Background—Little is known about innate immune mechanisms within the cardiac myocyte that determine susceptibility to enterovirus infection, an important cause of myocarditis and subsequent heart failure. Although interferon (IFN) generally plays a key role in innate immunity, ablation of IFN receptors has little or no effect on acute coxsackievirus B3 infection in the heart. Interestingly, gp130-cytokine–mediated stimulation of neonatal ventricular myocytes has a cytoprotective effect against virus infection in culture that can be inhibited by suppressors of cytokine signaling (SOCS)-3, a physiological inhibitor of gp130 signaling that does not affect IFN signaling. Therefore, we hypothesized that inhibition of gp130 signaling by SOCS3 would change cardiac myocyte susceptibility to virus infection without affecting IFN signaling.

Methods and Results—We generated cardiac-specific SOCS3 transgenic mice. Despite an intact IFN-mediated antiviral response in adult transgenic myocytes, there was a marked increase in susceptibility to viral infection in the SOCS3 transgenic mouse hearts. This indicated the presence of IFN-independent innate defense mechanisms within the cardiac myocyte. Subsequently, we demonstrated that cardiac-specific gp130-knockout mice also had increased susceptibility to viral infection. Furthermore, we demonstrated that the gp130-mediated increase in survival of infected myocytes occurred through a signal transducers and activators of transcription-3–dependent mechanism that did not affect viral replication. This was accompanied by a persistent expression of full-length dystrophin after coxsackievirus B3 infection. In addition, we found that both SOCS3 transgenic and gp130-deficient mice had a decrease in α-sarcoglycan.

Conclusions—SOCS3-mediated regulation of gp130 signaling can affect susceptibility to viral infection in the heart. Increased cardiac cell survival through gp130–signal transducers and activators of transcription-3 signaling appears to play an important role in preserving nondividing cardiac myocytes until specific immune responses begin to clear the virus. (Circulation. 2006;114:2364-2373.)

Key Words: heart failure ■ immune system ■ infection ■ molecular biology ■ myocarditis ■ signal transduction

Viral myocarditis and dilated cardiomyopathy can be caused by common viral pathogens such as enteroviruses and adenoviruses that can have a direct myocytopathic effect and can activate a potent T-cell immune response. Virus-mediated cardiac disease can be a debilitating and potentially lethal disease in adults and children and is one of the main indications for cardiac transplantation.1,2 Although our understanding of the pathogenesis of virus-mediated cardiomyopathy has improved, relatively little is known regarding factors that determine susceptibility to viral infection. What factors determine why most individuals

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infected with cardiomyopathic viruses do not develop significant cardiac disease whereas other individuals develop severe virus-mediated heart disease? Activation of the innate immune response can be mediated by cytokines such as the interferons (IFNs) and interleukin-6 and is one of the earliest defenses against infectious pathogens. Cytokines activate transcription of the cytokine-responsive genes, including suppressors of cytokine signaling (SOCS). This is often mediated through Janus kinase (JAK)
signal transducers and activators of transcription (STAT) signaling pathways. When induced, SOCS molecules inhibit JAK-mediated phosphorylation of the cytokine receptor, inhibiting activation of STAT signaling. This negative feedback via SOCS tightly regulates the duration and intensity of the cytokine-induced JAK-STAT signaling. Little is known of the importance of the innate immune response against viral infection within the cardiac myocyte.

Recently, we showed that coxsackievirus B3 (CVB3) infection is associated with activation of JAK-STAT signaling in the heart with an induction of SOCS mRNA. In addition, we demonstrated that SOCS1-mediated inhibition of JAK-STAT signaling in the intact heart markedly increased susceptibility to viral infection. This demonstrated the important role for JAK-STAT signaling within the cardiac myocyte in the prevention of viral infection.

A potential explanation for this finding is that SOCS1 increases susceptibility to viral infection by inhibiting IFN signaling in the heart. However, although CVB3 infection of global IFN-α/β (type I) receptor–knockout mice resulted in a marked increase in mortality, there was surprisingly little increase in viral infection of the heart in either IFN-α/β (type I) or -γ (type II) receptor–knockout mice. This raised the question of which innate signaling mechanisms against viral infection are important in the heart.

Among SOCS family proteins, there are clear differences in the effect of SOCS1 and SOCS3 on the innate immune response. Endogenous SOCS1 plays a key role in the negative regulation of IFN-γ whereas SOCS3 predominantly inhibits gp130 signaling. SOCS1 binds directly to the activation loop of all 4 JAKs, whereas SOCS3 initially binds to the cytokine receptor, thus inhibiting JAK activation of a subset of receptors such as gp130. Therefore, overexpression of SOCS1 inhibits activation of all 4 JAKs, whereas that of SOCS3 does not. Adenovirus-mediated overexpression of SOCS1 in neonatal rat cardiac myocytes inhibits both IFN and gp130 signaling, whereas that of SOCS3 inhibits only gp130 signaling and has no effect on IFN signaling. In the intact adult heart, it is not clear whether the inhibition of IFN or gp130 signaling is primarily responsible for the SOCS-mediated increase in susceptibility to viral infection.

To dissect the mechanisms by which SOCS overexpression in the heart could increase susceptibility to viral infection, we generated a transgenic mouse that overexpressed SOCS3 in the cardiac myocyte. We hypothesized that SOCS3 overexpression would increase susceptibility to viral infection without an effect on IFN signaling. Once we confirmed this, we further hypothesized that a mechanism by which SOCS3 increased susceptibility to viral infection was via inhibition of gp130 signaling. Our findings indicate that overexpression of SOCS3 in transgenic mouse myocytes effectively inhibited gp130 signaling. Furthermore, we found that cardiac-specific knockout of gp130 increased susceptibility to viral infection. The protective effect of gp130 signaling in isolated myocytes is mediated via STAT3 and is associated with a persistent expression of full-length dystrophin even after CVB3 infection. In addition, α-sarcoglycan protein is decreased in SOCS3 transgenic (SOCS3 Tg) hearts and in cardiac-specific gp130–knockout mice. These findings demonstrate the importance of gp130 signaling as a novel anti-viral mechanism within the cardiac myocyte and the minor contribution made by activation of IFN signaling during the early stages of enteroviral infection in the cardiac myocyte.

Methods

Mice

To generate SOCS3 Tg mice, the fragment carrying the α-myosin heavy chain promoter and myc-tagged SOCS3 cDNA was microinjected into (C57BL/6×Balb/c) F1 (C6Blf) zygotes. The cardiac-specific gp130-knockout mice were generated as described previously.

Viruses

The CVB3 used in this study was the cardiotropic H3 strain (Woodruff variant) of CVB3 (CVB3-H3). The CVB3 that expresses green fluorescent protein (GFP) (CVB3-GFP) was generated as described previously.

Antibodies

Western blot and immunofluorescence staining were performed as described previously with anti-STAT1, anti–phospho-STAT1, anti-STAT3, and anti–phospho-STAT3 antibodies (New England BioLabs Inc, Beverly, Mass); rabbit polyclonal anti-CVB3 antibody (a gift from Dr A. Henke, Friedrich Schiller University, Jena, Germany), anti–c-myc (A-14) antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif), anti-GFP antibody (Medical & Biological Laboratories, Nagoya, Japan), and anti–coxsackie-adenovirus receptor (CAR) antibody.

Echocardiogram

Avertin (2.5%) was administered intraperitoneally at 0.015 mL/g body weight. Recordings were performed as described previously.

Left ventricular end-systolic (LVESD) and end-diastolic (LVEDD) dimensions were measured from the left ventricular M-mode recording. Percent fractional shortening (%FS) of the LV was calculated as follows:

\[
%FS = \frac{100 \times (LVEDD - LVESD)}{LVEDD}
\]

Isolation of Adult Ventricular Myocytes

The heart was perfused retrogradely for 3 minutes using a calcium-free buffer (heart solution) containing (in mmol/L) 120 NaCl, 5.4 KCl, 1.2 MgSO4, 1.2 NaH2PO4, 20 NaHCO3, 5.6 d-glucose, 20 of 2,3-butanediol monoxime (Sigma-Aldrich, St Louis, Mo), 5 taurine, and 1 pyruvate. The enzymatic digestion was achieved by perfusing a collagenase type 2 solution (Worthington Biochemical, Lakewood, NJ; final concentration, 1 mg/mL in heart solution) at 37°C for 10 to 20 minutes. Isolated myocytes were allowed to pellet by gravity in heart solution containing 5 mg/mL bovine serum albumin (Sigma) (washing solution). The myocytes were resuspended in a series of washing solutions with increasing concentration of CaCl2 (0.125, 0.25, and 0.5 mmol/L). The myocytes were plated on laminin precoated plates (10 mg/mL, Sigma) and cultured in a minimal essential media (Gibco BRL) with 0.5 mmol/L CaCl2 for 12 hours.

Statistical Analysis

Data are expressed as mean ± SE unless otherwise noted. Statistical significance was evaluated with the unpaired Student t test for comparisons between 2 means. For multiple comparisons, 1-way or 2-way analysis of variance using the Tukey-Kramer post hoc test was used. For survival rate after CVB3 inoculation, the differences were assessed using the log-rank test. Where relevant, Student t test was used to compare differences between 2 groups.
between 2 groups were analyzed by log-rank (Mantel-Cox) test. Values of $P<0.05$ were considered significantly different.

The authors had full access to the data and take full responsibility for their integrity. All authors have read and agree to the manuscript as written.

**Results**

**Marked Increase in Susceptibility to CVB3-Mediated Cardiomyopathy in SOCS3 Tg Mice**

We generated transgenic mice overexpressing a **myc**-tagged SOCS3 under the control of the cardiac myocyte-specific α-myosin heavy chain promoter. Transgene expression was confirmed by immunoblotting with an anti-**myc** antibody in 3 separate founder lines. There was no evidence of embryonic lethality, and the mice grew to adulthood without increased mortality. Global cardiac structure and function were normal in the mice (data not shown). The mouse line that had a comparable level of expression of the transgene to the SOCS1 Tg mice was chosen for this study (Figure 1A).

To determine whether cardiac overexpression of SOCS3 affects the susceptibility to CVB3-mediated cardiomyopathy, we inoculated 6-week-old female Balb/c mice, SOCS3 Tg mice, and their wild-type (WT) littermates with CVB3. Mortality in the SOCS3 Tg mice was 80%, which compared with 10% in the infected WT littermates (n=16 mice for each group; $P<0.0001$). Cardiac-specific transgenic overexpression of SOCS3 leads to early mortality in CVB3-infected mice. Kaplan-Meier survival curves demonstrate that the survival rate in infected (10⁵ pfu of the virus) SOCS3 Tg mice was significantly lower than in infected WT littermates (n=16 mice for each group; $P<0.0001$). Evans blue dye uptake in the cardiac cells was markedly increased in the surviving SOCS3 Tg mice on day 5 after virus inoculation (red stain, left and middle). The percent Evans blue–positive area in SOCS3 Tg vs WT littermates was 38.3±9.9% vs 2.2±0.7%, respectively (right; mean±SE; n=5 mice for each group; $P<0.0001$). Increased viral titer in the heart but not the liver of SOCS3 Tg mice on day 5 after viral inoculation (mean±SE; n=6; $P<0.0001$, SOCS3 Tg mice vs WT littermates). Echocardiography was performed 4 days after virus inoculation (n=4 mice in each group). Left, LVESD was significantly elevated in SOCS3 Tg (solid bar) compared with WT (open bar). The percent fractional shortening (%FS; right), a parameter of cardiac function, was significantly decreased in SOCS3 Tg vs WT mice (mean±SE; $P<0.0001$, SOCS3 Tg mice vs WT littermates). F, Hematoxylin and eosin stains of representative WT littermate and SOCS3 Tg mouse heart 5 days after virus inoculation. Note the hemorrhage in the myocardium (right). Scale bars=1 mm (C), 100 μm (F, right).

**Figure 1.** Increased susceptibility to CVB3-mediated cardiomyopathy in SOCS3 Tg mice. A, The expression of **myc**-tagged SOCS3 in the heart was confirmed in SOCS3 Tg mice by immunoblotting with anti-**myc** antibody and compared with the level of SOCS1 Tg–mediated expression. B, Cardiac-specific transgenic overexpression of SOCS3 leads to early mortality in CVB3-infected mice. Kaplan-Meier survival curves demonstrate that the survival rate in infected (10⁵ pfu of the virus) SOCS3 Tg mice was significantly lower than in infected WT littermates (n=16 mice for each group; $P<0.0001$). C, Evans blue dye uptake in the cardiac cells was markedly increased in the surviving SOCS3 Tg mice on day 5 after virus inoculation (red stain, left and middle). The percent Evans blue–positive area in SOCS3 Tg vs WT littermates was 38.3±9.9% vs 2.2±0.7%, respectively (right; mean±SE; n=5 mice for each group; $P<0.0001$). D, Increased viral titer in the heart but not the liver of SOCS3 Tg mice on day 5 after viral inoculation (mean±SE; n=6; $P<0.0001$, SOCS3 Tg mice vs WT littermates). E, Echocardiography was performed 4 days after virus inoculation (n=4 mice in each group). Left, LVESD was significantly elevated in SOCS3 Tg (solid bar) compared with WT (open bar). The percent fractional shortening (%FS; right), a parameter of cardiac function, was significantly decreased in SOCS3 Tg vs WT mice (mean±SE; $P<0.0001$, SOCS3 Tg mice vs WT littermates). F, Hematoxylin and eosin stains of representative WT littermate and SOCS3 Tg mouse heart 5 days after virus inoculation. Note the hemorrhage in the myocardium (right). Scale bars=1 mm (C), 100 μm (F, right).
shown) were observed only in the infected SOCS3 Tg mice, a finding that is likely secondary to the extent of myocardial damage. Generally, the phenotype of infected SOCS3 Tg mice was very similar to that of infected SOCS1 Tg mice.12

Effect of SOCS3 Overexpression on IFN-Mediated Antiviral Activity in Isolated Transgenic Adult Cardiac Myocytes
To determine the functional effect of exogenously administered cytokines on viral replication in adult cardiac myocytes, we isolated ventricular cardiac myocytes from the adult heart and stimulated them for 12 hours with IFN-β, IFN-γ, or cardiotroponin (CT)-1. The myocytes were then infected for 24 hours with a recombinant CVB-3 GFP.23 IFN-β had a partial but significant antiviral effect in adult myocytes isolated from WT Balb/c mice. However, IFN-γ and CT-1 had no significant effect on the percent of infected myocytes (Figure 2).

We then sought to determine the effect of overexpression of SOCS3 on the cytokine-stimulated antiviral effect. The IFN-β–mediated antiviral effect was preserved in myocytes from SOCS3 Tg mice, whereas the effect was completely abolished in those from SOCS1 Tg mice, demonstrating the lack of inhibition of IFN antiviral effects by transgenic overexpression of SOCS3 (Figure 2). Incidentally, there was no significant difference in the level of CAR protein in the SOCS3 Tg myocytes as assessed by immunoblot (data not shown).

Effect of SOCS Overexpression on IFN-γ and CT-1 Signaling in Isolated Transgenic Adult Cardiac Myocytes
Because IFN-γ and CT-1 had no effect on coxsackieviral replication in the isolated adult myocytes, we sought to determine whether downstream signaling mechanisms were intact and whether SOCS1 or SOCS3 overexpression would affect downstream signaling for these cytokines.12 First, we stimulated adult cardiac myocytes from WT Balb/c mice with CT-1 or IFN-γ and found that CT-1 stimulated STAT3 but not STAT1 phosphorylation. However, IFN-γ induced both STAT1 and STAT3 phosphorylation 15 minutes after stimulation (Figure 3). As expected, in myocytes from SOCS1 Tg mice, STAT1 and STAT3 were not phosphorylated after CT-1 or IFN-γ stimulation. However, overexpression of SOCS3 did not prevent IFN-γ–stimulated STAT1 or STAT3 phosphorylation, but as hypothesized, it inhibited CT-1–mediated STAT3 phosphorylation.

Knockout of Cardiac gp130 Increases Susceptibility to CVB3 Infection
Because SOCS3 overexpression did not affect the IFN-β antiviral effects or IFN-γ–mediated STAT phosphorylation, we sought to identify IFN-independent, SOCS3-inhibitable,
The cytoprotective effect of CT-1 against CVB3 infection was examined by Western blots from 3 independent experiments. Activation of STAT1 and STAT3 was estimated by immunoblotting with anti-phospho-STAT1, STAT1, anti-phospho-STAT3, and STAT3 antibodies. Representative Western blots from 3 independent experiments are shown.

JAK-dependent signaling pathways that regulate cardiac cell susceptibility to viral infection. We focused on cardiac gp130 signaling as the best-known physiological target of SOCS3. Before cardiac-restricted gp130-knockout mice were generated, both the mice with homozygous floxed gp130 allele (gp130F/F) and the mice that express Cre under the direction of the myosin light chain-2v promoter (MLC-2vCre+) were backcrossed for 5 generations into a Balb/c background. The gp130-knockout efficiency in the MLC-2vCre+; gp130F/F cardiac myocytes was >80% compared with the WT control myocytes (Figure 4A). Knockout of gp130 did not affect the level of CAR expression (Figure 4B). However, 5 days after infection with CVB3, we found that the Evans blue dye area in 6-week-old female gp130-knockout mice was increased compared with WT littermates (Figure 4C), although the difference was not as dramatic as that observed in SOCS3 Tg mice (Figure 1C).

Subsequently, we examined the relationship between the virus distribution and Evans blue dye area by immunofluorescence staining. Generally, the myocytes that were positive for viral capsid proteins also were positive for Evans blue dye in both WT and gp130-knockout mice (Figure 4D, yellow in merged panel).

gp130 Signaling Has No Effect on CVB3 Replication but Has a Strong Cytoprotective Effect Through STAT3 Activation and Maintenance of Full-Length Dystrophin Expression

Because CT-1 did not have a direct antiviral effect in isolated adult cardiac myocytes (Figure 2), we sought to determine how gp130 signaling could affect viral pathogenesis. We have previously demonstrated that CT-1 has a strong cytoprotective effect in CVB3-infected neonatal rat cardiac myocytes.12 Because of the inherent time-dependent cytopathic effect that occurs after isolation of uninfected adult myocytes, the subsequent experiments were performed in cultured neonatal rat cardiac myocytes.12 First, we determined that the cytoprotective effect of CT-1 against CVB3 infection was selective to cardiac myocytes by demonstrating that it had no cytoprotective effect on nonmyocytes such as HeLa and 293 cells even though there was activation of STAT3 signaling (data not shown). Then, we found that there was no detectable change in CAR expression level 24 hours after CT-1 stimulation in the cardiac myocytes (Figure 5A). Subsequently, we found that CT-1 did not directly affect viral replication using a 1-step growth curve of the virus titer in CT-1–stimulated or nonstimulated neonatal myocytes (Figure 5B). These observations are consistent with the result from adult myocytes that CT-1 stimulation had no effect on infection efficiency (Figure 2).

We have previously demonstrated that one of the mechanisms by which CVB3 infection leads to death of cardiac myocytes is disruption of the sarcolemmal membrane. This is associated with dystrophin cleavage by the virus protease 2A.24 In addition, we have demonstrated that dystrophin cleavage has an important role in viral propagation in the heart.29 On the basis of this concept, we examined whether there is a difference in dystrophin cleavage in the myocytes stimulated with or without CT-1 after CVB3 infection (Figure 5C). Cleaved dystrophin bands were identified in CVB3-infected myocytes in both groups 12 hours after infection. However, both cleaved and noncleaved bands were barely detected 24 hours after infection in control myocytes, whereas uncleaved dystrophin was observed until at least 36 hours after infection in CT-1–stimulated myocytes. This indicated that activation of gp130 signaling prevented cleavage of all dystrophin by CVB3 infection (Figure 5C).

Finally, we sought to determine the gp130 downstream signaling pathway that is involved in the cytoprotective effect against CVB3 infection. Activation of gp130 signaling has been associated with activation of PI3K, MEK, and STAT3.30 To inhibit PI3K-Akt and MEK–extracellular signal–regulated kinase (ERK)-1/2 pathways, we added Wortmannin and PD98095, respectively, to the culture media before CT-1 stimulation. To inhibit the STAT3 pathway, we overexpressed dominant-negative STAT3 (dnSTAT3) using recombinant adenovirus vector. The specific inhibition of the PI3K-Akt, MEK-ERK1/2, and STAT3 pathways in our experimental conditions was confirmed by Western blot using anti–phospho-Akt, ERK1/2, and STAT3 antibodies, respectively (Figure 5D, top). In these conditions, cells were infected with CVB3 in the presence or absence of CT-1 stimulation. Although the inhibition of PI3K-Akt and MEK-ERK1/2 signaling pathways had no effect on CT-1–mediated cytoprotective effect against CVB3 infection (Figure 5D, bottom left), inhibition of the STAT3 pathway significantly abolished the cytoprotective effect (Figure 5D, lower right, white bar), indicating that the gp130-mediated cytoprotective effect against CVB3 is mediated through STAT3 signaling.

Both SOCS3 Overexpression and gp130 Deficiency in the Cardiac Myocyte Reduced α-Sarcoglycan in the Intact Heart

We have previously demonstrated that the susceptibility to CVB3-mediated cardiomyopathy can be increased in the absence of dystrophin-sarcoglycan complex in mdy mice.29 In this study, we found that CT-1 stimulation improved myocyte survival with an increase in full-length dystrophin. Therefore, we sought to determine whether inhibition of gp130 signaling by SOCS3 Tg overexpression or knockout of the gp130 signaling pathways were involved in the cytoprotective effect of CT-1.
receptor affected expression of dystrophin or α-sarcoglycan. We found no detectable changes in the cellular localization of dystrophin (data not shown) and α-sarcoglycan (Figure 6A) in the heart of SOCS3 Tg and gp130-knockout mice; however, the expression level of α-sarcoglycan by Western blot analysis was decreased in both SOCS3 Tg and gp130-knockout mice compared with WT littermate controls. We were not able to detect clear differences in dystrophin levels by immunoblot (Figure 6B, left). Based on serially diluted heart protein samples, the protein expression level of α-sarcoglycan in the heart of SOCS3 and gp130-knockout mice was approximately half of that in the hearts of WT littermates (Figure 6B, right).

**Discussion**

In the present study, we focused on innate defense mechanisms in the cardiac myocyte that are important determinants of susceptibility to viral infection in viral myocarditis. Like SOCS1, SOCS3 overexpression in the transgenic cardiac myocyte has a marked effect on the susceptibility of the heart to CVB3 infection; however, SOCS3 overexpression does not affect IFN-receptor signaling or the IFN antiviral effect in isolated myocytes. Finally, we showed that cardiac-specific knockout of gp130 increases susceptibility to CVB3, demonstrating a role for gp130 signaling via activation of STAT3.

It has previously been demonstrated that there is a potent cardiac myocyte–dependent innate immune response that can be inhibited by cardiac-specific overexpression of SOCS1. In addition, transgenic overexpression of SOCS1 in pancreatic islet cells affects susceptibility to CVB infection. A likely target for the SOCS1 protein that could affect susceptibility to viral infection in these models would be the IFN, JAK-STAT signaling cascade. The data from the present study, however, challenge that notion by demonstrating that both SOCS1 and SOCS3 overexpression increases susceptibility to viral infection despite the fact that SOCS3 overexpression does not inhibit IFN signaling or IFN-mediated antiviral effects. Although endogenous SOCS3 expression predominantly inhibits gp130 signaling, some have indicated that overexpression of SOCS3 can have inhibitory effect on IFN signaling. Differences in these results may be secondary to differences in cell types studied, mechanisms of overexpression, or levels of expression of SOCS3. To improve our understanding of the mechanisms by which SOCS3
overexpression in the heart alters susceptibility to infection, we isolated adult cardiac myocytes from transgenic mice overexpressing SOCS3 or SOCS1 and demonstrated that SOCS3 overexpression in the adult cardiac myocyte had no significant effect on IFN-mediated antiviral effects or IFN-γ STAT1 phosphorylation. As expected, IFN signaling in the SOCS1-overexpressing Tg cardiac myocytes was clearly inhibited as measured by IFN-α/β-mediated antiviral effects and IFN-γ-mediated STAT1 and STAT3 phosphorylation. At first glance, these data may seem contrary to previously published data demonstrating that global knockout of either the IFN-α/β receptor or IFN-

Figure 5. Cytoprotective effect of gp130-STAT3 signaling against CVB3 infection in neonatal rat cardiac myocytes via a STAT3-dependent mechanism. A, Expression level of CAR in the myocytes after CT-1 simulation was examined by immunoblotting with anti-CAR antibody using glyceraldehyde 3-phosphate dehydrogenase as an internal control. Lane 1 shows positive control protein from HeLa cells; lane 2, myocytes without CT-1 stimulation; and lane 3, myocytes with 24 hours of CT-1 stimulation. B, One-step growth curve of CVB3 in the myocytes with (solid line) or without (Control; dotted line) CT-1 stimulation. Myocytes were infected with CVB3 (multiplicity of infection, 100) for 1 hour, followed by washing 3 times with phosphate buffer saline. After the culture for indicated time on the x axis, cells were harvested to examine the virus titer by plaque forming assay. C, Effect of CT-1 stimulation on transition of structural and nonstructural proteins in the myocytes after CVB3 infection. Proteins from the myocytes stimulated with or without CT-1 (Control) were extracted at each time point indicated above the panel after CVB3 infection (multiplicity of infection, 100) and were subjected to immunoblotting with anti-dystrophin, sarcomeric α-actin, STAT1, enterovirus VP1, and enterovirus proteinase 2A antibodies. D, Downstream signaling pathway of gp130 that is involved in the cytoprotective effect. All experiments were performed in culture media with 5% serum to prevent spontaneous cell death. This resulted in baseline activation of Akt and ERK1/2 signaling. The specific inhibition of PI3K-Akt and MEK-ERK1/2 signaling pathways after CT-1 stimulation was accomplished in our experimental conditions using Wortmannin and PD98095, respectively (top left). To inhibit STAT3 activation specifically, HA-tagged dnSTAT3 was overexpressed with the use of recombinant adenovirus vector. The vector containing the LacZ gene was used as a control for adenoviral vector infection (top right). After inhibiting each downstream signaling pathway, cells were infected with CVB3 in the presence or absence of CT-1 for 24 hours. The number of cells that remained on the plate after CVB3 infection was quantified and reported as a percentage cell survival in the wells not infected with CVB3 (bottom right). *P<0.01, dnSTAT3-overexpressing cells vs LacZ-expressing control cells in the presence of CT-1 and CVB3 infection.
markedly increases mortality after CVB3 infection. Careful analysis of both models, however, fails to demonstrate an increase in the extent of viral infection in the hearts of CVB3-infected mice. Thus, although it is clear that IFNs are important innate immune molecules for viral infection, our data with transgenic SOCS3 overexpression in the heart strongly indicate that IFNs do not have a significant effect on the early intracardiac myocyte innate immune response to coxsackieviral infection.

This then raises the question of what mechanism is responsible for the SOCS3-mediated increase in enteroviral susceptibility in the cardiac myocyte. Our data demonstrate that transgenic SOCS3 overexpression in the isolated adult cardiac myocyte inhibited gp130 signaling. In addition, infection of mice with cardiac-specific knockout of gp130 also demonstrated an increase in susceptibility to viral infection. gp130 signaling is generally not known to have a direct antiviral effect, an observation confirmed in our experiments in neonatal and adult myocytes. Therefore, we wondered what mechanism could be responsible for the increased susceptibility to viral infection in the absence of gp130 signaling in the heart.

We have previously shown that the enteroviral protease 2A is able to directly cleave the cytoskeletal protein dystrophin. Furthermore, we have demonstrated that the presence of intact dystrophin has an important role on susceptibility to entero viral infection. The data in this article demonstrate that compared with unstimulated infected cells, stimulation of gp130 signaling with CT-1 leads to an increase in myocyte survival and the continued presence of full-length dystrophin in CVB3-infected cardiac myocytes. The maintenance of full-length dystrophin in gp130-stimulated infected myocytes is supportive of a role for gp130 signaling in maintaining membrane integrity in the presence of viral infection and thus decreasing susceptibility. This is supported in the intact heart by the increase in disruption of sarcolemmal integrity in CVB3-infected hearts that lack the gp130 receptor. It is interesting that α-sarcoglycan expression level is decreased in both SOCS3 and gp130-knockout mice even without infection (Figure 6B). Although further experiments are needed, the decreased α-sarcoglycan might be involved in the increased susceptibility to CVB3 infection as observed in dystrophin-sarcoglycan–deficient mdx mice.

ERK and Akt signaling have been shown to have a role in the cytoprotective effect conferred by stimulation of gp130 signaling. However, the cytoprotective effect that occurs...
in CVB3-infected cells after gp130 stimulation is dependent on STAT3 signaling.

Although these data demonstrate the importance of gp130 signaling, it is possible that other non-IFN innate signaling mechanisms that can be inhibited by SOCS3 also are important. This is suggested by the finding that the increased susceptibility to CVB3 infection in the cardiomyocyte gp130-knockout mouse was not as dramatic as that seen in the SOCS3 Tg mice (Figure 4). Although our data in isolated myocytes were obtained using CT-1 to stimulate gp130 signaling, it possible that other gp130-activating cytokines such as interleukin-6 may play a role in the innate immune response against viral infection in the intact heart.

The evidence that innate IFN signaling in the cardiac myocyte has little effect on inhibiting the early stages of adult cardiac myocyte CVB3 infection raises the question of whether there are differences in IFN signaling in the adult cardiac myocyte compared with the other cell types frequently used to study IFN signaling. Our results and those of others indicate that this is the case. Although exogenous IFN-β stimulation of isolated adult myocytes confers a partial antiviral effect, transgenic overexpression of SOCS3 has no impact on this effect, indicating that the increased susceptibility to viral infection in SOCS3 Tg mice is independent of IFN-β signaling. IFN-γ had no direct antiviral effect on the isolated adult cardiac myocytes despite induction of STAT1 and STAT3 phosphorylation. Taken together with these data, it is clear that caution should be exercised when the typical IFN antiviral paradigm is applied to adult cardiac myocytes.

Finally, these data conclusively demonstrate the presence of a potent innate immune response within the cardiac myocyte that can be inhibited by SOCS3. Surprisingly, IFN signaling has little or no role in this innate immune response, whereas gp130 signaling makes an important contribution to the innate immune response during the early stages of coxsackieviral infection in the heart.

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Disclosures

None.

References


**CLINICAL PERSPECTIVE**

One of the several causes of heart failure is viral infection of the heart that leads to dilated cardiomyopathy. Although there are many unresolved clinical questions related to viral myocarditis, one that is frequently asked by patients and physicians is, “What are the factors that determine why most individuals infected with cardiomyopathic viruses such as coxsackievirus do not develop significant cardiac disease yet other individuals develop severe virus-mediated heart disease?” To address this question, it is necessary to understand the molecular mechanisms that can affect susceptibility to viral infection. Previously, considerable attention in regard to this issue has focused on the cellular immune response that occurs with myocarditis. However, the data in the present study demonstrate that there is an antiviral defense mechanism within the cardiac myocyte that can affect susceptibility to viral myocarditis. Circulating cytokines such as interleukin-6 can affect many aspects of the host immune response. Receptors for cytokines such as gp130, the receptor for interleukin-6, are expressed on the surface of cardiac myocytes. The present article demonstrates that disruption of cytokine signaling by overexpressing the suppressor of cytokine signaling-3, an inhibitor to the receptor for interleukin-6 in the heart, or by disruption of the interleukin-6 receptor in the cardiac myocyte increases susceptibility to viral infection. This demonstrates that alterations in this signaling cascade through genetic polymorphisms or changes in gene expression could affect a person’s risk of developing viral myocarditis. It may also help to identify new targets for therapy of virus-mediated heart disease.
Innate Defense Mechanism Against Virus Infection Within the Cardiac Myocyte Requiring gp130-STAT3 Signaling

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