Reduced Degradation of the Chemokine MCP-3 by Matrix Metalloproteinase-2 Exacerbates Myocardial Inflammation in Experimental Viral Cardiomyopathy

Dirk Westermann, MD; Kostantinos Savvatis, MD; Diana Lindner, PhD; Christin Zietsch, PhD; Peter Moritz Becher, MD; Elke Hammer, PhD; Markus M. Heimesaat, MD; Stefan Bereswill, PhD; Uwe Völker, PhD; Felicitas Escher, MD; Alexander Riad, MD; Johanna Plendl, PhD; Karin Klingel, MD; Wolfgang Poller, MD; Heinz-Peter Schultheiss, MD; Carsten Tschöpe, MD

Background—Myocarditis is an important cause for cardiac failure, especially in younger patients, followed by the development of cardiac dysfunction and death. The present study investigated whether gene deletion of matrix metalloproteinase-2 influences cardiac inflammation and function in murine coxsackievirus B3 (CVB3)–induced myocarditis.

Methods and Results—Matrix metalloproteinase-2 knockout mice (MMP-2−/−) and their wild-type controls (WT) were infected with CVB3 to induce myocarditis. Three days after infection, an increased invasion of CD4+ activated T cells into the myocardium was documented, followed by an excess of inflammatory cells 7 days after infection, which was significantly higher in the MMP-2−/− animals compared with the WT animals. Moreover, cardiac apoptosis, remodeling, viral load, and function were deteriorated in MMP-2−/− animals after CVB3 infection. This overwhelming inflammation was followed by 100% mortality after 15 days. This was associated with increased levels of MCP-3 in the cardiac tissue of MMP-2−/− mice. Because MMP-2 cleaves the chemokine MCP-3, the loss of this cleavage lead to an overreaction of the immune system with pronounced myocardial damage mediated by the inflammatory cells. When a neutralizing antibody against MCP-3 was given to MMP-2−/− mice, this exaggerated reaction of the immune system could be normalized to levels similar to WT-CVB3 animals.

Conclusions—Loss of MMP-2 increased the inflammatory response after CVB3 infection, which impaired cardiac function and survival during CVB3-induced myocarditis. Matrix metalloproteinase-2–mediated chemokine cleavage has an important role in cardiac inflammation as a negative feedback mechanism. (Circulation. 2011;124:2082-2093.)

Key Words: inflammation ■ myocarditis ■ remodeling ■ metalloproteinases ■ viruses

Myocarditis is a common cause for inflammatory heart diseases, especially in children and young adults, and can lead to severe cardiac dysfunction and death.1 Common causal agents for myocarditis in this population are enteroviruses, such as the cardiotropic coxsackievirus B3 (CVB3).2 After infection of the cardiomyocytes with CVB3, a robust inflammatory response is provoked and can help to achieve the clearance of virus-infected cardiomyocytes. Often this is followed by a retraction of the immune cells from the heart, leading to a complete recovery from the acute disease.1,3 Nevertheless, this immune-cell infiltration can also cause detrimental effects by itself because inflammatory cells are known to mediate myocardial damage directly (eg, by CD8+ cytotoxic T lymphocytes)4–6 or indirectly (by the overexpression of proinflammatory cytokines or induction of fibrotic processes).7,8 This exaggerated reaction of the immune system with prolonged presence of inflammatory cells can induce cardiac apoptosis as well as an accumulation of extracellular matrix, both associated with detrimental cardiac remodeling resulting in cardiac dysfunction followed by ventricular dilatation and death.9,10 This is associated with an upregulation and activation of matrix metalloproteinases (MMPs), also triggered by cardiac inflammation.11 Nevertheless, whether this upregulation in the setting of viral myocarditis is beneficial or detrimental is not known.

Editorial see p 2052
Clinical Perspective on p 2093

It was shown that a knockout of MMP-2 and MMP-9 (both gelatinases) is beneficial in nonviral heart diseases.12–15 Moreover, a broad inhibition (not a complete inactivation due
to gene deletion) of all MMPs by overexpression of their tissue inhibitor was protective in CVB3-induced myocarditis. On the contrary, it could be shown that MMP-9 but not MMP-8 gene deletion was detrimental in CVB3-induced myocarditis and resulted in severe cardiac damage. Up to the present, it is known that MMP-9 degrades interferon-related cytokines, and therefore viral clearance might be decreased. This new role of MMPs, which involves not only processing of extracellular matrix proteins, but also degrading as well as activating cytokines and chemokines, has the potential to counterbalance an inflammatory response as negative feedback. Nevertheless, the immunomodulatory effects of MMP-2 are still unknown in the setting of inflammatory cardiomyopathies. The aim of this study was to investigate the effect of gene deletion of MMP-2 on the outcome of CVB3-induced myocarditis and whether possible antiinflammatory effects of MMP-2 by inactivation of immunomodulators during the development of myocarditis outbalance its matrix-degrading effects.

Methods

Study Design

Animals were bred and kept under specific pathogen-free conditions. Male MMP-2 knockout mice (backcrossed at least 7 times into the C57Bl/6b background) (MMP-2-/-) and their male wild-type (WT) littermates, based on a C57Bl/6b background, were kept under standard conditions (all mice were 7 weeks old at the day of infection). Mice were infected with 5 x 10^5 plaque-forming units of CVB3 intraperitoneally (i.p.) infected WT mice (WT-CVB3) and MMP-2-/- mice (MMP-2-/-–CVB3) 3 and 7 days after CVB3 infection. Furthermore, in an additional experiment, 18 MMP-2-/- mice were stimulated with 20 ng/mL concanavalin A (Biochrom AG) containing 10% fetal calf serum, 100 μg/mL streptomycin, and 100 μg/mL streptomycin (PAA). MMP-2-/- mice were treated with placebo (n=9, goat immunoglobulin G (control) i.p. injection day 2, 4, and 6 after CVB3 infection) or a MCP-3-neutralizing antibody (n=18, i.p. injection day 2, 4, and 6 after CVB3 infection) or a control group (n=10 per group). In addition, WT-CVB3 (n=25) and MMP-2-/-CVB3 (n=20) survival was investigated for 18 days after CVB3 infection. In brief, in an additional experiment, 18 MMP-2-/- mice were treated with placebo (n=9, goat immunoglobulin G (control) i.p. injection day 2, 4, and 6 after CVB3 infection) or a MCP-3-neutralizing antibody (n=18, i.p. injection day 2, 4, and 6 after CVB3 infection) or a control group (n=10 per group). In this study, the group of MMP-2 knockout mice and WT mice were compared with saline-treated mice of both groups (n=10 per group).

Physiological Measurements and Surgical Procedures

Cardiac output and stroke volume, as well as left ventricular (LV) contractility (dP/dt max, mm Hg/s) and LV relaxation (dP/dt min, mm Hg/s) as indices of LV function were recorded via a micromanoeuractometer (1.2F) system in closed-chest animals as described previously. Moreover, heart rate as well as mean blood pressure was analyzed. Cardiac tissue was harvested immediately after hemodynamic measurements. The hearts were rapidly removed, transversely dissected in 3-mm thick slices, cleaned in ice cold water, and snap frozen for later analysis.

Histological Measurements

In Tissue Tec–embedded LV-tissue sections, immunohistochemistry was carried out using primary antibodies for CD4 (Pharmin gen 1:50), CD8 (Pharmingen 1:50), neutrophils (Abcam 1:250), CD68 (Abcam 1:350) and CD80 (BD Biosciences 1:50), as well as receptor of activated protein kinase C (RACK1; Abcam 1:400) and MCP-3 (Abcam 1:500) followed by the DAKO Envision horseradish peroxidase technique (DAKO, Glostrup, Denmark). Quantification was performed by digital image analyses. In brief, the ratio between heart tissue area and the specific chromomogen-positive area was calculated (depicted here as area fraction in percentage). The amount of infiltrating cells was calculated by measuring the number of cells per area of heart tissue (cells/mm²).

Isolation and Cultivation of Primary Mouse Embryonic Cardiomyocytes

For the purpose of isolation and cultivation of primary mouse embryonic cardiomyocytes, WT and MMP-2-/- mice have been used. The pregnant mice were euthanized by CO₂ asphyxiation. The embryos (12 days old) were isolated from the uterus and then transferred into a dish with ice-cold sterile PBS without Ca²⁺ and Mg²⁺ (PAA, Cölbe, Germany). The embryos were released from the yolk sacs and then transferred into another dish with the same solution as described above. By the use of surgical instruments, every single heart was isolated under a microscope. The heart was incubated at 4°C for 20 hours and subsequently at 37°C for 15 minutes in a solution of 0.05% trypsin and 0.02% EDTA (wt/vol) (Biochrom AG). The tissue-trypsin-EDTA-mix has been included in 1 mL of DMEM (DMEM high glucose (4.5 g/L) without L-glutamine (PAA) with 10% FCS and 1% each of penicillin and streptomycin (Biochrom AG). In order to dissociate the heart, tissue was gently pipetted several times. The mix gained by this procedure contains fibroblasts and cardiomyocytes. The cells within the medium from each heart of the same genotype were transferred into a cell culture flask (TPP, Trasadingen, Switzerland) to seed the fibroblast. The cultures were maintained for 1 hour at 37°C in a humidified atmosphere with 5% CO₂. The supernatant containing the cardiomyocytes was removed gently, counted, and then plated out to an amount of 1 x 10⁵/cm² cardiomyocytes in a 12-well plate (TPP) coated with fibronectin (Biochrom AG).

Isolation and Cultivation of Primary Cardiac Fibroblasts

Primary cardiac fibroblasts were cultured in iscove medium (Biochrom AG) containing 10% fetal calf serum, 10% human serum (PAA), 100 μg/mL penicillin, and 100 μg/mL Streptomycin (PAA), as described earlier. Sixteen hours before the stimulation experiment, cells were starved in iscove medium containing 0.5% fetal calf serum, 100 μg/mL penicillin, and 100 μg/mL streptomycin. Then cells were stimulated with 20 μg/mL concanavalin A (Biochrom AG) for 72 hours. Cell culture supernatant was collected and stored at −80°C. The monocyte cell line THP-1 cells was cultured in RPMI medium (PAA) containing 10% fetal calf serum (PAA), 100 μg/mL penicillin, and 100 μg/mL streptomycin (PAA).

Zymography of MMP Activity

Gelatin zymography was performed to determine gelatinolytic activity of MMP-2. Protein (40 μg) from cardiac fibroblasts was treated with sampling buffer (0.5 mol/L Tris-HCl, 50% glycerol, 10% SDS, and 0.1% bromphenol blue) in a final solution of 20 μL. SDS-PAGE was performed using 10% polyacrylamid gel containing 0.1% gelatin at 125 V for 60 minutes. SDS was removed with Triton X-100 for 60 minutes, and the gel was incubated in a developing buffer (0.01 mol/L Tris-base, 0.4 mol/L Tris-HCl, 2 mol/L NaCl, 0.07 mol/L CaCl₂, and 0.6% Brij-35) overnight. Gels were stained for 3 hours with 0.5% coomassie G250 and destained for 60 minutes in 7% acetic acid and 35% methanol. The gelatinolytic activity was detected as a clear band against a blue background (arbitrary unit per centimeter) and analyzed as relative optical densities using Scion Image software.

Transferase-Mediated dUTP Biotin Nick-End Labeling Assay in LV Tissue

In Tissue Tec embedded LV tissue sections, we detected apoptotic cells by end-labeling the fragmented DNA using the DeadEnd Colorimetric Transferase–Mediated dUTP Biotin Nick-End Labeling (TUNEL) System (Promega, Madison, WI) according to the manufacturer’s instructions. The slides were examined in a blinded fashion. Transferase-Mediated dUTP Biotin Nick-End Labeling–
positive cells were calculated by cells per area of heart tissue at 200-fold magnification.

**Real-Time Reverse-Transcription Polymerase Chain Reaction**

The relative quantification of targets was performed using the comparative C_{\text{t}} (dC_{\text{t}}) method on the Applied Biosystems 7900HT System. The TaqMan Arrays were preloaded with TaqMan Gene Expression Assays (2 replicates per assay). One thousand ng of total RNA converted to complementary DNA, mixed with equal volume of TaqMan universal polymerase chain reaction master mix, were loaded into fill reservoirs of TaqMan Arrays. The real-time polymerase chain reaction was performed according to the manufacturer’s instructions. Each TaqMan gene expression assay contains a forward and reverse primer for each of the chosen genes (18S ribosomal, collagen I, fibronectin, CVB3, TNF-α, interleukin (IL)-1β, IL-6, IL-18, IL-23, CC chemokine receptor 2 [CCR2], CXC chemokine receptor 3 [CXCR3], Collagen type I, fibronectin, CVB3). 18S RNA were incorporated in our customized TaqMan low-density array as internal standards.

**Electron Microscopy**

After fixation in Karnovsky fixative followed by postfixation in 1% osmium tetroxide solution (0.1 mol/L phosphate buffer), small pieces of LV were rinsed and dehydrated in ascending alcohol series as described by Shakibaei. Next, samples were embedded in Epon and cut on a Reichert-Jung Ultracut E (Nussloch, Germany), followed by contrast staining with 2% uranyl acetate/lead citrate. A transmission electron microscope (Zeiss TEM 10, Jena, Germany) was used to examine the sections.

**Infection of Primary Cardiomyocytes With CVB3**

The embryonic cardiomyocytes were inoculated with CVB3 at a multiplicity of infection of 1 plaque-forming unit per cell in DMEM without FCS penicillin and streptomycin at 37°C for 30 minutes. After 30 minutes, the medium containing CVB3 was removed and replaced by DMEM containing 10% FCS and 1% of each penicillin and streptomycin. The cells were kept for another 24 hours before harvesting.

**In Situ Hybridization**

At the indicated time points after CVB3 infection, tissue sections from frozen hearts were used for detection of viral RNA with a 35S-labeled enterovirus-specific RNA probe as previously described. Briefly, tissue sections were exposed for 3 weeks and counterstained with hematoxylin and eosin. The detection of replication is a highly reproducible and sensitive method to document virus replication at the cellular level. Quantitation of CVB3 RNA following in situ hybridization was done by using the following score obtained in high power fields (×200): 0 = no positive cells; 1 = a few small foci with positive cells; 2 = a few foci with >100 positive cells; 3 = ≤10% of the tissue sections contain positive cells; and 4 = 10% to 30% of the tissue sections contain positive cells.

**MCP-3 Cleavage and Chemotaxis Assay**

For in vitro cleavage we used cell culture supernatant from human cardiac fibroblasts incubated with or without concanavalin A, which then contains the active MMP-2 or the inactive pro-MMP-2, respectively. Twenty μL of recombinant MCP-3 at a concentration of 50 μg/mL or MCP-1 at a concentration of 10 μg/mL (Peprotech, Hamburg, Germany) were incubated with 5 μL of cell culture supernatant from concanavalin A-stimulated human fibroblasts at 37°C for 48 hours.

Cell migration of THP-1 cells was performed using the FluoroBlok 24 well insert system with 8 μm pores (BD Falcon, Heidelberg, Germany). For the chemotaxis assay, the in vitro cleaved MCP-3 or MCP-1 were further diluted in iscove medium containing 0.5% FCS, 100 U/mL penicillin and 100 μg/mL streptomycin to a final concentration of 500 ng/mL or 100 ng/mL and added to the lower chamber. Cells of the THP-1 type were stained with Dil (Lonza, Basel, Switzerland) for 30 minutes in RPMI medium and were then also suspended in iscove medium containing 0.5% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin. A total amount of 100,000 Dil stained THP-1 cells were added to the upper chamber. After 3 hours incubation at 37°C in 5% CO₂, migrated cells in the lower chamber were counted. Using fluorescence microscopy, we recorded 10 different pictures during a scan through the whole well.

**MCP-3 Cleavage and Western Blot**

After activation of 1 ng MMP-2 (Calbiochem, Darmstadt, Germany) with P-aminophenyl-mercuric acetate at a final concentration of 2.5 mMol/L, the enzyme was mixed with 100 ng MCP-3 (Peprotech) in buffer (100 mMol/L NaCl, 5 mMol/L CaCl₂, 20 mMol/L Tris, pH 8.0) and incubated at 37°C for 24 hours. Samples were loaded into a 16.5% Tris-tricine gel followed by Western blot technique. To investigate a cleavage of MCP-3 by MMP-2, MCP-3 and possible additional bands were detected using a specific antibody (Abcam, Cambridge, UK). The blots were visualized with a chemiluminescence system (Amersham Bioscience, Buckinghamshire, UK).

**Enzyme-Linked Immunosorbent Assay for MCP-3**

Concentration of MCP-3 in the serum was determined using a murine MCP-3 ELISA kit (Peprotech) according to the manufacturer’s instructions.

**Statistical Analysis**

Statistical analysis was performed using SPSS Version 13.0. Data are expressed as box plots with median. Statistical differences were assessed by using the Kruskal-Wallis test in conjunction with the Mann-Whitney U posthoc test. Bonferroni correction was applied to adjust for multiple comparisons. Survival curves after CVB3 infection were created by using the Kaplan-Meier method and compared by using the log-rank test. Differences were considered statistically significantly at a value of P<0.05.

**Results**

**Severe Inflammation in MMP-2−/−–CVB3 After CVB3 Infection**

MMP-2−/− and WT demonstrate low numbers in inflammatory cells in the cardiac tissue under baseline conditions. Three days after infection, there was a small increment of CD4+ cells (P=0.0012) in the WT-CVB3 animals compared with baseline. This was aggravated in MMP-2−/−–CVB3 compared with WT-CVB3 after 3 days with a 2-fold increase (P=0.018) at this time point already. Seven days after infection, we found an exacerbation of cardiac inflammatory cells in MMP-2−/−–CVB3 compared with WT-CVB3 in all investigated cell types (all P<0.001), whereas in WT-CVB there was a milder invasion of inflammatory cells (Figure 1).

**Increased Cytokine Expression 7 Days After CVB3 Infection in MMP-2−/−–CVB3**

We found a cytokine gene overexpression 7 days after CVB3 infection in MMP-2−/−–CVB3 compared with WT-CVB3 animals when investigating IL-1β (9 fold, P<0.001), IL-6 (5 fold, P<0.0012), IL-18 (3 fold, P<0.001), IL-23 (10 fold, P<0.001) and tumor necrosis factor α (5 fold, P=0.028) as measured by real time polymerase chain reaction (Figure 2).

**Increased Chemokine Receptor Gene Expression 7 Days After CVB3 Infection in MMP-2−/−–CVB3**

In addition to increased transendothelial migration of immunocompetent cells 7 days after CVB3 infection, we found an
increase in CCR2 and CXCR3 in the WT-CVB3 animals. Nevertheless, there was a further increase in the MMP-2/-–CVB3 animals 7 days after infection compared with the WT-CVB3 animals in the cardiac tissue (Figure 2).

### Increased Apoptosis 7 Days After CVB3 Infection in MMP-2/-–CVB3

Seven days after infection, we documented higher numbers of TUNEL-positive cells in the WT-CVB3 group ($P=0.004$). A 100-fold increase ($P<0.001$) could be observed in the MMP-2/-–CVB3 7 days after infection compared with baseline conditions. The number of caspase-3+ cells was increased 3 ($P=0.019$) and furthermore 7 days ($P=0.002$) after infection in the MMP-2/-–CVB3, whereas this increment was lower in the WT-CVB3 after 7 days compared with MMP-2/-–CVB3 at day 7 ($P=0.002$). This was accompanied by an increase of RACK1 expression after 3 days in both MMP-2/-–CVB3 and WT-CVB3 (both $P<0.001$), but 7 days after infection RACK1 expression was further increased in MMP-2/-–CVB3 ($P<0.001$) but already downregulated in the WT-CVB3 ($P=0.003$) (Figure 3A). Moreover, electron microscopy revealed features of apoptotic cell death with disorganized fibrils and swollen degenerated mitochondria, as well as condensation of nuclear chromatin and membrane blebbing in the MMP-2/-–CVB3 7 days after CVB-3. These features were seen less frequently in WT-CVB3 animals (Figure 3B).

### Increased Accumulation of Extracellular Matrix in MMP-2/-–CVB3 7 Days After CVB3 Infection

Collagen type 1 gene expression ($P=0.02$) as well as protein of collagen type 1 ($P=0.003$) and fibronectin gene expression ($P=0.002$) were significantly increased 7 days after CVB3 infection in MMP-2/-–CVB3 compared with WT-CVB3 (Figure 4A).}

### Increased Mortality of MMP-2/-–CVB3 After CVB3 Infection

A low mortality could be observed after CVB3 infection in the WT-CVB3 animals after CVB3 infection. Contrarily, in the MMP-2/-–CVB3 group, all animals died after 15 days, revealing a 100% mortality after CVB3 infection. This was significantly higher than in the WT-CVB3 ($P<0.001$). In MMP-2/-–CVB3 treated with a MCP-3 neutralizing antibody, the mortality was significantly reduced ($P<0.001$) to MMP-2/-–CVB3 and not significantly different compared to WT-CVB3 animals ($P=0.78$). (Figure 4B).

### Increased CVB3 Load After 3 and 7 Days in CVB3-Infected MMP-2/- Mice

We found an increased virus replication by in situ hybridization with a radioactively labeled CVB3-specific probe not 3 (0.149) but 7 days ($P<0.001$) after infection in the hearts of MMP-2/- compared with WT mice (Figure 5A). Moreover, copy numbers were increased 7 days ($P=0.003$) after infec-
tion in the hearts of MMP-2−/− compared with WT mice (Figure 5B). Interestingly, there was no change in CVB3 genome quantity after in vitro infection of cardiomyocytes when embryonic myocytes of both strains were compared (MMP-2−/−: \(2.9 \pm 0.2\times10^8\) versus WT: \(3.2 \pm 1.8\times10^8\), mean±SEM, \(P=0.68\)). This indicates that no MMP-2–mediated effects influenced CVB3 replication in cardiomyocytes but that rather an overreaction of the immune system is responsible for higher CVB3 replication in MMP-2−/− mice compared with WT mice in the in vivo setting.

**Increased Protein Content of the MCP-3 Chemokine 7 Days After Infection in the MMP-2−/−–CVB3**

We found a significant increase in the protein content of the chemokine MCP-3 in the cardiac tissue as well as in the serum of the MMP-2−/−–CVB3 mice compared with WT-CVB3 mice 3 (cardiac tissue \(P=0.002\) and serum \(P=0.019\)) and 7 days after infection (cardiac tissue \(P=0.015\) and serum \(P=0.018\)) (Figure 6).

**In Vitro Cleavage of MCP-3 by Active MMP-2 and Low Chemotactive Activity of Cleaved MCP-3**

Active but not inactive MMP-2 cleaved recombinant MCP-3 in vitro, which can be seen by a second lower band in the Western blot (Figure 7A). Moreover, this cleaved MCP-3 showed significantly lower (\(P=0.001\)) chemotactic activity in a migration assay compared with uncleaved MCP-3 (Figure 7B). To document specificity of this cleavage, we incubated MCP-1 with active and inactive MMP-2 and could not show any differences in the chemotactic activity (MCP-1: 8.5±1.9; MCP-1 with active MMP-2: 9.1±0.5; MCP-1 with inactive MMP-2: 10.3±1.0, fold change of migrated cells against control medium; \(P=0.68\)). This indicates that MMP-2 cleaves MCP-3 but not MCP-1.

**Treatment of the MMP-2−/−–CVB3 With a MCP-3–Neutralizing Antibody In Vivo**

Treatment of the MMP-2−/−–CVB3 with a MCP-3–neutralizing antibody in vivo reduced the cardiac inflammatory
response after CVB3 infection compared with placebo-treated animals. There was a significant reduction of CD4+ (P=0.002) and CD8+ (P=0.012) as well as CD68+ cells (P=0.002) 7 days after infection when MMP-2-/-–CVB3 treated with the MCP-3–neutralizing antibody when compared with placebo-treated animals of the MMP-2-/-–CVB3 group (Figure 8A). Importantly, viral load was also significantly reduced in the cardiac tissue (P=0.002) (Figure 8C).

**Decreased LV Function in MMP-2-/-–CVB3 After 7 Days of CVB3 Infection**
MMP-2-/- and WT showed no significant differences in LV function under basal conditions. Seven days after CVB3 infection, we show a decrease of LV function in WT-CVB3 compared with baseline as evidence of myocarditis. There was a further deterioration in MMP-2-/-–CVB3 compared with WT-CVB3 with a further reduction of contractility (dP/dt max, P=0.043) and relaxation (dP/dt min, P=0.019) as well as global cardiac output (P=0.006). Treatment with the neutralizing antibody improved LV function compared with untreated MMP-2-/-–CVB3 (the Table).

**Discussion**
We present evidence that cardiac MMP-2 is beneficial in CVB3-induced myocarditis. Loss of MMP-2 resulted in cardiac apoptosis, severe inflammation with high numbers of invading inflammatory cells, and an excess of cytokine gene expression as well as increased CVB3 load, all of which contributed to LV dysfunction and high mortality after CVB3 infection. Matrix metalloproteinase-2–mediated chemokine degradation of MCP-3 seems to serve as a negative feedback during the development of myocarditis in WT animals and is responsible for the exacerbation of myocarditis in MMP-2 knockout mice.

Cardiac dysfunction during the development of myocarditis is caused not only by direct virus-induced myocyte damage but also by an overreaction of the inflammatory response of the immune system in order to control the viral infection. T cells especially were shown to be detrimental in the cause of CVB3-induced myocarditis.21,22 CD4+ as well as CD8+ T-cell knockout mice were protected from myocardial damage and necrosis in CVB3-induced myo-
carditis. In CD4⁺-CD8⁺ double knockout mice, survival was also improved. Interestingly, this T-cell depletion was associated with reduced numbers of invading macrophages as well as with reduced expression of TNF-α, known to be cardiotoxic. These data show that T cells in experimental myocarditis increase the severity of the disease. In line with this finding, increased cardiac inflammation impairs prognosis in patients with viral myocarditis. Nevertheless, in severe-combined-immunodeficiency mice (lacking all immune cells), CVB3 myocarditis was severe and uncontrolled. This suggests that a controlled response of the immune system is favorable in order to control viral infection.

Figure 4. Accumulation of extracellular matrix in MMP-2⁻/⁻-CVB3 and mortality. A, Increased accumulation of extracellular matrix with significantly higher protein content of collagen type 1 measured in histology as well as gene expression of collagen type 1 in MMP-2⁻/⁻-CVB3-7 days compared with WT-CVB3-7 days. Fibronectin gene expression was also significantly increased in MMP-2⁻/⁻-CVB3-7 days compared with WT-CVB3-7 days. The gene expression has been normalized to 18S messenger RNA levels as endogenous control according to the formula 2⁻ⁿ_{ΔΔCT} (n=10 in each group). *Significant differences between the groups indicated by bars. B, Significantly increased mortality in MMP-2⁻/⁻-CVB3 animals could be documented after CVB3 infection compared with WT-CVB3 animals. Significantly improved survival in MMP-2⁻/⁻-CVB3 treated with a MCP-3–neutralizing antibody (nAB), which was similar to survival in WT-CVB3. *Significant differences between MMP-2⁻/⁻-CVB3 compared with either WT-CVB3 or MMP-2⁻/⁻-CVB3 treated with a MCP-3–neutralizing antibody. WT indicates wild type; MMP2⁻/⁻, matrix metalloproteinase-2 knockout mice; CVB3, coxsackievirus B3; 7d, 7 days; and MCP-3 nAB, MCP-3–neutralizing antibody.

Figure 5. Increased viral load in MMP-2⁻/⁻-CVB3. A, Representative pictures of in situ hybridization with a probe specific for CVB3 showing replicating virus in myocytes. Note increased amount of virus-positive cells in association with myocardial lesions in the MMP-2⁻/⁻ mice especially 7 days after infection compared with the WT mice. B, Expression of CVB3 messenger RNA (given as copy numbers) in cardiac tissue after 3 and 7 days with significantly increased CVB3 messenger RNA after 7 days in MMP-2⁻/⁻-CVB3 compared with WT-CVB3. *Significant differences between the groups indicated by bars. WT indicates wild type; MMP2⁻/⁻, matrix metalloproteinase-2 knockout mice; CVB3, coxsackievirus B3; 3d, 3 days; and 7d, 7 days.
infection without too much T-cell–mediated myocyte damage.

In the present study, we found an increased upregulation of CD4<sup>+</sup>-activated T cells in the MMP-2<sup>−/−</sup>–CVB3 group already 3 days after infection, which was followed by an excessive invasion of further CD4<sup>+</sup> and CD8<sup>+</sup> T cells as well as neutrophils, CD68<sup>+</sup> macrophages, and CD80<sup>+</sup>-activated B cells 7 days after CVB3 infection compared with WT-CVB3 animals, where inflammation was less pronounced. This combination of different inflammatory cells, also suggested by upregulation of different chemokine receptors (CCR2 and CXCR3) in the cardiac tissue,

Figure 6. Increased MCP-3 in the cardiac tissue as well as serum of MMP-2<sup>−/−</sup>–CVB3. A, Increased protein abundance of MCP-3 can already be measured by histology after 3 days in the MMP-2<sup>−/−</sup>–CVB3. This was further aggravated 7 days after CVB3 infection. B, Increased protein of MCP-3 measured by ELISA in the serum of the myocarditis animals. After 3 days, a significantly higher increase could be documented in the MMP-2<sup>−/−</sup>–CVB3 animals compared with WT-CVB3. This was consistent after 7 days although the overall levels were lower when compared with day 3. C, Representative pictures of MCP-3 staining showing that MCP-3 is mostly expressed by inflammatory cells in the infiltrates in the MMP-2<sup>−/−</sup>–CVB3 animals. *Significant differences between the groups indicated by bars. WT indicates wild type; MMP2<sup>−/−</sup>, matrix metalloproteinase-2 knockout mice; CVB3, coxsackievirus B3; 3d, 3 days; and 7d, 7 days.

Figure 7. Cleavage to MCP-3 by active MMP-2. A, Zymography showing the inactive proform of MMP-2 (derived from cardiac fibroblast) and the active MMP-2 (derived from cardiac fibroblast stimulated with concanavalin A). B, Migration assay showing the significant increase in chemotactic activity of recombinant MCP-3 against THP-1 cells compared with medium only. Interestingly, using recombinant MCP-3 after incubation with active MMP-2 resulted in significantly lower migration compared with MCP-3 incubated with inactive MMP-2, demonstrating that the cleaved MCP-3 is less functional relative to chemotaxis. *Significant differences between the groups indicated by bars. C, Western blot showing MCP-3 after in vitro incubation with or without P-aminophenyl-mercuric acetate–activated MMP-2. Note the lower band after incubation with the active MMP-2 showing a degradation product of MCP-3. MMP2 indicates matrix metalloproteinase 2; and APMA, P-aminophenyl-mercuric acetate.
mediates cardiac damage by direct-cell toxic effects as well as by indirect actions of proinflammatory cytokines. Moreover, as shown by polymerase chain reaction as well as in situ hybridization experiments replicating virus was significantly increased in the MMP-2-/- mice compared with the WT animals. As documented by situ hybridization, the high virus load in MMP-2-/- mice was found to be in close association with increased myocardial lesions and inflammation, also suggesting that increased inflammation caused cardiac damage. Viral load was not different at day 3, which underscores that the differences in inflammation at day 7 are independent of virus load but suggests that this difference is mediated by changes in the immune response. Hence, viral load does not necessarily correlate to inflam-

Figure 8. Improvement of the phenotype after treatment with a MCP-3–neutralizing antibody. A, Significant decrease of inflammatory cells in the cardiac tissue (CD4” and CD68” left y axis; CD68” right y axis) when MMP-2-/-–CVB3–7 days was compared with MMP-2-/-–CVB3–7 days treated with a MCP-3–neutralizing antibody (nAB). B, Significant reduction of viral load in treated animals with reduced signal in situ hybridization for CVB3 and copy numbers of CVB3 with representative pictures of in situ for both groups. C, Significant reduction of MCP-3 in the cardiac tissue measured by histology with representative pictures when MMP-2-/-–CVB3–7 days was compared with the MMP-2-/-–CVB3–7 days treated with a MCP-3–neutralizing antibody. *Significant differences between the groups indicated by bars. MMP2-/- indicates matrix metalloproteinase-2 knockout mice; CVB3, coxsackievirus B3; 7d, 7 days; and nAB, neutralizing antibody.

Table. Hemodynamic Function

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>MMP-2-/-</th>
<th>WT-CVB3–7d</th>
<th>MMP-2-/-–CVB3–7d</th>
<th>MMP-2-/-–CVB3–7d + MCP-3 nAB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, bpm</td>
<td>473±28</td>
<td>423±32</td>
<td>302±27*</td>
<td>318±27*</td>
<td>335±29</td>
</tr>
<tr>
<td>Cardiac output, mL/min</td>
<td>9.5±0.6</td>
<td>9.3±0.55</td>
<td>6.23±0.47*</td>
<td>3.9±0.4†</td>
<td>5.9±0.8†</td>
</tr>
<tr>
<td>Stroke volume, µL</td>
<td>20.3±1.6</td>
<td>22.3±1.9</td>
<td>19.6±1.3</td>
<td>13.1±1.6†</td>
<td>17.6±1.7†</td>
</tr>
<tr>
<td>dP/dt max, mm Hg/s</td>
<td>8175±311</td>
<td>7050±379</td>
<td>5250±478*</td>
<td>3498±641†</td>
<td>4873±389§</td>
</tr>
<tr>
<td>dP/dt min, mm Hg/s</td>
<td>−6008±408</td>
<td>−6252±853</td>
<td>−4145±443*</td>
<td>−2898±329†</td>
<td>−3780±284‡</td>
</tr>
<tr>
<td>Mean blood pressure, mm Hg</td>
<td>102±4</td>
<td>105±5</td>
<td>75±9*</td>
<td>67±5†</td>
<td>71±4‡</td>
</tr>
</tbody>
</table>

WT indicates wild type, MMP-2-/-, matrix metalloproteinase-2 knockout mice; CVB3, coxsackievirus B3; 7d, 7 days; nAB, neutralizing antibody; dP/dt max, contractility; and dP/dt min, relaxation.

*Significantly different vs respective control (WT or MMP-2-/-).
†Significantly different vs WT-CVB3–7d.
‡Significantly different vs MMP-2-/-–CVB3–7d.
mation. This was associated with a high expression of cytokines 7 days after infection. These cytokines are all known to mediate cardiac damage directly and indirectly. Cytokines are known to induce pathological remodeling with increased cardiac collagen accumulation, which can be seen in the MMP-2/-/-CVB3 group compared with the WT-CVB3 animals. Also, fibronectin, known to be upregulated during inflammatory stress and to be proadhesive for activated T cells, was increased in the MMP-2/-/-CVB3.

Cardiac apoptosis is 1 prominent feature of myocarditis, and can also be induced by cardiac inflammation. We document only low-grade apoptosis in the WT-CVB3 animals 7 days after infection but an additional 10-fold increase in the MMP-2/-/-CVB3 group. Moreover, expression of caspase-3 favored apoptosis in the MMP-2/-/-CVB3, whereas caspase-3 was less activated in the WT-CVB3 animals, which is in agreement with the lower number of TUNEL-positive cells. RACK1 expression, known to disrupt the association of the proapoptotic Bax and antiapoptotic Bcl and therefore promote apoptosis by inducing Bax permeabilization into the mitochondria, was increased 7 days after infection in the MMP-2/-/-CVB3 as another sign of apoptotic processes. This proapoptotic pathology was also made evident by electron microscopy showing increased apoptotic features in the MMP-2/-/-CVB3 such as disarray of the myofilament and swollen as well as degraded mitochondria together with the condensation of nuclear chromatin and membrane blebbing of myocytes. This suggests that apoptosis is 1 part of the pathology in CVB3-induced myocarditis. Nevertheless, because only few myocytes undergo apoptotic processes, direct cardio-depressive effects of the inflammatory cells seem to be also important for dysfunction after CVB3 infection. Future studies have to reveal the exact consequence of apoptosis and cell death in this model.

Excessive inflammation, pathological remodeling, and apoptosis resulted in decreased LV function in the MMP-2/-/-CVB3 animals 7 days after infection. More importantly, this was followed by a 100% mortality of the MMP-2/-/-CVB3 animals compared with the WT-CVB3, in which only low mortality was documented. This increased mortality due to overwhelming inflammation highlights the need for more investigation of key mechanisms leading to excessive cardiac inflammation in human cardiac diseases.

In the course of heart failure, MMPs are upregulated by, e.g., cardiac inflammation and are also known to contribute to cardiac remodeling in CVB3-induced myocarditis. Increased activity of MMPs was thought to be important for the degradation of matrix proteins only. Nevertheless, it could be shown that the gelatinases MMP-9 and MMP-2 especially have other properties. Next to cleavage of matrix proteins, they also process cytokines and chemokines (reviewed in more detail elsewhere). For this reason, they can activate or inactivate those immunomodulators and therefore play an important role in inflammatory heart diseases. Recently it was shown that gene deletion of MMP-9 exacerbates CVB3-induced myocarditis, with high numbers of invading inflammatory cells and increased mortality. This role of MMP-9 is similar to its role during bacterial infection, in which a loss of MMP-9 results in increased bacterial load with increased joint inflammation in a murine model of Staphylococcus aureus–triggered septic arthritis. These findings might be explained by degradation of cytokines such as interferons, which are important for cell recruitment to inflammatory sites, by MMP-9.

Matrix metalloproteinase-2 is known to process chemokines such as MCP-3 and inactivate them or even convert them into receptor antagonists. MCP-3, 1 ligand for CCR2 on monocytes, is important for cell recruitment to inflammatory sites in a variety of inflammatory diseases, and this might be of special importance for early T-cell activation as well as differentiation in inflammatory responses. Here we show that active MMP-2 cleaves MCP-3 in vitro and that after cleavage MCP-3 has lower chemotactic activity compared with uncleaved MCP-3. This MMP-2–dependent degradation was specific for MCP-3 and could not be documented with MCP-1. Coherently, we show that the increased protein content of MCP-3 due to loss of MMP-2 facilitates the invasion of inflammatory cells and leads to the excess of the inflammatory response after CVB3 infection. Moreover, when treating MMP-2/-/-CVB3 with a MCP-3–neutralizing antibody in vivo, we could document reduced inflammation, lower CVB3 load, and improved cardiac function similar to what was documented in WT-CVB3 animals. This was associated with improved survival.

Therefore, an upregulation of MMP-2 in the setting of cardiomyopathies might induce degradation of matrix proteins, which can be detrimental, but it is also a negative feedback loop relative to chemokine degradation. In the future, it would be of interest to investigate the role of specific MMP-2 inhibitors (compared with a genetic knockout) in this disease. Pharmacological inhibitors might not completely block MMP-2 activity but rather normalize it to control values, and thus degradation of MCP-3 might still be effective to control inflammation. Nevertheless, we show here that a total inhibition of MMP-2 activity is detrimental in viral myocarditis, emphasizing that care must be taken when using MMP-2 inhibitors in this disease.

Our present study reveals an important and novel function of MMP-2 in viral myocarditis and increases our knowledge of MMPs in the immune modulation of cardiac diseases. Loss of MMP-2 exacerbates the inflammatory response after viral infection. Degradation of MCP-3 by MMP-2 seems to be a potent negative feedback in immunity and of special importance during myocarditis.

Sources of Funding

The present study was supported by the Deutsche Forschungsgemeinschaft (SFB-TR-19, TP A2 to Dr Westermann and Dr Schultheiss, Z3 to Dr Tschope, and Z4 to Dr Klingel, SFB 633, TP A7 to Dr Bereswill, and B6 to Dr Heimesaat), and Bundesministerium für Bildung und Forschung 01EZ0817.
Disclosures

None.

References


**CLINICAL PERSPECTIVE**

Cardiac inflammation is one important pathomechanism in the progression of heart failure. Although in some cardiac diseases inflammation might be helpful (e.g., after acute myocardial infarction), uncontrolled inflammation is detrimental in most forms of cardiomyopathy. Insights into the pathophysiological control of inflammation are needed to develop new interventions. Matrix metalloproteinases (MMPs) are deregulated in acute and chronic heart failure. In most studies, the gelatinases MMP-2 and MMP-9 are especially upregulated. Although MMPs were thought to process matrix proteins only and, therefore, trigger remodeling, there is recent evidence that some MMPs process cytokines as well as chemokines, thereby modulating the inflammatory process. This study enhances our knowledge about the immunomodulatory properties of MMPs, especially relative to inflammatory cardiomyopathies. Matrix metalloproteinase 2 degrades the chemokine MCP-3. We show here that this is one beneficial mechanism that controls inflammation during viral myocarditis. Loss of this degradation by MMP-2 knockout increases protein levels of the chemokine MCP-3, which fuels the inflammatory process leading to increased transendothelial migration of inflammatory cells into the cardiac tissue. This experimental overreaction of the immune system could be prevented by neutralizing MCP-3 in vivo. This shows that a complete inhibition of MMP-2 (and similar data exists for MMP-9) is detrimental in experimental viral heart disease. Preservation of MMP-2 should be one goal in treating heart failure with an increased inflammatory process because an uncontrolled inflammatory reaction leads to cardiac failure. This might be a double-edged sword given that future drug therapies target the MMP system to prevent adverse remodeling.
Reduced Degradation of the Chemokine MCP-3 by Matrix Metalloproteinase-2 Exacerbates Myocardial Inflammation in Experimental Viral Cardiomyopathy

Dirk Westermann, Kostantinos Savvatis, Diana Lindner, Christin Zietsch, Peter Moritz Becher, Elke Hammer, Markus M. Heimesaat, Stefan Bereswill, Uwe Völker, Felicitas Escher, Alexander Riad, Johanna Plendl, Karin Klingel, Wolfgang Poller, Heinz-Peter Schultheiss and Carsten Tschöpe

*Circulation*. 2011;124:2082-2093; originally published online October 10, 2011; doi: 10.1161/CIRCULATIONAHA.111.035964

*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/124/19/2082

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Circulation* is online at:
http://circ.ahajournals.org//subscriptions/