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BRIEF REPORT

Immunoenhancement with flagellin as an adjuvant to whole-killed rabies vaccine in mice

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Abstract Vaccination is the most effective method for preventing rabies virus (RABV) infection in both humans and animals; however, no satisfactory vaccine has been developed for use worldwide. In the present study, we investigated the immunoadjuvant properties of *Salmonella* Typhimurium flagellin (FljB, FliC, and FljB'-FliC) to improve immune responses against the rabies vaccine (RV) and the protective efficacy of the whole-killed rabies vaccine (WKRV) with or without flagellins in BALB/c mice. We also compared the differences among the three flagellins in terms of immunoadjuvant properties to RV. FljB can cause the WKRV to induce stronger humoral and cellular immune responses than WKRV alone or WKRV with FliC or FljB'-FliC can. Mice immunized with WKRV and FljB produced higher levels of virus-neutralizing antibody (VNA) against RABV than those in the other groups did. Although mice in all treatment groups survived RABV challenge, the body weight loss in the group immunized with WKRV and FljB was lower than in the other groups. These results indicate that FljB is a promising adjuvant for use in the development of effective rabies vaccines.

Keywords Rabies virus · Rabies vaccine · Flagellin · Adjuvant

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Rabies is a zoonotic disease caused by rabies virus (RABV), which belongs to the genus *Lyssavirus* of the family *Rhabdoviridae* and induces progressive fatal encephalomyelitis in humans and animals [6]. Approximately 55,000 humans die from rabies every year, and most of these cases are reported in Asia and Africa [16]. Stray dogs and wild animals, including bats, skunks, foxes and raccoons, are the natural reservoirs of field RABV. Bites or scratches by an infected animal are the primary means of transmitting rabies. Rabies in humans is largely attributed to bites from stray dogs in developing countries, where animal vaccination is limited [25].

The most effective method of preventing the onset of rabies and death is vaccination of both humans and animals. At present, commercially available rabies vaccines (RVs) are mainly derived from cell culture and are whole-killed rabies vaccines (WKRVs) that do not contain any adjuvants [14]. However, these vaccines are expensive; thus, most ordinary families cannot afford them and cannot prevent RABV infection as a result. Compounding the matter, the current post-exposure prophylaxis (PEP) schedule requires that the vaccine should be administered four to five times to be effective [7, 15]. These factors necessitate a vaccine that can induce a protective immune response with fewer administrations to aid accessibility and compliance with PEP. Therefore, a novel adjuvant must be developed to improve the efficacy of current RVs.

Bacterial flagellin is a major component of flagellar filaments and contributes to both the virulence of bacterial pathogens and bacterial motility [1, 21, 23]. Two genes (*fljB* and *fliC*) encode flagellar antigens in most *Salmonella* isolates. *fliC* encodes the phase I flagellin FliC, whereas *fljB* encodes the phase II flagellin FljB [26]. Flagellin is also a pathogen-associated molecular pattern (PAMP) that mediates signal transduction in mammalian cells through

Toll-like receptor 5 (TLR5) [10, 22]. The innate immune system perceives conserved regions (e.g., lipopolysaccharide and flagellin) in bacteria that are regarded as PAMPs by TLRs. These regions induce strong inflammatory responses and eventually activate the adaptive immune response [5, 11, 12]. Therefore, the TLR5 agonist flagellin can be used as a potent molecular adjuvant for vaccines.

The adjuvant properties of flagellin (FljB and FliC) of *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) have been demonstrated in diverse vaccine candidates against influenza virus [13, 24], uropathogenic *Escherichia coli* (*E. coli*) [2], *Clostridium difficile* [9] and West Nile virus [17]. However, the adjuvanting potential of flagellin in WKRVs against RABV has not been investigated previously. Moreover, no report has compared the adjuvant effects of flagellins (FljB, FliC, and FljB'-FliC) on RV. In the present work, we evaluate FljB, FliC, and FljB'-FliC as adjuvants to a whole-killed rabies vaccine (WKRV) and report on their effects on immunogenicity and protective efficacy against rabies in BALB/c mice.

S. Typhimurium *fljB* and *fliC* were obtained through polymerase chain reaction amplification using *Taq* DNA polymerase (TaKaRa, China). Primers were designed against sequences from the GenBank database: AF045151.1 (*fljB*) and KF589316.1 (*fliC*). FljB, FliC, and FljB'-FliC were expressed in *E. coli* BL21 (DE3) and purified using Ni-NTA Sefinose™ Resin. The purified proteins were confirmed by SDS-PAGE (Fig. 1A) and Western blot analysis (Fig. 1B). A 0.1 mg/ml aqueous solution of FljB, FliC, or FljB'-FliC was prepared with sterile phosphate-buffered saline (PBS, pH 7.4) before use. The WKRV (vaccine efficacy >2.5 IU/dose) without any adjuvants was purchased from Liaoning Chengda Biotechnology Co. (China) and prepared with PBS.

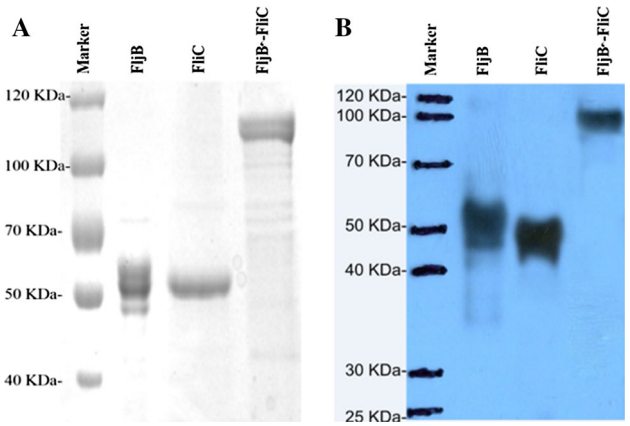

Fig. 1 Generation and analysis of flagellins (FljB, FliC, and FljB'-FliC). (A) Purified recombinant proteins were analyzed by SDS-PAGE on a gel stained with Coomassie blue. FljB, ~52.6 kDa; FliC, ~51.7 kDa; FljB'-FliC, ~104.4 kDa. (B) Purified recombinant proteins were analyzed by Western blot. One μg of purified proteins was transferred to a polyvinylidene fluoride (PVDF) membranes and probed with anti-His tag antibody

Six eight-week-old female BALB/c mice were purchased from the Animal Experimental Center of the Lanzhou Veterinary Research Institute, China. All animal care and use procedures were in accordance with the Guidelines for the Care and Use of Laboratory Animals presented by the Lanzhou Veterinary Research Institute. The mice were divided into in five groups (10 mice each) and injected intramuscularly in their posterior limbs with one of the following formulations: (1) PBS, (2) WKRV, (3) WKRV with 10 μg FljB, (4) WKRV with 10 μg FliC, (5) WKRV with 10 μg FliB'-FliC. WKRV was administered in doses of 100 μl per mouse. This dose was 1/40th of a human dose. Each group was immunized twice (days 0 and 14) at two-week intervals. Blood samples were obtained on days 0, 7, 14, 21, 28, and 35 by cutting the tails of the mice. Splenocytes were isolated from the BALB/c mice that were killed two weeks after the last immunization. Mice were challenged at three weeks after the last immunization. All challenged mice were observed daily for two weeks for disease signs, weight loss, and survival.

The serum IgG antibodies in the mice were measured using Animal RABV IgG Antibody Assay ELISA kits (Tianrun, China) according to the manufacturer's protocols. All treatment groups displayed humoral responses that were stronger than those of the control group (PBS) (Fig. 2). As shown in Fig. 2A, on days 21, 28, and 35 after immunization, the levels of IgG antibody induced by WKRV and FljB were higher than those induced by WKRV alone ($P = 0.0002$, 0.032, and 0.0004). WKRV and FljB also accelerated antibody production by seven days in comparison with WKRV alone. This accelerated antibody production enhanced the anti-RABV effects. As shown in Fig. 2B, the level of IgG antibody induced by WKRV and FliC was slightly higher than that induced by WKRV alone ($P < 0.05$) on day 21 after immunization. As shown in Fig. 2C, on day 28 after immunization, the level of IgG antibody induced by WKRV alone was slightly higher than that induced by WKRV and FljB'-FliC ($P < 0.05$). As shown in Fig. 2D, on days 21 and 28 after immunization, the levels of IgG antibody induced by WKRV and FljB were slightly higher than those induced by WKRV and FliC ($P < 0.05$). On day 35 after immunization, the level of IgG antibody induced by WKRV and FljB was significantly higher than that induced by WKRV and FliC ($P < 0.01$).

Subsequently, cellular immune responses were assessed by measuring IFN-γ and IL-4 production as an indicator of T cell immune responses. Approximately 100 μl cell splenocytes (1.0×10^5 cells) were added directly to the wells and stimulated with either 10 μl of stimulus (6 μg

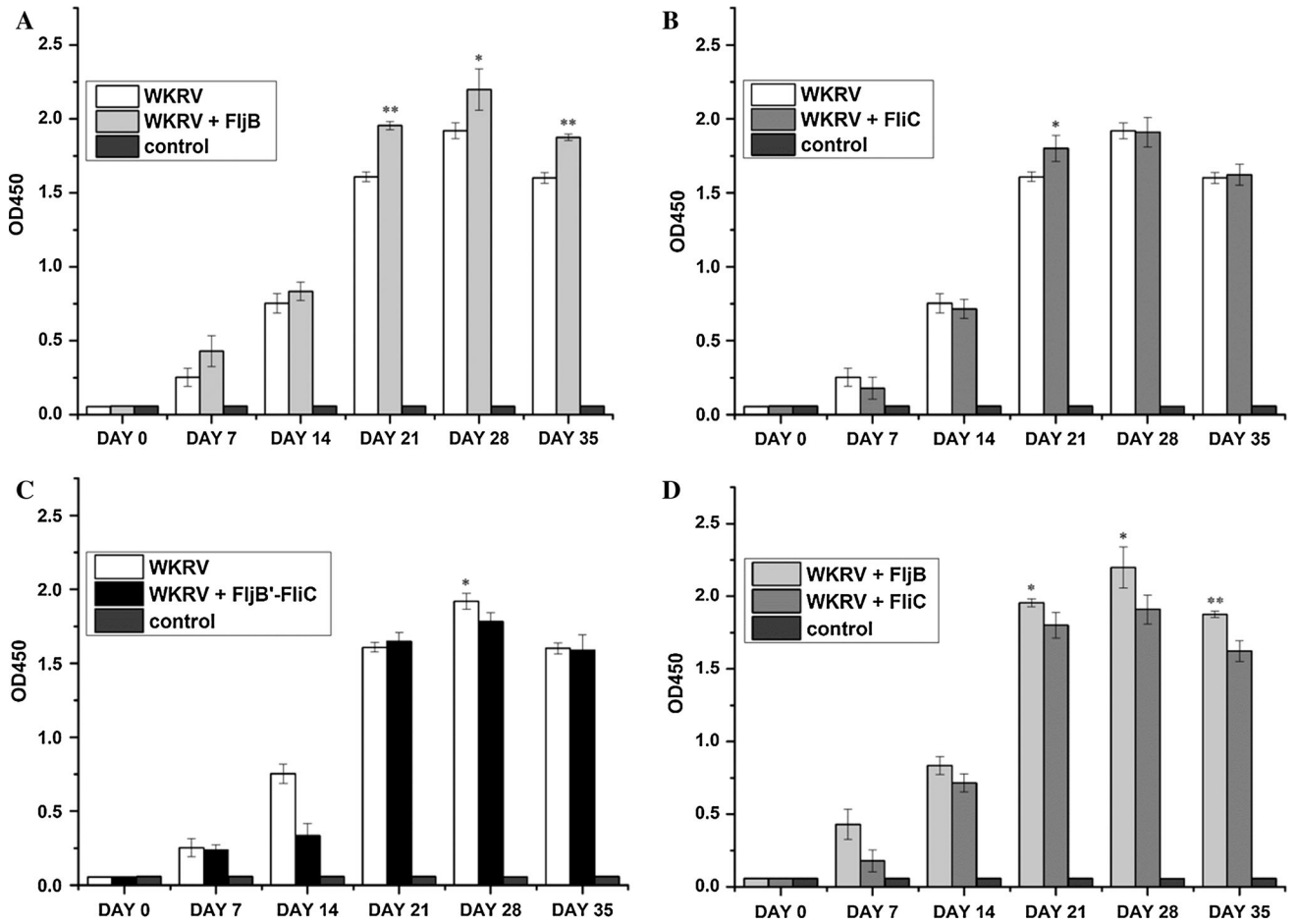

Fig. 2 RABV-specific IgG antibody titer determined by ELISA. RABV-specific antibody responses in mice induced by WKRV alone were compared with those induced by WKRV and FljB (A), FliC (B), or FljB'-FliC (C). RABV-specific antibody responses in mice induced by WKRV and FljB were also compared with those induced by WKRV and FliC (D). The mean and its standard deviation (SD) were determined for each group. Significant differences between WKRV and WKRV + FljB (A), between WKRV and WKRV + FliC (B), between WKRV and FljB'-FliC (C), and between WKRV + FljB and WKRV + FliC (D) were assessed using Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$)

synthetic peptide) or phytohaemagglutinin (PHA, positive control). The plates were then processed using mouse IFN-γ and IL-4 ELISPOT kits (DAKEWE, China) according to the manufacturer's protocol. All treatment groups exhibited significantly more IFN-γ- and IL-4-secreting cells than the PBS group did (Fig. 3). The number of IFN-γ- (Fig. 3A) and IL-4- (Fig. 3B) secreting cells induced by WKRV and FljB or FliC was significantly higher than that induced by WKRV alone ($P < 0.01$). Furthermore, FljB produced significantly more IFN-γ- and IL-4-secreting cells than FliC did ($P < 0.01$). However, no significant difference was observed between the WKRV group and the WKRV and FljB'-FliC group ($P > 0.05$). Furthermore, both FljB and FliC induced significantly more IFN-γ-secreting cells than IL-4-secreting cells ($P < 0.01$).

We also assessed splenocyte proliferation as another indicator of T cell immune responses. Splenocytes were cultured with 5 μg of concanavalin A (Con A; Sigma, USA) per ml for stimulation and then incubated at 37 °C in 5 % CO_2 for 44 h, followed by addition of 10 μl of Cell Counting Kit-8 (CCK-8, dojindo, Japan) solution and incubation for an additional 4 h. The stimulation index (SI) was determined by dividing the difference between the mean OD_{450} for cells stimulated with Con A and the mean OD_{450} for medium alone by the difference between the mean OD_{450} for cells stimulated without Con A and the mean OD_{450} for medium alone. All immunized mice showed a higher SI than those in the control group ($P < 0.01$) (Fig. 4). The SI of mice immunized with WKRV and FljB or FliC was significantly higher than that of mice immunized with WKRV alone ($P < 0.01$). Furthermore, the immunization of mice with WKRV and FljB resulted in a higher SI than that observed in mice vaccinated with WKRV and FliC ($P < 0.01$). However, no

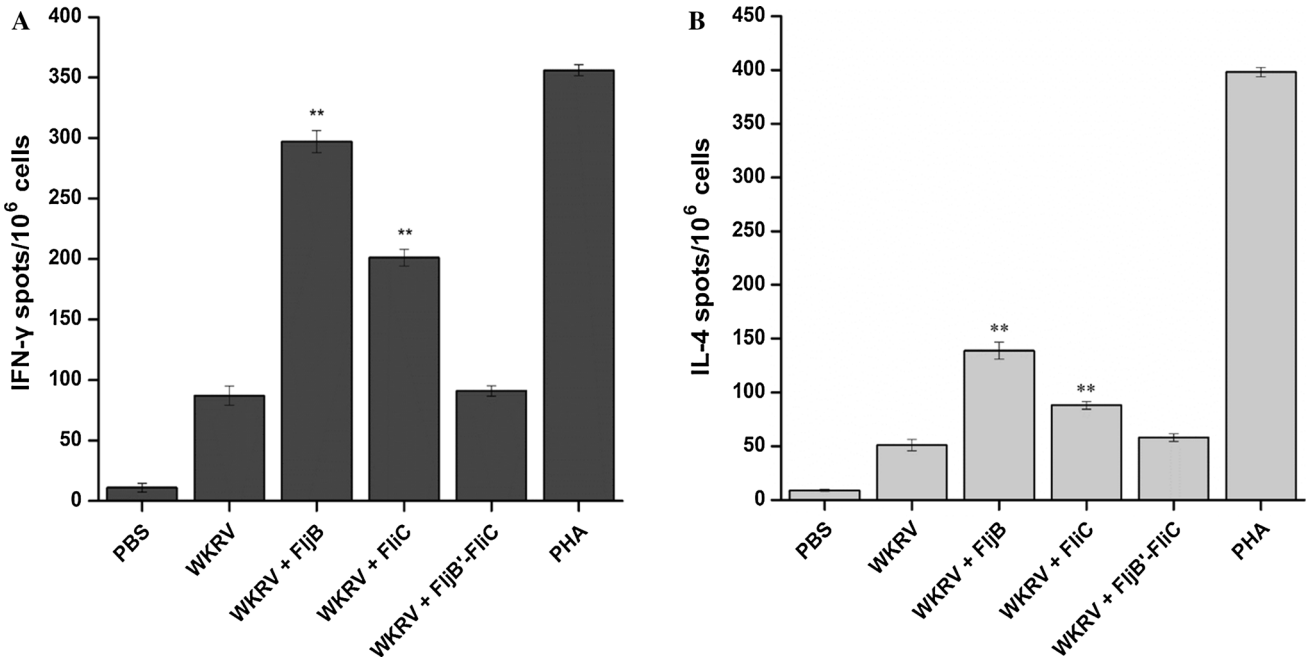

Fig. 3 IFN-γ and IL-4 production by splenocytes. Three mice in each group were killed two weeks after the last immunization. Amounts of synthetic-peptide-specific IFN-γ-producing cells (A) and of IL-4-producing cells (B) generated in the vaccinated mice were determined for each group, using an ELISPOT assay. PHA, which is a mitogen, can activate lymphocytes nonspecifically. Here, PHA was used as a positive control. The mean and its SD are presented for each group. Significant differences between WKRV and WKRV + FljB, between WKRV and WKRV + FliC and between WKRV + FljB and WKRV + FliC were assessed using Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$)

significant difference was observed between the WKRV group and the WKRV and FljB'-FliC group ($P > 0.05$).

To evalutate the protective efficacy of the vaccine preparations, all mice were challenged with 30 times the 50 % lethal dose (30 LD_{50}) of rabies virus strain CVS-24 by the intracerebral (i.c.) route. Serum samples were collected before challenge, and virus-neutralizing antibody (VNA) titers were measured using the rapid fluorescent focus inhibition test (RFFIT) according to the standardized test of the World Health Organization (WHO). The measured VNA titers were converted to international units (IU)/ml. All treatment groups produced significantly higher VNA titers than the control group did (Fig. 5). The level of VNA titers induced by WKRV and FljB was significantly higher than that induced by WKRV alone or WKRV and FliC or FljB'-FliC ($P < 0.01$). There were no differences in the level of VNA titers among the WKRV, WKRV and FliC and WKRV and FljB'-FliC immunization groups ($P > 0.05$). Two weeks after challenge, we monitored the mice daily for disease signs, weight loss, and survival. As shown in Fig. 6A, all mice in the PBS group died 6-10 days after challenge. These mice showed the classical rabies symptoms of severe neurologic dysfunction, including hind-leg paralysis, hunched-back posture, and circular motion. All mice immunized with WKRV alone or WKRV and FljB or FliC or FljB'-FliC were completely protected. However, mice immunized with WKRV and FljB or FliC lost less body weight (3 % and 6 %, respectively) than mice immunized with WKRV alone or WKRV and FljB'-FliC (10 % and 9 %, respectively) (Fig. 6B).

RABV is an important causative agent of rabies that results in acute infection of the nervous system and death [19]. At present, most commercially available RVs are WKRVs that do not contain adjuvants. Although RV adjuvants have been investigated since 1966 [8], there is still not an ideal adjuvant to RV. Many microbial products are currently employed as effective adjuvants because of their effects on antigen-presenting cells (APCs), which in turn promote the activation of immune responses. Cuadros et al. [5] demonstrated that FljB-enhanced green fluorescent protein fusion proteins can stimulate APCs, thus resulting in the maturation of these cells and the secretion of proinflammatory cytokines. This implies that FljB may be an effective adjuvant that induces and boosts immune responses against infectious diseases. McSorley et al. [18] reported that FliC, which can modulate $CD4^+$ T cell activation *in vivo*, is an effective adjuvant for $CD4^+$ T cells. Although the adjuvant properties of flagellin have been demonstrated in diverse vaccine candidates, few studies have examined the role of flagellin as a WKRV adjuvant.

In this study, we show that immunization of mice with WKRV and either FljB or FliC can induce stronger

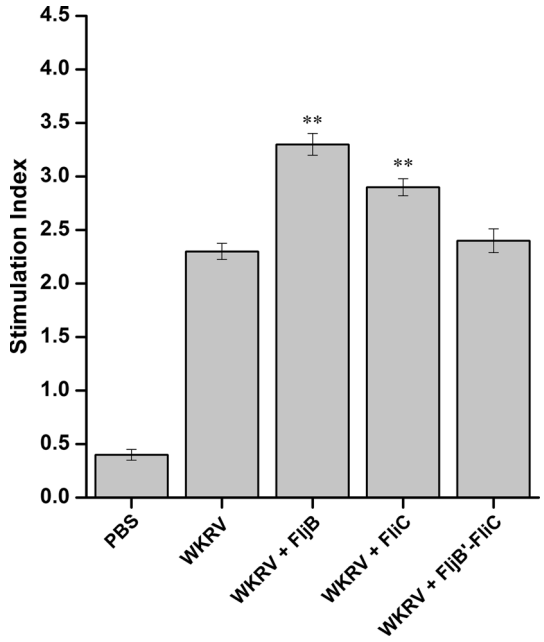

Fig. 4 Promotion of lymphocyte proliferation. Splenocytes were isolated from mice and cultured in triplicate wells at 2×10^5 cells/well in medium with or without Con A for 44 h. After stimulation, cells were treated with CCK-8 for an additional 4 h, and OD_{450} values were measured. Results are the mean stimulation index ± SD of three independent experiments. Significant differences between WKRV and WKRV + FljB, between WKRV and WKRV + FliC, and between WKRV + FljB and WKRV + FliC were assessed using Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$)

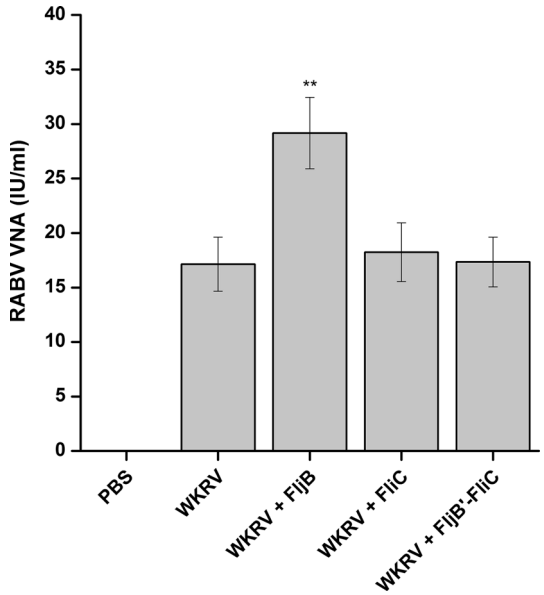

Fig. 5 Neutralizing antibody response elicited in all tested mice. Serum samples were collected three weeks after the last immunization and used for measuring VNA titers against rabies virus by RFFIT. Each column represents the mean VNA titer ± SD of seven mice per group. *, P < 0.05 vs. WKRV or WKRV + FliC or WKRV + FljB'-FliC

humoral immune responses than immunization with WKRV alone. Moreover, the adjuvant property of FljB was superior to that of FliC (Fig. 2D). Our findings also show that WKRV and FljB accelerated antibody production by seven days in comparison with WKRV alone (Fig. 2A), suggesting that FljB can provide early protection. FljB'-FliC reduced antibody production instead of promoting it (Fig. 2C). The reason for this phenomenon must be researched further, but the fusion protein FljB'-FliC may have influenced the formation of the TLR5-activating site and may have been immunodominant, thus blunting the anti-RABV response.

We also investigated cellular immune responses of the immunized mice. Cellular immunity is another important factor in eliminating virus infection, in addition to humoral immunity [20]. Our findings show that both FljB and FliC can promote the secretion of the cytokines IFN-γ and IL-4 (Fig. 3). Notably, FljB can induce significantly stronger Th1 and Th2 immune responses than FliC can, which explains why FljB caused stronger humoral responses than FliC. Our results also suggest that the type of cellular immune response induced by FljB and FliC is biased towards a Th1 response. This result is consistent with the results reported by McSorley et al. [18], but not consistent with those obtained by Karam et al. [2]. This discrepancy may be attributed to the method of vaccine administration and the nature of the antigen [3], both of which require further investigation. Our findings also show that both FljB and FliC can promote the proliferation of splenocytes. In addition, we found that the SI in mice immunized with WKRV and FljB was significantly higher than that in mice immunized with WKRV and FliC (Fig. 4). FljB'-FliC did not induce stronger cellular immune responses than the injection of WKRV alone, which is consistent with the humoral responses observed.

Here, we also observed that the immune response induced by WKRV and FljB provided better protection against challenge with strain CVS-24. Although mice in all treatment groups survived the challenge, they experienced different amounts of body weight loss after challenge. Not surprisingly, there was an inverse correlation between VNA titers and body weight loss (Figs. 5 and 6B), similar to what has been reported by Chen et al. [4].

In conclusion, the present work describes the first use of FljB and FliC as WKRV adjuvants. Results from our preclinical studies demonstrate that the mice immunized with WKRV and FljB have better immune responses than mice immunized with WKRV and FliC, suggesting that FljB is a promising adjuvant for the development of a new vaccination strategy that enhances the immune response against RABV.

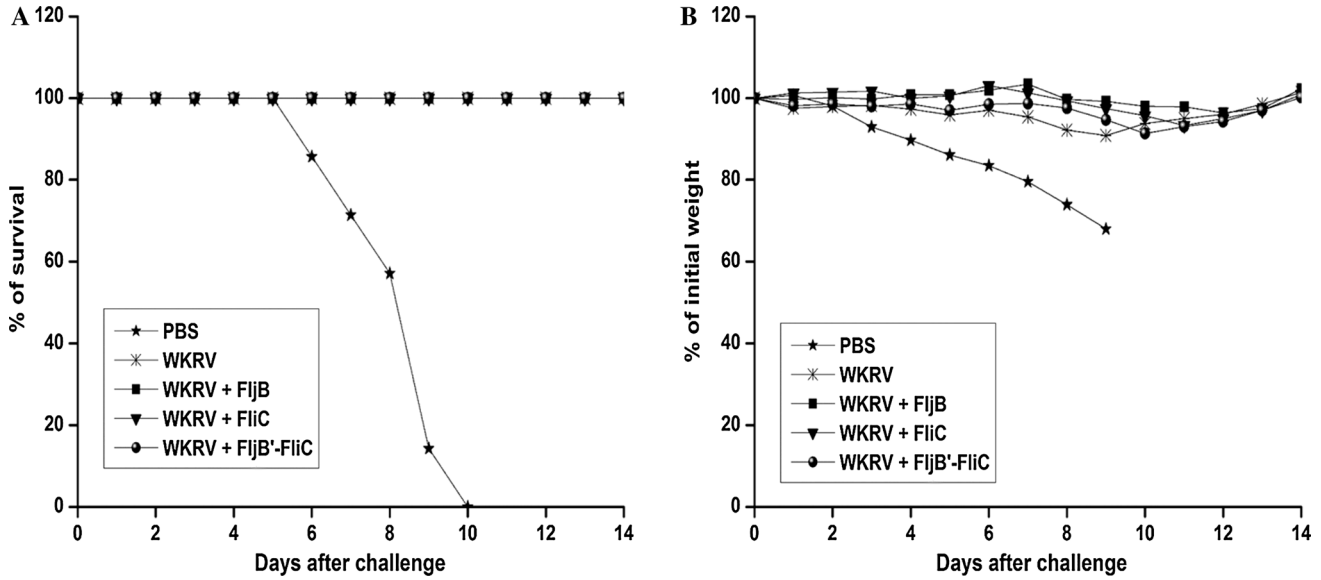

Fig. 6 Challenge study in mice. All tested mice were challenged three weeks after the last immunization. After viral challenge, survival (A) and body weight change (B) of mouse groups were monitored and recorded for two weeks. The mean body weight ± SD of each experimental group of seven mice was determined at each time point. Mortality is expressed as the percentage of mice surviving in each group at different times post-challenge. Body weight changes for each group are shown as ratios of the body weight at day 0, which was set as 100

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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