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AIM2 co-immunization favors specific multifunctional CD8$^+$ T cell induction and ameliorates coxsackievirus B3-induced chronic myocarditis

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Coxsackievirus B3 (CVB3) infection can cause acute myocarditis and chronic myocarditis, leading to dilated cardiomyopathy (DCM) with no effective therapeutic strategy. Therefore, we investigated the potential of absent in melanoma 2 (AIM2) to enhance the therapeutic efficacy of DNA vaccine against CVB3-induced chronic myocarditis. Mice were infected with CVB3 and then intranasally immunized with chitosan-pcDNA3.1 (mock), chitosan-pAIM2 (CS-pAIM2), chitosan-pVP1 (CS-pVP1), or chitosan-pAIM2 plus chitosan-pVP1 (CS-pAIM2/CS-pVP1) at 7, 21, and 35 d. Therapeutic efficacies of various vaccines were evaluated at day 56 d. Compared with CS-pVP1 immunization, CS-pAIM2/CS-pVP1 co-immunization significantly increased survival rate, improved cardiac function, as well as decreased myocardial injury and fibrosis, this result indicated that CVB3-induced chronic myocarditis was alleviated. CVB3-specific T lymphocyte proliferation and cytotoxic T lymphocyte responses of the CS-pAIM2/CS-pVP1 co-immunization group were also increased. More interestingly, CS-pAIM2/CS-pVP1 co-immunization could facilitate CVB3-specific multifunctional CD8$^+$ T cell induction in the intestinal mucosa, and this induction was closely correlated with myocardial scores, this result indicated that CS-pAIM2/CS-pVP1 vaccine exhibits therapeutic efficacy by enhancing multifunctional CD8$^+$ T cells. This study may represent a novel therapy for CVB3-induced chronic myocarditis.

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1. Introduction

Coxsackievirus B3 (CVB3) belongs to the genus *Enterovirus* of the family of picornaviruses; CVB3 contains a single-stranded RNA genome (Kandolf and Hofschneider, 1989; Kawai, 1999). CVB3-induced myocarditis is an inflammatory heart disease, which may be the precursor of heart failure caused by dilated cardiomyopathy (Gauntt and Huber, 2003; Kawai, 1999). Acute myocarditis can further develop into chronic myocarditis, resulting in myocardial fibrosis and cardiac dilated cardiomyopathy (DCM), thus, this condition poses a severe health problem (Caforio et al., 2013; Doolan et al., 2004; Fabre and Sheppard, 2006). Acute myocarditis may be clinically treated, but treatment of chronic myocarditis remains a challenge (Guglin and Nallamshetty, 2012). Thus, novel therapeutic strategies for chronic myocarditis should be developed.

Myocarditis is mainly treated using immunosuppressive and immunomodulating drugs that target immune responses (Kindermann et al., 2012). Previous studies demonstrated that treatment of myocarditis with azathioprine and prednisone improves cardiac function (Parrillo et al., 1989; Wojnicz et al., 2001). In a CVB3-induced myocarditis murine model, interferon (IFN)-beta and IFN-alpha2 therapy protects myocytes against injury and decreases inflammatory cell infiltration (Wang et al., 2007). In patients with viral myocarditis treated with IFN-beta, viral genomes are eradicated and cardiac function is improved (Kuhl et al., 2003). Although remarkable progress has been observed, treatment of patients with chronic myocarditis remains challenging (Guglin and Nallamshetty, 2012; Han et al., 2013; Maisch and Pankuweit, 2012). Therefore, we need to develop better therapeutic strategies for chronic myocarditis.

Abbreviations: CVB3, coxsackievirus B3; DCM, dilated cardiomyopathy; AIM2, absent in melanoma 2; $TCID_{50}$, tissue culture infective dose 50%; LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening; LVEDd, left ventricular end-diastolic dimensions; LVEDs, left ventricular endsystolic dimensions; TnI, troponin I; CVF, collagen volume fraction; PRRs, pattern recognition receptors.

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Therapeutic DNA vaccines are potent inducers of antigen-specific T cell immune responses through mucosal immunization at all mucosal sites (Fujkuyama et al., 2012; Holmgren and Svennerholm, 2012; Wang et al., 2014). Studies have shown that mucosal immunization approach has been successful applied to animal models (Wu et al., 2014; Ye et al., 2014; Zhao et al., 2013). Potent adjuvants are also necessary to use DNA vaccines (Hasegawa et al., 2014). Absent in melanoma 2 (AIM2) as a cytosolic DNA sensor recognizes double-strand DNA (ds-DNA), and then forms an inflammasome to activate the caspase-1 (Fernandes-Alnemri et al., 2010; Hornung et al., 2009). Pro-interleukin-1β is cleaved by activated caspase-1 resulting in the secretion of interleukin-1β (IL-1β) to mediate inflammatory responses (Fernandes-Alnemri et al., 2010; Hornung et al., 2009). Thus, AIM2 may benefit the potential therapeutic efficacy of VP1 vaccine against chronic myocarditis induced by CVB3.

This study aimed to determine whether AIM2 can enhance the therapeutic efficacy of VP1 vaccine against CVB3-induced chronic myocarditis. We demonstrated that CS-pAIM2/CS-pVP1 co-immunization could efficiently alleviate the severity of CVB3-induced chronic myocarditis. Therapeutic efficacy was closely associated with the enhanced induction of multifunctional $CD8^+$ T cells. Our study may represent a novel therapeutic approach for chronic myocarditis and provide reference for the development of other therapeutic vaccines which require the robust induction of multifunctional $CD8^+$ T cells.

2. Materials and methods

2.1. Animals and virus

3–4 week old male BALB/c mice ($H-2^d$) were purchased and remained in the pathogen-free conditions. All animal procedures were performed according to the guidelines for the Care and Use of Laboratory Animals (Ministry of Health, China, 1998) and the guidelines of the Laboratory Animal Ethical Committee of Soochow University. The animal use protocol was approved by the Laboratory Animal Ethical Committee of Soochow University. The CVB3 (Nancy strain) was propagated in HeLa cells (ATCC number: CCL-2), and virus titer was determined by a 50% tissue culture infectious dose ($TCID_{50}$) assay of HeLa cell monolayer and calculated by the Reed–Muench method.

2.2. CVB3-induced chronic myocarditis model

A chronic myocarditis model was established on the basis of a previously reported protocol (Zhang et al., 2013) with slight modification. Three-week-old to four-week-old male BALB/c mice were injected intraperitoneally (i.p.) with 100 µl of CVB3 ($10TCID_{50}$) diluted in phosphate-buffered saline (PBS) at 0 and 30 d; the mice in the control group were intraperitoneally treated with PBS. On day 56 post infection, the development of chronic viral myocarditis was evaluated by the indices (survival rate, cardiac function, fibrosis, and myocardial inflammation and damage).

2.3. DNA vaccine treatments

Murine AIM2 expression plasmid pEFBOS-AIM2 (pAIM2) or pcDNA3.1-VP1 (pVP1) was encapsulated with chitosan according to a previously described method (Yue et al., 2009). Groups of mice ($n = 20$ in per group) infected with CVB3 were mildly anesthetized with pentobarbital (40 mg/kg body weight, intraperitoneal) and intranasally immunized with CS-pAIM2/CS-pVP1, CS-pVP1, CS-pAIM2, or mock. Each group was immunized at a dose of 50 µg of each plasmid at three time points: (i) during the acute phase of immune response (at day 7 d), (ii) during the transition to the chronic phase of immune response (at 21 d), and (iii) during the chronic phase of the immune response (at 35 d). In CS-pVP1, CS-pAIM2, or mock-immunized group, the mice were treated with additional 50 µg of CS-pcDNA3.1 empty plasmid to ensure that the total amount of DNA was 100 µg.

2.4. Anesthesia and echocardiography

The mouse was positioned on a heating pad to maintain normothermia. Isoflurane (2%) in pure medical oxygen was administered using a vevo compact anesthesia system (VisualSonics). The mice were examined using an echocardiography system (Vevo2100, Visual Sonics) to determine left ventricular end-diastolic dimensions (LVEDd), left ventricular end-systolic dimensions (LVEDs), left ventricular ejection fraction (LVEF), and left ventricular fractional shortening (LVFS) according to the operator's manual.

2.5. Determination of cardiac troponin

Plasma cardiac troponin I levels were measured using a high-sensitivity mouse cardiac troponin-I ELISA kit (Life Diagnostics) according to the manufacturer's protocol. Plasma cTnI levels were determined in triplicate.

2.6. Histomorphological analysis

Heart tissues were fixed in 10% phosphate-buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE). The Histopathological changes were compared quantitatively by calculating the histopathological scores (Rezkalla et al., 1988). Two independent researchers individually scored the samples in a blinded manner.

The paraffin sections of myocardial tissue samples were subjected to collagen-specific staining by using picrosirius red F3BA (Abcam). Collagen volume fraction is an indicator of the degree of myocardial fibrosis and is the ratio of the area of interstitial fibrosis to the total area of connective and myocardial tissues (Querejeta et al., 2000). Collagen volume fraction (CVF) was calculated using Leica QWin image analysis software (Leica).

2.7. Immunofluorescence staining of VP1 expression

Heart tissues of immunized mice were dissected and frozen in OCT medium. Frozen sections (8 µm) were prepared, fixed, and stained with mouse anti-VP1 antibody (Dako) and then with Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody (BioLegend).

2.8. T lymphocyte proliferation assay

MLN cells were added to 96-well flat-bottomed tissue culture plates at 5×10^5 cells/well containing 50 U/ml IL-2 and 10 µg/ml recombinant VP1 protein. The plates were cultured at 37 °C in a humidified incubator with 5% CO2 for 5 d and the medium was changed medium at 3 d. Cell proliferation was determined through colorimetric immunoassay based on bromodeoxyuridine (BrdU) incorporation using ELISA BrdU kit (Roche Diagnostics) according to the manufacturer's.

2.9. Cytotoxic T lymphocyte (CTL) assays

MLN cells were cultured in RPMI 1640 medium containing 50 U/ml IL-2 and 10 µg/ml VP1 protein for 7 d at 37 °C in humidified air with 5% CO2. The processed MLN cells were washed and

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