Coxsackievirus B3 regulates T-cell infiltration into the heart by lymphocyte function-associated antigen-1 activation via the cAMP/Rap1 axis

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Coxsackievirus B3 (CVB3) infection can trigger myocarditis and can ultimately lead to dilated cardiomyopathy. It is known that CVB3-induced T-cell infiltration into cardiac tissues is one of the pathological factors causing cardiomyocyte injury by inflammation. However, the underlying mechanism for this remains unclear. We investigated the mechanism of T-cell infiltration by two types of CVB3: the H3 WT strain and the YYFF attenuated strain. T-cell activation was confirmed by changes in the distribution of lymphocyte function-associated antigen-1 (LFA-1). Finally, we identified which viral gene was responsible for LFA-1 activation. CVB3 could infect and activate T-cells in vivo and in vitro, and activated T-cells were detected in CVB3-infected mouse hearts. LFA-1 expressed on the surface of these T-cells had been activated through the cAMP/Rap1 pathway. Recombinant lentiviruses expressing VP2 of CVB3 could also induce LFA-1 activation via an increase in cAMP, whilst VP2 of YYFF did not. These results indicated that CVB3 infection increased cAMP levels and then activated Rap1 in T-cells. In particular, VP2, among the CVB3 proteins, might be critical for this activation. This VP2–cAMP–Rap1–LFA-1 axis could be a potential therapeutic target for treating CVB3-induced myocarditis.

INTRODUCTION

Coxsackieviruses are non-enveloped, ssRNA viruses in the family Picornaviridae (Esfandiarei & McManus, 2008; Kim et al., 2013). Coxsackievirus B3 (CVB3) infection is known to induce several diseases, including myocarditis and pancreatitis in neonates and young children (Tracy et al., 2000). Generally, coxsackievirus group B serotypes B1–B5 are responsible for about 5 million enteroviral infections every year in the USA (Kim et al., 2001). Among them, CVB3 is a major factor responsible for provoking mild to severe or even fatal cases of myocarditis (Henke et al., 2008). Most cases are mild, but up to 12% of patients with known CVB3 infections might have myocardial involvement (Gravanis & Sternby, 1991).

It has been suggested that the mechanism of CVB3-induced myocarditis falls into two categories: direct virus-triggered heart injury via a cytopathic effect, and indirectly triggered heart injury by activated immune cells such as T-cells and macrophages (Fairweather & Cihakova, 2009; Huber et al., 2002). However, how CVB3 triggers myocarditis remains unclear. According to previous studies, activated T-cells might play a major role in virus-induced myocarditis (Gebhard et al., 1991; Steinman, 2007). This is corroborated by the finding that CVB3 could not induce myocarditis in immunodeficient nude mice, which have no T-cells (Hashimoto & Komatsu, 1978). Moreover, p56Lck, one of the main factors involved in T-cell activation, is critical for CVB3-induced myocarditis (Grabie et al., 2003). However, the mechanism of T-cell infiltration remains unknown.

Lymphocyte function-associated antigen-1 (LFA-1) is part of the family of leukocyte integrins and is a heterodimer construct between common β-chains (β2 and CD18) and an α-chain (αL, CD11a) (Stanley et al., 2012). LFA-1 is found on the surface of T/B-cells, macrophages and neutrophils. In addition, LFA-1 is involved in the infiltration of lymphocytes and plays a key role in interacting with antigen-presenting cells (Roossien et al., 1989; Simonson et al., 2006). LFA-1 has three phases of the activated form (bent, alternative affinity and high affinity). The naive LFA-1 conformation is bent, and once the appropriate signal is received, LFA-1 is changed to either the alternative- or high-affinity form (Alon & Feigelson, 2012). Activated LFA-1 binds primarily to intercellular adhesion molecule-1 (ICAM-1), which is the main ligand. ICAM-1 is expressed on lymphocytes, monocytes, epithelial cells and fibroblasts (Marlin & Springer, 1987). Interestingly, previous reports showed that some viruses responsible for T-cell infections, such as human immunodeficiency virus type 1 (HIV-1) and human T-cell leukemia virus (HTLV), can activate LFA-1. Therefore, based on these reports, we speculated that CVB3 might activate LFA-1 and that these activated T-cells might infiltrate...
into the heart and ultimately induce heart injury from myocarditis.

We showed previously that CVB3 has a tyrosine-based activation motif (ITAM)-like immunoreceptor region on VP2 of CVB3 structural proteins (Kim et al., 2012). ITAM is present in some cellular receptor cytoplasmic tails, such as T/B-cell receptors, CD3 and Fc receptors, and has a role in transferring external signals into the lymphocyte for activation (Cantrell, 1996). When ligands bind to ITAM, the two tyrosine (Y) residues in the ITAM sequences become phosphorylated by Src-family kinases, leading to cellular activation and proliferation (Kim et al., 2012). We previously produced attenuated CVB3 (YYFF) by point mutation of the VP2 region, which involved changing the two Y residues of ITAM in VP2 to phenylalanine (F) (Park et al., 2009). This YYFF variant was significantly attenuated in murine models (Kim & Nam, 2010; Kim et al., 2010). Therefore, here we used the WT CVB3 H3 strain and YYFF as an attenuated strain to determine the mechanism of T-cell activation and infiltration following viral infection in a murine model.

RESULTS

CVB3 directly infects T-cells in vivo and in vitro, and causes them to infiltrate the heart

First, we investigated whether T-cells were present in mouse hearts after CVB3 or YYFF infection. As shown in Fig. 1(a), many immune cells were evident in CVB3-infected hearts at 7 days post-infection (p.i.) but not in mock- or YYFF-infected hearts. Among the infiltrating immune cells, T-cells were identified by immunohistochemistry using a mAb against a T-cell-specific surface marker, CD3. Fig. 1(a) (anti-CD3) shows that T-cells were present in most infiltrated heart cells after CVB3 infection. As expected, mock- and YYFF-infected hearts did not show any T-cell infiltration. Therefore, the infiltrating T-cells might have been in an activated state (Yndestad et al., 2003). We suspected that CVB3 might infect T-cells directly, leading to activation. To test this hypothesis, we confirmed the viral titres in T-cells from infected mouse spleens. Fig. 1(b) shows that the virus was present in T-cells from the infected mice. Additionally, the titre of CVB3 was approximately sevenfold higher than YYFF on a log scale, meaning that CVB3 infected T-cells more efficiently than YYFF. Moreover, CVB3 and YYFF could also secrete progeny viruses continuously to the extracellular space (Fig. 1c, upper panel) and could be detected inside infected Jurkat T-cells more than 96 h after infection (Fig. 1c, lower panel). The titre of CVB3 was also approximately 10-fold higher than that of YYFF in cell culture in vivo (Fig. 1c). Such prolonged viral progeny secretion suggests that CVB3-infected T-cells might function as virus reservoirs. Interestingly, although both CVB3 and YYFF could grow in T-cells, CVB3 showed a higher cytopathic effect compared with YYFF (14.4 vs. 4.0% in necrotic cells and 10.2 vs. 4.1% in apoptotic cells infected with CVB3 or YYFF, respectively; Fig. S1, available in the online Supplementary Material).

CVB3 infection affects the activation of LFA-1 in Jurkat T-cells and splenocytes

We expected that CVB3 infection would induce LFA-1 activation by conformational modification. In previous studies, Jurkat T-cells have been used for analysing viral growth and signal pathways (Luo et al., 2014, Rovira-Clavé et al., 2014; Russell et al., 2013). We expected that such cells would activate similarly to human peripheral T-cells and therefore investigated LFA-1 activation using them. The Kim127 mAb can detect LFA-1 activation (Shamri et al., 2005), as the Kim127 epitope is exposed as the extended active form of LFA-1 when T-cells are activated (Stanley et al., 2008). Using this antibody, we detected LFA-1 activation in infected Jurkat T-cells at 48 h and in infected mouse heart tissues at 7 days after CVB3 infection by immunofluorescence assay (Fig. 2a, upper panel) and immunohistochemistry (Fig. 2a, lower panel). The activation of LFA-1 was detected in CVB3-infected but not in YYFF-infected Jurkat T-cells in vitro and in infected mouse hearts in vivo (Fig. 2a). Moreover, flow cytometry also showed that LFA-1 activation could only be detected in the CVB3-infected cells in vivo (~20% positive cells) and in vitro (~14% positive cells) and not in the YYFF-infected cells (~8 and ~14%, respectively) (Fig. 2b, c). ICAM-1 is an LFA-1 ligand and only binds to activated LFA-1 (Stanley et al., 2008). Therefore, we measured the binding ability of virally infected Jurkat T-cells to ICAM-1 coated on a plate, which indirectly represented LFA-1 activation. CVB3-infected T-cells could bind to ICAM-1 at a 5% higher rate than YYFF-infected T-cells (Fig. 2d). Although this result does not guarantee any significance in vivo, it reinforces the results shown in Fig. 2(a–c). Above all, the data suggested that CVB3 infection in T-cells triggers LFA-1 activation and that these activated T-cells effectively bind to ICAM-1 on cardiomyocytes, leading to infiltration. However, YYFF infection did not induce these events in T-cells.

CVB3 infection increases cAMP and activates Rap1 in T-cells, whilst YYFF infection does not

Next, we investigated which signal factors are involved in LFA-1 activation by viral infection. Both cAMP and Rap1 have been shown to act as upstream factors for LFA-1 activation (Mor et al., 2007). As expected, CVB3 infection increased cAMP levels by approximately 1 fmol ml–1 more than the mock group (Fig. 3a) and activated Rap1 in Jurkat T-cells at 48 h p.i. (Fig. 3b). However, the attenuated strain YYFF did not show such results. The actual change in cAMP levels was slight but seemed to be sufficient, because it acts primarily as an upstream factor. To confirm these signal factors, we used a specific cAMP inhibitor (sq22536) and a Rap1 inhibitor (GGTI-298) in CVB3-infected Jurkat T-cells to investigate LFA-1 activation. FACs analysis showed that both inhibitors decreased LFA-1 activation in
CVB3-infected cells (Fig. 3c). The Rap1 inhibitor yielded a more significant decrease in LFA-1 activation by CVB3 infection than did the cAMP inhibitor. These results indicated that both cAMP and Rap1 are upstream molecules serving to activate LFA-1 following CVB3 infection.

VP2, a CVB3 capsid protein, can activate LFA-1

The YYFF strain is an attenuated virus made by the change of two Y residues into F residues in the ITAM-like region on the structural protein VP2 of CVB3 (Kim et al., 2012; Park et al., 2009). This represents the only difference between CVB3 and YYFF. In addition, based on the above data (Figs 2 and 3), YYFF did not activate LFA-1. Therefore, we hypothesized that VP2 of CVB3 might be responsible for LFA-1 activation. To test this hypothesis, we produced recombinant lentiviruses expressing CVB3-VP2 (Lenti-WT-VP2) and YYFF-VP2 (Lenti-YYFF-VP2). We injected these two viral strains three times into mice intraperitoneally with $1 \times 10^7$ p.f.u. ml$^{-1}$ in 100 μl. At 3 days after the last injection, we investigated LFA-1 activation in splenocytes. We confirmed VP2 expression in Lenti-WT-VP2- and Lenti-YYFF-VP2-injected mouse splenocytes by reverse transcription (RT)-PCR (Fig. 4a). Treatment with Lenti-WT-VP2 increased LFA-1 activation, whereas Lenti-YYFF-VP2 did not (Fig. 4b). Moreover, the level of cAMP – an LFA-1 stimulator – was also increased in lenti-WT-VP2-infected mouse serum (Fig. 4c). Therefore, we concluded that VP2 of CVB3 can trigger LFA-1 activation via an increase in cAMP.

DISCUSSION

According to previous studies, the mechanisms of CVB3-induced myocarditis are as follows: (i) direct virus-induced cardiomyocyte injury; (ii) autoimmune-mediated destruction of cardiac cells by circulating autoantibodies and/or
autoreactive immune cells or by the immune-mediated obliteration of cardiomyocytes arising from molecular mimicry between viral and host antigenic epitopes; and/or (iii) the excessive immune-mediated destruction of the myocardium by infiltrating immune cells targeting virus-infected cardiomyocytes (Esfandiarei & McManus, 2008). Here, we focused on the third mechanism, in which infiltration of immune cells is thought to be responsible for viral myocarditis. In particular, we hypothesized that, among the immune cells, T-cells would play a primary role in myocarditis, because of the following considerations: (i) T-cells can be used as carriers for virus delivery into the heart; and (ii) T-cells –primarily cytotoxic T-cells – activated by a CVB3 infection can attack heart tissue. Moreover, a previous study showed that the Src-family tyrosine kinase p56Lck, a T-cell upstream mediator, is required for efficient CVB3 replication in T-cell lines and for virus replication and persistence in vivo (Liu et al., 2000). In addition, CVB3 infection did not induce pathogenesis in p56Lck gene knockout mice (Liu et al., 2000). Moreover, p56Lck is known to act as a factor inducing T-cell activation (Veillette et al., 1989). Therefore, T-cell activation is a major factor for CVB3-induced pathogenesis. Here, we confirmed that CVB3 infection induced T-cell infiltration into the heart (Fig. 1a) and that T-cells might play a role as virus-delivery vehicles (Fig. 1b, c), because infected T-cells consistently secreted CVB3 viral progeny. However, although this T-cell delivery theory seems to be reliable, we require more intensive studies to confirm it.

Fig. 2. CVB3 infection activates LFA-1 on the T-cell surface. (a) LFA-1 activation was observed in Jurkat T-cells and mouse heart tissues after viral infection (48 h and 7 days p.i., respectively) by immunofluorescence assay (upper panel, Jurkat T-cells) and immunohistofluorescence (lower panel, heart tissue) using the anti-Kim127 mAb, which can detect LFA-1 in its active form. Magnification ×200. (b) Flow cytometry showing LFA-1 activation in Jurkat T-cells at 48 h p.i. by anti-Kim127 mAb binding. (c) Flow cytometry showing LFA-1 activation in mouse splenocytes at 7 days p.i. by anti-Kim127 mAb binding. The results of (b) and (c) are represented graphically below the plots. (d) Binding assay to ICAM-1 (an LFA-1 ligand) coated on a plate showing that CVB3-infected T-cells bound to ICAM-1 more than YYFF-infected T-cells. ‘Mock’ indicates non-infected cells. Binding is indicated as the percentage of cells remaining bound to the plate. *P<0.05. Error bars represent SD (n=3).
It is known that LFA-1 is responsible for T-cell infiltration (Aloisi et al., 2000). Here we showed that CVB3 infection changed the conformation of LFA-1 to its active form (Fig. 2). These T-cells bearing activated LFA-1 on their surfaces could easily bind to ICAM-1 on myocytes, leading to infiltration (Fig. 2d). Moreover, CVB3 infection activated T-cells directly leading to infiltration, and this T-cell infiltration might be a critical step for viral myocarditis. Many other viruses such as HIV-1 and HTLV-1 can affect the function of LFA-1 (Fortin et al., 1998; Hioe et al., 2001; Malbec et al., 2011). Thus, LFA-1 is known to play a role in the cell-to-cell transmission of HIV-1. In addition, active LFA-1 increases cell susceptibility to infection by HIV-1. The P8 protein of HTLV-1 increases LFA-1 clustering at the cell surface and affects viral transmission. Previous studies have shown that LFA-1 was activated by two pathways: the calpain–talin pathway and the Rap1 pathway (Katagiri et al., 2000; Shamri et al., 2005). We found that calpain activity was not significantly changed in cells infected with CVB3 (data not shown). However, Rap1 activation was increased through an increase in cAMP in CVB3-infected cells (Fig. 3). Interestingly, infection with the YYFF strain did not activate LFA-1 in T-cells and did not show any activation of related signal factors. In addition, although the YYFF strain showed low replication efficiency in T-cells compared with the CVB3 WT virus (Fig. 1), it could also infect and yield progeny viruses in T-cells. This indicates that the difference in T-cell activation between the CVB3 and YYFF strains might arise from specific signal effects and not from virus replication efficiency. Moreover, treatment with recombinant lentiviruses expressing VP2 (Lenti-WT-VP2 and Lenti-YYFF-VP2) clearly showed that only Lenti-WT-VP2 activated T-cells through an increase in cAMP, as was the case in CVB3 infection, whilst Lenti-YYFF-VP2 and YYFF did not. This finding indicates that VP2 of CVB3 is a critical factor for T-cell activation and infiltration.

Taken together, our data suggest the presence of a CVB3—VP2—cAMP—Rap1—LFA-1 axis (Fig. 5). Based on this axis, there are two possible therapies for viral myocarditis; namely, inhibition of cAMP and Rap1, which are upstream of LFA-1 activation. In addition, direct inhibition of LFA-1 activation might also be a good strategy for preventing viral myocarditis. These newly identified targets could be useful for the development of novel therapeutic drugs for treating CVB3-induced myocarditis and other T-cell activation-related diseases.
METHODS

Viruses. The attenuated infectious YYFF virus used in this study was derived from a cDNA copy of the Woodruff variant of CVB3, acquired from Dr E. S. Jeon (Knowlton et al., 1996; Lim et al., 2005). It was constructed on a WT CVB3 cDNA background by site mutagenesis (Park et al., 2009). Thus, CVB3 refers to the WT virus, whilst YYFF denotes the attenuated virus. Each virus was propagated in HeLa cells and titrated using plaque assays (Kim et al., 2004).

Cells and animals. Jurkat T-cells and HeLa cells were maintained in RPMI 1640 and Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) FBS, 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹ at 37 °C under 5% CO₂ in humidified air. The mouse experiments were reviewed and approved by the Animal Care and Use Committee of the Catholic University of Korea (Bucheon, Korea). Four-week-old BALB/c male mice were purchased from Orient. CVB3 and YYFF were injected intraperitoneally into each mouse, with 1 × 10⁷ p.f.u. in 100 μl. Lentiviruses lenti-WT-VP2, lenti-YYFF-VP2 and lenti-FUGW were injected intraperitoneally into each mouse three times with 1 × 10⁷ viral particles in 100 μl.

Purification of T-cells from mouse spleens. Splenocytes from infected BALB/c mice were harvested at 4 days p.i., and a single-cell suspension of splenocytes was prepared by homogenization, followed by lysis of red blood cells (10 min on ice, red blood cell lysis buffer). Sorting of lymphocyte subpopulations was performed by magnetic-activated cell sorting (Miltenyi Biotec) according to the manufacturer’s instructions. Briefly, splenocytes were resuspended at a concentration of 1 × 10⁶ cells in 40 μl. The antibody (10 μl) from the Pan T Cell Isolation kit II (Miltenyi Biotec) was added to the cell
suspension, mixed well and allowed to incubate at 4 °C for 15 min. Magnetic beads were added to the cell suspension, mixed and incubated at 4 °C for a further 15 min, and then washed with Variomacs buffer [PBS with 0.5 % (w/v) BSA and 5 mM EDTA]. The separation columns were placed in a magnetic field and prepared by washing with 500 μl Variomacs buffer. The washed cell pellet was carefully resuspended in Variomacs buffer and applied to the pre-filled column. Pan T-cells, which did not bind the antibody, passed through the column, whereas other cells were retained. The purity of the separated cells was determined by flow cytometry using an FITC-coupled anti-CD3 antibody (Becton, Dickinson; Fig. S2) (Mena et al., 1999).

Virus titration. Levels of infectious viruses were determined in isolated T-cells, prepared using a HT10 homogenizer (IKA-Korea), by plaque assays according to standard procedures. Briefly, samples were diluted serially 10-fold, added to 90–95 % confluent monolayers of HeLa cells in six-well plates and incubated for 90 min. The medium was removed, and 2 ml complete serum-free DMEM containing 0.5 % (w/v) agar was added to each well. The cells were incubated at 37 °C for 48 h, fixed with Carnoy’s fixative for 10 min and stained with crystal violet. Plaques were counted, and viral concentrations were calculated as p.f.u. ml⁻¹.

Immunohistochemistry. Mice infected with CVB3 and YYFF were euthanized on 7 days following injection. The heart tissues were fixed in 4 % (v/v) formalin, embedded in paraffin wax, dewaxed, sectioned and subjected to haematoxylin and eosin staining. Some sections were dewaxed in xylene blocked with 1 % (w/v) BSA in PBS at room temperature and incubated with an anti-CD3–FITC antibody at room temperature followed by washing in PBS. The infected Jurkat T-cells and sections blocked with BSA were incubated with anti-Kim127 mAb at room temperature followed by washing in PBS. The anti-Kim127 antibody was detected using an anti-mouse IgG–FITC antibody. The stained sections were subjected to light or fluorescence microscopy, and images were captured using a STOP charge-coupled device digital camera (Diagnostic Instruments).

Flow cytometry of LFA-1 activation. The harvested Jurkat T-cells and virus-infected splenocytes were incubated with anti-Kim127 mAb for 30 min at 4 °C. Cells were washed with PBS, incubated with FITC-labelled anti-mouse antibodies (Abcam) for 30 min, washed twice with PBS and fixed in 1 % (v/v) paraformaldehyde. A Beckman FC500 flow cytometer (Beckman Coulter) and Canto II software (Becton Dickinson) were used to record 10 000 events. We used unstained samples as negative controls and anti-CD3–FITC samples as positive controls.

ICAM-1 binding assay. We modified previously published methods to calculate ICAM-1 binding activity (Pyszniaik et al., 1994; Salas et al., 2006). Briefly, flat-bottomed 96-well microplates (Corning) were coated with recombinant human ICAM-1-Fc (R&D Systems) at 50 μg ml⁻¹ (25 μg per well) in 0.1 M sodium carbonate buffer (pH 9.5) or 0.1 M sodium bicarbonate buffer (pH 8.0) for 16 h at room temperature, and unbound ICAM-1-Fc was removed. Infected Jurkat T-cells (2 × 10⁵ cells per well) at 16 h p.i. in RPMI 1640 and 10 % (v/v) FBS were dispensed into the wells, centrifuged at 10 g for 2 min and incubated at 37 °C for 30 min. The wells were washed several times in the same medium, and the remaining bound cells were quantified by counting under a microscope.

cAMP quantification. CVB3-induced CAMP accumulation in T-cells was determined by seeding 2 × 10⁴ Jurkat T-cells per well in six-well plates. The cells were serum starved for 16 h. Thereafter, the cells were infected with CVB3 or YYFF. After infection, the cells were lysed in R&D Cell Lysis Buffer (R&D Systems). Recombinant lentivirus-induced CAMP accumulation in mouse serum was measured in circulating blood by cardiac puncture. Serum samples were prepared by a 10 min centrifugation step at 400 g, stored at −20 °C and used with a fivefold dilution in the buffer. The amount of cAMP released was determined by ELISA using CAMP kits (R&D Systems) according to the manufacturer’s protocol. Briefly, using 96-well microplates, 50 μl aliquots of the primary antibody solution were added to each well and incubated for 1 h at room temperature and then washed with the kit’s wash buffer. Aliquots of 50 μl conjugated CAMP, 100 μl each of the sample and standard, and 100 μl dilution buffer were added to each well. These were incubated for 2 h at room temperature and then washed with the wash buffer. Colorization was carried out by adding 200 μl of the kit’s substrate solution, incubating for 30 min in darkness and then adding 100 μl stop solution. The absorbance was measured at 450 nm using a microplate reader.

Measurement of Rap1 activity. The activity of Rap1 was determined using a Rap1 activation assay kit (Enzo Life Sciences) according to the manufacturer’s instructions. Briefly, infected Jurkat T-cells were harvested, centrifuged at 400 g for 5 min and washed with PBS. The pellet was resuspended with lysis/binding/wash buffer, incubated on ice for 5 min and centrifuged at 14 000 g at 4 °C for 15 min. The supernatant was tested using the Bradford method to quantify protein contents. Aliquots of 500 μg of the cell lysates were bound to glutathione-conjugated resin for 1 h at 4 °C and mixed with 20 μg glutathione S-transferase fusion protein containing the Rap1 binding domain of Rap1 guanine nucleotide dissociation stimulator. The beads were washed three times with lysis/binding/wash buffer and resuspended with SDS sample buffer including β-mercaptoethanol. For Western blotting, the proteins were separated by 12 % SDS-PAGE and transferred to nitrocellulose membranes. These were blocked with PBS containing 3 % (w/v) BSA at room temperature for 2 h and rinsed with 0.05 % (v/v) Tween 20. Rap1 antibody was added at a 1:1000 dilution and incubated at 4 °C overnight. Anti-rabbit IgG–HRP was diluted 1:4000 in Phosphate buffered saline tween 20 (PBS-T) containing 5 % (w/v) non-fat dry milk and incubated for 1 h at room temperature. The Rap1 signal is located at approximately 24 kDa.

Lentiviral system. The lentiviral expression vectors FUGW, VSVG, RSV and RRE with the FUGW construct were used. The VP2 gene was cloned into the BamHI and EcoRI sites of the FUGW expression vector. Production of the lentivirus particles was achieved by transient transfection of the cell line 293T with the four vectors using the calcium phosphate/DNA precipitate method. At 72 h after transfection, the medium was collected and filtered through a 0.45 μm sterile filter. We confirmed VP2 expression in splenocytes by RT-PCR (Fig. 4a).

RT-PCR. All procedures were performed as described previously (DiBàise et al., 2008). Total RNA was extracted from frozen splenocytes using TRIzol reagent (Invitrogen). The cDNA was synthesized from total RNA using RT & GO (MP Biomedicals Korea) with oligo(dT) nucleotides (Cosmo Genetech), and RT-PCR was performed. The samples were run in duplicate, and each transcript level was adjusted to that of a housekeeping gene (18S RNA gene). The primer set used for the genomic amplification of VP2 was: forward, 5'-CGTGGTGGTAGGCTATGGAGTG-3', and reverse, 5'-CGTCCTGTTGCGTGGTCG-3'.

Statistical analysis. All data are expressed as the mean ± SD. Unless otherwise noted, differences between mean values were analysed using Student’s t-tests, and P<0.05 was considered to be statistically significant.

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