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Amelioration of Coxsackievirus B3-Mediated Myocarditis by Inhibition of Tissue Inhibitors of Matrix Metalloproteinase-1

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Coxsackievirus B3 (CVB3) is a major cause of acute myocarditis, a serious condition that is refractory to treatment. Myocardial damage results in tissue remodeling that, if too extensive, may contribute to disease. Remodeling is achieved by extracellular proteolysis mediated by the matrix metalloproteinases (MMPs), and MMP activity is counterbalanced by tissue inhibitors of MMPs (TIMPs). We show herein that TIMP-1 expression is induced in the myocardium by CVB3 infection. Surprisingly, TIMP-1 knockout mice exhibited a profound attenuation of myocarditis, with increased survival. The amelioration of disease in TIMP-1 knockout mice was not attributable to either an altered T-cell response to the virus or to reduced viral replication. These data led us to propose a novel function for TIMP-1: its highly localized upregulation might arrest the MMP-dependent migration of inflammatory cells at sites of infection, thereby anatomically focusing the adaptive immune response. The benefits of TIMP-1 blockade in treating viral myocarditis were confirmed by administering, to wild-type animals, TIMP-1-specific siRNA or polyclonal antisera, both of which diminished CVB3-induced myocarditis. These unexpected findings indicate that increased TIMP-1 expression exacerbates, rather than ameliorates, CVB3-induced myocarditis and, thus, that TIMP-1 may represent a target for the treatment of virus-induced heart disease. ***(Am J Pathol 2007, 171:1762–1773; DOI: 10.2353/ajpath.2007.070179)***

Type B coxsackieviruses (CVBs) are the commonest infectious cause of human myocarditis. CVB infections are surprisingly prevalent, with an estimated 70% of the human population having been exposed to these viruses. Although CVB-induced acute myocarditis usually is asymptomatic or leads to only mild disease, it also can cause sudden cardiac arrest in young and vigorous individuals. Acute CVB myocarditis may result in serious long-term sequelae, including chronic myocarditis that can develop into dilated cardiomyopathy, a terminal condition requiring transplantation. At present, the treatment of viral myocarditis is primarily supportive[1]; specific therapies are lacking and are much needed.

The CVB group contains six members, of which CVB3 is the most frequent cause of human heart disease. CVB3 infection of mice provides an excellent model in which to study viral myocarditis because it recapitulates the clinical and histopathological features of both the acute and the chronic phases of human disease.[2] The mechanism(s) by which CVB3 induces acute and chronic myocarditis is unclear. During the acute infection, both direct virus-mediated cytolysis[3] and immune-mediated destruction of CVB3-infected myocardium[4,5] contribute to myocardial damage. The chronic disease is thought to be primarily immunopathological, but the antigenic target of the immune response is controversial[6]; evidence exists both for autoimmunity[7] and for ongoing responses to persistent viral materials.[8] The important part played by immunopathology in these diseases indicates that host factors play a key role. For example, in acute CVB3 myocarditis, the cytolytic protein perforin contributes to myocardial destruction; CVB3-infected perforin-deficient mice show diminished acute and chronic myocardial damage, although they clear the virus

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infection with normal kinetics,[9] perhaps using interferon-γ.[10] Furthermore, host signaling proteins play a role in CVB infection[11,12] and may be potential targets for therapeutic intervention.[1] The identification of additional host factors that are involved in CVB-induced disease offers the potential to abate the potentially devastating effects of acute myocarditis, and this, in turn, would diminish the likelihood of developing chronic cardiomyopathies.

Recovery from acute CVB3 infection is accompanied by fibrosis, caused by amplified remodeling of the myocardial extracellular matrix.[13] Matrix metalloproteinases (MMPs) are extracellular proteases responsible for the dynamic remodeling of the extracellular matrix. Enhanced activity of MMPs has been implicated as a maladaptive myocardial response to cardiac stress and injury, and this contributes to disease and to decline in heart function.[14] The proteolytic activities of MMPs can be regulated through direct interaction with the endogenous tissue inhibitors of MMPs (TIMPs).[15] It is well established that the ratio of MMP activity to the expression of TIMP proteins determines the rate of extracellular matrix remodeling in tissue.[16] An imbalance in the homeostatic ratio of MMP activity to TIMP gene expression has been associated with various forms of heart disease in humans.[17] Thus, TIMPs are key regulators of MMP activity, but their role in CVB3-induced myocarditis has not been previously characterized. Here, we have examined the expression of TIMP family genes in CVB3-induced myocarditis, and we have identified an unexpected role for TIMP-1 in CVB3-induced heart disease. Counter to the prevailing notions about the traditional protective role of TIMPs in disease, we have determined that TIMP-1 exacerbates CVB3-induced myocarditis, identifying this protein as a potential therapeutic target for this serious disease.

Materials and Methods

Mice and Virus

TIMP-1-deficient mice on the C57B/6 background[18] were generously provided by Dr. P. Soloway (Cornell University, Ithaca, NY) and were bred to homozygosity. Age-matched C57B/6 wild-type (WT) mice were used as controls. Mice were infected with 1×10^4 pfu (i.p.) CVB3 (Woodruff strain). Heart and pancreatic tissues were used for virus titers as determined by HeLa cell plaque assays. All animal protocols were approved by The Scripps Research Institute Department of Animal Resources in compliance with Institutional Animal Care and Use Committee guidelines.

Evaluation of CVB3-Induced Myocarditis

The severity of CVB3-induced myocarditis was determined using paraffin-embedded heart tissues stained by Masson's trichrome. To quantify differences in myocarditis, defined criteria were used on a 0 to 5 scale to allow for unbiased evaluation of myocarditis in murine cardiac tissues. This scale was used by individuals blinded to the experimental treatments for the determination of myocarditis scoring. A higher score is reflected by more severe myocarditis.

RNA Interference

Short interfering RNA (siRNA) directed against exon 2 of murine TIMP-1 (GenBank reference no. NM_011593) was obtained (siRNA database no. 69619; Ambion, Austin, TX). siRNA was administered to C57BL/6 mice by tail vein injection (30 nmol in 100 μl) once daily for 4 consecutive days starting 3 days after infection with CVB3 (1×10^4 pfu). The same amounts of control siRNA (silencer negative control no. 2, catalog no. 4613; Ambion) were administered to CVB3-infected control groups. Animals were monitored daily for clinical signs of illness and weight loss.

TIMP-1 Immunoneutralization

Goat polyclonal anti-TIMP-1 IgG antisera (catalog no. AF980; R&D Systems, Minneapolis, MN) was administered (0.5 mg/kg/day) for 4 consecutive days beginning the day immediately after inoculation of C57BL/6 mice with CVB3 (day 0). As a control, additional mice were given equivalent doses of an isotype-matched goat IgG (catalog no. AB-108-C, R&D Systems). Animals were monitored daily for clinical signs of illness and weight loss.

Multiprobe RNase Protection Assay (RPA)

RPA analysis of TIMP and cytokine mRNAs was performed as previously described.[19]

Flow Cytometry

Spleen and heart cells were prepared with red blood cell lysis using standard protocols. Briefly, mice were perfused intracardially with phosphate-buffered saline, and the organ was removed and minced manually with a Dounce homogenizer. Lymphocytes were separated by centrifugation after resuspending cellular homogenates in 44% Percoll and floating them onto a 56% Percoll cushion. Lymphocytes were identified as the primary cellular component at the interface of the two layers. Cells were stained directly *ex vivo* with anti-CD44 (clone IM7; eBioscience, San Diego, CA), anti-CD4 (clone RM4-5, eBioscience), or anti-CD8 (clone 53-6.7, eBioscience) antisera. Cells were acquired on a FACScalibur (BD Biosciences, Franklin Lakes, NJ) and analyzed using FlowJo software (Tree Star, Ashland, OR).

Histology

Tissues were collected into 10% zinc normal formalin and paraffin-embedded. Six-μm-thick microtome sections were cut and stained using hematoxylin and eosin (H&E) or Masson's trichrome stain. Measurement of tissue injury area was determined using ImageJ software (National Institutes of Health, Bethesda, MD). Briefly, H&E-stained sections were analyzed for the proportion of the area of

tissue using at least three fields of view (×20 magnification) from each subject. Tissues from uninfected mice were used to establish the measurable area covered by healthy tissues and sections from infected specimens were then quantified as a proportion of stained (healthy) to unstained (injured or inflamed) tissue.

Measurement of Apoptosis

Analysis of apoptosis in heart tissues from uninfected and CVB3-infected WT and TIMP-1KO mice was performed using an Apoptag Red kit according to the manufacturer's instructions (Millipore, Temecula, CA). Quantitative evaluation of apoptotic cells was performed on at least three sections per animal per treatment group by an investigator blinded to the experimental treatment. Data are presented as the average number of terminal dUTP nick-end labeling-positive ($TUNEL^+$) cells ± SEM.

Immunohistochemistry

Tissues were stained using primary antisera against TIMP-1 (Affinity Bioreagents, Golden, CO); secondary antibody was biotinylated anti-rabbit or anti-mouse, respectively (Vector Laboratories, Burlingame, CA). Staining was visualized using avidin-biotin peroxidase labeling with diaminobenzidine as the substrate (Vector Laboratories), according to the manufacturer's guidelines, and counterstained with hematoxylin (Accustain; Sigma-Aldrich, St. Louis, MO).

Enzyme-Linked Immunosorbent Assay (ELISA)

Detection of TIMP-1 protein from crude heart lysates was performed using a sandwich ELISA kit according to the manufacturer's instructions (R&D Systems).

Statistical Analyses

Data are presented as mean ± SEM. One-way analysis of variance with pair-wise posthoc tests or unpaired Student's *t*-test were used to determine group differences. Kaplan-Meier survival curves were compared by the Mantel-Haenszel log rank test. For all tests, $P < 0.05$ was considered significant.

Gelatin Zymography

Gelatin zymography of myocardial tissue was performed as follows. The volume of supernatant containing 18 g of protein was diluted in homogenizing buffer and mixed with an equal volume of sample buffer (80 mmol/L Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate, 10% glycerol, 0.01% bromphenol blue). For standardization, 50 pg of recombinant human MMP-2 and MMP-9 were diluted in collagenase buffer, mixed with equal volumes of sample buffer, and then loaded onto the gel. For electrophoresis, 8% sodium dodecyl sulfate-polyacrylamide resolving gels containing 1 mg/ml of gelatin were overlaid with 5% stacking gels, and samples were loaded and run at 4°C (25 mA per gel). After electrophoresis, gels were rinsed with distilled water briefly and washed three times in 150 ml of 2.5% Triton X-100 solution (15 minutes each) on a rotary shaker. The gels were then incubated in 250 ml of 50 mmol/L Tris-HCl (pH 7.5) containing 10 mmol/L $CaCl_2$ and 0.02% NaN_3 at 37°C for 42 hours before staining with Coomassie blue and destaining.

Results

Myocardial TIMP-1 Expression Is Up-Regulated during CVB3 Infection

RNase protection assay of metalloproteinase inhibitor mRNAs in the hearts of uninfected adult C57BL/6 mice showed that the four genes are expressed at varying levels (TIMP-3≈TIMP-2≫TIMP-4>TIMP-1; Figure 1A). The very low constitutive expression of TIMP-1 in the heart confirms a previous report.[20] After infection with CVB3 (1×10^4 pfu, i.p.), the TIMP-2, -3, and -4 mRNA levels increased marginally, but the greatest change occurred in TIMP-1 mRNA, which was significantly increased as early as 3 days after inoculation, peaking at 1 week, and returning to barely detectable levels by 30 days (Figure 1, A and B). These findings differ from a recent study that reported that TIMP-1 protein levels were readily detected in the uninfected myocardium and remained unaltered by CVB3 infection.[21] The reason for this discrepancy is unknown, but we considered it particularly important to verify that the changes that we observed in TIMP-1 mRNA after infection were reflected in the levels of TIMP-1 protein. To this end, an ELISA assay was performed on protein extracts from heart tissues of uninfected mice and of mice infected with CVB3 3 or 7 days previously. In each case, equivalent samples from TIMP-1 knockout (TIMP-1KO) mice were included as negative controls. TIMP-1 was undetectable in TIMP-1KO hearts and in the hearts of uninfected WT mice but was dramatically increased by day 7 after CVB3 infection ($P < 0.005$, Figure 1C). The heart tissues also were analyzed for TIMP-1 protein expression and distribution using immunohistochemistry; after CVB3 infection, TIMP-1 protein was present predominantly in and around areas containing acute inflammatory infiltrates (Figure 1D). These data indicate that the expression of TIMP-1 in the heart of CVB3-infected mice is spatially and temporally coincident with myocarditis, consistent with the concept that TIMP-1 expression might be up-regulated to counterbalance MMP-mediated dissolution of the extracellular matrix during viral infection. Therefore, we next investigated the consequences of TIMP-1 deficiency on CVB3-induced myocarditis.

TIMP-1KO Mice Show Reduced Morbidity and Mortality in Response to CVB3 Infection

As a first approach to evaluating the role of TIMP-1 in virus-induced heart disease, TIMP-1KO mice were stud-

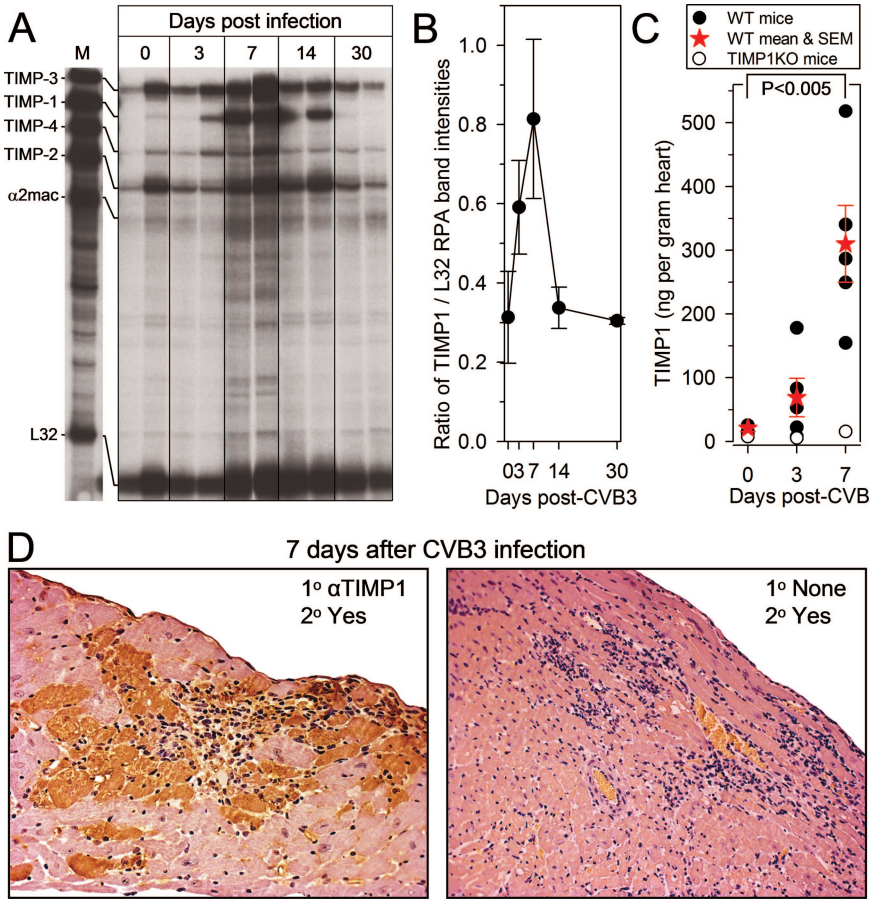

Figure 1. Myocardial TIMP-1 expression is upregulated during CVB3 infection. **A:** RNase protection assay of metalloproteinase inhibitor mRNA expression in cardiac tissues at varying times after CVB3 infection. Each lane represents RNA from individual animal. **B:** For each lane of the RPA, the band intensities of TIMP1 and L32 were determined. The mean TIMP1:L32 ratio at each time point after CVB3 is shown (±SE). **C:** TIMP-1 protein in myocardial extracts (ng/g, ±SEM) from uninfected mice (0 days) and mice at 3 or 7 days after CVB3 infection. **Black circles**, WT mice; **white circles**, TIMP-1KO mice. **Red stars** indicate the group means for WT mice; the difference between the day 0 and day 7 groups is statistically significant ($P < 0.005$). **D:** Immunohistochemistry for TIMP-1 in hearts of CVB3-infected WT mice demonstrates the colocalization of TIMP-1 protein expression with sites of myocardial inflammation (**left**). Immunoreactivity for TIMP-1 protein in cardiac tissues was absent when either the primary antisera was omitted (**right**) or in infected tissues from TIMP-1KO animals (not shown).

ied. These mice, and WT control mice, were given a normally lethal dose (10^4 pfu, i.p.) of CVB3, and were observed daily for clinical signs. WT mice quickly exhibited overt signs of distress (severe piloerection, lethargy, and weight loss), whereas CVB3-infected TIMP-1KO mice displayed few clinical signs (minor piloerection without obvious lethargy). As shown in Figure 2A, >90% of the WT mice ($n = 11$) succumbed by 6 days after infec-

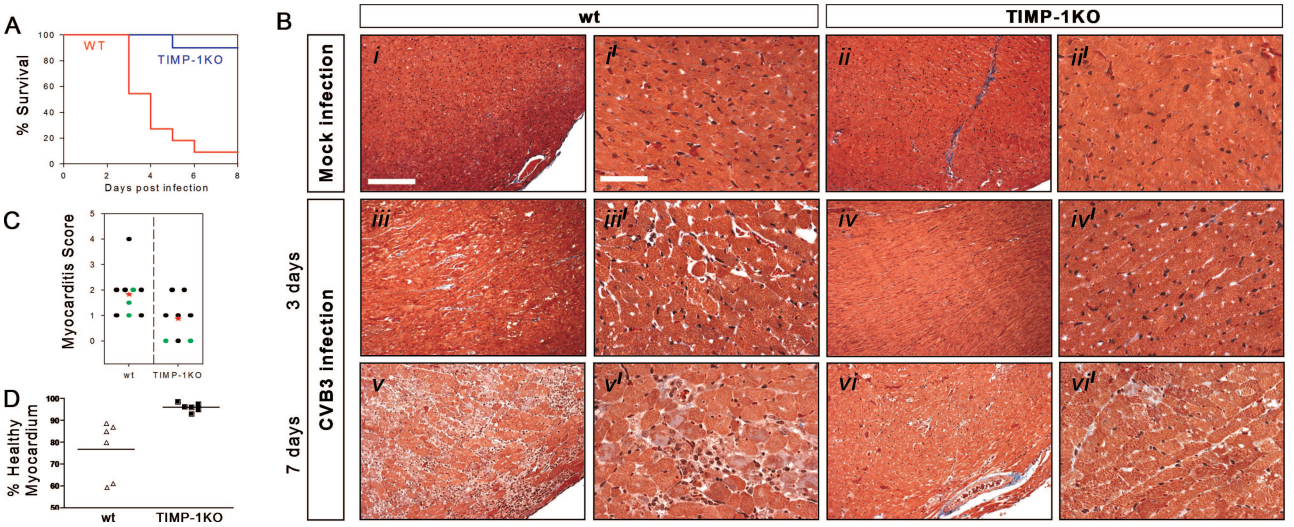

Figure 2. Myocarditis and mortality are reduced in the absence of TIMP-1. **A:** Survival graph of WT mice (red line, $n = 11$) and TIMP-1KO mice (blue line, $n = 10$) after CVB3 infection (1×10^4 pfu, i.p.); $P = 0.001$. **B:** Histological sections from the hearts of mock-infected and infected TIMP-1KO and WT mice. All mice survived until 3 days after infection, at which time point mice were randomly chosen for histological analysis. Many of the infected WT mice had died by 7 days after infection; surviving mice were sacrificed for histological analysis. **C:** Summary of myocarditis scores in WT and TIMP-1KO mice. **Green circles**, day 3 after infection; **black circles**, day 7 after infection. **Red stars** indicate the group means (combining both time points); $P = 0.078$. **D:** The percent area of healthy myocardium in infected WT and TIMP-1KO mice is shown (individual mice; means shown by horizontal lines); $P = 0.003$. Scale bars: 200 μm (**i**, applicable to **i–vi**); 30 μm (**i′**, applicable to **ii^I–vi^I**).

tion, whereas 90% of TIMP-1KO mice ($n = 10$) survived; this difference was highly statistically significant ($P < 0.0001$). To evaluate the extent of myocarditis, groups of WT and TIMP-1KO mice were infected and sacrificed 3 or 7 days later, and their hearts were evaluated histologically (Figure 2B). The hearts of mock-infected TIMP-1KO mice were histologically normal, indicating that TIMP-1 deficiency causes no gross myocardial abnormality; this was consistent with a previous demonstration that myocardial structure and function are primarily normal in TIMP-1-deficient mice.[22] At 7 days after infection, most of the WT mice exhibited florid myocarditis, whereas the cardiac tissues of TIMP-1KO mice showed reduced inflammation (Figure 2B). Although there was some variability among the mice in each group, quantification of the severity of myocarditis (Figure 2C) and the area of cardiac tissue involved (Figure 2D) at 7 days after infection revealed statistically significant differences between WT and TIMP-1KO mice ($P < 0.021$, Figure 2C; $P < 0.0025$, Figure 2D). Note that several WT mice had died between days 3 to 7, so the hearts of those mice were excluded from the evaluation of myocarditis at day 7.

T-Cell Activation after CVB3 Infection Is Minimal and Is Similar in WT and TIMP-1KO Mice

The myocardial damage caused by CVB3 infection results from a combination of direct, virus-mediated, damage and immunopathology caused by host T-cell responses,[3,23,24] so next we evaluated the T-cell responses to infection in WT and TIMP-1KO mice. The absence of known murine T-cell epitopes in CVB3 prevents the precise mapping of virus-specific immune responses. Therefore, the T-cell responses to CVB3 infection were, instead, determined by measuring the expression of a cellular activation marker, CD44, on $CD8^+$ and $CD4^+$ T cells before and during CVB3 infection. The validity of this approach for detecting virus-induced T-cell activation was demonstrated by the inclusion of a positive control group, comprising mice infected with lymphocytic choriomeningitis virus, which is known to induce a strong $CD8^+$ T-cell response. As shown in Figure 3A, lymphocytic choriomeningitis virus infection caused a dramatic increase in $CD44^+$ $CD8^+$ T cells, and the extent of the change was very similar in WT and TIMP-1KO mice. These data indicate that TIMP-1KO mice are capable of mounting adequate T-cell responses to virus infection. In contrast to lymphocytic choriomeningitis virus infection, CVB3 infection caused minimal activation of T-cell responses in either WT or TIMP-1KO mice (Figure 3A). This is consistent with a previous study showing that recombinant CVB3 induced very weak $CD8^+$ T-cell responses,[25] and others have reported that the 3A protein of the closely related poliovirus can interrupt trafficking of MHC class I molecules,[26] which is required for epitope presentation to $CD8^+$ T cells. In addition, and as was observed for $CD8^+$ T cells, the $CD4^+$ T-cell responses to CVB3 infection were similar in WT and TIMP-1KO mice (Figure 3B). Immunohistochemical analyses (not shown) of heart sections from WT and TIMP-1KO mice at 7 days after

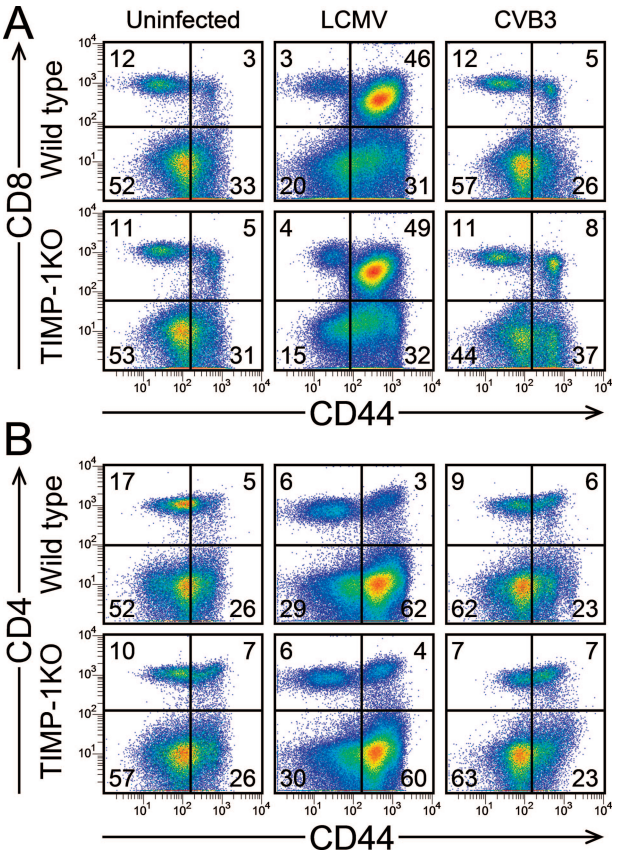

Figure 3. Systemic T-cell activation is limited, and similar, in WT and TIMP-1KO mice. WT and TIMP-1KO (KO) mice were infected with either lymphocytic choriomeningitis virus or CVB3, and 8 days later their splenocytes were analyzed using flow cytometry. Splenocytes were stained for CD44 and for either CD8 (**A**) or CD4 (**B**). The numbers represent the proportion of cells in each quadrant, as a percentage of all splenocytes.

infection indicated that, although the degree of myocarditis was reduced in the knockout animals, the cellular infiltrates in both genotypes were similar in composition, containing T cells, $CD45^+$ leukocytes, and $F4/80^+$ macrophages but few detectable B cells. Consistent with the T-cell analyses and the immunohistochemical data, flow cytometry analysis of macrophage and B-cell populations in CVB3-infected WT and TIMP-1KO spleens did not reveal any marked differences in proportion or activation that would point to a primary role for these cell populations in this model (data not shown). Together, these data suggest that attenuated myocarditis during CVB3 infection in TIMP-1KO mice was not correlated with any overt diminution in immune cell frequency.

Reduction in Myocarditis Is Associated with Decreased Myocardial Apoptosis

Next, as another measure of myocardial integrity, we determined the relative frequencies of apoptotic cells in WT and TIMP-1KO mice. Cardiomyocyte apoptosis has been previously shown to be important for both viral replication and virus-induced cardiac pathology after CVB3 infection.[9,27] Using fluorescent labeling of DNA strand breaks to identify apoptotic cells at 1 week after

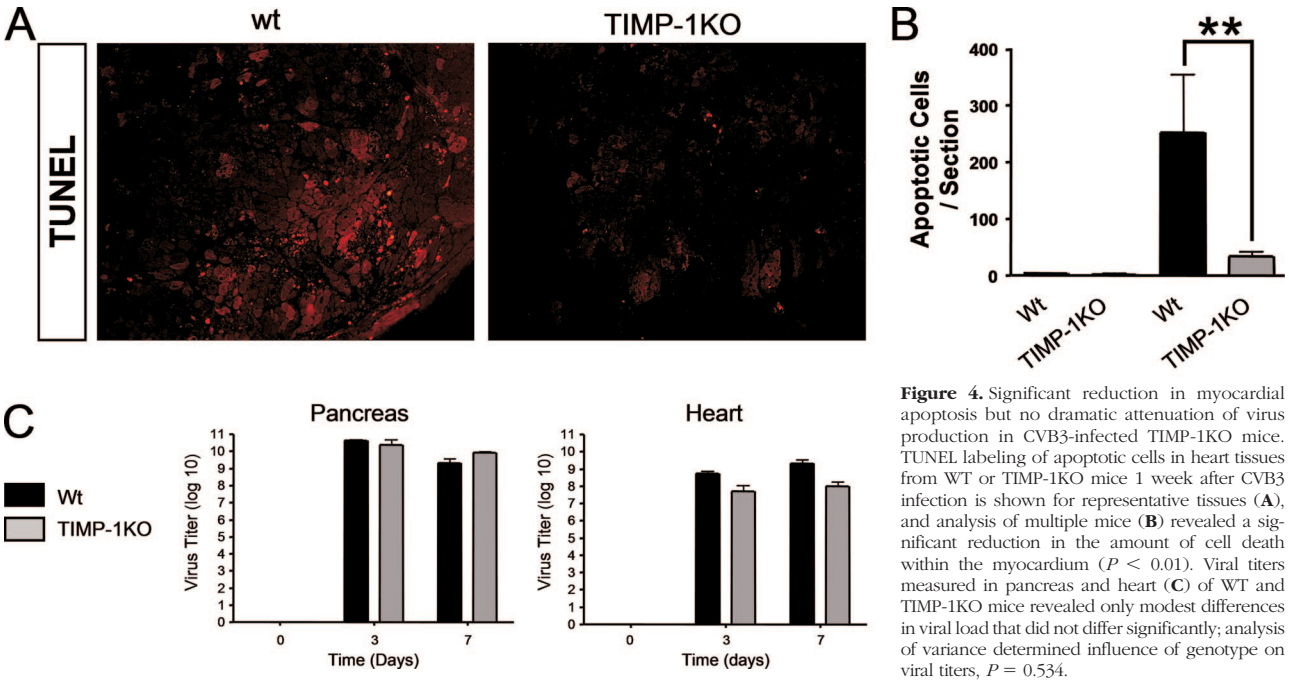

Figure 4. Significant reduction in myocardial apoptosis but no dramatic attenuation of virus production in CVB3-infected TIMP-1KO mice. TUNEL labeling of apoptotic cells in heart tissues from WT or TIMP-1KO mice 1 week after CVB3 infection is shown for representative tissues (**A**), and analysis of multiple mice (**B**) revealed a significant reduction in the amount of cell death within the myocardium ($P < 0.01$). Viral titers measured in pancreas and heart (**C**) of WT and TIMP-1KO mice revealed only modest differences in viral load that did not differ significantly; analysis of variance determined influence of genotype on viral titers, $P = 0.534$.

infection, we identified many more TUNEL$^+$ cells throughout the myocardium in WT mice compared with TIMP-1KO mice (Figure 4A). Apoptotic cells were counted, and the difference between the two mouse strains was statistically significant ($P < 0.01$, Figure 4B). One possible explanation for the dramatic attenuation of CVB3-induced myocarditis 7 days after infection in the TIMP-1KO mice was a difference in the level of virus replication. CVB3 titers in the pancreas (thought to be the first organ infected) and heart were determined, and, as shown in Figure 4C, viral titers in pancreas were almost identical; titers in the hearts of TIMP-1KO mice were high ($\sim 10^7$ to 10^8 per g) but slightly decreased compared with WT mice. This modest difference in virus titer raises the possibility that the data generated using TIMP-1KO mice reflect a common complication of knockout mouse studies; the possibility of developmental compensation in gene expression and/or unidentified differences in genetic background between knockout and WT animals.[28] Therefore, to circumvent completely this potential confounder, and to evaluate the potential therapeutic benefits of TIMP-1 blockade, we next used two different but complementary methods to block TIMP-1 expression and/or function in WT mice: we tested the effects of siRNA directed against TIMP-1 and of polyclonal anti-TIMP-1 antisera.

TIMP-1 siRNA Attenuates CVB3-Induced Myocarditis

Short interfering RNA (siRNA) can selectively disrupt the expression of a targeted gene,[29] and offers the therapeutic potential of affecting outcome from virus infections by silencing disease-related gene expression.[30] WT C57B/6 mice ($n = 9$) were inoculated with CVB3 (1×10^4 pfu, i.p.), and, starting 3 days after infection, either anti-TIMP-1 siRNA ($n = 6$) or a nonspecific (control) siRNA ($n = 3$) was administered daily (30 nmol, i.v.) for 4 consecutive days. Mice were sacrificed on day 8 after infection and TIMP-1 mRNA levels in the heart were determined by RPA. As shown in Figure 5A, the *in vivo* effects of siRNA on myocardial levels of TIMP-1 mRNA were quite variable, and marked depletion was observed in only two of six WT mice. Because the half-life of TIMP-1 mRNA ($\sim$14 hours[31]) is less than the periodicity of the siRNA dosing, we performed TIMP-1 ELISAs to determine whether the RPA results reflected the amount of TIMP-1 protein present in the hearts. A strong, statistically significant, correlation ($P < 0.0162$, $r = 0.766$) was observed between mRNA and protein levels (Figure 5B; green, anti-TIMP-1 siRNA-treated mice), indicating that siRNA administration successfully reduced TIMP protein in the hearts of some mice. There was a modest improvement in survival of mice that received anti-TIMP-1 at 1 week after CVB3 infection (Figure 5C) and, mirroring the data from the RPA, histological analysis revealed substantial variability in myocarditis. Tissue sections are shown in Figure 5D, together with a summary of data for all mice (Figure 5E; green, anti-TIMP-1 siRNA-treated mice). Those animals exhibiting significant TIMP-1 knockdown also showed reduced myocarditis, and there was a significant correlation between TIMP-1 mRNA levels and the severity of myocarditis ($P < 0.017$, $r = 0.762$). Consistent with the very low basal expression of TIMP-1 in the adult heart (Figure 1), TIMP-1 siRNA did not have any noticeable effects in sham-infected animals (data not shown). Finally, CVB3 virus titers were very similar in both groups of siRNA-treated mice (Figure 5F), indicating that the protective effects of TIMP-1 blockade are not related to changes in virus titer.

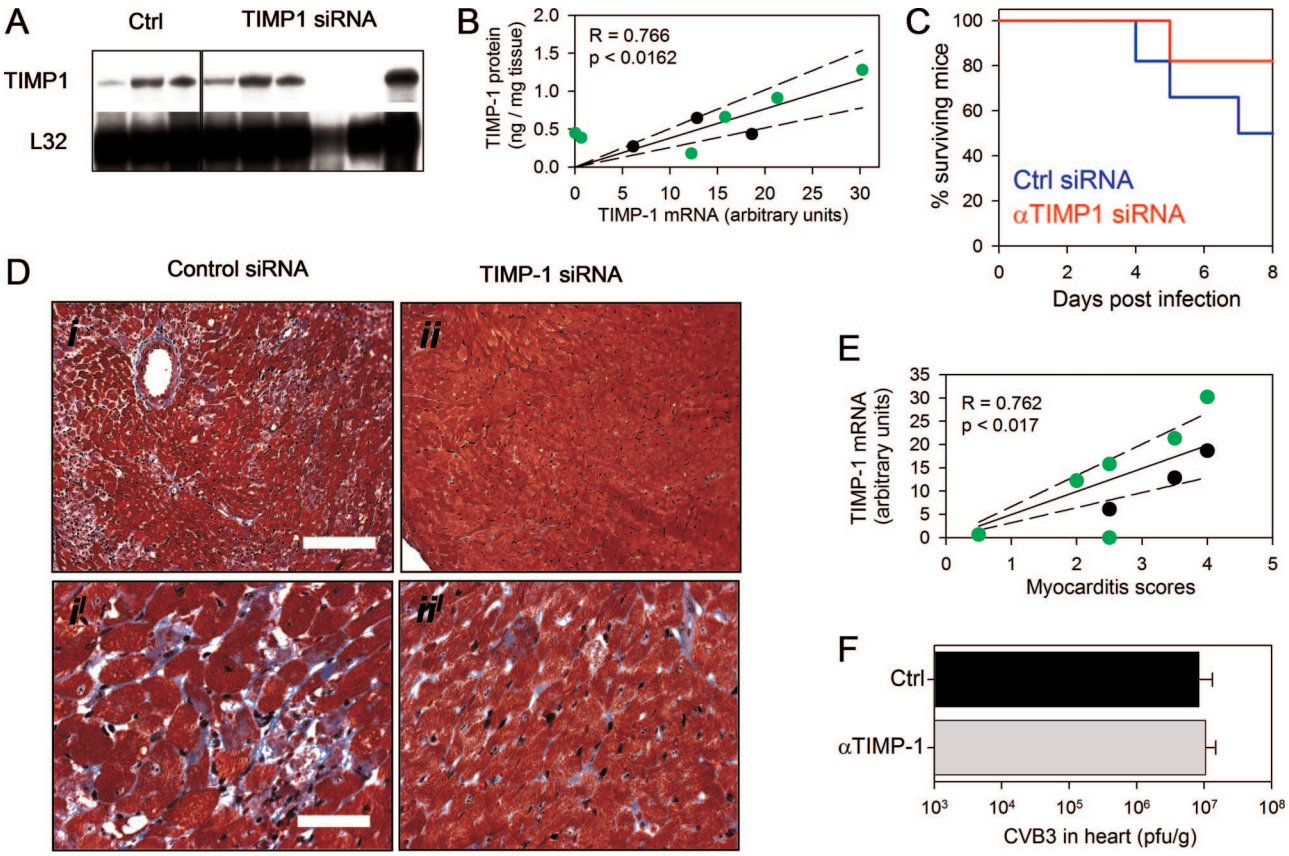

Figure 5. Anti-TIMP-1 siRNA ameliorates CVB3-induced myocarditis. **A:** RNase protection assay of TIMP-1 gene expression from heart tissues from day 8 CVB3-infected WT mice treated with either control siRNA or anti-TIMP-1 siRNA reveals variation in the efficacy of TIMP-1 siRNA-induced knockdown of TIMP-1 mRNA expression. **B:** TIMP-1 mRNA levels correlated with TIMP-1 protein expression as determined by ELISA assay. **Black circles**, control siRNA; **green circles**, anti-TIMP-1 siRNA. TIMP-1 siRNA treatment reduced CVB3 infection-related mortality (**C**) and resulted in a significant attenuation in the degree of myocarditis in some recipient mice (**D**). Representative images of control siRNA-treated (**i**, **i^{I}**) and TIMP-1 siRNA-treated heart tissues (**ii**, **ii^{I}**) at low and high magnifications, respectively. **E:** Quantification of myocarditis in control siRNA-treated or TIMP-1 siRNA-treated CVB3-infected mice revealed an overall attenuation in the severity of myocarditis with TIMP-1 siRNA treatment. **Black circles**, control siRNA; **green circles**, anti-TIMP-1 siRNA. **F:** CVB3 titers at 7 days after infection were similar in mice treated with control or anti-TIMP siRNAs ($P > 0.05$). The solid and dashed lines in **B** and **D** indicate linear regression analyses (solid), with 95% confidence limits (dashed). Scale bars: 180 μm (**i**, **ii**); 30 μm (**i^{I}**, **ii^{I}**).

Immunoneutralization of TIMP-1 Attenuates CVB-Induced Myocarditis

As an additional approach to investigating the effect of TIMP-1 blockade, antibodies specific for this secreted protein were used. WT mice were inoculated with CVB3 (1×10^4 pfu, i.p.) and received polyclonal murine α-TIMP-1 serum ($n = 13$) or isotype-matched IgG ($n = 11$) daily for 5 days (days 0 to 4). Histological analysis of cardiac pathology of mice 1 week after infection revealed a dramatic reduction in the degree of myocarditis in α-TIMP-1 antisera-treated animals ($n = 6$) compared with control IgG-treated mice ($n = 5$) (Figure 6A), and this treatment regime improved the 7-day survival of CVB3-infected WT mice compared with those administered control antisera (Figure 6B). α-TIMP-1 antisera treatment did not have any notable effect on uninfected animals (data not shown). Quantification of the severity of myocarditis in CVB3-infected animals revealed that treatment with α-TIMP-1 antisera significantly attenuated myocarditis at 7 days after infection in this treatment group ($P < 0.029$, Figure 6C), and, consistent with our prior analyses of viral titers in siRNA-treated mice, the beneficial effects of administration of α-TIMP-1 antisera did not rely on a reduction in virus titer (Figure 6D). However, the effects of short-term anti-TIMP-1 antiserum were transient; several of the treated mice succumbed to infection between days 8 and 11 after infection (Figure 6E). To test whether treatment with anti-TIMP-1 antisera initiated later during CVB3 virus infection could improve survival, WT mice were challenged with CVB3, and control IgG ($n = 6$) or anti-TIMP-1 antisera ($n = 4$) were administered 3 days later and then every other day until day 13 (0.5 mg/kg/day). Under these conditions, anti-TIMP-1 antisera significantly prolonged the survival of CVB3-infected mice ($P < 0.03$; Figure 6F).

Diminished MMP Activity in the Hearts of CVB3-Infected TIMP-1KO Mice

Increased expression and activation of metalloproteinases during CVB3 infection have been implicated in myocardial injury,[13,21,32–35] and a recent study described increased activity of both MMP-2 and MMP-9 in heart tissues during CVB3-induced myocarditis.[35] Because the

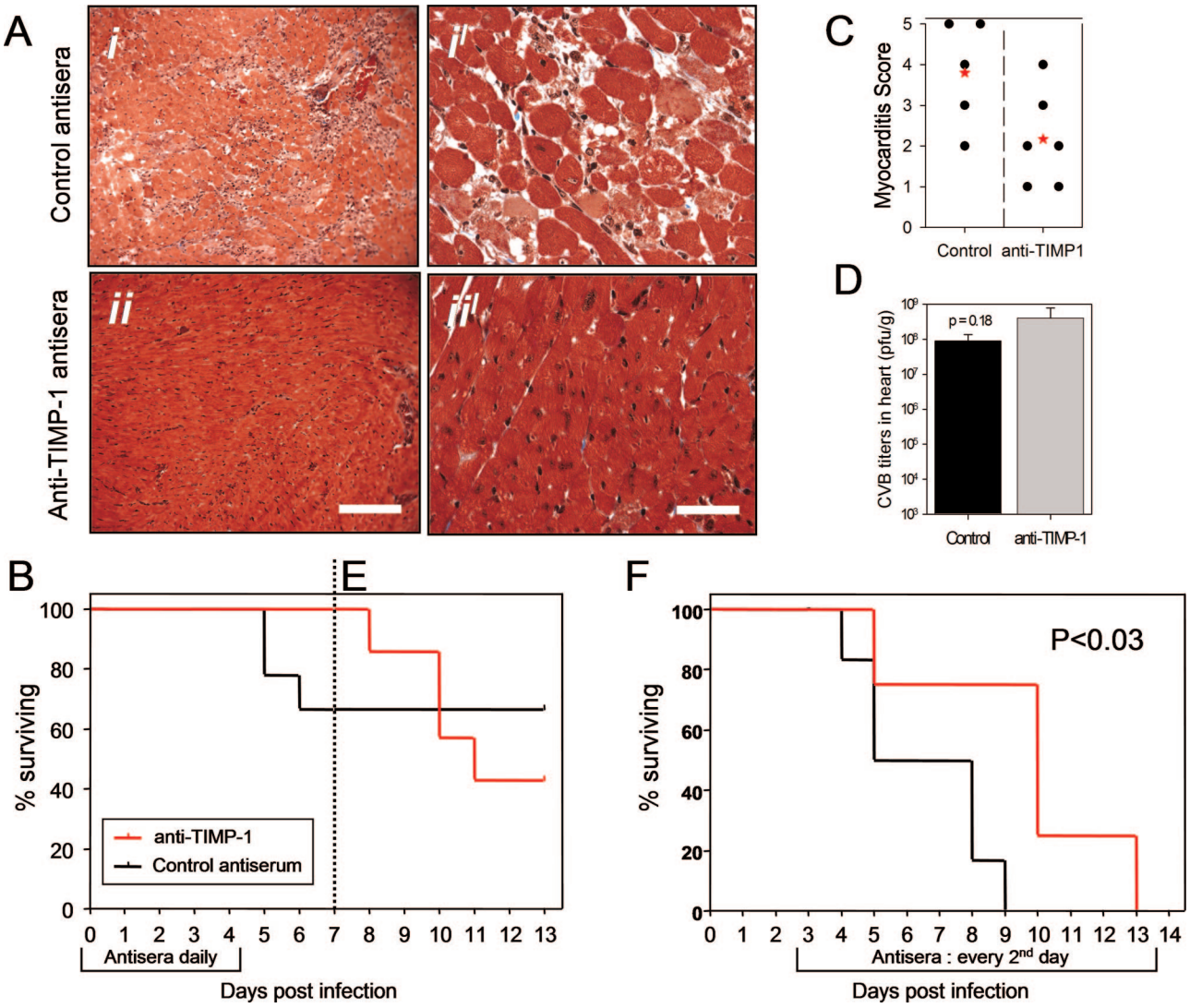

Figure 6. Antibodies against TIMP-1 attenuate CVB3-induced myocarditis. **A:** Anti-TIMP-1 antisera attenuates CVB3-induced myocarditis in adult WT C57B/6 mice infected with CVB3, compared with treatment of an IgG isotype-matched control for 5 consecutive days beginning on the day of infection. Representative cardiac pathology of CVB3-infected animals that were either treated with control IgG (**i**, **i^{I}**) or α-TIMP-1 antisera (**ii**, **ii^{I}**). Images taken at lower (**i**, **ii**) and higher magnifications (**i^{I}**, **ii^{I}**). **B:** Treatment of WT with α-TIMP-1 for the first 5 days of CVB3 infection increased 7-day survival. **C:** Quantification of myocarditis in IgG and α-TIMP-1 antisera-treated mice indicated that antibody-mediated blocking of TIMP-1 attenuated the severity of CVB3-induced heart injury 1 week after infection. **Red stars** indicate the group means; the difference between the groups is statistically significant ($P < 0.029$). **D:** Virus titers in the hearts of antisera-treated mice are shown. The difference between the two groups is not statistically significant ($P = 0.18$). **E:** Survival analysis of CVB3 infected mice for 2 weeks after short-term treatment with α-TIMP-1 antisera (days 0 to 5) revealed a transient benefit at day 7 ($P = 0.10$) but dropped away from significance by day 14 ($P = 0.56$). **F:** Administration of α-TIMP-1 antisera initiated 3 days after infection significantly delayed ($P < 0.03$), but did not prevent, virus-induced lethality. Scale bars: 300 μm (**i**, **ii**); 30 μm (**i^{I}**, **ii^{I}**).

primary biological function of TIMP-1 is thought to be its role as an endogenous MMP inhibitor, we determined whether TIMP-1 deficiency affected CVB3-induced MMP activity in the heart. Extracts from hearts of CVB3-infected or uninfected WT and TIMP-1KO mice were analyzed by gelatin zymography; the results are shown in Figure 7. Basal MMP activity was negligible in the uninfected hearts of both WT and TIMP-1KO mice, as expected, and CVB3 infection of WT mice resulted in a marked (approximately ninefold) increase in MMP-9 activity ($P < 0.001$); in contrast, CVB3 infection of TIMP-1KO mice led to only a minimal increase of myocardial MMP-9 activity (less than twofold, not statistically significant). MMPs are produced by infiltrating immune cells, so these findings are completely consistent with our observed reduction in myocarditis during CVB3 infection in TIMP-1KO mice (Figure 2).

Discussion

Herein, we have demonstrated that TIMP-1 is an important host factor involved in CVB3-mediated myocarditis. Previous studies have identified correlations between the expression of TIMP-1 and some forms of myocardial disease. Changes in TIMP-1 expression have been associated with chronic heart diseases leading to fibrosis and cardiomyopathy,[36,37] and elevated TIMP-1 is predictive

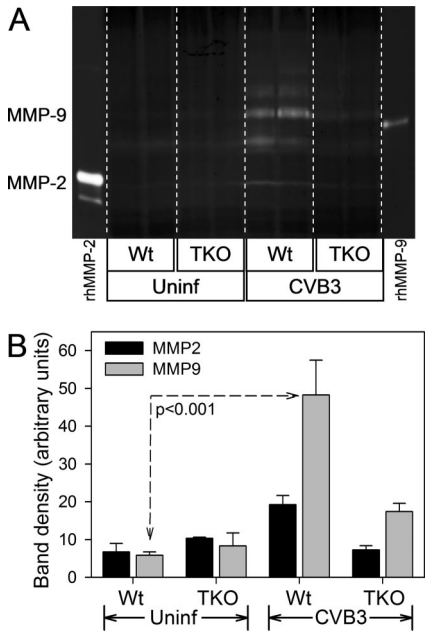

Figure 7. MMP activities in the uninfected and CVB3-infected hearts of WT and TIMP-1KO mice. **A:** Gelatin zymography of the uninfected or infected hearts of WT or TIMP-1KO (TKO) mice. Control MMP-2 and MMP-9 are shown (both recombinant human materials, as indicated by rh prefix). **B:** Band intensities were evaluated using the free National Institutes of Health software package ImageJ. $^{*}P < 0.001$ (when compared with MMP-9 activity in the hearts of uninfected WT mice).

of lethal outcome in patients with coronary artery disease.[17] TIMP-1KO mice, without experimental injury, have relatively normal heart physiology[38] but exhibit adverse ventricular remodeling after left coronary artery ligation ischemic injury.[22] Conversely, adenovirus-mediated expression of TIMP-1 was reported to mitigate the severity of ischemic injury.[39] These data suggested that TIMP-1 might exert a protective effect in the myocardium, consistent with the concept that increased MMP activity is a key process driving myocardial pathology after myocardial infarction or stress-related injuries[13,32]; by this reasoning, expression of TIMP-1 is a physiological response that counterbalances MMP proteolysis,[14,40,41] and TIMP-1 blockade should, in theory, exacerbate disease. We show here that TIMP-1 expression in the heart increases during viral myocarditis, consistent with many studies that show that TIMP-1 is up-regulated in response to myocardial injury. However, contrary to expectations, we found that the inhibition of TIMP-1 prevented, rather than worsened, CVB3-induced myocarditis and mortality (Figure 2). How might the unexpected benefits of TIMP-1 inhibition be explained?

Perhaps the simplest possible explanation for the lower mortality and diminished myocardial infiltration would be a reduction in viral titers in TIMP-1KO mice. Lower myocardial virus titers would cause less virus-mediated myocardiolysis and also might explain the reduced immune cell infiltrates that we observed. However, myocardial virus titers were only very modestly decreased in TIMP-1KO mice (Figure 4), and no antiviral effects were observed in WT mice that had been treated with anti-TIMP-1 siRNA or antibody (Figures 5 and 6). Taken together, our data suggest that the beneficial effects of TIMP-1 deficiency are unlikely to be related to altered virus replication. A second possible explanation is that TIMP-1KO mice have reduced immune responses, leading to reduced myocardial infiltration; in this scenario, the lower mortality might be attributable to reduced immunopathology. However, the $CD4^{+}$ and $CD8^{+}$ T-cell responses to CVB3 infection were similar in the spleens of TIMP-1KO and WT mice, as was the overall composition of the immunological infiltrates (Figure 3). Therefore, there appears to be little difference in the systemic T-cell response between the two mouse strains. We cannot exclude the possibility that differences exist in T-cell responses within the heart itself; nor have we evaluated the autoimmune T-cell responses that have been described by others.[7] Thus, we favor a third, and novel, explanation: that the absence of TIMP-1 may alter the anatomical distribution of activated immune cells, mitigating their immunopathological effects in the heart. A previous study has shown that activated immune cells produce and secrete MMPs to disrupt the extracellular matrix, thereby facilitating their entry into cardiac tissue[42]; another report showed that the administration of recombinant TIMP-1 arrests the migration of immune cells *in vitro*.[43] Furthermore, an adenovirus-mediated increase in TIMP-1 expression also resulted in diminished CVB3-induced myocarditis.[34] We show here that CVB3-infected WT mice express TIMP-1 protein in areas of immune cell infiltrates (Figure 1D) and that these mice also show high levels of MMP activity in the heart, as would be expected, because MMPs are produced by immune cells (Figure 7). In contrast, the hearts of CVB3-infected TIMP-1KO mice show no increase in MMP activity, consistent with the reduced immune cell infiltrate. Thus, we propose that, during virus infection, TIMP-1 may function not only to block the deleterious effects of MMPs but also to focus the antiviral response to infected areas in the myocardium by arresting the MMP-dependent migratory activity of activated cells, thereby resulting in their local accumulation, that is, in histologically evident myocarditis. Our novel hypothesis is depicted diagrammatically in Figure 8, and explains why eliminating TIMP-1 expression (our data) or enhancing TIMP-1 expression[34] may have similar outcomes on CVB-induced myocarditis, albeit through distinct mechanisms. By eliminating TIMP-1 (our data), we prevent the retention of immune cells at areas of CVB3 infection (Figure 8A), thereby reducing myocarditis, myocardial damage, and death. By enhancing TIMP-1 expression (others' data[34]), the deleterious effects of virus-induced metalloproteinase activities are blocked (Figure 8C), thereby diminishing myocardial damage. Hence, we suggest that our studies reveal a novel feature of the physiological function of TIMP-1, resulting in a parabolic relationship between TIMP-1 levels and myocarditis (Figure 8D).

The decreased inflammatory infiltration associated with inhibition of TIMP-1 does not necessarily imply a reduced antiviral effect; a previous study from this laboratory has shown that the immunopathological effects of the T-cell response can be uncoupled from the antiviral

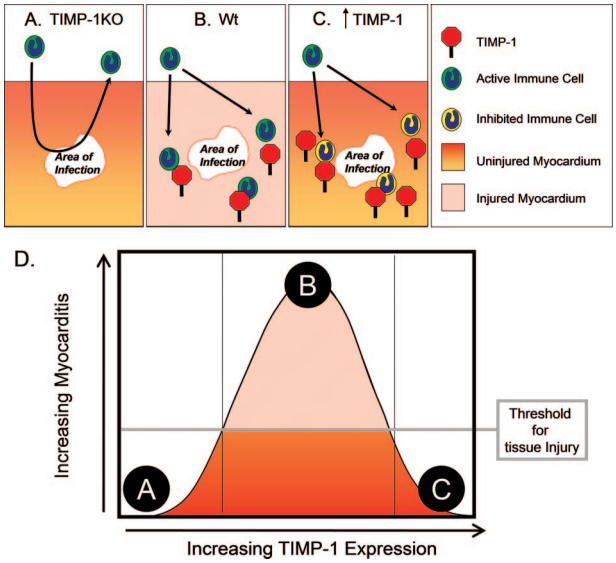

Figure 8. Diagrammatic representation of the proposed effects of TIMP-1 under- and overexpression. **A:** During CVB3 infection in the TIMP-1KO animal, immune cells traffic to sites of infection but, in the absence of TIMP-1, are not retained there. **B:** In the WT animal, immune cells trafficking into the heart are retained at sites of CVB3 infection by the local increase in TIMP-1 expression. Hence, TIMP-1 focuses the immune response at areas of infection, resulting in myocarditis and tissue injury. **C:** In contrast, artificially high TIMP-1 expression not only arrests immune cell trafficking but also inhibits the local destructive effects of MMPs, thus reducing myocardial injury. **D:** Hence, there may be a parabolic relationship between TIMP-1 expression and CVB-induced myocarditis.

effects, leading to effective viral control in the absence of myocardial immunopathology.[9] Furthermore, the nearly equivalent levels of CVB3 viral titers among the control and TIMP-1-inhibited groups also suggest that blocking TIMP-1 did not affect the virus infection per se; rather, we suggest that TIMP-1 had an influence on cardiac pathology via the trafficking of immune cells. Although both notions of blocking or enhancing TIMP-1 expression in heart improving myocarditis center on the role and activities of MMPs, alternative MMP-related explanations also must be considered. Although it is an MMP inhibitor, TIMP-1 also can bind the zymogen forms of pro-MMPs and facilitate their activation.[44] Therefore during the course of a CVB3 infection, a reduction in myocardial TIMP-1 may have also prevented the activation of a pro-MMP that causes myocardial pathology. Exploration of MMP-independent functions of TIMP-1 on virus-induced pathology warrant further investigation.

Finally, TIMP proteins also have less well characterized MMP-independent physiological functions.[45] The TIMP-1 protein was first described as a novel growth factor isolated from serum,[46] and this function caused it to be named erythroid potentiating activity. Its identification as an endogenous metalloproteinase inhibitor led to its being renamed TIMP-1, and this function subsequently dominated our understanding and interpretations of the role and biological activities of the protein.[47] However, the notion that TIMP proteins, and TIMP-1 in particular, have ancillary physiological functions that are exclusive of their inhibition of MMPs has gained standing with recent studies demonstrating MMP-independent functions for TIMP-2 and TIMP-3 as antagonists of β_1-integrin-mediating signaling[48] and VEGF receptor-induced angiogenesis,[49] respectively. Thus, we cannot exclude the possibility that TIMP-1 may act as a signaling molecule that promotes myocarditis in virus-infected mice.

In summary, we have identified a novel and unpredicted pathological role for TIMP-1 in CVB3-induced heart disease. We show that blockade of TIMP-1 expression (TIMP-1KO mice, siRNA) or function (anti-TIMP-1 antibodies) can ameliorate CVB3-induced acute myocarditis, suggesting that this protein may present a novel therapeutic opportunity for the treatment of human disease. Furthermore, given the prevalence of CVB3 infection in human populations, any drug that enhances myocardial expression of TIMP-1 might inadvertently trigger an exacerbation of myocardial injury, particularly during asymptomatic or acute CVB3 infection. Moreover, increased expression of TIMP-1 has been reported in a wide variety of infections,[50] suggesting that TIMP-1 may also facilitate immunopathology during infection by other pathogens as well. Hence, the traditional protective role of TIMP-1 in cardiac function should be reconsidered. Moreover, caution is warranted in the use of TIMP-1-inducing or TIMP-1 mimetic compounds as small molecule therapeutics for treatment of nonviral heart disease.

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