Viral infection induces the production of type I interferons (IFNs), which inhibit viral replication through a variety of mechanisms (1, 2). To establish effective infection, viruses need to develop strategies to evade the immune responses. Sindbis virus is a prototypic member of the Alphavirus genus, whose members include viruses such as eastern, western, and Venezuelan equine encephalitis viruses that can cause fatal encephalitis in humans and equines (3). Sindbis virus infection of mice has been used as a model to study the pathogenesis of acute viral encephalitis for many years (4). After peripheral inoculation, localized replication leads to viremia, with subsequent spread to various tissues (5). The outcome of infection is both age and virus strain dependent. Some strains of the virus display neuroinvasive properties and after peripheral inoculation can spread through the blood to infect the central nervous system, while others are neuroviral and cause disease only after direct inoculation into the brain. Similarly, age affects the outcome; some strains spread from the periphery and cause fatal encephalomyelitis in suckling mice but not in weanling mice (6). A strain of Sindbis virus that is both neuroinvasive and neuroviral (SVNI), adapted from extensive passage in mouse brains, can reach the brain and cause lethal encephalitis in weanling mice after peripheral inoculation (7, 8).

Sindbis virus infection induces type I IFN production in a manner dependent on RIG-I, MDAS, or PKR (9, 10). Type I IFN plays important roles in controlling Sindbis virus infection, as the absence of type I IFN signaling results in an otherwise avirulent virus gaining the ability to propagate, disseminate, and become rapidly fatal (11). Multiple IFN-stimulated genes have been reported to act as antiviral factors against Sindbis virus (12, 13).

ZAP is an IFN-stimulated host factor that specifically inhibits the replication of certain viruses in cell culture, including both RNA and DNA viruses such as Sindbis virus (14), Ebola virus (15), human immunodeficiency virus 1 (16), and hepatitis B virus (17). ZAP is not a universal antiviral factor; some viruses replicate normally in ZAP-expressing cells (14). Whether a virus is susceptible to ZAP seems to be determined by the presence of specific se-
quences in the viral mRNAs (18, 19). ZAP specifically binds to target viral mRNA and inhibits its expression by repressing the translation and/or promoting the degradation of the mRNA (16, 20). In addition to the antiviral activity, some cellular functions of ZAP have been reported. ZAP promotes the degradation of TRAILR4 transcript and thus increases the sensitivity of cells to TRAIL-induced apoptosis (21). ZAP is also involved in regulating microRNA-mediated gene silencing (22, 23).

ZAP efficiently inhibits the replication of Sindbis virus in cell culture (14), and the antiviral activity of ZAP requires the expression of other IFN-stimulated genes (ISGs) (24). Recently, Kozaki et al. reported that infection of 10-day-old suckling ZAP knockout (KO) mice with a Sindbis virus strain that is neurovirulent but does not cause lethal encephalitis resulted in elevated viral titers in the brain (25). In the present study, we show that consistent with their results, infection of suckling mice with SVNI caused faster animal death in the knockout mice. However, SVNI infection of 23-day-old weanling mice resulted in better survival of the knockout mice. We show that in ZAP knockout weanling mice the viral replication in lymphoid tissues is enhanced but the viral replication in the brain is considerably restricted. Further analyses revealed that enhanced viral replication in the lymphoid tissues induced higher levels of type I IFNs, which protected the central nervous system from viral infection in the knockout mice.

MATERIALS AND METHODS

Reagents. All of the antibodies for fluorescence-activated cell sorter (FACS) assays, including anti-CD3, anti-CD19, anti-NK1.1, anti-CD4, anti-CD8, and anti-CD69, were purchased from eBioscience.

Generation of ZAP knockout mice. A fragment of the Zc3hav1 (ZAP) gene was retrieved first. To construct the targeting vector, the first loxP-Neo-loxP cassette was inserted into the left arm of exon 2 and deleted with Cre recombinase to leave just one loxP site. The second loxP/FRT-Neo-FRT/loxP cassette then was inserted into the right arm of exon 2 (Fig. 1). Linearized targeting vector was electroporated into embryonic stem (ES) cells from 129 mice (Jackson Laboratories). After G418 selection and mini-Southern blot analyses, positive ES cell clones were identified. Pos-
itive clones were injected into C57BL/6I blastocysts, which were then uteri-
transferred to pseudopregnant recipients to produce 129/C57BL/6I
chimeras. By crossing the chimeric mice with C57BL/6I mice, ZAPloxP/+ 
mice carrying one conditional mutant form were obtained. In
order to generate conventional ZAP knockout mice, the ZAPloxP/+ 
mice were crossed with Ela-Cre transgenic mice (Jackson Laboratories) to
delete the second exon of the ZAP gene and backcrossed into the
C57BL/6I background for eight generations. The ZAP knockout mice and
wild-type mice used in this study were obtained by crossing the ZAP+/−
mice.

Genotyping of mice. The genotypes of ZAP knockout mice were an-
alyzed by PCR using mouse tail genomic DNA as the template. For ZAP
knockout mice, the 0.5-kb PCR product (using primer 1 and primer 2) was
detected. For wild-type mice, the 0.25-kb product (using primer 3 and
primer 2) was detected (Fig. 1B).

That ZAP knockout was successful was also verified by analysis of mRNa by reverse transcription-PCR (RT-PCR). PCR using primers
ZAP-Sp and ZAP-AP, flanking the 2nd exon of mRNA by reverse transcription-PCR (RT-PCR). PCR using primers
primer 2) was detected (Fig. 1B).

The following primers were used: primer 1, 5′-GGCGCCAGGACTCAGCTC; primer 2, 5′-GACGAGCTCTTTTCCATGCCCATACTGAGT-3′; ZAP-Sp, 5′-AGCGACCCCTTTCTTCATGCCTGAG; GAPDH-AP for genomic PCR, 5′-AAGCTCACTGGCATGGCCTT; ZAP-AP, 5′-ATGACCGATGCCGGAGGT ATTCTGTT; and ZAP-AP, 5′-GCAATGTCGCCCCGGGTAGTGCTCA.

Plasmid construction. pSVNI is an infectious clone of SVNI that has
been reported previously (7). Plasmid encoding SVNI-nsP3-nLuc, an
SVNI and the reporter

plasmid was constructed by in-frame insertion of the nLuc coding sequence (Promega) into the SpeI site within the nsP3-coding region of pSVNI.

Cell culture. All cell lines were maintained at 37°C in humidified chambers containing 5% CO2. Baby hamster kidney cells (BHK-21) (ATCC) used for SVNI production and viral titer measurement were cul-
tured in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) sup-
plemented with 10% fetal bovine serum (FBS) (Gibco). Mouse embryonic fibroblast (MEF) cells from wild-type and ZAP knockout mice were pre-
pared from day 13.5 embryos and cultured in DMEM supplemented with
10% FBS. Peritoneal macrophages were harvested from the peritoneal
cavity. In brief, 1× phosphate-buffered saline (PBS) (FBS) containing 2% FBS
was injected into the peritoneal cavity. After three rounds of aspiration
and injection with a syringe, the peritoneal fluid was collected. Cells were
washed 3 times with PBS and seeded in RPMI 1640 (Invitrogen) supple-
mented with 10% FBS. After 1 to 2 h, the unattached cells were washed
away and the remaining cells were cultured overnight for further use.

Virus production, titration, and infection. SVNI and the reporter
virus SVNI-nsP3-nLuc were produced and titrated essentially as previ-
sely reported (14). Briefly, the infectious clones pSVNI and pSVNI-
nsP3-nLuc were linearized with Xhol and in vitro transcribed into RNA with
SP6 RNA polymerase (Promega) in the presence of a Cap analog
(Promega). The transcripts were transcribed into BHK-21 cells using
Lipofectamine 2000 (Invitrogen) by following the manufacturer’s in-
structions. At 24 h posttransfection, the virus in the medium was har-
ested and stored at −80°C.

Virus stocks and virus samples were titrated in duplicate by infection
of BHK-21 cells at serial dilutions in DMEM supplemented with 1% FBS.
At 1 h postinfection, cells were covered with DMEM overlay containing
1.2% agarose and 2% FBS. Plaques were enumerated at 1 to 2 days postinfection.

MEF cells were seeded at 3×10^5 cells per well in a six-well plate
the day prior to infection. The cells were infected with the virus for 1 h at
a multiplicity of infection (MOI) of 0.01 in DMEM supplemented with 1%
FBS. Cells were washed twice with 1× PBS and cultured in DMEM with
2% FBS. At 48 h postinfection, supernatants were collected and titrated in
duplicate on BHK-21 cells. Macrophage cells were seeded at 3×10^5 per
35-mm dish the day prior to infection. Cells were infected with SVNI or
SVNI-nsP3-nLuc at an MOI of 5. At various time points postinfection,
cells were lysed for total RNA isolation or luciferase activity assay.

Mouse infection, tissue collection, and nLuc activity analysis. Twenty-
three-day-old mice were challenged with 13,000 PFU of SVNI by intra-
peritoneal injection or 3,000 PFU of SVNI by intracranial injection. Ten-
day-old mice were challenged intraperitoneally with 3,000 PFU of SVNI.
Eight-week-old mice were challenged intraperitoneally with 39,000 PFU
of SVNI. Disease progression and mortality were monitored daily. Blood
was collected by retro-orbital bleeding for viral titer determination and
IFN assays. To measure the viral titers in the central nervous system,
animals were perfused with PBS. The brain and spinal cord were then
extracted, weighed, frozen, and thawed twice, followed by homogeniza-
tion in PBS. Serial 10-fold dilutions of each homogenate were assayed on
BHK-21 cells to determine viral titers. For SVNI-nsP3-nLuc-infected
mice, tissues were homogenized in 1× passive lysis buffer (Promega) prior
to luciferase activity assay by following the protocol for the Nano-
Glo luciferase assay system (Promega).

To administer the neutralizing antibody against type I IFN receptor 1
(IFNAR1) (clone MAR1-5A3; BioXcell), 23-day-old mice were treated
by intraperitoneal injection with the antibody at the following time points
(and doses): day −1 (500 μg), day 0 (500 μg), and day 2 (250 μg). Mock-
infected animals received an equivalent volume of PBS. On day 0, mice
were challenged with SVNI (13,000 PFU per animal) by intraperitoneal
injection. Disease progression and mortality were monitored daily.

Real-time PCR. Total RNA from tissues or cells was extracted using
TRIzol reagent (Invitrogen) by following the manufacturer’s instructions,
followed by reverse transcription with murine leukemia virus (MLV) re-
verse transcriptase using random primers. The levels of mRNAs encoding
ZAP, ISG15, and IFI27 were measured by SYBR green real-time PCR in a
Rotor-gene 6000 (Corbett Life Science) using the following program: (i)
50°C for 2 min, 1 cycle; (ii) 95°C for 5 min, 1 cycle; (iii) 95°C for 15 s, 60°C
for 30 s, and 72°C for 30 s, 40 cycles; and (iv) 72°C for 10 min, 1 cycle.
The level of mRNA encoding glyceraldehyde-3-phosphate dehydrogenase
(GAPDH) served as an internal control. The primer sequences are the
following: qmZAP F, 5′-ATATCGAGCGGGCCTATTGTG-3′; qmZAP R, 5′-GCTCCTCATGGCCATCTGAGT-3′; qmISG15 F, 5′-GGTTGTCGCC
TGACTAACCTCAT-3′; qmISG15 R, 5′-TGAGAAAGGGTAAACCCGTC
CT-3′; qmIFI27 F, 5′-GCTGTTGAGGAAATGCCGGATTTG-3′; qmIFI27 R, 5′-GGATGGCATTTGTTGAG-3′; qmGAPDH F, 5′-CAGAG
CAACCTCACCTCCT-3′; and qmGAPDH R, 5′-GGTTTCAAGGTCTT
TATTCTCCT-3′.

Cytokine measurement. Mouse serum levels of IFN-α and IFN-β were determined using enzyme-linked immunosorbent assay (ELISA)
kits from eBioscience and PBL Assay Science, respectively. All measure-
ments were performed by following the manufacturer’s protocol.

Flow cytometry analyses. After harvest, the spleen was transferred to
a 6-cm dish containing 3 to 5 ml of ice-cold FACS buffer (2%, vol/vol, FBS in
1× Dulbecco’s PBS) and teased into a single-cell suspension by pressing
with the plunger of a 5-ml syringe in a cell strainer (BD). Cells were
collected by centrifuging the suspension for 5 min (350 ×g) at 4°C. Red
blood cells were removed with red blood cell lysis buffer (BioLegend) by
following the manufacturer’s instructions. About 1 million cells were
stained with labeled antibodies and analyzed with a BD FACS Calibur
analyzer.

Statistical analyses. All statistical analyses were performed in Prism
(GraphPad Software). Means between two groups were compared using
the two-tailed t test. Kaplan-Meier survival curves were compared using
the log-rank (Mantel-Cox) test. A P value of <0.05 was considered signif-
icant.

Ethics statement. All mice were housed under specific-pathogen-free
conditions in the animal care facilities at the Institute of Biophysics, Chi-
nese Academy of Sciences. All animal experiments were performed in
strict accordance with the Guide for the Care and Use of Laboratory An-
imals issued by the Ministry of Science and Technology of the People’s
Republic of China (www.lascn.net/Item/14.aspx). The project and protocol were approved by the Institutional Laboratory Animal Care and Use Committee (license no. DWSWAQ[ABSL-2]201303).

RESULTS
ZAP knockout increases the sensitivity of suckling mice but decreases the sensitivity of weanling mice to SVNI-induced disease progression. To study the role of ZAP in viral infection in vivo, ZAP knockout mice were generated. loxP sequences were first inserted to flank the second exon of the ZAP-encoding Zc3hav1 gene by gene targeting (Fig. 1A). The mice were then crossed with transgenic mice expressing EIIa-Cre to generate mice with complete deletion of the second exon (see Materials and Methods for details). Since the protein sequences encoded by the second exon are critical for target RNA binding and deletion of this exon results in a frameshift in the subsequent coding sequence, functional ZAP protein should be lost in the knockout mice. Genotypes were confirmed by PCR, as well as by analysis of the resulting mRNA (Fig. 1B and C). No obvious abnormal gross morphological and behavioral characteristics were observed in the ZAP knockout mice.

We first analyzed the antiviral activity of endogenous ZAP against SVNI in cell culture. Mouse embryonic fibroblast (MEF) cells were prepared from littermate wild-type and knockout mice and infected with SVNI. At 48 h postinfection, viral titers in the culture supernatants were measured. ZAP-deficient cells supported better virus replication than the wild-type cells (Fig. 2A).

To next infected mice with SVNI by intraperitoneal injection and analyzed virus-induced disease progression. Recently, Kozaki et al. reported that infection of 10-day-old suckling mice with a Sindbis virus strain that is neurovirulent but does not cause lethal encephalitis resulted in elevated viral titers in the brain of ZAP KO mice (25). To test whether SVNI and the Sindbis strain used in their study behave differently, we infected 10-day-old mice with SVNI. Consistent with their results, ZAP knockout mice died faster than wild-type mice (Fig. 2B), indicating that knockout of ZAP rendered the suckling mice more sensitive to Sindbis infection. Since the SVNI used here is both neuroinvasive and neurovirulent and causes lethal encephalitis in weanling mice, we used the virus to infect 23-day-old weanling mice, whose immune system is more developed than that of the suckling mice. Given the above-described results, we expected that the weanling knockout mice should also die faster from the viral infection. Surprisingly, however, the average survival time of the knockout mice was significantly longer than that of the wild-type mice (Fig. 2C).

To probe the role of ZAP in SVNI infection in the adult mice, 8-week-old mice were infected with the virus. No symptoms were
observed in either ZAP knockout or wild-type mice even at 20 days postinfection (Fig. 2D).

ZAP knockout results in increased SVNI replication in peripheral tissues but reduced viral replication in the central nervous system of weanling mice. To understand the phenotypic differences between ZAP knockout and wild-type mice of different ages, we first analyzed SVNI titers in the serum and central nervous system. The 10-day-old mice were infected by intraperitoneal injection. At the time points indicated, samples were collected, homogenized in PBS, and clarified by centrifugation. SVNI titers were measured on BHK-21 cells. (A) Mice (n = 4 per genotype; 10 days old) were infected with SVNI (3,000 PFU per mouse). (B to D) Mice (23 days old; n = 5 per genotype [B], n = 8 per genotype [C], and n = 4 per genotype [D]) were infected with SVNI (13,000 PFU per mouse). (E and F) Mice (n = 8 per genotype; 8 weeks old) were infected with SVNI (39,000 PFU per mouse). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ND, not detected.
knockout mice were about 10-fold higher than those in the wild-type mice, but at 36 h postinfection the titers were not significantly different (Fig. 3B). In contrast to the serum viral titers early after infection, at 2 and 5 days postinfection, the viral titers in the brain and spinal cord of the weanling knockout mice were much lower than the viral titers in the wild-type mice (Fig. 3C and D). Again, the difference in the viral titers in the central nervous system is consistent with the difference in the survival of these mice. For 8 week-old mice, higher viral titers were detected in the serum of ZAP knockout mice at 24 h postinfection (Fig. 3E). However, no virus was detected in the brain at 2 or 5 days postinfection (Fig. 3F). These results indicate that although the virus replicated better in the periphery in the adult knockout mice, they failed to enter the brain, which is consistent with the above-described survival phenotype (Fig. 2D).

It has been documented that Sindbis virus initially replicates in the CD11c–CD11b+ cells in the draining lymph nodes and spleen (11, 26). In addition, ZAP is mainly expressed in lymphoid tissues, including spleen, lymph nodes, and thymus (Fig. 4A), and SVNI infection further upregulates ZAP expression (Fig. 4B). Thus, we analyzed the replication of the virus in lymphoid tissues early after infection of 23-day-old mice. For more sensitive measurement of the viral replication in these tissues, a replication-competent reporter SVNI virus expressing nano-luciferase (SVNI-nsP3-nLuc) was constructed. Increased virus replication is expected to result in increased expression of the reporter. The results showed that in all lymphoid tissues examined, which included inguinal and mesenteric lymph nodes, spleen, and peritoneal exudates, the reporter was expressed significantly better in the knockout mice than in the wild-type mice (Fig. 4C to F). The difference in the reporter virus expression was significant (Fig. 4C to F).
expression levels in the lymphoid tissues early after infection is consistent with the difference in the early serum viral titers between the knockout and wild-type mice. Collectively, these results demonstrate that compared to the wild-type mice, ZAP knockout weanling mice support higher SVNI replication levels in the lymphoid tissues early after infection, but that viral spread to or replication within the central nervous system is restricted.

Higher viral titers in the periphery of ZAP knockout mice result in higher-level immune activation. To determine whether ZAP knockout affects the immune responses to SVNI infection, we analyzed distributions of the splenocyte subsets of both 10-day-old and 23-day-old mice with or without SVNI infection. In naive mice, no obvious difference between wild-type and ZAP knockout mice was observed in the total number of white blood cells (data not shown) or in the distributions of splenic lymphoid subsets (Fig. 5 and 6). Upon SVNI infection, the percentage of CD69+ cells increased significantly (Fig. 5 and 6). CD69 expression is rapidly induced in activated T and B cells, neutrophils, and NK cells (27, 28). Increased percentages of CD69+ cells indicated that SVNI infection activated the immune system. In both 10-day-old and 23-day-old mice, higher levels of CD69+ cells were observed in ZAP knockout mice (Fig. 5 and 6), which were in parallel with the higher serum viral titers in these mice (Fig. 3A and B). These results suggest that ZAP knockout generally does not affect the immune system in naive mice or activation of the immune system upon SVNI infection.

SVNI induces higher IFN levels in the peripheral tissues and higher ISG levels in the brain of weanling ZAP knockout mice. Viral infection induces the production of type I IFNs, which play important roles in determining the outcome of the infection. We next analyzed the levels of IFN-α and IFN-β at various time points postinfection. Without SVNI infection, the serum levels of IFN-α and IFN-β were not detectable (data not shown). In both knockout and wild-type weanling mice, the highest levels of serum IFN-α were detected at 12 h postinfection, and the IFN-α levels dropped thereafter over time (Fig. 7A). At 12 h and 24 h postinfection, the serum levels of IFN-α in the knockout mice were significantly higher than those in the wild-type mice (Fig. 7A). In comparison, at 36 h postinfection, the serum IFN-α levels were not significantly different (Fig. 7A). Compared with IFN-α levels, IFN-β levels were much lower in both types of mice (compare Fig. 7A and B). Nonetheless, IFN-β levels in the serum of knockout mice were still significantly higher than those in the wild-type mice (Fig. 7A). These results demonstrate that the serum levels of IFN-α paralleled the serum viral titers early after infection, consistent with the results previously reported by others (11, 29–34). These results also suggest that IFN-α is a major type I IFN induced by SVNI infection.

We next analyzed IFN-α levels in the central nervous system. At 2 days postinfection, IFN-α levels in the brain from either ZAP knockout or wild-type mice were below the detection limit of the
ELISA. Thus, we measured the mRNA levels of two representative ISGs, ISG15 and IFI27, to determine to what extent the cells in the brain have been exposed to type I IFN. The results showed that the brain mRNA levels of both ISG15 and IFI27 were higher in the knockout mice (Fig. 7C and D), implying greater exposure to IFN in the brains of knockout animals. At this same time point, however, the viral titers in the brain were lower in the knockout mice (Fig. 3C), suggesting that ISG expression limits viral spread to or replication in the brain. At 5 days postinfection, when the brain IFN-α protein levels could be easily detected by the ELISA, the IFN-α levels in both brain and spinal cord were lower in the knockout mice than in the wild-type mice (Fig. 7E and F), in parallel with the viral titers (Fig. 3D). This suggests that at this time point IFN-α levels are a consequence of viral replication levels and that IFN is locally produced.

IFN-α is induced mostly in cells of lymphoid origin, whereas IFN-β is produced by most cell types (35). It has been reported that upon infection by intraperitoneal injection the virus first primarily comes in contact with peritoneal macrophages and macrophage-derived dendritic cells (36), and that macrophage-like cells may serve as important early IFN-α producers after alphavirus infection (26). To further probe the role of ZAP in controlling SVNI infection and the subsequent production of IFN-α, macrophages were isolated from peritoneal exudates (n = 6 mice per genotype; 23 days old) and infected with SVNI-nsP3-nLuc reporter virus. At the time points indicated, cells were lysed and subjected to measurement of luciferase activity (G) or ISG15 mRNA levels (H). *, P < 0.05; **, P < 0.01; ***, P < 0.001.
surrogate, we again used ISG15 mRNA levels as an indicator of the type I IFN level. As expected, viral infection induced ISG15 expression, and the virus-induced ISG15 levels were much higher in the macrophages isolated from the knockout mice than in cells from wild-type mice (Fig. 7H). Collectively, these results define ZAP’s important role in the modulation of SVNI replication in macrophages and thereby in limiting virus-induced production of IFN-α in the host.

**Blocking of the type I IFN receptors renders weanling ZAP knockout mice more sensitive to viral infection than the wild-type mice.** The above-described results strongly suggest that SVNI replication in the lymphoid tissues of weanling mice induces the production of IFN-α, which restricts subsequent viral infection of the central nervous system. To substantiate this notion, we analyzed the relationship between serum IFN-α levels and disease progression in the wild-type mice. Indeed, the serum IFN-α level was statistically proportional to the survival time of the mice (Fig. 8A), with animals having the highest IFN-α levels also surviving the longest. To further prove that virus-induced IFN-α plays a critical role in the protection of the central nervous system, we used a neutralizing antibody against type I IFN receptor 1 (IFNAR1) to block type I IFN action. Mice were infected with SVNI, with or without administration of the neutralizing antibody before and during infection. Blocking of type I IFN receptor increased the susceptibility of both wild-type and knockout mice to SVNI-induced mortality (Fig. 8B), further demonstrating the importance of type I IFN in restricting SVNI infection. Consistent with the above-described results, in the absence of the antibody, knockout mice had increased survival after SVNI infection compared to the wild-type mice (Fig. 8B). In contrast, in the presence of the neutralizing antibody, ZAP knockout mice died much faster than the wild-type mice (Fig. 8B). To correlate the survival time and viral titers, the viral titers in the serum early after infection and in the central nervous system before animal death were measured. In the presence of the antibody, the viral titers in both serum and the central nervous system were significantly higher in the knockout mice than in the wild-type mice (Fig. 8C to E). The higher viral titers in the knockout mice are likely a result of the lack of restriction of SVNI replication by the basal levels of ZAP. Analysis of the mRNA levels of ISG15 and IFI27 in the brain revealed that they were higher in ZAP knockout mice (Fig. 8F and G), which paralleled the viral titers (Fig. 8D and E), suggesting that their expression was induced by the virus in the brain. These results are consistent with the notion that the extended survival time of ZAP knockout mice was a result of higher serum IFN-α levels.

To further substantiate this notion, 23-day-old mice were infected by direct intracranial injection, and the brain viral titers were measured. The results showed that the brain viral titers of knockout mice were higher than those in the wild-type mice (Fig. 9A). Consistent with this finding, the knockout mice died faster than the wild-type mice (Fig. 9B). The differences in the brain virus titers and survival times between knockout and wild-type mice were statistically significant but the magnitudes were relatively small. This could be accounted for by the fact that ZAP is expressed at a very low level in the brain (Fig. 4A).

**DISCUSSION**

Type I IFNs play very important roles in innate immunity against viral infections. Viruses have evolved multiple mechanisms to antagonize or evade IFN-mediated immunity. The most commonly reported mechanism is that the virus encodes antagonists to block the induction or to counteract the effectors of IFNs (37, 38). Here, we provide evidence showing that instead of antagonizing the antiviral activity of ZAP, SVNI exploits ZAP in weanling mice to limit its replication in lymphoid tissues and minimize IFN induction in order to evade immune surveillance for enhanced dissemination to the central nervous system.

When the mice were infected by intraperitoneal injection, SVNI replicated better in the lymphoid tissues of the weanling ZAP knockout mice than the wild-type mice. However, the viral titers in the central nervous system were much lower in the knockout mice (Fig. 8B). The differences in the brain viral titers between the knockout mice and the wild-type mice were statistically significant but the magnitudes were relative to SVNI replication in order to evade immune surveillance for enhanced dissemination to the central nervous system.

When blocking of the type I IFN receptors renders weanling ZAP knockout mice more sensitive to viral infection than the wild-type mice. The above-described results strongly suggest that SVNI replication in the lymphoid tissues of weanling mice induces the production of IFN-α, which restricts subsequent viral infection of the central nervous system. To substantiate this notion, we analyzed the relationship between serum IFN-α levels and disease progression in the wild-type mice. Indeed, the serum IFN-α level was statistically proportional to the survival time of the mice (Fig. 8A), with animals having the highest IFN-α levels also surviving the longest. To further prove that virus-induced IFN-α plays a critical role in the protection of the central nervous system, we used a neutralizing antibody against type I IFN receptor 1 (IFNAR1) to block type I IFN action. Mice were infected with SVNI, with or without administration of the neutralizing antibody before and during infection. Blocking of type I IFN receptor increased the susceptibility of both wild-type and knockout mice to SVNI-induced mortality (Fig. 8B), further demonstrating the importance of type I IFN in restricting SVNI infection. Consistent with the above-described results, in the absence of the antibody, knockout mice had increased survival after SVNI infection compared to the wild-type mice (Fig. 8B). In contrast, in the presence of the neutralizing antibody, ZAP knockout mice died much faster than the wild-type mice (Fig. 8B). To correlate the survival time and viral titers, the viral titers in the serum early after infection and in the central nervous system before animal death were measured. In the presence of the antibody, the viral titers in both serum and the central nervous system were significantly higher in the knockout mice than in the wild-type mice (Fig. 8C to E). The higher viral titers in the knockout mice are likely a result of the lack of restriction of SVNI replication by the basal levels of ZAP. Analysis of the mRNA levels of ISG15 and IFI27 in the brain revealed that they were higher in ZAP knockout mice (Fig. 8F and G), which paralleled the viral titers (Fig. 8D and E), suggesting that their expression was induced by the virus in the brain. These results are consistent with the notion that the extended survival time of ZAP knockout mice was a result of higher serum IFN-α levels.

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**DISCUSSION**

Type I IFNs play very important roles in innate immunity against viral infections. Viruses have evolved multiple mechanisms to antagonize or evade IFN-mediated immunity. The most commonly
fecting the as-yet poorly understood process of Sindbis virus neuroinvasion. For example, it has been reported that upon intranasal VSV instillation, early IFN response in the glomerular layer of the olfactory bulb is critically required to prevent viral spread over the entire CNS and thus confers survival (41). The mechanism by which increased early IFN-α levels lead to better control of Sindbis virus neuroinvasive disease is an interesting area for future investigation.

SVNI infection of suckling mice caused faster animal death in the knockout mice (Fig. 2B). However, SVNI infection of weanling mice resulted in better survival of the knockout mice (Fig. 2C). This difference could be caused by a few possibilities. Sindbis virus is known to be very sensitive to IFN (11). However, it has been reported that in neonatal mice, although the serum IFN level is relatively high, the virus titer is much higher than that in the adult mice, implying that the IFN system of

**FIG 8** Type I IFN receptor blockade renders ZAP knockout mice more sensitive to SVNI infection than the wild-type mice. (A) Wild-type mice (n = 8; 23 days old) were infected with SVNI by intraperitoneal injection. At 12 h postinfection, blood was collected by retro-orbital bleeding, and serum levels of IFN-α were measured. Disease progression and animal survival were monitored daily. The relationship between survival time and IFN-α levels was plotted (P < 0.05). (B) Wild-type and ZAP knockout mice (n = 8 mice per group; 23 days old) were mock treated or treated with blocking antibody against IFNAR1 by intraperitoneal injection (at day -1 and 0 and 2 days relative to infection), followed by SVNI infection by intraperitoneal injection. Disease progression and animal survival were monitored daily. Data presented are representative of two independent experiments. P < 0.001 for WT versus KO and for WT plus α-IFNR1 versus KO plus α-IFNR1. (C to G) Mice (23 days old) were infected with SVNI. At days -1 and 0 of infection, mice were mock treated or treated with a blocking antibody against IFNAR1 (n = 4 per group). At the times indicated, viral titers in the serum (C), brain (D), or spinal cord (E) were determined. ISG15 (F) and IFI27 (G) mRNA levels in the brain were measured by RT-qPCR. *, P < 0.05; **, P < 0.01.
neonates is not very effective and does not play a major role in controlling viral replication (11, 29). Second, permeability of the BBB is a critical factor that controls Sindbis virus invasion of mouse brain (42). In newborn and suckling mice, some non-neuroinvasive strains of Sindbis virus induce fatal encephalitis after intracranial or intraperitoneal inoculation, whereas in weanling mice the virus induces fatal encephalitis only after intracranial inoculation (43, 44). This age-dependent resistance to neuroinvasion often coincides with the maturation of the BBB (44). It is conceivable that, in weanling mice with mature BBB and relatively low serum viral titers, only a small number of the virions in the brain can enter the brain, so the virus would be controlled by the IFN in the brain. In suckling mice, which lack a developed BBB (43, 44), virus in the periphery would have much easier access to the brain, and the relatively high level of the virus in the brain would not be controlled by the relatively low level of IFN. Further investigation is needed to better understand the age-dependent phenotypes.

There is increasing evidence indicating that this is a widely used strategy for viruses to exploit host antiviral factors to evade immune surveillance. For example, North American eastern equine encephalitis virus, also an alphavirus, was demonstrated to make use of host microRNAs that target the viral genome to limit its replication in myeloid-lineage cells to avoid the induction of type I IFN (45). In another example, HIV-1 uses the cellular factor Trex1, which degrades the viral DNA, to evade immune detection (46). ZAP-mediated evasion of the immune surveillance by SVNI is conceptually similar to but mechanistically different from these reported processes.

In summary, we report here that in weanling mice SVNI exploits the IFN-inducible host antiviral factor ZAP to evade type I IFN-mediated innate immune surveillance. ZAP has been reported to inhibit the replication of other viruses, such as Ebola virus and HIV-1. It would be interesting to investigate the roles of ZAP in the inhibition of these viruses by IFN in vivo and in vivo.

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