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RESEARCH ARTICLE

Changing Trend of T lymphocytes in Mouse Spleen after Japanese Encephalitis Virus Infection

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ABSTRACT

Japanese encephalitis is caused by Japanese encephalitis viruses (JEV) with neurotropism. As one of the most important immune organ, spleen is directly involved in immune response against JEV. However, little research about JEV infection process in spleen has been reported. In this study, immunopathological changes in mouse spleen were analyzed every other day after subcutaneous injection of mice with JEV wild-type strain P3 by immunohistochemistry assay. Immunohistochemistry analysis demonstrated that the number of T lymphocytes was reduced from 0 to 3 DPI, increased from 3 to 7 DPI, and reduced again from 7 to 10 DPI. In addition, neurological dysfunction appeared at 6 DPI. These results suggested that spleen of mice suffered incontrovertible damages in influence of JEV infection. It can also be deduced that the cellular immunity took the crucial part in the first phase of transient viremia against JEV. Moreover, immune response was activated after the immune-depressed period in the first phase of viremia and neurological dysfunction appeared when cellular immunity was activated. Taking together, our research showed distinct immunopathological changes in mice after JEV infection, which enriched our understanding of Japanese encephalitis immunopathogenesis.

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INTRODUCTION

Japanese encephalitis (JE) is an acute zoonotic infection caused by Japanese encephalitis viruses (JEV) with neurovirulence and can result in permanent neurological sequelae or death. JEV, a member of flaviviridae, has spread through a vast geographic area including India, China, Japan and virtually all of Southeast Asia (van den Hurk *et al*., 2009), and it has recently been isolated from previously non-affected areas, such as Australia (Hanna *et al*., 1996). Globally, approximately 50,000 cases of JEV infection are reported every year and about 10,000 cases result in death (World Health Organization, 2007). Meanwhile, it also has serious consequences in sows reproduction and death in piglets (Mengeling *et al*., 1993).

As we know, there are usually two phases in course of the Japanese encephalitis. In general, when people or animals are bitten by mosquitoes carrying JEV, the virus may intrude into the skin with mosquito saliva, move through the lymphatic vessels or capillaries monocyte-monocyte-macrophage system, breed in Langerhans cells, dendritic cells or lymph nodes, liver, and spleen, and lead to the formation of the first phase of transient viremia (Mackenzie *et al*., 2004). Once people or animals suffer immune deficiency, JEV can invade the bloodstream again after proliferation to form the second phase of viremia. In this phase, the virus reaches the central nervous system (CNS) through blood-brain barrier (BBB) via leukocytes (probably T lymphocytes), where JEV virions then bind to the endothelial surface of the CNS and are internalized by endocytosis (Ghosh and Basu, 2009). After that, non-suppurative encephalitis occurs, and clinical manifestation is focal neurological deficits, generalized weakness and movement disorders. However, people or animals with well-developed immunity acquire the capacity to combat JEV and recover after the short-term viremia with mild symptom or no symptom.

As the biggest lymphoid organ, the residence of various kinds of immunocytes and the base to respond to blood-borne antigen and generate effector substance, spleen plays the irreplaceable role in immunity to endogenous or ectogenic pathogeny. T lymphocytes which account for about 40% of immunocytes in the

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spleen combat with pathogen through cellular immunity. Although many researchers have been making their endeavor to explore how JEV break through the BBB and the mechanism of JEV addiction to neurons and gliocytes, few researchers have focused on spleen, and the first phase of transient viremia always has been ignored. Therefore, little is known regarding spleen pathological changes in the immune response during the whole viremia after JEV infection.

It has been demonstrated that mice are the prevalent model in the study of flavivirus encephalitis (Kimura *et al*., 2010). Thus in this study, we focused on mouse spleen in the first phase and the second phase of viremia to explore the immunopathological response against JEV infection.

MATERIALS AND METHODS

Animal inoculation

JEV wild-type strain P3 was propagated in suckling mice brains and titered in BHK-21 cells. Fifty four-week old female BALB/c mice were purchased from Wuhan Institute of Biologic Products (Hubei Province, China) and randomly divided into two groups (25 mice per group): challenge group and control group. Mice in challenge group were inoculated subcutaneously with 100μl of Dulbecco's Modified Eagle Medium (DMEM) (high-sugar) containing 6.25×10^3 PFU of wild-type JEV P3. Mice in control group were inoculated subcutaneously with 100μl of DMEM without JEV. Three mice in both challenge group and control group were sacrificed every other day after inoculation, and spleen and brain were harvested.

Perfusion fixation

Perfusion fixation was done after clinical observation and anatomy observation. In perfusion fixation process, blood in the whole body was flushed with 5 ml PBS and then the body was fixed with 5ml 4% paraformaldehyde. Both PBS and 4% paraformaldehyde were injected to ventriculus sinister, and outflowed from atrium dextrum after systemic circulation. Before this operation, a small cut was carefully made by eye scissors on atrium dextrum for fluid outflow. PBS was injected firstly with perfusion speed of 20 ml/min. After liver and kidney became pallid, 4% paraformaldehyde was changed and the perfusion speed was 10 ml/min until mouse tail became stiff. The remains were discarded after bio-safety disposal and the used apparatus also underwent bio-safety disposal. The whole perfusion fixation operation was carefully done in the P2 laboratory in State Key Laboratory of Agricultural Microbiology and all procedures involving animals were carried out in accordance with the Animal Protection Regulations of China.

Histology and immunohistochemistry assay

Transversely 3 mm long Caput lienis each of three spleen on each sampling day was cut. In the same way, front half of brain was cut transversely on eacg sampling day. All tissues were embedded in paraffin wax and 3 μm successive sections were made, stained with H & E and immunohistochemistry (IHC) was carried out. For IHC, slides were stained after deparaffinating and hydration. Antigen was retrieved in citrate buffer (PH 6.0) with microwave, and endogenous avidin and biotin were blocked with Avidin/Biotin Blocking Kit (Vector, SP-2001). Rabbit polyclonal antibody CD3 (Chemicon) (dilution 1:200), GFAP (DakoCytomation) (dilution 1:1000) and IBA (Wako) (dilution 1:500) were used as the primary antibodies. Goat or horse serum was used as blocking antigen and then slides were incubated in 4°C for 15-18h with primary antibodies. Afterward, biotinylated secondary antibodies were incubated in 37°C for 30 min. Avidin and biotinylated alkaline phosphatases macromolecular complex (ABC) reagent was added and incubated for 20 min at 37°C. Goat or horse serum block liquid, second antibody and ABC reagent came from ABC-AP kit (Vector) or ABC kit (Vector). Finally, visualization was achieved by using Blue Alkaline Phosphatase Substrate Kit III (Vector) or DAB kit (Vector). Sections were counterstained with fast red or hematoxylin according to the need.

Sections were analyzed by the application of Zeiss microscope and accompanying software (Zeiss). Quantitative analyses of T lymphocytes were performed by optical density value (OD value) through analyzing the grayscale in positive region on IHC sections with Image-Pro Plus. There were three samples on each sampling day in both challenge group and control group, and at least three sections were made in each sample. Furthermore, pictures were collected under the same conditions and analyzed in the same camera shooting environment.

Statistics analysis

Variance was analyzed with two specimens. Statistical analysis was performed using the student t-test considering significant difference at $P<0.05$ and $P<0.01$.

RESULTS

Neurological dysfunction

From 3 to 5 days post infection (DPI), mice in challenge group showed depressed spirit and piloerection. At 6 DPI, mice in challenge group began to suffer hypokinesia, sluggish, frequent blinking, lethargy, and clustering, suggesting the neurological dysfunction of infected mice. Some mice lost weight and became unresponsive and stiff, and some of them were led to death. Four, 6, 4, and 2 mice died respectively at 7, 8, 9, and 10 DPI. Liquid feces adhesion was found on the buttocks of the two dying mice at 10 DPI. There was no death after 11 DPI, and the survival mice turned to be normal after 11 DPI. Control group showed no symptoms and no death (Fig. 1).

Congestion and hemorrhages on cranial arachnoid

Cranial arachnoid of mice in challenge group showed congestion and hemorrhage. The liver of one mouse which died at 10 DPI turned black. Pathological changes from 0 to 10 DPI became severer with age. However, no lesion was found in other organs and mice in control group had no pathological changes.

Non-suppurative encephalitis

During 8 to 10 DPI, obvious non-suppurative encephalitis was observed in brains of mice in the

challenge group. Necrosis and even acute liquefaction necrosis was seen in some neurons. It was also found that glial nodule appeared near the vessel (Fig. 2B). Moreover, gliocytes and microglia cells were activated in the challenge group. Astrocytes were also activated and interwove with each other. However, no pathological changes appeared in the control group (Fig. 2A & 2C).

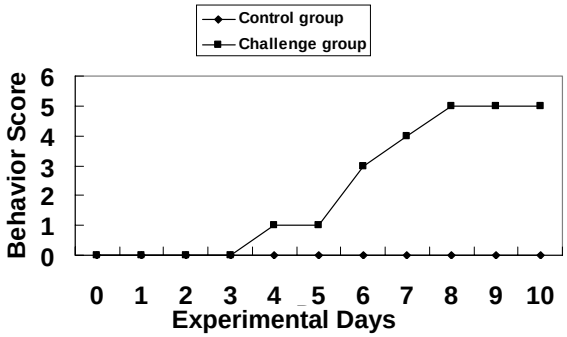

Fig. 1: Neurological dysfunction after JEV infection. 0=no depressed spirit, no piloerection, no frequent blinking, no movement disorder, no swelling eyelid; 1=depressed spirit, no piloerection, no frequent blinking, no movement disorder, no swelling eyelid; 2=depressed spirit, piloerection, no frequent blinking, no movement disorder, no swelling eyelid; 3=depressed spirit, piloerection, frequent blinking, no movement disorder, no swelling eyelid; 4=depressed spirit, piloerection, frequent blinking, movement disorder, no swelling eyelid; 5=depressed spirit, piloerection, frequent blinking, movement disorder, swelling eyelid.

T lymphocytes decreased from 0 to 3 DPI, increased from 3 to7 DPI and decreased again from 7 to 10 DPI

T lymphocytes were primly distributed around periarterial lymphatic sheath (PALS) in mice of both the challenge and the control groups, just as the regular distribution of T lymphocytes (Fig. 3). Trends of T lymphocytes number were analyzed by detecting the average OD value of CD3 positive area (Fig. 4). Compared with the control group, T lymphocytes decreased during the first three days post-infection in the challenge group, but the difference between these two groups was not significant. Subsequently, T lymphocytes gradually increased from 3 to 7 DPI, while the difference was also not remarkable. From 7 to 10 DPI, T lymphocytes decreased again with the significant difference compared to control group ($P<0.01$) (Fig. 4).

DISCUSSION

Since spleen is the biggest lymphoid organ which plays the crucial role in immunity to endogenous or ectogenic pathogeny, investigating immunopathological changes of host spleen with viral infection may be helpful for understanding the mechanism of JEV pathogenesis. Our study showed that neurological symptoms in JEV-infected mice appeared at 6 DPI, and T lymphocytes increased from 3 to 7 DPI and decreased in the next three days. It suggests that cellular immunity takes the crucial part in the first phase of viremia against JEV.

The increase of T lymphocytes in the first phase of viremia from 3 to 5 DPI before BBB break may provide significant protection against JEV replication. It has been demonstrated that $CD4^+$ T lymphocytes can inhibit the proliferation of JEV (Diagana *et al*., 2007). Moreover, the cellular immune response seemed to contribute to the repression of JE during acute infection by restricting viral replication before the CNS is invaded (Misra and Kalita, 2010). Therefore, the increase of splenic T lymphocytes in the first phase of viremia is an attempt to eliminate the virus. If so, the JEV entrance into brain will be inhibited and no neurological symptom will occur. However, cellular immunity has both positive and negative impact in the fight against JEV. Cytotoxic T lymphocytes rose rapidly to play a protective role to increase viral clearance. Whereas, the robust T lymphocyte response can be damaging to the host (Fujii *et al*., 2008) for it initiated an irreversible cellular immune activation leading to cell death. Mice lacking functional CD8+ T lymphocytes, or mice depleted of CD8+ T lymphocytes, show increased mortality, but survive longer than wild-type mice with low dose of WNV, indicating that CD8+ T lymphocytes have both protective and immunopathological effect (King *et al*., 2007). Unluckily, in this study, T lymphocytes increase failed to restrain JEV proliferation in this young mice model as most of the mice showed neurological symptoms. The robust T lymphocyte response from 3 to 7 DPI to resist JEV seemed also helpful to promote the first phase of viremia to turn to the second phase.

T lymphocytes had the initial immune-depressed period in the first phase of viremia in which organism was in the preparation for resistance to alien JEV. T lymphocytes decreased in the first three days in challenge group. The balance between cells and JEV interactions determines the outcome of the viral infection and disease progression (Pastorino *et al*., 2010). It may need about three days for the rebuilding of proper immunity balance after the immunity break in response to JEV-infection, which may be ascribed to the relative low increase of T lymphocytes compared to the high increase of JEV virulent strain with the incubation amount of 6.25×10^3 PFU.

The distinct reduction of splenic T lymphocytes from 8 to 10 DPI may be caused by T lymphocytes damage in spleen and its transfer to blood or brain. It indicates that the cellular immunity was not just out of existence in the second phase, but mainly transferred to other organs such as brain, the JEV target organ. The damage of T lymphocytes might have been caused by the cell apoptosis or death in the first and second phase of viremia. The examination of apoptosis of T lymphocytes and other cells in spleen under the influence of JEV is under investigation and will be reported later. T lymphocytes are usually transferred to brain out of spleen through blood circulation to provide immune surveillance and this may be hastened by neuro-inflammation. T lymphocytes injury in neuro-inflammation after entry into brain may accelerate T lymphocytes loss in the spleen, and may also cut off its return back to spleen as the normal T lymphocytes recirculation. This process consists of two steps as follows:

In the first step, neuro-inflammation was activated after JEV entered the brain. Non-suppurative encephalitis areas in JEV-infected mice showed inflammatory cell infiltration and edema, with a predominance of activated

T lymphocytes, macrophages and B lymphocytes (Gould *et al*., 2008). Increased astrocyte activation was shown in our model, which implicates the enhanced ability to detoxify glutamate, inactivate free radicals, and produce neurotrophic factors, although it is not sufficient in conferring protection against JEV-mediated pathogenesis (Mishra *et al*., 2007). It was also shown in our model that microglia cells were activated in response to JEV infection. However, it was verified that uncontrolled overactivation of microglia cells (Ghoshal *et al*., 2007) releases pro-inflammatory cytokines such as interleukin 6

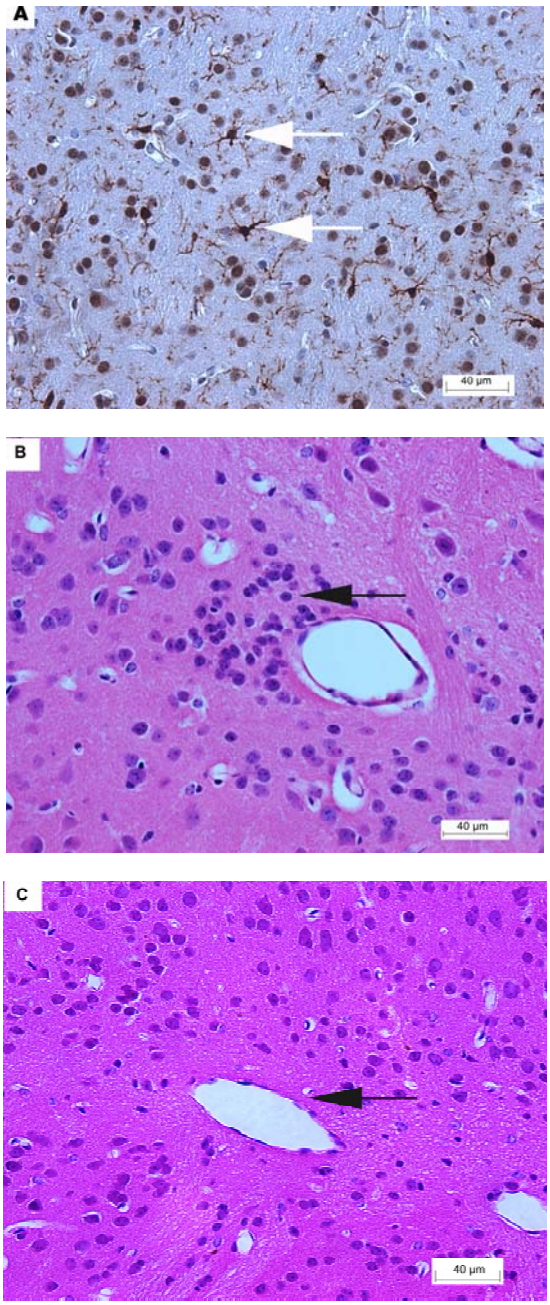

Fig. 2: Histology of brain in control group and pathological changes after JEV infection. (A) Microglia activation, IBA antibody, IHC, 400×, light microscope, arrows show activated microglia; (B) Glial nodule near vessel, H & E, 400×, light microscope, arrow show glial nodule near vessel; (C) Vessel in the control, H & E, 400×, light microscope, arrow show normal vessel without glial nodule around.

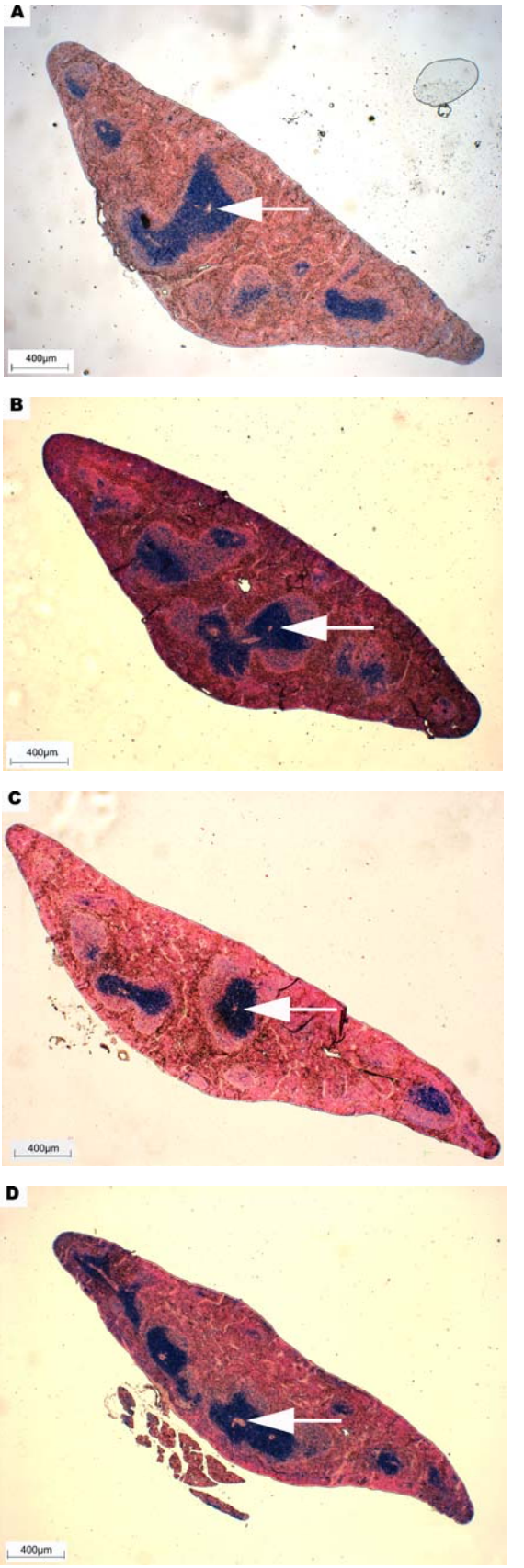

Fig. 3: T lymphocytes in challenge group dropped at 8 DPI and 10DPI. (A) 8 DPI, spleen IHC-infected; (B) 8 DPI, spleen IHC-control; (C) 10 DPI, spleen IHC-infected; (D) 10 DPI, spleen IHC-control. (A-D): CD3 antibody, IHC, 40×. Arrows show PALS (periarterial lymphatic sheath), T lymphocytes were all around PALS.

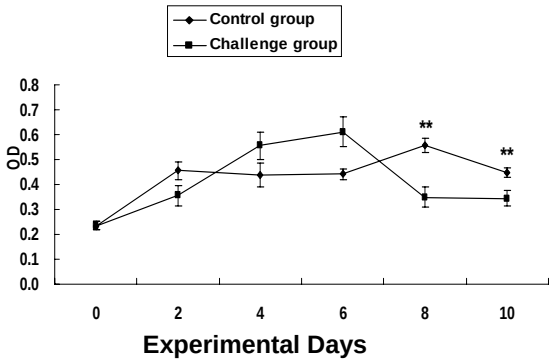

Fig. 4: The growth and decline trend of lymphocytes after JEV infected. OD: optical density of CD3 positive area; ** stands for highly significant difference (** P<0.01).

(IL-6), TNF-a monocyte chemotactic protein 1 (MCP1), and RANTES (regulated upon activation, normal T lymphocytes expressed and secreted), promoting massive leukocyte migration and infiltration into the brain in Japanese encephalitis (Gupta *et al*., 2010).

In the second step, T lymphocytes transferred into brain to provide immune surveillance and protect JEV-infected mice from death after neuro-inflammation was activated. Nevertheless, if cellular immunity is not strong enough, the entrance of T lymphocytes into brain can also aggravate the neuro-inflammation and result in JEV hidding in the T lymphocytes (Mathur *et al*., 1989). T lymphocytes entry into the brain has been demonstrated by many studies. For instance, Fujii *et al*. (2008) found that the high infiltration of T lymphocytes in the brain of mice infected with JEV. T lymphocytes entry into the brain for immune surveillance is not only reported in Japanese encephalitis but also in West Nile Fever and tick borne encephalitis caused by West Nile virus and tick-borne encephalitis virus which also belong to flavividae family (Sitati *et al*., 2007). Wang *et al*. (2003) proposed that CD8+ T lymphocytes mediated West Nile virus encephalitis recovery and it was also reported that the response of $CD4^+$ T lymphocytes was necessary for the CNS to remove West Nile virus (Sitati and Diamond, 2006). In tick borne encephalitis, it was reported that $CD8^+$ T lymphocytes could extend the survival of tick-borne encephalitis infected severe combined immune-deficiency (SCID) mice or $CD8^+$ deficient mice. It was also confirmed that $CD8^+$ T lymphocytes were involved in immunopathogenesis of tick-borne encephalitis (Růžek *et al*., 2009).

The sixth day post-infection on which neurological symptoms appeared was the critical turning point from the first phase of viremia to the second phase of viremia after JEV infection. Once JEV breakthrough the blood brain barrier (BBB), the neurons began to be under threat and perhaps the microglia cells and astrocytes were on their initiation of activation. At 6 DPI, the increase of lymphocytes was observed in JEV-infected mice, so 6 DPI may be the time point when cellular immunity strengthened. Since cellular immunity was failed to combat against ectogenic pathogeny JEV in spleen during the first phase of viremia, on this time point, some splenic T lymphocytes suffered damage and some began to migrate to brain, which was induced by neuro-inflammation. Therefore, cellular immunity was activated to resist the JEV infection when neurological dysfunction appeared.

In conclusion, mice pathological model with JEV infection was successfully constructed in our study. Spleen as the biggest immune organ suffered irreversible damage in influence of JEV. The quantity of T lymphocytes changed and took on some regular trends which represent the strength of cellular immunity in resistance to JEV infection. Our results demonstrated that the cellular immunity took the crucial part in the first phase of viremia against JEV. Nevertheless, it needed about three days for cellular immune preparation for resistance to ectogenic JEV. Therefore, cellular immunity had the initial immune-depressed period in the first phase of viremia. Then the immune response was activated after the immune-depressed period and neurological dysfunction appeared when cellular immunity was activated. In addition, T lymphocytes were damaged in spleen and transferred to brain in the second phase of viremia. This may be strongly beneficial for the mice survival after CNS infection. In a word, our research showed the elaborate immunopathology changes of host with JEV infection, which may provide some clues for understanding the mechanism of Japanese encephalitis pathogenesis.

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