Ubiquitin-Like Protein ISG15 (Interferon-Stimulated Gene of 15 kDa) in Host Defense Against Heart Failure in a Mouse Model of Virus-Induced Cardiomyopathy

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Background—Common causative agents in the development of inflammatory cardiomyopathy include cardiotropic viruses such as coxsackievirus B3 (CVB3). Here, we investigated the role of the ubiquitin-like modifier interferon-stimulated gene of 15 kDa (ISG15) in the pathogenesis of viral cardiomyopathy.

Methods and Results—In CVB3-infected mice, the absence of protein modification with ISG15 was accompanied by a profound exacerbation of myocarditis and by a significant increase in mortality and heart failure. We found that ISG15 in cardiomyocytes contributed significantly to the suppression of viral replication. In the absence of an intact ISG15 system, virus titers were markedly elevated by postinfection day 8, and viral RNA persisted in ISG15−/− mice at postinfection day 28. Ablation of the ISG15 protein modification system in CVB3 infection predisposed mice to long-term disease with deposition of collagen fibers, all leading to inflammatory cardiomyopathy. We found that ISG15 acts as part of the intrinsic immunity in cardiomyocytes and detected no significant effects of ISG15 modification on the cellular immune response. ISG15 modification of CVB3 2A protease counterbalanced CVB3-induced cleavage of the host cell eukaryotic initiation factor translation eIF4G in cardiomyocytes, thereby counterbalancing the shutdown of host cell translation in CVB3 infection. We demonstrate that ISG15 suppressed infectious virus yield in human cardiac myocytes and the induction of ISG15 in patients with viral cardiomyopathy.

Conclusions—The ISG15 conjugation system represents a critical innate response mechanism in cardiomyocytes to fight the battle against invading pathogens, limiting inflammatory cardiomyopathy, heart failure, and death. Interference with the ISG15 system might be a novel therapeutic approach in viral cardiomyopathy. (Circulation. 2014;130:1589-1600.)

Key Words: cardiomyopathy, dilated ■ immunology ■ infection ■ inflammation ■ interferon type I ■ ISG15 protein, human ■ models, animal ■ myocarditis ■ viruses

Myocarditis is a potentially serious and sometimes fatal disease. In developed countries, it commonly results from a virus infection, often by coxsackievirus B3 (CVB3).1,2 The disease can have long-term sequelae, including dilated cardiomyopathy in which progressive dilatation and dysfunction of heart chambers and tissue fibrosis lead to chronic heart failure.3,4 Inflammatory cardiomyopathy is both a histological and a functional diagnosis characterized by myocarditis in association with systolic cardiac dysfunction in dilated cardiomyopathy. Genetically predisposed individuals who fail to eliminate viral genomes in infected hearts4 are at risk for long-term inflammatory cardiomyopathy. Viral infection of the myocardium induces adverse autoimmunity directed against cardiac proteins.5 The innate immune system plays a key role in constraining the viral spread in viral infection. Consistent with several studies performed in the mouse model of CVB3-induced inflammatory cardiomyopathy, type I interferons (IFNs) are obvious candidates for this innate immune response.
response. Type I IFNs contribute to the suppression of viral titers and thereby ameliorate invasion of immune cells into the heart, contributing to improved survival in CVB3 infection.4–8 Type I IFN–dependent processes resulted in improved cardiac function during viral cardiomyopathy22 and ensured long-term survival in CVB3-positive dilated cardiomyopathy patients.10

Binding of type I IFNs to their cognate receptors results in the induction of IFN-stimulated gene of 15 kDa (ISG15), a small ubiquitin family protein consisting of 2 ubiquitin-like folds.11 ISG15 is involved in the struggle against pathogens,12–16 ISG15 modification, the process by which ISG15 is covalently attached to lysine residues of target proteins, is mediated through the sequential action of a type I IFN–induced E1-E2-E3 enzymatic cascade,17 involving the E1-activating enzyme Ube1L,18 E2-conjugating enzyme Ube2L6,19 and E3 ligases Herc5 and Herc620 in humans and mice, respectively. The isopeptidase USP18 specifically removes ISG15 from ISG15-modified substrates.21

Pursuing the aim to define host determinants that influence the pathogenesis of viral cardiomyopathy, we provide the first evidence for the impact of the intrinsic innate immune factor ISG15 in the myocardium. We found that ISG15 in cardiomyocytes contributed significantly to the suppression of viral replication and infectious virus yield in the heart. The absence of protein modification with ISG15 was accompanied by a profound exacerbation of myocarditis and by a significant increase in mortality and heart failure.

Methods

Mice and Virus Used

The CVB3 (cardiotropic Nancy strain) used in this study was prepared as previously described.21 Five- to 6-week-old mice (female and male at a ratio 1:1) were infected intraperitoneally with 1×10^9 plaque-forming units of CVB3 (USP18^G8A/A8A and respective wild-type [WT] control: 2×10^9 plaque-forming units of CVB3), C57Bl6/J mice were obtained from a stock breeding initially purchased from Jackson Laboratory. ISG15^−/− mice,22 Ube1L^−/− mice,23 and USP18^G8A/A8A transgenic mice were originally generated as described elsewhere. This study conforms to the Berlin State guidelines for animal welfare. The protocol was approved by the Committee on the Ethics of Animal Experiments of Berlin State authorities.

Endomyocardial Biopsies

Patients with clinical symptoms and signs of heart failure (New York Heart Association class II–III) underwent right ventricular endomyocardial biopsies after exclusion of coronary artery disease and valvular cardiomyopathy. Histological evidence for myocarditis is based on the Dallas criteria and immunohistochemistry. Inflammation in endomyocardial biopsies was considered positive if immunohistochemical detection revealed focal or diffuse mononuclear infiltrates of ≥14 CD3+ T lymphocytes or Mac1+ macrophages per mm², in addition to enhanced expression of HLA class II molecules.4 Control patients had the following: left ventricular ejection fraction >60%, left ventricular end-diastolic diameter ≤55 mm, and no inflammation. Patients with inflammatory cardiomyopathy had a left ventricular ejection fraction <60%, left ventricular end-diastolic diameter >55 mm, and no inflammation. Patients with dilated cardiomyopathy had left ventricular ejection fraction <60%, left ventricular end-diastolic diameter >55 mm, and no inflammation. The study protocol was set up in accordance with the ethics principles in the Declaration of Helsinki and was approved by the local ethics committee. Written informed consent was obtained from all patients.

Cell Culture and Plasmids

Primary mouse cardiomyocytes were isolated from embryonic mouse hearts.21 Human cardiac myocytes were purchased from ScienCell and cultured as described elsewhere.20 ISG15 was induced by IFN-β (100 U/mL) or by polyinosinic:polycytidylic acid (poly[I:C]) (50 μg/mL). For ISG15 modification assays, cDNA copies of HA-ISG15, Ube1L, UBE2L6, and Herc5 were transfected. For ISG15 rescue experiments, primary cardiomyocytes were transduced with AdV-pzS2 ISG15 at a multiplicity of infection of 25 for 48 hours.21 AdV-pzS2 lacking cDNA inserts served as control. For immune cell–based assays, splenocytes were obtained as described.24 For natural killer (NK) cell assays, the NK cell containing cell fraction was enriched by Ficoll density gradient centrifugation (Biocoll, Biochrom) and magnetic cell separation (MACS, Miltenyi).

Statistics

Results of continuous variables are expressed as mean±SEM. For 2-group comparisons, unpaired t tests were used. In cases of detection of unequal variances (F test), an unpaired t test with the Welch correction was applied. For multiple-group comparisons, unequal-variance versions of ANOVA (1-way or 2-way ANOVA) were performed, followed by a Tukey-Kramer multiple-comparison procedure. Survival curves were estimated from the Kaplan-Meier procedure with the log-rank (Mantel-Cox) test to compare survival among groups. The Fisher exact test was applied for analyzing differences between categorical data. The significance threshold was set at the 0.05 level. GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA; www.graphpad.com) was used for data analysis.

Results

CVB3-Induced Pathology Is Exacerbated in the Absence of ISG15

Differences in the genetic backgrounds in humans are mimicked by different inbred mouse strains. Transcriptomic profiling studies revealed an impaired induction of the ISG15 system in the A.BY/SnJ strain, which is susceptible to postviral inflammatory cardiomyopathy (Figure I in the online-only Data Supplement). The precise role of ISG15 was analyzed during CVB3 myocarditis in ISG15^−/− mice. At postinfection day 8, both unconjugated ISG15 and ISG15-modified protein substrates were upregulated in the hearts of WT controls (Figure 1A). Small areas of myocardial inflammation were detected in WT littermates, whereas ISG15^−/− mice developed large foci with cardiomyocyte death and infiltration of inflammatory cells (Figure 1B). Myocarditis scores (Figure 1C) and the areas of inflammation were increased in ISG15^−/− mice (Figure 1D). Mac-3+ macrophages were identified to be the most abundant invading cell type (Figure 1F). Immunohistology for CD3+ T lymphocytes revealed an equal abundance of CD3+ T lymphocytes in both ISG15^−/− mice and WT littermates (Figure 1E). In line with marked invasion of inflammatory cells to the site of myocardial infection, left ventricular function was deteriorated at postinfection day 8 in ISG15^−/− mice, as reflected by impaired cardiac contractility (dP/dmax) and relaxation (dP/dtmin), and decreased left ventricular end-systolic pressure compared with WT littermates (Table 1).

The invasion of immune cells is mediated by proinflammatory cytokines and chemokines. Cardiac mRNA expression profiling of proinflammatory cytokines (tumor necrosis factor-α, interleukin-6, and interleukin-1β), and chemokines (monocyte-attracting CCL3, CXCL10, and T cell/dendritic...
ISG15 protects against CVB3-induced heart failure and long-term inflammation

In agreement with impaired contractility at postinfection day 8 in CVB3-infected ISG15−/− mice, we observed increased lung

cell–attracting CXCL2) revealed increased expression levels for all these immune cell–attracting mediators in ISG15−/− mice during acute virus infection (Figure 1G).

ISG15 Acts via Modification of Target Proteins With ISG15

To discriminate whether the effects of ISG15 were attributed to unconjugated ISG15 or relied on ISG15 modification of target proteins, mice deficient in the ISG15 E1-activating enzyme Ube1L were investigated during CVB3 myocarditis. Unconjugated ISG15 was strongly induced in cardiac tissues during CVB3 myocarditis at postinfection day 8 (Figure 2A). The cardiac phenotype in Ube1L−/− mice during acute myocarditis resembled that observed in ISG15−/− mice (Figure 2B–2D). Increased myocarditis scores (Figure 2C) in Ube1L−/− mice demonstrated that the disease-attenuating effect of ISG15 during CVB3 myocarditis was attributed to ISG15 modification of target proteins. To evaluate whether increased ISG15-modification could be beneficial during CVB3 myocarditis, mice with inactivated catalytic activity of the ISG15-specific isopeptidase USP18 were studied on CVB3 infection. USP18C61A/C61A knock-in mice carry a mutation within the active site of the ISG15 deconjugating enzyme USP18 (described elsewhere). In cardiomyocytes obtained from these mice, we observed increased levels of ISG15-modified proteins on stimulation with the TLR3 agonist polyinosinic:polycytidylic acid (poly[I:C]) (Figure 2E). In CVB3-infected mice, we found increased levels of ISG15 conjugates in cardiac homogenates obtained from USP18C61A/C61A mice at postinfection day 8 (Figure 2E). This stabilization of ISG15-conjugated proteins in USP18C61A/C61A mice coincided with the detection of smaller myocarditis foci during CVB3 myocarditis (Figure 2F and 2G).

Lack of interferon-stimulated gene of 15 kDa (ISG15) results in exacerbation of coxsackievirus B3 (CVB3) myocarditis. ISG15−/− and ISG15+/+ mice in detail, immunohistology for CD3+ T lymphocytes (E) and Mac-3+ macrophages (F) was performed. Representative cardiac tissue sections are shown. Quantification of CD3+– and Mac-3+–positive signals was achieved by counting all CD3+ cells or Mac-3+ areas per visual field. Mac-3+ areas were related to the total AoI. Mac-3+ areas were related to the total AoI. Mac-3+–positive signals was achieved by counting all CD3+ cells or Mac-3+ areas per visual field. Mac-3+ areas were related to the total AoI. Mac-3+ areas were related to the total AoI. Mac-3+ areas were related to the total AoI.

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ISG15 Protects Against CVB3-Induced Heart Failure and Long-Term Inflammation

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Table 1. Analysis of Cardiac Function in CVB3 Cardiomyopathy

<table>
<thead>
<tr>
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<th>ISG15+/− Postinfection Day</th>
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<th>ISG15−/− Postinfection Day</th>
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<th>ISG15+/− Postinfection Day</th>
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<td></td>
<td>8 Control</td>
<td>8 CVB3</td>
<td>28 Control</td>
<td>28 CVB3</td>
<td>28 Control</td>
<td>28 CVB3</td>
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<td>Heart rate, bpm</td>
<td>497±30</td>
<td>492±18</td>
<td>384±19</td>
<td>413±36</td>
<td>525±25</td>
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<td>LV-Pmax, mm Hg</td>
<td>65.4±7.0</td>
<td>65.7±3.8</td>
<td>57.3±2.2</td>
<td>47.5±2.4*</td>
<td>72.7±2.4</td>
<td>69.5±3.6</td>
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<td>CO, µL/min</td>
<td>6808±2247</td>
<td>7655±1114</td>
<td>6647±1071</td>
<td>4287±505</td>
<td>8603±1049</td>
<td>6225±552</td>
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<tr>
<td>dP/dtmax, mm Hg/s</td>
<td>4492±1330</td>
<td>4077±478</td>
<td>3883±360</td>
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<td>5301±436</td>
<td>4081±400</td>
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<tr>
<td>dP/dtmin, mm Hg/s</td>
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<td>−2941±397</td>
<td>−2360±183</td>
<td>−1573±137</td>
<td>−3554±292</td>
<td>−3137±359</td>
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</tbody>
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Conductance catheter measurements were performed at the acute (postinfection day 8) and chronic (postinfection day 28) stages compared with sham-treated, age-matched controls (PBS). Postinfection day 8: 2-way ANOVA and Tukey multiple-comparison test were performed. All data are mean±SEM; n=6 mice per group. CVB3 indicates coxsackievirus B3; CO, cardiac output; dP/dtmax, contractility; dP/dtmin, relaxation; ISG15, interferon-stimulated gene of 15 kDa; and LV-Pmax, maximal left ventricular pressure.

*Significant differences between sham-treated and CVB3-infected ISG15−/− mice. No differences were detected between sham-treated ISG15+/+ and ISG15−/− mice. Thus, 1 group of sham-treated ISG15+/− mice was used as age-matched controls at postinfection day 28. One-way ANOVA and Tukey multiple-comparison tests were performed.

†Significant differences between CVB3-infected ISG15+/+ and CVB3-infected ISG15−/− mice.

and heart weights, which reflect congestive heart failure at this time point (Figure 3A). CVB3-induced death was observed in both ISG15+/− and Ube1L−/− mice between postinfection days 3 and 12 only (Figure 3B). In clear contrast, none of the WT littermates succumbed to CVB3 infection. Beyond ISG15-dependent amelioration of heart failure and improvement of survival, we addressed the question of whether ISG15 as part of cardiac innate immunity in CVB3 infection affects long-term disease progression. Therefore, ISG15+/− and Ube1L−/− mice were followed up to postinfection day 28, a time point at which inflammatory cardiomyopathy is observed in susceptible hosts such as A.BY/SnJ mice only and resistant mice...
such C57BL/6 reveal no signs of ongoing inflammation.29 At postinfection day 28, scattered residual lesions were detected in a small minority of WT littermates (Figure 3C), with mild signs of tissue fibrosis. In contrast, prominent inflammatory foci and large collagen-positive areas were evident in the majority of CVB3-infected ISG15−/− and Ube1L−/− mice at postinfection day 28 (Figure 3C and 3D and Figure IIB in the online-only Data Supplement). Analysis of left ventricular foci and large collagen-positive areas were evident in the majority of CVB3-infected ISG15−/− and Ube1L−/− mice at postinfection day 28, whereas CVB3 was not detected in any of the WT controls (Figure 4B). To provide evidence supporting the hypothesis that ISG15 represents an innate mechanism to fight viral infection in cardiomyocytes, viral replication was studied in primary embryonic cardiomyocytes. To discriminate ISG15-negative cells from ISG15-positive cardiomyocytes, ISG15 expression in ISG15−/− cardiomyocytes was restored via adenoviral gene transfer of ISG15. CVB3 infection of ISG15-positive cardiomyocytes resulted in drastically reduced viral copy numbers and viral titers compared with ISG15-negative cardiomyocytes (Figure 4E).

**ISG15 Modification Is Not Involved in the Activation of NK Cells and T Cells**

Immune surveillance in CVB3 myocarditis includes NK cells and T cells, both of which contribute to viral clearance in hearts.1 It was demonstrated only recently that ISG15 is involved in the activation of NK and T cells.12 Therefore, we addressed the function of ISG15 on NK- and T-cell immunity in CVB3 infection. NK cell function relies on immune surveillance in CVB3 myocarditis includes NK cells and T cells, both of which contribute to viral clearance in hearts.1 It was demonstrated only recently that ISG15 is involved in the activation of NK and T cells.12 Therefore, we addressed the function of ISG15 on NK- and T-cell immunity in CVB3 infection. NK cell function relies on activation of NK and T cells.12 Therefore, we addressed the function of ISG15 on NK- and T-cell immunity in CVB3 infection. NK cell function relies on activation and inhibitory receptors. Two of these receptors, the NK cell–activating receptor NKG2D and the inhibitory receptor killer lectin-receptor gene 1 (KLRG1), were monitored during acute CVB3 infection, and no effect of ISG15 was observed (Figure 5A and Figure IIA in the online-only Data Supplement). Activated NK cells induce granzyme- and perforin-mediated cytotoxicity. NK cytotoxicity assays demonstrated increased killing capacity of NK cells from
CVB3-infected mice compared with uninfected controls. NK cell–dependent cytotoxicity was independent of ISG15 (Figure 5B). NK cells are also the primary and very potent producers of IFN-γ. The increase in IFN-γ production by NK cells on CVB3 infection was found to be significantly impaired in ISG15−/− mice. With the induction of unconjugated ISG15 in Ube1L−/− mice on CVB3 infection, IFN-γ production by NK and T cells was efficiently induced, and no differences compared with WT controls were detected (Figure 5C). Among other functions, IFN-γ shapes the differentiation of naïve CD4+ T cells into T helper 1 effectors and stimulates CD8+ T-cell activation to control viral infection. Despite the fact that after ex vivo T-cell stimulation the IFN-γ production by ISG15−/− CD8+ and CD4+ T cells was impaired (Figure 5D), the absence of ISG15 did not affect the CD69 activation marker expression on T cells or the amount of antigen-experienced (CD137) CD4+ and CD8+ T cells (Figure 5E). Moreover, ISG15 had no impact on CD4+ T-cell differentiation (regulatory T-cell levels are shown in Figure 5F) or on antiviral humoral immunity determined by the amount of CVB3-specific IgG (Figure 5G).

ISG15 Interferes With CVB3 2A Protease Activity

Previously, it was reported that the ISG15 system is designed to target newly synthesized proteins. Therefore, viral proteins in particular represent principal targets of the ISG15 conjugation apparatus in CVB3-infected cardiomyocytes. Here, we focused on the role of ISG15 to modulate CVB3-protease 2A (2APro), a viral protease that interferes with host cell translation. 2APro-dependent cleavage of the mammalian eukaryotic translation initiation factor 4G (eIF4G) results in shutoff of host cell protein translation. First, we addressed the question of whether 2APro is a substrate for ISG15 conjugation. Immunoprecipitation and Ni-NTA pull-downs of His-tagged 2APro obtained from in vitro ISG15 conjugation assays demonstrated 2APro to be partially modified by ISG15 (Figure 6A and 6B). In addition, a 2APro K→R mutant was generated by site-directed mutagenesis whereby lysine residues that are needed for covalent ISG15 binding had been mutated to arginine. His-immunoprecipitation of this construct in ISG15 conjugation assays confirmed that 2APro is a target for ISG15 conjugation (Figure 6B, right).
Next, the question of whether ISG15 alters CVB3 2APro-dependent cleavage of eIF4G was addressed. HeLa cells as an established cell culture model of CVB3 infection were studied. ISG15 knockdown by siRNA increased the cleavage of eIF4G that was induced both by 2APro overexpression and on CVB3 infection (Figure 6C). Induction of the ISG15 machinery in HeLa cells reduced 2APro-dependent cleavage of eIF4G (Figure 6D). Further evidence for the key role of ISG15 modification in limiting proteolysis by CVB3 2APro was provided with 2APro K→R mutants. The failure to modify 2APro with ISG15 facilitated eIF4G cleavage in these mutants (Figure 6E).

We then investigated whether ISG15 as demonstrated in HeLa cells affects 2APro-dependent cleavage of eIF4G also in cardiomyocytes. eIF4G cleavage was reduced on induction of endogenous ISG15 in CVB3-infected primary embryonic cardiomyocytes (Figure 6F). In an alternative approach, ISG15 expression was restored in cardiomyocytes obtained from ISG15−/− mice by adenoviral gene transfer of ISG15 (AdV5-ISG15). Induction of the ISG15 apparatus resulted in reduced eIF4G cleavage in both CVB3-infected cardiomyocytes and 2APro-transfected cardiomyocytes (Figure 6G and 6H). Taken together, these data demonstrate that ISG15 modification of 2APro hinders the cleavage of eIF4G during CVB3 infection.

**ISG15 Controls Viral Titers in Human Cardiac Myocytes**

Next, we addressed the question of whether ISG15 may also affect viral replication in human cardiac myocytes. The ISG15 apparatus was induced in human cardiac myocytes by adenoviral gene transfer of ISG15 (Figure 7A and 7B). Infectious...
In this report, we demonstrated that the ISG15 conjugation system is a key intrinsic innate immune pathway in cardiomyocytes to fight the battle against invading viral pathogens. By suppressing viral titers in cardiomyocytes, ISG15 improves survival, protects against congestive heart failure, and prevents ongoing inflammation (Figure 8). Gene-targeting technologies were used to study the in vivo function of the ISG15 system in germline ISG15−/− and the Ube1L−/− mice. One potential limitation of this tool is the risk that phenotypes attributed primarily to the germline knocked out gene of interest could be attributed to tightly linked loci immediately flanking the ablated gene or unlinked loci located throughout the genetic background of the knockout strain. Application of 2 different germline knockout mouse models, namely virus yield was reduced in ISG15-expressing cardiomyocytes compared with controls (Figure 7C). This first demonstration of suppressed viral titers in ISG15-overexpressing primary human cells provoked us to study the ISG15 system in patients with viral cardiomyopathy. As depicted in Figure 7D and 7E, ISG15 was upregulated at both the mRNA and protein levels in patients with acute myocarditis and inflammatory cardiomyopathy.

**Discussion**

In this report, we demonstrated that the ISG15 conjugation system is a key intrinsic innate immune pathway in cardiomyocytes to fight the battle against invading viral pathogens. By suppressing viral titers in cardiomyocytes, ISG15 improves survival, protects against congestive heart failure, and prevents ongoing inflammation (Figure 8). Gene-targeting technologies were used to study the in vivo function of the ISG15 system in germline ISG15−/− and the Ube1L−/− mice. One potential limitation of this tool is the risk that phenotypes attributed primarily to the germline knocked out gene of interest could be attributed to tightly linked loci immediately flanking the ablated gene or unlinked loci located throughout the genetic background of the knockout strain.
ISG15−/− and Ube1L−/− mice with genetic ablation at different loci, ensured that the phenotype was in fact attributed to the function of the ISG15 system. Additional support came from the USP18<sup>C61A/C61A</sup> knock-in model and an adenoviral vector.

**Figure 7.** Interferon-stimulated gene of 15 kDa (ISG15) controls viral titers in human cardiac myocytes. **A,** Top, Immunofluorescence staining of primary human cardiac myocytes (first slide, anti–troponin I+GAR Alexa 488 [A488] merged with DAPI stain; second slide, negative control: GAR A488+DAPI stain). Flow cytometry analysis of primary human cardiac myocytes revealed 93% cardiac troponin I-positive cells (anti–troponin I+GAR A488). Negative control was GAR A488. **B,** Cardiac myocytes were transduced with adenovirus 5 (AdV5)–ISG15 or AdV5 lacking ISG15 cDNA at a multiplicity of infection of 25. **C,** After 48 hours, these cells were infected with coxsackievirus B3 (CVB3). Infectious virus yield was monitored by plaque assay. **D,** Endomyocardial biopsies (EMBs) were obtained from patients with acute viral myocarditis, inflammatory cardiomyopathy, and dilated cardiomyopathy. Patient characteristics are presented in Table 2. ISG15 expression was determined in EMBs by quantitative polymerase chain reaction after a preamplification step. **E,** Immunohistochemical detection of ISG15 (blue staining) in EMBs obtained from patients with acute myocarditis, inflammatory cardiomyopathy, and dilated cardiomyopathy (slides shown are representative of n=8 patients).

**Figure 8.** Protein conjugation to interferon-stimulated gene of 15 kDa (ISG15) attenuates viral cardiomyopathy. In coxsackievirus B3 (CVB3) infection, type I interferons (IFNs) induce the ISG15 system. Viral and potentially also host proteins become substrates of the ISG15 system in cardiomyocytes. ISG15 suppresses virus titers in cardiomyocytes and thus attenuates myocarditis, improves survival, protects against congestive heart failure, and prevents long-term inflammation in mice. Stabilization of ISG15 conjugation on enzymatic inactivation of de-ISGylating enzyme USP18 may provide a novel therapeutic approach in viral cardiomyopathy. 2APro indicates 2A protease.
A major initial response of mammalian cells to virus infection is the production of type I IFNs, which in turn induce the synthesis of a variety of antiviral effector systems. ISG15 is one of the most predominant among the several genes in 2 families with mendelian susceptibility to mycobacterial disease, the function of unconjugated ISG15 as a cytokine with the increased cytokine responses in ISG15−/− mice during infection, ISG15 deficiency contributed to elevated levels of proinflammatory cytokines, the latter being in agreement with the increased cytokine responses in ISG15−/− mice during CVB3 infection. Thus, ISG15 could contribute to the focal disease pattern in viral myocarditis.

ISG15 is known to interfere with various cellular pathways such as protein translation and signal transduction and thus regulates cytokines and chemokines such as CXCL-10. ISG15 counterbalanced the pathogenic cytokine response also in vivo in CVB3 myocarditis. This is particularly important because inflammation is related to poor outcome in patients with suspected myocarditis and persistent cardiac dysfunction. However, the molecular mechanisms of ISG15 action on the cytokine burden are not yet well defined. In Chikungunya virus, influenza virus B, and vaccinia virus infection, ISG15 deficiency contributed to elevated levels of proinflammatory cytokines, the latter being in agreement with the increased cytokine responses in ISG15−/− mice during CVB3 myocarditis described here. Because cytokine expression was increased in CVB3-infected Ube1L−/− mice also (data not shown), ISG15-dependent diminution of pathogenic cytokine levels in CVB3 myocarditis involved the process of ISGylation. Increased levels of tumor necrosis factor-α and interleukin-1β promote local tissue destruction and increase vascular permeability. Together with the chemokines CCL2, CXCL2, and CCL3, this cytokine milieu attracts both sensitized T cells and monocytes/macrophages. Therefore, detrimental effects even in remote uninfected areas of the myocardium occur, contributing to injury and remodeling of the heart, as observed in both CVB3-infected ISG15−/− and Ube1L−/− mice. An adverse inflammatory response was identified to be a requisite to progression to inflammatory cardiomyopathy in patients with viral myocarditis. Transition to sustained myocardial inflammation by persistence of viral genomes as observed in ISG15−/− mice propagates chronic inflammation and eventually leads to myocardial fibrosis in inflammatory cardiomyopathy. ISG15 suppressed infectious virus yield also in human cardiac myocytes. Therefore, defects within the ISG15 conjugation apparatus might also affect disease progression in patients with viral myocarditis.

The activation of innate immunity in CVB3 infection reflects a critical time point that determines the clinical outcome. If rescue of the ISG15 gene in primary cardiomyocytes that were obtained from ISG15−/− mice.

Once CVB3 interacts with the receptor on cardiomyocytes, the host immune system begins to counteract infection. A major initial response of mammalian cells to virus infection is the production of type I IFNs, which in turn induce the synthesis of a variety of antiviral effector systems. ISG15 is one of the most predominant among the several genes in 2 families with mendelian susceptibility to mycobacterial disease, the function of unconjugated ISG15 as a cytokine with the increased cytokine responses in ISG15−/− mice during infection, ISG15 deficiency contributed to elevated levels of proinflammatory cytokines, the latter being in agreement with the increased cytokine responses in ISG15−/− mice during CVB3 infection. Thus, ISG15 could contribute to the focal disease pattern in viral myocarditis.

ISG15 is known to interfere with various cellular pathways such as protein translation and signal transduction and thus regulates cytokines and chemokines such as CXCL-10. ISG15 counterbalanced the pathogenic cytokine response also in vivo in CVB3 myocarditis. This is particularly important because inflammation is related to poor outcome in patients with suspected myocarditis and persistent cardiac dysfunction. However, the molecular mechanisms of ISG15 action on the cytokine burden are not yet well defined. In Chikungunya virus, influenza virus B, and vaccinia virus infection, ISG15 deficiency contributed to elevated levels of proinflammatory cytokines, the latter being in agreement with the increased cytokine responses in ISG15−/− mice during CVB3 myocarditis described here. Because cytokine expression was increased in CVB3-infected Ube1L−/− mice also (data not shown), ISG15-dependent diminution of pathogenic cytokine levels in CVB3 myocarditis involved the process of ISGylation. Increased levels of tumor necrosis factor-α and interleukin-1β promote local tissue destruction and increase vascular permeability. Together with the chemokines CCL2, CXCL2, and CCL3, this cytokine milieu attracts both sensitized T cells and monocytes/macrophages. Therefore, detrimental effects even in remote uninfected areas of the myocardium occur, contributing to injury and remodeling of the heart, as observed in both CVB3-infected ISG15−/− and Ube1L−/− mice. An adverse inflammatory response was identified to be a requisite to progression to inflammatory cardiomyopathy in patients with viral myocarditis. Transition to sustained myocardial inflammation by persistence of viral genomes as observed in ISG15−/− mice propagates chronic inflammation and eventually leads to myocardial fibrosis in inflammatory cardiomyopathy. ISG15 suppressed infectious virus yield also in human cardiac myocytes. Therefore, defects within the ISG15 conjugation apparatus might also affect disease progression in patients with viral myocarditis.

The activation of innate immunity in CVB3 infection reflects a critical time point that determines the clinical outcome. If rescue of the ISG15 gene in primary cardiomyocytes that were obtained from ISG15−/− mice.
the initial immune response is ineffective, as shown here in ISG15-incompetent mice, viral genomes persist within the myocardium. Thus, proinflammatory and profibrotic cytokine release resulting from improper termination of the immune response facilitates cardiac remodeling, leading to long-term disease and decompensation in inflammatory cardiomyopathy. ISG15 modification at the critical stage of acute viral replication suppresses viral replication and ensures effective elimination of CVB3 from the heart (Figure 8).

Conclusions

The ISG15 conjugation system represents a critical innate response mechanism in cardiomyocytes to fight the battle against invading pathogens that initiate the fatal pathway to chronic viral cardiomyopathy. Further research is needed to evaluate the therapeutic potential to interfere with the ISG15 apparatus in viral heart disease.

Acknowledgments

We acknowledge the superb technical assistance of Nadine Albrecht, Karolin Voß, Xiaomin Wang, and Sandra Bundschuh. Dong-Ér Zhang kindly provided Ube1L-deficient mice, Ube1L, and Ube2L6 plasmid DNA. We appreciate the support of Nicole Lange and Ulrike Kuckelkorn (Institut für Biochemie, Charité Universitätsmedizin Berlin), Carmen Scheibenbogen and Madlen Löbel (Institut für Immunologie, Charité Universitätsmedizin Berlin), and Philippe Saikalai (DRFZ Berlin).

Sources of Funding

This study was supported by the Deutsche Forschungsgemeinschaft: DFG SPP1365 (VO 1602/1-1 to Dr Voigt and KN590/3-2 to Dr Kuckelkorn), DFG VO 1602/5-1 (Dr Voigt), and DFG SFBTR 19 (B3 to Dr Voigt, Z3 to Dr Tschöpe, Z4 to Dr Klingel); by a research grant to Dr Voigt, Z3 to Dr Tschöpe, Z4 to Dr Klingel); by a research grant from the Sonnenfeld Stiftung (Dr Voigt). ISG15-incompetent mice, viral genomes persist within the myocardium of adults with “idiopathic” left ventricular dysfunction. Circulation, 2005;111:879–893.

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Acute coxsackievirus B3 infection is one of the most common causes of acute myocarditis, a serious disease with a potential fatal outcome. The disease can have long-term sequelae, including inflammatory cardiomyopathy, in which the left ventricle decompensates, with resulting heart failure. To optimize treatment, it is vital to identify targets that control viral infection. Here, we show that the type I interferon induced interferon-stimulated gene of 15 kDa (ISG15) system acts in cardiomyocytes, limiting viral replication, myocarditis, death, and the development of inflammatory cardiomyopathy. Interference with this protein modification system might be a novel therapeutic approach in patients with virus-induced inflammatory cardiomyopathy.
Ubiquitin-Like Protein ISG15 (Interferon-Stimulated Gene of 15 kDa) in Host Defense Against Heart Failure in a Mouse Model of Virus-Induced Cardiomyopathy
Anna Rahnefeld, Karin Klingel, Anett Schuermann, Nicola L. Diny, Nadine Althof, Anika Lindner, Philipp Bleienheuft, Konstantinos Savvatis, Dorota Respondek, Elisa Opitz, Lars Ketscher, Martina Sauter, Ulrike Seifert, Carsten Tschope, Wolfgang Poller, Klaus-Peter Knobeloch and Antje Voigt

*Circulation.* 2014;130:1589-1600; originally published online August 27, 2014;
doi: 10.1161/CIRCULATIONAHA.114.009847
*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/130/18/1589

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2014/08/27/CIRCULATIONAHA.114.009847.DC1
SUPPLEMENTAL MATERIAL
Supplemental material

Expanded Methods

Ethics Statement

This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the German animal welfare act, which is based on the directive of the European parliament and of the council on the protection of animals used for scientific purposes. This study conforms to the Berlin State guidelines for animal welfare. The protocol was approved by the Committee on the Ethics of Animal Experiments of Berlin State authorities (Permit Number: G0311/06; G0269/08, H0076/08 and G0279/11). All efforts were made to minimize suffering. For diagnosis of human specimen, informed written consent was obtained from each patient. The study protocol complied with the Declaration of Helsinki.

Mice and virus used

All mice were backcrossed with C57BL6/J mice for 10 generations with the exception that for USP18\textsuperscript{C61A/C61A} transgenic mice speed congenics were performed (6 generations). Littermates were applied throughout the study for the analysis of cardiac function in myocarditis. All mice were kept at the animal facilities of the Charité University Medical Center.

Cell culture and plasmids

Primary cardiomyocytes were isolated from embryonic mouse hearts yielding > 93 % troponin I-positive cells (E13); these cells and other immortalized cell lines were cultured as described elsewhere\textsuperscript{1}. ISG15 in Hela cells was induced by 100U/ml IFN-β (Roche) for 24h-48h. Exogenous ISG15-modification was induced upon transfection of pcDNA3.1-HA-ISG15 (human), pcDNA3.1-\textit{h}UBE1L, pcDNA3.1-mouse (m) UBE2L6 and pTriEx2-\textit{h}Herc5 using X-treme Gene HP (Roche). CVB3 cDNA encoding the proteases 3C and 2A were cloned into pcDNA3.1-\textit{h}is\textsubscript{6}-V5 and transfected in the same manner. Human ISG15 siRNA (L-004235-00-0005; Dharmacon) was transfected into Hela cells with Lipofectamine\textsuperscript{®} RNAiMAX Transfection Reagent (Invitrogen) in OptiMEM. D0018101005 (Dharmacon) served as off-
target control. Cardiomyocytes isolated from ISG15−/− mice were transduced with AdV-pzS2-mISG15 at MOI 25 for 48h according to2. AdV-pzS2 lacking any inserted cDNA fragment served as a control. For all immune cell-based analyses, splenocytes were obtained as described elsewhere3. For NK cell assays, the NK cell containing cell fraction was enriched by ficoll density gradient centrifugation (Biocoll, Biochrom). Nkp46+ NK cells (NK cell kit to generate untouched NK cells, purity 80-90%) were isolated from splenic single cell suspensions using MACS-sorting (Miltenyi Biotec, Germany).

NK cell cytotoxicity assay

NK cell cytotoxicity was addressed in co-culture experiments with 1×104 DiOC18 (0.3 mM) -labeled YAC-1 cells. Purified NK cells from naive and CVB3-infected mice (d3 p.i.) were added at the indicated E:T ratios for three hours. Cells were then fixed with PBS / 2 % PFA (20 min) and LIVE/DEAD® Fixable Far Red Dead Cell staining (Invitrogen) was performed prior to FACS analysis.

Flow cytometric analysis

Cells were stained with combinations of the following antibodies in PBS / 0.5 % BSA: CD11c (APC), CD80 (FITC), CD86 (FITC), CD40 (PE), CCR7 (PE), CD3 (PE), CD8 (PB), CD4 (APC), CD137 (PE), CD69 (FITC), KLRG-1 (APC), CD4 (PE), CD25 (PE), NKp46 (PerCP Cy5.5), NKG2D (PE), purchased from BD Biosciences, Miltenyi Biotech (Germany), Biolegend and eBioscience, respectively. For intracellular cytokine staining experiments, T cells were cultured with PMA (25 ng/ml) / ionomycin (1 µg/ml). For NK cells assays, mIL-2 (50 U/ml) was added. Cytokine secretion was blocked with brefeldin A (10 µg/ml) for 4 h. Upon staining of cell surface markers, fixation was achieved with PBS / 2 % PFA (20 min). Cells were permeabilized using PBS /1 % BSA / 0.5 % Saponin and then stained with anti-IFN-γ (PE) (Miltenyi Biotech). FoxP3 (FITC) staining was performed with Foxp3 transcription factor staining buffer from eBioscience. Samples were analyzed by a CYAN-
ADP flow cytometer (Beckman Coulter, Germany) or a BD FACSCalibur flow cytometer using Summit4.3 and FlowJo7 software.

**Quantitative real time RT-PCR**

RNA preparation and cDNA synthesis were performed as described recently \(^1\). TaqMan PCR was performed using primers and probes of TaqMan® Gene Expression Assays (Applied Biosystems, Germany). mRNA expression was normalized to the housekeeping gene HPRT by means of the ΔCt method.

**In situ hybridization and histological staining**

*In situ* hybridization of genomic CVB3 RNA, histological staining with hematoxylin / eosin (HE) and Sirius red as well as immunohistochemistry for the detection of CD3\(^+\) T lymphocytes and Mac-3\(^+\) macrophages were carried out as described \(^4\), \(^5\). The area of inflammation (AoI) for each mouse was determined by averaging the inflammation-positive heart tissue \([\mu m^2]\) for each sample from 10 different individual pictures obtained from one slide at 100x magnification. CD3-positive T cells were counted in 10 different slides from each mouse. Absolute numbers were averaged. For Mac-3, marker-positive areas were calculated. To detect human ISG15 formaldehyde-fixed 5 mm paraffin sections from human endomyocardial biopsies were deparaffinized and after heat-induced epitope retrieval incubated with primary rabbit polyclonal ISG15 antibody (1:1000, lab stock) for 1hr at room temperature followed by incubation with biotinylated goat anti-rabbit IgG (Vector, Burlingame, USA). As a negative control, species-matched IgG were used in place of the primary antibody. Slides were processed using the Vectastain Elite ABC-Kit (Vector, Burlingame, USA) and HistoGreen (Linaris, Dossenheim, Germany) as substrate and counterstained with hematoxylin. Slides were viewed with a Zeiss Axioskop 40 microscope.

**Plaque Assay**

Plaque assays were performed on subconfluent green monkey kidney cell monolayers as described elsewhere \(^6\) and virus titers were calculated for each sample.
Immunoblot analysis / Ni-NTA pull down / Immunoprecipitation

Cell or tissue lysis was performed in 20 mM TRIS-HCl, pH 7.5, 10 mM EDTA, 100 mM NaCl, 1% NP40, 10 µM MG132, 5 mM NEM, Complete protease inhibitor cocktail (Roche, Germany). Homogenates were obtained as previously described7. Immunoblot analysis was performed according to standard protocols. Primary antibodies: ISG15 (lab stock), GAPDH (Santa Cruz), tubulin (GeneTex), β-actin (Millipore), meIF4G (Sigma Aldrich), Heif4G (antibodies online), HA (cell signaling), V5 (Invitrogen). ISG15-modification of 2APro: Hela cell extracts were subjected to Ni-NTA Magnetic Agarose Beads based pull-down assays of His-tagged proteins. Cells were lysed in RIPA Buffer (20mM Tris-HCl, 10mM EDTA, 100mM NaCl, 0.1% SDS, 1% NP40) and the lysate was diluted 1:10 in 8 M Urea, 0.1 M NaH2PO4, 0.01 M TrisHCl, 0.05% Tween 20, pH 8.0) and incubated with Ni-NTA-agarose (Qiagen) for four hours at 4°C. His6-tagged samples were eluted from Ni-NTA-agarose by heating in SDS-loading buffer for 5 minutes. Samples were subsequently subjected to SDS-PAGE.

Surgical Procedures

Mice were anesthetized, intubated, and artificially ventilated with a rodent ventilator type 7025 (Ugo Basile, Comerio VA, Italy). Heart rate, maximal left ventricular (LV) pressure (LV-Pmax), LV contractility (dP/dtmax), LV relaxation (dP/dtmin) and LV end diastolic pressure (LVEDP) were recorded via a microconductance catheter (1.2F) system in open-chest animals. After euthanization of mice, LV tissues were excised, immediately snap frozen in liquid nitrogen and stored at -80°C for further analysis.

Site-directed mutagenesis

Lysin-deficient mutants of pcDNA3.1-2APro were generated by subsequent site-directed mutation, thereby substituting lysine to arginine (K→R). PCR were performed with NEB Q5 High Fidelity DNA polymerase using pcDNA3.1-2APro as a template. Step-wise K→R-substitution was achieved using the following primers:
K149R (vector backbone): 5’-GCAATGGAACAGAgGGGCAATTCTGCAG-3’ (Fw), 5’-CTGCAGAATTGCCcTCTGGTCCATTGC-3’ (Rev);
K172R (V5-Tag): 5’- GTTCAAGGTAaGCCTATCCCTAACC-3’ (Fw), 5’-GGTTAGGGATAGGcTACCTTCGAAC-3’ (Rev);
K66R (2APro): 5’- GTACTTTTGTGCCTCCAaAACAAGCCTACCC-3’ (Fw), 5’-GGGTAGTGCTTTGTTCGGACGCACAAAGTAC-3’ (Rev);
K66R-K68R (2APro): 5’- CGGTCCAGAACAaGCACTACCCAAATTAC-3’ (Fw), 5’-CGAAATTGGGTAGTGcGTCTCTGGACGC-3’ (Rev).
Template plasmid-DNA was eliminated by incubation with DpnI.
Supplemental Figure 1

- ISG15
- USP18
- Ube1L
- Ube2L6
- Herc5

* indicates statistical significance.
Supplemental Figure 3
Expanded results and Additional figures

Supplemental Figure 1: Regulation of ISG15-machinery in the murine model of CVB3-cardiomyopathy

(A) mRNA expression of ISG15, UBE1L, UBE2L6, Herc6 and USP18 was monitored by quantitative real-time PCR (n=4 mice) in cardiac homogenates obtained from CVB3-infected C57BL/6 and A.BY/SnJ mice. Maximum mRNA expression levels were detected within the time course of infection as depicted here at 36h p.i.

(B) Representative immunoblots from pooled homogenates obtained from n=4 mice are shown indicating ISG15-modification in the infected heart at the indicated time points. α-actin indicates equal protein loading.

Supplemental Figure 2: Natural killer (NK) cell and dendritic cell (DC) maturation are independent of ISG15 in CVB3-myocarditis

NK cell and DC maturation were assessed at d3 p.i. (A) Maturation of NK cells in CVB3 infection. Immature NK46⁺ NK cells are CD11b⁻/CD27⁻. Premature NK cells: CD11b⁻/CD27⁺; mature NK cells: CD11b⁺/CD27⁺; terminally differentiated NK cells: CD11b⁺/CD27⁺. No strain differences were detected. (B) Representative NK cell sort shows approximately 80% NKp46⁺ NK cells. For NK cell cytotoxicity assay DiOC₁₈-labeled YAC-1 cells were gated and the percentage of Far Red positive YAC-1 cells was determined as shown here representatively. (C)/(D) Maturation and activation of CD₁₁c⁺ DCs in CVB3-infection. (C) The expression of co-stimulatory molecules and CCR7 on DCs was determined in splenic DCs by flow cytometry. MFI of respective cell surface markers gated on CD₁₁c⁺ DCs; n=4 mice, results are representative for 3 independent experiments. (D) The activation of DCs as represented by the expression of cytokines (here IL-6, IFN-β, IL-12 and MCP-1 are shown) was determined by quantitative PCR in CD₁₁c⁺ DCs. n=3 pools from 2 different mice each; the results are representative for two independent experiments.
Supplemental Figure 3: Recruitment of inflammatory cells depends on ISG15-modification

(A) UBE1L^{+/+} and UBE1L^{-/-} mice were infected with CVB3 1x10^{5} PFU; mice were sacrificed at d8 p.i. (wildtype littermates served as controls). Immunohistology for CD3^{+} T lymphocytes and Mac-3^{+} macrophages was performed (n=6 mice/group). Representative cardiac tissue sections are shown. Quantification of immunohistochemically positive signals was achieved by counting all CD3^{+} cells / Mac-3^{+} areas per visual field at a magnification of 100x in transverse heart tissue sections (refer to method section; n=6 mice, results obtained from 10 independent pictures were averaged for each individual mouse). Absolute CD3^{+} T cell numbers are shown; Mac3^{+} areas were related to the area of inflammation (AoI) as shown in figure 1E/F. (B) CVB3-infected UBE1L^{-/-} and controls were sacrificed at d28 p.i. Representative HE-stainings and myocarditis scores are shown.