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ORIGINAL ARTICLE

Immunopotentiator Thymosin Alpha-1 Promotes Neurogenesis and Cognition in the Developing Mouse *via* a Systemic Th1 Bias

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Abstract In early life, the immune system plays an essential role in brain development. In our study, the immunopotentiator thymosin alpha-1 (Ta1) was peripherally administered to neonatal mice to explore whether the peripheral immunopotentiator affects neurodevelopment and cognition, and to further investigate the relevant mechanism. Compared with the control group, the Ta1 mice displayed better cognitive abilities in early life. The numbers of 5-bromodeoxyuridine (BrdU)+, nestin+, T-box transcription factor 2 (Tbr2)+, BrdU+/doublecortin (DCX)+, BrdU+/ionized calcium-binding adaptor molecule 1 (Iba1)+, and BrdU+/neuronal nuclei (NeuN)+ cells in the hippocampus were increased in the Ta1 group, accompanied by increased interleukin-4 (IL-4), interferon-gamma, brain-derived neurotrophic factor, nerve growth factor, and insulin-like growth factor-1 as well as decreased IL-6 and tumor necrosis factor-α. Furthermore, the Ta1-group showed a Th1-polarized immune response, and the neurotrophic factors were positively associated with the Th1/Th2 ratio. More importantly, administration of Ta1 blocked lipopolysaccharide-induced impairment of hippocampal neurogenesis in early life. These findings suggest that peripheral Ta1 contributes to neurogenesis and cognition probably through a systemic Th1 bias, as well as neuroprotection against LPS infection by Ta1.

Keywords Microglia · Th1/Th2 bias · IGF-1 · Brain-derived neurotrophic factor · Nerve growth factor

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Introduction

An increasing number of studies have indicated that the peripheral immune response plays a vital role in neuronal plasticity and behavioral function. Environmental/psychological stimuli activate the immune system, which positively regulates neurogenesis and promotes learning, memory, and hippocampal long-term potentiation [1]. Moreover, previous research reported that T cells cross-talk with microglia to cause neural stem and progenitor cells to proliferate, migrate, and differentiate, and such microglia locally produce growth factors such as insulin-like growth factor-1(IGF-1) [2, 3]. Conversely, immune deficiency, brain aging, or inflammatory conditions result in the impairment of neurogenesis and cognitive dysfunction, but these effects can be reversed by immune activation [4–7].

Now, many immunomodulatory medicines, such as the immunopotentiator thymosin alpha-1 (Ta1), are widely

used in the clinic. Ta1, a 28 amino-acid peptide, is an active polypeptide produced by the thymus. It has been reported that thymectomy impairs learning and memory in mice [8], whereas Ta1 itself modulates excitatory synaptic transmission in cultured hippocampal neurons [9].

Early life is a crucial time in the development of the central nervous system [10]. In early postnatal life, the brain is at a stage in which immune activation can impact the developmental programming of the brain and behavior [11, 12]. Researchers have reported that early activation of the immune system may negatively affect an organism's neurodevelopmental trajectory. For example, neonatal immune activation with lipopolysaccharide (LPS) leads to an increase of reactivity to stress, decreased hippocampal neurogenesis, and behavioral alterations in adulthood [13–17]. However, we previously showed that neonatal immune activation with Bacillus Calmette-Guérin improves neurogenesis and behavior in early life [18]. Given the above facts, the aim of this study was to verify that Ta1, an immunopotentiator commonly used in the clinic, influences neurodevelopment, and to further investigate the mechanism by which neonatal immune activation affects neurodevelopment.

Materials and Methods

Animals and Study Design

Newborn (P0) C57BL/6J mice were purchased from the Sun Yat-Sen University Laboratory Animal Center (Guangzhou, China). The animals were housed under adequate temperature (22 ± 2 °C) and humidity (55%) control with a 12/12 h light/dark cycle in a specific pathogen-free facility. All procedures were approved by the Ethics Committee of Sun Yat-Sen University and carried out according to the animal care guidelines of the National Institutes of Health.

Our studies were divided into two parts (Fig. 1). In the first part, mice were randomly assigned into two groups (Ta1 and control groups) for behavioral testing as well as immunofluorescence and enzyme-linked immunosorbent assay (ELISA). The Ta1 (Zadaxin), in lyophilized powder form, was dissolved in sterile distilled water. Each mouse in the Ta1 group received daily subcutaneous injections on the back with 20 μL (0.4 mg/kg) Ta1/mouse from P0 to P4 [19]. Control animals were given normal saline injections in the same way for the same period of time.

In the other study, mice were randomly assigned into three groups (Control, LPS, and Ta1 + LPS groups) for assessing hippocampal neurogenesis. Mice in the LPS and control groups were injected intraperitoneally (i.p.) with a single dose of LPS (1 mg/kg, *Escherichia coli* 0111:B4, Sigma, St. Louis, MO) or normal saline on P9 [14]. Each mouse in the Ta1 group (Ta1 + LPS) received subcutaneous injections of Ta1 (0.4 mg/kg) from P0 to P4 and then given a single i.p. injection of LPS on P9.

Open Field Test (OFT)

The open-field apparatus consisted of a Plexiglas square arena (50 cm × 50 cm × 38 cm) on the floor in a quiet room. Each mouse ($n = 10$) was placed individually at the center of the arena and permitted free exploration. Behaviors were observed for a 10-min period. The time spent in the central area (30 × 30 cm^2), the frequency of entries into the central area and the total distance travelled were automatically recorded and analyzed using computerized tracking system (Noldus EthoVision XT, Guangzhou, China) [20]. The instrument was cleaned with 5% ethanol after each trial.

Morris Water Maze (MWM)

Spatial learning and memory were examined using the MWM [21, 22]. The MWM test began on the second day after the open-field test. The MWM consisted of a white circular pool (100 cm diameter, 60 cm height, and 30 cm depth) filled with water at 23 ± 2 °C and divided into four quadrants, painted black. The 9-cm hidden platform was positioned 1 cm below the water surface in one quadrant. The water was colored with non-toxic white dye to make the submerged platform invisible to the mice. Mice were placed into the water in one of the four starting positions facing the pool wall and allowed to swim to find the platform. Each mouse was given 4 successive trials per day for 4 consecutive days. Once the mouse reached the platform, it was permitted to stay on it for 15 s and the escape latency was recorded. If a mouse failed to find the platform within 60 s, it was guided to it for 15 s and the escape latency was recorded as 60 s. On the fifth day of the probe trial test, the platform was removed and the mice were allowed to swim freely in the pool for 60 s to search for the platform. The time spent in the target quadrant was recorded by an MT-200 Morris image motion system (Chengdu Technology & Market Corp., Chengdu, China).

Administration of 5-bromodeoxyuridine (BrdU) and Tissue Preparation

Mice received a once-daily i.p. injection of BrdU (50 mg/kg body weight, Sigma) for 5 consecutive days and then were sacrificed at different time points (2, 7, and 28 days after the first injection). Brains were removed and fixed overnight in 4% paraformaldehyde at 4 °C and then dehydrated in 10% sucrose followed by 30% sucrose for 24 h each at 4 °C. Serial coronal sections (40 μm) were cut

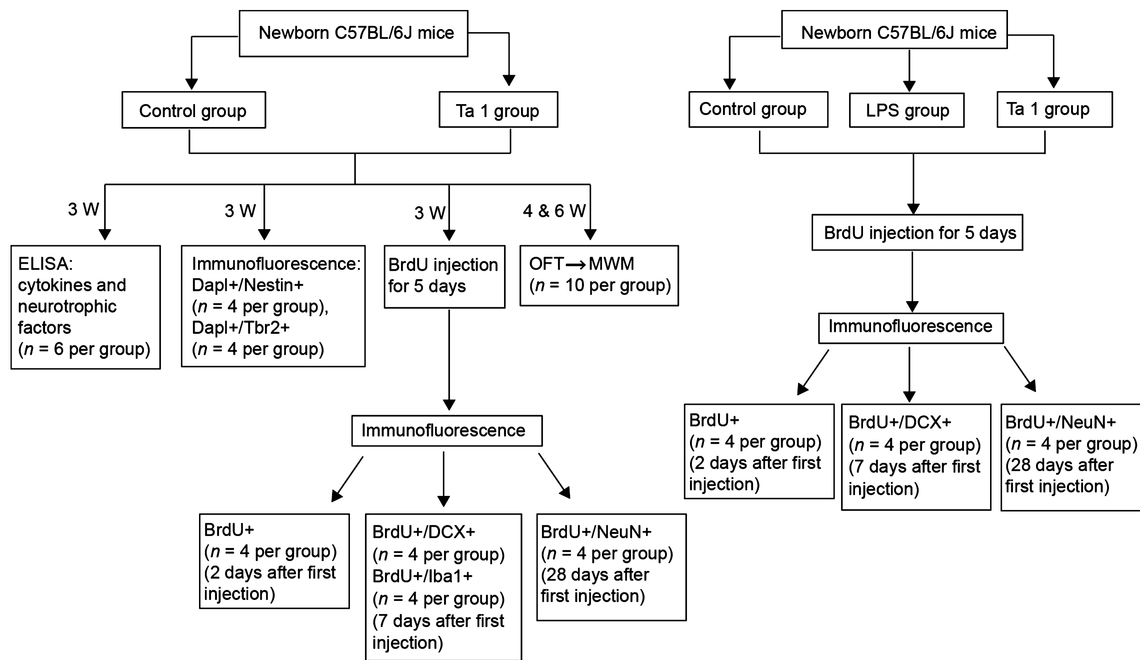

Fig. 1 Schematic of the experimental design. OFT, open field test; MWM, Morris water maze.

using a freezing microtome (Leica SM2000R, Richmond Hill, Ontario, Canada) and stored at 4 °C.

Immunofluorescence

The mice were sacrificed on day 2 after the first BrdU injection for BrdU+ cells ($n = 4$), on day 7 for BrdU+/DCX+ ($n = 4$) and BrdU+/Iba1+ cells ($n = 4$), and on day 28 for BrdU+/NeuN+ cells ($n = 4$). For BrdU labeling, brain sections were pre-treated with 2 N HCl at 37 °C for 30 min, then blocked for 1 h in phosphate-buffered saline (PBS) containing 1% bovine serum albumin and 0.25% Triton X-100 (Sigma-Aldrich,). Sections were stained with the primary antibodies overnight at 4 °C. The primary antibodies were rat anti-BrdU (1:400; Oxford Biotechnology, UK), mouse anti-nestin (1:400, Abcam, Cambridge, MA), rabbit anti-Tbr2 (1:400, Abcam), goat anti-DCX (1:400; Santa Cruz Biotechnology, CA), rabbit anti-Iba1 (1:1000; Wako Chemicals, Richmond, VA), and mouse anti-NeuN (1:800; Sigma). After washing 3 times with PBS on the next day, the specimens were incubated with the secondary antibodies Alexa Fluor 594 donkey anti-rat (1:400; Molecular Probes, Invitrogen, Eugene, OR), Alexa Fluor 488 donkey anti-goat (1:400; Molecular Probes, Invitrogen), Alexa Fluor 488 goat anti-mouse (1:400; Molecular Probes, Invitrogen) and Alexa Fluor 488 goat anti-rabbit (1:400; Molecular Probes, Invitrogen) at 37 °C for 2 h. The sections were counterstained with DAPI to observe nuclei. For quantitation of labeled cells in granule cell layer/subgranular zone (GCL/SGZ) by the optical-fractionator method of stereology, Stereo Investigator (MicroBrightField, Williston VT), we stained every 6th coronal section (240 μm apart) spanning the entire hippocampus.

Enzyme Linked Immunosorbent Assay

The hippocampi from mice were homogenized, followed by centrifugation for 10 min at 12,000 ×g at 4 °C. After quantification of the total protein level using a BCA protein assay kit (Beyotime Biotechnology, Shanghai, China) and adjusted to 4.5 mg/mL, the brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and insulin-like growth factor 1 (IGF-1) protein levels were measured using an ELISA kit (Promega, Madison, WI) according to the manufacturer's instructions. The production of cytokines (IFN-γ, TNF-α, IL-4 and IL-6) in the serum and hippocampus was also determined with an ELISA kit (eBioscience, San Diego, CA).

Statistical Analysis

Data from place navigation test in the MWM task were analyzed using two-way ANOVA with repeated measures. Comparisons between two groups were made with Student's unpaired *t*-test. Comparisons among three groups used one-way ANOVA followed by LSD *post hoc* comparisons. Data are expressed as mean ± SEM and $P < 0.05$ was considered statistically significant.

Results

Ta1 Improves Exploration

We tested C57BL/6J mice in a novel, open-field activity box to assess spontaneous motor activity and adaption in a new environment at 4 and 6 weeks. In this assessment, the Ta1-group spent more time in the central area ($P < 0.01$; Fig. 2A) and had a higher frequency of entry to the central area ($P < 0.05$) (Fig. 2B) than the control group at 4 weeks, but no significant difference was found at 6 weeks. The total distance in the Ta1-group was longer than the control group, but the difference was not significant ($P > 0.05$) (Fig. 2C). The representative images of movement on the OFT were recorded (Fig. 2D). These findings suggest that Ta1 improves exploration in mice at 4 weeks.

Ta1 Improves Learning and Memory

We compared the MWM performance of the Ta1 and control groups after the OFT. In the acquisition phase, the Ta1 group had a shorter escape latency than controls on day 4 at 4 weeks ($P < 0.05$) (Fig. 3A), but no significant difference was found at 6 weeks ($P > 0.05$) (Fig. 3B). In the probe phase, the Ta1-treated mice spent more time and a larger percentage of time in the target quadrant than the control group at 4 weeks ($P < 0.05$) (Fig. 3C). These data indicate that Ta1 improves mouse learning and memory at 4 weeks.

Ta1 Promotes Neurogenesis and Newborn Microglia

Reports have revealed that hippocampal neurogenesis affects cognitive abilities [23, 24]. Therefore, we examined the influence of immunomodulatory treatment on neurogenesis. We administered BrdU on day 21 to assess neurogenesis. The mice were sacrificed at 2, 7, or 28 days after the first BrdU injection and the brains were collected. There were more proliferating cells (BrdU+; $P < 0.01$; Fig. 4A, B, and M), neural stem cells (Nestin+; $P < 0.05$; Fig. 4C, D, and M), neuronal progenitors (Tbr2+; $P < 0.05$; Fig. 4E, F, and M), new progenitors (BrdU+/DCX+; $P < 0.01$; Fig. 4G, H, and M), and new neurons (BrdU+/NeuN+; $P < 0.01$; Fig. 4I, J, and M) in the dentate gyrus of the Ta1 group than controls.

In addition, the Ta1 mice had more BrdU+/Iba-1+ cells than controls ($P < 0.05$) (Fig. 4K, L, and N).

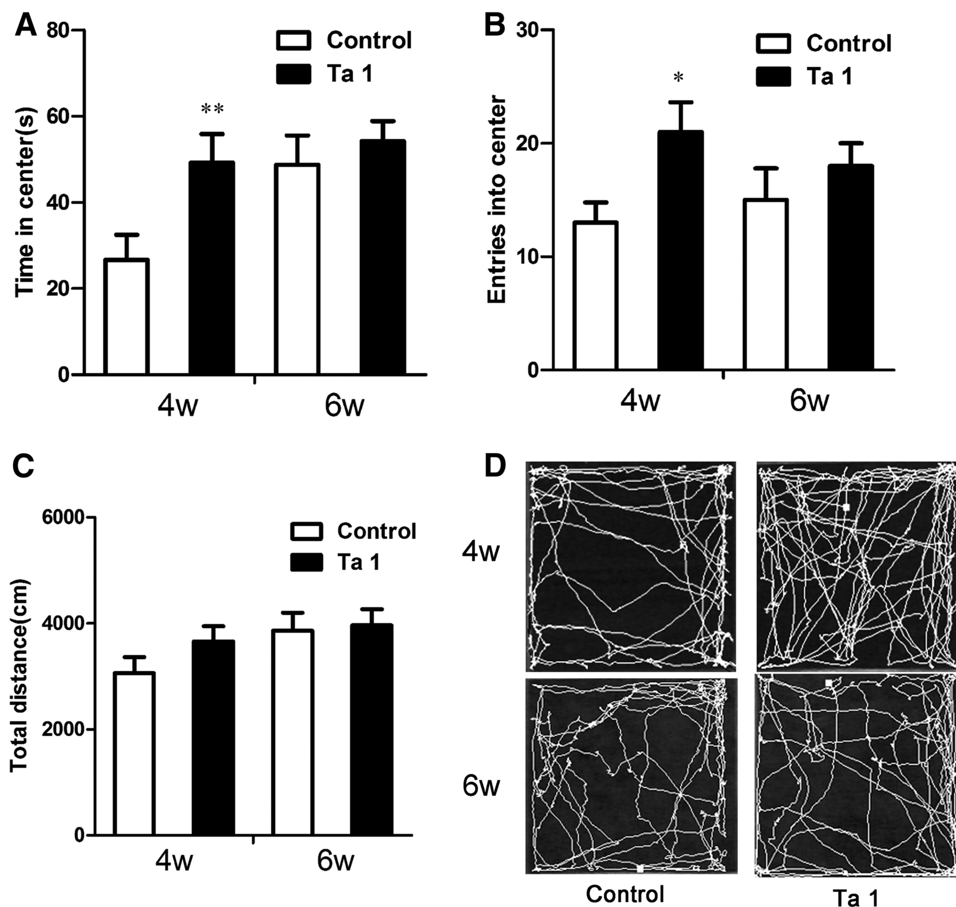

Fig. 2 Behavior in the open field test. **A–C** Time spent in center (**A**), frequency of entrances to center area (**B**), and total distance moved (**C**) (mean ± SEM; $n = 10$; $*P < 0.05$, $**P < 0.01$, compared to control; Student's t-test). **D** Representative images of movements at 4 and 6 weeks in control and Ta1-treated mice.

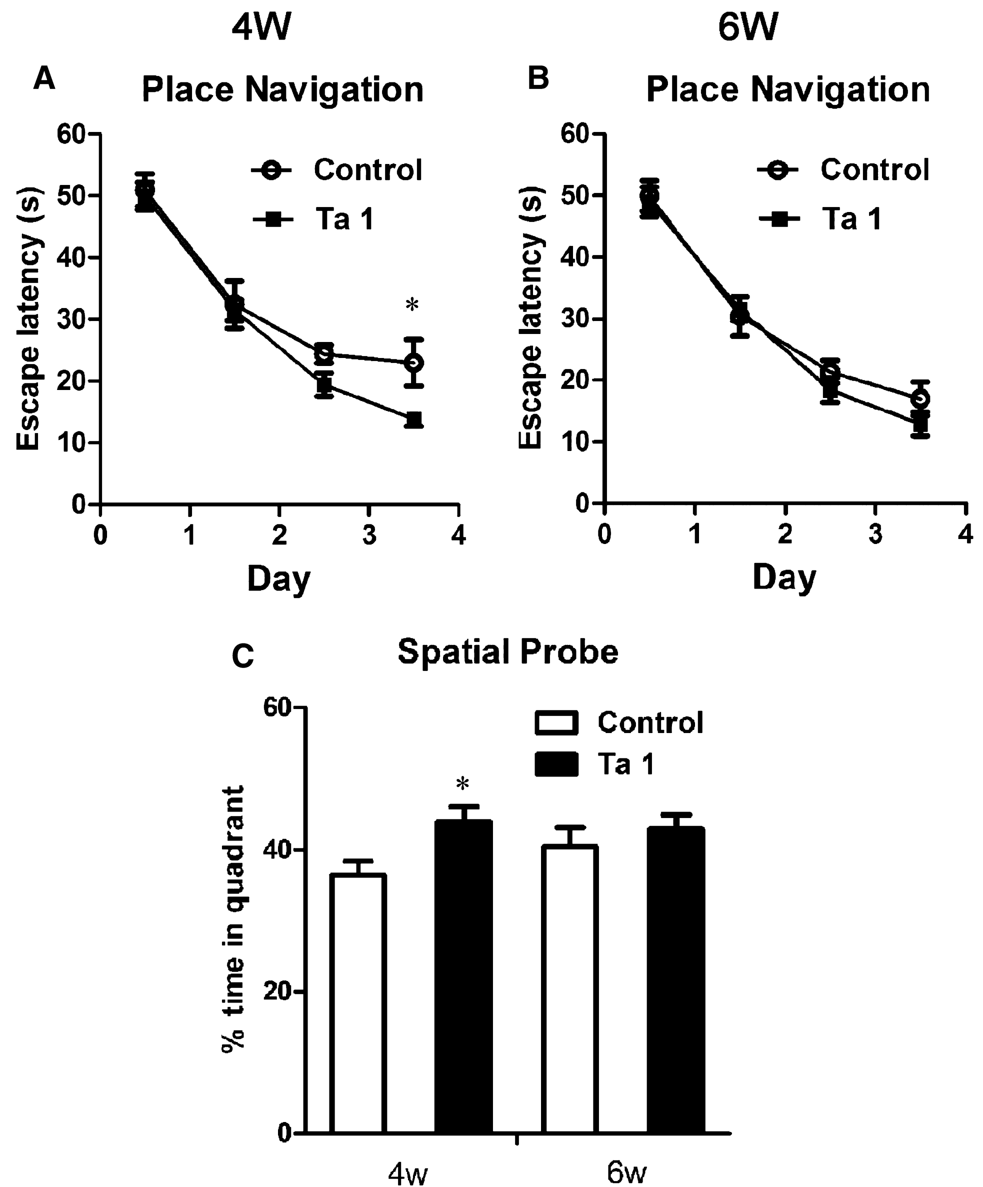

Fig. 3 Performance in the Morris water maze. **A** and **B** Escape latency in the hidden platform phase (days 1–4) at 4 (**A**) and 6 (**B**) weeks (analyzed using two-way repeated measures ANOVA). **C** Average percentage of time spent in the target quadrant during the probe trial (day 5) (analyzed using Student's *t* test). Data expressed as mean ± SEM ($n = 10$); *$P < 0.05$ compared with control.

These results show that Ta1 benefits proliferation and neuronal differentiation, and that peripheral immunoactivation leads to an increase of newborn microglia.

Ta1 Changes the Levels of Cytokines and Hippocampal Neurotrophic Factors and Induces a Th1-Polarized Immune Response

Cytokines play a critical role in mouse brain development [3, 25]. In our study, the Ta1-group showed more IFN-γ and IL-4 both in the serum ($P < 0.05$; Fig. 5A) and the hippocampus ($P < 0.05$; Fig. 5B) than the control on day 21. The levels of IL-6 and TNF-α in the hippocampus were lower in the Ta1-group ($P < 0.05$; Fig. 5A and B). The ratio of IFN-γ (a classical Th1-derived cytokine) to IL-4 (a classical Th2-derived cytokine), which represents the Th1/Th2 balance [26, 27], revealed a Th1-polarized immune response both in the peripheral blood and the hippocampus of the Ta1 group compared to the control ($P < 0.05$; Fig. 5C).

In addition, the neurotrophic factors BDNF, NGF, and IGF-1 in the hippocampus were increased in the Ta1-group compared with the control on day 21 ($P < 0.05$; Fig. 5D–F).

The Hippocampal IFN-γ/IL-4 Ratio is Correlated with BDNF, NGF, and IGF-1 Levels

Neurotrophic factors are important to neurogenesis. The association of the IFN-γ/IL-4 ratio with BDNF, NGF, and IGF-1 is thought to be equivalent to the Th1/Th2 ratio [18, 28, 29], and here we found a positive correlation

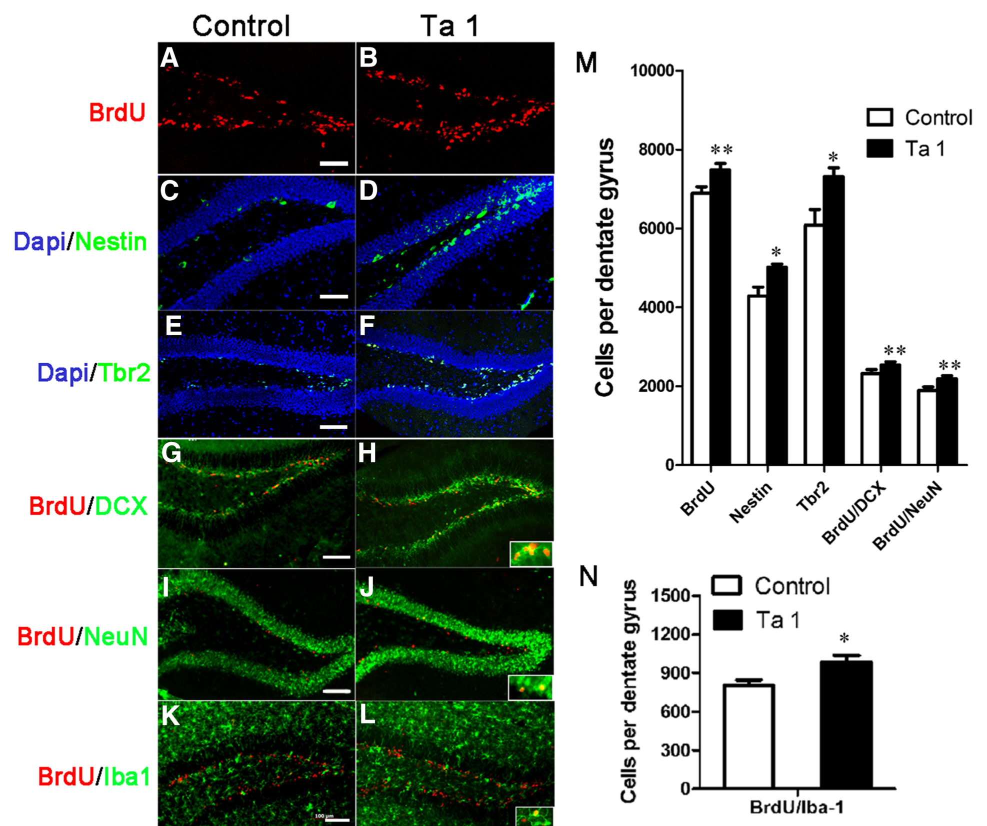

Fig. 4 Effect of Ta1 on neurogenesis in the dentate gyrus of mice assessed using fluorescence microscopy. **A–F** Representative images of newly-formed cells (BrdU) (**A** and **B**), Nestin (green) (**C** and **D**), and Tbr2 (green) (**E** and **F**); nuclear DAPI expression is shown in blue. **G** and **H** Double-staining for BrdU (red) and DCX (green). **I** and **J** Double-staining for BrdU (red) and NeuN (green). **K** and **L** Double-staining for BrdU (red) and Iba-1 (green) in the dentate gyrus. **M** Quantification of BrdU+, Nestin+, Tbr2+, BrdU+/DCX+, and BrdU+/NeuN+ cells in the dentate gyrus. **N** Quantification of BrdU+/Iba-1+ cells in the dentate gyrus. Data are expressed as the mean ± SEM in each group ($n = 4$). Scale bars, 100 µm. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control.

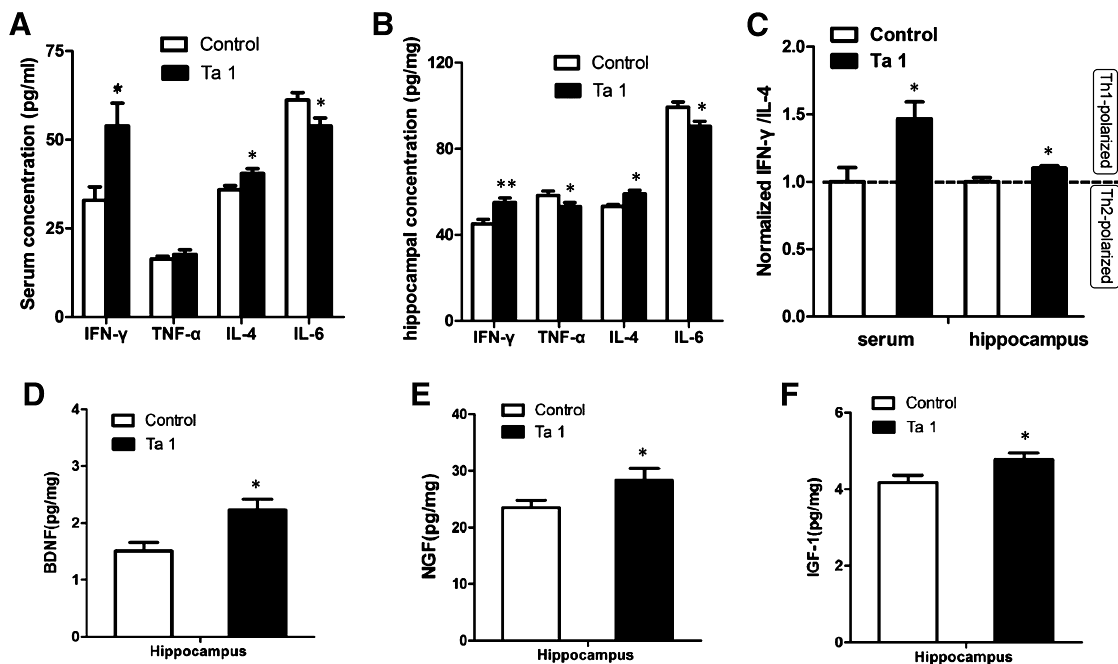

Fig. 5 Effects of Ta1 on cytokines and hippocampal neurotrophic factors. **A** and **B** Levels of IFN-γ, TNF-α, IL-4, and IL-6 in serum (**A**) and in the hippocampus (**B**) analyzed using ELISA. **C** Standardized ratio of IFN-γ to IL-4, representing the Th1/Th2 balance. **D–F** Protein levels of BDNF (**D**), NGF (**E**), and IGF-1 (**F**) in the hippocampus using ELISA. Data are expressed as the mean ± SEM in each group ($n = 6$; $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control).

between the hippocampal IFN-γ/IL-4 ratio and BDNF, NGF, and IGF-1 in the hippocampus (Fig. 6).

Ta1 Pretreatment Restores LPS-Induced Alterations of Hippocampal Neurogenesis in Early Life

Given the above results, we further investigated whether Ta1 has a protective effect on subsequent neurogenesis injury induced by LPS in early life. We found that LPS exposure induced decreases in BrdU+, BrdU+/DCX+, and BrdU+/NeuN+ cells in the hippocampus ($P < 0.05$; Fig. 7A and B). But Ta1 pretreatment increased hippocampal neurogenesis compared with the LPS group ($P < 0.05$; Fig. 7). These results indicate that Ta1 restores hippocampal neurogenesis affected by LPS in early life.

Discussion

Our behavioral results showed that immunopotentiator Ta1 improved exploration by mice in the OFT and enhanced spatial learning and memory in the MWM in early life. Also, the morphological results revealed that Ta1 increased the numbers of BrdU+, Nestin+, Tbr2+, BrdU+/DCX+, and BrdU+/NeuN+ cells, suggesting that Ta1 enhances proliferation and neuronal differentiation. These results indicate that neonatal immune activation with Ta1 promotes neural development.

Previous studies have shown that the immune system, especially in early life, plays an essential role in brain development [30]. Cytokines and neurotrophins are important mediators of the effects of peripheral immune activation on brain development [3, 25]. Here, we found that Ta1 increased the levels of the IFN-γ, IL-4, BDNF, NGF, and IGF-1 proteins, but decreased the levels of the TNF-α and IL-6 proteins in the hippocampus. First, the increased IL-4 and IFN-γ in serum and hippocampus were accompanied by increased numbers of microglia, indicating that the peripheral immune and central immune systems were activated by Ta1. Moreover, increasing evidence indicates that the immune system influences brain development *via* cytokines, which can cross the blood-brain barrier [25]. These two cytokines are beneficial to cognition and hippocampal neurogenesis [31], suggesting that Ta1 elevates neurogenesis and affects behavior possibly through the increases in IL-4 and IFN-γ. On the

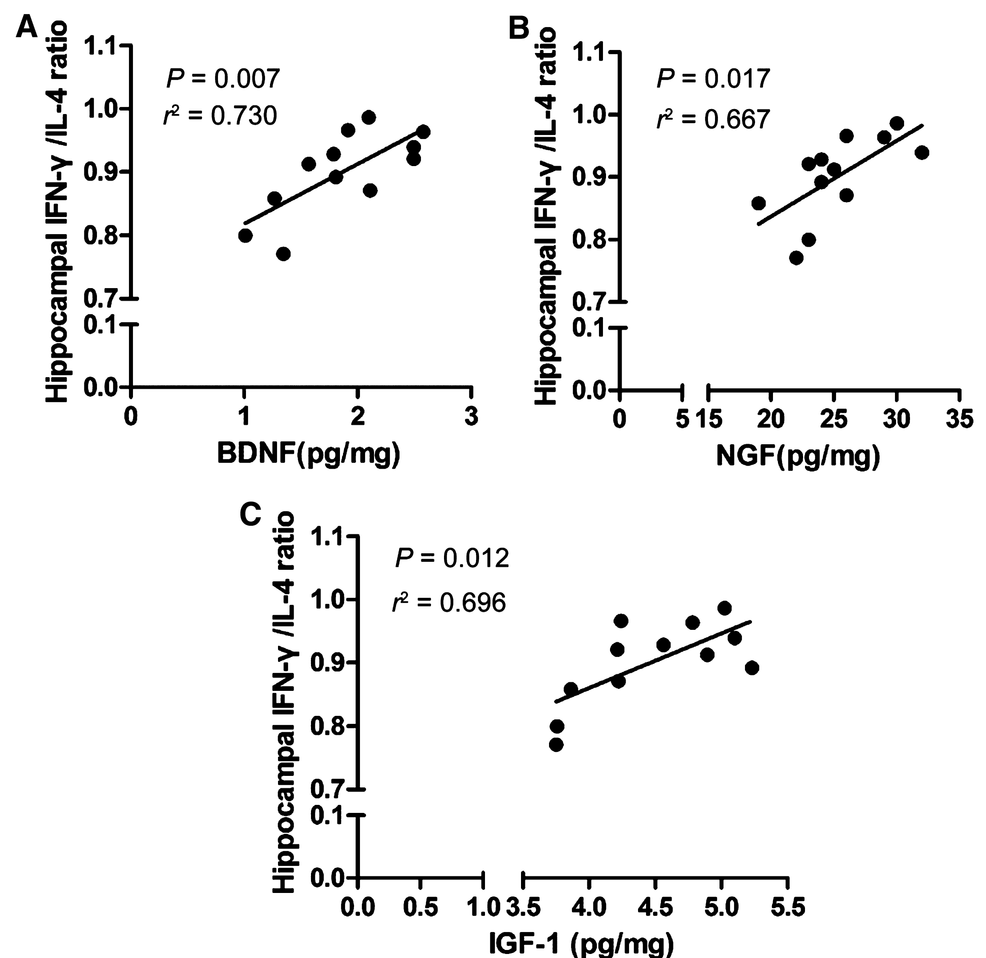

Fig. 6 Hippocampal Th1/Th2 ratio correlations with BDNF, NGF, and IGF-1. **A**–**C** Correlation analysis between the hippocampal IFN-γ/IL-4 ratio and BDNF (**A**; $n = 12$; Pearson $r^2 = 0.730$), NGF (**B**; $n = 12$; Pearson $r^2 = 0.667$), and IGF-1 (**C**; $n = 12$; Pearson $r^2 = 0.696$).

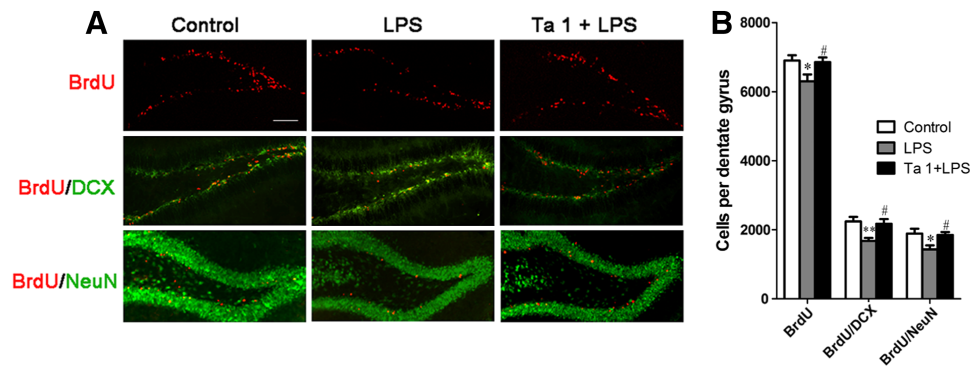

Fig. 7 Effect of Ta1 on hippocampal neurogenesis induced by LPS. **A** Representative images of newly-formed (BrdU), BrdU+ (red)/DCX+ (green), and BrdU+ (red)/NeuN+ (green) cells in the dentate gyrus (scale bar, 100 μm). **B** Quantification of BrdU+, BrdU+/DCX+, and BrdU+/NeuN+ cells as in **A**. The values are the mean ± SEM in each group ($n = 4$; *compared with control, #compared with LPS group; $*P < 0.05$, $**P < 0.01$, $^{\#}P < 0.05$, one-way ANOVA followed by LSD *post hoc* test).

other hand, TNF-α and IL-6, which are associated with a detrimental impact on cognition, were reduced in the Ta1 group [32, 33]. It has been demonstrated that BDNF, NGF, and IGF-1 are important for various aspects of hippocampal plasticity, including neurogenesis and spatial memory [34–37]. These studies prompted us to examine the influence of immunomodulatory treatment on neurotrophic factors. And our findings showed that BDNF, NGF, and IGF-1 were up-regulated after administration of Ta1, in line with the increase in cell proliferation. In brief, these alterations of cytokines and neurotrophins can be characterized as a neurotrophic molecular profile because IFN-γ, IL-4, BDNF, NGF, and IGF-1 are beneficial to neurogenesis and cognition, while TNF-α and IL-6 are neurotoxic factors. The neurotrophic molecular profile in the hippocampus can explain why Ta1 increases neurogenesis and improves cognition.

As the resident immune cells in the brain, newborn microglia increased after administration of the immunopotentiator Ta1. Microglia have neuroprotective or neurotoxic effects under different conditions. Although microglia were initially thought to be detrimental to neurogenesis, it is now clear that under quiescent conditions they play an important role in supporting neurogenesis [3, 38, 39]. Microglia activated by IL-4 or IFN-gamma differentially induce elevation in the number of newly-formed neurons in the hippocampus [31]. In our study, the increased microglia were accompanied with increased IL-4 and IFN-γ, implying that newborn microglia facilitate hippocampal neurogenesis.

Recently, the Th1/Th2 balance has been shown to be involved in hippocampal neurogenesis [40, 41]. Researchers have reported that decreased hippocampal neurogenesis and cognition are associated with a decreased Th1/Th2 balance in animal studies of aging. In our study, Ta1 induced a Th1-polarized immune response both in the serum and in the hippocampus. This suggested that Ta1 has beneficial effects in developing mice probably by inducing a Th1 bias. Also, we found that neurotrophic factors were positively associated with the IFN-γ/IL-4 ratio, and this demonstrated that the hippocampal neurogenesis and cognition enhanced by Ta1 in developing mice acts *via* inducing a Th1-shift of systemic cytokines and neurotrophins.

Our results showed that the significant changes in hippocampal neurogenesis and cytokines occurred at 3 weeks and the behavioral changes at 4 weeks. However, there were no evident differences in later life. These results suggest that Ta1 promotes transient neurogenesis and cognition. This is understandable because an organism has its own powerful homeostatic processes, especially during development.

Early life is a stage at which infection is more likely to occur than in the adult stage. And it has been reported that infection induced by LPS can lead to reduced neurogenesis and cognitive impairment [13–16]. Thus, in order to investigate the clinical application of Ta1 in neonates, we further assessed whether Ta1 prevents the damage to hippocampal neurogenesis in an early-life inflammatory challenge induced by LPS. We found that Ta1 restored the LPS-induced decline in hippocampal neurogenesis. Such data suggest that Ta1 may be a protective strategy to ameliorate or prevent the reduction of neurogenesis by LPS in early life.

In conclusion, our findings reveal a series of beneficial effects, including the elevation of cognitive function and hippocampal neurogenesis in early life, after administration of the peripheral immunopotentiator Ta1. And the underlying mechanism may involve a systemic Th1 bias. Perhaps more importantly, this work also showed that Ta1 restores the LPS-induced decline in hippocampal neurogenesis. Thus, Ta1 may play a preventive role against infection in early life.

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Compliance with Ethical Standards

Conflict of interest We declare no conflict of interest.

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