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Inhibition of IL-2 inducible T-cell kinase alleviates T-cell activation and murine myocardial inflammation associated with CVB3 infection

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ABSTRACT

Background: Coxsackievirus B3 (CVB3) infection causes myocarditis, pancreatitis, and aseptic meningitis. Targeting antigen-specific T cell reactions might be a promising way to alleviate the inflammatory response induced by CVB3 infection. IL-2-inducible T-cell kinase (ITK), a member of Tec kinase family expressed mainly in T cells, plays an important role in the activation of T cells. The role of ITK in viral myocarditis induced by CVB3 has not been documented.

Methodology: In this study, we inhibited the ITK expression in Jurkat cells, primary human peripheral blood mononuclear cells (PBMC), and mouse splenocytes by ITK-specific siRNA. The inhibition efficiently suppressed cell proliferation ($P<0.05$) and T-cell related cytokine secretion ($P<0.05$). In order to inhibit ITK in vivo, the pGCSIL plasmid containing short hairpin RNAs targeting ITK was constructed and transduced into mice infected with CVB3. ITK-inhibited mice showed reduced cell proliferation (3, 5, and 7 days post-challenge, $P<0.05$) as well as CD4+ and CD8+ T cells (5 days post-challenge, $P<0.05$). The altered production of inflammatory cytokines alleviated pathologic heart damage and improved mice survival rate ($P<0.05$).

Conclusion: ITK played an important role in the T cell development and represented a new target for the modulation of T-cell-mediated inflammatory response by CVB3 infection.

1. Introduction

CVB3 is one of the most common causes of viral myocarditis in infants and young children, often leading to acute heart failure and sudden death by direct cytopathic effects and pathologic immune response (Esfandiarei and McManus, 2008; Tam, 2006). The T cells play a crucial role in CVB3-induced immune reaction. Activated T cells enable clearance of the infectious virus and inhibition of CVB3 replication, (Huber, 2004; Van Houten and Huber, 1991). However, T-cell activation also affects the host adversely through immunopathologic mechanisms. T-cell mediated cytotoxicity leads to target organ damage and tissue destruction aggravated by elevated T cell cytokines even after viral clearance (Luppi et al., 2003). Therefore, it is important to downregulate the strong immune response in CVB3 infection by targeting the T cells.

T cells require two signals for activation. The antigen-specific T-cell receptor (TCR) delivers the first signal while binding of the ligands to T-cell surface molecule CD28 constitutes the second signal pathway. In the T cells, non-receptor kinase Src, Syk, and Tec families of tyrosine kinases, are activated. They amplify the signaling cascade that follows T-cell/antigen presenting cell (APC) interaction (Zhang et al., 1998; van Oers et al., 1996). ITK is a member of the Tec kinase family that is mainly expressed in the T cells, mast cells, and natural killer cells (Berg et al., 2005). It is located downstream of the T-cell receptor and plays a pivotal role in the development and activation of the T cells by transmitting signals from TCR and CD28 to the downstream effectors (Liao et al., 1997; Gibson et al., 1996). These downstream effectors include phospholipase C-gamma 1, which is a key regulator of PKC activation and intracellular Ca^{2+} mobilization, as well as GATA3, T-bet, NF-κB (Miller et al., 2004; Schaeffer et al., 1999; Grasis et al., 2003; Khurana et al., 2007) and NFAT transcription factors (Robert et al., 2012). ITK was found to promote Th2 differentiation and production of Th2-related cytokines IL-4, IL-5 in infectious diseases such as those caused by *Leishmania major*. Th1 differentiation was also impaired in the absence of ITK in *Leishmania major* and

Abbreviations: ITK, IL-2-inducible T-cell kinase; CVB3, coxsackievirus B3 strain; PBMC, peripheral blood mononuclear cell; RNAi, RNA interference; PFU, plaque-forming units; PHA, phytohemagglutinin; ConA, concanavalin.

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Schistosoma mansoni infection (Schaeffer et al., 2001). Previous studies also proved that ITK was required for efficient replication of HIV (Readinger et al., 2008) and influenza virus (Fan et al., 2012). Mice lacking ITK manifested reduced airway immunological symptoms in allergic asthma (Ferrara et al., 2006; Lin et al., 2004; Mueller and August, 2003) as well as reduced inflammatory response in skin disease (von Bonin et al., 2011). However, no data are available regarding the role of ITK in CVB3-induced myocarditis.

In this study, we designed the siRNA sequence targeting homologous sequences of human and mouse ITK. Using RNAi technology, we interfered with the ITK expression in vitro and in vivo, followed by evaluation of the ITK function in T cell response. We found that ITK played an essential role in cell proliferation and T-cell related lymphokine secretion in vitro and in vivo. We propose that ITK represents a new target for alleviation of inflammation by CVB3 infection.

2. Materials and methods

2.1. Ethical statement

The animal and human studies were approved by the Review Board of Capital Institute of Pediatrics with the permit number: DM2012001. The 6-week-old BALB/c male mice were purchased from the Institute of Laboratory Animal Sciences of China and were bred under conventional conditions. They were regularly screened for common pathogens and housed in compliance with the Regulations of Ethics Committee of Capital Institute of Pediatrics. The protocols for the blood collection from animals and post-viral infection survival rate assessment were approved by the Ethics Committee. All efforts were made to minimize animal suffering. Human blood samples were obtained from volunteers, with written informed consents.

2.2. Recombinant plasmid construction and siRNA synthesis

RNAi stem-loop DNA oligos containing the target sequences (5′-GCCTCTTCCTCCTACTCCTGA-3′) for both human (NM_005546) and mouse ITK (NM_010583) genes were chemically synthesized, annealed and cloned into the *AgeI*/*EcoR*I-digested pGCSIL to generate the plasmid pGCL-shITK. The vector pGCSIL containing a U6 promoter that continuously expressed shRNA was purchased from Shanghai GeneChem Co. Ltd (Shanghai, China). A scramble siRNA sequence (5′-UUCUCCGAACGUGUCACGU-3′) was used to generate the non-silencing control plasmid, designated as pGCL-shNC. The siRNA (siRNA-ITK) targeting the same sequences as pGCL-shITK and the scramble siRNA (siRNA-NC) were also synthesized by Shanghai GeneChem Co. Ltd.

2.3. Short hairpin RNA treatment in vitro

2.3.1. Cell culture

Jurkat cells, human peripheral blood mononuclear cells (PBMC) and mice splenocytes were maintained in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Human PBMC were isolated from the whole blood of healthy human donors using lymphocyte separation medium (Dakewei Biotech, Shenzhen, China), following the manufacturer's instructions. Mouse splenocytes were isolated from the spleens of the 6-week-old BALB/c male mice using lymphocyte separation medium (Dakewei Biotech). Cell viability was determined by Trypan blue dye exclusion test.

2.3.2. RNA interference (RNAi) and phenotype analysis

The siRNA-ITK (150 nmol/L) was mixed with purified cells (6×10^6) including Jurkat cells, the human PBMC, and mice splenocytes, followed by electrotransfection with Gene Pulser II (Bio-Rad, USA), at the optimized voltage of 230 V and capacitance of 975 μF. Cells were then cultured in 12-well plates with complete medium and stimulated for 24 h. The P form of phytohemagglutinin (PHA-P, for Jurkat cells and human PBMC, Sigma–Aldrich, USA) or concanavalin (ConA, for mice splenocytes, Sigma–Aldrich, USA) was used as stimulant (5 μg/ml). At 72 h post-activation, the cells were cultured for subsequent proliferation analysis or collected for ITK expression analysis. The secreted cytokines were measured in the supernatants using cytokine-specific ELISA (R&D, USA).

2.3.3. Western blot and cell proliferation analysis

Cells (4×10^6) were lysed using the M-PER Mammalian Protein Extraction Reagent (Pierce, USA) and Western blot was conducted as described previously (Li et al., 2007). Monoclonal antibodies to ITK (1:1000; monoclonal mice anti-human/mouse ITK, Millipore, USA), or GAPDH (1:5000; monoclonal mice anti-human/mouse GAPDH, Sigma–Aldrich, USA) were used. Cell proliferation analysis was conducted by using [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, an inner salt (MTS, Promega, USA), according to the manufacturer's instructions. All the experiments were performed in triplicate and separately repeated at least three times.

2.4. Short hairpin RNA treatment in vivo

2.4.1. Mice and CVB3 virus

Six- to eight-week-old male BALB/c mice were used for these experiments. All animal procedures were performed in accordance with protocols by the Capital Institute of Pediatrics Animal Research Committee and international guidelines for animals used in research. The CVB3m strain was provided by Dr. SA Huber from the University of Vermont, USA and was amplified in the HeLa cells by infection (Lodge et al., 1987). Virus titers were assessed by plaque-forming units (PFU), and a stock solution was stored at −20 °C until use.

2.4.2. Coxsackieviru-induced myocarditis model

The pGCL-shITK, incorporated with Entranster™-in vivo transfection reagent (Engreen Biosystem Co. Ltd., China), was delivered into the 6-week-old BALB/c male mice via their caudal vein, according to the manufacturer's instructions. Control groups were injected with an equal volume of pGCL-shNC or MEM. All mice were intraperitoneally inoculated with 2×10^3 PFU/mouse (LD50) or 8×10^3 PFU/mouse (LD100) of CVB3m virus 24 h later.

Some of the mice were observed for survival time and others were euthanized on the indicated days following the CVB3 challenge. Experiments were carried out three times.

2.4.3. RNA transcription and polymerase chain reaction

Mouse splenocytes were isolated as before. The total RNA was isolated from the cells using TRIzol (Invitrogen, USA), according to the manufacturer's instructions. For each sample analyzed, 50 ng DNase I-treated RNA was reverse transcribed using reverse transcriptase (Promega, USA). The resulting complementary DNAs (cDNAs) were used as templates for polymerase chain reaction (PCR). Real-time PCR was carried out by using Power SYBR Green PCR Master Mix (Applied Biosystems, USA). The forward and reverse primers used for the amplification of the ITK were 5′-GATGGATGGGCGGTGGAGGTG-3′ and 5′-TGAGGGTCGTTGGTTTGGTAGTCG-3′, which yielded a 188-base pair product. The GAPDH primers were 5′-GCCATCAC-TGCCACCCAGAAG-3′ and 5′-GTCAGATCCACGACGGACACATTG-3′, which were used to normalize the total cellular RNA. The PCR conditions were as follows: a 95 °C initial activation for 15 min followed by 45 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s. The

fluorescence determination was set at the melting temperature for 20 s on a real-time PCR System (Applied Biosystems, USA).

2.4.4. Detection of cytokine and PBMC proliferation

Surviving mice in each group were euthanized. The serum was obtained on days 2, 4, 7, 10, 14 and 21 after infection with 1 × LD50 CVB3 ($n = 10$). The serum samples were mixed, and analyzed for TNF-α, IFN-γ, IL-6, IL-10 protein expression as described above.

Whole blood was collected on days 3, 5, and 7 after infection of 1 × LD50 CVB3. The PBMC were isolated by standard Ficoll–Hypaque gradient centrifugation and cells were inoculated on 96-well plate at a density of 2×10^5/well. Cell viability assays were performed as described above. All experiments were performed in triplicate and repeated separately at least three times.

2.4.5. Flow cytometry

Cells were incubated for half an hour at 4 °C with antibodies in 100 ml PBS/2% FBS, followed by two washes in PBS/2% FBS for surface staining. Anti-CD4-FITC, anti-CD3-PerCP-Cy5.5, and anti-CD8a APC-Cy7 were purchased from BioLegend. Cells were analyzed using a FACSCalibur cytometer (BD Biosciences), and data were analyzed using FlowJo software.

2.4.6. Histological analysis

Mice were euthanized and their hearts were removed on days 5 and 7 after a lethal dose (LD_{50}) of CVB3 infection. The hearts were fixed in formaldehyde overnight and embedded in paraffin. Finally, the 5-μm sections were cut and stained with hematoxylin-eosin, and examined for infiltrating cells and inflammation.

2.5. Statistical analysis

All statistical analyses were performed using the SPSS 11.5 computer software program. The numerical parameters, including cytokine levels, and the absorbance (OD) values of the cell proliferation were expressed as mean ± SEM. The survival was analyzed using the Log-rank (Mantel–Cox) method. The significance of variability among the experimental groups was determined by Mann–Whitney *U* test. All the differences were considered significant at *P* values of <0.05.

3. Results

3.1. Inhibiton of ITK in vitro

3.1.1. Inhibition of ITK expression in Jurkat cells, human PBMC and mice splenocytes

Jurkat cells, human PBMC, and mice splenocytes were used to test for impaired T-cell activation following in vitro ITK knockdown. To inhibit the ITK expression, ITK-siRNA was electrotransfected under suitable conditions with transfection efficiency of more than

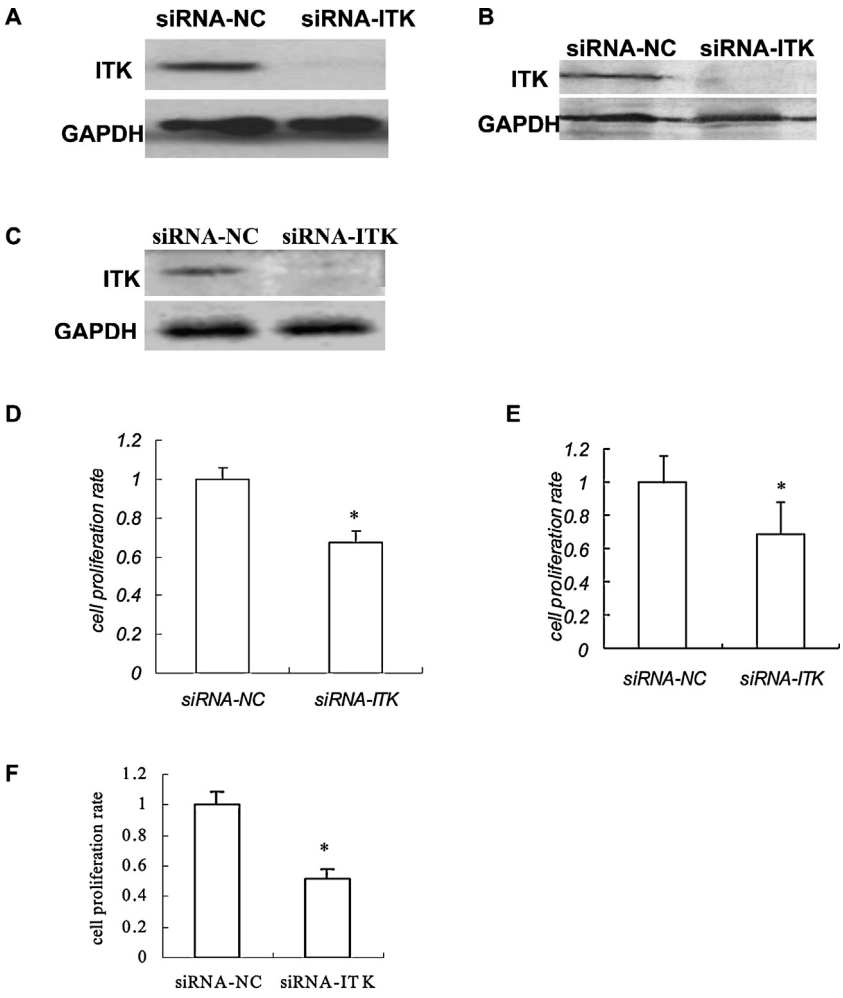

Fig. 1. ITK inhibition and cell proliferation of Jurkat cells, human PBMC and mice splenocytes. (A–C) siRNA-ITK or siRNA-NC (150 nM) were transfected into Jurkat cells, human PBMC and mouse splenocytes. Western blot was conducted to detect the ITK expression at 72 h post-activation. GAPDH was used as the loading control. (D–F) Proliferation of the Jurkat cells, human PBMC and mouse splenocytes, transfected with siRNA-ITK or siRNA-NC, were detected using the MTS assay at 72 h post-activation ($n = 5$, $^*P < 0.05$). Shown in this figure is a representative example from three independent experiments.

80% and a death rate of less than 40%. The transfection efficiency was confirmed by flow cytometry using FAM-labeled siRNA. Based on our results, 150 nmol/L was the lowest concentration of siRNA that was associated with high transfection efficiency and less toxicity to the cells (data not shown). Western blot was conducted following stimulation. As shown in Fig. 1A–C, the ITK protein expression was significantly inhibited compared to the control group.

3.1.2. Reduced cell activation by altered ITK expression in vitro

Jurkat cells and human PBMC were stimulated by PHA-P for 72 h following the ITK-siRNA transfection and mouse splenocytes were stimulated by ConA. The Th1- and Th2-related cytokine production was detected in the culture supernatant. The IL-2, IL-4, IL-5, and IFN-γ levels were found to be reduced in the ITK inhibition group compared to the control group ($P < 0.05$, Fig. S1–S3). However, the IL-4 expression was not detected in the Jurkat group. The IL-10 expression was also found to be reduced in the Jurkat and human PBMC groups ($P < 0.05$; Fig. S1 and S2). The MTS assay was performed to determine ITK inhibition of cell proliferation. It was found that Jurkat cells with the ITK inhibition showed nearly 35% reduction in their proliferation compared to the control group ($P < 0.05$, Fig. 1D). Furthermore, human PBMC and mice splenocytes showed nearly 30% and 50% ($P < 0.05$, Fig. 1E and F) reduction in their proliferation, respectively.

3.2. Inhibition of ITK in vivo

3.2.1. Inhibition of ITK expression and increased survival rate in mice CVB3 model by pGCL-shITK

The CVB3-induced myocarditis mice model was constructed to investigate the possible role of ITK in CVB3 infection. The pGCL-shITK (40 μg/mouse) was intravenously injected into the mice via caudal vein. It was incorporated with the transfection reagent followed by CVB3 infection. The PBMC were isolated on days 3, 5, 7 and 21 post-challenge, and the mRNA was extracted. Transfection of pGCL-shNC had no effect on the ITK expression compared to the infected but non-treated animals. The ITK mRNA was found to be significantly decreased on day 3 and 5 compared to the pGCL-shNC group as verified by real-time PCR assay, which suggested that the ITK expression was significantly inhibited by pGCL-shITK (Fig. 2). There was no difference on ITK expression between the two groups on day 21.

The pGCL-shNC transfection had no effect on the survival rate of the study group compared to the infected but non-treated animals. Half of the mice infected with lethal doses (LD_{50}) of CVB3, died on day 10 in the pGCL-shNC group while none of the mice in the pGCL-shITK group died up to day 25 ($n = 20$). Following the infection

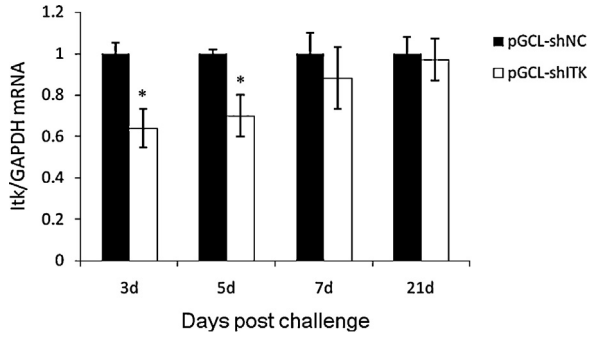

Fig. 2. Inhibition of ITK expression by pGCL-shITK plasmid A total of 40 μg of pGCL-shITK plasmid or pGCL-shNC was delivered into the BALB/c male mice via caudal vein incorporated with the transfection reagent and CVB3 were inoculated 24 h later. Mice were then euthanized for subsequent analysis. PBMC were isolated on days 3, 5, 7 and 21 post-challenge, RNA was extracted followed by real-time PCR to detect the ITK mRNA expression levels. GAPDH was used as control, ($n = 5$,*$P < 0.05$). pGCL-shITK: mice treated with pGCL-shITK; pGCL-shNC: mice treated with pGCL-shNC.

with LD_{100} of CVB3 virus, all the mice in the pGCL-shNC group died on day 10 while half of the pGCL-shITK group were alive 25 days after the CVB3 infection ($n = 20$, Fig. 3). The pGCL-shITK significantly improved the life span, suggesting a significant reduction of the mortality caused by viral myocarditis.

3.2.2. Reduced PBMC proliferation and altered CD4+, CD8+ T cell subsets

CVB3 infection of BALB-c mice results in the expansion of the T cells. To evaluate T cell proliferation in ITK inhibition group, equal amounts of PBMC from pGCL-shITK and pGCL-shNC groups were isolated on days 3, 5, and 7 post-challenge and were cultured for 24 h ($n = 5$). The MTS assays were performed to detect the cellular proliferation. The results showed that pGCL-shITK group exhibited a reduced proliferation compared to pGCL-shNC group (Fig. 4A). To illustrate the development of T cell subsets in mice with ITK inhibition post- CVB3 challenge, CD4+ and CD8+ T cells were detected in mice spleens. As shown in Fig. 4B and C, the percentage of CD4+ and CD8+ T cells decreased dramatically on day 5 after infection in pGCL-shITK group. ITK reportedly regulated Th17 effector cells. Th17 has a role in modulating inflammatory reactions in the CVB3 infection. Therefore, we analyzed the proportion of Th1, Th2 as well as Th17 in mice splenocytes (Fig. S4). The results showed that on day 5 post-challenge, development of Th1, Th2 as well as Th17 was affected by ITK inhibition.

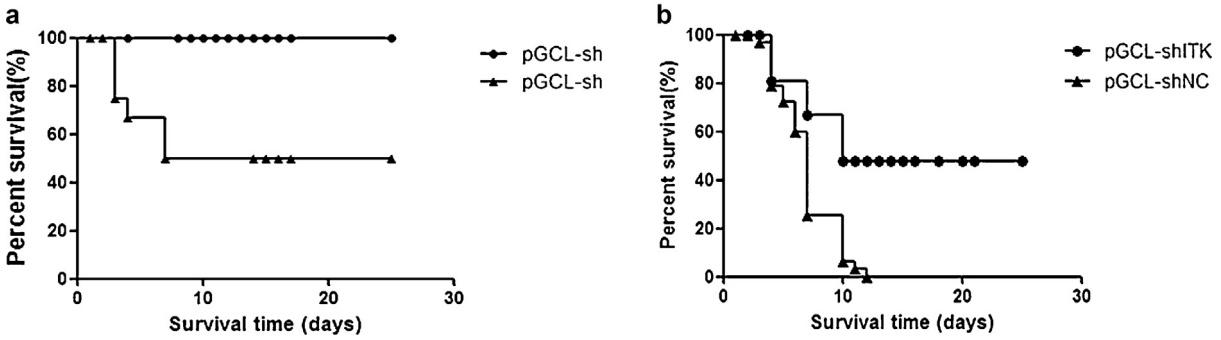

Fig. 3. Inhibition of ITK enhanced the survival rate in mice infected with CVB3. Mice were pretreated with plasmid expressing shRNAs under the conditions described in the legend to Fig. 2, followed by CVB3 infection 24 h later. The mice survival rates were analyzed. LD50 (a) or LD100 (b) CVB3 were used as challenge ($n = 20$). pGCL-shITK: mice treated with pGCL-shITK; pGCL-shNC: mice treated with pGCL-shNC.

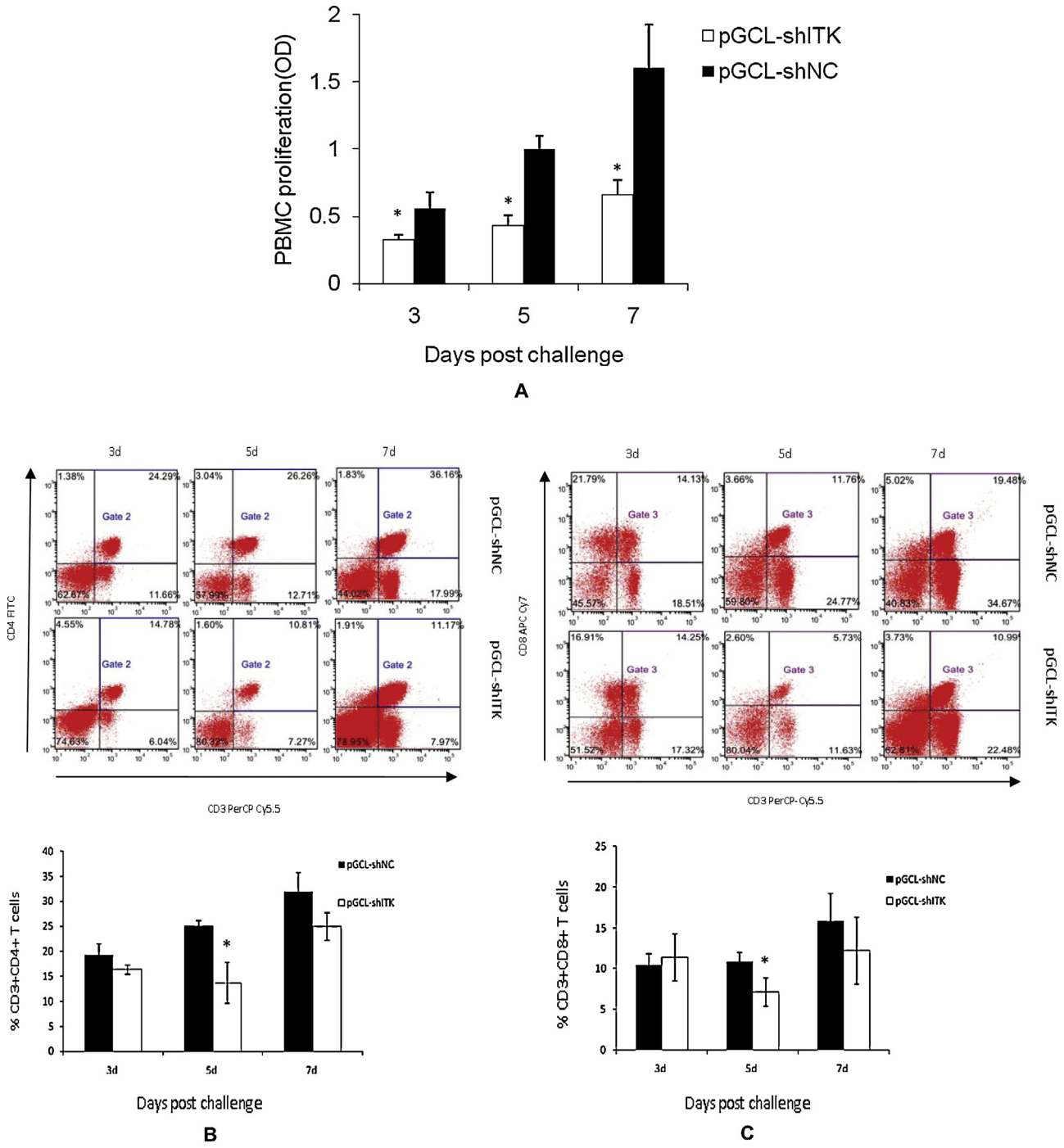

Fig. 4. Inhibition of ITK resulted in reduced PBMC proliferation and altered T cell subsets. Mice were pretreated with plasmid expressing shRNAs under the condition described in the legend to Fig. 2 followed by CVB3 infection 24 h later. (A) PBMC were isolated on the indicated days post challenge, followed by a 24 h culture for proliferation detection using MTS assay ($n = 6$). Lethal doses (LD_{50}) were used as a challenge, ($^*P < 0.05$). pGCL-shITK: mice treated with pGCL-shITK; pGCL-shNC: Mice treated with pGCL-shNC. (B and C) Mouse splenocytes were stained for surface expression of CD4 and CD8 and analyzed by flow cytometry on days 3, 5 and 7 post-challenge, numbers in the top right quadrant indicate the percentages of CD3+ CD4+ and CD3+ CD8+ T cells on the indicated days post-challenge ($n = 6$). Graphic representation is shown. Data shown are the arithmetic mean ± SEM for three independent experiments.

3.2.3. *Reduced cytokine production in response to CVB3 infection*

Mice were anesthetized with sodium pentobarbital (60 mg/kg, IP) followed by serum collection in order to detect the cytokines with specific ELISAs. The mouse eyeballs were removed under anesthesia to extract the whole blood on days 2, 4, 7, 10, 14, and 21 post-infection. The serum was separated from the whole blood by centrifugation. Ten mice were used at each time point and cytokines were detected using the mixed serum, which eliminates the difference between individuals. Transfection of pGCL-shNC had no effect on the cytokine production in the study group compared to the infected but non-treated animals. We found that Th2-related IL-6 and IL-10 levels were high during the first week in the pGCL-shNC group, and reached their peak levels on day four. The pGCL-shITK group showed lower levels of IL-6 and IL-10 on those days (Fig. 5). In the following days, IL-6 and IL-10 levels decreased in the pGCL-shNC group to a level similar to the pGCL-shITK group. The Th1-related IFN-γ and TNF-α showed lower level in pGCL-shITK group compared to the pGCL-shNC

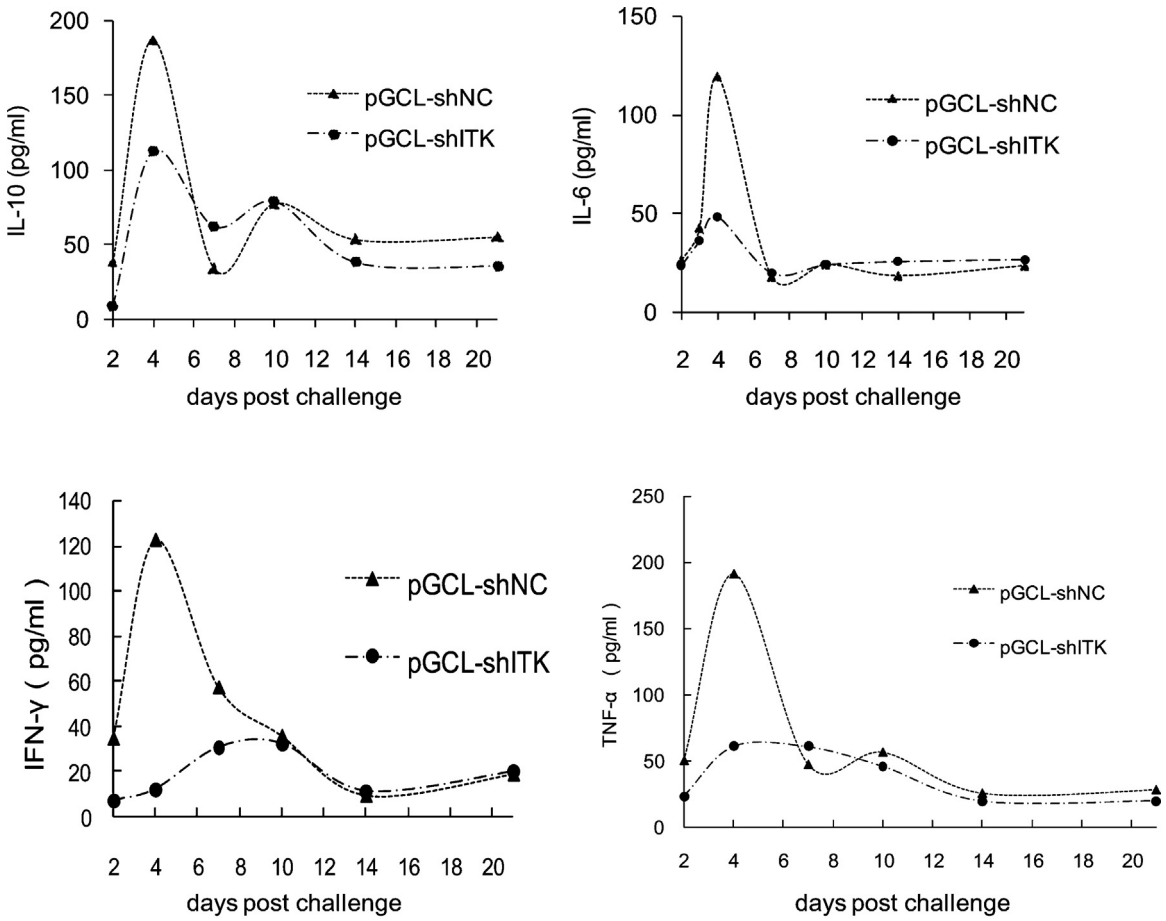

Fig. 5. Inhibition of ITK resulted in reduced inflammatory cytokine production. Mice were pretreated with plasmid expressing shRNAs under the conditions described in the legend to Fig. 2 followed by CVB3 infection 24 h later. The production of the inflammatory cytokines was detected in the serum of ten mice, on the indicated days post-challenge. The LD_{50} was used as a challenge. A representative example from three independent experiments is shown. pGCL-shITK: mice treated with pGCL-shITK; pGCL-shNC: mice treated with pGCL-shNC.

group, with peak expression on day 4 post-infection in both groups.

In this study, the maximum number of deaths was observed on days four to seven post-challenge that nearly correlated with the peak of the inflammatory cytokine production. The levels of Th1 and Th2-related cytokines were high in the pGCL-shNC group during these days, when ITK expression was inhibited and cytokine production was lower in the pGCL-shITK group. Therefore, an overly strong inflammatory reaction contributed to deaths in mice in the early phase. The inflammation was attenuated by pGCL-shITK.

3.2.4. Reduced cardiac inflammation in mice with ITK inhibition following CVB3 infection

Cardiac histopathology of CVB3-infected mice revealed that mice transfected with pGCL-ITK showed minimal to absent inflammation. In contrast, the pGCL-shNC group showed significant necrosis and signs of mononuclear cell infiltration on days five and seven following -infection (Fig. 6), suggesting that the inhibition of ITK expression blocked T-cell migration to the heart in this model system.

4. Discussion

Myocarditis frequently occurs as a pathogenic inflammation of the myocardium, blood vessels, and pericardium. It is characterized by the infiltration of inflammatory cells and cytokines in the target organs (Lundgren et al., 2009; Seko et al., 1997). Although patients with myocarditis generally recover with little or no permanent damage, a few die from persistent inflammation of the heart and pancreas, combined with dilated cardiomyopathy (DCM). CVB3 infection is among the most common causes of human myocarditis. It is estimated that more than 50% of the cases are attributable to the CVB3 infection. This study analyzed the important role of ITK in vitro, as well as the feasibility of attenuating inflammatory reaction in CVB3 mouse model by inhibiting ITK kinase expression using RNAi. It has been proved that mice lacking ITK have attenuated symptoms in allergic asthma (Mueller and August, 2003) and inflammatory skin diseases (Ferrara et al., 2006). However, the role of ITK in murine CVB3-induced viral myocarditis has not been documented.

T cells occupy a large proportion of PBMC and splenocytes. The PHA-P and ConA are T-cell-specific activators. In this study, Jurkat cells, human PBMC and mice splenocytes were used to investigate the role of ITK in T cell activation. Inhibition of ITK significantly reduced the cell proliferation and T-cell related cytokine production in Jurkat cells, human PBMC and mice splenocytes. This observation demonstrated the important role of ITK in T cell activation.

In subsequent animal experiments, we demonstrated that mice with ITK inhibition were largely resistant to immunopathological symptoms in the mouse model of CVB3 infection. The pGCL-shITK group showed effective decrease in morbidity of CVB3-induced myocarditis. Furthermore, the PBMC proliferation, and percentage of CD4+ and CD8+ T cells in splenocytes were found to be decreased post-challenge, which indicated a perturbed T-cell development and activation in ITK inhibition mice, and this may

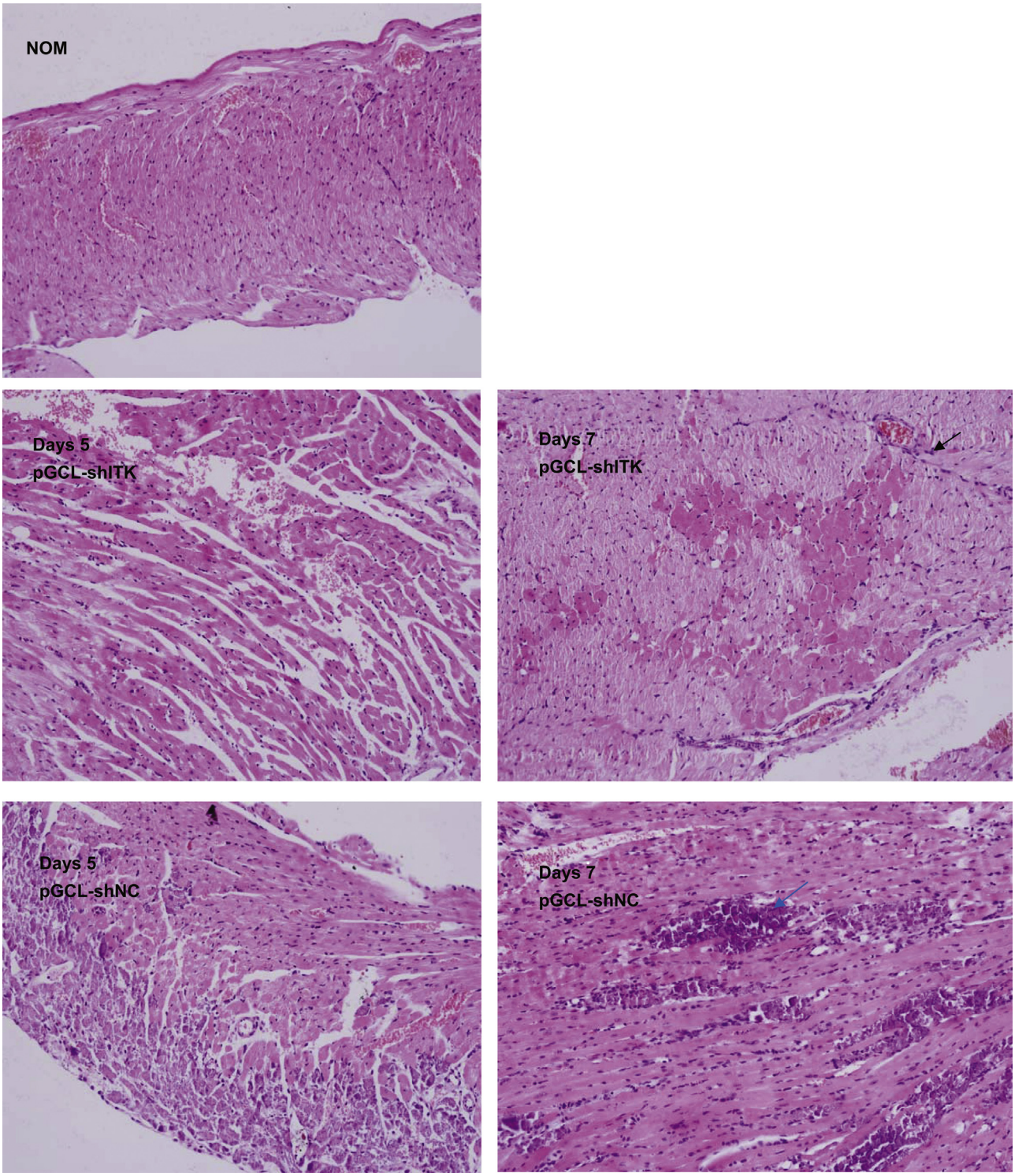

Fig. 6. Inhibition of ITK resulted in lowered inflammation. Mice were pretreated with plasmid expressing shRNAs under the conditions described in the legend to Fig. 2, followed by CVB3 infection 24 h later. Histology of the myocardial inflammation on days five and seven post-challenge (magnification of 100-fold) was analyzed. The black arrows show examples of infiltration and blue arrows show examples of necrosis and calcification. pGCL-shITK: mice treated with pGCL-shITK; pGCL-shNC: mice treated with pGCL-shNC.

induce a reduced influx of T cells into the inflamed tissue. T cells are important in antiviral immune response to CVB3 infection. However, T cell response might also harm the host through immunopathologic mechanisms, such as regulation of cytokine expression and lymphocyte infiltration. Downregulation of T cell activation might be achieved by ITK inhibition in CVB3 infection.

To further analyze the influence of ITK inhibition on the T helper cells, the Th2-related IL-6 and IL-10 as well as Th1-related IFN-γ and TNF-α in mice serum were detected. Homologous serum samples of ten mice were pooled and used in each group to detect cytokines production. IL-6 and IL-10 were found to be decreased in the pGCL-shITK group, which indicated an impaired Th2 response in the ITK inhibition group. IL-6 has been known

to induce inflammatory cell infiltration into the heart and pancreatic tissues, which are target organs for CVB3 infection (Heim and Weiss, 2004). Down-regulation of IL-6 in serum indicates an attenuated inflammatory symptom in the CVB3 mice models. The anti-inflammatory cytokine IL-10 was also found to be reduced on days 2, 3, and 4 post-challenge. The variation in IL-10 expression with in vitro conditions, suggested that ITK inhibition led to an overall deficit in the magnitude of the T-cell response post-challenge. Expression of IFN-γ and TNF-α may be a significant factor in determining susceptibility to CVB3 infection (Yue et al., 2011). IFN-γ and TNF-α might promote the viral clearance in case of virus infection (Huber et al., 2002). However, elevated expression of IFN-γ and TNF-α might enhance myocardial inflammation (Tam, 2006; Luppi et al., 2003). In our study, IFN-γ and TNF-α were found decreased 4 days post-infection in the pGCL-shITK group when pGCL-shNC group reached its peak. Our results indicated a reduced inflammatory cytokine production and impaired Th cell differentiation in the ITK inhibition group.

The pGCL-shNC group revealed typical virus-associated symptoms and severe cardiac pathology. In contrast, the pGCL-shITK group only manifested local cardiac inflammation. The findings were probably due to the altered T cell and cytokine proliferation (Han et al., 2013; Heim and Weiss, 2004; Bachmann et al., 1997).

ITK knockout mice were reportedly used in previous studies to investigate the role of ITK in allergic asthma (Mueller and August, 2003). However, compensatory mechanisms might occur in the embryonic development of the ITK knockout mice. The RNAi technology was used in vivo to inhibit the ITK expression. RNAi is an effective tool to inhibit specific gene expression, especially in the pathogenesis of viral infections (Yao et al., 2012; Yuan et al., 2005). RNAi inhibition depends on the efficacy of delivery of siRNA or shRNA. In our study, we inhibited the ITK expression by using an ITK-specific siRNA, in vitro. The pGCSIL plasmid used in our in vivo research contains a U6 promoter, and continuously expressed shRNA. The pGCL-shITK functioned well in vivo in our previous study (Yao et al., 2012) and successfully inhibited the ITK expression in this study. Lentiviral vector is a promising viral vehicle for the delivery of genes in vivo. Recombinant lentiviruses were first constructed in our study with pGCL-shITK and were designated as lenti-shITK. Lenti-shITK functioned well in vitro. However, it showed poor results in the ITK inhibition in vivo, even when 2×10^8 TU/mouse was used. To avoid possible toxicity, the pGCL-shITK was incorporated with the transfection regent and used in vivo. The ITK expression was successfully inhibited in mice. Furthermore, we observed attenuated pathogenic inflammation and increased survival rate in mice model of CVB3. Therefore, we confirmed the effectiveness of the in vivo plasmid-based gene delivery. We proposed the possible modulation of the strength of inflammatory response in CVB3-induced myocarditis by targeting ITK.

5. Conclusions

We concluded that the in vitro inhibition of ITK suppressed the T cell activation. Further, mice with ITK inhibition showed a prolonged survival, alleviated tissue damage, and attenuated T cell response compared with the control group after CVB3 infection. We further concluded that targeting ITK might be a potential way to reduce the magnitude of inflammatory response by CVB3 infection.

Competing interests

The authors declared that they had no competing interests.

Author contributions

FH carried out most experiments and drafted the manuscript. HY carried out the animal experiments. ZX & JH measured cytokines and performed the statistical analysis. JZ evaluated the pathology. ZL conceived the study, participated in its design and revised the manuscript. All authors read and approved the final manuscript. We also thank Mrs. Lingling Cai and Sha Wu for expert technical assistance.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molimm.2013.12.004.

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