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IL-4 mediates the delayed neurobehavioral impairments induced by neonatal hepatitis B vaccination that involves the down-regulation of the IL-4 receptor in the hippocampus

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ABSTRACT

We have previously verified that neonatal hepatitis B vaccination induced hippocampal neuroinflammation and behavior impairments in mice. However, the exact mechanism of these effects remain unclear. In this study, we observed that neonatal hepatitis B vaccination induced an anti-inflammatory cytokine response lasting for 4–5 weeks in both the serum and the hippocampus, primarily indicated by elevated IL-4 levels. Three weeks after the vaccination schedule, however, hepatitis B vaccine (HBV)-mice showed delayed hippocampal neuroinflammation. In periphery, IL-4 is the major cytokine induced by this vaccine. Correlation analyses showed a positive relationship in the IL-4 levels between serum and hippocampus in HBV-mice. Thus, we investigated whether neonatal over-exposure to systemic IL-4 influences brain and behavior. We observed that mice injected intraperitoneally with recombinant mouse IL-4 (mIL-4) during early life had similar neuroinflammation and cognition impairment similar to those induced by neonatal hepatitis B vaccination. Next, the mechanism underlying the effects of IL-4 on brain in mice was explored using a series of experiments. In brief, these experiments showed that IL-4 mediates the delayed neurobehavioral impairments induced by neonatal hepatitis B vaccination, which involves the permeability of neonatal blood–brain barrier and the down-regulation of IL-4 receptor. This finding suggests that clinical events concerning neonatal IL-4 over-exposure, including neonatal hepatitis B vaccination and allergic asthma in human infants, may have adverse implications for brain development and cognition.

1. Introduction

Hepatitis B vaccination, recommended for newborns to prevent hepatitis B virus infection and associated liver diseases globally [1], is administered to infants and children. This period is critical for brain development, and immune activation during the critical period can significantly alter brain development programming [2–4], which results in a long-lasting impact on the brain development and behavior [5]. Several studies have reported that early postnatal immune activation increased anxiety or vulnerability to later life cognition impairment in animal models [6–8].

Therefore, neonatal hepatitis B vaccination, which induces strong immune activation during the critical period of brain development, may be a risk factor for certain disorders of neuropsychological development. In fact, there has been a controversy about whether neonatal hepatitis B vaccination is associated with the occurrence of autism, multiple sclerosis and myelitis, with some reports argue for [9–11] and other reports argue against [12] this potential association. Moreover, the routine childhood vaccines have been worried by some parents to be associated with adverse neurological outcomes, specifically autism spectrum disorder [13]. Furthermore, our recent study revealed that neonatal hepatitis B vaccination led to impairments in mood- and cognition-related behaviors, neurogenesis and hippocampal long-term potentiation in mice [14].

We have given an initial explanation that the neonatal hepatitis B vaccination induced neurobehavioral impairments through a T helper (Th)-2 bias of systemic cytokines. However, the exact mechanism or pathway by which the systemic Th-2 bias influences the central nervous system (CNS) is still not clear. In our previous study, the peripheral Th-2 bias induced by HBV was assessed by the ratio value of IFN-γ/IL-4 in

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serum [14]. However, it was the alteration of the IL-4 level that contributed to the Th-2 bias without any significant alteration in the serum IFN-γ level [14]. Other studies concerning the immune responses to this HBsAg/alum vaccine, which was used by our previous work, also demonstrated that IL-4 is the major cytokine induced by this vaccine [15,16]. Hence, IL-4 may play a key role in the HBV-induced neurobehavioral influences.

There is a homeostasis between anti-inflammation and proinflammation in mice with normal development involving a series of anti-inflammatory and proinflammatory cytokines. Many of these anti-inflammatory and proinflammatory cytokines have important roles in neural development and function [17]. Any factor that disturbs this homeostasis may cause brain developmental and functional abnormalities [17]. IL-4 itself is a powerful anti-inflammatory cytokine that induces the anti-inflammatory response and inhibits the production of proinflammatory cytokines [18]. Thus, high levels of IL-4 exposure during the critical stages of brain development are likely to break the physiological anti-inflammatory/proinflammatory profile of the brain, altering brain developmental programming.

Our current study investigated the potential role of IL-4 induced by neonatal hepatitis B vaccination in affecting brain development and cognition and its possible mechanism.

2. Materials and methods

2.1. Animals and breeding

Litters of newborn C57BL/6 mice were purchased from the Laboratory Animal Center of Sun Yat-sen University (Guangzhou, China). They were housed in a specific pathogen-free conditions under 12-h light:12-h dark conditions with food and water available *ad libitum*. Male neonatal mice were used. This study was approved by the Institutional Animal Ethics Committee of Sun Yat-sen University.

2.2. Immunization procedures

Experiments in this study utilized one of the following two injection approaches: hepatitis B vaccine immunization and recombinant mouse IL-4 (mIL-4) injection. In the hepatitis B vaccine immunization approach, mice were immunized with HBV (yeast-derived, Kangtai Biological Pharmaceutical Company, China) in a three-dose series [19] or a same volume of PBS. The immunization procedure of HBV started on postnatal day 0 (P0) and was followed by two identical doses of HBV on P7 and P21 as the booster inoculations. The dosage (1 μg HBsAg/50 μl per pup) of HBV was used according to our previous research [14]. In the mIL-4 injection approach, mice were injected intraperitoneally with mIL-4 (PeproTech, once every other day; 25 ng/kg in 50 μl PBS from P0 to P20 and 50 ng/kg in 50 μl PBS once every other day from P22 to P34) or a same volume of PBS. The period and dosage for the mIL-4 injection were determined to imitate the period and amplitude of serum IL-4 elevation induced by hepatitis B vaccination. All mice were weaned on P21.

2.3. Cytokine analysis

A mouse cytokine/chemokine magnetic bead panel (MCYTOMAG-70K-06; Millipore, Billerica, MA, USA) was used to evaluate the levels of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-4, IL-6 and IL-10 in the serum and hippocampus, according to the manufacturer's instructions. The mice were anaesthetized deeply and the blood was collected immediately. Then the mice were transcardially perfused with 0.9% NaCl and hippocampi were collected. The serum samples were diluted 1:2 in assay buffer. Hippocampal homogenates were also assayed to evaluate the levels of mouse and rat IL-4 using the enzyme-linked immunosorbent assay kits as indicated by the manufacturer (Cusabio Biotech Co. Ltd., Wuhan, China). The hippocampi were homogenized in PBS at pH 7.4 supplemented with 1% BSA, 0.1% Triton X-100, and protease inhibitor cocktail (Sigma), according to the manufacturer's instructions (Milliplex MAP kit, Millipore). A BCA protein assay kit (Beyotime, Shanghai, China) was used to adjust the total protein concentration of each sample to 4.5 mg/ml. Then, the prepared serum and hippocampal samples were used strictly according to the manufacturer's protocols for the multiplex assays. The assays were run in triplicate. The data were collected on a Bio-Plex-200 system (Bio-Rad, Hercules, CA, USA) and analyzed using professional software (Bio-Plex Manager).

2.4. Morris water maze (MWM)

Another cohort of mice were subjected to the MWM task at 4, 8 and 12 weeks of age to evaluate the spatial learning and memory of the mice. A pool 100 cm in diameter and 60 cm in height was filled with water made opaque with non-toxic white dye at (22 ± 1) ˚C. The pool was divided into four equal imaginary quadrants and a white stationary circular platform 9 cm in diameter was below the water surface 1 cm. Each mouse was subjected to 4 trials per day and was given a maximum of 60 s to find the hidden platform for 5 consecutive days. Then, on the 6th day, each mouse was exposed to a probe trial with the platform removed and was allowed to swim for 60 s. The time spent in finding the platform and the distance traveled by each mouse to find the platform was recorded with a MT-200 Morris image motion system (Chengdu Technology & Market Corp., China).

2.5. Evans Blue injection

Evans Blue staining was applied to assess the permeability of the blood–brain barrier (BBB) as described previously [20] with minor modifications. In brief, the mice were injected intraperitoneally with a 2% solution of Evans Blue dye (Sigma-Aldrich, St. Louis, MO, USA) in 0.9% NaCl at a dose of 8 ml/kg on P0 or P14. Four hours later, all the mice were deeply anaesthetized and transcardially perfused with cold 0.9% NaCl until the outflow was clear. To assess Evans Blue leakage, their brain tissues were homogenized in 99% dimethylformamide and incubated for 48 h at 50 ˚C. After centrifuging at 12,000*g* for 30 min at 4 ˚C, the supernatant was collected to measure the absorbance of the samples at 620 nm using a microplate reader.

2.6. Immunofluorescence staining and cell quantification

Another cohort of mice were subjected to the morphological test. The mice were anaesthetized deeply and transcardially perfused with 0.9% NaCl, which was followed by 4% paraformaldehyde (PFA). The brains were removed and immediately post-fixed in 4% PFA overnight at 4 ˚C. Then, the brains were gradient dehydrated with 10%, 20% and 30% sucrose for 24 h each at 4 ˚C. Free-floating, serial coronal sections (40 μm) were obtained using a Leica SM2000R freezing microtome (Leica Microsystems, Richmond Hill, Ontario, Canada) and stored at 4 ˚C prior to immunostaining.

Free-floating sections were washed in PBS three times and then blocked in PBS containing 1% bovine serum albumin (BSA) and 0.25% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 37 ˚C. The slices were then incubated in the primary antibodies, including rabbit anti-Iba-1 (1:1000; Wako Chemicals), rat anti-CD68 (1:200; Bio-Rad) and rat anti-MHC-2 (1:100; IQ Products) at 37 ˚C for 2 h, followed by overnight incubation at 4 ˚C. The primary antibodies were diluted in PBS containing 1% BSA and 0.25% Triton X-100. The next day, the specimens were washed three times and then were incubated with secondary antibodies, including Alexa Fluor 555-conjugated goat anti-rabbit and Alexa Fluor 488-conjugated donkey anti-rat for 2 h at 37 ˚C. Both secondary antibodies (Invitrogen) were diluted to 1:400. A Zeiss LSM780 confocal laser-scanning microscope was used to capture the representative confocal micrographs of the labeled cells.

Quantitative analyses of the Iba-1$^+$ cells in the hippocampus of each mouse were done on a Stereo Investigator stereological system (MicroBrightField, Williston, USA). Measurements were made in an equidistant series of six coronal sections. To avoid oversampling, actual section thickness was measured, and the appropriate guard zones at the top and the bottom of the section were defined. The 40× objective of a Nikon microscope was used for all of the stereological analyses.

2.7. Real-time quantitative polymerase chain reaction (qRT-PCR)

Another cohort of mice were subjected to the qRT-PCR analyses. The qRT-PCR analyses were performed as previously described [14]. In brief, TRIzol reagent (Sangon Biotech, Shanghai, China) was used to extract the total RNA first. Then, a GoScript™ cDNA Reverse Transcription Kit (Promega, Madison, USA) was applied to convert the mRNAs (2 μg) into cDNAs. Fluorescence-based real-time quantitative PCR was used to assay the expression of several interested mRNAs. For each sample, the quantitative PCR reactions were conducted in triplicate using the TransStart Tip Green qPCR SuperMix (TransGen Biotech, Beijing, China). According to its stability, β-actin was selected to be the reference gene in the hippocampus. The amplification cycles were set as 94 °C for 5 s and 60 °C for 30 s. A melting curve was constructed to evaluate the specificity of the reaction. A Bio-Rad IQ5 Real-Time PCR System with the comparative Ct method was used to determine and analyze all the qRT-PCR reactions. The primers used in this study were as follows:

IL-1β, Forward 5′-TGTCTTTCCCGTGGACCTTC and
Reverse 5′-CTAATGGGAACGTCACACACC;
IL-6, Forward 5′-TCTGGGAAATCGGGAAATGAG and
Reverse 5′-TCTCTGAAGGACTCTGGCTTTGTC;
Ym1, Forward 5′-GGCATACCTTTATCCTGAG and
Reverse 5′-CCACTGAAGTCATCCATGTC;
ARG, Forward 5′-AGCCAATGAAGAGCTGGCTGGT and
Reverse 5′-AACTGCCAGACTGTGGTCTCCA; and
β-action, Forward 5′-GGTACCACCATGTACCCAGG and
Reverse 5′-ACATCTGCTGGAAGGTGGAC.

2.8. Western blot analysis

Another cohort of mice were subjected to the Western blot analyses. The protein levels of phospho-p65, p65, phospho-Stat6, Stat6 and IL-4R in the hippocampus of the mice were examined by Western blotting analyses. After being anaesthetized and perfused with PBS, the hippocampus of the mice were separated from the brains and then were homogenized in ice-cold RIPA buffer (Beyotime, Shanghai, China). The homogenate was centrifuged at 9000*g* for 15 min at 4 °C, and the supernatant was isolated for Western blot assay. The protein concentration was quantified using a BCA protein assay kit (Beyotime, Shanghai, China). Tissue homogenates (containing 50 μg total protein) were separated on 10% or 8% SDS-PAGE gels and were then transferred onto presoaked PVDF membranes (Millipore). The blots were then blocked in 5% no-fat milk in TBST (20 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.05% Tween-20) for 2 h at RT. The following primary antibodies were used: rabbit anti-phospho-p65 (Ser536) (bioworld, 1:1000), rabbit anti-p65 (bioworld, 1:1000), rabbit anti-phospho-Stat6 (Thr645) (bioworld, 1:1000), rabbit anti-Stat6 (CST, 1:1000) and rabbit anti-IL-4R (bioworld, 1:1000) overnight at 4 °C. HRP-conjugated secondary goat anti-rabbit antibodies (Fdbio science, Hangzhou, China) were used at 1:10,000. All blots were visualized using electrogenerated chemiluminescence (ECL) (Amersham Biosciences). Quantification was performed using the ImageJ software and normalized using β-actin.

2.9. IL-4 neutralization

Another new experiments were conducted, aiming to investigate whether IL-4 neutralization would block the effects on brain and behavior induced by neonatal hepatitis B vaccination. In this experiment, four groups of mice were prepared, namely CON-mice, HBV-mice, HBV + IgG1-mice and HBV + anti-IL-4-mice. The procedure of IL-4 neutralization was performed as previously described with minor modifications [21].

An IL-4-neutralizing antibody (11B11, BD Pharmingen) or isotype control antibody, IgG1 (BD Pharmingen) at 10 μg/g body weight was co-administered intraperitoneally with HBV on P0, P7 and P21. In addition, the administration of IL-4-neutralizing antibody or isotype control antibody was repeated intraperitoneally at 10 μg/g body weight on P1, P2, P8, P9, P10, P11, P22, P23, P24, P25. Mice in the CON group and HBV group received injection of PBS of a corresponding schedule. These injections (HBV, IL-4-neutralizing antibody, isotype control antibody or PBS) described above were actually performed in mice that were sacrificed for tests at ages larger than P25 and these injections were ceased when the mice were sacrificed at ages before P25 as shown in Figs. 9 and S6.

2.10. Statistical analyses

The data were statistically analyzed using the SPSS 23.0 statistical software for Windows (Chicago, IL, USA). The data are expressed as the mean ± standard error of the mean. The data in Fig. 5A-F, Fig. 6E, Fig. 10A-C and Fig. S3 were analyzed using two-way RM-ANOVA followed by the Bonferroni post hoc test. The data in Fig. 3 were analyzed using Pearson's correlation coefficient. The data in Figs. 7J-K, 9, 10D–E and S6 were analyzed using one-way ANOVA. Except the data in Fig. 3M and Fig. 6C and D, all the other data were analyzed using Student's *t*-test or Welch's *t*-test. $p < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Neonatal hepatitis B vaccination induced an instant anti-inflammatory cytokine response and a subsequent proinflammatory cytokine response in the hippocampus

We first observed the influences of neonatal hepatitis B vaccination on the levels of several major anti- and proinflammatory cytokines in the hippocampus from the neonatal period to adulthood. To be specific, we examined the levels of IL-4, IL-10, IFN-γ, IL-1β, IL-6 and TNF-α on P7, P14, P21, P28, P35, P42, P49, P56, P63 and P70 in the hippocampus of HBV- and CON-mice.

From P7 to P21, there were approximately 2.7–3.3-fold increases in the IL-4 concentrations in HBV-mice than CON-mice (Student's *t*-test; P7, $p < 0.001$; P14, $p < 0.001$; P21, $p < 0.001$; $n = 6$; Fig. 1A). On P28, there was only an increase of 1.6-fold compared to CON-mice (Student's *t*-test; $p < 0.05$; $n = 6$; Fig. 1A). However, no significant differences in the IL-4 concentrations were found between two groups from P35 to P70 (Fig. 1A).

From P7 to P21, both IL-1β and IL-6 showed opposite trends in the levels to IL-4 in HBV-mice, with significant decreases than the controls (Student's *t*-test; IL-1β: P7, $p < 0.05$; P14, $p < 0.05$; P21, $p < 0.05$; IL-6: P7, $p < 0.05$; P14, $p < 0.05$; P21, $p < 0.05$; $n = 6$; Fig. 1B and C). Interestingly, the levels of IL-1β and IL-6 in HBV-mice had no significant alterations on P28 and P35 but showed a period of rebounding increases from P42 to P56 or P63 (Student's *t*-test; IL-1β: P42, $p < 0.05$; P49, $p < 0.05$; P56, $p < 0.05$; IL-6: P42, $p < 0.01$; P49, $p < 0.01$; P56, $p < 0.05$; P63, $p < 0.05$; $n = 6$; Fig. 1B and C). TNF-α showed similar alterations to IL-1β and IL-6 from P28 but no significant alterations before this age (Student's *t*-test; P42, $p < 0.05$; P49, $p < 0.05$; P56, $p < 0.05$; $n = 6$; Fig. 1D).

There was a slight decrease (HBV: 35.46 ± 1.51 vs. CON: 42.15 ± 2.32; Student's *t*-test; $p < 0.05$) in the IFN-γ level only on P42 with no significant alterations at all the other ages (Fig. S1A) in HBV-

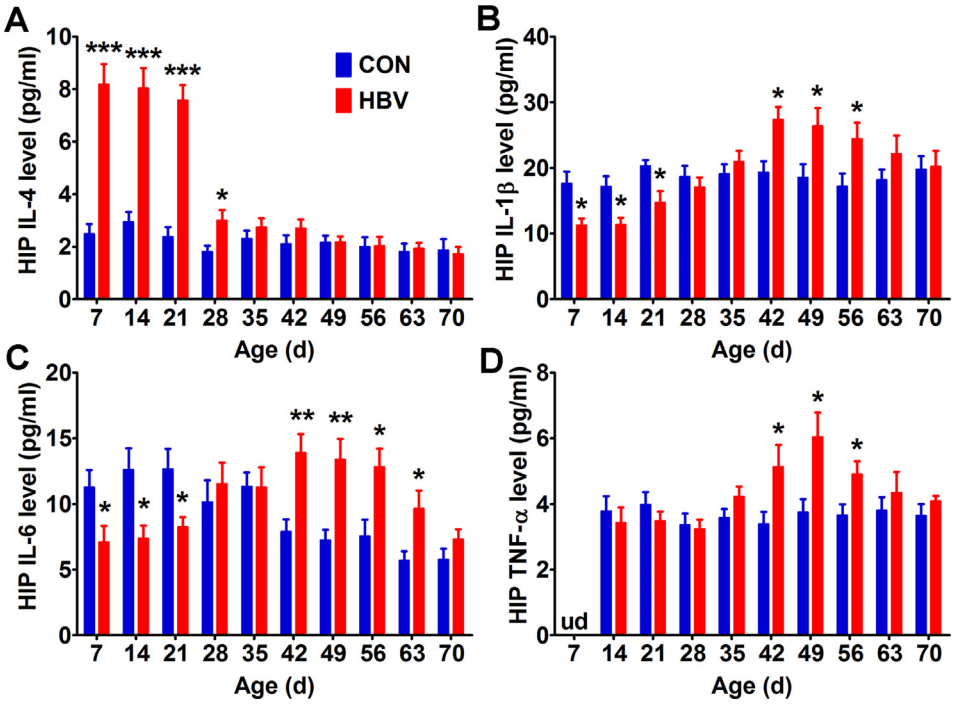

Fig. 1. Neonatal hepatitis B vaccination induced an instant anti-inflammatory cytokine response and a subsequent proinflammatory cytokine response in the hippocampus. (A-D) The bars represent the average levels of IL-4 (A), IL-1β (B), IL-6 (C) and TNF-α (D) in the hippocampus. The data represent the means ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $n = 6$/group; Student's *t*-test. HIP: hippocampus; ud: undetectable.

mice than CON-mice. At all ages, no significant alterations were found in the IL-10 levels between groups (Fig. S1C).

These findings suggest that the neonatal hepatitis B vaccination induced an anti-inflammatory cytokine response before P21 and a subsequent proinflammatory cytokine response that lasted from P42 to P63 in the hippocampus.

3.2. Neonatal hepatitis B vaccination elevated IL-4 levels and reduced the proinflammatory cytokine levels in serum

Having observed the effects of neonatal hepatitis B vaccination on the levels of IL-4, IL-1β, IL-6 and TNF-α in the hippocampus, we next examined the levels of these cytokines in the serum at same age points. The IL-4 concentrations in HBV-mice were significantly higher in serum than control mice from P7 to P35 (Welch's *t*-test; P7, $p < 0.001$; Student's *t*-test; P14, $p < 0.001$; P21, $p < 0.001$; P28, $p < 0.05$; P35, $p < 0.05$; $n = 6$; Fig. 2A) but showed no significant alterations from P42 to P70 (Fig. 2A).

The level of IL-1β in HBV-mice showed mild decreases in serum compared to CON-mice on P14 and P21 with no significant alterations at the other ages (Student's *t*-test; P14, $p < 0.01$; P21, $p < 0.05$; $n = 6$; Fig. 2B). The level of IL-6 in HBV-mice showed mild decreases in serum compared to CON-mice on P14, P21 and P28 with no significant alterations at other ages (Student's *t*-test; P14, $p < 0.05$; P21, $p < 0.01$; P28, $p < 0.05$; $n = 6$; Fig. 2C). The level of TNF-α in HBV-mice showed mild decreases in the serum than CON-mice on P14 with no significant alterations at other ages (Student's *t*-test; $p < 0.01$; $n = 6$; Fig. 2D). There were no significant alterations in the levels of IFN-γ (Fig. S1B) and IL-10 (Fig. S1D) in HBV-mice at all ages in contrast to CON-mice.

In summary, it is suggested that the decreased levels of proinflammatory cytokines might reflect the consequence of the elevation of the systemic IL-4 concentration.

3.3. The serum IL-4 levels positively correlated to the levels of the hippocampal IL-4 at the individual level

The findings above showed that IL-4, IL-1β, IL-6 and TNF-α were altered significantly both in the serum and in the hippocampus in HBV-mice at all or some of the age points before P28. To explore the relationship of the alterations in the cytokines in the hippocampus to that in the periphery, correlation analyses at the individual level were then performed between the hippocampal level and the serum level of each of the four cytokines tested on P7, P14, P21 and P28. Our results showed that the serum IL-4 levels positively correlated to the levels of the hippocampal IL-4 at all four time points (Pearson's correlation coefficient; P7, r = 0.925, $p < 0.01$; P14, r = 0.877, $p < 0.05$; P21, r = 0.907, $p < 0.05$; P28, r = 0.866, $p < 0.05$; $n = 6$; Fig. 3A-D). There was no significant correlation for IL-1β, IL-6 and TNF-α between serum and hippocampal levels (Fig. 3E-P).

3.4. Neonatal mIL-4 over-exposure imitated the HBV-induced instant anti-inflammatory cytokine response and the subsequent proinflammatory cytokine response in the hippocampus

IL-4 is the only cytokine that has a correlation between its levels in serum and the hippocampus (Fig. 3A-D). Moreover, IL-4 was the cytokine that had the greatest amplitude of change in both the hippocampus and serum in HBV-mice (Figs. 1 and 2). Therefore, we hypothesized that IL-4 might be the mediator by which neonatal hepatitis B vaccination induced the neuroinflammation found above (Figs. 1 and 2) and the behavior impairments reported previously [14]. To verify this hypothesis, mice were administered mIL-4 and were tested for their hippocampal cytokine levels. Mice that were subjected to testing before P28 were injected intraperitoneally with mIL-4 once every other day from P0 until they were sacrificed, and the mice that were subjected to assays after P35 were injected intraperitoneally with mIL-4 once every other day from P0 to P34. The dosage of 25 ng/kg body weight was used for injections from P0 to P20, and 50 ng/kg was used from P22 to P34. The whole time span (P0 to P34) and dosages selected for the mIL-4 injection were determined by imitating the period and extent of serum IL-4 elevation induced by hepatitis B vaccination.

Generally, neonatal mIL-4 over-exposure imitated the HBV-induced instant anti-inflammatory cytokine response and the subsequent proinflammatory cytokine response in the hippocampus. From P7 to P28, the IL-4 concentrations were significantly higher in the hippocampus (Student's *t*-test; P7, $p < 0.001$; P14, $p < 0.001$; P21, $p < 0.001$; P28, $p < 0.05$; $n = 6$; Fig. 4A) of mIL-4-mice compared to that of CON-mice. From P35 to P70, IL-4 returned to normal levels in

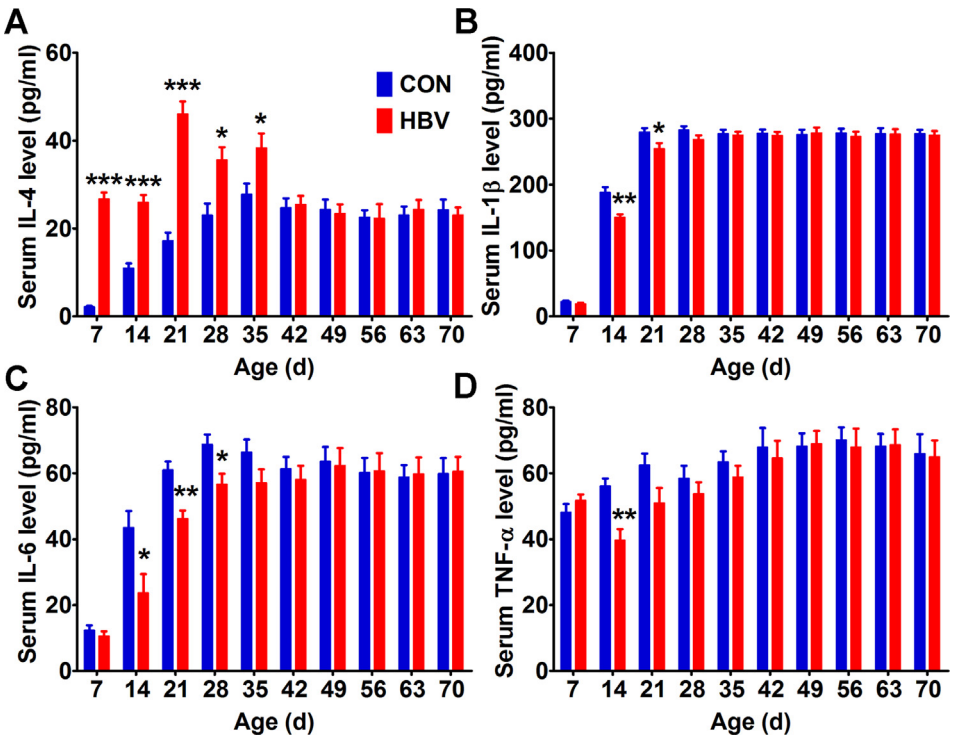

Fig. 2. The neonatal hepatitis B vaccination elevated the IL-4 level and reduced the proinflammatory cytokine levels in the serum. (A-D) The bars represent the average levels of IL-4 (A), IL-1β (B), IL-6 (C) and TNF-α (D) in the serum. The data represent the means ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $n = 6$/group; Student's *t*-test or Welch's *t*-test.

the hippocampus (Fig. 4A).

From P7 to P28, both IL-1β and IL-6 in mIL-4-mice showed an opposite trend in the levels compared to IL-4, with significant decreases than that of the controls (Student's *t*-test; IL-1β: P7, $p < 0.01$; P14, $p < 0.05$; P21, $p < 0.05$; P28, $p < 0.05$; IL-6: P7, $p < 0.01$; P14, $p < 0.05$; P21, $p < 0.05$; P28, $p < 0.05$; $n = 6$; Fig. 4B and C). The levels of IL-1β and IL-6 showed no significant alterations on P35 but had a period of rebounding increases from P42 to P63 (Student's *t*-test; IL-1β: P42, $p < 0.01$; P49, $p < 0.05$; P56, $p < 0.05$; P63, $p < 0.05$; IL-6: P42, $p < 0.01$; P49, $p < 0.05$; P56, $p < 0.05$; P63, $p < 0.05$; $n = 6$; Fig. 4B and C). The TNF-α level showed a trend in similar to the IL-1β and IL-6 levels mIL-4-mice from P14 to P70, but only two significant differences were found on P21 and P42 (Student's *t*-test; P21, $p < 0.05$; P42, $p < 0.05$; $n = 6$; Fig. 4D). At all ages, no significant alterations were found for the IFN-γ (Fig. S2A) and IL-10 (Fig. S2B) levels between groups.

These results showed that neonatal mIL-4 over-exposure induced an instant anti-inflammatory cytokine response and a subsequent proinflammatory cytokine response in the hippocampus of mice, similar to the neonatal hepatitis B vaccination.

3.5. Neonatal mIL-4 over-exposure imitated the HBV-induced impairments in spatial learning and memory in mice at 8-weeks-old

To observe whether neonatal mIL-4 over-exposure could imitate the HBV-induced transient spatial cognition impairment, three new sets of mice were administered with mIL-4 or PBS from P0 and were subjected to MWM tasks at 4, 8 or 12 weeks of age. The time points for testing were determined according to a previous report [14]. In the current study, we also observed the effects of neonatal hepatitis B vaccination on the MWM performances of mice at 8 weeks of age to show the similarity of the results from the mIL-4-mice and the HBV-mice in the same figure without duplicate publication of the data from HBV-mice in our previous work [14].

The results showed that in the acquisition phase, HBV-mice and mIL-4-mice showed a longer average escape latency (RM-ANOVA; HBV: groups × time: $F_{4,\ 112} = 1.665$, $p > 0.05$; groups: $F_{1,\ 28} = 10.225$, $p < 0.01$; time: $F_{4,\ 112} = 16.899$, $p < 0.001$; $n = 15$; mIL-4: groups × time: $F_{4,\ 112} = 2.663$, $p < 0.05$; groups: $F_{1,\ 28} = 18.461$, $p < 0.001$; time: $F_{4,\ 112} = 41.144$, $p < 0.001$; $n = 15$; Fig. 5A and D) than the control groups at 8 weeks of age. A longer average swimming path in both HBV-mice and mIL-4-mice (RM-ANOVA; HBV: groups × time: $F_{4,\ 112} = 1.548$, $p > 0.05$; groups: $F_{1,\ 28} = 9.418$, $p < 0.01$; time: $F_{4,\ 112} = 15.921$, $p < 0.001$; $n = 15$; mIL-4: groups × time: $F_{4,\ 112} = 2.649$, $p < 0.05$; groups: $F_{1,\ 28} = 17.642$, $p < 0.001$; time: $F_{4,\ 112} = 37.885$, $p < 0.001$; $n = 15$; Fig. 5B and E) than the control groups were also seen at 8 weeks of age. Neither HBV-mice nor mIL-4-mice had significant differences in velocity compared to the controls (Fig. 5C and F). In the probe phase, both HBV-mice and mIL-4-mice had less platform area crossings (Student's *t*-test; HBV: $p < 0.05$; $n = 15$; mIL-4: $p < 0.05$; $n = 15$; Fig. 5G and I) and spent less time in the target quadrant (Student's *t*-test; HBV: $p < 0.05$; $n = 15$; mIL-4: $p < 0.05$; $n = 15$; Fig. 5H and J) than the controls at 8 weeks of age. At 4 or 12 weeks, there were no significant different performances in the MWM tasks between mIL-4-mice and the control (Fig. S3). All these results were similar to the effects induced by neonatal hepatitis B vaccination, as reported previously [14].

These results indicated that neonatal mIL-4 over-exposure imitated the HBV-induced impairments in spatial learning and memory in mice.

3.6 Neonatal hepatitis B vaccination induced neuroinflammation, both through the penetration of IL-4 across the BBB into the brain before P14 and through the prolonged penetration period by neonatal IL-4 over-exposure

Then, we tried to explore the pathway by which IL-4 in the periphery affected the brain. The hippocampal IL-4 levels positively correlated to the levels of the serum IL-4 levels in HBV-mice (Fig. 3A–D). Moreover, the BBB is immature in the neonatal period [22,23]. Hence, we hypothesized that IL-4 permeating into the brain across the neonatal BBB mediated the influences of hepatitis B vaccination on the proinflammatory cytokine levels in the hippocampus.

Mice were injected intraperitoneally with Evans Blue on P0 or P14 to assess the integrity of the neonatal BBB. We observed the obvious presence of Evans Blue in the brain parenchyma, especially in the hippocampus on P0 (Student's *t*-test; prefrontal cortex (PFC): $p < 0.05$; hippocampus (HIP): $p < 0.001$; $n = 6$; Figs. 6A and B and S4). There

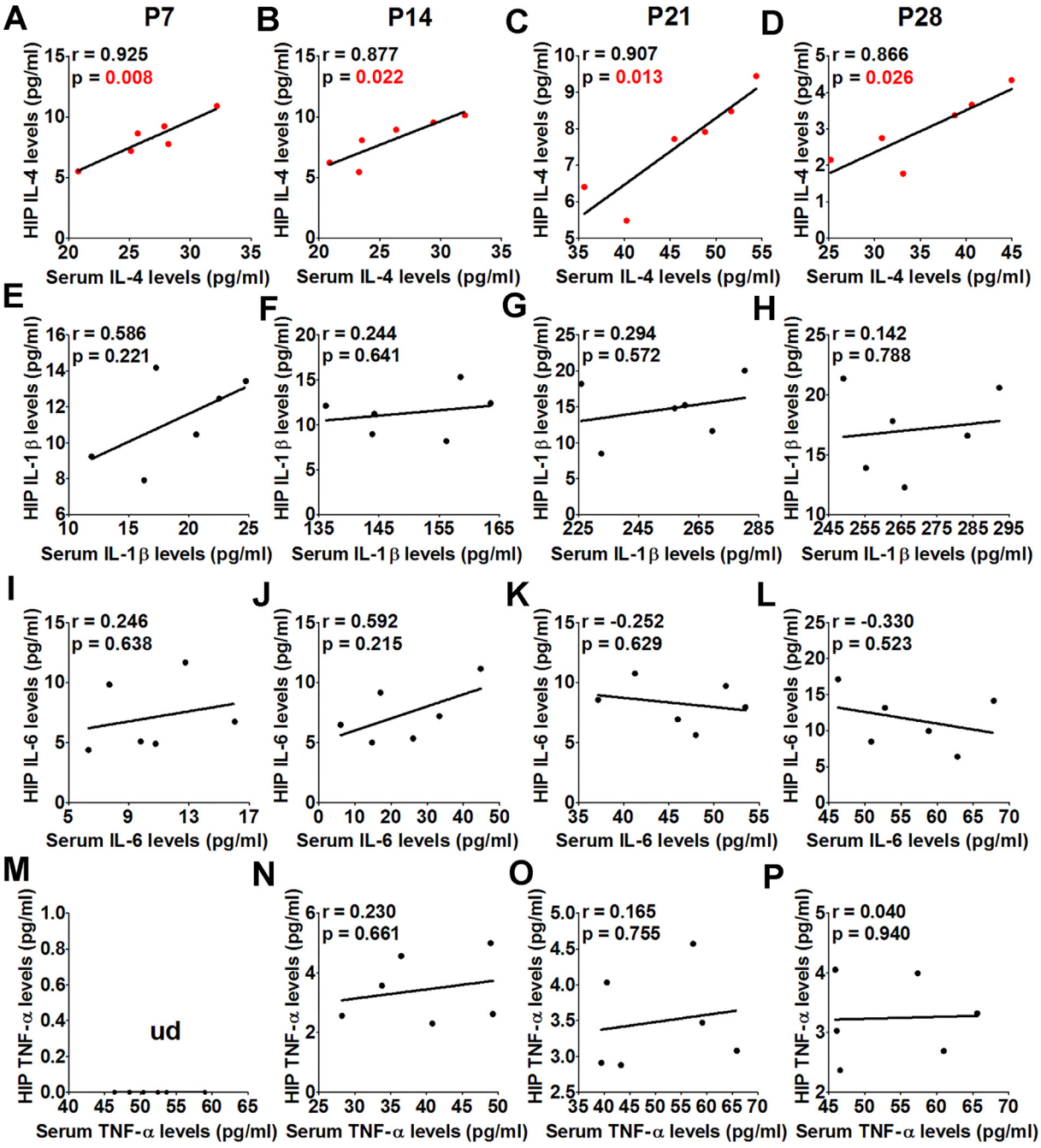

Fig. 3. The positive correlation of the serum IL-4 level with the hippocampal IL-4 level in HBV-mice. (A-D) Correlation analyses between the serum IL-4 level and the hippocampal levels of IL-4. (E-H) Correlation analyses between the serum IL-1β level and the hippocampal levels of IL-1β. (I-L) Correlation analyses between the serum IL-6 level and the hippocampal levels of IL-6. (M-P) Correlation analyses between the serum TNF-α level and the hippocampal levels of TNF-α. n = 6/group; Pearson's correlation analysis. HIP: hippocampus; ud: undetectable.

was no Evans Blue dye leakage in the brain on P14 (Fig. 6B). This finding confirmed that the BBB is permeable in the neonatal period.

We next examined whether IL-4 can infiltrate into the brain during the neonatal period. Mice were injected intraperitoneally with 100 ng/kg of recombinant rat IL-4 (rIL-4) on P0, P3, P7 or P14. A rat IL-4 ELISA kit was used to test the level of rIL-4 in the hippocampus of mice 4 h later. There was the presence of rIL-4 in the hippocampus on P0, P3 and P7, but this trend decreased with age (Fig. 6C). On P14, the last time point, there was no longer detectable rIL-4 in the hippocampus (Fig. 6C). These results indicate that IL-4 can cross the BBB into the brain before P14 in mice because mice produce only mouse IL-4 but not rIL-4.

The results in Fig. 6B and C showed that the BBB was mature and no longer permeable on P14. However, the findings that the levels of IL-4 in the hippocampus of HBV-mice (Fig. 1A) and mIL-4-mice (Fig. 4A) were still higher than control groups until P28 suggested that neonatal exposure to a high level of IL-4 might prolong the penetration period for IL-4. To verify this deduction, we conducted another experiment. Thirty-six mice were divided into two groups. One group was administered intraperitoneally with rIL-4 once every other day from P0 (P0-group), and the other group was administered from P14 (P14-group). At each of the three selected age points, P14, P21 and P28, twelve mice, 6 mice from the P0-group and 6 mice from the P14-group, were assayed for the level of rIL-4 in the hippocampus using an ELISA

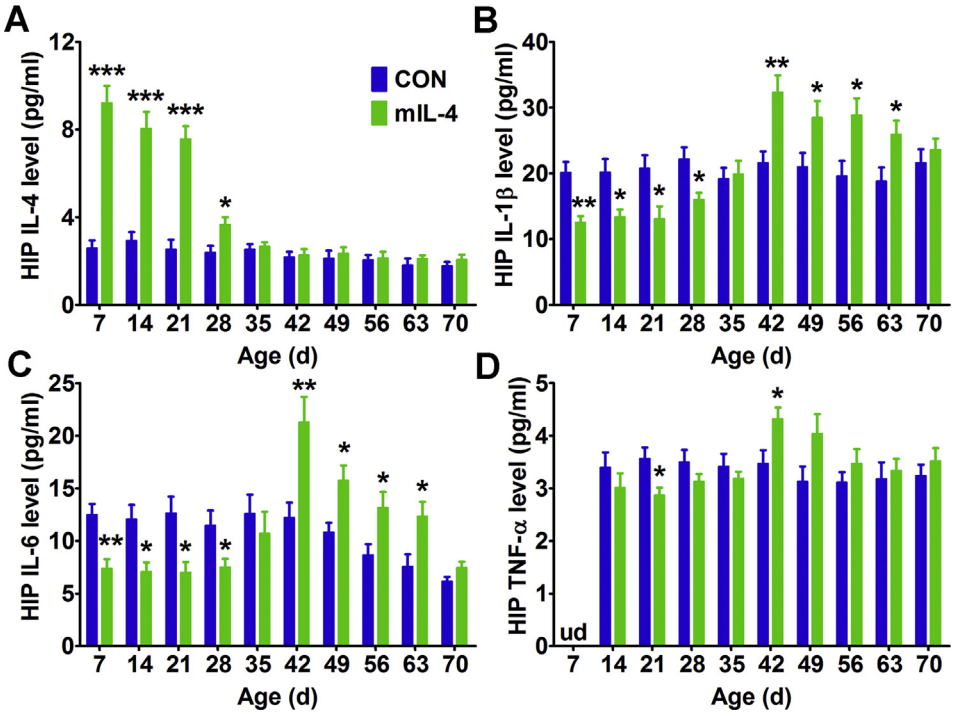

Fig. 4. Neonatal mIL-4 over-exposure imitated the HBV-induced instant anti-inflammation and subsequent proinflammation in the hippocampus. (A-D) The bars represent the average levels of IL-4 (A), IL-1β (B), IL-6 (C) and TNF-α (D) in the hippocampus. The data represent the means ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $n = 6$/group; Student's *t*-test. HIP: hippocampus; ud: undetectable.

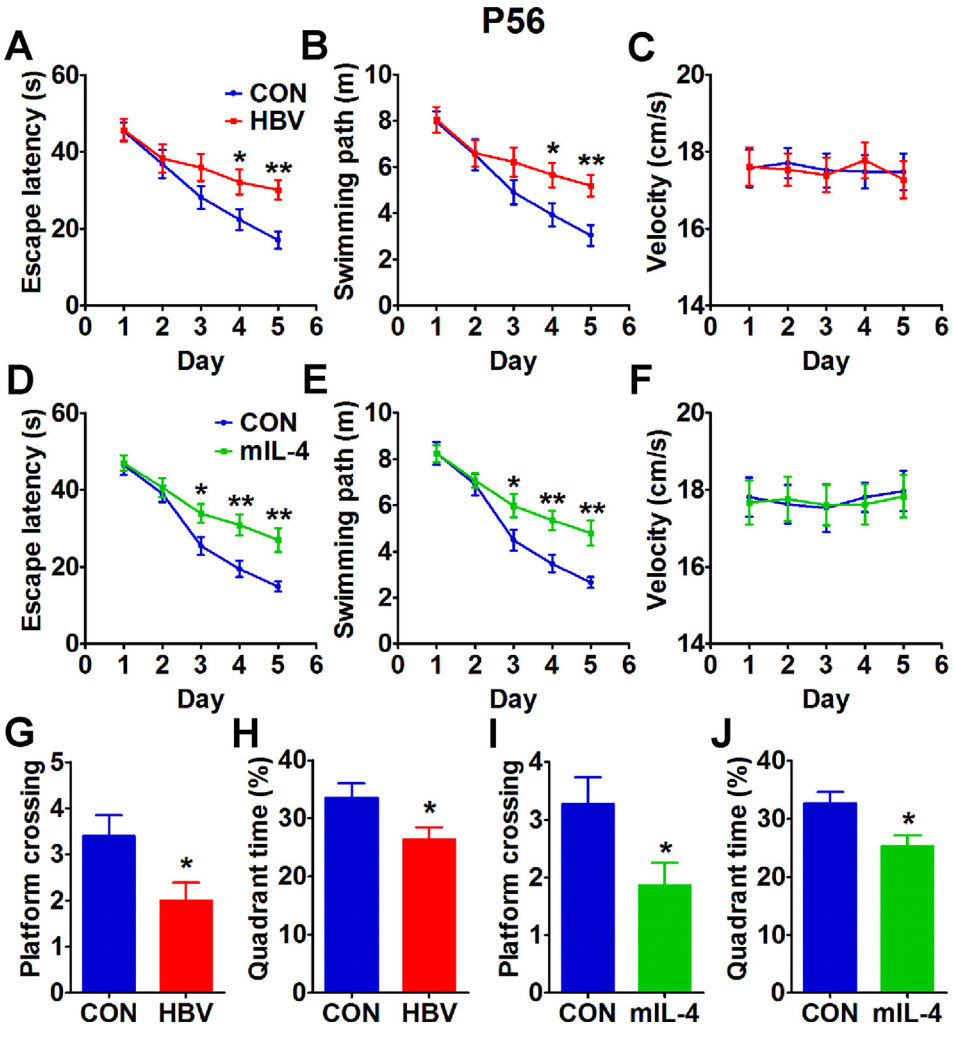

Fig. 5. Neonatal mIL-4 over-exposure imitated the HBV-induced spatial cognition impairment in the MWM task at 8 weeks of age. (A-B, D-E) The average escape latency (A, D) and average swimming path to reach the platform (B, E) of the HBV-mice (A, B) and mIL-4-mice (D, E) were longer than controls (Two-way RM-ANOVA followed by the Bonferroni post hoc test; $^{*}p < 0.05$, $^{**}p < 0.01$: significant post hoc differences). (C, F) The data showed the average swimming velocity of the HBV-mice (C) and mIL-4-mice (F) at 8 weeks of age. (G, I) The data showed the average numbers of platform area crossings in the HBV-mice (G) and mIL-4-mice (I) (Student's *t*-test; $^{*}p < 0.05$: significant differences). (H, J) The data indicate the average time proportion spent in the target quadrant by the HBV-mice (H) and mIL-4-mice (J). The data represent the mean ± SEM of 15 animals per group.

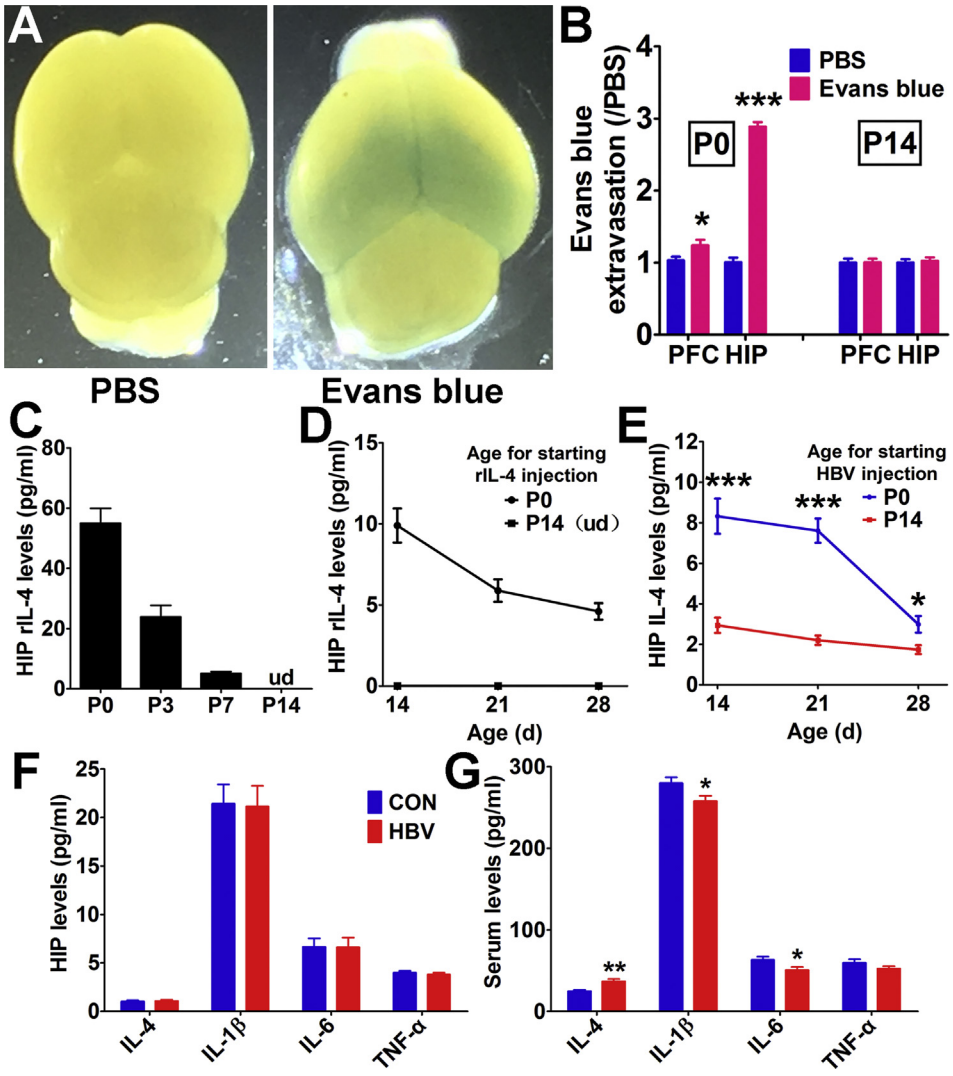

Fig. 6. Neonatal hepatitis B vaccination induced neuroinflammation, both through the penetration of IL-4 across the BBB into the brain before P14 and through the prolonged penetration of neonatal IL-4 over-exposure. (A) Visual inspection of the brains from PBS- and Evans Blue-administered mice, showing that neonatal mice had an immature and permeable BBB. (B) Evans Blue extravasation in the brain in both groups. PFC: prefrontal cortex; HIP: hippocampus. (C) The permeability of the BBB for rIL-4 before P14. ud: undetectable. (D) Exposure to high levels of rIL-4 before P14 increased the permeability of the BBB for rIL-4 infiltrating into the brain in the later life of mice. (E) Neonatal hepatitis B vaccination initiated before P14 increased the permeability of the BBB for IL-4 infiltrating into the brain in later life. The data represent the levels of mIL-4-mice compared to the controls. (F) Hepatitis B vaccination started on P14 had no significant influences on the cytokine levels in the hippocampus. (G) Hepatitis B vaccination started on P14 altered the levels of IL-4, IL-1β and IL-6 in the serum. The data represent the means ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $n = 6$/group; Student's *t*-test.

kit. The kit indicated the presence of rIL-4 in the hippocampus of the P0-group until P28, whereas there was no detectable rIL-4 in the hippocampus of the P14-group (Fig. 6D) at any age point. These results indicated that over-exposure to IL-4 during the neonatal period could prolong the penetration of IL-4 into the hippocampus, suggesting that HBV injection starting after P14 might not influence the brain any more.

To verify this possibility, another set of mice was divided into two groups (18 per group). One group was intraperitoneally administered HBV from P0 (P0-group), and the other group was administered from P14 (P14-group). At each of the three selected age points, P14, P21 and P28, twelve mice, 6 mice from the P0-group and 6 mice from the P14-group, were assayed for the level of IL-4 in the hippocampus using an ELISA kit. There was the mere basal level of IL-4 in the hippocampus of the P14-group on P14, P21 and P28 (Fig. 6E). As expected, there was a significantly higher level of IL-4 in the hippocampus of the P0-group than the P14-group at any of the three age points (RM-ANOVA; groups × time: $F_{2,\ 20} = 9.736$, $p < 0.01$; groups: $F_{1,\ 10} = 139.92$, $p < 0.001$; time: $F_{2,\ 20} = 20.047$, $p < 0.001$; $n = 6$; Fig. 6E). These results indicated that the peripheral IL-4 induced by hepatitis B vaccination starting after P14 did not penetrate into the hippocampus.

Next, the cytokine levels in the hippocampus and serum were examined in mice that received hepatitis B vaccination or PBS on P14, P21, and P35. The tests were done 4 h after the last injection. There were no significant alterations in the hippocampal levels of any of these cytokines between two groups (Fig. 6F), although there were slight differences in the serum levels of IL-4, IL-1β and IL-6 between the two groups (Student's *t*-test; IL-4: $p < 0.01$; IL-1β: $p < 0.05$; IL-6: $p < 0.05$; $n = 6$; Fig. 6G). These results indicated that hepatitis B vaccination starting after P14 induced no neuroinflammation in the hippocampus.

3.7. Neonatal mIL-4 over-exposure imitated HBV-induced microglia M1 polarization

Our previous study demonstrated that neonatal hepatitis B vaccination induced microglia M1 polarization in the hippocampus [14]. Therefore, we examined whether mere neonatal IL-4 over-exposure could imitate such a microglial response on P42. Mice were given HBV or mIL-4 as described above, and the CON-mice matched to them received PBS in the corresponding approaches. Immunofluorescence was used to detect the expression levels of microglial CD68, a classical marker of proinflammatory microglial activation [24]. The results showed a significant increase in CD68 expression in both HBV-mice and mIL-4-mice (one-way ANOVA; HBV- vs. CON-mice: $p < 0.001$; mIL-4- vs. CON-mice: $p < 0.001$; $n = 6$; Fig. 7A–J) compared with CON-mice. This result indicated more activated microglia and neuroinflammation. Moreover, MHC-2, another classical marker of proinflammatory microglial activation (M1 activation) [25], was also assessed in our present study. The results showed that both HBV-mice and mIL-4-mice had a few microglia (Iba-1^{+}) expressing MHC-2 (Fig. S5). In addition, there were no significant differences between groups in terms of the numbers

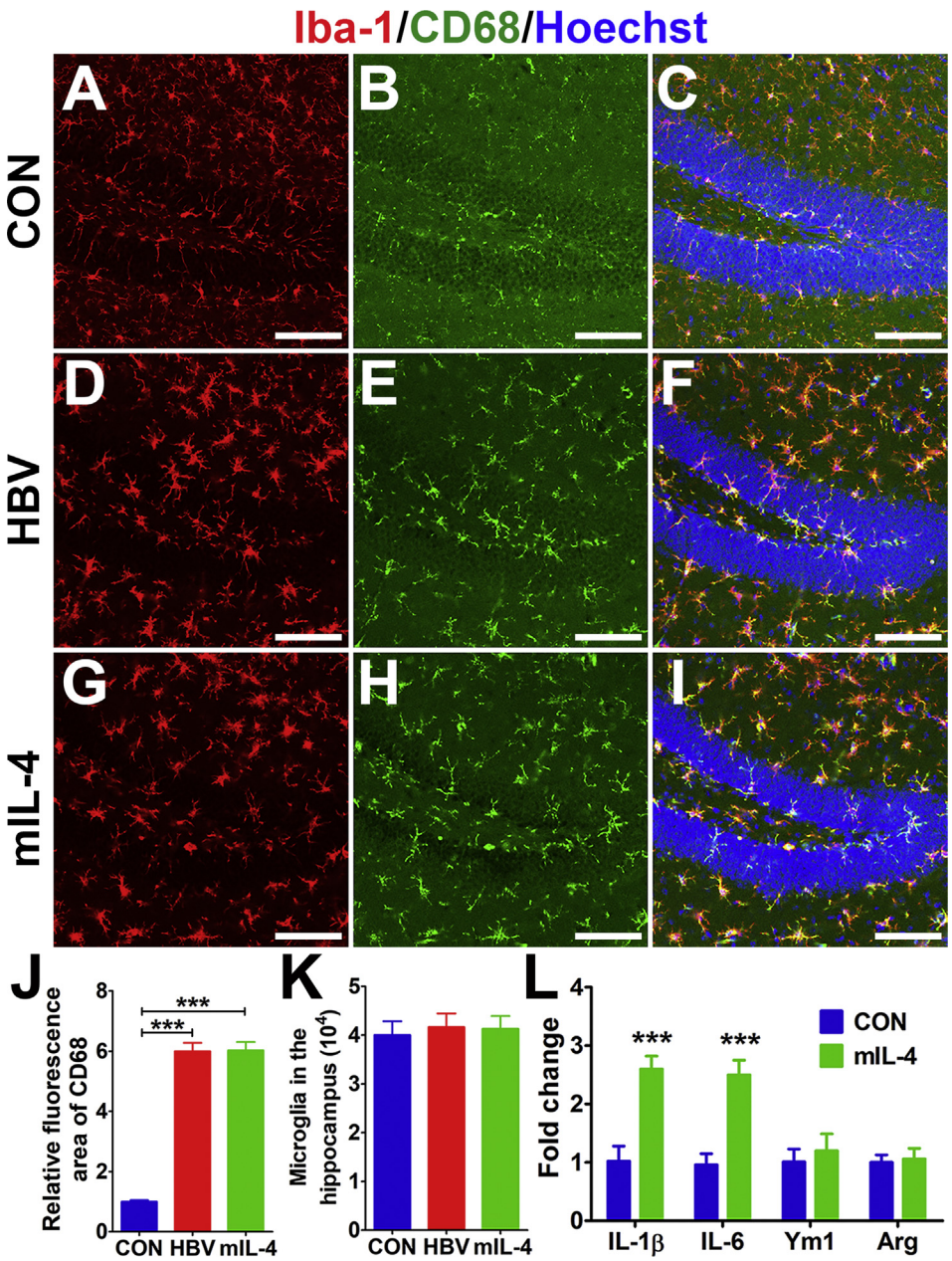

Fig. 7. Neonatal mIL-4 over-exposure imitated the HBV-induced microglia M1 polarization ($CD68^+$) without altering their numbers in the hippocampus. (A-I) Representative confocal micrographs of the M1-activated microglia ($Iba\text{-}1^+/CD68^+$) in CON– (A-C), HBV- (D-F) and mIL-4-mice (G-I) on P42. Scale bar, 100 μm. (J) The data represent the relative fluorescence area of CD68 in the hippocampus in all groups of mice. (K) The data represent the average numbers of microglia in the hippocampus in all groups of mice. (L) The data represent the relative mRNA expression of the M1- and M2-type genes in the hippocampus of mIL-4-mice compared to the controls. The data in (J) were analyzed using one-way ANOVA followed by the Dunnett's T3 post hoc test; the data in (K) were analyzed using one-way ANOVA followed by the Bonferroni post hoc test and the data in (L) were analyzed using Student's *t*-test. The data represent the means ± SEM. $^{***}p < 0.001$; $n = 6$/group.

of microglia (Fig. 7K), consistent with our previous report [14].

Another two groups of mice received mIL-4 or PBS and were subjected to qRT-PCR for assays of the mRNA levels of IL-1β and IL-6, representative of M1-type genes, as well as the mRNA levels of Ym1 and Arg, representative of M2-type genes [26,27]. The results showed that the mIL-4-mice had significantly increased mRNA levels of IL-1β (Student's *t*-test; $p < 0.001$; $n = 6$) and IL-6 (Student's *t*-test; $p < 0.001$; $n = 6$) in the hippocampus compared to the controls (Fig. 7L). However, the mRNA levels of Ym1 and Arg had no significant differences between both groups (Fig. 7L). The results showed that neonatal mIL-4 over-exposure induced microglia M1 polarization. All these findings indicated that neonatal mIL-4 over-exposure imitated the HBV-induced microglia M1 polarization without altering their numbers in the hippocampus. Given the sufficient morphological evidence that neonatal mIL-4 over-exposure imitated the effects of neonatal hepatitis B vaccination on the microglial activation type, qRT-PCR tests were not repeated for HBV-mice as in mIL-4-mice.

3.8. Neonatal mIL-4 over-exposure promoted NF-κB activation after causing a short inhibition in the hippocampus

The NF-κB signaling pathway plays a key role in initiating the expression of proinflammatory cytokines, including IL-1β, IL-6 and TNF-α [28,29]. This transactivation is mainly dependent on the activation of p65 (relA), one of the NF-κB family members [30]. Therefore, we tested the levels of activation of NF-κB p65. Western blot analyses revealed that the activation of p65 in the hippocampus, which was represented by the ratio of the phospho-p65 level to the total p65 level, decreased significantly (Student's *t*-test; $p < 0.01$; $n = 4$; Fig. 8A and C) in mIL-4-mice compared with CON mice on P21, when the mIL-4-mice had an anti-inflammatory status in the hippocampus (e.g., higher IL-4 level and lower IL-6 levels as shown in Fig. 4). Contrarily, NF-κB p65 activation was significantly increased (Student's *t*-test; $p < 0.001$; $n = 4$; Fig. 8B and D) in mIL-4-mice compared to the controls on P42, when the mIL-4-mice showed a proinflammatory status in the hippocampus (Fig. 4). These data indicate that neonatal mIL-4 over-exposure induced an instant anti-inflammatory cytokine response and the subsequent

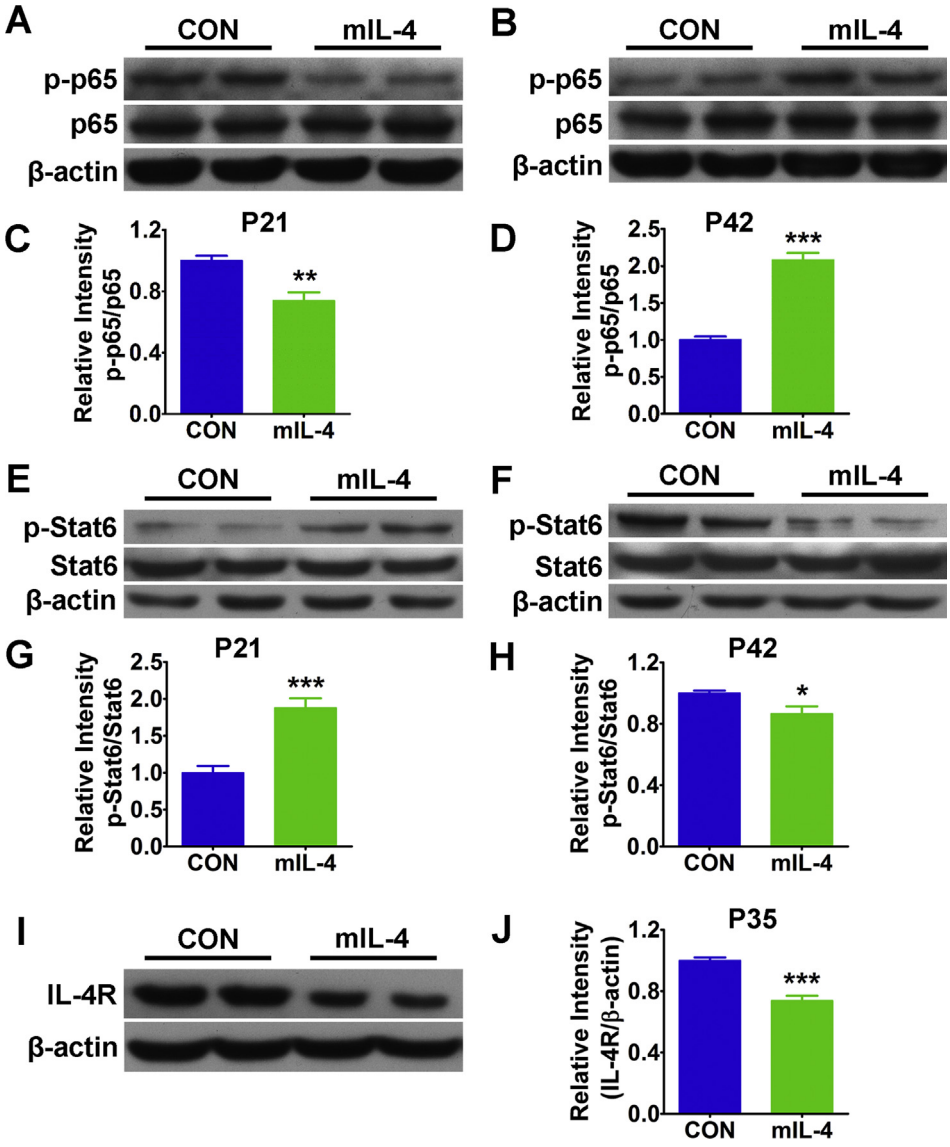

Fig. 8. The effects of mIL-4 administration on the NF-κB and Stat6 signaling pathways and IL-4R expression. Western blot analysis was used for the expression of p65, p-p65, Stat6, p-Stat6 and IL-4R. (A-D) Representative results for the Western blot analysis of p-p65 and p65 on P21 (A, C) and P42 (B, D). The relative quantification of p-p65 and p65 in each group of mice was normalized using the level of β-actin and presented as the ratio between p-p65 and p65. (E-H) Representative results of the Western blot analysis of p-Stat6 and Stat6 on P21 (E, G) and P42 (F, H). The relative quantification of p-Stat6 and Stat6 in each group of mice was normalized using the level of β-actin and presented as the ratio between p-Stat6 and Stat6. (I-J) Representative results of the Western blot analysis of IL-4R on P35. The data in (C), (D), (G), (H) and (J) represent the means ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $n = 4$/group; Student's *t*-test.

proinflammatory cytokine response in the hippocampus.

3.9. Neonatal mIL-4 over-exposure induced an increase and a subsequent decrease in Stat6 activation in the hippocampus

Stat6, a member of the signal transducer family and a transcriptional activator [31], is an essential component in mediating many biological functions of IL-4 [32–34], and IL-4 can antagonize NF-κB activation in a Stat6-dependent manner [35]. The Stat6 transcription factor blocks NF-κB transactivation by inhibiting the interaction of NF-κB with DNA. Thus, the Stat6 signaling pathway may have an important role in the IL-4-induced alteration of cytokines in the brain. The activation levels of Stat6 were detected using Western blotting on P21 and P42, when the mIL-4-mice had an anti-inflammatory and proinflammatory status in the hippocampus, respectively (Fig. 4). Compared with the controls, the Stat6 activation in the hippocampus, represented by the ratio of phospho-Stat6 level to the total Stat6 level, was increased significantly in mIL-4 mice on P21 (Student's *t*-test; $p < 0.001$; $n = 4$; Fig. 8E and G) but decreased significantly on P42 (Student's *t*-test; $p < 0.05$; $n = 4$; Fig. 8F and H).

3.10. Neonatal mIL-4 over-exposure induced the transient down-regulation of the IL-4 receptor

Stat6 acts as an immediate downstream signal transducer of IL-4 receptor activation [32–34]. Therefore, the alterations in Stat6 activation might result from the alteration of the function of IL-4R. Furthermore, receptor down-regulation often occurs when it is exposed to high levels of ligand [36]. Thus, we hypothesized that the down-regulation of IL-4R might be induced by the administration of exogenous mIL-4. The levels of IL-4R were detected using Western blotting on P35 in mIL-4-mice. Postnatal day 35 was chosen as the age for this analysis because it was the age when the elevated IL-4 levels in the hippocampus induced by either neonatal HBV or IL-4 injection restored to normal levels (Fig. 1A; Fig. 4A). As expected, the levels of IL-4R in the hippocampus decreased significantly in mIL-4 mice than controls (Student's *t*-test; $p < 0.001$; $n = 4$; Fig. 8I and J).

In addition, this down-regulation of IL-4R could not be observed in mIL-4-mice on P70 when neuroinflammation induced by HBV or IL-4 injection completely restored (Fig. 1; Fig. 4) (data not shown). These results demonstrated that neonatal mIL-4 over-exposure induced the transient down-regulation of the IL-4 receptor. This phenomenon explain the restoration of the hippocampal levels of proinflammatory cytokines as well as the spatial cognition impairments.

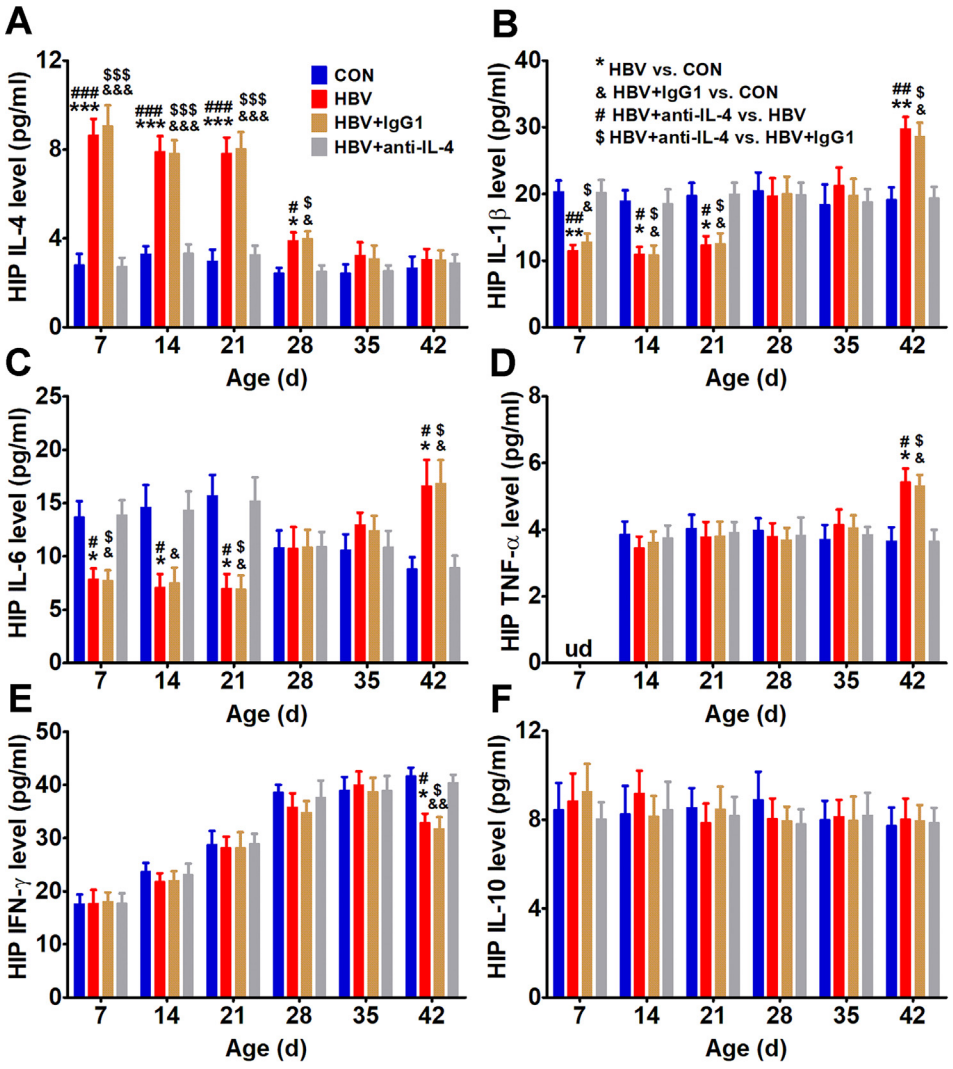

Fig. 9. Neutralization of IL-4 blocks the change of cytokines expression in the hippocampus induced by neonatal hepatitis B vaccination. (A-F) The bars represent the average levels of IL-4 (A), IL-1β (B), IL-6 (C), TNF-α (D), IFN-γ (E) and IL-10 (F) in the hippocampus. The data represent the means ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{\&}p < 0.05$; $^{\&\&}p < 0.01$; $^{\&\&\&}p < 0.001$; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$; $^{\$}p < 0.05$; $^{\$\$\$}p < 0.001$. The data were analyzed using one-way ANOVA followed by the Bonferroni post hoc test. $n = 6$/group. HIP: hippocampus; ud: undetectable.

3.11. Neutralization of IL-4 blocks the change of cytokines expression in the hippocampus and impairments in spatial learning and memory at 8-weeks-old induced by neonatal hepatitis B vaccination

To confirm the role of IL-4 in mediating the influences of neonatal hepatitis B vaccination on hippocampal cytokines expression and spatial cognition, we performed another experiment using anti-IL-4 neutralizing mAb and an isotype IgG1. We first found that administration of anti-IL-4 neutralizing mAb blocked the over-production of IL-4 in systemic blood at all detection age points (one-way ANOVA; HBV + anti-IL-4-mice vs. CON-mice: $p > 0.05$; $n = 6$; Fig. S6) but administration of the isotype IgG1 failed to block the HBV-induced peripheral IL-4 over-expression (one-way ANOVA; HBV + IgG1-mice vs. CON-mice: $p < 0.05$; HBV + IgG1 -mice vs. HBV-mice: $p > 0.05$; $n = 6$; Fig. S6).

Then, we found that neutralization of IL-4 blocked the HBV-induced alterations both in hippocampal cytokines expression (one-way ANOVA; HBV + anti-IL-4-mice vs. CON-mice: $p > 0.05$; $n = 6$; Fig. 9) and in the performance in the MWM tasks at 8-weeks-old in mice (RM-ANOVA; HBV + anti-IL-4-mice vs. CON-mice: $p > 0.05$; $n = 12$; Fig. 10), while administration of the isotype IgG1 showed no significant effects on blocking those HBV-induced alterations (Figs. 9 and 10).

4. Discussion

Our previous work showed neonatal hepatitis B vaccination led to spatial cognition impairment transiently at 8-weeks-old in mice, as well as a proinflammation profile of cytokine expression in the hippocampus [14]. IL-4 was the major cytokine in periphery induced by the HBV we used [15,16]. IL-4 itself is a powerful anti-inflammatory cytokine that induces the anti-inflammatory response and inhibits the production of proinflammatory cytokines [18]. This seems contrary to the hippocampal proinflammatory cytokine response found in mice that were immunized with HBV neonatally. Therefore, the present study was carried out to investigate the possible reason. We first found that HBV induced an anti-inflammation in the periphery, indicated by elevated IL-4 level and decreased levels of proinflammatory cytokines. Moreover, IL-4 had the largest amplitude and longest lasting alteration induced by HBV among these cytokines tested. After observing the positive correlation in the level of IL-4 in the periphery and that in the hippocampus, a series of experiments were carried out to examine the effects of neonatal IL-4 over-expression on the brain. Eventually, the present study provides direct evidence by means of neutralization of IL-4, supporting that IL-4 mediates a delayed neurobehavioral impairments induced by neonatal hepatitis B vaccination that involves the

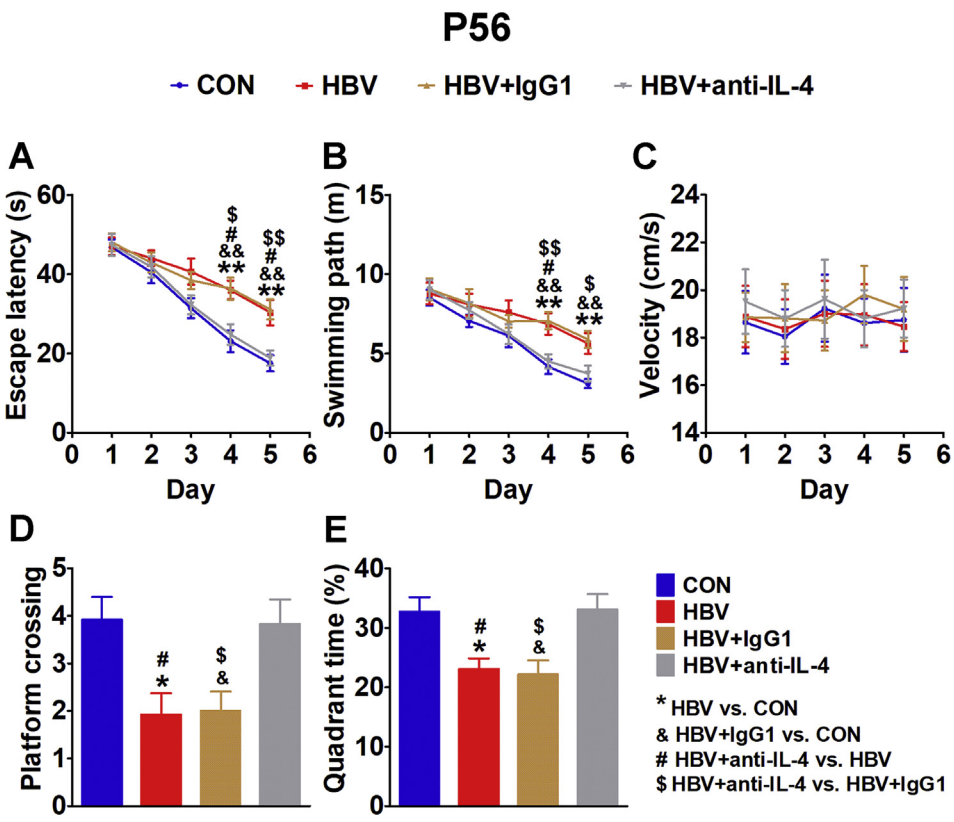

Fig. 10. Neutralization of IL-4 blocks the impairments in spatial learning and memory at 8-weeks-old induced by neonatal hepatitis B vaccination in mice. (A) The average escape latency of mice to reach the platform. (B) The average swimming path of mice to reach the platform. (C) The data showed the average swimming velocity of mice at 8 weeks of age. (D) The data showed the average numbers of platform area crossings by the mice. (E) The data indicate the average time proportion spent in the target quadrant by the mice. The data represent the means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$; $^{\&}p < 0.05$; $^{\&\&}p < 0.01$; $^{\#}p < 0.05$; $^{\$}p < 0.05$; $^{\$\$}p < 0.01$. The data in A-C were analyzed using two-way RM-ANOVA followed by the Bonferroni post hoc test and the data in D and E were analyzed using one-way ANOVA followed by the Bonferroni post hoc test; $n = 12$/group.

down-regulation of the IL-4 receptor in the hippocampus.

Notably, the behavioral impairment appeared in 8-week-old mice (Fig. 5), just during the age span (P42-P63) when the mice showed the delayed hippocampal neuroinflammation in HBV-mice as well as mIL-4-mice (Figs. 1 and 4). This phenomenon suggests that the latency for the emerging behavioral impairments in the HBV-mice and mIL-4-mice can be explained by the instant anti-inflammation caused by IL-4 (Figs. 1 and 4). The same phenomenon also suggests that the restoration of the behavioral impairments at 12 weeks of age in the HBV-mice and mIL-4-mice can be explained by the restoration of the proinflammatory response in the hippocampus from P70 (Figs. 1 and 4).

As stated in the Introduction section, early postnatal time is a critical period of brain development, when immune activation during this period could exert a long-lasting impact on brain development and behavior [2–5]. However, the exact mechanism of the critical period for immune activation affecting the brain is not fully understood yet. Here, we found not only the permeability of the neonatal BBB for IL-4 before P14 but also the prolonged permeability of the BBB for IL-4 by neonatal mIL-4 over-exposure. The present findings suggest that both the permeability of the neonatal BBB for cytokines and its regulation may provide some reasonable explanation for the nature of this critical period, although it needs further investigation to increase clarity on how the prolonging of BBB permeability happens.

The results in 3.8, 3.9 and 3.10 suggested that neonatal mIL-4 over-exposure induced the down-regulation of the IL-4 receptor in the hippocampus, which then led to a decrease in Stat6 activation and eventually resulted in a higher NF-κB activation level. These serial events concerning the IL-4R/Stat6 and NF-κB signaling pathways may account for the influences of the neonatal hepatitis B vaccination on the brain.

In our previous study [14], aluminum hydroxide adjuvant contained in the HBV had been verified to induce no significant alterations in the cytokine expression both in the periphery and in the brain and in the behavioral performances. These findings suggested that the immune responses by HBV is triggered by the whole vaccine (HbsAg + aluminum hydroxide adjuvant), but not by aluminum hydroxide adjuvant alone. Therefore, the effects of aluminum hydroxide adjuvant alone were not observed in our present study.

Given the reports that various neonatal immune stimuli have influences on the brain development and behavior [2,6,37], it is possible that over-exposure to other cytokines would also have a significant impact on long-term function. Addressing this issue would be significant. We will conduct further studies with different cytokines to that neonatal over-exposure often happens, such as IFN-γ and IL-6. However, the present study was designed to explore the mechanism by which neonatal HBV injection affected brain and behavior. Although vaccine may increase the levels of several cytokines in addition to IL-4, IL-4 was the only cytokine that has a significant correlation between its levels in the serum and its levels in the hippocampus (Fig. 3A–D) and was the cytokine with the greatest amplitude of increase both in the hippocampus and in the serum in HBV-mice (Figs. 1A and 2A). What's more, anti-IL-4 neutralizing mAb blocked both hippocampal cytokine expression alterations and behavior impairments induced by HBV. Accordingly, IL-4 is very likely to have a key role in mediating the delayed neurobehavioral impairments induced by neonatal hepatitis B vaccination.

An interesting question is which population of cells in the hippocampus are involved in the altered signaling process mentioned above, and addressing this question will contribute to the better understanding of the IL-4-induced neurobehavioral impairments at the cellular level. We are currently working on this issue. However, we will address it in the future because the scientific hypothesis in the present work that IL-4 can mediate the neurobehavioral impairments caused by neonatal hepatitis B vaccination has already been supported by both indirect and direct data provided by the current study.

5. Conclusions

Our current research demonstrates that IL-4 mediates the delayed neurobehavioral impairments induced by neonatal hepatitis B vaccination, which involves the permeability of the neonatal BBB and the

down-regulation of the IL-4 receptor. This conclusion was made according to these major results: 1) neonatal hepatitis B vaccination induced delayed hippocampal neuroinflammation and spatial cognition impairment after an instant anti-inflammatory cytokine response in the hippocampus; 2) neonatal IL-4 over-exposure imitated all the HBV-induced neurobehavioral effects; 3) peripheral IL-4 is able to penetrate into the hippocampus during the neonatal period; and 4) the permeability of the BBB in neonatal mice might explain the penetration of peripheral IL-4 into the brain and 5) the decreased levels of IL-4R in the hippocampus by neonatal IL-4 over-exposure.

These findings suggest that clinical events involving neonatal IL-4 over-exposure, including neonatal hepatitis B vaccination and asthma in human infants, may have adverse effects on neurobehavioral development.

6. Conflict of interest statement

The authors declare that there are no conflicts of interest.

7. Funding

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.cyto.2018.04.037.

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