RESEARCH PAPER

Human Vaccines & Immunotherapeutics 10:5, 1284–1294; May 2014; © 2014 Landes Bioscience

Mucosal co-immunization with AIM2 enhances protective SIgA response and increases prophylactic efficacy of chitosan-DNA vaccine against coxsackievirus B3-induced myocarditis

Dafei Chai[1], Yan Yue[1], Wei Xu[1], Chunsheng Dong[1], and Sidong Xiong[1,*]

[1]Jiangsu Key Laboratory of Infection and Immunity; Institutes of Biology and Medical Sciences; Soochow University; Suzhou, PR China

Keywords: Coxsackievirus B3, viral myocarditis, mucosal vaccine, AIM2, adjuvant, chitosan, SIgA

Abbreviations: CVB3, coxsackievirus B3; AIM2, absent in melanoma 2; SIgA, secretory immunoglobulin A; BAFF, B cell activating factor of the tumor necrosis family; APRIL, a proliferation-inducing ligand; iNOS, inducible nitric oxide synthase; RALDH1, retinaldehyde dehydrogenase 1; IL, interleukin; TGF-β, transforming growth factor β; MLN, mesenteric lymph node; PPRs, pattern-recognition receptors; LVEF, left ventricular ejection fraction; LVFS, left ventricular fraction shortening; CK, creatine kinase; CKMB, creatine kinase MB; LD_{50}, lethal dose 50%; $TCID_{50}$, tissue culture infective dose 50%

Coxsackievirus B3 (CVB3) infection is considered as the most common cause of viral myocarditis with no available vaccine. Considering that CVB3 mainly invades through the gastrointestinal mucosa, the development of CVB3-specific mucosal vaccine, which is the most efficient way to induce mucosal immune responses, gains more and more attention. In this study, we used absent in melanoma 2 (AIM2) as a mucosal adjuvant to enhance the immunogenicity and immunoprotection of CVB3-specific chitosan-pVP1 vaccine. Mice were intranasally co-immunized with 50 μg chitosan-pAIM2 and equal amount of chitosan-pVP1 vaccine 4 times at 2 week-intervals, and then challenged with CVB3 2 weeks after the last immunization. Compared with chitosan-pVP1 vaccine immunization alone, chitosan-pAIM2 co-immunization enhanced resistance to CVB3-induced myocarditis evidenced by significantly enhanced ejection fractions from 55.40 ± 9.35 to 80.31 ± 11.35, improved myocarditis scores from 1.50 ± 0.45 to 0.30 ± 0.15, reduced viral load from 3.33 ± 0.50 to 0.50 ± 0.65, and increased survival rate from 40.0% to 75.5%. This increased immunoprotection might be attributed to the augmented level of CVB3-specific fecal SIgA with high affinity and neutralizing ability. In addition, co-immunization with chitosan-pAIM2 remarkably facilitated dendritic cells (DCs) recruitment to mesenteric lymph nodes (MLN), and promoted the expression of IgA-inducing factors (BAFF, APRIL, iNOS, RALDH1, IL-6, TGF-β), which might account for its mucosal adjuvant effect. This strategy may represent a promising prophylactic vaccine against CVB3-induced myocarditis.

Introduction

Viral myocarditis is a myocardial inflammatory disease induced by viral infection and may lead to dilated cardiomyopathy,[1] and poses a major health problem for the higher mortality in young men.[2,3] Coxsackievirus B3 (CVB3) is a principle etiologic agent in acute myocarditis, whose infection is involved about half of myocarditis cases.[2,3] CVB3 is a non-enveloped, positive-sense, single-stranded RNA virus belonging to the picornaviridae family.[4-6] So far, there is no efficient vaccines or therapeutic reagents against CVB3-induced myocarditis in clinic.[7-13] Therefore, there is an obvious need to develop a new and efficient vaccine.

AIM2, as a cytosolic DNA sensor, is a member of pattern-recognition receptors (PRRs) with a PYRIN domain at its N-terminus and a HIN-200 domain directly involved in dsDNA recognition at its C-terminus.[14,15] After binding to dsDNA, AIM2 forms inflammasome to activate the procaspase-1.[14-18] The cleavage of pro-interleukin-1β by caspase-1 results in the secretion of interleukin-1β (IL-1β),[14,17,18] which is a key cytokine in the recruitment of immune cells and regulation of immune-cell proliferation, differentiation, and apoptosis.[19,20] The critical roles of AIM2 in host defense against bacterial and viral infections have been confirmed in AIM2-knockout mice.[15,18,21] These characteristics of AIM2 allow us to assume that mucosal co-immunization with AIM2 might benefit the induction of mucosal immunity against CVB3 infection.

In the present study, we demonstrated that co-immunization with chitosan-pAIM2 and chitosan-pVP1 could potentiate intrinsic adjuvant properties of AIM2. Co-immunization

*Correspondence to: Sidong Xiong; Email: sdxiongfd@126.com
Submitted: 01/13/2014; Revised: 02/12/2014; Accepted: 02/23/2014; Published Online: 03/10/214
http://dx.doi.org/10.4161/hv.28333

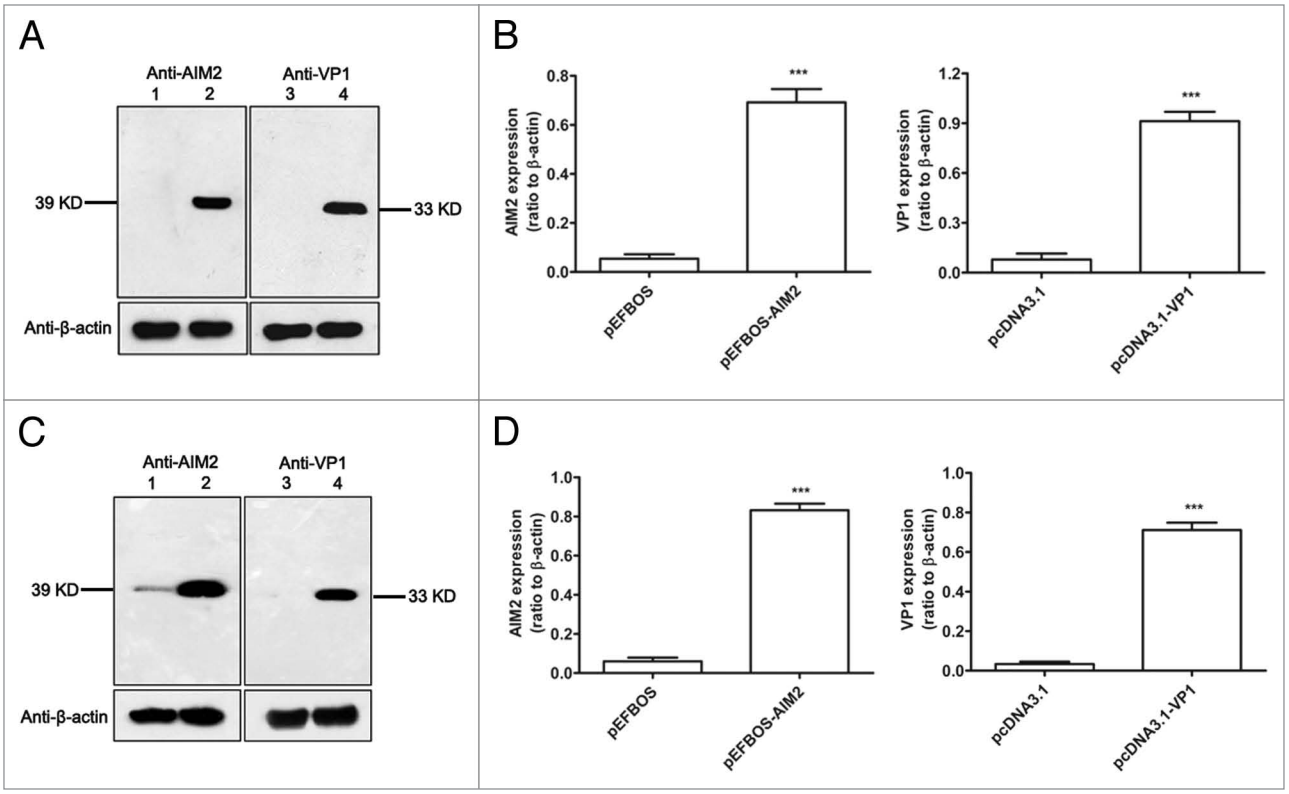

Figure 1. Expression of AIM2 and VP1 plasmids in vitro and in vivo. 293T cells were transfected with pAIM2, pVP1, or vector with lipofectamine for 48 h, and then cell lysates were subjected to western blot analysis using anti-AIM2, anti-VP1, or anti-β-actin antibody, respectively. (**A**) In vitro expression of AIM2 and VP1 plasmids. Lane 1: 293T cells transfected with pEFBOS vector; lane 2: 293T cells transfected with pEFBOS-AIM2; lane 3: 293T cells transfected with pcDNA3.1 vector; lane 4: 293T cells transfected with pcDNA3.1-VP1. (**B**) Quantification of pAIM2 and pVP1 expression by densitometry. Data are from one representative experiment of 3 performed and presented as the mean ± SD ***$P < 0.001$. BALB/c mice were intranasally immunized with chitosan-pAIM2 or chitosan-pVP1 vaccines, respectively, and intranasal mucosa biopsies were taken 3 d later for gene expression analysis. (**C**) Western blot analysis of AIM2, VP1, and β-actin expression for mucosal tissues from immunized mice. Lane 1: Mice immunized with pEFBOS vector; lane 2: Mice immunized with pEFBOS-AIM2; lane 3: Mice immunized with pcDNA3.1 vector; lane 4: Mice immunized with pcDNA3.1-VP1. (**D**) Quantification of AIM2 and VP1 expression by densitometry in (**C**). Data are from one representative experiment of 3 performed and presented as the mean ± SD (n = 10). ***$P < 0.001$.

with AIM2 enhanced mucosal immune responses elicited by chitosan-VP1 vaccine and protection against CVB3-induced viral myocarditis. Our results imply that the application of AIM2 as an adjuvant may be a new approach for improvement of mucosal vaccine for CVB3-induced acute myocarditis.

Results

Identification of AIM2 and VP1 plasmids expression in vitro and in vivo

To verify the expression of AIM2 plasmid (pAIM2) and VP1 plasmid (pVP1) in vitro, the plasmids were transfected into 293T cells. Western blot analysis of pAIM2-transfected cells showed an increased expression of AIM2 compared with empty vector-transfected cells at a predicted molecular weight 39KD (**Fig. 1A and B**). Robust expression of VP1 was also detected in pVP1-transfected cells at the size of 33KD (**Fig. 1A and B**). Next, the expression of pAIM2 and pVP1 was evaluated in intranasal mucosa of mice after intranasal application of 50 μg chitosan-DNA. Western blot analysis indicated that a higher expression of AIM2 and VP1 in chitosan-pAIM2-treated and chitosan-pVP1-treated mice, respectively, than those of chitosan-vector treated mice (**Fig. 1C and D**). Taken together, these data demonstrate the high expression efficiencies of pAIM2 and pVP1 plasmids in vitro and in vivo.

Enhanced resistance to CVB3-induced viral myocarditis by co-immunization of chitosan-pAIM2 and chitosan-pVP1

To evaluate the protection efficacy of co-immunization of chitosan-pAIM2 and chitosan-pVP1 vaccine, these 2 plasmids were used for intranasal immunization individually or in combination. Two weeks after the last immunization, mice were intraperitoneally infected with a normal lethal dose of CVB3 ($3LD_{50}$/mouse) to induce acute myocarditis. Seven days post-infection, the severity of myocarditis was evaluated. As shown in **Figure 2A**, chitosan-pVP1 immunized mice suffered significant weight loss, indicating a severe ongoing infection, while tiny change of weight was observed in chitosan-pAIM2 and chitosan-pVP1 co-immunized mice. Compared with chitosan-pVP1 immunized group, chitosan-pAIM2 and chitosan-pVP1 co-immunized group showed significant improvements on cardiac function reflected by left ventricular ejection fraction

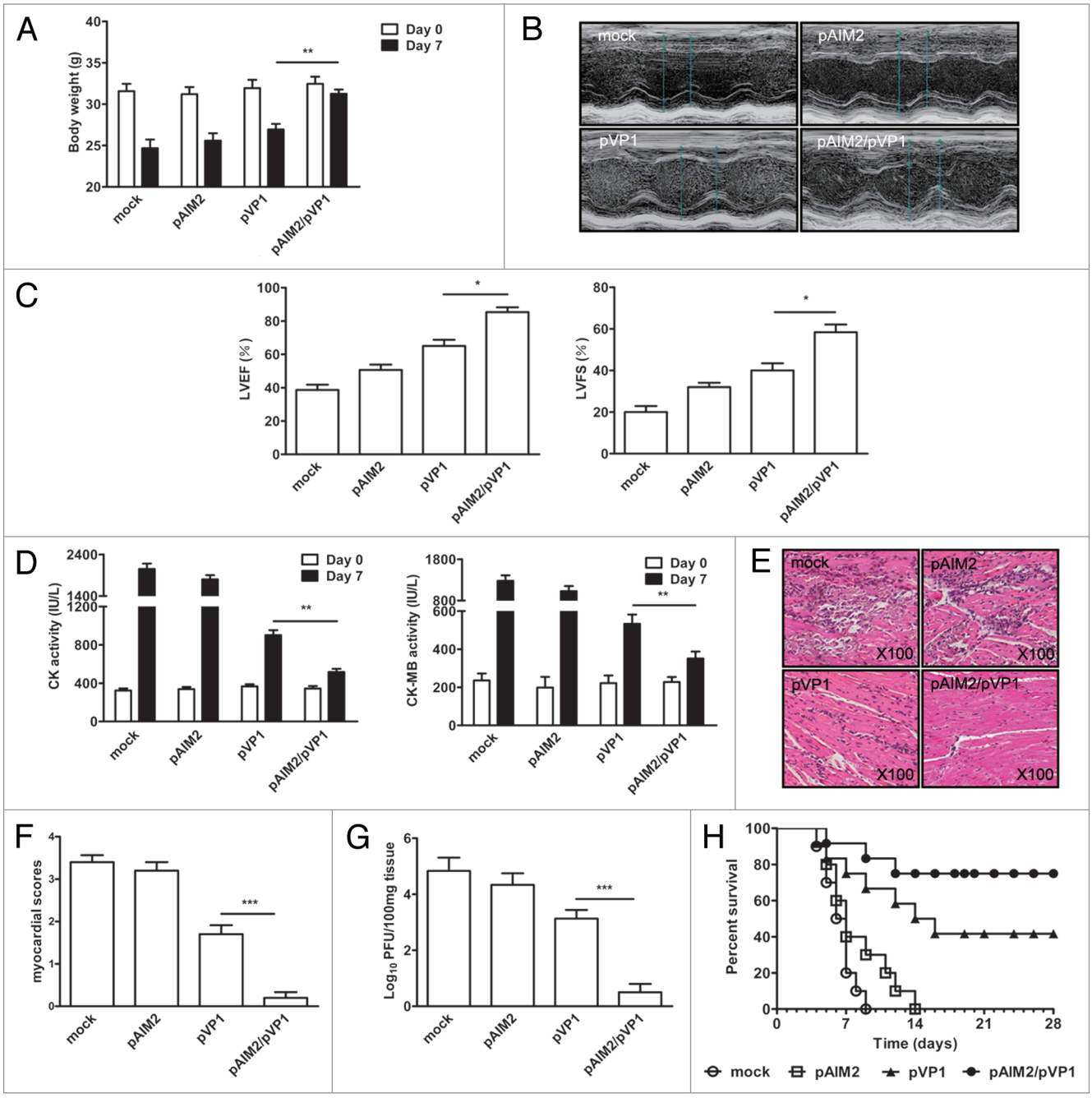

Figure 2. The protective effects against CVB3-induced acute myocarditis by co-immunization of chitosan-pAIM2 and chitosan-pVP1. Seven days after $3LD_{50}$ CVB3 challenge at week 2 after the last immunization, the protect effects were evaluated in mice. (**A**) Body weight loss. (**B**) Representative M-mode echocardiograms. (**C**) Left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) from echocardiographic data. (**D**) Serum CK, CK-MB activity. (**E**) The representative heart section was shown for each group (magnification: 100 ×). (**F**) Myocardial histopathological scores. (**G**) Viral titers in the heart tissues were measured by plaque assays. Data are from one representative experiment of 3 performed and presented as the mean ± SD (n = 10). **P* < 0.05, ***P* < 0.01, ****P* < 0.001. (**H**) The survival rate of mice was observed until day 28 following a lethal dose of CVB3 ($5LD_{50}$) infection. Data are from one representative experiment of 3 performed and presented as the mean ± SD (n = 12). ****P* < 0.001.

(LVEF) from 55.40 ± 9.35 to 80.31 ± 11.35 and left ventricular fractional shortening (LVFS) from 35.52 ± 7.40 to 55.54 ± 6.35 (**Fig. 2B and C**). In addition, the serological indexes of CK and CK-MB levels which reflect the myocardial injury was significantly lower in chitosan-pAIM2 and chitosan-pVP1 co-immunized mice compared with those in chitosan-pVP1 immunized mice (**Fig. 2D**). Consistently, histological analysis of HE-stained heart sections showed tiny areas of myocytes necrosis and infiltrating inflammatory cells in chitosan-pAIM2 and chitosan-pVP1 co-immunized mice compared with that of chitosan-pVP1 immunized mice (**Fig. 2E**). The myocardial pathology score of co-immunization group was also significantly

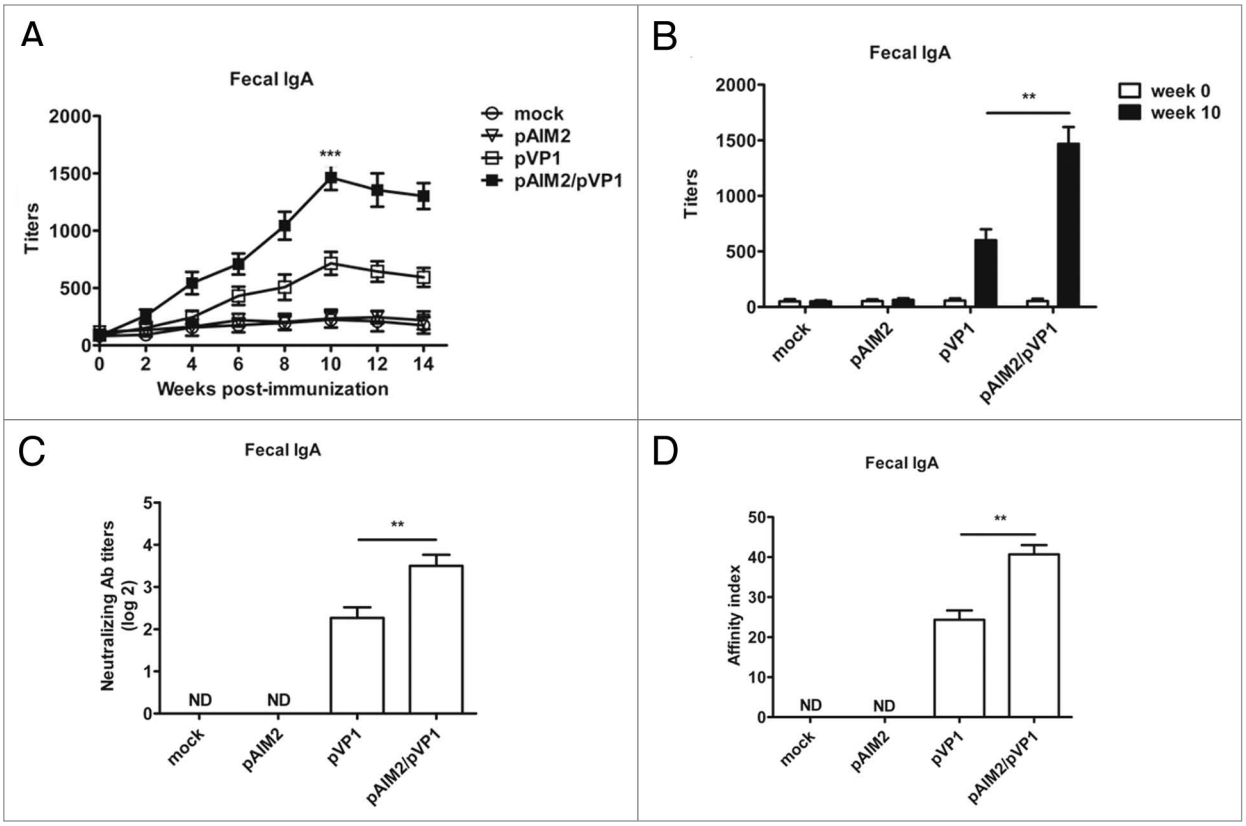

Figure 3. Mucosal SIgA responses induced by co-immunization of chitosan-pAIM2 and chitosan-pVP1. Mice were immunized 4 times with vaccines at 2-wk intervals. Two weeks after the initial immunization, mucosal SIgA antibody elicited by intranasal immunization was detected. (**A**) Fecal IgA levels were measured by ELISA at indicated time points. (**B**) Antibody titers, (**C**) Neutralizing titers, and (**D**) Affinity index were determined at week 10. Data are from one representative experiment of 3 performed and presented as the mean ± SD (n = 10). $^{**}P < 0.01$, $^{***}P < 0.001$, ND, not detected.

reduced from 1.50 ± 0.45 to 0.30 ± 0.15 (**Fig. 2F**). The above results were further supported by the remarkably decreased virus titer in the heart of co-immunized mice than that of chitosan-pVP1 immunized mice (0.50 ± 0.65 vs. 3.33 ± 0.50) at day 7 post-infection (**Fig. 2G**), indicating the more efficient control of viral replication in co-immunized mice.

To further evaluate the immune protection effect, mice were challenged with a lethal dose of CVB3 ($5LD_{50}$). The number of surviving mice was monitored for up to 28 d. All mock-immunized mice succumbed to severe illness and died within 10 d, while 40.0% of the chitosan-pVP1 immunized mice and 75.5% of the co-immunized mice survived to 28 d with no signs of illness (**Fig. 2H**), indicating that co-immunization of chitosan-pAIM2 and chitosan-pVP1 group significantly enhanced protection against CVB3 infection to compare control groups. The result indicates that chitosan-pAIM2 and chitosan-pVP1 co-immunization contributes to more efficient protection against CVB3-induced myocarditis.

Augmented production of CVB3-specific SIgA with higher affinity and neutralizing ability by co-immunization of chitosan-pAIM2 and chitosan-pVP1

SIgA antibody plays an important role in mucosal immune responses against CVB3 infection.[10,22] To investigate whether co-immunization of chitosan-pAIM2 and chitosan-pVP1 could promote mucosal antibody responses, SIgA antibody levels were determined in fecal samples of co-immunized mice. As shown in **Figure 3A**, the CVB3-specific fecal SIgA titers in chitosan-pAIM2 and chitosan-pVP1 co-immunized mice gradually increased after week 4 and achieved maximum at week 10, and then slightly decreased at week 14. The titer of SIgA antibody at week 10 was significantly higher in co-immunized mice than that of mice receiving chitosan-pVP1 alone (**Fig. 3B**). These results indicate that co-immunization of chitosan-pAIM2 and chitosan-pVP1 efficiently enhances CVB3-specific mucosal SIgA antibody production. Meanwhile, the neutralizing capacity and affinity of the antibody were also evaluated. The affinity indices of fecal SIgA elicited by co-immunization of chitosan-pAIM2 and chitosan-pVP1 were significantly higher than that of chitosan-pVP1 immunization (**Fig. 3D**). Consistently, the highest neutralizing titers of fecal SIgA were produced by co-immunized mice, and contributed to less cytolysis of Hela cells (**Fig. 3C**). These findings clearly indicate that intranasal co-immunization of chitosan-pAIM2 and pVP1 greatly enhances not only the quantity but also the quality of CVB3-specific SIgA antibody.

Increased MLN DCs recruitment and IgA-inducing factor expression by co-immunization with chitosan-pAIM2

Co-immunization of chitosan-pAIM2 and chitosan-pVP1 significantly enhanced mucosal SIgA antibody production,

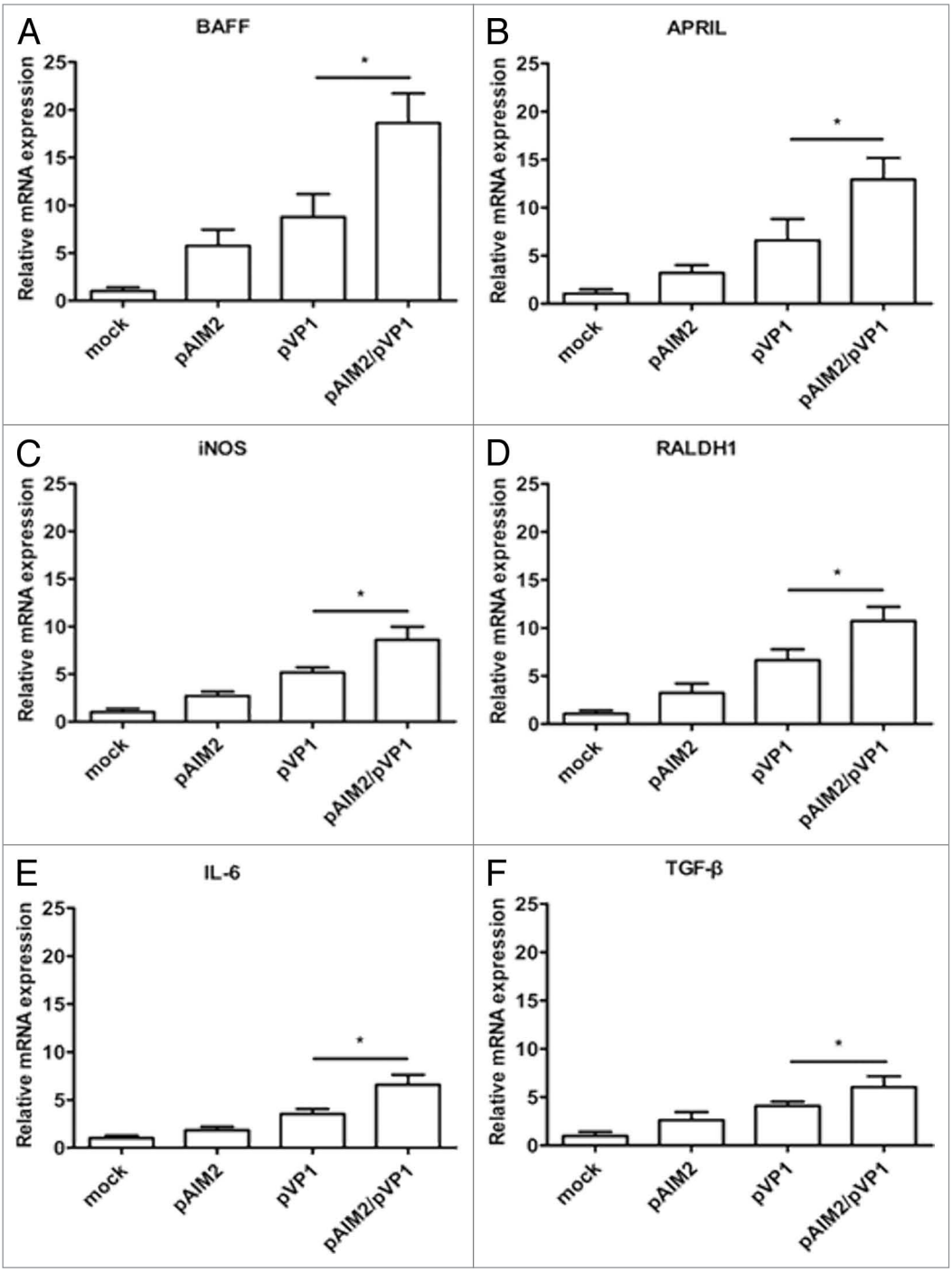

Figure 4. The expression of IgA-inducing factors in MLN by co-immunization of chitosan-pAIM2 and chitosan-pVP1. (**A-F**) Two weeks after the last immunization, the mRNA levels of IgA-inducing factors (BAFF, APRIL, iNOS, RALDH1, IL-6, TGF-β) were analyzed in MLN by real-time PCR using the assay specific primer. Data are from one representative experiment of 3 performed and presented as the mean ± SD (n = 10). *$P < 0.05$.

suggesting that expression of IgA-inducing factors by lymphocytes could be increased in these mice. Indeed, real-time PCR results demonstrated that the expression of BAFF, APRIL, iNOS, RALDH1, IL-6 and TGF-β were significantly higher in MLN cells from chitosan-pAIM2 and chitosan-pVP1 co-immunized mice than that from chitosan-pVP1 immunized mice (**Fig. 4A-F**). These results indicate that co-immunization with chitosan-pAIM2 effectively enhances SIgA production by promoting IgA-inducing factor expression in the CVB3-specific immune responses.

Dendritic cells (DCs) are the most potent secreting cells of IgA-inducing factors and are important in promoting IgA synthesis.[23-25] Thus, we determined the amount of DCs recruited into MLN during chitosan-pAIM2 and chitosan-pVP1

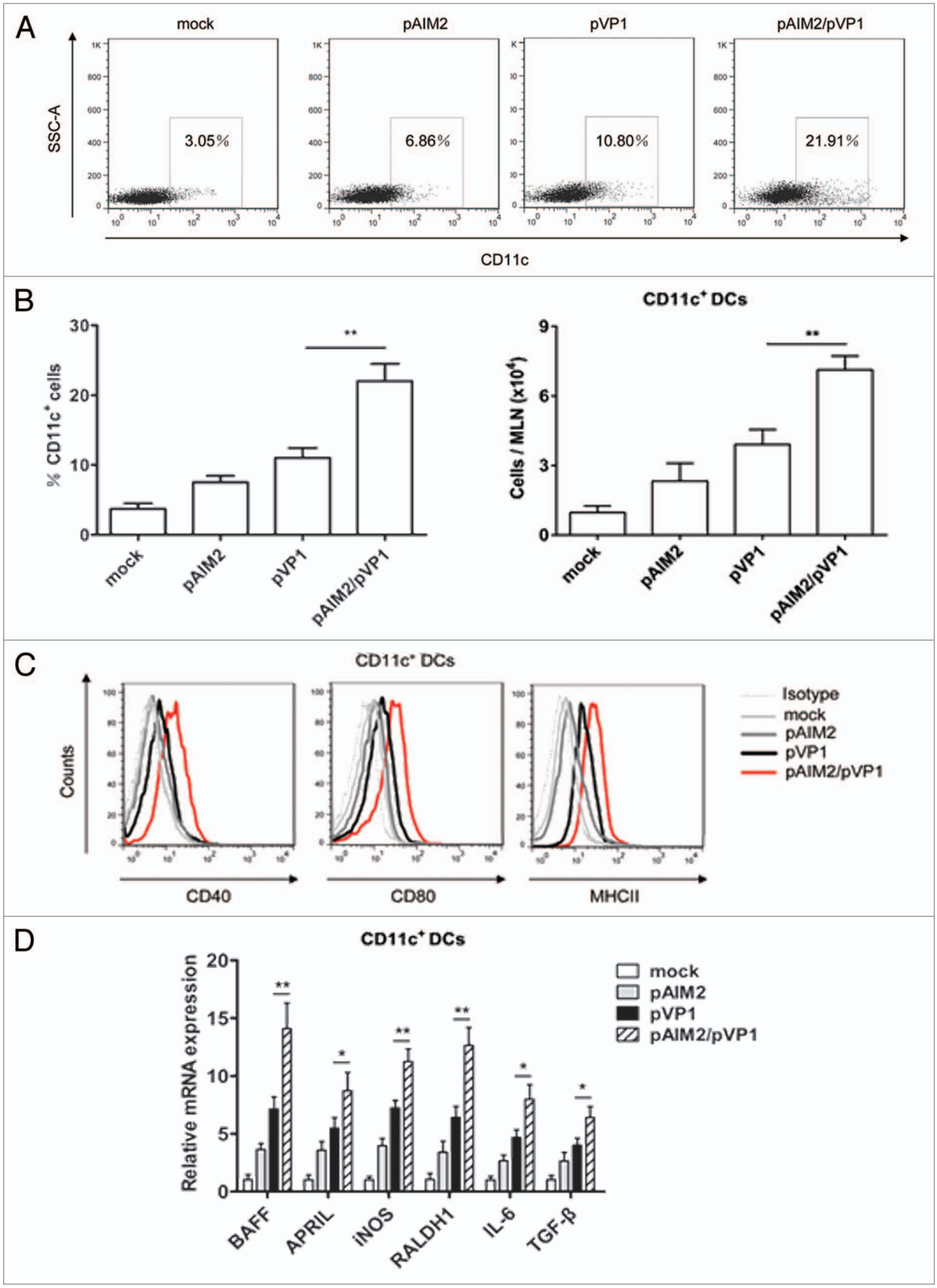

Figure 5. For figure legend, see page 1290.

Figure 5 (See previous page). Increased DCs recruitment and IgA-inducing factors expression by co-immunization of chitosan-pAIM2 and chitosan-pVP1. Two weeks after the last immunization, MLN cells from mice in each immunization groups were analyzed. (**A**) The percentages of $CD11c^+$ cells in MLN. One representative flow cytometry result was shown for per group. (**B**) Statistical analysis of the frequency and the absolute number of $CD11c^+$ cells in MLN. (**C**) Representative expression profile of $CD40^+$, $CD80^+$, $MHCII^+$ in DCs of MLN. (**D**) Real-time PCR analysis of BAFF, APRIL, iNOS, RALDH1, IL-6, and TGF-β relative to GAPDH for DCs cells from MLN. Data are from one representative experiment of 3 performed and presented as the mean ± SD (n = 10). $^*P < 0.05$, $^{**}P < 0.01$.

co-immunization. It was found that compared with chitosan-pVP1 immunization alone, chitosan-pAIM2 co-immunization not only increased the percentage (10.80% vs 21.91%, $P < 0.01$) and number (3.91×10^4 vs 7.16×10^4, $P < 0.01$) of $CD11c^+$ DCs in the MLN sites (**Fig. 5A and B**), but also significantly enhanced the expression of costimulatory and activation markers (CD40, CD80, and MHC II), indicative of the increased maturation of DCs (**Fig. 5C**). Then, the inducing factors of IgA, including BAFF, APRIL, iNOS, RALDH1, IL-6, and TGF-β were assessed by real-time PCR in DCs from MLN. Compared with the chitosan-pVP1 immunized mice, the gene expression of BAFF, APRIL, iNOS, RALDH1, IL-6, and TGF-β were markedly increased in chitosan-pAIM2 and chitosan-pVP1 co-immunized mice (**Fig. 5D**). These results indicate that co-immunization with AIM2 enhances DCs recruitment and IgA-inducing factors expression in intestinal mucosa.

Discussion

Coxsackievirus B3 (CVB3) is considered to be one of the important causative agents for acute or chronic viral myocarditis.[26] Currently, mucosal DNA vaccination is an attractive approach to induce mucosal immunity against CVB3 infection and reduce virus-associated myocardial dysfunction.[8-10,22] The potent adjuvants and delivery systems are needed for success of mucosal DNA vaccines to induce immune responses.[27-29] Our studies clearly demonstrate that protection against CVB3-induced myocarditis in mice can be effectively achieved by intranasal co-immunization of chitosan-pAIM2 and chitosan-pVP1.

PRRs that sense intracellular DNA have been proven to potentially enhance immunogenicity of DNA vaccines.[30,31] It has been demonstrated that DNA-dependent activator of IFN-regulatory factors (DAI) overexpression in vivo can boost DNA-sensing innate immune activation and generate a proinflammatory microenvironment, which is essential for effective CTL induction and long-lasting antitumor immunity.[32] Co-immunization with retinoic acid inducible-gene I (RIG-I) agonist enhances humoral immune response induced by influenza virus DNA vaccine.[33] Consistently, boosting innate immune PRR signaling by co-expressing intracellular adaptor molecules or downstream transcription factors as genetic adjuvants are efficient strategies for enhancing immunogenic of DNA vaccines.[34,35] These results imply that AIM2 might serve as an adjuvant and enhance the immunogenicity of pVP1 vaccine and efficiently induce CVB3-specific immune responses. However, whether AIM2 can enhance the immunogenicity of mucosal DNA vaccine is not yet known.

Mucosal immunization approach has been successful in animal models.[36-38] However, delivery systems of mucosal vaccines remain a challenge to induce maximal mucosal protection simultaneously. In recent years, chitosan has gained increasing interest as a safe delivery system for plasmid DNA (pDNA).[39] Chitosan is a natural polysaccharide bearing amino groups, its beneficial qualities include low toxicity, low immunogenicity, excellent biocompatibility, and the enhancing penetration of molecules across mucosal surfaces.[40,41] Therefore, chitosan can serve as a good vector for DNA vaccine delivery.[41-45] DNA encapsulated in chitosan has been proved to efficiently enhance the immunogenicity of mucosal vaccines.[46,47] In the present study, we used cytosolic DNA sensor AIM2 as an adjuvant to enhance the mucosal immune response, meanwhile we used chitosan to promote DNA uptake in nasal mucosal sites. Our results showed that AIM2 and VP1 plasmids encapsulated in chitosan could be efficiently expressed when chitosan-pAIM2 or chitosan-pVP1 was transfected into 293T cell line, indicating that a correctly translated in vitro. Further study confirmed that the chitosan-pAIM2 or chitosan-pVP1 could be efficiently expressed both in vitro and in vivo.

Here, we showed that co-immunization with AIM2 significantly decreased the body weight loss, improved cardiac function and reduced myocardial injury compared with chitosan-pVP1 immunization. Of note, co-immunized mice had dramatically decreased viral load in the heart tissue, reduced tissue destruction and inflammation in myocardial tissues and increased survival rate. Secretory immunoglobulin A (SIgA) is recognized as an important activity of mucosal surfaces and serves as a first line of defense against viral infection.[48] Co-immunization with chitosan-pVP1 and chitosan-pAIM2 enhanced production of CVB3 specific IgA antibody and neutralizing antibody titers in mice, indicating that AIM2 enhanced mucosal immune response against CVB3 infection. Antigens specific SIgA production is dependent on antigen processing by antigen-presenting cells such as DCs, T-cell activation, and ultimately B-cell class switch recombination in gut-associated lymphoid tissues (GALTs), mesenteric lymph nodes (MLN), and possibly neighboring lamina propria.[49,50] We found that co-immunization of chitosan-pAIM2 and chitosan-pVP1 effectively promoted DCs recruitment to MLN. In this study we mainly observed that chitosan-pAIM2 co-immunization could significantly recruite $CD11c^+$ mDC to mucosal site, which highly expressed IgA-inducing factors (BAFF, APRIL, iNOS, RALDH1, IL-6, and TGF-β). Both conventional dendritic cells (cDCs) and plasmacytoid dendritic cells (pDCs) are present in the mesenteric lymph nodes (MLNs). Previous studies have been made to identify the cDCs responsible for inducing IgA secretion by secreting IgA-inducing factors (BAFF, APRIL, iNOS, RALDH1, IL-6, TGF-β et al.).[23-25] However, whether co-immunization of chitosan-pAIM2 with chitosan-pVP1 could enhance pDCs recruitment and maturation as well

as the expression of IgA-inducing factors in MLN is unclear. According to our results, AIM2 co-immunization also enhances CD40, CD80, and MHCII expression on DCs in MLN. Mucosal SIgA antibody responses can be induced by a vaccine regimen through promoting IgA-inducing factors expression.[51] Multiple inducing factors of IgA, such as BAFF, APRIL, iNOS, RALDH1, IL-6, and TGF-β are involved in the activation and the terminal differentiation of IgA-secreting plasma cells and in intestinal stimulation of SIgA intracellular transport.[52,53] Co-immunization of chitosan-pAIM2 and chitosan-pVP1 effectively increased the expression of IgA-inducing factors in MLN DCs. These results were consistent with the results of increased SIgA production induced by AIM2 co-immunization strategy. Taken together, our findings demonstrated that AIM2 could be an effective adjuvant for mucosal DNA vaccine against CVB3-induced acute myocarditis.

In conclusion, our study showed that the use of cytosolic DNA sensor AIM2 as a mucosal adjuvant could efficiently enhance the immunogenicity of chitosan-pVP1 DNA vaccine and mucosal immune responses to confer protection. Thus, AIM2-adjuvanted DNA vaccines may have protection potential for virus induced diseases.

Materials and Methods

Experimental animals

Six-week-old male BALB/c (H-2^d) mice were purchased from experimental animal center of Chinese Academy of Science. All mice were bred and maintained under pathogen-free conditions. All animal experimental protocols were approved by the Care and Use of Laboratory Animals (Ministry of Health, China, 1998) and the guidelines of the Laboratory Animal Ethical Committee of Soochow University.

Transfection of AIM2 and VP1 plasmids in 293T cells

Murine AIM2 expression plasmid pEFBOS-AIM2 (pAIM2) was kindly provided by Dr Veit Hornung (Institute for Clinical Chemistry and Clinical Pharmacology, University of Bonn, Germany), and pcDNA3.1-VP1 (pVP1) has been described previously.[10] Plasmids were extracted from *Escherichia coli* (DH5a) grown overnight using Qiagen EndoFree Plasmid Mega kit. For cell transfection assays, plasmids were resolved in PBS at a concentration of 1mg/ml. In transfection assays, 5 × 10^5 293T cells at about 70–80% confluence were transfected with 4 μg of pAIM2 or pVP1 in 35mm dishes by lipofectamine (Invitrogen) according to the manufacturer's protocol. Same amount of empty vector pEFBOS (for pAIM2 transfection) or pcDNA3.1 (for pVP1 transfection) was used as a negative control. Forty-eight hours after transfection, cells were harvested for further experiment.

Western blot

Cells or tissues were lysed in cold lysis buffer containing 20 mM Tris–HCl pH7.4, 150 mM NaCl, 1 mM EDTA, and 0.5% NP-40. The cell debris was depleted by centrifugation at 6000 g for 5 min. Equal amount of protein was loaded to 10% SDS-PAGE gel for each sample, separated by electrophoresis, and transferred to PVDF membrane. The membrane was probed with anti-mouse AIM2 (Santa Cruz Biotechnology, 100 μg/mL, dilution 1:1000), mouse anti-enterovirus VP1 antibody (DAKO, 110 μg/mL, dilution 1:500) or anti-mouse β-actin (Cell Singaling Technology, 100μL, dilution 1:1000), followed by goat anti-Rabbit IgG-HRP (Southern Biotech, 1mg/mL, dilution 1:5000). The signals were developed with the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). β-actin was used as the internal marker for loading control.

Preparation and immunization of chitosan-DNA

Plasmid DNA was encapsulated with chitosan had described previously.[10,22] Briefly, plasmids were resolved in 5 mM Na2SO4 at a concentration of 400 μg/mL. Equal volumes of chitosan solution (0.02%, pH 5.5) and pVP1 or pAIM2 DNA solution were heated to 55 °C, and then vigorously mixed for 30s. The particle morphology was investigated using scanning electron microscopy (SEM). Meanwhile, the concentration of non-encapsulated DNA remaining in the aqueous suspension was determined spectrophotometrically at 260 nm with the intact particles or particles treated with chitosanase. DNA encapsulation efficiency was measured as (1-[amount of non-encapsulated DNA] / [total amount of DNA]) × 100%.

Groups of mice were mildly anesthetized by pentobarbital (40 mg/kg body weight, intraperitoneal) and intranasally immunized with chitosan-pAIM2 and chitosan-pVP1, chitosan-pVP1, chitosan-pAIM2, and chitosan-pcDNA3.1 (mock vaccine). Every group was immunized for 4 times biweekly at a dose of 50 μg of each plasmid 4 times biweekly. For pVP1 alone, pAIM2, or empty plasmid immunized group, mice were received additional 50 μg pcDNA3.1 to make sure that the total DNA amount was 100 μg. Mice were intranasally immunized with chitosan-pAIM2 plus chitosan-pVP1, chitosan-pVP1, chitosan-pAIM2, or mock vaccine comprising 50 μg DNA for 4 times at 2-wk intervals. Fecal extracts were collected every 2 wk. Fecal pellets were dissolved in PBS (containing 5% nonfat milk, 1 μg/mL aprotinin, 1 mM PMSF) at final concentration of 100 mg/mL. After centrifuging at 15 000 g for 10 min, supernatants were collected and stored at –70 °C.

Cells and virus

HeLa cells (ATCC number: CCL-2) were cultured in RPMI medium 1640 (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a CO2 incubator. The CVB3 (Nancy strain) was a gift from Professor Yingzhen Yang (Key Laboratory of Viral Heart Diseases, Zhongshan Hospital, Shanghai Medical college of Fudan University) and was maintained in HeLa cells with 2% FBS and used for viral challenge and virus neutralization assays after 3 passages. Viral titer was routinely determined prior to infection by a 50% tissue culture infectious dose ($TCID_{50}$) assay of Hela cell monolayer. Viral titer was also routinely determined prior to infection by a median lethal dose (LD_{50}) assay according to previously published procedures.[54]

CVB3 infection and evaluation of myocarditis

Mice were infected intraperitoneally with 3 × 50% lethal dose (LD_{50}) CVB3 (Nancy strain) at week 2 after the final

immunization. After 7 d later, assessment of cardiac function was performed using high-resolution ultrasound imaging system (Vevo2100, Visual Sonics) equipped with a 30-MHz microscan transducer. The echocardiographic measurements of left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) were performed according to the operator's manual. Serum creatine kinase (CK) and CK-MB were measured by Suzhou Kowloon Hospital. Heart tissues were fixed in 10% phosphate-buffered formalin, paraffin embedded, sectioned, and stained with hematoxylin and eosin (HE). The histopathological changes of immunized mice were compared quantitatively by calculating the histopathological scores.[55] 0, no lesions; 1, lesions involving <25%; 2, lesions involving 25–50%; 3, lesions involving 50–75%; 4, lesions involving >75%. Two independent researchers scored separately in a blinded manner. Quantization of viral burden in heart tissues 7 days after $3LD_{50s}$ CVB3 challenge, heart tissues were collected, weighed, and frozen at –70 °C in RPMI 1640 containing 10% FBS. Samples were later thawed, homogenized, serially diluted in 10-fold increments, and incubated on confluent Hela cell monolayers for 1 h at 37 °C and 5% CO2 to allow viral attachment, and then incubated for 7 d to allow plaque formation. Virus titers were expressed as the mean PFU/100 mg tissue ± SD.

Evaluation of CVB3-specific lgA antibody titers

The titers of CVB3-specific fecal IgA were detected by ELISA assays as described previously.[22] Briefly, Plates were coated with 10 μg/mL $VP1_{237–249}$ peptide at 4 °C overnight. The plates were then washed with 0.05% Tween 20 in PBS and blocked with 1% bovine serum albumin in 0.05% Tween 20/PBS at room temperature for 2 h. Five-fold serially diluted fecal samples were added in duplicate and incubated at 37 °C for 2 h. HRP-conjugated goat anti-mouse IgA (Southern-Biotech) was added, followed by TMB substrate addition. Absorbance at 490 nm was measured on an automated microplate reader (Thermo Scientific).

Micro-neutralization test

An equal volume of serial fecal dilutions was incubated with 100 × 50% tissue culture infectious dose ($TCID_{50}$) of CVB3 at 37 °C for 1 h. The mixture was added onto monolayer cultures of 10000 Hela cells/well in 96-well plates. After incubating for 1 h at 37 °C, the supernatantswere replaced by RPMI 1640 supplemented with 2% fetal calf serum (Gibco) and then cultured until the control cells exhibited complete destruction. The reciprocal of the sample dilution resulting in 50% cytopathic effects reduction compared with the control fecal sample was considered the neutralization titer.

Affinity of CVB3-specific lgA antibody

The affinity of fecal lgA was determined by ELISA with a urea-elution step as previously described.[56] Briefly, fecal samples (in 2-fold dilution) were tested in duplicate plates. In one of the plates, 6M urea (Sigma) was added after incubation with samples and incubated for 10 min. Results are expressed as affinity index calculated as: (endpoint titer in the presence of urea/endpoint titer in the absence of urea) × 100.

Isolation of RNA and quantitative real-time PCR

Total RNA was isolated from MLN cells or DCs cells from MLN by flow cytometry sorting, and cDNA was prepared with a cDNA synthesis kit (Takara) according to the manufacturer's instructions. Transcript levels were determined by quantitative real-time PCR using SYBR Green PCR Master Mix (Invitrogen) on the realplex Mastercycler (Eppendorf) according to the manufacturer's instructions. To normalize the amounts of input cDNA, the relative amount of the generated product was divided by the relative amount of GAPDH. All samples were analyzed in duplicate. Primers used were as follows: GAPDH, forward primer, 5′-GAGCCAAACG GGTCATCATC T-3′ and reverse primer, 5′-GAGGGGCCAT CCACAGTCTT-3′; BAFF, forward primer, 5′-AGGCTGGAAG AAGGAGATGA G-3′ and reverse primer, 5′-CAGAGAAGAC GAGGGAAGGG-3′; APRIL, forward primer, 5′-CCATGGCAGA GCCTCTGG-3′ and reverse primer, 5′-GAAGGATGGG GCGAAATCTC-3′; iNOS, forward 5′-GCTCCTGCCT CATGCCATT-3′ and reverse primer, 5′-CTACAGTTCC GAGCGTCAAAGA-3′; RALDH1, forward 5′-ATGGTTTAGC AGCAGGACTC TTC-3′ and reverse primer, 5′-CCAGACATCT TGAATCCACC GAA-3′; IL-6, forward primer, 5′-ACAACCACGG CCTTCCCTAC-3′ and reverse primer, 5′-CACGATTTCC CAGAGAACAT GTG-3′; TGF-β, forward primer, 5′-GTCTTTTGAC GTCACTGGAG TTGT-3′ and reverse primer, 5′-GGAGTTTGTT ATCTTTGCTG TCACA-3′.

Flow cytometry analysis

Single cell suspensions of MLN cells from immunized mice were incubated with PerCP5.5-conjugated anti-mouse CD11c antibody, PE-conjugated anti-mouse CD40, CD80, MHCII (dilution 1:100) (Biolegend) in PBS containing 2% FBS. Isotype-matched mouse immunoglobulin served as controls. Then labeled cells were analyzed and sorted by FACS using the BD Biosystems Aria III flow cytometer. All flow cytometry data were analyzed by FlowJo software (Tree Star Inc.).

Statistical analysis

Statistical analysis was performed with ANOVA followed by Tukey's post hoc test. Survival rates were analyzed by Kapla-Meier test using GraphPad Prism version 5.01 (GraphPad Software Inc.). All data are expressed as means ± SD of 3 independent experiments or from a representative experiment of 3 independent experiments. The statistical significance level was set as $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank Proffesor Veit Hornung (Institute for Clinical Chemistry and Clinical Pharmacology, University of Bonn, Germany) for providing plasmid pEFBOS-AIM2. This work was supported by grants from Major State Basic Research Development Program of China (2013CB530501, 2013CB531502), the National Science and Technology Key Projects during the Twelveth Five-Year Plan Period of China(2013ZX10003007, 2012ZX10003006–008), the National Natural Science Foundation of China (81072409), Jiangsu "333" project of cultivation of high-level talents, Qing Lan Project of the Jiangsu higher education institutions, Priority

Academic Program Development of Jiangsu Higher Education Institutions (PAPD), Jiangsu Provincial Innovative Research Team and the Program for Changjiang Scholars and Innovative Research Team in University of Ministry of Education of China (PCSIRT-IRT1075).

References

1. Kawai C. From myocarditis to cardiomyopathy: mechanisms of inflammation and cell death: learning from the past for the future. Circulation 1999; 99:1091-100; PMID:10051305; http://dx.doi.org/10.1161/01.CIR.99.8.1091
2. Maisch B, Ristić AD, Hufnagel G, Pankuweit S. Pathophysiology of viral myocarditis: the role of humoral immune response. Cardiovasc Pathol 2002; 11:112-22; PMID:11934603; http://dx.doi.org/10.1016/S1054-8807(01)00113-2
3. Huber SA, Born W, O'Brien R. Dual functions of murine gammadelta cells in inflammation and autoimmunity in coxsackievirus B3-induced myocarditis: role of Vgamma1+ and Vgamma4+ cells. Microbes Infect 2005; 7:537-43; PMID:15777711; http://dx.doi.org/10.1016/j.micinf.2004.12.011
4. Palmenberg AC. Proteolytic processing of picornaviral polyprotein. Annu Rev Microbiol 1990; 44:603-23; PMID:2252396; http://dx.doi.org/10.1146/annurev.mi.44.100190.003131
5. Tam PE. Coxsackievirus myocarditis: interplay between virus and host in the pathogenesis of heart disease. Viral Immunol 2006; 19:133-46; PMID:16817756; http://dx.doi.org/10.1089/vim.2006.19.133
6. Bailey JM, Tapprich WE. Structure of the 5′ nontranslated region of the coxsackievirus b3 genome: Chemical modification and comparative sequence analysis. J Virol 2007; 81:650-68; PMID:17079314; http://dx.doi.org/10.1128/JVI.01327-06
7. Fohlman J, Pauksen K, Morein B, Bjare U, Ilbäck NG, Friman G. High yield production of an inactivated coxsackie B3 adjuvant vaccine with protective effect against experimental myocarditis. Scand J Infect Dis Suppl 1993; 88:103-8; PMID:8390713
8. Henke A, Wagner E, Whitton JL, Zell R, Stelzner A. Protection of mice against lethal coxsackievirus B3 infection by using DNA immunization. J Virol 1998; 72:8327-31; PMID:9733878
9. Henke A, Zell R, Stelzner A. DNA vaccine-mediated immune responses in Coxsackie virus B3-infected mice. Antiviral Res 2001; 49:49-54; PMID:11166860; http://dx.doi.org/10.1016/S0166-3542(00)00132-7
10. Xu W, Shen Y, Jiang Z, Wang Y, Chu Y, Xiong S. Intranasal delivery of chitosan-DNA vaccine generates mucosal SIgA and anti-CVB3 protection. Vaccine 2004; 22:3603-12; PMID:15315839; http://dx.doi.org/10.1016/j.vaccine.2004.03.033
11. Dan M, Chantler JK. A genetically engineered attenuated coxsackievirus B3 strain protects mice against lethal infection. J Virol 2005; 79:9285-95; PMID:15994822; http://dx.doi.org/10.1128/JVI.79.14.9285-9295.2005
12. Kim JY, Jeon ES, Lim BK, Kim SM, Chung SK, Kim JM, Park SI, Jo I, Nam JH. Immunogenicity of a DNA vaccine for coxsackievirus B3 in mice: protective effects of capsid proteins against viral challenge. Vaccine 2005; 23:1672-9; PMID:15705471; http://dx.doi.org/10.1016/j.vaccine.2004.10.008
13. Henke A, Jarasch N, Martin U, Wegert J, Wildner A, Zell R, Wutzler P. Recombinant coxsackievirus vectors for prevention and therapy of virus-induced heart disease. Int J Med Microbiol 2008; 298:127-34; PMID:17897883; http://dx.doi.org/10.1016/j.ijmm.2007.08.010
14. Hornung V, Ablasser A, Charrel-Dennis M, Bauernfeind F, Horvath G, Caffrey DR, Latz E, Fitzgerald KA. AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. Nature 2009; 458:514-8; PMID:19158675; http://dx.doi.org/10.1038/nature07725
15. Schattgen SA, Fitzgerald KA. The PYHIN protein family as mediators of host defenses. Immunol Rev 2011; 243:109-18; PMID:21884171; http://dx.doi.org/10.1111/j.1600-065X.2011.01053.x
16. Roberts TL, Idris A, Dunn JA, Kelly GM, Burnton CM, Hodgson S, Hardy LL, Garceau V, Sweet MJ, Ross IL, et al. HIN-200 proteins regulate caspase activation in response to foreign cytoplasmic DNA. Science 2009; 323:1057-60; PMID:19131592; http://dx.doi.org/10.1126/science.1169841
17. Fernandes-Alnemri T, Yu JW, Datta P, Wu J, Alnemri ES. AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. Nature 2009; 458:509-13; PMID:19158676; http://dx.doi.org/10.1038/nature07710
18. Fernandes-Alnemri T, Yu JW, Juliana C, Solorzano L, Kang S, Wu J, Datta P, McCormick M, Huang L, McDermott E, et al. The AIM2 inflammasome is critical for innate immunity to Francisella tularensis. Nat Immunol 2010; 11:385-93; PMID:20351693; http://dx.doi.org/10.1038/ni.1859
19. Dinarello CA. Immunological and inflammatory functions of the interleukin-1 family. Annu Rev Immunol 2009; 27:519-50; PMID:19302047; http://dx.doi.org/10.1146/annurev.immunol.021908.132612
20. Sims JE, Smith DE. The IL-1 family: regulators of immunity. Nat Rev Immunol 2010; 10:89-102; PMID:20081871; http://dx.doi.org/10.1038/nri2691
21. Rathinam VA, Jiang Z, Waggoner SN, Sharma S, Cole LE, Waggoner L, Vanaja SK, Monks BG, Ganesan S, Latz E, et al. The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. Nat Immunol 2010; 11:395-402; PMID:20351692; http://dx.doi.org/10.1038/ni.1864
22. Yue Y, Xu W, Hu L, Jiang Z, Xiong S. Enhanced resistance to coxsackievirus B3-induced myocarditis by intranasal co-immunization of lymphotactin gene encapsulated in chitosan particle. Virology 2009; 386:438-47; PMID:19233446; http://dx.doi.org/10.1016/j.virol.2009.01.029
23. Mora JR, Iwata M, Eksteen B, Song SY, Junt T, Senman B, Otipoby KL, Yokota A, Takeuchi H, Ricciardi-Castagnoli P, et al. Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. Science 2006; 314:1157-60; PMID:17110582; http://dx.doi.org/10.1126/science.1132742
24. Massacand JC, Kaiser P, Ernst B, Tardivel A, Bürki K, Schneider P, Harris NL. Intestinal bacteria condition dendritic cells to promote IgA production. PLoS One 2008; 3:e2588; PMID:18596964; http://dx.doi.org/10.1371/journal.pone.0002588
25. Tezuka H, Ohteki T. Regulation of intestinal homeostasis by dendritic cells. Immunol Rev 2010; 234:247-58; PMID:20193023; http://dx.doi.org/10.1111/j.0105-2896.2009.00872.x
26. Feldman AM, McNamara D. Myocarditis. N Engl J Med 2000; 343:1388-98; PMID:11070105; http://dx.doi.org/10.1056/NEJM200011093431908
27. Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. Nat Med 2005; 11(Suppl):S45-53; PMID:15812489; http://dx.doi.org/10.1038/nm1213
28. Kumar H, Kawai T, Akira S. Pathogen recognition in the innate immune response. Biochem J 2009; 420:1-16; PMID:19382893; http://dx.doi.org/10.1042/BJ20090272
29. Fujkuyama Y, Tokuhara D, Kataoka K, Gilbert RS, McGhee JR, Yuki Y, Kiyono H, Fujihashi K. Novel vaccine development strategies for inducing mucosal immunity. Expert Rev Vaccines 2012; 11:367-79; PMID:22380827; http://dx.doi.org/10.1586/erv.11.196
30. Kumagai Y, Akira S. [Role of PRRs in vaccine]. Nihon Rinsho 2011; 69:1541-6; PMID:21922750
31. Olive C. Pattern recognition receptors: sentinels in innate immunity and targets of new vaccine adjuvants. Expert Rev Vaccines 2012; 11:237-56; PMID:22309671; http://dx.doi.org/10.1586/erv.11.189
32. Lladser A, Mougiakakos D, Tufvesson H, Ligtenberg MA, Quest AF, Kiessling R, Ljungberg K. DAI (DLM-1/ZBP1) as a genetic adjuvant for DNA vaccines that promotes effective antitumor CTL immunity. Mol Ther 2011; 19:594-601; PMID:21157438; http://dx.doi.org/10.1038/mt.2010.268
33. Luke JM, Simon GG, Söderholm J, Errett JS, August JT, Gale M Jr., Hodgson CP, Williams JA. Coexpressed RIG-I agonist enhances humoral immune response to influenza virus DNA vaccine. J Virol 2011; 85:1370-83; PMID:21106745; http://dx.doi.org/10.1128/JVI.01250-10
34. Sasaki S, Amara RR, Yeow WS, Pitha PM, Robinson HL. Regulation of DNA-raised immune responses by cotransfected interferon regulatory factors. J Virol 2002; 76:6652-9; PMID:12050378; http://dx.doi.org/10.1128/JVI.76.13.6652-6659.2002
35. Takeshita F, Tanaka T, Matsuda T, Tozuka M, Kobiyama K, Saha S, Matsui K, Ishii KJ, Coban C, Akira S, et al. Toll-like receptor adaptor molecules enhance DNA-raised adaptive immune responses against influenza and tumors through activation of innate immunity. J Virol 2006; 80:6218-24; PMID:16775309; http://dx.doi.org/10.1128/JVI.00121-06
36. Gao H, Yue Y, Hu L, Xu W, Xiong S. A novel DNA vaccine containing multiple TB-specific epitopes casted in a natural structure (ECANS) confers protective immunity against pulmonary mycobacterial challenge. Vaccine 2009; 27:5313-9; PMID:19615961; http://dx.doi.org/10.1016/j.vaccine.2009.06.093
37. Yue Y, Xu W, Xiong S. Modulation of immunogenicity and immunoprotection of mucosal vaccine against coxsackievirus B3 by optimizing the coadministration mode of lymphotactin adjuvant. DNA Cell Biol 2012; 31:479-88; PMID:21988406; http://dx.doi.org/10.1089/dna.2011.1367
38. Zhao F, Liu S, Zhang X, Yu J, Zeng T, Gu W, Cao X, Chen X, Wu Y. CpG adjuvant enhances the mucosal immunogenicity and efficacy of a Treponema pallidum DNA vaccine in rabbits. Hum Vaccin Immunother 2013; 9:753-60; PMID:23563515; http://dx.doi.org/10.4161/hv.23064
39. Mao S, Sun W, Kissel T. Chitosan-based formulations for delivery of DNA and siRNA. Adv Drug Deliv Rev 2010; 62:12-27; PMID:19796660; http://dx.doi.org/10.1016/j.addr.2009.08.004
40. Lee MK, Chun SK, Choi WJ, Kim JK, Choi SH, Kim A, Oungbho K, Park JS, Ahn WS, Kim CK. The use of chitosan as a condensing agent to enhance emulsion-mediated gene transfer. Biomaterials 2005; 26:2147-56; PMID:15576190; http://dx.doi.org/10.1016/j.biomaterials.2004.07.008

41. Bowman K, Leong KW. Chitosan nanoparticles for oral drug and gene delivery. Int J Nanomedicine 2006; 1:117-28; PMID:17722528; http://dx.doi.org/10.2147/nano.2006.1.2.117
42. Illum L. Chitosan and its use as a pharmaceutical excipient. Pharm Res 1998; 15:1326-31; PMID:9755881; http://dx.doi.org/10.1023/A:1011929016601
43. Dodane V, Amin Khan M, Merwin JR. Effect of chitosan on epithelial permeability and structure. Int J Pharm 1999; 182:21-32; PMID:10332071; http://dx.doi.org/10.1016/S0378-5173(99)00030-7
44. Kumar MN, Muzzarelli RA, Muzzarelli C, Sashiwa H, Domb AJ. Chitosan chemistry and pharmaceutical perspectives. Chem Rev 2004; 104:6017-84; PMID:15584695; http://dx.doi.org/10.1021/cr030441b
45. Baldrick P. The safety of chitosan as a pharmaceutical excipient. Regul Toxicol Pharmacol 2010; 56:290-9; PMID:19788905; http://dx.doi.org/10.1016/j.yrtph.2009.09.015
46. Dass CR, Choong PF. Chitosan-mediated orally delivered nucleic acids: a gutful of gene therapy. J Drug Target 2008; 16:257-61; PMID:18446603; http://dx.doi.org/10.1080/10611860801900801
47. Wang X, Zhang X, Kang Y, Jin H, Du X, Zhao G, Yu Y, Li J, Su B, Huang C, et al. Interleukin-15 enhance DNA vaccine elicited mucosal and systemic immunity against foot and mouth disease virus. Vaccine 2008; 26:5135-44; PMID:18462848; http://dx.doi.org/10.1016/j.vaccine.2008.03.088
48. Mantis NJ, Rol N, Corthésy B. Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. Mucosal Immunol 2011; 4:603-11; PMID:21975936; http://dx.doi.org/10.1038/mi.2011.41
49. He B, Xu W, Santini PA, Polydorides AD, Chiu A, Estrella J, Shan M, Chadburn A, Villanacci V, Plebani A, et al. Intestinal bacteria trigger T cell-independent immunoglobulin A(2) class switching by inducing epithelial-cell secretion of the cytokine APRIL. Immunity 2007; 26:812-26; PMID:17570691; http://dx.doi.org/10.1016/j.immuni.2007.04.014
50. Brandtzaeg P. Function of mucosa-associated lymphoid tissue in antibody formation. Immunol Invest 2010; 39:303-55; PMID:20450282; http://dx.doi.org/10.3109/08820131003680369
51. Marinaro M, Staats HF, Hiroi T, Jackson RJ, Coste M, Boyaka PN, Okahashi N, Yamamoto M, Kiyono H, Bluethmann H, et al. Mucosal adjuvant effect of cholera toxin in mice results from induction of T helper 2 (Th2) cells and IL-4. J Immunol 1995; 155:4621-9; PMID:7594461
52. Beagley KW, Eldridge JH, Lee F, Kiyono H, Everson MP, Koopman WJ, Hirano T, Kishimoto T, McGhee JR. Interleukins and IgA synthesis. Human and murine interleukin 6 induce high rate IgA secretion in IgA-committed B cells. J Exp Med 1989; 169:2133-48; PMID:2786548; http://dx.doi.org/10.1084/jem.169.6.2133
53. Boyaka PN, Marinaro M, Jackson RJ, Menon S, Kiyono H, Jirillo E, McGhee JR. IL-12 is an effective adjuvant for induction of mucosal immunity. J Immunol 1999; 162:122-8; PMID:9886377
54. Henke A, Huber S, Stelzner A, Whitton JL. The role of CD8+ T lymphocytes in coxsackievirus B3-induced myocarditis. J Virol 1995; 69:6720-8; PMID:7474082
55. Rezkalla S, Kloner RA, Khatib G, Smith FE, Khatib R. Effect of metoprolol in acute coxsackievirus B3 murine myocarditis. J Am Coll Cardiol 1988; 12:412-4; PMID:2839568; http://dx.doi.org/10.1016/0735-1097(88)90414-7
56. Capozzo AV, Ramírez K, Polo JM, Ulmer J, Barry EM, Levine MM, Pasetti MF. Neonatal immunization with a Sindbis virus-DNA measles vaccine induces adult-like neutralizing antibodies and cell-mediated immunity in the presence of maternal antibodies. J Immunol 2006; 176:5671-81; PMID:16622037