Outer membrane proteins of *Salmonella typhimurium* as an adjuvant in rabies vaccine

**Purpose:** The objective of the present study was to evaluate the immune-enhancing potential of *Salmonella typhimurium* outer membrane protein (OMP) and alum as adjuvants towards inactivated Vero cells rabies vaccine (FRV/K2).

**Materials and Methods:** Six groups of female Sprague Dawley albino rats (10/group) were used in the evaluation of immunogenicity and safety of vaccines and adjuvants. Total immunoglobulin G secreted interferon-gamma (IFN-γ), and the percentage of proliferated CD4+ and CD8+ T cells were measured. Biochemical analysis and histopathological examination were used to test safety profiles.

**Results:** OMP adjuvanted rabies vaccine (FRV/K2+OMP) (OMP combined locally prepared vaccine) induced significantly higher neutralizing antibodies on day 21 post-vaccination relative to free (FRV/K2) vaccine and alum adsorbed vaccine (FRV/K2+alum) (alum adsorbed locally prepared vaccine). (FRV/K2+OMP) induced a significantly higher level of IFN-γ on day 14 post-vaccination. CD8+ T cells were significantly higher post-vaccination with reference (RV), free (FRV/K2), and (FRV/K2+OMP) than (FRV/K2+alum). On the contrary, CD4+ T cells were significantly elevated post-vaccination with (FRV/K2+alum) at p<0.05. Biochemical analysis and histopathological examination revealed that OMP could be used safely as an adjuvant for the development of more effective rabies vaccines.

**Conclusion:** Outer membrane proteins adjuvanted rabies vaccines would be beneficial to induce rapid neutralizing antibodies and essential cytokines.

**Keywords:** Rabies, Outer membrane proteins, Adjuvant, Vaccine, Immune response

Introduction

Rabies is a fatal viral disease that is transmitted from animals to humans. It belongs to the Lyssavirus genus, family Rhabdoviridae. The virus infects a peripheral nerve and ascends within the spinal cord, then spreads rapidly to the brain, resulting in encephalitis that eventually leads to death [1]. Together, post-exposure vaccination and inoculation of anti-rabies immunoglobulins preferably human rabies immunoglobulin (HRIG) around the wound is the only effective way for protection against rabies in humans [2]. Although the post-exposure vaccination with current available inactivated adjuvant-free vaccines is relatively effective there are 55,000 annual deaths caused by rabies [3]. To prevent infection with rabies virus, both humoral and cellular immunity are required, to neutralize the biological effects of rabies virus and kill virus-infected cells [3-5].
As post-exposure rabies vaccination is the only treatment, a more protective and cost-effective vaccine that could induce an earlier, and higher titer of neutralizing antibodies (NAbs) associated by sufficient cellular immunity is needed [6]. One approach to improve the kind and strength of immune response towards inactivated rabies vaccine is the use of adjuvants. Vaccine adjuvants are used for several purposes: to enhance or accelerate the immune response, allow antigen-dose sparing, reduce the need for booster immunizations, increase the duration of protection induced by the vaccine, or improve efficacy in immunocompromised, neonates, and elders [7].

Although aluminum adjuvants can improve antibody titers to the rabies vaccine, several studies suggested that it might cause a delay of antibody production, which is unacceptable in rabies vaccines [8]. Besides, the usage of aluminum adjuvants caused many adverse reactions such as erythema, subcutaneous nodules, contact hypersensitivity, granuloma, and local tissue damage macrophagic myositis [9]. Testing inactivated rabies vaccines with a safe and effective new generation of adjuvants is necessary [10,11]. Natural or synthetic adjuvants that act through toll-like receptors (TLRs) signaling could induce rapid antigen-specific immune responses with long immunologic memory [12].

Outer membrane vesicles in Gram-negative bacteria consist of lipopolysaccharides, and periplasmic contents. Flagellin, peptidoglycans, lipoproteins, and other outer membrane proteins (OMPs), act as TLR ligands [13]. Activation of innate immune responses is always the first step in the induction of adaptive immune responses. TLR ligands bind to pattern recognition receptors of the antigen-presenting cells activate naïve T cells, hence stimulate the adaptive immune system. Salmonella Typhi OMPs OmpC and OmpF (porins) are immunostimulatory protective antigens for innate and adaptive immune responses [14].

Accordingly, the present work aimed to prepare a local rabies vaccine using FRV/K2 strain in Vero cells as host cells for virus replication, and evaluate the immune-enhancing potential of Salmonella typhimurium OMP and alum as adjuvants towards inactivated Vero cells rabies vaccine. The humoral and cellular immune response markers: total immunoglobulin G (IgG), secreted interferon-gamma (IFN-γ), and the percentage of proliferated CD4+ and CD8+ T cells were evaluated. Safety profiles of the tested vaccines were also studied.

Materials and Methods

Ethical considerations

The study protocol was approved by the research ethical committee at the Faculty of Pharmacy, Cairo University (approval no., MI2367). All animal procedures were performed as regulated by the international ethical guidelines and the National Institute of health guide concerning the care and use of laboratory animals.

Laboratory animals

Six groups of female Sprague Dawley albino rats 100–120 g (10/group) were used for evaluation of immune response, and testing safety profiles. Six groups of male BALB/c mice 10–12 g (10/group) were used for testing against challenging rabies virus strain (challenge virus standard, CVS).

Vaccines and antibodies

The commercially available rabies vaccine produced by VAC-SERA (batch no., 201716412; VACSER, Agouza, Egypt) was used as the reference vaccine (RV; commercially available rabies vaccine used as a reference positive control). Home reference of antirabies total IgG rat origin was kindly supplied from the research and development (R&D) sector VACSER as 5 IU/mL.

Strains

Fixed rabies virus strain FRV/K [15] was kindly supplied from the R&D sector of VACSER (The Egyptian Holding Company of Vaccines, Sera, and Drugs) at the power of 6.5 log10 LD50/mL, and it was adapted to grow on Vero cells (CCL-81) using mice–tissue culture alternating passaging. FRV/K was used to prepare local rabies vaccine FRV/K2 [16,17]. CVS was supplied as ampoules of freeze-dried preparation contain 0.5 mL of a 20% mouse-brain suspension for measuring ED50 [18] (the effective dose of vaccine that protects 50% of total mice against a challenging rabies virus strain) of the prepared vaccine. ED50 of both the prepared vaccine FRV/K2 and RV were insignificantly different and were ≥2.5 IU.

Adjuvants

Isolated outer membrane proteins of Salmonella enterica serovar Typhimurium strain (ATCC-131); 730 μg/mL was kindly supplied from the R&D sector of VACSER-Egypt. Imject Alum (aluminum hydroxide 40 mg/mL+magnesium hydroxide 40 mg/mL) Thermo Scientific CAS# 77161 was supplied from
the International Center for training and Advanced Researches (ICTAR-Egypt).

**Formulation of adjuvanted rabies vaccine**

Inject Alum was used as 200 μg/mL [19], and OMP was used as a final concentration of 20 μg/mL. The locally prepared rabies vaccine FRV/K2 was mixed with test adjuvants alum and OMP overnight at 37°C on a magnetic stirrer to facilitate the adsorption to alum and homogenization with OMP for 24 hours at +4°C.

**Immunization**

Female Sprague Dawley rats were randomly divided into six groups, 10/group. All groups were subcutaneously immunized using test rabies vaccines as 0.5 mL/rat according to a study of Liu et al. [20]. Test groups were identified as group 1 (RV), group 2 (FRV/K2), group 3 (FRV/K2+Alum), group 4 (FRV/K2+OMP), group 5 (OMP only), and group 6 (negative control group, phosphate buffer saline [PBS]).

**Immune sera preparation**

Blood samples were collected via the retro-orbital plexus of rats’ eyes after anaesthetization. Collected blood was incubated for 30 minutes at 37°C for blood coagulation then overnight at +4°C for retraction of blood clots followed by cold centrifugation for 15 minutes at 3,500 rpm (Jouan, Ki22 France). Sera were aliquoted and stored at -80°C until use.

**Measuring total anti-rabies IgG IU/mL**

Rabies-specific total IgG antibody in sera of immunized rats was measured at different time intervals using enzyme-linked immunosorbent assay (ELISA) [21]. Antibody titer was calculated using the following equation: antibody titer=optical density at wavelength 460 nm of test/optical density at same wavelength of home reference × home reference serum IU.

**Quantitative analysis of IFN-γ**

IFN-γ level was determined in sera of immunized rats. Previously prepared rat sera samples were withdrawn from the six groups at 14 days post-vaccination and they were quantitatively analyzed for (IFN-γ) using ELISA (Cloud-Clone Corp., Katy, TX, USA). The concentration of IFN-γ was determined using a standard curve.

**Quantitative evaluation of CD4+ and CD8+ T cells**

Two rats from each group were sacrificed on day 28 post-vaccination to measure the percentage of CD4+ and CD8+ T cells using flow cytometry. Splenic cells were harvested, and cell pellets were incubated for 30 minutes with an excess of fluorescein isothiocyanate-conjugated rat anti-CD4 monoclonal antibody (#96127) and PE/Texas Red rat anti-CD8 (Cat. no., ABIN1827180) [22].

**Testing protection in mice against viral challenge**

Protection against a challenge rabies virus was tested in BALB/c mice, and CVS-11 was prepared for infection at 50 LD50 injected intramuscularly in the posterior limb. After 2 hours, six groups (RV, FRV/K2, FRV/K2+alum, FRV/K2+OMP, sole OMP, and PBS) were immunized with 5 injections on 0, 3, 7, 14, and 28 days. Immunized mice were observed for 30 days, and rabies symptoms and survival rate were recorded starting from the 5th day after the first immunization.

**Testing safety profile of rabies vaccines and adjuvants**

**Acute toxicity test**

Rats in the six different groups (RV, FRV/K2, FRV/K2+alum, FRV/K2+OMP, sole OMP, and PBS) were observed for body weight, food intake, behavioral changes, and abnormal clinical symptoms.

**Biochemical analysis**

Liver and kidney functions: alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea, blood urea nitrogen (BUN), and creatinine were evaluated (Beckman Coulter AU 480; Beckman Coulter, Inc., Brea, CA, USA) in rat sera samples that were collected on the 28th and 60th days post-vaccination.

**Histopathological study**

The histopathological effects of both alum and OMP on different organs, i.e., kidney, liver, and spleen post-vaccination with test vaccine formulae were examined. Whereas the effects of commercially available reference (RV) and free FRV/K2 rabies vaccines without any adjuvants were recorded as well. Fresh liver, kidney, and splenic specimens were exited from the control and treated immunized rat groups and fixed in 10% neutral buffer formol and Carnoy’s fluid for the histological studies [23].

**Statistical analysis**

The data of antibody level, IFN-γ concentration, and CD4/8 cells count variation were conducted using GraphPad Prism.
Results

Total anti-rabies IgG (IU/mL)

The calculated Nab values for the sole outer membrane protein and the negative control relative to time were 0.139 and 0.1098 IU/mL, respectively. The commercially available reference (RV) LP (Louis Pasteur viral strain) strain showed a significantly elevated (p<0.05). Nab titer than both OMP combined FRV/K2+OMP and alum adsorbed FRV/K2+alum test vaccines. FRV/K2+OMP showed a faster release Nab level followed by gradual depletion of Nab titer relative to time till day 90. However, FRV/K2+alum showed a longer-lasting release of Nab (Fig. 1). FRV/K2+OMP induced enhanced, non-delayed Nab relative to free FRV/K2 vaccine and FRV/K2+alum (Fig. 2).

Quantitative analysis of IFN-γ

Regarding the cellular immune response markers, it was recorded that IFN-γ showed significant elevation (p<0.05) post-vaccination with RV administration than post-vaccination with the free locally prepared vaccine FRV/K2. IFN-γ was significantly higher in the case of FRV/K2+OMP followed by FRV/K2+alum. Sole injection of OMP showed a reduced level of secreted IFN-γ than in the case of free FRV/K2, FRV/K2/alum, and FRV/K2/OMP vaccines (Fig. 3).

Fig. 1. Rabies neutralizing antibodies (NAbs) post-vaccination with test vaccine candidates using enzyme-linked immunosorbent assay in female Sprague Dawley rats. RV, reference vaccine group; FRV/K2, free locally prepared vaccine group; FRV/K2+alum, alum adsorbed locally prepared vaccine group; FRV/K2+OMP, OMP combined locally prepared vaccine group.

Quantitative evaluation of CD4+ and CD8+ T cells

T-cell subtypes were evaluated on the 28th days post-vaccination. The absolute measured percentage of CD4+ T cell was highest in group 2 (free FRV/K2), followed by group 3 (FRV/K2+alum), then group 1 (RV) and group 4 (FRV/K2+OMP). The four vaccinated test groups showed significantly elevated CD4+ cells compared with its values in sole OMP and negative control groups. The measured CD8+ T cells were insigni-
Fig. 4. Dot blot showed the two populations of T cells in spleens of female Sprague Dawley rats on day 28 post-vaccination using flow cytometry. Percentage of CD4+ is shown in the upper left quadrant and CD8+ is shown in the lower right quadrant of the dot plot. (A) Sample 1: RV (reference vaccine group). (B) Sample 2: FRV/K2 (free locally prepared vaccine group). (C) Sample 3: FRV/K2+alum (alum adsorbed locally prepared vaccine group). (D) Sample 4: FRV/K2+OMP (OMP combined locally prepared vaccine group).

Significantly different in group 2 (free FRV/K2), group 4 (FRV/K2+OMP), and group 5 (sole OMP) than CD8+ induced post-vaccination with group 1 (RV). Meanwhile, group 3 (FRV/K2+alum) induced significantly lower CD8+ T cells (Fig. 4).

Protection in mice against viral challenge

Clinical symptoms or mortality in the first 5 days were not considered. All mice in group 5 (sole OMP) and group 6 (PBS) showed convulsions, irritability, and paralysis on day 6, by day 10 all animals in both groups died. The death occurred after 2–4 days of onset of clinical symptoms in all groups. The survival rate was recorded on day 30 at the end of the observation period. RV group 70%, free FRV/K2 group 50%, FRV/K2+alum group 60%, and 70% in FRV/K2+OMP group survived the challenge. The protection conferred by FRV/K2+OMP in the animal model was significantly higher than that conferred by free FRV/K2 and FRV/K2+alum.

Toxicity test

There were no abnormal clinical signs in immunized rats in different groups throughout the observation. A normal increase in body weight and food intake, no diarrhea, no behavioral changes, or any abnormal symptoms.

Biochemical analysis

Kidney and liver functions of rat sera in each tested group were measured twice at 28th and 60th days post-vaccination (Table 1). As shown in Table 1, there was insignificant elevated blood
urea and BUN urea level post-vaccination with RV, and both formulated vaccines FRV/K2-alum and FRV/K2-OMP were compared to its values in the control group. Creatinine was also in the normal range during the 28th and 60th days post-vaccination compared to control values. Similarly, there was an insignificant changed ALT value during test durations compared with negative control group values during the same durations. In contrast, there was a noticed significantly elevated AST level post-vaccination with RV/28, FRV/K2/28, FRV/K2+alum/60, and FRV/K2+OMP/28.

Histopathological alterations

Histopathological changes (photomicrographs of rat kidney tissue, photomicrographs of rat liver tissue, and photomicrographs of rat spleen tissue in six different groups) are presented in Supplements 1–3.

Discussion

More than 95% of human deaths due to rabies occur in Asia and Africa [17]. Rabies is fatal by 100% once the clinical symptoms begin. Post-exposure rabies vaccine is the only treatment together with the inoculation of HRIG to protect against viral replication and its entry to the central nervous system (CNS). Thus, a protective and cost-effective vaccine that induces faster, enhanced rabies-specific NAb together with sufficient cellular immune response is needed.

The prepared test vaccine FRV/K2 was immunogenic recording insignificant different ED50 than RV. Multiple proteins were reported in Salmonella Typhi outer membrane: Omp A, Omp C, Omp F, Omp S1, and Omp S2. They act through TLRs (TLR2, TLR4) that link the innate with the adaptive immune arms resulting in a more efficient immune response [14,24,25]. A recent study also reported that S. Typhi porins (Omp C and Omp F) increased IgG titers and led to antibody class switching when it was co-administered with ovalbumin (OVA) antigens (poor inducers of the immune response), inactivated H1N1 2009 pandemic influenza virus, and unconjugated Vi capsular polysaccharide vaccine (a T-independent antigen). Also, using Salmonella typhimurium proteins as adjuvants resulted in increased production of IFN-γ, interleukin (IL)-2, and IL-17A by OVA-specific CD4+ T cells suggesting that OMP is a promising adjuvant [14].

The peak of IgG in rats vaccinated with free (FRV/K2) and (FRV/K2+OMP) occurred one week after the fourth vaccination dose. FRV/K2+OMP showed rapid enhanced release of NAb on day 21 post-vaccination. Meanwhile, it occurred in rats of FRV/K2+alum group on day 28, 2 weeks after the fourth immunization, which suggests that alum postponed the maximum release of IgG relative to the free FRV/K2 vaccine. In agreement with previous studies, that using alum as an adjuvant in rabies vaccines may cause a delay of antibody production, which is unfavorable in the prevention of rabies [8,26].

Improvement in survival rate in the FRV/K2+OMP group than free-FRV/K2 and FRV/K2+alum groups suggested that induction of early, enhanced NAb and IFN-γ are significantly effective in protection against rabies. Previous studies reported that IFN-γ and antibodies are responsible for clearance of pathogenic rabies virus from the CNS [27] and could control viral replication in the CNS on the long term [28]. Venkatas-

### Table 1. Biochemical analysis of rat sera at 28th and 60th days post-vaccination

| Variable          | RV          | FRV/K2      | FRV/K2+alum | FRV/K2+OMP | OMP          | Negative control |
|-------------------|-------------|-------------|-------------|------------|--------------|-----------------|
| **Day 28 post-vaccination** |             |             |             |            |              |                 |
| Urea (mg/dL)      | 53.5±1.5    | 50±1.7      | 46.6±1.52   | 53±1       | 54±0.6      | 37.6±0.4        |
| Creatinine (mg/dL)| 0.53±0.15   | 0.4±0.1     | 0.3±0.1     | 0.2±0.01   | 0.3±0.1     | 0.23±0.1        |
| BUN               | 24.3±2.1    | 23±1.53     | 20±1        | 20.3±1.5   | 22±1.2      | 18.3±0.8        |
| ALT (U/L)         | 76±1        | 55±2.52     | 49±1        | 55.6±2     | 55±0.6      | 85.6±2.1        |
| AST (U/L)         | 313.3±11.59 | 195±2.4     | 136.7±3     | 163±1.2    | 111±4       | 131.6±3.5       |
| **Day 60 post-vaccination** | 31.43±0.52 | 42±1        | 31.7±1.96   | 46.5±1.5   | 45±1.3      | 35.3±1.2        |
| Urea (mg/dL)      | 0.13±0.1    | 0.2±0.1     | 0.26±0.12   | 0.17±0.1   | 0.3±0.1     | 0.2±0.1         |
| Creatinine (mg/dL)| 16±1        | 19.6±1.52   | 15±1        | 21.3±1.2   | 21±0.4      | 20.6±0.2        |
| BUN               | 42.3±2.2    | 45.3±0.58   | 66±2        | 45±1       | 45±0.6      | 84.6±1.7        |
| ALT (U/L)         | 114±1       | 124.3±1.1   | 185.6±2.2   | 124.7±1    | 120±2.5     | 112.3±7.3       |
| AST (U/L)         |             |             |             |            |              |                 |

Values are presented as mean±standard deviation.

RV, reference vaccine; OMP, outer membrane protein; BUN, blood urea nitrogen; ALT, alanine aminotransferase; AST, aspartate aminotransferase.
with TLRs to overcome the failure of aluminum to induce cellular cytokine responses (type 1 and type 2) and both are essential for vaccine-induced protective responses against rabies. Pro-inflammatory stimuli such as tumor necrosis factor-α, IL-6, and IFN-γ, could increase permeability in the blood-brain barrier allowing infiltration of immune effectors into the CNS to clear viral infection [20]. Thus, adjuvants as OMP that induce IFN-γ production and enhance earlier NAb would help in clearing rabies infection efficiently. Perez-Toledo et al. [14] reported that Salmonella Typhi porins (OMP C and OMP F) might drive the T-cell immune responses toward a Th1/Th17 rather than Th2. In agreement with our results, the high amount of secreted IFN-γ which was induced by FRV/K2+OMP relative to free-FRV/K2 and FRV/K2+alum tested groups suggested that OMP drives the CD4+ responses towards a Th1. Liu et al. [20] reported that rabies-specific Th1 responses which are characterized by both CD8+ IFN-γ T-cell responses and IgG2a-type were detected in mice immunized with Lipo-I or CE536 adjuvanted vaccine, where Lipo-I is a synthetic construct of CE536 to imiquimod (TLR7 agonist) induced better protection against rabies infection. Although alum adjuvanted test vaccine (FRV/K2+alum) group induced significantly elevated IFN-γ relative to negative control groups, the level of free FRV/K2 was significantly lower than IFN-γ induced in FRV/K2+OMP group. In addition, the percentage of CD8+ cells or T-cytotoxic cells with CD8 co-receptor recognize the major histocompatibility complex (MHC) I protein to kill virus-infected cells was significantly lower in FRV/K2+alum group [30]. Meanwhile, FRV/K2+alum induced a significantly higher percentage of CD4+ T cells in spleens on day 28 post-vaccination. CD4+ cells or T-helper cells have the CD4 co-receptor thus recognizes the MHC II protein, which is more beneficial in the protection against extracellular bacterial and fungal infections [30]. Data in our study suggested that alum drives a mixed response of Th1 and Th2. However, previous studies reported that aluminum directs the differentiation of CD4 T cells to Th2, not Th1 cells [31-33] and mainly induces IgG1 isotype [26]. Although it is believed that aluminum hydroxide-based adjuvants do not activate TLR-dependent signaling pathways, are unable to activate dendritic cells (DCs) directly, or cause the expression of co-stimulatory molecules and pro-inflammatory cytokines in vitro [9], Hogenesch [34] reported that aluminum adjuvants in vaccines activated DCs leading to the differentiation of CD4+ T cells into effector cells. He recommended the combination of aluminum adjuvants with TLRs to overcome the failure of aluminum to induce cellular immune response and differentiation of CD8+ into cytotoxic T-lymphocytes (CTL).

Although, ALT and AST levels are considered critical parameters that detect liver injury, the elevation of ALT is more indicative of liver toxicity rather than AST [6,35]. In our study, we did not consider the elevation in AST level only an indication for liver toxicity. Furthermore, the level of AST returned to normal at day 60 in RV/60, FRV/K2/60, and FRV/K2+OMP/60 except FRV/K2+alum/60.

Ayman et al. [36] recorded that both adjuvants used in their study, alum and monophosphoryl lipid (MPL), showed pathological changes in both liver and kidney cells compared to the control untreated group. Even, MPL adjuvanted vaccine exhibited more changes than that recorded in the case of alum adjuvanted. On the contrary, FRV/K2+OMP exhibited fewer adverse effects than FRV/K2+alum on both liver and kidney cells. Hyperplasia of the spleen and lymph nodes returned to normal by the end of the study period, showing that sole OMP and FRV/K2+OMP rabies vaccine did not induce toxicity in immune organs.

In conclusion, we introduced an alternative natural adjuvant OMP of Salmonella typhimurium other than the currently used aluminum adjuvants and compared their immunostimulatory mechanisms as adjuvants to rabies vaccine and their safety profiles in the animal model. FRV/K2+OMP induced enhanced, non-delayed rabies-specific NAb, and a significantly higher level of INF-γ. The safety study revealed that OMP and FRV/K2+OMP are well tolerated in rats. S. Typhi OMP could be an effective and safe adjuvant in rabies vaccines.

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**Supplementary Materials**

Supplementary materials are available at Clinical and Experimental Vaccine Research website (http://www.ecevr.org).

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Supplemental Materials

Evaluating the histopathological drawbacks of both alum and outer membrane protein (OMP) on different organs, i.e., kidney, liver, and spleen post-immunization with test vaccine formulae compared to non-immunized negative control rats. Whereas the effect of commercially available reference and free locally prepared rabies vaccines without any adjuvant was recorded as well.

Supplement 1. Photomicrographs of rat kidney section. Six different groups: C (negative control group), OMP (sole outer membrane protein [OMP]), V1 (RV group), V2 (FRV/K2 group), V3 (FRV/K2+alum group) and V4 (FRV/K2+OMP). (A) C group: A photomicrograph showing a normal architecture of kidney cortex of a control rat. Well-developed glomeruli (G), Bowman capsules (BC), Bowman spaces (Bs), distal (ds), and proximal (px) convoluted tubules with interstitial spaces (×250). (B) OMP group: A photomicrograph showing a normal architecture of kidney cortex of a rat treated with OMP only. Well-developed glomeruli (G), Bowman capsules (BC), Bowman spaces (Bs), distal (ds), and proximal (px) convoluted tubules with interstitial spaces (×250). (C, D) Photomicrographs showing a kidney cortex of a rat treated with rabies vaccine (RV). (C) V1-1: A photomicrograph showing highly elongated and thickened wall of the artery (a) which contains hemolyzed blood cells and enlarged nuclei of tunica intima (→), some degenerated and vacuolated cuboidal epithelial cells of the distal (ds) and proximal (px) convoluted tubules with ruptured brush borders of proximal (px) ones (×250). (D) V1-2: A photomicrograph showing kidney cortex of a rat treated with rabies vaccine (RV). lobulated glomeruli with many pyknotic nuclei (→) in the cuboidal cells of distal (ds) and proximal (px) convoluted tubules, the later show multilayered walls (×250). (E) V2: A photomicrograph showing a somewhat normal appearance of kidney cortex of a rat treated with FRV/K2 (×250). (F) V3-1: A photomicrograph showing highly affected kidney cortex of a rat treated with FRV/K2+alum. Notice: large interstitial hemorrhagic area, highly thickened arterial wall (a) with a narrow lumen, highly distorted (1) atrophied (2) lobulated glomeruli. Some cuboidal cells of convoluted tubules are degenerated (d) (×250). (G) V3-2: A photomicrograph showing highly affected kidney cortex of a rat treated with FRV/K2+alum. Large hemorrhagic area (h) in the interstitial spaces with hemolyzed blood cells and hemosiderin granules (hs) (×250). (H) V4: A photomicrograph showing a nearly normal appearance of kidney cortex of a rat treated with FRV/K2 vaccine+OMP, but brush borders of the proximal (px) convoluted tubules and some cuboidal cells are detected (×250).
**Supplement 2.** Photomicrographs of rat liver tissue. Six different groups: C (negative control group), OMP (sole outer membrane protein [OMP] only), V1 (RV group), V2 (FRV/K2 group), V3 (FRV/K2+inject alum group), and V4 (FRV/K2+OMP). (A) C1: A photomicrograph showing the central and portal areas of liver tissue of a control rat, central vein (cv), cords of hepatocytes (H), sinusoidal spaces (s), branch of the hepatic portal vein (hpv), bile ducts, and a branch of the hepatic artery (a) (×200). (B) C2: A photomicrograph showing central and portal areas of liver tissues of a control rat (×200). (C) OMP: A photomicrograph showing central and portal areas of liver tissues of a rat treated with OMP. Central vein (cv), cords of hepatocytes (H), sinusoidal spaces (s), branch of the hepatic portal vein (hpv), bile ducts (bd), and a branch of the hepatic artery (a) (×200). (D, E) V1-1 and V1-2: Photomicrographs showing the portal area of liver tissues of a rat treated with rabies vaccine (RV). (D) V1-1: A photomicrograph showing highly congested dilated and elongated hepatic portal vein (hpv) with some enlarged nuclei of the endothelial lining highly thickened arterial wall. It contains a large hemorrhagic area (h), increased proliferation and elongation of bd with lymphocytic infiltration and fibrosis (×200). (E) V1-2: A photomicrograph showing: highly dilated central vein (cv) with delaminated endothelial lining. It contains hemolyzed blood cells (×200). (F) V2: A photomicrograph showing somewhat normal central and portal areas of liver tissues of a rat treated with FRV/K2 vaccine (×200). (G) V3-1: A photomicrograph showing highly dilated and congested central vein of a rat treated with FRV/K2 vaccine+alum, which contains numerous hemolyzed red blood cells (×250). (H) V3-2: A photomicrograph showing dilated hepatic portal vein (hpv) of a rat treated with FRV/K2 vaccine+alum, with a thickened arterial wall (a) increased proliferation in walls of bile ducts (bd) with numerous fibroblasts around the portal area (×200). (I) V4: A photomicrograph showing a well-developed architecture of the central area of liver tissues of a rat treated with FRV/K2 vaccine+OMP, since normal appearance of cv, hepatocytes (H), and blood sinusoids (s) are detected (×200).
Supplement 3. Photomicrographs of rat spleen tissue. Six different groups: C (negative control group), OM (outer membrane protein [OMP] only), V1 (RV group), V2 (FRV/K2 group), V3 (FRV/K2+alum group), and V4 (FRV/K2+OMP group). (A) C: A photomicrograph showing splenic tissue of a control rat (×250). (B–D) Photomicrographs showing splenic tissue of rat injected with rabies vaccine (RV). (B) V1-1: A photomicrograph showing highly increased proliferation in the white pulp (W), numerous lymphocytes are detected in red pulps (×200). (C) V1-2: A photomicrograph showing subcapsular red pulps with congested blood sinusoids, numerous degenerated red blood cells with hemosiderin granules (hs), with common signs of vacuolation and degeneration (d) (×250). (D) V1-3: A photomicrograph showing highly thickened corrugated and elongated trabecular vein (V) which contains numerous hemolyzed blood cells, numerous hemosiderin granules (hs) are distributed through red pulps, most red pulps are invaded by WBCs (×250). (E, F) Photomicrographs showing splenic tissue of rat injected with locally prepared rabies vaccine (FRV/K2). (E) V2-1: A photomicrograph showing highly elongated trabecular vein (V) which contains numerous hemolyzed blood cells with lots of lymphocytes inside red pulps and hemosiderin granules (hs) (×250).

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Supplement 3. (Continued) (F) V2-2: A photomicrograph showing highly distorted red (r) and white (w) pulps, highly thickened wall of the central artery (a) with a highly reduced lumen, debris of degenerated cells (d) are detected in the red pulps (×250). (G, H) Photomicrographs showing splenic tissue of rat injected with (FRV/K2+alum). (G) V3-1: A photomicrograph showing corrugated and elongated wall of the trabeculum (T) with numerous hemosiderin granules in it (→) and in the red pulps, highly increased proliferation in the white pulp (w), the central artery shows undetected narrow lumens (×250). (H) V3-2: A photomicrograph showing splenic tissue of rat injected with (FRV/K2+alum), red pulps contain hemosiderin granules (hs) (×250). (I, J) Photomicrographs showing splenic tissue of rat injected with (FRV/K2+OMP). (I) V4-1: A photomicrograph showing highly elongated and thickened wall of the central artery (a) with numerous hemosiderin granules (→) in the red pulps with congested blood sinuses (×250). (J) V4-2: A photomicrograph showing highly increased proliferation in the white pulp (w) (×250).