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Abbreviations and acronyms

7-AAD	7-Amino-Actinomycin D
α-MHC	α-myosin heavy chain
ANF	atrial natriuretic factor
AT	angiotensin
BSA	bovine serum albumin
bFGF	basic fibroblast growth factor
CAR	coxsackievirus-adenovirus receptor
cDNA	complementary deoxyribonucleic acid
CSC	cardiac stem cell
CHO	Chinese hamster ovarian
CVB	coxsackievirus
DAF	decay accelerating factor
DAN	2,3-diaminonaphthalene
DCF	2',7'-di-chlorofluorescein
DCM	dilated cardiomyopathy
DMEM	Dulbecco's Modified Eagled Medium
DNA	deoxyribonucleic acid
eNOS	endothelial nitric oxide synthase
ERK1/2	extracellular signal-regulated kinase 1/2
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
HSC	hematopoietic stem cell
IFN	interferon
IDO	indoleamine 2,3-dioxygenase
IL	interleukin
iNOS	inducible nitric oxide synthase
i.v.	intravenously
L-NAME	N-omega-nitro-arginine methyl ester
LV	left ventricle
mRNA	messenger ribonucleic acid
MSC	mesenchymal stem cell
MMP	matrix metalloprotease
MTS	3-(4,5-dimethylthiazol-2-yl) -5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
NaOH	sodium hydroxide
NF-κB	nuclear factor-kappa B
NK	natural killer
NO	nitric oxide
NOS	nitric oxide synthase
p38MAPK	p38 mitogen-activated protein kinase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PES	phenazine ethosulfate
PGE	prostaglandin
PS	phosphatidylserine
RNA	ribonucleic acid
ROS	reactive oxygen species

RT-PCR	reverse transcription-polymerase chain reaction
SEM	standard error of the mean
STC-1	stanniocalcin-1
TGFβ	transforming growth factor-beta
TIMP-1	tissue inhibitor of metalloproteinase-1
TRAIL	tumor necrosis factor-related
UPS	ubiquitin proteasome system

CHAPTER ONE: INTRODUCTION

1.1. Inflammatory cardiomyopathy

1.1.1. Definition and incidence of (dilated) inflammatory cardiomyopathy

Cardiomyopathies are a group of disorders of the heart, which are associated with ventricular dysfunction and characterized as primary cardiomyopathies if the origin of contractile dysfunction is unknown and as secondary or specific cardiomyopathies if the heart is affected in association with specific infections or immunological, metabolic, neuromuscular or toxic diseases. Dilated cardiomyopathy (DCM) is one of the most common cardiomyopathy entities, the leading cause of heart failure, and contributes to the main mortality rate of cardiomyopathy.[1] The incidence of DCM is 5-10/100 000 persons. However, the „real“ incidence is probably much higher, since many patients with DCM die from sudden death, without having been clinically diagnosed.[2]

Patients who suffer from DCM have a five-year survival rate of 55% under contemporary heart failure treatment and the rate of morbidity of DCM is 36.5/100,000.[3] Inflammatory cardiomyopathy, which is characterized by idiopathic heart failure with evidence of immunocompetent infiltrates and cell adhesion molecule abundance in endomyocardial biopsy, is a specific cardiomyopathy entity, diagnosed in approximately 50% of DCM patients.[4]

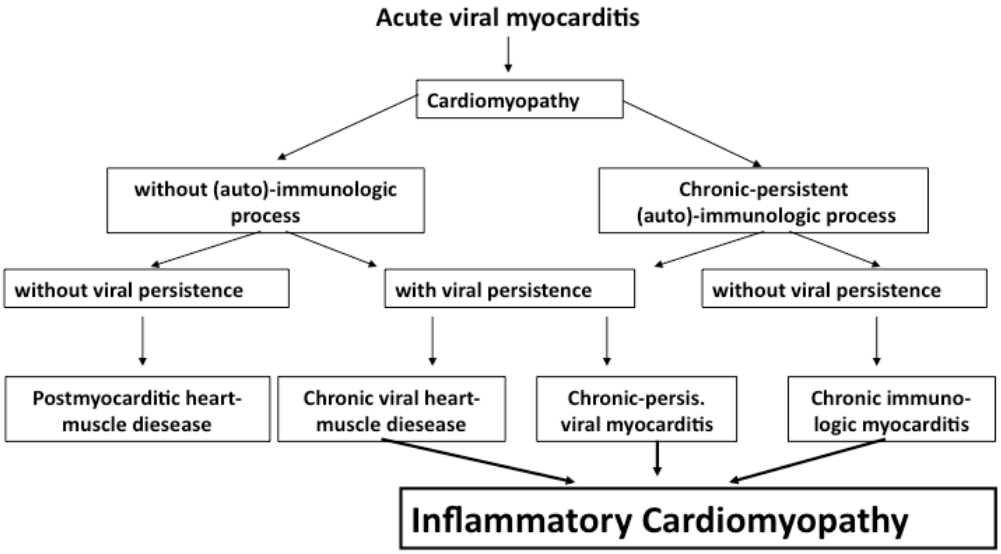


Figure 1.1. New classification of dilated cardiomyopathy (D'Ambrosio A et al., Heart. 2001; 85: 499-504)

1.1.2. Virus-induced inflammatory cardiomyopathy

In myocarditis and inflammatory cardiomyopathy, chronic myocardial damage is induced either by continued immune activity (autoimmunity) despite viral clearance, or with viral persistence despite activation of cellular and humoral defense immune mechanisms.

There conceptually exist 3 stages of viral myocarditis leading to DCM. The initial phase is viral, where the virus infects a susceptible host, and is able to engage the receptor and mediate viral proliferation. The appropriate host immune response will clear the virus and the infected cells. However, in the setting of inappropriate host immune controls, the inflammatory process will be unable to effectively clear the virus, leading to continued inflammation. The presence of additional viral proteins and damaged host tissue will expose the immune system to additional sources of antigen, and stimulate continued proliferation of inflammatory cells of the acquired immunity (T and B cells), as well as innate immunity. Finally, the inflammatory process including cytokine and matrix metalloproteinases (MMPs) will alter the structure of the myocardium to such an extent that DCM eventually results.[5]

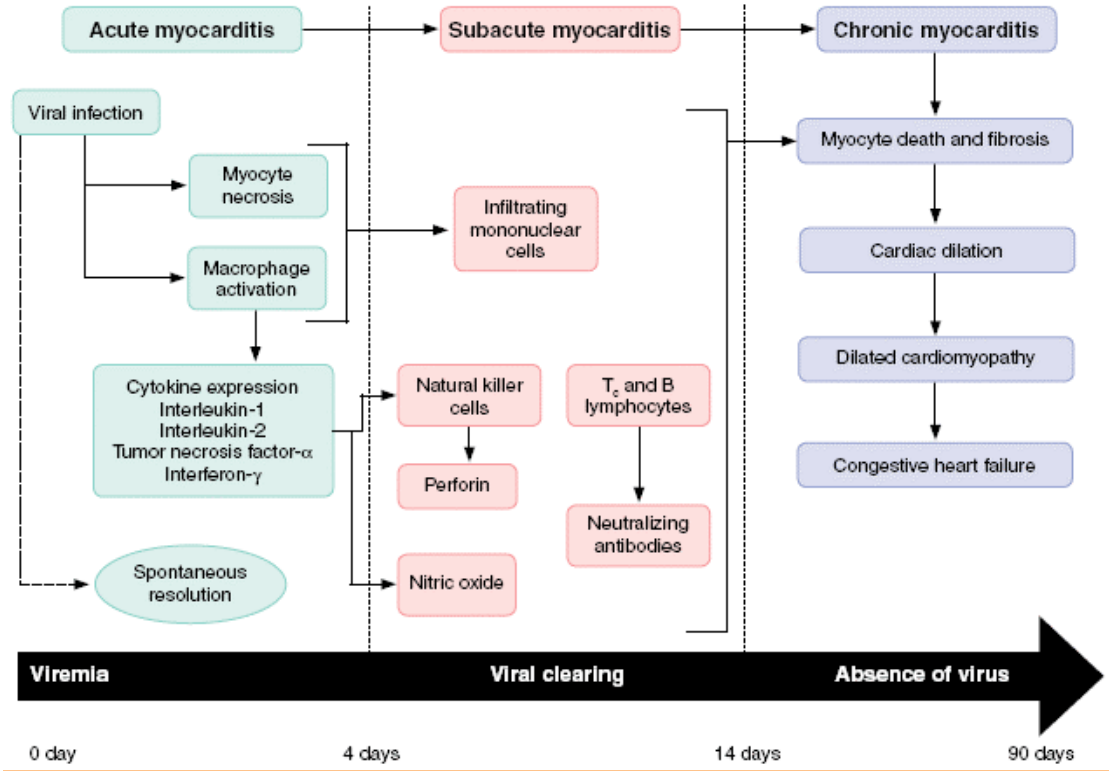


Figure 1.2. Treatment of viral myocarditis caused by coxsackievirus (Brunetti L et al.,

Many different infectious agents have been considered as the cause of viral myocarditis, including enteroviruses, adenovirus, cytomegalovirus, hepatitis C virus, parvovirus B19 and others. Among the most commonly identified infectious agents are the Coxsackie B viruses, members of the enteroviral family. Identification of enteroviral RNA and antigen of myocardial tissue provides evidence that enteroviruses are associated with DCM.

It has been reported that virus infection can cause a subset of cardiomyopathies in the form of acute myocarditis or chronic DCM. However, among the cardiotropic viruses, coxsackievirus is the most thoroughly studied virus that can induce acquired cardiomyopathies. Due to the lack of knowledge of exact pathogenesis of the highly variable natural course of inflammatory cardiomyopathy, the specific treatment is still a difficult issue.

1.1.3. Coxsackievirus

1.1.3.1. Coxsackievirus

Coxsackieviruses are divided into group A and group B viruses based on early observations of their pathogenicity in mice. At least 23 serotypes (1-22, 24) of group A and 6 serotypes (1-6) of group B are recognized.[6] In general, group A coxsackieviruses tend to infect the skin and mucous membranes, causing herpangina, acute hemorrhagic conjunctivitis, and hand-foot-and-mouth disease; Group B coxsackieviruses tend to infect the heart, pleura, pancreas, and liver, causing pleurodynia, myocarditis, pericarditis, and hepatitis. Both group A and group B coxsackieviruses can cause nonspecific febrile illnesses, rashes, upper respiratory tract disease, and aseptic meningitis.

The six serotypes of the group B coxsackieviruses (CVB1 to CVB6) are enteroviruses in the picornavirus family. The CVB genome is 7,400 nucleotides in length. The single open reading frame encodes 11 proteins and is flanked by non-translated regions. The 5'-terminal nucleotide is linked to a virus-encoded protein, and the 3' terminus is completed with a polyadenosine tail.[7]

30 nm. The structure of the B3 virus determined to 3.5 A resolution,[8,9] each of the 12 pentamers on the icosahedron is surrounded by a canyon which is the binding site on the picornavirus capsid for specific cellular receptor molecules.[10] The capsid is made up of 60 identical building units, each containing one copy of the four structural proteins VP1-VP4. The folding pattern of polypeptides VP1-VP3 is similar, resulting in an eight-stranded antiparallel b-barrel structure.

Once translation begins, host-cell protein synthesis is rapidly shut off, and the viral genome is translated into a large polyprotein. These include the proteins destined for incorporation into new virus capsids, as well as the RNA-dependent RNA polymerase and other enzymes involved in genome replication.

1.1.3.2. Coxsackievirus-adenovirus receptor and co-receptor

Coxsackievirus-adenovirus receptor (CAR) is a critical determinant for cellular uptake and pathogenesis of coxsackievirus and adenovirus. Non-permissive hamster cells have been shown to become susceptible to CVB attachment and infection when transfected with CAR complementary DNA[11]. In addition, it has been demonstrated that recombinant expression of CAR increases the adenoviral uptake in rat cardiomyocytes.[12]

CAR is a 46kDa integral membrane protein with a typical transmembrane region. It is structurally and functionally similar to the junctional adhesion molecules. CAR is an immunoglobulin superfamily protein, containing two immunoglobulin superfamily domains: an amino-terminal V-like module and a C2-like module.

In mammalian cells, CAR co-receptors determine the efficiency of host cell targeting by coxsackie and adenoviruses. CVB utilizes the complement-deflecting protein-decay-accelerating factor (DAF, CD55) as its co-receptor[13]; adenoviruses use the integrin αvβ1 as co-receptor.[14] Monoclonal antibody blockade, immunoprecipitation, and DNA transfection were used to identify DAF as a major cell attachment receptor for coxsackieviruses B1, B3, and B5.[15] DAF facilitates the viral internalization by CAR by significantly increasing the binding efficiency of coxsackievirus onto the DAF-CAR

from studies performed with anti-DAF antibodies which have been successful in blocking virus infection of susceptible cells *in vitro*.[16,17]

1.1.4. Coxsackievirus-induced inflammatory cardiomyopathy

Enterovirus (coxsackievirus) infections have been reported to be an important cause of morbidity and mortality during the natural course of DCM. Enterovirus (coxsackievirus) and adenovirus have been confirmed as cardiotropic viruses for myocarditis and DCM /inflammatory cardiomyopathy. CVB is the most commonly identified pathogen in patients with myocarditis and DCM, having been implicated in more than 50% of cases with infectious etiology.[18] These viruses enter the host primarily through gastrointestinal or respiratory tract and can reside in the reticulo-endothelial system in terms of extra-cardiac reservoir. Via viral specific proteases, such as coxsackieviral protease 2A, CVB3 can cleave dystrophin, resulting in the disruption of the cytoskeleton, contributing hereby to DCM.[19]

1.1.5. Importance of nitric oxide and interferon gamma in Coxsackievirus B3-induced inflammatory cardiomyopathy

Nitric oxide (NO) is a heterodiatomic free radical that can induce a variety of intra- and intercellular physiological actions.[20,21]

NO plays a critical role in the innate response by viral myocarditis. Coxsackievirus infection induces cardiac NO synthase (NOS) activity.[22,23] iNOS is expressed in the myocardium 3 days after infection, peaks 5 to 7 days after infection and disappears 14 days after infection.[24]Mice that lack iNOS have higher viral titers and viral RNA compared with wild-type mice and develop a much more severe myocarditis after coxsackievirus infection than infected wild-type mice.[24] NO can not only inhibit viral replication, [25,26] but also inhibit the cardiac dysfunction caused by cleavage of dystrophin and other cellular substrates.[27]

Interferons (IFNs) were discovered to be a natural defense system in the human body. This family of cytokines regulates anti-viral, anti-tumor, and immune responses and

stimulate apoptosis by inducing the expression of multiple genes that create a pro-apoptotic environment.[29] Myocarditis is much more pronounced in IFN-γ-receptor deficient mice compared to wild-type mice, which is associated with an impaired iNOS induction. [30] Taken together, these data demonstrate the importance of NO and IFN-γ in the defense against CVB3-induced myocarditis.

1.2. Stem cell therapy

1.2.1. Definition of stem cells

Stem cells are cells that have the ability to divide for indefinite periods, often throughout the life of the organism. Under the right conditions, or given the right signals, stem cells can give rise to the many different cell types that make up the organism. That is, stem cells have the potential to develop into mature cells that have characteristic shapes and specialized functions, such as heart cells, skin cells, or nerve cells.

Stem cells contribute to the body's ability to renew and repair its tissue. Unlike mature cells, which are permanently committed to their fate, stem cells can both renew themselves as well as create new cells of whatever tissue they belong to (and other tissues).[31,32]

1.2.2. Adult stem cells

The adult stem cell is an undifferentiated (unspecialized) cell that is found in a differentiated (specialized) tissue; it can renew itself and become specialized to yield all of the specialized cell types of the tissue from which it originated. Adult stem cells are capable of self-renewal for the lifetime of the organism. Sources of adult stem cells have been found in the bone marrow,[33] blood stream, cornea and retina of the eye[34], the dental pulp of the tooth,[35] liver,[36] skin,[37] gastrointestinal tract,[38] and pancreas.[39] Unlike embryonic stem cells, at this point in time, there are no isolated adult stem cells that are capable of forming all cells of the body. That is, there is no evidence, at this time, of an adult stem cell that is pluripotent.

Adult stem cells, like all stem cells, have two characteristics. First, they

make identical copies of themselves for a long period of time; this ability to proliferate is referred to as long-term self-renewal. Second, they can give rise to mature cell types or types before they achieve their fully differentiated state. The intermediate cell is called a precursor or progenitor cell. Progenitor or precursor cells in fetal or adult tissues are partly differentiated cells that divide and give rise to differentiated cells. Such cells are usually regarded as "committed" to differentiating along a particular cellular development pathway, although this characteristic may not be as definitive as once thought.[40]

Unlike embryonic stem cells, which are defined by their origin (the inner cell mass of the blastocyte), adult stem cells share no such definitive means of characterization. In fact, no one knows the origin of adult stem cells in any mature tissue. Some have proposed that stem cells are somehow set aside during fetal development and restrained from differentiating. Definitions of adult stem cells vary in the scientific literature, ranging from a simple description of the cells to rigorous set of experimental criteria that must be met before characterizing a particular cell as an adult stem cell.[41] Most of the information about adult stem cells comes from studies of mice. The list of adult tissues reported to contain stem cells is growing and includes bone marrow, peripheral blood, brain, spinal cord, dental pulp, blood vessels, skeletal muscle, epithelia of the skin and digestive system, cornea, retina, liver, and pancreas.

Ideally, adult stem cells should also be clonogenic. In other words, a single adult stem cell should be able to generate a line of genetically identical cells, which then gives rise to all the appropriate, differentiated cell types of the tissue in which it resides. Again, this property is difficult to demonstrate *in vivo*; in practice, scientists show either that a stem cell is clonogenic *in vitro*, or that a purified population of candidate stem cells can repopulate the tissue.[42]

An adult stem cell should also be able to give rise to fully differentiated cells that have mature phenotypes, are fully integrated into the tissue, and are capable of specialized functions that are appropriate for the tissue. The term phenotype refers to all the observable characteristics of a cell (organism); its shape (morphology); interactions

proteins that appear on the cell surface (surface markers); and the cell's behavior (e.g., secretion, contraction, synaptic transmission).[43]

1.3. Mesenchymal stem cells

1.3.1. Characteristics of mesenchymal stem cells

Mesenchymal stem cells (MSCs), which can be alternatively referred to as multipotent mesenchymal stromal cells or marrow stromal cells, are a heterogeneous population of cells which can proliferate *in vitro* as plastic-adherent cells, have fibroblast-like morphology, form colonies *in vitro* and can differentiate into bone, cartilage, adipose and stromal tissues.[44,45] MSCs are positive for many characteristic markers including CD29, CD44, CD71, CD90, CD106, CD120a, CD124, SH2, SH3 and SH4, and negative for CD14, CD34, and CD45, which are specific markers of hematopoietic stem cells.[46] Most surface markers have been found inadequate as a means to identify stem cells, because the putative markers may also be found on non-stem cells, or a particular marker may only be expressed on a stem cell at a certain stage or under certain conditions, such as with CD34 on hematopoietic stem cell (HSC). Nevertheless, surface markers or other attributes are useful in characterizing the stem cell as isolated or cultured, and as means to begin to understand its potential interactions with neighboring cells and the cell environment. To further identify the MSC, differentiation assays need to be performed. Differentiation into osteocyte can be detected by alkaline phosphatase staining;[47] differentiation into adipocyte can be evaluated by Oil red O staining;[48] differentiation into chondrocyte can be assessed by safranin O staining.[49]

MSCs are rare in bone marrow, representing ～1 in 10000 nucleated cells. It is isolated from the marrow by a simple process involving Ficoll centrifugation and adhering-cell culture in defined serum-containing medium. The resulting cells, while not immortal, have the ability to expand many times in culture, and keep their growth and multilineage potential (Figure 1.3.).

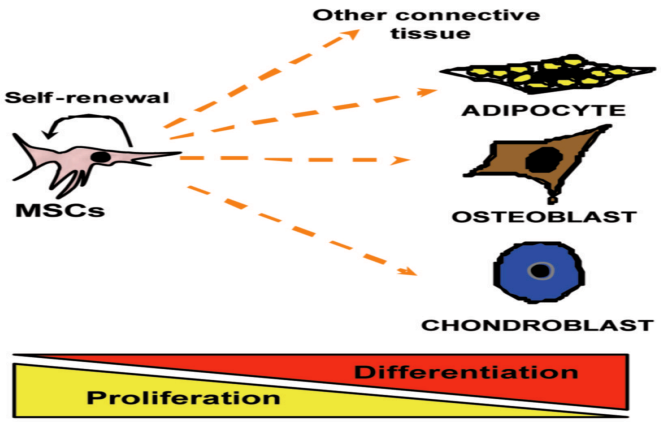


Figure 1.3. Models of mesenchymal stem cell differentiation (Baksh D et al., J Cell Mol Med. 2004; 8: 301-316)

1.3.2. Isolation of mesenchymal stem cells

To date, MSCs have been isolated from many tissues including marrow, muscle, fat, skin, cartilage, hair follicle and bone, although bone marrow is used more often.

For human beings, bone marrow can be obtained by needle from the iliac with local anesthetic; for small mammals such as rabbits, access to the bone marrow in the long bones is possible by a surgical cut-down, and using relatively stiff tubing placed over the end of the aspiration needle to gain access to marrow further down the bone shaft; for rodents, it is necessary to sacrifice the animal(s), dissect the long bones, and the bones of 10 rats or 20 mice can be processed as a single preparation. The ends of the femur or tibia are removed with sterile wire-cutter-type nippers and the bone marrow is extruded into a collection dish using a needle and a saline-filled syringe.

MSCs can be isolated by a variety of procedures. There are two common methods, the direct plating method and the density centrifugation method.[50] Both of them produce very similar populations of MSCs.

Adipose tissue, like bone marrow, is derived from the mesenchyme and contains a supportive stroma that is easily isolated.[51] Compared with bone marrow-derived MSCs, adipose tissue-derived stromal cells do have an equal potential to differentiate into cells and tissues of mesodermal origin, such as adipocytes, cartilage, bone, and skeletal muscle. However, the easy and repeatable access to subcutaneous adipose

tissue and the simple isolation procedures provide a clear advantage.

1.3.3. Cardiac protective effects of mesenchymal stem cells

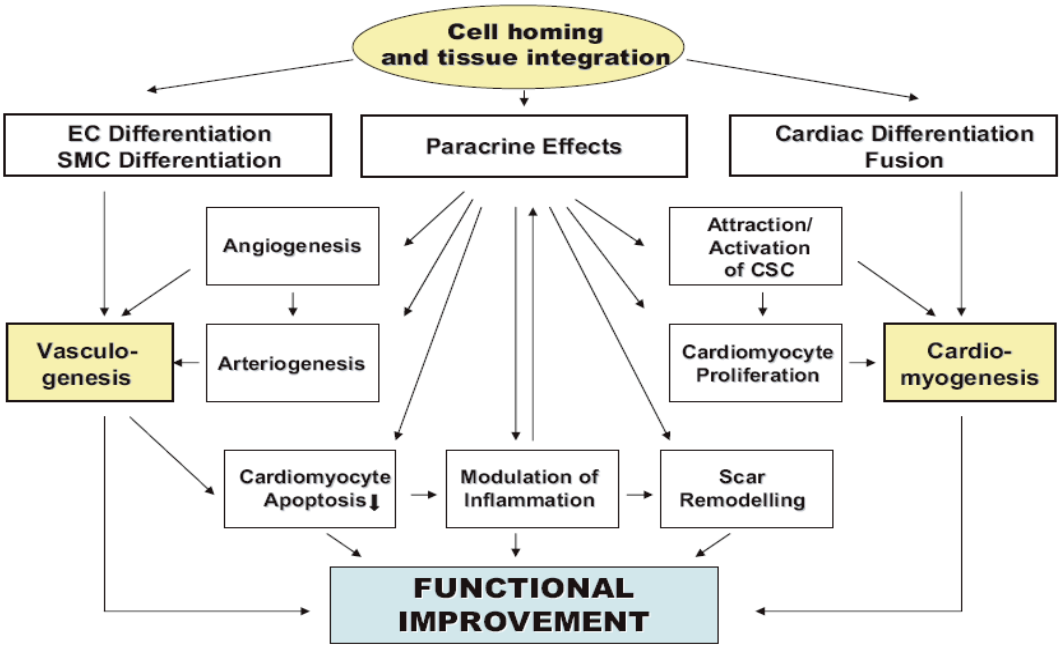


Figure 1.4. Proposed mechanisms of action of stem/progenitor cells in cardiovascular repair (Dimmeler S et al., Arterioscler Thromb Vasc Biol. 2008; 28: 208-216)

MSCs can be used allogeneically and differentiate into a cardiomyocyte-like phenotype when implanted in healthy myocardium. As opposed to the muscle precursor cells, allogeneic MSCs have the ability to be used immediately after acute injury; furthermore, MSCs appear to have the ability to home to the site of myocardial injury when administered intravenously after acute infarction. Engrafted MSCs differentiate toward a myogenic lineage after injection into the infarcted myocardium as evidenced by expression of muscle-specific proteins including α-actinin, troponin-T, tropomyosin, myosin heavy chain, phospholamban, and other muscle-specific proteins. Furthermore, the presence of connexin-43, a protein responsible for intracellular connection and electrical coupling between cells, suggests cardiomyocyte differentiation.[52] MSC implantation has been associated with significant functional improvements including prevention of pathologic wall thinning and improved post-infarcted hemodynamics in

cardiomyocytes and endothelial cells, it is believed that the cardiac-protective effects of MSC are predominantly due to facilitating endogenous repair processes including anti-fibrotic, anti-inflammatory, anti-apoptotic and pro-angiogenic processes via the paracrine actions of MSC.

Anti-fibrotic effects

Ohnishi et al.[54] demonstrated that cardiac fibroblast proliferation was slower when they were cultured in MSC-conditioned medium instead of cardiac fibroblast-conditioned medium. In parallel, the mRNA expression of collagen I as well as collagen III was downregulated when cardiac fibroblasts were cultured in MSC-conditioned medium. Guo et al.[55] reported that MSC transplantation in a rat model of myocardial infarction inhibited the deposition of types I and III collagen. Xu et al.[56] found that there was a marked up-regulation of collagen types I and III, tissue inhibitor of metalloproteinase-1 (TIMP-1) and transforming growth factor-beta (TGF-β) mRNA expression in myocardial infarcted rats. MSC transplantation significantly attenuated mRNA expression of the extracellular matrix genes, could inhibit left ventricle remodeling and improve heart function. Taken together, MSCs exert anti-fibrotic effects at least in part through regulation of cardiac fibroblasts proliferation and transcriptional downregulation of types I and III collagen, TIMP-1 and TGF-β. The anti-fibrotic features of MSC may be beneficial for the treatment of heart failure in which these specific fibrotic changes are involved, including myocardial infarction and DCM. In addition, Li et al.[57] showed that MSC transplantation can attenuate myocardial fibrosis in a rat model of global heart failure, and this may be at least partially mediated by paracrine signaling from MSCs via anti-fibrotic factors such as hepatocyte growth factor (HGF).

Anti-inflammatory / immunomodulatory effects

An emerging body of evidence indicates that MSC possess immunomodulatory properties, affecting T cells,[58] dendritic cells,[59] B cells,[60] and natural killer

which MSCs make physical contact with allogeneic tissue and release soluble factors, including interleukin-10 (IL-10), TGF-beta, Indoleamine 2,3-dioxygenase (IDO)[62] and prostaglandin E2. Moreover, the inflammatory environment/condition plays an important role in the ability of MSCs to exert their immunosuppressive effects. Ren et al. recently demonstrated the importance of IFN-γ in the induction of immunosuppression of T cells by MSCs.[58] Treatment of MSCs with IFN-γ decreases their susceptibility to natural killer-cell mediated lysis due to up-regulation of HLA class I molecules.[63] The immunosuppressive effects of MSC have also been demonstrated to be NO-dependent.[58]

Iyer et al.[64] reported that administration of MSC to animal models of lung injury attenuated inflammation and injury, revealing a central role for MSC in mitigating pro-inflammatory networks and amplifying anti-inflammatory signals. Molina et al.[65] demonstrated that in a model of pressure overload hypertrophy, intracoronary delivery of MSC during heart failure was associated with an improvement in hemodynamic performance, maximal exercise tolerance, systemic inflammation, and left ventricular reverse remodeling. Guo et al.[55] showed that the anti-inflammatory effects of MSCs might partly account for the cardiac protective effect in ischemic heart disease. From these data, the therapeutic effectiveness of MSC occurs in part through the release of anti-inflammatory cytokines, which promote the repair and protection of damaged tissues.

Anti-apoptotic and pro-angiogenic effects

Li et al.[66] reported that intramyocardial transplantation of MSC reduced apoptosis in the diabetic myocardium, indicated by a decrease in caspase 3 activity and an increased ratio of anti-apoptotic Bcl-2 to pro-apoptotic Bax expression, resulting in improved cardiac function. Schuleri et al.[67] demonstrated that MSC-treated animals showed larger, more mature vessels and less apoptosis in the infarct zone and improved regional and global LV function. Guo et al.[68] showed that overexpression of HGF in transplanted MSC improved ischemic cardiac function through angiogenesis and

demonstrated an improvement in LV function in a porcine model of myocardial infarction after intravenous MSC application, which was associated with an increase in von Willebrand factor-positive vessels and increased tissue vascular endothelial growth factor expression in the scar zone.

The transplantation of MSC has also been successfully used to repair injury in kidney, liver, lung, and brain models. Also here, the protective effects of MSC have been addressed to their anti-fibrotic, anti-inflammatory and anti-apoptotic characteristics. It was reported by Semedo et al.[70] that MSC have the potential to modulate renal ischemia and reperfusion injury via earlier regeneration of damaged renal tissue by an anti-inflammatory pattern. Abdel Aziz et al.[71] showed that MSC reduce liver fibrosis through their effect of minimizing collagen deposition in addition to their capacity to differentiate into hepatocytes. Gupta et al.[72] demonstrated that intrapulmonary MSC delivery resulted in a down-regulation of proinflammatory responses to endotoxin while increasing the anti-inflammatory cytokine IL-10. Iwanami et al.[73] reported that stimulation of angiotensin 2 (AT_2) receptor signaling in MSC played a pivotal role in the contribution of MSC treatment to brain protection after focal brain ischemia-reperfusion injury.

Finally, MSCs can be transduced by a variety of vectors and retain transgene expression after *in vivo* differentiation. Transgene expression by MSCs may be ultimately used to augment cell engraftment or the extent of differentiation. (Figure1.4.) Mangi et al. used a retroviral vector to overexpress the pro-survival gene Akt in MSCs before implantation in infarcted rat myocardium. Akt protein overexpression enhanced MSCs survival and prevented pathologic remodeling after infarction with impressive improvement in cardiac output.[74]

Overall, the current data indicate that although MSCs were first suggested as therapeutic tool in regenerative medicine on the basis of their stem-cell-like / differentiation qualities, their therapeutic effects can result from other characteristics, such as their anti-fibrotic, anti-inflammatory, anti-apoptotic and pro-angiogenic

cytokines and anti-fibrotic and anti-apoptotic molecules that promote the repair and protection of damaged tissues. MSCs represent an ideal stem cell source for cell therapies because of their easy purification and amplification and their multipotency. Taken together, the characteristics of MSC make them attractive tools for the treatment of heart disease.

1.4. Prospects and challenges

Current therapeutic approaches are primarily focused on limiting disease progression rather than repair and restoration of healthy tissue and function. In this perspective, stem cell therapy seems to be a potential new therapeutic strategy to achieve cardiac repair. Few questions in cardiac regeneration are definitively resolved, but it is widely agreed that the regenerative capacity of the human myocardium itself is grossly inadequate to compensate the severe loss of heart muscle presented by myocardial infarction or other myocardial diseases.

Although stem cell therapy has provided a broad prospect for cardiovascular diseases, several challenges still remain to be solved. First, perhaps the most attractive aspect of current progress towards cardiac regeneration, is the wide variety of cell types that have been considered as candidates for the therapeutic delivery in humans. The ideal cell type has not yet emerged, and few studies have directly investigated different stem cell types including myoblasts grown from skeletal muscle,[75] smooth muscle cells from blood vessels,[76] and HSCs or MSCs.[77] Second, the optimal route of delivery has to be determined. In the clinical setting, cells can be injected intravenously, intracoronary or transendocardially. The ultimate goal is the delivery of enough cells to the injured myocardium that will survive to restore cardiac function. Here, the engraftment of the cells is of importance, which is repressed due to the hostile microenvironment: the presence of fibrotic tissue, which is a physical barrier for the injected cells; the presence of oxidative stress and the reduced oxygen supply, which is hampered due to decreased angiogenesis. A reduction of MSC in the graft overtime has been reported.[78] In contrast, overexpression of SDF in MSC has been shown to reduce

the myocardial infarcted heart.[79] Lastly, transplantation of stem cells because of their paracrine effects remains a reasonable strategy since the beneficial paracrine factors remain unidentified and because multiple factors might be functioning synergistically. If improved cardiac function is due to paracrine mechanisms, identification of these cell-derived paracrine factors could lead to effective therapies, avoiding risks of stem cell transplantation complications.

Treatment of damaged myocardium by stem cell therapy is becoming an increasingly promising therapeutic approach. Ideally, the donor cells should be amplified efficiently in culture and would lead to regeneration of damaged myocardial tissue, including cardiogenic differentiation with local angiogenesis. Autologous cells exhibit a high proliferative potential *in vitro* and share a low potential for transplantation complications. However, the transplantation of autologous cells to repair the heart also has serious drawbacks; this procedure may delay the treatment because of intensive lab work. The ideal cell to treat the heart should be transplantable without delay to any patient without serious transplantation complications. In this regard, MSC have a main advantage over other cells, since due to their low immunogenicity, also allogeneic MSC can be used.[80]

1.5. OBJECTIVES

Under current heart failure treatment, the prognosis of DCM is poor, urging for alternative therapeutic strategies. Current pharmacological treatment is mainly focused on decreasing the activity of the neuroendocrine system. For viral-induced inflammatory DCM, additional pharmaca, blocking viral replication or stimulating the anti-viral directed immune response are used. These agents are not cardiac-specific and can be associated with negative side-effects. There is growing experimental evidence supporting the application of cellular transplantation as a strategy to improve myocardial function. MSC have immunomodulatory features and have been experimentally shown to be attractive tools for the treatment of myocardial infarction and cardiomyopathies. The present study focuses on investigating whether MSC are potential agents for the treatment of CVB3-induced inflammatory DCM. In view of clinical translation, safety aspects, including the possibility of MSC being infected by CVB3, are investigated. In addition, the study investigates whether and how MSC, independently of their immunomodulatory effects, i.e. in the absence of immunoregulatory cells, can have a direct protective effect on CVB3-induced cardiomyocyte apoptosis and oxidative stress.

CHAPTER TWO: MATERIALS AND METHODS

2.1. Materials

2.1.1. Laboratory equipments

Balance	Mettler Toledo, Giessen, Germany
BD FACSCalibur System	BD Sciences, Heidelberg, Germany
Centrifuge, 5415D,5415R,5810R	Eppendorf AG, Hamburg, Germany
Freezer, -80 °C	Thermo Forma, Marlotta, USA
Gelaire Laboratories Flow Hood	Labexchange, Burladingen, Germany
Leica DMI 4000B	Leica Microsystems, Wetzlar, Germany
Leica DMRBE	Leica Microsystems, Bensheim, Germany
NanoDrop™ 1000	Thermo Scientific, Wilmington, USA
Pipettes, adjustable	Eppendorf AG, Hamburg, Germany
SpectraMax Gemini microplate reader	Molecular Device GmbH, Munich, Germany
Thermocycler	Eppendorf AG, Hamburg, Germany
VersaMax microplate reader	Molecular Device GmbH, Munich, Germany
Vortexer	Beyer GmbH, Düsseldorf, Germany
7900HT Fast Real-time PCR System	Applied Biosystems, Foster City, USA

2.1.2. Chemicals, reagents, kits, media, and primers

Chemical and reagents:

Agarose	Gibco, Karlsruhe, Germany
Ascorbic acid	Sigma, Steinheim, Germany

BD FACSFlow™	BD Sciences, Franklin Lakes, USA
Bovine serum albumin (BSA)	Sigma, Steinheim, Germany
Chloroform	Sigma, Steinheim, Germany
5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acety ester (CM-H_2DCFDA)	Invitrogen, Heidelberg, Germany
Ethanol, 100%	Sigma, Deisenhofen, Germany
Fetal bovine serum (FBS)	Gibco BRL, Karlsruhe, Germany
Fibroblast Growth Factor-basic (bFGF)	PeproTech, Rocky Hill, USA
Fibronectin from bovine plasma	Sigma, Steinheim, Germany
Gelatin from bovine skin	Sigma, Steinheim, Germany
Glutamine	Sigma, Steinheim, Germany
Goat serum	PAA, Pasching, Austria
HEPES sodium salt	Sigma, Steinheim, Germany
Isopropanol	Merck, Darmstadt, Germany
M-MLV reverse transcriptase	Invitrogen, Karlsruhe, Germany
Nitro-L-Argininmethylesterhydrochloride (L-NAME)	Sigma, St. Louis, USA
Noble agar	Difco, Lawrence, USA
Norepinephrine	Sigma, Steinheim, Germany
Penicillin/streptomycin, 1%	Invitrogen, Grand Island, USA
Percoll gradient (1.073 g/ml)	Biochrom, Berlin, Germany
Phosphate buffered saline (PBS)	Biochrom, Berlin, Germany
Anti-mouse Interferon-gamma (IFN-γ) antibody	R&D systems, Minneapolis, USA
Trizol	Invitrogen, Heidelberg, Germany
Trypsin-EDTA	Invitrogen, Heidelberg, Germany
Vybrant® Dil Cell-labeling	Invitrogen, Heidelberg, Germany

solution	
Vybrant® DiO Cell-labeling solution	Invitrogen, Heidelberg, Germany
β-Mercaptoethanol	Sigma, Deisenhofen, Germany

Kits and other materials

BD Falcon™ Cell Culture Flasks	BD Sciences, Franklin Lakes, USA
BD Falcon™ Polypropylene Conical Tubes (15ml, 50ml)	BD Sciences, Franklin Lakes, USA
BD Falcon™ Polystyrene Round-Bottom Tubes (5ml)	BD Sciences, Franklin Lakes, USA
Cell Culture Plates (6-well, 96-well)	BD Sciences, Franklin Lakes, USA
CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay Kit	Promega, Madison, USA
High Capacity cDNA Reverse Transcription Kit	Applied Biosystems, Foster City, USA
MicroAmp optical 96-well reaction plates	Applied Biosystems, Foster City, USA
Nitric Oxide Assay Kit	Calbiochem, Darmstadt, Germany
NucleoSpin RNA II Kit	Macherey-Nagel, Düren, Germany
Polypropylene tubes (15 ml and 50 ml)	Greiner Labortechnik GmbH, Frickenhausen, Germany
qPCR MasterMix Plus for SYBR® Green I dNTP	Eurogentec, San Diego, USA

Media

Claycomb medium	SAFC Biosciences, Kansas, USA

Dulbecco's Modified Eagled Medium (DMEM)11966	Invitrogen, Heidelberg, Germany
Dulbecco's Modified Eagled Medium	Sigma, Steinheim, Germany
Ham´s F12	PAA, Pasching, Austria
Lung/Cardiac Fibroblast Basal Medium	Cell Applications, Inc. San Diego, USA
MEM2×	Gibco, Karlsruhe, Germany
RPMI 1640	Invitrogen, Heidelberg, Germany

Primers

L32 Murine	FOR 5´-TGCCCACGGAGGACTGACA-3´
	REV 5´-AGGTGCTGGGAGCTGCTACA-3´
L32 Human	FOR 5´-AGGAGAGACACCGTCTGAACAAG-3´
	REV 5´-GAACCAGGATGGTCGCTTTC-3´
CVB3	FOR 5´-CCCTGAATGCGGCTAATCC-3´
	REV 5´-ATTGTCACCATAAGCAGCCA-3´

2.1.3. Cell types and cultured medium

Cardiac Fibroblasts	Lung/Cardiac Fibroblast Basal Medium
Tebu-bio, Offenbach, Germany	Growth Supplement
CHO CAR^+	Ham´s F12 medium
kindly provided by Dr. M. Anders	10 % fetal bovine serum
	1 % penicillin/streptomycin
CHO CAR^-	Ham´s F12 medium
kindly provided by Dr. M. Anders	10 % fetal bovine serum
	1 % penicillin/streptomycin

HeLa cells	RPMI 1640
DSMZ, Braunschweig, Germany	10 % fetal bovine serum
	1 % penicillin/streptomycin
HL-1	Claycomb medium
kindly provided by Prof. U. Rauch	10 % fetal bovine serum
	1 % penicillin/streptomycin
	1 % norepinephrine
	2 mM glutamine
MSCs	Dulbecco's Modified Eagle's Medium
	10 % fetal bovine serum
	1 % penicillin/streptomycin
	2 % HEPES
	2 mM glutamine
	2 ng/ml basic fibroblast growth factor
RMCB	RPMI 1640
kindly provided by Dr. M. Anders	10 % fetal bovine serum
	1 % penicillin/streptomycin

2.1.4. FACS antibodies

Annexin V-PE Apoptosis Detection Kit	BD Sciences, Franklin Lakes, USA
Cy3 goat anti-mouse antibody	Dianova, Hamburg, Germany
FITC mouse anti-Human CD55	BD Biosciences, Franklin Lakes, USA
FITC IgG2A isotype control	BD Biosciences, Franklin Lakes, USA
FITC mouse anti-Human CD44	BD Pharmingen, Heidelberg, Germany
FITC mouse anti-Human CD45	BD Pharmingen, Heidelberg, Germany
FITC mouse anti-Human CD90	BD Pharmingen, Heidelberg, Germany
FITC mouse anti-Human CD105	Acris Antibodies, Hiddenhausen, Germany

PE mouse anti-Human CD14	BD Pharmingen, Heidelberg, Germany
PE mouse anti-Human CD34	BD Pharmingen, Heidelberg, Germany
PE mouse anti-Human CD73	BD Pharmingen, Heidelberg, Germany
PE mouse anti-Human CD166	BD Pharmingen, Heidelberg, Germany

2.1.5. Software

CellQuest	BD Sciences, Franklin Lakes, USA
Primer Express™ Version 2.0.0	Applied Biosystems, Foster City, USA
Instat 3.0.a.	GraphPad Software, Inc., La Jolla, USA

2.2. Methods

2.2.1. Cell culture

Isolation and culture of human mesenchymal stem cells

Human adult MSCs were isolated from iliac crest bone marrow aspirates of normal donors. In brief, aspirates (3–5 ml) were washed twice with phosphate buffered saline (PBS), and resuspended in complete DMEM containing 10% fetal bovine serum. Cells were purified using a percoll gradient at a density of 1.073 g/ml. Next, cells were washed with PBS and then resuspended in complete DMEM. Cells were plated at a density of 3×10^5 cells/cm^2 and cultured under standard cell culture conditions. Medium was changed after 72 h and every 3 days thereafter. Reaching 90% confluence, cells were trypsinized and replated at a density of 5×10^3 cells/cm^2. A representative FACS analysis picture of MSC is represented in Figure 2.1.

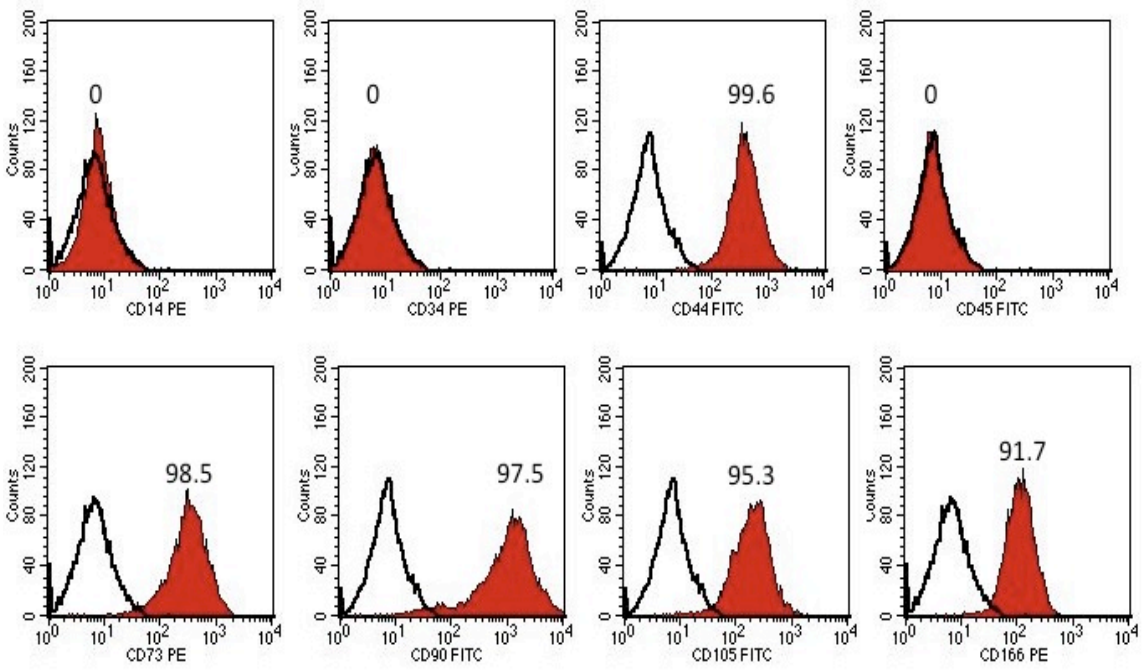


Figure 2.1. Representative FACS analysis of MSC. FACS histograms indicate that MSC are CD14-, CD34-, CD45- and CD44+, CD73+, CD90+, CD105+ and CD166+. Numbers above the histograms indicate the % of gated cells which are positive.

HL-1 culture

HL-1 is an immortal cell line with a phenotype, which is similar to adult cardiomyocytes. The cardiac characteristics of HL-1 cells include: an ultrastructure

similar to primary cultures of adult atrial cardiomyocytes and transplantable cells lines 1; cytoplasmic reorganizaition and myofibrillogenesis similar to that observed in mitotic cardiomyocytes of the developing heart; presence of highly ordered myofibrils and cardiac-specific junctions in the form of intercalated discs; the ability to undergo spontaneous contractions while remaining in a mitotic state typical of normal *in vivo* immature mitotic cardiomyocytes; expression of atrial natriuretic factor (ANF), α-myosin heavy chain (α-MHC), α-cardiac actin, desmin, and connexin43; and presence of several voltage-dependent currents that are characteristic of a cardiomyocyte phenotype, particularly the I_{Kr} current, which has not been identified in noncardiac tissues.[81]

HL-1, which were kindly provided by Prof. U. Rauch (Charité, Campus Benjamin Franklin, Department of Cardiology, Berlin), were cultured and under standard conditions (37°C, 5% CO_2) in Claycomb medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% norepinephrine and 2mM glutamine.

Cardiac fibroblast culture

Cardiac fibroblasts were cultured under standard conditions (37°C, 5% CO_2) in Lung/Cardiac Fibroblasts Basal Medium supplemented with Growth Supplement.

HeLa cell culture

HeLa cells were cultured under standard conditions (37°C, 5% CO_2) in RMPI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

CHO CAR^+ and CHO CAR^- cell culture

CHO CAR^+ and CHO CAR^- cells, kindly provided by Dr. Mario Anders (Department of Interdisciplinary Endoscopy, University Hospital Hamburg Eppendorf, Hamburg), were cultured under standard conditions (37°C, 5% CO_2) in Hams F12 medium with 10% fetal bovine serum and 1% penicillin/ streptomycin. Cells were split 1:3 every 3 days.

Hybridoma cell line RMCB cell culture for CAR antibody

The CAR antibody-producing hybridoma cell line RMCB, kindly provided by Dr. Mario Anders (Department of Interdisciplinary Endoscopy, University Hospital Hamburg Eppendorf, Hamburg), was cultured in suspension in RPMI 1640 medium with 10% FBS and 1% penicillin/ streptomycin under standard conditions (37°C, 5% CO_2). Cells were cultured at a high cell density. Next, the cell suspension was transferred to a T25 cell flask and cells were further cultured for 2 weeks without changing the medium. Then, the cell suspension was centrifuged at 1000 x g for 5 minutes and the antibody containing supernatant was collected. This antibody was used at dilution 1:2 for FACS analysis.

Pretreatment of MSCs with L-NAME

24h before co-culture, MSCs were plated into 6-well plates at a density of 200,000 cells/well in the presence or absence of L-NAME (10 mM). In a preliminary experiment, the concentration of L-NAME was determined: the effect of 1 mM versus 10 mM L-NAME on NO production in MSC was analyzed.

2.2.2. Coxsackievirus B3-infection and culture

Infection of HL-1, mesenchymal stem cells, and cardiac fibroblasts

HL-1, MSCs, and cardiac fibroblasts were plated into 6-well plates with 300,000, 200,000, and 165,000 cells in each well, respectively. After 24h of culture, HL-1, MSCs, and cardiac fibroblasts were infected with CVB3 at a m.o.i. of 5 for 1h. In parallel, non-infected cells were serum-starved. Then, cells were washed with PBS two times and Claycomb full medium, DMEM full medium, and cardiac fibroblasts full medium were added to the HL-1, MSCs, and cardiac fibroblasts, respectively.

Co-culture of MSCs or cardiac fibroblasts with HL-1

Four hours after CVB3-infection or serum starvation of HL-1 cells, untreated MSCs and MSCs pretreated with L-NAME were collected and added to the HL-1 cells for

MSC-mediated effects are IFN-γ dependent, anti-mouse IFN-γ antibody was added at a final concentration of 1 µg/ml. Cardiac fibroblasts were added to HL-1 cells at a ratio of cardiac fibroblasts to HL-1 1 to 10. For the purpose of apoptosis analysis via Annexin V/7AAD FACS analysis, HL-1 cells were labeled with DiO; for the purpose of reactive oxygen species (ROS) analysis via DCF FACS, HL-1 cells were labeled with DiL.

Collection of cells and medium

For monoculture experiments with HL-1, MSCs or cardiac fibroblasts, phase contrast pictures were taken 4h, 12h, 24h, and 48h after CVB3 infection. Next, medium was collected and frozen for later use.

For co-culture experiments, phase contrast pictures were taken 12h, 24h and 48h after CVB3 infection and medium was collected and frozen for later use. For RNA, cells were collected with and resuspended in Trizol, 12h, 24h and 48h after infection. For FACS analysis, cells were collected with 0.05% Trypsin-EDTA 12h, 24h, and 48h after infection.

2.2.3. Total ribonucleic acid extraction

Cells resuspended in Trizol were centrifuged. The supernatant was transferred to a new eppendorf tube and 200 µl of chloroform was added. The solution was shaken by hand for 15 sec, incubated at room temperature for 3 min and centrifuged at 13,200 rpm at 4°C for 15 min. The transparent aqueous phase was transferred to a new eppendorf tube containing 500 µl of isopropanol. The solution was incubated for 10 minutes at room temperature and centrifuged at 13,200 rpm at 4°C. Next, the supernatant was removed and the pellet was washed with 70% ethanol. The solution was vortexed, and centrifuged at 7500 rpm at 4°C for 10 min. Finally, the pellet was dissolved in 100 µl of RNase free water.

2.2.4. Deoxyribonucleic acidase treatment

RA1 buffer (10 µl β-mercaptoethanol/ 1ml RA1 buffer) was added to 100 µl of RNA. Then, 250 µl of 100% ethanol was added. The solution was mixed thoroughly by pipetting, then transfered to a column and centrifuged at 10,000 rpm at room temperature for 1 min. The column was moved on a new collection tube and 350 µl of MDB buffer was added on top of the columns. Then, the columns were centrifuged at 10,000 rpm at room temperature. 95 µl of DNase working solution was directly added onto the membrane of the column and incubated at room temperature for 15 min. Next, 200 µl of RA2 buffer was added to the column and the columns were centrifuged at 10,000 rpm at room temperature for 15 sec. 600 µl of RA3 buffer was added to the columns, and then the columns were centrifuged at 10,000 rpm at room temperature for 15 sec. Another 250 µl RA3 buffer was added, and the columns were centrifuged at 10,000 rpm at room temperature for 2 min. Next, the columns were centrifuged at full speed at room temperature for 1 min. The columns were transferred onto new 1.5 ml eppendorf tubes and 30 µl RNase free water was directly added onto the membranes and incubated at room temperature for 1-2 min. The tubes were centrifuged at 10,000 rpm at room temperature for 1 min and transferred onto ice. Then, 20 µl of RNase free water was added and the columns were incubated for 5 min. Finally, the tubes were centrifuged at 10,000 at room temperature for 1 min.

2.2.5. Complementary deoxyribonucleic acid preparation

cDNA preparation was performed with the High Capacity cDNA Reverse Transcription Kit. The reaction system was 20 µl, which consisted of 11 µl (300ng) of RNA, 2 µl of Random Primer, 0.8µl of 25x dNTP, 2µl of 10x Buffer, 1µl of reverse transcriptase, and 3.2µl of RNase free water. RNA and RNase free water were added to the PCR tube with the final volume of 11 µl. Random Primer/ dNTP mixture was prepared, and the mixture was added to the PCR tubes. The samples were vortexed and centrifuged. Then, the samples were heated at 65°C for 5 min. A mix of reverse transcriptase buffer and reverse transcriptase was prepared. The mixture was added to the PCR tubes and the samples were vortexed and centrifuged. Then, the following PCR programme for

85°C for 5 min.

2.2.6. Real-time polymerase chain reaction

Real-time PCR was conducted with the qPCR MasterMix Plus for SYBR® Green I dNTP Kit. Real-time PCR was carried out by amplification of samples in 96-well plates in a 7900HT Fast Real-time PCR System using specific PCR primer pairs. The reaction mixture was prepared with 12.5 µl qPCR MasterMix, 1 µl of the respective forward and reverse primers (7.5 µm stock solution) and 5.5 µl of sterile water in a total volume of 20 µl. The reaction mix was added to each well. Next, 5 µl of water (negative control), plasmid standards or samples were added. The plate was centrifuged and positioned in the 7900HT Fast Real-time PCR System. After initial incubation of 2 min at 50°C and 10 min at 95°C for HotGoldStar activation, 40 PCR-cycles were performed which consisted of 15 sec denaturation at 95°C, and 1 min annealing at 60°C, with an additional incubation of 15 sec at 95°C and 15 sec at 55°C after completion of the last cycle. Absolute quantification was performed according to the standards.

2.2.7. Viability Assay with MTS

Viability Assay was conducted with CellTiter 96® AQueous One Solution Cell Proliferation Assay Kit, which is composed of the novel tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS*), and an electron coupling reagent, phenazine ethosulfate (PES). MTS is bioreduced by cells into formazan, of which the absorbance at 490 nm can be directly measured without additional processing. The amount of formed formazan is directly proportional to the number of living cells in culture.

First, 10,000 cells (MSC, HL-1, cardiac fibroblasts) were plated with 100 µl medium in each well of a 96-well plate. After 24 h of culture, 20 µl of the CellTiter 96® AQueous One Solution Reagent was directly added to the culture wells and incubated for 2 h. The absorbance was recorded at 490 nm with a VersaMax microplate reader.

Nitric Oxide assay was conducted with a Nitric Oxide Assay Kit, which provided an accurate and convenient method for the measurement of total [NO_2^-] and [NO_3^-] in a simple two-step process. The first step involves the conversion of nitrate to nitrite by the enzymatic action of nitrate reductase. The second step involves the addition of 2,3-diaminonaphthalene (DAN) followed by sodium hydroxide (NaOH) to convert nitrite to a fluorescent compound 1 (*H*)-naphthotriazole. Measurement of fluorescence of 1 (*H*)-naphthotriazole provides an accurate assay of [NO_2^-].

Nitrate standard was prepared, and the standard gradient was made for the standard curve. 80 µl of assay buffer was added to the standard well designated as 0 µM, and 30 µl of assay buffer was added to the other standard wells containing 50 µl of standard solution. 20 µl of each sample was added to the wells in duplicate and the volume was adjusted to 80 µl with assay buffer. The enzyme cofactors were aliquoted and reconstituted in 1200 µl of assay buffer. Then, 10 µl of the enzyme cofactors solution was added to each well. Nitrate reductase was aliquoted. The nitrate reductase was reconstituted in 1200 µl of assay buffer, and 10 µl of the nitrate reductase solution was added to each well. Then the plate was covered and incubated at room temperature for 60 min. DAN was aliquoted and 10 µl of DAN was added to each well and incubated for 10 min. Then, 20 µl of NaOH was added to each well. The plate was read in a SpectraMax Gemini microplate reader at the excitation wavelength of 320 nm and an emission wavelength of 405 nm.

2.2.9. Fluorescence-activated cell sorting analysis

CAR analysis

Cells were trypsinized and neutralized with FBS. The cell suspension was centrifuged for 5 minutes at 1000 x g and washed 1x with PBS. The cell pellet was resuspended in 100 µl antibody solution (antibody diluted 1:2 in PBS with 2% BSA and 10% goat serum). The antibody was incubated in the dark for 45 minutes at 4°C. After this incubation, cells were washed 3x with PBS and the cell pellet was resuspended in the second antibody solution (Cy3 anti mouse 1:400 diluted with PBS with 2% BSA and

washed 1x with PBS and the pellet was resuspended in 300 µl PBS. As negative control, cells only incubated with the secondary antibody were used.

CD55 analysis

CD55 analysis was conducted with FITC mouse anti-human CD55 antibody. Cells were trypsinized and neutralized with FBS. The cell suspension was centrifuged (5min, 2500 rpm), and the supernatant was removed. Cells were washed with PBS and subsequently resuspended in 100 µl of PBS containing 1% BSA (PBS-BSA 1%) and 20 µl of CD55 antibody. The cells were incubated at 4°C for 15 min. Then, 900 µl of PBS-BSA 1% was added and cells were centrifuged for 5 min at 2500 rpm. Finally, cells were resuspended in 250 µl PBS. As negative control, IgG2A FITC isotype control was used.

CD14, CD34, CD73, CD166, CD44, CD45, CD90 and CD105 analysis

MSC were characterized by FACS analysis according to Binger et al.[82] with PE-labeled monoclonal mouse anti-human CD14, CD34, CD73, CD166 and FITC-labeled mouse anti-human CD44, CD45, CD90 and CD105 antibodies. Cells were washed with PBS-BSA 0.5%, resuspended in 100 µl of PBS-BSA 0.5% and incubated with titrated concentrations of antibodies at 4°C for 15 min. Prior to FACS analysis, cells were washed with PBS-BSA 0.5%.

Apoptosis analysis

Apoptosis analysis was conducted with the Annexin V-PE Apoptosis Detection Kit. The apoptotic program is characterized by certain morphologic features, including loss of plasma membrane asymmetry and attachment, condensation of the cytoplasm and nucleus, and internucleosomal cleavage of DNA. Loss of plasma membrane is one of the earliest features. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V is a Ca^{2+} dependent phospholipid-binding protein that has a high affinity for PS, and binds to cells with

(PE). This format retains its high affinity for PS and thus serves as a sensitive probe for flow cytometric analysis of cells that are undergoing apoptosis. Since externalization of PS occurs in the earlier stages of apoptosis, PE Annexin V staining can identify apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragment. PE Annexin V staining precedes the loss of membrane integrity which accompanies the latest of cell death resulting from either apoptotic or necrotic processes. Therefore, staining with PE Annexin V is typically used in conjunction with a vital dye such as 7-Amino-Actinomycin D (7-AAD) to allow the investigator to identify early apoptotic cells (7-AAD negative, PE Annexin V positive). Viable cells with intact membranes exclude 7-AAD, whereas the membranes of dead and damaged cells are permeable to 7-AAD. For example, cells that are considered viable are both PE Annexin V and 7-AAD negative, while cells that are in early apoptosis are PE Annexin V positive and 7-AAD negative, while cells that are in late apoptosis or already dead are both PE Annexin V and 7-AAD positive.

1x binding buffer was prepared, and PBS was positioned on ice. Cells were trypsinized and neutralized with FBS. The cell suspension was centrifuged (5min, 2500rpm), and the supernatant was removed. The cells were resuspended in 100 µl of 1x binding buffer to which 5 µl Annexin V-PE and 5 µl 7AAD was added. Cells were incubated for 15 min at room temperature in the dark. Next, 200 µl of 1x binding buffer was added and cells were centrifuged. The supernatant was removed, 2% formalin (4% formalin diluted by 1x binding buffer) was added, followed by 15 min incubation at room temperature in the dark. 200 µl of 1x binding buffer was added and cells were centrifuged (5min, 2500 rpm). Finally, the supernatant was removed and cells were resuspended in 200 µl of 1x binding buffer.

Reactive Oxygen Species analysis

Derivatives of reduced fluorescein can be used as cell-permeant indicators for reactive oxygen species (ROS). Chemically reduced and acetylated forms of 2',7'-di-chlorofluorescein (DCF) are nonfluorescent until the acetate groups are

cleavage of the lipophilic blocking groups yields a charged form of the dye that is much better retained by cells than is the parent compound. Oxidation of this probe can be detected by monitoring the increase in fluorescence with a flow cytometer. The carboxy derivative of fluorescein, carboxy-H_2DCFDA, carries additional negative charges that improve its retention compared to noncarboxylated forms. ROS indicator was reconstituted in 100 µl of DMSO to make a concentrated stock solution. Cells were collected and resuspended in pre-warmed PBS containing the probe with the final working concentration of 5µM dye, and then incubated at 37°C for 30 min. The loading buffer was removed, and 2% formalin (4% formalin diluted with 1x binding buffer) was added. Cells were next incubated for 15 min at room temperature in the dark. Then, 200 µl of pre-warmed PBS was added, and tubes were centrifuged (5min, 2500 rpm). Finally, the supernatant was removed and the cells were resuspended in 200 µl of pre-warmed PBS.

2.2.10. Virus plaque assay

HeLa cells were plated in 6-well plates (600,000/well). Dilutions (10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) of supernatant from CVB3-infected HL-1, cardiac fibroblasts and MSCs and from co-cultures of CVB3-infected HL-1 cells with MSC in the presence or absence of L-NAME or murine IFN-γ were prepared. After 24 h culture of HeLa cells, medium was removed and cells were washed with PBS. Next, 1ml of the prepared medium dilutions were added to each well and incubated for 30 min at 37°C. Then, 2 ml agar, consisting of an equal volume of MEM2x supplemented with 0.04% FBS and 1.3% noble agar, was added to each well. The plates were left under the hood for 15-20 min until the agar coagulated, and then kept in the incubator for 72 h. Finally, virus plaques were counted.

2.2.11. Statistical analysis

Data are presented as mean ± SEM. Paired and unpaired Student's t tests were used for statistical analysis with Instat 3.0.a. software. Differences were considered to be

CHAPTER THREE: RESULTS

3.1. Expression of coxsackievirus-adenovirus receptor and decay-accelerating factor on mesenchymal stem cells

Given the importance of the coxsackievirus-adenovirus receptor (CAR) and the co-receptor decay-accelerating factor (CD55) for the infectivity of cells by CVB3, we first analyzed the expression of CAR and CD55 on MSC by FACS analysis. As positive controls for CAR expression, we used Chinese Hamster Ovarian (CHO) cells, which are stable and transfected with CAR and which overexpress CAR (CHO-CAR+), and murine HL-1 cells. As negative controls, we used CHO cells lacking CAR receptor (CHO-CAR-) and cardiac fibroblasts, since it has been reported that primary fibroblasts express only low levels of CAR[83]. Compared to CHO-CAR+ and HL-1 cells, and to CHO-CAR- and cardiac fibroblasts, MSC only minimally express CAR at levels nearly comparable with secondary antibody controls (Figure 3.1.). As for CD55, MSC also only moderately express CD55 (Figure 3.2.).

3.2. Coxsackievirus B3 infectivity of mesenchymal stem cells

To investigate whether MSC can be infected with CVB3 and whether replication of CVB3 takes place, a time-frame experiment was performed. MSC were infected with CVB3 at a m.o.i. of 5 for 1 hour (h), which are the same conditions for CVB3-infection of HL-1 cells. Experiments were paralleled with HL-1 cells as positive control, since cardiomyocytes are the target cells of CVB3, and with cardiac fibroblasts as negative control. Cells for RNA isolation were collected 4h, 12h, 24h and 48h after infection. To determine the titer of CVB3, medium was collected 24h after infection and plaque assay was performed on HeLa cells, human cervix carcinoma cells, which are commonly used to detect viral plaque formation. CVB3 RNA copy number, expressed as CVB3 towards L32, decreased over time in MSC, with 4.1–fold ($p=0.0571$) and 7.2–fold ($p<0.05$) lower levels at 24h and 48h post-infection versus 4h post-infection, respectively (Figure 3.3.A). In contrast, CVB3 RNA copy number raised in HL-1 over time, with 9.0–fold ($p<0.01$) higher RNA levels at

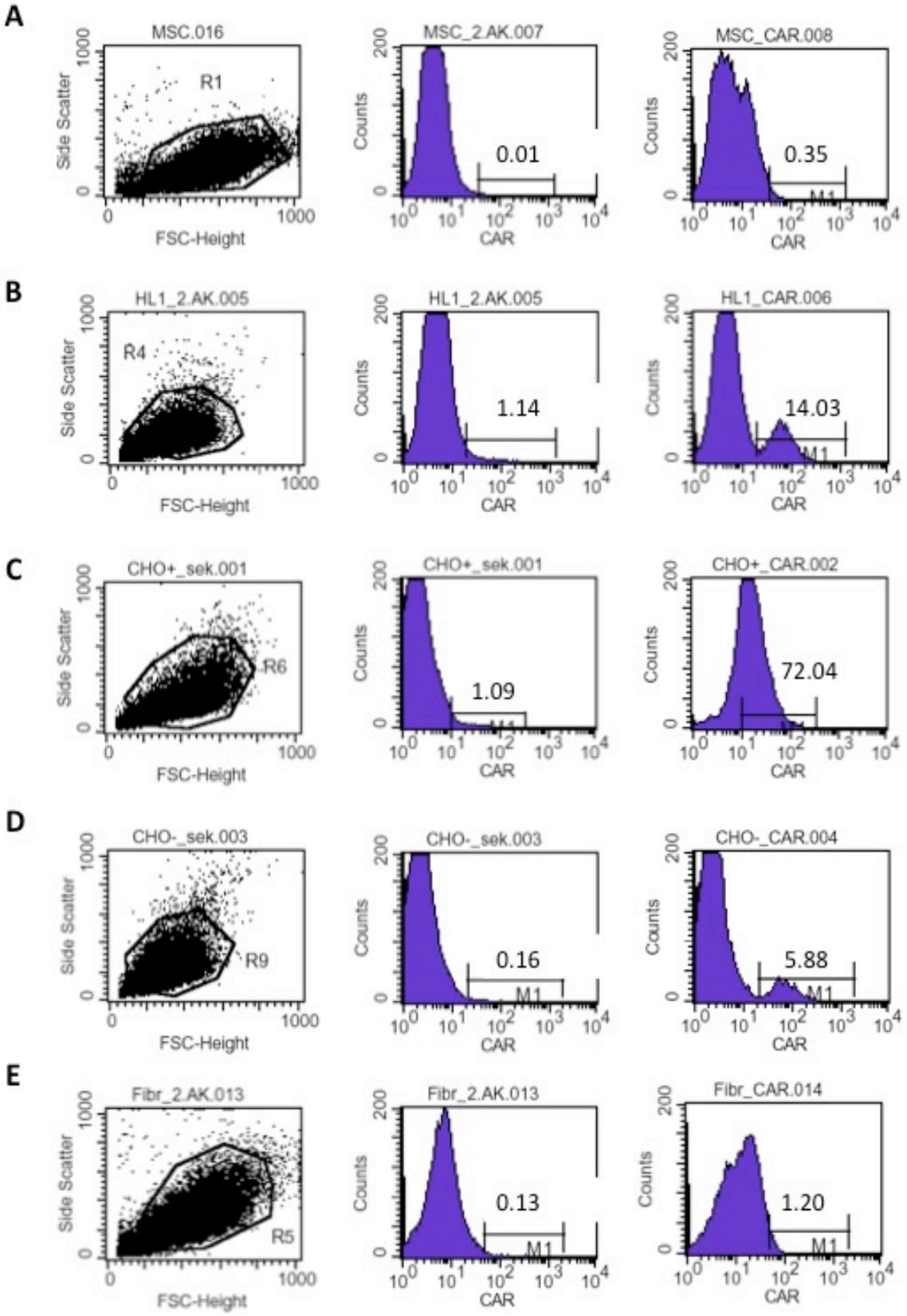


Figure 3.1. Mesenchymal stem cells minimally express coxsackievirus-adenovirus receptor. Panel **A** showing representative pictures of the Forward Scatter/Sideward Scatter (left) of MSC, of the % of secondary antibody positive MSC (middle) and of the % of CAR positive MSC (right)

Panel **B** showing representative pictures of the Forward Scatter/Sideward Scatter (left) of HL-1, of the % of secondary antibody positive HL-1 (middle) and of the % of CAR positive HL-1 (right). Panel **C** demonstrating representative pictures of the Forwards Scatter/Sideward Scatter (left) of CHO-CAR+, of the % of secondary antibody positive CHO-CAR+ (middle) and of the % of CAR positive CHO-CAR+ (right). Panel **D** demonstrating representative pictures of the Forwards Scatter/Sideward Scatter (left) of CHO-CAR-, of the % of secondary antibody positive CHO-CAR- (middle) and of the % of CAR positive CHO-CAR- (right). Panel **E** demonstrating representative pictures of the Forwards Scatter/Sideward Scatter (left) of cardiac fibroblasts, of the % of secondary antibody positive cardiac fibroblasts (middle) and of the % of CAR positive cardiac fibroblasts (right).

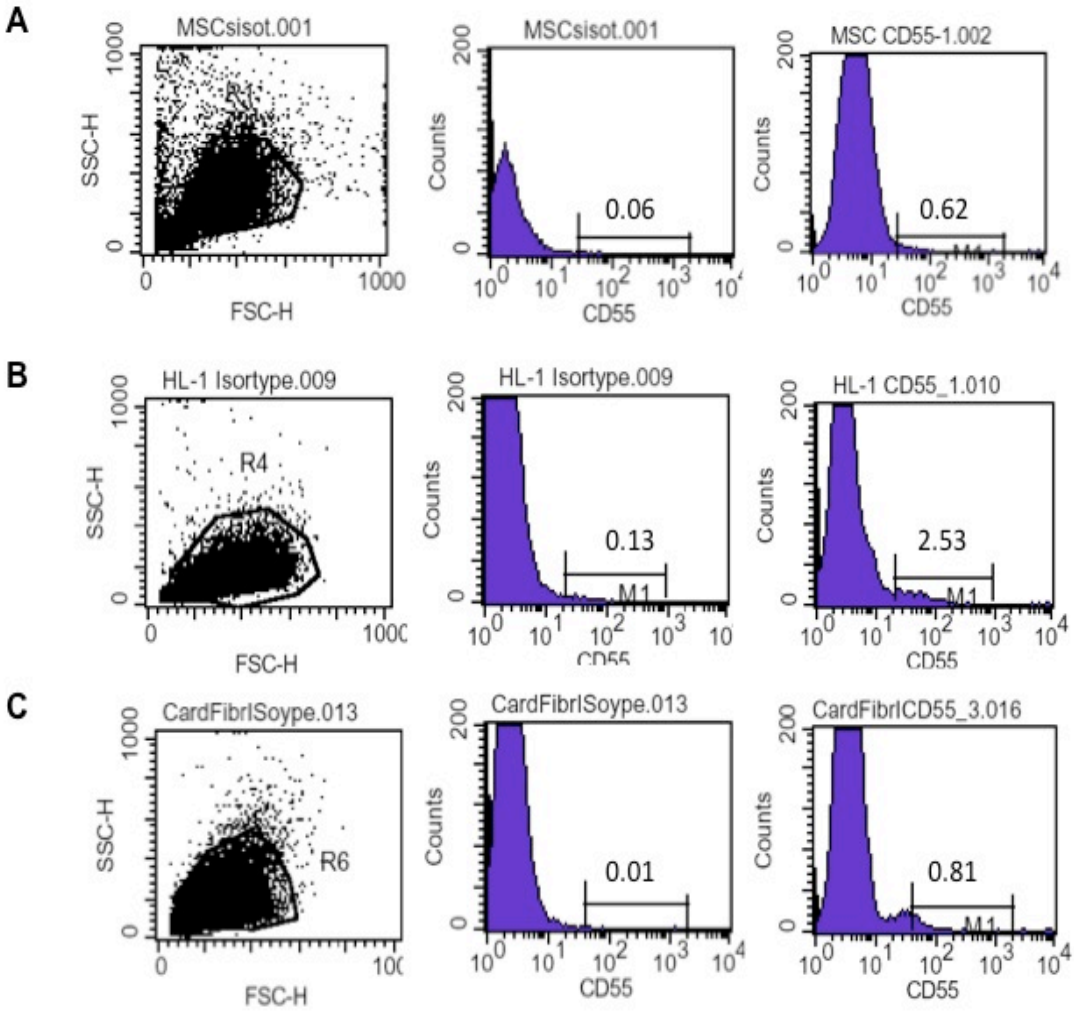


Figure 3.2. Mesenchymal stem cells minimally express CD55. Panel **A** showing representative pictures of the Forwards Scatter/Sideward Scatter (left) of MSC, of the % of isotype control positive MSC (middle) and of the % of CD55 positive MSC (right). Panel **B** demonstrating representative pictures of the Forwards Scatter/Sideward Scatter (left) of HL-1, of the % of isotype control positive HL-1 (middle) and of the % of CD55 positive HL-1 (right). Panel **C** demonstrating representative pictures of the Forwards Scatter/Sideward Scatter (left) of cardiac fibroblasts, of the % of isotype control positive cardiac fibroblasts (middle) and of the % of CD55 positive cardiac fibroblasts (right).

24h versus 12h post-infection (Figure 3.3.B). In cardiac fibroblasts, CVB3 copy number was 3.9-fold (p=0.05) higher at 48h compared to 4h post CVB3-infection (Figure 3.3.C). No plaques were detected on HeLa cells incubated with the medium collected from MSC 24h after CVB3 infection, nor at dilutions 10^{-5} and 10^{-4}, nor at dilution 10^{-3}. In contrast, 1.7±0.88 and 15±1.5 plaques were counted on HeLa cells incubated with medium of HL-1 cells 24h post CVB3-infection at dilution 10^{-5} and 10^{-4}, respectively. On HeLa cells incubated with medium collected from cardiac fibroblasts 24h post CVB3-infection, 5.3±1.2 plaques could be detected at the lowest dilution, i.e. 10^{-3}. In summary, medium of MSC, HL-1 cells and cardiac fibroblasts, collected 24h post CVB3-infection had a CVB3 titer of 0, 150±15 and 5.3±1.2 10^3 p.f.u./ml, respectively (Figure 3.3.D).

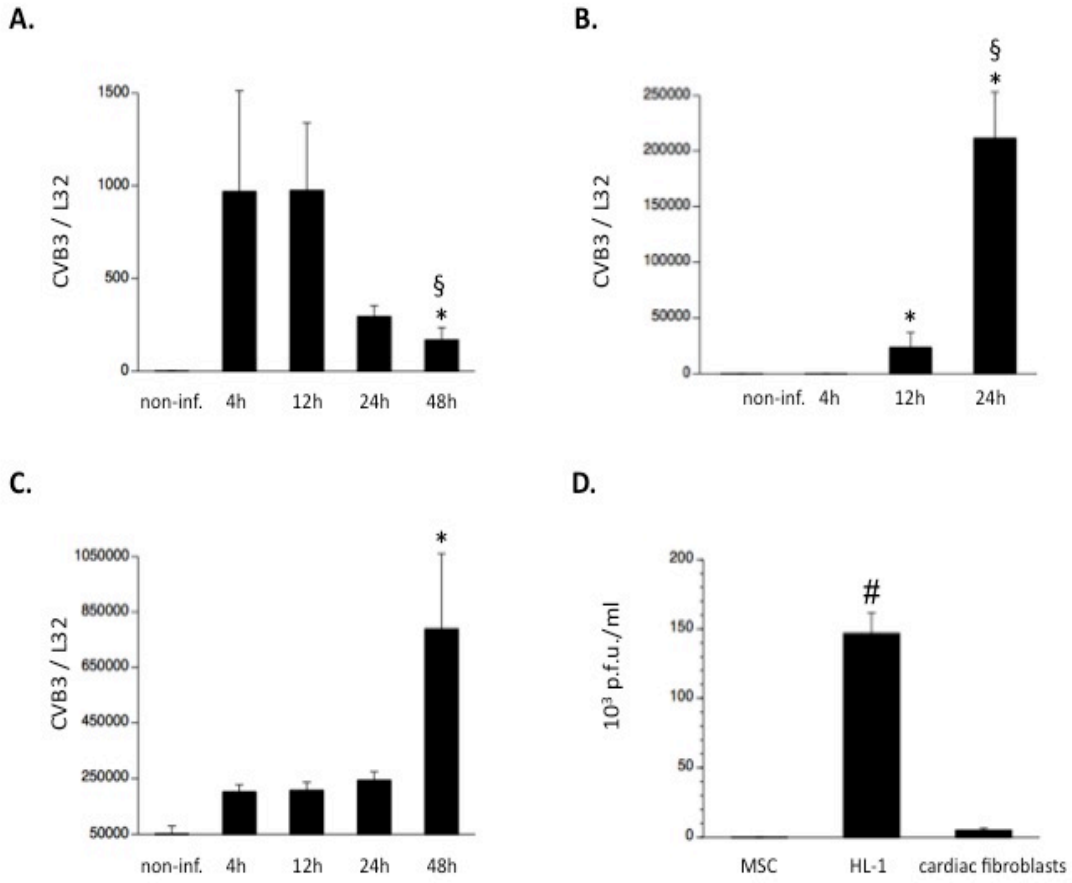


Figure 3.3. Coxsackievirus B3 does not replicate in mesenchymal stem cells. A. Bar graphs representing CVB3 RNA copy number in MSC 4h, 12h, 24h and 48h after CVB3 infection with CVB3 levels normalized towards ribosomal L32 with n=4/condition; *p<0.05 versus 4h and §p<0.05 versus 12h. **B.** Bar graphs representing CVB3 RNA copy number in HL-1 4h, 12h and 24h after CVB3 infection with CVB3 levels normalized towards ribosomal L32 with n=4/condition; *p<0.05 versus 4h and §p<0.01 versus 12h. **C.** Bar graphs representing CVB3 RNA copy number in cardiac fibroblasts 4h, 12h, 24h and 48h after CVB3 infection with CVB3

levels normalized towards ribosomal L32 with n=4/condition; *p=0.05 versus 4h. **D.** HeLa cells were incubated for 30 minutes with 1 ml of diluted supernatant of CVB3-infected MSC, HL-1 cells and cardiac fibroblasts. Then, cells were washed with PBS and covered with agar consisting of 50% 1.3% noble agar and 50% 2x MEM. Bar graphs represent the average amount of counted plaques 72h after incubation with diluted medium of CVB3-infected MSC (dilution 10^{-3}), HL-1 (dilution 10^{-4}) or cardiac fibroblasts (dilution 10^{-3}) with n=3/condition; #p<0.0005 versus cardiac fibroblasts.

3.3. Effect of coxsackievirus B3-infection on mesenchymal stem cell viability

To investigate whether MSC suffer from CVB3-infection, phase contrast pictures were taken from the time-frame experiment from paragraph 3.2. In addition, the same time-frame experiment was performed with MSC plated in a 96-well to measure cell viability via a MTS assay. All experiments were performed in parallel with HL-1 cells and cardiac fibroblasts. Phase contrast pictures did not show any significant changes in cell morphology between serum-starved and CVB3-infected MSC. In line with this observation, no significant differences were found in MSC viability between post-infection versus post-serum starvation at any time-point (Figure 3.4.). In contrast, compared to serum-starved HL-1 cells, CVB3-infected HL-1 showed gradually more cell death over-time post CVB3-infection. These visual observations were reflected in the MTS assay demonstrating 1.9–fold ($p<0.0001$), 1.5-fold ($p<0.0001$) and 1.2–fold ($p<0.05$) lower cell viability at 48h, 24h and 12h post CVB3-infection versus 4h post CVB3-infection, respectively. In addition, at 24h and 48h post CVB3-infection, cell viability was 1.5–fold ($p<0.0001$) and 2.0–fold ($p<0.0001$) reduced compared to serum starved HL-1 cells at respective times (Figure 3.5.). In cardiac fibroblasts, cell death was observed 48h post CVB3-infection, which was paralleled by 1.1-fold ($p<0.05$) lower cell viability at 48h post CVB3-infection versus 48h post serum starvation (Figure 3.6.).

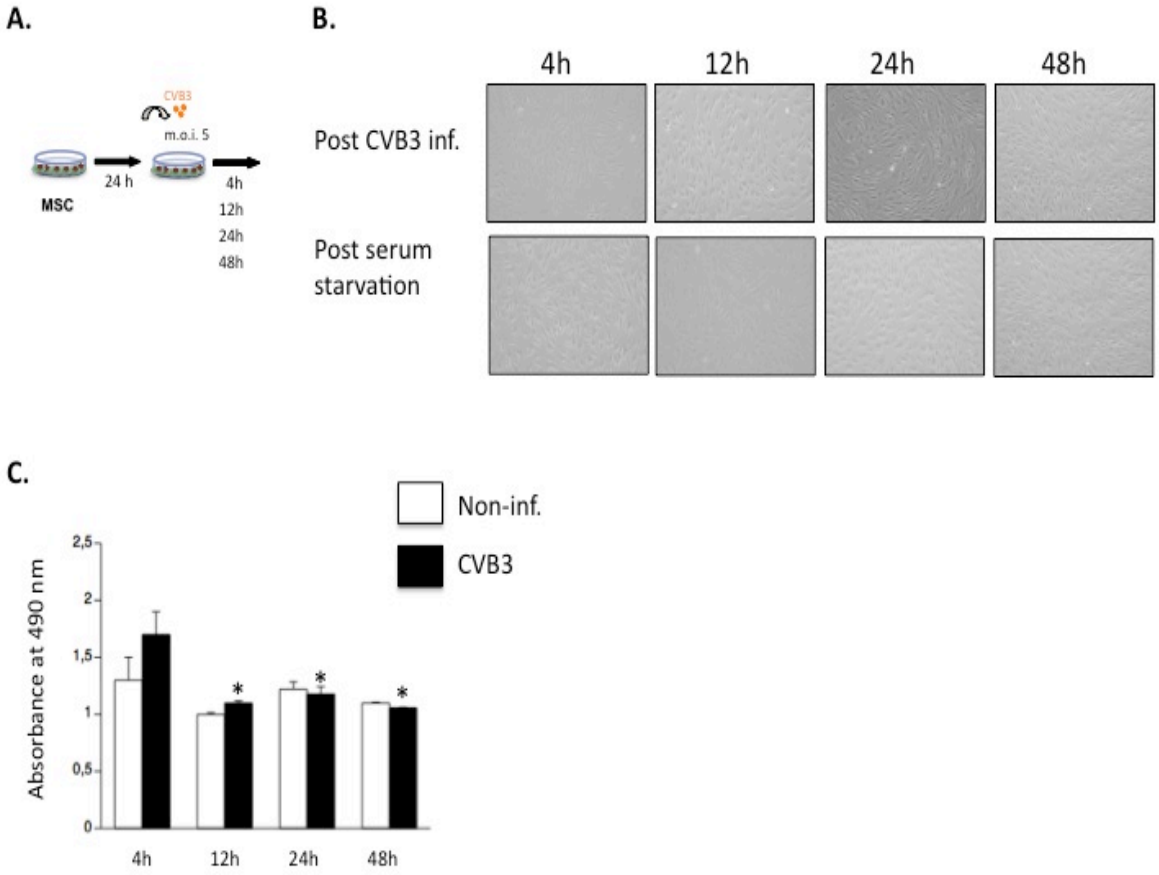


Figure 3.4. Mesenchymal stem cells do not suffer from coxsackievirus B3-infection. A. MSC were plated in a 6-well for phase contrast pictures or in a 96-well for MTS assay, respectively. After 24h, reaching 80% confluence, MSC were infected with CVB3 at a m.o.i. of 5 or serum starved. 4, 12, 24 and 48 hours after infection or serum starvation, phase contrast pictures were taken or MTS assay was performed. **B.** Phase contrast pictures of MSC, 4h, 12h, 24h and 48h after CVB3 infection (upper panel) or serum starvation (lower panel), at 100x magnification. **C.** Bar graphs representing the absorbance at 490 nm from non-infected (open bar graphs) and CVB3-infected (closed bar graphs) MSC 4h, 12h, 24h and 48h after serum starvation or CVB3-infection, respectively. n=6/condition; *p<0.05 versus 4h CVB3-infected group.

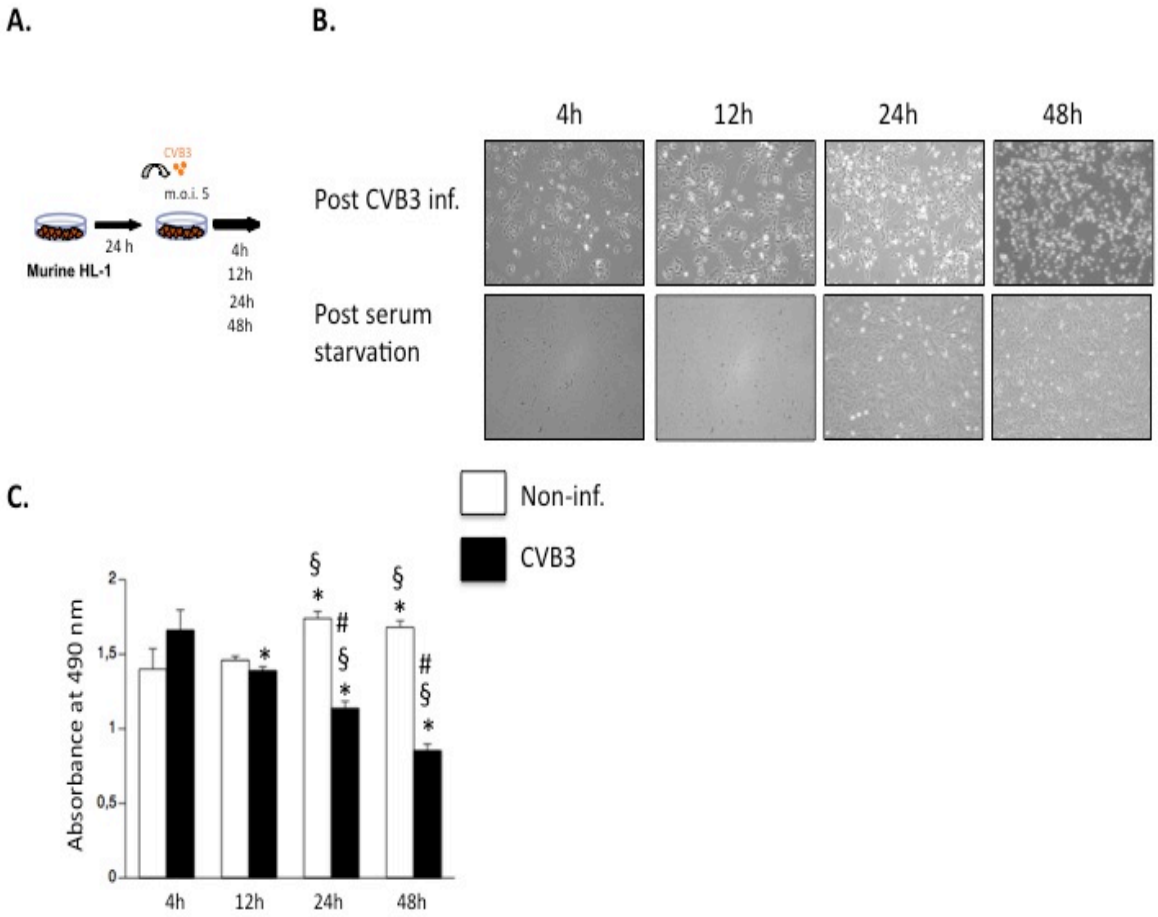


Figure 3.5. Murine HL-1 cells after coxsackievirus B3-infection. A. HL-1 were plated in a 6-well for phase contrast pictures or in a 96-well for MTS assay, respectively. After 24h, reaching 80% confluence, HL-1 were infected with CVB3 at a m.o.i. of 5 or serum starved. 4, 12, 24 and 48 hours after infection or serum starvation, phase contrast pictures were taken or MTS assay was performed. **B.** Phase contrast pictures of HL-1, 4h, 12h, 24h and 48h after CVB3 infection (upper panel) or serum starvation (lower panel), at 100x magnification. **C.** Bar graphs representing the absorbance at 490 nm from non-infected (non-inf.; open bar graphs) and CVB3-infected (CVB3; closed bar graphs) HL-1 4h, 12h, 24h and 48h after serum starvation or CVB3-infection, respectively. n=6/condition; *p<0.05 versus respective 4h non-infected (non-inf.) or CVB3-inf. group, §p<0.05 versus respective 12h non-inf. or CVB3-inf. group and #p<0.05 versus non-infected (non-inf.).

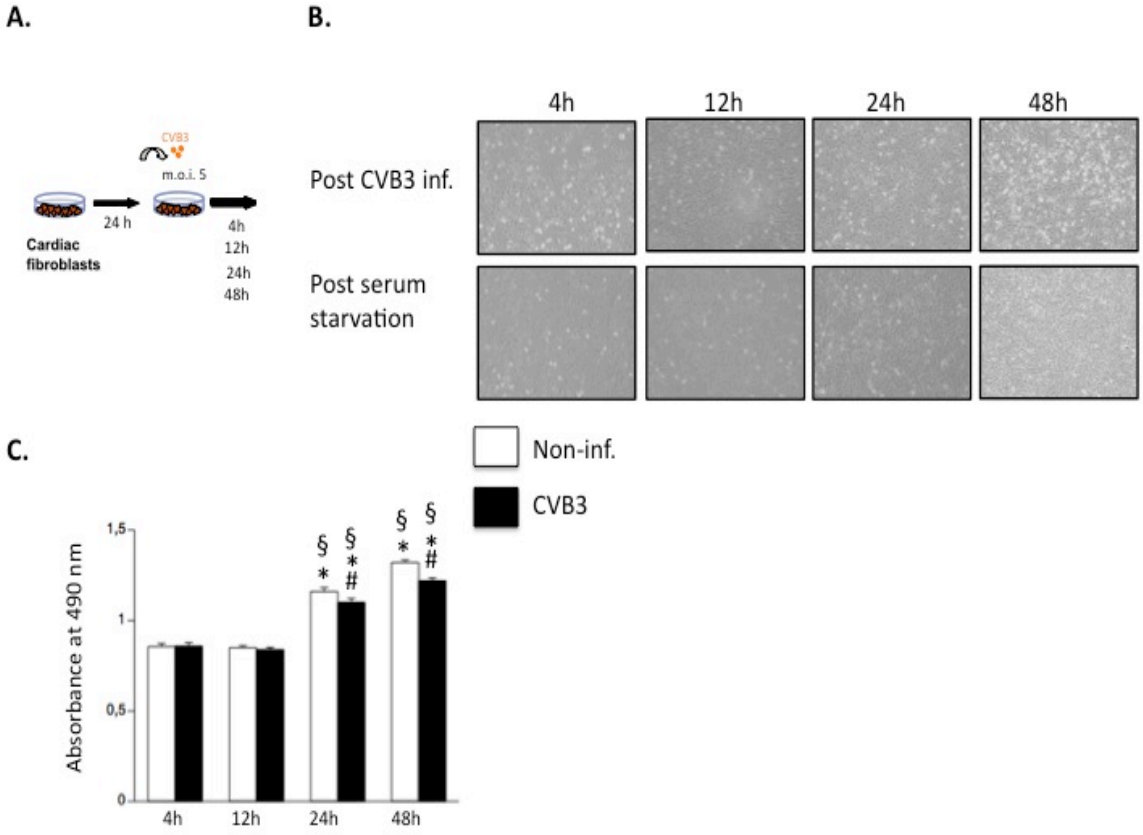


Figure 3.6. Cardiac fibroblasts after coxsackievirus B3-infection. A. Cardiac fibroblasts were plated in a 6-well for phase contrast pictures or in a 96-well for MTS assay, respectively. After 24h, reaching 80% confluence, cardiac fibroblasts were infected with CVB3 at a m.o.i. of 5 or serum starved. 4, 12, 24 and 48 hours after infection or serum starvation, phase contrast pictures were taken or MTS assay was performed. **B.** Phase contrast pictures of cardiac fibroblasts, 4h, 12h, 24h and 48h after CVB3 infection (upper panel) or serum starvation (lower panel), at 100x magnification. **C.** Bar graphs representing the absorbance at 490 nm from non-infected (non-inf.; open bar graphs) and CVB3-infected (CVB3; closed bar graphs) cardiac fibroblasts 4h, 12h, 24h and 48h after serum starvation or CVB3-infection, respectively. n=6/condition; *$p<0.0005$ versus respective 4h non-infected (non-inf.) or CVB3-inf. group, §$p<0.0005$ versus respective 12h non-inf. or CVB3-inf. group and #$p<0.05$ versus non-inf..

3.4. Mesenchymal stem cells reduce coxsackievirus B3-induced HL-1 apoptosis

To investigate whether MSCs exert anti-apoptotic effects on CVB3-infected HL-1 cells, we co-cultured MSC in the presence of CVB3-infected HL-1 and performed Annexin V/7AAD FACS analysis. To distinguish the presence of Annexin V/7AAD in HL-1 cells or MSC in the MSC-HL-1 co-cultures, HL-1 were prelabeled with the fluorescence dye DiO before plating (Figure 3.7.A). FACS analysis demonstrated that MSCs decreased the 4.2-fold ($p<0.05$) CVB3-induced HL-1 apoptosis to levels not

significantly different from those of non-infected cells Figure 3.7.

To assess whether the anti-apoptotic effects of MSCs are NO-dependent, we pre-treated MSCs with the iNOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) 24 hours before co-culture. In a preliminary experiment, we determined the concentration of L-NAME to block NO production in MSC. Culture of MSC in the presence of 1 mM L-NAME did not reduce NO production, whereas 10 mM L-NAME reduced NO by 1.4–fold ($p<0.05$) (Figure 3.8.). When MSCs were pre-treated with 10 mM of L-NAME, the anti-apoptotic effects of MSC were abrogated, suggesting that MSC reduce CVB3-induced HL-1 apoptosis in a NO-dependent way (Figure 3.7).

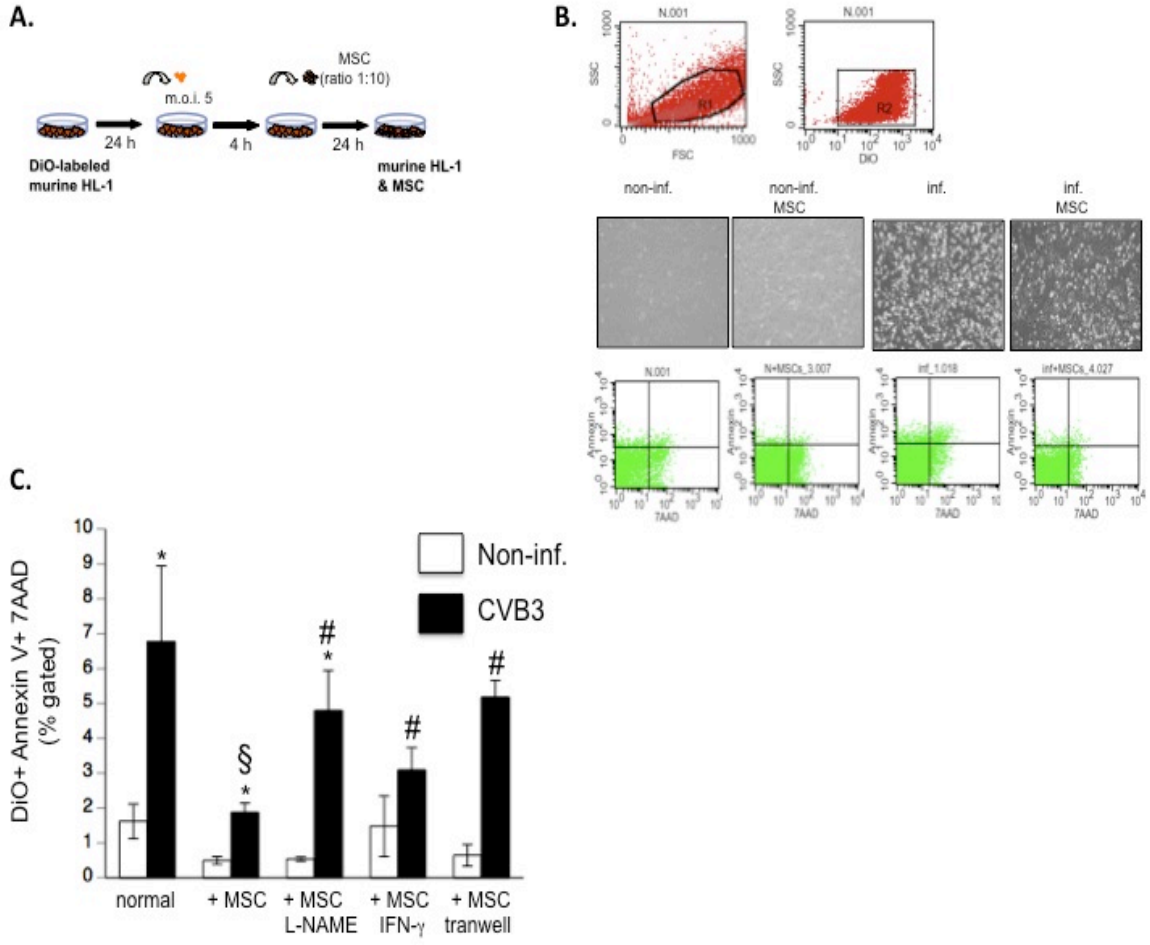


Figure 3.7. MSC reduce coxsackievirus B3-induced apoptosis in a nitric oxide-, interferon gamma- and cell-contact-dependent way. A. DiO-labeled HL-1 cells were cultured in a 6-well plate and 24 hours later infected with CVB3 at a m.o.i. of 5. Four hours later MSC were added at a ratio of 1 to 10 HL-1 cells. After 24 hours, cells were collected for Annexin V/7AAD FACS analysis. **B.** Upper panel left shows a Forward Scatter (FSC) Sideward Scatter (SSC) of HL-1 cells, and right shows the DiO-positive HL-1 cells; middle panel shows

infected HL-1 and infected HL-1 co-cultured with MSC; lower panel shows representative pictures of Annexin V/7AAD dot plots of preselected DiO+ HL-1 cells. **C.** Bar graphs represent DiO+ Annexin V+/7AAD- HL-1 cells in cultures of HL-1 with or without untreated or L-NAME-treated MSC or MSC in the presence or absence of 1 μg/ml of IFN-γ or with MSC cultured in a transwell; n=4/group; * $p<0.05$ versus respective non-inf. group, § $p<0.05$ versus respective normal group and # $p<0.05$ versus MSC.

Next, to investigate whether the anti-apoptotic effects of MSCs are IFN-γ-dependent, we co-cultured MSCs with CVB3-infected HL-1 cells in the presence of 1 μg/ml of anti-mouse IFN-γ neutralizing antibody. In this setting, MSCs could not decrease the CVB3-induced HL-1 apoptosis, suggesting that under the conditions of CVB3-infection, MSC exert their anti-apoptotic features in an IFN-γ-dependent way.

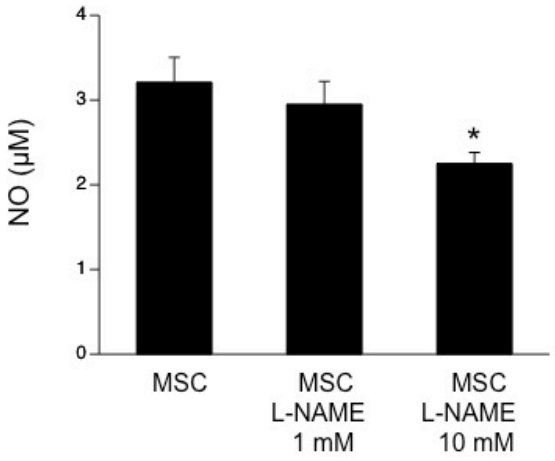


Figure 3.8. Effect of L-NAME treatment on nitric oxide production in mesenchymal stem cells. MSC were cultured in a 6-well plate in the presence of L-NAME at a final concentration of 0-1-10 mM. 24 hours later, medium was collected and frozen. Next, NO production in MSC medium was analyzed. Bar graphs represent NO production in supernatant of MSC in the presence or absence of 1 or 10 mM L-NAME. (n=3/condition; *$p<0.05$).

Finally, to evaluate whether the anti-apoptotic activity of MSCs was mediated by cell contact to HL-1 cells, MSCs were cultured in a transwell system, with 1 μm pore size, blocking cell contact to HL-1 cells and allowing the presence of secreting factors of the MSC in the total cell medium. Under these conditions, the anti-apoptotic effect of MSCs was abrogated (Figure 3.7).

To verify whether or not cell-contact *per se* leads to the reduction of CVB3-induced apoptosis of HL-1 cells, we co-cultured HL-1 cells in the presence of cardiac

HL-1 apoptosis by 1.4-fold ($p<0.05$) (Figure 3.9).

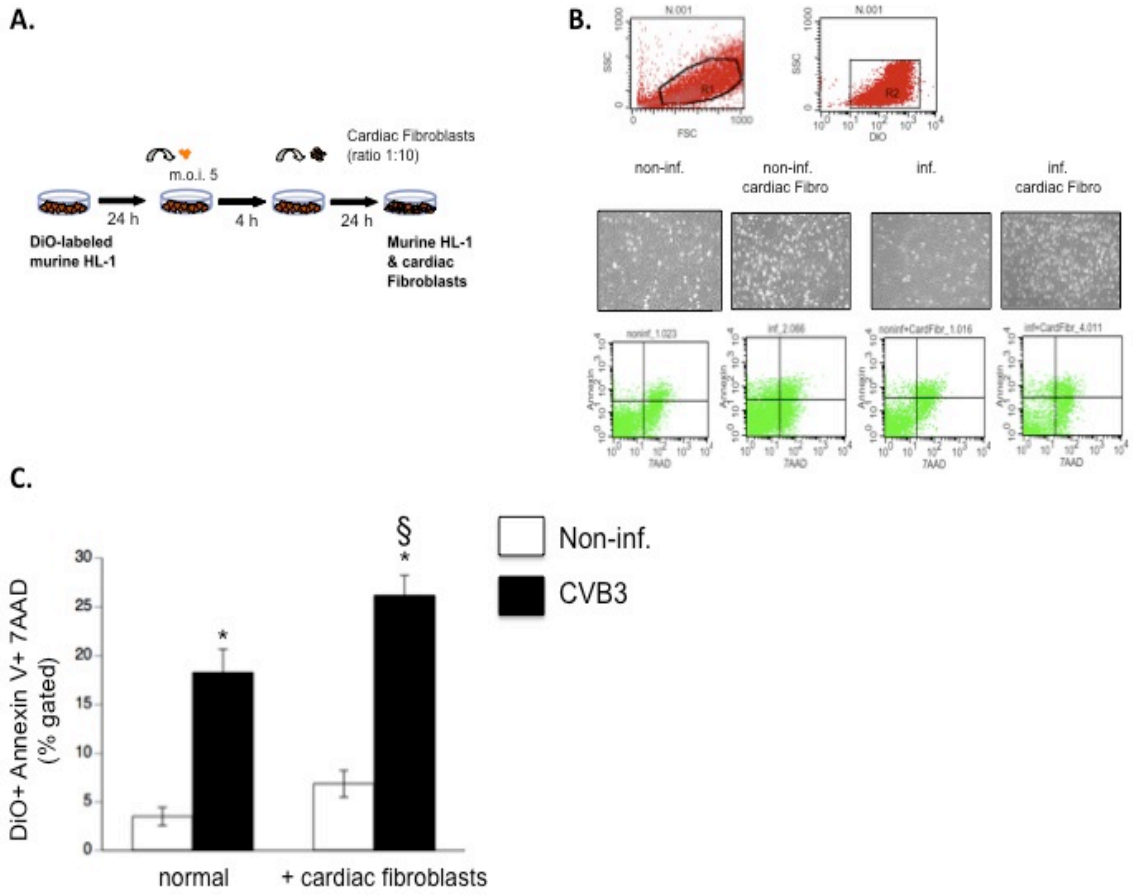


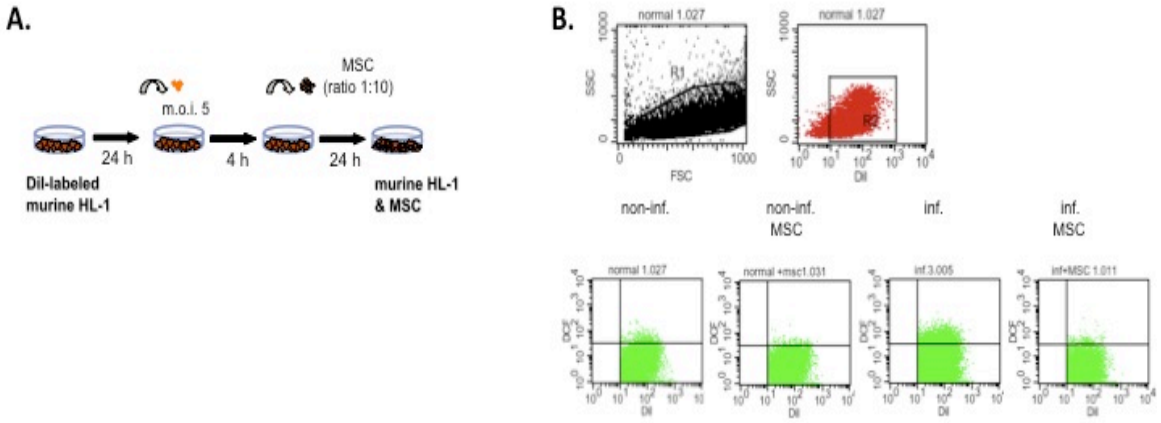
Figure 3.9. Cardiac fibroblasts increase apoptosis in coxsackievirus B3-infected HL-1 cells. **A.** DiO-labeled HL-1 cells were cultured in a 6-well plate and 24 hours later infected with CVB3 at a m.o.i. of 5. Four hours later cardiac fibroblasts were added at a ratio of 1 to 10 HL-1 cells. After 24 hours, cells were collected for Annexin V/7AAD FACS analysis. **B.** Upper panel left shows a Forward Scatter (FSC) Sideward Scatter (SSC) of HL-1 cells, and right shows the DiO-positive HL-1 cells; middle panel shows representative pictures of non-infected HL-1, non-infected HL-1 co-cultured with cardiac fibroblasts, infected HL-1 and infected HL-1 co-cultured with cardiac fibroblasts; lower panel demonstrates representative pictures of Annexin V/7AAD dot plots representing preselected DiO+ HL-1 cells. **C.** Bar graphs represent DiO+ Annexin V+/7AAD- HL-1 cells in cultures of non-infected or CVB3-infected HL-1 in the presence or absence of cardiac fibroblasts; n=8/group; * $p<0.05$ versus respective non-inf. group, § $p<0.05$ versus respective normal group.

3.5. Mesenchymal stem cells reduce coxsackievirus B3-induced oxidative stress in HL-1 cells

We next analyzed whether the MSC-mediated reduction in CVB3-induced HL-1

analyzed the presence of reactive oxygen species (ROS), markers of oxidative stress, in MSC-HL-1 co-cultures by FACS analysis. To distinguish the presence of ROS in HL-1 cells or MSC in the MSC-HL-1 co-cultures, HL-1 were prelabeled with the fluorescence dye DiI before plating (Figure 3.10.A). FACS analysis showed that CVB3 induced ROS production in HL-1 cells 6.3–fold ($p<0.01$) (Figure 3.10.B), whereas MSC reduced the increased ROS production 5.1–fold ($p<0.01$) to levels not significantly different from those of non-infected controls.

To assess whether these anti-oxidative stress effects of MSCs are NO dependent, we pretreated MSCs with L-NAME 24 hours before co-culture. Under these conditions, we found that MSCs could not abrogate the CVB3-induced oxidative stress in HL-1 cells.



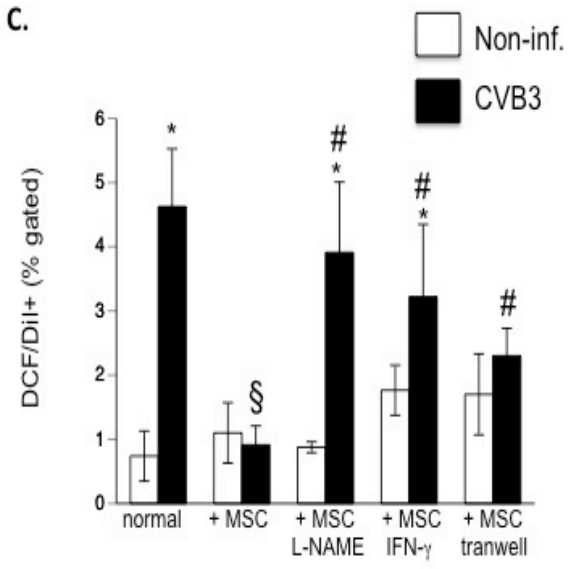


Figure 3.10. MSC reduce coxsackievirus B3-induced oxidative stress in a nitric oxide-, interferon gamma- and cell-contact-dependent way. A. Dil-labeled HL-1 cells were cultured in a 6-well or 24-well plate and 24 hours later infected with CVB3 at a m.o.i. of 5. Four hours later, MSCs were added at a ratio of 1 to 10 HL-1 cells. After 24 hours, cells were collected for DCF FACS analysis. **B.** Upper panel left demonstrates a Forward Scatter (FSC) Sideward Scatter (SSC) of HL-1 cells and right, selected on the Dil-positive HL-1 cells; lower panel demonstrates representative pictures of Dil/DCF dot plots. **C.** Bar graphs represent DCF+/Dil+ HL-1 cells in cultures of HL-1 with or without untreated or L-NAME-treated MSC or MSC in the presence or absence of 1 μg/ml of IFN-γ or with MSC cultured in a transwell; n=4/group; * $p<0.05$ versus respective non-inf. group, § $p<0.05$ versus respective normal group and # $p<0.05$ versus MSC.

To investigate whether the anti-inflammatory effect of MSCs are IFN-γ-dependent, we co-cultured MSCs with CVB3-infected HL-1 in the presence of 1 μg/ml of anti-mouse IFN-γ-neutralizing antibody. We observed that MSCs could not decrease ROS production of CVB3-infected HL-1 cells in the presence of IFN-γ antibody (Figure 3.10).

Finally, to evaluate whether the anti-oxidative features of MSCs was mediated by cell contact to HL-1 cells, MSCs were cultured in a transwell system, with 1 μm pore size, blocking cell contact to HL-1 cells and allowing the presence of secreting factors of the MSC in the total cell medium. Under these conditions, the anti-oxidative effect of MSCs was abrogated (Figure 3.10.).

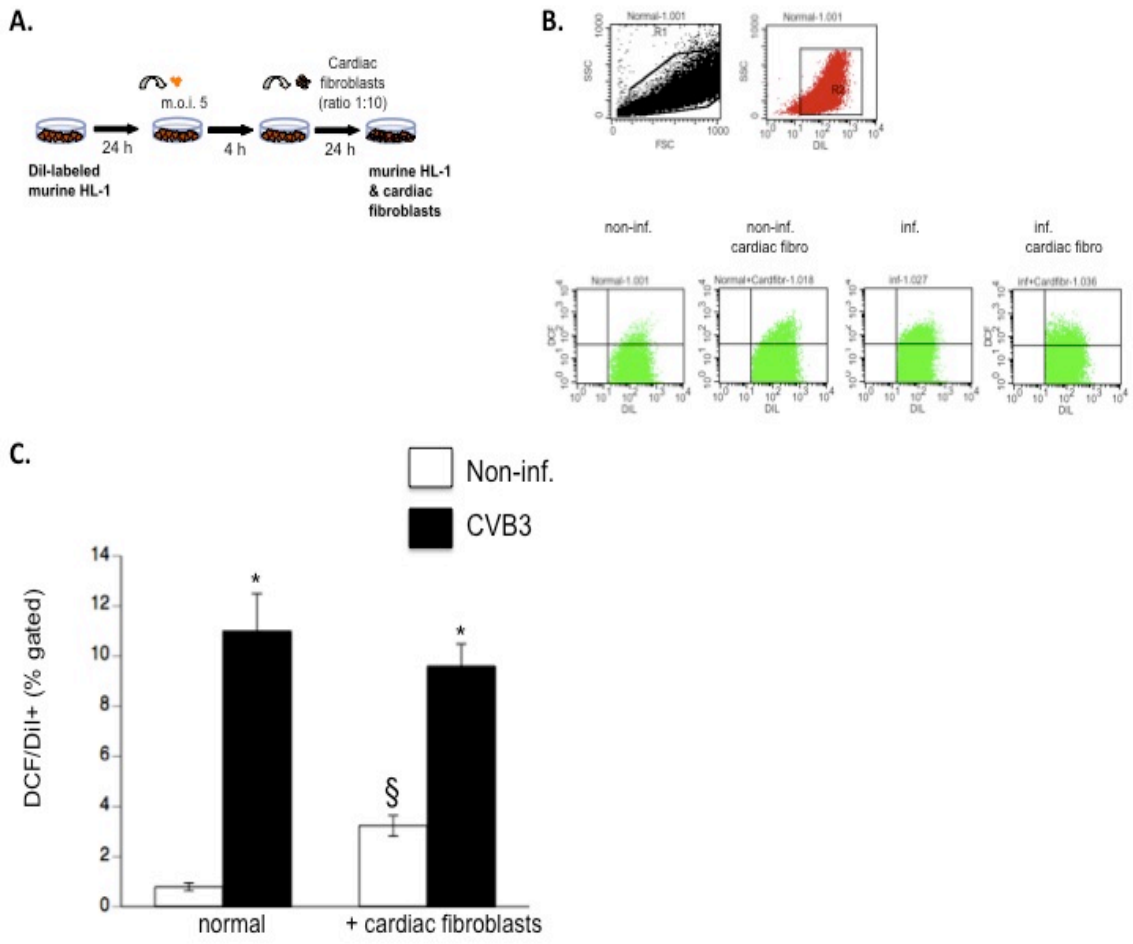


Figure 3.11. Cardiac fibroblasts increase oxidative stress in HL-1 cells. A. Dil-labeled HL-1 cells were cultured in a 6-well plate and 24 hours later infected with CVB3 at a m.o.i. of 5. Four hours hereafter cardiac fibroblasts were added at a ratio of 1 to 10 HL-1 cells. After 24 hours, cells were collected for DCF FACS analysis. **B.** Upper panel left demonstrates a Forward Scatter (FSC) Sideward Scatter (SSC) of HL-1 cells and right, selected on the Dil-positive HL-1 cells; lower panel demonstrates representative pictures of Dil/DCF dot plots. **C.** Bar graphs represent Dil+/DCF+ HL-1 cells in cultures of non-infected or CVB3-infected HL-1 in the presence or absence of cardiac fibroblasts; n=4/group; * $p<0.05$ versus respective non-inf. group, § $p<0.005$ versus respective normal group.

To verify whether or not cell-contact *per se* leads to the reduction of CVB3-induced ROS in HL-1 cells, we co-cultured HL-1 cells in the presence of cardiac fibroblasts. In contrast to MSC, cardiac fibroblasts did not reduce the CVB3-induced HL-1 oxidative stress (Figure 3.11). Under basal conditions, cardiac fibroblasts increased even ROS production in HL-1 cells by 4.1–fold ($p<0.005$).

3.6. Effect of mesenchymal stem cells on nitric oxide production in coxsackievirus

We next analyzed the effect of MSC on NO production in CVB3-infected HL-1 cells. CVB3 infection reduced NO production in HL-1 cells by 1.8–fold ($p<0.005$). MSC supplementation could not increase the impaired NO production under CVB3-infected conditions (Figure 3.12.).

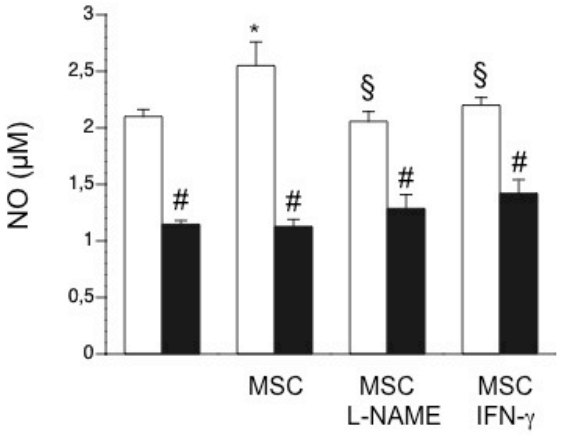


Figure 3.12. Effect of mesenchymal stem cells on nitric oxide production in coxsackievirus B3-infected HL-1 cells. Bar graphs representing NO production in non-infected (open bar graphs) and CVB3-infected (closed bar graphs) HL-1 cells in the presence or absence of MSC incubated with 10 mM L-NAME or 1 µg/ml IFN-γ antibody with n=4/group; #$p<0.005$ versus non-infected, $p<0.05$ versus non-infected HL-1; § $p<0.05$ versus non-infected HL-1+MSC.

3.7. Effect of mesenchymal stem cells on coxsackievirus B3 viral copy number and viral titer in coxsackievirus B3-infected HL-1 cells

To investigate whether the protective effects of MSC on CVB3-infected HL-1 is partly due to the ability of MSC to reduce CVB3 replication in HL-1 cells, CVB3 RNA copy number was determined 24 hours after CVB3 infection in cultures of HL-1 cells with or without MSC. In addition, medium was collected and plaque assay was performed on HeLa cells.

MSC could not reduce CVB3 RNA copy number in HL-1-MSC co-cultures (Figure 3.13.A.). In contrast, plaque assay demonstrated that co-culture with MSC reduced the viral CVB3 titer by 4.7–fold ($p<0.05$), an effect, which was blunted in the presence of L-NAME and by blocking murine IFN-γ (Figure 3.13.B.).

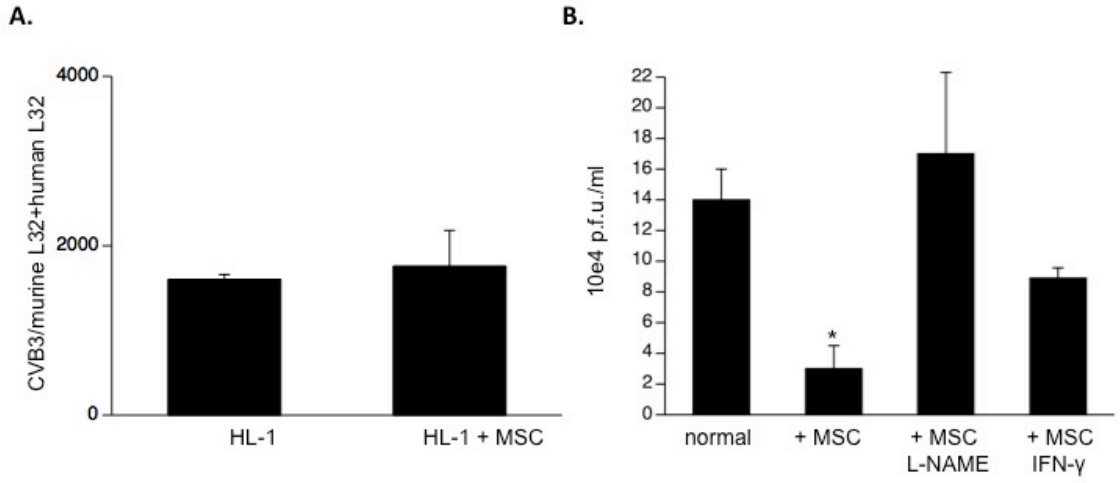


Figure 3.13. Mesenchymal stem cells reduce coxsackievirus B3 replication in coxsackievirus B3-infected HL-1 cells. A. Bar graphs representing CVB3 RNA copy number normalized towards the sum of murine and human L32 in CVB3-infected HL-1 cells (left bar graph) and CVB3-infected HL-1 cells co-cultured with MSC (right bar graph) 24 hours after CVB3 infection with n=6/group. **B.** HeLa cells were incubated for 30 minutes with 1 ml of diluted supernatant of CVB3-infected HL-1 cells or HL-1 cells co-cultured with MSC, in the presence or absence of L-NAME or murine IFN-γ antibody. Then, cells were washed with PBS and covered with agar consisting of 50% 1.3% noble agar and 50% 2x MEM. Bar graphs represent the average amount of counted plaques 72h after incubation with diluted medium of CVB3-infected HL-1 cells or HL-1 cells co-cultured with MSC, in the presence or absence of L-NAME or 1 μg/ml of murine IFN-γ antibody, (all at dilution 10^{-4}), with n=3/condition; #$p<0.05$ versus HL-1 cells.

CHAPTER FOUR: DISCUSSION

Under current pharmaceutical treatment, the prognosis of DCM is poor, urging for the search for alternative therapies. There is growing experimental support for the application of cellular transplantation as a strategy to improve myocardial function. Mesenchymal stem cells have anti-inflammatory, anti-apoptotic and pro-angiogenic features and have been documented to be succesful in experimental models of myocardial infarction. The present study demonstrates for the first time that MSC can be an attractive tool for the treatment of CVB3-induced DCM. We could show that 1) MSC only minimally express CAR and the co-receptor CD55, necessary for CVB3 viral uptake; 2) CVB3 cannot replicate in MSC and do not reduce the viability of MSC, which has important implications in view of clinical translation; 3) MSC can reduce CVB3-induced HL-1 apoptosis and oxidative stress in a NO-dependent, IFN-γ-dependent and cell-contact-dependent way; 4) MSC cannot reduce CVB3 RNA copy number but can decrease viral release in HL-1 in a NO-dependent and IFN-γ-dependent way.

4.1. Expression of CAR and DAF on mesenchymal stem cells

CAR and the co-receptor complement deflecting protein DAF (CD55) are critical determinants for cellular uptake and pathogenesis of CVB3. [11] [16,17] Analysis of CAR and CD55 by FACS analysis demonstrated that relative to the secondary antibody or isotype control, respectively, MSC only minimally express both receptors (see Figure 3.1. and 3.2.). CAR expression was even lower on MSC than on cardiac fibroblasts. Primary fibroblasts express low levels of CAR[83]. However, despite this low expression of CAR, it has been reported that cardiac fibroblasts can be infected with CVB3 and that viral replication can take place[84], suggesting a potential CAR-independent mechanism. In view of clinical translation, it is a prerequisite that no replication of CVB3 can take place in MSC or any other cell used for (cardiac) cell-therapy. To investigate this important safety aspect, we analyzed the presence of CVB3 RNA copy number 4h-12h-24h-48h in MSC after CVB3 infection.

4.2. Coxsackievirus B3 does not replicate in mesenchymal stem cells and does not impair the viability of mesenchymal stem cells

This timeframe study demonstrated that MSC can take up CVB3, but that CVB3 cannot replicate in MSC. CVB3 RNA copy number immediately increased after infection, but gradually decreased after 12h, indicating a potential self-defensive mechanism in MSC. In parallel, no cell stress or changes in cell morphology were observed and MTS assay demonstrated no differences between non-infected and CVB3-infected MSC. In addition, no plaques were formed on HeLa cells with medium collected 24h after CVB3 infection of MSC. In contrast, CVB3 RNA copy number gradually rise in HL-1 cells, indicative for viral replication, which was paralleled with cell stress, cell loss and a gradual reduction in cell viability over-time. Also in cardiac fibroblast CVB3 replication took place, which is in line with Klingel et al.[84], but it occurred at a later timepoint compared to HL-1 cells. In agreement with the low CAR expression on cardiac fibroblasts, the viral CVB3 titer of medium collected from cardiac fibroblasts 24h post infection was 28-fold ($p<0.05$) lower compared to the titer of HL-1 medium 24h after infection (Figure 3.3D). Moreover, the reduction in cell viability after CVB3 infection in cardiac fibroblasts was less pronounced compared to those in HL-1 cells and paralleled the late increase in CVB3 RNA copy number.

Similar to other viruses, CVB3 can modulate the pre-existing host signaling machinery to facilitate its own replication. Several signaling proteins, such as the extracellular signal-regulated kinase 1 and 2 (ERK1/2)[85-87] and protein kinase B/Akt (PKB/Akt)[88], are activated following CVB3 infection, and activation of these signaling proteins is important to successfully complete CVB3's life cycle. Their activation results in the activation of cell survival pathways, which could delay the onset of CVB3-induced apoptosis of infected cells and facilitate replication of CVB3.[87,89] Cunningham et al.[85] reported a link between CAR expression and ERK1/2 activation. Phosphorylation of ERK did not increase in CVB3-infected cells lacking CAR or expressing CAR with a truncated cytoplasmic domain. This suggests that ERK phosphorylation would be influenced by the efficiency of infection provided

model of ERK1/2 activation is given by Huber et al.[86], which demonstrated that ERK1/2 activation follows from viral protease cleavage of RasGAP.

Besides the importance of ERK and Akt activation for the viral life cycle of CVB3, recent studies have also revealed a pivotal role of the ubiquitin proteasome system (UPS) of the host cell in CVB3 viral infectivity. It has been demonstrated that the UPS can be utilized or manipulated by CVB3, to achieve successful viral infection.[90-92] For example, coxsackievirus infection facilitates ubiquitin-dependent proteolysis of cyclin D1, which is linked to CVB3-induced cell growth arrest.[90] Through ubiquitin-mediated degradation of β-catenin, CVB3 infection stimulates glycogen synthetase kinase 3β activity, which contributes to virus-induced cytopathic effect and apoptosis.[92] The importance of the UPS in CVB3 replication follows from an experimental study demonstrating that treatment of cardiomyocytes with proteasome inhibitors markedly reduces CVB3 replication through suppression of viral RNA transcription and protein synthesis.[91]

Further investigation is needed to determine whether the absence of CVB3 replication in MSC is due to or associated with the absence of ERK1/2 and/or Akt activation, or protection of the UPS, and whether there are differences in the kinetics of ERK1/2 and/or Akt activation and use of the UPS between HL-1 cells and cardiac fibroblasts.

4.3. Mesenchymal stem cells reduce apoptosis and oxidative stress in coxsackievirus B3-infected HL-1 cells independently of a reduction in viral RNA replication

CVB3-induced viral myocarditis, initially considered as a sole immune-mediated disease,[93] also results from a direct CVB3-mediated injury of the cardiomyocytes in infected hearts.[94] Besides its immunomodulatory effects, MSC have also been shown to have anti-apoptotic features. The aim of our study was to investigate whether MSC – in the absence of a potential influence on immuno-modulatory cells – could have a direct protective effect on CVB3-infected cardiomyocytes, HL-1 cells. In detail, we analyzed whether MSC could reduce CVB3-induced apoptosis and oxidative stress in

MSC significantly reduced apoptosis and ROS production in HL-1 cells (Figure 3.7 and 3.10, respectively), independently of reducing CVB3 viral replication (Figure 3.13A). The intracellular oxidation status has been implicated in the pathogenesis of CVB3 infections,[95] by which the ROS-mediated release of host cell nuclear transcription factor-kappa-B results in increased viral replication. Our data indicate that the MSC-mediated reduction in ROS production could not decrease viral replication, which is in line with Si et al.[96], who demonstrated that treatment with a potent ROS scavenger, NAC, prevented CVB3-induced intracellular ROS generation, but failed to inhibit CVB3 replication. This suggests that ROS generation is a relatively late event as compared to viral RNA synthesis and viral protein expression. On the other hand, taking the CVB3 viral cycle into account, it is foreseeable that the supplementation of MSC could not have resulted in a decrease in viral replication. MSC have been added 4h post infection, whereas viral replication takes place 8h post infection. At this time, i.e. 4h after seeding the MSC, MSC will just have attached to the culture plates. It is therefore understandable that at that time no appropriate protective effect of the MSC can be expected.

Despite no alteration in CVB3 replication, co-culture of CVB3-infected HL-1 with MSC resulted in a decrease in viral progeny release (Figure 3.13B), a process, which takes place 24h post infection. Inhibition of the stress-activated protein kinase p38 mitogen-activated protein kinase (MAPK) has been reported to reduce the viral progeny release, which was likely mediated by the reduction in CVB3-induced apoptosis and increased cell viability.[97] As in our findings, p38MAPK inhibition did not affect viral protein synthesis. Taken together, our data demonstrate that the anti-oxidative and anti-apoptotic features of MSC resulted in less viral progeny release in CVB3-infected HL-1. Whether these effects are mediated by a reduction in p38MAPK activity will require further investigation.

4.4. The anti-apoptotic/anti-oxidative effects of mesenchymal stem cells are nitric oxide-dependent

cytotoxic immune effector. In addition, NO is known to protect cardiomyocytes from apoptosis [98] and to have anti-viral properties. Recently, NO donors have been reported to inhibit CVB3 replication via S-nitrosylation of the proteinases 2A and 3C, with both the time course of NO release and the amounts of NO in the cell culture supernatant seeming to play an important role for antiviral efficacy.[99]

Consistent evidence has shown that MSC exert their immunosuppressive features in a NO-dependent way. Ren et al. demonstrated that MSCs induced cell-cycle arrest and apoptosis of activated T cells via NO.[58] Sato et al. reported that NO produced by MSCs could suppress phosphorylation of STAT5 and inhibit T cell proliferation.[100]

To investigate whether the anti-apoptotic and anti-oxidative features of MSC were NO-dependent, we pretreated MSC for 24h with the iNOS inhibitor L-NAME and next cocultured these MSCs in the presence of non-infected or CVB3-infected HL-1 cells. In contrast to untreated MSC, L-NAME treated MSC could not reduce the CVB3-induced apoptosis (Figure 3.7.) and oxidative stress (Figure 3.10.) in HL-1 cells, suggesting a NO-dependent mechanism. This finding is supported by the evidence that NO exerts anti-apoptotic effects on cardiomyocytes. [98] In contrast to NO donors, the supplementation of MSC could not reduce CVB3 replication, which can be due to the fact that the timepoint at which MSC was added was too late (see supra).

Addition of MSC to uninfected HL-1 increased NO production, which was abrogated in the presence of L-NAME as well as of murine IFN-γ antibody. The finding that the presence of the murine IFN-γ antibody blocked NO production by MSC is in line with Oh et al.[101] who demonstrated that IFN-γ is critical for NO production by MSC. Under conditions of CVB3-infected HL-1, the absence of a MSC-mediated increase in NO can be explained by the fact that under these severe conditions of oxidative stress, associated with impaired NO bioavailability,[102] MSC do not increase NO, but they increase NO bioavailability, in part by decreasing ROS production.

4.5. The anti-apoptotic/anti-oxidative effects of mesenchymal stem cells are IFN-γ-dependent

anti-tumor,[105,106] and immunomodulatory effects and regulation of cell differentiation.[107,108] The multiple functions of the IFN system are thought to be an innate defense against microbes and foreign substances. The IFN system consists first of cells that produce IFNs in response to viral infection or other foreign stimuli and second of cells that establish the antiviral state in response to IFNs. IFNs have also been used clinically in the treatment of viral diseases.[109]

The mechanisms by which IFN-γ can contribute to the immunosuppressive activity of MSCs have been widely investigated. It has been demonstrated that the enhancement of IDO activity, [62,110] and PGE2, induced by IFN-γ[111] are involved in this process. Ren et al. demonstrated that activation of MSC by IFN-γ plays a key role in the MSC-mediated immunosuppression showing that wild-type MSCs, but not IFN-γR1$^{-/-}$ MSCs, prevented graft-versus-host-disease in mice, an effect reversed by anti-IFN-γ inhibitors.[58]

To investigate whether IFN-γ produced by HL-1 cells is necessary to activate the MSC in the presence of CVB3-infected HL-1 cells, MSC were added to the HL-1 cells in the presence of an anti-mouse IFN-γ antibody. Under these conditions, the anti-apoptotic and anti-oxidative features of MSC were abrogated (Figure 3.7. and Figure 3.10., respectively), resulting in less viral progeny release (Figure 3.13). These findings suggest that MSC required the presence of IFN-γ to perform its protective effects. Taken together, our data together with the findings of Oh et al.[101] who demonstrated that IFN-γ is critical for NO production by MSC, suggest that upon activation with IFN-γ, MSC produce NO via which MSC exert their anti-apoptotic and anti-oxidative effects.

4.6. The anti-apoptotic/anti-oxidative effects of mesenchymal stem cells are cell contact-dependent

Since the well-known immunosuppressive effects of MSC occur most effectively under conditions in which MSCs make physical contact with allogeneic tissue[62,112-114], we also finally wanted to verify whether the protective effects of MSC co-cultured with

cultured in a transwell with pore diameter 1 μm, not allowing cell contact with the HL-1 cells. MSCs cultured under these conditions could not reduce the CVB3-induced apoptosis and oxidative stress in HL-1 cells (Figures 3.7. and 3.10., respectively), suggesting a cell-contact-dependent effect. Whether supplementation of MSC to CVB3-infected HL-1 cells influences the expression of cell contact markers in HL-1, such as the gap junction connexin 43, needs further investigation.

On the other hand, several studies [54,72] have reported that the MSC-mediated effects are paracrine- and not cell-contact-dependent. We suggest that, in the context of CVB3-infected HL-1 cells, MSC need cell contact to perform their protective effects, which are mediated - at least in part - via paracrine effects, including via the production of NO.

To verify whether cell contact *per se* of any cell is the prerequisite to reduce CVB3-induced HL-1 apoptosis and oxidative stress, we cultured cardiac fibroblasts in the presence of non-infected and CVB3-infected HL-1 cells. In contrast to MSC, cardiac fibroblasts did not reduce CVB3-induced apoptosis (Figure 3.9.) and ROS production (Figure 3.11.). Even an increase in apoptosis was observed under CVB3 conditions (Figure 3.9.), which could be caused by the presence and replication of CVB3 in cardiac fibroblasts. This latter finding underscores the importance of the use of cells lacking the susceptibility to CVB3 replication for cardiac repair.

In conclusion, this study demonstrates that CVB3 replication does not take place in MSC and that MSC do not suffer from CVB3 infection. The absence of CVB3 replication is a prerequisite for any cell used in a cell-based therapy for cardiac repair. Besides this important safety aspect, we could demonstrate that MSC have a direct protective effect on CVB3-infected HL-1 cells, i.e. in the absence of any immunomodulatory cells. MSC exert anti-apoptotic as well as anti-oxidative effects on CVB3-infected HL-1 cells in a NO-, IFN-γ-, and cell-contact-dependent way. Finally, MSC have the capacity to reduce the viral progeny release in CVB3-infected HL-1, without reducing CVB3 RNA copy number. This effect is NO- and IFN-γ-dependent

CVB3-infected HL-1.

CHAPTER FIVE: SUMMARY

Background - Under current heart failure treatment, the prognosis of dilated cardiomyopathy (DCM) is poor, urging for alternative therapeutic strategies. There is growing experimental support for the application of cellular transplantation as a strategy to improve myocardial function. Mesenchymal stem cells (MSC) have immunomodulatory features and have been experimentally shown to be attractive tools for the treatment of myocardial infarction and cardiomyopathies.

Aim - The aim of the present study was to investigate whether MSC are potential candidates for the treatment of CVB3-induced inflammatory DCM. In detail, the study focuses on analyzing whether coxsackievirus B3 (CVB3) replication can take place in MSC and whether CVB3 infection can influence MSC viability. In addition, the study investigates whether and how MSC, independently of their immunomodulatory effects, i.e. in the absence of immunoregulatory cells, can have a direct protective effect on CVB3-induced cardiomyocyte apoptosis and oxidative stress.

Methods/experimental design - To study the potential infectivity of MSC, the expression of the coxsackievirus-adenovirus receptor (CAR) and the co-receptor decay accelerating factor (DAF), both necessary for CVB3 uptake, on MSC was evaluated by FACS analysis. Chinese Hamster Ovarian (CHO) cells overexpressing CAR and HL-1 cells, since cardiomyocytes are the target cells of CVB3, were used as positive controls. CHO lacking CAR and cardiac fibroblasts, known to express only low CAR, were used as negative controls. Next, MSC were infected with CVB3 at a multiplicity of infection (m.o.i.) of 5 for 1h. Cell viability was determined 4h, 12h, 24h, and 48h after CVB3 infection via a MTS assay. To assess whether viral replication and viral progeny release take place, the same timeframe experiment was performed and cells and medium were collected to determine CVB3 RNA copy number and plaque formation, respectively. All experiments were performed in parallel with HL-1 and cardiac fibroblasts. To determine the effect of MSC on CVB3-infected HL-1, unlabeled, DiO-labeled or Dil-labeled MSC were cocultured with CVB3-infected HL-1 for RNA purposes, apoptosis (Annexin V/7AAD FACS) and oxidative stress (DCF FACS) analysis,

(NO)-dependent, MSC were pretreated with the iNOS inhibitor N-omega-nitro-arginine methyl ester (L-NAME). To determine whether MSC need the presence of IFN-γ or cell contact with HL-1, culture in the presence of anti-murine IFN-γ antibody or on a transwell membrane was performed, respectively. To determine whether cell contact *per se* leads to an improvement in the condition of the HL-1 cells, cocultures of HL-1 cells with cardiac fibroblasts instead of MSC were performed. Medium of coculture experiments was collected to analyze viral titer via plaque formation on HeLa cells.

Results - Compared to CHO-CAR+ and HL-1 cells, as well as to CHO-CAR- and cardiac fibroblasts, MSC only minimally express CAR at levels nearly comparable with secondary antibody controls. As for CAR, MSC also only moderately express DAF. CVB3 RNA copy number, expressed as CVB3 towards L32, decreased over time in MSC, with 4.1–fold ($p=0.0571$) and 7.2–fold ($p<0.05$) lower levels at 24h and 48h versus 4h post-infection, respectively. In parallel, no differences in cell viability were found between serum starved and CVB3-infected MSC. Medium of MSC collected 24h after CVB3 infection did not induce any plaques on HeLa cells. In contrast, CVB3 RNA copy number rised in HL-1 over time, with 9.0–fold ($p<0.01$) higher RNA levels at 24h versus 12h post-infection. Compared to serum-starved HL-1 cells, CVB3-infected HL-1 showed gradually more cell death over time post CVB3 infection. MTS assay demonstrated 1.9–fold ($p<0.0001$), 1.5-fold ($p<0.0001$) and 1.2–fold ($p<0.05$) lower cell viability at 48h, 24h and 12h post CVB3-infection versus 4h post CVB3-infection, respectively. In cardiac fibroblasts, CVB3 copy number was 3.9-fold ($p=0.05$) higher at 48h compared to 4h post CVB3-infection. In parallel, cell viability was significantly decreased in CVB3-infected compared to serum starved cardiac fibroblasts 48h post CVB3 infection. The viral titer of medium collected from cardiac fibroblasts 24h post infection was 28-fold ($p<0.05$) lower compared to the titer of HL-1 medium.

Annexin V/7AAD FACS analysis demonstrated that MSCs decreased the 4.2-fold ($p<0.05$) CVB3-induced HL-1 apoptosis to levels not significantly different from those of non-infected cells. In parallel, DCF FACS analysis showed that CVB3 induced ROS

ROS production by 5.1–fold ($p<0.01$) to levels not significantly different from those of non-infected controls. When MSCs were pre-treated with 10 mM of L-NAME or cultured in the presence of 1 μg/ml of anti-mouse IFN-γ antibody or cultured on a transwell, the anti-apoptotic and anti-oxidative effects of MSC were abrogated. MSC did not reduce CVB3 RNA copy number in CVB3-infected HL-1 cells, but decreased the viral CVB3 titer by 4.7-fold ($p<0.05$), an effect which was blocked in the presence of L-NAME or anti-mouse IFN-γ antibody. Co-culture of CVB3-infected HL-1 cells with cardiac fibroblasts aggravated CVB3-induced apoptosis.

Conclusion - In conclusion, we could demonstrate that CVB3 replication does not take place in MSC and that MSC do not suffer from CVB3 infection. Besides this important safety aspect, we could show that MSC have a direct protective effect on CVB3-infected HL-1 cells, i.e. in the absence of any immunomodulatory cells. MSC exert anti-apoptotic as well as anti-oxidative effects on CVB3-infected HL-1 cells in a NO-, IFN-γ-, and cell-contact-dependent way. Finally, MSC can reduce the viral progeny release.

CHAPTER SIX: STUDY LIMITATIONS AND PERSPECTIVES

This study was focused on investigating whether MSC are potential candidates for the treatment of CVB3-induced inflammatory DCM. We focused on investigating whether CVB3 replication could take place in MSC and whether CVB3 infection could influence MSC viability. Absence of CVB3 replication is an ultimate prerequisite for any cell use in a cell-based strategy for cardiac repair. No CVB3 replication took place in MSC. The finding that MSC do not suffer from CVB3 infection is important in view of engraftment of MSC in the CVB3-infected heart. Next, we focused on analyzing whether and how MSC, in the absence of any immunomodulatory cells, could directly protect CVB3-infected HL-1 cells. We found that MSC exerted anti-apoptotic and anti-oxidative effects on CVB3-infected HL-1 cells and could reduce CVB3 progeny release in CVB3-infected HL-1 cells without reducing CVB3 RNA copy number.

In view of clinical translation, a study limitation of the present study is the use of the HL-1 cell-line instead of primary cardiomyocytes. On the other hand, it is clear that further *in vivo* investigations have to take place, whereby it will be analyzed whether and how MSC can improve left ventricular function in CVB3-induced inflammatory DCM. Which dose of MSC is sufficient to reach this goal, which administration route has to be used, what is the engraftment of MSC in CVB3-infected hearts? Does MSC-mediated immunomodulation takes place in the CVB3-infected heart? What is the relevance of MSC-mediated immunomodulation in the spleen on the final improvement in left ventricular function? Does MSC, as in the *in vitro* studies, improve cardiomyocyte apoptosis in CVB3-infected hearts? All these questions need to be answered before any translation to the clinic can take place.

REFERENCES

1 Abelmann WH, Lorell BH: The challenge of cardiomyopathy. J Am Coll Cardiol 1989;13:1219-1239.

2 Bodo-Eckehard Strauer (federführend) RK, Gerhard Mall, Bernhard Maisch, Thomas Mertens, Hans Reiner Figulla, Bodo Schwartzkopff, Michael Brehm, Heinz-Peter Schultheiß: Update 2001 myokarditis-kardiomyopathie. Medizinische Klinik 2001;96:608-625.

3 Manolio TA, Baughman KL, Rodeheffer R, Pearson TA, Bristow JD, Michels VV, Abelmann WH, Harlan WR: Prevalence and etiology of idiopathic dilated cardiomyopathy (summary of a national heart, lung, and blood institute workshop. Am J Cardiol 1992;69:1458-1466.

4 Noutsias M, Pauschinger M, Poller WC, Schultheiss HP, Kuhl U: Current insights into the pathogenesis, diagnosis and therapy of inflammatory cardiomyopathy. Heart Fail Monit 2003;3:127-135.

5 Liu PP, Mason JW: Advances in the understanding of myocarditis. Circulation 2001;104:1076-1082.

6 Hyypia T, Hovi T, Knowles NJ, Stanway G: Classification of enteroviruses based on molecular and biological properties. J Gen Virol 1997;78 (Pt 1):1-11.

7 Iizuka N, Kuge S, Nomoto A: Complete nucleotide sequence of the genome of coxsackievirus b1. Virology 1987;156:64-73.

8 Muckelbauer JK, Kremer M, Minor I, Diana G, Dutko FJ, Groarke J, Pevear DC, Rossmann MG: The structure of coxsackievirus b3 at 3.5 a resolution. Structure 1995;3:653-667.

9 Muckelbauer JK, Kremer M, Minor I, Tong L, Zlotnick A, Johnson JE, Rossmann MG: Structure determination of coxsackievirus b3 to 3.5 a resolution. Acta Crystallogr D Biol Crystallogr 1995;51:871-887.

10 Rossmann MG, Palmenberg AC: Conservation of the putative receptor attachment site in picornaviruses. Virology 1988;164:373-382.

11 Bergelson JM, Cunningham JA, Droguett G, Kurt-Jones EA, Krithivas A, Hong JS, Horwitz MS, Crowell RL, Finberg RW: Isolation of a common receptor for coxsackie b viruses and adenoviruses 2 and 5. Science 1997;275:1320-1323.

12 Noutsias M, Fechner H, de Jonge H, Wang X, Dekkers D, Houtsmuller AB, Pauschinger M, Bergelson J, Warraich R, Yacoub M, Hetzer R, Lamers J, Schultheiss HP, Poller W: Human coxsackie-adenovirus receptor is colocalized with integrins alpha(v)beta(3) and alpha(v)beta(5) on the cardiomyocyte sarcolemma and upregulated in dilated cardiomyopathy: Implications for cardiotropic viral infections. Circulation 2001;104:275-280.

13 Selinka HC, Wolde A, Sauter M, Kandolf R, Klingel K: Virus-receptor interactions of coxsackie b viruses and their putative influence on cardiotropism. Med Microbiol Immunol 2004;193:127-131.

14 Li E, Brown SL, Stupack DG, Puente XS, Cheresh DA, Nemerow GR: Integrin alpha(v)beta1 is an adenovirus coreceptor. J Virol 2001;75:5405-5409.

15 Shafren DR, Bates RC, Agrez MV, Herd RL, Burns GF, Barry RD: Coxsackieviruses b1, b3, and b5 use decay accelerating factor as a receptor for cell attachment. J Virol 1995;69:3873-3877.

16 Martino TA, Petric M, Brown M, Aitken K, Gauntt CJ, Richardson CD, Chow LH, Liu PP: Cardiovirulent coxsackieviruses and the decay-accelerating factor (cd55) receptor. Virology 1998;244:302-314.

17 Shafren DR, Williams DT, Barry RD: A decay-accelerating factor-binding strain of

rhabdomyosarcoma cells. J Virol 1997;71:9844-9848.

18 Bowles NE, Richardson PJ, Olsen EG, Archard LC: Detection of coxsackie-b-virus-specific rna sequences in myocardial biopsy samples from patients with myocarditis and dilated cardiomyopathy. Lancet 1986;1:1120-1123.

19 Badorff C, Lee GH, Lamphear BJ, Martone ME, Campbell KP, Rhoads RE, Knowlton KU: Enteroviral protease 2a cleaves dystrophin: Evidence of cytoskeletal disruption in an acquired cardiomyopathy. Nat Med 1999;5:320-326.

20 Nathan C: Nitric oxide as a secretory product of mammalian cells. Faseb J 1992;6:3051-3064.

21 Ignarro LJ: Nitric oxide: A unique endogenous signaling molecule in vascular biology. Biosci Rep 1999;19:51-71.

22 Hiraoka Y, Kishimoto C, Takada H, Nakamura M, Kurokawa M, Ochiai H, Shiraki K: Nitric oxide and murine coxsackievirus b3 myocarditis: Aggravation of myocarditis by inhibition of nitric oxide synthase. J Am Coll Cardiol 1996;28:1610-1615.

23 Mikami S, Kawashima S, Kanazawa K, Hirata K, Katayama Y, Hotta H, Hayashi Y, Ito H, Yokoyama M: Expression of nitric oxide synthase in a murine model of viral myocarditis induced by coxsackievirus b3. Biochem Biophys Res Commun 1996;220:983-989.

24 Zaragoza C, Ocampo C, Saura M, Leppo M, Wei XQ, Quick R, Moncada S, Liew FY, Lowenstein CJ: The role of inducible nitric oxide synthase in the host response to coxsackievirus myocarditis. Proc Natl Acad Sci U S A 1998;95:2469-2474.

25 Zaragoza C, Ocampo CJ, Saura M, McMillan A, Lowenstein CJ: Nitric oxide inhibition of coxsackievirus replication in vitro. J Clin Invest 1997;100:1760-1767.

26 Lowenstein CJ, Hill SL, Lafond-Walker A, Wu J, Allen G, Landavere M, Rose NR, Herskowitz A: Nitric oxide inhibits viral replication in murine myocarditis. J Clin Invest 1996;97:1837-1843.

27 Badorff C, Fichtlscherer B, Rhoads RE, Zeiher AM, Muelsch A, Dimmeler S, Knowlton KU: Nitric oxide inhibits dystrophin proteolysis by coxsackieviral protease 2a through s-nitrosylation: A protective mechanism against enteroviral cardiomyopathy. Circulation 2000;102:2276-2281.

28 Baron S, Tyring SK, Fleischmann WR, Jr., Coppenhaver DH, Niesel DW, Klimpel GR, Stanton GJ, Hughes TK: The interferons. Mechanisms of action and clinical applications. Jama 1991;266:1375-1383.

29 Chawla-Sarkar M, Lindner DJ, Liu YF, Williams BR, Sen GC, Silverman RH, Borden EC: Apoptosis and interferons: Role of interferon-stimulated genes as mediators of apoptosis. Apoptosis 2003;8:237-249.

30 Eriksson U, Kurrer MO, Bingisser R, Eugster HP, Saremaslani P, Follath F, Marsch S, Widmer U: Lethal autoimmune myocarditis in interferon-gamma receptor-deficient mice: Enhanced disease severity by impaired inducible nitric oxide synthase induction. Circulation 2001;103:18-21.

31 Anderson DJ, Gage FH, Weissman IL: Can stem cells cross lineage boundaries? Nat Med 2001;7:393-395.

32 Morrison SJ: Stem cell potential: Can anything make anything? Curr Biol 2001;11:R7-9.

33 Caplan AI: Mesenchymal stem cells. J Orthop Res 1991;9:641-650.

34 Tropepe V, Coles BL, Chiasson BJ, Horsford DJ, Elia AJ, McInnes RR, van der Kooy D: Retinal stem cells in the adult mammalian eye. Science 2000;287:2032-2036.

35 Huang AH, Chen YK, Lin LM, Shieh TY, Chan AW: Isolation and characterization of dental pulp stem cells from a supernumerary tooth. J Oral Pathol Med 2008;37:571-574.

36 Suzuki A, Sekiya S, Onishi M, Oshima N, Kiyonari H, Nakauchi H, Taniguchi H: Flow cytometric isolation and clonal identification of self-renewing bipotent hepatic progenitor cells in adult mouse liver. Hepatology 2008;48:1964-1978.

37 Toma JG, Akhavan M, Fernandes KJ, Barnabe-Heider F, Sadikot A, Kaplan DR, Miller FD: Isolation of multipotent adult stem cells from the dermis of mammalian skin. Nat Cell Biol 2001;3:778-784.

38 Dekaney CM, Rodriguez JM, Graul MC, Henning SJ: Isolation and characterization of a putative intestinal stem cell fraction from mouse jejunum. Gastroenterology 2005;129:1567-1580.

39 Puglisi MA, Giuliani L, Fierabracci A: Identification and characterization of a novel expandable adult stem/progenitor cell population in the human exocrine pancreas. J Endocrinol Invest 2008;31:563-572.

40 Tosh D, Slack JM: How cells change their phenotype. Nat Rev Mol Cell Biol 2002;3:187-194.

41 Weiner LP: Definitions and criteria for stem cells. Methods Mol Biol 2008;438:3-8.

42 Watt FM, Hogan BL: Out of eden: Stem cells and their niches. Science 2000;287:1427-1430.

43 Alison MR, Islam S: Attributes of adult stem cells. J Pathol 2009;217:144-160.

44 Bianco P, Gehron Robey P: Marrow stromal stem cells. J Clin Invest 2000;105:1663-1668.

45 Wakitani S, Saito T, Caplan AI: Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. Muscle Nerve 1995;18:1417-1426.

46 Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR: Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143-147.

47 Chang PL, Blair HC, Zhao X, Chien YW, Chen D, Tilden AB, Chang Z, Cao X, Faye-Petersen OM, Hicks P: Comparison of fetal and adult marrow stromal cells in osteogenesis with and without glucocorticoids. Connect Tissue Res 2006;47:67-76.

48 Birk RZ, Abramovitch-Gottlib L, Margalit I, Aviv M, Forti E, Geresh S, Vago R: Conversion of adipogenic to osteogenic phenotype using crystalline porous biomatrices of marine origin. Tissue Eng 2006;12:21-31.

49 Im GI, Jung NH, Tae SK: Chondrogenic differentiation of mesenchymal stem cells isolated from patients in late adulthood: The optimal conditions of growth factors. Tissue Eng 2006;12:527-536.

50 Alhadlaq A, Mao JJ: Mesenchymal stem cells: Isolation and therapeutics. Stem Cells Dev 2004;13:436-448.

51 Gimble JM, Katz AJ, Bunnell BA: Adipose-derived stem cells for regenerative medicine. Circ Res 2007;100:1249-1260.

52 Nagaya N, Kangawa K, Itoh T, Iwase T, Murakami S, Miyahara Y, Fujii T, Uematsu M, Ohgushi H, Yamagishi M, Tokudome T, Mori H, Miyatake K, Kitamura S: Transplantation of mesenchymal stem cells improves cardiac function in a rat model of dilated cardiomyopathy. Circulation 2005;112:1128-1135.

53 Shake JG, Gruber PJ, Baumgartner WA, Senechal G, Meyers J, Redmond JM, Pittenger MF, Martin BJ: Mesenchymal stem cell implantation in a swine myocardial infarct model: Engraftment and functional effects. Ann Thorac Surg 2002;73:1919-1925.

54 Ohnishi S, Sumiyoshi H, Kitamura S, Nagaya N: Mesenchymal stem cells attenuate cardiac fibroblast proliferation and collagen synthesis through paracrine actions. FEBS Lett 2007;581:3961-3966.

55 Guo J, Lin GS, Bao CY, Hu ZM, Hu MY: Anti-inflammation role for mesenchymal stem cells

transplantation in myocardial infarction. Inflammation 2007;30:97-104.

56 Xu X, Xu Z, Xu Y, Cui G: Selective down-regulation of extracellular matrix gene expression by bone marrow derived stem cell transplantation into infarcted myocardium. Circ J 2005;69:1275-1283.

57 Li L, Zhang Y, Li Y, Yu B, Xu Y, Zhao S, Guan Z: Mesenchymal stem cell transplantation attenuates cardiac fibrosis associated with isoproterenol-induced global heart failure. Transpl Int 2008;21:1181-1189.

58 Ren G, Zhang L, Zhao X, Xu G, Zhang Y, Roberts AI, Zhao RC, Shi Y: Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. Cell Stem Cell 2008;2:141-150.

59 Jiang XX, Zhang Y, Liu B, Zhang SX, Wu Y, Yu XD, Mao N: Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells. Blood 2005;105:4120-4126.

60 Krampera M, Cosmi L, Angeli R, Pasini A, Liotta F, Andreini A, Santarlasci V, Mazzinghi B, Pizzolo G, Vinante F, Romagnani P, Maggi E, Romagnani S, Annunziato F: Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. Stem Cells 2006;24:386-398.

61 Sotiropoulou PA, Perez SA, Gritzapis AD, Baxevanis CN, Papamichail M: Interactions between human mesenchymal stem cells and natural killer cells. Stem Cells 2006;24:74-85.

62 Meisel R, Zibert A, Laryea M, Gobel U, Daubener W, Dilloo D: Human bone marrow stromal cells inhibit allogeneic t-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. Blood 2004;103:4619-4621.

63 Spaggiari GM, Capobianco A, Becchetti S, Mingari MC, Moretta L: Mesenchymal stem cell-natural killer cell interactions: Evidence that activated nk cells are capable of killing mscs, whereas mscs can inhibit il-2-induced nk-cell proliferation. Blood 2006;107:1484-1490.

64 Iyer SS, Rojas M: Anti-inflammatory effects of mesenchymal stem cells: Novel concept for future therapies. Expert Opin Biol Ther 2008;8:569-581.

65 Molina EJ, Palma J, Gupta D, Torres D, Gaughan JP, Houser S, Macha M: Improvement in hemodynamic performance, exercise capacity, inflammatory profile, and left ventricular reverse remodeling after intracoronary delivery of mesenchymal stem cells in an experimental model of pressure overload hypertrophy. J Thorac Cardiovasc Surg 2008;135:292-299, 299 e291.

66 Li JH, Zhang N, Wang JA: Improved anti-apoptotic and anti-remodeling potency of bone marrow mesenchymal stem cells by anoxic pre-conditioning in diabetic cardiomyopathy. J Endocrinol Invest 2008;31:103-110.

67 Schuleri KH, Amado LC, Boyle AJ, Centola M, Saliaris AP, Gutman MR, Hatzistergos KE, Oskouei BN, Zimmet JM, Young RG, Heldman AW, Lardo AC, Hare JM: Early improvement in cardiac tissue perfusion due to mesenchymal stem cells. Am J Physiol Heart Circ Physiol 2008;294:H2002-2011.

68 Guo Y, He J, Wu J, Yang L, Dai S, Tan X, Liang L: Locally overexpressing hepatocyte growth factor prevents post-ischemic heart failure by inhibition of apoptosis via calcineurin-mediated pathway and angiogenesis. Arch Med Res 2008;39:179-188.

69 Halkos ME, Zhao ZQ, Kerendi F, Wang NP, Jiang R, Schmarkey LS, Martin BJ, Quyyumi AA, Few WL, Kin H, Guyton RA, Vinten-Johansen J: Intravenous infusion of mesenchymal stem cells enhances regional perfusion and improves ventricular function in a porcine model of myocardial infarction. Basic Res Cardiol 2008;103:525-536.

70 Semedo P, Wang PM, Andreucci TH, Cenedeze MA, Teixeira VP, Reis MA, Pacheco-Silva A,

Camara NO: Mesenchymal stem cells ameliorate tissue damages triggered by renal ischemia and reperfusion injury. Transplant Proc 2007;39:421-423.

71 Abdel Aziz MT, Atta HM, Mahfouz S, Fouad HH, Roshdy NK, Ahmed HH, Rashed LA, Sabry D, Hassouna AA, Hasan NM: Therapeutic potential of bone marrow-derived mesenchymal stem cells on experimental liver fibrosis. Clin Biochem 2007;40:893-899.

72 Gupta N, Su X, Popov B, Lee JW, Serikov V, Matthay MA: Intrapulmonary delivery of bone marrow-derived mesenchymal stem cells improves survival and attenuates endotoxin-induced acute lung injury in mice. J Immunol 2007;179:1855-1863.

73 Iwanami J, Mogi M, Li JM, Tsukuda K, Min LJ, Sakata A, Fujita T, Iwai M, Horiuchi M: Deletion of angiotensin ii type 2 receptor attenuates protective effects of bone marrow stromal cell treatment on ischemia-reperfusion brain injury in mice. Stroke 2008;39:2554-2559.

74 Mangi AA, Noiseux N, Kong D, He H, Rezvani M, Ingwall JS, Dzau VJ: Mesenchymal stem cells modified with akt prevent remodeling and restore performance of infarcted hearts. Nat Med 2003;9:1195-1201.

75 Menasche P, Hagege AA, Scorsin M, Pouzet B, Desnos M, Duboc D, Schwartz K, Vilquin JT, Marolleau JP: Myoblast transplantation for heart failure. Lancet 2001;357:279-280.

76 Li RK, Jia ZQ, Weisel RD, Merante F, Mickle DA: Smooth muscle cell transplantation into myocardial scar tissue improves heart function. J Mol Cell Cardiol 1999;31:513-522.

77 Kocher AA, Schuster MD, Szabolcs MJ, Takuma S, Burkhoff D, Wang J, Homma S, Edwards NM, Itescu S: Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. Nat Med 2001;7:430-436.

78 Mazhari R, Hare JM: Mechanisms of action of mesenchymal stem cells in cardiac repair: Potential influences on the cardiac stem cell niche. Nat Clin Pract Cardiovasc Med 2007;4 Suppl 1:S21-26.

79 Zhang M, Mal N, Kiedrowski M, Chacko M, Askari AT, Popovic ZB, Koc ON, Penn MS: Sdf-1 expression by mesenchymal stem cells results in trophic support of cardiac myocytes after myocardial infarction. Faseb J 2007;21:3197-3207.

80 Prather WR, Toren A, Meiron M, Ofir R, Tschope C, Horwitz EM: The role of placental-derived adherent stromal cell (plx-pad) in the treatment of critical limb ischemia. Cytotherapy 2009;11:427-434.

81 Claycomb WC, Lanson NA, Jr., Stallworth BS, Egeland DB, Delcarpio JB, Bahinski A, Izzo NJ, Jr.: Hl-1 cells: A cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. Proc Natl Acad Sci U S A 1998;95:2979-2984.

82 Binger T, Stich S, Andreas K, Kaps C, Sezer O, Notter M, Sittinger M, Ringe J: Migration potential and gene expression profile of human mesenchymal stem cells induced by ccl25. Exp Cell Res 2009;315:1468-1479.

83 Hidaka C, Milano E, Leopold PL, Bergelson JM, Hackett NR, Finberg RW, Wickham TJ, Kovesdi I, Roelvink P, Crystal RG: Car-dependent and car-independent pathways of adenovirus vector-mediated gene transfer and expression in human fibroblasts. J Clin Invest 1999;103:579-587.

84 Klingel K, Rieger P, Mall G, Selinka HC, Huber M, Kandolf R: Visualization of enteroviral replication in myocardial tissue by ultrastructural in situ hybridization: Identification of target cells and cytopathic effects. Lab Invest 1998;78:1227-1237.

85 Cunningham KA, Chapman NM, Carson SD: Caspase-3 activation and erk phosphorylation

during cvb3 infection of cells: Influence of the coxsackievirus and adenovirus receptor and engineered variants. Virus Res 2003;92:179-186.

86 Huber M, Watson KA, Selinka HC, Carthy CM, Klingel K, McManus BM, Kandolf R: Cleavage of rasgap and phosphorylation of mitogen-activated protein kinase in the course of coxsackievirus b3 replication. J Virol 1999;73:3587-3594.

87 Luo H, Yanagawa B, Zhang J, Luo Z, Zhang M, Esfandiarei M, Carthy C, Wilson JE, Yang D, McManus BM: Coxsackievirus b3 replication is reduced by inhibition of the extracellular signal-regulated kinase (erk) signaling pathway. J Virol 2002;76:3365-3373.

88 Esfandiarei M, Luo H, Yanagawa B, Suarez A, Dabiri D, Zhang J, McManus BM: Protein kinase b/akt regulates coxsackievirus b3 replication through a mechanism which is not caspase dependent. J Virol 2004;78:4289-4298.

89 Opavsky MA, Martino T, Rabinovitch M, Penninger J, Richardson C, Petric M, Trinidad C, Butcher L, Chan J, Liu PP: Enhanced erk-1/2 activation in mice susceptible to coxsackievirus-induced myocarditis. J Clin Invest 2002;109:1561-1569.

90 Luo H, Zhang J, Dastvan F, Yanagawa B, Reidy MA, Zhang HM, Yang D, Wilson JE, McManus BM: Ubiquitin-dependent proteolysis of cyclin d1 is associated with coxsackievirus-induced cell growth arrest. J Virol 2003;77:1-9.

91 Luo H, Zhang J, Cheung C, Suarez A, McManus BM, Yang D: Proteasome inhibition reduces coxsackievirus b3 replication in murine cardiomyocytes. Am J Pathol 2003;163:381-385.

92 Yuan J, Zhang J, Wong BW, Si X, Wong J, Yang D, Luo H: Inhibition of glycogen synthase kinase 3beta suppresses coxsackievirus-induced cytopathic effect and apoptosis via stabilization of beta-catenin. Cell Death Differ 2005;12:1097-1106.

93 Mason JW, O'Connell JB, Herskowitz A, Rose NR, McManus BM, Billingham ME, Moon TE: A clinical trial of immunosuppressive therapy for myocarditis. The myocarditis treatment trial investigators. N Engl J Med 1995;333:269-275.

94 Chow LH, Beisel KW, McManus BM: Enteroviral infection of mice with severe combined immunodeficiency. Evidence for direct viral pathogenesis of myocardial injury. Lab Invest 1992;66:24-31.

95 Schwarz KB: Oxidative stress during viral infection: A review. Free Radic Biol Med 1996;21:641-649.

96 Si X, McManus BM, Zhang J, Yuan J, Cheung C, Esfandiarei M, Suarez A, Morgan A, Luo H: Pyrrolidine dithiocarbamate reduces coxsackievirus b3 replication through inhibition of the ubiquitin-proteasome pathway. J Virol 2005;79:8014-8023.

97 Si X, Luo H, Morgan A, Zhang J, Wong J, Yuan J, Esfandiarei M, Gao G, Cheung C, McManus BM: Stress-activated protein kinases are involved in coxsackievirus b3 viral progeny release. J Virol 2005;79:13875-13881.

98 Razavi HM, Hamilton JA, Feng Q: Modulation of apoptosis by nitric oxide: Implications in myocardial ischemia and heart failure. Pharmacol Ther 2005;106:147-162.

99 Zell R, Markgraf R, Schmidtke M, Gorlach M, Stelzner A, Henke A, Sigusch HH, Gluck B: Nitric oxide donors inhibit the coxsackievirus b3 proteinases 2a and 3c in vitro, virus production in cells, and signs of myocarditis in virus-infected mice. Med Microbiol Immunol 2004;193:91-100.

100 Sato K, Ozaki K, Oh I, Meguro A, Hatanaka K, Nagai T, Muroi K, Ozawa K: Nitric oxide plays a critical role in suppression of t-cell proliferation by mesenchymal stem cells. Blood 2007;109:228-234.

101 Oh I, Ozaki K, Sato K, Meguro A, Tatara R, Hatanaka K, Nagai T, Muroi K, Ozawa K: Interferon-gamma and nf-kappab mediate nitric oxide production by mesenchymal stromal cells. Biochem Biophys Res Commun 2007;355:956-962.

102 Kojda G, Harrison D: Interactions between no and reactive oxygen species: Pathophysiological importance in atherosclerosis, hypertension, diabetes and heart failure. Cardiovasc Res 1999;43:562-571.

103 Hiscott J, Grandvaux N, Sharma S, Tenoever BR, Servant MJ, Lin R: Convergence of the nf-kappab and interferon signaling pathways in the regulation of antiviral defense and apoptosis. Ann N Y Acad Sci 2003;1010:237-248.

104 Garcia-Sastre A: Mechanisms of inhibition of the host interferon alpha/beta-mediated antiviral responses by viruses. Microbes Infect 2002;4:647-655.

105 Thomas WJ, Thomas DL, Knezetic JA, Adrian TE: The role of oxygen-derived free radicals and nitric oxide in cytokine-induced antiproliferation of pancreatic cancer cells. Pancreas 2002;24:161-168.

106 Okamoto T, Tsuburaya A, Yanoma S, Yoshikawa T, Cho H, Takanashi Y, Noguchi Y: Inhibition of peritoneal metastasis in an animal gastric cancer model by interferon-gamma and interleukin-2. Anticancer Res 2003;23:149-153.

107 Lohoff M, Mak TW: Roles of interferon-regulatory factors in t-helper-cell differentiation. Nat Rev Immunol 2005;5:125-135.

108 Jego G, Palucka AK, Blanck JP, Chalouni C, Pascual V, Banchereau J: Plasmacytoid dendritic cells induce plasma cell differentiation through type i interferon and interleukin 6. Immunity 2003;19:225-234.

109 Masci P, Bukowski RM, Patten PA, Osborn BL, Borden EC: New and modified interferon alfas: Preclinical and clinical data. Curr Oncol Rep 2003;5:108-113.

110 Kang JW, Kang KS, Koo HC, Park JR, Choi EW, Park YH: Soluble factors-mediated immunomodulatory effects of canine adipose tissue-derived mesenchymal stem cells. Stem Cells Dev 2008;17:681-693.

111 Aggarwal S, Pittenger MF: Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 2005;105:1815-1822.

112 Sheng H, Wang Y, Jin Y, Zhang Q, Zhang Y, Wang L, Shen B, Yin S, Liu W, Cui L, Li N: A critical role of ifngamma in priming msc-mediated suppression of t cell proliferation through up-regulation of b7-h1. Cell Res 2008;18:846-857.

113 Najar M, Rouas R, Raicevic G, Boufker HI, Lewalle P, Meuleman N, Bron D, Toungouz M, Martiat P, Lagneaux L: Mesenchymal stromal cells promote or suppress the proliferation of t lymphocytes from cord blood and peripheral blood: The importance of low cell ratio and role of interleukin-6. Cytotherapy 2009;11:570-583.

114 English K, Ryan JM, Tobin L, Murphy MJ, Barry FP, Mahon BP: Cell contact, prostaglandin e(2) and transforming growth factor beta 1 play non-redundant roles in human mesenchymal stem cell induction of cd4+cd25(high) forkhead box p3+ regulatory t cells. Clin Exp Immunol 2009;156:149-160.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor, Prof. Dr. Carsten Tschöpe for offering the opportunity and providing support to carry out this study in Department of Cardiology and Pneumology in Charite Campus Benjamin Franklin, and for his continuous guidance, encouragement and care throughout the entire period of study. Also his critical reading of this manuscript and insight and thoughtful comments are highly appreciated.

I would also like to express my sincere gratitude to Dr. Sophie Van Linthout, who led me to the scientific research field, for her constructive and helpful directions throughout the entire period of study, and for her critical reading and revision.

I am very grateful to Kapka Miteva, Annika Köschel for their technical assistance in this study.

I own my sincere gratitude to my wife, my son and my parents for their love, encouragement and support throughout my study in Berlin.

Finally, I would like to thank the SFB (projects B5, B6) and the Berlin-Brandenburg Center for Regenerative Therapies (BCRT), without their financial support this study would not have been possible.

CURRICULUM VITAE AND PUBLICATIONS

STATEMENT IN LIEU OF OATH

I declare that the experiments described in the thesis were carried out by me.

Erklärung

„Ich, [Jun Peng], erkläre, dass ich die vorgelegte Dissertation mit dem Thema: [Effect of mesenchymal stem cells on Coxsackievirus B3-induced inflammatory cardiomyopathy] selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe."

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