Stem Cell Therapy Against Cardiac Inflammation

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Stem Cell Therapy Against Cardiac Inflammation

Stamceltherapie tegen Cardiale Inflammatie
(met een samenvatting in het Nederlands)

Proefschrift ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 15 september 2016 des middags te 12.45 uur

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Frederieke van den Akker

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to Niels and Ruari,
my two guys

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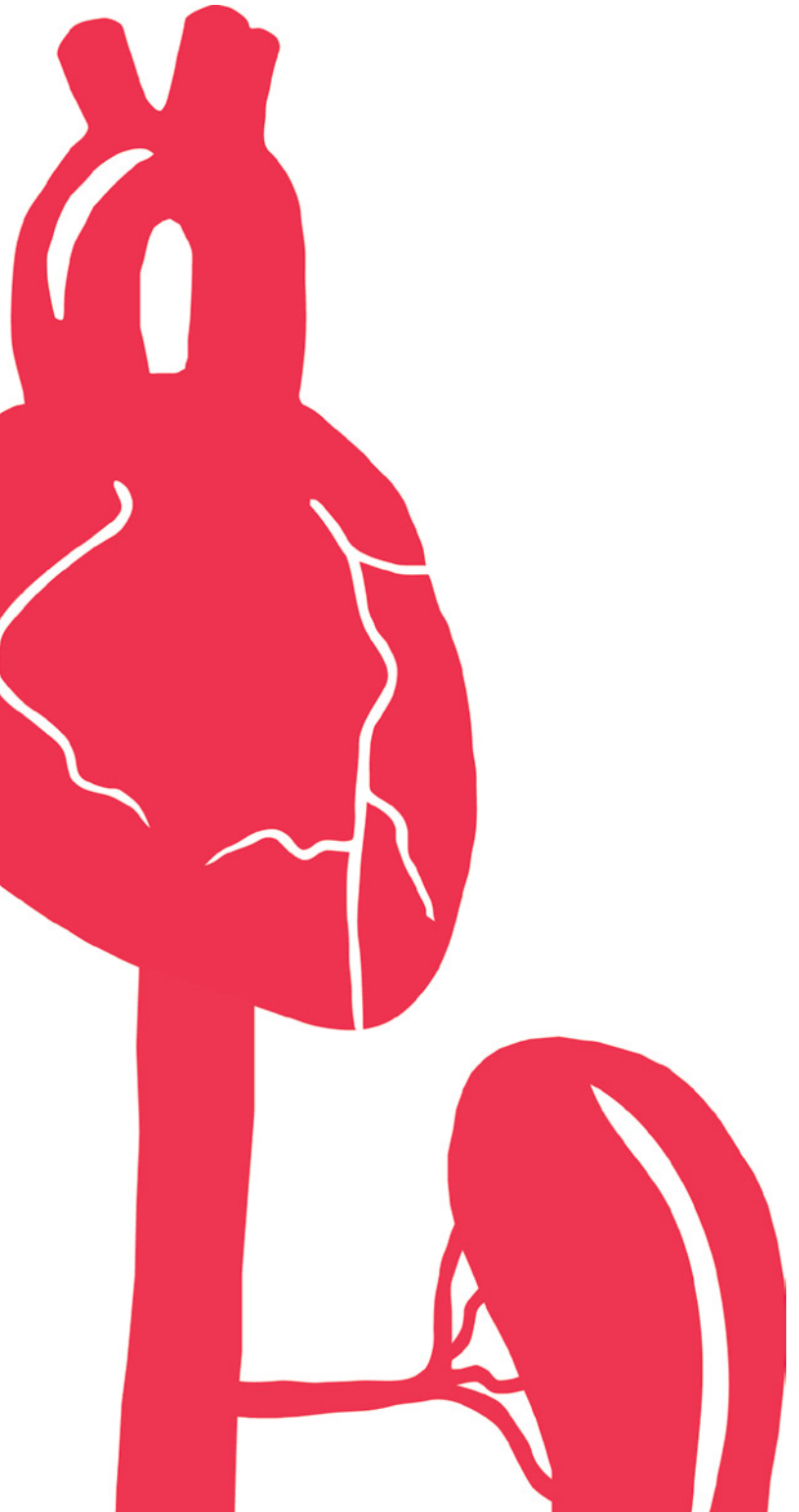
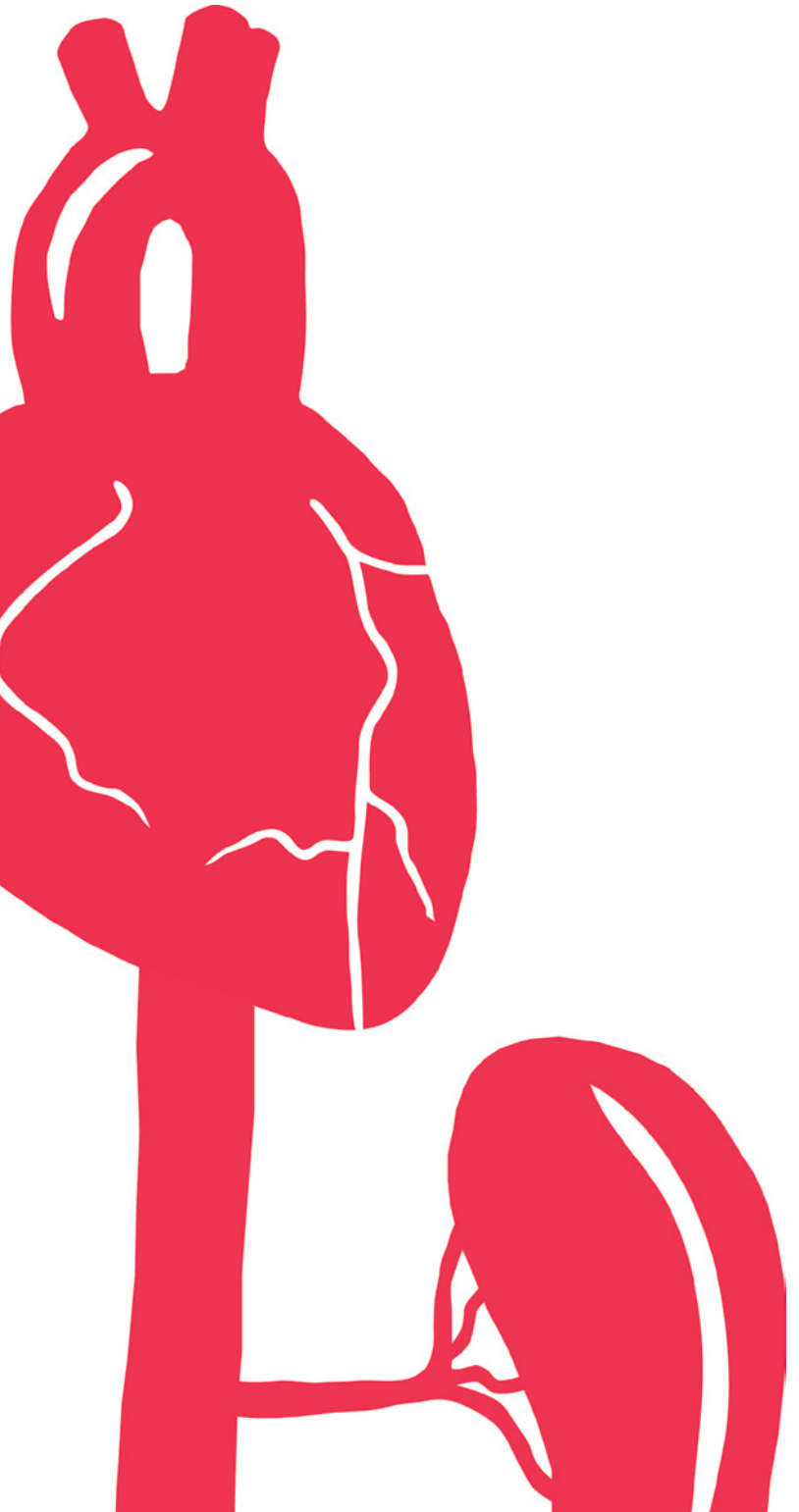


Part I

Immunomodulation by Stem Cells



Stem Cell Therapy Against Cardiac Inflammation

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F. van den Akker

Based on the reviews:

1 van den Akker F, Deddens JC, Doevendans PA, Sluijter JPG. Cardiac stem cell therapy to modulate inflammation upon myocardial infarction. Biochim Biophys Acta. 2013;1830(2):2449-2458.

2 van den Akker F, de Jager SCA, Sluijter JPG. Mesenchymal stem cell therapy for cardiac inflammation: immunomodulatory properties and the influence of toll-like receptors. Med Inflam. 2013: 181020.

Introduction

Ischemic heart disease is the number one killer worldwide[1]. During ischemia there is a shortage of oxygen and nutrients in the heart leading to cellular apoptosis and necrosis. One of the key steps to prevent further cardiac deterioration is to restore blood flow into the affected myocardium. Upon reperfusion the restored blood flow reintroduces oxygen, leading to the generation of damaging reactive oxygen species[2]. In addition to the ischemia induced cell death, this reperfusion progresses cell death even further.

As in all tissue damage, an inflammatory response is stimulated to remove cell remnant and debris. The cardiac ischemic response also triggers a strong immune reaction in the heart[3-5]. After initiation, this response is propagated by the damaged cells and matrix via the release of chemokines, cytokines, and a variety of endogenous proteins, referred to as damage-associated molecular patterns (DAMPs).

DAMPs can subsequently bind toll-like receptors (TLR) on immune and other cells to activate them, resulting in a strong inflammatory environment. This environment induces the activation of local macrophages and the attraction of other immune cells from the blood, such as neutrophils, monocytes and lymphocytes[3,4,6].

The inflammatory reaction clears the wound of dead cells and debris, thereby simultaneously providing key signals to activate reparative pathways. The different immune cells produce a large number of pro-inflammatory cytokines. The cytokines lead to a cascade in which more immune cells are attracted, causing further damage and stress on the surviving cardiomyocytes and leading to even more cell death.

Due to this strong response the inflammatory reaction has great influence on ventricular remodeling and cardiac function[7]. Resolution of the inflammatory response is currently thought to be an active process. It is mediated by factors produced by the cardiac cells, released from the matrix, and secreted by the infiltrated immune cells themselves[7].

The balance between inflammatory and reparative phases is delicate and needs proper fine-tuning in order to prevent excessive inflammation or inadequate stimulation of repair. Eventually this leads to adverse remodeling and subsequently decreased heart functions[2,7,8]. To adequately assess the role of immune cells in the progression of cardiac disease, it is important to be aware of the temporal profile and location of inflammatory cells after myocardial infarction (MI).

In the first part of this review we will provide an overview of the inflammatory processes at play in the heart during and after MI.

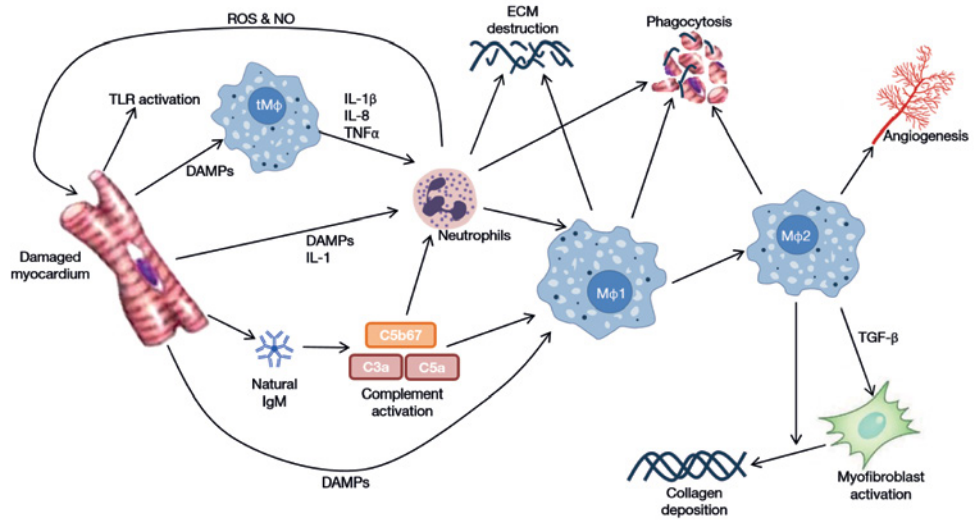

Figure 1.1 – Attraction and activation of the immune system after MI

The first cells activated after myocardial infarction are the local macrophages (*Mϕ*). Soon after, DAMPs, complement system and IL-1 attract neutrophils, which enter the damaged tissue to clear the debris. Within days large numbers of macrophages infiltrate the tissue, clearing both the debris and activating the reparative pathways. The M1 macrophages are the first to arrive and have a pro-inflammatory character. They are followed by the anti-inflammatory M2 macrophage. Lymphocytes arrive relatively late on the scene, due to the lengthy process of activation.

Post-MI inflammation

During cardiac ischemia there is a shortage in supply of oxygen and nutrients to the cardiomyocytes. As the heart is extremely sensitive for ischemia, the first ultrastructural changes, such as myofibrillar relaxation, glycogen depletion and mitochondrial swelling, are visible within minutes of onset[9]. In humans, the effects of the oxygen-shortage are still largely reversible within the first twenty minutes. After that time permanent damage occurs[9].

During the following hours, oxygen-deprived cells can undergo necrosis, apoptosis or remain in a so-called hibernating state[9,10]. In response to, or during either of these processes, the stressed myocardial cells release cytokines, such as IL-1, express damage-associated proteins or loose proteins and lipids, thereby displaying their hydrophobic portions.

After MI, these signals are all recognized as DAMPs and are important for attraction and activation of various components of the immune system[8,11,12] (*figure 1.1*). Additionally, the complement system plays an important role in post-MI inflammation. The complement system becomes activated via natural IgM classes, targeting non-muscle myosin heavy chain type II A and C[13]. This IgM leads to the activation of the complement system, leading to a strong attraction of neutrophils

and macrophages.

Initially, the immune response is aimed at cleaning up the debris of dead cells and matrix. Later on, the actual repair starts with myofibroblast activation, collagen deposition and angiogenesis stimulation[2,7].

TLR are of essential importance in the activation of the immune response after MI[7,14]. TLR are ubiquitously present receptors involved in perceiving danger signals. These danger signals were initially thought to be only pathogen-associated, yet recently it was found that DAMPs, released by stressed or damaged cells, can also activate TLR[11].

Although TLR were primarily found on immune cells, a number of TLR are also present on cardiomyocytes, endothelial cells, smooth muscle cells and fibroblasts[3,15]. Activation of several of these cardiac TLR has been described after MI and especially upon reperfusion. Activation of the TLR signaling pathways converges mainly on nuclear translocation of NF-κB and transcription of various genes involved in acute inflammation[3,8,15-17].

In the next sections, the early and late phase of post-MI inflammation as well as the role of the different types of immune cells will be described in more detail. A brief overview of the cardiac changes and the immune activity can be seen in *figure 1.3*.

Early inflammation

Two cell types of the immune system play a major role in early cardiac inflammation: the neutrophils and the macrophages. A schematic overview showing the presence and occurrence of various inflammatory cell types in the human post-MI heart is given in *figure 1.2*.

As is the case in many organs in the body, some local macrophages are present in the heart[4]. After MI, the cardiac macrophages are the first to be activated. Upon activation they secrete high levels of various pro-inflammatory cytokines and chemokines, such as IL-1, IL-6, IL-8, NO and TNF-α[2,4]. Together with DAMPs, released by damaged cells, these cytokines create a pro-inflammatory environment and attract neutrophils to the damaged area.

Neutrophils are circulating phagocytic granulocytes involved in the early inflammatory response. In the case of human cardiac inflammation, neutrophils enter the tissue in large numbers around six hours after infarction and remain the most prevalent immune cell for approximately three days. Migration of neutrophils into the injured tissue depends on chemotactic factors, of which IL-1 and IL-8 are among the most important.

When activated by these chemotactic signals, neutrophils produce reactive ox-

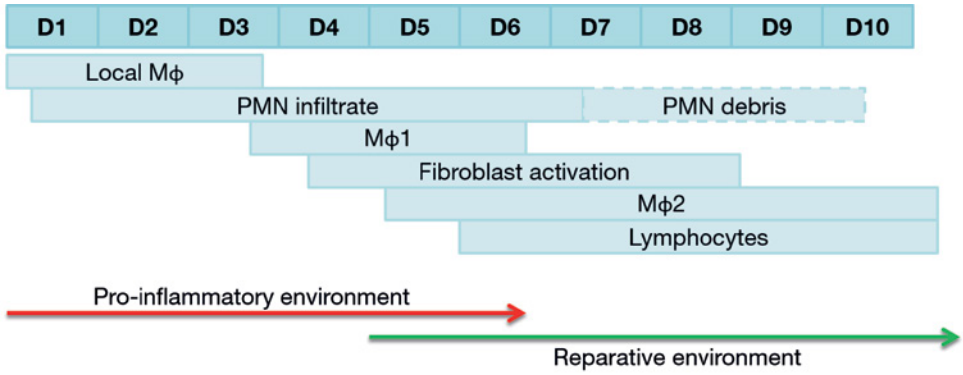

Figure 1.2 – Timeline showing various immune system components post-MI

The presence of neutrophils and M1 macrophages (*Mϕ)* in the first days after myocardial infarction and the cytokines they secrete, create a strong pro-inflammatory environment resulting in chemo-attraction of more immune cells and additional damage to the myocardium. When the switch to the M2 macrophage phenotype is made, the cytokine palate is changed to anti-inflammatory and healing and angiogenesis is promoted.

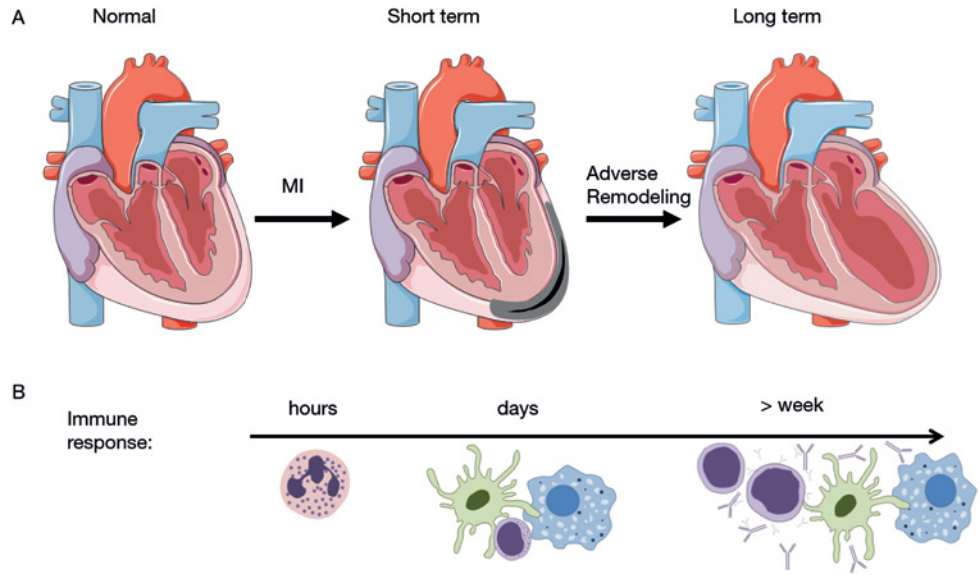

Figure 1.3 – Early and late remodeling and inflammation

A Shortly after MI the affected area of the cardiac wall is edematous (light grey) with areas of necrosis (black). In the long term, the walls of the dilated ventricle are thin and akinetic. B Immune cells involved in the different stages after MI. Immediately after, neutrophils are attracted, followed shortly by monocytes/ macrophages, dendritic and NK cells. Dendritic cells and macrophages activated in this phase proceed to activate the adaptive immune system, being the T- and B-cells. These cells can remain active for many years and influence the negative remodeling and progress to dilated cardiomyopathy.

ygen species (ROS) and reactive nitrogen intermediates. This respiratory burst is normally aimed at micro-organisms, however, in the post-infarct heart it progressively damages the cardiomyocytes. The additional damage is facilitated by ICAM-1 expression by cardiomyocytes, allowing the neutrophil to be in close proximity during the burst[18-20]. This neutrophilic discharge increases cardiac cell death, leading to the release of more inflammatory signals and thus further strengthening the immune response. In addition to the respiratory burst, neutrophils release a number of proteases to dissolve the extracellular matrix (ECM) and phagocytose cell debris, thereby starting to clean up of the infarcted area.

Approximately three days after MI, macrophages appear in large numbers at the infarct border and slowly make their way through the whole infarcted area[9]. Arriving monocytes can either mature into the classically activated M1 macrophage, or, become the alternatively activated M2 macrophage. In the post-MI heart, first an infiltration of M1 macrophages occurs, which stimulate local inflammation by secretion of pro-inflammatory cytokines, such as IL-1β, IL-6, TNF-α and IFN-γ[21,22].

These macrophages produce many matrix metalloproteinases (MMPs), which lead to further degradation of the extracellular matrix (ECM)[21,22], making it easier to phagocytose both the cellular debris and neutrophil remains in the infarcted area. Five days after MI, M2 macrophages become more prevalent[23]. The M2 macrophages secrete an anti-inflammatory mixture of cytokines, characterized by high levels of IL-4, IL-10 and TGF-β1 and low levels of IL-1, IL-6, TNF-α and IFN-γ[21,22].

Compared to the M1, M2 macrophages also secrete high levels of basic fibroblast growth factor (bFGF), thereby creating the right environment for wound healing and scar formation. In addition, vascular endothelial growth factor (VEGF) is produced, which stimulates angiogenesis[22].

Late or chronic inflammation

For a long time it was thought that after the acute phase post-MI, the inflammation would automatically be quenched and fibrosis combined with adverse remodeling was the culprit for deterioration towards heart failure. However, we know now that if the inflammatory phase is not resolved properly, immune activation may persist over a period of weeks and even years after the initial event[24].

The effectors in this auto-inflammatory state are mainly found in the adaptive immune system, namely the T-cells and B-cells. These lymphocytes get activated by antigen presenting cells (APC), such as dendritic cells and macrophages, shortly after the infarct[25]. Due to the ischemic chaos, these APC will present (parts of) normal cardiac antigens as if they were a potentially fatal threat. T-cells and B-cells responding to these antigens, will get activated and start multiplying. Helper-T-cells will further stimulate the maturation of B-cells and cytotoxic T-cells, by providing co-stimulatory signals and pro-inflammatory cytokines.

Meanwhile, cytotoxic T-cells will circulate in the blood to directly attack any cell bearing those same antigens, meaning any cardiomyocyte. B-cells mature into plasma cells, which produce antibodies against those cardiac antigens. When the antibodies attach to a cell displaying the antigen, it is tagged for destruction. Since the cardiac antigens will be present as long as there are cardiomyocytes, this process could continue for the rest of a patient's life. To resolve this phase of inflammation, it is assumed that more regulatory T-cells (T_{reg}) and B-cells (B_{reg}) are needed[26-28]. T_{reg} are most investigated and produce high levels of IL-10, aiding in the resolution of inflammation and the initiation of repair[28].

During *in vivo* studies, researchers have isolated lymphocytes from a mouse spleen after infarct, and have given these cells to a normal healthy mouse. Within 8 weeks, the ejection fraction of the healthy mouse started decreasing[29]. Similarly it was found that the vast majority of patients with heart failure, regardless of the initial cause, had their cardiac tissue coated with antibodies[30]. This suggests that these long-term auto-inflammatory processes are at play in other cardiac diseases, such as myocarditis, as well.

Another factor not to be overlooked in cardiac inflammation is the production of cytokines. Although it was long thought these cytokines served mainly as communication between immune cells, it was found that cardiac cells themselves also produce pro-inflammatory cytokines, such as TNF-α, IL-1 and IL-6. The production and release of these cytokines is increased during ischemia and stress[31].

Pre-conditioning the heart with these cytokines can reduce the damage caused by ischemia/reperfusion (I/R), leading to the hypothesis that these cytokines originally evolved as a short term cytoprotective mechanism[31,32]. However, a prolonged activation of these cytokines has deleterious effects on the heart. Long-term expression of TNF-α can lead to hypertrophy and eventually even dilated cardiomyopathy (DCM)[31]. Increased activation of MMP leads to increased interstitial fibrosis during long-term presence of cytokines, in particular IL-1β.

In addition to this, a number of these cytokines negatively influence the contractility of the cardiomyocytes. This negative reversible inotropic effect has been documented for TNF-α, IL-1β, IL-2, IL-6 and IL-8[31-34]. The cardiomyocyte depression is thought to be caused by a direct effect on calcium homeostasis, combined with B-adrenergic signaling, thereby inducing expression of genes involved in the remodeling process or apoptosis[34].

The decrease in cardiac function is initially reversible, but the induced remodeling might maintain these effects on the long term[32]. Interestingly, these cytokines are strongly linked to the pro-inflammatory subsets of the immune cells. The immunoregulatory cells, such as M2-macrophages or regulatory T-cells produce IL-4 and/or IL-10 instead. While IL-4 has no effect on cardiac contractility, IL-10 actually has

a positive inotropic effect, leading to an improved cardiac function[35].

Anti-inflammatory therapy

For over thirty years different ways of suppressing this immune reaction have been investigated to evaluate the effect on cardiac function. One of the most investigated approaches involves the administration of immunosuppressive drugs, usually corticosteroids[36]. Although the observed outcomes on cardiac performance differed strongly between studies, there was an overall decrease in leukocyte influx into the infarcted tissue.

A recent meta-analysis on all clinical trials with corticosteroids shows that there is a non-significant trend towards decreased mortality with corticosteroid treatment[36]. At the same time, corticosteroids were found to disrupt the healing process by delaying collagen deposition and scar formation. Smaller and weaker scars were formed, both in animal models and in humans, leading to an increased chance of LV rupture[36-38]. Non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen, were also investigated. Although a reduction in neutrophils en leukocyte infiltrate was observed, the broad suppression of the entire inflammation response resulted again in a thinner scar[39,40]. In addition, several NSAIDs were found to significantly increase the risk of recurrent myocardial infarction and death[41].

Depletion studies for circulating immune cells demonstrated that macrophages were essential for initiating healing after myocardial infarction[8,42]. Meanwhile, depletion of neutrophils showed a reduction in final scar size without adverse effects on cell survival or cardiac function, demonstrating that their role was unnecessary for healing and only led to additional damage[8,43-45]. However, in a cutaneous model of wound healing the absence of cytokines and growth factors from neutrophils did not affect granulation tissue formation[46].

Nowadays, neutrophil-depletion is actually applied in cardiothoracic surgery to prevent I/R-damage, with promising results, such as a reduction in reperfusion injury after coronary revascularization with cardiopulmonary bypass[47]. In contrast, depletion of macrophages impaired wound healing and scar formation in rats, and resulted in increased mortality[8,42]. This demonstrates that macrophages, or at least one of its subtypes, are necessary for cardiac repair. However, in the attempts complete depletion of macrophages was performed, and at this moment specific subgroup depletion is not possible.

In a recent rat study T-cell depletion was investigated after MI, using anti-thymocyte globulin to induce T-cell apoptosis. They showed smaller infarcts with reduced remodeling, maintenance of cardiac function and increased neoangiogenesis[48]. Depletion of B-cells using an anti-CD20 antibody also has beneficial effects on infarct size and heart function, by limiting myocardial inflammation[49]. Prevention of activation of the complement system can be achieved by a selective depletion

of natural IgM, which was found to protect the heart from I/R injury, and reduced the inflammatory response[13,50]. Monoclonal antibodies against C5a, which play a central role in complement activation, decreased infarct size[51]. Unfortunately, both neutrophil depletion and complement inhibition are quite invasive and therefore inconvenient for large scale clinical use.

Other animal models were created to investigate the role of different cytokines. Conventional knock-out mice were created for TNF receptor 1 and 2, a cytokine known for its potent pro-inflammatory effects. Surprisingly, these knock-outs resulted in a larger infarct size and increased apoptosis of cardiomyocytes[52]. This indicated that TNF-α also has cardio-protective functions after MI.

It is likely that the exact effect of TNF-α depends on the concentration as well as on the type of TNF receptor it binds[52,53]. One of the cytokines that attracts neutrophils to the site of injury is IL-8. Inhibition of this chemokine with neutralizing antibodies gave a significantly smaller infarct size, yet it did not affect the invasion of neutrophils in the cardiac tissue[54]. A knock-out model for IL-1R strongly diminished neutrophilic inflammation after cell death[8,55,56], while lacking the active form of IL-1β leads to improved survival with reduced cardiac (LV) remodeling[57].

Knock-out models were created for several TLR to determine their role in post-infarct inflammation. Most of these showed no effect on cardiac inflammation, except in the case of TLR2-/- and TLR4-/- mice, where a reduced mortality was observed as well as decreased LV remodeling[8,58,59]. It appears that especially TLR2 on the circulating cells, rather than the cardiomyocytes, was the mediator of TLR2-induced reperfusion injury[14].

Since all of the signaling via TLR depends on NF-κB activation and subsequent signaling, NF-κB subunit p50 -/- mice were used to follow-up these studies. These mice displayed less I/R damage and there was a strong reduction in the influx of neutrophils. This resulted in decreased LV remodeling with a preserved LV function[60]. This effect was completely based on the NF-κB-/- status of bone marrow cells[60]. NF-κB signaling by TLR2 and TLR4 leads to the activation of various downstream signaling pathways, one of which is MyD66. Knock-out models of MyD66 have almost no neutrophilic response to cell death, while macrophage response remains normal[58].

These studies are only the first steps towards understanding, as for other cells of the immune system or their interactions the role in clearing of cardiac cell debris and stimulation of reparative responses remains largely unknown.

What we have learned from these experiments, however, is that a general suppression of the immune response is not a therapeutic answer for modulating post-MI processes, and this approach has since been largely abandoned. An effective therapy that influences the immune response should reduce the length

and damage of the inflammatory response and not interfere with or even augment the activation of reparatory pathways. This shift in balance between inflammation and repair might be achieved by using stem cell therapy in the heart.

Clinical stem cell therapy

During the last decades the use of stem cell transplantation therapy, to repair or regenerate damaged tissue, has become increasingly popular in clinical research. In the mid-fifties, researchers could cure mice with leukemia by near-lethal whole body irradiation, after which bone marrow from a healthy donor was transplanted[61]. Within a few years this treatment was also given to humans and despite initial disappointments, it quickly developed into a successful therapy[62].

Since then more research has focused on stem cell therapy. Over the years many more adult stem cells and progenitor cells have been investigated for potential regenerative therapies. One of the most investigated cells in recent years is the mesenchymal stem or stromal cell (MSC).

MSC are present in the bone marrow, where they regulate the microenvironment. They secrete many paracrine factors, which can reduce cell death, fibrosis and even inflammation[6,63,64]. MSC are reputedly immuno-privileged, meaning that the host immune system will not attack them and allogeneic transplantation should be feasible[16,65]. The strength of the paracrine factors produced by MSC is demonstrated by experiments in which MSC-derived conditioned medium (CM) or components thereof are used. Such experiments demonstrated cytoprotective effects[66,67], reduced infarct size, improved cardiac function[38,68], stimulated human renal proximal tubule regeneration[69], and reduced autoimmune encephalitis[70].

Aside from numerous growth factors and cytokines produced by stem cells, exosomes have gained interest as one of the factors in the conditioned medium that serve an important role in these paracrine effects. Exosomes are small vesicles secreted by a large variety of cells containing a specific subset of proteins, mRNAs, and miRNAs, which can influence various biological processes[71].

For example, MSC-derived exosomes were found to reduce I/R injury[72], while tumor-derived exosomes stimulated angiogenesis[73] and cardiac progenitor-derived exosomes stimulated endothelial cell migration[74]. Within the immune system exosomes offer an important means of cross-talk between the various subsets of immune cells, thereby regulating the immune response[75]. It is therefore not unlikely that exosomes produced by MSC can modulate the immune system as well.

The notion that MSC are active immune-suppressors appeared about ten years ago[76], when a suppressive effect of MSC on T-cell proliferation was observed. This immunomodulatory capacity was further investigated and quickly applied in therapy, as patients with therapy-resistant severe acute graft-versus-host-disease

(GVHD) could be cured with repetitive MSC injections[77,78]. In fact, concomitant administration of MSC with the hematopoietic stem cell (HSC) transplantation enhanced engraftment of HSC and reduced the chances of acute GVHD[79].

These stunning results sparked an enormous interest to investigate their potential in other diseases. In the field of graft rejection, MSC were found to be able to prolong graft retention of skin grafts[80] as well as semi-allogeneic heart transplants[81]. MSC were also considered in auto-immune diseases. MSC applied in arthritis showed contrasting results, possibly related to the timing of administration[82-84], while research on inflammatory bowel disease (IBD) shows a positive response[83,85]. Interestingly, recently neural stem cells were also found to be immunosuppressive, raising the question whether immunomodulation is a general stem cell characteristic[86-89].

Current attempts in cardiac stem cell therapy

As cardiovascular disease is the most prevalent cause of death, still looking for new therapeutic modalities, MSC were quickly selected as a target population for stem cell therapy. In the past years, the focus of cardiac stem cell therapy has mainly been on the regeneration of damaged myocardium and angiogenesis, via paracrine signals or cellular differentiation.

Results of these stem cell therapies include an increase in ejection fraction, a measure of cardiac function, and positive effects on cardiac remodeling[90,91]. After a week, however, only less than 5% of the injected stem cells are still present in the heart[91,92], none of which show true differentiation towards the cardiac phenotype. This clearly indicated that possible differentiation and direct functional contribution is only a minor factor in the observed effects, whereas supportive or paracrine factors are likely more important.

These paracrine effects include a reduction in apoptosis and an increase in angiogenesis. However, a possible explanation, which has seen limited study, is the possible effect of MSC on post-MI inflammation. Since the immune response shortly after MI has a strong correlation with cardiac function and outcome[7], it is likely that these processes are affected by these paracrine factors of the transplanted MSCs soon after injection as well.

In the next section of this review, we provide an overview of the known interactions between the immune system and the MSC, one of the most abundantly studied stem cell types, in an effort to elucidate/predict the effects of immunomodulatory cardiac stem cell therapy. The pathways often assumed to be involved will be discussed first, followed by the different types of immune cells and the way(s) they are modulated by MSC.

Modulation of the immune system by MSC

The immune response after myocardial infarction is complex and still not entirely understood. It has however become clear that a general suppression of the immune system is not beneficial in the setting of post-MI inflammation, as many necessary and beneficial actions of the immune cells will also be suppressed.

The goal for treatment of post-MI inflammation should be to prevent collateral damage from the inflammatory response, while boosting the healing component. MSC are known to modulate the immune system and are currently being tried in various diseases to shift the balance of the immune response to a more tolerant type. Based on previously discovered interactions between MSC and the immune system, we can speculate what the beneficial effect of MSC treatment on post-MI inflammation can be. Although over the years many molecules and pathways have been implied in MSC-dependent immunomodulation, several pathways are reported, including indoleamine-pyrrole 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2), IL-10, nitric oxide (NO) and TGF-β1, not limited to specific immune cell types.

For a better understanding of the mechanism, through which MSC can modulate different immune cells, the major pathways will be shortly summarized.

Currently, one of the most popular enzymes in immunomodulation by MSC is IDO. IDO is an intracellular enzyme, which is up-regulated in pro-inflammatory environments and partly controlled by NF-κB[93-95]. IDO is involved in tryptophan metabolism and expression leads to local tryptophan depletion and accumulation of metabolites, such as kynurenic acid (KYNA) and kynurenine. Whether IDO only interacts with immune cells via the tryptophan-kynurenine pathway, is still unknown.

Another powerful modulator of the immune system is PGE2. PGE2 is a lipid compound produced by cyclooxygenases (COX-1 and COX-2) and secreted by MSC[94,96,97]. In response to pro-inflammatory stimulation, PGE2 production by COX-2 is sharply increased[94]. PGE2 can have many effects on different cells types and processes, such as cytokine secretion and T-cell differentiation, however the mechanism remains poorly understood.

NO, produced by the intrinsic nitric oxide synthase (iNOS), is another MSC mediator in immunomodulation. As NO is an unstable compound, signaling with NO only reaches those cells located near the MSC. Although initial reports on murine MSC attributed many effects to NO signaling, it was later found that in humans this pathway is of lower significance[95]. Transforming growth factor B1 (TGF-β1) is a protein known to play important roles in tissue regeneration, cell differentiation and immune regulation. TGF-β1 is produced by MSC but also by macrophages, which might explain why it can both stimulate and suppress the immune system, likely

depending on the local environment, concentration and the receptor it binds[93,98].

The last cytokine discussed here is IL-10, an anti-inflammatory cytokine produced by various cells of the immune system and MSC[7,94,99]. IL-10 can alter cytokine secretion from target cells and can also trigger immune cells to differentiate towards a specific, anti-inflammatory subtype[7,99].

Neutrophils

As mentioned previously, neutrophils are the first responders after myocardial infarction. Normally, neutrophils kill microorganisms and infected cells by production of reactive oxygen species (ROS) and clearance of the subsequent debris. They are also activated in response to local chemokines and DAMPs after sterile tissue damage, such as MI[100].

Within an hour an influx of neutrophils in the heart is visible and they remain the most prominent cell type for 1-2 days[101]. MSC produce high levels of IL-6, which activates STAT3 transcription factors, resulting in a longer life span of the neutrophils, as indicated in *figure 1.4*[101]. Although this appears counterintuitive at first, IL-6 also attenuates the neutrophil respiratory burst, so the neutrophils are less harmful to their environment[102]. MSC are able to suppress the degranulation of the enzyme–containing granules of neutrophils.

Monocytes/macrophages

Monocytes, which can differentiate into tissue macrophages, have a dual role in inflammation and tissue repair. After MI, two major subsets of macrophages can be found in the heart at different time points.

Shortly after MI, the classically activated M1 macrophage (inducible nitric oxide synthase (Nos2, iNOS), MHC Class II, CD80, CD86) is present in the heart, which is strongly associated with the clearing of debris, inflammation and the production of pro-inflammatory cytokines, such as IL-1β, TNF-α and IFN-γ[21]. After about five days the more prevalent type has switched to the alternatively activated M2 macrophage (Arginase 1 (Arg1); macrophage mannose receptor (Mrc1, CD206); Macrophage scavenger receptor (Msr1, SR-A, CD204))[21].

This macrophage subtype has an anti-inflammatory phenotype, reducing the release of pro-inflammatory cytokines, while stimulating cardiac reparative pathways, scar formation and angiogenesis[21,22,101,108]. In the presence of MSC, differentiation of macrophages into the M2 subtype was boosted (*figure 1.5*). Many pathways have been indicated in this process, such as IDO, PGE2 and MSC-derived IL-4 and IL-10[94,106,109,110]. MSC also secrete TGF-β1, which together with PGE2 was found to reduce the production of pro-inflammatory cytokines by the macrophages, such as IL-1β, IL-6, TNF-α and IFN-γ[109,110]. Meanwhile, anti-inflammatory cytokine

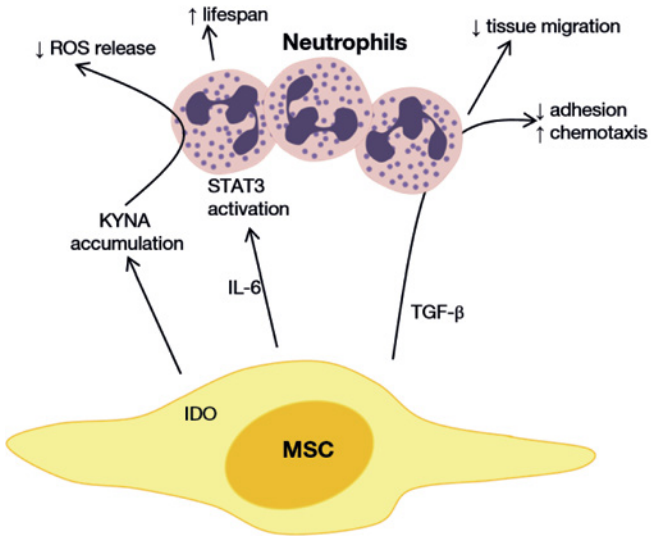

Figure 1.4 – Schematic overview of the interactions between MSC and neutrophils
MSC trigger an IL-6 dependent increase in neutrophil lifespan. In addition, they can reduce the amount of reactive oxygen species (ROS) production and release. One of the pathways involved in this process is IDO-related KYNA accumulation. Different reports exist on the effect on tissue migration and adhesion, although TGF-β1 appears to increase chemotaxis, while reducing adhesion to ICAM-1.

IL-10 was strongly increased, which in turn is said to boost formation of regulatory T-cells[106,109]. No negative effects on macrophage phagocytosis were observed in the presence of MSC, meaning their debris-clearing functions were still intact[7,110].

Two *in vivo* models confirm these *in vitro* findings[106,109]. A mouse model of inflammation was created by inducing sepsis in mice[106]. MSC therapy significantly reduced mortality during sepsis and it was found that these beneficial effects were related solely to the alternative activation of macrophages. In fact, macrophage depletion abrogated this effect[106]. In addition, in a mouse model of myocardial infarction, where MSC were infused 48 hours after MI[109], an overall reduction of monocyte levels was observed within 24 hours, while the proportion of M2 macrophages was strongly increased. After 16 weeks left ventricular remodeling was significantly reduced, while no difference in angiogenesis in the infarct area was observed.

A short-term beneficial effect was found on fractional shortening, yet no long term cardiac functional difference to control was found. These results were suggested to be related to the positive inotropic effect of IL-10 and a reduction in negative

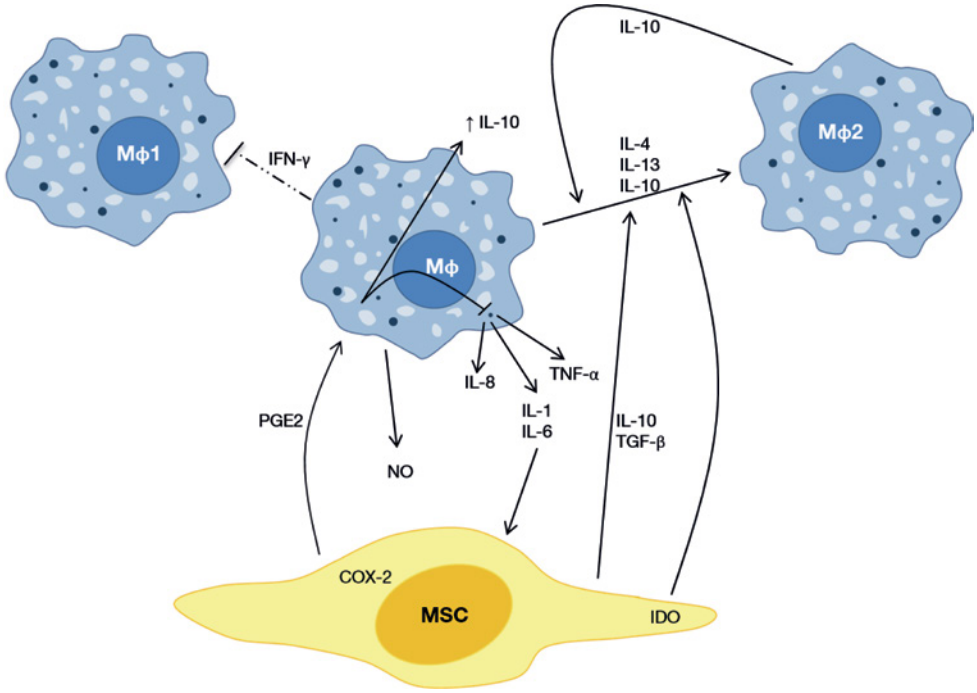

Figure 1.5 – Schematic overview of the interactions between MSC and macrophages
MSC trigger macrophages to differentiate towards the anti-inflammatory M2 macrophage. Different mechanisms appear to be involved in this process, amongst which IDO, TGF-β, IL-10 and PGE2 are the most important ones. In addition, the production and release of pro-inflammatory cytokines by macrophages is reduced.

inotropes, such as IL-1 and IL-6[109].

In short, MSC can trigger macrophages to create an anti-inflammatory environment, where the emphasis is on regeneration rather than destruction. This might explain why the removal of macrophages proved disastrous, as discussed earlier. Although more research on the effect of MSC on macrophages in the post-MI will be necessary, the switch to M2 macrophages appears promising.

Dendritic cells

Dendritic cells (DC) are the most potent antigen presenting cells of our immune system and after MI they present cardiac antigens, which activates the adaptive immune system[25]. Co-culture of MSC with DC progenitors, whether CD34+/- or monocyte-derived, prevented differentiation into mature DC, despite the fact that cells were grown in lineage-specifying growth conditions[111-114].

MSC also blocked maturation of DC, leading to a reduced expression or absence of antigens and co-stimulatory molecules CD40, CD80 and CD86, subsequently

necessary to activate T-cells (*figure 1.7*)[111,113,115]. This process is at least in part regulated via secretion of IL-6 by MSC[82]. MSC induce the production of IL-10 while suppressing IL-2, IL-12, IFN-γ, and TNF-α release by DC, resulting in impaired maturation, migration and antigen capture and processing[100,112,116]. These cytokines are also crucial for the activation of lymphocytes, which was therefore impaired as well. This suggests that MSC may induce a suppressive phenotype of DC which reduced the effector T-cells, but enhanced regulatory T-cell responses[100,112,117-119].

NK cells

Natural Killer (NK) cells are the innate immune system's subtype of cytotoxic lymphocytes. They usually react in response to viral antigens presented on MHC-I molecules, but can also recognize and lyse stressed cells, which many cardiac cells are shortly after MI[120]. In fact, levels of granzymes produced by NK cells peak shortly after MI[121-123]. MSC can suppress the proliferation of NK cells, as well as reduce the cytotoxic activity and pro-inflammatory cytokine profile (*figure 1.7*)[124].

Proliferation of NK cells is sharply reduced in the presence of both IDO and PGE2, thereby pointing to the possible synergistic effect of these two pathways[111,124,125]. MSC can also downregulate the NK activating receptors NKp30, NKp44, and NKG2D[125]. As NK receptors are correlated with the function of the NK cell, the downregulation of activating receptors leads to an altered cytotoxic activity and reduces secretion of pro-inflammatory cytokines[125]. The reduction in IL-2 and IFN-γ secretion leads to further suppression of NK cell proliferation[100].

B-cells

B-cells are part of the adaptive immune response and responsible for the production of antibodies during inflammation. The antibodies cover the cell displaying the specific antigen and allow easy engulfment by phagocytic cells, such as macrophages and neutrophils[126]. After MI, mature B-cells release Ccl7 to attract the pro-inflammatory M1 macrophages to the heart, which decreases cardiac function by enhancing tissue injury[127]. MSC were found to arrest B-cells in the G0/G1 phase, while simultaneously reducing the chemotactic capacity of these cells, as depicted in *figure 1.7*[100,118,128,129]. How this is exactly regulated remains unclear.

MSC can interact with B-cells via the PD-1 (programmed cell death protein-1) pathway, hereby reducing activation and proliferation of B-cells[130]. The co-stimulatory molecule CD40L is mainly present on activated T-cells, and plays a role in B-cell activation[105]. If this co-stimulatory signal is not obtained, B-cell activation will be reduced and antibody secretion will diminish. A reduction in T_H-cell activation by MSC, and especially the existence of T-cell anergy, could lead to decreased B-cell activity *in vivo*[117,131]. Finally, some research showed that MSC were able to suppress the production of antibodies by B-cells[128]. It is important to note here that

reports have also been published that MSC actually stimulate B-cell proliferation and differentiation[132,133].

T-cell proliferation

T-cells are a heterogeneous group of cells, consisting of many subtypes of which the T-helper cells (T_H-cells; CD4+), cytotoxic T-cells (T_C-cells; CD8+) and the regulatory T-cells (T_{reg}; CD4+ or CD8+, CD25+FoxP3+) are best known. Both T_H and T_C-cells recognize a specific antigen, but while T_C-cells directly induce apoptosis of the cell displaying that particular antigen, T_H-cells mobilize macrophages and B-cells to attack the antigen-displaying cell. T_{reg} are regulators of the immune response and capable of terminating T-cell mediated immunity. Upon MI, antigen-specific T-cells form against endogenous cardiac myosin and actinin, leading to a continuous assault of T_H-cells and T_C-cells on the remaining myocardium[134-136].

Several authors showed that MSC are quite potent suppressors of T-cell proliferation, although there is a lot of donor variability[76,94,116,137]. As shown in *figure 1.6*, MSC affect both T_H- and T_C-cells, by inducing cell cycle arrest of the T-cells in the G0/G1 phase[100]. Many different pathways were found to play a role in this interaction between MSC and T-cells, of which most studied are IDO and PGE2[101]. IDO is upregulated in MSC in co-culture with T-cells. The resulting tryptophan depletion and accumulation of KYNA and kynurenin are all thought to reduce T-cell proliferation[93]. Alternatively, induction of COX-2 expression also occurs in these co-cultures resulting in increased secretion of PGE2, thereby inhibiting T-cell proliferation[94,138].

Another pathway possibly involved is the interaction of inhibitory molecule PD-1 and the ligands PD-L1 and PD-L2[130]. The PD-1/PD-L1/PD-L2 pathway is a regulatory mechanism which controls T-cell receptor-mediated lymphocyte proliferation and cytokine secretion[130]. MSC expressing PD-L1 or PD-L2 can activate the PD-1 receptor on the target T-cell. This results in a decrease in production of pro-inflammatory cytokines IFN-γ, TNF-α and IL-2 and subsequent T-cell cycle arrest[116,130].

Another way in which T-cells could be kept inactive is related to the (inducible) expression of MHC (or HLA) molecules on MSC. With these molecules, MSC can play the role of antigen-presenting cell, which would normally activate T-cells[139]. However, due to the absence of an indispensable co-stimulatory signal from CD80, CD86, or CD40, T-cells might go into anergy instead of being fully activated[117,129,140]. In this state, T-cells are still alive, yet unable to be activated and therefore unable to mount a functional immune response.

T-cell differentiation

MSC are also able to influence differentiation of T-cells into different subtypes. In addition to the aforementioned pathways, several paracrine factors including HGF,

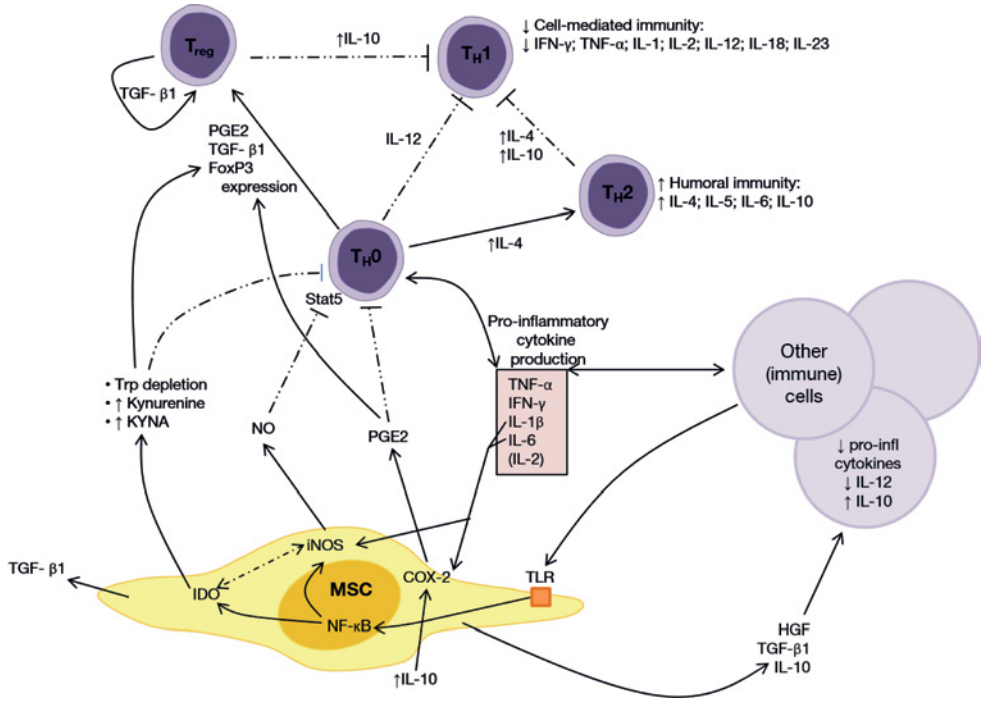

Figure 1.6 – Schematic overview of the interactions between MSC and T-cells

Co-culture of MSC and T-cells results in reduced T-cell proliferation and a decreased pro-inflammatory cytokine production. In addition, differentiation to T_H2 and regulatory T-cells is triggered, resulting in an anti-inflammatory environment.

TGF-β1, IL-6 and IL-10 have been implicated in this process, although the exact mechanisms still remain unknown[101]. MSC suppress the formation of T_H1 and T_H17 lymphocytes, which are essential for the activation of cytotoxic T-cells and the boost of phagocytic capacity of neutrophils and macrophages[141,142].

Meanwhile, MSC enhance the formation of T_H2 lymphocytes, which have a more immunotolerant phenotype and produce large amounts of IL-4 and IL-10[101,105,142]. Although these T_H2 cells normally induce B-cells, there are reports that the co-stimulatory molecules are down-regulated on the T_H-cells, resulting in a reduction in B-cell activation[143].

Besides reducing T-cell proliferation, MSC also induce formation of regulatory T-cells[141,142]. This provides a negative feedback loop for T-cell activation and proliferation and helps to regain a tolerance for the cardiac auto-antigens, such as myosin[144]. These regulatory T-cells are suggested to be formed via IDO-expression, secretion of PGE2, and TGF-β by interacting MSC. Interestingly, an increase in regulatory T-cells has been shown to attenuate ventricular remodeling after MI[145].

Targeting post-MI inflammation with MSC

The post-infarct inflammatory response is a necessary reaction, which clears cellular debris and initiates the healing pathways. However, this early infiltration causes cytotoxic injury to the surviving cardiomyocytes as well. Therefore clinicians and researchers have tried over thirty years to find a way to promote the beneficial effects of this immune response, while suppressing the detrimental results.

Where past pharmaceutical options have failed, cardiac stem cell therapy with MSC might provide a solution. The last decade the immunomodulatory potential of MSC has generated hope for treatment of many immune-related diseases. MSC have been found to interact with all cells of the immune system, where they gently steer the immune reaction towards an anti-inflammatory response. Although the exact mechanisms by which this is achieved remains unclear, it is quite likely that various dependent and independent pathways modulate different aspects of immunomodulation. This could also explain why different groups find different molecules or pathways to be the protagonist in immunomodulation. Additionally, the exact set up of the experiment, the types of immune cell(s) present and the mode of activation could augment these observed differences.

However, to use MSC as a treatment for post-MI inflammation it is essential to know the course of this process. Knowledge on the cell types present and processes at play can help pick the optimal time-frame for MSC-administration and achieve the best results. Interestingly, a recent meta-analysis on cardiac stem cell therapy[90] showed that the optimal effect of the therapy is reached with stem cell injection at least one week after MI[90]. This could imply that stem cell therapy is best given when the pro-inflammatory reaction in the heart has switched towards a reparative environment. Using stem cell therapy at an earlier time point could, besides reducing inflammation-related damage, create this healing environment sooner. A second stem cell injection within a few days could create a synergism, when both the endogenous repair mechanisms and the boost of the stem cells together improves cardiac repair.

Lastly, *in vitro* research elucidating potential pathways is interesting for scientific insight, however, it is impossible to recreate the immune system with its complex interactions between the different components *in vitro*. More focus should be on *in vivo* elucidation of immunosuppressive potential of MSC by measuring long- and short-term effects on tissue repair and function. Animal models can be created in which pathways thought to be involved are knocked-out, to estimate the true involvement of that mediator. Additionally, in human stem cell trials, the inflammatory status of the patients should be followed during the course of the study. Only in this way can the direct effects of stem cells on inflammation be studied.

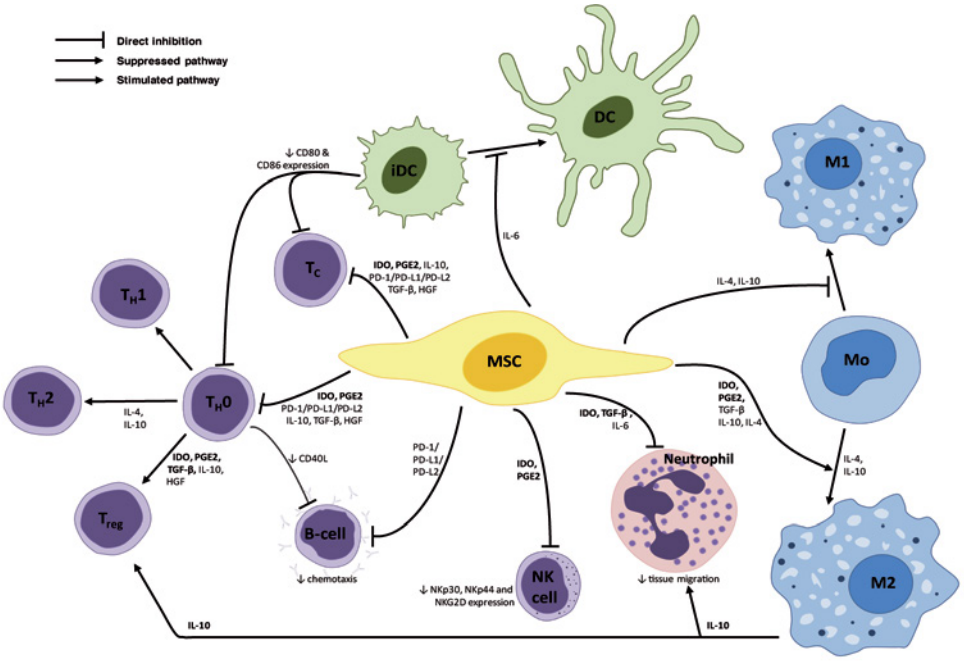

Figure 1.7 – Overview of the interactions between MSC and the immune system

Mesenchymal stem cells influence the functioning of many immune cells. Via multiple possible pathways MSC suppress proliferation of both helper (T_H) and cytotoxic T- cells (T_C). In addition, differentiation to T_H2 and regulatory T-cells (T_{reg}) is triggered, resulting in an anti-inflammatory environment. Maturation of immature dendritic cells (DC) is inhibited via IL-6, blocking upregulation of CD40, CD80 and CD86, which in turn can reduce T-cell activation. Monocytes are triggered by MSC to differentiate towards the M2 phenotype. Different mechanisms appear to be involved in this process, amongst which IDO, TGF-β, IL-10 and PGE2 are the most important ones. IL-10 produced by these M2 macrophages can boost the formation of T_{reg}, while reducing tissue migration of neutrophils. Neutrophils are allowed a longer life span by MSC-derived IL-6, while ROS production is decreased. Natural killer cell (NK cells) proliferation is suppressed, as well as cytotoxic activity and cytokine secretion. B-cell proliferation is inhibited and the production of antibodies is reduced.

***MSC**: mesenchymal stem cell, **T_H**: T-helper cell, **T_C**: cytotoxic T-cell, **T_{reg}**: regulatory T-cells, **iDC**: immature dendritic cell, **DC**: dendritic cell, **Mo**: monocyte, **M1**: classically activated type 1 macrophage, **M2**: alternatively activated type 2 macrophage, **NK-cell**: natural killer cell, **IDO**: indoleamine-pyrrole 2,3-dioxygenase, **PGE2**: prostaglandin E2, **PD-1**: programmed death 1 , **PD-L1 / 2**: programmed death ligand 1 or 2, **TGF-β**: transforming growth factor β*

Scope of This Thesis

The aim of this thesis was to explore the effects that mesenchymal stem cells (MSC) and their cardiac counterpart, the cardiomyocyte progenitor cells (CMPC), exert on the immune system. The direct *in vitro* effect of the progenitor cells on different immune cell types involved in cardiac inflammation is discussed in ***part I***. Chapter 2 discusses the effects MSC and CMPC have on neutrophils, which are the main mediator of the acute cardiac damage after MI. To investigate the possible effect on chronic cardiac inflammation, the focus is on the *in vitro* interactions between the progenitor cells and T-cells in **chapter 3**. These first two chapters demonstrated that the moment of intervention, i.e. the acute or the chronic phase, might be important for the selection of the progenitor cell used in stem cell therapy.

In ***part II*** the effect of progenitor cells is investigated in a murine model of chronic myocarditis. Myocarditis is introduced as a cardiac disease in **chapter 4**, where beside the clinical and pathophysiological progress of the disease also a potential role for microRNAs is discussed. In **chapter 5** the immunological and cardiac changes in this experimental model are described, while in **chapter 6** the focus is on the interventional effects of the injected progenitor cells.

In ***part III*** of this thesis related animal studies are described, aiming to increase the understanding of the immune response in cardiac disease and improve interventional strategies. In **chapter 7** the effect of time of cardiac damage on the ensuing immune response and endogenous progenitor cell mobilization is investigated. The reported immune-privileged status of MSC in (xeno)transplantation is examined in **chapter 8**. And lastly, in **chapter 9** we showed that current intracardiac stem cell injections are inefficient, as the majority of an intramyocardial injected fluid and co-injected cells it are flushed out of the tissue within heart beats.

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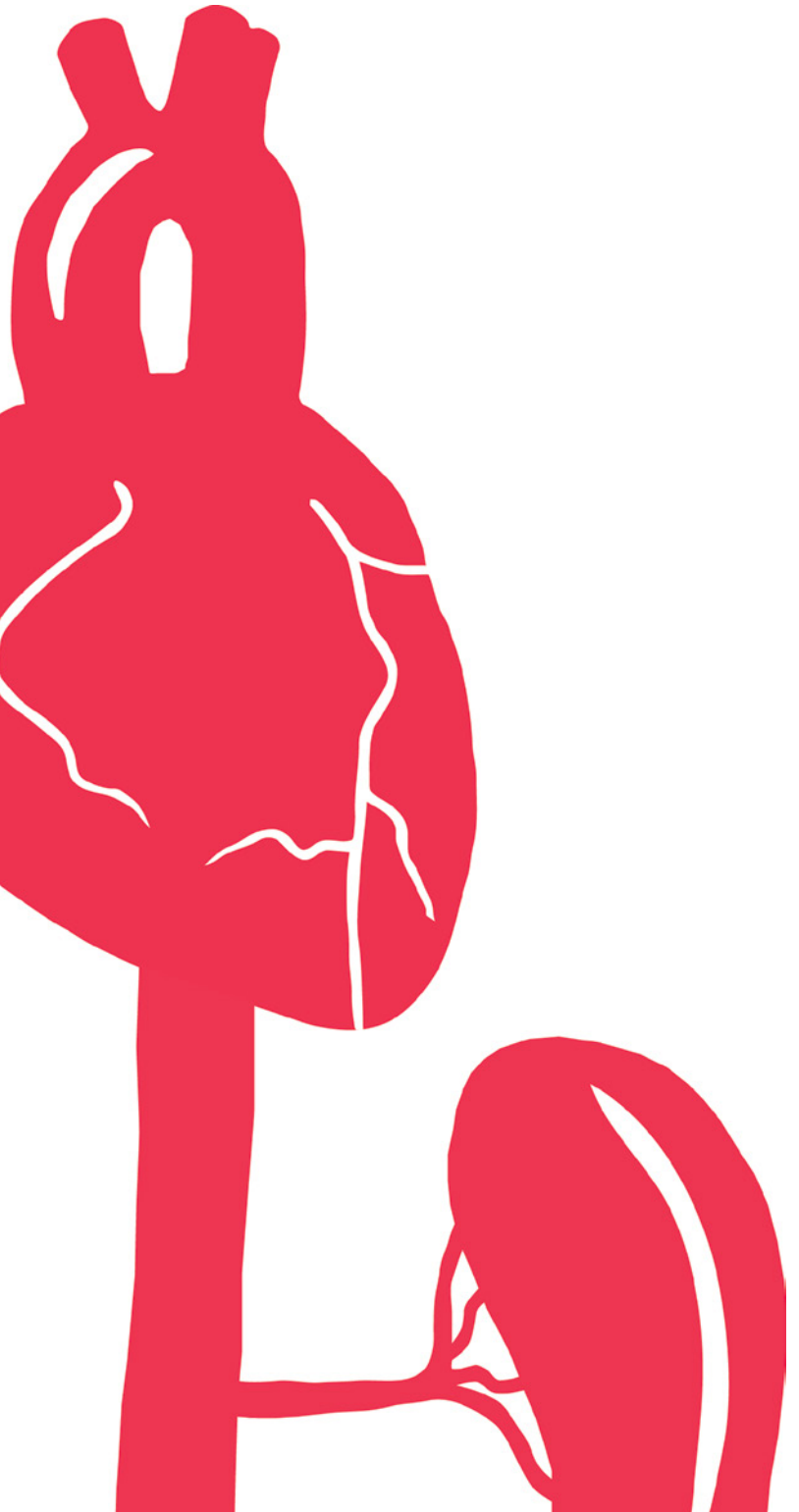

Differential Effects of Two Progenitor Cell Types on Neutrophils

2

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Abstract

Introduction

Neutrophils are the early responders after myocardial infarction. Neutrophil activation during reperfusion injury causes additional damage to cardiomyocytes. Interventions with mesenchymal stem cells (MSC) and cardiomyocyte progenitor cells (CMPC) are performed at this early time point, without knowledge on how these progenitor cells influence neutrophils directly.

Materials & Methods

Human neutrophils were cultured with human MSC and CMPC for up to 24 hours. Phenotype and functionality of neutrophils were compared using various assays.

Results

Both MSC and CMPC increased neutrophil viability, reduced NF-κB translocation and miR-223 expression, while increasing hypersegmentation. Neutrophils treated with MSC had a higher retention of CD62L and showed decreased ROS production.

Conclusion

During the acute phase after MI, intervention with MSC could create a more favorable neutrophil subtype and should be preferred over CMPC.

Introduction

Over the past years, the role of inflammatory processes in cardiac disease has become more evident. This includes the involvement of immune cells in the formation of atherosclerosis, their role in reperfusion injury after myocardial infarction (MI) and their contribution to the death of cardiomyocytes in all inflammatory cardiomyopathies[1-3]. In the acute phase of cardiac injuries, neutrophils are the most prominent immune cells that play a role. They infiltrate the heart in large numbers during, for example, reperfusion injury, attracted by chemo- and cytokines that are released due to the occurrence of cell death.

One of the main functions of the neutrophil is to scavenge cell debris and phagocytose fragments, in order to facilitate subsequent tissue healing. However, in the setting of acute cardiac damage, an abundance of pro-inflammatory signals is present, which activate the neutrophil into releasing reactive oxygen species (ROS)[1,3,4]. ROS are in turn toxic for surviving cardiac cells, leading to a negative spiral of increasing myocardial damage[5-7]. Although recognized by many, preventing neutrophil influx and ROS production remains challenging.

Within the immune system there is a need for complex interactions between the different immune cell subtypes, which combined might create the anti-inflammatory, reparative environment needed[8-10]. Complete inhibition of the immune response via corticosteroids or non-steroidal anti-inflammatory drugs (NSAIDs) leads to unwanted side-effects and harms the reparative signals in the heart[8,11].

Recently, the use of stem or progenitor cells has received a lot of attention for their cardiac regenerative effects and their immunomodulatory potential[8]. Cell therapies using progenitor cells, such as mesenchymal stem or stromal cells (MSC) or cardiac-derived cardiomyocyte progenitor cells (CMPC), achieve a reduction in acute myocardial damage and are able to preserve cardiac function at long term[12,13].

Although promising, the mechanism of action by which progenitor cells reduce damage remains ambiguous, as progenitor cells can influence many processes during the early post-ischemic period. Progenitor cells were suggested to have cytoprotective effects, which helps the stressed cardiomyocytes and supportive cells to survive[13]. Moreover, the cells induce angiogenesis, which will help the perfusion and thereby potentially prevent myocardial deterioration[12-14]. In addition, both MSC and CMPC have also been shown to have effects on the immune system[8], thereby make them interesting therapeutical options to counter inflammation in cardiac disease.

A shift in the field is that cardiac damage is better repaired using a cardiac progenitor cell. However, very little research has been performed to compare mesenchymal and cardiac progenitor cells, especially considering their immunomodulatory capacities. The broad range of effects of the progenitor cells makes the acute

inflammation hard to study *in vivo*: is the reduced inflammation a direct effect or a mere consequence of decreased damage?

To this end, we explored the MSC and CMPC effects on neutrophil activation and function, as the main effector in acute cardiac damage, *in vitro*.

Materials & Methods

Cell culture

Human fetal MSC and CMPC were isolated and characterized as described previously[14,15]. Cells from four different donors were used between passage 6 and passage 17. Cells were cultured in plastic culture flasks coated with 0.1% gelatin. MSC were cultured in MEM-α (Gibco, 22561) supplemented with 10% fetal bovine serum (Gibco, 10099-141), 100 U/ml Penicillin and 100 µg/ml Streptomycin (Lonza, 17-602E), 1 ng/ml bFGF (Sigma F0291) and 35.2 µg/ml ascorbic acid (Sigma A4034), as described before[16]. CMPC were cultured in CMPC culture medium (1 part endothelial basal medium (EGM-2; Lonza CC-3156) and 3 parts M199 (Lonza BE12-119F) supplemented with 10% FBS, 100 U/ml Penicillin and 100 µg/ml Streptomycin and 1% Non-essential Amino Acids (Lonza 13-114), as described before[14,17]. Both MSC and CMPC were passaged when reaching 80-90% confluence by trypsin digestion (0.25% Trypsin; Lonza, CC-5012) at 37°C for 2 minutes.

Neutrophil isolation and culture

Neutrophils were isolated by Ficoll-Paque PLUS (GE Healthcare) density gradient from the blood of healthy volunteers, according to the manufacturer's protocol[18]. The mononuclear cell layer was carefully removed. Erythrocytes in the granulocyte layer were lysed in isotonic ice-cold NH_4Cl solution (150 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM Na_2EDTA dissolved in ddH_2O). Neutrophils were typically >94% pure and were ~97% viable. Neutrophils were cultured in a 1:1 ratio with the progenitor cells, with $5x10^5$ or $1x10^6$ cells per well, depending om well size. When indicated, neutrophils were primed using 10^{-6} M N-Formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLF, Sigma F3506).

Flow cytometry

Monoclonal antibodies used for flow-cytometry are as follows: CD16 Alexa Fluor 647 (clone 3G8), CD11b-APC-Cy7 (clone ICRF44) and CD62L FITC (DREG56) were obtained from BD. Viability of the neutrophils was investigated using Annexin-V-PE and 7-Aminoactinomycin D (7-AAD, BD Pharmingen). Neutrophils were characterized by their distinct profile on the forward-sideward scatter plot. Within

this gate, only cells negative for both Annexin-V and 7-AAD were used for analysis of CD-marker expression.

NF-κB nuclear translocations

NF-κB nuclear translocation was studied on the level of the individual cell. Neutrophils from co-culture experiments were fixed with 0.1% formaline, permeabilized with 0.1% Triton X-100 and stained AF 488-anti NF-κB p65 (R&D, ic5078G). Nuclei were counterstained with Draq-5 (Invitrogen). Fluorescent photomicrographs were then taken of individual cells with an Amnis ImageStream Mark II Imaging flow cytometer. For each cell, the nuclear translocation was quantified by co-localization of NF-κB signal and DNA signal, data were expressed as Fisher's transformed Pearson's R.

Cytology

Before and after (co-)culture, cytospins were made and stained with May-Grunwald Giemsa. Nuclear lobes of 150 cells were counted during microscopical examination at ×100 magnification of 7 different co-culture experiments. Hypersegmentation was defined as nuclei containing ≥4 lobes.

miRNA

Total RNA was isolated with the Tripure isolation reagent (Roche Applied Science). Isolated RNA samples were used for cDNA synthesis using the qScript™ microRNA cDNA Synthesis Kit (Quanta Biosciences, Cat nº 95107-100) with up to 1000 ng of total RNA, according to the manufacturer's instructions. Human miRNA assays were performed for has-miR-223-3p, using up to 10 ng of cDNA.

The cDNA was subsequently used in the PerfeCTa® microRNA Assay quantitative PCR analysis. Data were normalized to the expression of the small nuclear RNA RNU6-1. Both miRNA primers were ordered from Quanta Biosciences.

mir-223-3p: UGUCAGUUUGUCAAAUACCCCA

RNU6-1: GUGCUCGCUUCGGCAGCACAUAUACUAAAAUUGGAACGAUACAGAGAA GAUUAGCAUGGCCCCUGCGCAAGGAUGACACGCAAAUUCGUGAAGCG UUCCAUAUUUU

Respiratory burst assay

In order to assess activation of neutrophils, production of reactive oxygen species in phagolysosomes was quantified by oxidation of dihydrorhodamine 123 (DHR 123) to rhodamine. $5x10^5$ neutrophils where stimulated for 4 hours with 4 μM fMLF alone or in combination with progenitor cells. Neutrophils where then transferred to conical tubes and loaded with DHR123 (BD Biosciences) at a final concentration

of 1 µM at 37°C for 10 minutes. Next, cells were fixed with 0.2% PFA, washed and nuclei were counterstained with Propidium Iodide to exclude dead cells. Intracellular fluorescence signal was then recorded on a BD Canto II flow cytometer.

External ROS production was measured after incubating neutrophils for 1 or 4 hours on progenitor cells. Neutrophils were then transferred to a 96-wells plate and stimulated with fMLF (4 µM; Sigma F3506) or fMLF/PAF-16 (4 µM/4 µM; Calbiochem 511075-1). As a positive control, stimulation with PMA (0.4 µg/mL; Sigma P8139) was also included. Amplex Red (25 µM; Molecular Probes, A-12222) and HRP (1 U/ml; Sigma, P-8250) were dissolved in HEPES medium. Production of fluorescent resorufin, formed from Amplex Red in the presence of HRP and hydrogen peroxide, was measured during the 90 minutes after addition of fMLF at 37°C using a fluoro-luminometer FluostarOptima (BMG LABTECH)[19]. Area under the curve (AUC) was calculated with Graphpad Prism 6.

Phagocytosis assay

Isolated neutrophils were cultured for 4 hours on MSC, CMPC or without stem cells. Subsequently, $1.5x10^7$ particles of FITC-labeled dead Escherichia coli (K12 Strain, Invitrogen) were added and cells were fixed after 20 minutes using 0.1% formaldehyde. All neutrophils were then washed and analyzed on an Amnis Imagestream Mark II imaging flow cytometer, which records brightfield and fluorescent images of individual cells.

A minimum number of 2000 cells were recorded and all images were analyzed using Ideas software (v6.2, Amnis). An image mask was created for each cell on the basis of the brightfield image and the number of fluorescent bacteria per cell was counted by a spot counting algorithm.

Statistics

All data are reported as mean ± SEM. Analysis was performed with IBM SPSS v20.0. For group comparison, parametric (one-way ANOVA) or non-parametric (Kruskal-Wallis) analysis was performed followed by an LSD or Mann-Whitney post-hoc analysis with a Bonferroni correction for multiplicity, respectively. For co-localization, correlation between NF-κB and Draq5 pixel intensities was quantified as Pearson's R and subsequently z-transformed by ln((1+r)/(1-r)) in order to obtain a normal distribution of co-localization values for statistical inference. The number of bacterial spots were analyzed by a nested generalized linear mixed effects model using a Poisson distribution; number of spots per cell were nested within a donor. P-values were obtained by Wald test. In all analyses, p-values of <0.05 were considered statistically significant.

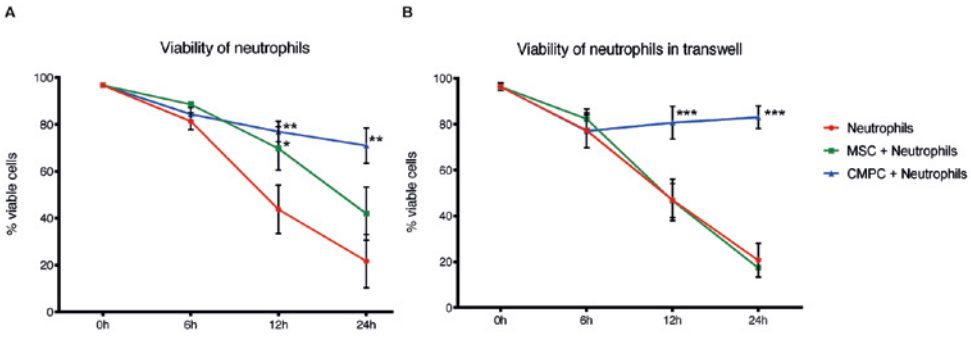

Figure 2.1 – Progenitor cells increase neutrophil survival

A Neutrophils that are kept in culture on top of MSC (green line) or CMPC (blue line) have a significantly higher survival compared to neutrophils alone (red line). B MSC (green line) and CMPC (blue line) were suspended in a transwell above the neutrophils. Only CMPC significantly prolonged survival compared to neutrophils alone (red line). ** p<0.05, ** p<0.01, *** p<0.001*

Results

Viability of neutrophils

Neutrophils were isolated from healthy donors and added to culture wells, which contained either no other cells or plated MSC or CMPC. Viability of the neutrophils was investigated at baseline and after 6, 12 and 24 hours (*figure 2.1A*). During the first 6 hours *in vitro*, the neutrophils in all groups experienced a mild decrease in viability with no significant differences between the groups (*figure 2.1A*). After 12 hours, however, over 50% of the neutrophils plated without stem cells showed signs of apoptosis and/or necrosis.

The viability of neutrophils plated on MSC ($p<0.05$) and CMPC ($p<0.01$) was significantly different: At 24 hours, the vast majority of neutrophils had died in the absence of stem cells (21.7% viability), while viability remained higher in MSC (41.9%) and CMPC (71%, $p<0.01$) co-cultures.

To study whether this was due to cell-cell contact, we also added the progenitor cells in a transwell system (*figure 2.1B*). The MSC no longer prolonged neutrophil survival, indicating that the MSC required cell-cell contact for their pro-survival effect. The CMPC, however, maintained their effect on neutrophil viability, suggesting a paracrine component for their effects.

Shedding of L-Selectin

Upon activation, neutrophils will shed the adhesion molecule L-selectin (CD62L). We measured CD62L expression on neutrophils after 0, 6, 12 and 24 hours of co-culture (*figure 2.2A*). Mesenchymal stem cell presence retained CD62L on

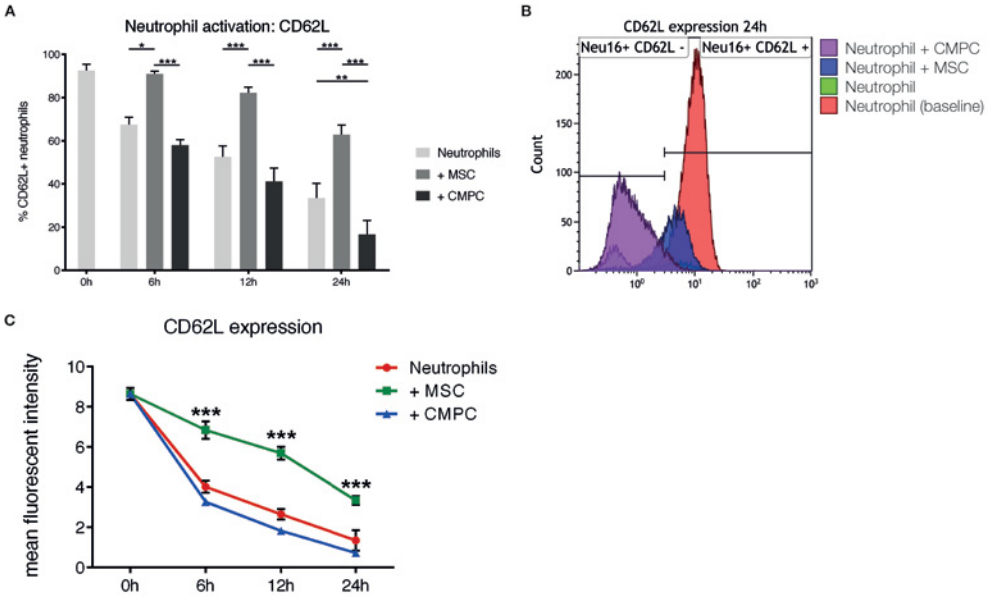

Figure 2.2 – L-selectin shedding

A L-selectin (CD62L) shedding occurs progressively during culture in the untreated and CMPC-treated neutrophils. MSC stimulate retention of CD62L. B Overlay graph showing the CD62L expression on neutrophils at baseline (red) and after 24 hours of treatment with MSC (blue), CMPC (purple) or control (green). C Median fluorescent intensity of CD62L is higher in the MSC treated group (green), compared to the untreated (red) or CMPC-treated (blue) neutrophils. Bars indicate mean of 7 experiments, error bars indicate SEM.
** p<0.05, ** p<0.01, *** p<0.001*

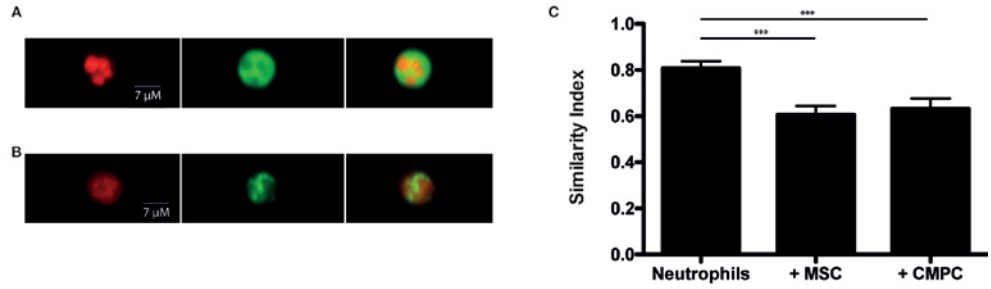

Figure 2.3 – Nuclear translocation of NF-κB

A The nuclear translocation in an neutrophil treated with MSC. NF-κB (AF 488; green) has a largely cytoplasmic distribution, being virtually absent in the nuclei (Draq-5; red). B Neutrophil in the control group, showing lower cytoplasmic signal of NK-κB (green) while more co-localization occurs. C Quantification of NK-κB nuclear translocation in the three treatment groups (data represent medians of 3 experiments).
**** p<0.001*

the neutrophil membrane, with >60% of the remaining neutrophils still expressing CD62L after 24 hours ($p<0.001$ compared to incubation without progenitor cells). Meanwhile, the CMPC did not reduce the shedding of CD62L. In fact, at all time-points measured, the neutrophils that were co-cultured on CMPC showed more shedding of CD62L, which even becomes significant after 24 hours ($p<0.01$).

The mean fluorescent intensity of CD62L (*figure 2.2C*) also differed strongly between the groups. Upon isolation, more than 95% of neutrophils have a high CD62L expression (*figure 2.2B*). After 24 hours, only 20% of the neutrophils cultured without progenitor cells retained high CD62L expression.

Co-culture with CMPC further reduced both median CD62L expression and the percentage of CD62L high neutrophils. In contrast, a large percentage of neutrophils co-cultured with MSC retained their high CD62L expression and had a higher MFI during the course of the experiment ($p<0.001$) (*figure 2.2C*).

Nuclear translocation of NF-κB

Subsequently, we measured the nuclear translocation of NF-κB, as an independent marker of cellular activation. After 4 hours, neutrophils cultured without stem cells show NF-κB localization in the nucleus, whereas neutrophils co-cultured with either MSC or CPMC show NF-κB to be localized largely in the cytoplasm (*figure 2.3A*). Quantification of NF-κB translocation with Pearsons correlation coefficient also shows a higher correlation between NF-κB and DRAQ-5 localization in neutrophils cultured alone, indicating a reduced activation state in presence of either MSC of CPMC (*figure 2.3C*, $p<0.001$).

Progenitor cells trigger neutrophil hypersegmentation, without T-cell suppressive capacities

Cytospins were made at various time points during the co-culture. After staining, the nuclei were examined for hypersegmentation (≥4 lobes), as a phenotypical characteristic of certain neutrophil subsets (*figure 2.4A*)[20]. As seen in *figure 2.4B*, healthy young adults already have about 50% of hypersegmented nuclei at baseline (0 hours), which decreases during culture of neutrophils. However, in the presence of both progenitor cells, the amount of hypersegmented nuclei increased over time ($p<0.05$).

Based on previous reports that hypersegmented neutrophils could be immunosuppressive[20], we examined the characteristics of these cells further. Since hypersegmentation is also linked to a low expression of miRNA-223 (miR-223)[21], we investigated miR-223 levels at the same time points. We found an increase in miR-223 levels in neutrophils during culture alone, peaking at 24 hours with a ~17-fold increase compared to baseline (*figure 2.4C*). In the presence of both MSC and CMPC, miR-223 levels remained low and comparable to baseline.

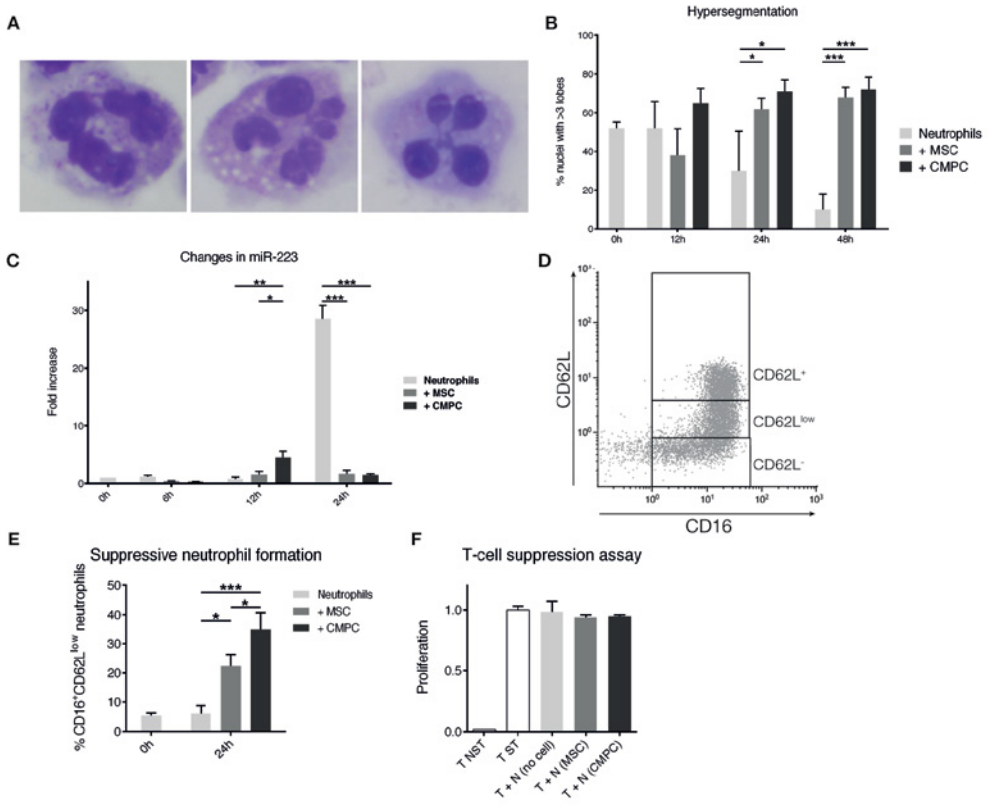

Figure 2.4 – Hypersegmentation and suppressive neutrophils

A Representative images of nuclear segmentation in the different treatment groups. **B** Quantification of hypersegmented nuclei (>3 lobes) over time in the three groups. **C** miR-223 is strongly upregulated at 24 hours in untreated neutrophils, while this is suppressed in the progenitor cell treated neutrophils. **D** Dot-plot showing CD62L and CD16 expression of neutrophils at 24 hours. The $CD62^{low}$ fraction (CD62L) is considered as possible immunosuppressive cells. **E** Quantification of $CD16^{+}CD62L^{low}$ neutrophils at baseline and after 24 hours of treatment. **F** After 16 hours of incubation, neutrophils from all three groups were added to activated, same-donor T-cells (T ST) and T-cell proliferation was measured after 4 days. T NST is the unstimulated control. ** p<0.05, ** p<0.01, *** p<0.001*

Although no definitive marker for suppressive neutrophils has been found, the combination of $CD16^{+}$ and $CD62L^{low}$ is considered to be the best surrogate[20,22]. Normal neutrophils in culture show only a low percentage of $CD16^{+}CD62L^{low}$. However, this percentage was increased in the vicinity of progenitor cells (*figure 2.4D* and *E*). Subsequently, we tested if these neutrophils had indeed suppressive effects on T-cells. For this, we pre-conditioned neutrophils for 16 hours and added them 1:1 to the same-donor, stimulated T-cells. However, despite all the suggestive characteristics, no effect on T-cell proliferation could be observed (*figure 2.4F*).

Functional analysis of neutrophils

In addition to the phenotypical changes, we examined the effect of the progenitor cells on neutrophil functions, such as the production of reactive oxygen species (ROS) and phagocytosis. ROS production can be measured both within the neutrophil (internal ROS) and upon cellular release (extracellular ROS). We incubated neutrophils for 4 hours on the progenitor cells, and subsequently measured the release of H_2O_2 (*figure 2.5A*). The neutrophils that had been exposed to MSC release significantly less ROS ($p<0.05$ at 1 and $p<0.001$ at 4 hours). CMPC, however, had no effect on the external ROS production by neutrophils, where levels remain similar to the neutrophils only group. Quantification of internal ROS production showed that neutrophils with MSC also produce significantly less internal ROS (*figure 2.5B and C*).

Lastly, we looked at the main immune-function of neutrophils by investigating phagocytosis of dead, FITC-labeled *Escherichia coli* by neutrophils that had been incubated for 4 hours on progenitor cells. As the bacteria were fluorescently labeled, the number of ingested bacteria per cell is quantified using flow cytometry (*figure 2.5D*). Phagocytosis of neutrophils treated with MSC is still similar to the normal neutrophil level, while the uptake is significantly decreased in neutrophils that have been in the presence of CMPC ($p<0.0001$, *figure 2.5E*). This is best seen by the increase in neutrophils that contain no bacteria in this group.

Discussion

In this study, we have investigated a potential immune modulatory effect of MSC and CMPC on neutrophils *in vitro* (*figure 2.5*). We have shown that both MSC and CMPC alter neutrophil phenotype and function, with some similar and some different effects (*figure 2.6*). As expected, the culture of neutrophils on either MSC or CMPC significantly prolonged neutrophil survival ex vivo. This corresponds to earlier findings in literature, where a prolonged survival of neutrophils is seen when kept in culture on other cell types[23-25]. However, in the case of MSC this is due to direct cell contact, rather than paracrine pro-survival signals. This is in contrast to the results observed by others, who noticed prolonged survival even in the absence of cell contact[23,24]. A paracrine-mediated increase in neutrophil life-span was in our experiments only observed during co-culture with CMPC.

In addition, both progenitor cell types suppress the nuclear localization of NF-κB. NF-κB is a transcription factor that regulates many pathways associated with inflammation[26]. Interestingly, in combination with Notch, NF-κB is able to activate the transcription of miRNA-223 (miR-223)[27]. The suppression in NF-κB translocation could therefore be responsible for the lower miR-223 levels observed. miRNAs are non-coding small RNAs, 20-24 nucleotides long, which regulate mRNA transla-

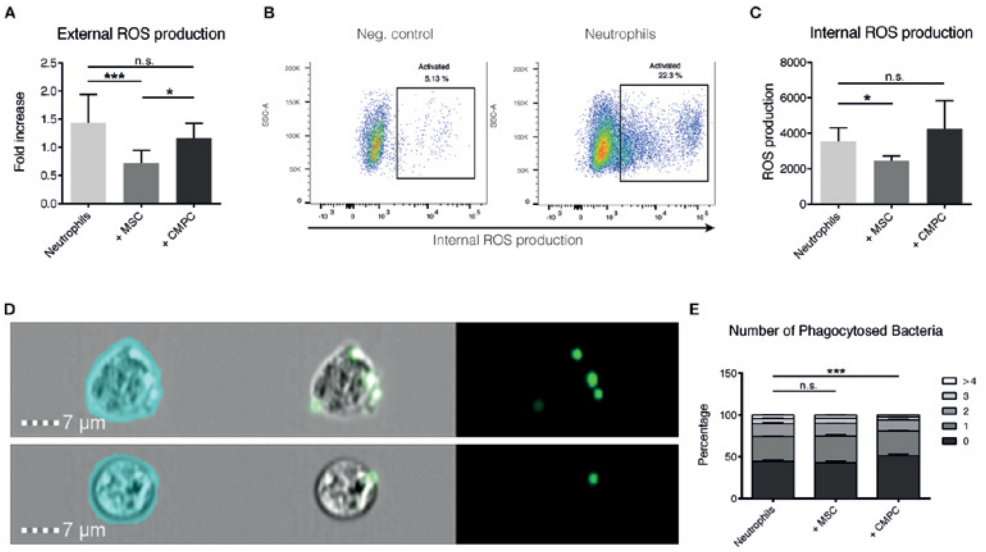

Figure 2.5 – ROS production and phagocytosis

A External ROS production measured by Amplex Red assay shows strong suppression of ROS production after 4 hours of incubation with MSC. **B** Internal respiratory burst production was analyzed oxidation of dihydrorhodamine 123 (DHR 123) to rhodamine. The process only occurs upon activation (right). **C** Quantification of internal ROS production during the respiratory burst. MSC again reduce ROS production by neutrophils. **D** FITC-labeled dead Escherichia coli were added to the treated neutrophils, after which uptake was determined by flow cytometry. Uptake of individual bacteria could easily be visualized. **E** Quantification of phagocytosis. In every neutrophil the number of phagocytozed bacteria was determined and stacked bars were created. A significantly larger number of CMPC-treated neutrophils did not ingest any bacteria. ** p<0.05, ** p<0.01, *** p<0.001*

tion[28,29]. miR-223 is known to play a role in neutrophil development and function[21]. In knock-out models of miR-223, no effects were seen on neutrophil mobilization, chemotaxis or extravasation, yet neutrophils of miR-223-/- mice showed increased hypersegmentation of the nucleus[21,30]. A study on ischemia-reperfusion injury showed that miR-223 expression is strongly increased after myocardial infarction, and administration of a miR-223 antagonist before reperfusion significantly reduced post-MI inflammation[28]. This makes miR-223 an interesting target in treatment of post-MI inflammation and more research should be performed to elucidate how progenitor cells prevent this increase in neutrophils.

In accordance with the lower NF-κB translocation and suppressed miR-223 levels, both progenitor cell types lead to increased hypersegmentation of neutrophil nuclei. The increased hypersegmentation of neutrophil nuclei might be important, as hypersegmented neutrophils have been described to have an immunosuppressive capacity, especially when displaying the markers $CD16^{+}CD62^{low}$ [20, 22]. However, hypersegmented neutrophils in our assays did not suppress T-cell

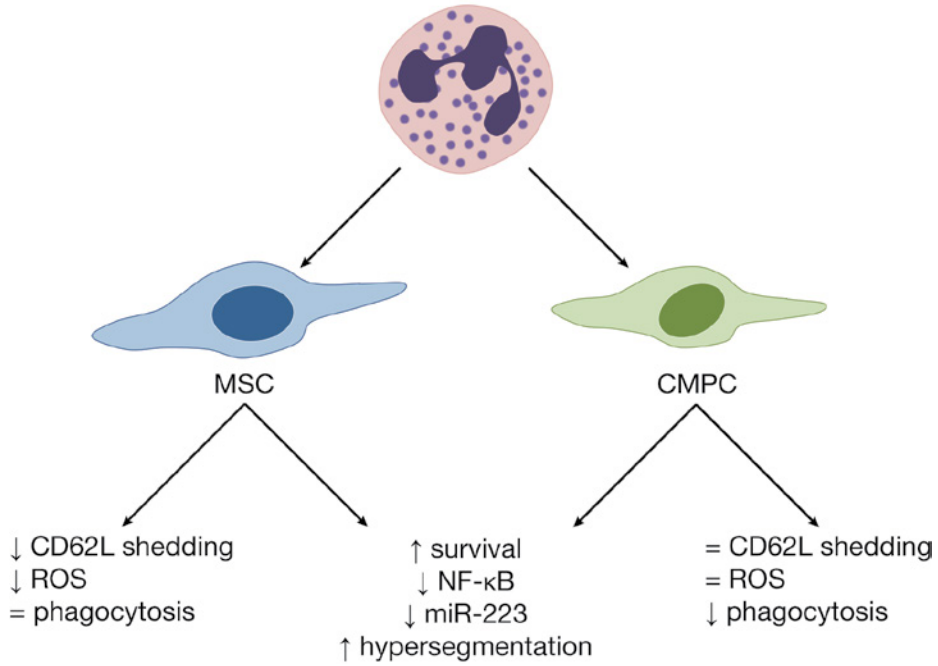

Figure 2.6 – The similar and different effects of progenitor cells on neutrophils
Both MSC and CMPC prolong neutrophils survival. They also reduce the nuclear translocation of NF-κB and the expression of miR-223. Hypersegmentation of neutrophil nuclei is increased by both progenitor cells. MSC reduce shedding of L-selectin (CD62L) and the production of ROS, whilst maintaining normal phagocytic capacity. CMPC-treated neutrophils have a decrease in phagocytosis, and a normal level of L-selectin shedding and ROS production.

proliferation. This could indicate that these two characteristics are only part of a larger panel that defines the neutrophil suppressive subtype, and that progenitor cells are not able to induce other necessary features. Alternatively, it could be that after 16 hours of co-culture, the neutrophils were simply too senescent to perform this task.

Despite the comparable suppression in NF-κB nuclear translocation in neutrophils by MSC and CMPC, only MSC have a beneficial effect on activation markers and function. This indicates that the regulation of neutrophilic inflammation is regulated on more levels than merely NF-κB[31]. One of the effects MSC had on neutrophils was a strongly increased retention of CD62L. CD62L is constitutively expressed on the cell surface of neutrophils, is essential for the rolling of neutrophils over the endothelium and during the process of extravasation it is enzymatically cleaved off and released[32].

The loss of CD62L is considered a strong activation marker[33,34]. However, the functional relevance of shedding of CD62L remains unclear[35]. Retention of CD62L

has been linked to reduced inflammation[36], while drug-induced, premature shedding of CD62L has also been associated with decreased neutrophil influx[33]. Most research points in the direction that subtle changes in CD62L expression can alter migration patterns and interactions with the vascular wall[37]. It is therefore important to not merely focus on the expression of CD62L, but also on the functional behavior of these neutrophils.

With the increased expression of CD62L, the MSC-treated neutrophils also had a strong reduction in ROS production, results similar to those observed in a study using feline cells[38]. As the production of ROS is considered to be one of the major causes of damage during reperfusion injury[7], reduction of these levels could very well decrease this additional injury. Interestingly, our results are in conflict with an earlier study, which showed that MSC actually prime neutrophils to produce ROS[24]. However, in that study neutrophils were kept in culture with MSC for 20 or 72 hours and our results clearly show poor viability of neutrophils at those time points, especially those without progenitor cells present.

Combining these results suggests that in the presence of MSC a less aggressive neutrophil is formed. The maintained scavenger function is an additional advantage of this induced neutrophil subset, as it leads to normal clearance of debris. Meanwhile, the CMPC-induced subset of neutrophils show an increased shedding of CD62L, while producing regular amounts of internal and external ROS. Additionally, this neutrophil subset showed diminished capacity for phagocytosis.

In conclusion, both MSC and CMPC strongly influence the phenotype and function of neutrophils in their proximity *in vitro*. Combined, the results suggest that the preferred intervention in the first 24 hours after myocardial infarction, when neutrophils are the most prevalent leukocyte in the heart, should be with MSC.

The administration of MSC before or during reperfusion might reduce the extent of reperfusion injury, by diminishing the neutrophilic production of ROS. In addition, the maintained phagocytic functions could boost cardiac repair. The use of CMPC, on the other hand, should preferably not be in this time frame, as it increases the incidence of long-living, ROS-producing neutrophils with lowered phagocytic function. Further *in vivo* studies focusing on neutrophils should be performed to further support this hypothesis.

Acknowledgements

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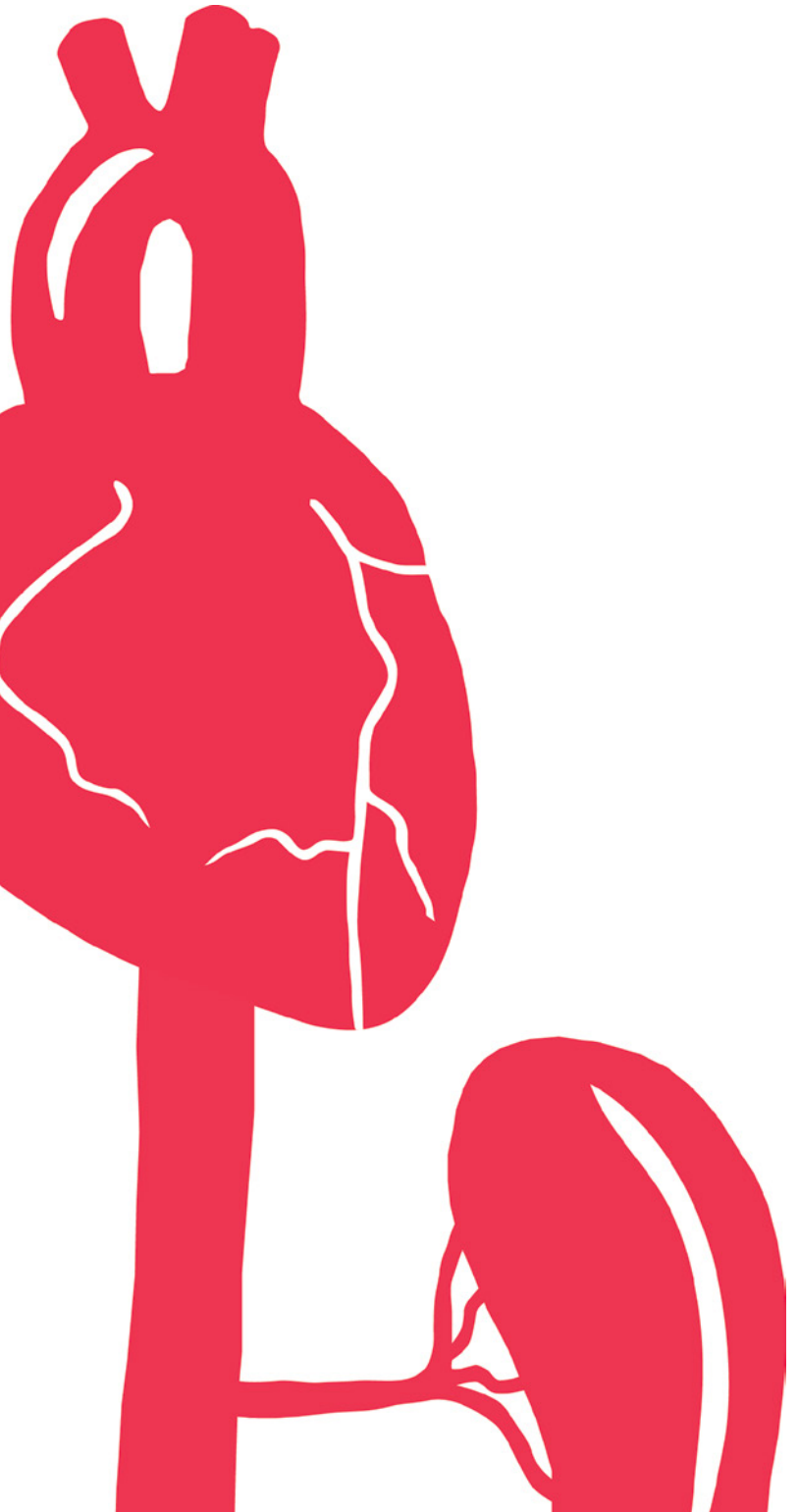

Suppression of the Adaptive Immune System by Mesenchymal and Cardiac Progenitor Cells

3

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Abstract

Introduction

Adverse remodeling after myocardial infarction (MI) is strongly influenced by T-cells. Stem cell therapy after MI, using mesenchymal stem cells (MSC) or cardiomyocyte progenitor cells (CMPC), improved cardiac function, despite low stem cell retention and only limited differentiation upon injection. As MSC are known to secrete many factors which affect T-cell proliferation and function, we hypothesized that the immune response could be affected as one of the targets of stem cell therapy. In this study, we compared the effect of MSC and CMPC on T-cells *in vitro*.

Material & Methods

Human MSC and CMPC were routinely characterized using flow cytometry and tested for multipotency. The immunosuppressive properties of CMPC and MSC and their extracellular vesicles (EVs) were tested in co-culture with freshly isolated, allogeneic human T-cells, stimulated with IL-2 and phorbol 12-myristate 13-acetate (PMA). Proliferation was measured by carboxyfluorescein succinimidyl ester dilution using flow cytometry. A luminex assay was performed to determine relevant cytokine profiles. Both full conditioned medium and EVs isolated from conditioned medium were tested for immunomodulative capacity. RNA-sequencing was performed to investigate alterations in activity of genes and pathways.

Results

The proliferation of T-cells was significantly reduced in the presence of MSC and CMPC. In addition, the inflammatory cytokine panel of the T-cells in co-culture changed, with strong downregulation of IFN-γ and TNF-α. The effect on proliferation was observed in both direct cell contact and transwell co-culture systems. Transfer of conditioned medium from the co-culture to unrelated T-cells abrogated proliferation in these cells to a similar extent as the original co-culture. EVs isolated from the conditioned medium of MSC and CMPC prevented T-cell proliferation in a dose-dependent fashion. The presence of progenitor cells induces up- and downregulation of multiple previously unreported pathways in T-cells.

Conclusion

Both MSC and CMPC have a strong capacity for *in vitro* immunosuppression. This effect is mediated by paracrine factors, one of which is extracellular vesicles. Besides proliferation, many additional pathways are influenced by both MSC and CMPC.

Introduction

In Europe ischemic heart disease remains the most common cause of death, responsible for the death of 19% of males and 20% of females[1]. After ischemia and the subsequent reperfusion, a strong immune response ensues[2-4]. Many types of circulating immune cells, such as neutrophils, monocytes, and dendritic cells are recruited to the heart[4-7], where cellular damage due to oxygen and nutrient deprivation is ubiquitous[5]. Some of these cells, including dendritic cells and macrophages, can pick up cardiac actin and myosin from the post-infarct debris and present them to T-cells, which can then become auto-reactive for these cardiac antigens[6,8-10].

The auto-reactive T-cells continue to attack cardiac cells displaying these antigens for a long time after the initial event, leading to adverse remodeling and a gradually decreasing heart function[11,12]. In fact, experimental models show the transfer of immune cells, including T-cells, from donors with cardiac disease will lead to decreased heart function in healthy recipients[12,13]. Currently, none of the heart failure therapies are aimed at modulating this ongoing process. The available immunosuppressive drugs suppress indiscriminately and can cause severe side effects, such as cardiac rupture[14-16]. Therapy aimed specifically at the suppression or modulation of these auto-reactive T-cells is still non-existent.

Recently a lot of focus has been on the use of stem cell therapy to regenerate the damaged heart. Studies using different kind of progenitor cells, such as mesenchymal stem cells (MSC) or cardiomyocyte progenitor cells (CMPC), show preservation of cardiac function[17-20], which is maintained up to at least three months after cell administration[17]. However, in the application of cardiac stem cell therapy poor engraftment has been observed for both MSC and CPMC[17-19,21] and, in the case of MSC, cardiac-differentiated cells have rarely been found[18].

This strengthens the hypothesis that most beneficial effects of cardiac stem cell injection arise from the secretion of paracrine factors by the injected cells[17,21,22]. Paracrine factors produced by stem cells can direct many processes, including stimulation of cardiomyocyte survival and angiogenesis, which could lead to improved outcome after MI[18,21].

An area of research which gained limited interest for the heart, is that MSC are also well known for their immunomodulating actions as described by DiNicola *et al.*[14,23-25]. MSC are able to reduce inflammation by suppressing the different cells of the immune system or force them into anti-inflammatory or even regulatory subtypes[14,24,25]. For this reason, we compared the effect MSC and CMPC have on the activated immune system and, specifically, how they can alter allogeneic T-cell responses *in vitro*.

Materials & Methods

Cell culture

Human MSC and CMPC, both adult and fetal, were obtained and characterized as described previously[26,27]. Of both MSC and CMPC four different donors were used between passage 6 and passage 17. Cells were cultured in plastic culture flasks coated with 0.1% gelatin. MSC were cultured in MEM-α (Gibco, 22561) supplemented with 10% fetal bovine serum (FBS; Gibco, 10099-141), 100 U/ml Penicillin and 100 µg/ml Streptomycin (Lonza, 17-602E), 1 ng/ml bFGF (Sigma F0291) and 0.2 mM L-ascorbic acid-2-phosphate (Sigma A4034), as described before[19].CMPC were cultured in CMPC culture medium (1 part endothelial basal medium (EGM-2; Lonza CC-3156) and 3 parts M199 (Lonza BE12-119F) supplemented with 10% FBS, 100 U/ml Penicillin and 100 µg/ml Streptomycin and 1% Non-essential Amino Acids (Lonza 13-114), as described before[26,28]. Both MSC and CMPC were passaged when reaching 80-90% confluence by trypsin digestion (0.25% Trypsin; Lonza, CC-5012) at 37°C for two minutes maximum.

Endothelial colony forming cells (ECFC) were isolated from human umbilical cord blood as previously described[29]. Briefly, the mononuclear cell (MNC) fraction was isolated from whole blood using Ficoll-paque density gradient centrifugation (GE life sciences, 17-1440-02). MNCs were plated on rat-tail collagen type I (BD Biosciences, 354236) coated six-well culture plates in a final concentration of $2x10^7$ cells per well in endothelial growth medium, consisting of EGM-2 supplemented with 10% FBS, 1% GlutaMax (Gibco, 35050038), 100 U/ml Penicillin and 100 µg/ml Streptomycin. Medium was refreshed daily for the first four days. After day seven the cells were trypsinized and plated on fresh collagen type I coated wells until colonies appeared. ECFC colonies were isolated and passaged at 90% confluency.

To generate conditioned medium (CM), cell cultures were maintained at 37°C for at least 3 days in a humidified atmosphere (5% CO_2 and 20% O_2). The CM was directly used in experiments, used for isolation of exosomes, or stored in -20°C for later use.

T-cell isolation

T-cells were freshly isolated from the blood of healthy volunteers. Peripheral blood mononuclear cells (PBMC) were purified by a Ficoll-Paque density gradient (1.077 g/mL, GE healthcare, 17-1440-02), according to the manufacturer's protocol[22,30]. Using anti-CD3 magnetic beads (BD Biosciences, 552593) and the BD IMagnet (BD Bioscience, 552311), T-cells were isolated from the PBMCs according to manufacturer's protocol. T-cells were labeled with 1.5 µM carboxyfluorescein succinimidyl ester (CFSE; Sigma, 21888), as described previously[22]. CFSE was diluted stepwise to the desired concentration and incubated with the cells for 10

minutes at 37°C in a dark, shaking water bath. Afterwards 5% FBS was added to block further uptake and cells were washed twice to remove excess CFSE.

Proliferation assay

T-cell proliferation was determined in the presence of MSC, CMPC, their conditioned medium or exosomes. For this, stem cells were plated at a concentration of $5.0x10^4$ cells per well in a 48-wells plate. In case of the pre-conditioning experiments, 20 ng/mL IFN-γ (Sigma I3265) was added. After 24 hours, the medium was removed and $5.0x10^4$ freshly isolated, CFSE-labeled T-cells were added in RPMI-1640 (Lonza, BE12-702F) supplemented with 10% autologous human serum, 100 U/ml Penicillin and 100 µg/ml Streptomycin.

T-cells were activated using phorbol 12-myristate 13-acetate (PMA 0.123 ng/ml; Sigma, P8139) and IL-2 (120 U/ml, BD Pharmingen, 554563) as described before[22]. After a six-day culture, cells were collected and Sytox blue (1 µM; Invitrogen, S34857) was added to determine viability. Cell viability and proliferation was measured by flow cytometry (Gallios, Beckmann Coulter) and analyzed using Kaluza Analysis Software (Beckman Coulter, v1.3) as followed: The fluorescent CFSE signal upon each cell division allowed us to count the number of cells present in each division. From this, we could calculate what percentage of the initial population had divided at the time of measurement (*suppl. figure 3.1*).

To allow comparisons between different donors, proliferation of the stimulated T-cell sample was used for normalization. Where indicated 1-MT (Sigma 447439), Indomethacin (Sigma I7378) and Alk5 (Sigma S4696) were added at the start of the co-culture.

Cytokine analysis

Conditioned medium (CM) was collected from CMPC, MSC and T-cells or from CMPC and MSC co-culture with T-cells after 6 days. Conditioned medium was centrifuged at 500 *g* for 10 minutes and supernatant was collected. After filtering through a 0.2 µm filter (Corning, 431219), the CMstored in -20°C for analysis and further experiments. Cytokines were measured using the multiplex immunoassay system (BioPlex, 200; Bio-Rad Laboratories) combined with the BioPlex Precision Pro Human Cytokine 10-Plex Panel (Bio Rad 171-A1001P), according to the manufacturer's protocol. This multiplex assay detects IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p70), IL-13, IFN-γ and TNF-α. For analysis, the data was normalized to stimulated T-cells to allow optimal comparisons between the different donors and groups.

Conditioned medium experiments

In the experiments where CM was used for T-cell suppression, $2.0x10^4$ freshly isolated, CFSE-labeled T-cells were added per well in a 96-wells plate and CM

was added 1:1 with fresh medium. T-cells were collected for analysis by flow cytometry after 4 days of culture.

Extracellular vesicle (EV) isolation and purification

For the isolation of EVs, CMPC and MSC were cultured in EV-free medium. Hereto all serum components were centrifuged 60 minutes at 150,000 *g*. EVs were isolated and validated from the CM by differential centrifugation as described before[31]. The CM was subsequently centrifuged for 15-30 minutes at 2,000 *g*, for 30 minutes at 10,000 *g* at 4°C (Beckman, Optima LE-80K Ultracentrifuge), and finally pelleted in a last centrifugation step (60 minutes at 100,000 *g* at 4°C). The EV pellet was washed with PBS and pelleted by another centrifugation step for 60 minutes at 100,000 *g* at 4°C. Finally, the washed pellets were re-suspended in PBS and before their use in functional experiments total EV protein concentration was determined with BCA protein assay (ThermoScientific). To determine the effect of CMPC and MSC derived EVs on activated T-cells, these exosomes were added immediately after PMA and IL-2 activation. After six days, the cells were collected and analyzed by flow cytometry. All functional tests were performed with unlabeled EVs.

To visualize uptake, the EV pellet was stained with PKH-26 (Sigma, PKH26GL), and after labeling excess PKH-26 was inactivated with EV-free FBS. PKH-26-labeled EVs and excess PKH-26 were separated by sucrose gradient purification and subsequently pelleted again by centrifugation at 100,000 *g*. The labeled EVs were added to CFSE-labeled T-cells. After incubation of various durations, T-cells were trapped on glass slides, the nucleus was stained with Hoechst 1:10,000 (Invitrogen H3570), fixed with 4% paraformaldehyde, and analyzed with fluorescence microscopy.

RNA sequencing

Samples for the RNA sequencing (RNAseq) were isolated and put in co-culture as described above. We included T-cells from three different donors and progenitor cells from two different donors. After 3 days, the non-adherent T-cells were collected, washed twice with PBS and stored at -80°C. In the end, 16 samples were selected based on quality, including both unstimulated (n=3) and stimulated (n=3) T-cells, as well as stimulated T-cells which had been in contact with MSC (n=5) or CMPC (n=5). RNA was isolated and libraries were created using the TruSeq Stranded Total RNA Sample Preparation LS according to manufacturer's protocol. An Illumina NextSeq500 and read-count analysis was performed by the Utrecht DNA Sequencing Facility (Utrecht, the Netherlands). RNAseq reads were aligned to the human reference genome GRCh37 using STAR v2.4.2a (http://bioinformatics.oxfordjournals.org/content/29/1/15). Read groups were added to the BAM files with Picard's AddOrReplaceReadGroups (v1.98). The BAM files are sorted with

Sambamba v0.4.5 and transcript abundances are quantified with HTSeq-count v0.6.1p1 (http://dx.doi.org/10.1093/bioinformatics/btu638) using the union mode. Subsequently, RPKM's are calculated with edgeR's rpkm() function (http://dx.doi.org/10.1093%2Fbioinformatics%2Fbtp616).

The resulting read counts per mRNA were subsequently analyzed according to the DESeq2 pipeline, to calculate differential expression (padj<0.05) between the 4 different groups of samples 32. PCA analysis was performed using DESeq2 command plotPCA() with "ntop = 5000" parameter.

Data analysis

All data are reported as mean ± SEM. Analysis was performed with IBM SPSS v20.0. For group comparison, parametric (one-way ANOVA) or non-parametric (Kruskal-Wallis) analysis was performed followed by a LSD and Mann-Whitney post-hoc analysis with a Bonferroni correction for significance respectively. A p-value<0.05 was considered significant.

Results

MSC and CMPC suppress proliferation of allogeneic T-cells

To examine and compare the immunomodulating capacities of both mesenchymal stem cells (MSC) and cardiomyocyte progenitor cells (CMPC), we performed co-culture experiments. Upon stimulation T-cells form proliferation clusters (*figure 3.1A*). In the presence of MSC and CMPC this cluster formation was strongly reduced or even absent, whereas in the presence of endothelial colony forming cells (ECFC) proliferating T-cell colonies still formed (*suppl. figure 3.2*). Proliferation of the T-cells was measured based on CFSE-signal intensity using flow cytometry (*figure 3.1B*).

Quantification of the proliferation of T-cells can be seen in *figure 3.1C*. In the non-stimulated conditions no proliferation could be observed, indicating the isolation process itself did not activate the T-cells. In IL-2/PMA-stimulated cultures, the percentage of proliferating T-cells was significantly lower in the presence of MSC (65% ± 8%; p<0.001) or CMPC (97% ± 0.6%; p<0.001; *figure 3.1C*). ECFC, on the other hand, did not influence the proliferation of T-cells (*suppl. figure 3.2*; 0% ± 0.2%). Comparing the two types of progenitor cells, the CMPC perform significantly better than the MSC (*figure 3.1C*; p<0.001).

We compared the effectiveness of the fetal progenitor stem cells with adult progenitor cells obtained from patients with cardiac disease (*figure 3.1D*). With respect to the MSC, the age or health status of the donor did not impact the suppressive capacities of the cells. This is not the case, however, for the CMPC, where the fetal

CMPC performed significantly better than the diseased adult CMPC ($p<0.001$). Even so, the adult CMPC still maintain immunosuppressive abilities comparable to fetal and adult MSC.

Several publications have shown that progenitor cells need to be activated by inflammatory molecules, such as cytokines, before they show any suppressive capacities: a process called *licensing*[33-35]. We investigated whether pre-conditioning using inflammatory cytokines had any effect on the suppressive potential of these cells. Incubation with IFN-γ for 24 hours had no effect on the progenitor cells of fetal origin (*figure 3.1E*). However, the adult progenitor cells perform better after the IFN-γ treatment, which is especially clear in the case of the adult CMPC ($p = 0.006$).

MSC and CMPC alter the inflammatory environment of stimulated T-cells

Since cell proliferation is only one element of inflammation, we also investigated the inflammatory status of the conditioned co-culture medium. Additionally, this allowed us to investigate to formation of specific T-cell subsets. To examine the production and release of cytokines, conditioned medium (CM) was collected from individual MSC, CMPC, and T-cell cultures and from co-cultures of MSC or CMPC with T-cells. Cytokines indicative of both T_H1 and T_H2 were strongly reduced in the presence of MSC and CMPC (*figure 3.2A*; *suppl. table 3.1*). Significant reductions were measured in the release of the T_H1 cytokines IFN-γ, TNF-α and IL-12-p70 ($p<0.001$ for all). Additionally, T_H2 cytokine secretion (IL-4, IL-5 and IL-13; *figure 3.2B*) was also significantly diminished. Interleukin-10 (IL-10), usually suggested to be anti-inflammatory and an inducer of T_{reg}, was produced in high levels by active and proliferating T-cells, whereas its release was strongly diminished in the presence of either MSC or CMPC ($p<0.001$ for both; *figure 3.2B*). The cytokine IL-1β was produced by MSC and in high levels by CMPC compared to stimulated T-cells ($p<0.01$). In the co-culture experiments, these levels were increased even further, to 13-fold for MSC and 52-fold for CMPC ($p<0.001$).

Paracrine factors are responsible for T-cell suppression

To investigate whether the immunosuppressive effect of MSC and CMPC is dependent on direct cell-cell contact, we subsequently used cell culture system in which an insert separated the different cell types from each other. This revealed that prevention of cell-cell contact led to an even stronger inhibition of T-cell proliferation. (MSC: 58 ± 10%, CMPC: 62 ± 9%, $p<0.05$ for both; *figure 3.3A*).

The next question was whether this observed immunosuppression could be mediated via soluble, stable compounds in cellular secretions. To this end we collected conditioned medium (CM) after six days of single- or co-culture. The obtained CM was added 1:1 with fresh medium to newly isolated and stimulated T-cells. A significant suppression of T-cell proliferation occurred in the presence of

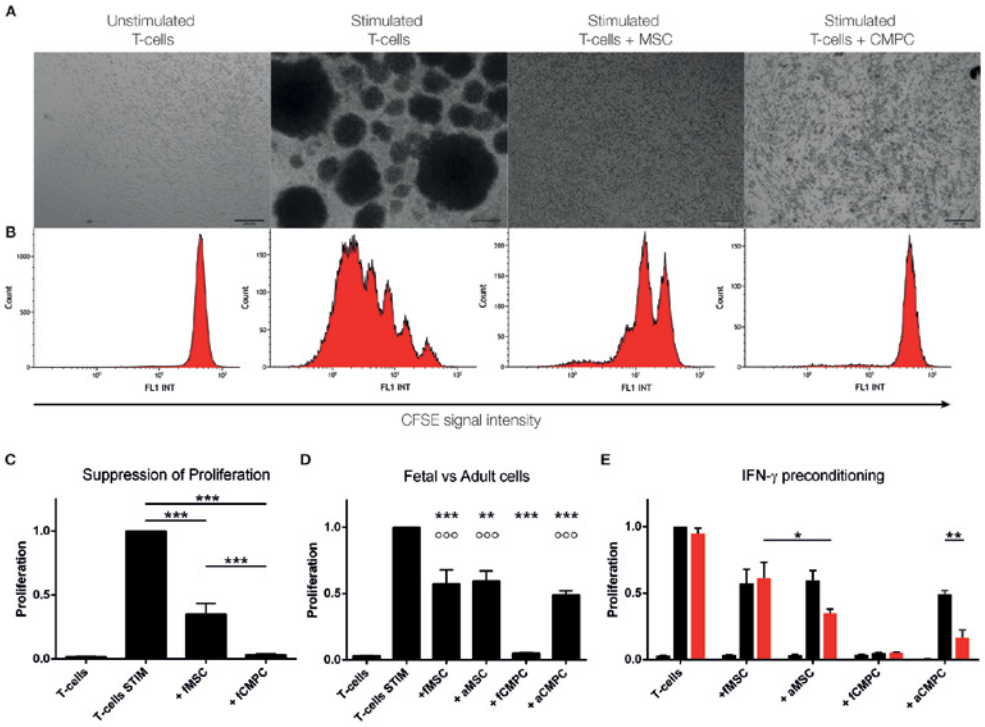

Figure 3.1 – MSC and CMPC co-cultures with T-cells after 6 days of stimulation

A Light microscopical representation of T-cell co-cultures after 6 days. In the non-stimulated samples, small individual cells are spread throughout the well. Upon stimulation, T-cells form proliferating colonies. In the presence of MSC and CMPC, the formation of these colonies is strongly reduced or even absent. Bar = 200 µm. B Proliferation of T-cells as measured by flow cytometry. Non-stimulated T-cells have a single FL1 peak at a high fluorescent intensity. Upon stimulation, lower intensity peaks form, halving the fluorescent signal upon each cell division. C Quantification of proliferation of T-cells in different co-cultures. The first bar represents the unstimulated control, while the second bar is the stimulated T-cells. Proliferation of T-cells is significantly reduced in the presence of MSC (65% ± 8) and CMPC (97% ± 0.6) (MSC and CMPC: n=12). D Differences in suppressive capacity based on donor age. Both fetal and adult cells suppress T-cell proliferation significantly. Adult MSC suppress similar to fetal MSC (40% ± 7.5 and 43% ± 11, resp.), while fetal CMPC perform better than adult CMPC (95% ± 0.4 and 51% ± 3.1, resp.). E The additional effect on T-cell suppression due to *licensing* was investigated. Progenitor cells were preconditioned with 20 ng/mL IFN-γ (red bars) and compared to the unconditioned cells (black bars). Preconditioning had no effect on fetal stem cells. Adult MSC improved to 65% ± 3 suppression, while adult CMPC improved to 83% ± 5.8 suppression (p=0.006).
** p<0.05, ** p<0.01, *** p<0.001, °°° p<0.001 compared to T-cells + fCMPC*

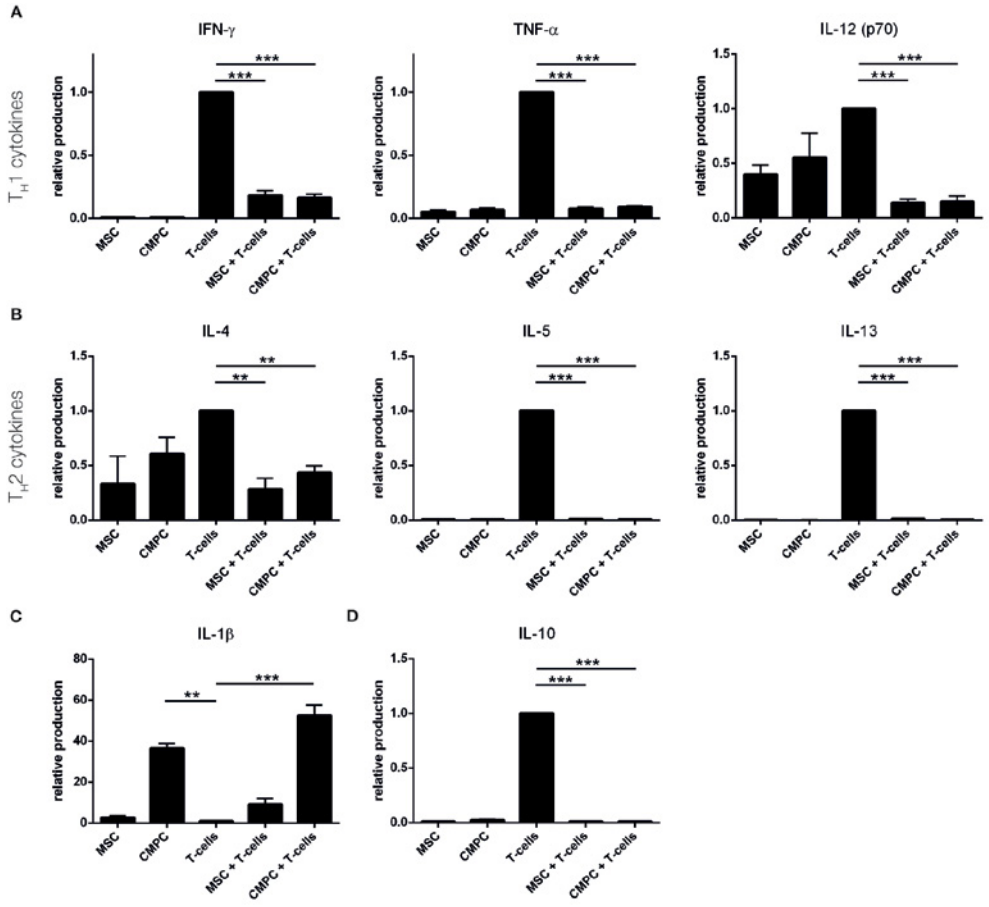

Figure 3.2 – Alteration of inflammatory environment

The cytokine production by MSC and CMPC only, by T-cells only, and upon co-culture of stem cells and T-cells was measured by Luminex assays. **A** Pro-inflammatory TH1 cytokines IFN-γ, TNF-α and IL-12 are produced by stimulated T-cells but down-regulated upon co-culturing with both MSC and CMPC. Indicating a suppression of T_H1 cell development. **B** The development of T_H2 cells is determined by the presence of IL-4, IL-5 and IL-13. These cytokines are all upregulated in response to T-cell activation and are significantly suppressed in the presence of progenitor cells. **C** The release of IL-1β, which is produced by MSC and CMPC yet hardly by T-cells, is further increased upon co-culture with T-cells. **D** Release of IL-10, a supposedly anti-inflammatory cytokine, is strongly suppressed when progenitor cells are present in the co-culture, yet highly produced by stimulated T-cells. For values see *suppl. table 3.1*. *** p<0.01, *** p<0.001*

CM obtained from MSC (40 ± 10%, p<0.01), while the CM from the co-culture of MSC with T-cells performed even better (51 ± 8%, p<0.01; *figure 3.3B*). This difference was, however, not significant. Even more pronounced effects were again observed with the CMPC, where the CM from both the CMPC and the co-culture completely suppressed T-cell proliferation (p<0.0001) (*figure 3.3C*). No significant effect, neither suppressive nor stimulatory, was seen after addition of CM derived from stimulated T-cells only (1.14 ± 17%, *figure 3.3B* and *C*).

Stem cell derived extracellular vesicles can inhibit T-cell proliferation

To investigate whether extracellular vesicles (EVs) secreted by stem cells can influence the proliferation of T-cells, we isolated EVs from conditioned medium of MSC and CMPC. To demonstrate uptake of EVs by stimulated T-cells, we added purified PKH-26 (red) labeled EVs to CFSE-labeled (green) T-cells. After overnight incubation, we observed by fluorescence microscopy a clear co-localization of the exosome-, T-cell- and nuclear labels (*figure 3.4A*). We then quantified the uptake of these EVs by T-cells over time (*figure 3.4B*) and noticed most EV uptake occurred in the first hour after addition. Around 35% of the T-cells is positive at this time-point, which rises to approximately 60% after 24 hours.

Next, we examined the effect of EVs on T-cell proliferation in a dose-response experiment using 0.0025 µg to 10 µg of EVs (*figure 3.4C*). A clear dose-dependent effect on T-cell proliferation was visible, as shown in *figure 3.4C*. We subsequently added 1.5 µg of MSC- or CMPC-derived EVs to stimulated T-cells. The addition of MSC- or CMPC-derived EVs resulted in a strong decrease of proliferation (73 ± 12%, p<0.01 and 77 ± 10%, p<0.01, respectively).

Pathway inhibitors

Several studies have reported a number of pathways to be involved in the immunosuppressive responses, amongst which indoleamine 2,3-dioxygenase (IDO) and prostaglandin E2 (PGE2)[14,33,36,37]. However, we studied the proteomics of our exosomes and did not find any components of the IDO or PGE2 pathways to be present (data not shown). Even so, we performed the co-culture assay in the presence of several inhibitors: 1-methyl-L-tryptophan (against IDO), indomethacine (against PGE2) and Alk5 (against TGF-β). Upon co-culture, however, we did not see any change in the T-cell suppression (*suppl. figure 3.3*). In addition to the inhibitor doses used by other research groups[38-40], we have performed a dose titration, as well as preconditioning and repetitive administration of the inhibitors to block the immunosuppression. However, we still did not find any effect of these inhibitors. We therefore changed to an unbiased approach using RNAseq.

RNAseq shows differentially regulated genes

In order to get an unbiased view on the changes induced in the T-cells, we

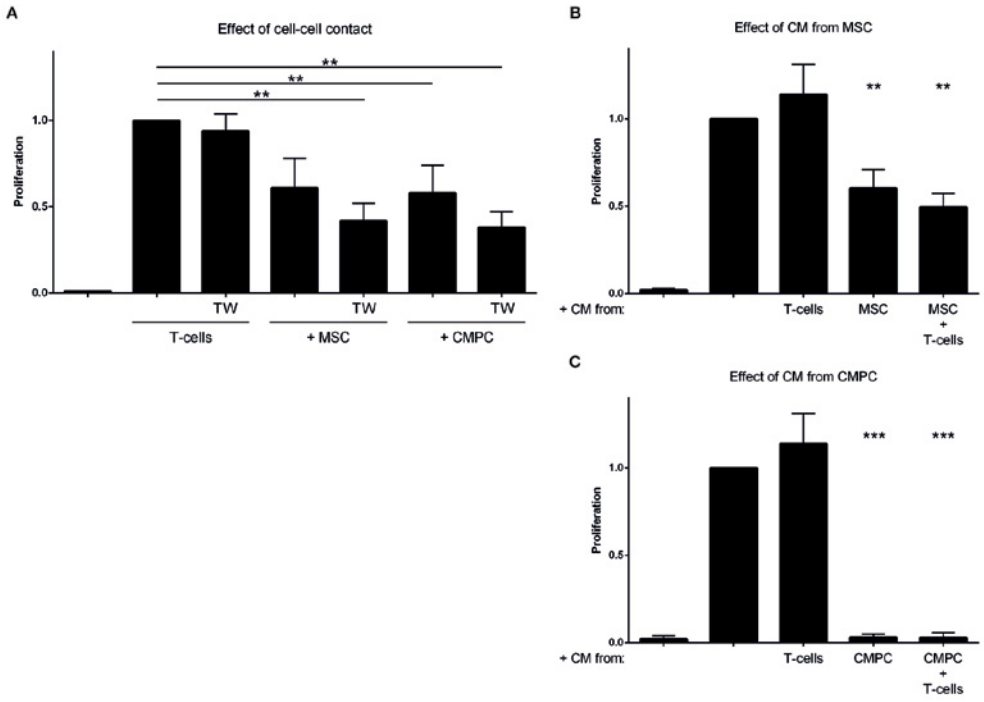

Figure 3.3 – Paracrine effect

A transwell experiment (TW) was performed where MSC or CMPC are located in the bottom part and activated T-cells on top of the 0.4 µm TW-filter. In control groups the cells were allowed cell-cell contact. **A** Stimulated T-cell co-cultures with MSC and CMPC (TW^-) and without (TW^+) direct cell-cell contact. Suppression of proliferation occurs in cell contact groups (MSC: 39% ± 17, n.s., CMPC: 42% ± 16, $p<0.05$). Reduction of proliferation still occurs in the absence of direct cell-cell interactions (MSC: 58% ± 10, CMPC: 62% ± 9, $p<0.05$ for both) (n=5). **B** Stimulated T-cells grown in the presence of CM from MSC or the MSC-T-cell co-culture have a significantly reduced proliferation (40% ± 10 (n=3) and 51% ± 10 (n=7), respectively), whereas CM from stimulated T-cell has no effect (14% ± 17 increase). **C** Stimulated T-cells proliferate significantly less after addition of CM from CMPC (97% ± 0.2 suppression, $p<0.0001$) or the CMPC-T-cell co-culture (97% ± 0.3).
*** p<0.01, *** p<0.001 compared to stimulated T-cells*

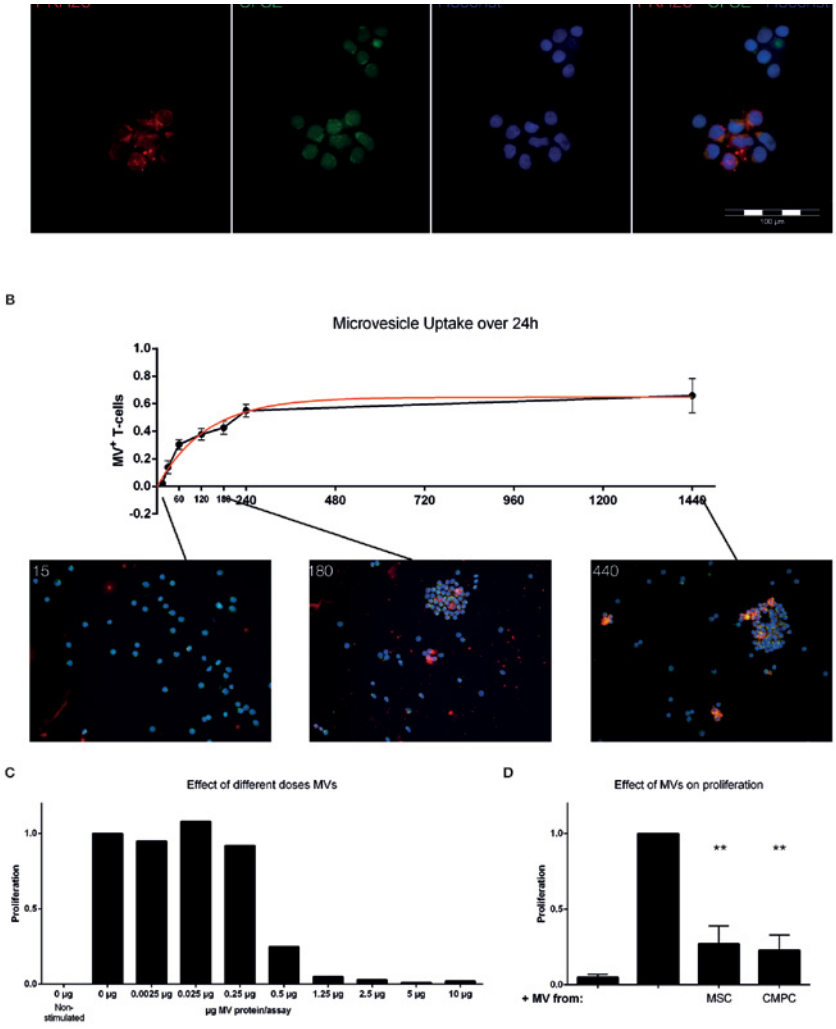

Figure 3.4 – Effect of stem cell derived EVs on T-cell proliferation

EVs were isolated from the conditioned medium of CMPC and MSC to investigate their immunomodulative potential. **A** Fluorescence microscope visualization of the EV uptake by T-cells. Red: EVs (PKH-26). Green: T-cells (CFSE). Blue: nucleus (Hoechst). **B** Titration curve of CMPC derived EV protein concentration and the effect on T-cell proliferation after stimulation. A cut-off point is reached around 1 µg of EV protein. **C** MSC- and CMPC-derived EVs significantly reduce T-cell proliferation (MSC-EV: 73 ± 12%, CMPC-EV: 77 ± 10%).

*** p<0.01 compared to stimulated T-cells*

performed an RNAseq on several individual T-cell donors that were exposed to different progenitor cell donors.

Unsupervised principal component analysis in the sixteen T-cell samples, color-coded by group (*figure 3.5A*) based on 5000 most variable transcripts (ref DESeq2), illustrated a close proximity of samples per group. Additionally, principal component analysis showed that 66% of the variation in the dataset can be explained by either the presence of the progenitor cells (PC1; 40%) and the T-cell activation (PC2; 26%).

Upon activation of T-cells, almost 1500 genes were significantly upregulated (>2-fold, padj<0.05). Of these genes, 416 were suppressed (>2-fold) in the presence of MSC, compared to only 100 genes in the presence of CMPC (*figure 3.5B*). 86 T-cell genes were upregulated upon activation and suppressed in the presence of either MSC or CMPC, as shown in the Venn diagram (see also *suppl. table 3.2*). Subsequently, we entered these genes in IPA, which showed pathways concerning inflammation and immune cell proliferation. However, we noticed all these pathways depended largely on a small number of the 86 genes. Therefore, we used the NBCI Gene database (http://www.ncbi.nlm.nih.gov/gene/) and the GeneCards database (http://www.genecards.org/) to investigate the function of these genes in T-cells. As depicted in the pie-chart in *figure 3.5C*, 20% of these genes (17 genes) has a known role in cell proliferation. Another 14% (12 genes) is related to the production and release of cytokines and/or their receptors. Lastly, only 3 genes (~3%) are associated with inflammation. This leaves 54 genes (63%) that have no clear role in proliferation/inflammation or no known function.

Discussion

In this study, we have investigated whether MSC and CMPC have a potential *in vitro* immune modulatory effect. We have demonstrated that both MSC and CMPC inhibit T-cell proliferation, with CMPC having a significantly stronger suppressive effect than MSC. There was no difference in low and high passage progenitor cells on suppressing T-cell proliferation (data not shown) but CMPC showed differences based on donor age (fetal versus adult). In addition to suppressing proliferation, both MSC and CMPC also suppressed the release of pro-inflammatory cytokines from T-cells. Although a shift towards a specific T-cell phenotype is occasionally reported[30,41], we found the cytokines of both T_H1 (IL-12, IFN-γ, TNF-α), T_H2 (IL-4, IL-5, IL-6) and T_{reg} (IL-10) subtypes to be suppressed, suggesting that the T-cells remain in a naïve, inactivated state.

Suppression of T-cell proliferation by MSC has been shown in a number of other previous studies[23-25,38,42], albeit with a lot of variation in suppression between different donors[42]. With our previous discovery that CMPC are also strong modulators

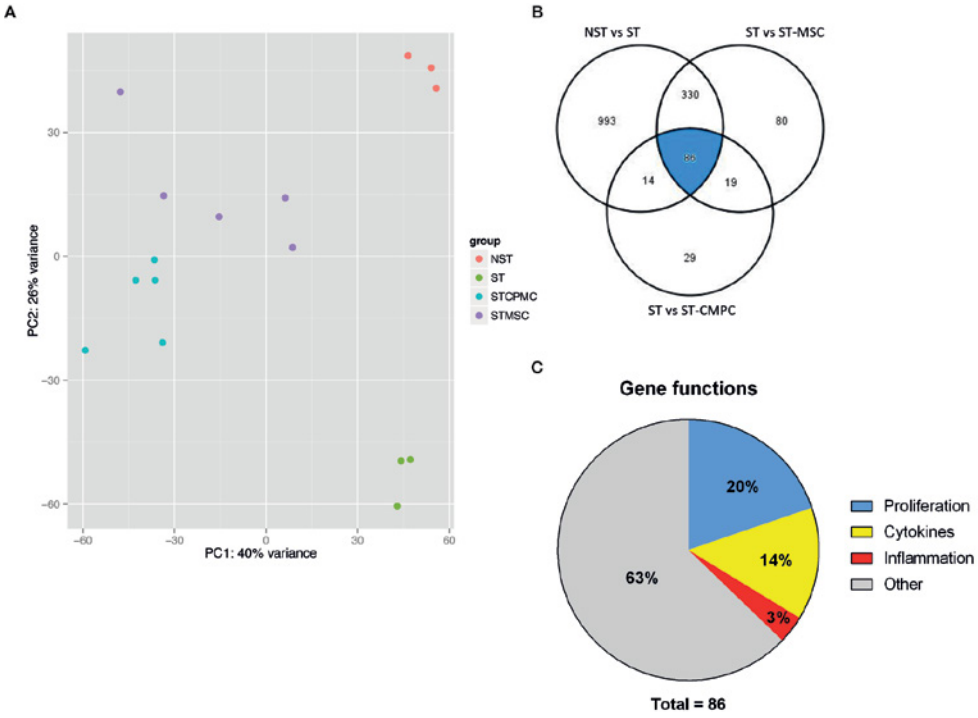

Figure 3.5 – RNA sequence of T-cell modulation

A Principal component analysis shows a division of samples in four groups, matching our experimental conditions. Most variation between the samples is explained by the presence of stem cells in the culture (PC1: 40%) and the activation of T-cells (PC2: 26%). **B** Venn-diagram showing the overlap between genes that are >2-fold upregulated upon activation (left-upper circle), and >2-fold downregulated in the vicinity of MSC (right-upper circle) or CMPC (lower circle). **C** Pie-chart showing the functions of the 86 genes found.

of the immune system and the reports of immunomodulative effects of neural stem cells (NSC)[42,43], we wondered whether immunomodulation is a more general stem cell trait. For this reason we included ECFC, a circulatory endothelial colony forming cell, which proved unable to alter the number of proliferating cells.

Both MSC and CMPC mediated immunomodulation by production of paracrine factors, which was readily demonstrated in our transwell experiments. This finding is in agreement with DiNicola *et al.*[23] who also found that cell-cell contact was dispensable for immunosuppression by MSC, while others[38] found reduced suppression in absence of cell-contact. A possible explanation for this is coined by English[33], who proposes that cell-cell contact is not necessary for the suppression itself, but for induction of the T-regulatory phenotype. Another explanation for these observed differences could be the different origins, isolation methods and (co-)culture methods in the different studies, which makes it hard to compare an already heterogenous group of progenitor cells[42].

The immunosuppressing paracrine factors were already present during normal expansion cultures without any immune cells being present (*figure 3.4B* and *C*). These findings correlate to the paracrine hypothesis, which states that the vast majority of MSC effects is exerted via paracrine mediators[21,44,45]. It is in contrast, however, with studies that claim the progenitor cells need to be 'licensed' by immune cells to release these suppressive factor[33-35]. Again, it is quite likely that the different origins of progenitor cells in different studies could be responsible there. Our study shows that adult progenitor cells appear to diminish in immunosuppressive capacity, but can be re-activated by exposing them to IFN-γ, while on the fetal progenitor cells this preconditioning has no effect.

Immunosuppression by exosomes derived from (modified) dendritic cells[46-48] or cancer stem cells[49] has been reported in the past and a recent study demonstrated effects of MSC-derived exosomes on hepatic inflammation and fibrosis[50]. Additionally, some reports were published on MSC-derived microvesicles and exosomes suppressing the proliferation of lymphocytes[51-54]. However, in these studies the whole peripheral blood mononuclear cell (PBMC) or splenocyte fraction was used, leaving it ambiguous whether observed effects were caused by direct interference with the T-cells or indirectly via another cell type, such as macrophages[53].

We examined the immunomodulating capacity of MSC- and CMPC-derived EVs on pure $CD3^+$ T-cells and observed a strong inhibition of proliferation *in vitro* when EVs were added to stimulated T-cells. Our titration experiment indicated this effect is dose-dependent. We do believe that, although MSC- and CMPC-derived EVs are important mediators of immunomodulation, they do not cover the complete suppressive effect, and will most likely function optimally in combination with several growth factors or cytokines produced by the progenitor cells.

Several potential mediators, produced by MSC, have been investigated for their involvement in the immunomodulatory effects, including interleukin-10 (IL-10), inducible nitric oxide synthase (iNOS), transforming growth factor-β (TGF-β), prostaglandin E2, and indoleamine 2,3-dioxygenase (IDO)[14,24,33,36,38,55]. Of these, the last two have been most investigated in different settings. Several studies have attempted to block these pathways, which resulted in a variable decrease of the immune suppressive effect of MSC. However, these experiments had variable outcomes and until now the exact mechanisms of immune suppression are controversial[14,33,38,55,56]. In our hands, addition of inhibitors for these pathways did not show any effect on the immunosuppressive effects of the progenitor cells. We did not include an inhibitor against iNOS in these experiments, as our experiments with the conditioned medium already demonstrated the effect is mediated by a stable compound, which nitric oxide (NO) is not.

An explanation for our observed ineffectiveness of pathway inhibition is suggested

by our RNA sequencing. We found 86 genes which are upregulated during T-cell activation and are suppressed in the presence of stem cells. Interestingly, less than half of these genes is directly linked to proliferation or inflammation, while the majority has either completely different or unknown functions. We believe these genes to play an important role in the modulation of T-cells and warrant further investigation.

We recognize some limitations of this study. The first is inherent in the study of the immune system *in vitro*, which forced us to use a non-physiological method of T-cell activation. However, in the MI-setting, activation of the immune system is a complex response mediated by various cytokines, damage-associated molecular patterns (DAMPs), natural antibodies and many other factors[14,57], which is impossible to simulate *in vitro*. This leads directly to the second limitation in our study, which applies to all *in vitro* immune research: the immune system is a complex and interactive system in which all components strongly influence each other and excluding a specific cell type could unbalance this system and possibly influence the interactions with MSC or CMPC.

Conclusion

We demonstrated that both mesenchymal stem cells (MSC) and cardiomyocyte progenitor cells (CMPC) strongly modulate the immune system by attenuation of T-cell proliferation *in vitro* and reducing release of pro-inflammatory cytokines. This suppression is not dependent on *licencing* of the progenitor cells, nor on cell-cell contact. It is mediated via paracrine factors, which are already produced during regular culture as can be seen by the effect of the conditioned medium on T-cell proliferation. EVs isolated from this conditioned medium were shown to be dose-dependently capable of suppressing T-cell proliferation and might be used as a new treatment for post-MI inflammation, to reduce damage to the heart in both short and long term. Lastly, despite earlier publications on pathways involved, we found a pallet of unstudied genes expected to play a major role in the activation and suppression of T-cells which need further investigation.

Acknowledgements

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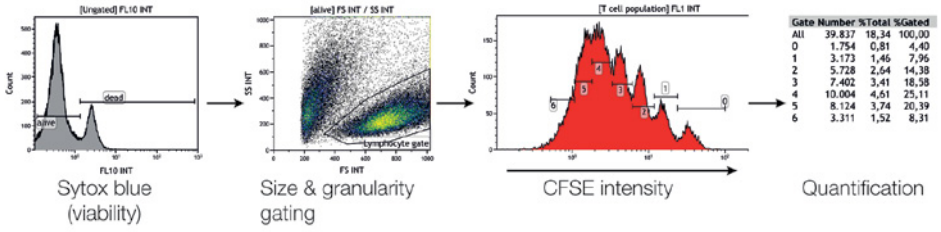
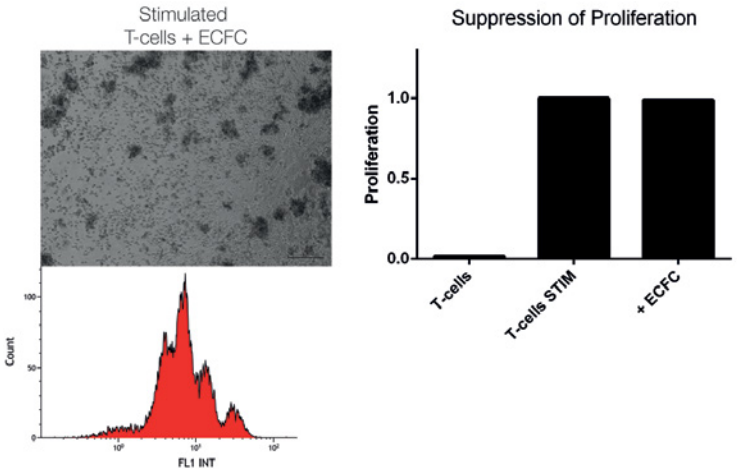
Supplement



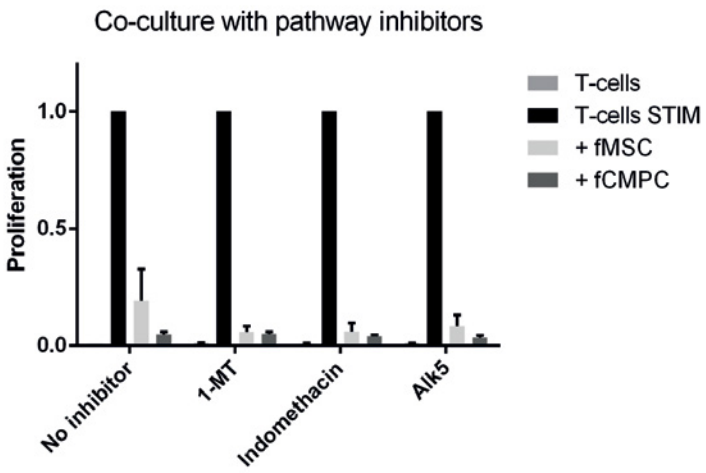
Suppl. Figure 3.1 – Flow cytometric gating and analysis

The non-adherent T-cells were collected from the culture plates and labeled with Sytox blue as a viability marker. These cells were then checked for viability and only the living fraction was plotted in a forward-sideward scatter. Subsequently, we created a lymphocyte gate based on size and granularity, and in these cells we visualized the CFSE signal. Peaks were clearly visible, allowing us to set gates per division. Taking into account the symmetrical division of T-cells, this allowed us to calculate the number of original T-cells that proliferated.



Suppl. Figure 3.2 – ECFC do not suppress number or proliferating T-cells

A similar co-culture was performed with several endothelial colony forming cell (ECFC) donors. Unlike the mesenchymal stem cell and the cardiomyocyte progenitor cell, the ECFC were not able to prevent the formation of the T-cell clusters or prevent proliferation (0 ± 0.2 suppression).



Suppl. Figure 3.3 – Effect of inhibitors on immunosuppression

Co-culture was performed as described, in the presence or absence of progenitor cells and inhibitors for the immunosuppressive pathways: 1-MT (1 mM; blocks IDO), Indomethacin (10 μM; block PGE2) and Alk5 (10 μM: competes with TGF-β signaling). None of these inhibitors showed an effect compared to the control group. This is one representative set of many experiments, which all showed no effect on T-cell proliferation.

Suppl. Table 3.1 – Cytokine release

		IL-1β	IL-2	IL-4	IL-5	IL-6	IL-10	IL-12-p70	IL-13	IFN-γ	TNF-α
T-cells	Unstimulated	3.64 ± 2.51	0.01 ± 0.01	0.08 ± 0.02	0.00 ± 0.00	0.26 ± 0.14	0.01 ± 0.01	0.04 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.01
	Stimulated	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
MSC	Unstimulated	0.43 ± 0.06	0.24 ± 0.17	0.33 ± 0.26	0.01 ± 0.00	1.25 ± 0.08	0.01 ± 0.00	0.37 ± 0.04	0.00 ± 0.00	0.01 ± 0.00	0.04 ± 0.00
	Stimulated	2.74 ± 0.85	27.24 ± 18.15	0.33 ± 0.25	0.01 ± 0.00	1.25 ± 0.11	0.01 ± 0.00	0.40 ± 0.09	0.00 ± 0.00	0.01 ± 0.00	0.05 ± 0.01
CMPC	Unstimulated	5.86 ± 2.34	0.01 ± 0.00	0.50 ± 0.12	0.01 ± 0.00	1.09 ± 0.03	0.02 ± 0.01	0.50 ± 0.19	0.00 ± 0.00	0.01 ± 0.00	0.06 ± 0.01
	Stimulated	36.49 ± 2.32	1.03 ± 0.22	0.61 ± 0.15	0.01 ± 0.00	1.09 ± 0.04	0.03 ± 0.00	0.55 ± 0.23	0.00 ± 0.00	0.01 ± 0.00	0.07 ± 0.01
MSC + T-cells	Unstimulated	0.51 ± 0.12	0.22 ± 0.07	0.21 ± 0.08	0.00 ± 0.00	1.26 ± 0.07	0.01 ± 0.00	0.17 ± 0.13	0.00 ± 0.00	0.01 ± 0.00	0.05 ± 0.02
	Stimulated	9.13 ± 2.93	31.71 ± 10.07	0.28 ±0.10	0.01 ± 0.00	1.29 ± 0.07	0.01 ± 0.00	0.14 ± 0.03	0.01 ± 0.00	0.18 ± 0.04	0.08 ± 0.01
CMPC + T-cells	Unstimulated	8.10 ± 1.84	0.01 ± 0.00	0.25 ± 0.03	0.00 ± 0.00	1.09 ± 0.02	0.06 ± 0.02	0.12 ± 0.04	0.00 ± 0.00	0.01 ± 0.00	0.06 ± 0.01
	Stimulated	52.41 ± 5.21	1.17 ± 0.07	0.44 ± 0.06	0.01 ± 0.00	1.07 ± 0.02	0.01 ± 0.00	0.15 ± 0.049	0.00 ± 0.00	0.16 ± 0.03	0.09 ± 0.01

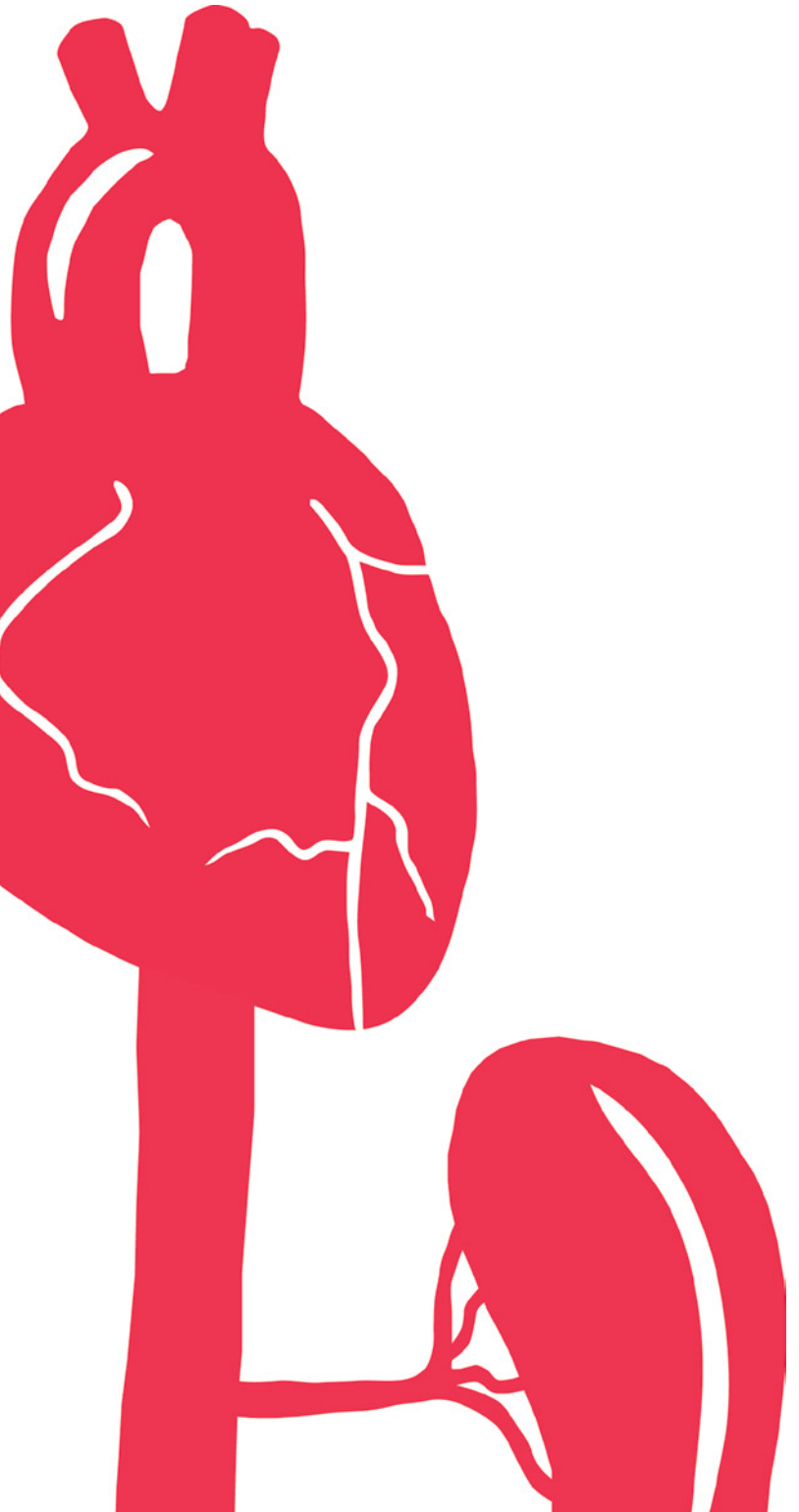
Suppl. Table 3.2 – 86 altered genes

This table contains the 86 genes significantly upregulated by activation of T-cells, and significantly downregulated in the presence of MSC or CMPC.

	Symbol	Entrez gene name	Location	Type(s)
1	ASB2	Ankyrin repeat and socs box containing 2	Nucleus	Transcription regulator
2	ASB9	Ankyrin repeat and socs box containing 9	Nucleus	Transcription regulator
3	DDX4	Dead (asp-glu-ala-asp) box polypeptide 4	Nucleus	Enzyme
4	DEPDC1	Dep domain containing 1	Nucleus	Transcription regulator
5	DHRS2	Dehydrogenase/reductase (sdr family) member 2	Nucleus	Enzyme
6	EBNA1BP2	Ebna1 binding protein 2	Nucleus	Other
7	GINS2	Gins complex subunit 2 (psf2 homolog)	Nucleus	Other
8	HIST1H1A	Histone cluster 1, h1a	Nucleus	Other
9	HIST1H2AI	Histone cluster 1, h2ai	Nucleus	Other
10	HIST1H2BC	Histone cluster 1, h2bc	Nucleus	Other
11	HIST1H2BL	Histone cluster 1, h2bl	Nucleus	Other
12	HIST1H3J	Histone cluster 1, h3j	Nucleus	Other
13	HIST2H2AB	Histone cluster 2, h2ab	Nucleus	Other
14	HIST2H2BF	Histone cluster 2, h2bf	Nucleus	Other
15	HIST3H2BB	Histone cluster 3, h2bb	Nucleus	Other
16	MCM2	Minichromosome maintenance complex component 2	Nucleus	Enzyme
17	POLR3G	Polymerase (rna) III (dna directed) polypeptide g (32kd)	Nucleus	Enzyme
18	RANBP1	Ran binding protein 1	Nucleus	Other
19	S100A2	S100 calcium binding protein a2	Nucleus	Other
20	TERT	Telomerase reverse transcriptase	Nucleus	Enzyme
21	APOBEC3B	Apolipoprotein b mrna editing enzyme, catalytic polypeptide-like 3b	Cytoplasm	Enzyme
22	BSPRY	B-box and spry domain containing	Cytoplasm	Other
23	CAMK1	Calcium/calmodulin-dependent protein kinase i	Cytoplasm	Kinase
24	CCNB1	Cyclin b1	Cytoplasm	Kinase
25	DAPP1	Dual adaptor of phosphotyrosine and 3-phosphoinositides	Cytoplasm	Other
26	GAD1	Glutamate decarboxylase 1 (brain, 67kda)	Cytoplasm	Enzyme
27	GALNT18	Polypeptide n-acetylgalactosaminyltransferase 18	Cytoplasm	Enzyme

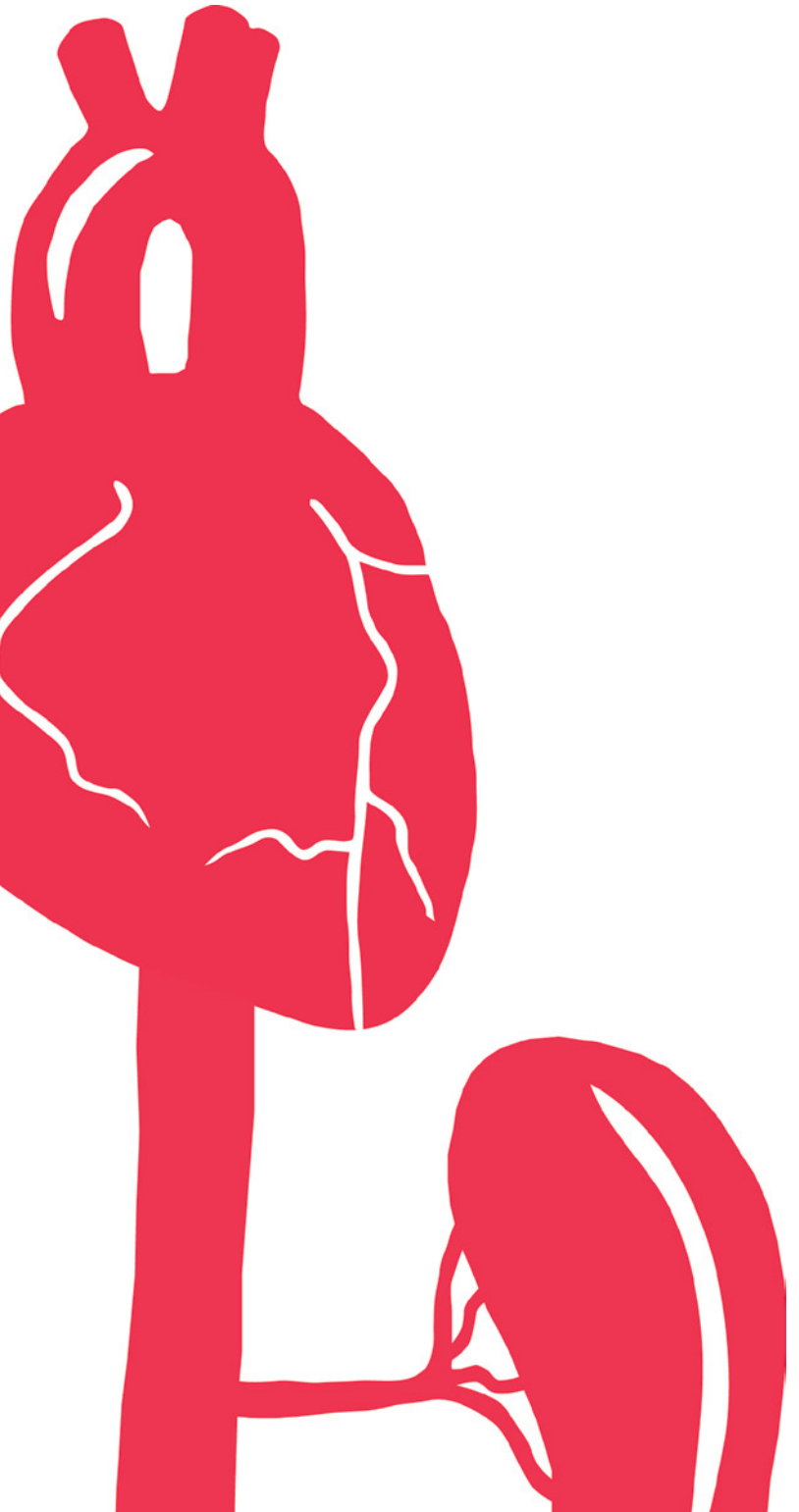
28	GLDC	Glycine dehydrogenase (decarboxylating)	Cytoplasm	Enzyme
29	KLK1	Kallikrein 1	Cytoplasm	Peptidase
30	MB	Myoglobin	Cytoplasm	Transporter
31	NCF2	Neutrophil cytosolic factor 2	Cytoplasm	Enzyme
32	NME1	Nme/nm23 nucleoside diphosphate kinase 1	Cytoplasm	Kinase
33	ODF1	Outer dense fiber of sperm tails 1	Cytoplasm	Other
34	PAICS	Phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase	Cytoplasm	Enzyme
35	PLCG2	Phospholipase c, gamma 2 (phosphatidylinositol-specific)	Cytoplasm	Enzyme
36	PTPN3	Protein tyrosine phosphatase, non-receptor type 3	Cytoplasm	Phosphatase
37	SERPINB10	Serpin peptidase inhibitor, clade b (ovalbumin), member 10	Cytoplasm	Other
38	STON2	Stonin 2	Cytoplasm	Other
39	TPRG1	Tumor protein p63 regulated 1	Cytoplasm	Other
40	ART3	Adp-ribosyltransferase 3	Plasma membrane	Enzyme
41	CCR2	Chemokine (c-c motif) receptor 2	Plasma membrane	G-protein coupled receptor
42	CHRNA6	Cholinergic receptor, nicotinic, alpha 6 (neuronal)	Plasma membrane	Transmembrane receptor
43	CLECL1	C-type lectin-like 1	Plasma membrane	Other
44	CPNE5	Copine v	Plasma membrane	Other
45	ENPP2	Ectonucleotide pyrophosphatase/ phosphodiesterase 2	Plasma membrane	Enzyme
46	GAP43	Growth associated protein 43	Plasma membrane	Other
47	GJB2	Gap junction protein, beta 2, 26kda	Plasma membrane	Transporter
48	GNA14	Guanine nucleotide binding protein (g protein), alpha 14	Plasma membrane	Enzyme
49	HCAR1	Hydroxycarboxylic acid receptor 1	Plasma membrane	G-protein coupled receptor
50	IGHM	Immunoglobulin heavy constant mu	Plasma membrane	Transmembrane receptor
51	IL17RB	Interleukin 17 receptor b	Plasma membrane	Transmembrane receptor
52	IL23R	Interleukin 23 receptor	Plasma membrane	Transmembrane receptor
53	MYO3B	Myosin IIIb	Plasma membrane	Kinase
54	NINJ2	Ninjurin 2	Plasma membrane	Other
55	TNFRSF8	Tumor necrosis factor receptor superfamily, member 8	Plasma membrane	Transmembrane receptor
56	CGREF1	Cell growth regulator with ef-hand domain 1	Extracellular space	Other
57	EBI3	Epstein-barr virus induced 3	Extracellular space	Cytokine

58	FBLN5	Fibulin 5	Extracellular space	Other
59	IFNG	Interferon, gamma	Extracellular space	Cytokine
60	IL17A	Interleukin 17a	Extracellular space	Cytokine
61	IL17F	Interleukin 17f	Extracellular space	Cytokine
62	IL5	Interleukin 5	Extracellular space	Cytokine
63	IL9	Interleukin 9	Extracellular space	Cytokine
64	LTA	Lymphotoxin alpha	Extracellular space	Cytokine
65	NAPSA	Napsin a aspartic peptidase	Extracellular space	Peptidase
66	PRG4	Proteoglycan 4	Extracellular space	Other
67	TNFSF15	Tumor necrosis factor (ligand) superfamily, member 15	Extracellular space	Cytokine
68	C4orf26	Chromosome 4 open reading frame 26	Other	Other
69	CDC20P1	Cell division cycle 20 pseudogene 1	Other	Other
70	CKS2	Cdc28 protein kinase regulatory subunit 2	Other	Kinase
71	COX17P1	Cox17 cytochrome c oxidase copper chaperone pseudogene 1	Other	Other
72	HMSD	Histocompatibility (minor) serpin domain containing	Other	Other
73	HPDL	4-Hydroxyphenylpyruvate dioxygenase-like	Other	Other
74	HSPE1P2	Heat shock 10kda protein 1 pseudogene 2	Other	Other
75	LINC00158	Long intergenic non-protein coding rna 158	Other	Other
76	LINC00877	Long intergenic non-protein coding rna 877	Other	Other
77	LINC00892	Long intergenic non-protein coding rna 892	Other	Other
78	LINC01132	Long intergenic non-protein coding rna 1132	Other	Other
79	LINC01281	Long intergenic non-protein coding rna 1281	Other	Other
80	NAPSB	Napsin b aspartic peptidase, pseudogene	Other	Other
81	PAICSP4	Phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase pseudogene 4	Other	Other
82	PHBP3	Prohibitin pseudogene 3	Other	Other
83	PIK3CD-AS2	Pik3cd antisense rna 2	Other	Other
84	RCAN2	Regulator of calcineurin 2	Other	Other
85	RNU5A-8P	Rna, U5a small nuclear 8, pseudogene	Other	Other
86	SLC16A9	Solute carrier family 16, member 9	Other	Other



Part II

Chronic Myocarditis



Heart Failure in Chronic Myocarditis: a Role for microRNAs?

4

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Abstract

Myocarditis is an inflammatory disease of the heart, which can persist over a long time. During this time, known as the chronic phase of myocarditis, ongoing inflammation damages the cardiomyocytes. The loss of cardiac cells culminates in the development of dilated cardiomyopathy, often followed by non-ischemic heart failure due to diminished cardiac function.

During the course of the disease, expression levels of non-coding small RNAs, called microRNAs (miRNAs), change. Although mainly studied in the acute setting, some of these changes in expression level appear to persist in the chronic phase. In addition to being a much-needed diagnostic tool, these miRNA could provide new treatment options.

miRNA-based intervention strategies already showed promising results in the treatment of ischemic cardiovascular diseases in preclinical animal models. By implementing more knowledge on the role of miRNAs in the progression towards heart failure, this can potentially be used in the development of miRNA-based therapeutic interventions in the treatment of myocarditis and thereby preventing the progression towards heart failure.

The first part of this review will focus on the natural course of myocarditis and the progression towards heart failure. Secondly, we will discuss the current knowledge on alterations of miRNA expression patterns, and suggest some possible future interventions.

Myocarditis

Myocarditis is an inflammatory disease of the heart, which often results in heart failure or sudden cardiac death[1]. The disease mostly occurs in young, healthy people in the age of 20-51 years[2]. Estimations of the incidence of myocarditis are variable due to the non-specific symptoms, leading to underestimation of the disease.

The incidence of myocarditis in cases with unexplained heart failure is estimated at 9,6%[2]. Bacterial infections, viruses, autoimmune diseases and other factors are able to induce myocarditis, with viruses being the most common cause[3,4]. Structural and functional damage of the myocardium, caused by these factors, activates the innate and adaptive immune response, which can lead to severe inflammation[5]. The immune response is eventually downregulated, however, myocardial inflammation can also persist. Persistent inflammation is characterized by an ongoing damage to the cardiomyocytes and ultimately results in non-ischemic heart failure[6]. In 30% of the cases, dilated cardiomyopathy (DCM) occurs, which is a major cause of heart failure and an important indication for cardiac transplantation[7]. Hence, the treatment of myocarditis is difficult due to late diagnosis and irreversible damage that has occurred[8].

Final clinical outcome of the disease depends on the host response, the amount of irreversible damage, and the use of therapeutic interventions[9,10]. Patients with severe acute myocarditis have a better prognosis, while patients with moderate chronic myocarditis are more prone to develop heart failure[1,2].

Phases

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

In the sub-acute phase (day 4-5), the innate immune response remains activated and immune cells infiltrate the heart[13]. Phagocytosis of dead cells and debris is initiated by monocytes, which augment the expression of pro-inflammatory cytokines[5]. Immune cells of the adaptive immune system, such as T-cells and B-cells, also accumulate in the infected heart[14]. Licensed cytotoxic T-cells ($CD8^+$) recognize virus-infected cardiomyocytes via the interaction and presentation of antigens

loaded on major histocompatibility complex type 1 (MHC-I)[15]. The cytotoxic T-cell directly kills the infected cardiomyocyte by releasing perforin and granzymes, triggering the caspase cascade and inducing apoptosis.

Antigen presenting cells (APCs), like dendritic cells (DCs), take up the debris of dead cardiomyocytes and present the ingested antigens on MHC-216. T-helper cells ($CD4^+$) are able to recognize these presented antigens via the interaction of the T-cell receptor and the presented antigen loaded on MHC-II. This subsequently leads to the activation of T-helper cells, which can license cytotoxic T-cells to kill infected cells or activate B cells, which produce neutralizing antibodies[16]. During the sub-acute phase, the immune response not only eliminates infected and dead cells, but also significantly contributes to irreversible damage of the myocardium by damaging of healthy cardiomyocytes[1].

In the chronic phase (day 14), repair and remodeling of the myocardium is initiated[1,12]. Regulatory T-cells ($CD4^+CD25^+FoxP3^+$) respond to the production of IL-2 by T-helper cells and start proliferating rapidly[1]. Anti-inflammatory cytokines are produced, such as transforming growth factor-beta (TGF-β) and IL-10, to down-regulate the immune response and to reduce cardiac damage[11,12]. Secondly, an alternative type of macrophage becomes activated, macrophage type 2 (M2). This type of macrophage produces more TGF-β and IL-10 to reduce inflammation and to stimulate repair mechanisms[17]. Fibroblasts start to proliferate and differentiate into myofibroblasts, which contain contractile properties like smooth muscle cells, to replace the lost cardiomyocytes[18].

Fibroblasts and M2-macrophages produce collagen to restore the extracellular matrix and form a permanent scar[1,12]. In some cases, the immune system fails to completely clear all the infected cells or auto-reactive immune cells persist in the myocardium, resulting in chronic inflammation[2,9]. The chronically activated immune system produces cytokines, which activates matrix metalloproteases (MMPs) that can digest interstitial collagen and elastin. Additionally, pro-fibrotic factors are produced and thereby facilitate the dilatation of the heart[11], which can lead to dilated cardiomyopathy (DCM), an irreversible disease with systolic and diastolic dysfunction[7,19].

Diagnosis

Clinical manifestation of myocarditis includes a broad spectrum of non-specific flu-like symptoms and signs of myocardial infarction[20]. Clinical presentation is usually not sufficient for the diagnosis of myocarditis. Myocarditis is considered in young patients with rapidly progressive cardiomyopathy, arrhythmias and acute myocardial-infarction-like symptoms in combination with normal healthy coronary arteries[20]. In these cases, symptoms often mimic myocardial infarction due to segmental wall abnormalities and elevated levels of troponin and creatine kinases

found in blood samples[8]. In patients with acute myocarditis, concentrations of troponin I and T are more elevated than creatine kinase[21]. In addition, inflammatory markers such as C-reactive protein and the number of circulating leukocytes can be increased[5]. In sub-acute and chronic myocarditis, anti-cardiac auto-antibodies and immunoglobulins can be detected in blood samples[19]. High levels of IgM are an indication for the presence of an active virus and can be used to determine the possible cause of myocarditis[11].

Electrocardiogram (ECG) is widely used as one of the diagnostic tools for the diagnosis of myocarditis, although it has a low sensitivity of only 47%[22]. Most of the patients with (acute) myocarditis show abnormalities in their ECG[6,23], which often mimics the ECG of a patient with a myocardial infarction[20]. Although ECGs can vary between patients, they often show non-specific T-waves, ventricular arrhythmias, PQ-segment depressions, and ST-segment changes by which high levels of Q waves are associated with higher rates of deaths and transplantations[5].

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

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

Non-Ischemic Heart Failure

Heart failure is a complex clinical syndrome that can result from an abnormal cardiac structure or function leading to failure of the heart to deliver oxygen and nutrients to metabolizing tissues[26]. According to the etiology and pathophysiology, heart failure can be categorized in ischemic or non-ischemic and in anatomical

designations as hypertrophic and dilated. Of these, dilated cardiomyopathy (DCM) is the most prevalent form of non-ischemic cardiomyopathy with a relative poor overall prognosis of 25% mortality in 5-years[27]. DCM is predominantly defined by left ventricular dilatation and reduced cardiac contraction in the absence of coronary artery disease and abnormal loading conditions. Amongst other secondary causes, e.g. chemotherapy, neuromuscular disease and alcohol abuse, myocarditis can result in DCM and eventually heart failure[7,28].

Early diagnosis of DCM is hindered by the fact that the majority of patients are asymptomatic in early stages of the disease, therefore patients often present themselves in the late stage of the disease with symptoms of exercise intolerance, breathlessness and edema[26]. Additionally, in some of these patients the conduction system is affected that lead to arrhythmias and an increased risk for sudden cardiac death.

For diagnosis of DCM, laboratory test and imaging techniques are important to define disease severity. Imaging often shows dilated cardiomyopathy, with general wall motion abnormalities and MRI using gallium-late-enhancement could show diffuse damage throughout the heart[21]. Since fast initiation of treatment is beneficial for the prognosis of heart failure, a clear diagnosis of DCM and also the contributing etiology should be known to give the best available therapy[27]. Accordingly, taking cardiac biopsies is indicated in patients with DCM from unknown etiology, and surprisingly, in 9-16% of patients presenting with DCM, traces of prior myocarditis are observed[7].

After diagnosis of heart failure, all patient with DCM are treated with standard heart failure therapy such as ACE-inhibitors, mineralocorticoid receptor antagonist, diuretics and β-blockers[10,26,29]. The primary aim of this treatment is to relieve symptoms of heart failure, to improve survival and to prevent ongoing remodeling and worsening of heart function[26,29]. For myocarditis induced DCM, additional immunosuppressive drugs are being used[3], however this is not beneficial for viral myocarditis and can even cause harm when used for this purpose[12].

Despite poor overall prognosis and lack of specific treatments, 25% of DCM patients with recent onset heart failure will undergo reverse remodeling and demonstrate a (partially) improved cardiac function[30]. The ability to recover is observed in the majority of dilated cardiomyopathy etiologies, including myocarditis. The fact that there is a certain degree of recovery and reversibility in cardiac function, attention should be focused on inducing this process to guide improvement.

Since prominent roles have been uncovered for miRNAs in the treatment of cardiovascular disorders[31], miRNAs might also have a potential role in treatment of myocarditis and reversibility of myocarditis induced-DCM.

The Role of miRNAs

microRNAs (miRNAs, miR) are small non-coding RNAs with a length of approximately 20-24 nucleotides, which are involved in the post-transcriptional regulation of protein expression by binding messenger RNAs (mRNAs)[32]. miRNAs are able to use two silencing mechanisms to down-regulate specific target genes: degradation or translational repression of the mRNA, depending on the complementarity with the target gene. If the miRNA and their target gene are complementary, the mRNA will be cleaved and degraded. If the miRNA and the target gene are not perfectly complementary, miRNAs suppress translation of the target gene, however without affecting the stability of the mRNA. miRNAs can be directly produced by cells themselves or secreted via e.g. microvesicles, such as exosomes[33].

miRNAs are involved in many processes, such as cell proliferation and apoptosis but also in the regulation of the immune response. Emerging evidence is found for the contribution of miRNAs in myocardial pathological processes by regulating angiogenesis, apoptosis and differentiation of cardiomyocytes[34,35]. The expression of certain miRNAs changes during cardiac disease and heart failure, which makes them interesting targets in the potential treatment of cardiovascular diseases, such as myocarditis[32,36].

Role of miRNAs in myocarditis

The role of miRNAs in human myocarditis is not fully elucidated. Currently, only a couple of miRNAs have been identified, which might be correlated with viral myocarditis, especially during the acute phase of myocarditis. Two identified microRNAs, miR-208b and miR-499, are elevated in viral myocarditis and can be detected in plasma of myocarditis patients[37]. miR-208b is expressed by cardiomyocytes and is involved in pathological processes, such as cardiac growth, fibrosis and inflammation by increasing MHC-expression[35]. Upregulation of miR-208b induces adverse cardiac remodeling. miR-499 is also expressed by cardiomyocytes and upregulated during cardiac disease[38]. Both of these microRNAs are released upon myocardial damage and can potentially be used in the diagnosis of myocarditis to determine the severity of the disease[37]. Elevated levels of miR-499 can be detected in all patients, whereas the expression of both miR-208b and miR-499 was only found in fulminant virus-induced myocarditis[37]. These miRNAs, however, are not specific for myocarditis but probably reflect myocardial injury in general, as was observed for ACS patients[39].

miRNAs involved in acute myocarditis

Current research is focusing on miRNA screenings in the different stages of myocarditis to identify additional microRNAs involved in the pathogenesis of the disease. In myocarditis patients, a cardiac miRNA profile was identified involved

in the acute viral phase and the inflammatory phase of acute myocarditis. The study of Corsten *et al*. showed that, for example, miRNA-155 is highly expressed in cardiac tissue of myocarditis patients[40]. miR-155 was found to be up-regulated in cardiac tissue in both human and mice with viral-induced myocarditis[40].

miR-155 is known to be pro-inflammatory and involved in multiple processes, such as immune cell functioning, and is expressed by inflammatory cells especially in the acute inflammatory phase of myocarditis[41]. In viral-induced myocarditis mice models, blockage of miR-155 by antagonists showed attenuated cardiac inflammation and less necrosis[41]. These results indicate that miR-155 plays an important role in the inflammatory response of viral-induced myocarditis. Next to miR-155, miR-21 and miR-146b were found to be upregulated in myocarditis patients, which also have a central role in immune activation and inflammation[40].

Silencing of these miRNAs by specific inhibitors showed a strong attenuation of myocarditis in viral-induced myocarditis mice[42]. The exact role of these miRNAs in the pathology of myocarditis is not elucidated, however it is shown that the expression of miR-21 and miR-146b is correlated with IL-17 expression[42]. These findings suggest that miR-21 and miR-146b are involved in the regulation of T_H17 differentiation and thereby control autoimmunity. The function of RORct, a transcription factor of T_H17 differentiation, is enhanced by increased expression of these miRNAs, which leads to the differentiation of mature T-cells towards T_H17 cells[42]. Furthermore, miR-21 is also involved in interstitial fibrosis and cardiac hypertrophy[43]. Interestingly, other studies showed contradictory results as significantly decreased levels of miR-21 expression were found.

The expression of miR-21 in the myocardium in coxsackievirus B3 (CVB3)-myocarditis mice was significantly reduced and a negative correlation was found between the severity of myocarditis and the expression of miR-21[44]. When mice were treated with miR-21 precursors, the myocarditis was alleviated and apoptosis was reduced, indicating that miR-21 also has a protective role by regulating programmed cell death 4 (PDC4)-mediated apoptosis[44]. Based on the different outcomes of these studies, it might be that the role of miR-21 in myocarditis depends on the temporal and spatial expression of its targets. In one study, additional elevated miRNAs, including miR-511 and miR-212[40], were found in patients with acute viral myocarditis. miR-511 functions as a positive regulator of Toll Like Receptor-4 (TLR) signaling, whereas miR-212 is involved in the hypertrophic responses of cardiomyocytes[40,45].

Next to upregulated miRNAs there is also evidence that miRNAs are involved by their down-regulation. Anti-inflammatory miR-106a and miR-93 were downregulated in the acute phase of human myocarditis[40]; both are involved in anti-inflammatory responses by inhibiting the production of inflammatory cytokines[43,46].

miRNAs involved in chronic myocarditis

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

Overall, miRNAs involved in inflammatory responses are disregulated and muscle specific miRNAs (myomiRs) are mostly upregulated during human myocarditis (*table 4.1*). Since the chronic phase of myocarditis can progress into heart failure, it is also hypothesized that miRNAs involved in the development of (non-ischemic) heart failure might also be involved.

Role of miRNAs in (non-ischemic) heart failure

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

miRNAs involved in inflammatory responses, leading to non-ischemic heart failure, are still subject of investigation. Currently, some potential miRNAs have been identified in patients with non-ischemic heart failure, such as miR-21, miR-146a/b, -155, -423-5p, and the miR-17-92 cluster[57–59]. Furthermore, miR-200b, -519, -520d, -558, and -622 are identified as biomarkers for non-ischemic heart failure with reduced ejection fraction (HF-REF), however, these miRNAs still have to be validated in other studies[58]. Some of the miRNAs that have been identified in heart failure patients are also aberrantly expressed in myocarditis patients (*table 4.1*),

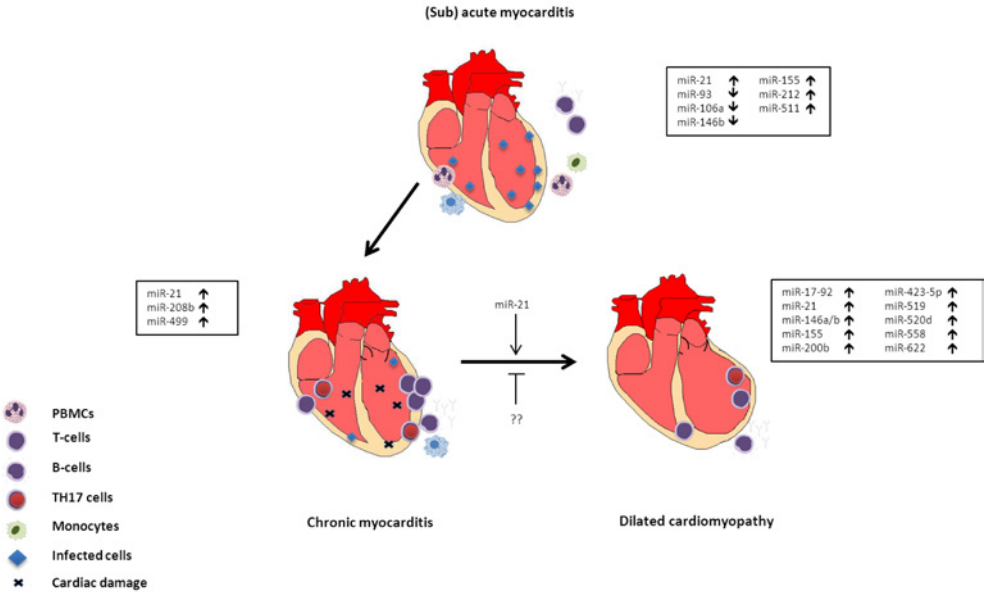

Figure 4.1 – Stages of myocarditis and progression towards dilated cardiomyopathy
In the (sub)-acute phase of myocarditis, infected cells are recognized by immune cells and inflammation is initiated. The immune response can either be downregulated or infected cells persist, which leads to chronic myocarditis. Chronic inflammatory reactions cause damage to the myocardium, which is further enhanced by autoimmune responses. Ultimately, chronic myocarditis can progress towards dilated cardiomyopathy (DCM). Multiple miRNAs have shown to be involved in the different phases of myocarditis and DCM, however miRNAs involved in the progression of myocarditis towards heart failure are not identified yet. These miRNAs can be of great importance for developing potential therapeutic interventions.

thereby pointing to a potential role of these miRNAs in the progression of myocarditis towards heart failure. However, additional research has to be performed to verify these miRNAs and to identify whether additional myocarditis-specific miRNAs can be identified and are specifically involved in the progression towards heart failure (*figure 4.1*).

Therapeutic options using miRNA-based Interventions

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

is restored.

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

Conclusion

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

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Table 4.1 – microRNAs involved in myocarditis and (non-ischemic) heart failure

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

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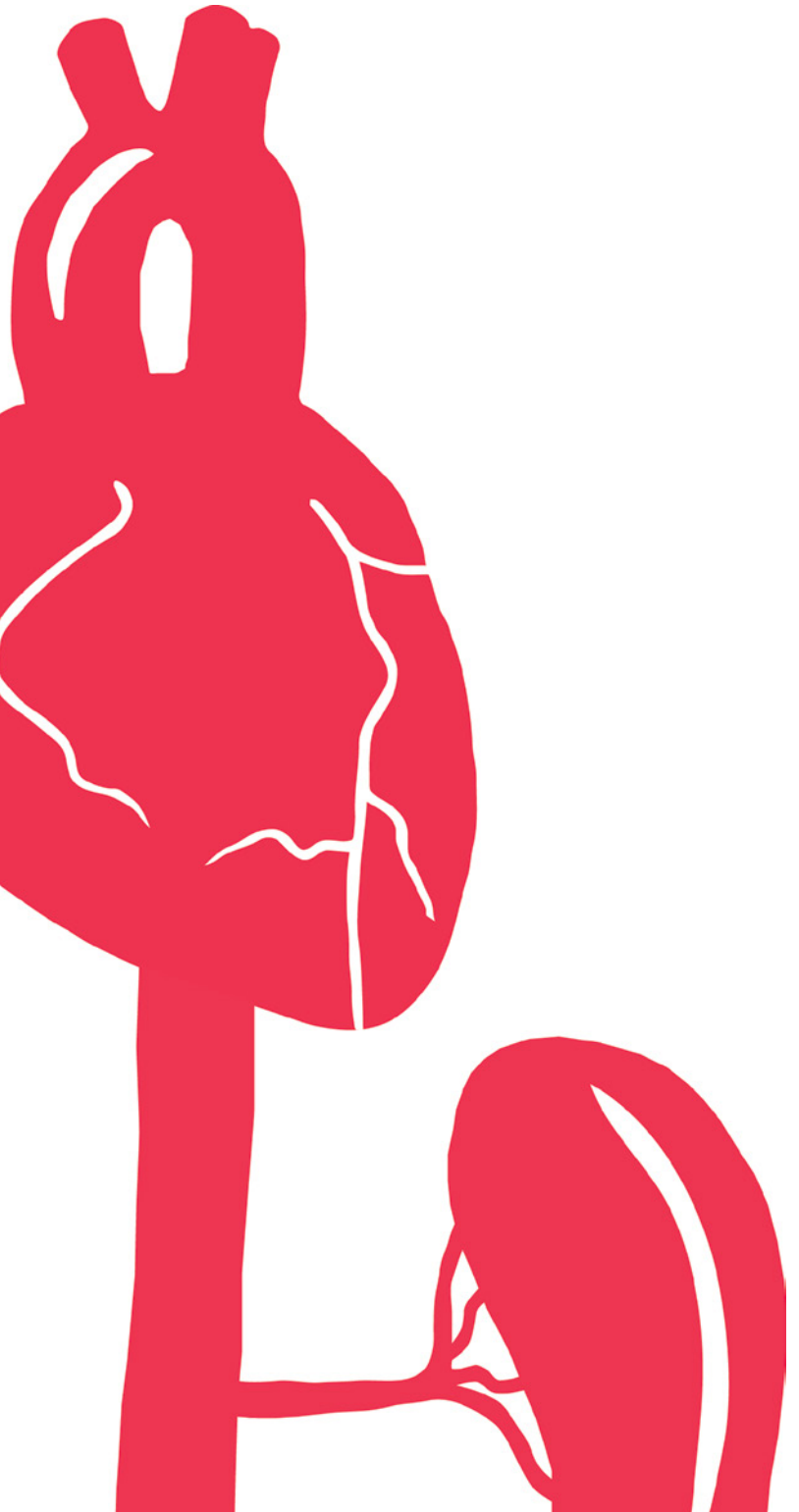

Cardio-Splenic Cross-Talk in Chronic Myocarditis

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Abstract

Rationale

Myocarditis is an unpredictable, inflammatory cardiac disease that mainly affects the young and healthy. It is well known that persisting viral load and sustained immune cell activation can trigger a chronic phase of this disease. However, little is known about the nature of this ongoing immune activation.

Objective

We aim to elucidate the active immune components involved in the progression of chronic myocarditis and retrieve the link with cardiac deterioration.

Methods & Results

Experimental auto-immune myocarditis (EAM) was induced in BALB/c mice and animals were terminated after 0, 7, 14 and 21 days. Immune cells obtained from the blood, lymph nodes, spleen and heart show distinct patterns at these time points, while over time cardiac function declines.

Conclusion

Chronic myocarditis is based on a complex interaction between the different components of the immune system, which appears to be self-sustaining. Overall, it leads to a progressive deterioration of cardiac function, which might be prevented by targeting specific immune processes.

Introduction

Myocarditis is a cardiac condition in which inflammatory cells attack the cardiomyocytes. Myocarditis occurs normally in a young and relatively healthy population, aged 20-51. This is in contrast to many other cardiac diseases, which generally strike the elderly or already diseased[1,2]. Additionally, no life style risk factors are known to make a person more susceptible to myocarditis[3].

Different etiologies are known, of which viral infection, especially with the Coxsackie B virus (CVB), is the most common one. Patients presenting with acute, fulminant myocarditis generally have acute heart failure due to massive immune activation, yet if they survive the acute setting their prognosis is relatively favorable[1-4]. The majority of patients, however, have a silent myocarditis. They do not experience any medical problems, but due to prolonged viral activity and auto-immune reactions against cardiac cells, both the left (LV) and right (RV) ventricular functions get impaired. These patients tend to present late, with an unexplained poor cardiac function and possibly even heart failure due to dilated cardiomyopathy (DCM).

In fact, of patients presenting with DCM an estimated 40% show evidence of previous or ongoing myocarditis[5]. These patients have a bad prognosis, as their 5-year survival is around 50%[6].

Current treatment options are restricted to the standard heart failure drugs, including ACE inhibitors, β-blockers, diuretics, and mineralocorticoid receptor antagonist[1,7]. No additive treatment is given to treat the inflammatory components of the disease, only an occasional patient receives plasmapheresis to remove auto-antibodies[8,9].

It is well known that the progression towards chronic myocarditis can either be due to persistent viral presence or to acquired cardiac auto-immunity[1,6], but in both circumstances there is an immunological dysfunction. In the case of persistent viral presence the immune system is unsuccessful in completely clearing the viral load.

Meanwhile, in the case of acquired cardiac auto-immunity the immune system has been triggered, either by molecular mimicry or due to damage associated activation, to respond to cardiac antigens[1,3,10]. It is the auto-immune category where there is little understanding of the disease progression and it remains unclear which immune cells are active during the chronic phase.

The rate of decrease in cardiac function and progression to heart failure is unknown, as is the possibility of reversal of the process. Therefore, we studied the normal course of disease in a murine model of experimental auto-immune myocarditis (EAM), mimicking this variant of chronic myocarditis[11,12]. As myocarditis is a systemic disease, we included not only the heart and its function, but also the

whole cardiosplenic axis in the data collection. Ultimately, we aim to elucidate the ongoing processes and hopefully find new targets for intervention.

Materials & Methods

Animals and experimental autoimmune myocarditis

Male BALB/c mice (8-12 weeks old, n=32) were purchased from Charles River. They were fed ad libitum and maintained in compliance with animal welfare guidelines of the UMC Utrecht. Mice were housed under controlled conditions in a 12 hours light/12 hours dark cycle. All research protocols were approved by the Animal Ethical Commission of Utrecht University.

To induce experimental auto-immune myocarditis, all mice were injected with 200 µg of α-MyHC peptide (Ac-SLKLMATLFSTYASAD-OH; NKI, Amsterdam, the Netherlands) emulsified with 100 µg Complete Freud's Adjuvans (Sigma: F5881) subcutaneously in the neck at day 0[11]. A booster containing the same amount of the α-MyHC peptide dissolved in PBS was given at day 7. After termination of the animals, hearts, spleens and thoracic lymph nodes were collected. Blood was collected in EDTA tubes and used for flow-cytometric analysis. Serum was separated from the blood and stored separately. Half of the spleen and heart was put in paraffin. The remaining part of the spleen, a quarter of the heart and the lymph nodes were used for flow cytometric analysis. The last quarter of the heart was snap frozen and stored at -80°C.

Echocardiography

3D-echocardiography (echo) was performed under isofluorane anesthesia on day 0, 7, 14 and 21 of the experiment, using a high resolution ultrasound system (Vevo 2100, VisualSonics) with an 18-38 MHz transducer (MS 400, VisualSonics). During the measurements temperature, heart rate and respiration were continuously monitored and kept stable within physiological range. A parasternal long axis view was used for acquisition of the B-mode images. Fractional shortening and wall thickening were measured in the 2D short-axis view (M-mode).

To obtain 3D-echo images, the transducer was positioned perpendicular to the long-axis view (short-axis view) and the left ventricle was scanned by consecutive images (0.064 mm intervals) of the short axis using a 3D-motor (VisualSonics). The echocardiogram and respiration gating were used to identify the end-diastolic volume (EDV) and end-systolic volume (ESV) triggers. Analysis of the reconstructed 3D images was performed with the manufacture's software (Vevo2100 v1.7.1). All measurements and analysis were performed by a blinded operator and investigator.

Flow cytometry

Lymph nodes and spleens were strained through a 40 µm nylon mesh (Greiner Bio-One 542040) to create a single-cell suspension. Hearts (except for the 7 days time point) were cut into small (1 mm) pieces and digested using Liberase TL (26 U/mL, Roche 05401020001) and DNase I ($10x10^3$ U/mL, Roche 04536282001) in HEPES buffer (Life Technologies 15630-080) at 37°C for 20 minutes. Subsequently, cells were also put through the 40 µm nylon mesh to make a single cell suspension.

Cells were washed twice with PBS containing 5% FBS and incubated for 30 minutes at room temperature with the appropriate combination and amount of fluorochrome-conjugated antibodies. After incubation, samples were washed and Optilyse C (Beckman Coulter A11895) was added to lyse the erythrocytes and fix the white blood cells.

Antibodies against the following markers were used: CD3e-PE (Clone 145-2C11; 12-0031-82), CD8a-APC-eFluor780 (Clone: 53-6.7; 47-0081-82), CD11b-AlexaFluor488 (Clone: M1/70; 53-0112-82), CD19-eFluor 450 (Clone: 1D3; 48-0193-82), CD25-APC (Clone: PC61.5; 17-0251-82), CD45-eFluor450 (Clone: 30-F11; 48-0451-82), F4/80-PE-Cy7 (Clone: BM8; 25-4801-82) and FoxP3-eFluor450 (Clone: FJK-16s; 48-5773-82) purchased from eBioscience. From BD Bioscience CD4-PerCP (Clone RM4-5; 553052), CD62L-PE-CF594 (Clone MEL-14; 562404), Ly6G-APC (Clone 1A8; 560599) and Ly6C-PE-CF594 (Clone AL-21; 562728). Viability was determined using Sytox blue (Invitrogen s34857). Cell fluorescence was measured using the Gallios Flow Cytometer (Beckman Coulter) and all analyses were performed with Kaluza Analysis Software (Beckman Coulter, v1.3).

Histopathological examination

Hearts and spleens embedded in paraffin were cut and stained to detect infiltrating immune cells. Sequential slices were labeled with Hematoxylin-eosin and CD45 (BD Pharmingen 550539). Representative pictures were taken with a fluorescent microscope (Olympus) at 10 or 20 times magnification. In addition, a picrosirius red staining was performed to detect collagen using circularly polarized light microscopy. Five representative images of the heart were taken per animal and converted into mean grey values. Collagen density was analyzed using the average number of grey values expressed per square micrometer.

Serum measurements

High sensitive Troponin I levels were measured in serum of the mice using a clinical chemistry analyzer (AU5811, Beckman Coulter). Serum levels of 36 cyto- and/or chemokines were measured with a Luminex-200 instrument (Bio-Plex 200) using a 36-multiplex panel (eBioscience, EPX360-26092-01). The luminex assay was

performed according to manufacturer's protocol.

Statistical analysis

All statistical analysis were made in SPSS statistics v20 (IBM, Armonk, NY). After verifying normal distribution, the groups were compared using an ANOVA analysis with an LSD post-hoc correction. A p-value < 0.05 was considered significant.

Results

Experimental auto-immune myocarditis was induced by injecting BALB/c mice with α-MyHC-peptide, emulsified with Complete Freud's Adjuvans (*figure 5.1A*). Echo measurements and blood samples were collected at 0, 1, 2 and 3 weeks. During the study no animals died prematurely or showed signs of severe heart failure (i.e. shortness of breath, inactivity, weight loss or peripheral cyanosis). The circulating white blood cells increased in number after myocarditis induction and peaked at one week (*figure 5.2B*). After the peak, the number of circulating cells decreased again but remained elevated compared to baseline. Heart and body weight showed small increases likely related to growth, further supported by the fact that the heart-to-body-weight ratio did not change (*figure 5.1C*).

Weekly measurements showed Troponin I increased significantly over the course of the experiment, indicating ongoing death of cardiac cells (*figure 5.1D*). Analysis of collagen deposition at baseline and at the end of the experiment, showed a trend towards increased collagen levels three weeks after induction of myocarditis (*figure 5.1E*).

Functional measurements

Echographic measurements during the course of the experiment showed a decrease in cardiac function over time. The strongest decline was observed in the first week after immunization, followed by a period of slow, ongoing decrease in function (*figure 5.2A*). Over the three weeks, end-systolic volume (ESV) was increased by 37%, while end-diastolic volume (EDV) also increased with 14%. Consequently, left ventricular ejection fraction (LVEF) decreased with 10%. Wall thickening after 3 weeks was identical compared to baseline (*figure 5.2B*). Fractional shortening of the heart remained at baseline level for one week after the induction, but decreased significantly during week two and three (*figure 5.2C*).

Monocytes in chronic myocarditis

During the immunization process, the Complete Freud's Adjuvans attracts monocytes from the blood, after which they take up the injected cardiac myosin and present it to the adaptive immune system. This makes the monocyte the first

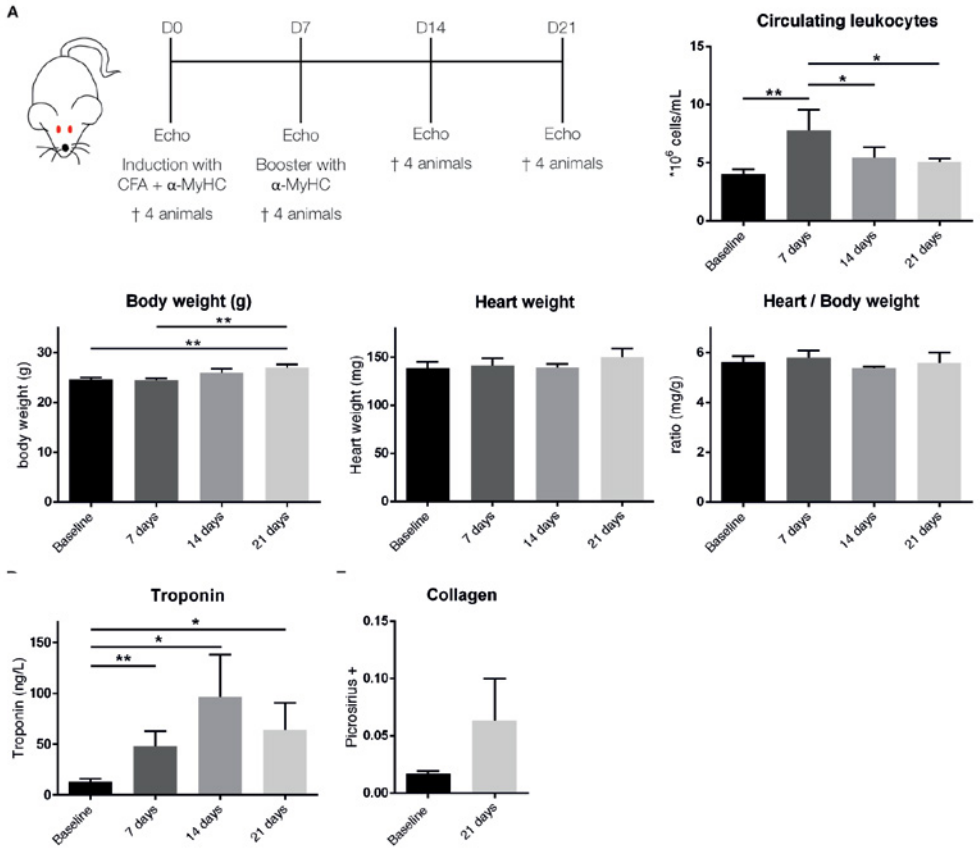

Figure 5.1 – Experimental auto-immune myocarditis

A The set-up of the experimental model and the timing of samples. B White blood cell count (WBC) increases after induction of myocarditis. C Body weight increases slightly, corresponding to normal growth, while heart weight and the heart/body weight ratio remain stable. D Troponin becomes elevated after induction and remains this way during the whole course of the experiment. E Collagen depositions at baseline and three weeks after induction of myocarditis. ** p<0.05, ** p<0.01, *** p<0.001*

responder of the immune system and in fact the catalyzer of the whole disease model. Monocytes in circulation can have a high or low expression of Ly6C, giving them a pro-inflammatory or anti-inflammatory phenotype, respectively[13,14]. The pro-inflammatory Ly6C^{high} monocytes were present in the blood at very low numbers at baseline, and appear to either form in or migrate to the cardiac lymph nodes (*figure 5.3B*), before they re-enter the bloodstream in week 2 (*figure 5.3A*).

In week three, we found these pro-inflammatory cells significantly upregulated in the spleen (*figure 5.3C*). In the heart, low numbers of these cells were present, and remained relatively constant for the duration of the experiment with a mild

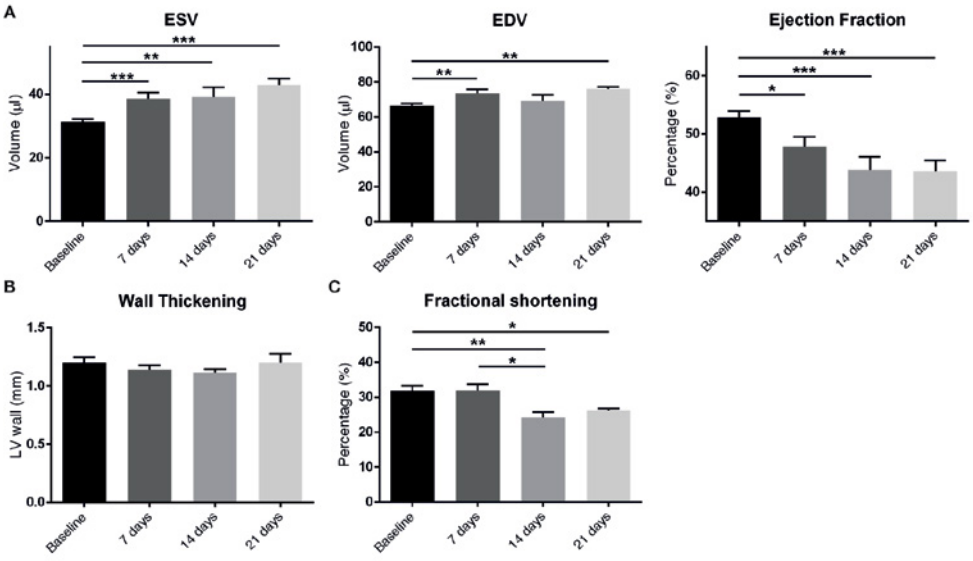

Figure 5.2 – Functional cardiac measurements

A In the weeks following myocarditis induction, end systolic volume (ESV) and end-diastolic volume (EDV) increase, leading to a drop in ejection fraction (EF). B Wall thickening remains stable during the course of the experiment. C Fractional shortening decreases during the progression of myocarditis ** p<0.05, ** p<0.01, *** p<0.001*

increase after 21 days (*figure 5.3D*).

We found the numbers of anti-inflammatory Ly6C^{low} monocytes to increase slightly in the blood shortly after induction and remain elevated during the whole experiment (*figure 5.3E*). An initial peak at one week could also be seen in the lymph nodes, after which the level strongly decreased (*figure 5.3F*). In the spleen the number of Ly6C^{low} monocytes increased slowly over time (*figure 5.3G*).

T_c-cells in chronic myocarditis

Cytotoxic T-cells (T_C-cells), characterized as CD3$^+$CD8$^+$, can directly kill their target cells when they are activated. The number of T_C-cells was most elevated in the blood one week after induction, but remained higher than baseline in the weeks after (*figure 5.4A*). In the lymph nodes, the increase after one week was followed by a decrease in week two and three (*figure 5.4B*). In the spleen, the amount of T_C-cells was decreased at all time points compared to baseline, suggesting the cells might have gone into the circulation (*figure 5.4C*). In the heart, a significant increase in T_C-cells was seen two weeks after induction of myocarditis, which seemed to normalize at day 21 (*figure 5.4D*).

Although less-known, T_C-cells also have a regulatory subtype (T_{reg}), defined as

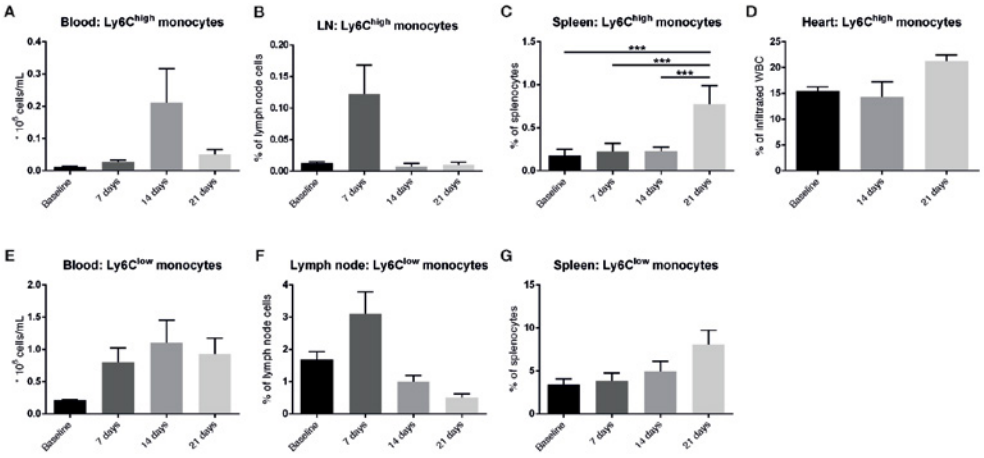

Figure 5.3 – Monocytes in chronic myocarditis

The numbers of pro-inflammatory Ly6C^{high} monocytes in the blood (**A**) or percentages in the lymph nodes (**B**), spleen (**C**) and heart (**D**) are compared, where the pro-inflammatory Ly6C^{high} cells are initially seen in the lymph nodes before entering the blood and migrating the spleen and the heart. Anti-inflammatory Ly6C^{low} monocytes in blood (**E**), in lymph nodes (**F**) and the spleen (**G**) show a different pattern, suggesting an initial mild increase in blood and lymph nodes, which draws to the spleen in a later stage. ** p<0.05, ** p<0.01, *** p<0.001*

CD8$^+$CD25$^+$FoxP3$^+$ [15,16]. The baseline levels of circulating cytotoxic T_{reg} were very low, yet after three weeks the levels were 4-fold increased (*figure 5.4E*). A similar increase was visible in the lymph node, where the occurrence of cytotoxic T_{reg} was increased 5-fold (*figure 5.4F*). In the spleen, the peak in cytotoxic T_{reg} started already after two weeks and remained elevated in week three (*figure 5.4G*).

T_H-cells in chronic myocarditis

CD3$^+$CD4$^+$ T-cells, commonly known as T-helper (T_H) cells, play a role in the activation of B-cells. They appeared to maintain a relatively stable level in the blood, with a small increase seven days after induction of myocarditis (*figure 5.5A*). Like with the T_C-cells in the lymph nodes, the number of T_H-cells decreased significantly two weeks after induction and remained low (*figure 5.5B*). In the spleen the level of T_H-cells appeared to be constant, showing no differential numbers over time (*figure 5.5C*). Analysis of the cardiac tissue showed hardly any infiltration of T_H-cells (*figure 5.5D*).

However, the regulatory T_H-cell subset, defined as CD4$^+$CD25$^+$FoxP3$^+$ (T_{reg}), showed a different trend. Although present at very low levels in the blood, the number of T_{reg} was increased nearly 10-fold at day 21 (*figure 5.5E*). Additionally, this increase in T_{reg} was also observed to occur gradually in the lymph nodes

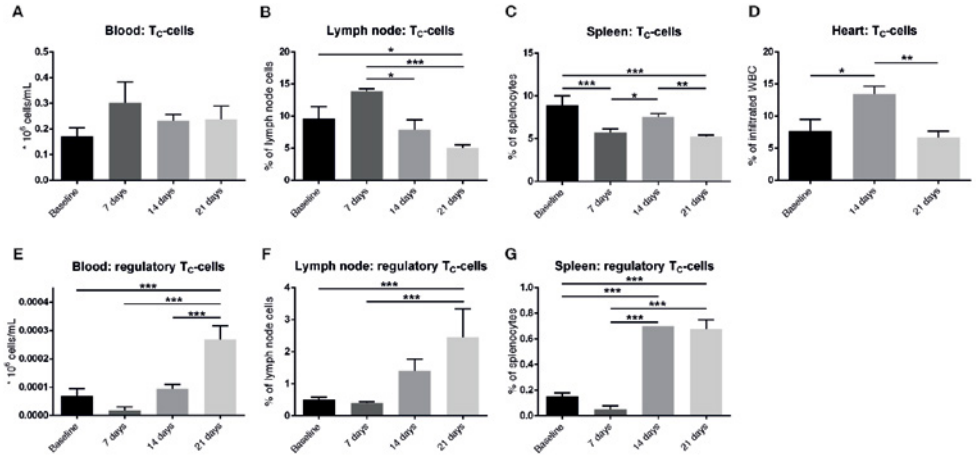

Figure 5.4 – Cytotoxic T-cells in chronic myocarditis

The numbers of $CD8^+$ T-cells in the blood (**A**) or percentages in the lymph nodes (**B**), spleen (**C**) and heart (**D**) are compared, suggesting a primary increase in blood and lymph nodes, and an influx in the heart at two weeks. Spleen levels remain relatively stable. Cytotoxic regulatory T-cells ($CD8^+CD25^+FoxP3^+$) in blood (**E**), in lymph nodes (**F**) and the spleen (**G**) indicate a late formation of regulatory cells, possibly starting in the spleen.

** p<0.05, ** p<0.01, *** p<0.001*

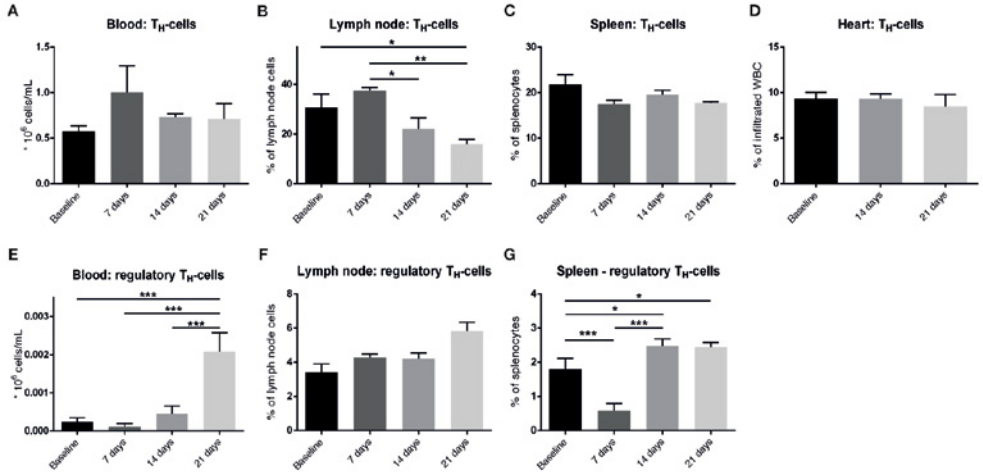

Figure 5.5 – T-helper cells in chronic myocarditis

The numbers of CD4+ T-cells in the blood (**A**) or percentages in the lymph nodes (**B**), spleen (**C**) and heart (**D**) are compared, and despite a small peak in the blood and lymph nodes after one week remain relatively stable in all organs. Regulatory T-cells ($CD4^+CD25^+FoxP3^+$) in blood (**E**), in lymph nodes (**F**) and the spleen (**G**) hint at a late formation of regulatory cells, possibly starting in the lymph nodes or spleen.

** p<0.05, ** p<0.01, *** p<0.001*

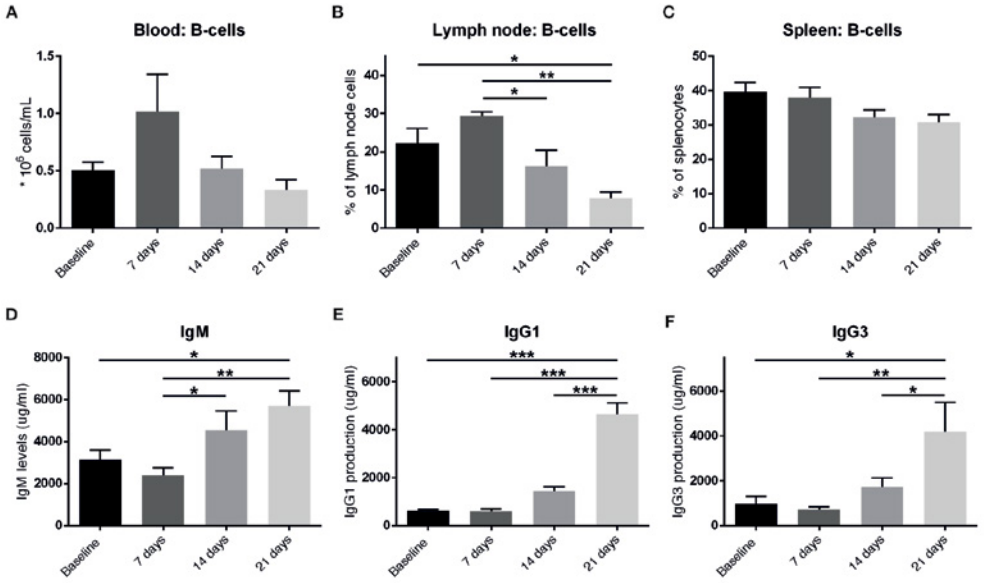

Figure 5.6 – B-cells in chronic myocarditis

The numbers of CD19⁺ B-cells in the blood (**A**) or percentages in the lymph nodes (**B**) and spleen (**C**) are compared, showing a peak in the blood and lymph nodes, before decreasing in all organs. This possibly reflects maturation into plasma cells. This correlates with antibody production, where IgM (**D**) is detected in the blood at two and three weeks, while IgG1 (**E**) and IgG3 (**F**) are strongly present at three weeks. ** p<0.05, ** p<0.01, *** p<0.001*

(*figure 5.5F*). In the spleen there was a drop in T_{reg} at one week after induction, after which the levels normalized again (*figure 5.5G*).

B-cells and antibodies

The number of B-cells in the blood increased rapidly after induction of myocarditis (*figure 5.6A*). After this first week, the levels decreased again. A similar trend was visible in the cardiac lymph nodes, where a small increase after 7 days was followed by a strong decline in B-cells at two and three weeks (*figure 5.6B*). In the spleen the amount of B-cells did not increase, but actually showed a small decrease at two and three weeks (*figure 5.6C*). We were not able to determine whether this decrease was due to the shedding of CD19 upon maturation into plasma cells, or to migration of the CD19⁺ B-cells to other tissues. We did, however, measure the production of circulating antibodies over the course of the experiment (*figure 5.6D-F*). IgM, usually the first Ab produced in response to inflammation, showed increased levels two weeks after induction. These levels remained elevated during week three (*figure 5.6D*). IgG, the late response Ab, exists in various subtypes. Of these subtypes, IgG1 and IgG3 are usually produced in response to foreign antigens. Both IgG1 (*figure 5.6E*) and IgG3 (*figure 5.6F*) had low baseline levels,

that were strongly increased at three weeks, indicating a mature, ongoing immune response to the injected antigens.

Neutrophils

Although not considered to play a major role during chronic myocarditis, we also determined the number and location of the neutrophils. Neutrophils in the blood were increased in number at all time-points after induction of myocarditis (*figure 5.7A*). In the lymph nodes, an increase in neutrophils was only found at week 2, while the cells were virtually absent at the other time points (*figure 5.7B*). In the spleen a gradual increase in neutrophils was registered over time (*figure 5.7C*). In the heart there appeared to be some infiltration of neutrophils three weeks after induction of myocarditis (*figure 5.7D*).

Cytokines

In addition to measuring the presence of different immune cells in each organ, we also investigated the systemic inflammatory environment by analyzing the levels of cytokines in the blood. Due to the strong increase in end systolic volume, the cytokines that negatively influence contractility were of special interest (*figure 5.8A*). Of these cytokines, Il-1β and IL-2 did not show any increase in level after induction of myocarditis, and circulating IL-2 even dropped after two weeks. IL-6 and TNF-α, however, did increase strongly in week 2 and remained elevated during the course of disease.

IFN-γ, a typical pro-inflammatory cytokine, that corresponds among others to a T-cell subset known as T_H1 cells, was slightly elevated in the course of disease (*figure 5.8B*), while IL-4, linked to the T_H2-subtype, did not change at all (*figure 5.8C*). IL-17A, associated with the aggressive T_H17 subset of T-cells increased strongly over time (*figure 5.8D*), while the anti-inflammatory IL-10, indicative of functional T_{reg}, actually went down (*figure 5.8E*). MCP-3, also known as CCL7, plays a role in monocyte attraction and accordingly peaked at 7 days (*figure 5.8F*). G-CSF is involved in granulocyte release and was found slightly elevated from baseline onwards, corresponding to the levels of neutrophils in the blood (*figure 5.8G*). The other cytokines measured can be seen in *suppl. figure 5.1*.

Discussion

In this study, we investigated the effect of experimentally induced auto-immune myocarditis on cardiac function and the mobilization of immune cells. Immediately after induction of the disease, a mobilization of white blood cells was noticeable, in which practically all types of leukocytes were involved. The increasing levels of troponin at this point suggest that the heart is already damaged by this immune

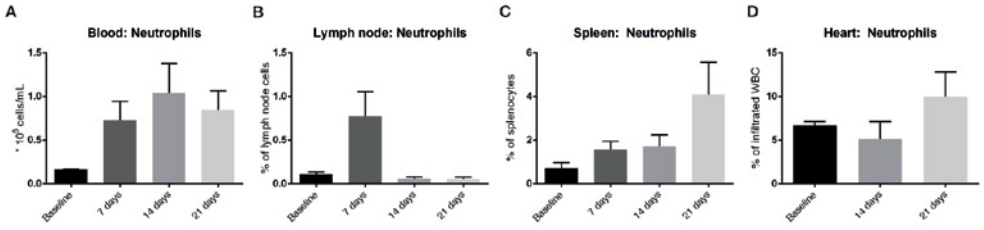

Figure 5.7 – Neutrophils in chronic myocarditis

The numbers of neutrophils in the blood (**A**) or percentages in the lymph nodes (**B**), spleen (**C**) and heart (**D**) are compared, showing an immediate and sustained increase in the blood (**A**). In the lymph nodes (**B**), normally devoid of neutrophils, a peak is observed at 1 week, while in the spleen (**C**) an increase is seen at three weeks. In the heart (**D**) a minor increase is seen at three weeks as well. ** p<0.05, ** p<0.01, *** p<0.001*

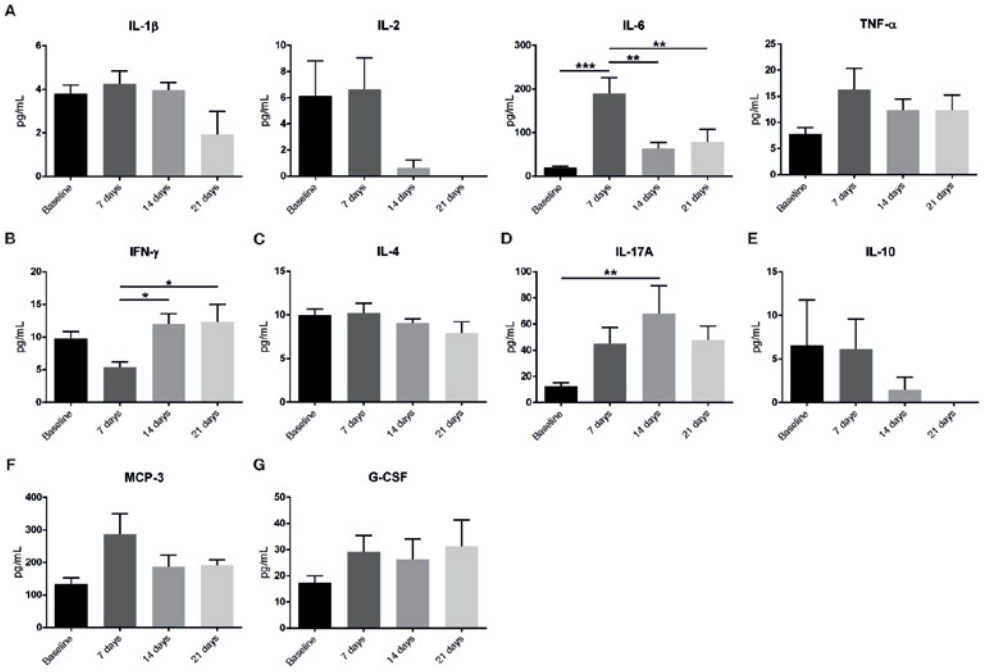

Figure 5.8 – Cytokines in chronic myocarditis

A Negative inotropic cytokines. IL-1β and IL-2 do not increase in response to induction of myocarditis, while IL-6 and TNF-α peaked after induction. **B** IFN-γ is slightly elevated at later time points. **C** IL-4 stayed stable throughout the whole experiment. **D** IL-17A increased significantly at later time points after induction. **E** IL-10 is present at low levels that drop even further during chronic myocarditis. **F** MCP-3 (CCL7) peaks one week after induction. **G** G-CSF remains slightly elevated during the course of disease. ** p<0.05, ** p<0.01, *** p<0.001*

response one week after induction.

The fact that the troponin levels remain elevated throughout the studied period clearly suggests an ongoing nature of this immune attack. Even so, in line with clinical practice, troponin increases in our model were mild, especially when compared to the acute infarction setting[17]. The troponin release is likely reflecting the slow and chronic nature of myocarditis, where damage occurs over time and does not give one single peak of release. Another indication for the chronic progression of this disease and thereby inducing myocardial damage is the small increase in collagen deposition.

Monocytes, the first cells activated in the immunization process, peak in the first week after induction of myocarditis. The pro-inflammatory Ly6C^{high} cells are especially elevated in the local lymph nodes at this time, likely migrating here to perform antigen presentation[18,19]. Afterwards the levels drop in the lymph nodes, and become elevated in the blood. This may indicate that the monocytes migrate or have a scavenger role for clearing-up debris. This could also explain the small, non-significant increase in monocyte levels in the heart in the third week. Interesting is the strong increase at day 21 in Ly6C^{high} cells in the spleen, where the main reservoir of the adaptive immune system is localized[20]. Ongoing antigen presentation in the spleen could suggest the beginning of chronic inflammation.

Meanwhile, the anti-inflammatory Ly6C^{low} cells are mildly increased in the blood and lymph nodes after one week. This could indicate that upon activation of the immune system, the regulatory counterparts are already formed in the same organs. At day 14, the levels are back to baseline in the lymph nodes, but in the blood this increase in Ly6C^{low} cells is maintained. At three weeks these anti-inflammatory monocytes are mainly localized in the spleen, just like their pro-inflammatory counterparts.

Interestingly, the formation of regulatory T-cells, by both T_H- and T_C-cells is also observed at these later time points, but in a reversed pattern. The increase after two weeks in the spleen suggest the spleen as origin of the formed T_{reg}-cells, after which the cells spread via the blood to the lymph nodes. Interestingly, the levels of the main cytokine produced by T_{reg}, IL-10, remains low at the time points when these cells are present, namely day 14 and 21. The low IL-10 could indicate that these T_{reg} have not yet been fully activated or matured[21].

It is likely that the formation of regulatory cells reflects an attempt to control this ongoing immune response. As Ly6C^{low} cells and T_{reg} are likely to interact, it would be interesting to elucidate if either one of them induces the other subtype, and if they successfully alter the adaptive immune response. If so, stimulating the formation of regulatory subtypes could be an interesting target in resolving the inflammatory response during chronic myocarditis. However, at this stage this is speculative and

more research on these migration patterns and subsequent changes in adaptive response are necessary to fully understand the process.

Shortly after induction of myocarditis, cytotoxic T-cells appear easily mobilized from the spleen to the blood and lymph nodes. At later time points, the circulating number decreases and an influx into the heart is observed. The influx of active T_C-cells could account for a component of the decrease in cardiac function, but is unlikely to be solely responsible due to their low numbers and timing.

Meanwhile, the main role of the helper T-cells (T_H-cells) is to provide a co-stimulatory signal for the B-cells. The similar pattern of T_H-cell and B-cell presence in lymph node and spleen is therefore unsurprising. The data suggest the T_H-cells and B-cells migrate to the lymph nodes after induction of myocarditis, where the T_H-cells might activate the B-cells. Subsequently the B-cells can start maturing into plasma cells, which explains the drop in B-cells observed in all tissues on 14 days onwards. More evidence of B-cell maturation is provided by the measurements of the immunoglobulins. The initial antibody produced in response to foreign antigens is IgM, which increases at the same time the number of $CD19^+$ B-cells declines[22].

The plasma cells continue with class switching, demonstrating the maturation of the immune response, as seen by the increase in IgGs[22]. Opsonized cardiomyocytes can be recognized and are phagocytosed by neutrophils, explaining the peak of neutrophils at three weeks in the heart. Although we did not measure activity, IgG1 and especially IgG3 are involved in complement activation and as such it is probable that at 3 weeks the complement system will be involved in the immune response as well[23].

Lastly, the neutrophils are slightly elevated in the blood shortly after induction of myocarditis and remain increased for the duration of the experiment. This could very well reflect a response to ongoing damage, as the neutrophil is the main scavenger of debris in the body. More surprising is the increase in neutrophils in the lymph nodes at one week. This could reflect an antigen-presenting role of neutrophils in this model[24]. Similarly, there is an unexpected increase in neutrophils in the spleen at three weeks which could also be due to antigen presentation, yet the spleen has also been suggested as location for extra-medullary granulopoiesis[25]. To determine which of these options is correct demands further investigation.

As a result of these immune activations, changes in the cardiac function are observed. Most noticeable is the increase in end systolic volume (ESV) by 37%. ESV is influenced by merely two factors, being afterload and inotropy. Although it was not measured in these experiments, an increase in afterload by hypertension seems an unlikely consequence of this disease model and has in fact never been reported[26]. This leaves a decrease in inotropy as explanation for the increased ESV. A drop in inotropy can be due to the death of cardiomyocytes as confirmed

by the elevated troponin, but certain cytokines (IL-1β, IL-2, IL6 and TNF-α) have also been described to have negative inotropic effects. Interestingly, in the course of disease in this model, IL-6 and TNF-α become and remain elevated and as such could affect the contractility of the cardiomyocytes.

Assuming that a portion of the decreased contractility is due to the negative inotropic effects of cytokines, this implies that removal of the cytokines might partially restore cardiac function. Blocking cytokines could be an interesting consideration both in acute and chronic myocarditis.

Naturally, there are limitations to this study. The induced myocarditis, although demonstrating clear immune-infiltrations in the heart, was not as dramatic as anticipated which suggests that the end point of three weeks is too short. The use of four animals per group might lead to some variation and thereby have impacted on the significance of studied populations. However, despite these drawbacks we do believe the data to accurately reflect the ongoing inflammation found in chronic myocarditis. Additionally, we have identified several processes that could prove to be interesting as new therapeutical targets to treat chronic myocarditis.

In conclusion, we have shown that during chronic myocarditis the whole immune system is and remains involved. The cytokines released by the activated immune cells stimulate the release and chemotaxis of other immune cells, keeping this inflammatory spiral going. Interventional strategies could aim at the possible cross-talk between both $Ly6C^{low}$ and $Ly6C^{high}$ monocytes and T_{reg} in the spleen, in order to induce more, mature anti-inflammatory cells. An approach could be to apply plasmapheresis to remove antibodies, which can reduce the number of neutrophils in the heart and might prevent complement activation. Meanwhile, the use of targeted pharmaceuticals against specific cytokines might partially restore the cardiac function.

Acknowledgements

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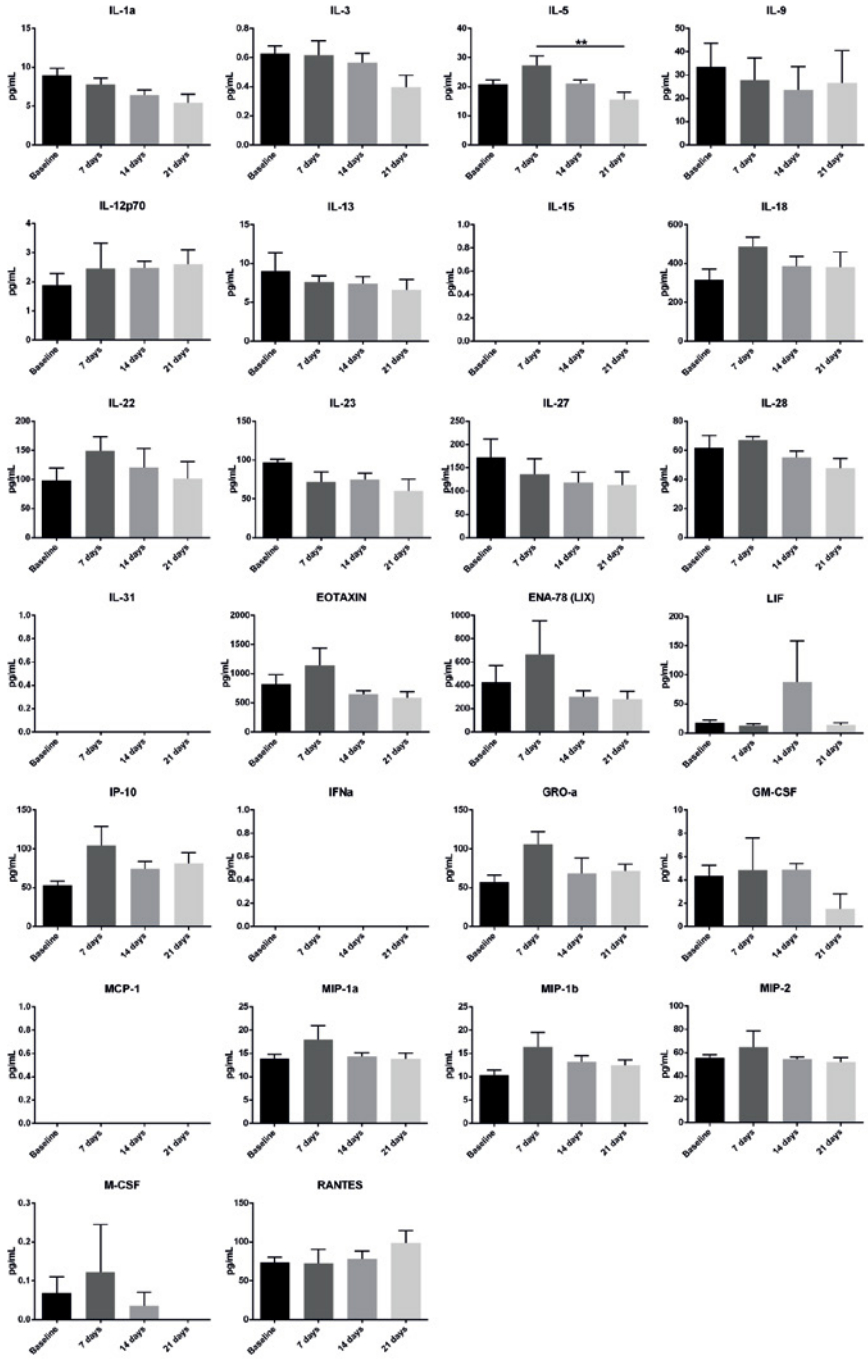
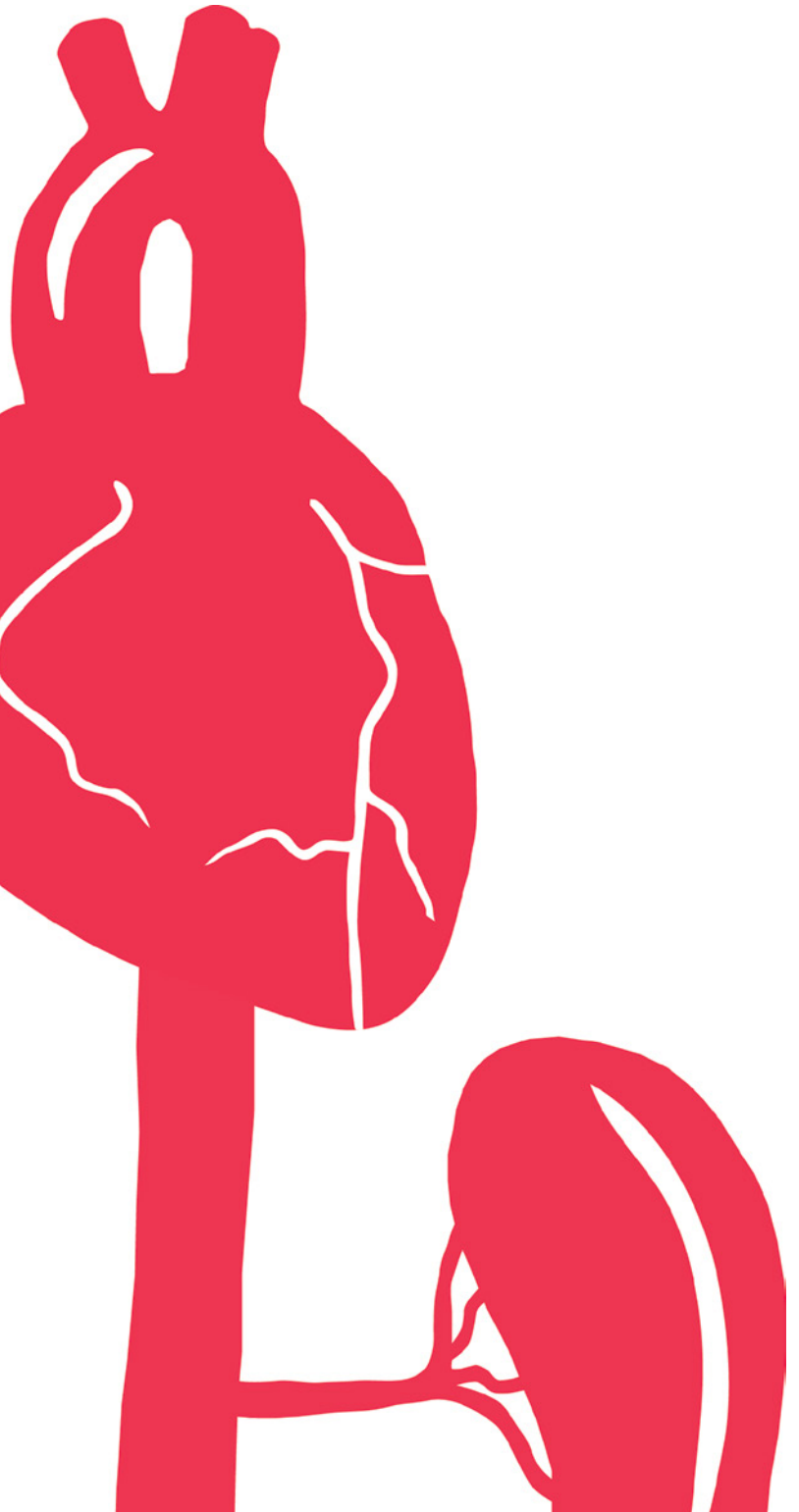
Supplement



Suppl. Figure 5.1 – Cytokines in myocarditis

*** p<0.01*



Stem Cell Therapy Against Chronic Myocarditis

6

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Abstract

Rationale

Myocarditis is an inflammatory cardiac disease that can lead to dilated cardiomyopathy and heart failure. Few treatments exist for the chronic auto-immune phase of this disease.

Objective

We aim to evaluate the effectiveness of cell therapy on the progression of chronic myocarditis using mesenchymal stem cells (MSC) and cardiomyocyte progenitor cells (CMPC).

Methods & Results

Experimental auto-immune myocarditis was induced and intervention with MSC, CMPC or PBS was performed at 7 days. Animals were terminated 0, 7, 14 and 21 days after induction of myocarditis. Immune cell analysis in heart, spleen, lymph nodes and blood showed small changes induced by the progenitor cells, and cardiac function is slightly better upon intervention with CMPC.

Conclusion

Cell intervention using MSC and CMPC leads to subtle changes in myocarditis progression. CMPC perform slightly better than MSC.

Introduction

Chronic myocarditis is an inflammatory condition of the heart muscle. The most common cause is a viral infection which can spread to the heart, thereby eliciting an immune response against cardiomyocytes[1-3]. If the myocarditis does not have a fulminant clinical presentation, it can go unnoticed for many years. During these years, however, auto-reactive lymphocytes and macrophages can cause ongoing myocardial inflammation[1,3].

Patients in this chronic phase will complain about fatigue and decreased exercise tolerance, largely due to an effectively decreased cardiac function[1,4,5]. Initially, this reduced cardiac output is reversible, but progressively it can lead to dilated cardiomyopathy. Prognosis of diagnosed dilated cardiomyopathy is poor; over 50% will die within five years unless cardiac transplantation is performed[2,3,6].

Limited treatment options exist in the chronic phase. Most drugs developed aim to prevent or treat ischemic damage, rather than an excessive inflammatory response[3]. Additionally, early experiments with immunosuppressive drugs in cardiac disease had dramatic adverse side effects, i.e. increased incidence of cardiac rupture[7]. This made research into inhibiting immunological responses during cardiac disease unpopular for quite some time.

In recent years the use of stem cells as a treatment for heart failure has been suggested. However, the effects in these studies were low to moderate, which is not surprising considering most interventions took place in end-stage ischemic cardiomyopathy, when advanced adverse remodeling has already taken place [8-10]. Moreover, true regeneration of cardiac tissue, by injected donor cells or stimulated endogenous stem cells, has hardly been convincingly shown thus far[8].

The last decade ample evidence has been gathered, showing that many stem or progenitor cells can modulate the immune system[5,7,11]. Potentially, stem cell treatment for chronic myocarditis, a disease based on ongoing inflammation, could be an interesting new target.

Most promising cell types for clinical application would be the mesenchymal stem cell (MSC), having a long history of immunomodulatory capacity[5,7], and the cardiomyocyte progenitor cell (CMPC), a progenitor cell originating from the heart and showing favorable effects on the adaptive immune system as well[12,13].

To this end, we explored the effectiveness of MSC and CMPC in a murine model of Experimental Auto-immune Myocarditis (EAM).

Materials & Methods

Cell isolation and culture

Human fetal mesenchymal stem cells (MSC) and cardiomyocyte progenitor cells (CMPC) were obtained and characterized as described previously[14,15]. Both cell types were cultured in plastic culture flasks coated with 0.1% gelatin. For MSC medium MEM-α (Gibco, 22561) supplemented with 10% fetal bovine serum (FBS; Gibco, 10099-141), 100 U/ml Penicillin and 100 µg/ml Streptomycin (Lonza, 17-602E), 1 ng/ml bFGF (Sigma F0291) and 0.2 mM L-ascorbic acid-2-phosphate (Sigma A4034) was used[11].

CMPC culture medium consisted of 1 part endothelial basal medium (EGM-2; Lonza CC-3156) and 3 parts M199 (Lonza BE12-119F) supplemented with 10% FBS, 100 U/ml Penicillin and 100 µg/ml Streptomycin and 1% Non-essential Amino Acids (Lonza 13-114)[14,16]. Upon reaching 80% confluency, both cell types were passaged by trypsin digestion (0.25% Trypsin; Lonza, CC-5012) at 37°C for two minutes maximum.

Animals and experimental autoimmune myocarditis

All research protocols were approved by the Animal Ethical Commission of Utrecht University and animals were maintained in compliance with animal welfare guidelines of the UMC Utrecht. Male BALB/c mice (8-12 weeks old, n=32) were purchased from Charles River. Housing occurred under controlled conditions in a 12 hours light/12 hours dark cycle and the animals were fed ad libitum.

Experimental auto-immune myocarditis was induced by injecting 200 µg of α-MyHC peptide (Ac-SLKLMATLFSTYASAD-OH; NKI, Amsterdam, the Netherlands) emulsified with 100 µg Complete Freud's Adjuvans (Sigma: F5881) subcutaneously in the neck of the mice at day 0. At day 7, a booster solution containing the same amount of the α-MyHC peptide dissolved in PBS was given.

Before administration of the booster, mice were randomly assigned to receive 100 µL phosphate-buffered saline (PBS), or PBS containing 1×10^6 MSC or CMPC. The total interventional volume was administered slowly via the tail vein.

All animals were terminated by exsanguination, after which hearts, spleens and thoracic lymph nodes were collected. Blood was collected in EDTA tubes and used for flow-cytometric analysis. Serum was separated from the blood by centrifugation at 2,000 *g* for 20 minutes and stored separately at -80°C. Half of the heart was put in paraffin for histochemical analysis, a quarter was digested for flow cytometry and the last quarter was snap frozen and stored -80°C. The spleen and lymph nodes were used completely for flow cytometry.

Echocardiography

3D-echocardiographic analysis (echo) was done under isofluorane anesthesia on day 0, 7, 14 and 21 of the experiment. A high resolution ultrasound system (Vevo 2100, VisualSonics) with an 18-38 MHz transducer (MS 400, VisualSonics) was used, with constant monitoring of temperature, heart rate and respiration. M-mode (2D short-axis view) was used to measure fractional shortening and wall thickening, while B-mode images were obtained using the parasternal long axis view.

3D-echo images were obtained by placing the transducer perpendicular to the long-axis view (short-axis view) and scanning the left ventricle by consecutive images (0.064mm intervals) of the short axis using a 3D-motor (VisualSonics).

Both the echocardiogram and respiration gating were used to adequately identify the triggers for end-diastolic volume (EDV) and end-systolic volume (ESV). Analysis of the reconstructed 3D images was performed with the manufacturer's software (Vevo2100 v1.7.1). All measurements and analysis were performed by a blinded operator and investigator.

Flow cytometry

Hearts (except for the 7 days time point) were cut into small (1 mm) pieces and digested using Liberase TL (26 U/mL, Roche 05401020001) and DNase I ($10x10^3$ U/mL, Roche 04536282001) in HEPES buffer (Life Technologies 15630-080) at 37°C for 20 minutes. Subsequently, cells were put through a 40 µm nylon mesh (Greiner Bio-One 542040) to make a single cell suspension. To create single cell suspensions, lymph nodes and spleens were also strained directly through a 40 µm nylon mesh.

Cells were washed with PBS containing 5% FBS and each sample was incubated for 30 minutes at room temperature with the appropriate mixture of fluorochrome-conjugated antibodies. After incubation, samples were washed with 5% FBS, after which Optilyse C (Beckman Coulter A11895) was added to lyse the erythrocytes and fix the labeled white blood cells.

The following antibodies were used from eBioscience: CD3e-PE (Clone 145-2C11; 12-0031-82), CD8a-APC-eFluor780 (Clone: 53-6.7; 47-0081-82), CD11b-AlexaFluor488 (Clone: M1/70; 53-0112-82), CD19-eFluor 450 (Clone: 1D3; 48-0193-82), CD25-APC (Clone: PC61.5; 17-0251-82), CD45-eFluor450 (Clone: 30-F11; 48-0451-82), F4/80-PE-Cy7 (Clone: BM8; 25-4801-82) and FoxP3-eFluor450 (Clone: FJK-16s; 48-5773-82).

Additionally, from BD Bioscience we used CD4-PerCP (Clone RM4-5; 553052), CD62L-PE-CF594 (Clone MEL-14; 562404), Ly6G-APC (Clone 1A8; 560599) and Ly6C-PE-CF594 (Clone AL-21; 562728). Sytox blue (Invitrogen s34857) was added before measuring to determine cell viability.

Cell fluorescence was measured using the Gallios Flow Cytometer (Beckman Coulter) and all analysis were performed with Kaluza Analysis Software (Beckman Coulter, v1.3). The gating strategies used for analysis can be seen in *suppl. figure 6.1*.

Histopathological examination

The hearts embedded in paraffin were cut in 7 µm sections. Collagen was detected with a picrosirius red staining using circularly polarized light microscopy. Of each animal, five representative images of the heart were taken and converted into mean grey values. Collagen density was analyzed using the average number of grey values expressed per square micrometer.

Serum measurements

Serum was used for detection of cardiac damage by measuring high sensitive Troponin I levels. Samples were processed in a clinical chemistry analyzer (AU5811, Beckman Coulter) and compared to baseline. To analyze the systemic inflammatory reaction, the serum levels of 36 cyto- and/or chemokines were determined using a Luminex-200 instrument (Bio-Plex 200), combined with a 36-multiplex panel (eBioscience, EPX360-26092-01). The luminex assay was performed according to manufacturer's instructions.

Statistical analysis

All statistical analysis were made in SPSS statistics v20 (IBM, Armonk, NY). After verifying normal distribution, the groups were compared using a one-way ANOVA analysis with a LSD post-hoc correction. A p-value < 0.05 was considered significant.

Results

Chronic myocarditis

One week after induction of experimental auto-immune myocarditis, all mice received intervention by intravenous injection of PBS, 1×10^6 mesenchymal stem cells (MSC) or 1×10^6 cardiomyocyte progenitor cells (CMPC).

At 0, 1, 2 and 3 weeks, four animals in each group were analyzed by 3D-echo and terminated (*figure 6.1A*). Premature death due to severe heart failure was not seen in any animal. Circulating leukocyte levels increased slightly upon induction of chronic myocarditis at 7 days, whereas levels at 3 weeks stayed minimally elevated in all three groups (*figure 6.1B*). Immune cell infiltration in the heart, defined by $CD45^+$ cells, was observed at three weeks, with the MSC and CMPC treated

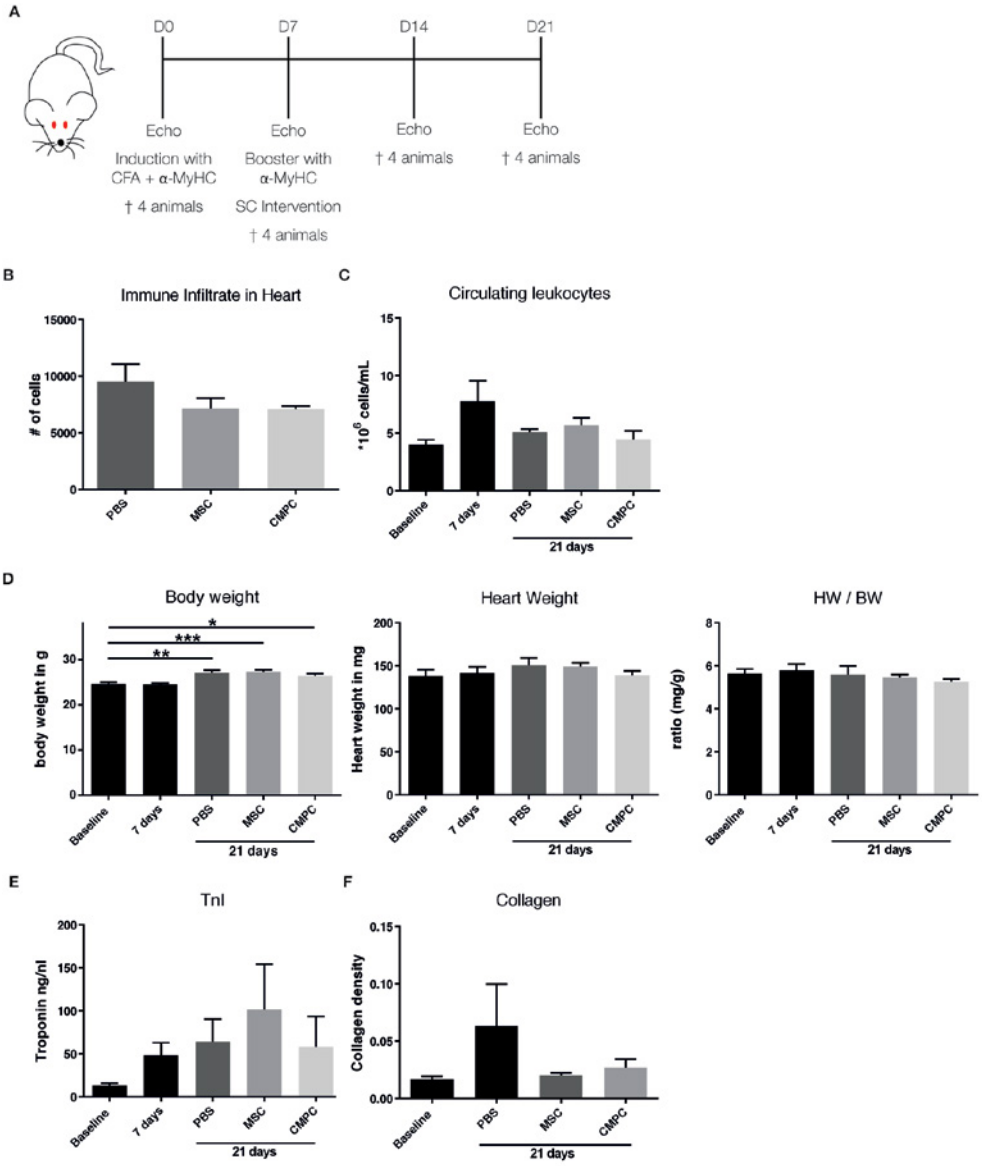

Figure 6.1 – Experimental auto-immune myocarditis

A Experimental set up and timing of sample collection. B Circulating leukocytes at baseline, moment of intervention and at 3 weeks. C Immune infiltrate in the heart at three weeks in the three treatment groups. D Body weight, heart weight and the body-weight heart weight ratio. E Troponin levels became elevated after induction and remained elevated at three weeks in all groups. F Collagen depositions at baseline and three weeks after induction of myocarditis. ** p<0.05, ** p<0.01, *** p<0.001*

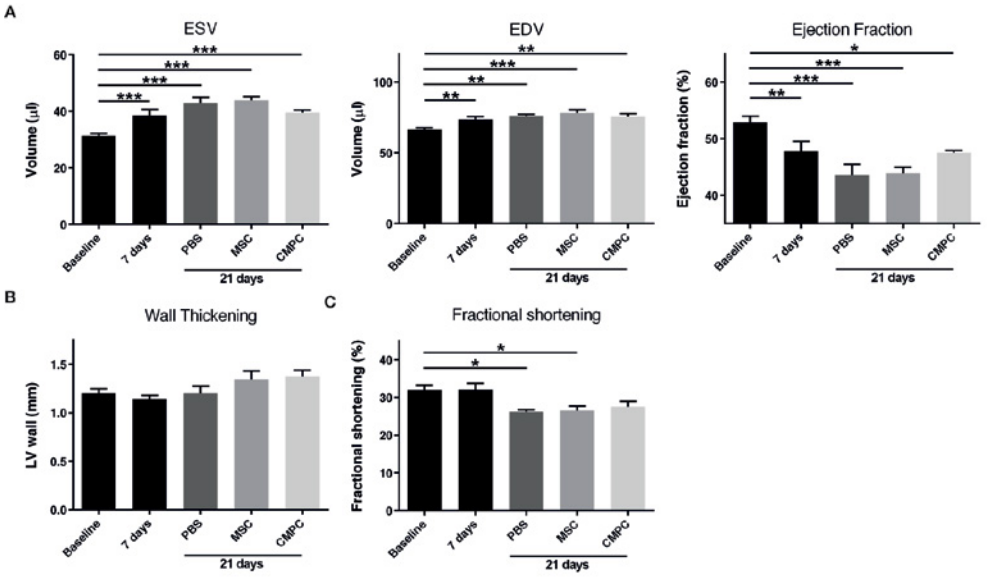

Figure 6.2 – Functional cardiac measurements

A End-systolic volume (ESV) and end-diastolic volume (EDV) in all groups over time. Ejection fraction was calculated from these volumes. B Wall thickening remains stable during the course of the experiment. C Fractional shortening was decreased after three weeks of myocarditis in the PBS and MSC group, not in the CMPC group. ** p<0.05, ** p<0.01, *** p<0.001*

groups showing slightly less infiltration (*figure 6.1C*). Body weight increased over time, reflecting normal growth of the mice (*figure 6.1D*).

Meanwhile both heart weight and the heart weight/body weight ratio did not differ between time points or groups. Troponin I levels were measured in the serum and showed increased levels after induction of myocarditis, and this elevation is maintained in all three groups after 21 days (*figure 6.1E*). Interestingly, histological examination of collagen deposition after 3 weeks showed an increase in collagen deposition in the PBS group, while both the MSC and CMPC group had levels comparable to baseline (*figure 6.1F*).

Cardiac function

Both end-systolic (ESV) and end-diastolic volumes (EDV) increased during the course of chronic myocarditis, as measured by 3D-echo (*figure 6.2A*). In the CMPC-treated group, these volume increases appeared less pronounced, which resulted in a slightly higher ejection fraction (48%) compared to the PBS and MSC-treated groups (44%). Wall thickening was not significantly altered during

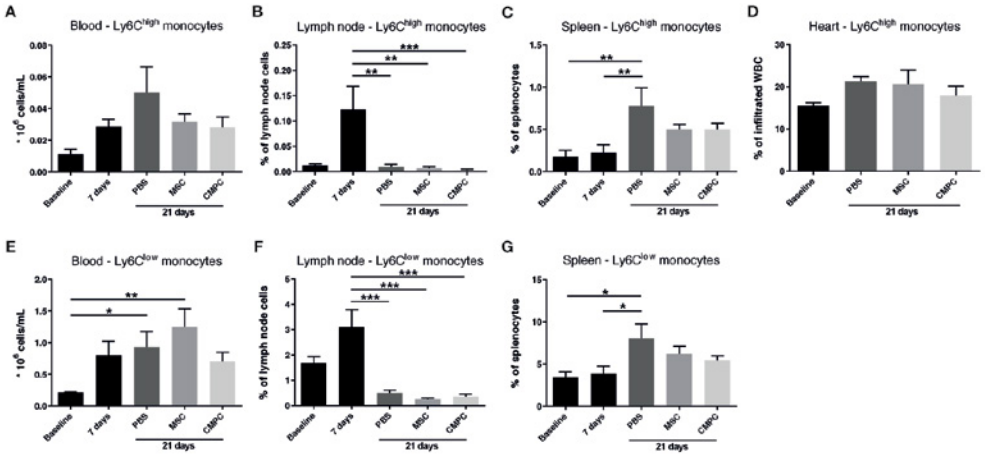

Figure 6.3 – Effect of MSC and CMPC on monocytes in chronic myocarditis

The numbers of pro-inflammatory Ly6C^{high} monocytes in the blood (**A**) or percentages in the lymph nodes (**B**), spleen (**C**) and heart (**D**) are compared. The pro-inflammatory Ly6C^{high} cells were initially seen in the lymph nodes. At later time points, blood and spleen had most Ly6C^{high} monocytes, with slightly lower amounts after treatment with MSC or CMPC. Anti-inflammatory Ly6C^{low} monocytes in blood (**E**), in lymph nodes (**F**) and the spleen (**G**) showed a different pattern, suggesting an initial mild increase in blood and lymph nodes, which draws to the spleen in a later stage. * p<0.05, ** p<0.01, *** p<0.001

chronic myocarditis, although the cell-treated groups showed a slightly increased wall thickening (*figure 6.2B*). Meanwhile, fractional shortening was decreased after 3 weeks of chronic myocarditis in the PBS and MSC group, while in the CMPC group fractional shortening was preserved (*figure 6.2C*).

Monocytes in chronic myocarditis

Two subsets of monocytes can be distinguished; the pro-inflammatory Ly6C^{high} monocytes and the more anti-inflammatory Ly6C^{low} monocytes[17]. Fluctuations in the levels of both Ly6C^{high} and Ly6C^{low} monocytes were observed, however, due to low number of animals these changes were not significant. The pro-inflammatory Ly6C^{high} cells were increased in the blood after induction of myocarditis and were maintained at high levels at three weeks in the PBS-treated group (*figure 6.3A*).

In the progenitor cell-treated groups, slightly lower levels were observed. In the lymph nodes, the Ly6C^{high} cells were mainly seen after induction of myocarditis, reflecting the antigen presentation here causing the chronicity of the disease (*figure 6.3B*). At the later time points, baseline numbers of Ly6C^{high} cells were observed

in all groups. In the spleen, a peak in Ly6C^{high} cells was seen at three weeks in the PBS-treated animals, suggesting an ongoing stimulation of immune response (*figure 6.3C*).

Again, these levels in Ly6C^{high} cells in the spleen were slightly decreased in the MSC- and CMPC-treated animals. In the heart low numbers of Ly6C^{high} monocytes were present and did not alter much over time in any group (*figure 6.3D*).

The anti-inflammatory Ly6C^{low} cells showed roughly a similar pattern. The induction of myocarditis induced an increase in Ly6C^{low} monocytes, which remained elevated in all groups during the experiment (*figure 6.3E*). However, the highest levels of these cells were observed in the MSC-treated animals.

In the lymph nodes an increase in Ly6C^{low} monocytes was observed at seven days, after which the levels dropped markedly (*figure 6.3F*). In the spleen, the percentage of Ly6C^{low} monocytes was increased at three weeks, with the highest levels obtained by animals treated with PBS only (*figure 6.3G*).

Cytotoxic t-cells

Cytotoxic T-cells (T_C-cells) were increased in the blood upon induction of myocarditis and remained elevated in all three groups during the experiment (*figure 6.4A*). In the lymph nodes an initial increase in T_C-cells was seen at seven days after induction, which decreased again in all groups (*figure 6.4B*). In the spleen, the number of cytotoxic T-cells dropped after myocarditis induction, suggesting their mobilization (*figure 6.4C*), although no differences between groups could be observed. The levels of T_C-cells remained low in the spleen in all groups at three weeks. In the heart, a small increase in T_C-cell infiltration was seen in animals treated with CMPC (*figure 6.4D*).

The prevalence of regulatory cytotoxic T-cells, defined as $CD8^+CD25^+FoxP3^+$, was also investigated in blood, lymph nodes and spleen. In the blood, an increase in regulatory T_C-cells was seen at three weeks in all groups, with the MSC-group having the most pronounced increase (*figure 6.4E*). In the lymph nodes, a similar increase was seen at three weeks, however, the PBS-group had a higher percentage than the cell-treated groups (*figure 6.4F*). In the spleen, also a strong increase in regulatory cytotoxic T-cells was observed, with the progenitor cell-treated animals attaining the highest levels (*figure 6.4G*).

T-helper cells

T-helper (T_H-cells) cells are essential in the activation of B-cells. In the blood, T_H-cells showed a minor increase at 7 days, the moment of cell intervention, after which the levels drop back to baseline in all three groups (*figure 6.5A*). A similar increase was seen in the lymph node samples at day 7, however, in all treatment

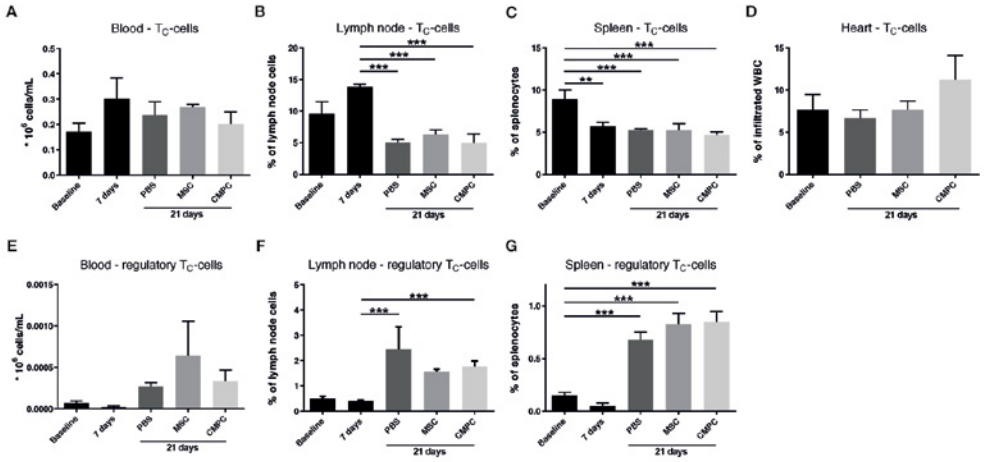

Figure 6.4 – Effect of MSC and CMPC on cytotoxic T-cells in chronic myocarditis

The numbers of $CD8^+$ T-cells in the blood (**A**) or percentages in the lymph nodes (**B**), spleen (**C**) and heart (**D**) were compared with an without treatment with MSC or CMPC. Cytotoxic regulatory T-cells ($CD8^+CD25^+FoxP3^+$) in blood (**E**), in lymph nodes (**F**) and the spleen (**G**) show increased levels in especially the lymph nodes and spleen in all groups. *** p<0.01, *** p<0.001*

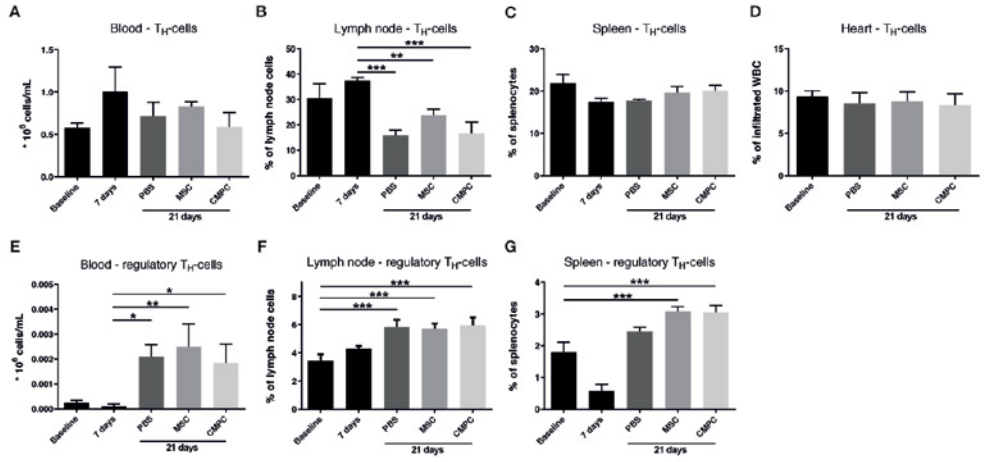

Figure 6.5 – Effect of MSC and CMPC on T-helper cells in chronic myocarditis

The numbers of $CD4^+$ T-cells in the blood (**A**) or percentages in the lymph nodes (**B**), spleen (**C**) and heart (**D**) were compared between the different treatment groups. Regulatory T-cells ($CD4^+CD25^+FoxP3^+$) in blood (**E**), in lymph nodes (**F**) and the spleen (**G**) showed at a late formation of regulatory cells in all groups. ** p<0.05, ** p<0.01, *** p<0.001*

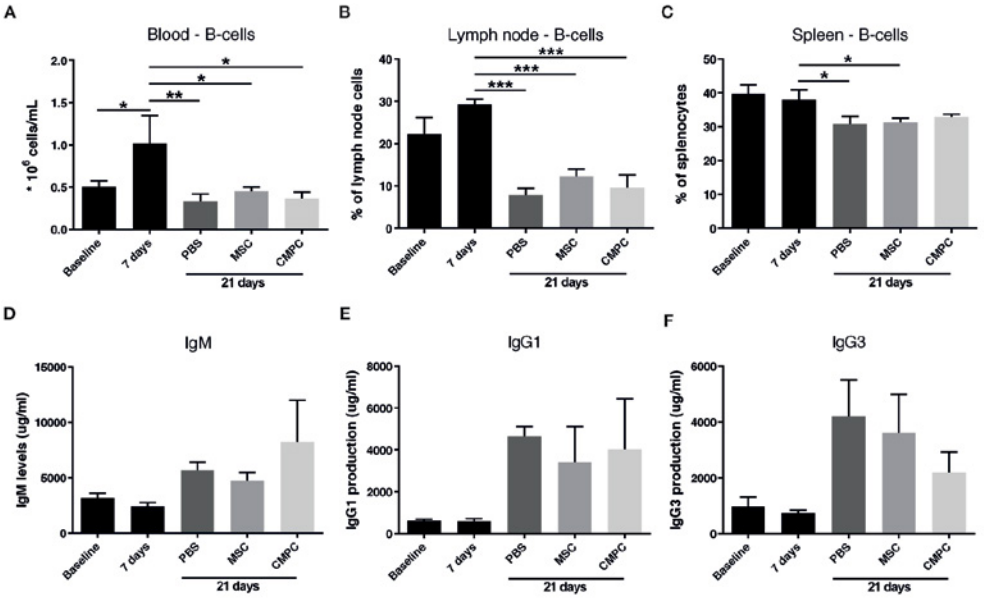

Figure 6.6 – Effect of MSC and CMPC on B-cells in chronic myocarditis

The numbers of CD19⁺ B-cells in the blood (**A**) or percentages in the lymph nodes (**B**) and spleen (**C**) were compared. At three weeks, levels had decreased compared to the moment of intervention in all three groups. Antibody production was active at three weeks, with IGM (**D**), IgG1 (**E**) and IgG3 (**F**). ** p<0.05, ** p<0.01, *** p<0.001*

groups the numbers at three weeks were even lower than at baseline (*figure 6.5B*). In both the spleen and the heart, little changes in T_H-cell levels could be observed over time (*figure 6.5C* and *D*).

Regulatory T-cells, defined as $CD4^+CD25^+FoxP3^+$ (T_{reg}), are the anti-inflammatory subset of T-cells. In the blood, a strong and comparable induction of these T_{reg} is observed in all groups after 21 days (*figure 6.5E*). In the lymph nodes, these cells increased more gradually in percentage but again no differences between the intervention groups was observed (*figure 6.5F*). In the spleen an initial drop in T_{reg} was seen at one week, after which the levels increased again (*figure 6.5G*). In the animals treated with MSC or CMPC, a slightly higher percentage of T_{reg} was reached than in animals treated with PBS.

B-cells and antibodies

The number of B-cells ($CD19^+$) in the blood increased after induction of myocarditis, while at three weeks all treatment groups were at comparable low levels again (*figure 6.6A*). A similar pattern was observed in the lymph nodes (*figure 6.6B*).

Meanwhile, in the spleen no initial increase was seen, only a minor reduction in

B-cells was observed at three weeks in all intervention groups. A decrease in $CD19^+$ B-cells can be caused by maturation of these cells into $CD19^-$ plasma cells, which is the reason we determined the levels of antibody production (*figure 6.6D-F*). The levels of IgM (*figure 6.6D*), IgG1 (*figure 6.6E*) and IgG3 (*figure 6.6F*) were low at baseline and at the time of intervention, yet elevated serum levels were found in all three treatment groups at 21 days.

Neutrophils

Although often neglected in myocarditis, neutrophils are involved in clearing dead cell debris and phagocytose opsonized cells. The number of neutrophils in the blood increased shortly after induction and remained elevated in all three treatment groups (*figure 6.7A*). In the lymph nodes, neutrophils were only found at the moment of intervention (*figure 6.7B*). At later time points no neutrophils were found in the lymph nodes in all groups. In the spleen there was a mild increase in neutrophils over time in the PBS treated groups, while those that received progenitor cell intervention retained lower (*figure 6.7C*). In the heart, a slight increase in neutrophils was observed, which did not significantly differ between the three treatment groups (*figure 6.7D*).

Cytokine production

Cytokines influence many inflammatory processes in the body and therefore we determined the levels of cyto- and chemokines at different time points during the experiment. Due to their intrinsic negative inotropic effect, IL-1β, IL-2, IL-6 and TNF-α were of special interest. IL-6 and TNF-α showed an increase in the first week after induction, yet at 21 days the levels had largely normalized again (*figure 6.8A*). In the case of IL-6 and TNF-α, the mice treated with MSC or CMPC had still slightly lower levels. IFN-γ, associated with T_H1 cells, did remain elevated in the PBS-treated animals at 21 days, while the levels in both progenitor cell-treated groups were significantly lower (*figure 6.8B*). IL-4, a cytokine linked to alternative macrophages and T_H2-cells, showed significantly lower levels in mice treated with MSC (*figure 6.8C*).

In addition, MSC apparently stimulated the production of IL-17A, indicative of the formation of T_H17-cells (*figure 6.8D*). IL-10, produced by mature regulatory T-cells, is not detected in any of the groups during the chronic phase of myocarditis (*figure 6.8E*). MCP-3 (CCL-7) is involved in monocyte chemotaxis and showed a mild increase at the time of intervention, before returning to baseline in all groups (*figure 6.8F*). G-CSF, responsible for the mobilization of neutrophils, showed a trend towards reduction in the animals treated with MSC and CMPC (*figure 6.8G*). Levels of other cytokines are presented in *suppl. figure 6.1*.

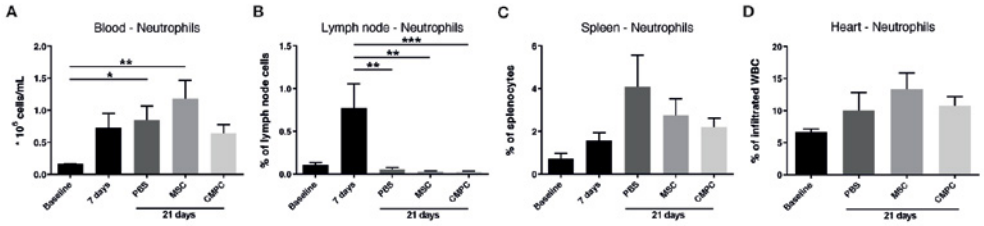

Figure 6.7 – Effect of MSC and CMPC on neutrophils in chronic myocarditis

The numbers of $CD11b^{+}Ly6G^{+}$ neutrophils in the blood (**A**) or percentages in the lymph nodes (**B**), spleen (**C**) and heart (**D**) were compared. We observed an increase in circulating neutrophils that remained in all groups at 21 days (**A**). In the lymph nodes (**B**) a peak was only observed at 1 week, while in the spleen (**C**) an increase was seen at three weeks. In the heart (**D**) a minor increase was seen at three weeks in all groups.
** p<0.05, ** p<0.01, *** p<0.001*

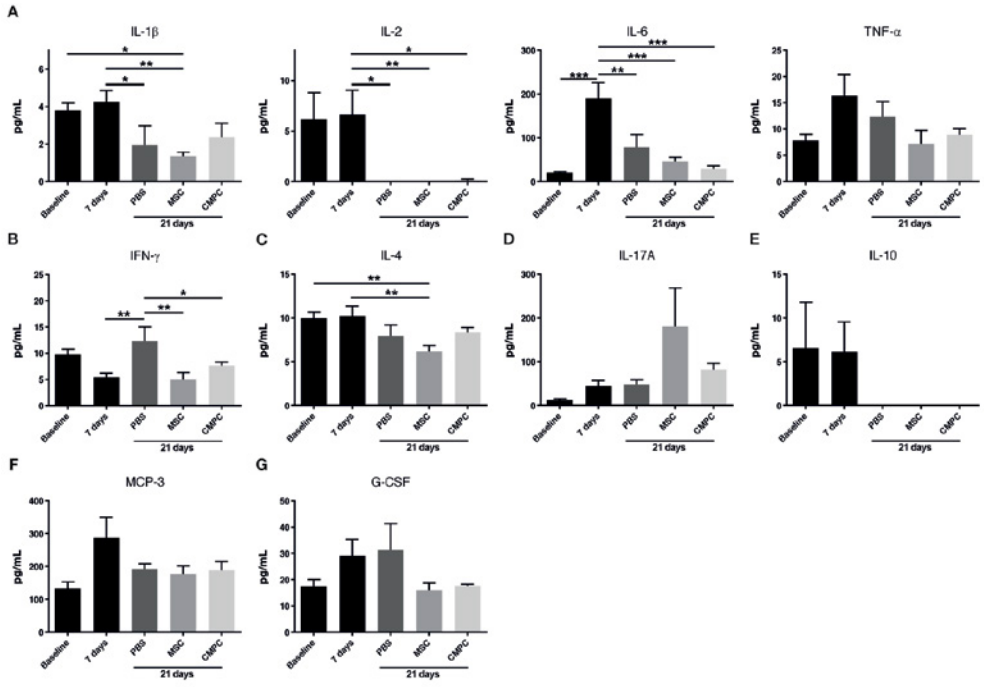

Figure 6.8 – Cytokines in chronic myocarditis

A Negative inotropic cytokines. IL-1β and IL-2 did not increase during chronic myocarditis. IL-6 and TNF-α peaked at one week. **B** IFN-γ was slightly elevated at 21 days in the PBS group. **C** IL-4 lowers in the MSC group. **D** IL-17A was strongly increased after treatment with MSC. **E** IL-10 was released at low levels at baseline, but it suppressed in all groups at three weeks. **F** MCP-3 (CCL7) returned to baseline in all groups at 21 days. **G** G-CSF remained slightly elevated in the PBS group.
** p<0.05, ** p<0.01, *** p<0.001*

Discussion

In this work, we investigated the effect of cell therapy on Experimental Auto-immune Myocarditis (EAM) by using mesenchymal stem cells (MSC) and cardiomyocyte progenitor cells (CMPC).

We have described the changes in the cardiosplenic axis in response to EAM previously[18]. No large effects of cell therapy with MSC or CMPC could be observed, yet several parameters were altered in a beneficial way. Although there were no differences in the number of circulating leukocytes, the infiltration of these cells into the heart was diminished after treatment with MSC or CMPC. The decrease in cardiac function over time in the PBS-treated group is a clear indicator of the chronicity of this disease.

Although no significant differences could be observed with regard to ESV and EDV, there is a trend towards lower ESV and EDV after intervention with CMPC, resulting in a slightly higher ejection fraction. Additionally, while fractional shortening is significantly reduced in the PBS and MSC treated groups, this is not the case in the mice treated with CMPC. In addition, lower amounts of collagen deposition were detected. This possible reduction in cardiac damage was, however, not visible in the serum troponin concentration, which was similar across all groups.

Additionally, both cell types were able to reduce the number of pro-inflammatory Ly6C^{high} monocytes in the blood and spleen. As the continued presence of Ly-6C^{high} cells in the spleen could be a symptom of ongoing antigen presentation, a reduction in these levels could reflect a lower disease burden. However, both cell types also reduced splenic levels of the anti-inflammatory Ly6C^{low} monocytes, with CMPC also moderating their blood levels. As Ly6C^{high} monocytes can differentiate into Ly6C^{low} monocytes[17], a suppression in the former could by extension lead to lower levels of Ly6C^{low} monocytes in the cell treated groups. More research will be needed to further investigate this relationship.

Considering the lymphocytes, little variation was observed in the levels of T-cells and B-cells. Interestingly, a link between B-cells and MSC has previously been described, claiming that MSC can reduce B-cell activation and proliferation[19]. However, in this dataset we could not reproduce this effect of MSC or CMPC on B-cells. This is different for the regulatory T-cells, both the CD4$^+$ (T_H) and the CD8$^+$ (T_C). Although the levels of T_{reg} do not differ much in the blood and the lymph nodes, higher levels are seen in the spleen after treatment with MSC and CMPC. The induction of regulatory T-cells has often been described for MSCs[20,21], while the induction of T_{reg} by CMPC is new.

Although the increase in T_{reg} in the spleen are small, much influence has been attributed to these cells, and small increases are thought to have major effects[22,23]. Interestingly, the increase in T_{reg} correlated to the lower number of Ly6C^{high} cells

observed in the spleen in these groups, suggesting that possibly the anti-inflammatory pathways have been induced. However, upon the formation of mature regulatory T-cells, we expected increased levels of IL-10, the main effector cytokine of T_{reg}. Instead we noticed that the levels of IL-10 dropped dramatically in all three treatment groups.

It should be kept in mind that the cytokine levels shown here were determined in serum, and different levels might have been present locally in the spleen and heart. Although very few alterations were seen in the number of T_H-cells, there could still be differences in the prevalence of the different T-helper subtypes.

One clue for shifts in the T_H-subtypes is the reduction of IFN-γ. IFN-γ has a strong pro-inflammatory effect and is produced in high quantities by T_H1-cells. T_H1-cells can activate cytotoxic T-cells and boost the killing capacities of phagocytes[7]. A reduction in IFN-γ in the stem cell treated groups could therefore reflect a reduction in pro-inflammatory T_H1-formation.

Interestingly, in the MSC-treated mice the IL-4 levels were reduced, with an increase in IL-17A levels. This could suggest an additional shift towards induction of the T_H17-subtype instead of the T_H2-subtype. T_H17-cells have been linked to the development of auto-inflammatory conditions[24], hinting that MSC treatment might not be a favorable therapeutic option. However, as no specific surface markers have been described to conclusively measure the number of T_H1, T_H2- and T_H17-cells, we should be careful when interpreting the alterations in cytokine levels.

Finally, we explored the number of neutrophils, the levels of which are slightly decreased in the blood upon CMPC-treatment. As the main function of neutrophils in myocarditis is thought to be the clearing of debris and the phagocytosis of antibody-covered cardiomyocytes, a reduction in neutrophil levels could reflect a lower inflammatory burden. Interestingly, this lower amount of circulating neutrophils is reflected in the lower levels of G-CSF, a cytokine involved in neutrophil activation[25].

Limitations

This study had various limitations, which could have affected both the results and the calculated significance of outcomes. The number of animals per group was low, and in certain measurements individual variations hampered analysis. Additionally, the end point of three weeks might have been too early to show a definitive switch towards resolution of inflammation in this model. Also, only one dose of progenitor cells was injected in the circulation. It could very well be that ongoing inflammation needs multiple doses, or perhaps intra-splenic injection is preferable to directly target the immune reservoir.

Lastly, due to the lack of markers for CMPC in mice, we were forced to use hu-

man stem cells in immune-compromised mice, which could have influenced the inflammatory status. Despite these shortcomings, however, we have analyzed the data critically and found several effects that could be attributed to the intervention with progenitor cells.

In conclusion, we have shown subtle changes in myocarditis progression in response to intervention with mesenchymal stem cells and cardiomyocyte progenitor cells. The latter one seems specifically linked to several beneficial effects. Mice treated with CMPC maintained a slightly better cardiac function, reduce T_H1 formation and circulating neutrophils, and might experience delayed B-cell maturation. Numbers of regulatory T-cells are similarly increased by MSC and CMPC, yet the formation of T_H17-cells appears boosted by MSC. However, a more long-term study with larger groups of animals will be needed to confirm these results and to determine conclusively whether cellular therapy is a serious option to treat myocarditis during chronic phases.

Acknowledgements

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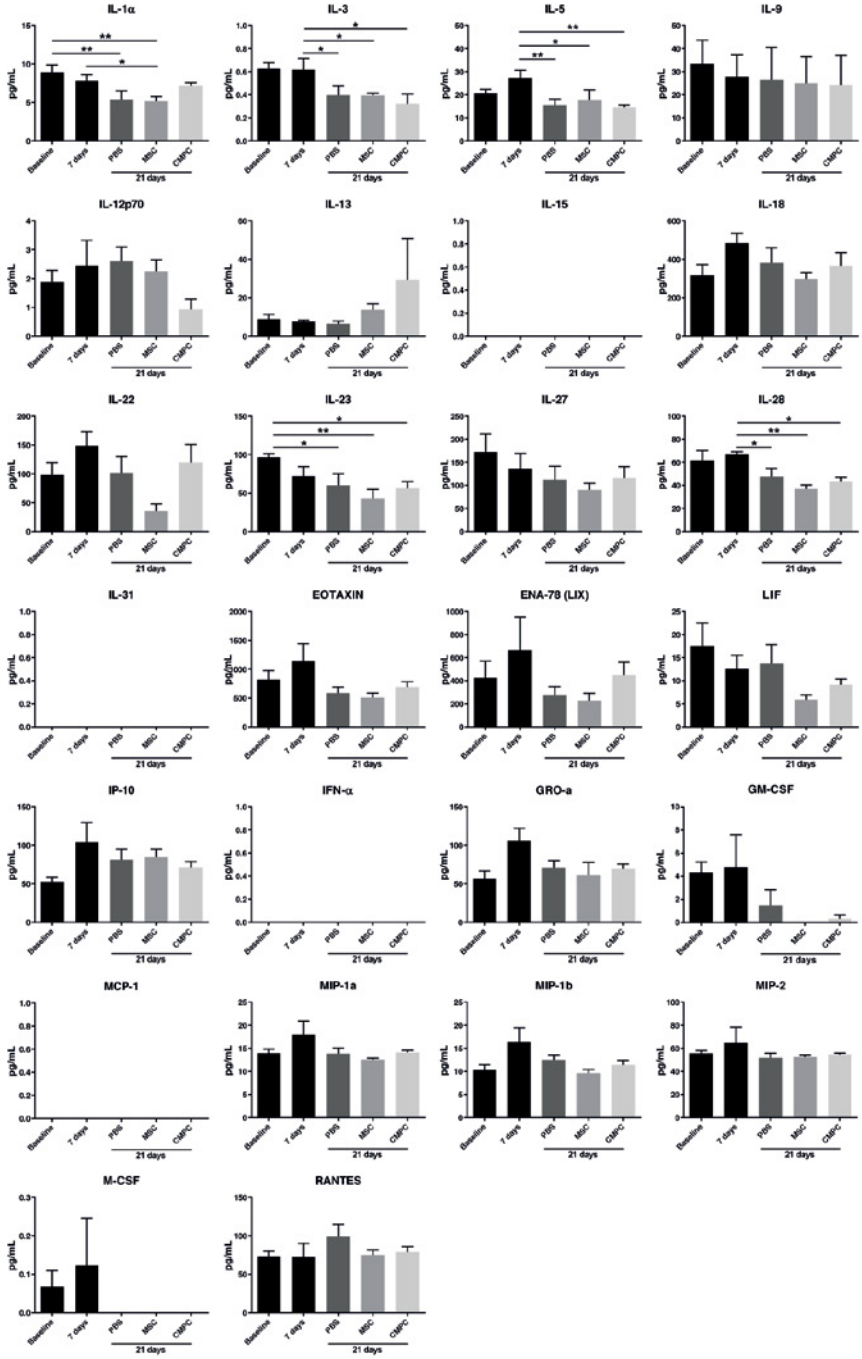
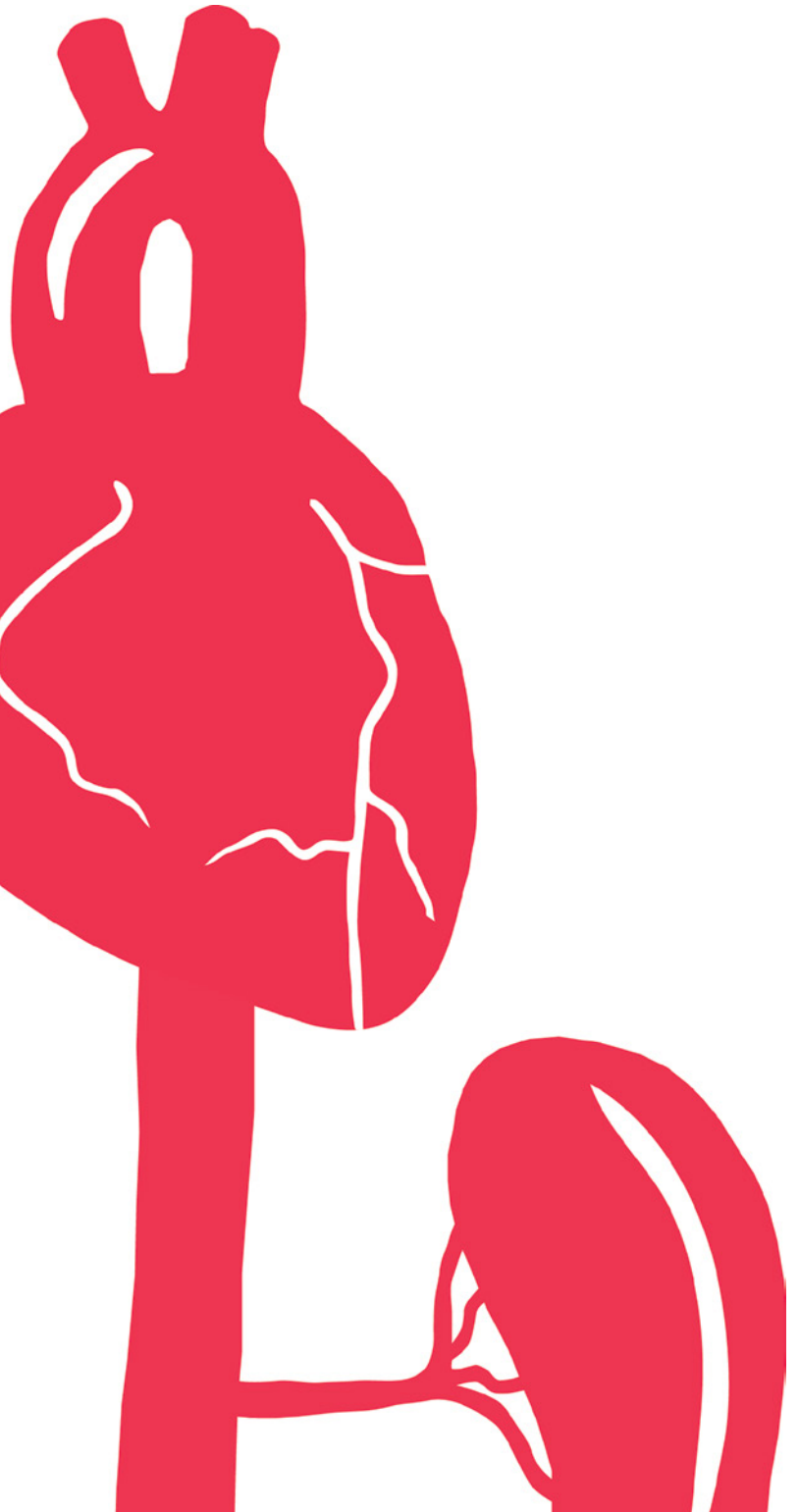
Supplement



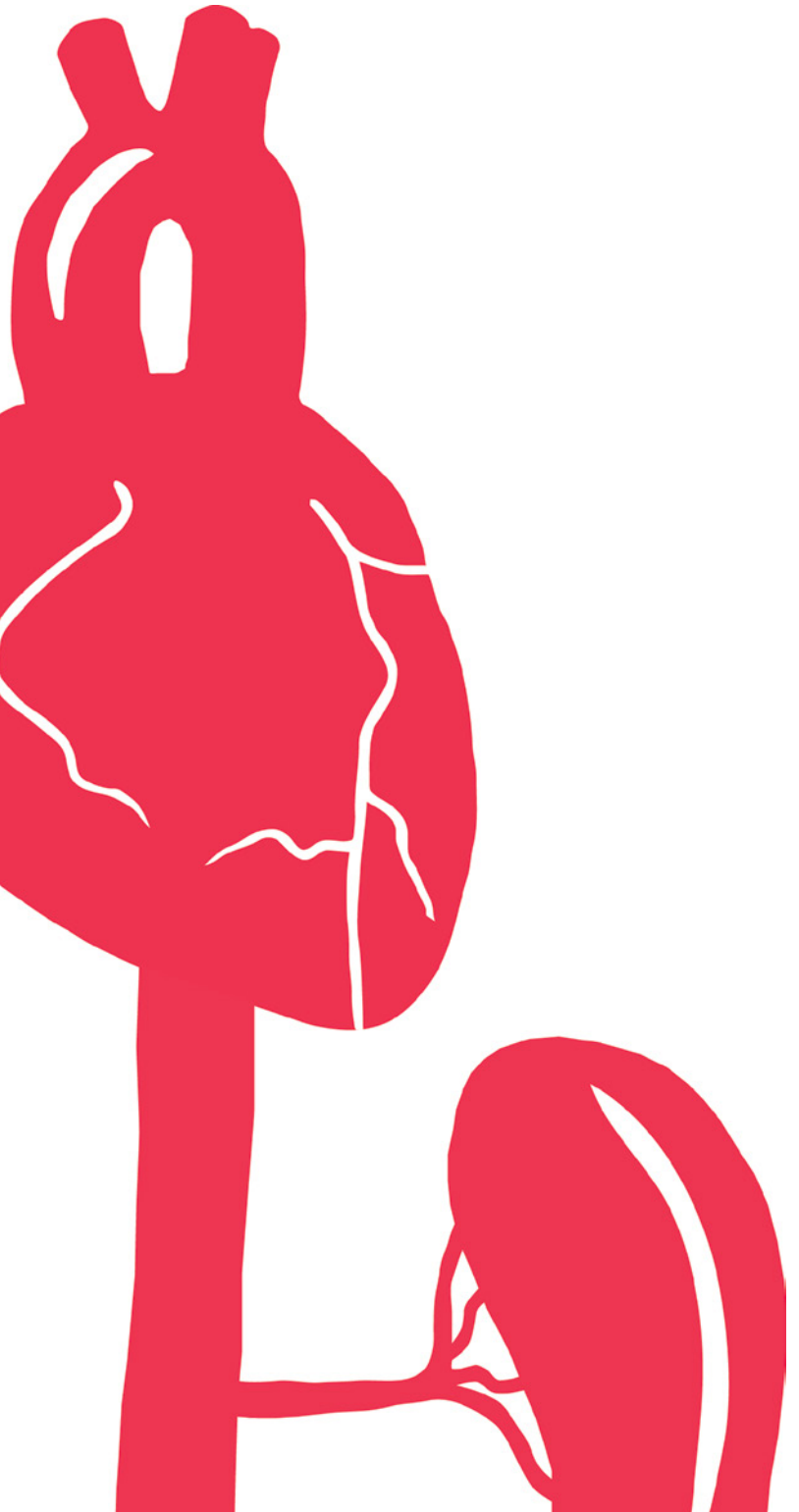
Suppl. Figure 6.1 – Cytokines in myocarditis after treatment with progenitor cells

** p<0.05, ** p<0.01*



Part III

Inflammation and Stem Cell Therapy



Circadian Dependence of the Immune Response to Myocardial Infarction

7

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Abstract

Rationale

Circadian rhythms govern many physiological processes, including immune responses. Whether these immunological oscillations affect the damage occurring during ischemic injury, such as myocardial infarction (MI), remains unclear.

Objective

We aim to investigate if circadian rhythms are present in inflammatory responses after MI damage.

Methods & Results

BALB/c mice were operated at Zeitgeber Time (ZT) 2, 8, 14 and 20. Three hours after MI, animals were terminated and blood and hearts were collected to assess immunological status and damage. Circadian patterns in leukocyte number and neutrophil tissue invasion are found.

Conclusion

Circadian patterns in immune cell circulation, cyto- and chemokine release and adhesion molecule expression are associated with increased cardiac extravasation upon damage at ZT14. This is also reflected in damage markers.

Introduction

Living organisms have evolved to efficiently interact with their surrounding environment. In particular the time of day is a critical factor that dictates the function of organisms in nature and for which specific-diurnal physiological processes are controlled by endogenous circadian rhythms[1].

These internal biological "clocks" help anticipate daily changes in the environment (e.g temperature fluctuation), but also prepare the body for behavior such as prolonged periods of activity and rest. This allows the organism to use energy supplies more efficiently on processes that are crucial during specific time periods, while suppressing others required at the opposing end of the circadian cycle. Nonetheless, these cyclical perturbations to physiology also have an effect on the ability of the body to handle stress[2,3].

In this regard, the incidence of cardiovascular related events is dependent on circadian rhythmicity, with the highest prevalence of myocardial infarction (MI)[4], sudden cardiac death[5], and arrhythmias[6] during the rest-to-activity transition, which for humans falls in the morning hours. This morning predisposition is also seen in the outcome of MI. In both mouse[7] and human studies[8,9], the damage caused by an ischemic insult is greater during the sleep-to-wake transition period, where larger infarct size and higher levels of creatine kinase are observed.

Although studies performed in cardiomyocyte specific clock-mutant mice (CCM mice; with a defective molecular circadian clock in cardiomyocytes) have suggested that cardiomyocytes are more vulnerable to stress during this transition period[7], the exact mechanisms underpinning these findings remain unclear.

One unexplored aspect in this phenomenon is the immune system, which plays a major role in the pathophysiology of heart failure. After a myocardial infarction (MI), immune cells are attracted to the site of injury in order to clear dead cells and debris[10,11]. This process stimulates the healing of tissue after injury. Although necessary, this immune response is known to "over-react", leading to additional cardiac damage[10]. Especially so called "early-responders" such as neutrophils can get hyper-reactive during both the ischemia and reperfusion phase and produce many factors that enhance the myocardial injury[10,12,13].

Interestingly, recent studies have revealed that the immune system is in part regulated by circadian oscillations. The presence of hematopoietic cells in the blood exhibits a cyclical pattern, with a peak in the circulation during the rest phase and decrease during the subsequent active phase. The oscillation is to some extent regulated by the diurnal expression of CXCL12[14], a major retention factor in the bone marrow, which sharply decreases in the beginning of the rest phase and coincides with the reduction in CXC-chemokine receptors on immune cells and reduced expression of adhesion molecules on the endothelial wall.

During the active phase these processes are reversed, which leads to lower hematopoietic cell content in blood and migration towards organs, such as the bone marrow and skeletal muscles[15]. Despite these profound diurnal changes in the immune system, the implication on immune cell recruitment to the heart after myocardial infarction remains unexplored.

In the present study, we aimed to characterize the circadian dependence of the immune response to myocardial infarction. By using the acute setting of cardiac injury (three hours after MI), we mirror the timeframe in which patients arrive in the hospital. This way we aim to provide insights in the potential relation between circadian rhythms and acute inflammatory processes, leading to increased understanding of the effect of time on (inflammatory) damage after MI.

Material & Methods

Animal experiments

All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, with prior approval by the Animal Ethical Experimentation Committee, Utrecht University. Male BALB/c mice (Jackson) aged 10-12 weeks mice were housed under controlled conditions in a 12 hours light/12 hours dark cycle (lights on at ZT0, lights off at ZT12). Water and food were provided ad libitum. Mice were anesthetized (i.p.; fentanyl 0.05 mg/kg; midazolam 5 mg/kg; medetomidine 0.5 mg/kg) and myocardial infarction (MI) was induced by ligation of the left coronary artery, as described previously[16].

Before any incision was made, the adequacy of anesthesia was monitored by testing rear foot reflexes. Continual observation of respiratory pattern, rectal temperature, and responsiveness to manipulations was carried out throughout the procedure. Baseline blood samples were collected by tail-clip prior to the start of surgery. After termination (3 hours after surgery) of the mice, blood and heart were collected. Blood was collected in EDTA tubes and used for flow-cytometric analysis. Serum was separated from the blood and stored at -80°C. Half of the heart was inserted in paraffin for staining, the other half was snap frozen and stored at -80°C for further analysis. N=5-6 per group.

Flow cytometry

Blood cells were incubated with the appropriate combination and amount of fluorochrome-conjugated antibodies for 30 minutes at room temperature. After incubation, samples were washed with PBS containing 5% FBS, after which Optilyse C (Beckman Coulter A11895) was added to lyse the erythrocytes and fix the white blood cells. Antibodies against the following markers were used: CD3e-PE

(Clone 145-2C11; 12-0031-82), CD8a-APC-eFluor780 (Clone: 53-6.7; 47-0081-82), CD11b-AlexaFluor488 (Clone: M1/70; 53-0112-82), CD19-eFluor 450 (Clone: 1D3; 48-0193-82) and F4/80-PE-Cy7 (Clone: BM8; 25-4801-82) purchased from eBioscience. From BD Bioscience CD4-PerCP (Clone RM4-5; 553052) and Ly6G-APC (Clone 1A8; 560599) were used. The Gallios Flow Cytometer (Beckman Coulter) was used to measure cell fluorescence and all analyses were performed with Kaluza Analysis Software (Beckman Coulter, v1.3).

Serum measurements

High sensitive Troponin I was measured in the collected serum using a clinical chemistry analyzer (AU5811, Beckman Coulter). The serum levels of inflammatory cytokines and chemokines were determined using a 36-multiplex panel (eBioscience, EPX360-26092-01), measured with a Luminex-200 instrument (Bio-Plex 200). The luminex assay was performed according to manufacturer's protocol.

Histological analysis

Upon termination, hearts were excised and fixated in 4% formaldehyde and embedded in paraffin. Paraffin sections were stained for Ly6G (for neutrophils; rat anti-mouse Ly-6G 1:100, Abcam), MAC-3 (for macrophages; rat anti-mouse MAC-3 1:30, BD Pharmingen), CD3 (for T-cells; rat anti-mouse CD3 1:100). Before staining, sections were deparaffinized and antigen retrieval was performed by boiling in citrate buffer (MAC-3, Ly6G) or in pepsin buffer (CD^+). After incubation with alkaline phosphatase conjugated secondary antibody, staining was visualized with Liquid Permanent Red substrate kit following the manufacturer's instructions (DAKO). All sections were counterstained with Mayer's hematoxylin stain.

RNA isolation and real-time PCR

Total RNA and protein were isolated from snap frozen heart sections using 1 ml TripureTM Isolation Reagent (Roche) according to the manufacturer's protocol. After DNase treatment, 500 ng total RNA was used for cDNA synthesis using the iScriptTM cDNA synthesis kit (Bio-Rad). Amplification was performed using 10 µl iQTM SYBR Green supermix and 10 µl cDNA.

CXCL1	forward: 5'-ATGAGCTGCGCTGTCAGTGC-3'
	reverse: 5'-CACCAGACGGTGCCATCAGA-3'
CXCL2	forward: 5'-GCGTCACACTCAAGCTCTG-3'
	reverse: 5'-GCGCTGTCAATGCCTGAAGA-3'
ICAM-1	forward: 5'-CAGTGAGGAGGTGAAT GTATAAG-3'
	reverse: 5'-GATGTGGAGGAGCAGAGAAC- 3'

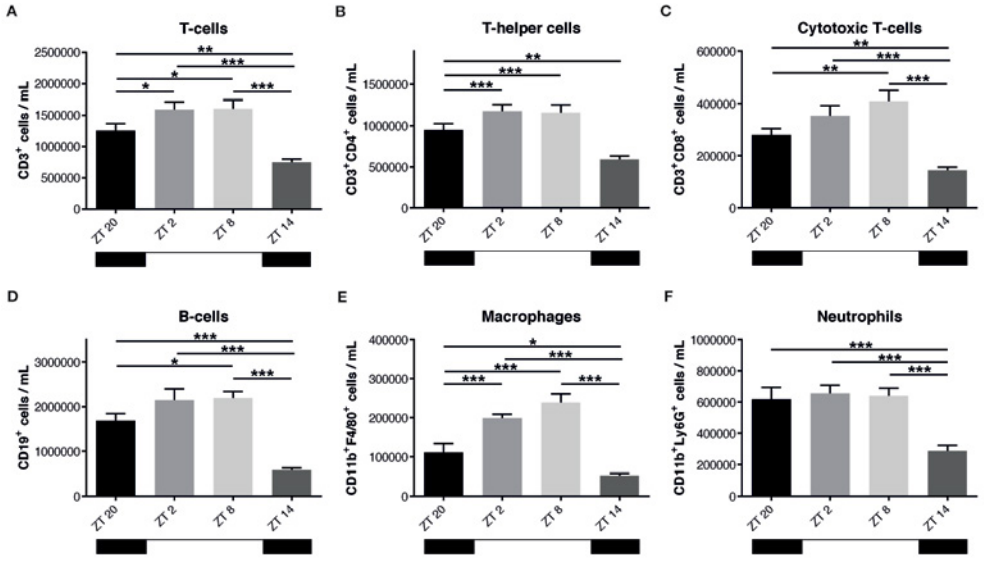

Figure 7.1 – Baseline leukocyte count in the blood

T-cells (**A**), further subdivided in helper T-cells (T_H-cells) (**B**) and cytotoxic T-cells (T_C-cells) (**C**) showed an oscillating pattern, reaching the highest points at ZT2-8 (T_H-cells) and ZT8 (T_C-cells). B-cells also had a clear cyclical pattern, reaching highest circulatory levels at ZT2-8 (**D**). Macrophages (**E**) showed a pattern similar to B- and T-cells. Neutrophils had stable high levels, which dropped at ZT14 (**F**). ** p<0.05, ** p<0.01, *** p<0.001*

VCAM-1	forward: 5'-CACCCTCACCTTAATTGCTATG-3'
	reverse: 5'-CGTCAGAACAACCGAATCC-3'
p0	forward: 5'-GGACCCGAGAAGACCTCCTT-3'
	reverse: 5'-GCACATCACTCAGAATTTCAATGG-3'

Statistical analysis

All statistical analysis were made in SPSS statistics v20 (IBM, Armonk, NY). After verifying normal distribution, the groups were compared using an ANOVA analysis with an LSD post-hoc correction. 24-hour rhythmicity was analyzed using Cosinor analysis. A p-value < 0.05 was considered significant.

Results

Steady-state oscillations of leukocytes in the blood

Prior to the operations, we collected blood from the mice to investigate the existence of circadian circulation patterns at baseline. We observed clear oscillation

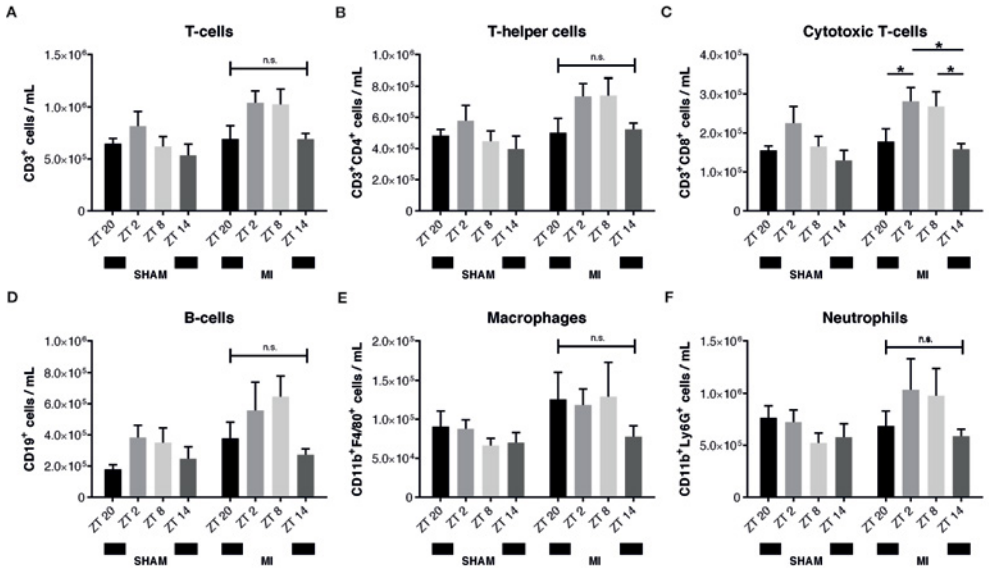

Figure 7.2 – Leukocyte numbers in blood after operation

Amount of circulating T-cells (**A**), both T_H-cells (**B**) and T_C-cells (**C**), increased after SHAM and MI operation. MI-animals reached highest levels of cells. In T_H-cells, the oscillating patterns were no longer significant (**B**), while in T_C-cells (**C**) this circadian pattern was maintained. Circulatory B-cells (were) increased in number as well, more pronounced with induction of MI, while losing their significant circadian pattern (**D**). Macrophages showed increased numbers after MI at ZT20-8, while experiencing a drop at ZT14 (**E**). Similarly, neutrophils have increased circulatory numbers, with the lowest values at ZT14-20 (**F**). ** p<0.05, n.s. non-significant*

patterns in all circulating leukocytes. T-cells, further subdivided in the T-helper cells (T_H-cells) and cytotoxic T-cells (T_C-cells) had the lowest levels at ZT14 (*figure 7.1A-C*). T_H-cells had their highest point at ZT2-ZT8 (*figure 7.1C*), while T_C-cells peaked at ZT8 (*figure 7.1C*). The number of circulating B-cells was also lowest at ZT14, while their peak ranged from ZT2-ZT8 (*figure 7.1D*). Macrophages showed a clear pattern, slowly increasing from ZT14 onwards until they peaked at ZT8 (*figure 7.1E*). Neutrophils, on the contrary, showed relatively stable levels throughout ZT20-ZT8, after which the number of circulating neutrophils dropped at ZT14 (*figure 7.1F*).

Leukocytes in the blood after myocardial infarction

Three hours after induction of myocardial infarction (or sham) the mice were terminated and their blood was collected. We again established the patterns in the circulating cells at different time points. At all time-points, the animals that suffered from myocardial infarction showed higher levels of circulating leukocytes than the sham-operated group (*figure 7.2A-F*). The number of circulating T-cells increased over 4-fold (*figure 7.2A*), with an especially strong increase in the number of cytotoxic T-cells (*figure 7.2C*). T_H-cells also showed clear increase, although the

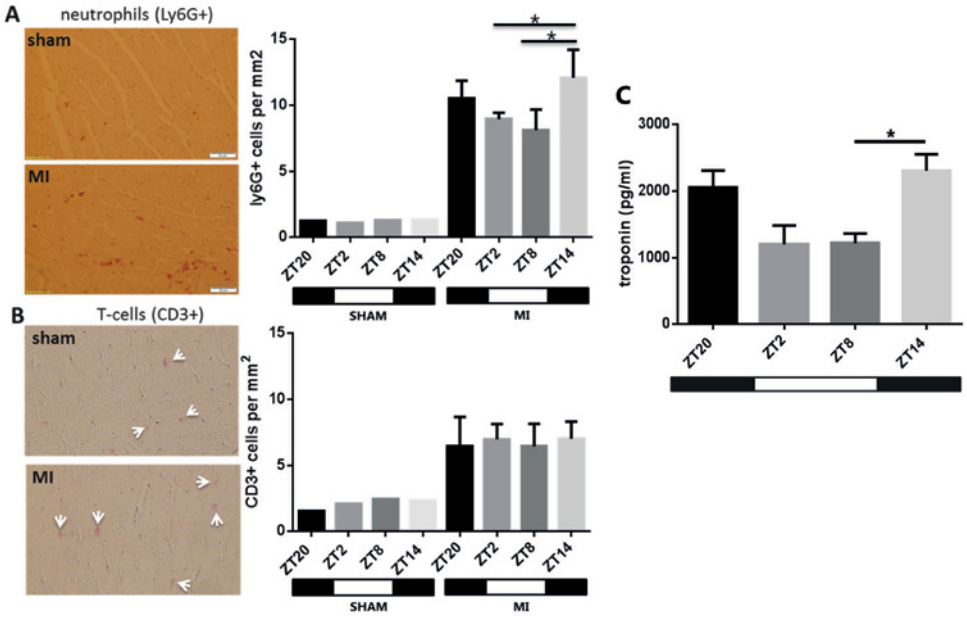

Figure 7.3 – Immune cell infiltration and heart damage 3 hours after MI

Neutrophil (Ly6G$^+$) infiltration into the myocardium (**A**) was dependent on the time of day. Higher neutrophil presence was observed after MI at ZT14 compared to when injury occurred during the rest-phase (ZT2 and ZT8). Although T-cells did respond to the myocardial injury compared to sham, their infiltration (**B**) did not exhibit any circadian dependence. Troponin-I levels (**C**) in the blood serum samples were twice as high during the active phase as compared to the serum samples taken from mice that received a MI during the rest-phase.
** $p<0.05$*

circadian pattern stayed similar (*figure 7.2B*).

The obvious circadian patterns observed in T-cells before was less pronounced after MI, and was therefore no longer significant, except in the case of cytotoxic T-cells whose peak shifted to ZT2 (*figure 7.2C*). Circulating B-cells also increased upon damage and their highest level was reached with a myocardial infarction at ZT8, although a lot of variation between animals was present (*figure 7.2D*).

In macrophages, the clear oscillatory/circadian pattern visible at baseline was completely disrupted (*figure 7.2E*). Stable high levels of macrophages were now seen from ZT20 through ZT8. Still, the lowest circulating numbers of macrophages was still observed at ZT14. In neutrophils the pattern visible at baseline had also been disrupted (*figure 7.2F*). Where at baseline stable, high levels of neutrophils were observed in circulation from ZT20-ZT8, we now found a lower amount of neutrophils at ZT20, leading to a diphasic pattern of low numbers of neutrophils from ZT14-20, and elevated neutrophils from ZT2-ZT8.

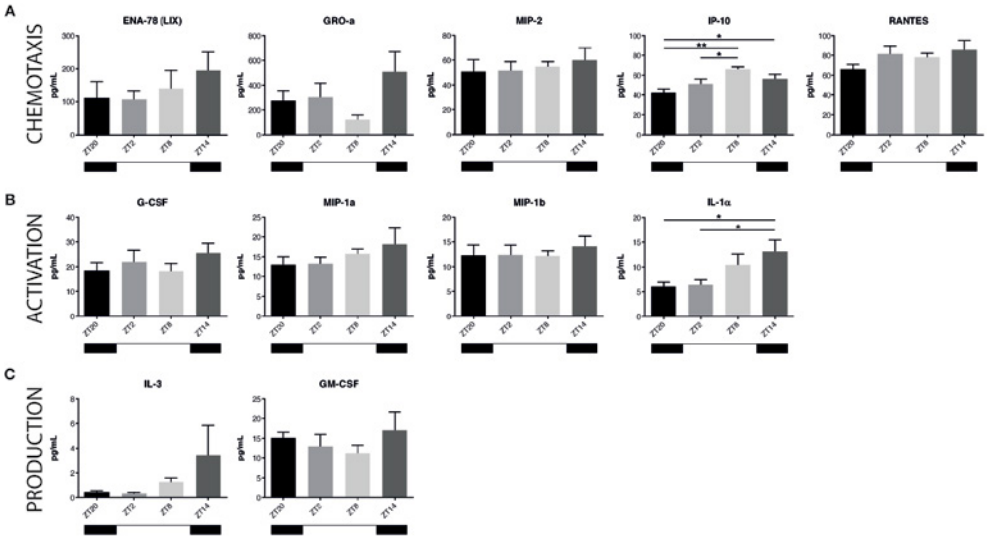

Figure 7.4 – Cyto- and chemokines in the blood after MI

Most chemokines involved in chemotaxis of neutrophils (ENA-78, GRO-a, MIP-2 and RANTES) reached the highest levels at ZT14, with the exception of IP-10, which peaked early at ZT8 (**A**). Cytokines involved in neutrophil activation (G-CSF, MIP-1a, MIP-1b and IL-1α) all peaked at ZT14 (**B**). Cytokines that play a role in the stimulation of neutrophil production and release (IL-3 and GM-CSF) peaked at ZT14 (**C**). ** p<0.05, ** p<0.01*

Leukocyte invasion into the myocardium after MI

Next, we investigated whether the observed circadian oscillations of immune cells in the blood had an effect on the ability of leukocytes to infiltrate into myocardial tissue. We examined the heart sections for several immune cell types that have been implicated in the acute response to myocardial injury; macrophages ($MAC3^+$), neutrophils ($Ly6G^+$), and T-cells ($CD3^+$).

In both sham- and MI-operated hearts, we were unable to detect the presence of MAC3-positive cells (data not shown), indicating that macrophages do not play a key role in this early phase of injury. However, both neutrophils and T-cells were found in the myocardium under normal physiological conditions, as shown by the presence of $CD3^+$ and $Ly6G^+$ cells in sham-operated hearts (*figure 7.3A and B*). Furthermore, three hours after MI both cells types showed a clear response to the injury (~10-fold increase in $Ly6G^+$ cells/ ~2-fold increase in $CD3^+$ compared to sham) with an observed accumulation in the left ventricle in proximity to the site of coronary ligation.

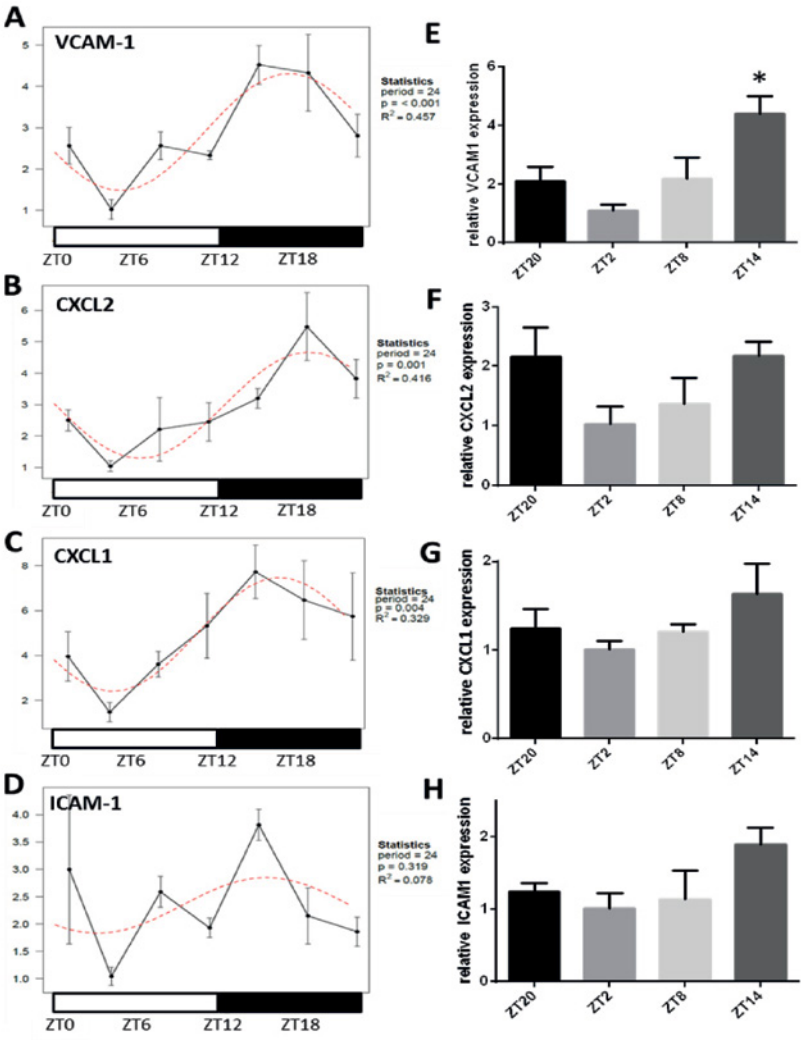

Figure 7.5 – Gene expression in the heart

In healthy mice, the expression of VCAM1 (**A**), CXCL2 (**B**), and CXCL1 (**C**) followed strict circadian oscillation with higher levels present during the active phase. Similar oscillation was not detected in the expression of ICAM1 (**D**), however we did observe a peak expression of ICAM1 during the early active phase. In the RNA isolated from MI-hearts, a clear increase in VCAM1 (**E**) expression was noticed at ZT14 compared to hearts operated during the early rest-phase (ZT2). Although non-significant, similar trends are observed with CXCL2 (**F**), CXCL1 (**G**), and ICAM1 (**H**). ** p<0.05*

Interestingly, the neutrophil response was dependent upon the time of day, with the highest myocardial presence of $Ly6G^+$ cells observed when the MI was performed at ZT14, while the lowest neutrophil infiltration was seen at ZT2 and ZT8. The infiltration of $CD3^+$ cells remained constant over the four time points and did not follow a circadian pattern. The peak in neutrophil infiltration was correlated to highest level of troponin I in the serum of the operated mice, indicating that greater myocardial damage was found during the active period of the day (*figure 7.3C*).

Chemokine oscillation in the blood

As shown above, neutrophils are clearly the first responders early after myocardial infarction, and their tissue infiltration showed a remarkable circadian dependence. Therefore, we decided to examine the neutrophilic axis further, by measuring the levels of neutrophil-related cyto- and chemokines in the serum at the different time points. We roughly divided the cytokines in three groups based on their main effect on neutrophils, although several, including IP-10, can influence more than one cell type. The cytokines involved in neutrophil chemotaxis were all highest at ZT14, except for IP-10 which peaked already at ZT8 (*figure 7.4A*). A similar pattern was visible in cytokines involved in neutrophil activation or degranulation, which all reached their highest values at ZT14, after which they dropped to their lowest level at ZT20 (*figure 7.4B*). Likewise, the cytokines stimulating the differentiation of new neutrophils, IL-3 and GM-CSF, also showed the highest serum levels at ZT14, before decreasing rapidly in concentration at ZT20.

Chemokine and adhesion molecule expression in the heart

The attraction and uptake of leukocytes from the blood into the myocardial tissue is orchestrated by the expression of several chemotactic proteins and adhesion molecules. In order to better understand the expression of these factors under normal physiological conditions, we isolated RNA from hearts of healthy mice at 7 time points across day and night. Gene expression in these samples clearly showed that CXCL1, CXCL2, and VCAM-1 followed a circadian oscillatory pattern, with the highest expression achieved during the active period, which dropped in the subsequent rest-phase of the circadian cycle (*figure 7.5A-C*).

Although ICAM-1 expression did not follow a clear circadian oscillation, we noticed the peak expression of ICAM-1 to be in the early-active phase (*figure 7.5D*). Similarly, gene expression in the hearts of operated mice (both sham and MI) showed a similar trend towards highest presence in ZT14 (*figure 7.5E-H*). In particular, VCAM-1 exhibited robust oscillation with a 4-fold higher expression in the early active phase. The data suggests that the recruitment of neutrophils in the first hours after MI might be mediated by circadian oscillations of chemokines and adhesion molecules.

Discussion

The time of the day can determine the clinical outcome for patients that suffer from a myocardial infarction. Although previous studies investigating this phenomenon have focused on the underlying cardiomyocytes, the immune system plays an important role in the pathophysiology of heart failure and has shown to be robustly controlled by circadian rhythms. Therefore, in the present study we aimed to characterize the circadian oscillation of the immune cells and their response to myocardial damage.

From the blood samples collected prior to the start of the experiment, we observed strong oscillations of leukocytes that followed a strict rest-to-activity pattern. High levels of circulating immune cells were present during periods of rest, while a strong reduction in these levels was observed after the start of the active period. After operation, these oscillations were not as clearly defined, especially in the sham operated animals where the circadian rhythms were vastly suppressed.

The suppression is likely due to the invasive thoracotomy operation, which in itself is likely to have created enough damage to activate the immune system and potentially "mask" the normal physiological processes. Nonetheless, in mice that underwent LAD ligation there was a clear additional response to the myocardial damage as shown by the higher number of immune cells (B-cells, T-cells, and phagocytes) in the blood compared to sham-operated animals. Furthermore, the immune response in the MI-mice followed a circadian oscillation similar to that observed in the baseline data, with higher leukocyte numbers during the rest-phase and a sharp decrease upon entering the active period.

From our histological analysis, it is clear that within three hours after MI the vast majority of immune cells found in the heart were neutrophils. Surprisingly, the infiltration rate of the neutrophils to the myocardium followed the opposite diurnal rhythm than that observed in the blood. We observed a peak infiltration rate when MI occurred at ZT14, which is concurrent with a major decrease in leukocyte count in the blood. Conversely, lower neutrophil presence in the myocardium was observed when injury arose during the rest-phase (ZT2 and ZT8), during which time high levels of neutrophils are present in the circulation. Interestingly, our present finding of peak neutrophil infiltration during the early active period correlates with the other studies that have shown the heart to be most susceptible to myocardial injury during this phase of the circadian cycle[8,9].

In this regard, we measured 2-fold higher troponin levels in serum when the ligation was placed during the active period of the day, demonstrating a strong correlation between neutrophil presence and the extent of heart injury. Future neutrophil depletion studies should shed more light on the precise role of these immune cells in driving the observed circadian dependent-myocardial damage.

In order to better understand the discrepancies between neutrophil levels in the blood and tissue, we analyzed various parameters that could be involved in the enhanced recruitment of the neutrophil from the bloodstream during the early-active phase. In this regard, the chemokine levels in the blood involved in neutrophil chemotaxis and activation reached the highest levels in mice operated on at ZT14. Furthermore, in the heart itself the expression of chemokines and adhesion molecules showed a strong up-regulation in the early activity hours (peaking on average around ZT15).

The increased expression would allow immune cells not only to respond to but also infiltrate into the damaged tissue at higher rates during this part of the circadian cycle. In this respect, it seems likely that even though higher levels of neutrophils are present in the blood during rest, they are unable to leave the circulation and extravasate into the myocardial tissue. During the rest-to-activity transition, the high presence of chemokines and adhesion molecules can lead the neutrophils, although less abundant in the blood, to more efficiently infiltrate into the damaged tissue.

Implication for the clinic

The adverse inflammatory response after MI can partially be attributed to biological day/night rhythms that influence the ability of the heart to attract the immune cells to the site of injury and potentially exacerbate the complication of the disease. So far, studies that performed general immune suppressive therapy during or after MI have not shown any beneficial effects[17]. On the contrary, general immunosuppression using NSAIDs appeared to increase the incidence of cardiac rupture[17,18]. However, these studies included patients with MI at all times, leveling out potential benefits in a time-dependent subgroup[17].

Our data clearly suggests that neutrophil extravasation into the damaged myocardium is strongest during the sleep to wake transition period, making patients immunologically vulnerable at that time. It is exactly at those time points in patients that the administration of drugs that reduce or prevent extravasation of neutrophils should be considered[19-21].

Acknowledgements

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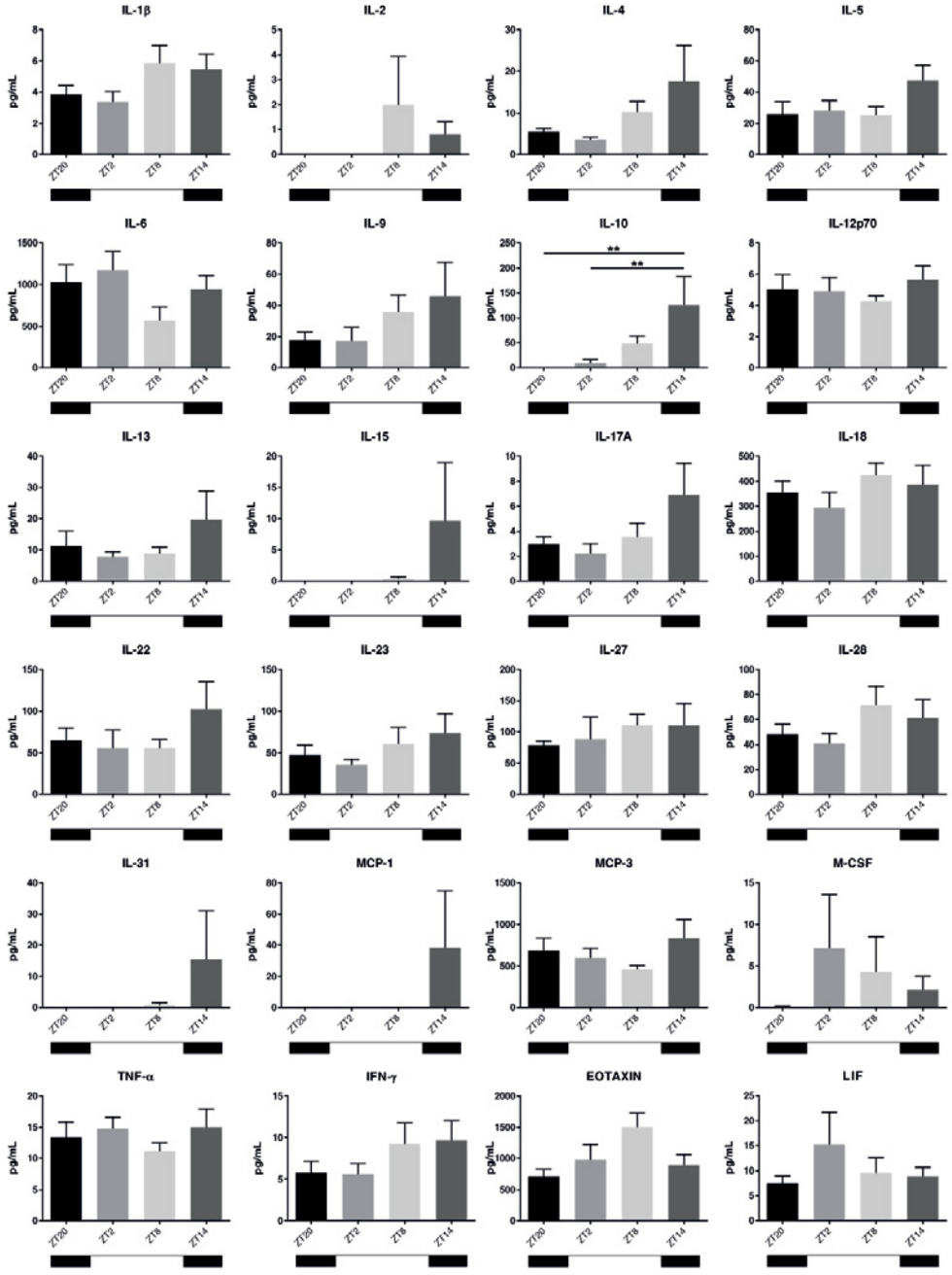
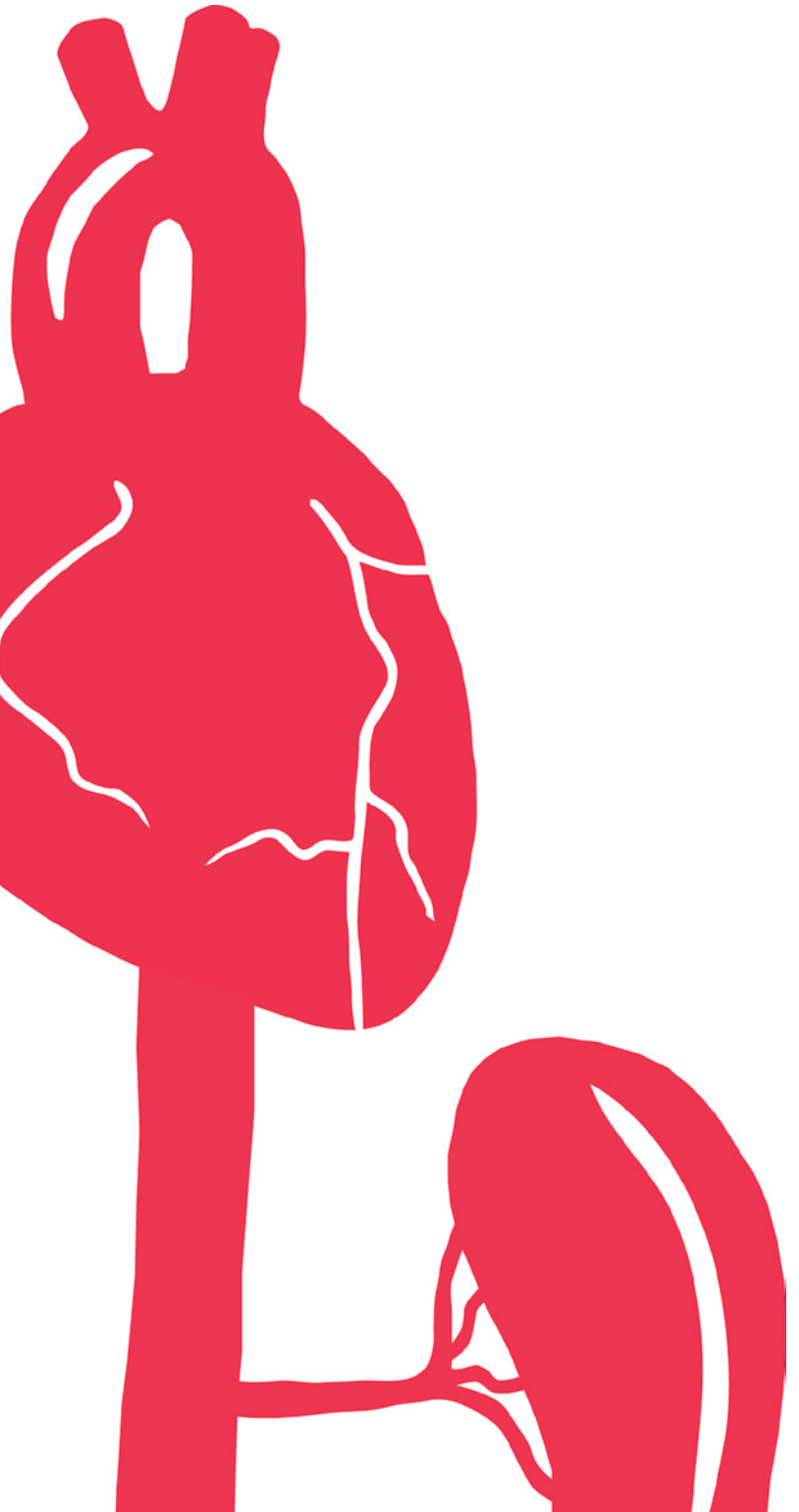
Supplement



Suppl. Figure 7.1 – Diurnal cytokine variations after MI

*** p<0.01*



Rapid Immune Activation After Human Stem Cell Injection in BALB/C and NOD-SCID Mice

8

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Introduction

During a myocardial infarction, millions of cardiomyocytes die[1]. Although the heart has a small reservoir of dividing progenitor cells, the sheer volume of lost tissue is too large for endogenous repair and the demand for novel therapies is high. In the past decades, different types of stem or progenitor cells have been investigated for their regenerative capacity[2]. After successful isolation and characterization of progenitor cells, animal studies were conducted to investigate their reparative potential in animal models of myocardial infarction[3]. Interestingly, directly after the first promising results in animal studies, clinical trials were started[4].

As observed and reviewed many times, the functional outcome of these clinical trials was marginal at most[4]. Moreover, as was observed for many drugs[5,6], a poor translation of laboratory work to clinical practice applies to cardiac cell therapy as well. The characterization of a progenitor cell population is time consuming and species differences in functional effects do exist. Additionally, even if the same progenitor cell can be found in multiple species, e.g. mesenchymal stem cells (MSC), the mechanisms of action can differ between species. For example, ample research initially indicated that MSC suppress T-cell proliferation using nitric oxide[7,8]. Later it turned out this pathway of immunomodulation was specific for murine MSC only[7,9].

These observations led many researchers to focus on human stem and progenitor cells, as these are the cells ultimately to be used in clinical stem cell therapy. However, this creates other hurdles by using human cells in animal models. One of the most obvious problems is the animal's immune system, which recognizes and eliminates everything that is recognized as being non-self. Initial reports claimed that many progenitor cells, including MSC, do not display HLA-antigens[10], and therefore had an immune privileged position. Yet, later publications showed the presence of HLA-antigens on the cell membranes of these progenitor cells[11-13]. Interestingly, researchers either continued to use human cells in animal disease models[14-16], switched to the use of immunodeficient mice[17,18] or administered immunosuppressive drugs[19,20]. However, in cardiac diseases the immune system plays a strong role in determining short-term damage (reperfusion injury) and long-term outcome[8].

Despite the known interactions between stem and progenitor cells and the immune system, it was hardly investigated if cross-species transplantation actually led to immune cell activation. In this short observational study, we injected mesenchymal stem cells (MSC)[21] and cardiomyocyte progenitor cells (CMPC)[22] into the myocardium of immunocompetent BALB/c mice and immunodeficient NOD-SCID (SCID) mice. We investigated the survival of the cells and the immune activation to determine if the immune system was activated by the cells in this cardiac application of cell therapy.

Material & Methods

Cell culture

Human fetal tissue was obtained by individual permission using standard informed consent procedures and prior approval of the ethics committee of the University Medical Center Utrecht, the Netherlands. This procedure is in accordance with the principles outlined in the Declaration of Helsinki for the use of human tissue or subjects. CMPC and MSC were isolated and characterized as previously described[22,23]. Cells were cultured in flasks coated with 0.1% gelatin (Sigma). CMPC were cultured in SP++, containing 1 part EGM-2 (Lonza CC-3156) and 3 parts M199 (Lonza BE12-119F) supplemented with 10% fetal bovine serum (FBS; Gibco), 1% Penicillin/Streptomycin (P/S; Lonza 17-602E) and 1% Non-essential Amino Acids (Lonza 13-114 MSC were cultured in MEM-alpha (Gibco) supplemented with 10% FBS, 1% P/S and 0.2mM L-ascorbic acid-2-phosphate (Sigma A4034).

Both cell types were transduced with pLV-CMV-luc-GFP, a lentiviral construct, to facilitate their identification *in vivo*[24]. After transduction, CMPC and MSC were passaged and cultured until 80% confluency before injection at passage 14 and 13, respectively.

Animals

All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, with prior approval by the Animal Ethical Experimentation Committee, Utrecht University, the Netherlands.

Intramyocardial cell injection

Male NOD.CB17-Prkdc^{scid} mice (Harlan Laboratories) and wildtype (WT) male BALB/cAnNcrl (Charles River), aged 10 weeks, were injected intramyocardially with either CMPC (n=3) or MSC (n=3). Intramyocardial injections were performed via a percutaneous transthoracic approach with echo guidance (Vevo 2100, VisualSonics). Mice were anesthetized with 4% isoflurane and anesthesia was maintained with 1.5% isoflurane during echocardiography. Mice were positioned in an adjusted parasternal long axis view and 0.5 million cells were injected with a 27 Gy needle in a volume of two times 5 µl.

Bioluminescent imaging (BLI)

To determine the retention of the injected CMPC-luc and MSC-luc *in vivo*, mice were imaged at 2 and 7 days after injection, as previously described[24]. In short, mice were injected intraperitoneally with Luciferase (Promega E1605) and emitted photons by CMPC-luc or MSC-luc were detected with a photon imager from Biospace Laboratory. Data was analyzed by PhotoVision software. Injections were

considered successful based on location (mid-thorax) and a threshold of BLI signal at day 2 (> 20.000 ph/s/cm^2/sr), as established with previously performed titrations[24]. The absolute BLI values in the individual mice were used as primary outcome parameters and were denoted as BLI Signal Units (BSU; ph/s/cm^2/sr).

Analyses of immunological response

Mice were terminated by exsanguination 7 days after intramyocardial injection of either CMPC or MSC. Blood was collected in EDTA tubes. After flow cytometric samples were taken, the blood was centrifuged at 2,000 *g* for 20 minutes and the serum was stored separately at -80 °C for cytokine analysis. After flushing with PBS, spleens were collected and strained through a 40 µm nylon mesh (Greiner Bio-One 542040), creating a single cell suspension and washed twice with 5% FBS. Blood cells and splenocytes were incubated for 30 minutes at room temperature with a cocktail of fluorochrome-conjugated antibodies, allowing differentiation between the different types of immune cells. After incubation, cells were washed and subsequently Optilyse (Beckman Coulter A11895) was added to fix the cells and remove any erythrocytes.

Antibodies against the following markers were used: CD8a-APC-eFluor780 (Clone: 53-6.7; 47-0081-82), CD11b-AlexaFluor488 (Clone: M1/70; 53-0112-82), CD19-eFluor 450 (Clone: 1D3; 48-0193-82) and CD25-APC (Clone: PC61.5; 17-0251-82) purchased from eBioscience. From BD Bioscience CD4-PerCP (Clone RM4-5; 553052), CD62L-PE-CF594 (Clone MEL-14; 562404), and Ly6C-PE-CF594 (Clone AL-21; 562728). Historical controls in BALB/c AnNcrl (Charles River) between 10-12 weeks of age were used for baseline. The Gallios Flow Cytometer (Beckman Coulter) was used to measure cell fluorescence and all analysis were performed with Kaluza Analysis Software (Beckman Coulter, v1.3).

Serum measurements

A 36-multiplex panel (eBioscience, EPX360-26092-01) was used to measure the concentration of 36 cyto- and chemokines in the isolated serum using a Luminex-200 instrument (Bio-Plex 200). The luminex assay was performed according to manufacturer's instructions.

Statistical analysis

Statistical analyses were performed by GraphPad Prism 6.0 software. Data are presented as mean ± SEM and were compared using the two-tailed Student's T-test. Flow cytometric data and cytokines were compared using one-way ANOVA with LSD post-hoc correction, after determining normal distribution. A value of $p<0.05$ was considered statically significant.

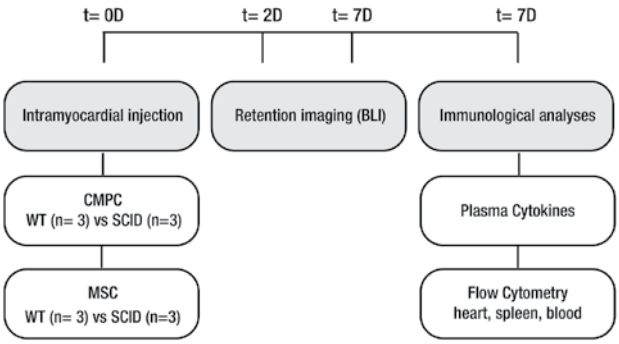

Figure 8.1 – Study protocol

Results

Injection of CMPC and MSC in WT mice resulted in an accelerated reduction of cell retention

To determine the course of human stem cell retention in immune competent and immune compromised mice, MSC-luc or CMPC-luc were injected intramyocardially in WT and SCID mice, respectively (*figure 8.1*).

In vitro analysis of the luciferase activity of CMPC-luc and MSC-luc showed a linear dose dependency for cell count and BLI signal (*figure 8.2A-B*). Consequently, higher BLI signals correspond to a higher cell retention. Two days after injection with CMPC-luc, cell retention appeared higher in SCID mice than in WT mice (1.2 ± 0.5 vs. 0.5 ± 0.2 BSU x 10^5, p=0.18). Likewise, injection with MSC-luc resulted in a higher signal in SCID mice compared to WT mice (5.4 ± 0.6 vs. 2.1 ± 1.0 BSU x 10^5, p=0.05) (*figure 8.2C-E*).

Although cell retention was decreased dramatically in both SCID and WT mice after 7 days, the difference in cell retention between SCID and WT was even more pronounced than at day 2. In CMPC-luc injected mice, cell retention decreased to 1.3 ± 0.3 BSU x 10^4 in SCID mice and to background values of 0.3 ± 0.02 BSU x 10^4 in WT mice, p=0.08. Also for MSC-luc injected mice, retention was significantly higher in SCID mice compared to WT mice (2.3 ± 0.5 vs. 0.4 ± 0.01 BSU x 10^4, p=0.05) (*figure 8.2F-H*). Notably, the overall BLI signal at day 2 was higher for MSC-luc injected mice than for CMPC-luc injected mice (~4-fold) (*figure 8.2C and F*).

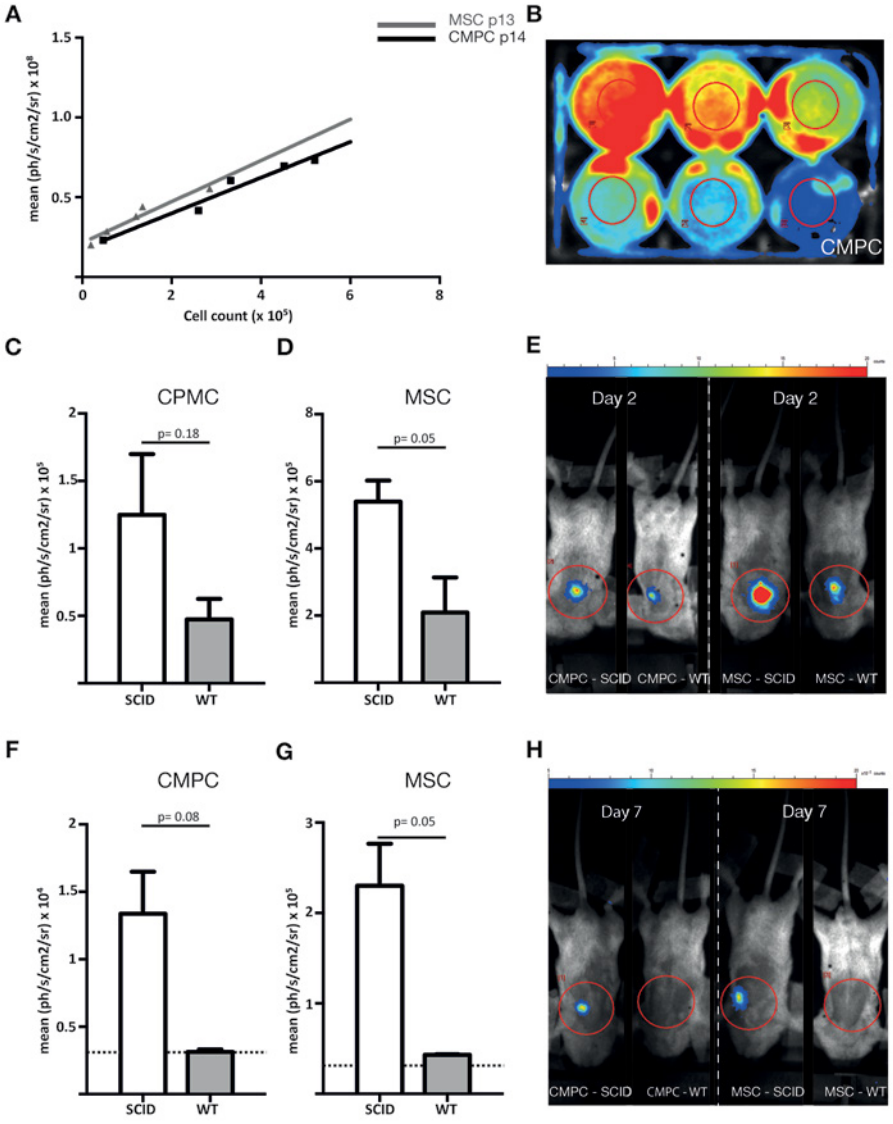

Figure 8.2 – Stem cell retention is affected by the immunological status of mice

(**A**) BLI signal of MSC-luc and CMPC-luc is linear proportional to cell count *in vitro*. Grey line represent MSC-luc. Black line represent CMPC-luc, as shown in (**B**). At day 2 (**C-E**) and day 7 after injection (**F-H**), BLI signals are lower in WT mice compared to SCID mice for both MSC-luc and CMPC luc.

Immune activation after cell injection

After the animals were terminated, we studied the blood and spleen for signs of systemic inflammation. The amount of T-helper cells in the blood, necessary for B-cell activation, remained constant in WT after injection with CMPC, while the WT animals receiving MSC had a significantly decreased level of T-helper cells (*figure*

8.3A). On the other hand, cytotoxic T-cells, capable of directly killing their target after activation, appeared to increase mildly in the CMPC treated WT, while levels in MSC-treated animals were comparable to historical baselines (*figure 8.3B*). The level of circulating B-cells showed a similar trend, with the CMPC-treated animals reaching slightly higher levels than MSC treated WT mice (*figure 8.*3C). As expected, the SCID mice had virtually no lymphocytes (*figure 8.3A-C*). However, this black and white difference was less obvious when examining the myeloid-derived immune cells. The neutrophils in WT mice were strongly increased, and again the CMPC-injected mice reached slightly higher levels (*figure 8.2D*). Interestingly, this slight elevation was also visible in CMPC-treated SCID mice although at a lower absolute number. With regard to monocytes, no significant differences were observed between WT and SCID in the circulating numbers (*figure 8.3E*).

Interestingly, the spleen showed the opposite trend from the blood. After intramyocardial injection of MSC, the spleen contained slightly higher amounts of T_H-cells (*figure 8.3F*) and T_C-cells (*figure 8.3G*). CMPC-treated animals showed hardly any variation in the level of T-cells compared to the historical baseline. The elevated $CD4^+$ immune cells in the spleens of SCID mice was surprising. However, as macrophages can also display CD4, this might consist largely of non-lymphocytic $CD4^+$ cells. In addition, the absolute number of B-cells in the spleen remained constant during the experiment.

Production of antibodies and inflammatory cytokines

In addition to measuring the leukocytes and splenocytes, we also collected serum to investigate the levels of antibodies and pro-inflammatory cytokines. IgM is the first antibody secreted after B-cell mature into plasma cells, and the levels observed here are comparable to our historical baseline (*figure 8.4A*). Meanwhile, the levels of the more mature IgG antibodies were strongly elevated, indicating a mature and ongoing immune response[25]. This elevation was noticeable along the whole range of IgG subtypes (*figure 8.4A*) in WT mice receiving either MSC or CMPC. Obviously, no antibody production was observed in SCID mice. The panel of general inflammatory cytokines showed an increase in IFN-γ in SCID mice, obtaining even higher levels than the WT mice (*figure 8.4B*). Although less pronounced, a similar trend was visible in TNF-α and IL-1β. IL-6 showed a slight elevation in the CMPC-treated WT mice. Cytokines associated with neutrophil chemotaxis (GRO-a) and activation (G-CSF) showed a marked increase in the WT group treated with CMPC, corresponding to the high levels of circulating neutrophils found in that group (*figure 8.4C*). Lastly, the cytokines corresponding to macrophage activation (IL-18) and differentiation (M-CSF) showed a significant increase in the SCID mice, regardless of which progenitor cell had been injected (*figure 8.4D*; *suppl. figure 8.1*).

Discussion

Small animal models are of great importance to study the mechanistic effects of human stem cell therapy to improve clinical efficacy. However, this necessitates clinically translatable animal models that resemble specific aspects of human disease[26]. As presented here, intramyocardial injection with either CMPC or MSC resulted in a fast decline in cell retention after the initial engraftment. Interestingly, this decline was more pronounced in WT mice then in SCID mice for both injected stem cell populations. At the same time, activation of the immune system was observed as measured by flow cytometry and plasma cytokines. Immune rejection occurred in both immune competent and incompetent mice, and for both MSC and CMPC.

For a long time MSC were considered to be immune-privileged cells, allowing allogeneic transplantation and even xenotransplantation[27]. This was initiated by publications claiming MSC to be MHC class I positive, protecting them from the innate immune system, while avoiding T-cell-mediated death by being class II negative[10]. Others have found, however, an inducible or constitutive expression of both types of MHC molecules on MSC[11-13] with the implication that transplanted cells can be recognized by the host immune system leading to graft rejection. This notion is supported by our results, which showed a clearance of transplanted cells and activation of the immune system on all levels. The most likely explanation for this is an expression of MHC-antigens on both cell types. Although an immunomodulatory capacity has been described for MSC and CMPC[8], this is most likely not strong enough to prevent the host-versus-graft-response triggered by xenogeneity.

As stated earlier, it is necessary to perform preclinical trials using the same cells that are used in the clinical setting, to avoid species-specific effects. In our study, a higher retention rate was observed in MSC injected mice than in CMPC injected mice. Since several studies showed a higher cell retention with increasing cell or vehicle size[28,29], it is possible that the larger cell size of MSC contributed to this initial difference[30]. Additionally, we did observe host responses, although several other studies suggest the immune privileged nature of stem cells[10]. Apparently, xenotransplantation from human to mouse is not optimal for either CMPC or MSC in the currently used mouse models. Therefore, (partly) immune deficient mice (NOD-SCID) were developed, which are now widely used for xenotransplantation studies[17,18,31]. Although we did observe a higher cell retention in SCID mice, even here an immune response was triggered by the transplanted human cells. Despite being immunodeficient on many levels, SCID mice do still have functional immune cells patrolling their tissues. Both phagocyte counts and cytokines indicate an innate immune response, although weak, is launched against the foreign cells. To overcome this problem, another opportunity is provided by the generation of humanized mouse models, which are generated by engraftment of human he-

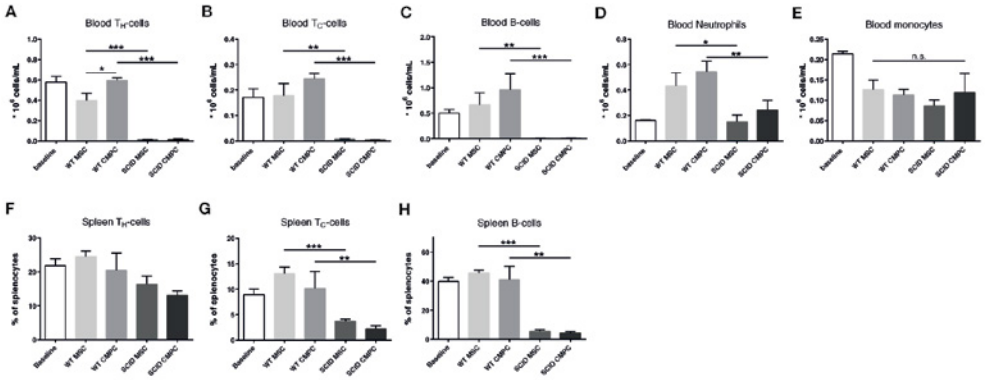

Figure 8.3 – Activation of inflammatory cells in WT mice upon stem cell transplantation

Number of T_H-cells (**A**), T_C-cells (**B**), B-cells (**C**), neutrophils (**D**) and monocytes (**E**) in the blood after injection of MSC or CMPC in Wildtype (WT) and SCID mice. All circulating immune cells are elevated upon progenitor cell injection, and highest in the CMPC-injected animals. SCIDs only upregulate the myeloid-derived cells. Percentage of splenocytes formed by T_H-cells (**F**), T_C-cells (**G**) and B-cells (**H**). ** p<0.05, ** p<0.01, *** p<0.001*

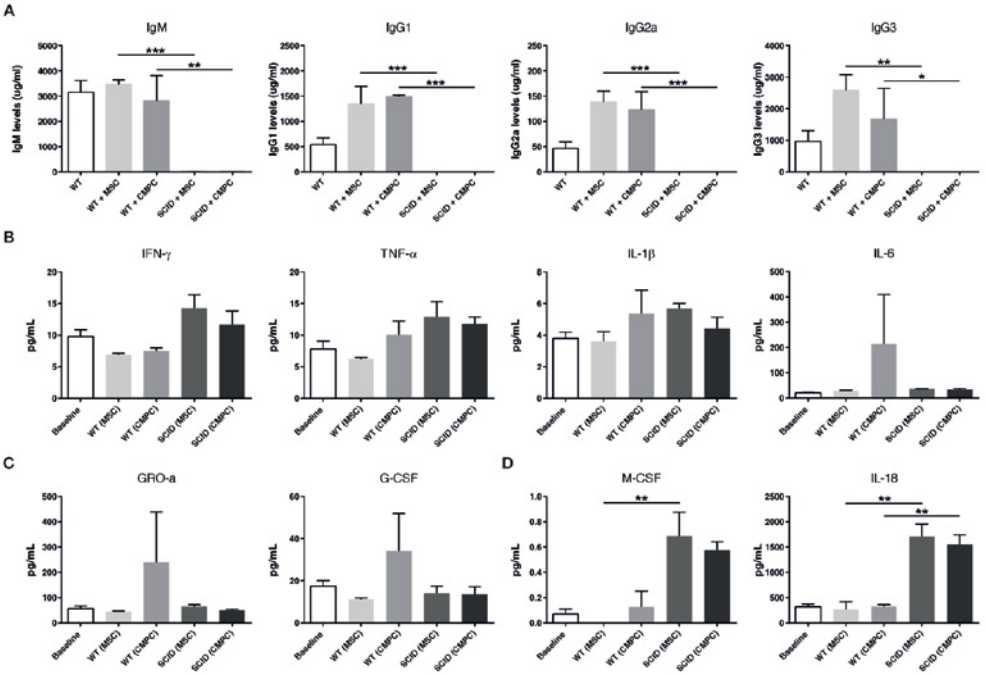

Figure 8.4 – Stem cell injection result in elevated cytokine levels

A Production of IgGs is stimulated following the injection of progenitor cells in WT mice. **B** The general inflammatory cytokines are induced in both WT and SCID animals. **C** Neutrophil-associated cytokines peak especially in WT mice injected with CMPC. **D** Monocyte-related cytokines are strongly induced in SCID mice.
** p<0.05, ** p<0.01, *** p<0.001*

matopoietic cells or tissue in severe immune deficient mice[32]. Reconstruction of the human immune system provides the opportunity to study human biological processes, including allograft rejection upon cell transplantation and the potential immune modulatory effects of CMPC and MSC[33].

With the recognition of the role of the immune system in cardiac disease[34], it is obvious that animal models in immunodeficient mice are unrepresentative. In combination with the knowledge that even SCID-mice can induce a mild immune response after xenotransplantation, we believe a shift towards humanized animals is necessary to obtain proper pre-clinical rodent models for cardiac human cell therapy. Even when using fully humanized pre-clinical animal models, it could be worthwhile to perform HLA-matching before injecting stem cells. Ultimately, any feasible approach to reduce cell rejection only a little, can increase the effectiveness of the cardiac cell therapy.

Acknowledgements
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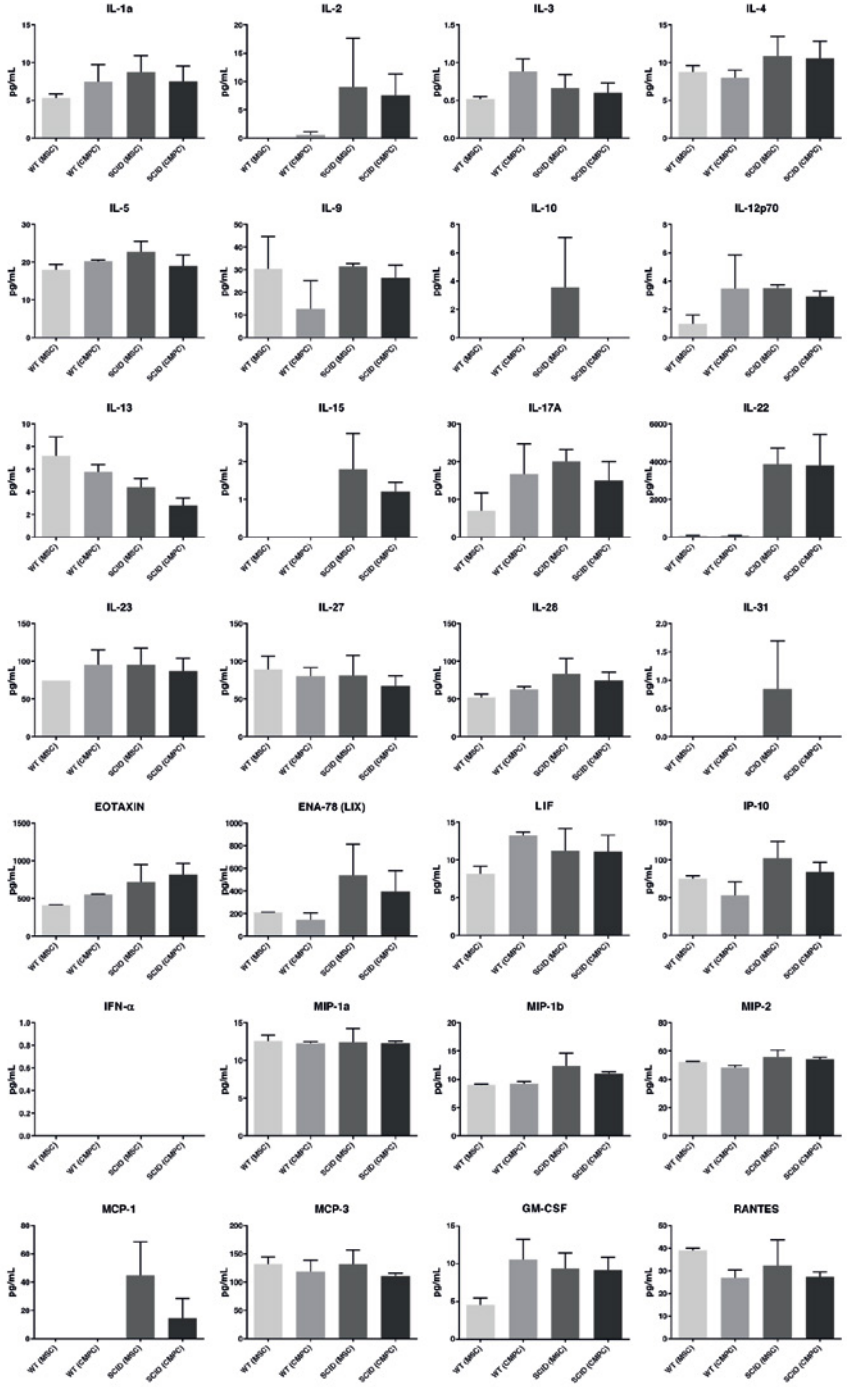
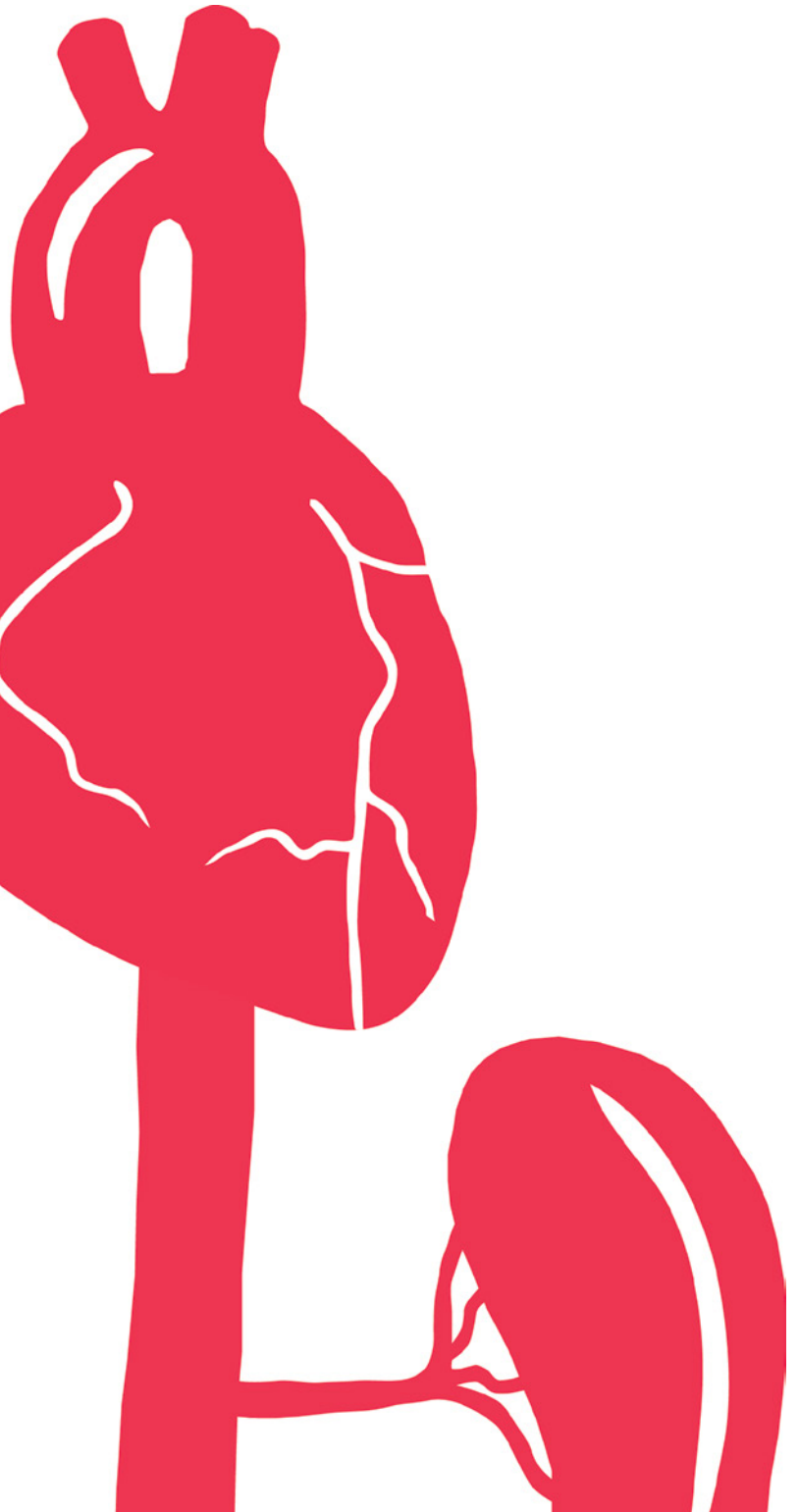
Supplement



Suppl. Figure 8.1 – Cytokine production by WT and SCID mice after progenitor cell injection



Intramyocardial Stem Cell Injection: Go(ne) with the Flow

9

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To the editor:

Many preclinical and clinical trials aim to deliver stem or progenitor cells in the heart, to protect or alleviate patients from the symptoms of myocardial infarction (MI) or heart failure. Although some beneficial effects on the heart have been reported[1], the magnitude of effect is moderate and cellular retention is consistently low. Therefore, improvements in delivery strategies are needed in order to truly assess the regenerative potential of different injected cell types.

Intracoronary and intramyocardial injections have been compared extensively, with no major differences detected in stem cell retention[2]. Interestingly, the majority of injected cells was found in the lungs rather than the heart, even after intramyocardial injection[3]. The mechanism underlying this observation remains unknown, as is the time window in which the cells are lost from the heart. The aim of this present study was to observe the events during and shortly after injection of stem cells in the myocardium.

For this, we injected two healthy, anesthetized, female pigs with a mixture of one million mesenchymal stem cells (MSCs) and 0.2 million inert, cell-sized (15 μm) fluorescent microspheres using a 26G catheter placed in the center of the cardiac muscle layers (*figure 9.1A*)[2]. To enable real-time visualization of fluid dynamics upon injection, the MSCs and microspheres were resuspended in contrast fluid (Telebrix) and fetal bovine serum (1:1 volume), without it affecting cell viability. After filling the injection catheter, total volumes of 500 μL or 200 μL cell suspensions were used for regular and concentrated injection volumes, respectively, thereby preventing an effect of the dead volume of the catheter. During and after injection, the localization of the contrast agent was recorded using C-arm fluoroscopy. In a third pig, we mounted one million MSC on large (60 micron) gelatin beads and injected these without contrast via the same method as described before. Five minutes after injection, all pigs were terminated by exsanguination.

Fluoroscopic monitoring of the contrast-resuspended cell suspension revealed the initial formation of a small depot at the tip of the injection catheter (red arrow in *figure 9.1B, video material is available on the article's EHJ website*)[4]. After 2-3 heartbeats this depot was quickly emptied into nearby veins with each following contraction (green arrow in *figure 9.1B*). This venous drainage is strongest during systole and decreases during diastole, when it is hardly visible using fluoroscopy. After 5 minutes, the depot was no longer visible (*figure 9.1B*, lower-right panel).

Before, during and after the injection, blood samples were taken from the coronary sinus to detect the presence of the injected MSCs, which we had fluorescently labeled[2]. Upon blood sample collection, red blood cells were lysed and flow cytometric analysis was used for MSC detection. This demonstrated that wash-out of injected fluorescently labeled MSCs from the myocardium was most noticeable immediately upon injection (*figure 9.1C* and *D*). This cell clearance was highest

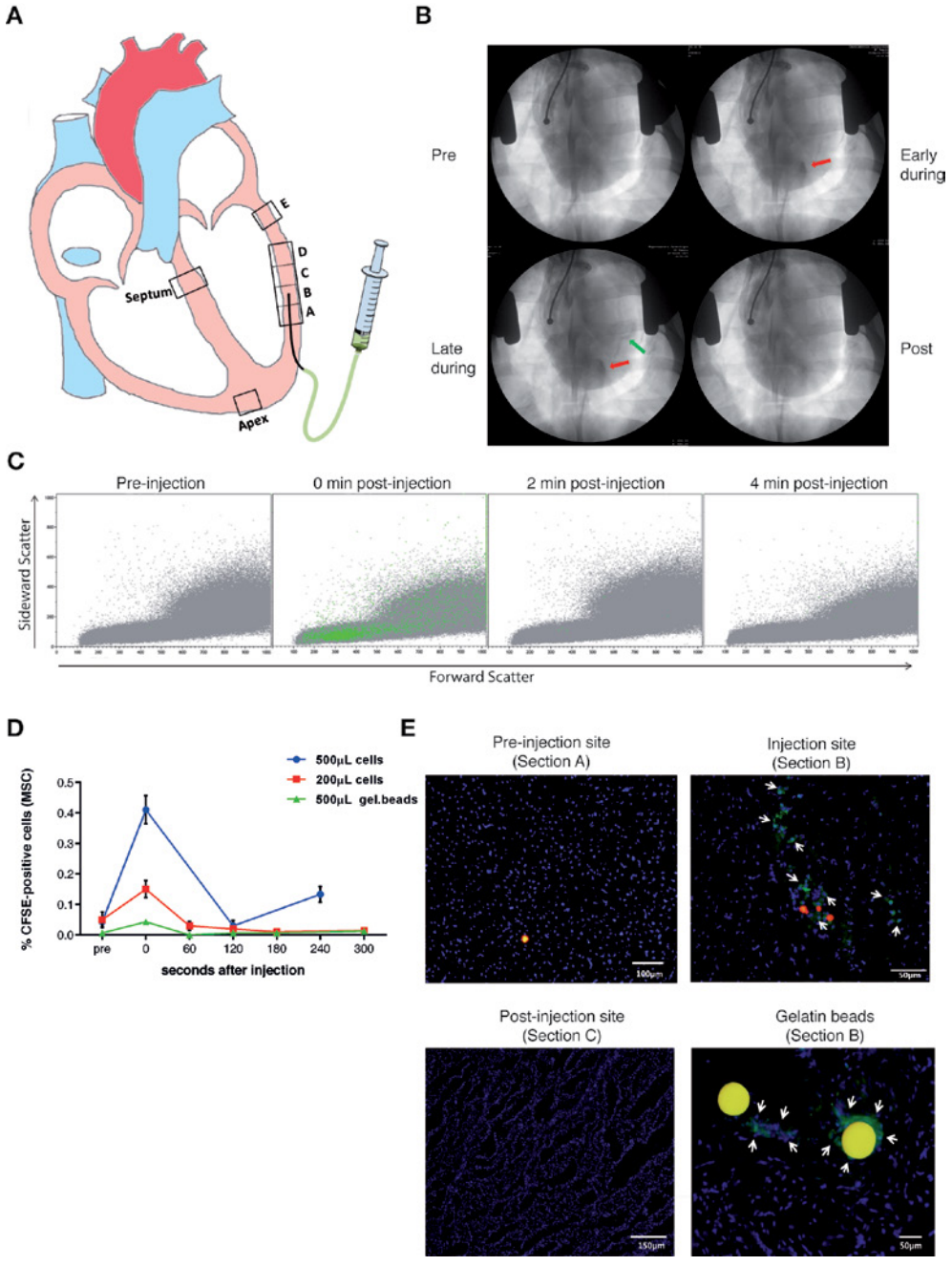
with the 500 µL injection (blue line), whereas the more concentrated sample (200 µL, red line) led to a lower immediate wash-out.

After collection of the heart, tissue samples were taken near the injection area, as well as from remote areas to search for the MSCs and the fluorescent inert microspheres, as shown in *figure 9.1A*. Part of these samples were cryopreserved and used for microscopical detection of the labeled MSC, while the remainder were degraded for quantitative detection of the fluorescent microspheres[5].

The location of the co-injected inert fluorescent microspheres in the two hearts was determined by tissue degradation, where the majority of retrieved fluorescent beads was found near the injection site (data not shown). Small amounts were visible upstream of the catheter tip. This could be due to backflow along the catheter, or possibly an artifact due to catheter removal. The amount of fluorescent beads from the downstream areas from the injection site (areas *C*, *D* and *E*) was comparable to the negative controls, apex and septum, indicating limited diffusion of the fluorescent microspheres into distal myocardial tissue, including tissue along the 'outflow route'. In part of the tissue used for sectioning, MSCs were also found exclusively near the tip of the injection catheter, corresponding to the location of the depot previously imaged with contrast fluoroscopy. The local myocardial structure in this area was slightly disrupted and both the fluorescently labelled MSCs and the inert fluorescent microspheres were located in small clumps in these areas (*figure 9.1E*, second pane). Like the microspheres, the MSC did not migrate to adjacent areas.

However, when MSCs were applied when attached *in vitro* to small gelatinous carriers (gelatin beads), the direct outflow into the coronary sinus was reduced ten-fold compared to its cells-only control (*figure 9.1D*, blue vs. green line). Microscopical imaging of the injection site shows the increased presence of the MSCs, in close proximity to their gelatin carriers (*figure 9.1E*).

Despite extensive pre-clinical and clinical trials, cardiac stem cell therapy displayed so far only modestly increased cardiac performance[1]. In the present observational study of the injection process, substantial numbers of MSCs were immediately flushed out of the heart via the venous system within a few heart beats after the start of injection. We realize that a limitation of this case report study is the small sample size of merely one animal per group. Furthermore, the contrast can only be optimally imaged with open chest and dilution by natural muscle perfusion might lower the signal in the course of time. Still, to our knowledge, this is the first report that demonstrates the dramatic wash-out of intramyocardially injected cells via these visualizations. This study clearly demonstrates the major limitation of current cell delivery approaches and thereby potentially also their observed limited functional benefits to the heart. Although positive effects on cardiac function have been reported, increasing the retention of the cells will likely improve these effects.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Sideward Scatter
Forward Scatter
D
% CFSE-positive cells (MSC)
500µL cells
200µL cells
500µL gel.beads
pre
0
60
120
180
240
300
seconds after injection
E
Pre-injection site
(Section A)
Injection site
(Section B)
Post-injection site
(Section C)
Gelatin beads
(Section B)

Figure 9.1 – Stem cell retention in a porcine heart

A Experimental set-up: 1 million fluorescently-labeled (CFSE) MSCs and $0.2x10^6$ red fluorescent beads were injection in the lateral wall of the porcine left ventricle (open thorax, Dutch Landrace pigs, 3 months of age, ±70 kg). All animal experiments were performed in accordance with the Directive 2010/63/EU of the European Parliament, national guidelines on animal care and with prior approval by the Animal Experimentation Committee of Utrecht University). Labeled black squares indicate where tissue samples were taken for degradation and histology. **B** Contrast images depicting the localization of the injected fluid pre-, early during-, late during-, and post-injection. The red arrow points at the depot formed at the catheter tip, while the green arrow shows the venous outflow tract. **C** Forward-Sideward scatterplot of nucleated cells with fluorescently-labeled MSCs marked in green. Coronary sinus samples were taken at different time-points before and after injection. **D** Quantification of the outflow of fluorescently-labeled MSCs in the coronary sinus at different time-points after injection. The blood was exclusively sampled from the cardiac vein, as the hemi-azygos vein was ligated. N=1 per group, error bars are standard deviations of technical replicates. **E** Histological analysis of pig heart samples. Images show the beads in the different sites of the heart. Colors indicate nuclei (blue; Hoechst), MSCs (green; CFSE-label) and fluorescent microspheres (red) or gelatin beads (yellow). Both cells and fluorescent beads are visible near the injection site, present in the disrupted tissue. In more remote areas, no cells are retrieved. Bar = 100 µm.

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Supplementary Methods

Animals

All animal experiments were performed in accordance with the Directive 2010/63/EU of the European Parliament, national guidelines on animal care and with prior approval by the Animal Experimentation Committee of Utrecht University. In three healthy pigs (Dutch Landrace pigs, 3 months of age, ±70 kg), brought under anesthesia, a thoracotomy was performed as previously described[1]. The vena azygos was ligated and a coronary sinus sampling line was inserted via the auricle of the right ventricle. The sampling line balloon was half-inflated to maintain it in location, while still permitting regular blood flow. Subsequently, a 26G catheter placed under echographic guidance in the center of the cardiac muscle layers of the left ventricle as indicated in *figure 9.1A*. Special care was taken not to insert the catheter directly in a vein.

Mesenchymal stem cell preparation

One million human fetal mesenchymal stem cells were isolated and cultured as described previously[2]. Before the experiment, MSC were harvested, labeled with 3 μM carboxyfluorescein succinimidyl ester (CFSE; Sigma, 21888), according to manufacturer's protocol. A single cell suspension containing one million MSC and 0.2 million fluorescent microspheres (Invitrogen F8891, Red colour) per injection volume (500 μL or 200 μL) was created, suspended in contrast fluid (Telebrix) and fetal bovine serum (1:1 volume). Viability of MSC in this mixture was not affected, based on Annexin-V and 7-AAD (BD Pharmingen) staining and analyzed via flow cytometry. To load the gelatin microspheres (MS) with MSCs, 5 mg gelatin MS were mixed with 8 million MSCs in a 10 cm ultra-low attachment plate. The gelatin microspheres and MSCs were cultured together for 24 hours to let the cells attach to the MS. MSC-laden gelatin microsphere were size selected and collected by running the suspension through a 100 μm cell strainer.

Gelatin microsphere preparation

Gelatin microspheres were prepared by water-in-oil emulsion. Briefly, an aqueous 10 wt% solution of gelatin type B (Sigma; G9382) was made. This solution was added to heated olive oil, stirred at 400 rpm and chilled on ice while stirring. Speed was reduced to 300 rpm, chilled acetone was added and the solution was filtered. The gelatin microspheres were collected, washed in chilled acetone and sieved to separate 50-75 μm gelatin MS. MS were cross-linked with 25% glutaraldehyde (Sigma; G5882) solution overnight and the reaction was stopped with glycine (100 mM). Next, the gelatin microspheres were washed in chilled acetone again and freeze dried.

Injection and Analysis

Total volumes of 500 µL or 200 µL cell suspensions were used for regular and concentrated injection volumes, respectively. During and after injection, the localization of the contrast agent was recorded using C-arm fluoroscopy. Simultaneously with the moment of injection, blood samples were taken from the coronary sinus in EDTA-tubes. Upon blood sample collection, red blood cells were lysed and flow cytometric analysis (GALLIOS, Beckman Coulter) was used for MSC detection. Five minutes after injection, the pigs were terminated by exsanguination. The heart was collected and tissue samples were taken near the injection area and remote areas to search for the MSCs and the fluorescent microspheres.

Part of these samples were cryopreserved using Tissue-Tek. Tissue sections of 7 microns were cut in the pre-defined areas (*figure 9.1A*) to screen for the presence of the labeled MSC. Slices were co-stained with Hoechst for nuclear visualization. Representative pictures were taken with a fluorescent microscope (Olympus) at 10 or 20 times magnification.

Statistics

Due to the observational nature of this study, having only n=1 in each group, no statistical analyses were performed.

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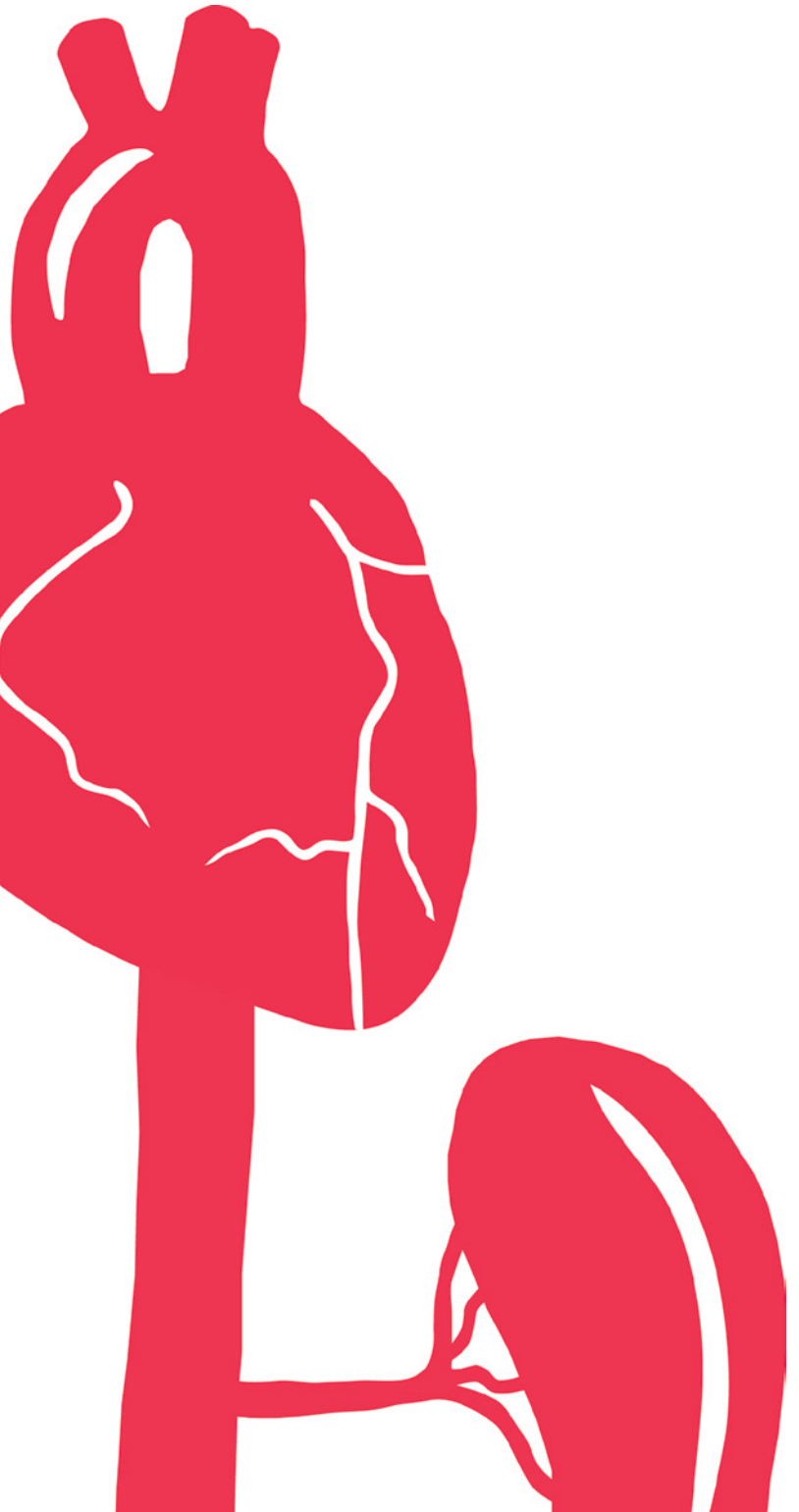


Discussion

10

Discussion

In recent years, the role of inflammation in cardiac disease has become more and more established. The immune system plays a role in the pathogenesis of cardiac disease. Well-established examples are atherosclerosis and myocarditis[1,2]. Immune system activity can also be an immediate consequence of a cardiac event, i.e. the neutrophil activation after both ischemia and reperfusion[3]. Lastly, the immune response can develop into an auto-immune disease aimed at the heart, thereby influencing the progression of the disease[4].

In this thesis the role of cardiac inflammation in cardiac disease has been extensively studied, as well as the potential role for cell therapy in this process. In this discussion we will visit the ideas and ideals behind cardiac stem cell therapy. Afterwards, we will highlight several aspects of inflammation in both acute and chronic cardiac disease and the potential roles for stem cells as treatment modality. Subsequently, will consider the role of stem cell therapy in animal models in a broader sense, before finishing with the clinical implementations and future research suggestions.

Cell therapy in cardiac disease

The original concept of cardiac stem cell therapy was that injected or infused stem cells would repopulate damaged areas of the heart[5]. In the first attempts, intravenous administration of the suspended cells was applied, with little effect, as cells did not home to the heart in sufficient numbers[6,7]. This method of administration was then quickly abandoned in favor of intracoronary and later intracardiac administration. Still, delivery using these techniques is meagre at best[8]. The heart is a heavily vascularized organ and all fluids injected are immediately drained via the venous system (**chapter 9**)[9]. Although the intracardiac injection is still popular, focus appears to be shifting towards the development of 3D constructs containing progenitor or differentiated cells[10]. By using these constructs, it is possible to create both a more beneficial environment for stem cells to thrive in, as well as provide structural support for the scarred cardiac wall.

The few cells that remain in the heart after intracoronary or intracardiac injection have a hard time thriving in an adverse environment. This adverse environment is created by stress signals from the surviving myocardium (both cardiomyocytes and myofibroblasts) and the infiltrated immune cells, which produce pro-inflammatory signals[11]. These factors combined maintain a suboptimal climate, in which surviving injected cells do not manage to fulfill the regenerative promise. While applying the progenitor cells in a cardiac patch solves the problem of cells washing out, it does not necessarily alter the state of the local environment. Although one could argue that the progenitor cells can alter the environment using their paracrine powers. The production of paracrine factors will likely diminish upon their

	 MSC	 CPC	 iPS
PRO	- pro-angiogenesis - pro-survival - immunomodulation - autologous - years of clinical experience	- cardiac differentiation - pro-angiogenesis - immunomodulation	- autologous - cardiac differentiation
CON	- no cardiac differentiation	- hard to obtain autologous	- need 100% differentiation before injection - paracrine effects unknown

Figure 10.1 – Advantages and disadvantages of MSC, CPC and iPS cells during cardiac cell therapy

differentiation into mature and functional cardiomyocytes.

What the optimal stem or progenitor cell will be for cell therapy in cardiac disease is still the subject of debate. Different cells have been considered[12], of which the most relevant are as shown in *figure 10.1*:

Mesenchymal stem (or *stromal*) *cells* (MSC), a progenitor cell type found mainly in the bone marrow and adipose tissue. They differentiate into cartilage, bone or fatty tissue, and were also found to have many supportive functions[7]. As such, MSC can stimulate the formation of new blood vessels (angiogenesis), promote survival of surrounding cells and modulate the immune system to create a more favorable environment[7]. Other advantages of MSC are the ease of autologous transplantation and the many years of clinical experience with these cells. Their main disadvantage is the inability of mature cells to differentiate into functional cardiomyocytes[13].

Cardiac progenitor cells (CPC), such as the cardiomyocyte progenitor cells (CMPC), are multipotent cells derived from the heart. They are predisposed to differentiate into cardiomyocytes or vascular cells and as such should be able to form both the new heart muscle and support blood supply[14]. In addition, using paracrine factors the CPC can also induce angiogenesis. CPC are often considered to be the better cell type for the heart, as their normal function is to repair the daily wear and tear of the heart[5,14]. However, these cells have been less clearly characterized and consensus about their surface markers and paracrine capacities is yet to be reached[15]. Moreover, it is very invasive to obtain autologous CPC from patients, so

that therapy would be more feasible with allogeneic cells.

Induced pluripotent cells (iPS) are a recent addition to the injectable cells. iPS are created by taking somatic cells and de-differentiating them into pluripotent stem cells by re-expressing certain 'stem cell genes'[16,17]. The newly induced pluripotent cells can then be re-differentiated into whichever cell type desired, by adding a precisely composed cocktail of differentiation factors[18]. The advantage of iPS cells is that they are donor-specific, thereby minimizing the odds of rejection although it is still not excluded[19]. However, the differentiation process of these cells needs to be closely monitored to prevent non-differentiated cells to become teratomas, which is why only differentiated cells can be injected[19]. So far it is still unclear what the paracrine effects of these cells might be.

Despite a decade of experience with cell therapy, it has become apparent that we have a very limited idea about what the injected cells actually do in the body. Cells are administered during cardiac disease and we subsequently determine some cardiac functional parameters as the main outcome measure. However, improvements of cardiac function will always be an indirect effect of cell therapy, as it is the result of the modulation of one or more specific processes.

The use of a particular stem or progenitor cell is usually based on availability, rather than comparative research on which cell is most suitable. However, this decision should be determined by the goal of the intervention, and should preferably be better defined than the broad goal of 'improving cardiac function'. This then will enable more accurate outcome measurements, which could subsequently be related to cardiac function. It is also important to keep in mind whether the goal is reached mainly by paracrine or structural incorporation. If a cell needs to be integrated in host tissue, correct differentiation and prevention of rejection is essential.

The goals of stem cell therapy can roughly be divided into four categories (*figure 10.2*):

I Angiogenesis

II Cardiac regeneration

III Scar modulation

IV Immunomodulation

As during ischemic cardiac disease, the blood supply is inadequate, stimulation of angiogenesis can lead to new vessel formation, thereby improving the oxygenation of the tissue. Improved circulation can help cardiomyocytes or injected cells survive and can reduce a patient's angina complaints. It should be remembered though, that revascularization via angiogenesis is a slow process and multiple doses of cells might be required to adequately revascularize tissue.

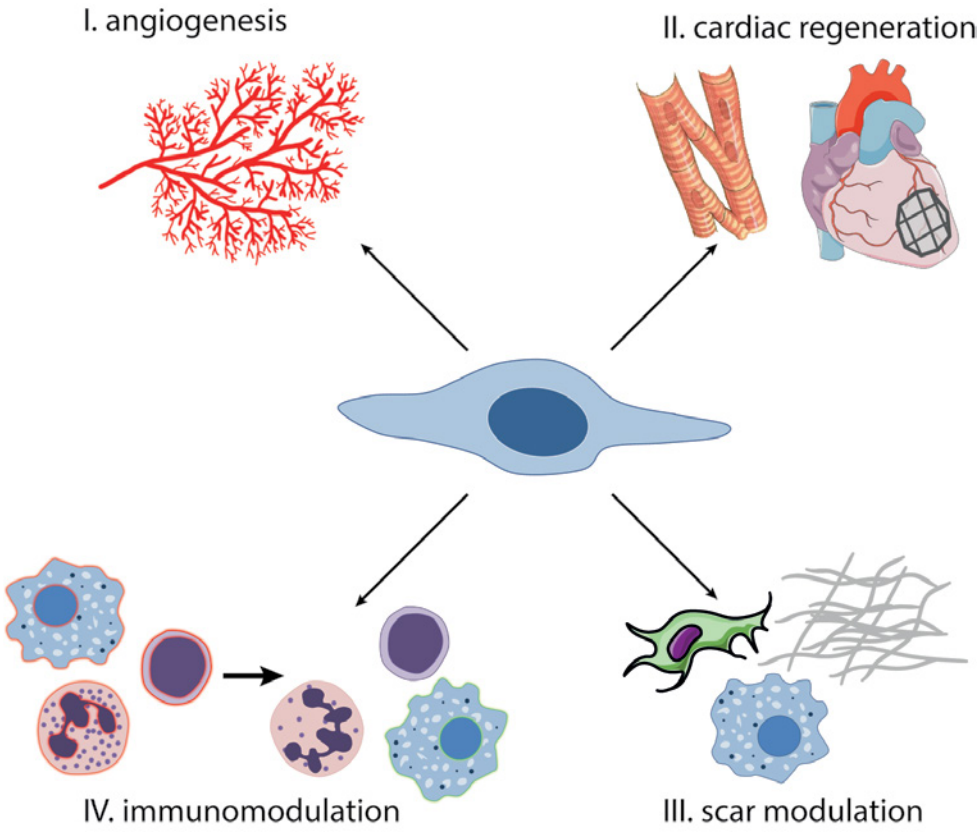


Figure 10.2 – The four main goals of cell therapy in cardiac disease
Cell therapy should induce angiogenesis (**I**) to provide oxygen and nutrients to the cardiac tissue. Cardiac tissue can be regenerated (**II**), thereby restoring the heart to normal function. Scar modulation (**III**) can improve scar structure and diminish adverse remodeling. Lastly, immunomodulation (**IV**) can improve the environment for surviving cardiomyocytes and trigger the reparative phase.

Cardiac regeneration can be obtained by (**1**) injecting cardiac progenitor cells that differentiate into cardiomyocytes, (**2**) applying a construct containing differentiated cardiomyocytes or (**3**) paracrine stimulation of the endogenous stem cells. Cardiac regeneration is a goal that is hard to achieve, but it is considered the holy grail of cardiac stem cell research as it can restore the heart and cardiac function to a normal, healthy level.

Modulation of the scar, while challenging, might be essential for patients with ischemic cardiomyopathy. It is well known that without scar formation there is a high incidence of cardiac rupture and death[20]. However, there are reports indicating that scar formation after MI can proceed suboptimally, aided by adverse remodeling[21,22]. This can even lead to fat depositions in the scar and misaligned collagen depositions[22-25]. This may lead to insufficient support and subsequent scar bulging

during contraction, thereby effectively reducing cardiac output. Post-MI fibrosis is a complex process that has not yet been fully elucidated[20,22]. Processes known to play a role include the differentiation of cardiac fibroblasts into myofibroblasts and the formation of alternatively activated (M2) macrophages[20,26]. Additionally, TGF-β appears to be a major regulator of post-MI fibrosis[27].

Immunomodulation to reduce the inflammatory burden in cardiac disease is a relatively new target. Inflammation contributes to the death of cardiomyocytes, either by killing directly or by targeting cells for destruction[28,29]. Additionally, the production of negative inotropic cytokines can decrease the cardiac function even further[30-32]. Modulation of the immune system can prevent ongoing damage, and trigger the transition to the reparative phase[4,33].

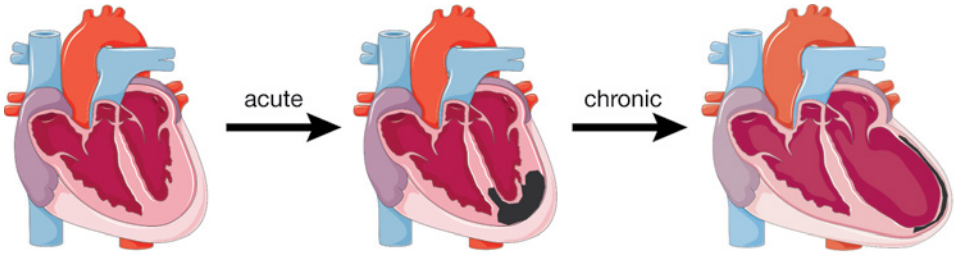
Inflammation in cardiac disease

In this thesis, the role of inflammation in both in the early hours after myocardial infarction (MI) and chronic myocarditis has been studied. The reperfusion injury after MI is probably one of the best-recognized immunological events in cardiac disease[3]. With the development of percutaneous coronary intervention people realized that the newly restored blood flow itself caused additional myocardial injury. The extent of reperfusion injury turned out to depend strongly on the infiltration and activation of neutrophils[3]. This is also supported by our data, where shortly after MI a strong leukocytosis is observed, in which all circulating white blood cells are increased in number (**chapter 7**). Interestingly, the actual number of white blood cells appears to be closely linked to the time of the day. What surprised even more was the observation that the circulating number of leukocytes is not directly correlated to the level of immune infiltration. Circadian rhythms influencing chemokine release and adhesion molecule expression are probably critical for the attraction and subsequent extravasation of the neutrophils. This suggests a high-risk period, at the transition of sleeping to waking, where the heart is most susceptible to an immunological overkill by neutrophils after MI.

The pattern of immune activity after MI differs greatly from the one observed during chronic myocarditis. Chronic myocarditis is commonly considered a T-cell mediated disease, triggered by acquired cardiac auto-reactivity, however, we actually found the whole immune system to be involved via complex interactions (**chapter 5**). The resulting immune response is mild, yet ongoing, leading to a slow accumulation of damage. Shifting the balance towards resolution of inflammation will depend on a multifactorial approach. Simply suppressing one immune cell type will not be sufficient to break this downward spiral.

Stem cell therapy in cardiac inflammation

As mentioned before, the aim of this thesis was to investigate inflammation in



Primary intervention with	MSC	CMPC
Affected immune cells	Neutrophils	T-cells
Other processes influenced	Stimulation angiogenesis Cardiomyocyte survival	Stimulation angiogenesis Regeneration
Administration	Single dose	Multiple doses

Figure 10.3 – Phases of cell intervention

In the acute setting intervention with MSC would be advised, as they suppress neutrophil activity while inducing cardiomyocyte survival and angiogenesis. In the chronic phase, intervention with CMPC is more suitable, as they suppress T-cells, while stimulating angiogenesis and possibly contributing to cardiac regeneration.

cardiac disease and the effect of cell therapy on this process. To this end, we wanted to compare the two best candidates for cardiac stem cell therapy and determine their suitability at different intervention moments. The immunological processes involved both after MI (**chapter 1**) and during myocarditis (**chapter 4**) are extensively described earlier in this manuscript and at the beginning of this discussion. It is obvious that the immunological fingerprints differ between the two diseases and different immune cells are active at different time points. During the early phase after MI, the neutrophil is the first and strongest respondent. This prompted us to investigate the effects of both MSC and CMPC on neutrophils (**chapter 2**).

Quite unexpectedly, we found a clear difference in effect of these progenitor cells on neutrophils. Although several processes were similarly influenced, only MSC reduced ROS production and L-selectin shedding. These suppressive effects were not visible in neutrophils treated with CMPC, which produced ROS just as easily and even had a reduced phagocytic function. In addition, injection of CMPC in BALB/c or SCID mice elicited a stronger neutrophil response and a quicker clearance of the cells compared to MSC (**chapter 8**). Combined, these results indicate that intervention shortly after myocardial damage, in which a mobilization of neutrophils is expected, should preferably be with MSC, not CMPC (*figure 10.3*). Since MSC have paracrine effects that also promote survival of surrounding cells, they can help the struggling myocardium by stimulating the cardiomyocytes to survival[7], while reducing the pro-inflammatory effects of infiltrating neutrophils. The local environment shortly after MI will be hostile to the MSC and as such, they

will not survive there for long. However, as spontaneous cardiac differentiation has not been observed in these cells, this might not be a great loss. Moreover, differentiation of MSC into fatty or bone tissue could be detrimental to the heart[24]. It appears best if MSC are applied to reduce the acute inflammation, after which the environment is more receptive to regenerative therapy using a different stem or progenitor cell.

Interestingly, during the chronic setting when T-cells are the main effector cells, the progenitor cell preference is reversed. Although MSC have been studied for over ten years for their capacity to suppress T-cell proliferation[4,34], we found CMPC to have a similar effect. Moreover, CMPC proved to be better T-cell suppressors than MSC both in vitro (**chapter 3**) and *in vivo* during chronic myocarditis (**chapter 6**). Our results therefore indicate that during the chronic phase of cardiac disease, administering CMPC is the more appropriate intervention. In addition to suppression of the T-cells and potentially shifting the balance towards more T_{reg}, the CMPC can also induce angiogenesis and participate in cardiac tissue regeneration[35]. In this chronic setting, however, it should be strongly considered to have multiple administrations of the cells. The initials progenitor cells will have to deal with a hostile environment as well, which could shift their secretory and differentiating capacities. After establishing a favorable environment, CMPC can be administered to aid regeneration.

Clinical perspective

Unfortunately, until recently there was little attention in the field of cardiology to treat the inflammatory components in cardiac disease. This is partly related to the complexity of immunological interactions during cardiac disease, which prohibit broadly suppressive therapy[11,36]. Furthermore, there is also a lack of awareness among clinicians about the role of inflammation. Over the years a few studies have been performed aimed at the suppression of the immune system[37-39], yet often without little previous study of the immune process to be interfered with. The immune response often goes in phases and a therapy that might have excellent results when administered at the right time, will show no effect when given at another time point.

Based on the work in this thesis, and related literature, several suggestions for implementation in the clinic will be discussed. As described in **chapter 7**, and the beginning of this discussion, neutrophil extravasation is stimulated in the early morning hours. Patients who have a MI at these hours are more likely to suffer from large ischemia-reperfusion injuries compared to people who have their MI in the evening. Specifically targeting the early-morning patients with drugs blocking or reducing neutrophil extravasation might reduce the myocardial damage in these patients.

Similarly, in patients who have suffered an MI and show rapid deterioration of cardiac function in the absence of new cardiac events, it could be considered to investigate their inflammatory status based on cytokines and cytotoxic T-cell activation. T-cell suppression, using either stem cells or perhaps a drug like tacrolimus or ciclosporin, could prevent immune-based progression of cardiomyopathy. Moreover, it is known that people with certain haplotypes, especially the HLA-DR4[40-42] and HLA-DRB1[43,44], are more prone to develop cardiac auto-immune disorders. Interestingly, HLA-DRB1 is also linked to endothelial dysfunction and increased risk of MI[44,45]. Screening for haplotype in patients with intermediate to large MI, could identify patients who would benefit most from immunomodulative therapy. These haplotypes also play a role in dilated cardiomyopathy (DCM). In dilated cardiomyopathy, 70% of the patients have their cardiomyocytes coated with antibodies, regardless of initial etiology. This indicates a strong and ongoing immune response during DCM. In patients with DCM it might therefore be worthwhile to screen for immune activation and treat with either rituximab (against the CD20 on B-cells) or by targeting the T-cells. As there is very little treatment for DCM and having a bad prognosis, immunomodulative therapy might provide a much-needed therapy.

Future directions in research

While various future research suggestions have already been described or hinted at in this chapter, it is crucial to examine stem- or progenitor cells in a laboratory before going to clinical trials. By examination, the analysis of the cell itself and its effects on the different processes at play in the heart are indicated, as described in *figure 10.2*. In this respect, it would also be of value to further compare adult progenitor cells from patients with cardiac disease or diabetes to those from healthy adults. Especially in diabetes many processes in the body, including inflammation, are in disarray. Are the cells of these people still suitable for autologous transplantation?

The autologous/allogeneic discussion is another area that deserves attention. Despite initial reports, progenitor cells, including MSC, might not be as HLA-free as originally imagined. Activation of the immune response will result in the rejection of injected cells unless proper measures are taken to match donor and recipient. Donor matching seems like an obvious solution, especially if the goal is regeneration and the structural incorporation of cells, or if the goal is achieved by multiple injections. In the last case, the innate immune system will destroy the first delivery of mismatched cells, which will train the adaptive immune response. Subsequent doses of the same donor will be immediately attacked by T-cells and B-cells.

A similar caution extends to research on human cells in animal models. Although the need for animal models remains, we always have to consider the differences on a molecular level in cardiac injury and repair that might exists in mice or pigs,

as compared to those in humans. Especially when deciding to use immunodeficient mice to reduce the speed of cell rejection in experiments, we cannot ignore how the lack of a functional immune system disrupts our model. From our own observations we have seen that NOD-SCID mice, if they survive the first day after the MI operation, have a better prognosis than wildtype mice. This is most likely linked to the absence of an adaptive immune system, which will only start to invade the heart a few days after MI. In addition, even in the often used NOD-SCID mice immune-detection of stem cells is not completely circumvented and immune activation can be observed. Although they are not ubiquitously available yet, the use of humanized animals, in which animals are given a human immune system, likely reduces rejection problems and can provide a better study model than wildtype or immunodeficient mice.

Aside from the immune-related research suggestions, it became glaringly obvious during the work towards the completion of this thesis that for optimal research good communication between basic researchers and clinicians is invaluable. While basic researchers understand the stem cell behavior and the molecular/cellular processes at play better than the clinicians, the clinicians know the clinical presentation of cardiac disease and can improve the relevance of molecular targets and interventions. I would strongly advice both sides to ask each other's input in both basic and (pre-)clinical studies. This should help prevent us from starting studies that in retrospect were doomed to fail. Moreover, intense collaboration between basic scientists and clinicians will greatly increase clinical translatability.

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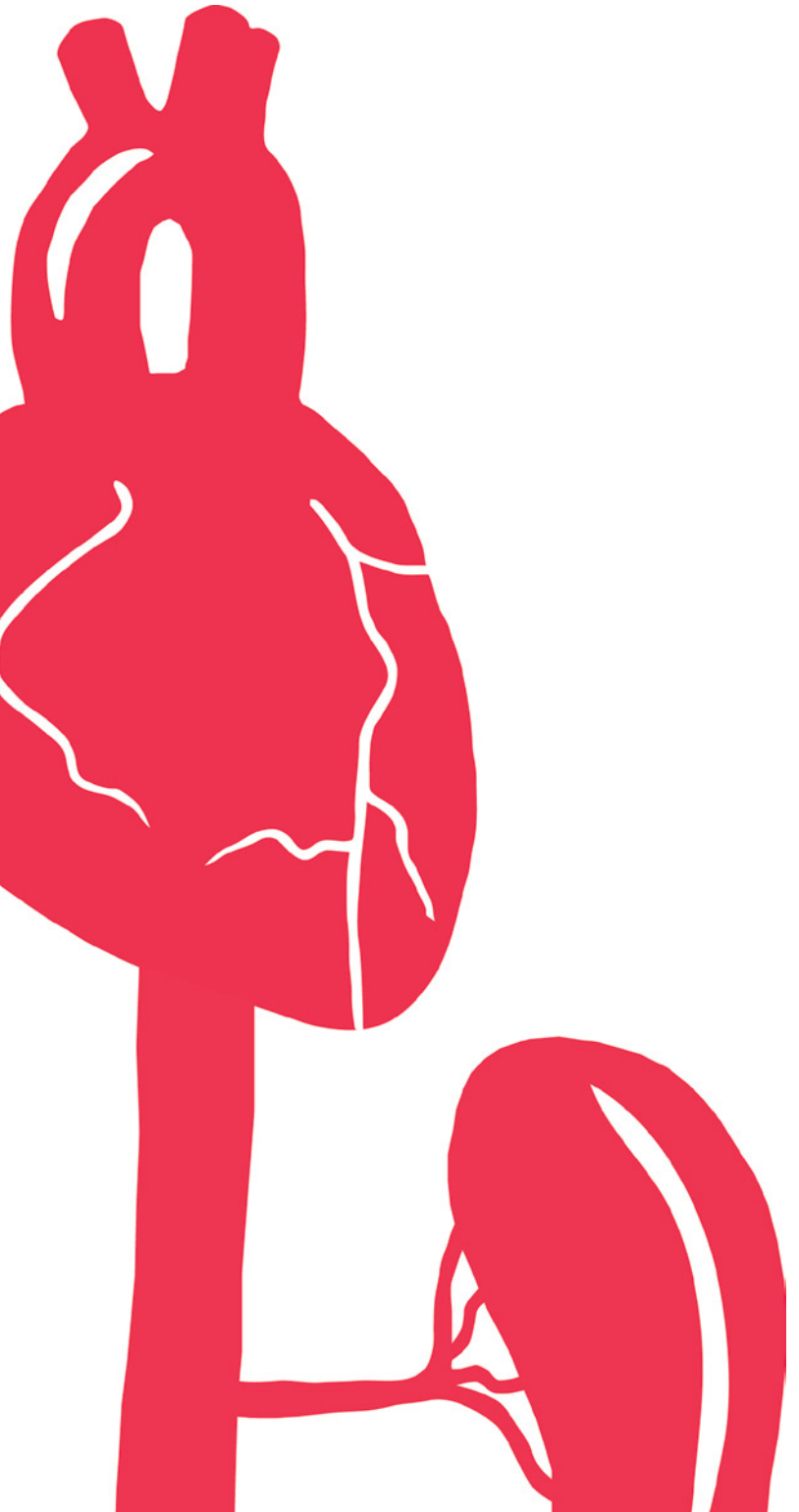

Appendix

Nederlandse Samenvatting

Acknowledgements

List of Publications

Curriculum Vitae

Nederlandse Samenvatting

Hart- en vaatziekten zijn een grote oorzaak van sterfte wereldwijd. De grote meerderheid van mensen met hart- en vaatziekten krijgt vroeg of laat een hartaanval. Tijdens een hartaanval gaat een van de hartslagaders dicht zitten, meestal door een bloedstolsel. Zolang het bloedvat dicht zit, kunnen er geen zuurstof of voedingsstoffen bij de hartspier komen.

Na zo'n 20 minuten leidt dit tot onomkeerbare schade en beginnen er hartspiercellen, ook wel cardiomyocyten genoemd, dood te gaan. Voor en tijdens het sterven zenden deze hartspiercellen stress-signalen uit, die immuuncellen aantrekken en activeren. Dit proces wordt verergerd tijdens een interventie, bijvoorbeeld een dotter-behandeling waarbij het vat weer wordt opengemaakt.

De snelle instroom van zuurstofrijk bloed heeft een paradoxale reactie tot gevolg, waarbij zuurstofradicalen extra schade veroorzaken. Ook hierbij komen weer extra stoffen vrij die immuuncellen aantrekken, en daardoor nog meer schade veroorzaken.

Immuuncellen na hartaanval en myocarditis

In de eerste dag na een infarct zijn vooral neutrofielen in grote getale aanwezig in het hart. Neutrofielen zijn de immuuncellen die het snelst reageren op een stress-oproep in het lichaam. Hun primaire taak is normaliter het doodmaken van bacterieen die in het lichaam binnendringen en het opruimen van dode cellen. Echter, door de onstuimige omgeving in het gebied van de hartaanval vergeten de neutrofielen dat er geen bacterieen zijn om te doden, en beschadigen ze in plaats daarvan de nog levende hartspiercellen. Teglijkertijd beginnen ze met het opruimen van dode cellen, zodat er mettertijd een litteken op die plaats kan terugkomen.

Kort daarna komen de macrofagen aan in het hart. De macrofagen zijn onder te verdelen in een inflammatie- en reparatiestimulerende soort. In eerste instantie komen de inflammatiestimulerende macrofagen, die zowel extra schade veroorzaken aan hartspiercellen en dode cellen opruimen. Pas een paar dagen na de hartaanval komen de reparatiestimulerende macrofagen, die de complexe processen van littekenvorming en de aangroei van nieuwe bloedvaten in werking stellen.

Men zou verwachten dat als de hartaanval eenmaal voorbij is, en al het dode materiaal is opgeruimd, het immuunsysteem weer rustig wordt. Echter, dat blijkt bij een groot aantal mensen niet te gebeuren. Tijdens de acute fase kunnen eiwitten van de hartspiercellen gepresenteerd worden aan T- en B-cellen als vijandige, bacteriele eiwitten. Deze T- en B-cellen worden dan getraind om alle cellen die deze eiwitten bevatten, te doden. Dit is een langzaam proces, maar het betekent dat mensen in de jaren na een hartaanval steeds meer hartspiercellen kwijtraken.

Uiteindelijk krijgen deze mensen hartfalen.

Deze getrainde immuuncellen zijn ook de oorzaak van problemen in chronische myocarditis. Myocarditis is een ontsteking van de hartspiercel, vaak uitgelokt door virussen die een eiwit hebben dat heel veel lijkt op een eiwit van hartspiercellen. Nadat T- en B-cellen getraind zijn om het virus op te ruimen, zien ze eenzelfde eiwit ook op hartspiercellen en gaan er dan van uit dat deze cellen geinfecteerd zijn. Als gevolgd daarvan maakt het getrainde immuunsysteem vervolgens gezonde hartspiercellen dood. Aangezien dit chronische effect van immuuncellen op het hart lange tijd niet (h)erkend is, zijn hier tot dusver weinig medicijnen tegen ontwikkeld.

Stamceltherapie

Een mogelijke therapie om de acute en chronische ontstekingsreacties in het hart tegen te gaan is het gebruik van stamcellen. Stamcellen, of voorlopercellen, zijn cellen in het lichaam die nog niet een specifieke weefselcel zijn geworden, maar kunnen uitgroeien tot verschillende soorten cellen. In dit boek is veel gewerkt met twee soorten stamcellen: afkomstig uit het hart en beenmerg.

De *beenmerg-stamcel*, MSC, wordt al tientallen jaren gebruikt in verschillende soorten therapie. Hoewel deze cel niet kan uitgroeien tot hartspiercel, is het wel een interessante cel om mee te werken. Hij produceert namelijk veel stoffen die allerlei processen in het lichaam beïnvloeden. Zo heeft de MSC een gunstig effect op het vormen van nieuwe bloedvaten, het onderdrukken van een immuunreactie en stimuleert het cellen om te overleven.

De *stamcel uit het hart*, de CMPC, kan daarentegen wel uitgroeien tot zowel hartspiercel als bloedvatcel. Daarnaast blijkt ook deze cel stoffen te produceren die immuunreacties onderdrukken en de groei van bloedvaten bevorderen.

De afgelopen jaren zijn er verschillende manieren bedacht op deze cellen in het hart te krijgen. De twee meest populaire manieren zijn om de stamcellen in te spuiten in de hartslagaders, bijvoorbeeld terwijl er een dotter-procedure wordt gedaan, óf de stamcellen worden met een mini-naaldje direct in de hartspier gespoten. Het voordeel van deze twee manieren is dat iemand niet geopereerd hoeft te worden om cellen te krijgen, maar het nadeel is dat er weinig cellen in het hart achterblijven. Een derde manier die gebruikt wordt om stamcellen in het hart te krijgen is door een zogenaamde 3D-constructie te maken. Hierbij wordt er een soort pleister gemaakt van stamcellen en collageen, die tijdens een operatie op het beschadigde deel van het hart geplakt wordt. Hoewel de ingreep een stuk invasiever is dan bij een stamcel injectie, blijven er op deze manier veel meer stamcellen zitten.

In dit boek staan de resultaten van vier jaar onderzoek naar de interacties tussen

immuuncellen en stamcellen, en hoe dit gebruikt kan worden tegen ontstekingsreacties in het hart. Het eerste deel van dit werk richt zich op de directe interacties tussen stamcellen en belangrijke immuuncellen.

Een uitgebreide beschrijving van de immuunreacties na een hartaanval staat beschreven in **hoofdstuk 1**. Hier worden vervolgens ook de rollen van immuuncellen stuk voor stuk bekeken, en wordt er een literatuur onderzoek gedaan om te achterhalen hoe MSC deze immuuncellen kunnen beinvloeden. Er zijn veel aanwijzingen dat MSC de aggressieve immuuncellen onderdrukken, of omvormen tot een subtype immuuncel dat helpt bij de regulatie van de immuunreactie en het starten van weefselherstel.

De effecten van MSC en CMPC op neutrofielen, de belangrijkste immuuncel gedurende acute schade, worden verder onderzocht in **hoofdstuk 2**. Neutrofielen hebben verschillende functies in het lichaam, waarvan het merendeel getest is in de aan- en afwezigheid van stamcellen. Op een deel van de processen hebben de MSC en CMPC hetzelfde effect. Dit geldt voor een verlenging van de levensduur van de neutrofiel, een verlaging van de NF-kB (een signaal-eiwit dat immuunreacties stimuleert) en een onderdrukking van de productie van miR-223 (een stukje RNA dat mogelijk van belang is voor de agressiviteit van neutrofielen). Daarentegen onderdrukken alleen MSC de productie van schadelijke zuurstofradicalen door neutrofielen en zorgen ze dat de neutrofielen langer inactief blijven.

In **hoofdstuk 3** worden de effecten van stamcellen op de T-cel, de belangrijkste cel van de chronische immuunreactie, bekeken. Zowel MSC als CMPC zorgen ervoor dat deze immuuncel niet meer kan delen, wat de CMPC nog effectiever doet dan de MSC. Daarnaast onderdrukken ze de productie van inflammatie-stimulerende stoffen. De stamcellen doen dit niet door zelf de T-cellen aan te raken, maar door bepaalde stoffen te produceren die op afstand deze effecten veroorzaken.

Een van deze factoren zijn exosomen, een soort blaasje dat door de stamcel wordt gemaakt en gevuld met specifieke eiwitten. Deze exosomen worden opgenomen door de T-cel, waarna de eiwitten in de T-cel vrijkomen en daar het delen van de cel onderdrukken. De onderdrukking van T-cellen verloopt niet via de onderdrukking van één gen, maar juist van vele genen waarvan van sommige de rol nog niet geheel duidelijk is in dit proces.

Het tweede deel van dit proefschrift richt zich op myocarditis. Myocarditis is een ontsteking van de hartspier, meestal uitgelokt door een virus (**hoofdstuk 4**). Myocarditus veroorzaakt hartschade door twee mogelijke mechanismes. Het virus kan in de hartspier gaan zitten, waardoor het een immuun aanval op het hart uitlokt. Of het virus camoufleert zich als hartspiercel maar wordt toch herkend, waardoor hartspiercellen vervolgens foutief herkend worden als virus. Op lange termijn ontstaat dan een chronische immuunreactie tegen het hart, welke hartfalen

vaak tot gevolg heeft. Er wordt gedacht dat verschillende stukjes microRNA hier een rol in kunnen spelen en mogelijk ook als therapie ingezet kunnen worden.

Ter verdere beoordeling van myocarditis wordt er in **hoofdstuk 5** gekeken welke immuuncellen precies actief zijn tijdens deze chronische fase. Hiervoor werden muizen 'gevaccineerd' met een harteiwit, waarna ze dezelfde immuunreactie tegen de hartspiercellen kregen. Vervolgens is op verschillende momenten in tijd gekeken welke immuuncellen actief waren in welke organen. Hieruit hebben we kunnen concluderen dat het in stand houden van een chronische immuunreactie afhankelijk is van meerdere soorten immuuncellen, die zich mettertijd steeds meer in het hart of de milt lokaliseren. In het bloed is dus niet altijd te zien of iemand een chronische ontsteking heeft.

Vervolgens worden MSC en CMPC ingespoten bij muizen met chronische myocarditis (**hoofdstuk 6**), om te bestuderen of dit effect heeft op de voortgang van de ziekte. De effecten van de stamcellen waren klein, maar niet verwaarloosbaar. Vooral de muizen behandeld met CMPC behielden een iets betere pompkracht van het hart en verminderde chronische immuunrespons. Dit komt goed overeen met de sterkere effecten van CMPC op T-cellen, zoals eerder beschreven in hoofdstuk 3. De geobserveerde effecten waren echter klein, wat aangeeft dat de studie wellicht met grotere aantallen muizen of met vaker toedienen van stamcellen herhaald moet worden.

Het laatste deel van dit proefschrift is gericht op het verbeteren van ons begrip van de immuunrespons in cardiale ziekte en stamceltherapie, en geeft suggesties voor de verbetering van stamcel interventies. In **hoofdstuk 7** is er gekeken naar de relatie tussen het tijdstip waarop iemand een hartaanval krijgt en de hevigheid van de immuunreactie. We ontdekten dat de immuunmobilisatie het sterkst is wanneer de hartaanval plaatsvindt in de vroege ochtend.

Op dit tijdstip is de productie van neutrofiel-aantrekkende stoffen het hoogst en zijn er ook veel eiwitten aanwezig die helpen om de neutrofielen in het hartweefsel te krijgen. Dit is ook het tijdstip dat er de meeste hartschade ontstaat. Concluderend hebben mensen die in de vroege ochtend een hartaanval hebben de sterkste immuunreactie en lopen daardoor veel risico op extra schade veroorzaakt door het immuunsysteem.

Het zou interessant zijn om in een volgende studie te kijken of een specifiek immuun-onderdrukkende therapie op deze tijdstippen van toegevoegde waarde kan zijn.

In **hoofdstuk 8** wordt gekeken naar de immuunreactie tegen stamcellen in proefdieren. Proefdieren worden vaak gebruikt als model voor menselijke ziekten. Resultaten van zulke stamcel studies bij proefdieren wil men graag 'vertalen' naar mensen. Een obstakel hierbij is dat van sommige stamcellen geen muizen-variant

is gevonden. Aangezien stamcellen een reputatie hebben dat ze het immuunsysteem onderdrukken, wordt veelal aangenomen dat dit geen enkel probleem moet opleveren. Een alternatieve oplossing is dat het 'ziektemodel' wordt gemaakt in een muis met een incompleet immuunsysteem.

In deze studie hebben wij bestudeerd wat er gebeurt na het inspuiten van MSC en CMPC in zowel gezonde muizen als die met een incompleet immuunsysteem. We zagen dat in beide gevallen de overgrote meerderheid van stamcellen na twee weken is verdwenen. Bij gezonde muizen, met een intact immuunsysteem, gaat dit sneller. Wel zagen we dat ook muizen met een incompleet immuunsysteem, nog steeds een immuunreactie hebben met de neutrofielen die ze nog wél hebben.

Concluderend is het werken met menselijke cellen in muizen, zelfs al zijn het muizen met bijna geen immuunsysteem, niet aan te raden. Een oplossing kan zijn om muizen te maken die een menselijk immuunsysteem hebben.

In **hoofdstuk 9** wordt gekeken wat er gebeurt tijdens het injecteren van stamcellen in de hartspier. Hiervoor hebben we MSC opgelost in een contrast-vloeistof, waarmee we precies kunnen zien hoe het vocht zich tijdens en na de injectie door het hart beweegt. Hiermee zien we dat tijdens injectie het vocht eerst een lokaal depot vormt, waarna dat via een netwerk van kleine adertjes wordt weggepompt bij elke samentrekking van het hart. Uiteindelijk stromen de cellen via het veneuze verzamelvat weer terug de bloedstroom in, waarna ze zich verspreiden door het lichaam.

Twee manieren om het weglekken van MSC te voorkomen is door het inspuit volume te verkleinen, of door de cellen op een grotere gelatine-bubbel te kweken en in te spuiten. Deze kan dan niet door de aders afgevoerd worden.

Concluderend is er veel bewijs dat er een verband bestaat tussen het hart en het immuunsysteem. Wanneer er massale celdood optreedt in het hart, om wat voor reden dan ook, kan het immuunsysteem daarop ontsporen. Dit leidt zowel op korte als lange termijn tot extra schade. Hoewel dit immunologische component van hartziekten al langer bekend is, wordt er in het ziekenhuis vooralsnog weinig gedaan met deze kennis.

Met behulp van stamceltherapie kan ook een gunstig effect bereikt worden op de inflammatie. In dat geval is het belangrijk om na te denken over welk soort stamcel op welk moment gegeven wordt.

In het geval van inflammatie kort na een hartaanval, waarbij er veel neutrofielen geactiveerd raken, is het beter om te behandelen met MSC (of de stofjes geproduceerd door MSC). MSC hebben een sterker onderdrukkend effect op neutrofielen en zorgen dat zij de beschadigende zuurstofradicalen minder produceren.

Op de langere termijn, als de T-cellen de voornaamste immuuncel zijn, is een behandeling met CMPC verstandiger. Deze cellen zijn veel beter in staat om T-cellen te onderdrukken, en daarbij kan in deze chronische fase begonnen worden door het lichaam met de reparatie van het hart. Dan is het altijd handig om extra cellen in de buurt te hebben die ook hartspiercel of bloedvatcel kunnen worden. Al met al is het dus erg belangrijk om een stamcel goed te bestuderen onder verschillende omstandigheden om daarmee een optimale therapie te kunnen ontwikkelen.

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List of Publications

Published

Feyen DA, **Van den Akker F**, Noort W, Chamuleau SA, Doevendans PA, Sluijter JP. *Isolation of Pig Bone Marrow-Derived Mesenchymal Stem Cells*. Methods Mol Biol. 2016;1416:225-32.

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In preparation

Van den Akker F, Tak T, Gremmels H, Van Eeuwijk ECM, Koenderman L, Van Laake LW, Doevendans PA, Sluijter JPG. *Differential Effects of Two Progenitor Cell Types on Neutrophils*.

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Curriculum Vitae

Frederieke van den Akker was born September 14th, 1985 in Vlissingen, the Netherlands. After completing the Gymnasium track at the Nehalennia Scholengemeenschap in 2003, she moved to Tulancingo, in Hidalgo, Mexico. There she lived with a local Mexican family, and attended Colegio Jorge Berganza. Upon returning to the Netherlands in 2004, she enrolled at University College Utrecht, where she followed the pre-med track. During her time at UCU, she spent a semester abroad at UC Irvine, California. In summer 2007 she graduated UCU *summa cum laude*, and continued on to the Selective Utrecht Medical Master (SUMMA) — a Master aimed at training MDs suited for clinical and research work.

During her master she started working with dr. Six and dr. Backus on the HEART-score, continuing until she completed SUMMA. Her SUMMA scientific internship landed her in the lab of dr. Sluijter, at the University Medical Center Utrecht, as she had a particular interest in stem cell therapy. During this internship she started exploring the interactions between stem cells and the immune system. The work performed here led to an Alexandre Suerman stipendium, allowing her to continue her line of research after graduating in 2011.

For over four years she investigated the interactions between different stem cells and the immune system, and tried to figure out how all of this could aid in cardiac inflammation. After many happy years in the lab, she could no longer resist the call of the clinic. Frederieke is currently working in the Cardiology department of the Isala Ziekenhuis in Zwolle.