanding this, different sensitivity to colchicine-induced toxicity between different species or between different strains cannot be excluded. To the best of our knowledge, colchicine was not yet studied in the C3H mouse strain that we used.

**Study Limitations**
Several important limitations must be noted. We were unable to provide a mechanism through which colchicine treatment resulted in enhanced viral load in the heart and pancreas, nor can we adequately explain the opposite effects of colchicine on macrophage and neutrophil numbers in analyzed tissues. As we terminated the experiment before significant damage can be observed in the myocardium after CVB3 infection (day 3), we were not able to study how colchicine would have affected further progression of acute myocarditis.
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REFERENCES


### Supplementary table 1. Antibodies, sera and blocking reagents in order of application.

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<th>Stain</th>
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<th>Dilution</th>
<th>Application time (RT)</th>
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<td>Overnight, 4°C</td>
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*Antibodies and sera were diluted in Normal Antibody Diluent (ImmunoLogic, Duiven, the Netherlands) unless mentioned otherwise. *= Diluted in PBS. †= Diluted in M.O.M. Diluent. RT= room temperature unless mentioned otherwise.
Lymphocytic myocarditis occurs with myocardial infarction and coincides with increased inflammation, hemorrhage and instability in coronary artery atherosclerotic plaques

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ABSTRACT

Objective: Although lymphocytic myocarditis (LM) clinically can mimic myocardial infarction (MI), they are regarded as distinct clinical entities. However, we observed a high prevalence (32%) of recent MI in patients diagnosed post-mortem with LM. To investigate if LM changes coronary atherosclerotic plaque, we analyzed in autopsied hearts the inflammatory infiltrate and stability in coronary atherosclerotic lesions in patients with LM and/or MI.

Methods: The three main coronary arteries were isolated at autopsy of patients with LM, with MI of 3-6 hours old, with LM and MI of 3-6 hours old (LM+MI) and controls. In tissue sections of atherosclerotic plaque-containing coronary segments inflammatory infiltration, plaque stability, intraplaque hemorrhage and thrombi were determined via (immuno)histological criteria.

Results: In tissue sections of those coronary segments the inflammatory infiltrate was found to be significantly increased in patients with LM, LM+MI and MI compared with controls. This inflammatory infiltrate consisted predominantly of macrophages and neutrophils in patients with only LM or MI, of lymphocytes in LM+MI and MI patients and of mast cells in LM+MI patients. Moreover, in LM+MI and MI patients this coincided with an increase of unstable plaques and thrombi. Finally, LM and especially MI and LM+MI patients showed significantly increased intraplaque hemorrhage.

Conclusions: This study demonstrates prevalent co-occurrence of LM with a very recent MI at autopsy. Moreover, LM was associated with remodeling and inflammation of atherosclerotic plaques indicative of plaque destabilization pointing to coronary spasm, suggesting that preexistent LM, or its causes, may facilitate the development of MI.
INTRODUCTION

Lymphocytic myocarditis (LM) is an inflammatory disease of the heart, predominantly characterized by diffuse focal aggregates of inflammatory infiltrate in the myocardium that in majority is associated with viral infection [1]. The prevalence of LM in the general population is uncertain as it often may have a clinically silent course. The clinical presentation of LM is very diverse and varies from mild flu-like symptoms to acute heart failure and sometimes sudden death [1]. In addition, patients with LM can present with a variety of clinical symptoms suggestive of myocardial infarction (MI), such as chest pain, electrocardiographic ST-segment elevation, wall motion abnormalities and increased blood levels of cardiac enzymes [2-10]. Moreover, coronary artery spasms have been reported to occur in patients with LM [11-13]. In fact coronary vasospasm was demonstrated in 70.9% of patients with proven LM on endomyocardial biopsy (EMB) who presented with chest pain [11].

In clinical practice, LM is only considered as potential underlying cause of infarct-like complaints when MI is ruled out, based on the absence of coronary narrowing or obstruction as detected by coronary angiography. In case of normal or non-obstructed coronary arteries, cardiac magnetic resonance (CMR) imaging is often employed as a complementary imaging tool to further differentiate between MI and LM, wherein myocardial injury is mainly located in the subendocardium with MI as opposed to a more (sub)epicardial location with LM [14-16].

The general consensus in the literature is that although LM and MI can be similar in clinical presentation they are distinct clinical entities. However, there is accumulating evidence suggesting an interrelatedness between LM and MI. For instance, recent respiratory tract infections of the cardiotropic influenza virus, which is commonly associated with LM, are significantly associated with MI also [17]. In addition, markers of infection of another group of cardiotropic viruses, i.e. enteroviruses, were detected in the hearts of 40% of patients who died of sudden MI versus only 4% of matched subjects without cardiac disease [18]. In line herewith, as we will show below, we have observed in autopsied cases of patients diagnosed post-mortem with LM a high prevalence of very recent MI, demonstrating that LM and MI can be present simultaneously. Even more, these data may suggest that LM or its cause can facilitate the development of MI.

MI most often is the result of coronary plaque complication, either through rupture or erosion of atherosclerotic plaques. Inflammation has been found to be an important mediator of atherosclerotic plaque destabilization that renders them more vulnerable for complication [19]. For instance, in autopsied cases MI was found to be associated with increased inflammatory cell infiltration in the coronary arteries, including macrophages, T-lymphocytes [20, 21] as well as mast cells [22]. Interestingly, via the secretion of vasoactive factors such as histamine, chymase and tryptase, mast cells have been associated with coronary vasospasm also [23, 24].

However, knowledge regarding coronary artery changes associated with LM is scarce. In EMB-proven myocarditis, impairment of endothelium-dependent vasodilation of the epicardial coronary arteries correlated with the number of T-lymphocytes in the myocardium [25], indicative of endothelial dysfunction in the coronary arteries. Conversely, in autopsied hearts of patients LM did not coincide with increased infiltrate of T-lymphocytes in the coronary arteries [26]. However, infiltration of other inflammatory cell types was not analyzed.
Therefore, the aim of this study was to analyze in autopsied hearts the inflammatory infiltrate, plaque bleeding and plaque stability in coronary atherosclerotic lesions in patients with LM coinciding with very recent MI, in patients with only LM or very recent MI and control patients without heart disease.

**METHODS AND MATERIALS**

**Patient material**
A total of 38 autopsied cases were retrospectively selected and divided into four groups:

1) A group with lymphocytic myocarditis (LM; \(n=10\)): In these patients LM was diagnosed based on immunohistochemical analysis of multiple heart slides of both the left ventricle (the septum, the anterior, lateral and posterior wall) and right ventricle (anterior wall). LM was diagnosed based on the presence of multiple aggregates of extravascular lymphocytes (CD45+ cells) with or without cardiomyocyte damage (based on presence of complement activation product C3d). MI was excluded in these patients based on absence of localized nitro blue tetrazolium (NBT) decoloration and the absence of thrombi in the epicardial coronary arteries.

2) A group with a MI of 3-6 hours old (\(n=10\)): In these patients a MI was diagnosed of 3-6 hours old based on reduced localized nitro blue tetrazolium (NBT) staining that appears after three hours after infarction on a mid-ventricular macroscopic cross section of the heart and/or a thrombus in the epicardial coronary artery, in the absence of neutrophil infiltration in the NBT staining-identified infarction area. MI-induced neutrophil infiltration in the infarcted myocardium was shown previously to start after 6 hours after onset of MI (19). In addition, in four cases a thrombus was present in the epicardial coronary artery.

3) A group with lymphocytic myocarditis and a MI of 3-6 hours old (\(n=13\)): In these patients in addition to LM, a MI of 3-6 hours old was diagnosed based on reduced localized NBT staining of the heart. Moreover, in four cases a thrombus in the epicardial coronary artery was found. In one patient no NBT decoloration was found, but a thrombus was found in the epicardial coronary artery indicative of a MI of less than 3 hours old.

4) A control group (\(n=5\)): Control patients were selected whose death was not related to cardiac disease. Patients had no NBT decoloration of the heart and patients with diseases that theoretically could coincide with cardiac inflammation were excluded.

In all groups, patients that used prednisolone were excluded. The patient characteristics are shown summarized in Table 1 and in detail in the supplementary table.

The infarct area in patients with MI was determined on a mid-ventricular macroscopic cross section stained with NBT. The percentage of the infarct area was calculated by the dividing the infarct area with the total area of the left ventricle.

From all patients, the three main epicardial coronary arteries were dissected from the heart (left anterior descending, left circumflex and right coronary artery). Segments with macroscopically the most profound stenosis were microscopically analyzed. The number of segments per coronary artery varied between 1 and 6. In total 307 segments were fixed in formalin and embedded in paraffin: 85 from LM, 98 from LM+MI, 86 from MI, 38 of control patients. The coronary segments of the two patients groups with MI were further subdivided in the infarct- and non-infarct-related coronary arteries. The infarct-related coronary arteries were first identified based on the occurrence of a thrombus. In the absence of a thrombus,
the infarct related coronary arteries were defined related to location of the NBT staining-identified infarct area.

This study was approved by and performed according to the guidelines of the ethics committee of the VU University Medical Center, Amsterdam, and conforms to the principles of the Declaration of Helsinki. Use of the leftover material after the pathological examination has been completed is part of the patient contract in our hospital.

**Immunohistochemistry**

Paraffin tissue sections (4 μm) of the coronary segments were stained with antibodies detecting CD45 (lymphocytes), CD68 (macrophages), MPO (neutrophils), tryptase (mast cells) and Glut-1 (erythrocytes). Sections were first deparaffinized, rehydrated and blocked for endogenous peroxidases by incubation in H2O2 (0.3%) diluted in methanol for 30 minutes. As an antigen retrieval step the slides were heated in 10mM citrate buffer (pH 6.0) for 10 minutes to boiling and then cooled for 20 minutes for slides to be stained for CD68, MPO and tryptase or were heated in a Tris/EDTA buffer (pH 9.0) for 10 minutes to boiling and then cooled for 20 minutes for slides to be stained for Glut-1. Slides to be stained for CD45 required no antigen retrieval step. The sections were subsequently incubated with either mouse-anti-human CD45 (1:100, Dako M0701), rabbit-anti-human CD68 (1:400, Dako M0814), mouse-anti-human MPO (1:500, Dako A0398), mouse-anti-human Tryptase (1:500, Dako RB9052-P) or rabbit-anti-human Glut-1 (1:100, Thermo Scientific RB-9052-P) for 60 minutes. The primary antibodies were diluted in normal antibody diluent (ImmunoLogic ABB500). The slides were then incubated with antimouse/rabbit Envision (Dako, K5007) for 30 minutes. The staining was visualized via

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**Table 1. General patient characteristics.**

<table>
<thead>
<tr>
<th></th>
<th>Control (n=5)</th>
<th>LM (n=10)</th>
<th>LM + MI (n=13)</th>
<th>MI (n=10)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years), median (IR)</strong></td>
<td>66.0 (55.0-71.5)</td>
<td>63.5 (52.5-77.3)</td>
<td>68.0 (44.5-69.0)</td>
<td>63.0 (53.25-90.0)</td>
<td>0.582</td>
</tr>
<tr>
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<td>6/4</td>
<td>11/2</td>
<td>8/2</td>
<td>0.556</td>
</tr>
<tr>
<td><strong>MI 3-6 hours old LM</strong></td>
<td>0/5</td>
<td>0/10</td>
<td>13/13</td>
<td>10/10</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Clinical history and medication §:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes Mellitus type 2</td>
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<td>0/9</td>
<td>2/12</td>
<td>1/10</td>
<td>0.503</td>
</tr>
<tr>
<td>COPD</td>
<td>0/5</td>
<td>2/9</td>
<td>3/12</td>
<td>2/10</td>
<td>0.689</td>
</tr>
<tr>
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<td>5/12</td>
<td>1/8</td>
<td>0.219</td>
</tr>
<tr>
<td>Diuretics</td>
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<td>2/9</td>
<td>4/12</td>
<td>0/8</td>
<td>0.120</td>
</tr>
<tr>
<td>Anti-coagulation</td>
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<td>2/9</td>
<td>2/12</td>
<td>3/8</td>
<td>0.418</td>
</tr>
<tr>
<td>Ace inhibitors</td>
<td>0/5</td>
<td>3/9</td>
<td>2/12</td>
<td>1/8</td>
<td>0.214</td>
</tr>
</tbody>
</table>

LM = lymphocytic myocarditis; MI = myocardial infarction; NA = not applicable; § = data included if available; COPD = chronic obstructive pulmonary disease.

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5
incubation in 3,3'-diaminobenzidine (0.1 mg/ml, Dako K3468) for 10 minutes. Subsequently, the slides were counterstained with haematoxylin, dehydrated and covered. With each staining slides were included incubated with antibody diluent without a primary antibody as a negative control and all these controls showed no staining (not shown).

Quantification of immune cells and morphometric analysis
In all individual segments of each coronary artery the numbers of extravascular inflammatory cells were quantified separately in the intima, media, adventitia. The total surface area of the intima, media and adventitia were determined on the scanned slides using the Panoramic Desk scanner and analyzed with Pannoramic Viewer 1.15.2 software (3DHistech, Budapest, Hungary). The surface area of adventitia was measured from the external elastic lamina until 100 μm into the tunica externa.

The numbers of macrophages, neutrophils and mast cells were counted using a light microscope using a 250x magnification objective (Zeiss, Germany) and then calculated per mm² of the surface areas of the intima, media and adventitia, as well as the surface area of the complete coronary segment composed of the three coronary wall layers together.

The amount of lymphocyte infiltration was determined on scanned anti-CD45 stained slides (using the Panoramic Desk scanner and Pannoramic Viewer software). The amount of lymphocyte infiltration was assessed using a color threshold to determine the surface area of positive anti-CD45 staining as well as the surface areas of the of the intima, media and adventitia using ImageJ 1.47 software (National Institutes of Health, USA). The percentages of positive anti-CD45 staining were then calculated of the surface areas of the intima, media and adventitia, as well as the surface area of the three coronary wall layers together.

The amount of stenosis of each individual segment was determined as the percentage of the surface area of the lumen compared to the surface area of the lumen and the intima.

Plaque stability, thrombi and intraplaque hemorrhage
Paraffin tissue sections (4 μm) of the coronary segments were stained with haematoxylin-eosin (HE) and then evaluated microscopically to verify the presence of a thin fibrous cap. Plaques were classified as unstable if the fibrous cap was thin and/or if multiple inflammatory cells reached up to the luminal endothelial layer of the plaque (based on anti-CD45 staining) [27]. If neither of these characteristics were present, plaques were classified as stable.

To classify intraplaque hemorrhage (IPH) all coronary segments where stained for erythrocytes with the immunohistochemical Glut-1 staining (see immunohistochemistry section above) and for iron using the histochemical Perls staining. Using a microscope with 250x magnification, fresh IPH was identified by the presence of > 10 extravascular localized intact erythrocytes, while old IPH was identified by the presence of iron and/or erythrocyte fragments [28]. Segments were classified as having fresh IPH (only fresh hemorrhage present), as ongoing (both fresh and old hemorrhage present) or absent IPH (no hemorrhage present).

Thrombi were histologically identified in the coronary segment [29].

Statistics
Differences between groups were analyzed for statistical significance using SPSS software (version 20). Statistical significance between groups were compared with a Mann-Whitney
test or an independent T-test if data was normally distributed. Patients characteristics, plaque instability, thrombi and IPH was analyzed with the chi-square test. P value of <0.05 was considered significant. Unless otherwise stated, numeral data in the text are expressed as median with interquartile range, unless.

RESULTS

Coincidence of recent MI and LM in patients
In the period of January 2010 to May 2013 at the department of Pathology of the VU University Medical Center 50 cases were diagnosed post mortem with LM according to the Dallas criteria supplemented with immunohistochemistry. In 16 (32%) of these cases a recent MI was also diagnosed. Of these 16 cases 13 were selected of which all three coronary arteries were available. Patients with only LM (n=10), only MI (n=10) and controls (n=5) were also included (Table 1 and supplementary table of detailed patients characteristics). There were no significant differences in age, sex, prevalence of diabetes or medication use between the four groups (Table 1).

Table 2. Histopathological findings

<table>
<thead>
<tr>
<th></th>
<th>Control (n=5)</th>
<th>LM (n=10)</th>
<th>LM + MI (n=13)</th>
<th>MI (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NBT staining (n)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Localized decoloration</td>
<td>0</td>
<td>0</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>No localized decoloration</td>
<td>5</td>
<td>10</td>
<td>1 §</td>
<td>0</td>
</tr>
<tr>
<td><strong>Infarct location (n)</strong></td>
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<td>NA</td>
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<td>3</td>
</tr>
<tr>
<td>LVPW</td>
<td></td>
<td></td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>LVPW/septum</td>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>LVPW/LVLW</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>LVLW</td>
<td></td>
<td></td>
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</tr>
<tr>
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<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>LV</td>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>LVLW/RV</td>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Coronary thrombus (n)</strong></td>
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<td>NA</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>LAD</td>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>LAD + Cx</td>
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<td>1</td>
<td></td>
</tr>
<tr>
<td>LAD + RCA</td>
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<td>1</td>
</tr>
<tr>
<td>RCA</td>
<td></td>
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</tr>
<tr>
<td>Cx</td>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>Intramyocardial vascular thrombus (n)</strong></td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

LM = lymphocytic myocarditis; MI = myocardial infarction; NBT = nitro blue tetrazolium; § MI based on occluding thrombus in the epicardial coronary artery; NA = not applicable; LVPW = left ventricular posterior wall; LVLW = left ventricular lateral wall; LVAW = left ventricular anterior wall; LV = left ventricle; RV = right ventricle; LAD = left anterior descending artery; Cx = circumflex artery; RCA = right coronary artery.
In all cases of the MI group and in 12 of the 13 cases in the LM+MI the MI was diagnosed based on a localized area of NBT decoloration on a mid-ventricular macroscopic cross section of the heart typical of a MI older than 3 hours (Figure 1A) (Table 2). In one of these LM+MI patients no NBT decoloration was found, but instead a thrombus was found in the epicardial coronary artery indicative of a MI of less than 3 hours old. Also, in three other LM+MI patients and 4 MI patients a thrombus was found in one or more of the epicardial coronary arteries (Figure 1C). There was no significant difference in infarct area between the LM and LM+MI groups (Figure 1B). In LM patients without MI and controls no localized decoloration of NBT was found in the heart nor thrombi in the epicardial coronary arteries. All LM patients with or without MI were diagnosed with LM based on the presence of multiple aggregates of extravascular CD45+ lymphocytes in the myocardium (Figure 1D). In LM and LM+MI patients these lymphocyte aggregates were present in multiple locations within the heart. Importantly, in LM+MI patients these aggregates were observed both in the infarcted and in the non-infarcted myocardium. Two of the LM and three of the LM+MI cases had borderline LM i.e. myocarditis without cardiomyocyte damage in the area of the lymphocytes aggregates. Furthermore, in three patients with LM+MI, one with MI and one with LM thrombi were found in the intramyocardial vasculature of the left ventricular wall. These data thus show that LM and a very recent MI can occur simultaneously.

The level of stenosis of the coronary arteries
First the level of stenosis of the different coronary segments of all coronary arteries were compared between the patients groups (Figure 2A). The level of coronary stenosis of the control group (60; 46-73) was comparable to that in the LM group (54; 43-64) and the LM+MI group (67; 49-82). Contrarily, the amount of stenosis of the MI group (71; 52-83) was significantly higher than the control group (p=0.011). Moreover, the amount of stenosis in the LM group was significantly lower than in the LM+MI and MI groups (p<0.001). In the LM+MI group, the stenosis of the infarct-related coronary arteries (71; 61-88) was significantly higher than of the non-infarct-related coronary arteries (57; 44-76)(Figure 2B; p=0.008). In the MI group, the stenosis of the infarct- (72; 63-83) and non-infarct-related (68; 51-85) coronary arteries was comparable.

Increased infiltration of macrophages and neutrophils in coronary lesions of patients with only LM or MI
Next the inflammatory infiltrate in the coronary segments both in the total wall as well as in the individual wall layers was quantified (Figure 3A). First macrophages and neutrophils were analyzed.
In the total wall of the LM group (11.10; 6.50-15.30) significantly more infiltrated macrophages per mm² were found than in the control (6.40; 4.25-9.95, p=0.005) and LM+MI group (6.50; 3.18-12.73, p=0.003), but not the MI group (8.50;4.35-15.35) (Figure 3B). Overall this pattern was observed in the intima, the media and the adventitia, wherein the most macrophages were recorded in the LM group(Figure 3C). Albeit, in the media the number of macrophages in the LM and the MI group were comparable. Also the number of neutrophils per mm² that infiltrated the total wall in the LM group (1.80; 1.10-4.63, p=0.004) and the MI group (2.30; 0.80-5.00, p=0.002) were significantly higher than in the control group (1.30; 0.65-1.75)(Figure 3D). However, no significant differences
Figure 1. Histopathological findings. A) Representative NBT staining of a patient with lymphocytic myocarditis (LM) and a myocardial infarct (MI) of 3-6 hours old. The infarction area as distinguished by decoloration of NBT staining outlined at the lateral posterior wall. B) The percentage of infarct area of patients with LM+MI and with MI. Data is presented as the mean with standard deviation. C) Example of a CD45 staining with a thrombus (asterisk) coinciding with an atherosclerotic plaque (double asterisk) in the left anterior descending coronary artery of a LM+MI patient. D) Example of an aggregate of CD45+ lymphocytes in the myocardium of a LM patient. NBT: Nitro Blue Tetrazolium.

were observed with the LM+MI group (1.50; 1.10-2.95) and the control group. These differences between groups were observed in the intima (albeit not significant) and adventitia. However, in the media the LM+MI and the MI groups had the highest number of neutrophils per mm² (Figure 3E). Thus increased numbers of macrophages and neutrophils were found particularly in coronary lesions of LM patients and MI patients.
Increased infiltration of lymphocytes and mast cells in coronary lesions of patients with LM+MI

Subsequently, lymphocytes and mast cells were analyzed. In contrast to macrophages and neutrophils, the percentage of lymphocytes in the total wall was significantly higher in the LM+MI group (0.38; 0.145-0.90) compared to both the control group (0.12; 0.07-0.27, p<0.001) and the LM group (0.16; 0.08-0.37, p<0.001) (Figure 4A). Also, the MI group (0.29; 0.07-0.76) had a significantly higher percentage of lymphocytes compared to the control (p=0.013) and the LM groups (p=0.041). Overall, especially for LM+MI patients, these differences between groups for the total coronary wall were also observed within the intima, media and adventitia (Figure 4B).

Moreover, the number of mast cells per mm² in the total wall was significantly increased in the LM+MI group (6.10; 3.50-9.48) compared to the control (4.50; 3.30-6.00, p=0.019), the LM group (3.90; 2.70-4.80, p<0.001) and the MI group (2.80; 1.80-4.60, p<0.001) (Figure 4C). Also, the MI group had a significantly higher number of mast cells compared to the control (p=0.001) and the LM group (p=0.009). This increase of mast cells in the LM+MI group was especially found in the adventitia and to a lesser extent in the media (Figure 4D). Thus increased numbers of lymphocytes and mast cells were found particularly in coronary lesions of patients with LM coinciding with MI.
Figure 3. Quantification of macrophages and neutrophils in the coronary artery segments. A) An example of the coronary layers of an anti-CD68 (macrophage) staining. The intima (I), media (M), adventitia (A) and total segment (T) are indicated. B) The number of CD68+ macrophages per mm² in the total coronary segments and C) subdivided in the different coronary layers of the coronary segments of controls, patients with LM, LM+MI and MI. D) The number of MPO+ neutrophils per mm² in the total coronary segments and E) subdivided in the different layers of the coronary segments of controls, patients with LM, LM+MI and MI. All the data points are shown combined with a boxplot showing the median and the interquartile. *p<0.05, **p<0.01, ***p<0.001. LM: lymphocytic myocarditis, MI: myocardial infarction.
Figure 4. Quantification of lymphocytes and mast cells in the coronary artery segments. A) The percentage of surface area positive of lymphocytes in the total coronary segments and B) subdivided in the different layers of the coronary segments of controls, patients with LM, LM+MI and MI. C) The number of tryptase+ mast cells per mm² in the total coronary segments of controls, LM patients, LM+MI patients and MI patients and D) subdivided in the different layers of the coronary segments of controls, patients with LM, LM+MI and MI. All the data points are shown combined with as a boxplot showing the median and the interquartile range. *p<0.05, **p<0.01, ***p<0.001. LM: lymphocytic myocarditis, MI: myocardial infarction.

Comparable inflammatory cell infiltration in infarct- versus non-infarct-related coronary arteries
In the LM+MI and MI groups the inflammatory cell density was compared between the infarct-related arteries and non-infarct-related arteries. However, no significant differences were found in the density of infiltrated macrophages, neutrophils and lymphocytes between infarct- and non-infarct-related coronary arteries of the total wall layer (data not shown). Only in the non-infarct-related coronary arteries of the LM+MI group, but not of the MI group, there were significantly (p=0.045) more mast cells compared to the infarct-related coronary arteries (data not shown).
Higher prevalence of coronary plaque instability in patients with LM+MI and patients with MI

Increased inflammation in coronary atherosclerotic lesions has been associated with plaque instability. For this we compared the percentages of stable (Figure 5A) and unstable plaques (Figure 5B) between the groups. The percentage of unstable plaques in the LM+MI group (44 out of 98 coronary segments: 44.9%) was significantly higher than in the LM group (25 out of 60 coronary segments: 29.4%; p=0.031) and in controls (8 out of 30 coronary segments: 21.1%; p=0.010) (Figure 5C). The highest percentage of unstable plaques was found in the MI group (57 out of 86 coronary segments: 66.3%), which was significantly higher compared to the control group (p<0.001), the LM group (p<0.001) and LM+MI group (p=0.004). Thus, patients with LM+MI and only MI have a higher prevalence of unstable plaques and may as a consequence be at an increased risk to develop plaque complication, such as plaque rupture and thrombus formation.

Indeed, the percentage of epicardial coronary segments wherein thrombus formation was found was significantly higher in the LM+MI group (11 out of 98 segments: 11.2%) than in the LM group (0%; p<0.05), the control group (0.0%; p<0.01), but also the MI group (2 out of 84 segments: 2.3%; p<0.05) (Figure 5D). In none of the patients plaque rupture was identified.

Thus LM+MI patients and MI patients have an increased prevalence of unstable plaques and LM+MI patients have increased coronary thrombus formation.

Higher prevalence of intraplaque hemorrhage in patients with LM+MI and patients with MI

Intraplaque hemorrhage (IPH) may also contribute to plaque instability and complication that could facilitate MI development in which vasospasm is hypothesized to play an important role [30]. Therefore, occurrence of IPH in the coronary segments was determined as a percentage of the total number of segments and compared between the patient groups. IPH was classified as absent (Figure 6A), fresh IPH (presence of intact extravascular erythrocytes) (Figure 6B) or ongoing IPH (fresh IPH in combination with old IPH (presence of extravascular erythrocyte fragments, Figure 6C, or iron, Figure 6D). IPH was only determined when intact erythrocytes were present alone or in combination with old IPH.

The LM group had significantly more IPH (22.3%) compared to controls (2.6%; p=0.023) (Figure 6E). The highest percentage of IPH was found in the LM+MI group (47.4%). Both the LM+MI group and the MI group (40.7%) had significantly more IPH than the control group (p<0.001) and the LM group (p<0.001). In the LM group the IPH was predominantly fresh (84.3%) and only a small percentage of the IPH was ongoing (15.7%). In the LM+MI group (45.6%; p=0.004) and the MI group (40.1%, p=0.003) the percentage of ongoing IPH was significantly higher compared to the LM group (Figure 6F).

In conclusion, significantly more IPH was observed in the coronary lesions of LM patients and especially of LM+MI patients than of controls. Additionally, IPH was significantly increased in MI patients compared to controls.
Figure 5. Plaque instability and thrombi in the coronary artery segments. A) Example of a haematoxylin-eosin (HE)-stained stable atherosclerotic plaque of a coronary artery segment. Characteristic is the thick fibrous cap of the intima (I) and absence of inflammatory cells at the luminal endothelial layer of the plaque. B) Example of a HE-stained unstable atherosclerotic plaque of the intima coronary artery segment. Characteristic is the thin fibrotic cap (arrow), the inflammatory cells that reach up to the luminal endothelial layer of the plaque (asterisk) and the ceroid that is present within the plaque (double asterisk). C) The graph shows the percentage (%) of unstable plaques and stable plaques in the of the total atherosclerotic plaques in the coronary segments of controls, patients with LM, LM+MI and MI. D) The graph shows the percentage (%) of coronary segments wherein thrombi were present in controls patients with LM, LM+MI and MI. *p<0.05, **p<0.01, ***p<0.001. LM: lymphocytic myocarditis, MI: myocardial infarction.

DISCUSSION

In clinical autopsies we observed a high prevalence (32%) of very recent MI (3-6 hours old) coinciding with LM in a cohort of patients diagnosed post mortem with LM. To the best of our knowledge we are the first to describe the co-occurrence of LM and MI. Patients with LM, LM+MI or MI, showed significantly increased infiltration of inflammatory cells in atherosclerotic lesions of the coronary arteries, albeit with differences in the types of inflammatory cells between the groups. In LM patients without MI this inflammatory infiltrate consisted predominantly of macrophages and neutrophils, while in LM patients with MI it consisted predominantly of lymphocytes and mast cells. In patients with MI mostly lymphocytes were increased. In LM+MI and MI patients this coincided with an increased prevalence of unstable coronary lesions and thrombus development. Finally, LM patients
and especially LM+MI and MI patients showed significantly increased intraplaque hemorrhage.

In our study only patients with a very recent MI, i.e. 3-6 hours old, were included. It is namely known that MI itself can induce infiltration of immune cells in the remote non-infarcted myocardium in time post MI. Abbate et al. showed infiltration of T lymphocytes in the remote unaffected myocardial regions in approximately two thirds of patients with MI [31]. However, these observations were made in patients who died between 1 and 12 weeks after MI, while infarcts younger than 6 days were specifically excluded. Moreover, the first inflammatory cells to infiltrate the myocardium in response to MI are neutrophils, which do not appear in the myocardium before 6 to 12 hours after onset of MI [32]. Therefore, it is very unlikely that the inflammatory infiltrate we observed in the myocardium of the LM+MI patients, somehow was the result of MI. All the more since the pattern of the inflammatory infiltrate, i.e. multiple focal aggregates of extravascular lymphocytes coinciding with focal cardiomyocyte death, are typical for LM. Moreover, given the short duration of the MI this strongly suggests that these patients developed LM prior to the MI. This in turn raises the question whether the LM or its causes did facilitate the development of MI.

MI most often results from coronary plaque rupture or erosion, leading to coronary artery occlusion. It is now generally accepted that inflammation is an important mediator of atherosclerotic plaque destabilization and that unstable plaques are more prone to complication [19]. Our observation of an increased inflammatory infiltrate in the coronary lesions of LM patients clearly argues for the possibility that, via induction of increased plaque inflammation, LM or its causes may induce plaque instability and thereby facilitate MI development. This hypothesis is supported by our finding of a high prevalence of unstable coronary plaques in the LM patients that did develop MI. At present we do not know what triggers this increased inflammatory infiltrate in LM patients. Notably, cardiotropic viruses such as Influenza A were shown in atherosclerotic mice to directly infect atherosclerotic lesions and promote inflammation, albeit the presence of myocarditis in these models was not determined [33, 34]. Unfortunately, no viral genome analyses could be performed on the coronary arteries in our patients as the routine tissue processing protocol for calcified coronary arteries in our institute includes a decalcification step that interferes with DNA/RNA isolation. In theory, the increased inflammation in the coronary plaques could also be a spill-over of the LM-related increased systemic inflammation or the inflammatory response in the heart.

Patients with only MI also showed increased plaque inflammation and instability. It is known that MI itself can affect remodeling and inflammation of atherosclerotic plaques as was also shown in mouse models [35, 36]. However, in these studies the MI-induced effects were recorded 3 and 4 weeks after MI, whereas in the present study the changes in coronary plaque morphology and inflammation were observed only a maximum of 6 hours after onset of MI. This thus makes it likely that these changes occurred prior to MI. Interestingly, the prevalence of unstable plaques in LM+MI patients was significantly lower than in MI patients. This suggests that LM may increase the likelihood of developing MI even at lower unstable plaque burden.

At present we do not know how to exactly interpret the differences in the types of inflammatory cells that infiltrate the coronary lesions between LM patients with and without MI. In LM patients without MI increased intraplaque content of macrophages and neutrophils
Figure 6. Quantification of intraplaque hemorrhage. A-C) Examples of immunohistological anti-Glut 1 staining identifying extravascular erythrocytes indicative for intraplaque hemorrhage (IPH). In coronary segments wherein no Glut 1+ staining was found IPH was deemed absent (A); Presence of intact Glut 1+ erythrocytes indicates fresh IPH (B); Presence of Glut 1+ residual fragments of erythrocytes indicates old IPH (C). D) Presence of iron based on a histological Perls staining indicates an old IPH. E) The graph shows the percentage (%) of the coronary artery segments in which IPH was present (based on fresh or ongoing IPH) in controls, patients with LM, LM+MI and MI. F) The graph shows the percentage (%) of the coronary artery segments in which either only fresh IPH or ongoing IPH was present in controls, patients with LM, LM+MI and MI. * P<0.05, **p<0.01, *** P<0.001. LM: lymphocytic myocarditis, MI: myocardial infarction.
was observed. Both are important sources of pro-inflammatory cytokines, extracellular proteases and reactive oxygen species and hence may lead to plaque instability via enhanced intraplaque inflammation, fibrous cap thinning and intraplaque hemorrhage [37]. Albeit no significant increase in the prevalence of unstable plaques was observed in our LM patients, a significant increase in fresh intraplaque hemorrhage was noted compared to control patients. In LM patients with MI, increased intraplaque content of lymphocytes and mast cells was observed. Also lymphocytes and mast cells have been associated with plaque development and destabilization [38, 39] and were found in increased numbers in the coronary arteries of MI patients before [20-22]. Indeed, their increased presence was associated with more prevalent unstable coronary lesions in our LM+MI patients. Moreover, mast cells have been implicated in provoking coronary spasms [23, 24] that have been reported to occur in myocarditis patients [11]. This is in line with our finding of increased intraplaque hemorrhage, that can be the result of vasospasm [30], in the LM+MI patients. These coronary spasms can lead to MI directly or indirectly via spasm-induced destabilization of coronary lesions followed by plaque complication [24, 40-42]. The risk of thrombotic plaque complication may even be higher in LM patients as LM can induce a hypercoagulable state as part of the antiviral immune response [43, 44]. The presence of intramyocardial vascular thrombi in the LM patients with MI may underscore such hypercoagulable state.

Differences in plaque morphology or inflammation noted between control patients and LM or LM+MI patients were not related to the grade of stenosis, since in this study the control patients had comparable grades of coronary stenosis to the patients with LM and LM+MI. Only the LM group had a lower grade of stenosis, and segments of the infarct related coronary artery of the LM+MI had a higher grade of stenosis.

Given the high prevalence of MI (32%) in patients with LM we have observed at autopsy, we believe it is advisable to include immunohistochemical analyses for the diagnosis of LM in patients in whom MI has been diagnosed at clinical autopsy or in patients in whom MI was suspected or diagnosed prior to death. This especially is advisable in cases of very recent infarcts of up to 12 hours old, as in older infarcts putative diagnosis of LM may be hampered by the infiltration of lymphocytes and possibly other inflammatory cells also in the remote non-infarcted myocardium [31]. We therefore recommend that after macroscopical confirmation of MI, via NBT staining and/or putative coronary artery thrombi, as well as confirmation of a very recent infarct by the absence of MI-induced inflammatory cell infiltration in the infarction area on HE stained slides, tissue sections of the whole heart are analyzed for putative presence of LM via immunohistochemistry using CD45. How our autopsy findings regarding the prevalence of MI coinciding with LM translate to the clinic is speculative at present. In MI patients with angiographically normal coronary arteries myocarditis was diagnosed in 33% of cases based on the subepicardial location of CMR abnormalities versus 24% subendocardial MI [45]. However, in the present study we also found LM in patients with a thrombus in the epicardial coronary artery. In the clinic these patients would be classified as having MI and putative co-occurrence of LM would most likely remain undiagnosed. Although, a case of MI with thrombus in the LAD occurring with biopsy proven viral myocarditis has been reported previously [46]. It is also speculative whether patients with preexisting LM have a worse prognosis after MI and are therefore more likely to die as a result. This would explain the high prevalence we observed at autopsy.
Future perspective
The present study adds to the large body of evidence supporting a role of inflammation in the progression of atherosclerosis and the development of acute MI. In addition, findings of our autopsy study may suggest that LM, or its underlying causes, can precipitate acute MI by destabilization of atherosclerotic plaques. These observations raise awareness that LM and MI may co-occur, and that LM may play a role in the pathogenesis of MI. However, future clinical research in patients with LM is needed to further unravel the putative role of LM in the pathogenesis of MI, and to investigate clinical and prognostic implications of disease co-occurrence.
REFERENCES

### Supplementary table. Specific patient characteristics. The clinical data was obtained prior to the death and the infarct size was obtained after autopsy.

<table>
<thead>
<tr>
<th>Age</th>
<th>Sex</th>
<th>Clinical presentation</th>
<th>Clinical history</th>
<th>Recent viral infection</th>
<th>Antecedent symptoms</th>
<th>Rhythm monitor</th>
<th>12 Lead ECG</th>
<th>Echocardiography</th>
<th>Relevant medication</th>
<th>Cause of death after autopsy</th>
<th>Findings within the heart at autopsy</th>
<th>Infarct area (%)</th>
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<tbody>
<tr>
<td>40</td>
<td>F</td>
<td>IHCA</td>
<td>DCM, Asthma, Pulmonary HT, Bronchectasis.</td>
<td>No.</td>
<td>None.</td>
<td>LBBB, Secondary ST-T changes.</td>
<td>&quot;&quot;</td>
<td>Beta blockers. Diuretics.</td>
<td>LM and MI.</td>
<td>MI &lt; 6 hours old.</td>
<td>LM. Old MI.</td>
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<tr>
<td>69</td>
<td>M</td>
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<td>Smoking</td>
<td>No.</td>
<td>None.</td>
<td>VF, Asystole.</td>
<td>&quot;&quot;</td>
<td>LM, MI and cardiac arrhythmias.</td>
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<td>LM. Positive cytomegalovirus.</td>
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<tr>
<td>44</td>
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<td>CHCA</td>
<td>&quot;&quot;</td>
<td>Yes.</td>
<td>Chest pain</td>
<td>VF, Asystole.</td>
<td>&quot;&quot;</td>
<td>&quot;&quot;</td>
<td>LM and MI.</td>
<td>MI 3-6 hours old.</td>
<td>LM.</td>
<td>63</td>
</tr>
<tr>
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<td>M</td>
<td>CHCA</td>
<td>DM II, HT, AF, Peripheral artery disease.</td>
<td>Yes.</td>
<td>Dyspnea.</td>
<td>VT, AF, SR.</td>
<td>Normal.</td>
<td>Poor LV function, regional WMA in the LAD territory.</td>
<td>LM and MI.</td>
<td>MI &lt; 3 hours.</td>
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<td>39</td>
<td>M</td>
<td>CHCA</td>
<td>&quot;&quot;</td>
<td>No.</td>
<td>Chest pain</td>
<td>VF, Asystole.</td>
<td>&quot;&quot;</td>
<td>&quot;&quot;</td>
<td>LM and MI.</td>
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<td>Borderline LM.</td>
<td>36</td>
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<tr>
<td>68</td>
<td>M</td>
<td>IHCA</td>
<td>DCM, 3-vessel CAD, DM II, Renal insufficiency, ICD.</td>
<td>No.</td>
<td>None.</td>
<td>Asystole.</td>
<td>Q wave in III, poor R wave progression in the precordial leads, negative T wave in I, III, aVF and aVL.</td>
<td>Poor LV function, regional WMA in the RCA and LAD territory.</td>
<td>Diuretics. Anticoagulation.</td>
<td>LM and MI.</td>
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<td>Gender</td>
<td>Age</td>
<td>Diagnosis</td>
<td>Symptoms</td>
<td>Treatment</td>
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<td>58 M HF.</td>
<td>Ischemic cardiomyopathy. CABG. No. Pacemaker. ICD. Chronic AF. COPD. Chronic renal insufficiency.</td>
<td>None</td>
<td>*Biventricular paced rhythm, secondary ST-T changes. Poor LV function, no valvular vegetations, pericardial effusion.</td>
<td>Diuretics. Beta blockers.</td>
<td>LM and MI</td>
<td>MI 4-5 hours old.</td>
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<td>46 F OHCA</td>
<td>Hodgkin. Dyspnea of unknown cause.</td>
<td>No</td>
<td>Dyspnea. PEA.</td>
<td></td>
<td></td>
<td>MI</td>
<td>MI 3-6 hours and 1 week old.</td>
<td>20</td>
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<td></td>
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<tr>
<td>60 M Respiratory insufficiency.</td>
<td>MI. Parkinson disease.</td>
<td>No</td>
<td>Dyspnea. SR. Negative T waves V3-V4.</td>
<td>Antiplatelet therapy. Statin.</td>
<td></td>
<td>MI</td>
<td>MI 3-6 hours old.</td>
<td>64</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>95 F SD.</td>
<td>Congestive HF. Paroxysmal AF.</td>
<td>No</td>
<td>Collapse.</td>
<td>*</td>
<td>Beta blockers. Ace inhibitors. Anticoagulation. Immunosuppressive.</td>
<td>MI and/or trauma injury.</td>
<td>MI &lt;6 hours old.</td>
<td>Old MI.</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>90 M IHCA.</td>
<td>Chronic AF. HT. Cardiac amyloidosis. Streptococci.</td>
<td>Yes</td>
<td>Dyspnea. AF. VF. AF with fast ventricular rate, diffuse ST-segment depression, negative T waves V4-V6.</td>
<td></td>
<td></td>
<td>MI</td>
<td>MI 3-6 hours old.</td>
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<td>51 M SD.</td>
<td>*</td>
<td>No</td>
<td>None. Asystole.</td>
<td>*</td>
<td></td>
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<td>38</td>
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<td>*</td>
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<td>*</td>
<td></td>
<td></td>
<td>MI</td>
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<tr>
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<td>Congestive HF. AF. HT. DM II. Angina pectoris. Peripheral artery disease. COPD.</td>
<td>No</td>
<td>None.</td>
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<td></td>
<td>MI &lt;6 hours old.</td>
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<tr>
<td>54 M OHCA</td>
<td>*</td>
<td>No</td>
<td>Chest pain. SR: VF. Slow VT.</td>
<td>*</td>
<td></td>
<td></td>
<td>MI</td>
<td>MI 1-3 hours old.</td>
<td>Old MI.</td>
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<td>82 M Respiratory insufficiency.</td>
<td>Prostate carcinoma. Sigmoid resection for diverticulitis. HF.</td>
<td>None</td>
<td>Non-sustained VT. Normal. Mildly reduced LV function. No regional WMA.</td>
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<td>MI and respiratory insufficiency.</td>
<td>MI 3-6 hours old.</td>
<td>Old MI.</td>
<td>46</td>
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<td>F</td>
<td>Upper gastrointestinal bleeding due to esophageal varices</td>
<td>Pancreatic/cholangiocarcinoma. Pneumonia. HT Upper-extremity deep vein thrombosis.</td>
<td>No.</td>
<td>None.</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>Anticoagulation</td>
<td>LM and metastatic cholangiocarcinoma.</td>
<td>LM.</td>
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<td>53</td>
<td>M</td>
<td>SD. Alcohol abuse.</td>
<td>COPD.</td>
<td>No.</td>
<td>None.</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>Immunosuppressive.</td>
<td>LM.</td>
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<td>None.</td>
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<td>*</td>
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<td>F</td>
<td>SD.</td>
<td>Yes.</td>
<td>Diarrhea. Nausea.</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>Beta blocker. Ace inhibitor.</td>
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<td>Septic shock. HT. Chronic AF. Right sided HF. Pulmonary HT.</td>
<td>Yes.</td>
<td>Diarrhea. Abdominal pain.</td>
<td>AF with incomplete RBBB. No new ECG abnormalities.</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>Diuretic.</td>
<td>LM.</td>
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<td>Yes.</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
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<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
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<td>47 M SD.</td>
<td>None.</td>
<td>None.</td>
<td>No.</td>
<td>None.</td>
<td>Asystole</td>
<td>*</td>
<td>*</td>
<td>Diffuse ST-segment depression.</td>
<td>*</td>
<td>*</td>
<td>Moderate LV function. No regional WMA. No pericardial effusion.</td>
<td>Encephalitis</td>
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<tr>
<td>63 M OHCA.</td>
<td>None.</td>
<td>No.</td>
<td>None.</td>
<td>*</td>
<td>*</td>
<td>Atrial rhythm.</td>
<td>*</td>
<td>Asystole</td>
<td>*</td>
<td>*</td>
<td>NSAIDS.</td>
<td>Emphysema, pulmonary edema and novice ARDS.</td>
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<tr>
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<td>No.</td>
<td>*</td>
<td>Asystole.</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>Rupture of abdominal aortic aneurysm.</td>
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<td>70 M Respiratory insufficiency after traumatic paraplegia.</td>
<td>None.</td>
<td>No.</td>
<td>None.</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>Paraplegia and tetraplegia with respiratory insufficiency.</td>
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<tr>
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<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
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<td>Aortic dissection.</td>
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</tr>
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</table>

*AF = atrial fibrillation; CABG = coronary artery bypass graft; COPD = chronic obstructive pulmonary disease; DCM = dilated cardiomyopathy; DM II = diabetes mellitus type 2; ECG = electrocardiogram; F = female; HF = heart failure; HT = hypertension; ICD = implantable cardioverter-defibrillator; IHCA = in-hospital cardiac arrest; LAD = left anterior descending; LBBB = left bundle branch block; LM = lymphocytic myocarditis; LV = left ventricle; M = male; MI = acute myocardial infarction; OHCA = out-of-hospital cardiac arrest; PEA = pulseless electrical activity; RBBB = right bundle branch block; RCA = right coronary artery; RV = right ventricle; SD = sudden death; SR = sinus rhythm; VF = ventricular fibrillation; VT = ventricular tachycardia; WMA = wall motion abnormalities; * not available*
Development of a new therapeutic technique to direct stem cells to the infarcted heart using targeted microbubbles: StemBells

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\(^7\) Interuniversity Cardiology Institute of the Netherlands (ICIN), Utrecht, the Netherlands.
\(^8\) Department of Biomedical Engineering, Erasmus MC, Rotterdam, the Netherlands.
\(^9\) Department of Acoustical Wavefield Imaging, Technical University of Delft, Delft, the Netherlands.

ABSTRACT

Successful stem cell therapy after acute myocardial infarction (AMI) is hindered by lack of engraftment of sufficient stem cells at the site of injury. We designed a novel technique to overcome this problem by assembling stem cell-microbubble complexes, named ‘StemBells’.

StemBells were assembled through binding of dual-targeted microbubbles (~3 μm) to adipose-derived stem cells (ASC) via a CD90 antibody. StemBells were targeted to the infarct area via an ICAM-1 antibody on the microbubbles. StemBells were characterized microscopically and by flow cytometry. The effect of ultrasound on directing StemBells towards the vessel wall was demonstrated in an in vitro flow model. In a rat AMI-reperfusion model, StemBells or ASC were injected one week post-infarction. A pilot study demonstrated feasibility of intravenous StemBell injection, resulting in localization in ICAM-1-positive infarct area three hours post-injection. In a functional study five weeks after injection of StemBells cardiac function was significantly improved compared with controls, as monitored by 2D-echocardiography. This functional improvement neither coincided with a reduction in infarct size as determined by histochemical analysis, nor with a change in anti- and pro-inflammatory macrophages.

In conclusion, the StemBell technique is a novel and feasible method, able to improve cardiac function post-AMI in rats.
INTRODUCTION

Adult mesenchymal stem cell therapy has been proposed as a promising therapy for regenerative tissue repair, for example to prevent heart failure development after acute myocardial infarction (AMI).\[1, 2\] However, one of the major problems of stem cell therapy is lack of engraftment of sufficient stem cells at the site of injury.[3, 4] We hypothesized that when retention and engraftment of stem cells is increased, the therapeutic effect of stem cells will improve. Therefore, we designed a novel targeting technique to direct adipose-derived stromal/stem cells (ASC) specifically to the activated endothelium of blood vessels within the infarct area in the heart by coating them with dual-targeted microbubbles.

We used ASC, as adipose tissue is a rich source of mesenchymal stem cells, which can be harvested easily, show high proliferation rates in culture and have the capacity to differentiate into several cell types amongst which cardiomyocytes.[5-8] Furthermore, it has been shown that ASC have a beneficial effect on cardiac function post-AMI in several preclinical studies.[3, 4, 9] Early clinical trials using ASC therapy post-AMI, however, show that there is room for improvement for ASC therapy.[10-13]

To be able to direct the ASC to the infarct area we employed so-called microbubbles, which are small (2-4 μm) gas-filled bubbles originally developed as contrast agents for echocardiography.[14] Nowadays, microbubbles can also be designed as targeting agents by conjugating antibodies, ligands or peptides to the microbubble shell.[15, 16] We have constructed stem cell-microbubble complexes, named ‘StemBells’, by coating ASC with microbubbles using a CD90 antibody via biotin-streptavidin bridging (Figure 1A). Additionally, an antibody against ICAM-1, an adhesion molecule expressed on activated endothelium of blood vessels within the infarct area[17], was simultaneously conjugated to the microbubble shell to create a bridge and improve attachment of the StemBells specifically in the infarct area. Application of the microbubbles has several benefits. First, it allows coupling of a targeting antibody to the ASC without modifying the stem cell itself. Second, the microbubbles cause buoyancy and susceptibility of the ASC to acoustic radiation force exerted by diagnostic ultrasound, as we previously showed in an chicken embryo using intravital microscopy.[18] StemBells can thus be pushed from the center of the blood stream to the vessel wall by ultrasound, further enhancing the effect of targeting.

Here, we describe the development and validation of this novel StemBell technique. We demonstrate its safety, as well as its positive effect on cardiac function in a rat AMI study.

MATERIALS AND METHODS

Isolation and culture of the stromal vascular fraction from human and rat adipose tissue

For isolation of the human stromal vascular fraction (SVF), subcutaneous abdominal adipose tissue samples were obtained as waste material after elective surgery and donated upon informed consent of the patients from three clinics in the Netherlands (Tergooi Ziekenhuis, Hilversum; ‘Jan van Goyen’ clinic, Amsterdam; VU University Medical Center, Amsterdam). This study complied with the principles of the Declaration of Helsinki. SVF was isolated as described previously.[5] Adipose tissue was stored in sterile phosphate-buffered saline (PBS; Braun, Melsungen, AG, USA) at 4°C and processed within 24h after surgery as described previously.[5] In brief, adipose tissue was enzymatically digested using 0.1%
collagenase A (Roche Diagnostics GmbH, Mannheim, Germany) in PBS containing 1% bovine serum albumin (BSA; Roche Diagnostics) for 45 min at 37°C under intermittent shaking. To remove contaminating erythrocytes, the cells were subjected to Ficoll density centrifugation (Lymphoprep, ρ01.077 g/ml, osmolarity 280±15 mOsm; Axis-Shield, Oslo, Norway).

For rat SVF isolation, adipose tissue from the inguinal fat pad of 30 male Wistar rats (Harlan Laboratories, Horst, the Netherlands; 300-400g) was resected, pooled per 5 rats, collected in sterile PBS and processed immediately after resection as described previously.[3] Animals were treated according to national guidelines and with permission of the Institutional Animal Care and local Animal Ethical Committee of the VU University Medical Center (Amsterdam, The Netherlands), which conforms with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996).

Both human and rat SVF cells were seeded at 1×10^5 cells/cm^2 in Dulbecco’s modified Eagle’s medium (DMEM; BioWhittaker, Cambrex, Verviers, Belgium) containing 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (all from Gibco, Invitrogen, Carlsbad, CA, USA). Culture medium for rat SVF cells was supplemented with 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA). For human SVF cells, culture medium was supplemented with 5% human platelet lysate and 10 IU/mL heparin (Leo Pharma, Amsterdam, the Netherlands).21 Cells were cultured in a humidified atmosphere of 5% CO₂ at 37°C. Media were changed twice a week.

The percentage of ASC within the SVF was determined by a colony forming unit assay. SVF cells were seeded in 6-wells culture dishes (Greiner Bio one, USA) at a density of 10 and 100 cells/cm^2 (in triplicate) in ASC growth medium which consisted of low glucose Dulbecco’s modified Eagle’s medium (DMEM). After 14 days, cells were washed with PBS, fixed with 4% formalin for 10 minutes, and subsequently stained in a 1% toluidine blue solution in borax buffer for 1 minute and washed twice with H₂O. Colonies containing at least 50 cells were scored using a Stereomicroscope (Zeiss, Germany).

Prior to in vitro experiments or in vivo injection SVF from liquid nitrogen storage was thawed and seeded at 1x10^5 cell/cm^2 in ASC growth medium. When ASC reached 90% confluency, cells were detached with 0.5 mM EDTA/0.05% trypsin (Gibco). Cell size was determined using a Scepter handheld automatic cell counter (Millipore, Billerica, MA, USA). For in vitro experiments ASC in passage 1 or 2 were used, for in vivo experiments ASC were used in passage 1, cultured for six days.

**StemBell assembly**

Biotinylated microbubbles with a perfluorobutane (C₄F₁₀) gas core were made by sonification, as described by Klibanov.15 The shell was composed of 1,2-distearoyl-sn-glycerol-3-phosphocholine (DSPC; 59.4 mol%; Sigma-Aldrich, the Netherlands); polyethylene glycol (PEG)-40 stearate (35.7 mol%; Sigma-Aldrich); 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine (DSPE)-PEG(2000) (4.1 mol%; Avanti Polar Lipids) and DSPE-PEG(2000)-biotin (0.8 mol%; Avanti Polar Lipids). Microbubbles (10⁶ per ml) had an average diameter of 3.5 μm and were stored in sealed glass vials with a C₄F₁₀ gas headspace to prevent deflation. To make dual-targeted microbubbles, 100 μl biotinylated microbubbles were washed twice with PBS/C₄F₁₀ by centrifugation (400 g, 1 min, 4°C) to remove superfluous biotin and resolved in PBS/C₄F₁₀. Next, streptavidin (1 mg/ml; Sigma) was added and the mixture was incubated at 4°C for 25 min. Microbubbles were again
washed to remove superfluous streptavidin and resolved in PBS/C₄F₁₀. Next, biotinylated mouse-anti-rat-CD90 (1 μg; BD Bioscience) and biotinylated mouse-anti-rat-ICAM-1 (1 μg; ACRIS) were added and the mixture was incubated at 4°C for 25 min. These dual-targeted microbubbles were again washed and resolved in DMEM. Final concentration of the microbubbles was determined using a Multisizer 3 Coulter Counter (Beckman Coulter, Fullerton, CA, USA). The presence of both antibodies was verified using a Cy3-labeled anti-IgG1-mouse (Invitrogen) and a FITC-labeled anti-IgG2a-mouse (Invitrogen) by fluorescence microscopy (Marianas, I.I.I., Denver, CO, USA) with a 40x objective (Zeiss, Germany).

Prior to incubation with the dual-targeted microbubbles, ASC were labeled with Hoechst 33342 (10 μg/mL; Invitrogen) at 37°C for 30 min. In vitro testing demonstrated that this concentration provided adequate labeling up to 42 days, as determined by fluorescence microscopy. In addition, this concentration had no effect on cell viability, determined by the addition of propidium iodide (PI) to the cells and counting of PI-positive, i.e. dead cells. Hoechst labeling also had no effect on cell proliferation as determined cell proliferation assay (data not shown). Hoechst-labeled ASC were then incubated with dual-targeted microbubbles in a 100:1 ratio under continuous rotation at room temperature for 25 min. A minimum of 10 StemBells per preparation was analyzed for the number of microbubbles per StemBell, using differential interference contrast microscopy (Marianas) with a 40x objective providing 3D-images of a StemBell.

**Ultrasound application protocol**

Ultrasound was applied using a 1-MHz unfocused transducer (V303-SU, Panametrics Inc, Waltham, MA, USA) or a 500kHz PZT transducer (V318, Panametrics Inc) coupled to an arbitrary waveform generator (33220A, Agilent, Palto Alto, CA, USA) and a linear 60-dB power amplifier (150A100B, Amplifier Research, Bothell, WA, USA). The ultrasound signal was monitored by a synchronized digital oscilloscope (GOULD DSO 465, Valley View, OH, USA). Peak negative acoustic pressure was 100 kPa as verified with a calibrated hydrophone (PA076; Precision Acoustics, Dorchester, UK). ASC or StemBells in suspension were exposed to sine-wave ultrasound bursts with a 10% duty cycle and 1 kHz pulse repetition frequency for one minute. Rats were exposed to the same ultrasound protocol by positioning the transducer parasternal at the anterior wall and location of the infarct area.

**Cell viability assay**

Cell viability was analyzed by flow cytometry using an Annexin-V-FITC and Propidium Iodide (PI) Apoptosis Detection Kit (eBioscience, San Diego, USA), as described by the manufacturer, to test whether the assembly of StemBells, as well as application of ultrasound affected cell viability. In short, following StemBell formation with or without ultrasound treatment, cells were labeled with Annexin-V in the dark for 30 min. Prior to analysis, PI was added for at least 30 sec. Fluorescence of both Annexin-V and PI was measured with a FACS Calibur flow cytometer (BD Biosciences). Stem cells negative for both Annexin-V and PI were scored as viable. Data was analyzed with CellQuest-Pro software (BD Biosciences).

**Attachment assay**

To investigate the ability to attach to a surface despite the numerous microbubbles bound to the cell surface 48x10³ StemBells with and without exposure to ultrasound were seeded in a
culture dish. After seeding, cells were allowed to attach in a humidified incubator for 5, 10, or 20 min, followed by removing unattached cells by washing with PBS. The number of attached cells was quantified using CyQuant Cell Proliferation Kit (Invitrogen) according to the manufacturer’s protocol.

**In vitro flow system to assess acoustic radiation force**

A VI-slide flow chamber (Ibidi, Martinsried, Germany) was mounted on the Marianas microscope allowing real-time visualization of acoustic radiation force acting on flowing StemBells. Shear stress on the StemBells (1x10^6 cells/50 mL) was 0.2 dyn/cm^2. Sequel bright field images were made at 20 Hz. After 2 sec the ultrasound was switched on for the total duration of the image capture, i.e. 20 sec.

**Rat model of acute myocardial infarction**

An acute myocardial infarction was induced as described previously[3, 19] in eight-week old male Wistar rats (300-400 grams, Harlan, the Netherlands) that were housed under constant temperature (21-22°C), humidity (60-65%) and light-dark periodicity (L:D 12:12). Experimental procedures started after two weeks of acclimatization. Rats were anesthetized using subcutaneous hypnorm/dormicum (fentanyl and fluanisone 0.5 ml/kg, midazolam 5 mg/kg) injection, and were ventilated at 75 breaths/min, 10-0.4 mbar (Zoovent ventilator, Netherlands). Heart rate was monitored using Einthoven I ECG. A left thoractomy in the fourth intercostal space was made, and the left anterior descending coronary artery was ligated using a 6.0 prolene suture (Ethicon, Germany). Ischemia was maintained for 40 min, followed by reperfusion and chest closure. This procedure results in relatively small non-aneurysmatic infarcts, comparable to what occurs in the majority of patients suffering from AMI.[20] StemBells or ASC (both 1x10^6 600 µL DMEM) or vehicle (an equal volume of DMEM alone) were injected intravenously (i.v.) in the tail vein one week post-AMI under isoflurane (3%) anesthesia. Directly prior to injection the suspension was vigorously resuspended to prevent clustering of the ASC or StemBells.

A pilot short-term experiment was carried out in five rats to test whether ICAM-1 targeted StemBells (~20 µm ⌀) injected intravenously were able to bind in the infarcted myocardium and whether they co-localized with ICAM-1 positivity in the infarct area three hours post-injection.

In the long-term functional experiment 27 rats were anesthetized one week post-AMI and injected with either ASC (n=11), StB (n=9) or Vehicle (n=7), followed by ultrasound exposure. Animals were sacrificed five weeks post-injection and putative effects on stem cell retrieval and differentiation, cardiac function, infarct size and macrophage infiltration were analyzed. For analysis of cardiac function, 2D-echocardiography was performed prior to AMI (baseline), prior to injection (day 7) and prior to sacrifice (day 42), using a 13 MHz linear-array transducer (ProSound SSD-4000 PureHD, Aloka, Tokyo, Japan). Short-axis images were analyzed to determine wall thickness and lumen diameter of the left ventricle, in order to calculate fractional shortening (FS), wall thickness and stroke volume (SV). After sacrifice hearts were isolated and cut into five equal slices of approximately 2.5 mm thickness. The middle two slices were snap frozen in liquid nitrogen, the apex and the top two slices (as seen from the base of heart) were embedded in paraffin.
**Fluorescence microscopy**

Determining the number of fluorescent stem cells in frozen heart slides (10 μm thickness) was performed as described previously[3], with a minor adaptation that we now used the nuclear stain Hoechst in order to retrace engrafted stem cells. To retrieve Hoechst-positive stem cells and determine putative co-localization with ICAM-1 in the pilot study, four frozen heart slides per rat were stained with monoclonal antibodies against goat-anti-ICAM-1 (1:100, Abcam, Cambridge, UK) and mouse-anti-α-actinin (1:200, 4°C overnight, Sigma), followed by incubation with a rabbit-anti-goat-FITC and a donkey-anti-mouse Cy5 secondary antibody (both 1:100, BD) for 30 minutes in the dark. In the functional study Hoechst-positive cells were also checked for putative differentiation towards cardiomyocytes. For this, four frozen heart slides per rat were counterstained with mouse monoclonal antibodies against troponin T (1:25, 4°C, overnight; AbD Serotec, UK) or connexin43 (1:1000, 4°C, overnight; Abcam), both followed by incubation with a goat-anti-mouse-FITC secondary antibody (1:100, BD).

Fluorescence microscopy (Marianas I.I.I.) was performed with a 10x and a 40x objective (Zeiss). Fluorescent images were analyzed using SlideBook software (I.I.I.). Due to the Hoechst labeling of the ASC no nuclear counterstain could be used for the whole heart tissue. However, the tissue morphology was clear from both autofluorescence as well as from the cardiomyocyte counterstains. Lung and liver were also screened.

**Histological staining to determine infarct size**

To determine the infarct size a phosphotungstic acid haematoxylin (PTAH) staining was performed as described previously[19], on four heart slides per rat distal from the suture: two frozen and two paraffin embedded heart slides. Paraffin embedded slides were deparaffinized and dehydrated, whereas frozen slides were fixed for 10 min in 100% acetone. After washing with PBS, the slides were incubated in Bouin at 60°C for 30 min. After a cooling down period of 15 min and a wash step in water of 10 min, slides were incubated in PTAH at 60°C for 60 min. After cooling down, slides were dehydrated, washed in xylene and covered. PTAH stains viable cardiomyocytes purple and infarcted cardiomyocytes pink, allowing infarct area measurements on scanned slide (Pannoramic Desk scanner and Pannoramic Viewer 1.15.3 software (3DHistech, Budapest, Hungary). Infarct size was expressed as the percentage of the surface area per whole heart slide, averaged for four cross sections.

**Immunohistochemical staining of macrophages**

To determine the inflammatory healing process post-AMI, the number and subtype of macrophages was determined by immunohistochemistry on serial paraffin slides. For total numbers of macrophages a mouse-anti-rat CD68 antibody (1:100, RT, 60 min; Serotec) was used after antigen retrieval with 0.1% pepsin (in 0.02M HCl, 37°C, 30 min). For the late, pro-healing M2 subtype of macrophages a mouse-anti-rat ED2 antibody (1:200, RT, 60 min; a gift from prof. C.D. Dijkstra, VUmc, Amsterdam, the Netherlands) was used after antigen retrieval with 10mM sodium citrate buffer, pH 6.0, by boiling the slides in this buffer for 10 min. A secondary antibody Envision-HRP (1:200, 30 min, DakoCytomation, USA) was used. Staining was visualized using Envision-diaminobenzidin (DakoCytomation). Control slides incubated with PBS instead of primary antibody yielded no staining (not shown). CD68 and
ED2 positive cells in the infarct area were scored microscopically using a 20x objective (Zeiss, Germany). Infarct area was measured using Pannoramic Viewer.

Statistical analysis
All in vitro experiments were performed at least six times. For all in vivo data non-parametric Mann-Whitney or Kruskal-Wallis rank sum test was used, followed by Dunn’s multiple comparison (GraphPad Prism 6.0). The echocardiographic data at baseline and at day 7 were analyzed for equal variances, and were subsequently taken together to provide a single value for all measurements at baseline and day 7. These values are displayed in the graphs as dotted lines. A p-value smaller than 0.05 was considered to represent a statistically significant difference. Data is described as mean ± standard error of the mean (SEM).

RESULTS

In vitro characterization of StemBells
Rat SVF cells were isolated from inguinal fat. The percentage of colony forming cells indicative of the percentage of stem cells in the stromal vascular fraction was 11.1±1.8%. After a culture period of six days, ASC were characterized and showed stem cell morphology with an average cell size of 14.3±0.3 μm (not shown) in accordance with our previous studies.[3, 21] Dual-targeted microbubbles were successfully assembled showing presence of both anti-CD90 and anti-ICAM-1 antibodies on the microbubble shell (Figure 1B). Next, these dual-targeted microbubbles were added to rat ASC in a 100:1 ratio to form stem cell-microbubble complexes, named “StemBells” (Figure 1C). ASC were coated with on average 32.5±4.2 microbubbles/cell (Figure 1D). This number was not affected by ultrasound exposure (30.6±3.7 microbubbles/cell).

The procedure to assemble StemBells, using rat ASC, did not significantly affect cell viability (ASC 85.7±2.3% viable cells; StB 87.1±1.4% viable cells). Although exposure to ultrasound slightly, but non-significantly decreased cell viability (StB+US 80.0±2.3% viable cells; p=0.09), as shown in Figure 2A. In addition, StemBells with or without exposure to ultrasound were similarly capable of attachment to culture plastic (Figure 2B), with already 60% attachment after 5 min, increasing up to 95% at 20 min. Next, it was studied, using human ASC, whether StemBells with dual-targeting microbubbles were susceptible to acoustic radiation force in an in vitro flow assay. Ultrasound displaced StemBells to the side of the flow channel in the direction of the ultrasonic wave propagation and perpendicular to the direction of flow with a translation speed of 23.2 mm/s orthogonal to the flow (Figure 2C). ASC alone were not susceptible to ultrasound (not shown).

To summarize, StemBells were successfully assembled, susceptible to acoustic radiation force, without affecting the number of microbubbles per StemBell, and without affecting cell viability and attachment rate. These in vitro results made application of the StemBell technique suitable for first in vivo usage.
Figure 1. StemBells. A) Schematic drawing of a StemBell: a stem cell-microbubble complex coupled via strepavidin-biotin-antibody bridging. B) Microscopic images demonstrating presence of two antibodies on one microbubble. Upper left panel: bright field image; upper right panel: anti-CD90 in red; lower left panel: anti-ICAM-1 in green, and lower right panel is an overlay. C) Microscopic brightfield images showing three planes of a 3D stack of a StemBell. D) Quantification of the number of microbubbles per stem cell includes the effect of ultrasound. Data is shown as mean ± SEM (n=6).

Intravenously delivered StemBells target to the infarcted myocardium

In a pilot study StemBells were injected intravenously in five rats 7 days after AMI. No shortness of breath was observed and no rats died as a consequence of injecting StemBells. Using fluorescence microscopy on frozen heart slides to retrieve Hoechst-positive cells it was shown that the StemBells were able to extravasate and bind to the myocardium, specifically in the infarct area (8.4±3.3 cells/mm²), co-localizing with ICAM-1 (Figure 3A). Virtually no cells were found in the non-infarct areas (0.1±0.02 cell/mm²; Figure 3B).
Figure 2. In vitro functionality. A) Quantification of stem cell viability using flow cytometry with Annexin V and propidium iodide (PI). B) Attachment rate of StemBells with or without ultrasound (US) at 5, 10 and 20 min after seeding to tissue culture plastic. Data is shown as mean ± SEM (n=3). C) Sequential microscopic brightfield images demonstrating susceptibility of a StemBell in a flow system. Direction of flow is from bottom to top. Ultrasound exerts acoustic radiation force from right to left. Arrow indicates a StemBell in focus displacing with a translational speed of 23.2 mm/s orthogonal to the flow.

StemBells have long term beneficial effects on heart function after AMI

Following the pilot study, we determined in a long term functional study whether injection of StemBells 7 days after AMI improved cardiac function at 42 days post-AMI. To determine putative effects on cardiac function 2D-echocardiography was performed prior to AMI, immediately prior to stem cell injection and immediately prior to sacrifice at day 42 post-AMI. Analysis of the fractional shortening (FS), a measurement reflecting left ventricular contractile capacity showed that contractility declined from 50.3±1.2% at baseline to 34.3±1.7% on day 7 (both represented by dotted lines, Figure 4A). The vehicle group displayed a further decline to 27.0±1.8% on day 42. The ASC group showed minor improvement (36.5±3.1% on day 42), and only the StB group showed a significant improvement in FS (40.5±3.8% on day 42; p<0.01) compared with the vehicle group. Stroke
**Figure 3. In vivo pilot study.** A) Fluorescence microscopy image showing a Hoechst-positive stem cell (blue) within ICAM-1 positive region (red) next to cardiomyocytes (green). B) Quantification of the number of Hoechst-positive StemBells in non-infarct area and infarct area. Data is shown as mean ± SEM (n=5).

**Figure 4. In vivo functional data.** Analysis of echocardiography short-axis images for A) Fractional shortening (%) on day 42, with dotted lines representing baseline (day 0) and day 7 post-AMI. B) Stroke volume (mL). C) Posterior wall thickness (cm). Data is shown as mean ± SEM (n=7 for Vehicle, n=11 for ASC, n=9 for StB).
volume analysis showed similar results (Figure 4B), with a significant improvement of stroke volume in the StB group (0.44±0.03 mL) as compared with the vehicle group (0.32±0.02 mL; p<0.01), and compared with baseline (0.32±0.01 mL; p<0.05). Subsequently, the left ventricle wall thickness of the non-infarcted posterior wall was determined to study putative post-AMI hypertrophy (Figure 4C). No changes in wall thickness were observed on day 7 (0.18±0.01 cm) compared with baseline (0.19±0.01 cm; p=0.99). However, in the vehicle group significant thickening of the posterior wall was found on day 42 (0.24±0.02 cm; p<0.01 vs. both baseline and day 7), indicative for cardiac remodeling towards hypertrophy. This thickening was also present, but to lesser degree in the ASC group (0.22±0.01 cm on day 42; p<0.01 vs. baseline and day 7). Notable, the posterior wall in the StB group did not show any changes on day 42 compared with baseline and day 7 (0.19±0.01 cm on day 42; p=0.99), and was significantly different from both the vehicle (p<0.05) and ASC group (p<0.05). No changes were observed in the anterior wall where the infarction was induced (data not shown).

Thirty-five days after injection Hoechst-positive cells were still visible and were predominantly found as solitary cells specifically within the infarct area (Figure 5A). These labeled stem cells were not found in the lungs or liver. A small, non-significant increase in the number of stem cells was found in the infarct area of StB-treated rats (4.6±1.1 cells/mm²) compared with ASC-treated rats (3.3±1.0 cells/mm², p=0.29), as shown in Figure 5B. Hardly any cells were found in the non-infarct area (ASC 0.03±0.01; StB 0.13±0.08 cells/mm²). All retrieved stem cells showed expression of the cardiomyocyte markers[21] Connexin 43 (Figure 5C, left panel) and Troponin T (Figure 5C, right panel), with no difference between the ASC and StB group. Albeit that intensity levels of these markers were markedly lower in the retrieved stem cells than in healthy cardiomyocytes.

Next, infarct sizes were determined by a PTAH staining on the heart slides.[3, 19] A small infarct size of 5.9±0.8% was found in the vehicle-treated animals, 5.7±1.2% in ASC-treated animals and 8.0±1.6% in StB-treated animals, with no significant differences between the groups (Figure 6A; p=0.40).

Finally, the number and subtype of macrophages within the infarct area were quantified to determine a putative effect of the stem cells on the healing process post-AMI. However, also here no differences were found in the total number of macrophages (CD68 positive) per mm² (Figure 6B), or the number of pro-healing ED2 positive macrophages (Figure 6C), nor in the ratio of ED2/CD68 (Figure 6D).

DISCUSSION

In this study we described the development of a novel technique to target stem cells specifically to the infarct area by the assembly of stem cell-microbubble complexes, named StemBells. These StemBells were first in vitro characterized, showing high viability after assembly and ultrasound exposure, as well as susceptibility to acoustic radiation force. In vivo this new stem cell delivery technique proved to be safe for intravenous injection with StemBells reaching the infarct area of the heart. In addition, StemBells significantly improved cardiac function, independent of an effect on infarct size or inflammatory macrophages five weeks after injection.
Figure 5. In vivo stem cell retrieval. A) Fluorescence microscopy image of Connexin 43 (green) stained heart slide. Arrows indicate presence of Hoechst-positive stem cells (blue) within the infarct area. B) Quantification of the number of stem cells per mm². C) Retrieved stem cells at day 42 stained positive for cardiomyocyte specific markers Connexin 43 (left panel) and Troponin-T (right panel). Data is shown as mean ± SEM (n=3).

We developed our novel targeting technique in order to overcome one of the major problems with stem cell therapy, namely the high wash-out of injected stem cells by the blood and low engraftment rate. This high-washout of over 90% by the blood is irrespectively of the delivery route of the stem cells.[22-25] In the pilot short-term study the retrieved cells co-localized with ICAM-1 positive infarct area three hours after StemBell injection. This
demonstrated safe passage of the StemBells through the pulmonary microcirculation and extravasation within the infarct area, without any complications such as shortness of breath or suffocation. Although intravenous delivery has been put away as non-effective due to pulmonary first-pass effect, recent animal studies demonstrated that stem cells do safely traverse the pulmonary circulation in mice[26], rats[27] and pigs[28]. In addition, we previously demonstrated that culturing ASC for several passages does result in obstruction in the lungs[3], indicating that culturing changes the stem cells in such a way that they are entrapped in the lungs. Whereas coupling microbubbles to ASC which were cultured for only one passage did not lead to entrapment in the lungs. Naturally, smaller cells or cell-constructs traverse the pulmonary circulation easier and faster than larger particles and cells.[27] With future clinical application in mind, we deliberately chose for intravenous delivery. For the patient a minimally invasive, intravenous drop with a StemBell suspension at the out-patient clinic is more attractive than an additional catheterization for intracardiac delivery, from both patient and economical view. The patient could even receive multiple treatments of StemBells in combination with ultrasound exposure, something that one would not readily do for intracardiac delivery.

Previously it was described that bi-directional antibodies against myosin light chain in mice[29] or VCAM in rats[30] that were used to increase homing of hematopoietic stem cells to the heart. The results from our pilot study now demonstrated that our ICAM-1 targeted StemBells had the same effect as the retrieved stem cells co-localized with ICAM-1 upregulated in the infarct area. Even more, StemBells - in addition to their targeting moiety - are susceptible to ultrasound. This was demonstrated by the ability of ultrasound to displace StemBells over a relevant distance in vitro in this study, as well as previously in a vitelline vein of a chick embryo[18], locally aiding the StemBells to slow down in the bloodstream and enhance contact to the vessel wall. The application of microbubbles in the aid of improving stem cell delivery over the endothelium has been studied before.[31, 32] In the study performed by Ghanem et al. (2009) ultrasound-mediated microbubble destruction was applied in rats four days post ischemia-reperfusion, and 15 minutes prior to intra-aortic bone-marrow mesenchymal stem cell (BM-MSC) injection. One hour after injection it was shown that destruction of microbubbles, focused at the infarct site, resulted in significantly enhanced regional adhesion and transendothelial migration BM-MSCs: 14.6 cells/heart slide vs. 9.5 cells/heart slide in animals not pretreated with US and microbubbles.[31] The other study, performed by Toma et al. (2011), showed that non-targeted microbubbles in combination with ultrasound were able to direct BM-MSC to mechanically injured endothelium of a rabbit aorta in vivo, resulting in 3.3 cells/mm² at the ultrasound-exposed segments of the aorta.[32] Although different experimental models, the number of stem cells we retrieved was in the same order of magnitude: 8.4 cells/mm² (13.4 cells/heart slide) in the infarct area three hours after injection.

Following the safety pilot study, a long term functional study was performed where treatment of the rats with StemBells significantly improved their cardiac function as demonstrated by echocardiography. A functional improvement was found in a significantly better fractional shortening in the StemBell group, concordantly, the StemBell group had the highest stroke volume. Finally in the StemBell group thickening of the non-infarcted posterior left ventricle wall was prevented. These functional effects could not be explained by a difference in infarct size between the groups. In the literature several studies have shown improved cardiac function without an effect on infarct size, e.g. in rats where ASC were injected 24h[33] or
1 week post-infarction in a model with a permanent ligation, as well as in pigs where ASC were injected 1 week post-infarction induced by a balloon angioplasty for 3h followed by reperfusion. Besides studying infarct size, we also analyzed other mechanisms that might explain the improved cardiac function by StemBells, such as cardiac differentiation or an effect on inflammatory cells. We did find cardiomyocyte specific markers Connexin 43 and Troponin-T on the retraced stem cells, which may be indicative for partial differentiation towards cardiomyocytes. However, the low number of retrieved stem cells makes it highly unlikely that differentiation is a key mechanism.

Recently it was described that the ratio between the early and late subtype of macrophages provides information about the course of infarct repair, especially as macrophages in the infarct area can release substances that are of influence on the non-infarct healthy cardiomyocytes. Therefore, we analyzed macrophage subpopulations within the

**Figure 6. In vivo analysis on the long term.**

A) Quantification of PTAH staining of heart slides showing infarct size. B) Quantification of the number of macrophages: Total number of CD68 positive cells per mm². C) Number of ED2 positive, type 2 macrophages per mm². D) ED2 positive cells expressed as percentage of total number of macrophages. Data is shown as mean ± SEM.
infarct area by ED2, the sole marker for rat type 2 anti-inflammatory macrophages identified. It was observed, that only very focally macrophages were present in the non-infarct area in all groups. However, no difference between the groups was found for the type 2 macrophages within the infarct area.

The fact that we found only a small difference in the number of retrieved stem cells five weeks after injection may indicate that the mechanism behind improved heart function might be explained by substances produced by StemBells exposed to ultrasound directly after injection, but not by stem cells exposed to ultrasound. It has been demonstrated that BM-MSCs produce and secrete a wide range of cytokines, chemokines and growth factors (extensively reviewed by Gnecchi et al. 2008), and that hypoxia, e.g. the infarct area, increases this production and secretion.[39, 40] According to Gnecchi et al. (2008) some of the paracrine factors released may alter the extracellular matrix, resulting in more favorable post-infarction remodeling and strengthening of the infarct scar.[40] In addition, there is data suggesting that factors secreted by stem cells may positively influence cardiac contractility. For example, insulin-like growth factor (IGF-1) improved myocardial function in both normal and infarcted adult rat hearts.[41] Another suggestion is the positive effect of secreted substances on bioenergetics in the post-infarcted heart. Feygin et al. (2007) demonstrated in pigs that hearts in the AMI group developed severe contractile dyskinesis in the infarct and border zone, whereas BM-MSC transplantation significantly improved contractile performance. Interestingly, they also did not find an effect on infarct size. Because of low cell engraftment, the authors concluded that the observed beneficial effects were most likely attributable to paracrine repair mechanisms.[42] It is also known that an infarction results in a decrease in cardiomyocyte contractility outside of the infarct area.[43] It can be speculated that the paracrine effects of StemBells exposed to ultrasound may positively affect cardiac contractility in the remote areas, thereby preventing cardiac remodeling, and improving fractional shortening, although further research in necessary to confirm this.

The results described here demonstrate the potential of this novel targeting technique. Although we focused on cardiac repair in AMI, the StemBell technique bears the perspective to be applicable in a variety of diseases that may require regenerative cellular therapy.

In conclusion, the StemBell technique is a novel and feasible technique, able to improve cardiac function post-AMI in rats.
REFERENCES


A comparison in therapeutic efficacy of several time points of intravenous StemBell administration in a rat model of acute myocardial infarction

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ABSTRACT

Background. Adipose-derived stromal cells (ASCs) are a promising new therapeutic option for acute myocardial infarction (AMI) patients. Previously, we found that ASCs coupled to antibody targeted microbubbles (StemBells; StB) improved cardiac function when administered intravenously 7 days post-AMI in rats. In this study, we compared the efficacy of intravenous StB administration at different administration time points following AMI in rats.

Methods. AMI, followed by reperfusion, was induced in four groups of male Wistar rats, which subsequently received an intravenous 1x10^6 StB bolus 1 day post AMI (StB1, n=8), 7 days post-AMI (StB7, n=9), at both time points (StB1+7, n=7) or neither (Control, n=7). The effect on cardiac function was determined using echocardiography prior to AMI, 7 days post-AMI and 42 days post-AMI. The effect on infarct size and macrophages in the infarct core were determined (immuno)histochemically 42 days post-AMI.

Results. At 42 days post-AMI, all three StB groups had a significantly improved fractional shortening compared to the control group. Between the StB-treated groups, the effects did not differ significantly at 42 days post-AMI. At 7 days post-AMI, the StB1 group had a significantly improved fractional shortening compared to the control and StB7 groups. No significant changes in infarct size or macrophage numbers were found compared to the control group for any StB group.

Conclusions. StB administration resulted in long-term improvement of cardiac function, independent of the time point of administration. When administered at 1 day post-AMI, this improvement was already evident at 7 days post-AMI.
INTRODUCTION

Acute myocardial infarction (AMI) is a major cause of death and morbidity worldwide.[1] A potential treatment option for AMI is mesenchymal stromal cell administration.[2] Adipose tissue forms an attractive source to obtain mesenchymal stromal cells (adipose-derived mesenchymal stromal cells, ASCs), as it is easily accessible and stromal cell-rich.[3] ASCs have been demonstrated to differentiate into several intracardiac cell types,[4, 5] and secrete paracrine factors which can stimulate cardiac regeneration.[6, 7] Therapeutic application of ASCs for AMI has been investigated thoroughly in pre-clinical animal studies.[8] In general, an important limitation of cytotherapy for AMI is poor stromal cell retention and survival in the infarcted myocardium.[9, 10] We recently developed a technique to increase ASC engraftment into the post-AMI myocardium, referred to as ‘StemBells’ (StB).12 StB are ASCs coupled to gas-filled microbubbles coated with antibodies targeting CD90 (ASC surface marker) and ICAM-1 (adhesion molecule present on activated endothelial cells during inflammation). This facilitates a binding of the StBs to the activated endothelial cells in intramyocardial blood vessels following AMI. Furthermore, StBs can be pushed against vessel walls using ultrasound, as previously demonstrated intravitally in chicken embryos.[11]

We demonstrated that intravenous StB administration in a rat model of AMI at 7 days post-AMI significantly improved cardiac function compared to regular ASCs. A reduction in infarct size however was not found for StBs or ASCs administered at day 7 post-AMI.[12] Between day 1 and day 7 post-AMI, a strong inflammatory response is induced in the infarcted myocardium. This inflammatory response clears the wound of necrotic cells and debris and provides signals to initiate reparative pathways. This inflammatory response however also causes further damage to cardiomyocytes, resulting in an increased infarct size and adverse ventricular remodelling.[13-15] We hypothesized that StBs can protect against this inflammatory damage when administered 1 day post-AMI, and thus decrease infarct size and restore cardiac function even more compared with StB administration 7 days post-AMI. For this, we compared in a rat AMI model the efficacy of intravenous StB administration at 1 day post-AMI with administration 7 days post-AMI.

METHODS AND MATERIALS

Laboratory animals

All animals were treated according to national guidelines and with permission of the Institutional Animal Care and local Animal Ethical Committee of the VU University Medical Center (Amsterdam, The Netherlands), which conforms with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996). All animals were housed under constant temperature (21-22°C), humidity (60-65%) and light-dark periodicity (L:D 12:12). Experimental procedures started after two weeks of acclimatization.

Isolation and expansion of adipose-derived stromal cells

Adipose tissue was resected from the inguinal fat pads of 18 male Wistar rats (Harlan, Horst, the Netherlands), and was pooled prior to processing. The tissue was washed in sterile phosphate-buffered saline (PBS), minced manually with a sterile surgical blade and
digested with 0.0125% Liberase TM Research Grade medium Thermolysin (Roche Diagnostics, Indianapolis, USA, dissolved in PBS with 1% Bovine Serum Albumin) under intermittent shaking for 25 min at 37ºC. The mixture was filtered (100μm; Codan, Deventer, the Netherlands) and centrifuged (10 min, 600g). The supernatant was discarded and the cell pellet was re-suspended in PBS with 1% Bovine Serum Albumin, and washed by centrifugation (5 min, 600g). Finally, the cell pellet (stromal vascular fraction; SVF) was harvested, frozen in Recovery cell culture freezing medium (Gibco, Thermo Fischer Scientific, Waltham, MA), and subsequently stored in liquid nitrogen.

Prior to StB assembly, the SVF from liquid nitrogen storage was thawed and seeded at 100,000 cells/cm² in low glucose Dulbecco’s modified Eagle’s medium (DMEM, Gibco) containing 100 U/ml penicillin, 100 μg/ml streptomycin (all Gibco) and 10% foetal bovine serum (FBS; Hyclone, GE Healthcare, Logan, UT), and cultured in a humidified atmosphere of 5% CO2 at 37ºC. When the ASCs reached 90% confluency, cells were detached with 0.5 mM EDTA/0.05% trypsin (Gibco). ASCs in passage 1 were used for StB assembly.

**StemBell assembly**

Biotinylated microbubbles with a C₄F₁₀ gas core were made by sonification, as described by Klibanov et al.[16] The shell was composed of DSPC (59.4 mol %; Sigma-Aldrich, St Louis, MO); PEG-stearate (35.7 mol %; Sigma-Aldrich); DSPE-PEG (2000) (4.1 mol %; Avanti Polar Lipids, Alabaster, AL) and DSPE-PEG (2000)-biotin (0.8 mol %; Avanti Polar Lipids). Microbubbles (10⁹ per ml) had an average diameter of 3.5 μm and were stored in sealed glass vials with a C₄F₁₀ gas head-space to prevent deflation.

Dual-targeted microbubbles were made according to protocols as described previously by Woudstra et al.[12] Briefly, microbubbles (100 μl) were washed twice with PBS/C₄F₁₀ by centrifugation (1500 rpm, 1 min, 4°C) to remove superfluous biotin, and resolved in PBS/ C₄F₁₀. Next, streptavidin (1 mg/ml; Sigma-Aldrich) was added and the mixture was incubated at 4°C for 25 min. Microbubbles were again washed to remove superfluous streptavidin, and resolved in PBS/ C₄F₁₀. Next, biotinylated mouse-anti-rat-CD90 (1 μg; BD Biosciences, Franklin Lakes, NJ) and biotinylated mouse-anti-rat-ICAM-1 (1 μg; Acris antibodies, Herford, Germany) were added, and the mixture was incubated at 4°C for 25 min. These dual-targeted microbubbles were again washed, and resolved in DMEM. To assemble the StB, ASCs were incubated with dual-targeted microbubbles in a 1:100 ratio under continuous rotation at room temperature for 25 min. A minimum of 10 microbubbles were coupled to each ASC.

**Induction of AMI in rats**

Male Wistar rats (Harlan) were anaesthetized using subcutaneous hypnorm/dormicum (fentanyl and fluanison 0.5 ml/kg, midazolam 5mg/kg) injection, and were ventilated at 75 breaths/min, 10-0.4 mbar (Zoovent ventilator, Instruvet, Amerongen, The Netherlands). Heart rate was monitored using Einthoven I ECG. A left thoracotomy in the fourth intercostal space was made, and the left anterior descending coronary artery (LAD) was ligated using a 6.0 prolene suture (Ethicon, Somerville, NJ). Ischaemia was maintained for 40 min, followed by reperfusion and chest closure. This procedure results in relatively small non-aneurysmatic infarcts, comparable to what occurs in the majority of patients suffering from AMI. A total of 48 rats was subjected to the procedure, of which 31 were used for analysis.
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The other 17 rats either died during surgery, or were excluded due to an unsuccessful LAD ligation.

Experimental procedures
After induction of AMI, the rats were subdivided into four groups: Control (n=7), StB1 (n=8), StB7 (n=9) and StB1+7 (n=7). For ethical reasons concerning animal use, we compared the StB1 and StB1+7 groups to the same control and StB7 groups used previously.[12] One day post-AMI, the rats in the StB1 and StB1+7 group received a bolus of 1x10^6 StB in 600 µl DMEM in the tail vein. Seven days post-AMI, the rats in the StB7 and StB1+7 group received a bolus of 1x10^6 StB in 600 µl DMEM in the tail vein as well. At both time points, the injections were performed under 3% isoflurane anaesthesia, and rats received ultrasound exposure following injection. Ultrasound was applied using a 1-MHz unfocused transducer (V303-SU, Panametrics Inc, Waltham, MA) or a 500kHz PZT transducer (V318, Panametrics Inc), coupled to an arbitrary waveform generator (33220A, Agilent, Palto Alto, CA) and a linear 60-dB power amplifier (150A100B, Amplifier Research, Bothell, WA). The transducer was placed parasternal at the anterior wall, and location of the infarcted area and rats were exposed to sine-wave ultrasound bursts with a 10% duty cycle and 1 kHz pulse repetition frequency for one minute. The ultrasound signal was monitored by a synchronized digital oscilloscope (GOULD DSO 465, Valley View, OH). Peak negative acoustic pressure was 100 kPa, as verified with a calibrated hydrophone (PA076; Precision Acoustics, Dorchester, United Kingdom). Rats were sacrificed 42 days post-AMI, where after the hearts were isolated, fixed in 4% formaldehyde, sliced transversally and embedded in paraffin. For infarct size determination and macrophage quantification, two sections were examined per heart, one section approximately 2 mm above the tip of the apex and the other approximately 2 mm below the site where the LAD was ligated.

Analysis of cardiac function
For analysis of cardiac function, transthoracic short-axis 2D-echocardiography was performed immediately prior to AMI, immediately prior to the second administration time point (7 days post-AMI), and immediately prior to sacrifice (42 days post-AMI), using a 13 MHz linear-array transducer (ProSound SSD-4000 PureHD, Aloka, Tokyo, Japan). Echocardiographic images were analysed using Image-Arena 2.9.1 (TomTec Imaging Systems, Unterschleissheim, Germany). To ensure that the (repeated) measurements in the rats were taken at the same mid-ventricular location, the echocardiographic images were recorded using the attachment site of the papillary muscle for orientation. For each rat and time point, lumen diameter and wall thickness was measured in three separate images, and the average values of the three images were used for the analysis. The fractional shortening (FS) was determined by calculating the degree of shortening of left ventricular diameter between end-systole and end-diastole. Contraction of posterior and anterior walls was determined by calculating the difference between end-systolic and end-diastolic wall thickness.

Infarct size determination
To determine the infarct size of the StB1 and StB1+7 groups, an Elastica von Giesson (EvG) staining was performed on 4 µm thick paraffin-embedded heart slides. Slides were placed in ethanol and washed in regular tap water. Next, the slides were placed in Lawson’s solution...
(Klinipath, Duiven, the Netherlands) for 30 minutes, followed a brief (several seconds) immersion in 100% ethanol. The slides were washed, stained with haematoxylin, washed again, and placed in Van Giesson solution (saturated picric acid solution with 0.075% fuchsin acid) for 5 minutes. Surplus fluid was drained after which the slides were air dried and covered. The slides were scanned using a Pannoramic DESK digital slide scanner (3DHistech Ltd., Budapest, Hungary), and surface areas of the magenta-stained infarct areas, as well as the total cross-sectional surface areas of the hearts, were measured using Pannoramic Viewer v1.15.3 (3DHistech Ltd.). The infarct size was determined as the mean relative infarct size of both sections, and compared with infarct sizes of the control and StB7 groups, which were determined previously.\[12\]

Immunohistochemical staining of macrophages

The number and the subtype of macrophages in the infarcted area was determined by immunohistochemistry on serial 4 μm thick paraffin-embedded heart slides. A mouse-anti-rat CD68 antibody was used to identify macrophages (1:100, RT, 60 min; AbD Serotec, Puchheim, Germany) after antigen retrieval with 0.1% pepsin (in 0.02M HCl, 37°C, 30 min). A mouse-anti-rat CD163 antibody (ED2) was used to identify the anti-inflammatory subtype of macrophages (1:200, RT, 60 min; a gift from prof. C.D. Dijkstra, VUmc, Amsterdam, the Netherlands) after antigen retrieval through boiling the slides in 10mM sodium citrate buffer, pH 6.0 for 10 min. As secondary antibody, Envision-HRP (1:200, 30 min, Dako, Glostrup, Denmark) was used. Staining was visualized using Envision-diaminobenzidin (Dako Cytomation). Control slides incubated without primary antibody yielded no staining (not shown). All CD68- and CD163-positive cells in the infarcted area were scored microscopically using a 20x objective (Zeiss, Oberkochen, Germany). For each rat, the CD68- and CD163-stainings were done on serial tissue slides. For the number of cells per mm² infarct area, the infarct area was measured again on the CD68-stained sections using Pannoramic Viewer v1.15.3, and these infarct sizes were used specifically for both CD68- and CD163-stainings.

Statistical analysis

Statistical analysis was performed with Prism 6.0 (Graphpad software, La Jolla, CA). Normality of each data set was analysed using the Shapiro-Wilk normality test. Infarct sizes, macrophage numbers, and echocardiographic changes in anterior wall contraction and posterior wall thickness were analysed with the Mann-Whitney U-test, as not all data sets were normally distributed. Echocardiographic data compared between different time-points within the same experimental group were analysed with a paired T-test. Echocardiographic data compared between different experimental groups within the same time point were analysed with an independent T-test. A p-value below 0.05 was considered statistically significant for all analyses. Data-values in text are displayed as mean ± standard error of the mean.

RESULTS

The effect of different StemBell administration time points on fractional shortening

The effect of the StB administration on the fractional shortening (FS) was determined at baseline (day 0, prior to induction of AMI), 7 days post-AMI and 42 days post-AMI (Figure...
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1A). At baseline, the FS did not differ between the groups (control: 0.50±0.02, StB1: 0.50±0.03, StB7: 0.50±0.02, StB1+7: 0.49±0.02).

At 7 days, the FS in the control group and StB7 group (0.31±0.03 and 0.33±0.02 respectively) was significantly decreased compared to baseline (Figure 1B) as result of AMI. Interestingly, at 7 days the StB1 and StB1+7 groups showed an improvement in FS (0.45±0.02 and 0.42±0.04 respectively) compared to the control and StB7 groups, which was significant for the StB1 group (Figure 1C).

At 42 days, the FS of the control group (0.28±0.02) did not differ significantly compared to day 7 and remained significantly lower compared to baseline (Figure 1B). Compared to the control group, at 42 days the StB1 group (0.46±0.04), the StB7 (0.41±0.04) and the StB1+7 group (0.44±0.03) all showed a significantly improved FS, but did not differ significantly between each other (Figure 1C).

Summarized, on the long term (42 days post-AMI) StB administration at 1 day post-AMI, 7 days post-AMI or at both time points all similarly counteracted the AMI-induced effects on FS. In addition, StB administered at 1 day post-AMI resulted in a higher FS on the short-term (7 days post-AMI) compared to the control group.

Figure 1. Fractional shortening. A) Time-lapsed echocardiographic image (left ventricular short-axis view) of a control rat created at day 0 and day 42, displaying the lumen diameter (yellow line) during systole (S) and diastole (D). AW: anterior wall. L: lumen. PW: posterior wall. B) Mean fractional shortening (systolic lumen diameter relative to diastolic lumen diameter) compared between different time points within each experimental group. C) Mean fractional shortening compared between different experimental groups within each time point. Error bar: Standard error of the means. *: p<0.05 compared to control group. **: p<0.01 compared to control group. ***: p<0.001 compared to day 0. #: p<0.05 compared to StB 7 group. The star colour above the data points in Figure B indicates the group for which a significant difference was found. Of one control rat (all time points) and two rats from the StB1 group (one rat: all time points. one rat: day 7), echocardiographic images were of insufficient quality for determination of fractional shortening.
The effect of different StemBell administration time points on cardiac muscle contraction

Next, we investigated the effects of StB administration on the contraction of the anterior wall (containing the infarction) and the (non-infarcted) posterior wall (Figure 2A), expressed as the end-systolic wall thickness relative to the end-diastolic wall thickness. At baseline, the anterior wall contraction did not differ between groups.

Compared to baseline, at 7 days post-AMI a significant decrease in anterior wall contraction was observed for the control (from 1.73±0.05 to 1.24±0.14) and StB7 groups (from 1.78±0.09 to 1.26±0.08) (Figure 2B). For the StB1 (from 1.68±0.04 to 1.52±0.04) and StB1+7 (from 1.71±0.05 to 1.51±0.05) the anterior wall contraction was significantly decreased as well compared to baseline, although it remained significantly higher compared to the StB7 group (Figure 2C).

At 42 days, the anterior wall contraction was similar for all three StB groups (StB1: 1.46±0.09, StB7: 1.30±0.09, StB1+7: 1.37±0.04). Importantly, these values were all increased compared to the anterior wall contraction of the control group (1.09±0.04), although only significantly for the StB1 and StB 1+7 groups (Figure 2C). Contraction of the posterior wall did not significantly differ between the groups, nor between different time points within the same group (Figure 2D).

Summarized, at 42 days post-AMI all StB groups had a significantly improved anterior wall contraction compared to the control group. In addition, StB administration at 1 day post-AMI resulted in a significantly improved anterior wall contraction at 7 days post-AMI.

The effect of different StemBell administration time points on ventricular remodelling

We were also interested whether the StB therapy had an effect on the end-diastolic ventricular wall thickness. In the (infarcted) anterior wall, the control group had a slight decline in wall thickness from baseline to 42 days post-AMI. In the StB1 and StB1+7 groups the wall thickness showed a slight increase from baseline to 42 days post-AMI. However, no significant differences were found between the groups compared per time point, nor between the different time points (Figure 2E). The posterior wall thickness was similar for all groups at baseline (control: 0.19±0.01 cm, StB1: 0.20±0.01 cm, StB7: 0.19±0.01 cm, StB1+7: 0.20±0.01 cm) and at 7 days post-AMI (control: 0.16±0.01 cm, StB1: 0.18±0.01 cm, StB7: 0.17±0.01 cm, StB1+7: 0.17±0.01 cm). At 42 days post-AMI, the control group had a significantly higher posterior wall thickness compared to earlier time points (0.24±0.02 cm)(Figure 2F). Remarkably, at day 42, all three StB groups did not display this increase in posterior wall thickness compared to earlier time points (all 0.19±0.01 cm). For the StB1 and StB7 group, the posterior wall thickness at 42 days post-AMI was significantly decreased compared to the control group (Figure 2G).

Summarized, StB treatment prevented long-term (42 days post-AMI) posterior wall thickening independent of administration time point.
Figure 2. Wall contraction and thickness. A) Time-lapsed echocardiographic image (left ventricular short-axis view) of a control rat created at day 0 and day 42, displaying the wall thickness (yellow line) during systole (S) and diastole (D). AW: anterior wall. L: lumen. PW: posterior wall. B+C) Mean contraction (systolic wall thickness relative to diastolic wall thickness) of the anterior wall, compared between different time points within each experimental group (B) and compared between different experimental groups within each time point (C). D) Mean contraction (systolic wall thickness relative to diastolic wall thickness) of the posterior wall, compared between different time points within each experimental group. E+F) Diastolic wall thickness of the anterior wall (E) and posterior wall (F) compared between different time points within each experimental group. G) Diastolic wall thickness of the posterior wall compared between different experimental groups within each time point. Error bar: Standard error of the means. *: p<0.05 compared to control group. **: p<0.01 compared to day 0. ***: p<0.001 compared to day 0. #: p<0.05 compared to 7 days post-AMI. The symbol colour above the data points in Figure B indicates the group for which a significant difference was found. Of one control rat (all time points) and one rat from the StB1 group (day 0), echocardiographic images were of insufficient quality for wall analysis.
The effect of StemBell administration on infarct size

To determine whether the long-term beneficial functional effects of StB therapy were related to a decrease in infarct size, the infarct areas were quantitatively analysed using an EvG-staining at 42 days post-AMI (Figure 3A). Compared to the control group (5.83±0.85%), in the StB1 group (3.63±1.25%), the StB7 group (7.72±1.56%) and the StB1+7 group (4.24±1.50%), the infarct size did not differ significantly (Figure 3B). However, for the StB1 group the infarct size was significantly decreased compared to the StB7 group. Taken together, the long-term improvement of cardiac function does not correspond to a significant reduction in infarct size.

![Figure 3. Infarct size. A) Elastica von Giesson staining of a cardiac cross section of a rat with an infarction (arrow) in the left ventricular anterior wall (stained magenta). B) Mean infarct sizes (relative to the total area of the tissue slide) compared between experimental groups at day 42. Error bar: Standard error of the means. *: p<0.05.](image)

The effect of different StemBell administration time points on macrophages

Finally, we analysed whether the long-term beneficial effects of StB therapy were related to effects on cardiac inflammation. For this, we quantified the density of macrophages present in the heart 42 days post-AMI, as macrophages are known to remain present in increased numbers for an extended period of time in the infarcted myocardium while other inflammatory cells do not.[17] Virtually no macrophages were observed outside of the infarct area in any of the groups (not shown). In the infarct area, the total number of macrophages in the control group (225±46 cells/mm²) did not significantly differ compared to the StB1 group (220±41 cells/mm²), the StB7 group (132±27 cells/mm²) or the StB1+7 group (182±31 cells/mm², Figure 4A). Compared to StB1 however, the total number of macrophages was significantly decreased in the StB7 group. Similar to the total number of macrophages, the number of CD163-positive macrophages (the anti-inflammatory M2 macrophage subset) did not differ
A comparison of several time points of StemBell therapy

significantly between the control group (193±35 cells/mm²), the StB1 group (161±21 cells/mm²), the StB7 group (115±29 cells/mm²) and the StB1+7 group (134±23 cells/mm²) (**Figure 4B**). Also, the percentage of CD163-positive macrophages relative to the total number of macrophages was comparable between groups (87±6%, 82±9%, 81±6% and 75±9% respectively for control, StB1, StB7 and StB1+7, **Figure 4C**).

Summarized, the long-term functional improvement of the heart does not relate to the state of cardiac inflammation (macrophages) at day 42.

**Figure 4. Macrophages. A)** Mean macrophage numbers per mm² of infarcted tissue, compared between experimental groups at day 42. **B)** Mean CD163-positive macrophage numbers in the infarct area compared between experimental groups at day 42. **C)** CD163-positive macrophage numbers, relative to total macrophage numbers, compared between experimental groups at day 42. Error bar: Standard error of the means. *: p<0.05. Of two control rats and one rat of the StB1+7 group, cardiac tissue was insufficient for macrophage analysis.

**DISCUSSION**

The aim of this study was to compare in rats the therapeutic efficacy of StB administration 1 day post-AMI with administration 7 days post-AMI. At 42 days post-AMI, both time points of administration resulted in a similar improvement of cardiac function. Additionally, StB administration prevented long-term posterior wall hypertrophy independent of administration time-point. However, despite that these long term effects were equal, StB administration 1 day post-AMI resulted in an improved cardiac function, measured at 7 days post-AMI. A
second StB bolus administered at 7 days post-AMI provided no additional long-term functional benefits. Interestingly, the observed functional benefits did not correlate with a significant reduction in infarct size, nor a significant reduction of residual cardiac inflammation at 42 days post-AMI.

Theoretically, the improved cardiac function observed at day 7 post-AMI after StB administration 1 day post-AMI could have been caused by an attenuated infarct expansion during the first week following AMI. However, we did not find significant differences in infarct size between the groups at 42 days post-AMI. Despite the absence of a significant infarct size reduction, the contraction of the (infarcted) anterior wall was significantly improved at day 42 for the StB1 and StB1+7 groups compared to the control group. In line with this, cardiac hypertrophy in the non-infarcted myocardium is a well-known compensatory response to the reduced contractile function of the infarcted myocardium.[18] The observed reduction in posterior wall hypertrophy for all StB groups is therefore possibly an indirect effect caused by the StB-induced improvement of anterior wall contraction.

It has been demonstrated before that AMI also results in a decrease in cardiomyocyte contractility outside of the infarct area.[19] Paracrine factors secreted by the StBs may have improved cardiac function by improving cardiomyocyte contractility. Indeed, Takahashi et al. previously demonstrated that bone marrow-derived mesenchymal stromal cell (BMSC)-treated culture medium increased in vitro contraction of individual cardiomyocytes.[20] Which secreted factors caused this increased contractility was not determined however, nor has such an effect been demonstrated yet for ASCs.

In several animal studies, ASC administration at 1 day post-AMI has been studied before. Danoviz et al injected 1x10^6 rat ASCs directly in the cardiac muscle 1 day post-AMI in rats, and observed a decreased susceptibility to afterload stress 28 days post-AMI.[21] In contrast, Zhu et al administered a large number of human ASCs (1x10^8) intravenously 1 day post-AMI in rats and observed improvements in cardiac function (FS), as well as a reduction in infarct size 28 days post-AMI.[22] Our data show that using the StB technique, it is possible to improve cardiac function after ASC administration 1 day post-AMI, using a low cell quantity (1x10^6) and a low-invasive administration route (intravenously).

We previously compared intravenous administration of uncultured adipose tissue cell isolates (the SVF, 5x10^6 cells) also between 1 day and 7 days post-AMI in rats.[23] Here we found no change in infarct size for SVF administered at day 1, while SVF administration at day 7 did result in a significant infarct size reduction. At day 1, the inflammation in the infarcted rat myocardium starts, while at day 7, inflammation is subsiding and reparative mechanisms have been activated.[24, 25] We previously hypothesized that these differences in cellular environment caused SVF administration to be less effective at day 1 compared to day 7.

However, for StBs we found no indication in our current study that this inflammatory environment reduces the efficacy of administration at day 1 post-AMI. We are thus far unsure why StBs appear to be more resilient against inflammation compared to ASCs as part of the SVF. In addition, it is known that AMI causes inflammation in remote areas of the myocardium, and this is hypothesized to induce adverse remodelling outside of the infarct area.[26] The paracrine effects of the StBs may attenuate this remote inflammation, and hereby improving contraction of the anterior wall. Further research is needed to confirm this
however, as at 42 days post-AMI we saw no signs of inflammation outside the infarcted areas in any group.

Between day 3 and day 5 following AMI in mice, a macrophage phenotype switch occurs in the infarcted myocardium, where pro-inflammatory M1 macrophages are replaced by M2 macrophages, which suppresses inflammation and stimulates repair.[27] In AMI patients, higher levels of M2 monocytes in blood are associated with decreased infarct size and increased left ventricular ejection fraction.[28] Importantly, BMSCs are known to influence this switch in favour of the anti-inflammatory M2 macrophage.[29] In our study, StB administration did not appear to influence macrophage numbers or M1/M2 subtype composition compared to controls at day 42 post-AMI, independent of administration time point. Nevertheless, we cannot exclude that ASCs did accelerate the macrophage transition towards a M2 phenotype in our StB-treated groups, although cardiac tissue obtained at earlier time points is needed to study this further.

Compared to StB administration at 1 day post-AMI alone, a second administration at 7 days post-AMI did not result in additional benefits. We were unable to find a reason why no cumulative improvement was observed for the StB1+7 group. For BMSCs, including a second administration was previously described to enhance the therapeutic efficacy in a rat AMI model, as compared to a single BMSC administration.[30] To the best of our knowledge however, dual administration of ASCs in an AMI animal model has never been studied before.

In our study, we did not measure cardiac function at the first time point of StB administration (1 day post-AMI). Therefore, based on our data alone we cannot rule out that the near-baseline FS measured at 7 days post-AMI after StB administration on day 1 is an inhibited decline of FS rather than a ‘fast recovery’. However, from other studies using rat AMI models we do know that FS declines significantly already in the first day following AMI induction.[31, 32] Therefore, we consider a fast recovery a more likely scenario than a reduced decline. A second limitation is the fact that we chose to obtain cardiac tissue only at 42 days post-AMI, as the primary objective was to determine the effects of StB administration on cardiac function. However, because of this we could not study putative effects of StB administration on cardiac inflammation at earlier time points. Finally, the low number of rats and large variance in some datasets may have contributed to an underestimation of differences between groups and time points.

The improved cardiac function after StB administration at 1 day post-AMI may be beneficial for patients. A large population-based study on AMI patients determined that mortality can be as high as 6.5% 7 days post-AMI, and more than half of these deaths occur after day 1.[33] Therefore, our data indicate that StB administration 1 day post-AMI is preferable over 7 days. An additional practical advantage of StB administration 1 day post-AMI is that this allows the administration during the initial hospitalization period of the AMI patients, which in modern times is generally less than 4 days following the ischemic event.[34]
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General discussion
Myocarditis and myocardial infarction (MI) are important contributors to the worldwide burden of cardiovascular disease. Inflammation plays a crucial role in the pathogenesis of both diseases. We have studied cardiac inflammation in more detail in MI and/or myocarditis to develop new diagnostics and therapeutics.

**LYMPHOCYTIC MYOCARDITIS (LM)**

**Alternative diagnostic options for LM**

Endomyocardial biopsies (EMB) are still the gold standard to diagnose myocarditis. The immunohistochemical examination of EMB is crucial for diagnosing LM. According to the guidelines of the European Society of Cardiology (ESC) Working Group LM is diagnosed in case the inflammatory infiltrate consists of ≥14 leukocytes per mm², composed of ≥7 activated CD3 positive T-lymphocytes and up to 4 macrophages per mm².[1] Since there is a risk of underdiagnosing LM, we wondered whether a more common leukocyte marker rather that a specific T-lymphocyte marker would increase the pathological diagnostic sensitivity for LM. A common leukocyte marker that is expressed on all lymphocytes is CD45. In Chapter 2 we compared the sensitivity of the recommended pan T-lymphocyte marker CD3 with the leukocyte common antigen CD45 to diagnose LM. For this we quantified CD3 and CD45 positive lymphocytes within the heart to discover which marker reaches the recommended threshold of ≥14 leukocytes per mm², that is necessary to diagnose LM. We chose to quantify the lymphocytes within 2.0 mm thick intact endocardium of the left ventricular posterior wall of autopsy patients with proven LM, as in this area EMB are usually taken. We found within this EMB sampling area that the diagnosis of LM increased from 17% to 50% when quantifying CD45 instead of CD3 positive cells using the recommended threshold (Figure 1, number 1). Moreover, we found that the aggregates of lymphocytes, which are characteristic for LM, could be observed more clearly with the use of CD45 than with CD3. When lymphocytes attach to cardiomyocytes and are activated they degrade. However, in contrast to CD3 the CD45 marker is still expressed on epitopes attached to cardiomyocytes that originate from degraded lymphocytes. Therefore, the use of CD45 staining is preferred to distinguish aggregates of lymphocytes and to demonstrate the presence of activated, but now degraded lymphocytes. We propose to stain heart tissue of patients with suspected myocarditis with CD45 to improve the diagnosis of LM. Nevertheless, CD3 staining is still important as high numbers of T-Lymphocytes indicate a viral infection of the heart.

For the diagnosis LM in autopsy material there are no clear guidelines available that recommend a threshold of lymphocytes as is the case for EMBs. Therefore, we were also interested how the diagnosis of LM using CD45 would relate between the EMB sampling area and the remaining myocardium of the left ventricular posterior wall using the threshold of ≥14 leukocytes per mm². In addition, with the use of late gadolinium enhancement using cardiovascular magnetic resonance (CMR) it has been shown that myocardial injury related to LM is primary detected in the subepicardial layer and almost never in the subendocardial compartment as seen with ischemia-mediated injury.[2-4] This suggests that the endocardium, albeit the only practically available site to collect biopsies, may in fact be a poor location to diagnose LM. We therefore compared the inflammatory infiltrate in the EMB sampling area with the remaining myocardium of the left ventricular posterior wall in autopsy material and found that with the use of CD45 there was almost a 3-fold higher inflammatory
infiltrate within the EMB sampling area compared to the remaining myocardium. This indicates that despite the risk of underdiagnosing LM in EMB, related to the patchy inflammatory infiltrate in the myocardium characteristic for LM, the endomyocardial sampling area still has the highest chance to diagnose LM. Moreover, this may indicate that the aberrations detected using CMR do not necessarily correspond to infiltrated inflammatory cells in the heart. Indeed, in a study on deceased patients, myocarditis could not be detected by post-mortem CMR in patients with autopsy proven myocarditis.

Therefore, to properly validate aberrations found with CMR in patients with LM, ideally CMR results obtained from LM patients shortly before death should be compared with pathological (immuno)histochemical analysis of the heart after autopsy.

Next to the risk of underdiagnosing LM in EMBs, as depicted above, an EMB has also the risk of potentially serious complications, such as cardiac perforation or valvular damage. Moreover, imaging diagnostics do not provide evidence of viral etiology of the disease. Therefore, new additional tools are necessary to diagnose LM. From patients with chronic heart failure it is known that degenerative alterations occur in the skeletal muscle, referred to as muscle wasting. Muscle wasting has been linked to increased levels of pro-inflammatory cytokines, indicating that the immune system has a potential role in the skeletal muscle alterations also. Interestingly, in the Atlantic salmon it was found that piscine reovirus infection of the heart coincided with heart and skeletal muscle inflammation. Moreover, several human case reports have demonstrated that cardiotropic viruses can infect not only cardiac but also skeletal muscle tissue. In Chapter 3 we studied in...
post-mortem patients with autopsy proven LM whether the quadriceps muscle would reflect cardiac inflammation also. We indeed found a significant increase of lymphocytes in the quadriceps muscle of patients with LM, with a sensitivity of 71% and specificity of 100%, suggesting that the skeletal muscle might be a peripheral ‘mirror’ of cardiac inflammation. The exact mechanism of this phenomenon however is still unknown. We and others have shown in mice models with Coxsackievirus B3 induced myocarditis that the virus can also infect the skeletal muscle causing local inflammation herein.[13, 14] Moreover, not only Coxsackievirus but also influenza virus have been shown to result in myocarditis and myositis simultaneously.[12, 15]

To extrapolate the autopsy findings that quadriceps muscle biopsy is a new diagnostic tool for LM in living patients, we are currently conducting the perspective clinical ‘Improving the diagnosis of viral myocarditis’ (INFLaMe) trial. In this trial inflammation within the quadriceps muscle biopsy will be compared with inflammation within the EMB of patients with clinically suspected LM. Moreover, viruses will be analyzed on the EMB, quadriceps biopsy, blood and feces and will be compared with MRI, ECG, an immunohistological analysis of EMB. We will also analyze putative new biomarkers in these quadriceps biopsies, blood and feces. Lastly we will also analyze the quadriceps muscle with MRI. Through this and future research we hope to identify biomarkers in the blood, feces and skeletal muscle to improve the diagnosis of viral myocarditis that at the same time form new targets for therapy.

Colchicine as treatment for viral myocarditis
Infectious agents, mainly viruses, but also bacteria, protozoans and fungi can cause myocarditis, with or without a coinciding autoimmune response.[1] The clinical presentation of myocarditis is highly diverse, varying from shortness of breath and flu-like symptoms to chest pain, heart failure and sometimes sudden death.[16-18] The highly variable etiology of LM engender that there is no specific treatment. In case of reduced cardiac function standard heart failure therapy is applied for symptom repression[1], albeit the underlying disease then is not treated. Since inflammation plays an important role in the pathogenesis of myocarditis, immunosuppressive therapy, such as prednisone, azathioprine and cyclosporine, have been explored. However, in one of the largest clinical myocarditis trials, prednisone with either azathioprine or cyclosporine, next to heart failure therapy, did not improve heart function nor mortality rates 1 year after treatment.[19] Therefore, other more specific treatment options are necessary.

Pericarditis, i.e. inflammation of the pericardium, often coincides with myocarditis[20, 21] and can also be induced by viral infection[22]. For pericarditis the immunosuppressive drug colchicine is rapidly becoming standard therapy.[23] We wondered therefore whether colchicine would also be effective in viral myocarditis. In Chapter 4 we studied colchicine therapy in mice with Coxsackievirus B3-induced myocarditis and surprisingly found that colchicine therapy induced severe illness and even mortality within three days after the mice were injected with Coxsackievirus B3. These mice also suffered from severe pancreatitis coinciding with increased virus titers in the pancreas. Increased virus titers were also detected in the heart. Colchicine did influence inflammatory cells also as the number of neutrophils increased and the number of macrophages decreased within the heart (Figure 1, number 3). In none of the colchicine trials for pericarditis in humans viral infection was
reported and no serious adverse effects related to the colchicine treatment were observed.[24] Some study limitations have to be mentioned of our mouse model. We started colchicine treatment one day after Coxsackie virus infection. Theoretically this could have prevented the initiation of a proper immune response enabling uncontrolled viral spread, replication and high viral loads. Nor can we exclude that other viruses will induce the same effects as found for Coxsackie virus B3 in mice or even humans. On the contrary, it is known that in patients with LM having a proven co-infection of Epstein-Barr virus and Cytomegalovirus, treatment with a low dose of colchicine (0.5 mg twice daily), as adjunct to conventional therapy improved heart function and no complications were found.[25] It has to be emphasized that in our mice model we used higher dose of colchicine (2 mg/kg) compared to what is used in clinical practice (0.5 mg/day)[26], however mice have a much higher metabolic rate compared to humans and therefore a higher dose is necessary. Our study thus suggests that more research is necessary to analyze the potential harm of colchicine therapy in patients with an active virus infection.

Influence of myocarditis on the cardiac vasculature
The general consensus is that although LM can mimic MI in clinical presentation, by presenting with symptoms such as chest pain, electrocardiographic ST-segment elevation, wall motion abnormalities and increased blood levels of cardiac enzymes[16, 27, 28], they are distinct clinical entities. Notwithstanding, there is also accumulating evidence suggesting that LM and MI may be related. For instance, recent respiratory tract influenza virus infections, a virus commonly associated with myocarditis, are significantly associated with the development of MI also[29, 30], while vaccination against influenza is associated a decreased risk of MI.[31] In addition, markers of infection of another group of cardiotropic viruses, i.e. enteroviruses, were detected in the hearts of 40% of patients who died of sudden MI versus only 4% of matched subjects without cardiac disease.[32] Moreover, a case report also demonstrated in a patient the co-occurrence of EMB confirmed Parvovirus B19-related myocarditis with acute MI caused by a thrombus-occluded epicardial coronary artery.[33] In line herewith, in Chapter 5 we confirmed for the first time in autopsy material that MI coincided with LM. In 50 autopsied cases, diagnosed post-mortem with LM using immunohistochemical CD45 staining, a recent MI of 3-6 hours old was diagnosed in 16 cases (32%).

In 25% of the patients with both LM and MI a thrombus in one or more of the epicardial coronary arteries was found. MI most often is the result of atherosclerotic plaque complications of which inflammation has been found to be an important mediator.[34] We subsequently analyzed whether LM has also influenced the arteriosclerotic plaque composition (Chapter 5). We found in patients with LM, just as in patients with solely MI (<6 hours old) that the inflammatory infiltrate consisted predominantly of macrophages and lymphocytes coinciding with an unstable fibrous cap of the atherosclerotic plaque (Figure 2A, number 1). Although atherosclerotic plaques in patients with both LM and MI also predominantly constituted of macrophages and lymphocytes they had an increased number of mast cells and displayed a more stable fibrous cap 2. Interestingly, the prevalence of this more stable plaques in LM with MI patients might suggests that LM increases the likelihood of developing MI despite lower unstable plaque burden. Moreover, both LM and LM with MI patients presented intraplaque hemorrhage and thrombi. However, patients with both LM and MI developed more thrombi compared to MI alone, suggesting that the LM
might induce thrombi formation and thereby the MI. One plausible cause of the thrombi in patients with LM is that the risk of thrombotic plaque complication may be higher in LM patients as LM can induce a hypercoagulable state as part of the antiviral immune response.[35, 36] The presence of plaque hemorrhage in patients with LM may be explained by vasospasm within coronary arteries.[37] Vasospasms indeed have been reported to occur in myocarditis patients.[38] The increased number of mast cells could be a possible explanation for this, since mast cells have been implicated in provoking coronary spasms.[39, 40] Our findings thus suggest that LM can precipitate acute MI by destabilization of atherosclerotic plaques in the epicardial coronary arteries. How our autopsy findings regarding the prevalence of MI coinciding with LM translate to the clinic is speculative at present. In patients who present with evidence of MI but angiographically normal coronary arteries, estimated to occur in 6% of patients who present with evidence of MI, myocarditis was diagnosed in 33% of patients based on the subepicardial location of CMR abnormalities versus 24% subendocardial MI.[41] Nonetheless, in the present study we also found LM in patients with a thrombus in the epicardial coronary artery. In the clinic these patients would be classified as having MI and putative co-occurrence of LM would most likely remain undiagnosed.

The study depicted in Chapter 5 clearly argues that myocarditis changes the composition of the inflammatory infiltrate within epicardial coronary arteries. In general blood vessels of the heart, both epicardial coronary arteries and intramyocardial microvasculature play a role in myocarditis. They not only provide a natural barrier function to prevent blood borne pathogens entering the heart but at the same time they are also a prime target for infection. The importance of structural and functional changes of the cardiac vasculature in patients with infectious myocarditis as known form the literature is discussed in a review article in Chapter 6.

**MYOCARDIAL INFARCTION (MI)**

Inflammation not only can induce atherosclerotic plaque complications resulting in MI, but also plays an important role post-MI.[42] After MI inflammatory cells namely infiltrate the infarcted area to remove dead cells and to repair the myocardium. Neutrophilic granulocytes infiltrate the infarcted heart within 24 hours post-MI and monocytes/macrophages shortly thereafter.[43] Pro-inflammatory monocytes/macrophages dominate on days 1 to 4 and promote digestion of infarcted tissue and removal of necrotic debris, whereas anti-inflammatory monocytes/macrophages dominate during the resolution of inflammation on days 4 to 8 and propagate repair.[44] Even more, a strong inflammatory response during the first week post-MI also causes additional damage to cardiomyocytes, resulting in an increased infarct size and adverse ventricular remodeling.[45, 46] In general, the first two weeks post-MI the inflammatory response is the most active in wound healing and additional therapies in this period may prevent putative heart failure development.
**Figure 2. A)** The influence of lymphocytic myocarditis on the atherosclerotic plaque within the epicardial coronary artery with MI. **B)** The influence of StemBells therapy after MI on the inflammation within the atherosclerotic plaque and within the infarct area. See text for explanation of the numbers.

**StemBell therapy for MI**

An important therapeutic option to restore the loss of cardiomyocytes is stem cell therapy, such as adipose derived stem cells (ASC). ASCs have the advantage that they can be easily harvested and can differentiate towards several cell types, including cardiomyocytes.[47] Post-MI ASC therapy was shown to improve cardiac function in several pre-clinical studies.[48-50] One of the major problems of stem cell therapy however is a lack of engraftment of sufficient stem cells at the site of injury.[48, 50] Therefore, in Chapter 7 we designed a novel targeting technique to improve the engraftment of the administrated ASC by coupling them to duel-targeted microbubbles and using ultrasound, the so-called StemBell technique (Figure 2B, ③). Microbubbles are small (2-4 μm) ultrasound-sensitive
gas-filled bubbles that were originally developed as contrast agents for echocardiography.[51] We coated these microbubbles with an antibody against the ASC cell surface marker CD90 and with an antibody against Intercellular Adhesion Molecule 1 (ICAM-1) that is expressed on activated endothelium of blood vessels within the infarct area.[52] The microbubbles were then complexed to the ASC to create StemBells. By doing so, the StemBells acquired susceptibility to the acoustic radiation force exerted by diagnostic ultrasound and in Chapter 7 we indeed have shown \textit{in vitro} that StemBells can be pushed against the vessel wall with the use of ultrasound, improving the opportunity for the ASCs to infiltrate the infarct area.

To study the potential therapeutic effect of StemBells \textit{in vivo} we compared StemBell and ASC therapy in a rat model. As administration of ASC during the first days post-MI might result in cell death of the ASC due to the harsh environment of the infarcted area[48, 53], we administrated StemBell, ASC and vehicle therapy 7 days post-MI. We showed the StemBells could be injected safely and that they reached the infarct area. However, there was no difference in the number of engrafted stem cells within the myocardium between StemBell and ASC therapy. Moreover, StemBell or ASC therapy did not significantly reduce infarct size nor did it influence macrophages within the infarct area compared to the vehicle treated group. However, in contrast to ASC therapy, StemBell therapy did significantly improve cardiac function, measured via fractional shortening and stroke volume using \textit{echocardiography}. In addition, StemBell therapy prevented significant thickening of the heart compared to ASC and vehicle therapy. This thus demonstrates the potential of the novel and feasible targeting StemBell technique to improve cardiac function post-MI.

We can only speculate how StemBells improve cardiac function. We did find cardiomyocyte specific markers Connexin 43 and Troponin-T on retraced stem cells in the heart, which might be indicative for partial differentiation towards cardiomyocytes[54, 55], although these numbers were extremely low, making it unlikely that differentiation is a key mechanism for the improved cardiac function. Moreover, the paracrine effect of ASC might play a crucial role. ASC namely can secrete growth factors, cytokines and signaling molecules that contribute to angiogenesis[56, 57], inhibition of apoptosis[57] and reduction of the inflammatory response[58-60].

As mentioned above, during the first week post-MI a strong inflammatory response is induced in the heart. Nevertheless, several studies have also shown positive cardiac regenerative effects of stem cell therapy administrated during the first day post-MI.[55, 61, 62] As theoretically stem cell therapy applied at day one post-MI could also protect against the inflammatory response at that time point, protecting putative cell death of cardiomyocytes, we compared in \textbf{Chapter 8} StemBell therapy applied 1 day with 7 days post-MI in rats. Interestingly, StemBell therapy at day 1 post-MI had comparable effects on infarct size and inflammatory content of the infarct area as applied at day 7 post-MI. Moreover, StemBell therapy on day 1 had the same cardiac function improvements five weeks post-MI as StemBell therapy administrated at day 7. Even more, StemBell therapy applied 1 day post-MI already resulted in improved cardiac function on day 7 post-MI and remained improved. Since on the long term the cardiac effects were similar with StemBell therapy administrated on day 1 or day 7 they can be given at either time point.
General discussion

StemBell therapy for atherosclerosis post-MI

It is known that post-MI the atherosclerotic plaque can grow more rapidly due to increased inflammatory cell content inside the plaque, further destabilizing the plaque as found in animal models[63-65], that theoretically could induce re-infarction. Re-infarction is a common event in MI patients, which is shown to occur in 54% of patients within the first year after MI.[66] Hence, new therapeutic strategies to prevent this are warranted. Animal studies have shown favorable results of mesenchymal stem cell therapy on the stability of the atherosclerotic plaques.[67-71] Plaque stability depends on a thick fibrous cap and/or a low amount of inflammatory cells, especially macrophages.[72] Mesenchymal stem cell namely improved cap thickness[67-69], reduced intraplaque macrophage number[69, 70], increased levels of anti-inflammatory macrophages in the adventitia of the aortic root[71] and reduced pro-inflammatory cytokines.[68] However, all these studies were not performed in relation with MI.

In Chapter 9 we studied StemBell therapy applied 6 days post-MI on the atherosclerotic plaques of the aortic root in an atherosclerotic model of apolipoprotein E-deficient mice. The StemBell were targeted to ICAM-1 which is expressed, next to the infarcted area, on activated endothelial cells of atherosclerotic plaques.[52] We found that StemBell therapy increased the stability of the atherosclerotic plaque, by increasing the cap thickness (Figure 2B, number 5), decreasing the total number of macrophages and increasing the percentage of anti-inflammatory macrophages content within the atherosclerotic plaque 6 28 days after MI. Even more, within the circulation and the infarcted heart 28 days post-MI an increased percentage of respectively anti-inflammatory monocytes 7 and macrophages 8 was found. StemBell treatment in this mouse model however did not have an effect on cardiac function, infarct size nor cholesterol and triglycerides levels 28 days post-MI. Thus, StemBells may become a promising therapy to prevent re-infarction post-MI.
CONCLUSIONS

In this thesis, we studied the role of inflammation in LM and MI towards improving both diagnostic and treatment options.

In LM we found increased inflammation within the quadriceps muscle implying that pathological analysis of skeletal muscle biopsies may be a viable alternative for EMB to diagnose myocarditis. Moreover, the immunohistological diagnosis of LM can be improved by staining CD45 lymphocytes within the heart, since we found that the inflammatory infiltrate consists predominately of CD45 compared to CD3 positive lymphocytes. Importantly, in contrast to the widely held belief that LM and MI are distinct clinical entities, we found that LM can coincide with MI and that LM, or its viral cause, may precipitate the occurrence of MI. This may be facilitated through intensified inflammation, bleeding and instability coronary atherosclerotic plaques.

Cardiac tissue loss and inflammation are important denominators of the outcome after MI. Moreover, inflammation also accelerates atherosclerosis after MI, which heightens the chance of re-infarction. We aimed to improve stem cell therapy through the development of a targeted stem cell technique; StemBell therapy. Intravenously administered StemBells after experimental MI in animal models resulted in long-term improved cardiac function, prevention of maladaptive cardiac remodeling in rats, and decreased MI-induced atherosclerotic plaque instability in mice. All this was most likely related to the cell-protective and immunomodulating paracrine effector function of ASC. Therefore, StemBell therapy may be a promising therapy for MI.
REFERENCES


Summary
Nederlandse samenvatting (Summary in Dutch)
List of publications
Curriculum vitae
Dankwoord (Acknowledgements)
Summary

Chapter 1: Introduction
Worldwide cardiovascular disease is the number one cause of death. Both myocardial infarction (MI) and infectious inflammation of the heart muscle, i.e. lymphocytic myocarditis (LM), are important contributors to cardiovascular mortality. Inflammation plays a pivotal role in the pathogenesis of viral myocarditis and in the induction and the outcome of MI. This thesis describes inflammation both in viral myocarditis and MI aiming at better understanding of its mechanisms in search of improving and therapy.

In general myocarditis is defined as inflammation (itis) of the heart muscle (myocardium). The cause of myocarditis can be infectious agents such as viruses, bacteria, protozoans and fungi, but also an autoimmune reaction. In Europe most cases of myocarditis are caused by a viral infection. Due to the large varieties in clinical presentation, viral myocarditis is a complicated clinical entity and is difficult to diagnose. Moreover, to date there is a lack of specific therapies to treat viral myocarditis. Therefore, there is a need for a better understanding of the pathogenesis of viral myocarditis to enable the development of improved diagnostic tools and potential new therapies.

A MI is usually an acute event as a result of an obstruction within the coronary arteries that supply the heart with oxygenized blood. Such obstructions are generally the result of erosion, inflammation and/or rupture of atherosclerotic plaques in the coronary artery leading to blood clot formation. The clinical presentation of MI is more clear than that of myocarditis. The diagnosis of MI is based on specific changes in electrocardiogram, biomarkers, echocardiography and angiography. Although treatment of MI has improved significantly in recent years there still is a demand for improved therapies to prevent loss of cardiomyocytes and/or to regenerate myocardial tissue.

Chapter 2: For the diagnosis of lymphocytic myocarditis CD45 is more sensitive than CD3
The gold standard for diagnosing LM is the pathological examination of endomyocardial biopsies (EMB). In these heart biopsies various inflammatory cells can be detected via immunohistochemistry. To diagnose LM with immunohistochemical stainings clear guidelines have been suggested: in the EMB ≥14 leukocytes per mm² need to be present, composed of ≥7 (CD3-positive) T-lymphocytes and maximum 4 macrophages per mm². We hypothesized that a more common leukocyte marker, CD45, instead of CD3 could increase the diagnostic sensitivity. In hearts of mice with acute viral myocarditis we found that, using the guidelines of ≥14 leukocytes per mm², only 33% of the mice classified for the diagnosis of LM with the CD3 T-lymphocyte marker, while 89% classified with the CD45 lymphocyte marker. Also in human autopsy material of deceased patients with proven LM we found that the diagnosis of LM could be increased from 17% to 50% with the use of CD45 instead of CD3 in the EMB sampling area using the guideline of ≥14 leukocytes per mm². Thus, the use of the common leukocyte marker CD45 increases the sensitivity of the diagnosis of LM. Moreover, we also found more inflammatory cells within the EMB area compared with the rest of the ventricular wall, indicating that the endomyocardial sampling area constitutes the highest chance for positive histological diagnosis of LM.
Chapter 3: Lymphocytes infiltrate the quadriceps muscle in lymphocytic myocarditis patients: A potentially new diagnostic tool

As described above, the gold standard to diagnose LM is via endomyocardial biopsies (EMB). Since EMBs are small there is a chance that the inflammation is not present within the EMB (sampling error). Moreover, with the collection of EMBs there is a small risk of potentially serious complications, such as cardiac perforation and valvular damage. Taken together new diagnostic methods are necessary to improve the diagnosis of LM. We know that in other diseases if the heart changes in the heart muscle may sometimes be reflected in other muscles also. In this chapter we examined whether the inflammatory cells in a biopsy of the quadriceps skeletal muscle can serve as a potential new diagnostic tool for LM. In autopsy material of patients with proven LM and of healthy controls various inflammatory cells were quantified within a biopsy of the quadriceps muscle. Compared to the controls, LM patients had significant more lymphocytes within the quadriceps muscle. Using the number of lymphocytes it was possible to diagnose LM with a specificity of 100% and a sensitivity of 71%. This study clearly shows that immunohistochemistry on a biopsy of the upper leg thigh muscle is a potential new method of diagnosing LM.

Chapter 4: Colchicine aggravates coxsackievirus B3 infection in mice

Currently there is no specific therapy for patients with LM. For LM patients that have reduced cardiac function, treatment usually consists of supportive therapy and cardiovascular stabilization therapy. Although the standard heart failure therapy is useful in symptom repression and preventing on-going decline of cardiac function, the underlying disease is not treated (the LM). Therefore, there is a clinical need to find effective treatments for LM. In this chapter we investigated the effect of the immunosuppressive drug colchicine, that is often used to treat patients with pericarditis i.e. inflammation of the pericardium, as treatment option for LM. We treated mice with colchicine that had acute coxsackievirus B3 induced myocarditis. We found that colchicine treatment rapidly resulted in severe disease symptoms, such as weight loss, inactivity and in some cases even led to death. Tissue investigation revealed that colchicine treatment induced massive degeneration of the pancreatic acini, which was most likely the cause of the severe disease symptoms. Moreover, we found in the heart that colchicine treatment induced an increased the number of neutrophils and decreased the number of macrophages. It was also found that in mice with coxsackievirus B3-induced myocarditis, that colchicine treatment, in contrast to placebo treatment increased the level of coxsackievirus B3 in both the heart and the pancreas.

Chapter 5: Lymphocytic myocarditis coincides with myocardial infarction and concurs with increased inflammation, hemorrhage and instability in coronary artery atherosclerotic plaques

Patients with LM can present with clinical symptoms similar to MI. In clinical practice, LM is only considered as a potential underlying cause of infarct-like complaints when MI is ruled out, based on the absence of coronary artery narrowing or obstruction. The general consensus is that although LM and MI can be similar in clinical presentation they are distinct clinical entities. In this chapter we have observed in clinical autopsies a high prevalence (32%) of very recent MI coinciding with LM in a cohort of patients diagnosed post mortem with LM. To investigate whether LM affects coronary atherosclerotic plaques, we analyzed the inflammatory infiltrate and stability in coronary atherosclerotic lesions in autopsied patients.
Summary

patients with LM and/or MI. Compared to controls, patients with LM or MI showed increased numbers of macrophages and neutrophils in the tissue sections of the coronary segments, while patients with both LM and MI or MI alone showed an increase in lymphocytes and mast cells. Moreover, in patients with both LM and MI or MI alone, this coincided with an increase of unstable plaques and thrombi. Finally, LM patients showed more intraplaque hemorrhage than controls, which was even more pronounced in patients with MI, with and without LM. This study demonstrates prevalent co-occurrence of LM with a very recent MI at autopsy, and inducibility of inflammation and remodeling of the atherosclerotic plaques by LM.

Chapter 6: Infectious myocarditis: the role of the cardiac vasculature

In this chapter we reviewed the existing literature regarding the role of infectious myocarditis on the vessels of the heart. First we found that the blood vessels of the heart, both the intramyocardial microvasculature and the large epicardial coronary arteries, play an important role in the pathogenesis of infectious myocarditis. Moreover, endothelial cells, which form the inner lining of the heart vessels, are direct targets for infection. The blood vessels assist in shaping the cellular immune response in infectious myocarditis through the expression of adhesion molecules and antigen presenting Major Histocompatibility Complex molecules. In addition, damage and dysfunction of the cardiac (micro)vasculature are associated with thrombus formation as well as aberrant regulation of vascular tone including coronary vasospasm. These in turn can cause cardiac perfusion abnormalities and even MI.

Chapter 7: Development of a new therapeutic technique to direct stem cells to the infarcted heart using targeted microbubbles: StemBells

Stem cell therapy has been proposed as a promising therapy for regenerative tissue repair and to prevent heart failure development after MI. One of the major problems of stem cell therapy is lack of engraftment of sufficient stem cells at the site of injury. To overcome this problem we designed a novel targeting technique by assembling adipose-derived stem cell-microbubble complexes, named ‘StemBells’. Microbubbles are small gas-filled bubbles originally developed as contrast agents for echocardiography. In this research the microbubbles were labeled with an antibody against CD90 to bind them to the CD90 marker of the adipose-derived stem cells. Additionally, the microbubbles were labelled with an ICAM-1 antibody. ICAM-1 is expressed on activated endothelium of blood vessels within the infarct area, allowing the StemBells to theoretically target the infarct area. To investigate the therapeutic effect of StemBells rats were injected with StemBells 7 days after MI. We proved that is was possible to inject StemBells safely. Moreover, StemBells were found, albeit in small numbers, within the infarct area. Using echocardiography we also found that StemBell therapy induced improved cardiac function on the long term (5 weeks after therapy). This functional improvement neither coincided with a reduction in infarct size nor with a change in anti- and pro-inflammatory macrophages within the infarct area. This study shows that StemBell technology is a novel method to improve cardiac function after MI.

Chapter 8: A comparison in therapeutic efficacy at several time points of intravenous StemBell administration in a rat model of acute myocardial infarction

StemBell therapy is a promising new therapeutic option to improve cardiac function after MI (see chapter 7). MI leads also cell death and inflammation in the heart. This inflammatory
response is beneficial, since inflammatory cells remove the dead cells from the infarcted area allowing the heart to recover. However, this response may be unfavorable for the survival rate of stem cells entering the infarct area. Therefore, we initially chose to administrate StemBell therapy 7 days after MI. However, since 7 days after MI a lot of damage is already initiated there may be more therapeutic effect when StemBell therapy is given earlier. In this study we compared the efficacy of StemBell therapy in rats 1 and 7 days after MI. Using echocardiography, we observed that StemBell therapy given 1 day after MI improved cardiac function already at one week after MI. At 6 weeks after MI StemBells administrated either 1 or 7 days after MI resulted in a similar improvement of cardiac function. Furthermore, there was no difference in infarct size or the number of pro- and anti-inflammatory macrophages in the infarct area between the different times of StemBell administration. Thus, StemBell therapy leads independently from the time of administration to prolonged improvement in cardiac function and can be administrated either 1 or 7 days after MI.

Chapter 9: StemBell therapy stabilizes atherosclerotic plaques after myocardial infarction

After MI the development of atherosclerotic plaques is accelerated and the amount of inflammation (mainly consisting of macrophages) is increased within the plaque, leading to destabilization of the atherosclerotic plaque. In theory, stem cell therapy is a therapeutic option for atherosclerosis. In this chapter we investigated the use of StemBell therapy on the atherosclerotic plaque after MI. Mice with atherosclerosis received StemBell or vehicle treatment 6 days after MI. Compared to the vehicle group, StemBell therapy resulted in a thicker (more stable) fibrous cap of the aortic plaque and a decreased number of macrophages within the plaque 4 weeks after MI. Additionally, StemBell treatment induced an increased percentage of anti-inflammatory macrophages both in the atherosclerotic plaques and in the infarct area of the heart, coinciding with a trend to increased percentage of anti-inflammatory monocytes within the blood. Moreover, we found that the effect of StemBells on atherosclerosis was independent of cholesterol and triglyceride levels, since these were similar in the blood after both treatments. Furthermore, StemBell treatment did not affect cardiac function nor infarct size 4 weeks after MI. Since StemBell therapy did result in more anti-inflammatory macrophages in the tissue and a trend towards more anti-inflammatory monocytes in the blood this point to a systemic effect. Thus, in atherosclerotic mice after MI, StemBell therapy not only has a positive result on the development of the atherosclerotic plaque, but also on the inflammation within the infarct area.

Chapter 10: Conclusion

In this thesis, we studied the role of inflammation in LM and MI in search of improving both diagnostic and treatment options.

In LM we found that increased inflammation was not limited to the heart, but was also found in the atherosclerotic plaques of the coronary arteries and even within the skeletal quadriceps muscle. The increased inflammation in the skeletal quadriceps muscle may be a viable alternative for EMB to diagnose myocarditis. Moreover, the immunohistological diagnosis of LM can be improved by staining CD45 lymphocytes within the heart, since we found that the inflammatory infiltrate consists predominately of CD45 compared to CD3 positive lymphocytes. Importantly, we have provided evidence that LM can coincide with MI.
and that LM, via destabilization effects on atherosclerotic plaques, may facilitate MI development.

After MI an inflammatory response develops within the heart and the atherosclerotic plaques of the coronary arteries. We have developed a so-called StemBells technique that not only improves inflammation in the atherosclerotic plaque and the infarct area, but also improves cardiac function. Therefore, StemBell therapy may be a promising therapy after MI.
Nederlandse samenvatting

Inflammatie in hartziekten: 
De verbetering van diagnose en nieuwe therapieën

Hoofdstuk 1: Introductie
Hart- en vaatziekten zijn wereldwijd verantwoordelijk voor een belangrijk aantal doodsoorzaken. Zowel het hartinfarct, ofwel myocard infarct (MI), als een infectieuze ontsteking van de hartspier, ofwel lymfocytair myocarditis (LM), behoren tot deze doodsoorzaken. Ontsteking speelt als mechanisme een belangrijke rol bij zowel LM als bij MI. In dit proefschrift hebben wij de ontsteking bij LM en MI bestudeerd om de diagnostiek te verbeteren en om nieuwe therapieën te vinden.

In het algemeen wordt myocarditis gedefinieerd als een ontsteking (itis) van de hartspier (myocardium). De oorzaak van myocarditis kan een infectie zijn, bijvoorbeeld door virusen, bacteriën en schimmels, maar kan ook veroorzaakt worden door een auto-immuunreactie. In Europa wordt een infectieuze myocarditis meestal veroorzaakt door virusen. Door de grote diversiteit in klinische presentatie is het diagnosticeren van LM moeilijk. Bovendien is er tot op heden geen specifieke therapie voor LM. Het is daarom belangrijk beter te begrijpen hoe LM ontstaat zodat er adequatere diagnostische hulpmiddelen en mogelijk nieuwe therapieën ontwikkeld kunnen worden.

Een MI is meestal het gevolg van een obstructie van de kransslagader die het hart van zuurstofrijk bloed voorziet. Dergelijke obstructies kunnen het gevolg zijn van een zogenaamde erosie, ontsteking en/of ruptuur van de atherosclerotische plaque met als gevolg dat zich in de kransslagader een bloedstolsel ontwikkelt. De klinische presentatie van MI is duidelijker dan bij LM. De diagnose van MI is gebaseerd op specifieke veranderingen van elektrocardiogram, biomarkers, echocardiogram en angiogram. Hoewel de behandeling van MI de afgelopen jaren aanzienlijk is verbeterd, is er nog steeds vraag naar therapieën om het verlies van hartweefsel te voorkomen en hartspier regeneratie te verbeteren.

Hoofdstuk 2: Voor de diagnose van lymfocytaire myocarditis is CD45 gevoeliger dan CD3
De gouden standaard om LM te diagnosticeren is pathologische onderzoek van endomyocard (hart) biotpien. In deze hartbiotpien kunnen middels immuunhistochemie verschillende ontstekingscellen aangetoond worden. Om LM te diagnosticeren met behulp van deze immuunhistochemische kleuringen zijn duidelijke richtlijnen opgesteld; in het endomyocard biop tieven ≥14 ontstekingscellen per mm² aanwezig te zijn, bestaande uit ≥7 (CD3 positieve) T-lymfocyten en maximaal 4 macrofagen. Onze hypothese was dat de ontstekingsmarker CD45 veel beter LM kon diagnosticeren dan CD3. In harten van muizen met virus geïnduceerde myocarditis hebben wij met behulp van de richtlijn van ≥14 ontstekingscellen per mm² gevonden dat zich slechts 33% classificeerde voor de diagnose LM bij het gebruik van de CD3 T-lymfocyt marker, en 89% bij het gebruik van de CD45 lymfocyt marker. Ook in humaan obductie materiaal van overleden patiënten met bewezen LM vonden wij dat de diagnose van LM met behulp van de ≥14 ontstekingscellen per mm² richtlijn, in het bioptiegebied verhoogd kon worden van 17% naar 50% bij het gebruik maken van CD45 in plaats van CD3. Het gebruik van de CD45 lymfocyt marker verhoogt
Hoofdstuk 3: Infiltratie van ontstekingscellen in de bovenbeen dijspier bij mensen met lymfocytaire myocarditis: een potentiële nieuwe diagnostische methode

Zoals boven beschreven is de gouden standaard om LM te diagnosticeren het afnemen van endomyocard (hart) biopten. Aangezien dit kleine biopten zijn is er een kans dat de ontsteking zich niet in het hartbiopt bevindt. Bovendien is het afnemen van een hartbiopt niet geheel zonder risico, aangezien er een kleine kans is op potentieel ernstige complicaties, zoals hartspierperforatie en schade aan de hartkleppen. Dit alles maakt dat nieuwe diagnostische methodes nodig zijn voor het verbeteren van de diagnose van LM. Van andere hartziekten is bekend dat veranderingen in de hartspier soms ook in andere spieren terug te vinden zijn. In dit hoofdstuk is onderzocht of het aantonen van ontstekingscellen in de bovenbeen dijspier een potentiële nieuwe methode is om LM te diagnosticeren. In obductie materiaal van patiënten met bewezen LM en van gezonde controles zijn verschillende ontstekingscellen gekwantificeerd in de bovenbeen dijspier. Ten opzichte van de controles bevonden zich in de patiënten met LM aanzienlijk meer lymfocyten. Het aantal lymfocyten in de bovenbeen dijspier kon LM diagnosticeren met een specificiteit van 100% en een gevoeligheid van 71%. Deze studie toont duidelijk aan dat immunohistochemie op biopten van de bovenbeen dijspier een potentiële nieuwe methode is om LM te diagnosticeren.

Hoofdstuk 4: Colchicine verergert coxsackievirus B3 infectie in muizen

Momenteel is er geen specifieke therapie voor patiënten met LM. Indien LM patiënten een verminderde hartfunctie hebben worden ze vaak behandeld met ondersteunde medicijnen voor de stabilisatie van het hartfalen. Hoewel dit nuttig is om de symptomen te onderdrukken en de hartfunctie te verbeteren, behandelen ze niet specifiek de onderliggende oorzaak (de LM). Er is dus een klinische noodzaak om effectieve therapieën te vinden om LM te behandelen. In dit hoofdstuk is onderzocht of het immunosuppressieve geneesmiddel colchicine, dat veelal gebruikt wordt bij patiënten met pericarditis (ontsteking van de buitenkant van het hart), gebruikt kan worden bij LM. Hiervoor hebben wij muizen met coxsackievirus B3 geïnduceerde myocarditis behandeld met colchicine. Wij vonden dat colchicine therapie ernstige ziekteverschijnselen vertoonden, zoals gewichtsverlies, inactiviteit en in sommige gevallen zelfs leidend tot de dood. Uit weefselonderzoek bleek dat colchicine therapie in de alvleesklier resulteerde in de vernietiging van de acini, waardoor de muizen hoogstwaarschijnlijk deze ernstige ziekteverschijnselen ontwikkelden. Daarnaast zagen wij in het hart dat colchicine behandeling resulteerde in een verhoging van het aantal neutrofielen en verlaging van het aantal macrofagen. Tevens bleek dat colchicine in tegenstelling tot de placebo behandeling in muizen met coxsackievirus B3 geïnduceerde myocarditis, leidde tot verhoging van het coxsackievirus B3 level in zowel het hart als de alvleesklier.
Hoofdstuk 5: Lymfocytaire myocarditis treedt gelijktijdig op met een hartinfarct en gaat gepaard met verhoogde ontsteking, bloeding en instabiliteit van de atherosclerotische plaques in de kransslagader

Patiënten met LM kunnen ook klinische symptomen hebben die passen bij MI. In de praktijk wordt LM alleen beschouwd als mogelijke oorzaak van infarctklachten wanneer MI is uitgesloten op basis van afwezigheid van een obstructie in de kransslagaders. De algemene consensus is dat hoewel LM en MI vergelijkbare klinische symptomen kunnen hebben, ze beschouwd worden als afzonderlijke ziektebeelden. In dit hoofdstuk hebben wij in obductie patiënten met een recente MI aangetoond dat er een hoge prevalentie is (32%) van patiënten die gelijktijdig een LM vertonen. Om te onderzoeken of LM invloed heeft op atherosclerotische plaques is gekeken naar ontsteking en stabiliteit van de atherosclerotische plaques in de kransslagaders van patiënten met LM en/of recente MI. In kransslagaders van patiënten met alleen LM of MI was een toename te zien van macrofagen en neutrofielen ten opzichte van controle patiënten, terwijl in patiënten met zowel LM als MI of alleen MI, een toename te zien was van lymfocyten en mastcellen. Bovendien hadden patiënten met zowel LM en MI, als met alleen MI een toename van het aantal onstabiele plaques en bloedstolsels. Ten slotte hadden patiënten met LM een verhoogd aantal plaque bloedingen ten opzichte van controles en hadden patiënten met MI, al dan niet gepaard met LM, nog een groter aantal plaque bloedingen. Deze studie toont dus aan dat LM gelijktijdig met MI kan optreden en dat LM kan leiden tot ontsteking en veranderingen in de atherosclerotische plaque.

Hoofdstuk 6: Infectieuze myocarditis: de rol van het vaatstelsel in het hart

In dit hoofdstuk hebben wij de bestaande literatuur gereviewd met betrekking tot de rol van infectieuze myocarditis op de vaten van het hart. Allereerst hebben we beschreven dat de bloedvaten van het hart, zowel de kleine bloedvaten als de grote kransslagaders, een belangrijke rol spelen in de pathogenese van infectieuze myocarditis. Zo zijn endotheelcellen, die de binnenste bekleding vormen van de hartvaten, het directe doelwit voor infectie. Daarnaast spelen de bloedvaten door middel van de expressie van adhesiemoleculen een rol bij de vorming van de cellulaire immuunrespons bij infectieuze myocarditis. Bovendien wordt de schade en dysfunctie van vaten in het hart geassocieerd met de vorming van bloedstolsels en vasospasmen in de kransslagaders. Deze kunnen op hun beurt leiden tot perfusie problemen en zelfs MI.

Hoofdstuk 7: De ontwikkeling van een nieuwe therapeutische techniek om stamcellen naar het geinfarceerde hart te sturen met behulp van microbellen: StemBells

Stamceltherapie wordt gezien als een veelbelovende therapie voor weefsel regeneratie om de ontwikkeling van hartfalen na een MI te voorkomen. Eén van de belangrijkste belemmeringen van stamceltherapie is het lage percentage stamcellen dat het infarctgebied bereikt en daar weet te overleven. Om dit probleem te verhelpen hebben wij een nieuwe techniek ontwikkeld waarbij vetstamcellen gekoppeld worden aan gelabelde microbellen ofwel ‘StemBells’. Microbellen zijn kleine gasbelletjes die oorspronkelijk zijn ontwikkeld als contrastmiddel voor echocardiografie. De microbellen werden in dit onderzoek gelabeld met een antilichaam dat ervoor zorgt dat ze hechten aan CD90 marker op de stamcellen. Daarnaast werden de microbellen gelabeld met een ICAM-1 antilichaam. ICAM-1 komt tot expressie in het infarctgebied, waardoor de StemBells theoretisch gestuurd kunnen worden.
naar het infarctgebied. Om het therapeutische effect van StemBells te onderzoeken zijn ratten 7 dagen na een MI geïnjecteerd met StemBells. Het bleek mogelijk om StemBells veilig te injecteren. Bovendien werden ze in kleine hoeveelheden teruggevonden in het infarctgebied. Met behulp van echocardiografie zagen wij dat StemBell therapie op langere termijn (5 weken na therapie) een betere hartfunctie induceerde. StemBell therapie had geen effect op de infarctgrootte noch op het aantal pro- en anti-inflammatoire macrofagen in het infarctgebied. Deze studie toont aan dat de StemBell techniek een nieuwe methode is om de hartfunctie na MI te verbeteren.

**Hoofdstuk 8: Analyse van het effect van toediening van StemBells op verschillende tijdstippen in een rat model met een acuut hartinfarct**

StemBell therapie is een nieuwe therapie om de hartfunctie na MI te verbeteren (zie *hoofdstuk 7*). Als gevolg van een MI ontstaat in het hart celdood en ontsteking. Deze ontstekingsreactie is gunstig aangezien ontstekingscellen de dode cellen verwijderen uit het infarctgebied zodat het hart kan herstellen. Dit zou echter ongunstig kunnen zijn voor de overlevingskans van stamcellen die het infarctgebied binnentreten. Daarom is in eerste instantie gekozen om StemBell therapie te geven 7 dagen na MI. Echter aangezien er binnen 7 dagen na MI al veel schade is opgetreden, is er eventueel nog meer ruimte voor verbetering. Daarom hebben wij onderzocht wat het verschil in effect is van StemBell therapie 1 en 7 dagen na MI in ratten. Met behulp van echocardiografie zagen wij dat StemBell therapie 1 dag na MI zorgde voor een verbeterde hartfunctie één week na MI. Echter, was 6 weken na MI geen verschil in hartfunctie tussen de twee tijdstippen van StemBell toediening. Ook was er geen verschil in infarctgrootte en het aantal ontstekingsremmende en -stimulerende macrofagen in het infarctgebied tussen de verschillende tijdstippen van StemBell therapie. StemBell therapie leidt dus onafhankelijk van het tijdstip van toediening tot langdurige verbetering van de hartfunctie en kan worden toegepast op zowel 1 of 7 dagen na MI.

**Hoofdstuk 9: StemBell therapie verbetert atherosclerose na een hartinfarct**

Het is bekend dat na een MI de atherosclerotische plaque sneller groeit en de hoeveelheid ontsteking (voornamelijk bestaande uit macrofagen) in de plaque toeneemt, hetgeen kan resulteren in een destabilisatie van de plaque. Theoretisch is stamceltherapie een therapeutische optie bij atherosclerose. In dit hoofdstuk hebben wij onderzocht of StemBell therapie na MI effect heeft op atherosclerose. Hiervoor kregen muizen met atherosclerose StemBell of placebo therapie 6 dagen na MI. Vergeleken met de placebo groep had StemBell therapie vier weken na MI gezorgd voor een dikkere (stevigere) buiten laag van de plaque en er bevonden zich minder macrofagen in de plaque. Daarnaast bevonden zich in de atherosclerotische plaque, maar ook in het infarctgebied en zelfs in de bloedbaan in verhouding meer ontstekingsremmende dan -stimulerende macrofagen/monocyten, wat kan wijzen op een versnelde genezing na MI. Bovendien vonden wij dat het effect van StemBells op atherosclerose onafhankelijk was van cholesterol en triglyceriden, aangezien deze spiegsels gelijk waren in het bloed. Daarnaast had StemBell therapie geen invloed op infarctgrootte en hartfunctie, maar aangezien StemBell therapie wel zorgde voor meer ontstekingsremmende macrofagen in het weefsel en een trend naar meer monocyten in het bloed wat kan wijzen op een systemisch effect. StemBell therapie na MI in
atherosclerotische muizen heeft dus niet alleen een positief resultaat op de ontwikkeling van de atherosclerotische plaque, maar ook op de ontsteking in het infarctgebied.

**Hoofdstuk 10: Conclusies**

In dit proefschrift hebben wij de rol van ontsteking in LM en MI bestudeerd om zowel diagnostische als therapeutische opties te verbeteren.

In LM hebben wij gevonden dat de verhoogde ontsteking zich niet beperkt tot het hart, maar ook kan worden teruggevonden in de atherosclerotische plaques van kransslagaders en zelfs in de bovenbeen dijspier. De verhoogde ontsteking in de bovenbeen dijspier is derhalve een potentiële nieuwe diagnostische optie voor het aantonen van LM. Bovendien kan de immunohistologische diagnose van LM worden verbeterd door het aantal CD45 positieve cellen te kwantificeren in het hart, aangezien wij hebben ontdekt dat het ontstekingsinfiltraat van het hart vooral bestaat uit CD45 lymfocyten in plaats van CD3 positieve lymfocyten. Daarnaast hebben we aangetoond dat LM gelijktijdig kan optreden met MI en dat LM, via destabilisatie van de atherosclerotische plaque, de ontwikkeling van MI kan vergemakkelijken.

Na een hartinfarct ontstaat een ontstekingsreactie in het hart en in de atherosclerotische plaque van de kransslagaders. Wij hebben een zogenaamde StemBell techniek ontwikkeld die niet alleen de ontsteking verbetert in de atherosclerotische plaque en in het infarctgebied, maar die ook de hartfunctie verbetert. Derhalve is StemBell therapie een veelbelovende therapie na een hartinfarct.
List of publications


Woudstra L, Juffermans LJ, van Rossum AC, Niessen HW, Krijnen PAJ. Infectious myocarditis: the role of the cardiac vasculature. Submitted for publication.


Linde Woudstra was born on December 16th 1987 in Groningen. In 2006 she received her VWO diploma at the Lindenborg in Leek. That same year she started the study BioMedical Sciences in Groningen, of which she achieved her master's degree at the end of 2011. In June 2013 she began her PhD at the Department of Pathology at the VU University Medical Center, Amsterdam, under the supervision of prof.dr. Hans Niessen, prof.dr. Bert van Rossum, dr. Paul Krijnen and dr. Lynda Juffermans. In early 2018 she successfully completed her promotion.
Dankwoord

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Pikerje net, it komt doch oars