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RESEARCH PAPER

Interleukin-25 enhances humoral immune responses caused by the rabies virus

Yue Zhang, Mengwei Zhang, Xilan Liao, Yunsong Yu, Qing Liu, Yongwen Luo, Jun Luo, and Xiaofeng Guo

College of Veterinary Medicine, South China Agricultural University, Guangzhou, China

ABSTRACT

Rabies is an important zoonotic disease caused by the rabies virus (RABV). Currently, no effective treatment is available for this condition. The prevention and control of rabies mainly depend on effective vaccination. Therefore, it is crucial to enhance the immune responses induced by the rabies vaccine. Virus neutralizing antibodies (VNA) induced by rabies vaccines are important for the clearance of RABV. Interleukin-25 (IL-25) has been demonstrated to activate T helper type 2 cells that contribute to humoral immune responses. The IL-25 gene was inserted into the genome of RABV, and the immunogenicity of recombinant RABV with IL-25 gene was investigated to develop more efficient rabies vaccines. Here, we found that the expression of IL-25 did not affect RABV production *in vitro* and pathogenicity *in vivo*. However, recombinant RABV expression of IL-25 induced a better VNA level than the parental virus in mice. In addition, expression of IL-25 enhanced the IgG1 level induced by RABV. Furthermore, mice immunized with recombinant RABV showed a higher survival rate and milder clinical signs than those immunized with the parent strain after challenge with CVS-11. Thus, these results showed that IL-25 could enhance the humoral immune responses induced by RABV, suggesting that IL-25 can be used as a viral vaccine adjuvant.

Introduction

Rabies is a zoonosis with mainly damages in the central nervous system (CNS) and still an important public health problem worldwide. At least 55,000 humans died after infection by rabies virus (RABV) each year and most cases occurred in the developing countries, especially in Asia and Africa. Dogs with rabies virus are the main infection source [1]. Rabies has a high mortality rate in both humans and dogs without effective treatment. However, rabies vaccines can effectively prevent this medical condition. Therefore, it is crucial to improve the immune responses induced by the vaccine.

Rabies is caused by the RABV. RABV is a nonsegmented, single-stranded, negative-sense RNA virus, belongs to the genus *Lyssavirus* of the family *Rhabdoviridae*. The RABV genome is approximately 12 kb in size. From 3' to 5,' the genome of RABV encodes nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and an RNA-dependent RNA polymerase (L). RABV has been applied as an excellent vector to express exogenous proteins since the first recombinant RABV was produced [2]. Previous studies have reported that many foreign proteins are expressed between N and P, P and M, and G and L [3–6]. Furthermore, researches have indicated that immunomodulatory molecules expressed between G and L genes significantly enhance the immunogenicity of RABV [3,7]. Therefore, the expression of immune-enhancing molecules in RABV is a good strategy to develop more efficient rabies vaccines.

Interleukin-25 (also known as IL-17E) is a member of the IL-17 family of cytokines based on amino acid sequence homology [8]. IL-25 is secreted by various cells, including CD4+ T cells, CD8+ T cells, macrophages, dendritic cells, mast cells, eosinophils, and epithelial cells [9]. Overexpression of IL-25 increases IL-4 and IL-5 cytokines production, activating Th2 immune responses [10]. IL-25-deficient mice infected with pathogenic microorganisms cannot to eradicate the infection, which coincides with a failure in Th2-type immunity [11,12]. Therefore, IL-25 plays an essential role in infectious diseases.

This study investigated whether IL-25 could increase the immunogenicity of RABV. IL-25 was expressed between G and L genes in RABV, and its immunogenicity was determined in mice. The results indicated that IL-25 expressed in RABV did not inhibit viral replication *in vitro* and enhanced viral immunogenicity *in vivo*.

CONTACT Jun Luo ✉ junluo@scau.edu.cn; Xiaofeng Guo ✉ xfguo@scau.edu.cn

Materials and methods

Cells, viruses, antibodies, and animals

Baby hamster kidney (BHK-21) cells and BSR cells (a clone of baby hamster kidney-21 cells) (Wuhan Institute of Biological Products, Wuhan, China) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Suzhou, China). In addition, mouse neuroblastoma (NA) cells (Wuhan Institute of Biological Products, Wuhan, China) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Suzhou, China). Both DMEM and RPMI were supplemented with 10% foetal bovine serum (FBS) (Gibco, Australia). HEP-Flury was rescued from the plasmid pHEP-3.0 (provided by Dr. Kinjiro Morimoto), which contains the full-length cDNA of HEP-Flury in BHK-21 cells, and propagated in NA cells. In addition, challenge virus standard 11 (CVS-11) (a gift from Dr. Xianzhu Xia, Academy of Military Medical Sciences, Beijing, China) was propagated in BHK-21 cells. Fluorescein isothiocyanate (FITC)-conjugated antibodies against RABV N were purchased from Fujirabio Inc. (Malvern, PA, USA). Anti-IL-25 antibody was purchased from Signalway Antibody LLC (Maryland, USA). HRP-conjugated antibodies against IgG1 and HRP-conjugated antibodies against IgG2a were purchased from ABclonal Biotechnology Co., Ltd. (Wuhan, China). Anti-RABV M antibody was produced in our laboratory (unpublished data). Female Kunming (KM) mice were purchased from Zhuhai BesTest Bio-Tech Co., Ltd. (Zhuhai, China). The mice were housed in the Laboratory Animal Center of the South China Agricultural University. The animal experiments were allowed by the Ethics Committee for Animal Experiments of the South China Agricultural University (under the code 2019B190).

Construction of recombinant full-length cDNA clones and rescue of the viruses

The *mus musculus* IL-25 gene was synthesized (Sangon Biotech Co., Ltd., Shanghai) based on the published sequence on NCBI (NM_080729.3). Recombinant RABV infectious clones were developed by inserting IL-25 gene between G and L genes using restriction sites *Bsi*w I and *Nhe* I with the primers IL-25-forward (5'-AAG CGTACGATGTACCAGGCTGTTGC-3;' the underlined segment indicates *Bsi*w I restriction site) and IL-25-reverse (5'-AAAGCTAGCCTAAGCCATGACCCG-3;' the underlined segment indicates *Nhe* I restriction site). Sequences corresponding to IL-25 were inserted into the vector pHEP-3.0, which carried the full-length cDNA of HEP-Flury. The recombinant plasmid was named pHEP-IL-25. Insertion of IL-25 was confirmed by restriction analysis and DNA sequencing. The recombinant RABV expressing IL-25 was rescued from BHK-21 cells [13,14]. The rescued recombinant RABV expressing IL-25 was named rHEP-IL25. The rescued virus was confirmed in NA cells by a direct fluorescent antibody assay (dFA) with FITC-labelled anti-RABV N antibodies, as described previously [15]. The IL-25 and RABV M expression was confirmed by western blotting as described previously [16] using mouse anti-IL-25 and anti-RABV M antibodies. Protein fingerprints were shown using Fine-do 96 (Tanon, China).

Detection of the recombinant virus with fluorescent antibody

NA cells cultured in 96-well plates were infected with the recombinant RABV at 37°C for 48 h. The supernatant was removed, and the cells were fixed with 80% acetone at −20°C for 30 min. Then, the cells were washed with PBS three times. The anti-IL-25 antibody was added and the fixed cells were incubated at 37°C for 1 h and washed with PBS three times. The cells were then incubated with FITC-conjugated anti-rabbit IgG at 37°C for 2 h. The cells were washed with PBS three times, and antigen-positive foci (green) were observed under a fluorescence microscope (AMG).

The growth characteristics of RABV

Recombinant virus rHEP-IL25 and its parental virus HEP-Flury were cultured in NA cells, and their titres were determined as focus-forming units (FFU) per millilitre (FFU/mL) using the Karber method. The proliferation curves of rHEP-IL25 in NA cells and BSR cells were built. NA or BSR cells cultured in cell culture dishes were infected with virus either at a multiplicity of infection (MOI) of 0.01 or 1. Culture supernatants were collected daily for four days after inoculation. Virus titres of the collected supernatants were determined in BSR or NA cells by dFA.
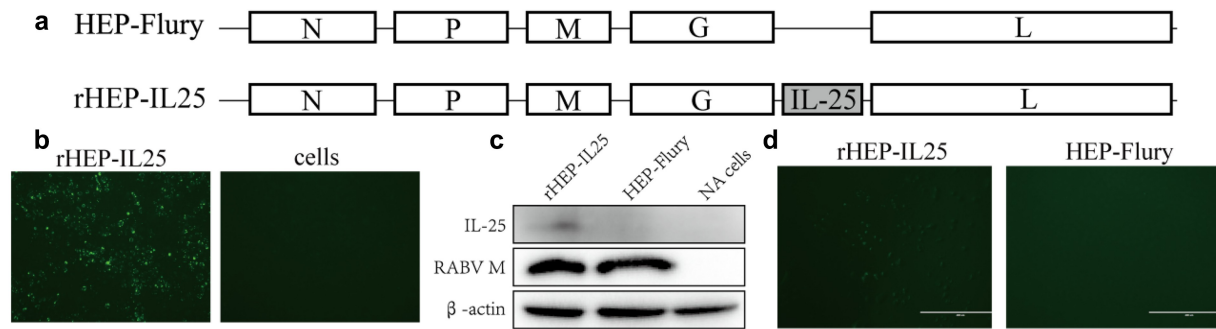
Figure 1. Confirmation of recombinant rHEP-IL25. (A) Schematic diagrams of the RABV genomes. N, nucleoprotein; P, phosphoprotein; M, matrix protein; G, glycoprotein; L, RNA-dependent RNA polymerase. (B) the rescued recombinant RABV expression of IL-25 was investigated by dFA with fluorescent antibody anti-RABV N antibody. (C) IL-25 expression and RABV M was determined by the western blot assay with anti-IL-25 and anti-RABV M antibodies. (D) IL-25 expression in NA cells was determined by iFA with the anti-IL-25 antibody.

The spread of RABV

A virus spread assay was performed in NA cells as described previously [17]. Briefly, NA cells were infected with rHEP-IL25 or HEP-Flury at an MOI of 0.01 and incubated at 37ºC for 2 h. The inoculum was discarded, and the cells were lavated with PBS three times. Next, the cells were fixed with 80% acetone and washed with PBS three times. The fixed cells were inoculated with FITC-conjugated anti-RABV-N antibodies at 37ºC for 2 h. The treated cells were then stained with DAPI to display the nucleus. Fluorescence was observed under a fluorescence microscope.

Pathogenicity of RABV in adult mice

To determine the pathogenicity of rHEP-IL25 on adult mice, 1.0×10^5 FFU rHEP-IL25 or HEP-Flury was intramuscularly (i.m.) injected into adult female KM mice (8–10 mice in each group). The medium injection was used as a mock group. Body weight and clinical symptoms were recorded daily for 21 consecutive days. The data were analysed using GraphPad Prism 6 software (GraphPad Software, USA).

Immunization and *in vivo* challenge To observe the immunogenicity of rHEP-IL25, 6–7 weeks old female KM mice were used in this study. Groups of 20 KM mice were i.m. immunized with 1.0×10^5 FFU of rHEP-IL25 or HEP-Flury, respectively, 100 μL each mouse. An equal volume of cell culture medium control group was set at the same time. Peripheral blood was collected on days 14 and 21 after immunization, and serum was used to determine VNA levels using fluorescent antibody virus neutralization (FAVN) tests as described previously [18]. The IgG1 and IgG2a in serum were determined with anti-IgG1 and anti-IgG2a antibodies using western blot as described previously [16]. Levels of IgG2c in serum were determined using Enzyme-Linked Immunosorbent Assay (ELISA) Kits (Meimian Biotechnology, Jiangsu, China) according to the manufacturer's instructions. The mice were intracerebrally (i.c.) challenged with 1.0×10^5 FFU of CVS-11 in a total volume of 30 μL for challenge experiments 21 days after immunization. The number of survived mice was recorded daily for consecutive 21 days. The survived rates were analysed using GraphPad Prism 6 software. Body weight and clinical symptoms of all the challenged mice were recorded daily for 16 days. With regards to clinical symptoms observed in mice, scores were assigned as follows: 0, no clinical symptoms observed; 1, loss of initial body weight >5% or/and piloerection; 2, paralysis, coma; 3, death. The data were analysed using GraphPad Prism 6 software.

Results

Identification of recombinant RABV

Recombinant cDNA clones carrying IL-25 were constructed, and recombinant RABV was generated in BHK-21 cells. IL-25 was expressed between G and L genes, and the recombinant RABV was designated as rHEP-IL25 (Figure 1a). NA cells were inoculated

with this recombinant strain to confirm the infectivity of rHEP-IL25. Green foci for antigens were confirmed by dFA (Figure 1b). In addition, the expression of RABV M or IL-25 was identified by western blotting with anti-IL-25 and anti-RABV M antibodies. IL-25 protein was detected in NA cells infected with rHEP-IL-25 (Figure 1c). Furthermore, IL-25 expression was identified by iFA after infection with rHEP-IL25 in NA cells (Figure 1d).

Not significantly effect on the RABV production in vitro by IL-25 insertion

NA cells were infected with rHEP-IL25 or HEP-Flury at an MOI of 0.01, followed to stain by fluorescent antibody to investigate whether the expression of IL-25 altered virus spread ability between cell and cell. As shown in Figure 2a, the number of the fluorescent foci (green) in the cells infected with rHEP-IL25 was not different from the cells infected with HEP-Flury. Thus, IL-25 expressed in RABV did not affect the
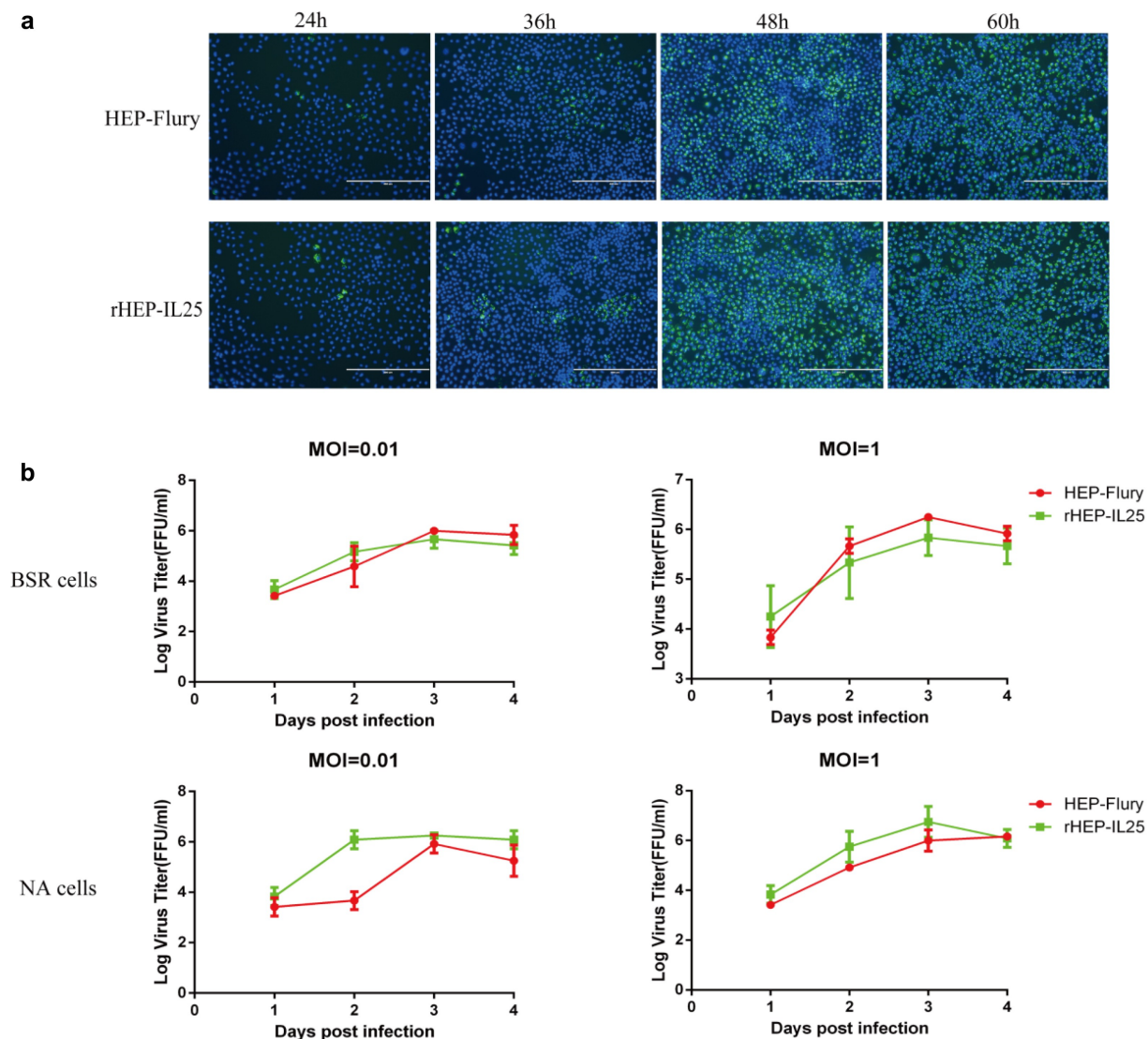

Figure 2. The characteristics of rHEP-IL25 in vitro. (A) Spread assay of RABV in cells. rHEP-IL25 or HEP-Flury were inoculated to a monolayer of NA cells at an MOI of 0.01 and followed by incubated at 37ºC. The cells were fixed with 80% acetone at 24-, 36-, 48-, and 60-hours post-infection intervals and stained with fluorescent antibody anti-RABV N protein and DAPI. (B) the growth characteristics of RABVs in BSR and NA cells. rHEP-IL25 or HEP-Flury were inoculated to BSR or NA cells at an MOI of 0.01 or 1. The supernatants of the medium were collected at 1-, 2-, 3-, and 4-day post-infection intervals and virus titres were detected.
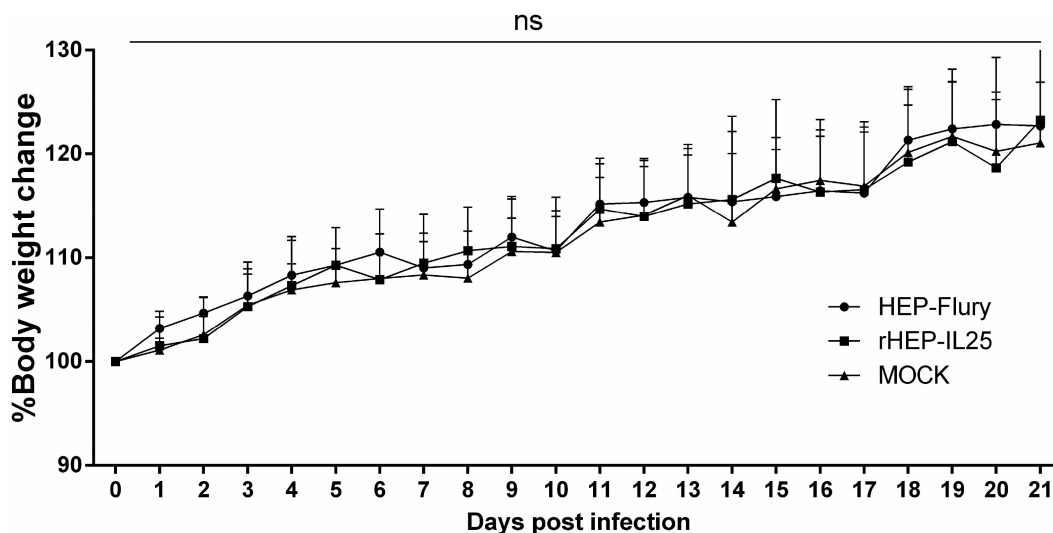
Figure 3. Pathogenicity of RABV in adult mice. rHEP-IL25, HEP-Flury, or medium were i.M.-inoculated to KM mice. Body weight was recorded daily for 21 days. The results showed as the body weight ratio on day 0, and data were presented as mean value ± SD (n = 8 ~ 10; ns, non-significant).

virus spread in cells. In addition, the growth kinetics of rHEP-IL25 and parental virus HEP-Flury were investigated in NA and BSR cells infected at MOI of 0.01 and 1. As shown in Figure 2b, rHEP-IL25 exhibit the same growth curves and virus titres in both NA and BSR cells compared with the parent strain. Therefore, the expression of IL-25 did not significantly change the growth of RABV. Taken together, the overexpression of IL-25 did not influence RABV production.

Il-25 expression does not increase the virulence of RABV

RABV HEP-Flury strain was apathogenic for adult mice and used as live vaccine before 2018 in China. To investigate whether IL-25 increased the pathogenicity of HEP-Flury, KM mice were infected intramuscularly (i.m.) with 1.0×10^5 FFU of RABVs or medium (mock). The body weights and clinical symptoms of all the mice were monitored daily for 21 days. During the infection, no mice showed significant clinical symptoms of rabies. Furthermore, as shown in Figure 3, the body weight changes of mice were not significantly different between rHEP-IL25 and HEP-Flury infection. Therefore, the expression of IL-25 in RABV did not increase the virus pathogenicity.

IL-25 expression enhances VNA level in the periphery

1.0×10^5 FFU of rHEP-IL25 or HEP-Flury were injected to KM mice i.m to investigate whether IL-

25 improved the immune response induced by RABV. Peripheral blood samples were collected on days 14 and 21 after (post) immunization (dpi), and serum was separated to determine VNA as described above. As shown in Figure 4a, titres of VNA in the immunized mice with rHEP-IL25 or HEP-Flury were >0.5 IU at 14 and 21 dpi. Interestingly, at both 14 and 21 dpi rHEP-IL25 induced significantly higher levels of VNA in the immunized mice than HEP-Flury. These findings indicated that rHEP-IL25 enhanced more humoral immune response in mice than parent HEP-Flury.

To further investigate whether increased VNA by IL-25 expression was associated with Th2 response, IgG1 and IgG2a in serum were determined by western blot and IgG2c was determined by ELISA. As shown in Figure 4b, rHEP-IL25 induced significantly higher levels of IgG1 than HEP-Flury in serum at both 14 and 21 dpi. Less IgG2a were determined in the serum of mice infected with rHEP-IL25 than those infected with HEP-Flury (Figure 4b). The IgG2c levels were not significantly different between rHEP-IL25 and HEP-Flury infection (Figure 4c). Therefore, the expression of IL-25 in RABV might enhance Th2 activation in infected mice.

rHEP-IL25 provides better protection against lethal RABV challenge

To explore the relation between the VNA titres and infection protection, the immunized mice with either rHEP-IL25 or HEP-Flury were challenged with
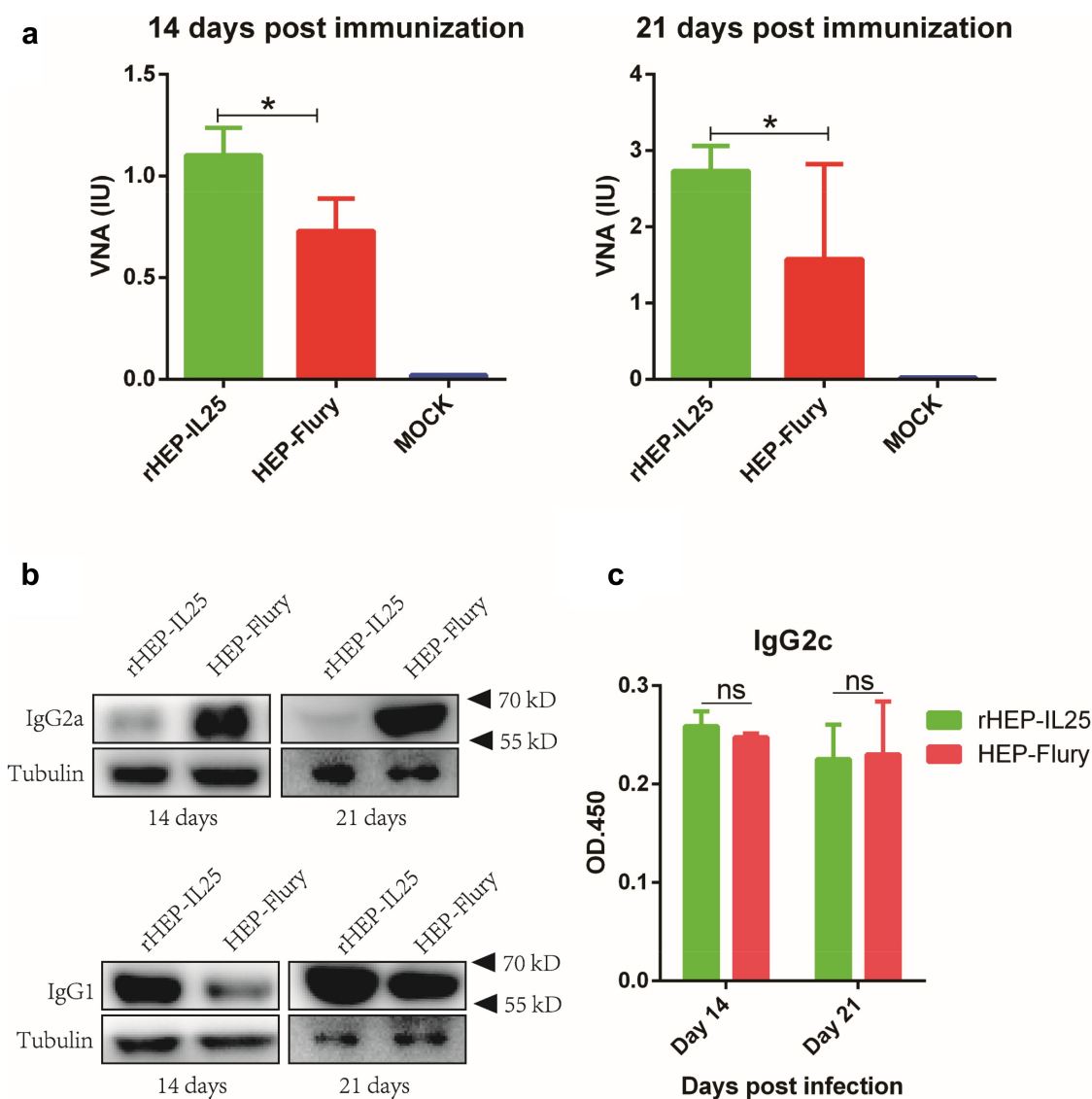
Figure 4. Immune response in the periphery induced by rHEP-IL25. (A) VNA levels. KM mice were i.M.-immunized with rHEP-IL25, HEP-Flury, or medium (mock infections). Peripheral blood was obtained on days 14 and 21 after immunization, and serum VNA levels were determined by FAVN. The data were presented as mean ± SD (n = 20). The data were analysed with ANOVA (*p < 0.05). (B) IgG1 and IgG2a levels. IgG1 and IgG2a in peripheral blood obtained on days 14 and 21 after immunization were determined by western blot with anti-IgG1 and anti-IgG2a antibodies. (C) IgG2c levels. IgG2c in peripheral blood obtained on days 14 and 21 after immunization was determined by ELISA assay. The results were presented as the mean of OD_{450} ± SD (n = 3). The data were analysed by the Student's *t* test (ns, none significant).

virulent CVS-11 through i.c. infection. As shown in Figure 5a, the mice immunized with rHEP-IL25 exhibited a significantly higher survival rate than those immunized with HEP-Flury. Clinical symptoms of all challenged mice observed are shown in Figure 5b. rHEP-IL25 immunized mice showed milder clinical symptoms than those immunized with HEP-Flury after CVS-11 challenge. Thus, IL-25 expression could enhance the protective effect of the rabies vaccine.

Discussion

Molecular adjuvant expressed by viruses should not diminish the virion production to decrease the vaccine costs. Previous studies have indicated that the pseudogene between G and L exerts no discernible effect on the virus replication of RABV [19,20]. In this study, IL-25 was expressed between G and L genes, and the recombinant RABV exhibited growth curves and replication similar to those of
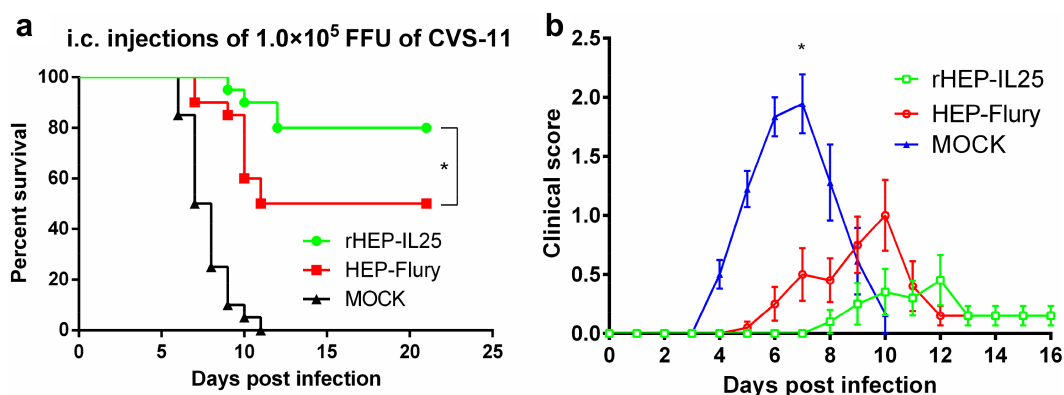
Figure 5. Challenged assay. Immunized mice were i.C. challenged with lethal strain CVS-11. (A)survival rates were recorded daily for three weeks. The results were presented as the mean ± SD (n = 20). The data were analysed by the log-rank Mantel-Cox test (*p < 0.05). (B) Clinical symptoms presented by challenged mice were scored according to severity on a scale from 0 to 3 and are presented here as mean values of scores ±SD (n = 18 ~ 20). Asterisks indicate significant differences among groups, as calculated by Student's t test (*P < 0.05).

the parent strain. Furthermore, previous studies reported that foreign genes expressed between G and L change viral growth and replication [21–23]. However, some studies have indicated that the insertion of foreign genes does not significantly affect viral growth and replication [24–28]. Thus, different foreign proteins expressed between G and L of RABV exert different effects on viral growth and replication. Here, IL-25 did not change RABV growth and replication in NA and BSR cells.

A study showed that chemokine MIP-1α expressed between G and L significantly enhanced immune responses induced by RABV while inactivated MIP-1α did not affect the immunogenicity of recombinant RABV [29]. Thus, only the nucleotide sequence cloned into pseudogene sequence between G and L did not affect the immunogenicity of recombinant RABV. In this study, RABV expressing IL-25 between G and L induced higher IgG1, which is the product of Th2 response. Previous study indicated that IL-25 mediates Th2 activation [10]. Therefore, expression of IL-25 may contribute to the higher VNA induced by RABV.

Previous studies have shown that VNA induced by the G of RABV is critical to preventing RABV infection [30,31]. Therefore, the primary objective when we focus on developing rabies vaccines is to improve the level of VNA. In this study, for the first time, we presented evidence that IL-25 expressed in RABV could enhance the humoral immune responses induced by RABV. Furthermore, we found that IL-25 expression augmented IgG1 levels in serum. Therefore,

we suspect that IL-25 improves the VNA concentration by promoting a Th2-type immune response after RABV infection [32,33]. Th2 cells could stimulate high titres of antibodies during infections [34,35]. Consistent with this, we showed that IL-25 could lift the protective effect of recombinant RABV immunization, raising the survival of the mice challenged by a RABV CVS-11 and diminishing clinical signs of rabies.

In conclusion, IL-25 expressed in RABV did not interfere with virus replication in vitro and did not increase the pathogenicity of RABV in mice. rHEP-IL25 enhanced viral immunogenicity and provided excellent immune protection against infection with virulent RABV. The findings indicated that IL-25 could enhance the humoral immune responses induced by viral vaccines. Therefore, rHEP-IL25 has the potential to be an attenuated vaccine. In addition, this study provides a reference that IL-25 can be used as a viral vaccine adjuvant.

Disclosure statement

Funding

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article or its supplementary materials.

Author contributions

Jun Luo, Xiaofeng Guo, and Yue Zhang designed the experiments. Yue Zhang, Mengwei Zhang, Xilan Liao, Yunsong Yu and Qing Liu carried out the experiments and collected data. Jun Luo and Yue Zhang wrote the manuscript. Xiaofeng Guo and Yongwen Luo checked and revised the manuscript.

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