Wnt11 gene therapy with adeno-associated virus 9 improves the survival of mice with myocarditis induced by coxsackievirus B3 through the suppression of the inflammatory reaction

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ABSTRACT

The wnt signaling pathway plays important roles in development and in many diseases. Recently several reports suggest that non-canonical Wnt proteins contribute to the inflammatory response in adult animals. However, the effects of Wnt proteins on virus-induced myocarditis have not been explored. Here, we investigated the effect of Wnt11 protein in a model of myocarditis induced by coxsackievirus B3 (CVB3) using recombinant adeno-associated virus 9 (rAAV9). The effect of Wnt11 gene therapy on a CVB3-induced myocarditis model was examined using male BALB/c mice. Mice received a single intravenous injection of either rAAV9-Wnt11 or rAAV9-LacZ 2 weeks before intraperitoneal administration of CVB3. Intravenous injection of the rAAV9 vector resulted in efficient, durable, and relatively cardiac-specific transgene expression. Survival was significantly greater among rAAV9-Wnt11 treated mice than among mice treated with rAAV9-LacZ (87.5% vs. 54.1%, $P < 0.05$). Wnt11 expression also reduced the infiltration of inflammatory cells, necrosis of the myocardium, and suppressed the mRNA expression of inflammatory cytokines. This is the first report to show that Wnt11 expression improves the survival of mice with CVB3-induced myocarditis. AAV9-mediated Wnt11 gene therapy produces beneficial effects on cardiac function and increases the survival of mice with CVB3-induced myocarditis through the suppression of both infiltration of inflammatory cells and gene expression of inflammatory cytokines.

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1. Introduction

Viral myocarditis is an important cause of human morbidity and mortality for which specific and effective therapy is lacking. It has been commonly associated with cardiotropic viruses, such as coxsackievirus, with histological inflammation of the myocardium [1, 2]. It is also one of the primary causes of acute heart failure and may progress to dilated cardiomyopathy and chronic heart failure [3–6]. Viral myocarditis has been investigated in the murine model of coxsackievirus B3 (CVB3)-induced myocarditis. Studies in these models have revealed that direct viral injury and the subsequent immune response in the acute to subacute phase determine the severity of viral myocarditis [5,7]. The inflammatory response has the beneficial effect of viral clearance; however, an overly prolonged immune reaction can cause further damage to salvaged cardiomyocytes. CVB3-induced myocarditis results in excessive Th1 immune responses that proved to play a critical pathogenetic role in the initiation and progression of CVB3 myocarditis [8,9].

Myocarditis is a disease with variable clinical manifestations ranging from asymptomatic electrocardiogram abnormality to cardiogenic shock or fatal outcome. Supportive care is the first line of treatment for acute myocarditis due to the lack of specific therapy. Fulminant myocarditis shows severe hemodynamic compromise requiring high dose vasopressor support or mechanical circulatory support, which was identified in 10% of myocarditis patients [6]. These patients typically have severe global left ventricular dysfunction. On the other hand, only 45% of patients with acute (nonfulminant) myocarditis survived without having received heart transplantation for 11 years [6]. This report suggests that specific therapies to modulate the inflammation induced by myocarditis should be investigated. More than 20 treatment trials for myocarditis or inflammatory cardiomyopathy have been reported using immunosuppressive, immunomodulating, anti-inflammatory, or immunoadsorption therapies. Immunosuppressive therapy using prednisolone, azathioprine, or cyclosporine failed to improve survival in myocarditis patients [10]. Specific therapies that can improve prognosis in conjunction with supportive treatment are expected to be developed.

The Wnt glycolipoproteins are best characterized as regulators of cell proliferation, cell polarity, and cell-fate determination during

embryonic development; however, components of the Wnt signaling pathway have also been linked to hematological malignancies (e.g., acute and chronic myeloid leukemia, acute lymphoblastic leukemia, multiple myeloma) and to inflammatory diseases such as type 2 diabetes, in adults [11–14]. Within the immune system, the canonical Wnt-β-catenin signaling appears to have an important role in the self-renewal of hematopoietic stem cells and progenitor cells, in T- and B-cell development, in peripheral T-cell activation, and in the maturation of dendritic cells [15–17], whereas non-canonical signaling by Wnt5a may counteract canonical Wnt signaling to inhibit B- and T-cell development. Wnt5a also regulates the inflammatory response by modulating interleukin production in monocytes and macrophages [18,19]. Additionally, Wnt11, which regulates the development of heart and kidney through the non-canonical Wnt signaling pathway, suppresses inflammation in intestinal epithelial cells [20–22]. Collectively, these reports suggest that Wnt proteins participate in the inflammatory response and consequently, that patients recovering from heart diseases with inflammation might benefit from therapies that alter Wnt signaling.

Myocarditis is one of the representative heart diseases that inflammation is a major constituent of the disease. Acute viral-induced myocarditis is accompanied by severe inflammation of the infected heart, with high mortality. Wnt11, which regulates heart development in embryonic stage and can modulate inflammation in adult organs, is one of intriguing candidates for the therapy of heart diseases with inflammation, especially virus-induced myocarditis. We explored the possibility that Wnt11 gene therapy exerts a therapeutic effect in a CVB-induced model of myocarditis.

2. Materials and methods

2.1. Coxsackievirus B3 (CVB3)

CVB3 (Nancy strain) was a kind gift from Dr. Y. Seko (Asahi Life Foundation, Tokyo, Japan). It was grown in cultures of Vero cell (ATCC), then tittered by plaque assay and stored at −80 °C [23]. Aliquots from the same stock were used for all animals.

2.2. Mouse model of viral myocarditis

Six-week-old male BALB/c mice were purchased from SLC Co. Ltd. (Nagoya, Japan). Mice were inoculated intraperitoneally with 1×10^6 PFU of CVB3 in 100 μl PBS on day 0 to induce acute viral myocarditis. Mice were observed for spontaneous death until day 14. Mice were weighed on days 0, 4, 7, 10, and 14 after CVB3 infection and average body weight was calculated. Surviving mice were examined by echocardiography on days 0, 4, 7, 10, and 14 post-CVB3 infection. All experimental procedures and animal-care protocols were approved by the Institutional Animal Care and Use Committee of Nagoya University.

2.3. Production of recombinant virus

cDNA encoding mouse Wnt11 (Open Biosystems, Inc.) was subcloned into the EcoRI/SalI sites of a pAAV-MCS vector using the AAV Helper-Free System (Agilent Technologies) to create pAAV-Wnt11. pAAV2/9 containing AAV2 rep and AAV9 capsid genes was kindly provided by the Penn Vector Core (University of Pennsylvania School of Medicine).

The rAAV9 viruses were produced by the triple transfection method, using AAV293 cells (Agilent Technologies) [24,25]. The pAAV-LacZ (Agilent Technologies) and pAAV-Wnt11 plasmids were co-transfected with the pHelper and the pAAV2/9 plasmids into AAV293 cells using the CaPO$_4$ method (Agilent Technologies) according to the manufacturer's instructions. At 48 h post-transfection the cells were lysed by freeze–thaw cycles and Benzonase (SIGMA) was added to a final concentration of 200 μm/ml, before incubating at 37 °C for 1 h. The extracted recombinant AAV9 viruses were purified by two rounds of cesium chloride (CsCl) isopycnic gradient centrifugation. Virus sample was dialyzed using a 10,000 MWCO Slide-A-Lyzer Dialysis Cassette (Thermo Scientific) and phosphate-buffered saline containing 5% sorbitol. The titer was determined by quantitative polymerase chain reaction (PCR) using primers for the cytomegalovirus (CMV) promoter.

2.4. AAV9 infection

For assessment of gene expression, 3×10^{11}-genome copy (GC) doses of rAAV9-LacZ were administered from the tail vein of mice. For experiments performed in the CVB3-induced myocarditis model, 3×10^{11}-GC doses of AAV9-LacZ or rAAV9-Wnt11 in 150 μl PBS were administered 2 weeks before CVB3 infection via tail vein injection.

2.5. Cardiac functional assessments

Cardiac function was evaluated with an echocardiographic system (ACUSON Sequoia c512, SIEMENS). Mice in subgroups were anesthetized with pentobarbital and examined by echocardiography for left ventricular end-diastolic dimensions (LVEDd), left ventricular end-systolic dimensions (LVEDs), left ventricular ejection fraction (LVEF) and left ventricular shortening fraction (FS) on days 0, 4, 7, 10, and 14 after CVB3 infection by two independent observers in a blinded manner. LVEF was calculated by the following equation; LVEF = {Left ventricular end-diastolic volume (LVEDV) − left ventricular end-systolic volume (LVESV)} / (LVEDV) × 100 (%). LVEDV and LVESV were estimated by Teichholz formula: $(7.0/2.4 + D) (D^3)$, where D is the internal dimension of left ventricular end-diastole and end-systole measured from M-mode tracing.

2.6. Histopathological evaluation

Mice were killed on days 4, 7, 10, and 14 post-CVB3 infection. Hearts were embedded in O.C.T. compound, snap-frozen in liquid nitrogen, and cut into 7-μm sections. Sections were stained with hematoxylin and eosin to evaluate the level of inflammation. Evidence of myocarditis was evaluated according to a 6-tier scoring system: grade 0, no inflammation; grade 1, cardiac infiltration in up to 5% of the cardiac sections; grade 2, 6–10%; grade 3, 11–30%; grade 4, 31–50%; and grade 5, >50% [26].

To evaluate CD45, CD68, CD3 and Ly6G expression, sections were fixed with cold acetone and then stained with anti-CD45 (BD Pharmingen), anti-CD68 (Bio-Rad), anti-CD3 (Abcam) and anti-Ly6G (BD Pharmingen) primary antibodies and Alexa-Fluor 488 or Alexa-Fluor 594 secondary antibodies (Invitrogen Corporation); nuclei were counterstained with DAPI.

Fibrosis was evaluated by treating sections with 0.05% picrosirius red (Direct Red 80; Sigma-Aldrich Corp.) solution for 1 h as previously described [27].

2.7. Real-time, reverse transcription polymerase chain reaction (RT-PCR), and gene array analyses

RNA was isolated from frozen, homogenized organ tissues using ULTRA-TURRAX T8, as instructed by the manufacturer. For quantitative RT-PCR analyses, total RNA was reverse transcribed with a ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO Co. Ltd.), and amplification was performed on a Mx3000p QPCR system (Stratagene). Primer sequences are reported in the Supplementary Table. The relative expression of each mRNA was calculated by the comparative threshold cycle method and normalized to β-actin RNA expression. Data were shown as a relative value for WT heart without any virus infection.

2.8. ELISA (enzyme-linked immunosorbent assay)

Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (100 mg/kg) on day 14. Blood samples were collected immediately. Blood samples were centrifuged (1500 rpm for 10 min) and then supernatants were stored as serum at − 80 °C used for analysis. Mouse IFN-γ was measured by commercially available enzyme-linked immunosorbent assay (Enzo Life Sciences).

2.9. Statistical analysis

Survival was summarized via Kaplan–Meier analysis and evaluated for significance with the log-rank test. Other results are presented as mean ± SEM. Comparisons between two groups were evaluated for significance with the Student's t-test and measurements obtained in the same animal at multiple time points were evaluated via repeat measure analysis. A P value less than 0.05 was considered significant.

3. Results

3.1. Systemic rAAV9-Wnt11 administration leads to persistent, cardiac-specific gene expression

The efficiency, duration, specificity, and dose-dependence of rAAV9-mediated transduction was evaluated by injecting rAAV9-LacZ into the tail vein of BALB/c WT mice and monitoring LacZ expression for 4 weeks. X-gal-stained sections of cardiac tissue indicated that cardiomyocytes expressed LacZ in the heart and that a substantial proportion of cardiomyocytes expressed the transduced gene within 2 weeks of
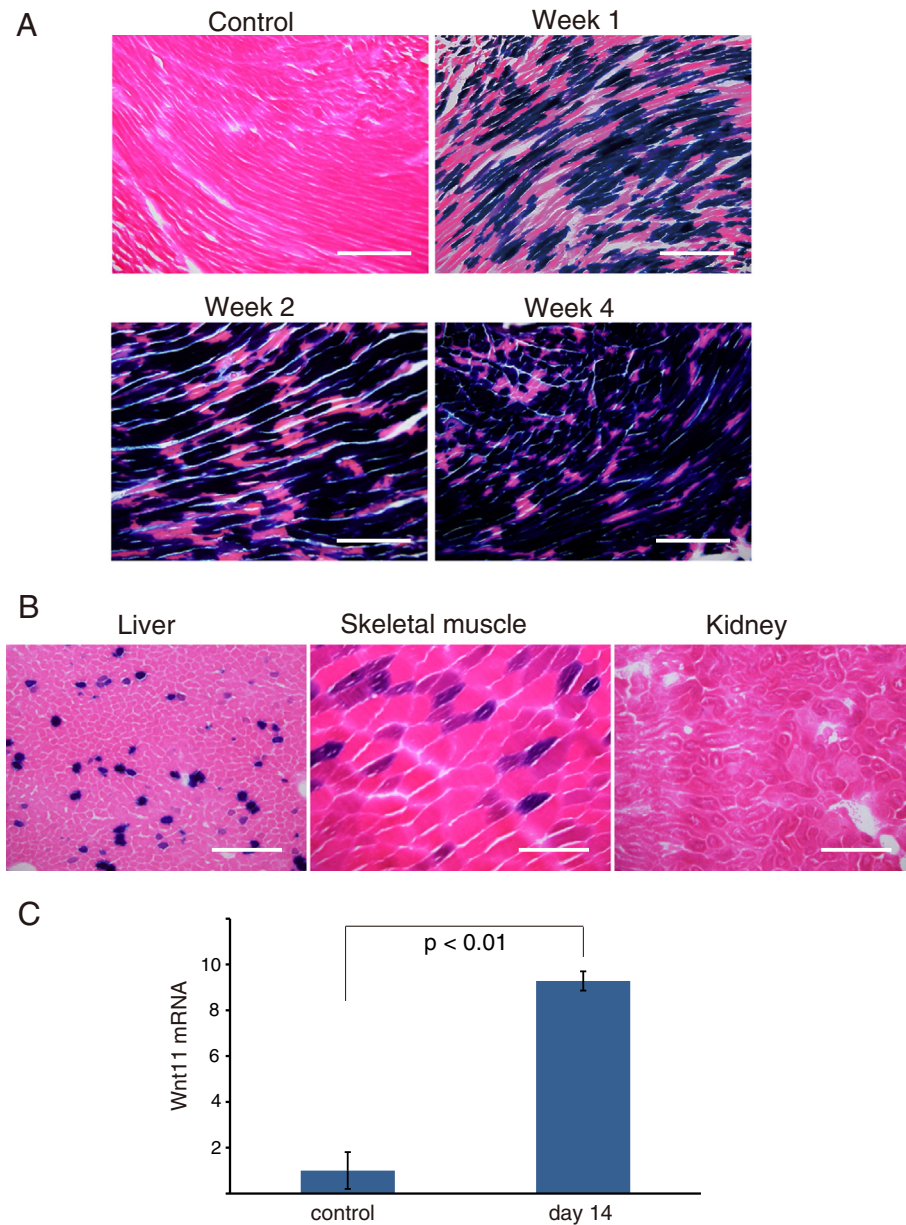

Fig. 1. The rAAV9 vector induces robust, durable, and cardiac-specific gene expression. 3.0×10^{11} GC of AAV9-LacZ expression vector was injected into the tail vein of BALB/c WT mice. (A) LacZ expression was identified in the hearts of mice sacrificed 1–4 weeks after treatment by 3.0×10^{11} GC of rAAV9-LacZ via X-gal staining; scale bar = 200 μm. (B) LacZ expression in the liver, skeletal muscle, and kidney at 2 weeks after treatment of 3.0×10^{11} GC of rAAV9-LacZ (scale bar = 200 μm). (C) Gene expression of Wnt11 in heart tissue was measured 2 weeks after rAAV9-Wnt11 injection via RT-PCR and normalized to β-actin. n = 4.

injection (Fig. 1A). LacZ expression in the heart was more robust than that in the liver, skeletal muscle, and kidney (Fig. 1B).

Next gene expression of Wnt11 in the heart tissue was evaluated 2 weeks after rAAV9-Wnt11 administration and compared to control sample. Systemic administration of rAAV9-Wnt11 resulted in strong gene expression of Wnt11 in the heart tissue (Fig. 1C).

Moreover, we checked RNA expression of endogenous Wnt11 after CVB3 infection by RT-PCR. Virus infection of CVB3 induced no significant increase of Wnt11 for 28 days (Supp. Fig. 2).

3.2. Systemic rAAV9-Wnt11 administration protects mice from CVB3-induced myocarditis

To evaluate the effect of Wnt11 gene expression on CVB3-induced myocarditis, we injected 3.0×10^{11} GC of rAAV9-Wnt11 or rAAV9-LacZ 2 weeks before CVB3 infection. More than 40% of mice treated with rAAV9-LacZ died within 14 days post-infection, while mice receiving rAAV9-Wnt11 treatment showed a significantly higher 14-day survival rate at 87.5% ($P = 0.0127$) (Fig. 2A).

Body weight is a parameter reflecting the overall health status of mice. Mice were weighed on days 4, 7, 10, and 14 after CVB3 infection and average body weight was calculated. Mice treated with rAAV9-LacZ developed hypophagia after CVB3 infection, resulting in body weight loss during the course of the study, while mice treated with rAAV9-Wnt11 showed a steady increase in body weight gain reflecting a better general condition (Fig. 2B).

To determine whether rAAV9-Wnt11 treatment enhanced cardiac function after CVB3 infection, echocardiographic assessments were performed on days 0, 4, 7, 10, and 14 after CVB3 infection. There was no difference in LVEDd between either groups. However, compared to rAAV9-LacZ treated mice, LVEDs was significantly lower and LVEF and FS were significantly higher in mice treated rAAV9-Wnt11 on days 7, 10 and 14 post CVB3 infection (Fig. 2C, D).

3.3. Administration of rAAV9-Wnt11 reduces infiltration of inflammatory cells during CVB3-induced myocarditis

Moderate to severe inflammation as well as widespread degeneration and necrosis of the myocardium were observed in rAAV9-LacZ treated mice on days 10 and 14 after CVB3 infection. On the other hand, fewer infiltrating inflammatory cells were observed in rAAV9-Wnt11 treated mice, resulting in an improved pathological score of the heart tissue (Fig. 3A, B). Furthermore, sections stained for expression of the inflammatory marker CD45 indicated that prolonged inflammation was present in the heart tissue of mice treated with rAAV9-LacZ until 14 days after CVB3 infection, while rAAV9-Wnt11 administration was associated with fewer inflammatory cells in the heart tissue after day 7. The difference in the cardiac mRNA level of CD45 between groups also supports this phenomenon (Fig. 4A, B).

We also examined the infiltration of macrophages, T cells and neutrophils by immunohistochemistry on day 14 after CVB3 infection to evaluate what inflammatory cells are affected by Wnt11. Surprisingly, CD68 positive macrophages, CD3 positive T cells and Ly6G positive neutrophils were dramatically suppressed in the heart treated with rAAV9-Wnt11 (Fig. 5). These data indicate that Wnt11 expression can reduce infiltration of inflammatory cells globally. Fibrosis which can be induced by inflammation was not enhanced in both samples of LacZ and Wnt11 groups on day 14 (Supp. Fig. 1).
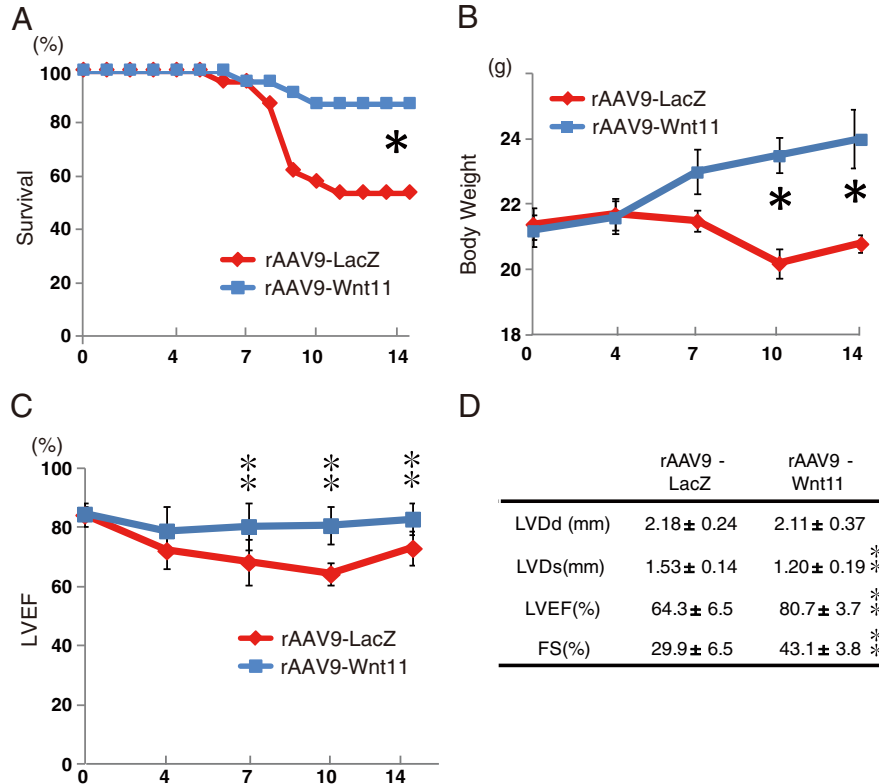

Fig. 2. Wnt11 expression improves survival, cardiac function, and preserves the general condition post-CVB3 infection. At 2 weeks before CVB3 infection, 3.0×10^{11} GC of a rAAV9 vector coding for Wnt11(rAAV9-Wnt11) or LacZ (rAAV9-LacZ) expression was injected into the tail vein of BALB/c WT mice. (A) Survival was monitored for 14 days after CVB3 infection; n = 24 per treatment group at day 0. *$P < 0.05$ (B) Body weight was measured at day 0, 4, 7, 10, and 14 post-CVB3 infection. n = 5 *$P < 0.05$. (C, D) Echocardiographic assessments of left ventricular end-diastolic dimensions (LVEDd), left ventricular end-systolic dimensions (LVEDs), left ventricular ejection fraction (LVEF) and left ventricular shortening fraction (FS) were performed on days 4, 7, 10, and 14 after CVB3 infection. (D) Data of day 10. n = 6 for each time points. ‡$P < 0.01$.
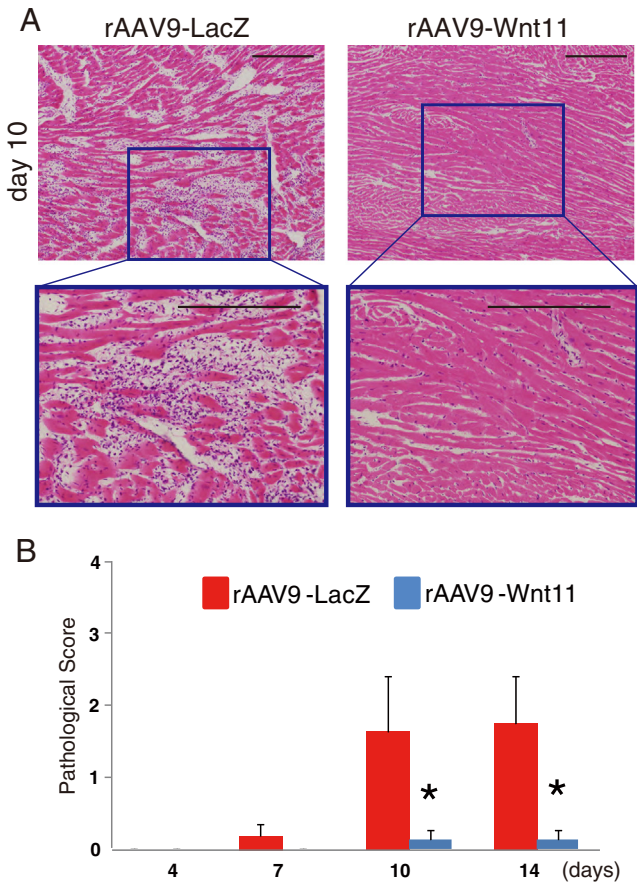
Fig. 3. Wnt11 expression reduced inflammation and necrosis of the myocardium post-CVB3 infection. Mice in subgroups were sacrificed on days 4, 7, 10, and 14 post-CVB3 infection. (A) Cardiac inflammation was examined by H&E stained sections of heart tissue (scale bar = 200 μm). Higher magnifications of the boxed areas in the top panels are shown in the bottom panels. (B) The severity of myocarditis was evaluated according to a 6-tier scoring system. n = 6 for each time points. *$P < 0.05$.

3.4. Administration of rAAV9-Wnt11 reduces gene expression and protein expression of inflammatory cytokines during CVB3-induced myocarditis

To determine how rAAV9-Wnt11 administration affects the cytokine response in cardiac tissue post-CVB3 infection, cardiac cytokine mRNA expression was evaluated by RT-PCR on days 4, 7, 10, and 14 after CVB3 infection. Cardiac mRNA levels of IL-1β, IL-6, and IFN-γ were significantly decreased in rAAV9-Wnt11 treated mice compared to those of mice treated with rAAV9-LacZ. Similarly, the mRNA expression of IP-10, which plays a critical role on CVB3-induced myocarditis, was also significantly suppressed in rAAV9-Wnt11 treated mice (Fig. 6A).

Concentration of IFN-γ, which is a cytokine that is critical for innate and adaptive immunity against viral was examined in blood samples 14 days after CVB3 infection using enzyme-linked immunosorbent assay. rAAV9-Wnt11 treatment showed a tendency to suppress the concentration of IFN-γ in circulating blood compared to rAAV9-LacZ treatment (Supp. Fig. 3).

4. Discussion

Viral infections like coxsackievirus B3 are the most commonly indentified cause of myocarditis in developed countries [28]. CVB3 infection can lead to viral myocarditis through a direct virus-mediated reaction and autoimmune-mediated responses [29,30]. In the acute phase, direct injury of cardiomyocytes due to virus infection can be induced in conjunction with secondary injury of cardiomyocytes by
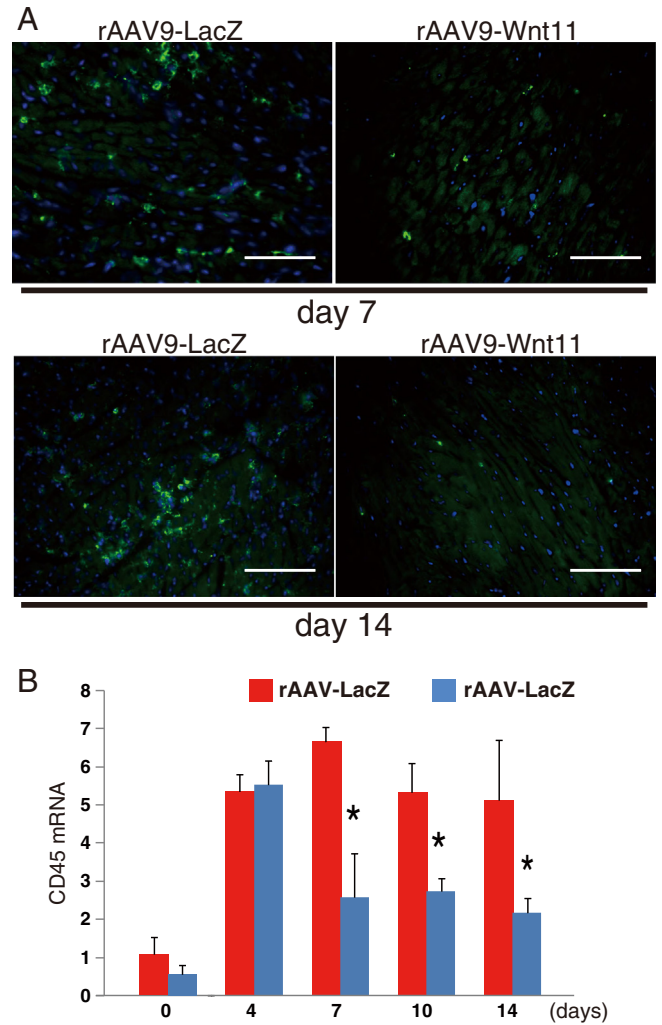
Fig. 4. Wnt11 expression reduces infiltration of inflammatory cells into the myocardium post-CVB3 infection. Mice in subgroups were sacrificed on days 4, 7, 10, and 14 post-CVB3 infection. (A) Inflammatory cells were identified by staining sections for expression of the inflammatory cell marker CD45 (green). Nuclei were counter-stained with DAPI (scale bar = 200 μm). (B) The mRNA level of CD45 at every time point was measured via RT-PCR and normalized to β-actin; n = 6 for each time points. *$P < 0.05$.

the inflammation through innate immune cells such as dendritic cells (DCs), natural killer (NK) cells and macrophages [31]. During the same phase, the expression of various cytokines, such as IL-1β, IL-6, TNF-α, IFN-γ, and IP10, can be upregulated in the heart [31,32]. These cytokines play a crucial role in the defense against virus infection [33, 34], however, overly robust expression of these cytokines can cause severe inflammation and widespread necrosis of the myocardium [35].

Although treatment of myocarditis should be focused on the causal pathophysiology, specific treatment options for viral myocarditis are not established. For patients who deteriorate despite optimal medical treatment with cardiogenic shock due to acute fluminant myocarditis, mechanical circulatory support may be required to bridge the patient to recovery or heart transplantation. On the other hand, investigational treatment options using immunosuppressive or anti-inflammatory agents as well as immunoadsorption therapy have been reported. However, immunosuppressive therapy has not been shown to be effective as routine treatment for acute myocarditis [36].

Wnt signaling is not only essential in T- and B-cell development but may also have an important role in the activation of immune cells in the periphery. T-cell migration and the induction/maintenance of a regulatory immune response are partly controlled by Wnt signaling [16]. Both the canonical and the non-canonical Wnt pathways may influence the
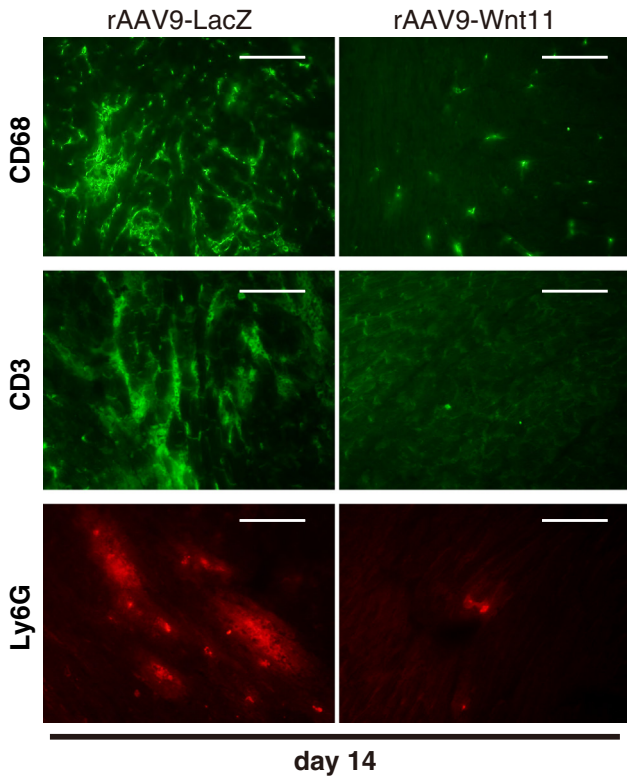
Fig. 5. Wnt11 expression reduces infiltration of macrophages, T-cells and neutrophils into the myocardium post-CVB3 infection. Mice in subgroups were sacrificed on days 14 post-CVB3 infection. Macrophages, T-cells and neutrophils were identified by staining sections for expression of CD68, CD3 and Ly6G, respectively (scale bar = 100 μm).

balance between inflammatory and tolerogenic immune responses [37]. Given its widespread importance in the immune system and hematopoiesis, both activators and inhibitors of the Wnt pathway could be used to treat pathological conditions with inflammation.

Recombinant adeno-associated virus has become increasing common as a vector for use in human clinical trials [38]. The popularity of AAV vectors reflects the appreciation of the long-term gene expression in post mitotic cells and the relative lack of deleterious immune response. To date over a hundred naturally occurring primate AAVs have been reported. Among them, rAAV9 has a high natural affinity for myocardium [25]. Our results confirmed that rAAV9-mediated gene delivery is efficient, cardiac-specific, and durable: gene expression was robust in the heart, much lower in the liver, skeletal muscle and kidney. These data show that rAAV9 is suitable vector to examine the effects of Wnt11 in virus-induce model of myocarditis.

Here, we investigated the effect of rAAV9-mediated Wnt11 gene therapy on a myocarditis model induced by CVB3. rAAV9-Wnt11 treated mice showed a significantly improved 14-day survival rate at 87.5% in contrast to the low survival rate of 54.1% in the rAAV9-LacZ treated mice. Wnt11 expression was also associated with significant improvements in cardiac function and appeared to reduce the infiltration of inflammatory cells into the heart. Interestingly, diverse populations of inflammatory cells including macrophages, T-cells and neutrophils were affected by Wnt11. Moreover, the expression of inflammatory cytokines such as IL-1β, IL-6, and IFN-γ was significantly suppressed in rAAV9-Wnt11 treated mice compared to rAAV9-LacZ treated mice after day 7 of CVB3 infection. IP-10 expression was also dramatically suppressed in rAAV9-Wnt11 treated mouse. IP-10 is considered to be a Th-1 mediated chemokine that plays a critical role in CVB3-induced myocarditis [39–41]. Therefore, suppression of the expression of IP-10 can lead to a reduction in chemokine-directed inflammation and the resultant myocardial injury [42]. Collectively, Wnt11 gene expression
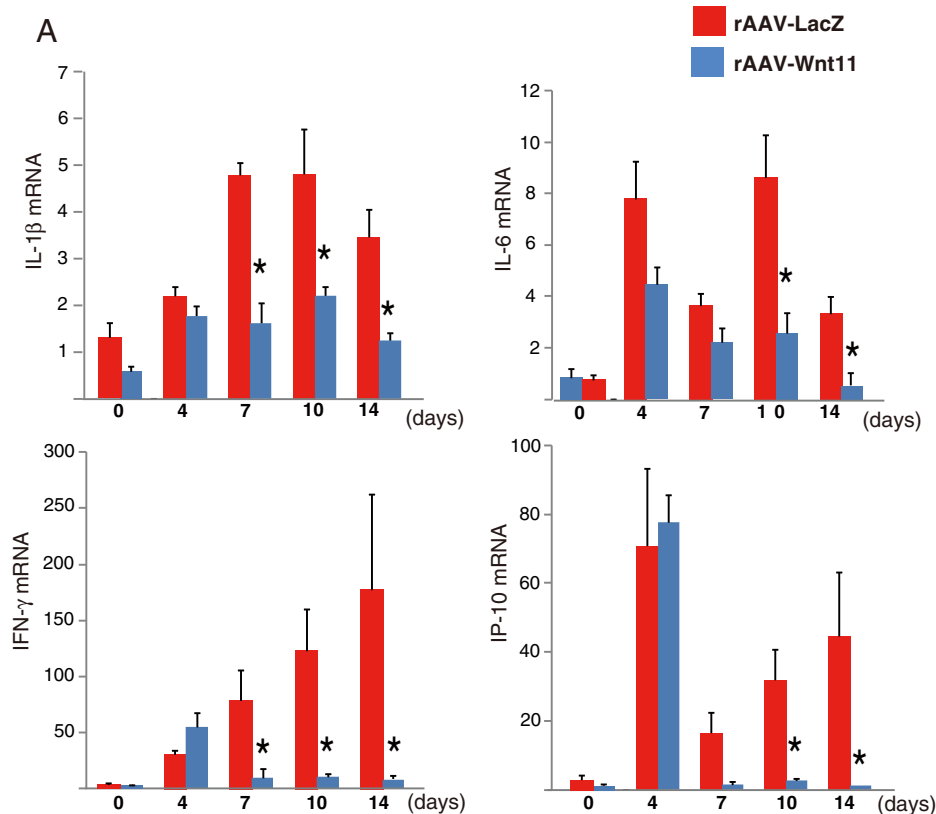

Fig. 6. Wnt11 expression reduces the expression of inflammatory factors post-CVB3 infection. (A) On days 4, 7, 10, and 14 post CVB3 infection, mRNA levels of inflammatory factors (IL-1β, IL-6, IFN-γ, and IP-10) were measured via RT-PCR and normalized to β-actin; n = 6 per treatment group.

improves survival and cardiac performance after CVB3 infection by suppressing the inflammatory response. However, we cannot deny the possibility that cardiomyocytes were directly protected by Wnt11 protein against inflammation, though it is difficult to distinguish between direct effect and indirect effect through the suppression of inflammation.

In this study, we clearly showed the beneficial effects of Wnt11 in virus-induced myocarditis. We expect to be able to apply a modified rAAV9 vector with the property of rapid gene expression or recombinant protein of Wnt11 with high bioactivity for human Wn11 therapy in the near future. Our data proved the concept that Wnt11 has beneficial therapeutic effects in virus-induced myocarditis.

In conclusion, this is the first evidence for the importance of Wnt11 signal in CVB3-induced myocarditis. AAV9-mediated Wnt11 gene therapy protects against viral myocarditis, as documented by a dramatic reduction in the histopathologic evidence of myocardial injury and gene expression of inflammatory cytokines, and improved survival of mice with CVB3-induced myocarditis. Wnt11 therapy could be a promising novel approach for treating CVB3-induced myocarditis.

Conflicts of interest

No potential conflicts of interest are reported.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2015.04.009.

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