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## Identification of human cytomegalovirus phosphoprotein 65 in C57BL/6 and BXSB mice as a potential trigger of systemic lupus erythematosus related serum markers

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## PEER REVIEW

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## Comments

The work is an interesting experimental study on proteomics of cytomegalovirus infection. The standard animal experimental study and proteomics research are done and they give a new original data. The work produce a good new data in virology, infectious medicine and bioinformatics. The work can be further referenced in tropical biomedicine.

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## ABSTRACT

**Objective:** To investigate the potential role of human cytomegalovirus lower matrix phosphoprotein 65 (HCMV-pp65) in murine systemic lupus erythematosus (SLE).

**Methods:** The prokaryotic plasmid pET-28b-pp65 was constructed to express the HCMV-pp65 protein. BXSB mice and C57BL/6 mice were inoculated with pp65 eukaryotic plasmid pcDNA3.0-pp65 intramuscularly 5 times at 2-week intervals, and then the blood of the mice was subsequently collected via the retro-orbital vein. Indirect ELISAs were used to evaluate the concentration of anti-pp65 immunoglobulin G, anti-double-stranded DNA and antinuclear antibodies. Interleukin-1 $\beta$  and tumor necrosis factor- $\alpha$  were also determined by competitive ELISA. At the same time, 3 major SLE-related circulating microRNAs were examined by quantitative RT-PCR.

**Results:** The early production of autoantibodies was observed in pp65-immunized male BXSB as well as C57BL/6 mice. Overexpression of interleukin-1 $\beta$  and tumor necrosis factor- $\alpha$  were detected in pp65-immunized male BXSB mice. Quantitative RT-PCR analyses showed that three SLE related microRNAs (microRNA-126, microRNA-125a, and microRNA-146a) were down-regulated in peripheral blood mononuclear cells of pp65-immunized mice.

**Conclusions:** Our findings indicate that HCMV-pp65 immunization strongly triggers the development and progression of SLE-like disease in both BXSB and C57BL/6 mice, which indicates that the immune responses induced by HCMV-pp65 may be involved in the development of SLE.

## KEYWORDS

Systemic lupus erythematosus, Autoantibody, Human cytomegalovirus, Lower matrix phosphoprotein 65, Cytokine, MicroRNA

## 1. Introduction

Systemic lupus erythematosus (SLE) is a chronic inflammatory

connective tissue disorder that involves joints, kidneys, mucous membranes, and blood vessel walls. Although genetic predisposition is crucial in the development of SLE, attention has been recently

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focused on the contribution of environmental factors, among which infection is essential[1]. The relationship between SLE and infection with viruses, bacteria, or parasites, has been well known in the literature[2]. Some viruses, such as human cytomegalovirus (HCMV)[3], Epstein–Barr virus and parvovirus B19 may play a pivotal role in SLE[4,5].

HCMV is a common DNA virus of the herpes family with a high prevalence in human. The potential role of HCMV in the etiology of SLE has been extensively studied. Recent reports have shown the increasing levels of anti-HCMV immunoglobulin G (IgG) and immunoglobulin M antibodies in sera of SLE patients compared with healthy controls[6-10]. It has also been suggested that an association between the presence of HCMV antibodies and anti-Smith/ribonucleoprotein was found for patients with SLE but not those with mixed connective-tissue disease[11]. Moreover, it was reported that in a case, acute HCMV infection may have been the precipitating event in the development of SLE[12]. Based on these findings of reported cases, a potential role of HCMV in the development and/or progression of SLE has been suggested.

In particular, the lower matrix phosphoprotein 65 (pp65), a dominant tegument protein of the HCMV virion, plays a significant role in immune evasion, and thereby, in controlling the innate and adaptive immune responses via HCMV-specific cytotoxic T lymphocytes[13]. Thus, the effect of pp65 is mostly attributed to preventing infected cells from being destroyed. Several studies have shown that pp65 may be associated with various autoimmune responses. These observations highlight the fact that immunization of either BALB/c or NZB/W F1 mice with one cytomegalovirus-specific peptide results in multiple autoreactive antibodies[14,15]. However, it would be of high priority to learn whether those autoantibodies also exist isolated from individuals with a background predisposed to SLE, especially whether more SLE related factors, such as cytokines and microRNAs, could be regulated by cytomegalovirus pp65 affection.

To further clarify the possible role of HCMV in SLE, authors chose the HCMV-pp65 recombinant plasmid as the vaccine to study its effect in C57BL/6 and SLE-prone BXSB mice by detecting a series of SLE-related serological makers and microRNAs (microRNA-125a, microRNA-126, and microRNA-146a), which served as potential biomarkers of SLE.

## 2. Materials and methods

### 2.1. Plasmids

Prokaryotic expression plasmid pET28b-pp65 encoded the full-length HCMV-pp65 (National Center of Biotechnology Information reference sequence: NC\_006273.2, synthesized by Sangon Biotech. Co., Ltd, Shanghai, China). Subsequently, the HCMV-pp65 fragment (1705 bp) was generated by digesting pET-28b-pp65 with *Bam*H1/*Xho*1 and ligated in pcDNA3.0 at the same sites using T4 DNA ligase (Takara Bio Inc., Kyoto, Japan). Recombinant plasmid, namely pcDNA3.0-pp65, was verified by agarose gel electrophoresis and sequencing.

### 2.2. Immunization of mice

Ten male BXSB mice (6 weeks old) were purchased from Jackson

Laboratory (Bar Harbor, ME, USA), and 14 C57BL/6 mice (6 weeks old) were purchased from the Animal Center of the Academy of Military Medical Sciences (Beijing, China). In this study, BXSB and C57BL/6 mice constituted SLE-prone and healthy control groups, respectively. The study was approved by the Ethics Committee of Peking Union Medical College. All the BXSB and C57BL/6 mice were raised in a specific pathogen free animal facility and were then divided randomly into Groups BP (BXSB-pp65,  $n=5$ ), BC (BXSB-control,  $n=5$ ), CP (C57BL/6-pp65,  $n=7$ ), and CC (C57BL/6-control,  $n=7$ ). Treatment involved five times 100- $\mu$ L intramuscular injections at 2-week intervals, with 100  $\mu$ g of either pcDNA3.0 plasmids encoding HCMV-pp65 (for Groups BP and CP) or with no insert (for Groups BC and CC) dissolved in sterile saline. Serum was separated after blood collection from the tail vein 1 d prior to each immunization. All the mouse were sacrificed 2 weeks after the last inoculation. Serum and peripheral blood mononuclear cells (PBMCs) of each sample used for the following detection were stored at 80 °C within 1 h of collection.

### 2.3. Prokaryotic expression, purification, and western blot analysis of HCMV-pp65 protein

Plasmid pET28b-pp65, which contains a N-terminal 6His-tag, was transformed into *Escherichia coli* strain BL21 (DE3). Expression of HCMV-pp65 proteins was induced at 37 °C in Lysogeny broth medium supplemented with 0.8 mmol/L isopropyl 1-thio- $\beta$ -D-galactoside for 10 h. After induction, cells were harvested and lysed by sonication in lysis buffer (KCl 22.4 g/L,  $\text{KH}_2\text{PO}_4$  6.8 g/L, lysozyme 0.1 mg/mL, phenylmethanesulfonyl fluoride 1 mmol/L, urea 8 mol/L; pH 8). The expressed N-terminal His-tagged pp65 protein (72 kDa) was then purified using Ni-NTA Magnetic Beads (Bio-Rad Inc., Hercules, CA, USA) and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 10% gels. Subsequently, the purified HCMV-pp65 protein was refolded at 4 °C in urea gradient (from 8 mol/L to 1 mol/L) refolding buffer (Tris-HCl 50 mmol/L, KCl 100 mmol/L,  $\text{MgCl}_2$  12.5 mmol/L, ethylene diamine tetraacetic acid 1 mmol/L, DL-Dithiothreitol 1 mmol/L, glycerin 10%, phenylmethanesulfonyl fluoride 0.1 mmol/L), which was changed every 6 h. After evaluating the antigenicity of refolded HCMV-pp65 protein by western blot using anti-cytomegalovirus pp65 antibody (Abcam Inc., Cambridge, MA, USA), the protein concentration was measured with the Bradford assay (Bio-Rad Inc., Hercules, CA, USA) using bovine serum albumin as a standard.

### 2.4. Indirect ELISA for the immunological response, anti-double stranded DNA (anti-dsDNA) and antinuclear antibodies (ANA)

To detect the titer of anti-pp65 IgG of immunized mice, 100  $\mu$ L/well of 2  $\mu$ g/mL refolded pp65 protein diluted in 0.05 mol/L carbonate buffer (pH 9.6) was coated onto ELISA plates (Corning-Costar Corp., Corning, NY, USA) at 4 °C overnight. The plates were then washed 5 times with phosphate buffered saline (PBS) and blocked at 37 °C for 2 h with blocking buffer (1.5% bovine serum albumin and 0.05% Tween 20 in PBS). After washing with PBS containing 0.05% (w/v) Tween 20, each well was incubated with 100  $\mu$ L of anti-sera (diluted by blocking buffer at 1:200) for 1 h at room temperature. Afterwards, each well was washed and incubated with anti-mouse IgG, horseradish peroxidase-

linked secondary antibody. Finally, the absorbance at 450 nm for each well was measured after incubating with o-phenylenediamine dihydrochloride as a substrate. In addition, anti-dsDNA antibody and ANA in serum were measured with precoated ELISA plates (R&D Systems, Inc., MN, USA). All tests were performed in duplicate.

### 2.5. Indirect immunofluorescence (IIF)

Two weeks after the last immunization, the serum samples in each group was tested by IIF for ANA according to the manufacturer's instruction (Euroimmun, Lübeck, Germany). In summary, sera were diluted to 1:100 and applied to commercially available slides coated with fixed Hep-2 cells (Euroimmun, Lübeck, Germany) for 30 min at room temperature. After washing for 5 min with PBS containing 0.05% (w/v) Tween 20, fluorescein isothiocyanate-labeled goat anti-rabbit IgG (Santa Cruz Biotechnology, Inc., CA, USA) were added at 1:100 dilution to each spot and incubated for 30 min. Slides were then embedded and visualized by a fluorescence microscope (Olympus, Tokyo, Japan).

### 2.6. ELISA for cytokines

Mouse competitive ELISA kits (Dakewe, Beijing, China) were used to measure the concentration of serum cytokines interleukin (IL)-1 $\beta$  and tumor necrosis factor (TNF)- $\alpha$  according to the manufacturer's instructions. For each assay, a standard curve was established by calibrators supplied with the kits and then used to calculate the concentrations of IL-1 $\beta$  or TNF- $\alpha$ . All tests were performed in duplicate.

### 2.7. Extraction of RNA and evaluation of microRNA levels

To isolate total RNA from PBMCs, TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) was used according to the manufacturer's protocol. For RT and real-time PCR, the PrimeScript™ RT reagent Kit (Takara Bio Inc., Kyoto, Japan) and the SYBR® Premix Ex Taq™ II (Takara Bio Inc., Kyoto, Japan) were purchased and used according to the manufacturer's instruction. U6 small nuclear RNA was used as an endogenous control. The stem-loop RT primers and PCR primer sets specific for each microRNA and U6 are shown in Table 1. All reactions were performed in

triplicate. Differences in the microRNA levels in each sample were normalized by U6. The comparative C<sub>t</sub> method ( $2^{-\Delta C_t}$ ) was used to calculate fold changes of microRNAs expression, where  $\Delta C_t = (C_{t \text{ microRNA}} - C_{t \text{ U6}})$ . To simplify the presentation of the data, the relative expression values were multiplied by 100.

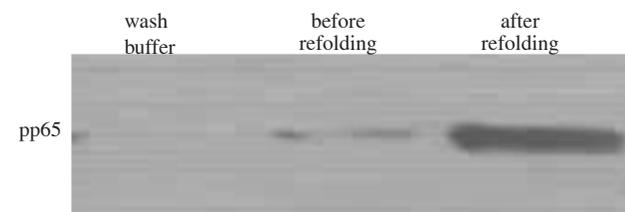
### 2.8. Statistical analysis

Comparison of categorical variables was performed using Fischer's exact test. The Mann-Whitney U test (GraphPad Prism; GraphPad Software Inc., San Diego, CA, USA) was used for comparison of continuous data which were expressed as mean $\pm$ SEM.  $P < 0.05$  was considered to be statistically significant.

## 3. Results

### 3.1. Analysis of HCMV-pp65 production and antigenicity

After digestion by *Bam*H1 and *Xho*1, the purified pcDNA3.0-pp65 was subjected to 1% agarose gel electrophoresis and a band between 1000 bp and 2000 bp was observed for the sample. Subsequently, the pcDNA3.0-pp65 was verified by sequencing. To confirm that pp65 was produced in the *Escherichia coli* strain BL21 expression system, a purified sample was analyzed by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The molecular weight of the proteins in the sample was approximately 72 kDa. After refolding, the purified protein was then analyzed by western blot using anti-cytomegalovirus pp65 antibody (Figure 1). These results indicated that the HCMV-pp65 protein was successfully expressed and displayed antigenic properties *in vitro*.



**Figure 1.** Identification of HCMV-pp65 protein.

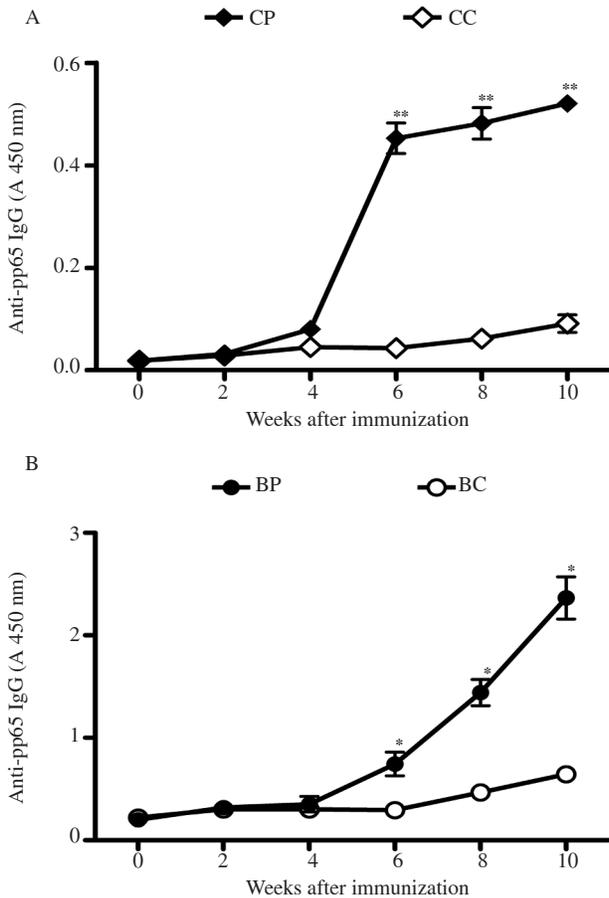
Western blot analysis of purified pp65 protein after refolding. Lane 1: The non-specific proteins in wash buffer; Lane 2: pp65 Protein in eluent before refolding; Lane 3: pp65 Protein in eluent after refolding.

**Table 1**  
Primers for miRNAs quantitative RT-PCR.

ID	Primer	Sequence
Micro-125a	Stem-loop RT primer	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT CAC AG
	Forward primer	GGC TCC CTG AGA CCC TTT A
	Reverse primer	GTG CAG GGT CCG AGG T
Micro-126	Stem-loop RT primer	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC GCG TA
	Forward primer	GGC GGC ATT ATT ACT TTT GG
	Reverse primer	GTG CAG GGT CCG AGG T
Micro-146a	Stem-loop RT primer	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA ACC CA
	Forward primer	GGC GTG AGA ACT GAA TTC CA
	Reverse primer	GTG CAG GGT CCG AGG T
U6	RT primer	CGC TTC ACG AAT TTG CGT GTC AT
	Forward primer	GCT TCG GCA GCA CAT ATA CTA AAA T
	Reverse primer	CGC TTC ACG AAT TTG CGT GTC AT

### 3.2. Analysis of anti-pp65 response

Antibody responses were elicited by immunization with the recombinant plasmid pcDNA3.0-pp65 (Groups BP, CP) and blank plasmid (Groups BC, CC) were all assessed 2 weeks after each immunization. It is showed that 6 weeks after the first incubation, anti-pp65 antibody was significantly higher in pcDNA3.0-pp65 immunized C57BL/6 [0.45 (0.39 to 0.50) vs. 0.28 (0.25 to 0.33),  $P<0.001$ ] and BXSB [0.74 (0.49 to 0.94) vs. 0.29 (0.24 to 0.34),  $P<0.05$ ] mouse than pcDNA3.0 vector immunized C57BL/6 and BXSB controls, respectively (Figure 2A). By contrast, the sera of blank plasmid groups presented negative reactivity (Figure 2B). These results indicated that the pcDNA3.0-pp65 construct, which was used to immunize the mice, expressed pp65 protein and induced specific antibodies in mice.



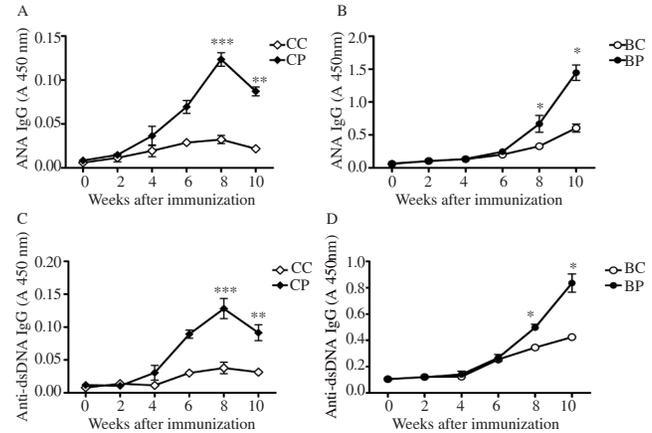
**Figure 2.** Mean levels of IgG against HCMV-pp65 ( $\pm$ SEM) in sera from mice (BXSB and C57/BL6).

A: Anti-pp65 activities of C57BL/6 mice; B: Anti-pp65 activities of BXSB mice. Anti-pp65 activities were determined by UV absorbance at 450 nm. The error bars indicate the SEM. Values in tests are expressed as mean $\pm$ SEM. \*:  $P<0.05$ ; \*\*:  $P<0.01$  (Mann-Whitney  $U$  test, compared with control).

### 3.3. Analysis of autoantibody development

On performing the ELISA to quantify levels of anti-dsDNA antibody and ANA in the sera of mice every 2 weeks after each immunization, we found a considerable increase in autoantibodies in all pp65-immunized mice. Treatment with pp65 significantly increased the levels of ANA [0.67 (0.48 to 0.89) vs. 0.33 (0.30 to

0.36),  $P<0.05$ ] and anti-dsDNA antibody [0.50 (0.45 to 0.55) vs. 0.34 (0.31 to 0.37),  $P<0.05$ ] in BXSB mice by 8 weeks post inoculation (Figures 3B and 3D), and the titers increased in the next 2 weeks. Surprisingly, serum of pp65-immunized C57BL/6 mice also showed statistically higher levels of ANA [0.13 (0.10 to 0.15) vs. 0.04 (0.03 to 0.04),  $P<0.001$ ] and anti-dsDNA antibody [0.13 (0.10 to 0.15) vs. 0.04 (0.01 to 0.06),  $P<0.001$ ] (Figures 3A and 3C), but the titers decreased in the next 2 weeks. Notably, the change of autoantibody titer in BP group was more dramatic than that in CP group compared with respective controls.



**Figure 3.** Mean levels of IgG against nuclear components ( $\pm$ SEM) and dsDNA ( $\pm$ SEM) in sera of mice (BXSB and C57BL/6) immunized with plasmids encoding HCMV-pp65 and vector only.

A: Antinuclear antibodies activity of C57BL/6 mice; B: Antinuclear antibodies activity of BXSB mice; C: Anti-dsDNA activity of C57BL/6 mice; D: Anti-dsDNA activity of BXSB mice. Activities of antibodies were determined by UV absorbance at 450 nm. The error bars indicate the SEM. Values in tests are expressed as mean $\pm$ SEM. \*:  $P<0.05$ ; \*\*:  $P<0.01$ ; \*\*\*:  $P<0.001$  (Mann-Whitney  $U$  test, compared with vector control).

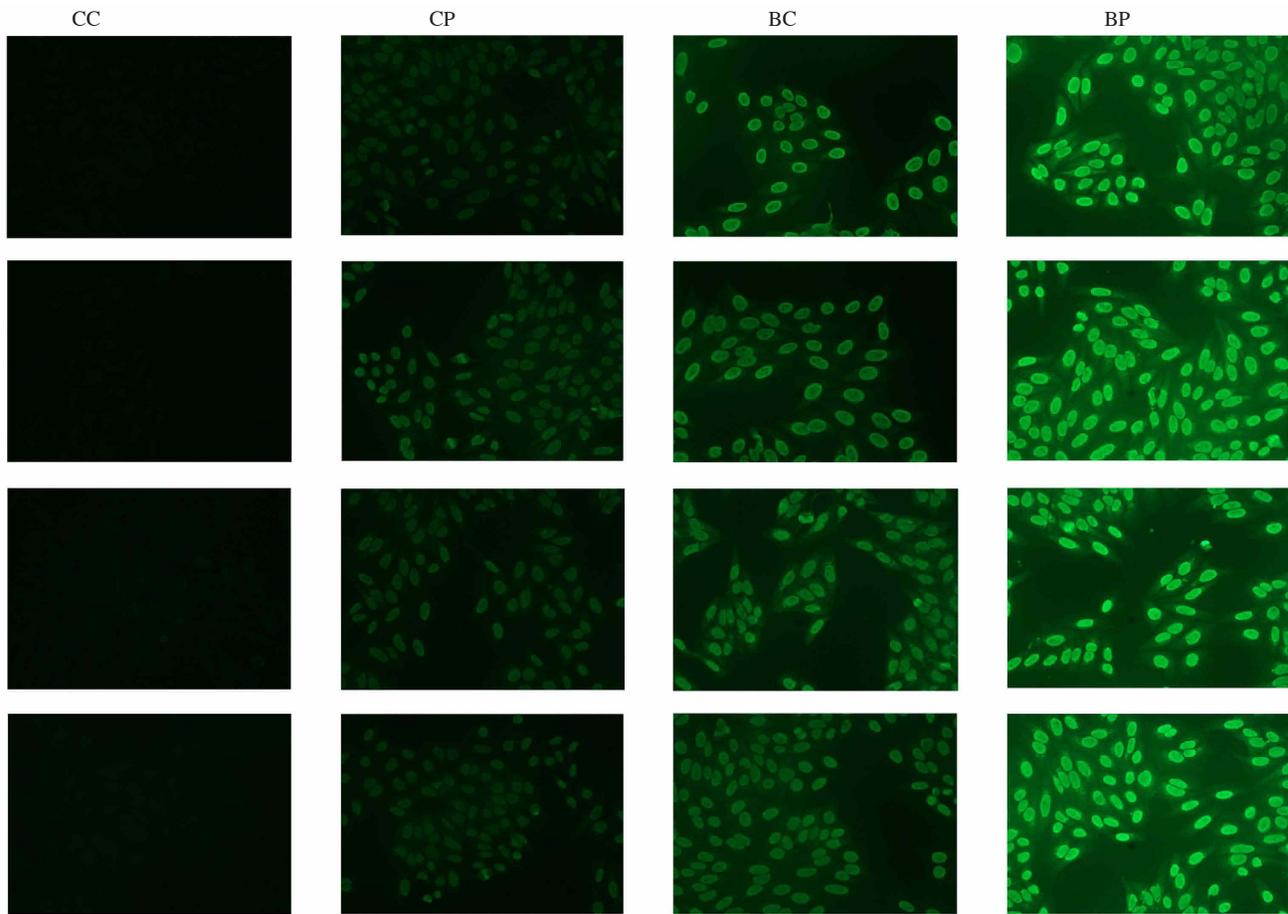
Moreover, 2 weeks after the last immunization, the serum samples in each group was tested by IIF and determined to be positive or negative for ANA based on known controls. Routinely, intensity of IIF was evaluated by titers (threshold value 1:40). Because low titer positive ANAs are fairly common in BXSB mice, the threshold titer of strongly positive ANAs was defined as 1:160 in our study. As summarized in Table 2, the serum of Groups BP and CP showed significantly stronger reacting ANA than Groups BC (threshold value 1:160,  $P<0.05$ ) and CC (threshold value 1:40,  $P<0.05$ ), respectively. ANA immunofluorescence staining of samples from each group are shown in Figure 4. We also found that ANA of these positive samples yielded a homogeneous fluorescence staining of the nuclear.

**Table 2**

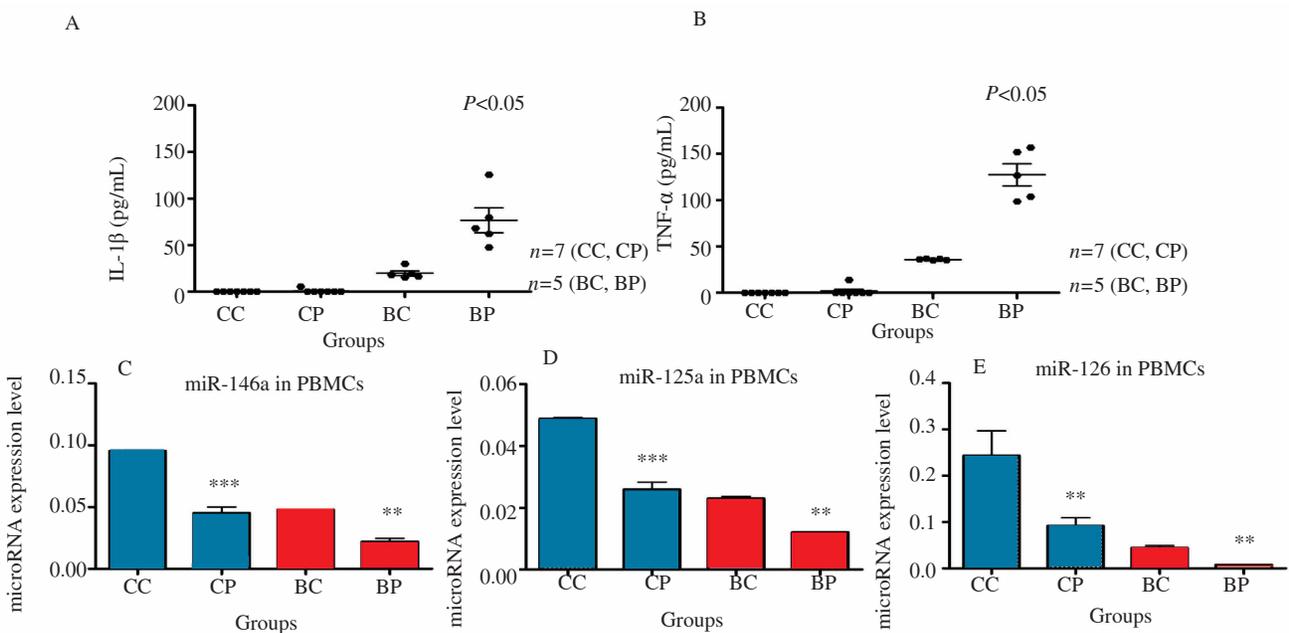
Detection of ANA by indirect immunofluorescence.

Groups	Negative	Positive		P value
	( $\leq$ 1:40) [n (%)]	Weakly (1:40<titre $\leq$ 1:160) [n (%)]	Strongly (>1:160) [n (%)]	
CC	7 (100.00)	0 (0.00)	---	<0.05
CP	2 (28.57)	5 (71.43)	---	
BC	---	4 (80.00)	1 (20.0)	<0.05
BP	---	1 (20.00)	4 (80.0)	

Taken together, the levels of anti-dsDNA antibody and ANA in Group BP were significantly higher than those in the Group BC after the fourth incubation. Similarly, the serum in the Group CP



**Figure 4.** ANA immunofluorescence staining in sera from mouse (BXSB and C57/BL6) immunized with plasmid encoding HCMV-pp65 and vector only. The staining patterns shown are representative for each group. Sera were incubated with Hep-2 cells at a dilution of 1:100. ANA were detected with fluorescein isothiocyanate-labeled goat anti-rabbit IgG secondary antibody. Magnification was  $\times 20$ .



**Figure 5.** The effect of immunization with plasmid encoding HCMV-pp65 and vector with no insert on the content of cytokines (IL-1 $\beta$  and TNF- $\alpha$ ) in sera and relative expression levels ( $2^{-\Delta Ct} \times 100$ ) of mature microR-146a, miR-125a, and microR-126 in PBMCs from mice (BXSB and C57/BL6) after the last injection.

A: The concentration (pg/mL) of IL-1 $\beta$  in each group; B: The concentration (pg/mL) of TNF- $\alpha$  in each group; C: The expression level of miR-146a (normalized by U6) in each group; D: The expression level of miR-125a (normalized by U6) in each group; E: The expression level of miR-126 (normalized by U6) in each group. The error bars indicate the SEM. Values in tests are the mean $\pm$ SEM. \*\*:  $P < 0.01$ ; \*\*\*:  $P < 0.001$ .  $P$  values are listed at the top (Mann-Whitney U test, compared with vector control).

had higher levels of the 2 autoantibodies than the CC group did after the same time of immunization. These findings showed that the humoral autoantibody response could be induced by pcDNA-3.0-pp65 plasmid in both C57BL/6 and BXSB mice.

### 3.4. Analysis of cytokine secretion

All the sera were diluted in 1:2 and analyzed by competitive ELISA. IL-1 $\beta$  and TNF- $\alpha$  were negative in C57BL/6 mice (Group CC), and were not significantly increased in pp65-treated C57BL/6 mice (Group CP). However, IL-1 $\beta$  was detectable in 80% of Group BC mice and 100% of Group BP mice (Figure 5A). The expression of IL-1 $\beta$  in Group BP mice reached statistical significance compared with Group BC controls [76.50 (64.66 to 92.53) vs. 19.70 (18.83 to 21.46),  $P < 0.05$ ]. In addition, sera of all the Group BP and Group BC mice produced TNF- $\alpha$ . As shown in Figure 5B, mice treated with pcDNA3.0-pp65 had a significantly higher TNF- $\alpha$  level than those treated with vehicle [127.50 (101.15 to 154.30) vs. 35.95 (35.11 to 36.73),  $P < 0.05$ ]. Thus, these data indicated that BXSB mice stimulated by pp65 had higher IL-1 $\beta$  and TNF- $\alpha$  production.

### 3.5. Analysis of differential microRNA expression

BXSB mice had significantly lower levels of microRNA-146a, microRNA-125a, and microRNA-126 compared with C57BL/6 mice (0.50-fold, 0.47-fold, and 0.18-fold;  $P < 0.01$ , 0.01, and 0.01, respectively). Moreover, the expression of microRNA-146a, microRNA-125a, and microRNA-126 were also markedly decreased in PBMC from the Group BP compared with the Group BC (0.46-fold, 0.52-fold, and 0.17-fold,  $P < 0.01$ , 0.01, and 0.01, respectively). The down-regulation of microR-146a, microR-125a, and microR-126 were also observed in PBMC from the Group CP compared with the Group CC (0.48-fold, 0.53-fold, and 0.38-fold;  $P < 0.001$ , 0.001, and 0.01, respectively). In summary, quantitative RT-PCR analysis clearly indicated that the expression of microR-125a, microR-126, and microR-146a was significantly down-regulated in PBMC from pp65-immunized BXSB and C57BL/6 mice compared with control BXSB and C57BL/6 mice, respectively (Figures 5C, 5D, and 5E).

## 4. Discussion

This study was performed to investigate the association between HCMV-pp65 immunization and the development of SLE in mice. In the present study, by detecting antibodies, cytokines, and microRNAs related with SLE, we verified that HCMV-pp65 could contribute to both initiation and exacerbation of SLE in non autoimmune-prone C57BL/6 mice and lupus-prone BXSB mice, respectively.

The spontaneous lupus-like syndrome was advanced in BXSB

males, which are the offspring of a cross between C57BL/6 females and SB/Le males. The Y chromosome of BXSB mice is derived ultimately from the strain SB/Le. Thus, we selected BXSB and C57BL/6 mice as SLE-prone and healthy control groups, respectively.

The results presented above indicated that the immunization of pcDNA3.0-pp65 induced humoral activity to the pp65 antigen in both BXSB and C57BL/6 mice. After 5 times of infection by pp65, the fluorescence intensities of ANA staining in serum of BP and CP groups were significantly higher than BC and CC groups, respectively. The positive samples which yielded a homogenous pattern of ANA staining-interphase cells showed homogeneous nuclear staining while mitotic cells showed staining of the condensed chromosome regions. Therefore, we knew that if pp65 could induce the higher level of not only ANA, but also the anti-dsDNA antibody. Subsequently, we tested both antibodies after each immunization and found that pcDNA3.0-pp65 could induce the significant increase of ANA and anti-dsDNA antibody production in both strains of mice by 8 weeks postinfection. Anti-dsDNA antibody is a very specific marker for SLE. Similar to these results in BXSB mice, a recent study found that pp65 increased the levels of ANA and anti-dsDNA antibody in another strain of SLE-prone mice (NZB/WFI), and the titers increased persistently[14]. Accordingly, pp65 immunization is likely to trigger the autoimmune responses and increase the level of anti-dsDNA antibodies highly associated with SLE in mice with or without a background predisposed to SLE.

Additionally, some results have aroused our interest. Firstly, the anti-pp65 antibody level was found to be lower in pp65-immunized C57BL/6 mice than in pp65-immunized BXSB mice. Moreover, the autoimmune responses induced by pp65 vaccine were more powerful in BXSB mice than in C57BL/6 mice. One possible explanation is that the cytotoxic T lymphocytes responses initiated by pcDNA3.0-pp65 vaccine could restrain the humoral responses in normal individuals[16], whereas an imbalance of T-helper 1/T-helper 2 in autoimmune patients makes the T-helper 2-mediated humoral responses greater[17]. Lastly, the titers of these autoantibodies increased in BXSB-pp65 mice while decreasing in C57BL/6-pp65 mice in the last 2 weeks of immunization. However, it was reported that pp65 could increase the levels of anti-dsDNA antibody continuously in C57BL/6 mice[14], which was not the same as results of ours. Thus, further studies are needed to explain this discrepancy.

To further analyze the possible link between pp65 vaccine and SLE, 3 well-studied microRNAs (microR-125a, microR-126, and microR-146a) closely related to SLE were chosen and examined by quantitative RT-PCR. We noted that the expression of microR-125a, microR-126, and microR-146a in PBMCs were all down-regulated significantly more in BXSB mice than in C57BL/6 mice. Similarly, several groups of investigators have shown that microR-146a and microR-125a were down-regulated in PBMCs of SLE patients[18,19].

Nevertheless, another recent report showed that the microR-126 level was much higher in SLE patients than in controls[20]. To the best of our knowledge, various disease stages, treatment histories, samples types, or detection methods are possible causes for the discrepancy in data reported by different studies, though the most important difference is likely in the genetic backgrounds of diverse species. Thus, to a certain extent, the down-regulated expressions of microR-125a, microR-126, and microR-146a, which served as potential biomarkers of SLE, may accompany the onset of SLE in mice. Furthermore, our findings revealed that immunization of pcDNA3.0-pp65 sharply decreased expressions of microR-125a, microR-126, and microR-146a in the PBMCs of both BXSB and C57BL/6 mice. Hence, we deduced that immunization with pp65 extends the progress of SLE in mice at the level of microRNA, though the precise roles of these down-regulated microRNAs still need to be identified in future researches.

We also observed that levels of TNF- $\alpha$  and IL-1 $\beta$  were higher in SLE-prone BXSB than in normal C57BL/6 mice. The observed discrepancy between C57BL/6 and BXSB mice could be explained by the mechanism that anti-dsDNA antibody induced the secretion of IL-1 $\beta$  and TNF- $\alpha$ [21,22]. Although pp65 immunization was able to induce ANA and anti-dsDNA antibody production even in the normal C57BL/6 mice, the secretion levels of TNF- $\alpha$  and IL-1 $\beta$  in C57BL/6 mice stimulated by pp65 were maintained. On the contrary, after immunization with pp65-DNA vaccine, the secretion levels of TNF- $\alpha$  and IL-1 $\beta$  in BXSB mice increased dramatically. In particular, IL-1 $\beta$  and TNF- $\alpha$  were not only verified as negative regulators of microR-146a, but were also suppressed by microR-146a expression[23,24]. microR-125a was also found to decrease the secretion of TNF- $\alpha$ [25]. From these results in C57BL/6 mice, it is speculated that the immunized effects of pp65 in SLE-prone mice go stronger and faster than in healthy mice, and immunization with pp65 may cause responses earlier at the microRNA level rather than at the cytokine level. Accordingly, the slight down-regulations of these microRNAs in C57BL/6 mice are not enough to influence the secretion of cytokines. Another explanation may be the repression of TNF- $\alpha$  and IL-1 $\beta$  secretion by pp65 vaccine in normal individuals. Taken together, we suggest that pp65 may have negatively regulated microR-125a and microR-146a expression, and subsequently increased the TNF- $\alpha$  and IL-1 $\beta$  secretion in BXSB mice. Although the relationship of HCMV-pp65 and SLE-related autoantibodies, cytokines, as well as microRNAs have been investigated in the present research, the precise mechanism still needs to be determined. Therefore, it is of great need for further exploration in this area by analyses of cellular immunity and pathology.

In conclusion, the present study confirms that pp65 contributes to the onset and aggravation of SLE in C57BL/6 and BXSB mice, respectively. It is of interest that pp65 also acts as a suppressor of SLE-related microR-125a, microR-146a, and microR-126 in both strains of mice. The autoimmune effect caused by pp65 in BXSB mice was more potent than that in C57BL/6 mice. Thus, inhibition of active pp65 effects may be helpful in the treatment of SLE

patients, and the microRNAs can potentially be applied as novel targets for SLE treatment.

### Conflict of interest statement

We declare that we have no conflict of interest.

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### Comments

#### Background

The lower matrix phosphoprotein 65 (pp65), a dominant tegument protein of the HCMV virion, plays a significant role in immune evasion, and thereby controlling the innate and adaptive immune responses via HCMV-specific cytotoxic T lymphocytes. Thus, the effect of pp65 is mostly attributed to preventing infected cells from being destroyed. Several studies have shown that pp65 may be associated with various autoimmune responses.

#### Research frontiers

This is an interesting proteomics study in tropical biomedicine. The work is a good information on cytomegalovirus. The standard animal model experiment is reported.

#### Related reports

There are some related reports but not on the same topic. The present work has high originality. Chang *et al.*, has reported that pp65 increased the levels of ANA and anti-dsDNA antibody in another strain of SLE-prone mice (NZB/WFI), and the titers increased persistently.

#### Innovations and breakthroughs

The work shows new data from protein study. The work has a good new data in virology, infectious medicine and bioinformatics.

#### Applications

The work can be further applied in future protein research of cytomegalovirus infection. The work can further applied in human study and can be further referenced in tropical biomedicine.

#### Peer review

The work is an interesting experimental study on proteomics of cytomegalovirus infection. The standard animal experimental study and proteomics research are done. The work produces good new data in virology, infectious medicine and bioinformatics. The work can be further referenced in tropical biomedicine.

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