The microRNA-221/-222 cluster balances the antiviral and inflammatory response in viral myocarditis

Maarten Corsten†, Ward Heggermont†,2,3, Anna-Pia Papageorgiou1,2, Sophie Deckx1, Aloys Tijssma4, Wouter Verhesen1, Rick van Leeuwen1, Paolo Carai1,2, Hendrik-Jan Thibaut4, Kevin Custers1, Georg Summer1, Mark Hazelgroek1, Fons Verheyn5, Johan Neyts4, Blanche Schroen1‡, and Stephane Heymans1,2‡*

1Center for Heart Failure Research, Cardiovascular Research Institute Maastricht, Maastricht University, P. Debyelaan 25, Maastricht AZ-6202, The Netherlands; 2Center for Molecular and Vascular Research, University of Leuven, Leuven B-3000, Belgium; 3Department of Internal Medicine, Service of Cardiology, University Hospitals Leuven, Leuven B-3000, Belgium; 4Rega Institute for Medical Research, University of Leuven, Leuven B-3000, Belgium; and 5Electron Microscopy Unit, Maastricht University, Maastricht AZ-6202, The Netherlands

Received 11 September 2014; revised 25 May 2015; accepted 21 June 2015; online publish-ahead-of-print 23 July 2015

Aims
Viral myocarditis (VM) is an important cause of heart failure and sudden cardiac death in young healthy adults; it is also an aetiological precursor of dilated cardiomyopathy. We explored the role of the miR-221/-222 family that is up-regulated in VM.

Methods and results
Here, we show that microRNA-221 (miR-221) and miR-222 levels are significantly elevated during acute VM caused by Coxsackievirus B3 (CVB3). Both miRs are expressed by different cardiac cells and by infiltrating inflammatory cells, but their up-regulation upon myocarditis is mostly exclusive for the cardiomyocyte. Systemic inhibition of miR-221/-222 in mice increased cardiac viral load, prolonged the viraemic state, and strongly aggravated cardiac injury and inflammation. Similarly, in vitro, overexpression of miR-221 and miR-222 inhibited enteroviral replication, whereas knockdown of this miR-cluster augmented viral replication. We identified and confirmed a number of miR-221/-222 targets that co-orchestrate the increased viral replication and inflammation, including ETS1/2, IRF2, BCL2L11, TOX, BMF, and CXCL12. In vitro inhibition of IRF2, TOX, or CXCL12 in cardiomyocytes significantly dampened their inflammatory response to CVB3 infection, confirming the functionality of these targets in VM and highlighting the importance of miR-221/-222 as regulators of the cardiac response to VM.

Conclusions
The miR-221/-222 cluster orchestrates the antiviral and inflammatory immune response to viral infection of the heart. Its inhibition increases viral load, inflammation, and overall cardiac injury upon VM.

Keywords
Myocarditis • Coxsackievirus B3 • Adverse inflammation • Dilated cardiomyopathy • Viral replication

Translational perspective
While viral myocarditis (VM) is responsible for considerable morbidity and mortality, causal therapies are painfully lacking. Insight in the molecular underpinnings and therapeutic targets are therefore essential. Our results identify a microRNA cluster (miR-221/-222) with a completely novel protective role in myocarditis. Besides implicating therapeutic potential for targeting miR-221/222 or its direct targets, our data additionally emphasize that early viral clearance per se may be a fruitful therapeutic strategy in myocarditis. Since VM is an aetiological precursor of dilated cardiomyopathy (DCM), this research also raises the question whether viral clearance in DCM might prove beneficial and limit the development of concomitant heart failure.

* Corresponding author. Tel: +31 433 88 29 49; fax: +31 433 88 29 52, Email: s.heymans@maastrichtuniversity.nl
† These authors contributed equally and thus share the first authorship position.
‡ These authors share the last authorship position.
Published on behalf of the European Society of Cardiology. All rights reserved. © The Author 2015. For permissions please email: journals.permissions@oup.com.
Introduction

Viral myocarditis (VM) is an inflammatory condition of the heart causing heart failure and sudden death in young, previously healthy individuals. Evidence-based therapies for VM are currently lacking.1 Myocarditis has been estimated to account for up to 12% of sudden cardiac deaths in patients < 40 years of age. Signs of VM are found in ~10% of biopsies from patients with unexplained heart failure.2,3 Cardiac injury in VM is initiated by infection of the heart by cardiotropic viruses, such as the enteroviral CVB3, Human Herpesvirus 6, or Parvovirus B19 (PVB19). These viruses may trigger an adverse immune response, initially aimed for viral elimination, but when uncontrolled causing myocardite destruction, reparative fibrosis, and heart failure. It remains elusive why some patients develop a strong adverse inflammatory response with massive injury of the heart, while others only have mild, aspecific, and transient symptoms which do not even warrant a doctor’s visit. While immune activation is an important contributor to cardiac injury, broad, and aspecific inhibition of the immune response does not result in clinical improvement in patients.2,4 Rather, targeting specific signalling pathways may protect against cardiac inflammatory injury without compromising viral elimination.5,6

MicroRNAs (miRs) are important regulators of gene expression in a wide range of biological processes, including cardiac biology and regulation of immune responses.6–8 The co-transcribed miR-221/-222 family was previously reported to promote tumour growth by targeting p27 (kip1) and p57 (kip2)9,10 and to affect vasculature by promoting smooth muscle cell proliferation and inhibiting angiogenesis.11–13 However, the role of miR-221 and miR-222 in the heart or during viral infection has not been studied so far. In this study, we establish that cardiac miR-221/-222 expression during VM is functionally important for the pathogenesis and propagation of cardiac injury. Both miRs are strongly up-regulated in response to infection with CVB3 and overexpression of miR-221/-222 inhibited viral replication in cardiomyocytes in vitro, while inhibition of the miR cluster augmented viral replication. Systemic inhibition of miR-221/-222 in vivo resulted in strongly aggravated cardiac injury, increased cardiac viral load, and a prolonged viraemic phase. Our study identifies miR-221/-222 expression as a protective factor in acute VM because it delicately fine-tunes the balance between an efficient antiviral and inflammatory response in VM.

Materials and methods

Detailed study design, animal characteristics, patient data, experimental materials, methods, and statistical analysis are available in Supplementary material online. Animal studies were approved by the Ethical Commission for Animal Experiments of KU Leuven, Belgium. The experimental protocols used on the animals all comply with the Declaration of Helsinki. The collection of patient material (myocardial biopsies) was only performed after written informed consent by the patient or his/her legal representative, consistent with the Declaration of Helsinki. The protocol for studies on human myocardial biopsies was approved by the Medical Ethical Committee of Maastricht University Hospital, The Netherlands. Briefly, mice were treated with synthetic oligonucleotides directed against miR-221/-222 (or their respective scrambled}

Results

Cardiac expression of miR-221/-222 is increased during acute viral myocarditis

We previously studied the cardiac expression of miRs during VM in two mouse models.14 C3H mice are susceptible to the development of VM after inoculation with CVB3 (Nancy strain), while C57Bl6N mice are rather resistant to cardiac inflammation from this specific CVB3 strain despite similar viral titres.14 This difference originates from the CVB3 strain used as well as distinctive innate and adaptive immune responses between mouse strains.15–17 Quantitative real-time PCR revealed the dynamic regulation of both miR-221 and miR-222 in both mouse strains at day +7 (Figure 1A and B). In addition, levels of miR-221 were already elevated in C3H mice at day +4, whereas miR-222 levels were elevated by trend. MiR-221 and -222 were among the strongest and most significantly up-regulated miRs in a VM microarray depicted by the Volcano plot (Figure 1C).14 These data indicate that miR-221 and miR-222 are significantly elevated during acute VM. We next asked what cardiac cell types express miR-221 and miR-222 under basal and inflamed conditions, both in vivo and in vitro. In situ hybridization of miR-221 in the normal mouse heart revealed its expression by multiple cell types in the heart, including cardiomyocytes, endothelial cells, and interstitial immune-like cells while no signal was detected using a scrambled probe. During VM (7 days), staining was strongly apparent in zones of inflammation. Similarly, in cardiac biopsies from VM patients, we observed miR-221 expression in areas of the heart infiltrated by immune cells as shown by in situ hybridization (Figure 1D). A significant and biologically relevant increase of miR-221 and miR-222 levels was confirmed with qPCR on adult mouse cardiac myocytes isolated 7 days after infection with CVB3 (Figure 1E). Furthermore, we determined miR-221 and -222 levels in the immune cell fraction (CD45+ cells) in the infected heart, and found that miR-221/-222 levels did not significantly alter. The same was true for the splenic leukocytes (Figure 1E). We conclude that miR-221/-222 expression during murine VM likely reflects a combination of their up-regulation
MiR-221 and miR-222 are up-regulated in mouse viral myocarditis. (A and B) Quantitative real-time PCR measurement of miR-221 (A) and miR-222 (B) expression showed elevated levels of both miRs in C3H mice at day +7 after viral infection, and additionally showed elevation of miR-221 at day +4 in C3H mice and at day +7 in C57Bl/6N mice (mean ± SD, n = 5–6 mice per time point). (C) Volcano plot showing fold changes (log2 values) and P-values (−log10 values) compared with baseline for individual miRs in C3H mice at day +7 post-infection (n = 6 mice). (D) In situ hybridization of miR-221 (blue) on representative left ventricular sections of a healthy mouse, a viral myocarditis mouse at day +7 and a viral myocarditis patient, showing blue cellular staining in multiple cardiac cell types, including cardiomyocytes, endothelial cells, interstitial cells, and infiltrating white blood cells (scale bars: 50 μm). (E) miR-221 and miR-222 increase in freshly isolated adult cardiomyocytes 7 days after infection with CVB3 (n = 11–15 mice), while there is no significant regulation of these miRs in cardiac CD45+ cells (n = 3–4 mice) nor in splenic leucocytes (n = 3–4 mice) (mean ± SD, experiment was performed twice).
in cardiomyocytes and of the influx of miR-221/-222 expressed by macrophages and T cells.

As a validation of these in vivo findings, we demonstrated that both miR-221 and miR-222 were up-regulated by CVB3 infection in a dose-dependent manner in neonatal rat cardiomyocytes. In contrast, miR-1 and miR-208a, normally highly expressed by cardiomyocytes, were unchanged upon viral infection (Supplementary material online, Figure S1A). To mimic in vivo CVB3-induced inflammatory conditions, we stimulated nRCMs, fibroblasts (nRCFs), and MCECs, with TNF-α, BMDMs with lipopolysaccharide (LPS), and splenic T lymphocytes with ConCanavalin A. MiR-221/-222 expression responses to TNF-α stimulation in cardiac cell types were mild: both miRs were up-regulated in nRCFs after 24 h of TNF-α and only slightly and not significantly elevated in nRCMs (Supplementary material online, Figure S1B and C). In addition, TNF-α caused no miR-221/-222 expression changes in endothelial cells, and also macrophages and T cells were unresponsive to their respective activating stimuli (Supplementary material online, Figure S1C), in line with previous reports.\(^\text{18,19}\) Similar results were obtained by stimulation of nRCMs, nRCFs, and MCECs with IL-1β, and after TNF-α stimulation of the fibroblast 3T3 cell line and the endothelial cell line SVEC (Supplementary material online, Figure S1B and C). Average raw C\(_v\) values that help to compare baseline miR levels in different cell types can be found in Supplementary material online, Figure S1D.

**In vivo inhibition of miR-221/-222 strongly increases cardiac injury and immune cell infiltration during acute viral myocarditis**

To investigate whether expression of miR-221/-222 affects the course of VM in mice, we systemically administered antagoniMiRs against miR-221/-222 throughout the first 7 days of VM in male C3H mice (Figure 2F). We detected increased miR-221 levels upon VM and sharply reduced miR-221 levels after antagoniR-221/-222 treatment, indicative of knockdown by miR degradation (Figure 2E). Quantitative real-time PCR analysis of miR-221 and miR-222 expression confirmed up-regulation of both miRs during VM in scrambled control groups and revealed strong and significant knockdown of both miRs in sham and VM conditions after antagoniR treatment (Figure 2C). At day +7, cardiac necrosis was significantly aggravated in antagoniR-221/-222 treated C3H mice when compared with controls (Figure 2A and B), with also a trend wise increase of cardiac damage in ‘resistant’ C57BL6N mice (Figure 2B). Heart weight significantly increased in VM mice, reflecting cardiac oedema, and influx of inflammatory immune cells (Figure 2D and Supplementary material online, Table S1). MiR-221/-222 inhibition indeed promoted the infiltration of both CD3+ T-cells and CD68+ monocytes/macrophages in the heart after VM (Figure 3A), while not affecting numbers in sham hearts (Figure 2G–H). Circulating leucocyte numbers were increased in antagoniR-treated infected mice. Contrarily, in uninfected mice, miR-221/-222 inhibition did not cause higher circulating leucocyte numbers (Figure 2I) and there were no other aberrant findings (cardiac immune cell infiltration, necrosis) in the uninfected mice. Electron microscopy confirmed necrotic degeneration of cardiomyocytes and influx of mononuclear cells in antagoniR-221/-222 VM hearts after 7 days. No ultrastructural differences were observed between sham hearts of different treatments (Figure 3C). MiR-221/-222-inhibited VM hearts contained >3-fold higher CVB3 genome copies than scrambled-treated VM controls, and expressed significantly higher amounts of both pro-inflammatory (interferon gamma, IFN-γ) as well as anti-inflammatory (IL-10) cytokines (Figure 3B). We did not find any indication of an altered Th1/Th2 response in the CVB3-infected mice treated with antagoniRs (Supplementary material online, Figure S5C). Together, these data show that inhibition of miR-221 and miR-222 during VM in mice aggravates cardiac inflammatory infiltration by macrophages and T cells, which increases cardiac injury.

**Inhibition of miR-221/-222 in vivo increases viral replication at Days 4 and 7 post-infection**

MiR-221/-222-inhibited C3H mice displayed higher cardiac CVB3 levels at day +7 of VM. However, since differences in inflammation might distort the observation of viral replication per se, we next investigated whether antagoniRs against the miR cluster directly affected viral load in vivo at 4 days post-infection, before the onset of inflammatory infiltration (Supplementary material online, Table S2). Inflammatory patches were absent in all infected hearts at 4 days, and both miR-221 and miR-222 were efficiently suppressed by antagoniR administration (Figure 4A and B). At day +4, miR-221/-222 inhibition significantly increased CVB3 titres in the heart, without affecting the number of apoptotic cells in the myocardium (Figure 4C). Increased viral loads after miR-221/-222 inhibition were associated with significantly higher expression mRNA transcript levels of IL-6, macrophage chemo-attractant protein-1 (MCP1), IFN-β, and with a trend to higher expression of CXCL12, but not TNF-α (Figure 4D). These data indicate that miR-221/-222 directly impacts on viral replication and cardiac CVB3 load before onset of the inflammatory phase in the heart. In addition, we aimed to characterize the systemic viraemic response upon miR-221/-222 inhibition. We found very high titres of CVB3 in the plasma of both scrambled control and antagoniR-treated VM mice at day +2 (Figure 4E, green and blue bars, respectively). However, the viraemic phase in the scrambled control VM mice was quenched at day +4, whereas viral titres were still highly significantly present in the plasma of antagoniR-treated VM mice (Figure 4E, red and purple bar, respectively). This was not the case in C57Bl6N mice, generally resilient to CVB3-induced myocarditis (Figure 4E, right panel). At day +7, viruses were not present anymore in the plasma in both conditions. In conclusion, we state that inhibition of miR-221/-222 in vivo prolongs the viraemic state and subsequent cardiac viral replication upon CVB3 infection in C3H mice.

**The miR-221/-222 family directly targets regulators of the antiviral and the inflammatory response**

We queried predicted miR-221/-222 targets in mouse and human using the TargetScan algorithm (www.targetscan.org) and searched the overlapping conserved gene set for putative targets with a
Figure 2  In vivo inhibition of miR-221 and miR-222 strongly aggravates cardiac injury during acute viral myocarditis. (A) Left ventricular H&E stainings at day +7 of CVB3-induced viral myocarditis showing aggravated cardiomyocyte necrosis after miR-221/-222 inhibition (scale bars: 100 μm). (B) Left ventricular infiltration by inflammatory cells, quantified on haematoxylin-eosin stainings. Left ventricular necrotic area 31% in scrambled control viral myocarditis C3H mice compared with 52% in antagoniR-221/-222 viral myocarditis C3H mice; 3% in scrambled control viral myocarditis C57Bl6N mice compared with 11% in antagoniR-221/-222 viral myocarditis C57Bl6N mice (mean ± SD; n = 22–25 mice in the C3H group, n = 10 in the C57Bl6N group). (C) Quantitative real-time PCR analysis of cardiac miR-221/-222 expression, demonstrating significant knockdown of both miRs by antagoniR treatment in both sham and viral myocarditis groups, in agreement with Northern blot (mean ± SD; n = 5 in the sham groups, n = 10 in the viral myocarditis groups). (D) Heart weights significantly increase during viral myocarditis, both in scrambled and antagoniR-221/-222-treated conditions, a sign of cardiac oedema and immune cell infiltration (mean ± SD; n = 22–25 mice in the C3H group, n = 10 in the C57Bl6N group; also see Supplementary material online, Table S1). (E) Northern blot analysis showed markedly reduced band intensity after antagoniR treatment. (F) Study design: antagoniRs targeting miR-221 and miR-222 were administered by three intravenous injections throughout the first 7 days of CVB3-induced VM, 20 mg/kg/injection for each antagoniR vs. 40 mg/kg/injection for the scrambled control. (G and H) The number of CD68+ macrophages in sham hearts was not affected by antagoniR-221/-222 administration (mean ± SD; n = 5 per group; scale bar: 100 μm). (I) MIR-221/-222 inhibition increased white blood cell counts in the systemic circulation (mean ± SD; n = 5 per group). (J) Cardiac IL-4 transcript levels were not affected by antagoniR-221/-222 administration (mean ± SD; n = 7–8 per group). (K) CXCL12, TOX, and BCL2L11 were significantly elevated in the VM hearts of the antagoniR-treated mice compared with the scrambled control viral myocarditis mice, while ETS1, ETS2, and IRF2 levels were only elevated by trend (mean ± SD; n = 7–8 per group).
Figure 3  Inhibition of miR-221/-222 promotes cardiac immune cell infiltration and inflammatory cytokine expression in viral myocarditis. (A) MiR-221/-222 inhibition promoted cardiac infiltration of CD68+ macrophages and CD3+ T lymphocytes during viral myocarditis. Representative cryosections scrambled control and antagomiR viral myocarditis hearts (mean ± SD; n = 10 per group; scale bars: 100 μm). (B) Quantitative real-time PCR analysis showing that miR-221/-222-inhibited viral myocarditis hearts contained 3-fold higher CVB3 genome copies than viral myocarditis controls, and expressed significantly higher amounts of both pro-inflammatory (IFN-γ) cytokines. MiR-221/-222 inhibition also caused significantly higher expression levels of ICAM1, and elevation by trend of IL-6 and PSEL, while not affecting TNF-α, IFN-α, IFN-β, and vascular cell adhesion molecule (mean ± SD; n = 7–8 per group). (C) A normal ultrastructural morphology of cardiomyocytes and capillary blood vessels is observed in hearts of sham animals treated with antagomiR-221/-222 or control. In control viral myocarditis hearts, several mononuclear cells have infiltrated in the interstitial space between the cardiomyocytes. In viral myocarditis hearts treated with antagomiR-221/-222, several mononuclear cells and a plasma cell are seen around the cardiomyocyte, as well as degenerating cardiomyocytes (IC, interstitial cell; scale bars represent 5 μm, except for the top right panel where it represents 2 μm).
Figure 4  Inhibition of miR-221/-222 increases cardiac CVB3 load but does not affect cardiomyocyte apoptosis. (A) Left ventricular H&E stainings at day +4 of CVB3-induced viral myocarditis show absence of inflammatory patches at this time point (scale bars: 1 mm). (B) Quantitative real-time PCR measurements of cardiac miR-221 and miR-222 levels, showing significant knockdown for both miRs at day +4 (mean ± SD; n = 10 per group). (C) Photographs and quantification of terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL, green signal with DAPI nuclear counterstain in blue). No difference in the percentage of cardiac apoptotic cells was observed between scrambled control and antagomiR treatments (n = 5 in sham groups, n = 10 in viral myocarditis groups; scale bar represents 100 μm). (D) Quantitative real-time PCR showing significantly higher CVB3 levels (2.7-fold) in antagomiR-221/-222-treated viral myocarditis hearts compared with controls. In addition, miR-221/-222 inhibition caused significantly higher expression levels of IL-6, MCP1, IFN-β, and with a trend to higher expression of vascular cell adhesion molecule and ICAM, but not CXCL12, IL-1β, and TNF-α (mean ± SD; n = 9–10 per group). (E) Bar chart (bars represent mean) with individual data points superimposed, showing the quantification of CVB3 genome copies (log10) per millilitre plasma in C3H mice. There is no viral presence in all control groups (n = 5 per group except for C3H viral myocarditis day +2 and C57Bl6N viral myocarditis day +7 where n = 10 per group). The viraemic state is maximal at day +2 in both scrambled control (4-log CVB3 copies/mL, green bar) and antagomiR-221/-222 (3.8-log CVB3 copies/mL, blue bar) groups. However, whereas there is no viral presence anymore in the scrambled control viral myocarditis group at day +4 (except for one specimen, red bar), there is still considerable viral presence (2.4-log CVB3 copies/mL, purple bar) in the antagomiR-221/-222-treated group, while there is no viral presence in the plasma at day +7. Additionally, in C57BL6N mice, there is no difference in viral load at day +4 in scrambled control viral myocarditis vs. antagomiR viral myocarditis mice (also see Supplementary material online, Table S2).
potential role during VM. Based on this search strategy, we selected eight potential targets. The pro-inflammatory transcription factors ETS1 and ETS2 are predicted targets that promote the expression of cytokines, adhesion molecules, and chemokines such as MCP1.20–22 In addition, ETS1 promotes replication of the human immunodeficiency virus 1 and human T-cell leukemia virus 1 in lymphoid cells.13–15 The predicted target IRF2 dampens the transcription of type I IFNs, which are protective in VM.26 IRF2 also plays a role in the maturation of natural killer cells, an important defence line against several viruses.27,28 The chemokine CXCL12 is a strong lymphocyte attractant, while thymocyte selection-associated high mobility group box protein (TOX) and CD4 are important in T-cell biology. Finally, we selected BCL2-like 11 (BCL2L11) and BCL-2-modifying factor (BMF) as two anti-apoptotic proteins. All predicted targets harbour a single 3′UTR 8-mer conserved binding site, except for ETS1, which has a 7-mer conserved binding site and an additional 8-mer non-conserved binding site (Supplementary material online, Figure S3). Luciferase reporter assays revealed that the 3′UTRs of all these potential targets except for CD4 were functional, as evidenced by suppression of the firefly luciferase signal by overexpression of miR-221/-222 (~20-fold, data not shown) but not by overexpression of a control sequence or the unrelated miR-29b (Figure 5). Moreover, mutation of three single bases within the binding site for the miR-221/-222 seed sequence abrogated luciferase suppression by pre-miR-221/-222 in all targets. For ETS1, both sites were functional as only double, but not single binding site mutation could rescue the reporter signal from miR-221/-222 repression. To see whether these confirmed miR-221/-222 targets were de-repressed at the mRNA level by in vivo miR-221/-222 inhibition, we measured their cardiac transcript levels using qPCR at 4 and 7 days of VM in animals treated with miR-221/-222 or control antagoniRs. Levels of BCL2L11 and TOX were significantly increased by miR-221/-222 inhibition, while ETS1, ETS2, IRF2, and BMF were only increased by trend. MiR-221/-222 inhibition in VM mice also caused significantly higher mRNA transcript levels of its direct targets CXCL12 and intercellular adhesion molecule-1 (ICAM1), and elevation by trend of IL-6, MCP1, and P-selectin (PSEL) (Figures 2K and 3C), while no difference in IL-4 levels was observed (Figure 2J).

**MiR-221/-222 overexpression suppresses viral replication in cardiomyocytes in vitro**

Finally, we overexpressed or inhibited miR-221 and miR-222 in neonatal rat cardiomyocytes. Control experiments showing the specificity of the oligonucleotides can be found in Supplementary material online, Figure SSA and B. In agreement with the increased viral loads after antagoniR-221/-222 treatment in vivo, knockdown of miR-221/-222 in vitro significantly aggravates viral replication, while overexpression of miR-221/-222 markedly suppressed viral replication, compared with control pre-miRs (after 72 h) (Figure 6A and B). Noteworthy, we also investigated how miR-221/-222 overexpression or knockdown affects the behaviour of fibroblasts and endothelial cells but did not find any relevant suppression of cytokine expression and IFN responses to TNF-α (Supplementary material online, Figure S2A–C). These in vitro experiments indicate a role for miR-221/-222 in CVB3 replication, while not supporting a role in endothelial cell adhesion molecule expression, or fibroblast IFN responses. Together with the effect on the inflammatory response, we posit that miR-221/-222 balances the antiviral and inflammatory response in VM (Figure 6C).

We then performed siRNA experiments to study the functional contribution of the validated miR-221/-222 targets in cardiomyocytes upon CVB3 infection, focusing on those targets known to be expressed by cardiomyocytes and potentially associated with viral infection. We inhibited CXCL12, TOX, and IRF2 separately in nRCMs and as a result found a strong suppression of the inflammatory response in neonatal rat cardiomyocytes infected with CVB3 (Figure 6D). From these data, we conclude that miR-221/-222 is of paramount importance for the myocyte response to CVB3 infection, and controls a network of targets including CXCL12, TOX, and IRF2 to dampen the adverse inflammatory response associated with viral infection.

**Discussion**

Acute VM is an important cause of heart failure and sudden death in previously healthy young persons. A detrimental combination of enhanced cardiac viral replication and an exaggerated immune response against these cardiotropic viruses causes myocardial injury and tissue loss. The present study identifies increased miR-221/-222 levels during CVB3 myocarditis as a protective mechanism to prevent adverse cardiac viral replication, inflammation, and injury in mice (Figure 6C). Systemic inhibition of miR-221/-222 both enhanced cardiac CVB3 replication and increased the inflammatory response.

How does the miR-221/-222 cluster impact on the process of CVB3-induced myocarditis, and the major changes in cardiac CVB3 load? First, miR-221/-222 up-regulation, which we found both in vivo during VM and in vitro in isolated cardiomyocytes in a CVB3 dose-dependent manner, has antiviral properties. Inhibition of miR-221/-222 with antagoniRs stimulated CVB3 replication in isolated cardiomyocytes, whereas further overexpression using mimics decreased viral load. Concordantly, systemic inhibition increased cardiac viral load and prolonged the viraeic state of CVB3-infected mice. Secondly, miR-221/-222 may also regulate the immune response upon viral infection. Their systemic inhibition increased the influx of T cells and macrophages upon CVB3 infection with a resulting depletion of these immune cells in the circulation. No influence of miR-221/-222 on the Th1/Th2 balance was observed.

Up-regulation of miR-221/-222 appeared to be cardiomyocyte specific and dependent of CVB3 infection, since LPS or TNF-α stimulation of T-cells, monocytes, endothelial cells, or fibroblasts did not increase miR-221/-222 expression. MiR-levels increased significantly after viral infection in adult mouse cardiomyocytes, while there was no significant change in the leucocyte population of the infected heart. These data position the ‘immune response’ of the cardiomyocyte to CVB3 replication as pivotal in VM. With respect to the upstream signalling pathways regulating miR-221/-222 up-regulation in the cardiomyocyte, in silico analysis (Supplementary material online, Table S4) predicted STAT1 and STAT3, of which respective
implications in VM have been documented elsewhere,\textsuperscript{29–31} to be able to bind the miR-221/-222 promotor region. Importantly, also IRF1 and IRF2, the latter being a direct miR-221/-222 target validated in our study, were a top predicted transcription factor for miR-221/-222, suggesting a feedback-signalling cascade with miR-221/-222 in a crucial position.

Which messenger targets may have a function in the observed anti-viral properties of miR-221/-222? We identified a number of direct targets that may affect viral replication upon miR-221/-222 manipulation in VM but also may modulate other functional pathways including apoptosis (BCL2L11 and BMF), cell adhesion (ETS1 and ETS2), and cytokine secretion (ETS1 and ETS2, CXCL12). The...
Figure 6 MiR-221/-222 overexpression suppresses viral replication in vitro, while knockdown of miR-221/-222 aggravates viral replication, and inhibition of the direct targets IRF2; CXCL12 or TOX attenuates the pro-inflammatory response upon viral infection. (A) Neonatal rat cardiomyocytes were transfected with mimics for miR-221/-222 or (B) inhibitors against miR-221/-222 vs. scrambled control and infected with CVB3 (MOI = 1). Overexpression of miR-221/-222 significantly suppressed CVB3 replication at 48 h after infection. Conversely, inhibition of miR-221/-222 caused higher CVB3 loads at 72 h after infection (mean + SEM; n = 8 per group; experiment was performed at least two times). (C) Schematic representation of the proposed mechanism of action of CVB3-induced miR-221/-222. (D) Validation experiments where neonatal rat cardiomyocytes were infected with CVB3 after inhibition of IRF2, CXCL12, or TOX. Although there was a mixed response with respect to CVB3 levels, there was a clear blunting of the pro-inflammatory response with very significantly lower IFN-γ levels in the siIRF2, siCXCL12, and siTOX conditions. The overall response appeared to be most pronounced upon IRF2 inhibition as both IL-6, TNF-α, and IFN-γ response was blunted (mean + SD; n = 6–8 per condition; experiment was performed twice).
miR-221/-222 target TOX is important for T-cell biology but is also expressed in cardiomyocytes; and IRF2 is fine-tuning the antiviral response, also in virus-induced cardiac damage.32 Most likely, the miR-221/-222 cluster functions through a combination of different targets, affecting the immune response against viral infection and subsequent inflammation. We however decided to limit our siRNA experiments to CXCL12, TOX, and IRF2. We excluded ETS1 and ETS2 because of their predominant function in oncogenesis, and although we indeed show that the pro-apoptotic factors,33 BCL2L11 and BMF are bona fide targets of miR-221/-222, the observed effects on viral load were not parallelized by differences in cardiac apoptosis, neither in vitro nor in vivo. Inhibition of CXCL12, TOX, or IRF2 clearly dampened the pro-inflammatory response in neonatal rat cardiomyocytes, and the derepression of IRF2, CXCL12, and TOX upon miR-221/-222 inhibition in vivo integrates the observed effects on viral replication on the one hand, and on immune cell infiltration on the other hand. It is to be expected that the effect on the antiviral response in myocytes would even be greater when the siRNAs were to be combined, an effect that is observed in vivo by the up-regulation of miR-221/-222 in VM. All together, the proven targets help to explain the CVB3-suppressive and immune modulatory properties of miR-221/-222.

What role does the miR-221/-222 cluster have in human cardiac diseases, where its expression is down-regulated in chronic heart failure,34,35 in contrast to its up-regulation in acute cardiac injury.36–39 Given the marked conservation of miR-221 and miR-222 throughout evolution (mature miR-221/-222 sequences are perfectly evolutionarily conserved from zebra fish to rodents and humans; Supplementary material online, Figure S4), comparable biological functions in humans and mice are expected. There is vast evidence of miR-221/-222 up-regulation in heterogeneous acute murine heart disease related to acute stress responses to ischaemia and pressure overload,36,38,39 in line with our up-regulation upon acute VM. Importantly, one of the most frequent causes of dilated cardiomyopathy is VM.40 Thus whereas the increase in VM reflects both the influx of inflammatory cells expressing miR-221/-222 and its up-regulation in cardiomyocytes upon viral infection, conversely its down-regulation in chronic cardiac disease may mirror cardio-myocyte loss or a maladaptive response resulting in a chronic inflammatory state of the failing heart. One could even speculate that this maladaptive down-regulation of miR-221/222 might further impair viral clearance in the chronic phase after VM; however, this hypothesis definitely remains to be explored.

Obtaining early viral clearance in VM, for example with miR-221/-222 mimics, may help to prevent exaggerated and chronic cardiac inflammation. Anti-viral therapy could consist of a combination of direct viral clearance and an indirect modulation of inflammatory pathways involved in virus elimination. Since specific immune modulating, anti-viral therapy against two of the most frequent viruses provoking myocarditis (PVB19 and CVB3) is currently lacking, miR-221/-222-targeted therapy might form an interesting alternative for this unpredictable yet often detrimental condition. The drawback of an miR-221/-222 mimics-based anti-viral approach is that it would need to be administered in the very early phase of the disease, which is clinically challenging because patients often present only after the acute phase has seized and symptomatic heart failure has developed. Furthermore, we were not able to design an experiment to demonstrate these therapeutic effects. Firstly, experience with the use of microRNA mimics for in vivo applications is rather limited and there is lack of organ and cell targeting. Second-ly, overexpression of miR-221/-222 in an adenov-associated viral vector was not pursued because of the lack of translational perspective regarding this approach.

Altogether, we conclude that the miR-221/-222 cluster acts as a protective factor in VM and has therapeutic potential both at the miR-level and at the level of its direct targets.

**Supplementary material**

Supplementary material is available at European Heart Journal online.

**Funding**

This work was supported by the Scientific Research Fund of Flanders (FWO) to W.H. (1183211N, 1183213N) and S.H. (G074009N), and by grants from the Dutch Scientific Research Fund (NWO) to S.H. (2008N01). This work also received funding from the European Union Commission’s Seventh Framework programme under grant agreement n° 261409 (MEDIA), and the Marie Curie Industry Academy Pathways and Partnerships (CARDIOMIR) n° 285991. It was supported by research grants from the Netherlands Organization for Scientific Research (NWO) Vidi 91796338 (Dr Heymans). We acknowledge the support from the Netherlands Cardiovascular Research Initiative; the Dutch Heart Foundation; the Dutch Federation of University Medical Centres, the Netherlands Organization for Health Research and Development, and the Royal Netherlands Academy of Sciences (CVON 2011-11 ARENA). W.H. wants to gratefully mention the Frans Van de Werf Fund for Cardiovascular Research, Leuven, Belgium.

**Conflict of interest:** None declared.

**References**


17. Huber SA. Coxsackievirus-induced myocarditis is dependent on distinct immunopathogenic responses in different strains of mice. Lab Invest 1997;76:691–701.


