Arrhythmogenic Mechanisms of Acute Cardiac Infection

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Translational Biology, Medicine, and Health

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March 11th, 2022
Roanoke, VA

Keywords: adenovirus, arrhythmias, Connexin43, gap junctions, ion channels

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Abstract

Cardiovascular disease is the leading cause of death world-wide, with 42% of sudden cardiac death in young adults caused by myocarditis. Viruses represent the main cause of myocarditis, with adenovirus being a leading pathogen. However, it is not understood how adenoviruses cause sudden cardiac arrest. Myocarditis is defined by two phases, acute and chronic. The acute phase involves viral-mediated remodeling of subcellular structures in the myocardium, which is thought to contribute to arrhythmogenesis. The chronic phase is immune response-mediated, where the host immune system causes damage that induces gross remodeling of the heart, which can result in cardiac arrest or heart failure. Electrical impulses of the heart are propagated by cardiomyocytes, via gap junctions, ion channels, and intracellular junctions, creating the healthy heartbeat. Cx43, the primary gap junction protein in the myocardium, not only propagates electrical signals, but also anti-viral molecules. Viral targeting of gap junction function leads to reduced anti-viral responses in neighboring cells. However, reduced cellular communication would dangerously alter cardiac conduction. Using a cardiotropic adenovirus, MAdV-3, we find that viral genomes are significantly enriched in the heart, with a decrease of gap junction and ion channel mRNA in infected hearts, however, their protein levels were unchanged. Phosphorylation of Cx43 at serine 368, known to reduce gap junction open probability, was increased in infected hearts. *Ex vivo* optical mapping illustrated decreased conduction velocity in the infected heart and patch clamping of isolated cardiomyocytes revealed prolonged action potential duration, along with decreased potassium current density during infection. Pairing mouse work with human induced pluripotent stem cell-derived cardiomyocytes, we found that human adenovirus type-5 infection increased pCx43-Ser368 and perturbation of intercellular coupling, as we observed with *in vivo* MAdV-3 infection. Allowing adenovirus infection to progress *in vivo*, we find myocardium remodeling and immune cell infiltration. Together, these data demonstrate the complexity of cardiac infection from viral-infection induced subcellular alterations in electrophysiology to immune-mediated cardiomyopathy of cardiac adenoviral infection. Our data describe virally induced mechanisms of arrhythmogenesis, which could lead to the development of new diagnostic tools and therapies, to help protect patients from arrhythmia following infection.

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General Audience Abstract

Viral infection has long thought to be a cause of unexplained sudden cardiac death, especially in young adults. Viruses have been identified to cause many cases of deleterious remodeling of the heart, which can result in heart failure. The heart relies on electrical signaling that moves in a coordinated fashion to contract and pump blood throughout the body. The cells within the heart that do this are called cardiomyocytes, and they join end-to-end to communicate with each other via gap junctions. Gap junctions are tunnels that allow for ions that create electrical impulses to pass, and molecules, such as ones that are important in immune responses to infection. In addition to gap junctions in the heart, ion channels, which are highly selective to allow only one ion flow, unlike gap junctions, create the healthy heartbeat. The most common gap junction in the heart comprises Cx43 proteins. If a virus were to alter how Cx43 connects to a neighboring cell, this would cause a better environment for the virus, as this would keep anti-viral surveillance low, however, this would change how the electrical signal moves throughout the heart, creating arrhythmias. Adenoviruses are a common cold virus, but have been found in the hearts of many cardiac arrest patients. However, little is known on how adenoviruses may cause cardiac arrest, because human adenoviruses are only successful in humans, and mouse adenoviruses are only successful in mice. This creates a challenge when studying the dynamic heart, which does not translate well to cells in a dish. A mouse adenovirus, called Mouse Adenovirus Type-3 (MAdV-3) was reported to favor infecting the heart in mice, but no research has been published on if this virus can answer how adenoviruses change the heart. Because of this virus, and our prior research that adenoviruses can decrease Cx43 within skin cells in a dish, we used MAdV-3 to understand if, how adenoviruses could cause sudden cardiac arrest, and if longer infection could change the overall structure of the heart. We find that MAdV-3 infection prefers the heart to other organs, and that early stages, reduce both the speed of the electrical signal moves through heart and, looking within a cardiomyocyte, how it creates that electrical signal. These changes are arrhythmogenic and accompany modification of Cx43 that would close the gap junction between two cells, changing how ions and molecules move between cells. Using a human adenovirus infection in human cardiomyocytes created from stem cells, this result is also observed. If infection is allowed to continue in the mouse to cause chronic infection, the heart itself changes shape and is diseased. Together, this work shows that adenoviruses create a diseased heart, first the virus changes how the electrical signal moves and then later, causes thinning of the heart muscle. These data illustrate the role viruses play in causing cardiac arrest and could lead to diagnostic or drug targets.

Dedication

This body of work is dedicated to my family. My parents, John and Lisa, have constantly supported me throughout my life and instilled a curiosity for the world around me from a young age. My sister, Katie, whose grit and determination is as infectious as the viruses I worked with. My grandparents, Mary and Jim, who have been my most steadfast advocates, believers, backers, all of these words and more.

Acknowledgements

Graduate school is rough, and for some reason, I decided to go through it twice. Both times, the people around me have been vital in my success; whether they were directly involved with my research or are some of the best people I have had the pleasure of calling friends.

My advisor, Jamie Smyth deserves every ounce of my gratitude. He challenges me to become the best scientist and we may never agree on specific uses of effect vs. affect, I wouldn't have wanted any other advisor for this journey.

My committee members; Drs. Shihoko Kojima, Steve Poelzing, and Stefanie Robel, for your critical eye and support for my career development.

For my family, who have never questioned why I'm still in school, just supported me along the way.

The two cats who reside in my house, Link and Luna. They are very cute and cheer me up with their silly antics. Although, I could do without being woken up at weird hours.

All of the Smyth lab members along the way, Mike Zeitz, Carissa James, Patrick Calhoun, Alec Carlson, and Kenneth Young. Mike, for being a sounding board for some random question I've thought of and always entertaining it with an answer. Carissa, for being the first person in the Smyth lab to teach me literally anything and a running buddy outside of lab. Patrick, for showing me the way of the virus. Alec, for putting up with me learning how to mentor a new student. Lastly for Kenny, for filling the years-long void in our lab bay with great conversation.

And, to some pretty stellar TBMH members. Maria Solares, formerly TBMH Class 2017, for not ending me when I got locked out of our apartment for the billionth time or blocking me on social media after I sent something absurd. Mark Orloff for creating a "healthy" appreciation for SPAM, pinball, and rock climbing in my life. Bharath Sreekumar, for being an honorary Smyth Lab member for a semester or two and indulging in a lot of half-priced soup with me. Ryan King, for making me write a lot of collaboration pieces that will likely never see the light of day. Finally, my cohort mates, Rachana Deven Somaiya, Gabby Carrillo, Paras Patel, and Kijana George for the many hikes, ice cream outings, and beers throughout the years. You guys really made for the best cohort.

Table of Contents

List of Figures

Abbreviations

ID- Intercalated disc
Cx43- Connexin43
GJIC- Gap junction intercellular communication
HAdV- Human adenovirus
MAdV- Mouse adenovirus
CAR- Coxsackie and Adenovirus Receptor
AKT- Protein Kinase B
PKC- Protein Kinase C
cGAMP- cyclic guanosine monophosphate-adenosine monophosphate
cGAS- cGAMP synthetase
IFN- Interferon
ISG- Interferon stimulate genes
CVB3- Coxsackievirus B3
DCM- Dilated cardiomyopathy
IP_3- Inositol triphosphate
iPSC-CMs- induced Pluripotent Stem Cell CardioMyocytes
HIV- Human Immunodeficiency Virus
CVB3- Coxsakievirus B3
ACM- adult mouse cardiomyocytes

Attribution

Chapter 2 is co-authored with Grace Blair, Mira Tanenbaum, and Sharon Swanger. Grace performed and analysis the optical mapping experiments, Mira performed the echocardiography, and Sharon performed and analyzed the patch clamping. I performed all other experiments, wrote, and revised the manuscript with James Smyth.

Introduction

Viral Myocarditis

Viral infection is the most common etiological cause of myocarditis [1]. Myocarditis is the leading cause of sudden cardiac death in young adults, by either viral, bacterial, or fungal infection or drug use. In many cases, there is little structurally abnormal with the heart, however, there are detectable viral and/or bacterial antigens present [2]. Sudden infant death syndrome (SIDS) is also postulated to be caused by sudden cardiac arrest in some cases, and postmortem infant cardiac biopsies often detect viral genomes present, again without structural change to the heart [3, 4]. A search on PubMed for "virus" and "cardiac arrest" shows around 15 publications a year before 2020, and 195 for 2020, and 207 for 2021. With the advent of the Covid-19 pandemic, there is an increased interest in the role of viruses and the electrical activity of the heart [5]. Generally, however, it is unknown how acute viral infection causes the arrhythmias that lead to cardiac arrest. Prior to end-stage disease, subcellular structures in the heart undergo remodeling, including cases of heart failure [6]. Even during acute trauma, such as ischemia and reperfusion, insult to the myocardium can modify subcellular structures of the heart [7]. Research into acute viral myocarditis is an underdeveloped field when compared to understanding the chronic stage of disease. Disease during the acute stage in the clinic ranges from atrioventricular block, fulminant myocarditis, and sudden cardiac arrest [8].

While treatment for chronic viral myocarditis resembles standard of care for dilated cardiomyopathy and heart failure, if the acute stage of viral infection is better understood, we could potentially halt progression into late disease and additionally reduce cases of sudden cardiac arrest.

Chronic myocarditis

The intrinsic innate immune system reacts to viral presence as soon as viruses enter the cell. Subsequent extracellular innate- and adaptive- immune responses involve recruitment of infiltrating immune cells that cause inflammation. Specific viruses can trigger particular immune cell types that cause immunopathology, such as T lymphocytes which release perforin during Coxsackievirus B cardiac infection but do not effect viral clearance [9]. Chronic infection could develop by way of the immune system reacting to lingering viral genomes, which are detected in a large portion of dilated cardiomyopathy (DCM) cases, or can response to cell death caused during inflammation [10, 11]. While current treatment for chronic myocarditis is generally uniform, regardless of the pathogen that caused it, distinct immune cell populations' trigger chronic response and is dependent on the viral agent, thus may open targeted therapies once we understand specific disease progression [12]. Thus, understanding the pathobiology leading to chronic myocarditis may alter treatment to specifically target host immune cell populations to restore the healthy heart.

Adenoviruses

Adenoviruses are one of the leading viral causes of myocarditis, and myocarditis is the leading cause of sudden cardiac arrest in young adults[13]. Typically, adenoviral infection causes cold-like symptoms and is the most common cause of viral conjunctivitis [14]. However, adenoviruses can cause more severe disease, such as lethal pneumonia, and have been a

causative agent of localized outbreaks in the military, preschools, colleges, and nursing homes [15-17].

Adenoviruses are part of the *Adenoviridae* family, which contain genera such as *Mastadenovirus*, *Ictadenovirus*, and *Aviadenovirus*, that infect mammals, fish, and birds, respectively. Adenoviruses were first described in 1953 by Wallace Rowe after their isolation from human adenoid tissue [18, 19]. They are double stranded (ds) DNA viruses that are non-enveloped. Currently, there are over 100 different human adenoviruses (HAdV) described which are classified into subgroups A-G [20].

Adenoviruses are commonly studied for viral mechanisms of tumorigenesis. Adenoviruses used to transform cell lines were discovered to be oncogenic via their E1A protein in 1985 by Sawada et al [21]. Since then, many of adenoviral proteins have been studied in the context of cancer. Interestingly, there is no evidence so far that human adenoviruses are the causative agent of cancer [22]. While they are commonly used to understand oncogenic mechanisms and cell cycle control, present-day research has focused on using them as tools for transduction, namely in vaccine research and gene therapy. The depth and scope of adenoviral transduction enabled the creation of adenoviral vector vaccines during the SARS-CoV-2 pandemic. Several marketed adenoviral vaccines are available, although there have been plenty of research into adenoviral vector vaccines outside of SARS-CoV-2 [23]. These vectors are replication-incompetent, commonly lacking at least E1/E3 genes, which are responsible for early infection stages (covered below), but have the SARS-CoV-2 spike gene inserted [24]. The Janssen (Johnson & Johnson) Ad26-nCoV vaccine was approved for emergency use by the United State Federal Drug Administration (FDA) and 106 other countries with over 18 million doses administered around the world as of early February 2022 [25]. However, with this success in human tooling of adenovirus, there are still many infectious adenoviruses in circulation that can cause significant disease.

HAdV-2 and 5 primarily use the Coxsackie Adenovirus Receptor (CAR) for viral entry into the host's cell via attachment by its fiber knob protruding from the viral particle (**Figure 1.1**) [26, 27]. CAR is typically found on the basolateral side of polarized epithelial cells within their tight junctions [28]. However, adenovirus is not solely reliant on this receptor for cellular invasion [29, 30]. Additionally, these viruses utilize the α_v integrin-RGD motif to aid in endocytosis, but not direct attachment to the host's cell [31]. The penton protein at the base of the adenoviral fiber protein interacts and can bind with clusters of integrins, and this clustering of integrins signals for endocytosis via activation of phosphatidylinositol 3-kinase (PI3K)/Akt [32]. Viral particle uncoating occurs here at the cell surface and fiber proteins are released into the extracellular space [33]. This release of fiber is also important, as if adenoviruses retain fiber, it fails to escape endosomes to achieve replication [34]. Release of viral proteins, such as hexon, causes acidification of the cytosol, which is important in activation of the viral protease VI packaged within the virion [35, 36]. However, the exact role between viral protein release and pH change remains unclear. The partially dissembled virus is trafficked to the nucleus via dynein, which is recruited by the hexon protein in the newly acidic environment and targeting is aided by Protein Kinase A (PKA)/Mitogen-Activated Protein Kinase (MAPK) pathways [37-39]. Adenoviral core protein pVII's role in the next steps are two-fold, as it completes the uncoating

of viral particles, and, bound with viral DNA, is imported through the nuclear pore complex into the nucleus [40].

Once in the nucleus, adenoviral DNA transcription occurs in a temporally controlled fashion. Transcription of the 'early' units E1A, E1B, E2, and E3 occur first. This transcriptional stage is responsible for producing viral proteins key in pushing the host cell into S phase, favoring viral protein translation, and hindering host immune responses [41-44]. E1A protein performed a variety of functions in the host cell, but it importantly binds and inhibits the function of the cellular retinoblastoma protein (Rb), in order to effect cell cycle activation [45]. E1B-55k and E4orf6 interact to limit the host's response to infection by targeting p53 for degradation [46]. p53 is a key regulator in controlling the cell cycle and signaling for apoptosis in deregulated cells, thus adenoviral targeting of this protein uncouples the cell cycle and reduces cell death [47]. The intermediate transcription stage follows, producing IX, Iva2, L4 intermediate, and E2 late [48, 49]. The key responsibility of these products is transcriptional activation of late stage viral genes. The late transcriptional stage produces the major late promoter (MLP), which creates protein products that are important for viral packaging and condensing viral DNA [50].

The varied tissue tropisms and pathologies associated with adenoviruses is due to different adenovirus strains having amino acid differences among fiber proteins which bind to a host cell, and differences within their genome, leading to functional variation. This can create a challenge when used in the research setting, for example, in the case of myocarditis, as mouse adenovirus only successfully replicates in mice and human adenovirus only successfully replicates in humans.

Mouse Adenovirus Type-3

Mouse Adenovirus Type-3 (*Murine Adenovirus C*, MAdV-3) was isolated from a wild field mouse in 2009 by the Stang laboratory in Germany [51]. This adenovirus was reported to be highly cytopathic *in vitro* and cardiotropic when administered via retro-orbital injection [51]. Sequence comparisons showed that MAdV-3 is more similar to Mouse Adenovirus Type-1 (*Murine Adenovirus A*, MAdV-1), with Mouse Adenovirus Type-2 (*Murine Adenovirus B*, MAdV-2) being the least conserved of the three [52]. The MAdV-3 genome is 30,570 bp, compared to around 35,870 bp for HAdV-5 and 30,900 for MAdV-1 [53, 54]. MAdV-1 and MAdV-3 are most dissimilar in their fiber, pV, 100k proteins in amino acid length [51, 52]. MAdV-3 was most similar compared to MAdV-1 in the following order from most to relatively similar: hexon, 52k, pX, E4 ORFA, E1A, and pIX proteins [51]. BLAST alignment of the MAdV-3 (Ascension number EU83553.1) and HAdV-5 (Ascension number M73260.1) genomes reveals a similarity of 67.34% while comparing MAdV-3 to MAdV-1 (Ascension number NC_000942.1) shows a similarity of 71.08%. While there is little comparison of MAdV-3 to HAdV-5 previous work with MAdV-1 tropism compared to HAdV-3 displayed different cellular binding, perhaps due to the RGD-binding motif within MAdV-1's fiber protein when compared to this motif in the penton base of Adenovirus [55, 56]. This appeared to favor the endothelia tropism of MAdV-1 compared to HAdV-5. It is unknown what cell type MAdV-3 preferentially infects, but with its unique fiber signature, this could underlie the altered tissue tropism observed with MAdV-3.

Bieri and Hemmi investigated the receptors important for MAdV-1 & -3 binding during infection and found that MAdV-1 & -3 fiber knob utilizes the RGD domain on integrins αvβ6 and αvβ8, and that blocking or removing these motifs and receptors significantly reduces viral pathogenesis [56]. MAdV-1 primarily infects the endothelial cells of the nervous system, however, is detectable within all organs during infection [51, 57, 58]. MAdV-2 infects primarily the gut of mice but causes no appreciable disease [59]. MAdV-3 preferentially infects the myocardium and viral genomes are not significantly detected in other organs (Chapter 3) [51]. While Bieri and Hemmi illustrated that MAdV-1 & -3 utilize the same integrin receptors for infection, it is unclear what causes the tropic differences between the viruses, as αvβ6 is expressed in epithelial cells throughout the body and αvβ8 is highly expressed on non-proliferating cell types (such as cardiomyocytes several days past birth) [60, 61].

With the discovery of a cardiotropic mouse adenovirus, this opens the avenue to develop a murine model for adenoviral myocarditis and understand the role of adenoviral infection in arrhythmogenesis.

Cardiomyocyte Structure and Communication

Sudden cardiac arrest arises from sustained and uncorrected abnormal electrical activity of the heart, which precipitates arrhythmia and loss of normal function [62]. The individual cells of the heart, cardiomyocytes, are responsible for anisotropic action potential propagation, which they achieve by precise intercellular electrical coupling at intercalated discs (ID). Through such intercellular communication, the heathy heartbeat is achieved, and is dependent upon the coordinated contraction of the cardiac muscle to pump blood throughout the body [63]. Such proper electrical propagation relies upon the ion channels and specialized intercellular junctions of cardiomyocytes and modifications intercellular junctions can result in rapid changes to cardiac function, without altering gross heart structure. As mentioned above, the structure that couples cardiomyocytes together is the ID and an overview of this region in **Figure 1.2**.

Gap Junctions

The gap junctions of the ID are responsible for intercellular exchange of small molecules (less than 1 kDa in size) and ions. Gap junctions were first characterized in 1966 and their identifying characteristic was they allowed passage of molecules from cell-to-cell [64]. In 1985, Neyton *and* Trautmann employed patch clamping of isolated rat lacrimal gland cells to describe the electrical activity of intercellular junctions [65]. Within the heart, the electrical properties of the gap junction were demonstrated in 1986 by Veenstra and DeHann in two connecting chick ventricle cells [66]. Additionally, various scaffolding proteins that maintain structure and aid in intracellular signaling support gap junctions at the ID, which are covered below.

The classical role of gap junctions at the ID is to propagate electrical signals from one cardiomyocyte to the next. In addition to electrical coupling however, gap junctions provide metabolic coupling and the exchange of many factors including second messengers, for example Inositol triphosphate (IP_3) to trigger calcium release, regulate development, and spread immune signaling molecules including cGAMP [67-69].

Connexin proteins comprise gap junctions. Within the heart, Connexin43 (Cx43, gene name *Gja1*), Cx45 (gene name *Gjc1*), and Cx40 (gene name *Gja5*) are the major connexin

proteins, with Cx43 being most highly expressed in the myocardium [70]. Six connexin proteins oligomerize to form a connexon, or hemichannel, before exiting the Golgi apparatus, then traffic to the plasma membrane via the cytoskeleton. At the cell surface, connexons dock between apposing cells and coalesce in vast arrays to form the gap junction [71, 72]. Cx43 has a surprisingly short life cycle, with half-lives measured at less than two hours at the plasma membrane and 1-5 hours in total, and thus gap junctions are highly dynamic and responsive to acute stress [73, 74]. Gap junctions are regulated at various levels including transcription, translation, and post-translational modifications [75-77]. Post-translational modifications represent one of the most rapid mechanisms by which the cell can alter its function to stimuli [78-80].

One such post-translational modification known to have a major impact on gap junctional regulation is phosphorylation. In fact, the C-terminus of Cx43 harbors at least 19 residues subject to dynamic phosphorylation [81, 82]. Phosphorylation of $Cx43^{S373}$ by Akt regulates the interaction between ZO-1, a scaffolding protein, and Cx43, which alters gap junction size and gap turnover [83-86]. Phosphorylation of $Cx43^{S368}$ occurs downstream of Akt signaling on S373 and occurs via Protein Kinase C (PKC) [80]. Phosphorylation of Cx43 at Ser368 is well described in reduction of open channel probability [87]. Phosphorylation at S373 and S368 sites is increased during heart failure and acute ischemia [77]. With the diversity of function provided by the phosphorylation sites of the C-terminus tail of Cx43, many of which are not focused in this dissertation, I refer the reader to Solan and Lampe, 2015 [81].

Ion Channels

Sodium, calcium, and potassium channels cooperate in formation of the cardiac action potential. This section will only focus on the major ion channels in the ventricle action potential (**Figure 1.3**). Voltage-gated sodium channels generate the I_{Na} in the working myocardium with $Na_v1.5$ (gene name: *Scn5a*), the membrane subunit comprising voltage-gated sodium channels, is expressed highest in the developed myocardium [88]. Activation of voltage-gated sodium channels is controlled by the outward movement of charge across the membrane [89]. Inactivation follows quickly and the channel is blocked by an intracellular loop, like a hinged lid on a jar [90]. $Na_v1.5$ and Cx43 traffic together to the ID via interaction of the C-terminal tail of Cx43 and the α-subunit of $Na_v1.5$ [91]. Altered localization or modification of $Na_v1.5$ can affect I_{Na} and lead to the development of arrhythmias [92]. I_{Ca} is mainly achieved through the L-type voltage-dependent calcium channels in the ventricle, which control the flux of Ca^{2+}. $Ca_v1.2$ (gene name: *Cacna1c*) is the pore-forming subunit of the of the L-type calcium channel and is tightly regulated by several mechanisms, including Protein Kinase A phosphorylation [93]. $Ca_v1.2$ is localized to the T-tubules, which are organized throughout the cardiomyocyte to enable close proximation to intracellular ryanodine receptors to effect calcium-induced calcium release [94]. The I_K is complex and encompasses several channels. Three potassium currents repolarize the cardiomyocyte and maintains resting polarization, I_{Ks}, I_{Kr}, I_{K1}, in that order [95]. I_{Ks}, the slow delayed rectifier current is created by $K_v7.1$, the interaction between KCNQ1 and KCNE1 form this channel, and function can be modified by PKC phosphorylation of KCNE1, reducing their interaction [96]. I_{Kr} is mainly achieved by the hERG proteins, and alterations in the current contribute to cardiac developmental disorders leading to long QT syndromes and heritable arrhythmias [97]. The K_{ir} family of potassium channel proteins generate I_{K1}, which regulates action potential duration [98]. Changes in the K^+ currents in the ventricular cardiomyocyte

subjugate the duration of action potentials, long QT intervals, and increased risk of arrhythmias [99].

Scaffolding Proteins

Scaffolding proteins are proteins that associate with two or more proteins to create a stable configuration. They can additionally act as transducers between membrane-bound proteins and the nucleus within signaling cascades [100]. A major scaffolding protein family that regulates gap junctions and ion channels at the ID are zonula occuldens. ZO-1 (gene name: *Tjp1*), which is colocalized with Cx43 and can alter the size and trafficking of Cx43 during normal physiological function and during disease [86, 101, 102]. ZO-1 also complexes with CAR [103, 104]. Both CAR and Cx43 interact with ZO-1 via PDZ domain binding motifs on their C-terminus tails to create a link to the cytoskeleton [105]. Mutations in *Tjp1* genes were identified in arrhythmogenic cardiomyopathy patients, and cardiomyocyte-specific knockout was found to alter AV node conduction [106, 107]. Cx43 also complexes with plakophilin-2, an important competent of the desmosome structure, along with CAR [108]. CAR conditional knockout mice showed a loss of interaction with β-catenin, another scaffolding protein at the intercalated disc [109]. B-catenin, while a scaffolding protein, also translocates to the nucleus and acts as a transcription factor for various proteins, including Cx43, and larger signaling cascades, such as Wnt signaling during development and disease [76, 110, 111]. Cx43 associates with $Na_v1.5$ at the intercalated disc, and more specifically, the nanodomain called the perinexus, that also contributes to cardiac conduction [112, 113]. The perinexus region controls ephaptic coupling, a classic function in neurons but newly identified in the heart, which controls electrical propagation based on cell distance, outside of gap junctions [114]. The conjunction of the perinexus along with gap junctions, control conduction velocity across the myocardium [115]. It is postulated that Cx43/$Na_v1.5$ interaction may regulate each other, or Cx43 could act as a scaffolding protein itself and form a macrocomplex of scaffolding, adhesion, and other proteins to localize at the ID. Regardless, loss or alterations in Cx43 at the ID correlates with impaired and/or downregulation of $Na_v1.5$, and is arrhythmogenic [116-118]. Several other scaffolding proteins create a stable ID, although only a few are discussed here, please see Chudasama, Marx, and Steinberg's book chapter for more detail [119].

Combined, gap junctions, ion channels, and scaffolding proteins at the ID facilitate cardiac action potential formation and propagation of cardiac conduction from one cell to the next. In addition, these proteins play important roles in cellular homeostasis and responses to changes in stimuli. This role in communication and response creates a favorable site for viral targeting to halt a cell's response to infection and increase success of replication and spread.

Viral targeting of intercellular communication

Intercellular communication is an integral aspect of cell function and homeostasis. Cyclic GMP-AMP synthase (cGAS) is activated by the presence of cytosolic DNA and produces 2'3'-cyclic GMP-AMP, a secondary molecule that activates STimulator of Interferon Genes (STING) [67]. Previously, it was discovered that cGAMP can be spread from virally infected cells to uninfected cells via gap junctions [120, 121]. cGAMP serves in STING activation and is an important early protein in the innate immune response to viral infection. Activation of STING triggers the IRF3/NF-κB pathway and induction of Interferon-Stimulated genes (ISG), encompassing over a hundred pro-inflammatory proteins [122]. Given that gap junctions couple

almost all cell types together, including immune cell synapses, and cardiomyocytes and macrophages in the heart, they are a powerful avenue for propagation of immune responses across tissues [123, 124]. This spread of antiviral signaling molecules creates a firewall for neighboring cells by eliciting an inhibitory environment for viral replication, thus, a potential target for direct or indirect viral modification during early infection. A cellular mechanism regulating many processes which is discussed at length in this dissertation is phosphorylation. Some viruses, such as retroviruses, encode for their own kinases to either phosphorylate viral or host proteins to shutdown antiviral responses and/or favor replication and production of viral progeny [125]. In the case of phosphorylation by adenoviruses, in addition to E1B-55k/E4orf6 limiting eIF2α phosphorylation, the E1A protein is known to activate PKC signaling during infection, which could be either an advantage or disadvantage, as PKC signaling would close gap junctions [126]. Thus, phosphorylation plays a complex role in viral infection and is an expansive field of research which many consequences on our understanding of viral pathogenesis. Dynamic phosphorylation may favor the virus in the short-term, but in the context of the heart, could it play a fatal role for the host?

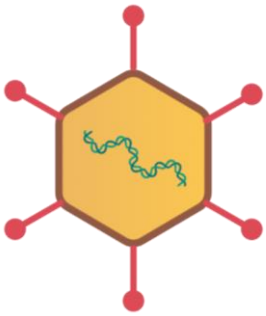

Figure 1.1 General Adenovirus Structure. Fiber protein (red) that binds to host's cell receptor for infection. Hexon protein (brown) compose most of the capsid shell. Adenovirus is a double stranded DNA (green/blue) virus. Figure created using biorender.com.

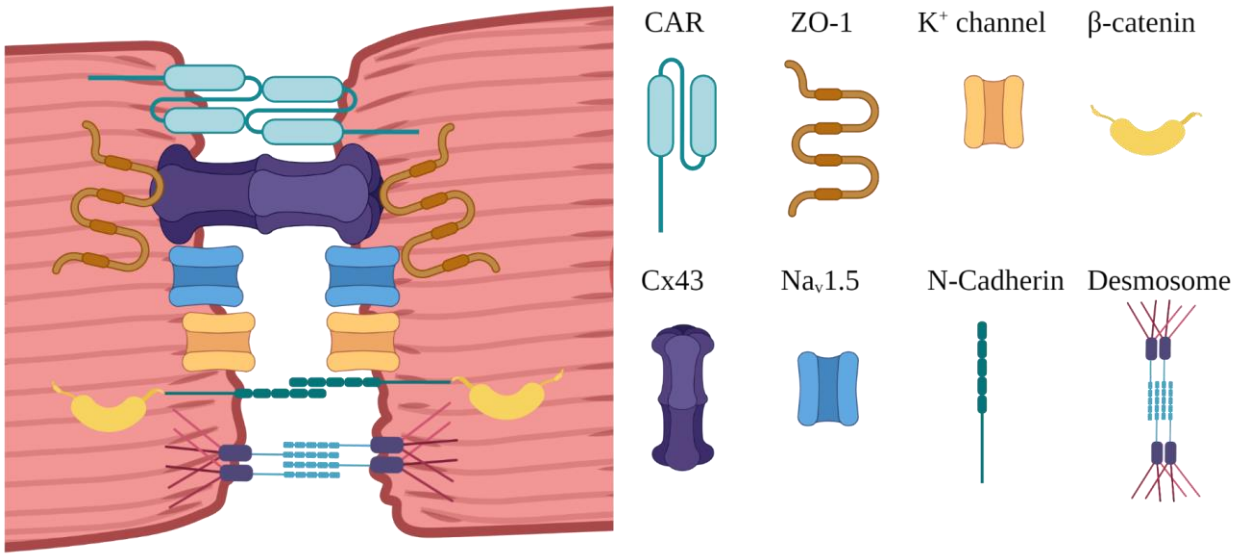

Figure 1.2 The cardiomyocyte intercalated disc. Cardiomyocytes are coupled together to propagate the electrical signal generated in the atria down to the apex, and then up through the ventricles which results in the heart to contract and pump blood. While protein expression is important, the localization of proteins that comprise the disc is vitally important for its function. Gap junctions, like Cx43, allow for the passage of small molecules and ions from one cell to the next. ZO-1 is a scaffolding protein that colocalizes with Cx43 and is important in localization of Cx43. Ion channels like $Na_v1.5$ and K^+ channels are found at the intercalated disc, and throughout the membrane. CAR, an important binding receptor for adenoviral infection, is a cellular adhesion molecule. N-Cadherin is hallmark protein in adherens junctions at the disc, and along with β-catenin, can regulate cardiomyocyte function. Figure created using biorender.com.

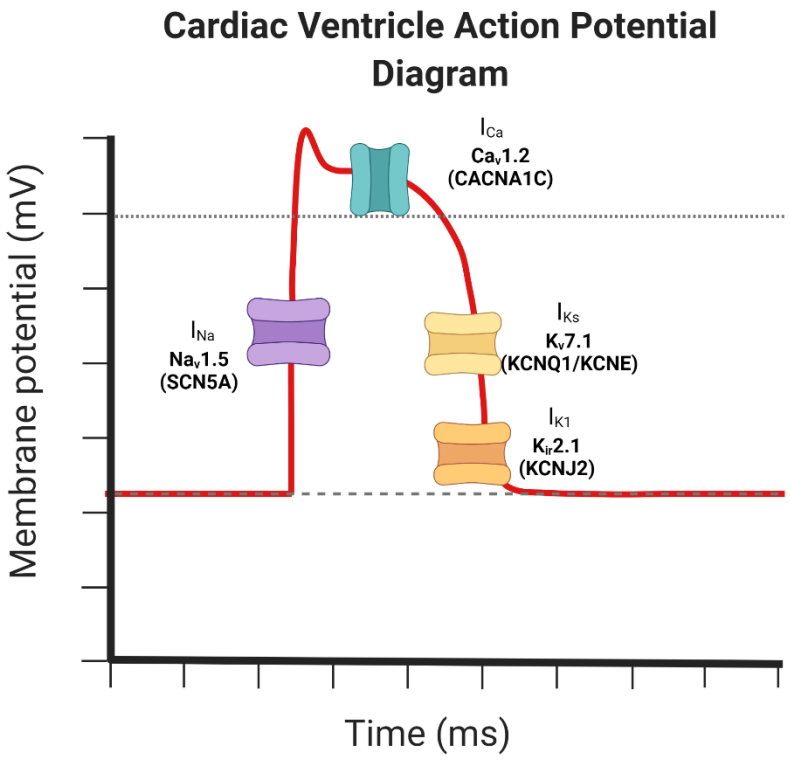

Figure 1.3 Cardiac ventricle action potential. Different cardiomyocytes have different action potential shapes, with various ion channels involved. The ventricle action potential is the "classic" action potential shape, with rapid depolarization upstroke, rapid repolarization, then a delayed repolarization that sets the stage for the next action potential. Figure created using biorender.com.

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Chapter 1: Viral Myocarditis: from arrhythmogenic remodeling to chronic disease

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Abstract

Viral infection is the leading cause of myocarditis, particularly in young adults and children. Several viruses can infect the myocardium and cause a variety of disease presentation due to the diversity of viral replication mechanisms in the host. During the acute stage of disease, it is hypothesized that the heart is susceptible to arrhythmogenesis due to subcellular modifications by the virus. The chronic stage of viral myocarditis takes place during/after immune infiltration into the myocardium where inflammation can cause damage to tissue fibrosis, for example, that can perpetuate the arrhythmogenic state. Moreover, chronic disease can often underlie progression to cardiomyopathies, and eventually, heart failure. In this review, we summarize the pathogenesis of common viral agents during both the acute and chronic stages of viral infection and myocarditis.

Introduction

Myocarditis is the leading cause of sudden cardiac death in young adults and also a significant number of dilated cardiomyopathy/heart failure patients. Viral myocarditis is commonly separated into two phases: the acute phase, which is viral-mediated remodeling of the myocardium; and the chronic phase, classified by immune-mediated damage to the myocardium. Acute viral myocarditis is associated with electrical changes of the heart, without significant structural pathology. Chronic viral myocarditis involves inflammation and damage to cardiac tissue due to host immune responses to infection [1]. Several viruses can cause myocarditis, however much is unknown regarding viral family-specific pathogenesis, presentation of illness, and treatment.

Viral myocarditis is a global disease caused by numerous viruses including common sources such as picornaviruses, influenza, adenoviruses, and coronaviruses [2-4]. Young adults are more prone to cardiac infection, several reports have implicated outbreaks in children, and, viral genomes have been found post-mortem in cardiac tissue from neonates that succumbed to sudden infant death syndrome (SIDS) [5]. Regardless, the pathogenesis and course of disease is varied due to differences in viral mechanisms evoking illness. This review will cover acute and chronic viral myocarditis by discussing distinct viral agents in subcellular remodeling, immune-mediated signaling and cellular infiltration into the cardiac tissue, and what is known to be specific to viral families.

Pathology

Virus-mediated manipulation

others including fibroblasts, smooth muscle cells, and endothelial cells [6]. The working myocardium relies heavily on cardiomyocytes that are coupled via gap junction complexes at intercellular structures known as intercalated discs (ID). The ID is essential in the propagation of cardiac conduction that results in contractile function [7]. Proteins localized to the ID play several roles, including maintaining structural integrity and propagating small molecules, metabolites, second messengers, and ions from one cardiomyocyte to the next [8, 9]. Gap junctions, such as Connexin43 (Cx43), ion channels including $Na_v1.5$, and scaffolding proteins, such as ZO-1, localize to the ID [10-12]. Modifications of proteins at the disc alter the ability of cardiomyocytes to create and handle action potentials, and can also change how the cell responds to infection, creating an advantageous site for viral targeting [8, 13].

Direct damage to cardiac cells occurs during active viral infection of the tissue, and while lytic effects are unequivocal, more pervasive and specific effects of virally encoded factors can elicit dangerous pathologies. Virally encoded proteins target sub-cellular pathways and machinery to facilitate replication and immune-evasion while viral nucleic acids and genomes can precipitate antiviral signaling cascades that are detrimental to cardiac function. Active viral replication therefore causes direct damage to cardiac tissue before significant immune infiltration and cytokine responses. Such mechanisms are likely to underlie sudden cardiac death events where viral genomes were detected in cardiac tissues with little or no abnormalities detected by histopathology or gross cardiomyopathy [14, 15]. As we gain further molecular insight into the cardiac viral infection process and the development of myocarditis, it is hoped that identification of pathways common to several viruses and/or those specific to viral species will inform

development of antiviral therapeutics protecting cardiac function. Similarly, parsing out viral- *vs* immune-mediated pathological changes in the heart is critical to improve early diagnosis and efficacious treatment/management of myocarditis. Currently, there are a variety of different animal models used in the research setting, with advantages and disadvantages to each. We have outlined common ones in **Figure 2.1**. This section will focus, in brief, on select cardiotropic viral agents and their roles in modifying subcellular mechanisms that have potential to trigger sudden cardiac disease, before an appreciable immune response and subsequent damage.

Enteroviruses

The pathobiology of cardiac Coxsackievirus, a single-strand (ss) RNA virus, infection is historically the most studied and best understood, in part due to permissive infection of the mouse providing a relevant and useful model. The cell entry stage of Coxsackievirus infection relies on the Coxsackie Adenoviral Receptor (CAR), an adhesion protein that localizes to intercellular junctions, including the ID structures that adjoin cardiomyocytes [16]. Of note, the developing myocardium has high levels of CAR expression, possibly contributing to higher occurrence of myocarditis in infants compared to adults. Interestingly, another leading viral agent of myocarditis, adenovirus, share exploitation of CAR as a primary receptor. In the epithelium, CAR is essentially inaccessible as it is within the tight junction complex. Coxsackievirus has been reported to interact with Decay Accelerating Factor (DAF) on the apical surface of cells to activate Abl kinase, which allows access to intracellular junctions by way of actin remodeling. Additionally, this viral interaction with DAF activates Fyn kinase, which is ultimately facilitates transport of viral particles across the cell surface through caveolar vesicles. Disruption of actin filaments is well described in pro-arrhythmogenic situations, supporting the concept that this mechanism of viral entry can be detrimental in the heart [17-19]. Outside of structural changes brought on by Abl/Fyn kinase activation, it has been reported that the downstream signaling of DAF can trigger intracellular calcium release to enable Coxsackievirus to enter the cytosol [20]. Alterations in calcium handling are a quintessential hallmark of arrhythmias and heart failure, so such viral manipulation of this process could potentially impact normal electrophysiology and contractility [21]. CAR itself is known to play a significant role in the establishment and maintenance of normal cardiac conduction. Mouse cardiomyocyte-specific knockout of CAR was found to induce atrioventricular (AV) block via disruption of Cx45 at intercalated discs within the AV node [22]. In patch clamping of heterozygous CAR cardiomyocytes, reduced sodium current at the intercalated disc was reported and *in vitro* immunoprecipitation supports interaction of CAR with both Cx45 and $Na_v1.5$ [22, 23].

Turning to subcellular effects within the infected cardiomyocyte, Coxsackievirus protease 2A protein directly cleaves and impairs the function of the protein dystrophin during Coxsackievirus B3 infection [24]. Dystrophin is a cytoplasmic protein that forms the critical costamere complex with additional proteins to anchor contractile machinery to the sarcolemma [25]. Improper function ofdystrophin underlies both the development of dilated cardiomyopathy and increased susceptibility to arrhythmia, both of which can occur during viral myocarditis [26-28]. Additionally, Coxsackievirus protein 3B can disrupt mitochondrial function and in cardiomyocyte studies, acutely reduced function of respiratory chain complexes I-III, and suppresses transcripts encoding components of complexes I-IV [29-31]. With the heart being one of the most metabolically active organs, disruptions in mitochondrial function have clear and

serious implications to cardiac function. Indeed, cardiomyocytes and cardiac biopsies from acute viral myocarditis patients have elevated levels of biomarkers for oxidative stress [30]. Oxidative stress, or the uncontrolled production of reactive oxygen species, is known to alter calcium handling in cardiomyocytes by way oxidizing the type 2 ryanodine receptor (RyR), and causing leaky calcium into the sarcoplasmic reticulum, leading to an increased susceptibility of atrial fibrillation [32, 33]. Moreover, increased oxidative stress is linked to perturbed Cx43 trafficking through displacement of microtubule plus-end binding proteins, thus reducing gap junction formation and contributing to an arrhythmogenic substrate [13]. Other ion channels, including $Na_V1.5$, are transported by similar mechanisms and so would likely be affected under these conditions, further exacerbating disruption to normal cardiac electrophysiology [34].

Adenovirus

Adenoviral myocarditis has a high clinical incidence as diagnosed by biopsy in cases of sudden cardiac death. However, in the context of cardiac infection, our understanding of the contribution of these viral mechanisms to evoke disease are limited, due to the species-specificity of adenovirus limiting development of animal disease models. Adenovirus subgroups A, C, E, and F, a double stranded (ds) DNA virus, favors binding of CAR and integrin $\alpha v\beta 5$ by adenoviral fiber protein for cellular entry, similar to Coxsackievirus [35, 36]. Human Adenovirus Type-2 (HAdV-2) infection can produce excess fiber protein that escapes the infected cell, which then binds CAR at neighboring cells, disrupts junctional structure and allows to viral migration across cell layer [37]. Use of *in vitro* modeling of human Adenovirus Type-5 (HAdV-5), one of the most common cardiotropic adenovirus, shows impaired interaction of Cx43, the most common gap junction expressed in the heart, and ZO-1, a scaffolding protein regulating gap junction size and localization. Additionally, post-translational modification by phosphorylation of Cx43 both the Serine373 and 368 residues occurred in induced pluripotent stem cell derived cardiomyocytes (iPSC-CMs), which lowers the open probability of gap junctions and alters cardiac conduction [38-40]. The adenoviral protein E4orf1 has been shown to activate AKT, by which this pathway phosphorylates Cx43 Ser373 and β-catenin during early infection [41, 42]. β-catenin can play a multitude of roles in maintaining cardiomyocyte homeostasis and cardiac conduction, as it is a scaffolding protein for N-cadherin at the ID, and is a transcriptional regulator of Cx43 [43, 44]. Interestingly, a previous study has reported that increased calcium flux and increased protein kinase C (PKC) was observed when adenoviral protein E1A level's increased, using an Ad5-E1A-Luc vector [45]. PKC activation is a global upstream regulator of Cx43 Ser368 phosphorylation, with increased phosphorylation at this site is a arrhythmogenic mechanism [46, 47]. Outside of phosphorylation of Cx43, increased phosphorylation of PKC followed elevated Adenoviral E1A protein levels, which altered the interaction of PKC and Caveolin-1 (Cav-1) in corneal fibroblasts [48].Caveolins are localized to the plasma membrane and regulate signaling in localization of other proteins, cell survival and apoptosis, and an alternative pathway of endocytosis outside of clathrin-coated pits [49]. Cav-1 associates with Cx43 at the post-Golgi apparatus and traffic together to the plasma membrane, and reduction in Cav-1 protein levels accompanies a decrease in gap junction intercellular communication [50, 51]. Early adenoviral proteins can cause a cascade of downstream effects, which culminate in modifications to intercellular communication across various cell models. In regards to an *in vivo* model of adenoviral myocarditis, there is promise of Mouse Adenovirus Type-3 as a modeling mechanism. In 2009, this virus was isolated out of a field mouse and shown to be cardiotropic, however, there has been limited research using this virus since [52]. While the majority of this

research was not performed in the context of cardiac modeling, it paints the picture of how early adenoviral infection alters proteins important for intercellular communication and how deleterious early stages of infection could be on cardiac conduction.

SARS-CoV-2

SARS-CoV-2 has spurred further investigation into viral-mediated cellular damage as there is some evidence demonstrating direct cardiomyocyte remodeling and cytopathic death by SARS-CoV-2 infection [53]. Viral genomes have been detected in patient population biopsies in the working myocardium in a variety of cardiac cell types, including cardiomyocytes [54]. *In vitro* experiments have demonstrated that wild-type SARS-CoV-2 productively infects adult cardiomyocytes and induced Pluripotent Stem Cell- Cardiomyocytes (iPSC-CMs), which leads to reduced contractile function, slowed contractions, and impaired ability to relax while also reducing the expression of ACE2, the host receptor exploited for viral entry [55-59]. Chen *et al* further demonstrated that SARS-CoV-2 infects cardiomyocytes in a hamster model and separately evaluated the role of macrophages during the pathogenesis of SARS-CoV-2 myocarditis, demonstrating that macrophage co-culture with cardiomyocytes reduced the infection of cardiomyocytes when compared to the infection of cardiomyocytes grown in isolation [60]. The role of macrophages in the heart has come into a particular light, as they couple with cardiomyocytes via Cx43 and are important in maintaining cardiac conduction and propagation [61]. This group further suggested that detection of viral particles in human cardiac tissue may be impeded by the delayed autopsy and sample collection weeks after infection [60]. Initial evidence indicating direct cardiomyocyte damage from SARS-CoV-2 infection included cardiomyocyte necrosis and degenerative vacuolization of the cytoplasm on postmortem examination of patients with clinically diagnosed myocarditis in the context of SARS-CoV-2 infection. Further examination of these hearts revealed spike and nucleocapsid RNA in the myocardium with viral gene products localizing to the cytoplasm and perinuclear area within cardiomyocytes, providing evidence of active cardiomyocyte infection during the natural course of infection in humans [56]. Finally, Dolhnikoff *et al* visually demonstrated the presence of viral particles inside cardiomyocytes, along with capillary endothelium, endocardial endothelium, fibroblast, macrophages, and neutrophils using electron microscopy with tissue from a child with SARS-CoV-2 infection [62]. Currently, it is thought that direct viral infection of cardiomyocytes is limited, however, infectious virus may be spread to the myocardium via immune cell migration [63]. The role of indirect myocardium damage and inflammation will be discussed in the next section.

While only major viral agents of myocarditis are described here, it illustrates the contribution of direct viral modification of subcellular remodeling during early infection, without immune-cell infiltrate and damage due to inflammation. Understanding mechanisms of virus-mediated damage in acute viral infection is critical, but must also be considered in the context of the area of host immune-mediated damage during viral myocarditis disease progression. As we elucidate the molecular biology of such early stages in infection, it is likely that common pathways will be uncovered that result in elicitation of maladaptive immune responses and subsequent pathological remodeling of the heart.

Immune-mediated damage

Host immune-responses elicit a variety of direct and indirect effects considered to contribute significantly to pathological cardiac remodeling during the progression of viral myocarditis. Ranging from development of lesions on cardiac tissue, infiltration of reactive immune cells, and finally to development of dilated cardiomyopathy and heart failure, therapeutic modulation of such immunoreactivity may alleviate and prevent disease progression. This stage of viral myocarditis is comparatively more understood than to the acute phases, given the higher chance of clinical presentation and accompanying diagnosis and research tools available.

Adeno- and Entroviruses

Development of myocardial lesions is a classic sign of chronic viral myocarditis, and often progresses to induction of dilated cardiomyopathy (DCM) and/or hypertrophy [64, 65]. Within the context of Coxsackievirus myocarditis and subsequent development of dilated cardiomyopathy, leucocytic infiltration, particularly T-cell infiltration, and chronic inflammation are reported as a key pathogenic player [66]. Mice lacking CD4+ T cells display a reduction in pathological remodeling of the heart, but an increase in viral genomes associated with infected organs, whereas mice lacking CD8+ T cells present with both a reduction in pathological remodeling and viral load [66]. Recently, STAT3 signaling was identified to increase IL-6-induced differentiation of Th17 cells in myocarditis. In a reovirus-based model of cardiac infection in primary cardiac cells, NF-κB, a transcriptional activator of many inflammatory cytokines, undergoes a unique activation and translocation in cardiomyocytes which may play into the development of DCM [67]. While reovirus is not known to cause viral myocarditis in humans, it has been an important tool in understanding the role of the immune response and resulting inflammation in the progression of virally induced dilated cardiomyopathy. In the context of DNA viruses, Mouse Adenovirus Type-1 (MAdV-1) induces IFN-γ *in vivo*, which was identified to play a key role in the development of chronic viral myocarditis by the development of necrosis of the tissue [68]. Modulation of the inflammatory response during Coxsackievirus and adenovirus infection has been a topic of intense research and we refer the readers to a more in-depth review by authors Lasrado and Reddy, Reviews in Medical Virology, 2020 [69].

Herpesvirus

Within the past decade, human herpesvirus 6 (HHV-6), a dsDNA virus, has been associated with an increasing number of pediatric cases of inflammatory myocarditis and dilated cardiomyopathy [70]. A comprehensive review of clinical reports published by Reddy et al. 2017 found that a population of under 2 years old patients with viral myocarditis at HHV-6 genomes detected and that, while compared to other viral agents such as Coxsackievirus B, mortality and progression into DCM due to inflammatory damage was significantly higher (64% with HHV-6 vs 25% with Coxsackievirus B). Myocardial biopsies of cardiac tissue typically reveal both cardiomyocyte necropsy and leukocyte infiltration via endothelial leakage during the later acute stages of disease [70]. HHV-6 and other members of the *herpesviridae* family appear to be significant causes of pediatric chronic viral myocarditis, however, there is a lack of laboratory modeling of disease in the literature. With direct infection of cardiomyocytes observed in the patient population accompanying significant immune cell infiltration leading to rapid progression towards heart failure in HHV-myocarditis patients, there is a need for understanding viral entry

and replication in cardiac cells, the requirement of immune cells, and antiviral treatment during the early stage of disease.

Influenza

Seasonal hospitalization of severe influenza cases is rarely accompanied by myocarditis but often have a particularly high lethality [71, 72]. However, little is known about the disease pathology that leads to inflammation of cardiac tissue due to limited laboratory tools to model cardiac infection in adults. Wang et al. found that in young mice inoculated with influenza A (H1N1) had increased levels of proinflammatory cytokines, such as TNF-α, IL-1β, and trypsin in the lung, brain, and heart tissue. This leads to loss of tight junctions via decreased ZO-1, resulting in increased vascular permeability and unregulated intracellular calcium levels. They concluded that this increase in cytokines caused multi-organ failure [73]. An interferon-induced transmembrane protein 3 (FITM3) KO mouse was created that induced reliable influenza-susceptible myocarditis. Influenza A-infected IFITM3 KO mice had significant viral loads in cardiac tissue, increased fibrosis, inflammation, and presented with prolonged heart rate, measured by ECG [74].

Human Immunodeficiency virus (HIV)

A recent clinical study of HIV-infected patients made the interesting finding that Cx43 expression in ventricular cardiomyocytes was increased, and the majority was localized laterally, rather than localized at the intercalated disc. This was true, regardless of viral loads, CD4+ T-cell counts, or antiretroviral use. Patients with HIV have an increased rate of heart disease in comparison to the general public, along with chronic systemic inflammation. This increase and pathological localization of Cx43 in conjunction with inflammation could explain the significant rates of heart disease within this patient population [75]. As the role of immune cells, such as macrophages, play in the cardiac conduction system and their function with connexin proteins become more understood, changes to the immune system during viral infection outside of direct inflammation will contribute to our understanding of pathological mechanisms and remodeling [61, 76].

SARS-CoV-2

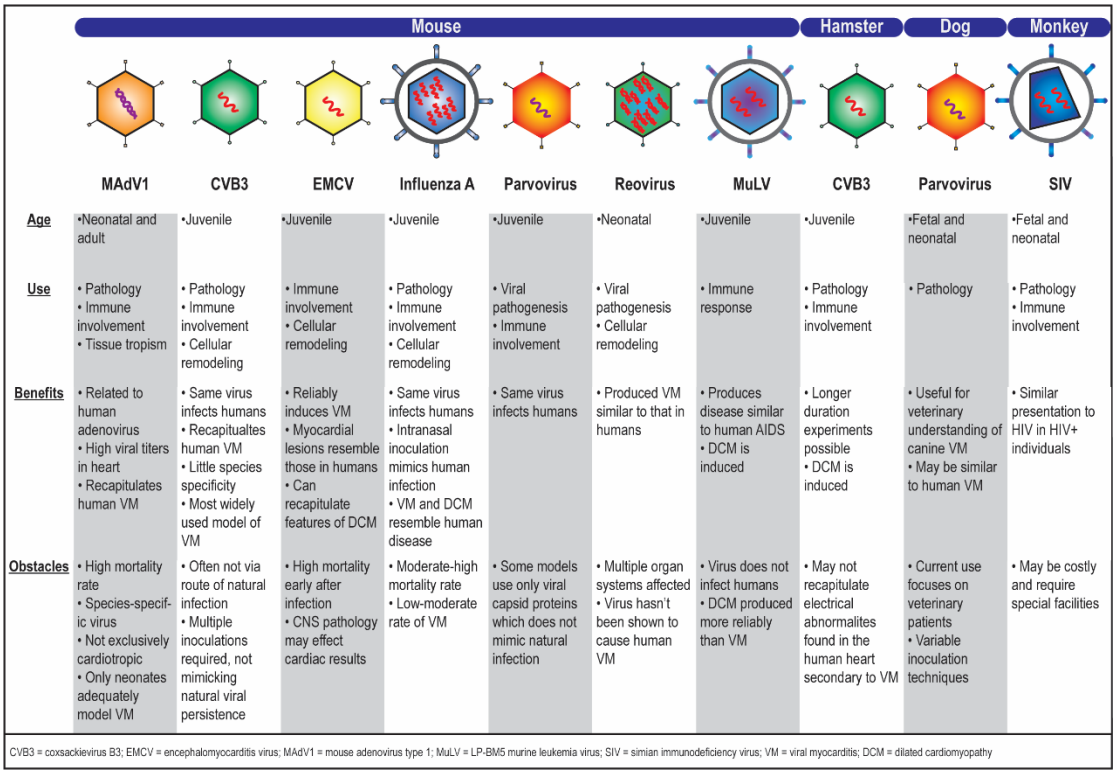
Increasingly, more research and clinical reports have highlighted the role of the immune system during SARS-CoV-2 infection on damage accumulated in the heart. Within patient populations, direct infection of cardiomyocytes is rare and indirect damage due to inflammation and immune cell infiltration in the heart is more common [54]. Both systemic cytokine storm and vascular leakage are observed clinically. SARS-CoV-2 has been shown to directly infect myocardial macrophages, lymphocytes, and endothelial cells at a higher proportion that cardiomyocytes in post-mortem studies, while infiltrating populations are comprised of CD8+ T-cells and non-myocardial, CD11b+ macrophage groups [77]. Proinflammatory cytokine release accompanies infiltrating immune cells and directly infected cardiac cells, and cytokines such as TNF-α, IFNγ, and IL-4 are released during infection [78]. While the prominent idea that comes to mind with the mass release of cytokines, is cytokine storm, which is a significant cause of morality with Covid-19, there are other roles that cytokines can function as to contribute to disease [79]. TNF-α treatment to iPSC-CMs was found to increase viral entry into cardiomyocytes, thus this interplay between immune response and direct viral entry and damage creating a symphony of challenges for the heart to overcome [80]. Previously, increased

extracellular TNF- α has also been found to modify cardiac conduction and alter cardiomyocyte coupling [81]. The interplay between cytokines, inflammation, and direct remodeling of cells illustrate that infections do not function in a vacuum and both viral- and immune- function during infection can exacerbate cardiac disease.

The interplay of the immune system and the heart during viral infection is important to understand, as this has led to the development of therapeutics during inflammation, such as soluble CAR, which can bind to both extracellular CAR and CVB3 capsid protein, and anti-inflammatory drugs [82, 83]. Late stage treatment for chronic viral myocarditis is similar to other causes of DCM, as there is typically no active viral replication, however, viral genomes may still be detectable. Additionally, many causes of DCM are unknown, however, detectable viral genomes are present in ranges of up to 42% patients with idiopathic dilated cardiomyopathy [84, 85]. Together, this suggests that detection and treatment during the acute stage of viral myocarditis is a worthwhile avenue of investigation.

Conclusion

While viral myocarditis is often undetected due to asymptomatic infections, it can lead to life threatening cardiac complications. A number of viruses have been found to cause viral myocarditis and biochemical techniques have made identification of the causative virus increasingly possible. Furthermore, post-mortem analysis and *in vitro* experiments have shed light on the mechanisms by which viruses exert their detrimental effects on the myocardium despite the variety of mechanisms employed across the viral etiologies. Further work should continue to elucidate the modes of myocardial injury associated with each infection to shed light on the natural history of the disease as a whole and to provide potential therapies for both the acute and chronic complications of viral myocarditis.



	MAdV1	CVB3	EMCV	Influenza A	Parvovirus	Reovirus	MuLV	CVB3	Parvovirus	SIV
Age	•Neonatal and adult	•Juvenile	•Juvenile	•Juvenile	•Juvenile	•Neonatal	•Juvenile	•Juvenile	•Fetal and neonatal	•Fetal and neonatal
Use	• Pathology • Immune involvement • Tissue tropism	• Pathology • Immune involvement • Cellular remodeling	• Immune involvement • Cellular remodeling	• Pathology • Immune involvement • Cellular remodeling	• Viral pathogenesis • Immune involvement	• Viral pathogenesis • Cellular remodeling	• Immune response	• Pathology • Immune involvement	• Pathology	• Pathology • Immune involvement
Benefits	• Related to human adenovirus • High viral titers in heart • Recapitulates human VM	• Same virus infects humans • Recapitualtes human VM • Little species specificity • Most widely used model of VM	• Reliably induces VM • Myocardial lesions resemble those in humans • Can recapitulate features of DCM	• Same virus infects humans • Intranasal inoculation mimics human infection • VM and DCM resemble human disease	• Same virus infects humans	• Produced VM similar to that in humans	• Produces disease similar to human AIDS • DCM is induced	• Longer duration experiments possible • DCM is induced	• Useful for veterinary understanding of canine VM • May be similar to human VM	• Similar presentation to HIV in HIV+ individuals
Obstacles	• High mortality rate • Species-specific virus • Not exclusively cardiotropic • Only neonates adequately model VM	• Often not via route of natural infection • Multiple inoculations required, not mimicking natural viral persistence	• High mortality early after infection • CNS pathology may effect cardiac results	• Moderate-high mortality rate • Low-moderate rate of VM	• Some models use only viral capsid proteins which does not mimic natural infection	• Multiple organ systems affected • Virus hasn't been shown to cause human VM	• Virus does not infect humans • DCM produced more reliably than VM	• May not recapitulate electrical abnormalites found in the human heart secondary to VM	• Current use focuses on veterinary patients • Variable inoculation techniques	• May be costly and require special facilities

CVB3 = coxsackievirus B3; EMCV = encephalomyocarditis virus; MAdV1 = mouse adenovirus type 1; MuLV = LP-BM5 murine leukemia virus; SIV = simian immunodeficiency virus; VM = viral myocarditis; DCM = dilated cardiomyopathy

Figure 2.1 Animal Models of Viral Myocarditis. There are several animal models of viral myocarditis, many address the role the immune system plays in causes chronic disease. There are limited models on the arrhythmogenic potential of viruses in the myocardium.

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Chapter 2: Arrhythmogenic mechanisms of acute cardiac adenoviral infection

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Abstract

Myocarditis is the leading cause of sudden cardiac death in young adults, which viral infections being the most common causative agent. However, little is known how virus can induce an arrhythmogenic state in the heart. Viruses are known to alter gap junction intercellular communication in various cell types, except for cardiomyocyte coupling. Adenovirus is a leading cause of myocarditis, but due to being species-specific, it is not understood how adenoviruses cause sudden cardiac arrest. Mouse Adenovirus Type-3 (MAdV-3) was reported to be cardiotropic, yet has not been utilized to understand viral-mediated remodeling of cardiomyocytes that could alter cardiac conduction. We hypothesize that adenovirus infection alters intercellular communication in the heart, causing an arrhythmogenic substrate. We find that human adenovirus type-5 (HAdV-5) increases phosphorylation of Cx43-Ser368 and disrupts calcium handling in human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs). *In vivo* infection of MAdV-3 is cardiotropic in murine modeling and before an appreciable immune response, decreases conduction velocity and this correlates with increased phosphorylation Cx43-Ser368. In infected isolated adult mouse cardiomyocytes (ACMs), prolonged action potential duration is observed, with decreased I_{K1} and I_{Ks} current density. These findings show that adenoviruses create an arrhythmogenic substrate by increased phosphorylation and altered ion channel function that would cause sudden cardiac arrest.

Introduction

Viral infection of the heart and subsequent myocarditis causes significant morbidity and mortality in patients. Viruses have been recognized as a major cause of sudden cardiac death, with 42% of young adult patients having viral-associated myocarditis [1-3]. Viral myocarditis is classified into two distinct phases, the acute, which includes viral-mediated damage to the myocardium, which can progress to the second phase, chronic myocarditis [4]. Chronic viral myocarditis can progress to dilated cardiomyopathy (DCM) and heart failure and the later stages of chronic viral myocarditis are typically treated like other causatives of DCM in the clinic [5, 6]. However, it remains unclear how viruses in the myocardium cause sudden cardiac death before an appreciable immune response and development of chronic infection occurs. A better understanding of myocarditis during viral infection and replication stage could lead to therapies to prevent both the progression into chronic viral myocarditis and cases of sudden cardiac arrest that arise during the acute stage.

Several viruses have been identified to be causative agents of myocarditis, but progress of disease for many viral families remain elusive, due to the diversity of viruses and their mechanisms of subcellular modifications that allow for infection and spread during the acute stage. Common viruses identified in the myocardium after sudden cardiac death include: SARS-CoV-2, parvovirus B19, human herpesvirus 6, adenoviruses, and enteroviruses, with enteroviruses being the most common viral agent used in the research setting [7-9]. Coxsackievirus B3 (CVB3) is a non-linear, positive-sense RNA virus from the picornavirus family. CVB3 is both a common childhood illness and is a significant causative agent of viral myocarditis [10]. CVB3 infects across species, lending itself as a valuable research model. Historically, most viral myocarditis research has been conducted using CVB3 as the viral substrate with an emphasis on the chronic phase of disease [11]. However, RNA viruses use the host's cytosol for viral replication, whereas another significant causative agent of viral myocarditis, adenovirus, requires the host's nucleus for replication [12]. Differences such as these during the infectious stage of viral myocarditis illustrate the need to investigate virus family-specific disease progress and pathology.

Adenoviruses are non-enveloped, double-stranded DNA viruses comprising over 50 different serotypes causing a range of illnesses, from mild cold-like symptoms to more severe disease and death [13]. CVB3 and adenoviruses primarily utilize the Coxsackie and adenovirus receptor (CAR) for binding and viral entry into the host cells for replication and propagation [14, 15]. CAR is expressed in several tissue types, including the heart, in which, is primarily expressed on endothelial, epithelial cells, and cardiomyocytes and is essential in development [16, 17]. In cardiomyocytes, CAR is localized to the intercalated disc (ID) in ventricular cardiomyocytes, where CAR is an integral component of coupling cells together for electromechanical and metabolic signaling [18, 19].

The ID is a structure that creates a junction between two cardiomyocytes where gap junctions, ion channels, and scaffolding molecules stabilize the cell to allow for the propagation of small molecules and ions across the myocardium [20-22]. Subcellular remodeling at the ID impairs electrophysiological activity of cardiomyocytes and can lead to arrhythmogenesis, cardiac failure, and other pathologies [23-25]. Three main structures at the ID coupling cells are: adherens, desmosomes, and gap junctions, and along with the localization of ion channels like $Na_v1.5$, the ID is a complex structure with cross-talk between ions and small molecules [20, 21]. Changes to gap junction intracellular communication (GJIC) via remodeling or modifications to gap junctions are well-characterized to underlie arrhythmias and sudden cardiac arrest [26].

A rapid and modifiable mechanism of regulating structures at the ID is phosphorylation of proteins by major signaling pathways. A common outcome of increased phosphorylation of gap junctions and ion channels results in the temporary reduced function of that channel and/or junction [27]. Increased phosphorylation by Protein Kinase C (PKC) on KCNE1 can impair trafficking of KCNQ1/KCNE1 complex forming the ion channel, Kv7.1, and reduces I_{Ks} current [28, 29]. A similar mechanistic outcome of increased PKC activity on Connexin43 (Cx43), the most common gap junction protein in the human body, including the working myocardium, is reduced GJIC [30]. Phosphorylation represents a flexible and powerful target for electrical activity and communication between cardiomyocytes that is important in homeostasis but can lead to severe consequences on the myocardium, such as arrhythmogenesis.

Arrhythmias are the leading cause of sudden cardiac arrest in children and young adults [3, 7, 31]. Proper electrical conductance requires two major components: ion channels localized throughout the sarcolemma and the ID, and cell-to-cell coupling via gap junctions localized to the ID [32, 33]. The action potential (AP) generated from the coordination between these groups of ion channels and is subject to regulation through a variety of mechanisms, for example, ion flux and phosphorylation [32]. Ion channels like $Na_v1.5$, Kir2.1, and Kv7.1 create ion pores that are vital in creating the functional AP duration. Changes in the AP duration can be lethal, as these can lead to sudden cardiac arrest if uncorrected [34]. APs are then propagated between cardiomyocytes by their IDs to create the functional heartbeat, however, even small yet deleterious changes to the cardiomyocytes can alter action potential propagation, such as viral infection.

Adenoviral infection rapidly alter host-signaling cascades as a way to increase viral invasion and replication [35, 36]. For example, early in infection increased expression of Adenoviral protein E1A correlates with increased Protein Kinase C (PKC) levels, a family of kinases, activity, which is needed in endosome escape by the virus [37, 38]. However, increased PKC is known to phosphorylate a multitude of host proteins, such as $K_v7.1$ and Cx43 [28, 39]. It is unknown how adenoviral infection can affect phosphorylation of proteins such as these that are vital for cardiac function.

Human adenovirus serotype 2 and 5 (HAdV-2 and -5) are the most predominant causes of adenoviral myocarditis, however, there is a dearth of understanding regarding cardiotropism and viral-effect on the heart due to the host-pathogen species-specific nature of adenoviruses [13]. Because of this, adenoviral myocarditis research has been limited historically and in scope, as most of our current understanding comes from research using CVB3 and Mouse adenovirus type-1 (MAdV-1) [40-42]. MAV-1 has been typically used for modeling of adenoviral-induced respiratory disease and encephalitis [43, 44]. Prior studies have modeled chronic adenoviral myocarditis using MAV-1 to understand the role of viral-induced inflammation and macrophage recruitment during infection and the role age plays *in vivo* [40, 45].

While this research has contributed much to our understanding of viral infection and inflammation of the heart, previous studies show that there are viral-specific effects to the host cell at the molecular level, thus limiting the value of extrapolating viral-mediated effects, especially during acute myocarditis [46]. Additionally, previous research has primarily focused on the effects of the immune system generating damage to cardiac tissue, leaving the role of viral-mediated alterations to the host's cellular environment and electrophysiology of the heart largely uncharacterized. We have shown human adenovirus type-5 (HAdV-5) infection disrupts gap junction function *in vitro* within 12 hours post infection (h.p.i) in lung epithelial cells and in human induced pluripotent stem cell-derived cardiomyocytes (HiPSC-CMs). GJIC was

decreased during early infection, occurring with increased phosphorylation of Cx43 in lung epithelial cells [47].

In 2008, Mouse Adenovirus Type-3 (MAdV-3) was isolated and reported to be cardiotropic when mice were infected. MAdV-3 was found to be a distinct virus from MAV-1 and MAV-2, with tropism for cardiac tissue and substantial differences in early regions of the genome when compared to other *Mastadenoviruses* [48, 49]. However, there has been little research into adenoviral mechanisms that induce arrhythmogenesis. As acute infections are hypothesized to be a significant cause of sudden cardiac arrest, we propose the use of MAdV-3 as a novel acute adenoviral myocarditis model and characterize the effects of viral-mediated changes on subcellular cardiomyocyte remodeling and electrophysiological defects leading to an arrhythmogenic substrate. This work in acute mouse adenoviral infection is paired with studies in HiPSC-CMs using HAdV-5 to compliment human physiological relevance.

We demonstrate that acute HAdV-5 infection in iPSC-CMs increases Cx43-Ser368 phosphorylation, and this occurs concurrently with uncoupling of infected cells. *In vivo* infection with MAdV-3 is cardiotropic without an appreciable immune response 7 d.p.i., however, there is decreased conduction velocity and increased Cx43 phosphorylation in the heart. Infected isolated adult mouse cardiomyocytes have decreased I_{K1} and I_{Ks} currents that elongate action potential deration. Mouse adenovirus type-3 causes a primed arrhythmogenic state in the heart by decreasing conduction velocity and action potential deration during acute infection. These findings describe, for the first time, how adenoviral infection in the heart could cause sudden cardiac death.

Methods

Cell Culture

Normal murine mammary gland cells (NMuMG) (ATCC, Manassas, VA, USA) were maintained and passaged in Dulbecco's Modified Eagle Medium, high glucose, with L-glutamine (Genesee Scientific, San Diego, CA, USA) supplemented with 10% FBS (Gibco, Waltham, MA, USA), non-essential amino acids (Life Technologies, Carlsbad, CA, USA) and Mycozap Plus-CL (Lonza, Basel, Switzerland).

iPSC-CM differentiation, expansion, and maturation

iPSC (Cell Applications, San Diego, CA, USA) were differentiated into cardiomyocytes following per publication [50] with modifications. Briefly, iPSCs were seeded into matrigel coated 12well plates in E8 medium with daily medium changes for 3 days. On day 0, medium was changed to CDM3, defined by publication [51] with 3 μM CHIR-99021 (Selleckchem) for 2 days. On day 2, medium was changed to CDM3 with 2 μM Wnt-C59 (Cayman). On day 4, medium was replaced with CDM3. On day 6, medium was changed to RPMI1640 + B27 (- insulin) and refreshed on day 8. On day 10, medium was changed to RPMI1640 without glucose + B27 (- insulin). On day 12, cardiomyocytes were expanded as in [52]. Briefly, cells were lifted with TrypLE Select Enzyme 10X, washed with PBS 20% FBS and centrifuged at 200xg for 4 minutes, resuspended in RPMI 1640 + B27 1X with 10% Knock Out Serum Replacement (GIBCO) and Thiazovivin 1.0 μM (Selleckchem), and transferred into 10 cm dishes. 24 hours later medium was replaced with RPMI 1640 + B27 1X supplemented with 2 μM CHIR-99021. Cells were further expanded for 4 passages prior to cryopreservation. IPSC-CMs were verified by the presence of cardiac troponin-T, N-cadherin and contraction. Prior to experiments IPSC-CMs were matured in a defined maturation medium composed of oxidative substrates and low glucose for 3 weeks according to [53].

Virus propagation and titering

Mouse adenovirus Type-3 (a generous gift from D. H. Krüger, M.D., Ph.D., Institute of Med. Virology, University Hospital Charité, Berlin) was propagated in NMuMG cells. MAdV-3 was purified by PEGlyation and CsCl ultracentrifugation as previously described with slight modifications and titer was determined by immunofluorescence confocal microscopy in 3T6 cells (Thermo Fisher, Waltham, MA, USA) [54].

Ad5 was obtained from ATCC (Manassas, VA, USA) and propagated in A549 cells. AdlacZ was generated according to manufacturer's instructions from pAd/CMV/V5-GW/LacZ (Thermo Fisher, Waltham, MA, USA). Virus was purified by CsCl ultracentrifugation as previously described and titer was determined by immunofluorescence confocal microscopy in HEK293A cells [47, 54].

Adult mouse cardiomyocyte isolations

Adult mouse cardiomyocytes (ACMs) were isolated as previously described [55]. In brief, mice were anesthetized with isoflurane and received a 500 U heparin (Sigma, St. Louis, MO, USA) i.p. injection shortly after. Heart was excised from the chest cavity and cannulated within <4 min after aortic dissection with perfusion buffer. Perfusion buffer comprised of (mM) 120.4 NaCl, 12.7 KCl, 0.6 KH_2PO_4, 0.6 Na_2HPO_4, 1.2 $MgSO_4$-$7H_2O$, 4.6 $NaHCO_3$, 30 Taurine, 5.5 Glucose, 10 2,3-Butanedione 2-monoxime, and 10 HEPES brought to pH 7.4 with HCl was allowed to flow via gravity for 2-3 min. Digestion buffer comprised of Type II Collagenase at 2.4 mg/mL (Worthington Biochemical Corporation, Lakewood, NJ, USA) and perfusion buffer (above) was then perfused with $CaCl_2$ reintroduction towards end of solution flow. Aorta, vena cava, and atria were removed, and the ventricles were mechanical broken apart into single cells. Three stop buffers comprised of perfusion buffer and fetal bovine serum + $CaCl_2$ reintroduction steps were completed and isolated cells were plated on 100 µg/mL laminin (Gibco, Waltham, MA, USA) -coated dishes. Cardiac myocyte media (CMM) (ScienCell, Carlsbad, CA, USA) with provided cardiac myocyte growth supplement, 5% FBS, 50 mM 2,3-Butanedione monoxime (Acros Organics, Fair Lawn, NJ, USA), and MyocoZap Plus-PR (Lonza, Basel, Switzerland) was changed on cardiac myocytes at 2 hr post-plating. Cells were infected during this 2-hr media change. All infection experiments in isolated cardiomyocytes were concluded at 24 h.p.i..

Infections

In vivo infections

8-12 week old C57Bl/6 (Jackson Laboratory, Bar Harbor, ME, USA) male and female mice were anesthetized with isoflurane (VetOne) then infected via retro-orbital injection with MAdV-3 at 5×10^5 infectious units (i.u.) diluted in 0.9% sterile saline (Teknova, Hollister, CA, USA) using U-100 Insulin syringes (BD, Franklin Lakes, NJ, USA). Mock infection was performed with 0.9% sterile saline solution. Mice were monitored daily for weight, fur ruffling, and neurological pathology, and sacrificed if weight loss exceeded 25% of starting mass. All experiments were carried out under NIH guidelines and animal protocols were approved by the Virginia Tech Institutional Animal Care and Use Committee.

In vitro infections

All infections were performed at a multiplicity of infection (M.O.I.) of 10 in Cardiac Myocyte Media (ScienCell, Carlsbad, CA, USA) for isolated ACMs and media described above for iPSC-CMs. *In vitro* experiments were conducted at 24 h.p.i..

Echocardiography

Echocardiography was performed and analyzed using VisualSonics Vevo 660 High-Resolution Imaging System (Visual Sonics, Toronto, Canada) with a MS250 transducer at 7 days post-infection. Mice were anesthetized with 2% isoflurane with 2% supplemental O_2. Heart rate, electrocardiography, and left ventricle (LV) dimensions of ejection fractions (EF%), fractional shortening (FS%), left ventricular developed pressure (LVDP) at diastole were measured.

Histology

All histological preparation, complete blood counts and serum chemistry analysis were performed according to standard operating procedures in the Virginia Tech Animal Laboratory Services (ViTALs) diagnostic laboratory accredited by the American Association of Veterinary Laboratory Diagnosticians (AAVLD) at Virginia-Maryland College of Veterinary Medicine. Tissues for histology were fixed in 10% neutral buffered formalin for 24 hr, then trimmed, processed, paraffin-embedded, and cut with a microtome into 5 µm sections prior to staining with hematoxylin and eosin. Histopathologic examination was performed in a blinded fashion by a board-certified veterinary pathologist. Tissues evaluated included heart, brain, lungs, thymus, salivary glands, esophagus, tongue, eyes, spleen, adipose tissue, liver, kidneys, adrenal glands, pancreas, stomach, duodenum, jejunum, ileum, colon, skeletal muscle, bone, bone marrow, and haired skin.

DNA extraction and qPCR

Tissue was homogenized in TRIzol (Thermo Fisher, Waltham, MA, USA) and DNA was extracted according to manufacturer's instructions in combination with ethanol precipitation. For detection of viral genomes, quantitative PCR was performed with SYBR Select Master Mix for CFX (Thermo Fisher, Waltham, MA, USA) on a QuantStudio 6 Flex System (Thermo Fisher, Waltham, MA, USA). Cellular reference gene C1 primer forward- 5'-CCT TCC AGT TGA GTC AGT GG-3' reverse- 5'-CTG CTG CTG TTG GGT ACT TC-3'. For detection of MAdV-3 viral genomes MAdV-3 E1A primer forward- 5'-TCG TAC CCG CTT TTT CTG TT-3' reverse- 5'-AGG AGC AAT AAA AAT AAC CAA CCG-3'.

RNA extraction and RT-qPCR

Tissue was homogenized in TRIzol (Thermo Fisher, Waltham, MA, USA) and clarified by phenol-chloroform phase separation according to manufacturer's instructions. cDNA was generated with iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad) according to manufacturer's instructions. Real-time PCR was performed with SYBR Select Master Mix for CFX (Thermo Fisher, Waltham, MA, USA) on a QuantStudio 6 Flex System (Thermo Fisher, Waltham, MA, USA). Primers: *gapdh*, Mm.PT.39a.1 IDT; *hrpt* Mm.PT.39a.22214828 IDT; *cacna1c*, Mm.PT.58.8608981 IDT; *scn5a*, Mm.PT.58.30418114 IDT; *kcnh2*, Mm.PT.58.42865919 IDT; *cdh2*, Mm.PT.58.12378183 IDT; *pkp2*, Mm.PT.58.11914062 IDT; *tjp1*, Mm.PT.58.12952721 IDT; *gja1*, Mm.PT.58.5955325 IDT; *gjc1*, Mm.PT.58.8383900 IDT; *gja5*, Forward: 5'-TAG ACA CCC AGC TCG TAA GG- 3' Reverse: 5'- GGT GAC TTT GGA CAT TCC TG -3'.

Langendorff heart preparations and optical mapping

Cervical dislocation was performed upon loss of peripheral stimuli response and was immediately followed by thoracotomy and excision of the heart. Hearts were cannulated and Langendorff perfused within 4 min of excision, as previously described [56]. Hearts were perfused with a baseline crystalloid solution containing (in mM): 144.5 NaCl, 1.8 CaCl2, 5.5 NaOH, 4 KCl, 5.5 Dextrose, 1.0 MgCl2, 10.0 HEPES, 1.2 NaH2PO4. The perfusate was equilibrated to pH 7.4 using NaOH or HCl, as necessary, at 37.0 °C. A second perfusate was prepared containing the baseline solution with the addition of 26.1g/L mannitol. Solutions were bubbled with 100% O_2 for the duration of the experiment. Perfusions were run at a constant flow to maintain a pressure of ≈70-90mmHg, and the perfusates and bath were maintained at ~37 °C.

Following a 15 min stabilization period, all hearts (n=14) were perfused with the voltage-sensitive dye, Di-4-ANEPPS, at a concentration of 8 μM, and excess dye was washed out over a 20 min period. Images were acquired during perfusion with the baseline solution (after completion of dye washout), and then after 20 minutes of perfusion with 143 mM mannitol-containing solution. The electromechanical uncoupler blebbistatin (10 μM), as well as slight pressure on the posterior surface of the heart, were used to minimize cardiac motion and stabilize the heart against the glass of the imaging window. Hearts were paced with a unipolar silver wire placed on the anterior epicardium of the heart and a reference wire place in the back of the bath. Stimulation of 1.5x the threshold voltage was applied with a basic cycle length of 150 ms and 1 ms pulse duration. Hearts were paced for 30 sec before each image was collected. Action potentials were recorded as previously described [57]. Briefly, the anterior epicardium of the heart was exposed to a 520/35 nm band-pass filtered halogen light source (MHAB-150W, Moritex) reflected onto the heart via epi-illumination. Emitted light was collected via a tandem lens system and transmitted through a 610 nm long-pass filter (Andover Corp.) before detection by a MiCam Ultima L-type CMOS camera (SciMedia: 100x100 pixels, field of view – 6.25x6.25mm). Action potentials were recorded at a sample rate of 1 kHz for a duration of 2 seconds during intrinsic activity and steady state pacing. Conduction velocity (CV) was collected in the transverse and longitudinal direction (CVT and CVL, respectively), and was calculated as previously described [56]. Briefly, activation time for each pixel was designated as the maximum rate of rise of the optical action potential. Conduction in each direction was quantified by selecting vectors up to 5 pixels away and within an angle of ± 8° from a user-defined line indicating the direction of action potential propagation. Conduction vectors within 2 rows of the pacing site were excluded to reduce pacing artifacts. Data presented here are representative isochrone maps with 2 ms time steps.

Western blotting

Mouse hearts were lysed in RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100 (Sigma Aldrich, St. Louis, MO, USA), 1% sodium deoxycholate, 2 mM NaF, 200 μM Na_3VO_4, 0.1 % sodium dodecyl sulfate, 5 mM n-ethylmaleimide) supplemented with HALT protease and phosphatase inhibitor cocktail (Thermo Fisher, Waltham, MA, USA). Protein was homogenized and then clarified by sonication and centrifugation and concentration determined by DC protein assay (Bio-Rad, Hercules, CA, USA). 4X Bolt LDS sample buffer supplemented with 400 mM DTT was added to samples then heated to 70 °C for 10 min and subjected to SDS-PAGE using NuPAGE Bis-Tris 4-12% gradient gels and MES (Thermo Fisher, Waltham, MA, USA) running buffer according to manufacturer's instructions.

Proteins were transferred to PVDF (Bio-Rad, Hercules, CA, USA) membrane and fixed in methanol and dried. PVDF membranes were reactivated in methanol followed by blocking in 5% nonfat milk (Carnation, Los Angeles, CA, USA) or 5% bovine serum albumin (Fisher Scientific, Waltham, MA, USA) in TNT buffer (0.1% Tween 20, 150 mM NaCl, 50 mM Tris pH 8.0) for 1 h at room temperature. Primary antibody staining was performed at 4 °C overnight using the following antibodies: rabbit anti-Cx43 (1:3000; Sigma-Aldrich, St. Louis, MO, USA), mouse anti-GAPDH (1:2000; Santa Cruz Biotechnology, Dallas, TX, USA), rabbit anti-phospho-Cx43 (Ser368) [D6W8P] (1:1000; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-Cx43 (Phospho-Ser373) (1:1000; Singalway Antibody, College Park, MD, USA). Membranes were washed six times with 1X TNT before secondary antibody labeling. Membranes were labeled for 1 h at room temperature with secondary antibodies conjugated with Alexa Fluor 555 or 647 (1:5000; Thermo Fisher, Waltham, MA, USA) or HRP (1:5000; Abcam, Cambridge, UK). Membranes were stripped for 30 minutes in ReBlot Plus Strong Antibody Stripping Solution before reprobing (Millipore Sigma, Burlington, MA, USA). Membranes were imaged by a Chemidoc MP imaging system (Bio-Rad, Hercules, CA, USA).

Patch clamping

Electrophysiological recordings from dissociated cardiomyocytes were performed at room temperature using whole-cell voltage-clamp and current-clamp techniques. Recordings were made with an Axopatch 200B amplifier, Digidata 1550B digitizer, and pClamp 11 software (Molecular Devices) with a 20 kHz sampling rate and low-pass filtering at 5 kHz. Series resistance was monitored and compensated at >70% during voltage-clamp recordings. Recording electrodes with a tip resistance of 2-4 MΩ were pulled from borosilicate glass. Action potentials and potassium currents (I_{K1}, I_{Ks}, and I_{Kr}) were recorded with an internal pipette solution containing (in mM) 120 K-aspartic acid, 20 KCl, 10 NaCl, 2 MgCl2, and 5 HEPES brought to pH 7.3 with KOH, and extracellular solution containing (in mM): 120.4 NaCl, 12.7 KCl, 0.6 KH_2PO_4, 0.6 Na_2HPO_4, 1.2 $MgSO_4$-$7H_2O$, 4.6 $NaHCO_3$, 30 Taurine, 5.5 Glucose, and 10 HEPES brought to pH 7.4 with HCl. Sodium current (I_{Na}) and calcium currents were recorded with an internal pipette solution containing (in mM) 120 $CsMeSO_3$, 20 tetraethylammonium chloride (TEA-Cl), 2 $MgCl_2$, 10 HEPES, 10 EGTA, and 4 Mg-ATP brought to pH 7.3 with CsOH. Sodium currents were recorded in extracellular solution containing (in mM) 25 NaCl, 120 N-methyl-D-glucamine (NMDG), 1.8 $CaCl_2$, 1.8 $MgCl_2$, 10 glucose, and 10 HEPES, pH 7.3, supplemented with 1 μM nisodipine to block voltage-gated calcium channels. Calcium currents were recorded in extracellular solution containing (in mM): 137 NaCl, 5.4 CsCl, 1.8 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 HEPES, pH 7.3, supplemented with 10 μM TTX to block voltage-gated sodium channels.

Action potentials were recorded in current-clamp mode and elicited by a 2 ms current pulses between 200 pA to 2 nA at 1 s intervals. Action potential shape analysis was performed on action potentials elicited in response to the lowest current injection using the Action Potential module in Clampfit 11 (Molecular Devices). I_K, I_{Na}, and I_{Ca} were recorded in voltage-clamp mode, and the 10 mV liquid junction potential was corrected for during the recordings. IK_1 was elicited from a holding potential of -40 mV with 2.5 s depolarizing voltage steps from -100 mV to 0 mV and quantified by measuring the peak steady-state current amplitude during the voltage steps. To measure I_{Ks} and I_{Kr}, cardiomyocytes were held at a holding potential of -40 mV holding potential, given depolarizing voltage pulses from -30 to 60 mV for 2.5 s, and then stepped back to -40 mV in the absence and presence of 5 μM E4031. I_{Ks} was determined by measuring the

peak amplitude of the tail current in the presence of E4031, and I_{Kr} was determined by measuring the peak amplitude of the E4031-sensitive current. I_{Na} was elicited from a holding potential of -90 mV with 20 ms depolarizing steps from -80 mV to +60 mV. I_{Ca} was recorded from a holding potential of -40 mV with 300 ms depolarizing steps from -30 mV to +60 mV. I_{Na} and I_{Ca} were determined by measuring the peak current amplitude relative to baseline during the voltage steps. Three runs were completed per cell for all recordings.

Immunocytochemistry

Isolated ACMs were fixed with -20°C methanol for 5 min, followed by three washes of PBS. Cells were blocked with 5% normal donkey serum (Invitrogen, Carlsbad, CA, USA) and 0.1 % Triton X-100 (Sigma Aldrich, St. Louis, MO, USA) for HiPSC-CMs and 0.5% Triton X-100 for ACMs in PBS for 1 hr at room temperature. Primary antibody labeling was performed for 1 hr at room temperature using; mouse anti-Cx43 (1:500; EMD Millipore, Burlington, MA, USA) and rabbit anti-phospho-Cx43 (Ser368) [D6W8P] (1:250; Cell Signaling Technology, Danvers, MA, USA). Cells was washed 6 times prior to secondary antibody labeling for 1 hr at room temperature with secondary antibodies conjugated to AlexaFluor 488, AlexaFluor 555 (1:500; Thermo Fisher, Waltham, MA, USA). During secondary antibody labeling, cells were counterstained with DAPI and wheat germ agglutinin (WGA) conjugated AlexaFluor 647 (1:500; Thermo Fisher, Waltham, MA, USA). Slides were kept in PBS for imaging. Confocal imaging was performed on Nikon SoRa Spinning Disk confocal and image processing was done in Fiji software [58].

Statistics

All quantification was performed on experiments and repeated at least three times (see figure legends for specific replicate values). Echocardiography analysis was performed by a blinded investigator. Data are presented as mean ± SEM. Statistical analysis was conducted with GraphPad Prims 8.0.2 (GraphPad Software, Inc. La Jolla, CA). Data were analyzed for significance using Student's t-test or one-way ANOVA correcting for multiple comparisons. A value of $p<0.05$ was considered statistically significant.

Results

Human Adenovirus Type-5 increases Cx43 pS368 and disrupts calcium handling 24 h.p.i. in iPSC-CMs.

Previously, we found that HAdV-5 infection increased Cx43 phosphorylation, downregulated total Cx43 protein levels by Adenoviral protein E4orf1 causing translocation of host's β-catenin from the cell membrane to the nucleus in HaCaT cells, immortalized human keratinocytes, and remodeled the colocalization of Cx43/ZO-1 in iPSC-CMs [47]. Building on these findings, we asked if gap junction function was changed in the context of adenoviral infection in cardiac systems. HiPSC-CM were cultured and infected with human adenovirus type-5 (HAdV-5) or with HAdLacZ, a replication incompetent human adenovirus vector to serve as a control virus. Western blotting of iPSC-CMs 24 h.p.i. with HAdLacZ or HAd5 at an MOI 10 showed a significant increase of phosphorylated Cx43 at Ser368 residue **(Figure 3.1, quantified 3.1B**). Phosphorylation at this residue is known to reduce gap junction opening probability [39, 59].

Mouse adenovirus type-3 is cardiotropic and does not invoke an appreciable immune response 7 d.p.i.

Given the arrhythmogenic implications of our *in vitro* cardiac HAdV-5 infection caused a decrease in conduction velocity during live calcium imaging, understanding the role of adenovirus in altering cardiac function *in vivo* is critical. We validated the previous finding that MAdV-3 viral genomes are significantly enriched in heart tissue 7 days post-infection (d.p.i.). Using C57BL/6 male and female mice, 8-12 weeks of age, we administered $5x10^5$ i.f. via retro-orbital injection and quantified MAdV-3 viral genomes across major organs. Consistent with previous publications, MAdV-3 viral genomes are significantly enriched in the heart (**Figure 3.2A**). Viral time course over 7 days was performed as before in heart tissue. We find that viral genomes are detected throughout the time course of experiments (**Figure 3.2B**). Unlike chronic viral myocarditis patients, acute myocarditis patients do not typically present with significant inflammation and/or dilated cardiomyopathy (DCM) [60]. Using echocardiography, there were no detectable changes in ejection fraction, left ventricle developed pressure at diastole, or fractional shortening, indicating no changes to gross cardiac structure (**Figure 3.2C**). Infiltration of leukocytes into the infected tissue and development of lesions is a hallmark of chronic infection which can be measured by histopathology [61]. This symptom is not typically observed in cases of acute viral myocarditis [62]. Histopathology was performed on hearts and no gross pathology changes were reported in cardiac tissue (**Figure 3.2D**). Gross spleen weight was increased in infected mice, which is consistent with an immune response mounted towards infection and associated with the increase in spleen node size in infected mice (**Figure 3.S1A & B**). However, there was no detectable cardiac troponin enzymes measured in the circulating serum of infected mice, which is elevated in roughly one-third of myocarditis patients (**Figure 3.S1C**) [63]. As there was no appreciable immune cell infiltration into cardiac tissue, nor gross structure change at least at 7 dpi, delineating viral-mediated remodeling of cardiac tissue is more straightforward than parsing out immune-mediated effects.

Acute adenoviral cardiac infection reduces conduction velocity.

As adenoviral DNA was significantly enriched in the heart 7 d.p.i. and no gross structural changes, we turned to measuring basal cardiac electrical activity *in vivo* to ask if cardiac infection altered the electrophysiology of the mice. Using electrode pads, baseline electrical activity was measured in lightly anesthetized mice. No significant difference in the PR, QRS, and QT interval in MAdV-3-infected mice compared to mock-infected was measured (**Figure 3.3A**). Although, there was a significant increase in the RR interval in infected mice (**Figure 3.S2A**).

Defects in conduction velocity are known to predicate arrhythmias [64, 65]. To identify the effect of MAdV-3 infection on conduction velocity, 8-12 week old male and female mice were mock- or MAdV-3-infected and then hearts were isolated for optical mapping (n=8,9). CV_L and CV_T (conduction velocity longitudinal; transverse) were measured during steady-state perfusion of hearts. Representative isochrones illustrate the decreased conduction velocity in the transverse direction in MAdV-3-infected hearts compared to mock-infected hearts (**Figure 3.3B**). CV_T was significantly decreased in MAdV-3-infected hearts via Student's t-test comparison (38.75 ± 2.60 and 29.33 ± 2.88 cm/s, mean ± SEM, respectively) (**Figure 3.3C**). No significant change in CV_L was detected upon MAdV-3 infection (**Figure 3.S2B**). Reduction in ventricular conduction velocity is well described in the literature to predispose the heart to arrhythmias [66, 67]. A significant mechanistic cause for conduction velocity defects are due to

changes in gap junction function and/or expression, which changes to gap junction proteins preceding decreases in conduction velocity leading to an arrhythmogenic substrate [68, 69].

Acute adenoviral infection elongates action potential duration in isolated ACMs.

Conduction velocity in the heart is the summation of billions of action potentials across the myocardium, and our finding that acute MAdV-3 infection decreased conduction 7 d.p.i., there could also be viral-mediated impacts on the electrophysical properties of individual cardiomyocytes. Organized and tightly regulated ion channels in cardiomyocytes create action potentials. Because changes in ion channels precede arrhythmias, we performed RT-qPCR in cardiac tissue 7 d.p.i. to measure mRNA in infected ventricles. Ion channels *Cacna1c* ($Ca_v1.2$) and *Scn5a* ($Na_v1.5$) transcripts are significantly reduced in the infected heart, however, *Kcnh2* (hERG) was not reduced in infected cardiac tissue (**Figure 3.4A**). Because of the reduction of several ion channel mRNAs *in vivo*, we performed patch clamping experiment to investigate if acute adenoviral infection alters the electrical activity of cardiomyocytes. MAdV-3 24 h.p.i. causes a prolongation of Phase 3/4 in the repolarization of a cardiomyocyte and an increase of APD90 (**Figure 3.4B, 3.4C**). With arrhythmogenic prolonged action potential duration, voltage-gated patch clamping for individual ion channel currents were then measured in infected cardiomyocytes.

MAdV-3 infection decreases potassium current density 24 h.p.i. in cardiomyocytes.

Given the prolonged action potential, we assessed the properties of individual currents during MAdV-3 infection. We find no significant difference in I_{Na} and I_{Ca}, even as these mRNA transcripts were downregulated in our *in vivo* model (**Figure 3.5A&B**). MAdV-3 infection did significantly decreases I_{Ks} tail current, but not I_{Ks} peak current, and I_{K1} peak current density with no changes to I_{Kr} peak current (**Figure 3.5C**), raw current traces of I_{Ks} and I_{K1} (**Figure 3.S3A&B**). Importantly, both *in vivo* and *in vitro* infection illustrate the generation of an viral-mediated arrhythmogenic substrate, absent of an appreciable immune response.

Gap junctions are remodeled during acute *in vivo* MAdV-3 infection.

Abnormal conduction velocity is often associated with alterations of gap junctions and uncoupling of cardiomyocytes [21, 68, 70]. Using RT-qPCR, we measured mRNA levels of the major gap junctions and scaffolding proteins found in the working myocardium. *Cdh2* and *Tjp1* were decreased in acute infection in cardiac tissue, and *Gja1* and *Gjc1* were also decreased (**Figure 3.6A**). Changes were not significant in, *Pkp2*, and *Gja5*, interestingly in cardiac tissue. Using Western Blotting, we probed for protein levels of Cx43, based on the decrease of mRNA, however, in infected cardiac tissue, no change in Cx43 levels were detected at 7 d.p.i. (**Figure 3.6B, quantified 3.6C**).

Because of changes in conduction velocity but not overall change in Cx43 expression in cardiac tissue, we assessed post-translational modifications of Cx43. Alterations of phosphorylation at Cx43 S368 are known to cause change conduction velocity and the development of reentrant arrhythmias. Increased phosphorylation at S368 caused by PKC results in reduced open channel probability [39, 71]. With a decrease in conduction velocity, we turned to phosphorylation events at Cx43 that could lead to this arrhythmogenic phenotype in MAdV-3-infected mice. Western blotting in ventricle tissue harvested from mice infected 7 d.p.i. showed increased pCx43Ser368 when compared to total Cx43 protein levels, we find a 313% increase of phosphorylated Ser368 (**Figure 3.6D, quantified in Fig. 3.6E**).

Discussion

Adenoviruses are a leading causative agent of myocarditis in young adults and children [60]. Although there has been little study on how viruses evoke arrhythmias, adenoviral genomes have been detected in cardiac tissue in cases of sudden cardiac arrest where gross structural change of the myocardium is not observed [72]. We report that, prior to an appreciable immune response in cardiac tissue; adenoviral infection decreases potassium current density leading to prolonged action potential duration with concurrent reductions in gap junction intercellular communication affecting conduction.

Viral remodeling of host pathways during infection is diverse and generally virus-specific. Viruses hijack host machinery to allow for viral replication and hinder host's stress and immune responses [73, 74]. Previous research has shown that cGAS, a signaling molecule for anti-viral response, can pass from one cell to a connected cell, and this can prime an anti-viral state in an uninfected cell [75]. Previously, we have reported that human adenoviral E4orf1 can decrease Cx43 protein expression through transcriptional repression via β-catenin, resulting in decreased gap junction intercellular communication in human epithelial cells [47]. In addition to reduced Cx43 expression, there was an increase in phosphorylation of Cx43 C-terminal sites known to be modified by AKT and PKC, retaining Cx43 at the cell membrane and reducing GJIC in cells infected with HAdV-5 [47]. We sought to build on these findings by investigating the effects of viral modification of GJIC in the whole heart using a cardiotropic adenovirus with the aim of better understanding the role of infection in inducing an arrhythmogenic substrate.

We performed optical mapping on hearts infected with the cardiotropic adenovirus MAdV-3. Slow conduction velocity is a well-known arrhythmogenic determinant and is closely linked to gap junction function in the heart [70, 76, 77]. We report that adenoviral infection causes decreased conduction velocity in ventricles of mouse hearts (Figure 3.3C). Previous publications have demonstrated that uncoupling of cardiomyocytes favors a decreased conduction velocity in the transverse direction [77, 78]. This phenomenon is due to the higher density of gap junctions per a myocyte area in the transverse direction, as such, the increased intercellular junctions at a given area result in increased resistance, and decreased velocity [79-82]. Thus, reductions in conduction velocity in the transverse direction point to loss and/or alteration of gap junction function and can precipitate arrhythmias. This propensity supports our observations of a decrease of CV_T in infected mice but no change in CV_L as being due to gap junction dysfunction.

Other viral agents have been shown to alter intercellular communication by way of phosphorylation of connexins during infection. Various RNA viruses are known to decrease intercellular communication during disease, such as herpes simplex virus (HSV), feline immunodeficiency virus, and bovine papillomavirus [83-85]. Herpes simplex virus type-2 infection in rat liver epithelial cells increased serine phosphorylation 24 h.p.i. and reduce GJIC, resulting in eventual loss of Cx43 localization at the membrane [85]. In murine fibroblasts, Rous sarcoma virus, an oncogenic virus, increased phosphotyrosine and phosphoserine and reduced GJIC [86]. While changes to connexin phosphorylation caused by acute viral infection is most commonly studied in the context of cancer biology, changes to gap junction phosphorylation will also impact cardiomyocyte coupling.

With the decreased conduction velocity and increased phosphorylation at Cx43-Ser368 in the heart during adenovirus infection, this is in line with previous research of the interplay between viruses and PKC signaling [87, 88]. Given that PKC isoforms are known to regulate

many endosomal functions, such as endocytosis and trafficking from the membrane, it is unsurprising that many viruses target this pathway for host cell invasion [89]. The direct interaction with PKC family members is vital for influenza assembly, replication machinery function, and endosomal exit [90, 91]. For example, West Nile virus replication is inhibited when PKC activity is blocked [87]. Viral fusion of Respiratory Syncytial Virus (RSV) requires PKCα membrane translocation [92]. Thus, modification of the PKC family is a common mechanistic target across viral families. However, these are master regulatory pathways, and altering their activity has severe effects on the host's cellular mechanisms.

Adenoviral E1A is the first protein expressed in the adenoviral life cycle and increased protein levels are correlated with increased levels of viral protein along with increased PKC protein levels [38]. However, the E1A activation of PKC signaling in adenoviral early infection needs further delineation. PKC and downstream protein signaling regulate the cell cycle, which has been well-studied in both the fields of cancer and immune cell proliferation [93, 94]. PKC exerts control over many cyclin genes, a particular note Cyclin D, via transcription and thus can uncouple cells to start cell cycle from S phase [95]. Adenoviruses push cells into S phase via E1A, E1B, and E4 protein direct interaction with host mechanisms, and this is required for viral replication [96-98]. Cardiomyocytes, themselves, are typically quiescent several days after birth, and therefore, are particularly recalcitrant to entering the cell cycle [99]. Adenoviruses that infect the myocardium may disrupt such cell cycle arrest and cause arrhythmogenesis via activation of AKT/PKC pathways, for example, during infection.

Prolonged action potential duration (APD) is also an arrhythmogenic substrate that can cause early and/or delayed afterdepolarization in cardiomyocytes, leading to tachycardia/fibrillation [100]. The APD is determined by several distinct cardiomyocyte ionic currents such as sodium (I_{Na}), calcium (I_{Ca}), together with several potassium currents including I_{K1}, I_{Kr}, and I_{Ks}. This study provides evidence that adenoviral infection prolongs action potential duration, through decreased I_{K1} and I_{Ks} current densities in adult ventricular cardiomyocytes, further perturbing ventricular electrophysiology in addition to conduction delay (**Figure 3.4 & Figure 3.5).**

While there is a paucity of research investigating the effects of viral infection on action potential duration, turning to models of Long QT syndromes (LQT), we can interpret how alterations to these currents contribute to sudden cardiac arrest. Anderson-Tawil syndrome (LQT7) is caused by a monogenic defect in *KCNJ2* and presents as ventricular arrhythmias, prolonged QT intervals, together with physical malformations [101, 102]. The ventricular arrhythmias in LQT7 are due to defects in *KCNJ2*, the protein responsible for the Kir2.1 inward rectifier K+ current of I_{K1}, which lead to spontaneous action potential durations and early/delayed afterdepolarizations [102-104].

The KCNQ1 and KCNE1 proteins form the Kv7.1 channel, creating the slow activating delayed rectifier current, I_{Ks} [105]. Genetic modeling of LQT1 syndrome by loss-of-function in KCNQ1 causes decreased I_{Ks} that result in ventricular arrhythmias and sudden cardiac death [106]. Several mutations causing LQT1 impair trafficking of KCNQ1 to the cell surface, which could inform future directions in understanding effects on I_{Ks} we have identified during viral infection [107].

Viral altering of ion channel function has previously been described across different viral agents and host cell types. In a cardiac system model, CVB3 viral titers were increased in the mayday mutant murine model; these mice are deficient in Kir6.1, a subunit of K_{ATP} channels, and had decreased survivability after infection [108, 109]. Additionally, CVB3 infection in

Xenopus oocytes modified calcium channel currents in patch clamp experiments. Additionally, hERG and $Ca_v1.2$ ion channels were redistributed in murine myocardium 4 d.p.i. and continued until 12 d.p.i [41]. Outside of the heart, patch clamping experiments of neuroblastoma cells that were infected with rabies virus displayed decreased I_{Kir} and I_{Na} amplitude 3 d.p.i with no observable morphological changes [110]. While many of these studies lack a viral mechanistic cause of the change in K^+ currents, it is plausible to consider this as a viral advantage, as viruses across different realms alter ionic currents.

We find that adenoviral infection increases phospho-Ser368 Cx43 both during *in vivo* and *in vitro* infection (**Figure 3.1A & Figure 3.6D**). This Cx43 phosphorylation event occurs in concert with both uncoupling and altered calcium handling in iPSC-CMs *in vitro* and decreased conduction velocity *in vivo* (Figure 3.1C). Huke *et al.* reported a relationship between reduced intercellular coupling of cardiomyocytes, the development of focal arrhythmias, and increased phosphorylated Cx43 [111]. PKC is understood to be responsible for phosphorylation of Cx43 at Ser368 and results in reduced channel opening probability [39, 71, 112]. PKC signaling is also known to regulate I_{Ks} by promoting Kv7.1 endocytosis through increased phosphorylation of KCNE1 [28]. When broadly looking at the role of kinases during ischemic stress and heart failure, PKC signaling has been found to be sufficient in I_{K1} reduction [113]. The commonality between negative regulation of Cx43 and I_{K1} and I_{Ks} currents by PKC suggests that a potential common mechanism is activated during viral infection concurrently, and dangerously, affecting distinct arms of cardiac electrophysiology.

Viral targeting of potassium channel function may be advantageous for replication and infection. Potassium channels are ubiquitously found across almost all cell types, including non-excitable cells. This included the epithelium, a common entry point for most viruses, and other cell types such as immune cells, such as macrophages and T-cells [114-117]. K^+ channels in the epithelium are localized along the basolateral membrane, coincident with the CAR and integrin receptors utilized by human adenoviruses for entry [115].

Mouse adenovirus Type-1 has previously been employed in understanding the role of adenoviral-specific cardiac infection. The Weinberg group has shown significant inflammation of cardiac tissue, immune cell infiltration, and cardiac output during chronic MAdV-1 infection [40, 45]. This response occurred along with high levels of IFN-γ and the immunoproteasome induced in a dose-dependent and inverse age-dependent manner that followed IFN-γ levels [40, 45, 118]. These models are beneficial to understand the role of immune-mediated adenoviral response that also afflicts the respiratory system [119]. Another common virus utilized for myocarditis modeling are reoviruses, ds RNA viruses, which do not typical cause disease in humans but cause myocarditis in neonate animals [120]. Acute reovirus infection causes cytopathic effect specifically in cardiomyocytes, as compared to fibroblasts or epithelial cells [121]. This increases inflammation and infiltration of host immune cells, which is a common pathogenesis found in CVB3 infection [122]. Because of the overlapping primary host cell viral entry receptor of CAR, CVB3 infection has also been a valuable tool for understanding chronic cardiac myocarditis. However, when turning to the arrhythmogenic potential of viral infection, understanding subcellular modifications of infected tissues is key. MAdV-3 represents a powerful viral tool with which to understand how adenoviral infection remodels cardiomyocytes and the myocardium to induce an arrhythmogenic substrate. By combining MAdV-3 infection studies with human iPSC-CM infection of HAdV-5, we can answer questions as to how adenoviruses cause sudden cardiac death in young adults and test novel therapies to prevent these outcomes.

Acknowledgments

The authors thank Dr. Sarah Barrett (Virginia-Maryland College of Veterinary Medicine) for performing the organ histopathology. This work was supported by a NIH NHLBI R01 grant (HL132236 J.W.S.) and NHLNI F31 grant (HL152649 R.L.P.).

Author contributions

J.W.S. and R.L.P. designed this study. R.L.P. performed all infections, viral propagation, PCR, confocal, western blotting, troponin ELISA. G.A.B. performed the optical mapping and analysis. M.N.T. performed and analyzed the echocardiograms. S.A.S. performed and analyzed the patch clamping experiments. J.W.S. performed the cytokine array and live cell imaging analysis. J.W.S. and R.L.P. analyzed the data and interpretation. R.L.P. composed the manuscript and along with J.W.S. revised the manuscript.

Declaration of interests

The authors declare no competing interests.

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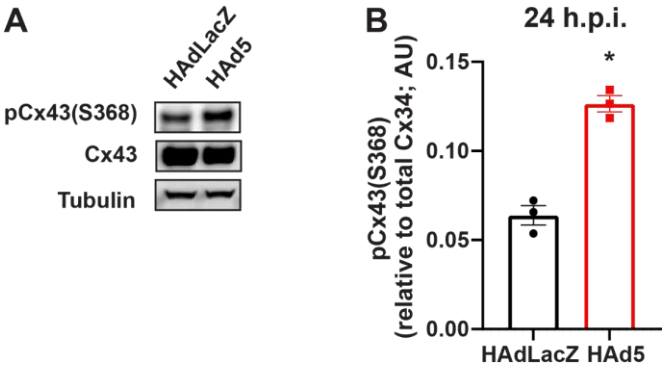

Figure 3.1 Human Adenovirus Type-5 increases Cx43 pS368 and disrupts calcium handling 24 h.p.i. in iPSC-CMs. iPSC-CMs were infected with HAdV-5 or HAdLacZ at an MOI 10. A) Western blotting of ventricle tissue probed for pCx43(Ser368) and total Cx43 to measure phosphorylation during infection, GAPDH serves as a loading control. B) Densitometry analysis of A. p≤0.5, **p≤0.01, ***p≤0.001, ****p≤0.0001, Student's T-test. Data are represented as mean ± SEM.

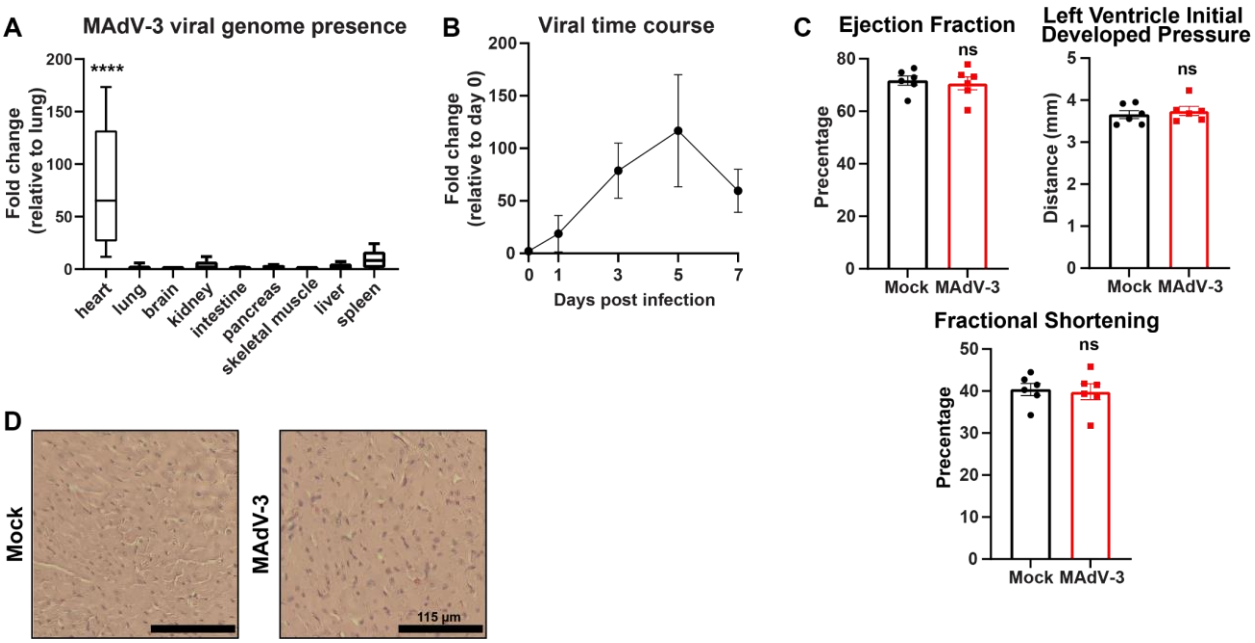

Figure 3.2 Mouse adenovirus type-3 is cardiotropic and does not invoke an appreciable immune response 7 d.p.i. Mice were infected $5x10^5$ i.u./mL and tissue was harvested 7 d.p.i. A) Using quantitative PCR, MAdV-3 viral genomes were measured in major organs and compared to organ lung tissue as a reference. (n=12) B) A viral time course of MAdV-3 viral genomes in heart tissue using qPCR. (n=3) C) H&E staining on cardiac sectioning to detect immune cell infiltration in mock- and MAdV-3-infected hearts. (n=6) D) Echocardiography was used on lightly anesthetized mock- and MAdV-3-infected mice which cardiac function was measured. (n=6). *p≤0.5, **p≤0.01, ***p≤0.001, ****p≤0.0001, One-way ANOVA, Student's T-test. Data are represented as mean ± SEM.

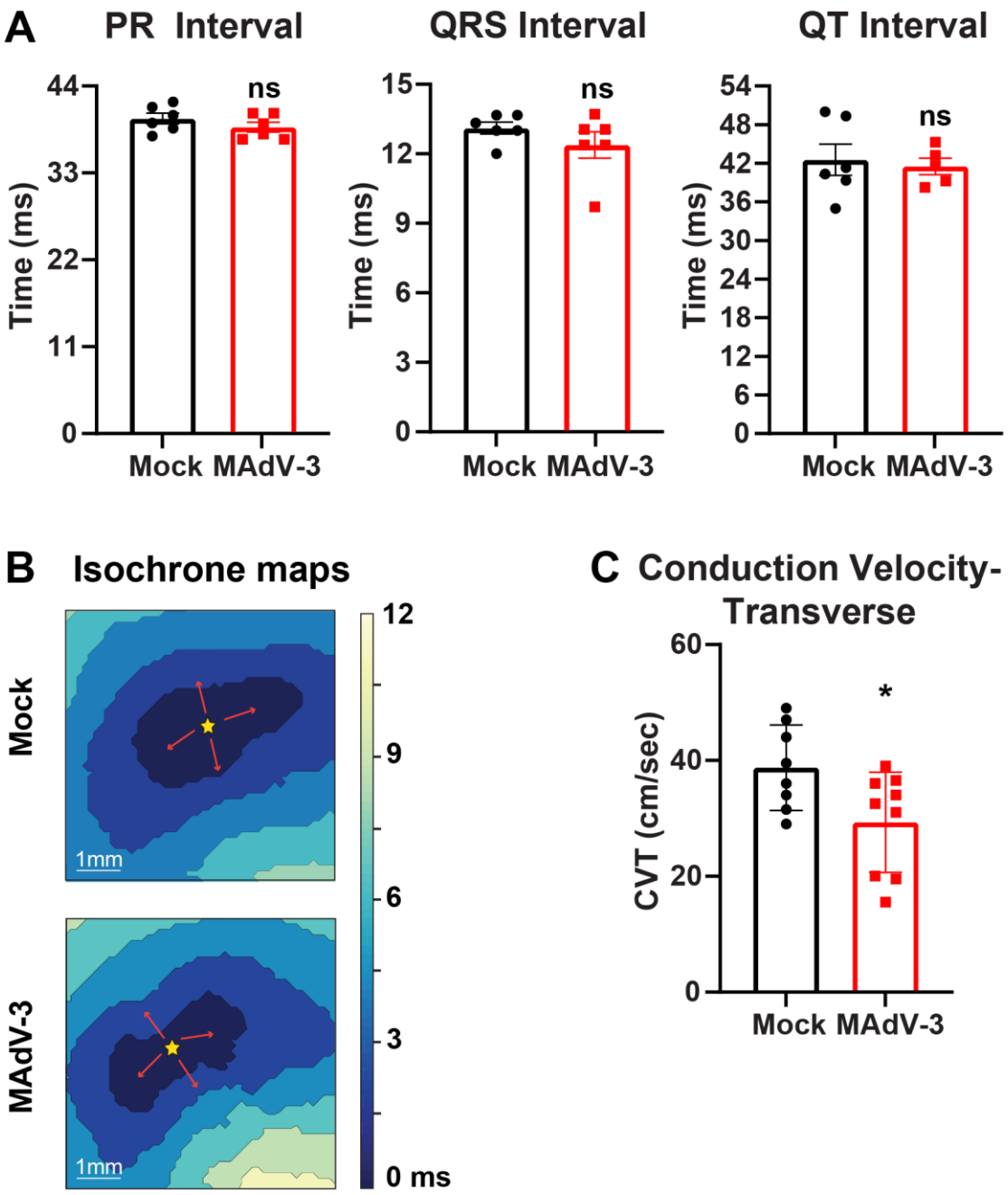

Figure 3.3 Acute adenoviral infection reduces conduction velocity. *In vivo* electrocardiography or ex vivo optical mapping was performed 7 d.p.i. in adult mice either mock- or MAdV-3-infected. A) *In vivo* electrocardiography measurement were taken on lightly anesthetized mock- and MAdv-3-infected mice using paw pad electrodes. (n=6). B) Conduction velocity was measured using optical mapping on ex vivo heart from infected mice. Example isochrones maps with the MAdV-3 map paced at a cycle length of 150 ms with 1 ms pulse duration. C) Conduction velocity in the transverse direction (CV_T) was quantified in mock- and MAdV-3-infected hearts 7 d.p.i. (n=8,9). *p≤0.5, **p≤0.01, ***p≤0.001, ****p≤0.0001, Student's T-test. Data are represented as mean ± SEM.

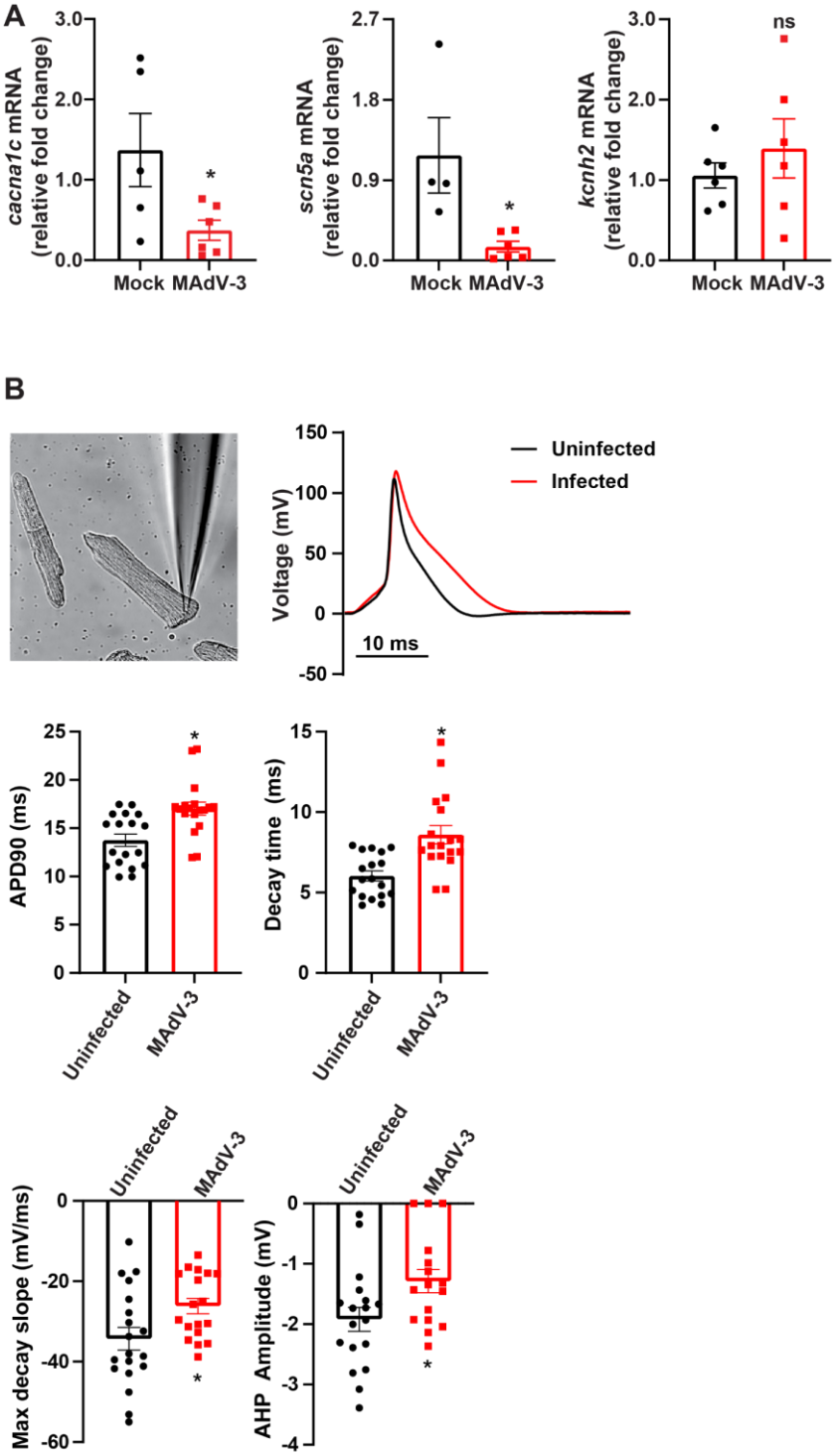

Figure 3.4 Acute mouse adenovirus infection prolongs cardiac action potential duration. Mice or isolated ACMs were infected with MAdV-3 at an i.u./mL of 5×10^5 or MOI 10 and was harvested or patch clamped at 7 d.p.i. *in vivo* or 24 h.p.i. *in vitro*. A) RT-qPCR of heart tissue for cardiac ion channels at 7 d.p.i. in ventricle tissue (n= 4: Mock, n=6: MAdV-3). B) Action potential duration data collected on uninfected and MAdV-3-infected isolated ACMs. *p≤0.5, **p≤0.01, ***p≤0.001, ****p≤0.0001, Student's T-test. Data are represented as mean ± SEM.

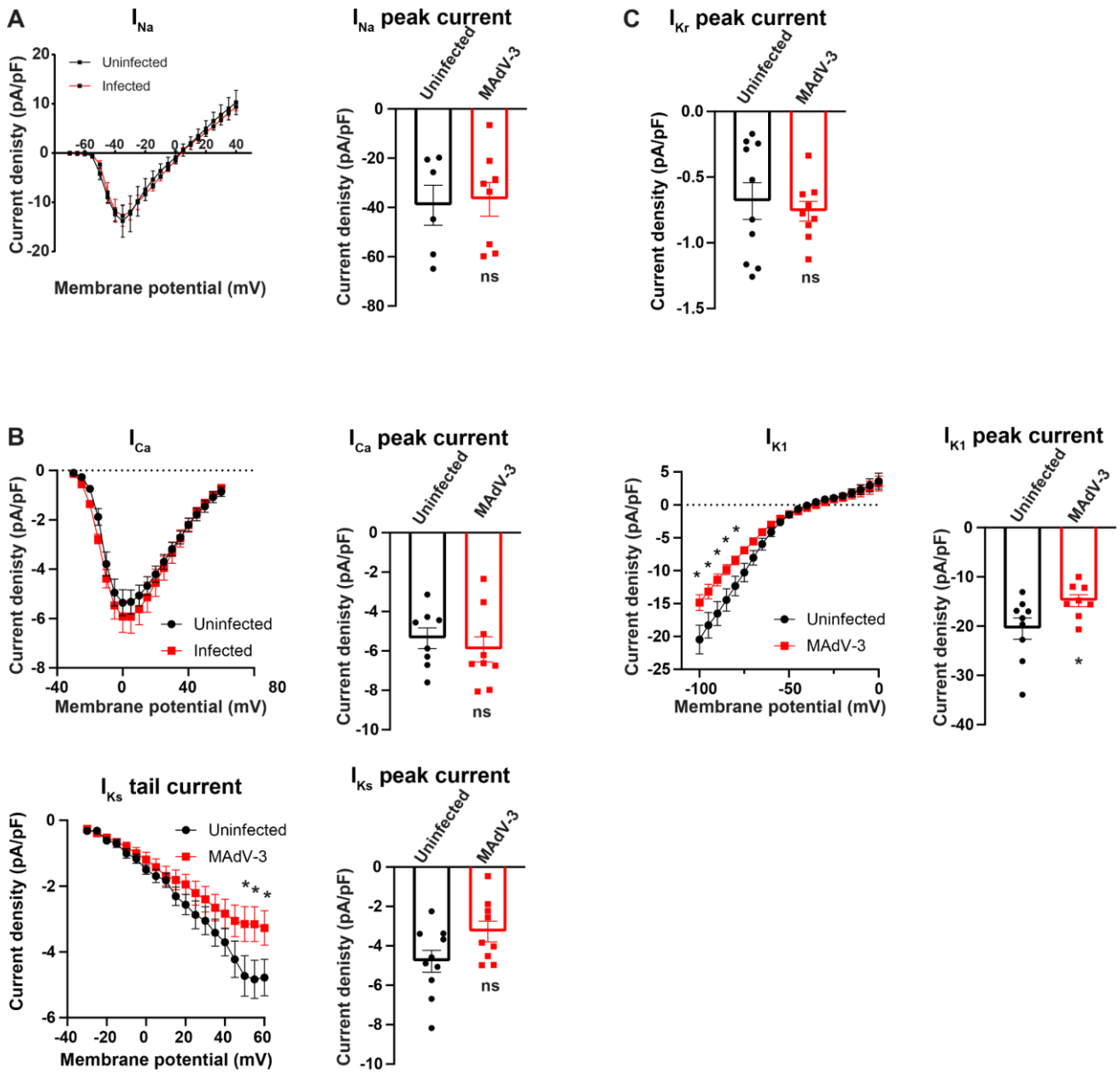

Figure 3.5 Acute mouse adenovirus infection decreases IK1 and IKS current density in isolated adult mouse cardiomyocytes. Mice or isolated ACMs were infected with MAdV-3 at an i.u./mL of 5×10^5 or MOI 10 and was harvested or fixed at 7 d.p.i. *in vivo* or 24 h.p.i. *in vitro*. A) Current density measured isolated ACMs at 24 h.p.i. measuring I_{Na}, n=6,8. B) Current density measured isolated ACMs at 24 h.p.i. measuring I_{Ca}, n=8,9 C) Current density measured isolated ACMs at 24 h.p.i. measuring I_{Ks},n=10, 9 D) Current density measured isolated ACMs at 24 h.p.i. measuring I_{Kr}, n=10,9 E) Current density measured isolated ACMs at 24 h.p.i. measuring I_{K1}, n=9,8. p≤0.5, **p≤0.01, ***p≤0.001, ****p≤0.0001, Student's T-test. Data are represented as mean ± SEM.

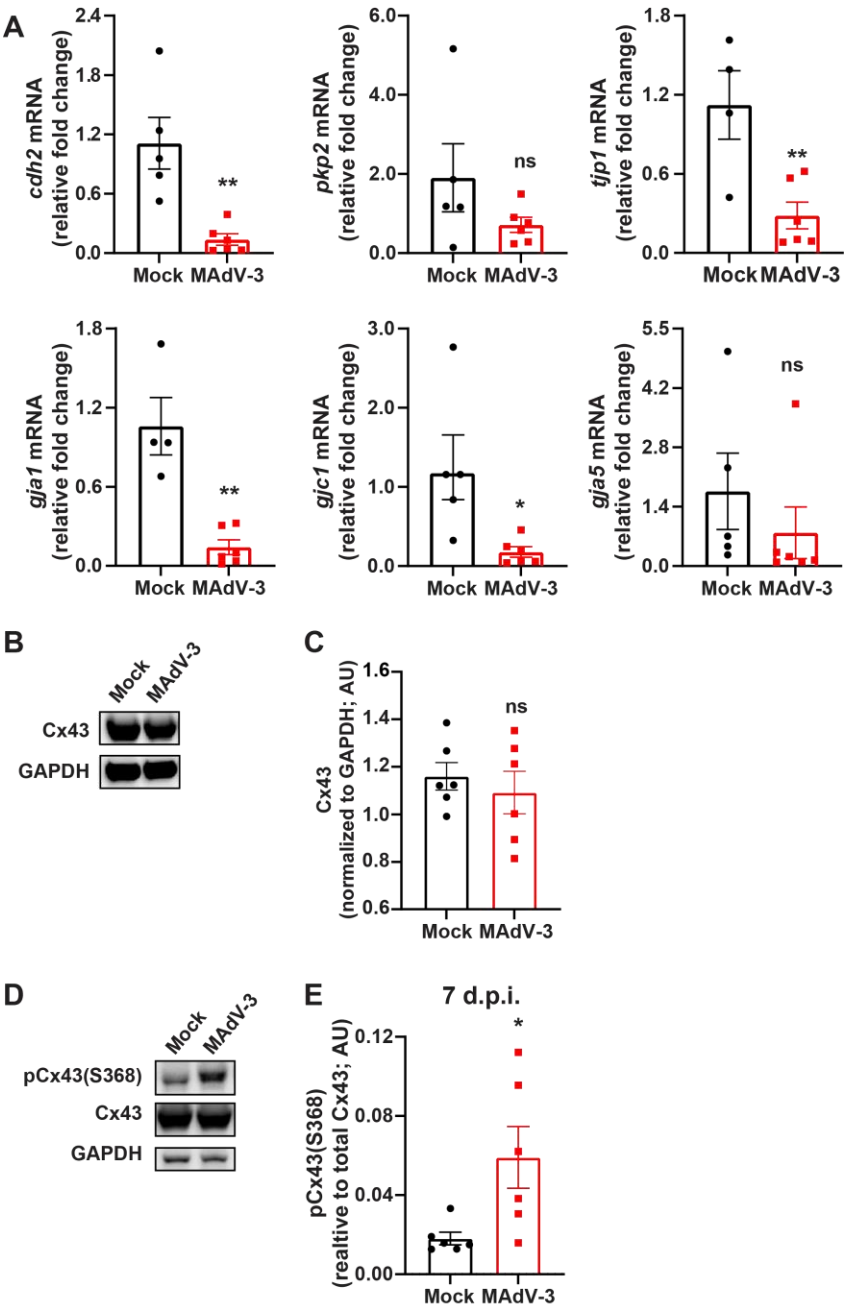

Figure 3.6 Phosphorylated Cx43 is increased in vivo 7 d.p.i and in vitro. Mice or isolated ACMs were infected with MAdV-3 at an i.u./mL of 5×10^5 or MOI 10 and was harvested or fixed at 7 d.p.i. *in vivo* or 24 h.p.i. *in vitro*. A) RT-qPCR of heart tissue for cardiac scaffolding and gap junction genes. B) Western blotting of ventricle tissue probed for total Cx43 to measure total Cx43 during during infection, GAPDH serves as a loading control. C) Densitometry analysis of B. D) Western blotting of ventricle tissue probed for pCx43(Ser368) and total Cx43 to measure phosphorylation during infection, GAPDH serves as a loading control. E) Densitometry analysis of D. p≤0.5, **p≤0.01, ***p≤0.001, ****p≤0.0001, Student's T-test. Data are represented as mean ± SEM.

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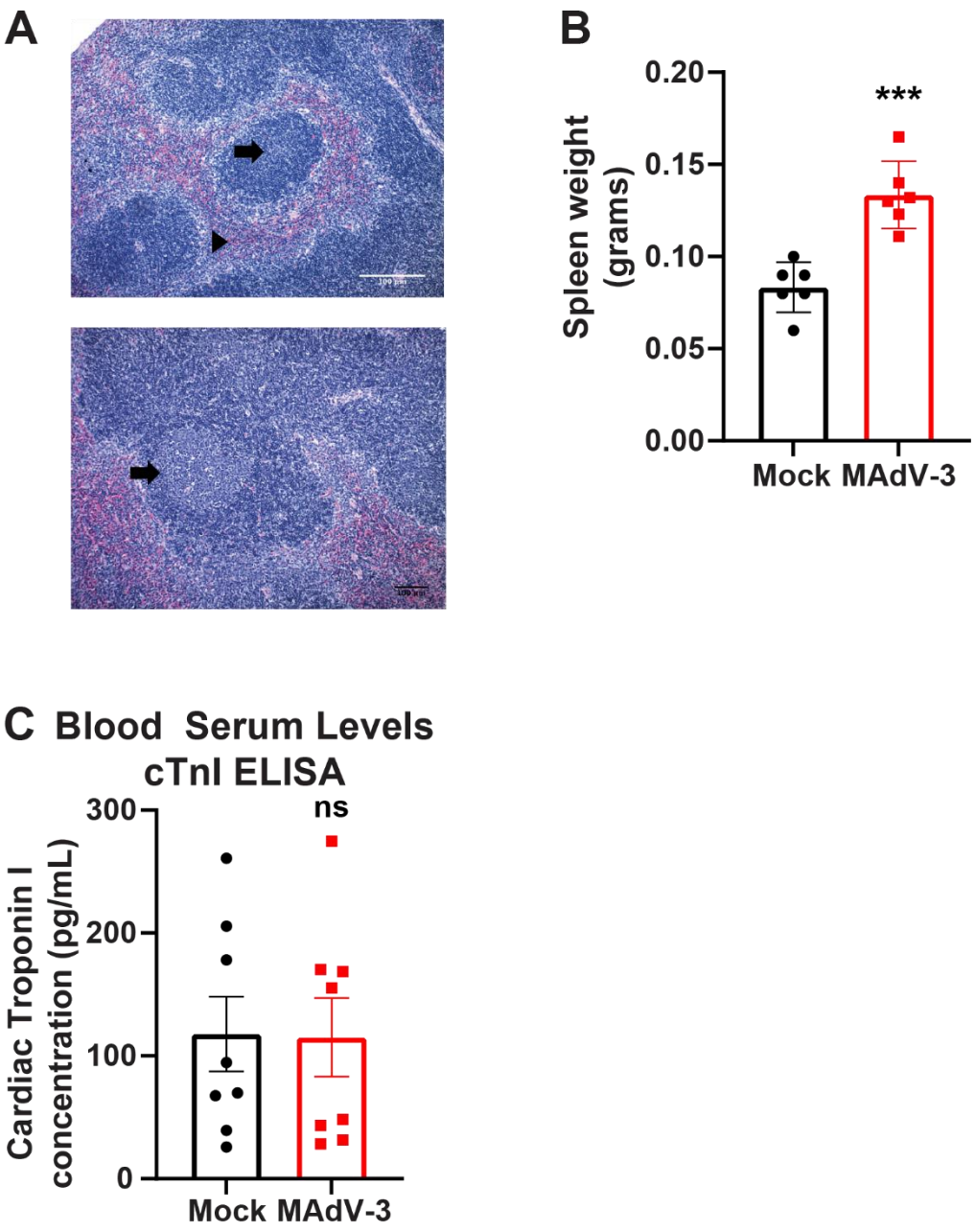

Figure 3.S1 No significant immune cell infiltration in the brain or cardiac damage markers 7 d.p.i. Mice were infected $5x10^5$ i.u./mL and tissue and serum were harvested 7 d.p.i. A) H&E staining on brain sectioning to detect immune cell infiltration in mock- and MAdV-3-infected brains. (n=6) B) Gross spleen weight was measured at endpoint. (n=6). C) Cardiac Troponin I ELISA was performed on blood serum collected at endpoint. (n=8). *p≤0.5, **p≤0.01, ***p≤0.001, ****p≤0.0001, Student's T-test. Data are represented as mean ± SEM.

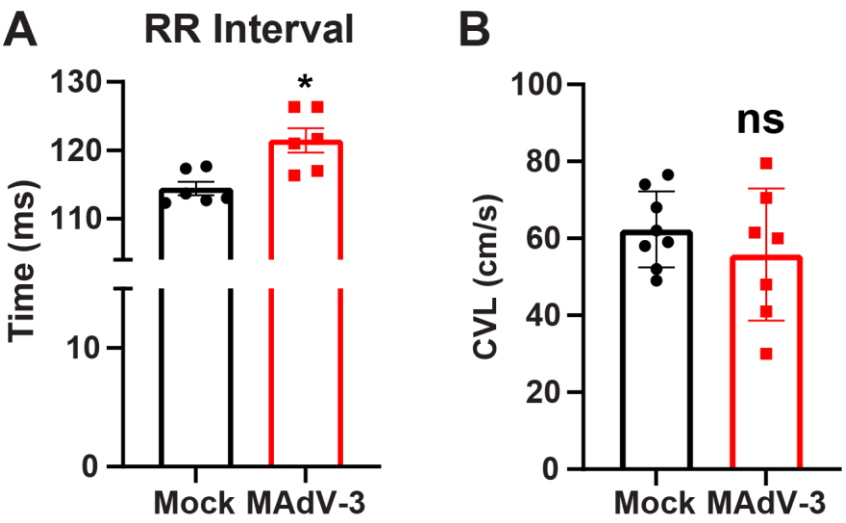

Figure 3.S2 RR interval was increased 7 d.p.i. with no change in longitudinal conduction velocity. **.** *In vivo* electrocardiography or ex vivo optical mapping was performed 7 d.p.i. in adult mice either mock- or MAdV-3-infected. A) *In vivo* electrocardiography measurement were taken on lightly anesthetized mock- and MAdv-3-infected mice using paw pad electrodes. (n=6). B) Conduction velocity was measuring using optical mapping on ex vivo heart from infected mice Conduction velocity in the longitudinal direction (CV_L) was quantified in mock- and MAdV-3-infected hearts 7 d.p.i. (n=8,9). *p≤0.5, **p≤0.01, ***p≤0.001, ****p≤0.0001, Student's T-test. Data are represented as mean ± SEM.

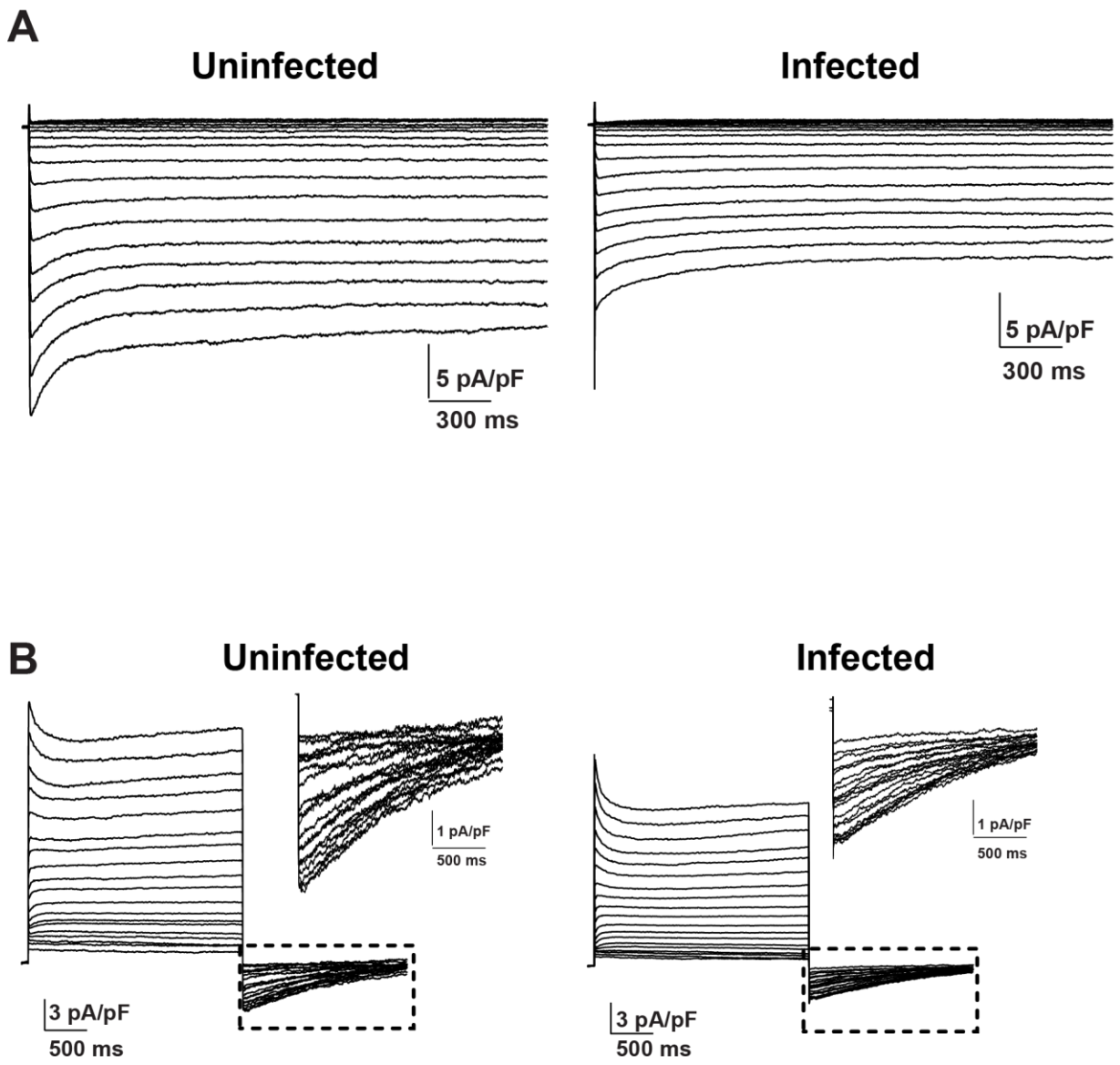

Figure 3.S3 Raw traces of IK1 and IKs currents Isolated ACMs were infected with MAdV-3 at a MOI 10 and were patch clamped at 24 h.p.i. A) Full current traces of I_{K1} in uninfected and infected cardiomyocytes B) I_{Ks} insert currents measured in the presence of 5 uM E4031. N=19

Chapter 3: Investigating adenoviral myocarditis disease progression

Rachel L. Padget, James W. Smyth

Abstract

Chronic myocarditis is a life threatening condition that decreases the quality of life of a patient and can progress into heart failure. Viral infection is one of the leading causes of myocarditis, however, little is known about disease progression by common viral agent, adenovirus. Specific viral families can elicit distinct reactive immune cells that create chronic inflammation and damage, thus, it is important to understand viral-specifics of disease progression to create targeted therapies. We used Mouse Adenovirus Type-3, a cardiotropic adenovirus, to characterize the immune response and pathology of disease in heart tissue. Using RT-qPCR, we find that 7 days post-infection (d.p.i.), there is an increase of *Irf7* mRNA, and a significant increase of both circulating and heart tissue IgG protein measured by ELISA and western blot. Using cardiac magnetic resonance imaging at 14 d.p.i., there is ventricle wall thinning in the infected hearts. These findings show that cardiotropic adenovirus can be used to understand myocarditis disease progression and the development of dilated cardiomyopathy.

Introduction

Chronic myocarditis

Myocarditis is typically separated into two stages, the acute stage occurring during viral infection before there is an appreciable immune response and the chronic phase, where the host's immune system reacts to an infection and, in the process, can cause damage and hypertropic remodeling the myocardium to the heart [1]. Immune-mediated responses to infection span from the rapid response of the innate immune system to the priming and infiltration of the adaptive response several days later.

The heart is not as immune-privileged as once believed and has a coordinated system of infiltrating immune cells in response to infection [2]. This system can exacerbate initial inflammation in the heart stemming from viral clearance of the tissue, which can compound into additional immune cell infiltration in response to cellular damage and death. This amplification of host immune responses can result in lesions of fibrotic tissue, cell death, and eventually, heart failure. Viral genomes are detected in a significant number of dilated cardiomyopathy cases that can progress into heart failure or lethal arrhythmias [3]. However, the specific characteristics of chronic disease development for different viral families are unknown.

Adenovirus

Adenoviral infection is one of the leading etiological agents of myocarditis. Adenoviridae is a family of double stranded DNA viruses that commonly present as pink eye or cold-like illnesses. However, in cardiac biopsies of recently deceased heart failure patients, adenoviral genomes are detected at a significant rate. Adenoviruses have been detected via PCR in up to 22% of dilated cardiomyopathy patients, with the majority adenoviral strains being from adenovirus group C, and genomes are also present in cases of idiopathic left ventricular dysfunction [4, 5].

Modeling of adenoviral myocarditis in the laboratory has been limited historically due to the species-specificity. Most of our current understanding comes from extrapolation of Coxsackievirus B3 (CVB3) infection in mouse models, as CVB3 uses CAR for cellular entry, similar to human adenoviruses [6]. However, CVB3 is an RNA virus, while adenovirus is a DNA virus, thus replication and therefore modification of host cellular mechanisms are quite distinct. When turning to adenovirus infection to understand myocarditis, the genes in adenovirus that modify the host's immune response are located in the early 3 (E3) region. This region produces proteins that suppress MHC I binding to cytotoxic T-cells, suppress Tumor Necrosis Factor (TNF) production, and other speculative immune evasion functions that may be species-specific [7]. McCarthy and Weinberg utilized Mouse Adenovirus Type-1 (MAdV-1); *Murine Adenovirus A*, infection in neonatal and adult mice to study pediatric myocarditis, and found significant $CD3^+$ T-cell infiltration, along with IFN-γ signaling causing increased cardiac mass and cardiomyocyte necrosis [8]. Additionally, as early as 10 days post infection (d.p.i.), adult mice had reduced ejection fraction in the heart, however, infected neonates had peak pro-inflammatory cytokines at 10 d.p.i. while this was not observed in adults [8]. MAdV-1 is not cardiotropic, and causes significant disease in the respiratory and central nervous system (CNS), which could compound disease burden in the cardiovascular system [9]. When understanding clinical presentation, there is some crossover of Human Adenovirus (HAdV) families that can cause both

central nervous system and cardiac effects, however, CNS symptoms of adenoviral infection are extremely rare [10].

Immune signaling from infected tissue

The cell-intrinsic innate immune response is the first step in combating viral infection. This response can occur as early as endocytosis of the virus and is facilitated by pattern recognition receptors (PRR) responding to dsDNA and nucleic acid presence in the cytosol and endosomes [11]. A well-studied innate signaling pathway important for viral clearance is the cGAS/STING cascade that precipitates the type I interferon (IFN) response [12, 13]. The production of interferon transcriptional activators are important in STAT signaling and bridging innate signaling to innate/adaptive cell recruitment, such as natural killer (NK) cells, macrophages, and ultimately, T- and B-cells tailored towards adenoviral infection [14].

Immune cells involved in chronic myocarditis

Much of our understanding of chronic adenoviral myocarditis comes from CVB3 disease models. This is due to the ability of CVB3 to infect several animal species and retain similar tropism and disease [15]. CVB3 can elicit development of T helper cells that are autoreactive to antigens not only in the heart, but to other organs, including the liver [16-18]. It is unknown however, if adenoviral infection precipitates an autoreactive response from the immune system in myocarditis.

Here, we study the role of Mouse Adenovirus Type-3 (MAdV-3), a cardiotropic mouse adenovirus that was recently described and has had little subsequent research into the development of an *in vivo* model of adenoviral myocarditis [19]. As there are viral-specific immune cell infiltrates that cause chronic disease, studying an adenovirus that directly infects the heart could illustrate novel immune cell populations that contribute to chronic myocarditis and if viral genomes specifically in the myocardium are enough to cause structural remodeling of the heart [19, 20].

Methods

Cell Culture

Normal mouse mammary gland cells (NMuMG) (ATCC, Manassas, VA, USA) were maintained and passaged in Dulbecco's Modified Eagle Medium, high glucose, with L-glutamine (Genesee Scientific, San Diego, CA, USA) supplemented with 10% FBS (Gibco, Waltham, MA, USA), non-essential amino acids (Life Technologies, Carlsbad, CA, USA) and Mycozap Plus-CL (Lonza, Basel, Switzerland).

Virus propagation and titering

Mouse adenovirus Type-3 (a generous gift from D. H. Krüger, M.D., Ph.D., Institute of Med. Virology, University Hospital Charité, Berlin) was propagated in NMuMG cells. MAdV-3 was purified by PEGlyation and CsCl ultracentrifugation as previously described with slight modifications and titer was determined by immunofluorescence confocal microscopy in 3T6 cells (Thermo Fisher, Waltham, MA, USA) [21].

Infections

8-12 week old C57Bl/6 (Jackson Laboratory, Bar Harbor, ME, USA) male and female mice were anesthetized with isoflurane (VetOne) and infected via retro-orbital injection with MAdV-3 at 5×10^5 infectious units (i.u.) diluted in 0.9% sterile saline (Teknova, Hollister, CA, USA) and injected using U-100 Insulin syringes (BD, Franklin Lakes, NJ, USA). Mock infection was performed with 0.9% sterile saline solution. Mice were monitored daily for weight, fur ruffling, and neurological pathologies, and were sacrificed if weight loss exceeded 25% of starting mass. All experiments were carried out under NIH guidelines and animal protocols were approved by the Virginia Tech Institutional Animal Care and Use Committee.

RNA extraction and RT-qPCR

Tissue was homogenized in TRIzol (Thermo Fisher, Waltham, MA, USA) and clarified by phenol-chloroform phase separation according to manufacturer's instructions. cDNA was generated with iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad) according to manufacturer's instructions. Real-time PCR was performed with SYBR Select Master Mix for CFX (Thermo Fisher, Waltham, MA, USA) on a QuantStudio 6 Flex System (Thermo Fisher, Waltham, MA, USA). Primers: *oas1*, forward 5' ATGGAGCACGGACTCAGGA 3' reverse 5' TCACACACGACATTGACGGC 3'; *irf7* forward 5' GAGACTGGCTATTGGGGGAG 3', reverse 5' GACCGAAATGCTTCCAGGG 3'; *ifna4* forward 5' GGATGTGACCTTCCTCAGACTC 3' reverse 5' ACCTTCTCCTGCGGGAATCCAA 3'; *ifnb1* forward 5' ATCAACCTCACCTACAGGGC 3', reverse 5' ATCTCTTGGATGGCAAAGGCA 3'; *18s* forward 5' GGC CCT GTA ATT GGA ATG AGT C 3' reverse 5' CCA AGA TCC AAC TAC GAG CTT 3'; *gpadh* Mm.PT.39a.1 IDT.

Immunocytochemistry

Tissue was fixed with -20°C acetone for 10 min, followed by three washes of PBS. Tissue was blocked with 5 % normal donkey serum (Invitrogen, Carlsbad, CA, USA) and 0.5 % Triton X-100 (Sigma Aldrich, St. Louis, MO, USA in PBS for 1 h at room temperature. Primary antibody labeling was performed for 1 h at room temperature using; goat anti-CD45 (1:500; Novus, Centennial, CO, USA), rabbit anti-CD3 (1:250; Novus, Centennial, CO, USA), mouse anti-N-cadherin (1:500; Cell Signaling Technology, Boston, MA, USA), Guinea pig anti-$Ca_v1.2$ (1:250; Alomone, Jerusalem, Israel). Tissue was washed 6 times prior to secondary antibody labeling for 1 h at room temperature with secondary antibodies conjugated to AlexaFluor 488, AlexaFluor 555 (1:500; Thermo Fisher, Waltham, MA, USA), AlexFluor 647 (1:500, Biotium, Hayward, CA, USA). During secondary antibody labeling, cells were counterstained with DAPI and wheat germ agglutinin (WGA). Slides were mounted with Prolong Gold (Thermo Fisher, Waltham, MA, USA).

ELISA

MAdV-3 purified virus was diluted 1:1000 and plates were coated with 100 µL diluted virus and incubated overnight at 4°C. Wells were washed and blocked for 1 hr at room temperature. Serum harvested from MAdV-3 infected animals at various time points was diluted 1:500 in blocking buffer. Diluted sera were added to coated plates in triplicate and incubated for 1 h at room temperature. After several washes, HRP-conjugated secondary antibody was diluted 1:3000 in blocking buffer and incubated on plates for 30 min at room temperature. After several

washes, substrate solution was applied and absorbance was measured via Spectramax (Molecular Devices, San Jose, CA, USA).

Western blot

Mouse hearts were lysed in RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100 (Sigma Aldrich, St. Louis, MO, USA), 1% sodium deoxycholate, 2 mM NaF, 200 μM Na3VO4, 0.1 % sodium dodecyl sulfate, 5 mM n-ethylmaleimide) supplemented with HALT protease and phosphatase inhibitor cocktail (Thermo Fisher, Waltham, MA, USA). Protein was clarified by sonication and centrifugation and concentration determined by DC protein assay (Bio-Rad, Hercules, CA, USA). 4X Bolt LDS sample buffer supplemented with 400 mM DTT was added to samples then heated to 70 °C for 10 min and subjected to SDS-PAGE using NuPAGE Bis-Tris 4-20% gradient gels and MES (Thermo Fisher, Waltham, MA, USA) running buffer according to manufacturer's instructions. Proteins were transferred to PVDF (Bio-Rad, Hercules, CA, USA) membrane, fixed in methanol, and dried. PVDF membranes were reactivated in methanol followed by blocking in 5% nonfat milk (Carnation, Los Angeles, CA, USA) in TNT buffer (0.1% Tween 20, 150 mM NaCl, 50 mM Tris pH 8.0) for 1 h at room temperature. Secondary antibody was incubated with membranes for 1 h at room temperature with goat secondary against mouse IgG conjugated with Alexa Fluor 647 (1:5000; Thermo Fisher, Waltham, MA, USA). Membranes were imaged with BioRad Chemidoc (Bio-Rad, Hercules, CA, USA).

Cardiac MRI

MRI images were acquired with 9.4 Tesla high field MR scanner (BioSpec 94/20 USR) with high power gradient amplifier (the gradient strength is 660 mT/m and the max. linear slew rate is 4570 Tm/s) at Small Bore Preclinical Imaging Core at Fralin Biomedical Research Institute at Virginia Tech Carilion. A surface coil with an internal diameter of 2.0 cm was used for mice cardiac imaging. The anatomical structure of the mice hearts was imaged using a 2D Rapid Imaging with Refocused Echoes (RARE) sequence with the following parameter: TE 25 ms, TR 2.0 s, RARE factor 8, FOV 1.92 × 1.92 × 0.42 (0.63, short axis images) cm, matrix 192 × 192, slice thickness 0.5 mm, slice gap 0.2 mm. There are 6 slices for long-axis heart images, or 9 slices for short-axis heart images.

Results

Limited Type I Interferon response at 7 d.p.i.

Adenoviruses are double stranded DNA viruses that activate host cell innate immune responses upon infection. Toll-like receptors in the cytosol and endosomes, cGAS, STING, and AIM2 are common mechanisms for stimulating the innate immune response and activating Type I Interferon response genes [22]. We sought to characterize the Type I Interferon response at 7 days post infection (d.p.i.) using RT-qPCR using RNA isolated from left heart ventricle of mock and MAdV-3 infected animals. At 7 d.p.i. there is a significant increase in *Irf7*, interferon regulatory factor 7, a transcription factor for Type I Interferon genes member, and no significant increase in other members of this response (**Figure 4.1**). This limited response could be due to a dynamic innate immune signaling response to initial infection. Interferon response genes are regulated differently during the time course of an infection [23].

B- cell infiltration is significant by confocal 7 d.p.i. infection.

We used confocal microscopy to quantify the number of infiltrating immune cells into the ventricle tissue of MAdV-3 7 days post-infection. Previously, we have reported that there is not appreciable immune cell infiltration detected by Hematoylin & Eosin staining (**Chapter 2, Figure 3.2**). Confocal immunofluorescence microscopy provides a more sensitive and quantitative approach over H&E staining [24]. Using antibodies targeting B220 (CD45R), a surface marker for mature B-cells, we found there was a significant increase in B- cells in MAdV-3 infected ventricle tissue 7 d.p.i. (**Figure 4.2**) [25]. It would be valuable to follow up this microscopy with an extended assessment of immune cell populations over time, as persistent T-cell presence is considered a trigger for chronic infection, while there is evidence that chronic levels of viral genomes can trigger a strong protective B-cell response [7, 26]. The interplay between reactive and protective immune cells is an important balance between development of permanent damage or not.

Circulating IgG levels remain elevated 28 days post MAdV-3 infection.

IgG produced by the infected host are critical for an effective response to bacterial or viral infection. Circulating IgG that is specific towards the pathogen can remain elevated for weeks to months after initial infection [27]. While elevated IgG is important for combating infection, prolonged levels are tied to chronic infection [28]. Using ELISA, we quantified the serum IgG response in MAdV-3 infected mice over 28 days. IgG levels in the circulating blood steadily rose until end point collected (**Figure 4.3A**). We probed using western blotting of mock and MAdV-3 infection myocardium protein lysate with anti-mouse IgG to detect if there was localized IgG production in the infected myocardium at 7 d.p.i. and found there was increased IgG in MAdV-3 infected hearts (**Figure 4.3B**). Future work to extend this protocol into longer time points will be important in understanding the role of IgG in chronic adenoviral myocarditis.

14 days post MAdV-3 infection causes myocardial wall thinning.

A classic diagnosis of chronic myocarditis is gross pathological remodeling of the heart, which is characterized by several metrics, including ventricle wall thinning [29]. Previously, we found no pathological remodeling to the heart 7 d.p.i. (**Chapter 2, Figure 3.2**). To measure if there was myocardium remodeling due to chronic infection, we used cardiac MRI on hearts excised from mock or MAdV-3 infected mice 14 d.p.i. Mouse hearts infected with MAdV-3 show ventricle wall thinning in the short axis view (**Figure 4.4**).

Discussion

Adenovirus is a significant causative agent of myocarditis [1, 29]. Adenoviral genomes are detected in many cases of end-stage heart failure and dilated cardiomyopathy [29, 30]. However, due to the species-specific nature of adenovirus infection and replication, research on adenoviral myocarditis is limited. This work utilizes a recently described cardiotropic adenovirus, MAdV-3 to characterize the progression of chronic myocarditis and build on literature that has used MAdV-1 to model of myocarditis [8, 19, 31]. This study finds that cardiotropic adenovirus causes immune cell infiltration, increases both a circulating and cardiac tissue IgG response, and alters the myocardial structure, with more work in progress.

Increased B-cell infiltration into left ventricle tissue is consistent with clinical reports of chronic myocarditis and heart failure [32]. During cardiac viral infection, it is expected in

healthy mice all leukocytes filtrate the tissue, and these can be circulating or resident cells, like macrophages [33]. However, persistent infection can cause autoreactive responses to self-antigens. Evidence has been found in human patients for self-antigens against laminin and intermediate filaments in cases of myocarditis and DCM [34, 35]. Mature B-cells are responsible for the generation of immunoglobulins, with IgG a primary antibody to viral infection [36]. The significant circulating and tissue IgG is consistent with the observed increase of B-cells in the infected myocardium.

The interferon response during viral infection are temporally controlled and increase or decrease during infection [23]. We found that at 7 d.p.i., *Irf7* only was significantly increased in cardiac tissue. *Irf7* is a potent transcription factor during infection and irregular expression of these transcript is common in autoimmune disorders and cancer. Expression of *Ifr7* is produced by lymphocytes, and such, could explain the significant increase later in infection when compared to other anti-viral transcripts measured, as those transcripts are expressed in all cell types, which react to initial infection [37, 38].

Myocardium remodeling is a definitive characteristic of dilated cardiomyopathy. Chronic myocarditis is commonly diagnosed when there is structure change to the heart that results in reduced function [39]. Ventricle wall thinning is a sign of dilated cardiomyopathy, which can result in heart failure or cardiac arrest [40-42]. Our finding was a sample size of one, but bares worth repeating. This result of MAdV-3 recapitulating what is observed in the clinic is a positive sign of the value in this virus for explaining our understanding of adenoviral myocarditis.

Future directions with this work would expand on the early immune response to MAdV-3 infection, and accessing the functional changes of the heart. Echo- and electrocardiography is needed at later time points during infection to describe functional and electrical changes to the myocardium. Continuing immune cell infiltration measurements in tissue at extended time points is needed and could be performed using flow cytometry. Because edema is observed in clinical cases of myocarditis, and previous studies demonstrated that edema interferes with ephaptic couple of cardiomyocytes, both microscopy and optical mapping can be paired to understand the arrhythmogenic substrate of chronic infection [43, 44]. Finally, applying therapeutics to target cytokine production or autoreactive antibodies to test if this could halt development of sustained myocarditis and stopping progression into heart failure.

Acknowledgments

The authors thank Dr. Maoson Wang (Virginia Tech Fralin Biomedical Research Institute) for performing the cardiac MRI. This work was supported by a NIH NHLBI R01 grant (HL132236 J.W.S.) and NHLNI F31 grant (HL152649 R.L.P.).

Author contributions

J.W.S. and R.L.P. designed this study. R.L.P. performed the experiments. J.W.S. performed the blood serum ELISA experiments. J.W.S. and R.L.P. analyzed the data and interpretation.

Declaration of interests

The authors declare no competing interests.

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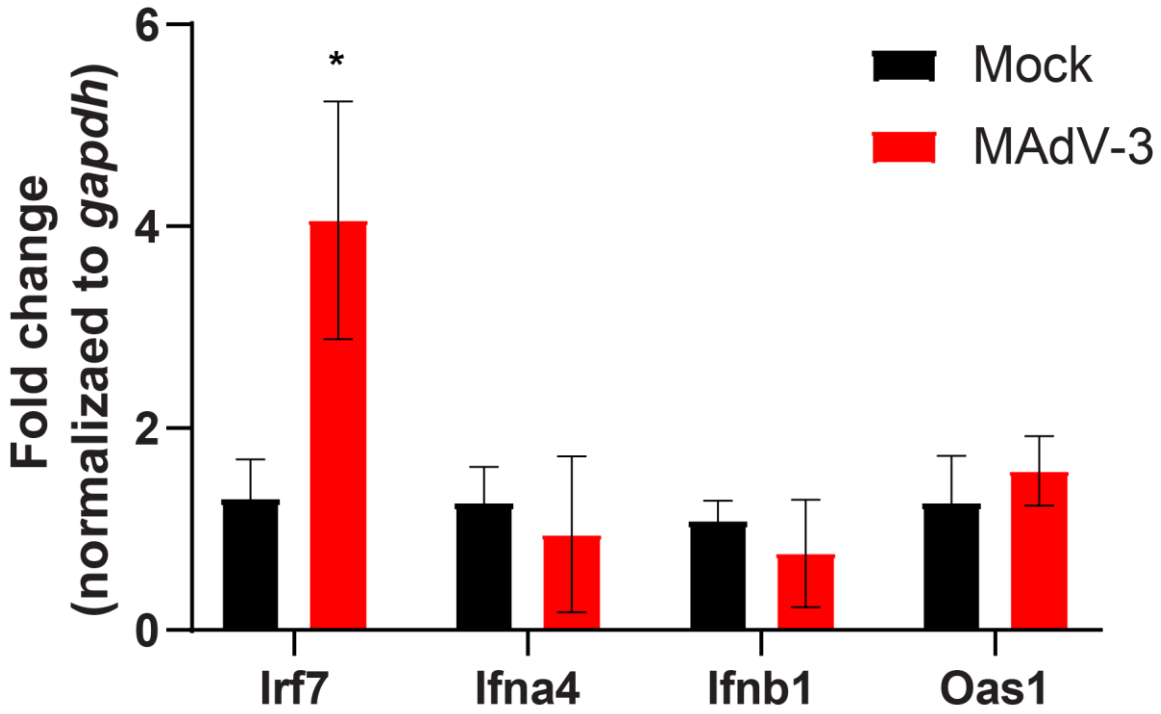

Figure 4.1 Characterizing the Interferon response in MAdV-3 infection. Mice were infected retro-orbitally with 5×10^5 i.u. and tissue was harvested 7 d.p.i. RT-qPCR was performed on ventricle mRNA on Type I Interferon cascade transcripts. N=6, Student's T-test, data are presented as mean ± SEM.

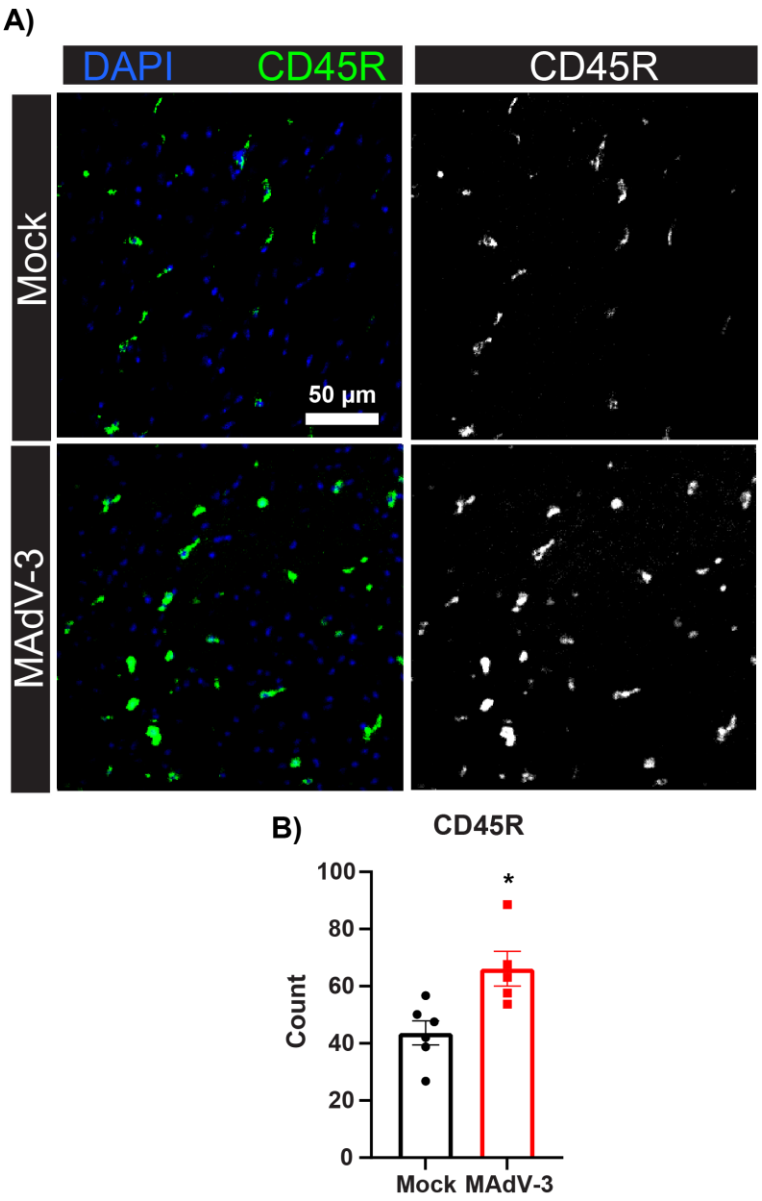

Figure 4.2 B-cell infiltration in mock and MAdV-3 cardiac tissue . Mice were infected retro-orbitally with 5×10^5 i.u. and tissue was harvested 7 d.p.i. **A)** Confocal imaging and count of CD45R+ (green) cells in ventricle tissue, 20x magnification. **B)** Quantification of A), N=6, Student's T-test, data are presented as mean ± SEM.

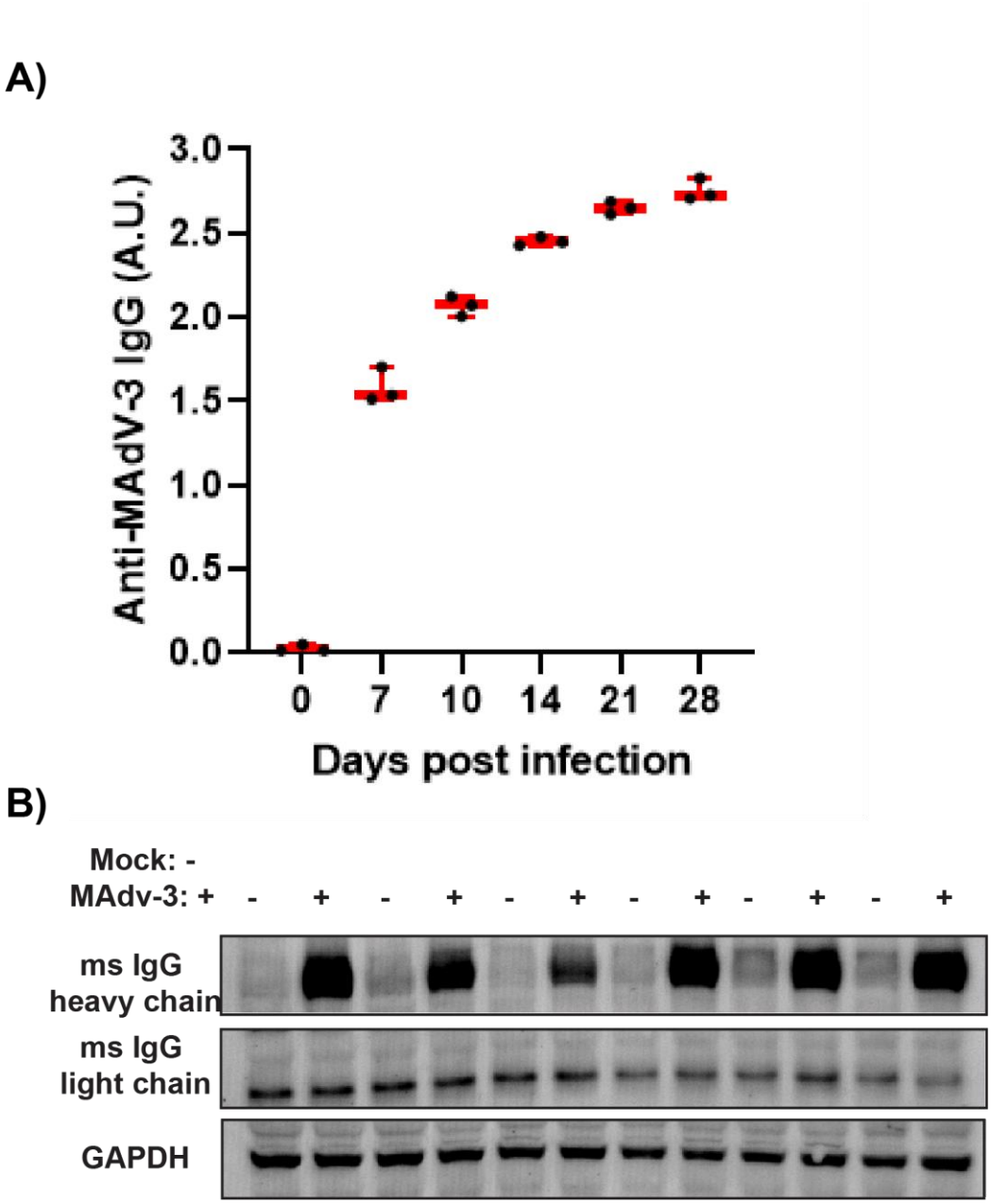

Figure 4.3 MAdV-3 IgG response in a time-dependent manner in blood serum . Blood and myocardium was collected from MAdV-3 infected mice at various time points. A) ELISA of IgG in circulating blood serum specific to anti-MAdV-3 from 0 to 28 d.p.i. N=3 animals at each time point. B) Western blot against mouse IgG in myocardium protein lysate, GAPDH serves as a loading control, N=6.

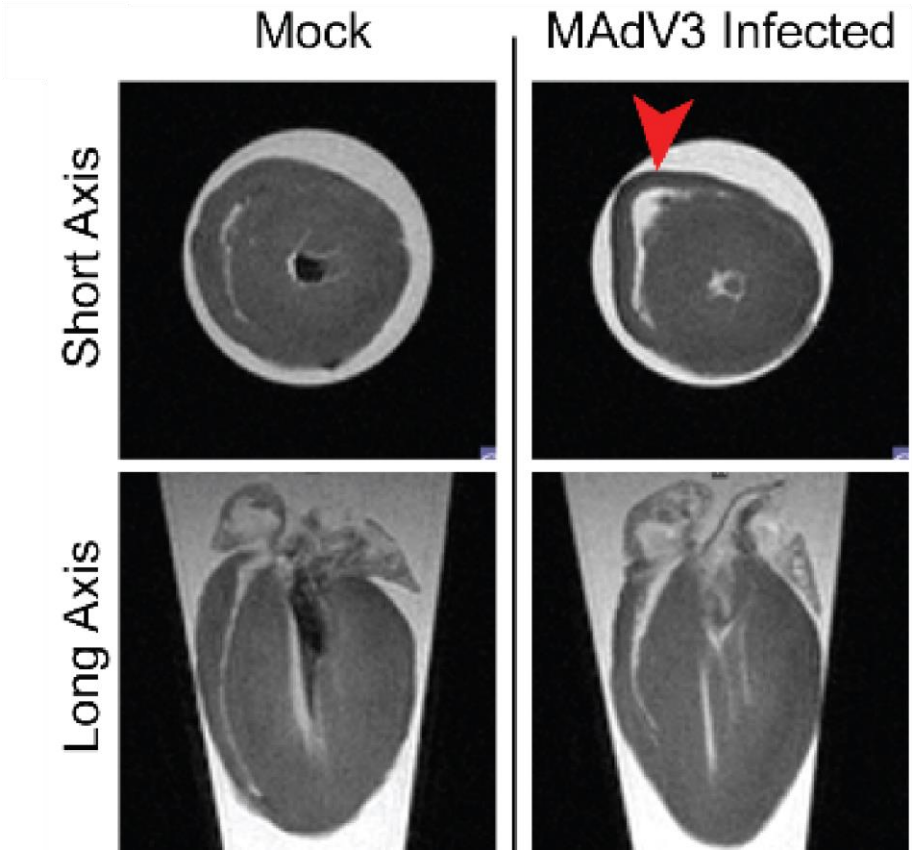

Figure 4.4 Myocardium wall thinning of MAdV-3 infected heart. Cardiac MRI was performed on excised hearts at two weeks post-infection. MAdV-3 infected heart displays right ventricle wall thinning (red arrow) in short axis view. N=1.

Conclusions

Viruses have long been suspected to cause sudden cardiac death in cases where there is no discernable change to the structure of the heart [1]. However, discerning viral causes of sudden cardiac arrest has been elusive due to the difficulty in parsing out viral vs immune causality of disease. Adenoviruses are commonly detected in the myocardium following sudden cardiac death events and dilated cardiomyopathy patients, yet, it is unclear how these viruses contribute to arrhythmia and/or cardiac remodeling [2, 3]. This dissertation has utilized a murine cardiotropic adenovirus to demonstrate that acute viral infection is sufficient to cause arrhythmogenesis *in vivo* without appreciable immune cell infiltration and prior to subsequent pathological remodeling of the myocardium.

Cardiac conduction requires the coupled communication of billions of cardiomyocytes, their gap junctions, and ion channels, which pass the electrical signal from one cell to the next. Disruption of this coupling can cause arrhythmias, which if uncorrected by the body or by intervention, cause cardiac arrest [4]. Viruses rapidly and dynamically alter a broad array of host cellular mechanisms to replicate and this can have major consequences at the organ level. Gap junctions are known to propagate both innate and adaptive anti-viral immune responses [5, 6]. As such, viral targeting of intercellular communication would be advantageous for replication and spread. We previously reported that adenoviral infection rapidly increases phosphorylation of Cx43, the most ubiquitously expressed gap junction protein, shutting down intercellular communication in human epithelial cells [7]. Here, we built on existing work by using an animal adenoviral infection model to understand an underlying mechanism for the viral-mediated arrhythmogenic substrate in adenoviral myocarditis patients.

We found that acute adenoviral infection in the heart phosphorylates Cx43 *in vivo* and causes decreased conduction velocity, and, in isolated cardiomyocytes, alters action potential duration by reducing potassium channel current density. This work is first-of-its-kind in demonstrating that adenoviral infection can create an arrhythmogenic substrate in the heart prior to immune cell infiltration and cardiac remodeling. Cx43 and potassium channel regulation have post-translation modification crossover via PKC phosphorylation, which reduces gap junction open probability and decreases K^+ channel trafficking to the cell membrane [8-10]. Many viruses modify global PKC pathways during infection, adenoviruses being no exception [11-13].

Generally, myocarditis is diagnosed in the chronic stage of disease, due to diagnostic tools presently available. With the species-specific nature of adenoviruses, understanding the cytokines, infiltrating immune cells populations, and gross myocardium remodeling is important as these can vary from virus-to-virus [14-16]. Chronic infection of cardiotropic adenovirus showed significant IgG response in both the circulating sera and in the myocardium, with gross remodeling of the myocardium 14 days post infection. Viral genomes are commonly detected in cardiomyopathies and there is a depth of knowledge of viruses causes chronic disease, however, there is limited research on adenoviral myocarditis disease progression [17-20]. These data, in conjunction with our observed acute arrhythmogenesis before appreciable immune response, describe the disease burden of adenoviruses in the heart, and how viruses could elicit cardiac arrest by altering intercellular communication and ion channel function in the heart.

Translational Perspectives

Gap junction and ion channel dysregulation underlie multitudes of cardiomyopathies and arrhythmia diseases [21, 22]. There are currently limited diagnostic and treatment options available for acute viral myocarditis, partly due to the paucity of research in this area. Acute viral myocarditis can be difficult to diagnosis, as the only preceding symptoms may be flu-like, which do not generally call for cardiac investigation. If abnormal cardiac rhythm is detected, there are also limited anti-viral and anti-arrhythmic drugs available. Changes in cardiac electrical propagation could be highly specific to viral families, as the limited work on viruses altering ion currents and gap junctions showed a variety outcome between viruses [23-25]. This confounds the potential use of cardiac modulators as applying an inappropriate therapy could exacerbate disease [26].

Adenovirus genomes are detectable in up to 23% of myocarditis or idiopathic ventricle dysfunction, and thus, adenoviruses represent a significant causative agent of cardiac disease [27, 28]. We describe how adenoviruses cause an arrhythmogenic substrate before an appreciable immune response is mounted against the virus. This infection causes post-translational modification of Cx43 in the heart during infection which can lead to decreased conduction velocity and prolonged action potential duration. This work will lead to future investigation of how to target either cardiac dysfunction or viral mechanisms that could restore the healthy cardiac electrical system following infection.

Concluding Remarks

The Covid-19 pandemic has exposed how little we really understand regarding these pieces “of bad news wrapped up in protein,” as Sir Peter Medawar called them in his 1960 Nobel Prize address. Viruses have long been speculated as a causative agent of arrhythmogenesis, especially in young adults. In this work, we demonstrate how acute adenoviral infection causes an arrhythmogenic substrate in the myocardium and alters action potential duration in isolated cardiomyocytes. This work gives validation to the hypothesis that viruses can cause sudden cardiac death without appreciable immune damage to the heart and gross myocardial remodeling. Additionally, we have started assessing how cardiotropic adenoviral infection can develop into chronic disease in the heart.

This work is just the beginning of our understanding of viral-mediated effects on the heart that underlie arrhythmias. Many other viruses are thought to cause cardiac arrest, along with the mechanism behind how adenovirus change potassium currents is still unknown. Our work described here shows that viruses can alter cardiac conduction and the action potential, and that acute infection increases phosphorylation of Cx43 in the working myocardium. Future work is needed to understand how adenoviruses alter action potential duration and if healthy cardiac conduction can be restored after acute infection.

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