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Spontaneous activation of a MAVS-dependent antiviral signaling pathway determines high basal interferon-β expression in cardiac myocytes

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ABSTRACT

Viral myocarditis is a leading cause of sudden death in young adults as the limited turnover of cardiac myocytes renders the heart particularly vulnerable to viral damage. Viruses induce an antiviral type I interferon (IFN-α/β) response in essentially all cell types, providing an immediate innate protection. Cardiac myocytes express high basal levels of IFN-β to help pre-arm them against viral infections, however the mechanism underlying this expression remains unclear. Using primary cultures of murine cardiac and skeletal muscle cells, we demonstrate here that the mitochondrial antiviral signaling (MAVS) pathway is spontaneously activated in unstimulated cardiac myocytes but not cardiac fibroblasts or skeletal muscle cells. Results suggest that MAVS association with the mitochondrial-associated ER membranes (MAM) is a determinant of high basal IFN-β expression, and demonstrate that MAVS is essential for spontaneous high basal expression of IFN-β in cardiac myocytes and the heart. Together, results provide the first mechanism for spontaneous high expression of the antiviral cytokine IFN-β in a poorly replenished and essential cell type.

1. Introduction

Viral myocarditis is recognized as the second leading cause of sudden death in young adults [25,29] and is found in approximately 10% of patients with unexplained heart failure [7]. Although most cases resolve spontaneously, viral myocarditis can progress to dilated cardiomyopathy and cardiac failure [16,29]; the latter representing one of the major causes of morbidity and mortality worldwide [11]. The unimpeded cardiac access afforded to viruses during viremia as well as the severely limited capacity of cardiac myocytes to enter the mitotic cycle [92] contribute to the particular vulnerability of the heart to viruses.

Type I interferon (IFN-α/β) treatment can be an effective therapeutic for human myocarditis [18,39,62,76], and defects in the host or cardiac myocyte IFN-α/β response aggravate virus-induced damage in mouse models of myocarditis and in primary cultures of cardiac myocytes [1,40,41,45,85]. While viral myocarditis can reflect immune-mediated damage to cardiac myocytes, most myocarditic viruses, including reovirus, induce direct cytopathic effects in these cells [7,29] and can induce myocarditis in the absence of the adaptive immune response [5,82–84]. For reovirus, the primary determinant of protection against virus-induced cardiac damage is the host IFN-α/β response [5,40,45,54,64,81,82,85,112]. Reovirus-induced cytopathic effect in primary cultures of murine cardiac myocytes accurately recapitulates both reovirus strain-specific differences in induction of myocarditis and the critical role for IFN-α/β [5,45,85]. We have previously shown that cardiac myocytes express higher basal levels of IFN-α/β than cardiac fibroblasts do, whereas the latter are more responsive to IFN-α/β signaling [54,87,111]. This integrated signaling network helps to pre-arm poorly replenishable cardiac myocytes and amplify antiviral signaling in cardiac fibroblasts to eliminate the viral infection [111]. However, the mechanism by which cardiac myocytes express high basal levels of IFN-β has not been elucidated.

Viral induction of IFN-α/β expression is initiated when a stimulus such as viral RNA is recognized by its sensors, in this case retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs), including RIG-I itself and melanoma differentiation-associated gene 5 (MDA5) [48,49,105]. RLRs then translocate from the cytosol to intracellular membranes to interact with the mitochondrial antiviral signaling (MAVS; also known as IPS-1,

Abbreviations: ER, endoplasmic reticulum; IFN, interferon; IKK, IκB kinase; ISG, interferon-stimulated gene; MAM, mitochondrial-associated ER membranes; MAVS, mitochondrial antiviral signaling; MDA5, melanoma differentiation-associated gene 5; MFN2, mitofusin2; MOI, multiplicity of infection; PFU, plaque-forming unit; PLA, proximity ligation assay; RIG-I, retinoic acid inducible gene-I; RLR, RIG-I-like receptors; SR, sarcoplasmic reticulum; TBK1, TANK-binding kinase 1; TRAF3, tumor necrosis factor (TNF) receptor-associated factor 3; TNF, tumor necrosis factor

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Cardif, and VISA) adapter protein and promote MAVS oligomerization [42,44,78,101,102]. MAVS localizes primarily to the outer mitochondrial membrane [78], but also to peroxisomes [22] and to specialized domains of the endoplasmic reticulum (ER) that are in constant communication with the mitochondria [42]. These mitochondrial-associated ER membranes (MAM) are an important site for induction of IFN-α/β expression and inflammatory signaling [42,43,110]. When components of the MAVS pathway translocate to the MAM they induce IFN-α/β expression [42,43,55]. In contrast, peroxisomal MAVS plays a role in induction of IFN-λ expression in mucosal and epithelial cells [6,21,30,42,94]. Interactions of MAVS at the MAM activates the E3 ubiquitin ligase tumor necrosis factor (TNF) receptor-associated factor 3 (TRAF3) [38,65,91], which activates TANK-binding kinase 1 (TBK1) [32,37,79] and members of the IκB kinase (IKK) family [71,103,107]. These kinases then phosphorylate and activate the transcription factors IRF3, ATF-2/c-Jun and NF-κB; all of which are required for maximal IFN-α/β expression [57,97]. Secreted IFN-α/β then binds its receptor and induces expression of IFN-stimulated genes (ISGs), most of which have direct antiviral properties [20,75].

The identification of MAVS as a critical mitochondrial adapter for IFN-β expression introduced mitochondria as essential components in the IFN-β response [2,61,78,102]. While MAVS is ubiquitously expressed, its expression is much higher in cardiac and skeletal muscle compared to other organs and tissues [102], suggesting cells with abundant mitochondria might have an enhanced IFN-β response. Indeed, ectopic expression of MAVS in other cell types induces IFN-β expression through spontaneous activation of IRF3 and NF-κB [61,78,102]. We therefore hypothesized that differences in MAVS abundance and localization in cardiac myocytes might drive spontaneous downstream signaling and expression of IFN-β in the absence of viral infection.

Here, we used primary cultures of murine cardiac and skeletal muscle cells to identify differences between skeletal and cardiac muscle cells, and to identify the mechanism for high basal IFN-β expression in cardiac myocytes. First, we found that skeletal muscle cells do not express high basal levels of IFN-β, indicating that this is not characteristic of muscle cells in general. Remarkably, in unstimulated cardiac myocytes but not cardiac fibroblasts or skeletal muscle cells, MAVS is spontaneously activated to associate with the MAM resulting in activation of TRAF3 and TBK1. Accordingly, MAVS expression is required for high basal IFN-β expression in cardiac myocytes and in the adult heart. Together, results provide the first mechanism for spontaneous high expression of the antiviral cytokine IFN-β in a poorly replenished and essential cell type, and highlight a novel cell type-specific mitochondrial role in antiviral protection.

2. Materials and methods

2.1. Primary cardiac cell cultures

Timed-pregnant Cr:NIH(S) mice from the National Cancer Institute were maintained as a colony in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Wild-type C57BL/6, MAVS$^{-/-}$ [88], and RIG-I$^{-/-}$MDA5$^{-/-}$ [27] mice were maintained as an in-house colony and used for timed matings. The hearts of MAVS$^{-/-}$ mice were confirmed to be histologically and functionally normal (Fig. S1). All animal procedures were approved by the North Carolina State University Institutional Animal Care and Use Committee (IACUC). Primary cardiac cell cultures were generated as previously described [80] from 1-day-old neonatal and term fetal mice resulting from timed pregnancies. For RNA or protein harvest, cardiac myocyte and fibroblast cultures in Dulbecco MEM (DMEM; Gibco #11965-092) supplemented to contain 7% fetal calf serum (FCS; Atlanta Biologicals) and 10 μg/ml gentamycin (Sigma, #G1272) were plated at 1×10^6 or 5×10^5 cells per well in 24-well or 48-well clusters, respectively. For confocal microscopy, cardiac myocyte or cardiac fibroblast cultures were plated at 3.5×10^5 or 1.75×10^5 cells per chamber in 8-well poly-D-lysine-coated chamber slides (Corning), respectively. Cardiac myocyte cultures were also supplemented to contain 0.06% thymidine (to inhibit cardiac fibroblast growth). Cardiac myocyte and cardiac fibroblast cultures were ≥ 90% and ≥ 95% pure, respectively, as estimated by immunostaining against sarcomeric actin (alpha Sr-1) and vimentin, respectively (data not shown). In addition, RT-qPCR results (Fig. S2) confirmed that, as expected, cardiac fibroblast cultures expressed high levels of vimentin [9,53] and DDR2 [35,53,109] but expressed lower levels of α-myosin heavy chain than did cardiac myocyte cultures and lower levels of CD31 (a.k.a. PECAM-1) and VE-Cadherin (a.k.a. CDH5, CD144) than did vascular endothelial cells. For all experiments, cultures were incubated at 37 °C in 5% CO_2 for two days post-seeding before use, and were never passaged.

2.2. Primary skeletal muscle cultures

Timed-pregnant mice and mouse colonies for timed matings were maintained as above, and all procedures were IACUC-approved. Muscle from the limbs of neonatal mice < 24 h old was dissected away from other tissues into Hanks' balanced salt solution (HBSS; Corning, #21-023-CV), the HBSS was aspirated, and the muscle was minced with sterile scissors and incubated in freshly prepared 2% Type II collagenase (Worthington Biochemical, #LS004174) in HBSS for 30 min with vortexing every 10 min. Cells were pelleted by centrifugation at 1800 × *g* for 5 min at room temperature, resuspended in HBSS, and re-pelleted as above. Cells were resuspended in DMEM supplemented to contain 6% FCS, 2 mM L-glutamine (Corning #25-005-Cl), and 10 μg/ml gentamycin, and passed through a 100 μm cell strainer (Falcon, #352360). Cells resuspended to 4×10^5/ml in supplemented DMEM were plated at 8×10^5 cells per well in 24-well clusters for RNA or protein harvest, or 2.8×10^5 cells per chamber in 8-well poly-D-lysine-coated chamber slides for confocal microscopy. After incubation at 37 °C in 5% CO_2 for two days, media was replaced with DMEM supplemented as above (for undifferentiated skeletal muscle cell cultures) or DMEM supplemented as above but at 3% instead of 6% FCS (for differentiated skeletal muscle cell cultures). Cultures were incubated an additional two days before use to allow differentiation, and were never passaged.

2.3. Viral infections

CsCl-purified reovirus type 3 Dearing (T3D) was maintained as a low-passage laboratory stock and stored at − 80 °C. T3D was chosen as a strong inducer of IFN-α/β expression in cardiac cells [54,85,87] through the RIG-I/MAVS pathway [36,41]. For infections, cardiac cultures plated in 8-well chamber slides were inoculated with reovirus T3D at a multiplicity of infection (MOI) of 25 plaque forming units (PFU) per cell in 200 μl of supplemented DMEM. An additional 300 μl of supplemented DMEM was added after 1 h of incubation at 37 °C, and cells were fixed at 24 h post-infection. Mock-infected cultures were treated similarly but received supplemented DMEM as inocula.

2.4. Stimulation with Poly(I:C)

Cardiac fibroblasts were stimulated with 25 μg/ml Poly(I:C) (Invivogen; #tlrl-pic) using Lipofectamine 2000 (Thermo Fisher Scientific; #11668027) for a total of 4 h according to the manufacturer's instructions.

2.5. Antibodies

Antibodies used for immunoblotting and their corresponding dilutions were anti-MAVS (Santa Cruz Biotech sc-365334; 1:500), anti-TOM20 (Santa Cruz Biotech sc-11415; 1:300), anti-alpha Sr-1 (Abcam ab28052; 1:1500), anti-β-actin (Santa Cruz Biotech sc-1615-hrp,