Ablation of Matrix Metalloproteinase-9 Increases Severity of Viral Myocarditis in Mice

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Background—Coxsackievirus B3 (CVB3) causes human myocarditis, which can result in cardiac damage, maladaptive remodeling, and heart failure. Matrix metalloproteinases (MMP)-8 and -9 have been identified in virus-infected myocardium, but their particular roles and underlying mechanisms of effect are unknown. For the first time, we examine the severity of CVB3-induced myocarditis in MMP-8–and MMP-9–deficient mice.

Methods and Results—CVB3-infected MMP-8 and MMP-9 knockout (KO) mice and corresponding wild-type (WT) mice were euthanized and harvested at 9 days after infection. Expression of MMP-2, -8, -12, and -13 and tissue inhibitors of MMPs was assessed by zymography or immunoblotting on harvested hearts, and in situ hybridization was performed to detect active infection. Infected MMP-9 KO mice had greater myocardial injury and foci of infection than WT mice despite similar pancreatic infection. Increased fibrosis (10.6±2.7% versus 7.1±2.6%, P<0.04), viral titer, as well as decreased cardiac output, were evident in MMP-9 KO compared with WT mice as assessed by picrosirius red staining, plaque assay, and echocardiography, respectively. Immune infiltration was also greatly increased in MMP-9 KO compared with WT mice (15.2±12.6% versus 2.0±3.0%, P<0.002). Myocardial interferon-β1, interferon-γ, interleukin-6, interleukin-10, and macrophage inflammatory protein-1α expression was elevated in MMP-9 KO mice as measured by quantitative real-time polymerase chain reaction and ELISA. In contrast, MMP-8 KO mice had the same degree of cardiac injury, fibrosis, and viral infection as their WT counterparts.

Conclusions—During acute CVB3 infection, MMP-9 appears necessary to halt virus propagation in the heart, promote proper immune infiltration and remodeling, and preserve cardiac output. (Circulation. 2008;117:1574-1582.)

Key Words: inflammation ■ metalloproteinases ■ myocarditis ■ infection

Infection with coxsackievirus B3 (CVB3) is the most common cause of human myopericarditis.1 Viral myocarditis can occur as an acute, fulminant illness or progress as a chronic disorder that is attended by fibrotic scarring and eventual dilated cardiomyopathy.2 Virus replication elicits leukocytic infiltration, including macrophages, T cells, natural killer cells, and granulocytes.3,4 Ineffective viral clearance may lead to virus persistence in the myocardium, protracted virus-induced cytolysis, and greater chronic inflammation.5

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Studies indicate that both early direct, virus-mediated injury and the immune response to viral infection contribute to the progression of CVB3-induced myocarditis.5,6 Our laboratory has shown that CVB3 infection of severe immune-deficient mice, which lack both T and B lymphocytes, results in early and extensive myocyte coagulation, contraction band necrosis, and cytolysis compared with wild-type (WT) counterparts.6 Immune infiltration is a critical reflection of host defense in response to viral infection, but an inappropriate and exaggerated immune response may also lead to tissue damage.7 Woodruff and Woodruff8 first demonstrated that T-cell depletion results in a decrease in inflammatory infiltrate and mortality after CVB3 infection. Transfer of mononuclear cells from CVB3-infected mice or patients with myocarditis into genetically identical or immunodeficient mice, respectively, also exacerbates myocardial damage.9 Conversely, inhibition of natural killer cells in virus-infected mice or CVB3 infection of B-cell–knockout (KO) animals exacerbates disease.10,11
Matrix metalloproteinases (MMPs) are important immunomodulators through their matrix-degrading functions, affecting cytokine processing and cell migration.12,13 MMP-8 and MMP-9 regulate both innate and adaptive immunities. They can modulate the inflammatory reaction through both proteolytic activation (interleukin [IL]-1β)14 and inactivation (interferon [IFN]-β)15 of chemokines and cytokines. Our laboratory and others have demonstrated the upregulation of MMP-2, -9, -8, and -12 during the inflammatory phase of viral myocarditis, which suggests that MMPs play a role in the evolution of the disease.16–18 In the present study, we used MMP-8 and MMP-9 KO mouse models of acute murine myocarditis to further discern the effects of these MMPs.

**Methods**

**Experimental Groups**

MMP-9 KO mice were derived from their WT 129SvEv counterpart by Vu et al19 (Taconic, Germantown, NY). MMP-8 KO mice were generated as described previously.20 Their WT counterparts (C57BL/6) were homozygotes derived from the same parental line.

**Virus Infection and Detection**

Virus stocks were propagated and quantified.21 Five-week-old adolescent male mice from all 4 genotypes were infected intraperitoneally with 10^3 plaque-forming units of CVB3 (Gauntt strain) or phosphate-buffered saline. Mice were euthanized at 9 days after infection (PI). In total, 26 WT 129SvEv, 24 MMP-9 KO, 11 C57BL/6, and 11 MMP-8 KO mice were used in the present study. The presence of active virus in cardiac tissue was assessed with plaque assay and in situ hybridization.21 All animal procedures were performed in accordance with the guidelines of the Animal Care Committee, University of British Columbia.

**Gelatin Zymography**

Heart tissue was assessed with gelatin zymography to detect the activity of MMP-2 and -9, as described previously.16,22

**Western Blot**

Frozen heart apices were homogenized, and 100 μg of total protein was resolved with SDS-PAGE under reducing conditions. MMP-8 antibody was a gift from Dr Overall; MMP-12 antibody was purchased from BIOMOL (Plymouth Meeting, Pa); and MMP-13 antibody was from Neomarkers/Labvision (Fremont, Calif).

**Histological Assessment**

Midventricular samples were fixed in 10% neutral-buffered formalin and processed for standard histological staining.16

**Immunohistochemistry**

Paraffin-embedded heart sections were immunolabeled for the following proteins: CD3 (Dako, Mississauga, Ontario, Canada), neutrophil marker (clone 7/4, Serotec, Raleigh, NC), and CD45 (BD Biosciences, Mississauga, Canada) and detected as described previously.23
Quantitative Real-Time Polymerase Chain Reaction
Expression of IFN-β, IFN-γ, IL-1β, IL-5, IL-6, IL-10, IL-12, macrophage inflammatory protein (MIP)-1α, transforming growth factor-β, and tumor necrosis factor-α was assessed. Total mouse RNA was isolated from the basal portion of the heart with an RNeasy kit (Qiagen, Mississauga, Canada) per the manufacturer’s instructions.16 Quantitative real-time polymerase chain reaction was run according to the manufacturer’s protocol on the Applied Biosystems 7900HT Fast RT-PCR System with GAPDH expression as the internal control. Polymerase chain reaction primers and probes were used from the TaqMan Gene Expression Assays collection (Applied Biosystems, Foster City, Calif).

ELISA Analysis
Protein expression levels were measured by ELISA. The following molecules were assessed: IFN-γ (Quantikine, R&D Systems, Minneapolis, Minn); monocyte chemotactic protein; and IFN-α and IFN-β (Invitrogen-BioSource, Carlsbad, Calif). Absorbance was measured according to the manufacturer’s suggestions on a RainbowReader (Tecan, Zurich, Switzerland).

Echocardiography
Echocardiography images were obtained from infected MMP-9 KO and WT mice at days 0, 3, 5, and 9 PI with the Vevo 770 (VisualSonics, Toronto, Canada) while the mice were under 1.5% isoflurane anesthetic. Short-axis, 2D M-mode views were obtained at the level of the papillary muscles. From these images, left ventricular posterior wall thickness and left ventricular internal diameter in systole and diastole were measured; cardiac output and ejection fraction were calculated with the manufacturer’s software. Doppler examination of the mitral valve was also performed.

Results

Activation Status of MMP-2 and MMP-9
Absence of MMP-9 in MMP-9 KO mice was confirmed with gelatin zymography (Figure 1A and 1B). The proform and active form of MMP-2 were detected in all samples, whereas a single 100-kDa MMP-9 band was detected only in WT hearts. The activities of both proteases were upregulated in infected WT mice compared with sham. As expected, only MMP-2 was upregulated in infected MMP-9 KO mice (Figure 1A and 1C). Despite absent MMP-9 activity, MMP-2 expression did not differ between WT and MMP-9 KO, which suggests that compensation did not occur (Figure 1C).

Protein Expression of MMPs
To determine compensation by other MMPs, we investigated the protein expression of MMP-8, -12, and -13. The mesenchymal-derived forms of MMP-8 (45 to 55 kDa) were predominantly detected (Figure 2A), and active MMP-8 (45 kDa) was strongly expressed in both WT and MMP-9 KO mice (Table 1). Multiple lower bands (22 to 35 kDa) were detected in both genotypes, which were thought to be degradative peptides of this enzyme. ProMMP-12 and active MMP-12 (55 and 45 kDa, respectively) were expressed in comparable amounts in both WT and MMP-9 KO mice (Table 1). Glycosylated proMMP-13 (70 kDa), unglycosylated proform (55 kDa), and active MMP-13 enzyme (40 kDa) did not differ between WT and MMP-9 KO mice (Table 1).

Table 1. Protein Expression of MMPs

<table>
<thead>
<tr>
<th>MMP</th>
<th>Molecular Weight, kDa</th>
<th>MMP-9 KO</th>
<th>WT</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-8 Total</td>
<td>1.23±0.37</td>
<td>1.32±0.43</td>
<td>0.58</td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>0.10±0.10</td>
<td>0.17±0.16</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>1.10±0.31</td>
<td>1.04±0.33</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>0.01±0.01</td>
<td>0.01±0.01</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>0.03±0.02</td>
<td>0.05±0.04</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>0.04±0.03</td>
<td>0.16±0.12</td>
<td>0.02*</td>
<td></td>
</tr>
</tbody>
</table>

MMP-9 KO and WT values are expressed as arbitrary densitometry units. *P<0.05.
MMP-9 KO Mice Show Increased Susceptibility to CVB3 Infection, Viral Load, and Immune Infiltration in the Heart

No difference in mortality rates was found between groups of KO mice and controls. Also, no major histological abnormalities were observed in sham MMP-9 KO and MMP-8 KO mice compared with their respective uninfected WT counterparts (Figure 3A and 3B). CVB3-infected MMP-9 KO mice exhibited increased morbidity and histological scores for cardiac injury, as evidenced by an increase in myocytolysis, calcification, and cellular infiltration (3.6±0.9 versus 2.4±1.3, P<0.004; Figure 3A and 3C). Plaque assay revealed that MMP-9 KO mice had significantly increased viral load within the myocardium compared with controls (P=0.04; Figure 3C and 3D).

To determine the sites of active CVB3 infection in the organ systems of MMP-9 KO and WT mice, we performed in situ hybridization for the presence of CVB3 genome. There was extensive and concordant CVB3 staining in the pancreas of both MMP-9 KO and WT mice (Figure 3D), whereas there was considerably more CVB3 replication detected within MMP-9 KO myocardium than in WT mice. MMP-9 KO mice demonstrated large disseminated regions of virus-infected myocardium, whereas the WT hearts showed rare or weak and punctate staining (Figure 3D). Infected MMP-8 KO mice had increased morbidity as reflected by increased weight loss (data not shown), with no significant differences in the degree of cardiac injury, calcification, and viral load between MMP-8 KO and WT mice (Figure 3B and 3E).

Virus-Infected MMP-9 KO Hearts Revealed a Marked Increase in Fibrosis Compared With WT

Picosirius red was used to detect fibrosis. Sham MMP-9 KO and WT hearts had similar amounts of collagen (Figure 4A). Virus-infected MMP-9 KO hearts revealed a marked increase in collagen deposition compared with WT (10.6±2.7% versus 7.1±2.6%, P=0.04; Figure 4A). MMP-9 KO myocardium had abundant replacement fibrosis, and reactive fibrosis was noted adjacent to viable cells. Perivascular fibrosis was a common observation in both groups.

MMP-8 KO and WT sham-infected hearts were similar with respect to the degree of fibrosis (Figure 4B). After virus infection, collagen accumulation was detected in areas of myocyte dropout. Perivascular and reactive fibrosis were also detected, albeit not common. There was no significant differ-

Figure 3 (Continued). eosin-stained hearts were scored for myocardial injury and inflammation, and MMP-9 KO mice had a higher pathological score than WT (3.6±0.9 versus 2.4±1.3). Viral load within MMP-9 KO mouse hearts was higher than WT, as determined by plaque assay. D, In situ hybridization of CVB3 in WT and MMP-9 KO heart and pancreas shows increased susceptibility of MMP-9 KO to cardiac CVB3 infection. Scale bars denote 50 μm (original magnification ×50) and 4 μm (original magnification ×400). E, Viral load within MMP-8 KO mouse hearts was identical to WT. Mean±SD. *P<0.05. ‡P<0.005. n=10 to 15. Significance was determined by the Wilcoxon–Mann–Whitney rank sum U test. PFU indicates plaque-forming units.
ence in the amount or quality of the collagen between the MMP-8 KO and WT hearts.

**CVB Infection Results in Greater Immune Infiltration in MMP-9 KO Mouse Hearts Than in WT Hearts**

A major role of MMP-9 and MMP-8 is immune regulation by modulation of immune-cell migration and activity. Therefore, we compared total leukocyte (CD45, a pan-leukocyte marker), phagocyte (neutrophils and macrophages), and T lymphocyte (CD3, a pan–T-cell marker) infiltration in WT and KO mice.

Immune infiltration was increased in MMP-9 KO mice compared with WT mice (15.2 ± 12.6% versus 2.0 ± 3.0%, respectively, $P = 0.002$) as measured by CD45 immunostaining (Figure 5A and 5B). Immunopositivity in MMP-9 KO hearts consisted mostly of focal lymphoid aggregates localized to areas of extensive injury, whereas the inflammatory foci were smaller and infrequent in the WT.

We next performed immunostaining for phagocytes, innate cells responsible for the first line of defense against viruses that are important in launching wound repair. Both neutrophils and macrophages were detected in the myocarditic heart, and no apparent difference in infiltration of these subtypes was found between MMP-9 KO and WT mice (2.7 ± 2.5% versus 1.5 ± 1.5%, $P = 0.26$; Figure 5C and 5D).

T-cell infiltration was increased significantly in MMP-9 KO animals compared with controls (4.2 ± 2.0% versus 1.8 ± 1.5%, $P = 0.004$). In both groups, most T cells were localized to inflammatory foci (Figure 5E and 5F). Morphometric analyses of the immunostaining for CD45, phagocytes, and CD3 in MMP-9 KO and WT are represented as a ratio of positively stained cells to the total number of cells in Figure 5G.

In MMP-8 KO mice, phagocytic infiltration in the myocardium was not significantly different from that in WT animals (2.2 ± 0.5% versus 3.2 ± 0.5%, $P = 0.6$). Phagocytes localized to similar-sized lesions in the heart (Figure 5H and 5I). However, T-cell infiltration into the myocarditic tissue of MMP-8 KO mice was decreased significantly compared with WT mice (0.9 ± 1.0% versus 7.2 ± 6.9%, $P = 0.0006$; Figure 5J and 5K). The T lymphocytes localized mostly to inflammatory lesions in the WT mice compared with more diffuse localization in the MMP-8 KO mice. Morphometry of immunostaining for phagocytes and CD3 in MMP-8 KO and WT mice is represented in Figure 5L.

**Regulation of Cytokines During CVB3 Infection**

We then investigated the transcriptional regulation of cytokines to determine whether the increase in CD45 invasion of MMP-9 KO myocardium is related to an alteration in cytokine expression. We used quantitative real-time polymerase chain reaction to compare the expression of IFN-β, IFN-γ, IL-1β, IL-6, IL-10, MIP-1α, and tumor necrosis factor-α between WT and MMP-9 KO animals. Previous studies have shown these cytokines play important roles in myocarditis and wound healing. In the infected MMP-9 KO hearts, IFN-β (1.36 ± 1.34 versus 0.32 ± 0.69 target gene Cγ/GAPDH Cγ, $P = 0.003$), IFN-γ (0.22 ± 0.19 versus 0.10 ± 0.07, $P = 0.04$), IL-6 (0.50 ± 0.72 versus 0.09 ± 0.07, $P = 0.01$), IL-10
Table 2. Expression of Cytokines: RT-PCR

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>MMP-9 KO</th>
<th>WT</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-β</td>
<td>1.36±1.34</td>
<td>0.32±0.69</td>
<td>0.003†</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.22±0.19</td>
<td>0.10±0.07</td>
<td>0.04*</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.95±0.08</td>
<td>0.89±0.03</td>
<td>0.06</td>
</tr>
<tr>
<td>IL-5</td>
<td>0.91±0.47</td>
<td>0.88±0.64</td>
<td>0.58</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.50±0.72</td>
<td>0.09±0.07</td>
<td>0.01†</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.25±0.13</td>
<td>0.12±0.08</td>
<td>0.04*</td>
</tr>
<tr>
<td>IL-12</td>
<td>0.23±0.14</td>
<td>0.16±0.11</td>
<td>0.24</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>1.68±1.26</td>
<td>0.65±0.56</td>
<td>0.04*</td>
</tr>
<tr>
<td>TGF-β</td>
<td>3.70±1.64</td>
<td>4.38±2.00</td>
<td>0.43</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.70±1.47</td>
<td>1.05±0.73</td>
<td>0.26</td>
</tr>
</tbody>
</table>

Values are expressed as target gene CT/GAPDH CT.

* $P<0.05$; † $P<0.01$; ‡ $P<0.005$.

(0.25±0.13 versus 0.12±0.08, $P=0.04$), and MIP-1α (1.68±1.26 versus 0.65±0.56, $P=0.04$) were increased compared with WT controls (Table 2). IL-1β, transforming growth factor-β, tumor necrosis factor-α, IL-5, and IL-12 were not significantly different between MMP-9 KO and WT mice.

To confirm whether these differences translated into protein expression, we performed ELISA quantification of select immune mediators. Measurement of IFN-α produced no significant results (28.9±1.8 pg/mL for MMP-9 KO versus 30.1±2.9 pg/mL for WT, $P=0.77$). Meanwhile, a significant increase in the amount of IFN-γ protein was apparent in MMP-9 KO myocardium 5 days PI compared with WT

Table 3. Expression of Cytokines: ELISA

<table>
<thead>
<tr>
<th>Infection Status</th>
<th>Wild-Type Concentration, pg/mL</th>
<th>MMP-9 KO Concentration, pg/mL</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interferon-α</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham infected</td>
<td>23.3±0.57</td>
<td>25.31±0.81</td>
<td>0.11</td>
</tr>
<tr>
<td>Infected</td>
<td>30.09±2.95</td>
<td>28.94±1.79</td>
<td>0.77</td>
</tr>
<tr>
<td>Interferon-β</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham infected</td>
<td>6.52±1.99</td>
<td>2.56±2.81</td>
<td>0.25</td>
</tr>
<tr>
<td>Infected</td>
<td>8.05±1.54</td>
<td>14.99±3.89</td>
<td>0.03*</td>
</tr>
<tr>
<td>Interferon-γ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham infected</td>
<td>1.51±0.26</td>
<td>1.46±0.57</td>
<td>0.93</td>
</tr>
<tr>
<td>Infected</td>
<td>5.61±1.07</td>
<td>11.52±2.43</td>
<td>0.002†</td>
</tr>
<tr>
<td>MCP-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham infected</td>
<td>57.23±0.16</td>
<td>57.74±0.71</td>
<td>0.51</td>
</tr>
<tr>
<td>Infected</td>
<td>65.3±2.23</td>
<td>64.95±1.09</td>
<td>0.89</td>
</tr>
</tbody>
</table>

* $P<0.05$; † $P<0.005$.  

Figure 5. Increased T-cell infiltration in MMP-9 KO hearts after CVB3 infection. Representative micrographs showing immunostaining for CD45 in (A) MMP-9 KO and (B) WT mice; phagocytes in (C) MMP-9 KO and (D) WT mice; and CD3 in (E) MMP-9 KO and (F) WT mice. G, Morphometric quantitation of immunostaining showed an increase in overall inflammation, notably T cells, in MMP-9 KO myocardium. Representative micrographs show immunostaining for phagocytes in (H) MMP-8 KO and (I) WT mice and for CD3 in (J) MMP-8 KO and (K) WT mice. G, Quantitation of immunostaining showed a relative decrease in T cells in MMP-8 KO myocardium. Mean±SD. † $P<0.01$, ‡ $P<0.005$. Scale bars denote 50 μm. n=10 to 15. Phag indicates phagocytes. Significance was determined by the Wilcoxon–Mann–Whitney rank sum $U$ test.
mice (11.5±2.4 versus 5.6±1.1 pg/mL, *P*=0.002; Table 3). Furthermore, IFN-β measurements by ELISA were also significantly increased in MMP-9 KO mice (14.99±3.89 versus 8.05±1.54 pg/mL, *P*=0.03), whereas this was not the case in MMP-8 KO mice compared with their WT counterparts (14.4±0.9 versus 14.0±1.1 pg/mL, *P*=0.74), which reflects the pattern seen in quantitative polymerase chain reaction measurements (Table 2).

**Echocardiography**

To determine the global cardiac effect of MMP-9 deficiency during CVB3 infection, we conducted echocardiographic examinations on WT and MMP-9 KO animals on days 1, 3, 5, and 9 PI. M-mode measurements of the left ventricle showed that the infected mice exhibited a decreased cardiac function (ejection fraction) by day 3 PI that decreased further at day 9 PI (Figure 6A), as described previously.23 Direct measurement of left ventricular dimensions reflected the progression of disease: Thickening of the left ventricular posterior wall was more evident in infected MMP-9 KO mice than in WT (0.92±0.05 versus 0.75±0.03 mm, *P*=0.018 [systole] and 0.88±0.09 versus 0.64±0.03 mm, *P*=0.025 [diastole]) at 9 days PI (Figure 6B). Similarly at this stage, the MMP-9 KO mice experienced greater thickening of the interventricular septum (0.98±0.09 versus 0.73±0.06 mm, *P*=0.04 [systole] and 0.95±0.10 versus 0.68±0.04 mm, *P*=0.03 [diastole]).

As a corollary, the MMP-9 KO mice exhibited a trend toward a more rapidly decreasing ejection fraction by day 3 (12.4%) compared with a decrease of 7.5% in WT mice. On day 9 PI, there was a 28% decrease in ejection fraction in the MMP-9 KO mice, which was a significantly greater loss than the 15% (*P*=0.03) decrease in the WT mice. Blood flow through the mitral valve into the left ventricle was detected with the Doppler E wave, an assessment of active relaxation of the left ventricle. We show in Figure 6C that peak E-wave velocity of MMP-9 KO mice decreased significantly (−3.30×10^2 mm/s) more than in the WT mice (−5.71×10^1 mm/s) by day 3 PI, followed by a stabilization of the E wave in both groups on day 5. However, MMP-9 KO mice subsequently experienced a considerable decline in E-wave velocity (−5.16×10^2 mm/s) compared with WT mice (−8.00×10^1 mm/s) at 9 days PI.

**Discussion**

In the present study, we infected 2 different strains of MMP-deficient mice, MMP-9 KO and MMP-8 KO, with CVB3 and compared cardiac injury, viral load, and inflammation with their corresponding WTs. Infected MMP-9 KO mice experienced considerably more severe cardiac injury despite similar infection of the pancreas compared with the corresponding WT. This correlated with increased disease, as reflected by increased left ventricular myocardial thickness, decreased cardiac function, increased virus abundance, fibrosis, and inflammation in the MMP-9 KO mice. We observed widespread, disseminated CVB3 replication in the myocardium of MMP-9 KO mice, whereas WT hearts exhibited sparse and discrete sites of viral replication. We observed increased migration of T cells and CD45-positive cells into the MMP-9 KO myocardium with no difference in phagocytic infiltration, which corresponded to an increase in IFN-β, IFN-γ, IL-6, IL-10, and MIP-1α cytokines.

Meanwhile, MMP-8 KO mice experienced the same degree of cardiac injury, fibrosis, and viral infection as their WT
counterparts. Consistent with the extracellular matrix degradation activity of MMP-8, there was a decrease in T-cell infiltration in the MMP-8 KO myocardium.

The present data are consistent with what is known about the role of MMP-9 during microbial infection. During bacterial infection, MMP-9 deficiency has been reported to increase severity of disease and bacterial titers through impairment of the immune response. In Staphylococcus aureus–triggered septic arthritis, bacterial titers, disease severity, and immune-cell infiltration were also increased, possibly due to impairment of the immune response during early phases of infection, with resultant delays in microbial clearance and repARATION. The present results show for the first time the importance of MMP-9 in controlling viral load and susceptibility to cardiac infection in a CVB3 myocarditis model.

These results contrast with studies in noninfectious settings, in which MMP-9 has been associated with maladaptive remodeling in both animal models and humans: MMP-9 levels are increased in the plasma of patients presenting with hypertension-induced hypertrophy or congestive heart failure, and after ischemic attacks, which correlates with decreased cardiac function and adverse structural alterations. In light of these studies, it is apparent that MMP-9 plays a key role in innate immunity and clearance of cardiac infection, but it is also associated with cardiac injury.

We found increased viral titers despite an increase in immune-cell infiltration, specifically T lymphocytes, in CVB3-infected MMP-9 KO mice. This suggests that innate immunity was also impaired, and virus was not eliminated efficiently; adaptive immune responses may have been delayed, and viral persistence promoted. These findings are supported by previous work that shows the critical importance of innate immunity in controlling virus infection. In the MMP-8 model, we did not observe any significant difference in inflammation between MMP-8 KO and WT mice; however, MMP-8 KO mice experienced relatively lower levels of T-cell infiltration than WT mice, which is consistent with the collagenase specificity of this MMP.

Thus, the role of MMP-8 may not lie in innate immunity but rather in modulation of the adaptive immune response during viral myocarditis.

MMP-9 is involved in cytokine and chemokine processing, effects that directly affect the recruitment, activation, and function of immune cells. Previous reports show that this enzyme cleaves cytokines and chemokines; MMP-9 can regulate the synthesis of IL-1β by cleavage of the proforms to generate active cytokines and chemokines. We did not see a significant change in IL-1β levels, but we did show a significant increase in levels of IFN-β and IFN-γ in the MMP-9 KO mice during infection. The interferons are exquisite at recruiting immune cells to the site of insult. In addition, the dramatically increased level of IFN-β over IFN-γ is consistent with the specificity and ability of MMP-9 to cleave IFN-β, which results in inactivation of this cytokine. Therefore, the pattern of cytokine expression agrees with the observed increase in CD45 cells in the myocardium of infected MMP-9 KO mice.

The present results suggest that MMP-9 is uniquely involved in a complex network of cytokines/chemokines, and ultimately immune-cell regulation, wherein this enzyme is necessary for proper inflammatory control. Infected MMP-9 KO mice did not have compensatory increases in any other investigated MMPs, which suggests that they exhibit no redundant function in lieu of MMP-9. Subtle downregulation of TIMP-1, TIMP-2, and TIMP-3 in MMP-9 KO mice may reflect the feedback loop between MMPs and TIMPs, and their reduction may ultimately favor increased remodeling and fibrosis, as seen in MMP-9 KO mice, by increased left ventricular wall dimension coupled with decreased cardiac function. No differences in collagen deposition and organization were observed between the MMP-8 KO and WT mice, which further suggests that this enzyme functions during late remodeling and immunomodulation.

In the present study, we assessed 2 MMPs that are both involved in extracellular matrix regulation. Although previous reports have shown that global MMP inhibition through either TIMP-1 or plasmin (a major MMP activator) overexpression ameliorated viral myocarditis, we show here that specific MMP-9 deficiency results in profoundly deleterious outcomes. MMP-9 plays a major role in the immune response to CVB3 infection, via mediation of innate immunity, which leads to increased control viral titers.

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Disclosures
None.

References
MMP-9 in a knockout (KO) mouse model enhances susceptibility to cardiac infection by coxsackievirus. There was a lower foci count of 102 versus 19 in WT mice) and significantly decreased cardiac output. Immune infiltration was increased in myocardium. The MMP-9 KO mice had significantly more pancreatic and myocardial infection (cross-sectional myocardial destruction and tumor growth.

Importantly, this study suggests that MMP-9 plays a role in suppressing viremia to the point of protecting the heart from infection, while mediating an appropriate immune response. Therefore, treatments meant to prevent the onset of infectious myocarditis would be best to preserve MMP-9 function.
Ablation of Matrix Metalloproteinase-9 Increases Severity of Viral Myocarditis in Mice
Caroline Cheung, David Marchant, Elizabeth K.-Y. Walker, Zongshu Luo, Jingchun Zhang, Bobby Yanagawa, Maziar Rahmani, Jennifer Cox, Christopher Overall, Robert M. Senior, Honglin Luo and Bruce M. McManus

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