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Enhancement of autophagy as a strategy for development of new DNA vaccine candidates against Japanese encephalitis

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ABSTRACT

For decades, an on-going concerted effort has been made to develop a universal DNA vaccine to combat the looming threat of a potential outbreak of the emerging Japanese encephalitis virus (JEV) infection. However, effective strategies are urgently required to counter poor immunogenicity and insufficient long-term protection. Recent reports have confirmed the critical role of autophagy in antigen presentation, long-term immune memory and immune responses against JEV. In this study, JEV prM and E protein with strong immunogenicity were fused with microtubule-associated protein 1 light chain 3 (LC3) encoding gene to construct an autophagy-mediated pJME-LC3 DNA vaccine. Researches indicated significant increase of autophagosomes or LC3 Ⅱ expression in pJME-LC3 transfected cells. Furthermore, prME-LC3 fused protein was observed co-localized with GFP-LC3 to autophagosomes, which means it was successfully targeted to autophagosomes. After immunizing with pJME-LC3, mice were detected highest proportion of $CD3^+CD8^+$ T lymphocytes, $CD8^+$ effector memory T cells (TEMs) and JEV specific cytotoxic T lymphocyte (CTL) activity to eliminate JEV. pJME-LC3 also enhanced IgG2a antibody in serum and cytokines IFN-γ, IL-12 produced by splenocytes, thus skew toward Th1 type immune response by activating the JAK2/STAT1 signaling pathway and upregulating expression of transcription factor T-bet. Notably, mice immunized with pJME-LC3 showed highest survival rate and long-lasting neutralizing antibody when challenged with virulent JEV, which were consistent with augment in percentage of $CD4^+$ central memory T cells (TCMs). In brief, our studies suggested that autophagy can be used as a optimization strategy to enhance JEV specific immune response and long-term immune memory. Our attempt will contribute towards future efforts to develop an efficacious JEV vaccine.

1. Introduction

Approximately 30–50% of patients with Japanese encephalitis (JE), a zoonosis caused by JEV, suffer from long-lasting neurological sequelae, with a case fatality rate of 20–30% [1,2]. The disease is endemic predominantly in southeast and east Asia, where an estimated 67,900 cases occur annually [1]. In recent years, the annual incidence of JE increased up to 50,000–175,000 cases, depending on the age group, geographical area, and immunization status [3]. According to the Chinese Centers for Disease Control and Prevention (CDC), there were 1934 new cases of JE in 2018, including 138 deaths, which was significantly higher than that reported (1147 new cases, 79 deaths) in 2017 [4]. The ineffectiveness of insect-vector control protocols and lack of specific treatments make effective vaccines the key to defense against JEV. Although cell-derived inactivated JE vaccine (JEV-I) and live attenuated JE vaccine (JEV-L) have made great contributions to active immunization over the past half-century, poor immunogenicity or anaphylaxis are defects that cannot be ignored [5,6]. JE used to occur primarily among children under the age of 15. However, incidence in older children and adults in recent years had increased with the decrease in protective immunity, which indicating a lack of long-term protection from existing vaccines [7]. DNA vaccine against JEV has been extensively studied in the past few decades because of safety and other advantages, but the immune efficacy and long-term immune protection to the host require further improvement [8]. In summary, there is an urgent need to explore a novel strategy to optimize the JE DNA vaccine.

Autophagy is a conserved lysosomal degradation pathway essential for cellular homeostasis and adaptation to stress. Not only that, it also plays an important role in the process of virus infection and immunity [9,10]. English et al. [11] believed that autophagy promote endogenous viral protein presentation to

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MHC class I molecules and initiate CTL activity to eliminate the virus during herpes simplex virus-1 (HSV-1) infection. Munz et al. [12] confirmed that autophagy increase MHC II molecules loaded with LC3-influenza matrix proteins to deliver antigens by 20 times, thus enhance antigen recognition by virus-specific $CD4^{+}$T cells. Furthermore, cross-presentation is also an autophagy-dependent process [13]. In addition, T cell memory pool has previously been shown to be controlled by PI3K/Akt and AMPK signaling as well as mTOR (mammalian target of rapamycin) inhibition, all of which also control autophagy [14]. Autophagy is also crucial for survival of T memory cells (TMs) and production of long-term antibody in the host [15,16]. Therefore, we believe that introduction of JE DNA vaccine into the autophagy pathway may contribute to induce strong and long-term immune protection by participating in antigen presentation process.

In this research, for the first time, pJME-LC3 DNA vaccine was constructed using autophagy as optimization strategy and identified *in vitro*. We further analyzed immune efficacy and long-term immune protection of this autophagy-mediated DNA vaccine in mice.

2. Methods

2.1. Cell lines, animals and other important materials

Chinese hamster ovary cell subline (CHO-K1), mouse mastocytoma cell strain (P815) and baby hamster kidney cell strain (BHK) were obtained from Shanghai Institutes for Biological Sciences (Shanghai, China) and cultured in F12K medium (Sigma, USA), supplemented with 10% fetal bovine serum (FBS, Gibco, USA). AAV-LC3-GFP was purchased from Hanbio (Shanghai, China). JEV-L (derived from JEV SA 14-14-2 strain, Batch number: 201608054, Wuhan Institute of Biological Products, China) was presented by Shenyang Disease Control and Prevention Center. JEV-YN2016-1 strain was presented by Changchun Institute of Military Veterinary Medicine. Female BALB/c mice (4–6 weeks old) were purchased from Beijing Laboratory Animal Research Center, raised and carried out in accordance with the National Institutes of Health Guidelines on Animal Care. All experimental procedures were approved by the Ethics Committee of Shengjing Hospital of China Medical University (Permit number: 2015PS105K).

2.2. Construction of pJME-LC3 plasmid

JEV prM and E protein with strong immunogenicity had been used to develop recombinant plasmid, termed pJME and stored in our Laboratory [17]. Mouse LC3B coding gene (Sequence ID: NM_026160.5) were searched from GenBank to synthesis single stranded small fragments DNA and amplified with specific primers (sense-5′-tggttgtgcctttattagtgcatc-3′, anti-sense-5′-cactgctctgtcttgt gtaggttg-3′) by PCR. Amplified fragments of DNA were purified with DNA fragment purification kit (TaKaRa, Japan). The agarose gel DNA extraction kit (TaKaRa, Japan) was used to extract target fragments (390 bp). pJME plasmid was digested by restriction endonuclease EcoR I and amplified by PCR as vector DNA. To construct pJME-LC3 fusion plasmid, LC3B coding gene was then subcloned into pJME vector DNA using In-Fusion® HD Cloning Kit (TaKaRa, Japan). pJME-LC3 plasmid was identified by sequencing with primer BGHrev: 5′-tagaaggcacagtcgagg-3′. All primers were synthesized by TaKaRa.

2.3. Cell transfection

CHO-K1 cells were plated in 6-well plates and transfected transiently with pJME-LC3 plasmid for different time-points (12, 24, 36, 48, and 72 h) or gradient concentrations (1, 2, 4 and 8 μg/mL) using Lipofectamine™ 3000 reagent (Invitrogen, USA) to observe the expression of prME-LC3 *in vitro*. Autophagy activation in CHO-K1 cells was evaluated by transfecting with pJME, pJME-LC3 or pcDNA3.1(+). Cells incubated in complete medium containing 50 nM rapamycin (Sigma, USA) for 4 h were used as positive controls and non-transfected cells were used as blank controls. Cells transfected stably with pJME-LC3 were screened by G418.

2.4. SDS-PAGE and western blotting

Total proteins were isolated from CHO-K1 cells using RIPA lysis buffer (Beyotime Biotechnology, China) according to the instructions. Equivalent protein (50 μg) were resolved by SDS-PAGE and transferred onto PVDF membranes. Membranes were then blocked with 5% BSA followed by incubating with rabbit anti-mouse Flag, LC3, p62, pJAK2, pSTAT1, T-bet or β-actin antibody (all from CST, USA) overnight at 4 . Primary antibody-bound membranes were incubated with goat anti-rabbit IgG-HRP antibody (Abcam, USA) for 2 h at room temperature. The target protein was visualized with enhanced chemiluminescence system (ECL, Thermo, USA), followed by analysis using Amersham Imager 600 (GE, USA). β-actin was used as internal control. All evaluations were performed at least triplicate for independent experimental condition.

2.5. Transmission electron microscope (TEM)

After being transfected with pJME/pJME-LC3 for 24 h, CHO-K1 cells were collected and fixed in a solution containing 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at 4 °C for 2 h, then fixed by 1% osmic acid at 4 °C for 90 min. After washing with ddH_2O, cells precipitation were dehydrated with gradient ethanol and acetone, then soaked in a mixture of 100% acetone and Epon-Araldite resin embedding agent (1:1) overnight at room temperature. Ultrathin sections (50–70 nm) were cut on a Reichert ultramicrotome after polymerizing and solidifying in an embedded mold. Slices on copper wire were double stained with 3% uranium acetate and 0.3% lead citrate to observe on HT7800 electron microscope (Hitachi, Japan).

2.6. Confocal laser scanning microscopy (CLSM)

To investigate whether prME-LC3 can be targeted to autophagosomes, CHO-K1 cells were first transfected with AAV-LC3-GFP for 24 h to label endogenous LC3, and then transfected with pJME-LC3 or pJME for 24 h. After being fixed with 4% paraformaldehyde for 30 min, cells were washed with PBS and blocked in 1 × PBS/5% goat serum/0.3% Triton X-100 for 1 h. Then anti-Flag antibody diluted with 1 × PBS/1%BSA/0.3%Triton X-100 were added to stain overnight at 4 °C. After three washes, ALexa Fluor 594 conjugated secondary antibodies were added and incubation at room temperature for 2 h. At the time indicated, the cell nuclei were mounted with DAPI at 37 °C for 20 min. Fluorescence measurements were performed using an FV1200 confocal microscope (Olympus, Japan).

2.7. Murine model immunization and challenge with JEV

Female BALB/c mice aged 4–6 weeks were randomly divided into six groups: For immunoassay: groups of mice (n = 15/group) were injected intramuscularly(i.m.) in the quadriceps with 100 μg of plasmid DNA encoding prME or prME-LC3 in 100 μL phosphate-buffered saline (PBS). In addition, mice in pJME-LC3 + 3-MA group received intraperitoneal injection of 3-methyladenine (3-MA) (60 μg/d) over immunization period [18]. For the positive control group, each mouse of the JEV-L vaccinated

group was given an intraperitoneal injection of 100 μL. The negative control mice were immunized i.m. with either 100 μg of pcDNA3.1(+) control vector or 100 μL of PBS. The same procedure of immunization employing the same dose of immunogen was used for immunization three times at two week intervals. In order to significantly validate the protective efficacy of pJME-LC3, we modified the inoculation doses in viral challenge assay on the basis of previous experiments. For viral challenge assay: Groups of 20 female BALB/c mice were immunized i.m. with 200 μg of each DNA construct of interest in 100 μL PBS. JEV-L was still given an injection of 100 μL as above. For immunoassay: 1, 3, 5, 6, 7 weeks after the initial immunization, blood of posterior orbital vein in mice was collected and serum was isolated for detection of anti-JEV specific IgG antibody. Three weeks after the final immunization, 15 mice/group were decapitated, spleens were dissected out for further immunological analysis. For viral challenge assay: mice received intraperitoneal injection with 10^5 PFU/100 μL of JEV-YN2016-1 strain three weeks after the final immunization. Viral challenge assay was performed triplicate for independent experimental condition.

2.8. Flow cytometry

Three weeks after the final immunization, mice were sacrificed and isolated splenic lymphocytes using Ficoll-Paque™ PLUS Media (GE, USA). After washing with PBS, cell suspension (1×10^5 cells) was incubated with APC-CD3e, FITC/PerCP-CD4, BB700-CD8a, BB515-CD44, or PE-CD62L antibodies (all from BD, USA), followed by detecting with flow cytometry and analyzing with FlowJo software.

2.9. Lactate dehydrogenase (LDH) release assay

Splenic lymphocytes of mice were collected as previous mentioned and stimulated in duplicate with JEV (1×10^5 PFU) and 20U/mL recombinant murine IL-2 (rmIL-2, Peprotech, USA) as effector cells. P815 cells were co-incubated with JEV (10 PFU/cell) for 17 h as target cells. The ratio of effector and target cells were adjusted at 10:1. Moreover, triplicate of effector cell spontaneous release, target cell spontaneous release, target cell maximum release and medium correction controls were all set. LDH released into supernatant was measured using Cyto Tox 96 assay kit (Promega, USA) according to the instructions. The percentage of specific lysis was calculated as follows: (experimental LDH release – effector cells spontaneous LDH release)/(target cells maximum LDH release – target cells spontaneous LDH release) × 100.

2.10. Antibodies and cytokine response to DNA vaccine

Three weeks after the final immunization, anti-JEV IgG1 and IgG2a antibody titers in the serum from immunized mice were detected by ELISA. Briefly, JEV (10^3 PFU/100 μL) were incubated in 96-well plates overnight at 4 °C followed by blocked with 5% BSA. Individual serum were added in triplicate and HRP conjugated rabbit anti-mouse IgG1 or IgG2a (Abcam, USA) was incubated. Tetramethylbenzidine substrate was added for a color reaction, followed by stopped with 1 M H_2SO_4. The optical density (OD) was measured at 450 nm. The relative antibody titer in serum was determined by calculating the ratio among samples. Culture supernatant of splenic lymphocytes stimulated by JEV for 72 h were harvested, and the presence of cytokines, including IFN-γ, IL-12, IL-4, and IL-10 were detected using commercial mouse cytokine immunoassay ELISA kits (R&D, USA), according to the manufacturer's instructions.

2.11. 80% plaque reduction neutralization test ($PRNT_{80}$)

Serum was first incubated in hot water (56 °C) to inactivate complements followed by made a two-fold gradient dilution from 1:10 to 1:320. Subsequently, serum was mixed with isometric JEV (10^3 PFU/mL). 24-wells plates covered with monolayer BHK cells were added with serum-virus mixture and incubated in DMEM culture medium, which contains 1% carboxymethylcellulose sodium and 1% FBS. Finally, the number of plaques were counted following by 0.1% crystal violet staining, and the maximum serum dilution that resulted in 80% reduction of plaque was considered the neutralization antibody titer of serum.

2.12. Statistical analysis

The plotted data are represented as mean ± SD. All statistical analyses in the present study were concluded using SPSS software (version 19.0).

Statistical analyses of the experimental data and controls were performed by Graph Pad Prism 7 and one-way factorial analysis of variance. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Design and in vitro expression of pJME-LC3 DNA vaccine

The pJME synthetic DNA vaccine was constructed previously based on JEV-Beijing-1 strain and had been showed protective efficacy in mice [17]. In this study, we designed an autophagy-mediated pJME-LC3 DNA vaccine by fusing pJME with LC3 coding gene. Then gradient concentrations of pJME-LC3 were transfected into CHO-K1 cells for different time. Results showed that prME-LC3 fusion protein expressed significantly at 12 h post-transfection and appeared dose-dependent in the range of 1–4 μg/mL (Fig. 1A and B). In addition, it also expressed stably in CHO-K1 cells up to 48 generations after G418 selection (Fig. 1C).

3.2. pJME-LC3 induced an increase in autophagy activity

According to recently studies, intracellular JEV can be eliminated by induction of autophagy [18,19]. We observed that autophagosomes increased significantly in CHO-K1 cells transfected with pJME-LC3 compared to pJME group (Fig. 2A). Moreover, expression of LC3 Ⅱ and p62 was dose-dependent in pJME-LC3 treated cells (Fig. 1B), which had significant difference with other groups as shown by internal control β-actin (Fig. 2B).

3.3. prME-LC3 fusion protein co-localize with autophagosomes

As seen in Fig. 2C, autophagosomes, marked as green puncta by AAV-LC3-GFP, were localized in cytoplasm and membrane, and overlapped with prME-LC3 fusion protein, resulting in yellow puncta in pJME-LC3 transfected cells. However, the green puncta were diffused and did not overlap with autophagosomes in pJME transfected cells.

3.4. Analysis of splenic T lymphocytes

The enhancing effects of LC3 encoding gene on the generation of $CD4^+$T cells, $CD8^+$T cells and TMs were analyzed by flow cytometry three weeks after the final immunization. We found that there was no statistically significant difference in the percentage of $CD4^+$T cells among pJME, JEV-L, pJME + LC3 and pJME + LC3 + 3-MA vaccinated groups. Meanwhile, we also observed a significant enhancement of percentage of $CD8^+$T cells (30.23 ± 1.43)% in pJME-LC3

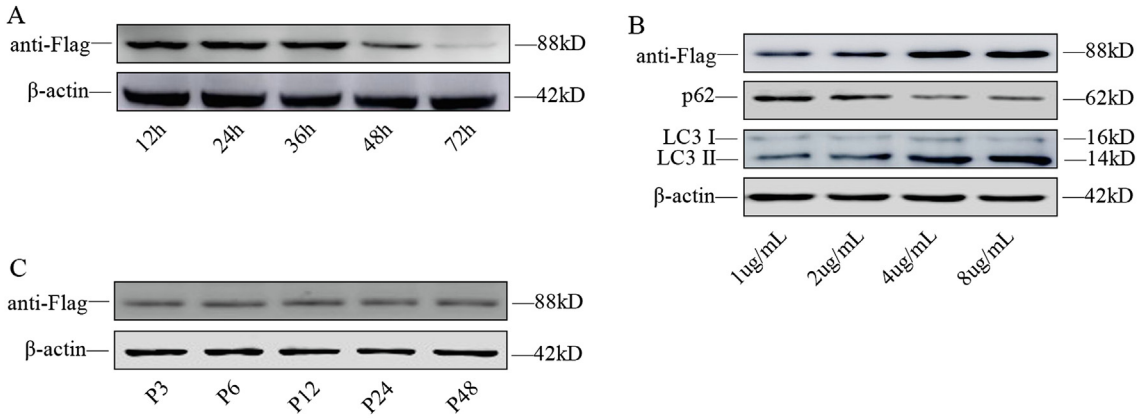

Fig. 1. *In vitro* expression of fusion protein prME-LC3, which was encoding protein of pJME-LC3. (A) pJME-LC3 was transiently transfected into CHO-K1 cells for different time to confirm its rapid expression *in vitro*. prME-LC3 expressed rapidly at 12 h post-transfection and showed time-dependent (12–36 h).(B) prME-LC3 had a dose-dependent expression in gradient concentration of pJME-LC3 plasmid (1–4 μg/mL). Expression of autophagy marker LC3 Ⅱ increased gradually, while p62, a crucial autophagy substrate decreased gradually. (C) CHO-K1 cells which expressed prME-LC3 stably were screened by G418 and showed no attenuation in different generation. All indicators were performed in at least triplicate for each experimental condition. β-actin was used as internal control.

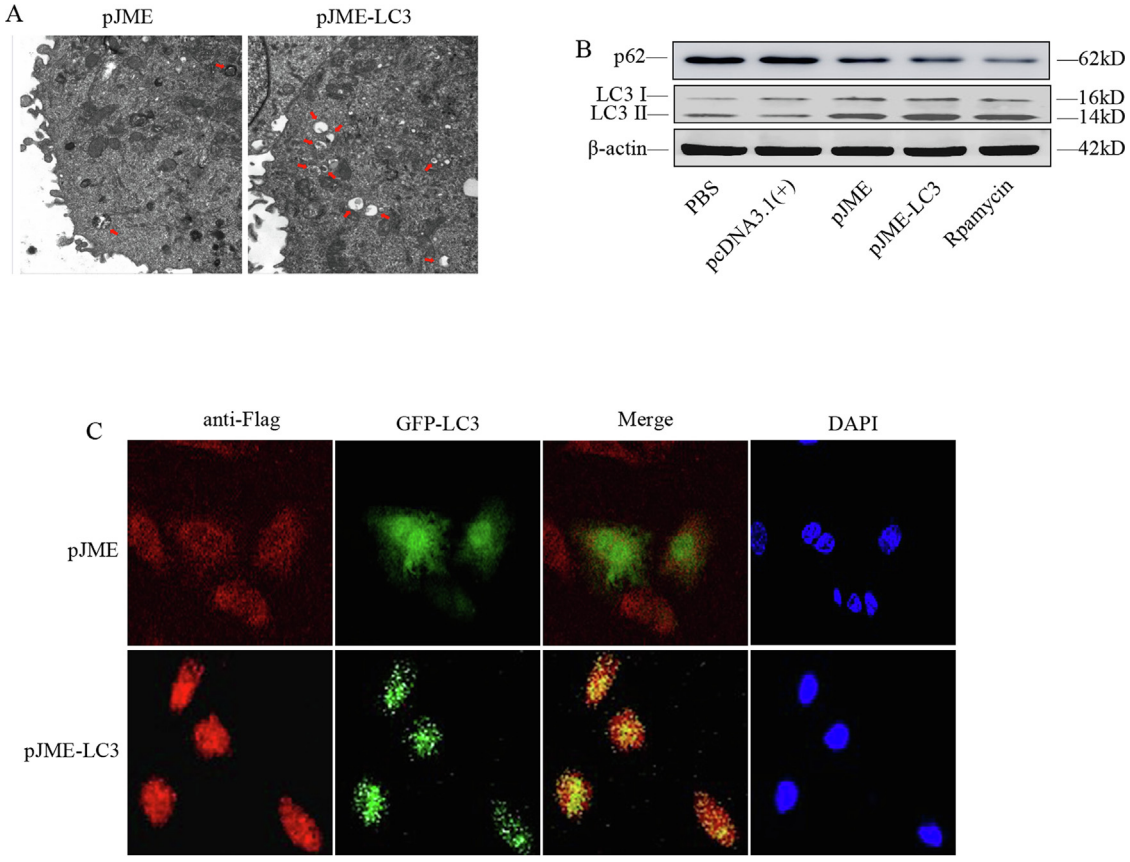

Fig. 2. pJME-LC3 enhance autophagy activity and target the encoding protein prME-LC3 to autophagosomes. (A) More autophagosomes were found in pJME-LC3 treated CHO-K1 cells rather than pJME by transmission electron microscopy. (Red arrows, 20,000×). (B) Like rapamycin, pJME-LC3 significantly enhanced the expression of LC3 Ⅱ in CHO-K1 cells comparing with other groups, while p62 decreased. β-actin was used as internal control. (C) Endogenous LC3 labeled by green puncta in pJME-LC3 transfected group aggregated in cytoplasm and membrane, and co-located with pJME-LC3 so as to present yellow fluorescence. Green puncta diffused and did not overlap with autophagosomes in pJME transfected cells. All indicators were performed in at least triplicate for each experimental condition.

immunized group comparing with other groups immunized with pJME (15.89 ± 1.45)%, JEV-L (15.07 ± 0.96)% or pJME-LC3 + 3-MA (13.42 ± 1.58)% (Fig. 3A and B) ($P < 0.05$). As for TMs, there was no significant difference in the levels of $CD4^+$ TEMs among pJME, JEV-L, pJME + LC3 and pJME + LC3 + 3-MA immunized groups. However, the percentage of $CD4^+$TCMs in pJME-LC3 group (66.34 ± 0.79)% was the highest comparing with pJME (57.68 ± 2.64)%, pJME-LC3 + 3-MA(56.86 ± 2.02)% and JEV-L (48.87 ± 1.08)% ($P < 0.05$) (Fig. 3C and D). Interestingly, our results showed that there was no significant difference in the levels of

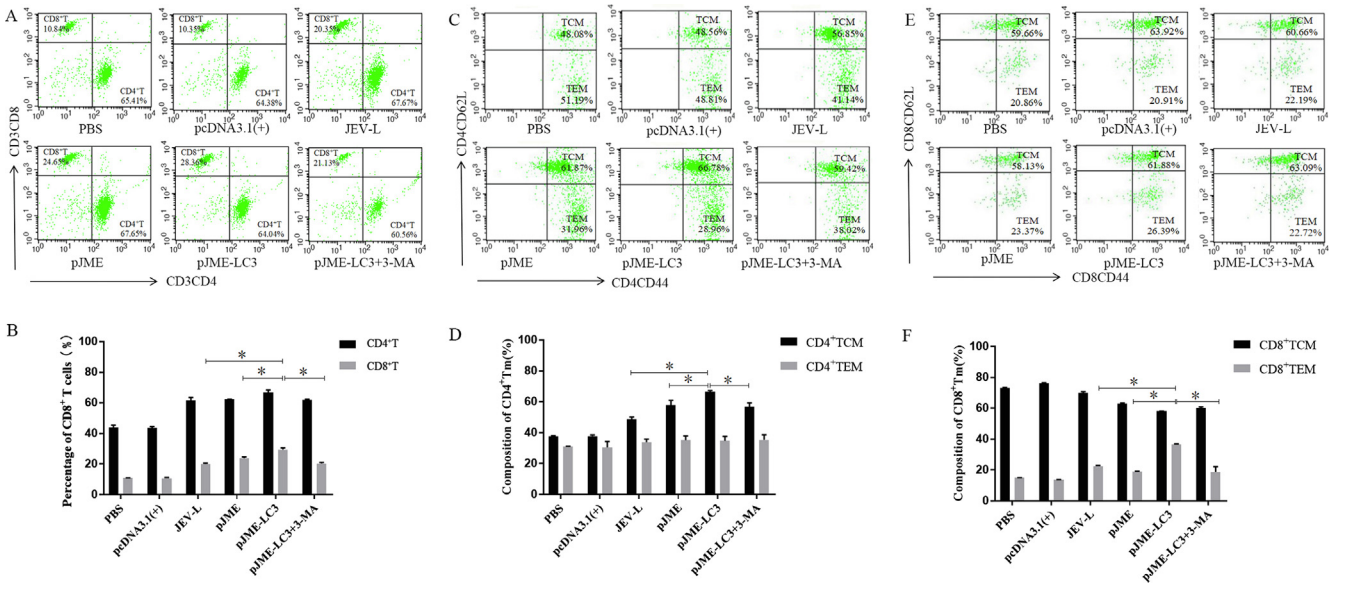

Fig. 3. The percentage of splenic $CD3^+CD4^+/CD3^+CD8^+$ T lymphocytes or Memory T Cells (TMs) of immunized mice (n = 15 mice per group). $CD3^+CD4^+/CD3^+CD8^+$ T lymphocytes or TMs in different vaccinated groups were detected three weeks after the final immunization. (A) Flow cytometry analysis on percentage of $CD4^+/CD8^+$ T cells. Representative dot plots and similar results were obtained in 4 independent experiments. The percentage of $CD4^+$ or $CD8^+$ T cells is indicated in the lower right quadrant or upper left quadrant of each dot plot. (B) The percentage of $CD4^+/CD8^+$ T lymphocytes of immunized mice. Each bar represents the mean ± SD, and each sample was analyzed in triplicate (*$P < 0.05$). (C) The percentage of $CD4^+$ central memory T cells (TCMs) ($CD4^+CD44^{high}CD62L^{high}$) or $CD4^+$ effector memory T cells (TEMs) ($CD4^+CD44^{high}CD62L^{low}$) cells is indicated in the upper right quadrant or lower right quadrant of each dot plot by flow cytometry. (D) Percentage of $CD4^+$ TCMs/TEMs of immunized mice. Each bar represents the mean ± SD and each sample was analyzed in triplicate (*$P < 0.05$). (E) Flow cytometry assay investigating the percentage of $CD8^+$ TCMs ($CD8^+CD44^{high}$-$CD62L^{high}$) or $CD8^+$ TEMs ($CD44^{high}CD62L^{low}$) in spleen. The percentage of $CD8^+$ TCMs or $CD8^+$ TEMs cells is indicated in the upper right quadrant or lower right quadrant of each dot plot. (F) Percentage of $CD8^+$ TCMs/TEMs of immunized mice. Each bar represents the mean ± SD and each sample was analyzed in triplicate (*$P < 0.05$).

$CD8^+$TCMs among all groups, whereas the percentage of $CD8^+$TEMs in pJME-LC3 group was (36.6 ± 0.31)%, much higher than JEV-L (22.43 ± 0.43)%, pJME (18.87 ± 0.29)%, and pJME-LC3 + 3-MA (18.72 ± 2.83)% ($P < 0.05$) (Fig. 3E and F).

3.5. CTL activity

Virus-specific CTLs have been shown to be crucial in determining viral load and rate of disease progression [20]. As shown in Fig. 4A, CTL activity of splenic lymphocyte in pJME-LC3 immunized mice was (55.72 ± 2.34)%, much higher than JEV-L (39.43 ± 2.85)%, pJME (37.88 ± 2.47)% and pJME-LC3 + 3-MA (28.96 ± 3.21)% ($P < 0.05$).

3.6. JEV-specific antibody and cytokine response to vaccines

Antibodies of IgG1 and IgG2a respectively indicate Th2- and Th1-type immune responses in mice [21]. When titers of JEV specific IgG isotypes were investigated, mice immunized with pJME-LC3 showed highest IgG2a titer whereas there were no obvious differences in IgG1 titer (Fig. 4B and C), which suggested that immunized with pJME-LC3 induce the immune response skewed toward Th1-type dominance. Furthermore, functional phenotype of the antigen-specific T cell response in mice was confirmed after stimulating splenocytes with JEV. As shown in Fig. 4D–G, cytokines IL-12 and IFN-γ (Th1-like) responded to antigen stimulation in pJME + LC3 vaccinated group were the highest comparing with pJME ($P < 0.05$), JEV-L ($P < 0.01$), pJME + LC3 + 3-MA groups ($P < 0.05$), and levels of IL-12 and IFN-γ in pJME vaccined group was significantly higher than JEV-L group ($P < 0.05$), whereas no significant difference was found in IL-4 and IL-10 (Th2-like) levels. Production of cytokines in different group indicated that pJME-LC3 induce Th1 immune response, which was consistent with previous results. Further experimentation showed that the expression of phosphorylated JAK2/STAT1 protein (pJAK2/pSTAT1) and downstream transcription factor T-bet in pJME-LC3 group increased significantly comparing with pJME, JEV-L and pJME + LC3 + 3-MA vaccinated groups (Fig. 4H).

3.7. pJME-LC3 DNA vaccine induced protective immunity against lethal JEV challenge in mice

BALB/c mice (n = 20/group) received 3 injections of different vaccines were challenged three weeks later with JEV-YN2016-1 strain (10^5 PFU/100 μL). We evaluated protective effects of these vaccines on immunized mice, and found that the mean survival rate in pJME-LC3 group (91.67 ± 2.36)% was statistically different from the JEV-L group (81.67 ± 2.36)%, pJME group (61.67 ± 2.36)% and pJME-LC3 + 3-MA group (56.67 ± 2.36)% as shown in Table 1. Moreover, serum collected at different time before and after challenge from immunized BALB/c mice were used for $PRNT_{80}$. Results indicated that neutralizing antibody titer in pJME-LC3 immunized mice increased at day 2 before challenge, and reached 1:160 at day 14 after challenge, which was higher than that in pJME or pJME-LC3 immunized group. The neutralizing antibody titer of all other groups decreased at day 21 after the challenge while that of pJME-LC3 group showed no attenuation (Table 2).

4. Discussion

Although many progress had been made, the goal of developing a novel anti-JEV vaccine that can induce strong immune protection and long-term immune memory is not yet to be achieved. In this study, we aimed to design a novel recombinant DNA vaccine for JE that utilize the autophagy pathways. We selected prM and E protein coding genes, which have strong antigenicity in the JEV Beijing-1 strain to construct pJME plasmid, as reported previously [17], and fused it with coding gene of autophagy marker LC3.

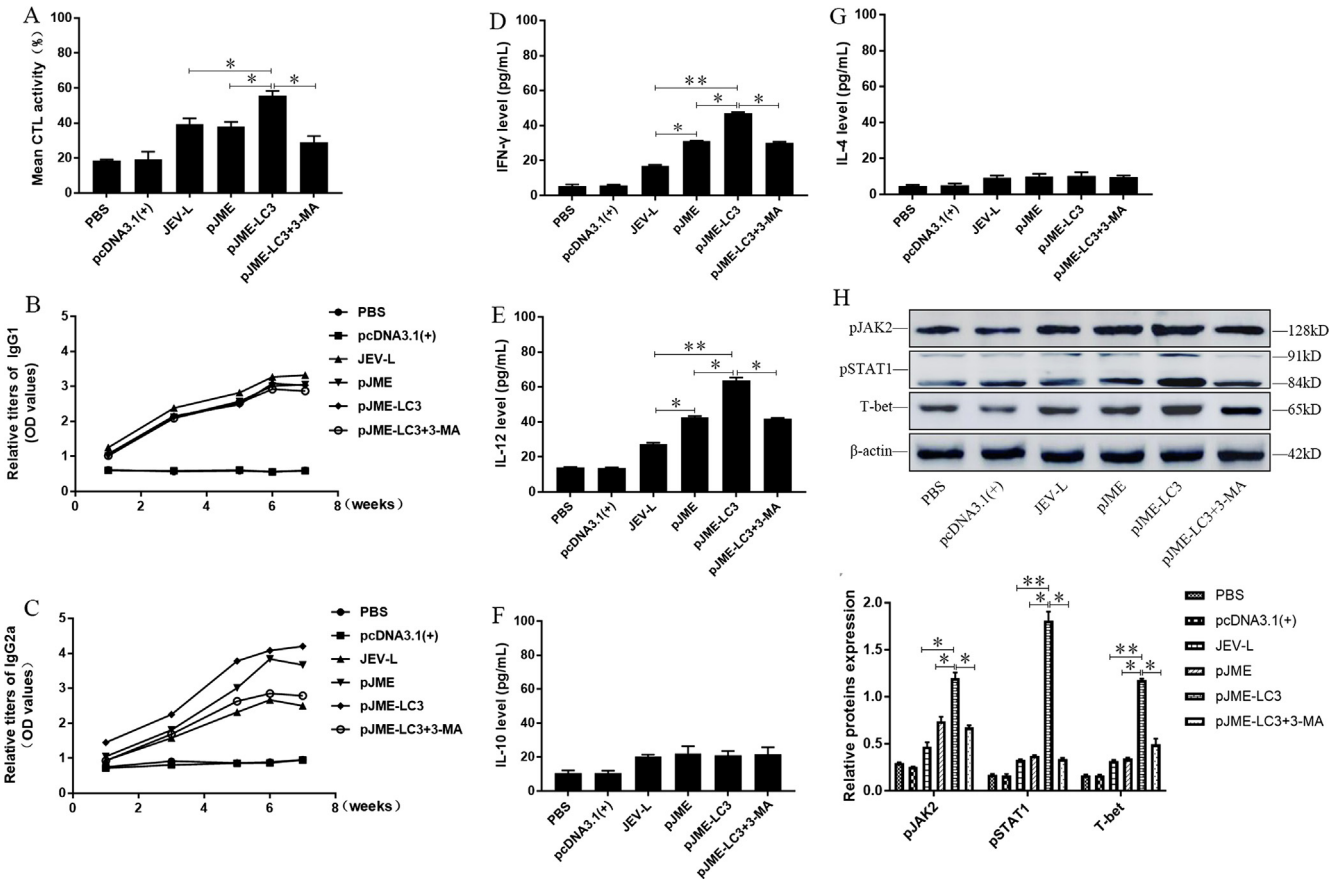

Fig. 4. CTL activity, antibody titer and cytokine production. Mice were randomly assigned into six groups for different immunization regiments: PBS, pcDNA3.1(+), JEV-L, pJME, pJME-LC3 and pJME-LC3 + 3-MA. (A) CTL activity was detected three weeks after the final immunization by LDH release assay as the ratio of effector and target cells was set at 10:1. (B and C) The relative level of anti-JEV IgG1 and IgG2a in sera was determined by ELISA at 1, 3, 5, 6, 7 weeks after the initial immunization. (D–G) For detecting cytokine responses to vaccination, bulk splenocytes from vaccinated mice were stimulated *in vitro* with JEV for 72 h and the supernatants were harvested for cytokine determination using ELISA. (H) The proteins of signaling pathway JAK2/STAT1 and transcription factors T-bet levels were detected using western blotting(upper)and semi-quantitative analysis by comparing with β-actin(lower). All indicators were performed at least triplicate for independent experimental condition ($^{*}P < 0.05$, $^{**}P < 0.01$).

Table 1
Survival percentage of immunized mice after viral challenge.

Different time after viral challenge[a]	Percent survival (%)					
	PBS	pcDNA3.1(+)	JEV-L	pJME	pJME-LC3	pJME-LC3 + 3-MA
Day7	55 ± 4.08	53.33 ± 2.36	88.33 ± 2.36	73.33 ± 2.36	93.33 ± 2.36	71.67 ± 2.36
Day14	20 ± 4.08	18.33 ± 2.36	83.33 ± 2.36	68.33 ± 2.36	91.67 ± 2.36	61.67 ± 2.36
Day21	1.67 ± 2.36	3.33 ± 2.36	81.67 ± 2.36○◇	61.67 ± 2.36○	91.67 ± 2.36○◇*	56.67 ± 2.36○

Groups of twenty 4-week-old BALB/c mice were immunized with indicated immunogens at two week intervals. 100 μL of PBS containing 200 μg of DNA vaccine plasmids was inoculated directly into the quadriceps of mice. For positive control group, each mouse of JEV-L vaccinated group was still given an intraperitoneal injection of 100 μL. Mice were challenged 3 weeks after the final immunization and monitored for 21 days for survival. The data shown are representative of three independent experiments. All values are shown as means ± SD.

[a] The JEV-YN2016-1 strain (10^5 PFU/100 μL i.p) was used for viral challenge assay.

○ Compared with PBS and pcDNA3.1(+) groups, $P < 0.001$.

◇ Compared with pJME and pJME-LC3 + 3-MA groups, $P < 0.001$.

* Compared with JEV-L group, $P < 0.05$.

Analysis by western blotting indicated that prME-LC3 fusion protein expressed rapidly, stably and showed time/dose-dependent relationship with plasmid concentration, which implied that pJME-LC3 DNA vaccine may provide swift and continuous antigen stimulation for the host, thus induce long-term immune protection. One of important aspects of constructing autophagy-mediated DNA vaccines is to utilize their ability to activate autophagy. In this study, we observed that the formation of autophagosomes was enhanced. Furthermore, western blotting results also showed a significant increase in membrane-bound LC3 II which transformed from cytoplasmic LC3 I. Previous studies have emphasized the key role of autophagy in enhancing antigen process and presentation [22]. Therefore, vaccines which mediated to autophagy pathways may provide better immune protection. Through CLSM, we confirmed that pJME-LC3 successfully induce autophagy activity and target prME-LC3 into autophagosomes, thus facilitated the further processing and presentation of antigens.

The ideal immune response of pJME-LC3 depends on protective immunity that imparts to human or animal models. As an important effector cell in anti-infection immunity, $CD8^{+}$T cells can be activated and proliferated after receiving antigen stimulation followed by differentiate into natural killer cells (NK) and CTLs for

Table 2
Neutralizing antibody titer at different time before or after challenge.

Immunogen	Neutralizing antibody titer[a]			
	day-2	day 7	day 14	day 21
PBS	<1:10[b]	1:10	1:10	1:10
pcDNA3.1(+)	<1:10	<1:10	1:10	1:10
JEV-L	1:10	1:40	1:160	1:80
pJME	<1:10	1:20	1:80	1:40
pJME-LC3	1:20	1:80	1:160	1:160
pJME-LC3 + 3-MA	1:10	1:20	1:80	1:40

Serum specimens of mice at day 2 before or at day 7, 14 and 21 after challenge were separated for $PRNT_{80}$. Neutralizing antibody titer in pJME-LC3 group began to rise at day 2 before challenge, then continued to rise up to 1:160, and did not decline during the observation period. In comparison, neutralizing antibody titer in other groups were lower than those in pJME-LC3 group at all time, and all of them showed attenuation at day 21 after challenge.
[a] Mice were challenged 3 weeks after the final immunization with JEV-YN2016-1 (10^5 PFU/100 μL i.p.).
[b] Each titer described represents the highest serum dilution yielding a 80% reduction in plaque number.

identifying and eliminating pathogens [23]. Interestingly, we found that proportion of $CD3^+CD8^+$T cells in pJME-LC3 immunized mice was highest compared with other groups, which highly consistent with CTLs cytotoxic activity. Results suggested that autophagy-mediated DNA vaccine pJME-LC3 enhance the recognition and elimination of JEV.

Intracellular bacterial, fungal, and viral infections induce the differentiation of naive $CD4^+$T cells into Th1 or Th2 according to their cytokine expression patterns [24]. Th1 cells induce activation of macrophages, NKs, and $CD8^+$T cells, enhance the differentiation of CTLs and promote synthesis of IgG2a antibody and cytokines IL-12, IFN-γ, thus regulate cellular immune response. Th2 cells induce cytokines IL-4, IL-10 and IgG1 antibody, so as to activate eosinophils and mast cells for inducing inflammation and allergic reactions [25]. We found that levels of IgG2a antibody, cytokine IL-12, and IFN-γ (Th1-like) were highest in pJME-LC3 immunized mice whereas there were no significant differences in IgG1 antibody, IL-4, or IL-10 (Th2-like) levels among groups. We concluded that pJME-LC3 DNA vaccine induces Th1 type cell immune response mainly via promoting the differentiation of naive $CD4^+$ T toward Th1 rather than inhibiting its differentiation toward Th2. Therefore, only differentiation mechanism of naive $CD4^+$T to Th1 was investigated. IFN-γ induces the activation of STAT1 and transcription factors T-bet, which in turn generate a positive feedback loop of Th1 response [26,27]. Western blotting analysis of splenic lymphocytes in immunized mice showed that pJAK2 and pSTAT1 were significantly increased in pJME-LC3 group along with T-bet. Results all above demonstrated that pJME-LC3 induced a cellular immune response skewed towards Th1 type dominance by activating IFN-γ-JAK2/STAT1 signaling pathway and up-regulating the expression of its downstream transcription factor T-bet.

In addition to provide strong immune response, an ideal vaccine should also induce long-term immune memory. TCMs that stably proliferate and differentiate into TEMs after antigen stimulation are dominantly differentiated from $CD4^+$T cells and participate in regulating long-term reactive memory to ensure high sensitivity counter antigen stimulation. TEMs mainly differentiate from $CD8^+$T cells to regulate short-term effector immune memory [28]. Anthony et al. [29] confirmed that antiviral $CD8^+$TEMs, which act in a manner similar to CTLs, were capable of rapidly mobilizing potent antigen-specific cytotoxic activity *in vivo* to eliminate virus. Our research showed that proportion of $CD8^+$TEMs in pJME-LC3 immunized mice enhanced significantly, which supports previous CTL activity assay. Moreover, proportion of $CD4^+$TCMs in pJME-LC3 immunized mice was highest, which indicated that this autophagy-mediated vaccine may induce long-term immune memory.

It had been confirmed that expression of prME-LC3 in CHO-K1 cells increased gradually with the augment of pJME-LC3 (1–4 μg/mL) *in vitro*. Moreover, we also successfully confirmed that pJME-LC3 (100 μg/100 μL) has immune enhancement effect in previous *in vivo* experiments. Therefore, we predicted that the immunity efficacy of pJME-LC3 could be obviously enhanced by up-regulating its vaccination dose. Viral challenge assay is considered to be a gold standard to evaluate efficacy of vaccine. In order to significantly validate the protective efficacy of pJME-LC3, we modified the viral challenge assay on the basis of previous experiments. 100 μL of PBS containing 200 μg of DNA vaccine plasmids was inoculated directly into the quadriceps of mice. Meanwhile, each mouse in JEV-L group was still given an intraperitoneal injection of 100 μL as before. The survival rates indicated that pJME-LC3 can provide stronger protective immunity response against lethal JEV challenge compared to pJME, JEV-L or pJME-LC3 + 3-MA. It has been reported that CTLs response alone is not sufficient to protect mice from JEV challenge, neutralizing antibody following challenge also provides critical protective component [19]. Our studies demonstrated that neutralizing antibody titers in pJME-LC3 group increased swiftly rising up to 1:160 and showed no attenuation during the entire experimental duration. In conclusion, it has been confirmed that immune efficacy and long-term immune memory induced by the novel anti-JEV DNA vaccine are enhanced by targeting autophagy pathways.

Although our results demonstrate that pJME-LC3 is a novel and effective anti-JEV vaccine, it should be noted that these results were obtained in murine models intraperitoneally infected with virus; therefore, these data cannot be directly used to assess the protective effect of the vaccine against infection by other routes. The molecular mechanism by which pJME-LC3 DNA vaccine enhances JEV antigen presentation and induces a long-term immune response by autophagy activation still needs to be studied. While more research needs to be carried out on this novel autophagy-targeted anti-JEV DNA vaccine, the use of autophagy to optimize DNA vaccines provides a new approach for vaccine development.

5. Contribution

FFZ, YZZ and GHF conceived and designed research. FFZ, YZZ and PPX performed the experiments. JYZ contributed materials/-analysis tools. FFZ and YZZ analyzed the data and wrote the manuscript. All authors read and approved the final version of the manuscript.

Conflict of Interests

Fangfang Zhao and Yongzhen Zhai are co-first authors for equal attribution to the manuscript. All authors and contributors meet

authorship criteria as published by the ICMJE and declare no competing financial interests.

Declaration of Competing Interest

We declare that all authors have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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