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The pediatric anti-viral immune response impairs neural stem/ progenitor cell activity

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1. INTRODUCTION:

Viral infections of the brain are one of the major causes of central nervous system (CNS) disease. Although the brain is well protected by the blood brain barrier, neurotropic viruses can gain entry into the brain via many routes like retrograde transport through motor and olfactory neurons, direct infection of the endothelial cells, and by crossing the blood brain barrier [1]. Several viruses like measles virus (MV), cytomegalovirus (CMV), lymphocytic choriomeningitis virus (LCMV), herpes simplex virus (HSV) enter the brain and cause disease. Some of the pathological outcomes of these viral infection are immediate (e.g. meningitis and encephalitis), while conditions like epilepsy, blindness, hearing loss, and cognitive deficits occur subsequently [1, 2]. Neurotropic infections can also lead to neuropsychiatric dysfunction like memory loss, bipolar disorder, schizophrenia, and hallucinations [2].

The age of the host plays an important role in the outcome of CNS disease. Neurotropic infections may impair brain development if it occurs in the prenatal and postnatal stages [1, 3-6]. A number of animal models have been shown to recapitulate this age-dependent pathology during neurotropic infections [5, 7]. Sindbis virus, MV, LCMV, and borna disease virus (BDV) induce mortality in neonates while adults survive the infection [5, 8-10]. Although majority of the brain development occurs in the fetal and neonatal periods, developmental processes are ongoing in the pediatric age. In humans, the brain reaches 90-95% of its adult size by ~3 years of age, which corresponds to post-natal day 21 (P 21) in a rodent. Development of the prefrontal cortex, synaptogenesis, and pruning occurs majorly from pediatric to juvenile phase in humans and rodents [11, 12]. Additionally, the immune system also continues to develop postnatally. The two major arms of the immune response (innate and adaptive) mature with age [13]. As such, neonates have limited capability to mount a robust immune response to infections

compared to adults. In rodents, peak antigen responsiveness and changes in the adaptive immune system has been shown to occur between postnatal week 2-3 [14-16]. Thus, a CNS viral infection during this period could lead to substandard immune response that might fail to control the infection and ultimately affect CNS development.

Neural stem/progenitor cells (NSPCs) are the multipotent progenitor cells of the CNS, which differentiate to form neurons, astrocytes, and oligodendrocytes. With age, the NSPC pool becomes restricted to two neurogenic niches of the brain, namely the hippocampus and the sub-ventricular zone (SVZ) [17, 18]. In the hippocampus, the NSPC pool is involved in neurogenesis that helps continue memory and learning process while in the SVZ, the NSPC pool plays a role in olfaction [19, 20]. CMV, HSV, West Nile virus (WNV), LCMV, MV, BDV, and rabies virus are known to impact NSPC activity either by inhibiting their proliferation or altering their differentiation [2, 21-23]. As NSPCs are responsible for formation of neurons and glia, impairment of NSPC activity can impact overall neurogenesis and gliogenesis. This impairment in the hippocampus might impact memory and learning. This alteration in NSPC activity can be direct; where viruses infect NSPCs or indirect; via the anti-viral immune response. Our lab focuses on the indirect impact of viral infections on the NSPCs.

In the current study, our goal was to define the impact of a pediatric anti-viral immune response on NSPCs. To accomplish this, we used the transgenic CD46+ mouse model. In this model, the human isoform of the MV receptor CD46 is under the control of a neuron-specific enolase (NSE) protomer and is expressed only on mature neurons. Hence, only the neurons are infected in our model [24]. Thus, any alteration in NSPC activity will be due to the anti-viral immune response and not the infection itself. Using this model of CNS infection, we show that the NSPC pool is significantly reduced in the pediatric hippocampus and SVZ during an active viral infection.

2. METHODS AND MATERIALS:

2.1 Animals and ethics statement:

Mice were maintained and treated in accordance with the Institutional Animal Care and Use Committee of Duquesne University and the *NIH Guide for the Care and Use of Laboratory Animals.* CD46+ mice (a gift from Dr. Glenn Rall; Fox Chase Cancer Center, Philadelphia, PA) were maintained on a 12:12 light/dark cycle under controlled temperature conditions (20±2°C) with free access to food and water. Mating pairs were established for CD46+ mice in order to generate pediatric pups for our experiments.

2.2 Measles virus infection:

CD46+ mice were infected with MV-Edmonston obtained from the ATCC (American Type Culture Collection, Cat. No: VR-24). The virus was passaged thrice in Vero fibroblasts. The inoculum was diluted to 10,000 plaque forming units (PFU)/10µl with phosphate-buffered saline (PBS).

On postnatal day 10 (P10), pediatric mice were injected intracerebrally (IC) with MV (either 20,000 or 40,000 PFU) with a 1 cc syringe and 27½ gauge needle. The uninfected control group was infected with 20µl PBS at the same anatomical location. The pups were monitored daily for any signs of illness (ruffled fur, hunched poster, problems with movement and weight loss) and survival up to 90 days post infection (*dpi*).

2.3 Flow cytometric analysis for neural cells:

At the indicated dpi, pediatric mice were deeply anesthetized using isoflurane. The whole brains were harvested, and the hippocampus and SVZ were dissected. These brain regions were then processed to obtain a single cell-suspension. Briefly, the hippocampi and SVZ were incubated for 30 mins at 37°C in an enzyme mixture containing 15 U/ml papain, 1 U/ml dispase, 5mM L-cysteine and 1mM of DNase. The tissue was mechanically dissociated by pipetting 15-20 times in 500µl of Dulbecco's Modification of Eagle's Medium (DMEM) and incubated for 10 mins to allow for undissociated tissue to settle at the bottom. Then, 400µl of the supernatant was collected, and again subjected to mechanical dissociation in an additional 400µl DMEM. After allowing the sample to stand for 10 mins, 700µl of the supernatant was

collected and centrifuged to obtain a pellet of single cell isolates. The single cell isolates were then fixed with Cytoperm/Cytofix solution for 30 mins at 4°C in dark. The fixed cells were stained with the following antibodies for 1 hour at 4°C: Alexa Fluor 647-Nestin (1:1), PE-Doublecortin (DCX) (1:10), βIII tubulin (1:50), GFAP (1:50), A2B5 (1:10) and Alexa Fluor 488-secondary goat anti-rabbit (1:1000). The cells were then analyzed on Attune NxT. For each panel of antibodies, $1x10^6$ events were counted. Gating was applied based on Fluorescent Minus One (FMO) which were used as staining controls as described previously [25, 26].

2.4 Flow cytometric analysis for immune cells:

After dissecting the hippocampus and SVZ as mentioned above, the rest of the brain was processed to detect the immune cells infiltrating the brain during infection. Briefly, the remaining brain tissue was passed through a nylon mesh cell strainer in PBS. The dissociated tissue was run over a 30/70% discontinuous Percoll gradient for 20 mins at room temperature. Mononuclear cells were collected from the interface, washed with PBS, and then treated with 0.84% ammonium chloride to remove contaminating red blood cells (RBCs), and washed again in PBS. Primary antibodies were applied in 1% fetal bovine serum (FBS) in PBS for 1 hour in dark at 4°C. The following antibodies were used to identify T cells: APC CD8a (600ng/ml), FITC CD19 (600ng/ml), PE CD4 (600ng/ml), and PerCP-CY™ 5.5 CD3 Molecular complex (600ng/ml). To identify NK cells, APC NK1.1 (600ng/ml), PE CD49b (600ng/ml), PerCP-CY™ 5.5 CD3 Molecular complex (600ng/ml), and FITC CD19 (600ng/ml) were used. To identify neutrophils, PerCP CD45 (1:50), APC CD11b (1:50), FITC Ly6G (1:50), and PE F4/80 (1:50). As described above, the stained cells were analyzed on Attune NxT. Gating was applied based on FMO using splenocytes. Single antibody stains were used for color compensation.

2.5 Statistical analysis:

Statistical analysis for the Kaplan-Meier plots was done by log rank test to compare the survival rate between infected and uninfected groups. Two-way Student t-test was done to compare between

neural cells and immune cells between infected and uninfected mice. Pearson's correlation test was used to understand if NSPC numbers correlate with immune cell infiltration. Differences were significant when the p value was less than 0.05. All graphs and statistical analysis were done using GraphPad Prism software.

3. RESULTS:

3.1: Pediatric mice develop neurological symptoms and succumb after MV infection:

To understand the impact of a CNS viral infection during the pediatric age, we used a neuron-restricted MV infection model [24]. Previous studies in our model have shown that neonates succumb to the infection, while the adults survive [5, 27, 28]. The infected neonates also showed severe CNS disease while the adults do not show any signs of sickness during the infection [5, 29]. The impact of viral infections can be disastrous even during the pediatric age group. However, not much is known about the pathology of neurotropic infections in them. Thus, we aim to characterize a pediatric model to study the neuropathological impacts of a CNS viral infection which is still understudied. To study this, we injected either 20,000 PFU MV or 40,000 PFU MV intracranially at post-natal day 10 (P10). The animals were monitored for any sickness (*e.g*. ataxia, seizures) till 90 days post infection (*dpi*). As shown in **Fig. 1**, survival was dose-dependent, where the pups infected with the high dose (40,000 PFU of MV) showed 75% mortality while those infected with the low dose (20,000 PFU of MV) showed 22% mortality. Death occurred between 10-15 dpi and 7-15 dpi for pups infected with 20,000 PFU MV and 40,000 PFU MV respectively. Regardless of the viral inoculum, mice that reached 16 dpi survived until 90 dpi. The sick pups showed varying degree of symptoms, ranging from crusty eyes, ruffled fur, hunched posture, and tremors. The control group, which was injected with an equivalent volume of PBS, did not show any signs of illness or weight loss throughout the study. Based on these findings, we selected 20,000 PFU MV as the dose for future pediatric studies, as this group showed some degree of pathology while also having a group of survivors for our long-term studies. Overall, our survival studies showed that a CNS viral

infection in the pediatric age groups is associated with mortality rate between that of the neonates and the adults.

Figure 1. Survival curve

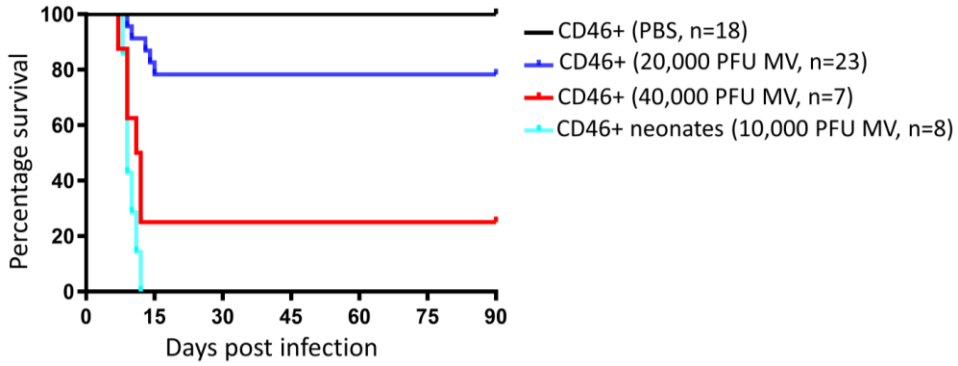

Figure 1. MV infection in the CNS of pediatric mice shows dose-dependent survival: Kaplan-Meier plot of pediatric (P10) mice after MV infection or PBS to 90 days post infection (dpi). Log rank test was used to compare between infected and control groups. The PBS injection mice (black line) shows 100% survival (n=18). Pups injected with a low dose of virus (20000 PFU MV, purple line) showed 78% survival ($p=0.0381$, n=23). Pups injected with a high dose of virus (40,000 PFU MV, red line) showed 25% survival ($p<0.0001$, n=7). Neonates injected with 10,000 PFU MV succumb to the infection (light blue line) by 12 dpi ($p<0.0001$, n=8), as supported by our prior studies.

3.2 Innate and adaptive immune cells infiltrate the brain early during infection

The anti-viral immune response has been viewed as central to viral clearance and control in the CNS [30-34]. Earlier studies in MV infected CD46+ adults have shown that infiltrating immune cells from the periphery are important for viral control. Specifically, CD4+ T cells along with CD8+ T cells or B cells were seen to be of prime importance in controlling the viral load in the infected adults [29]. In neonates, the CD4 T cell population is low in the brain during infection, which is believed to contribute to the failure of viral control in newborns. To determine the immune cell profile at the pediatric age, we carried out flow cytometry to determine the innate and adaptive immune cells infiltrating the brain. We selected 9 dpi as our time point in early infection for the following reasons: (a) at this time point, we could distinguish between sick and non-sick pups in the group and (b) mortality occurred mainly from 10-15 dpi. We observed that both the innate and adaptive immune cells infiltrated the brain at 9 dpi. As seen

in **Fig. 2**, significant differences were seen in innate immune cells [e.g. macrophage/microglia (****p<0.0001), NK cells (**p=0.0006), neutrophils (***p=0.0002)] and adaptive immune cells [CD4+ T cells, CD8+ T cells and B cells (****p<0.0001)] in the infected group compared to the PBS group. Thus, we observed that early during infection both the innate and adaptive immune system is active in the CNS.

Figure 2. Immune cells in the brain at 9 dpi

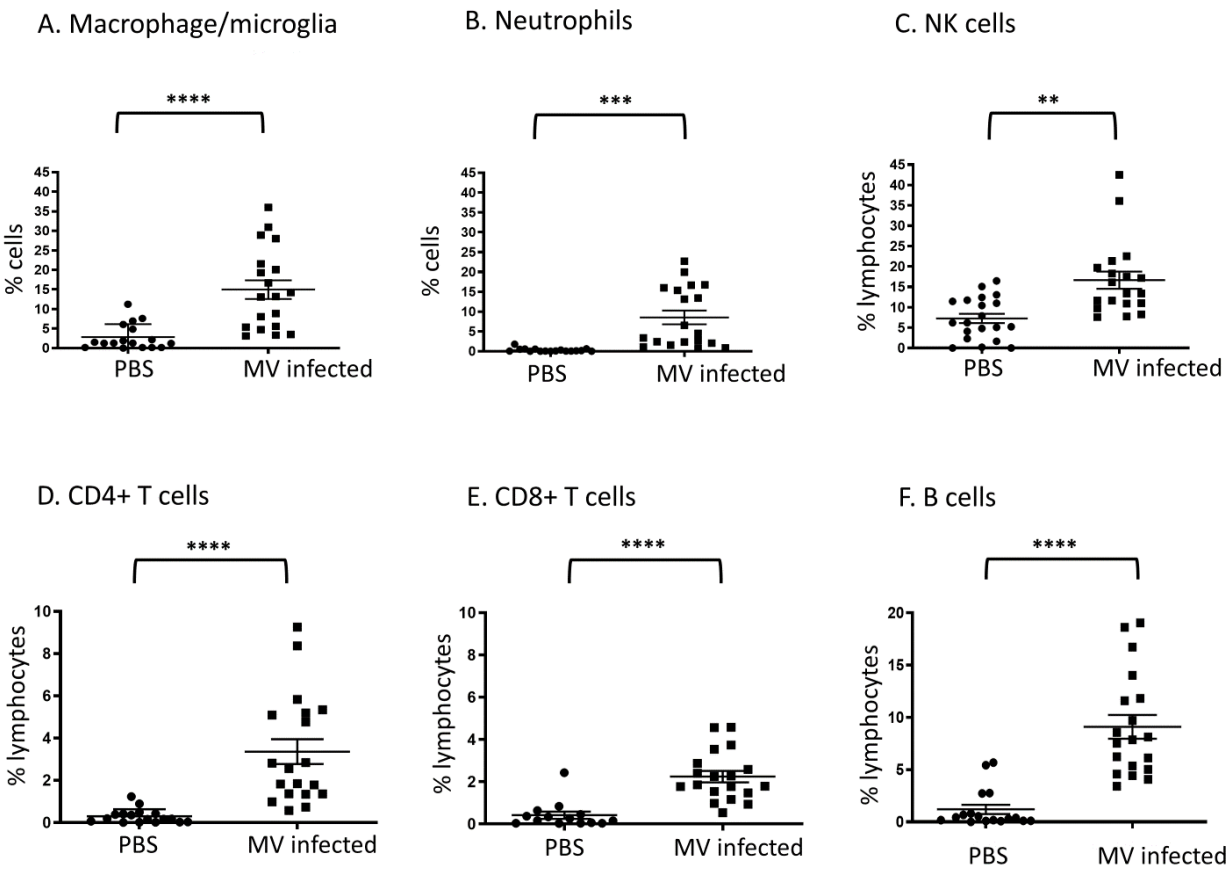

Figure 2. Innate and adaptive immune cells infiltrate the brain at 9 dpi: Whole brain tissue (with the hippocampus and the SVZ removed for neural cell flow cytometry) was processed to obtain a single cell suspension and analyzed for both innate and adaptive immune cells. Macrophage/microglia (2A) in the MV infected brain was significantly increased compared to PBS (****p<0.0001). Neutrophils (2B) and NK cells (2C) also showed significantly higher infiltration in the MV infected animals than PBS (***p=0.0002, and ***p=0.0006). Along with innate immune cells, at 9 dpi, adaptive immune cells such as CD4+ T cells (2D), CD8+ T cells (2E), and B cells (2F) were also significantly increased in the infected group (****p<0.0001). Two-way student t-test was used for statistical comparison (n=17-19). Both male and female, as well as sick and non-sick animals, were included together.

3.3 NSPC pool decreases in pediatric mice early during infection

The brain is under ongoing development during the pediatric age in both humans and rodents [11, 35-37]. Thus, we hypothesized that CNS viral infection at this stage might impact the developing neural cells. We examined the number of NSPCs, neurons, and astrocytes in infected and uninfected pediatric mice using flow cytometry. The hippocampus and SVZ were analyzed separately to account for the main neurogenic niches in adulthood [17]. In the hippocampus, we observed that NSPCs (**Fig. 3A**, nestin+) and immature neurons (**Fig. 3B,** DCX+) showed a significant decrease with infection (****$p<0.0001$ and ***$p=0.0002$). We did not see any differences in mature neurons, immature glia, or astrocytes during infection. Similarly, in the SVZ, we saw a significant reduction in the NSPC pool (**Fig. 4A,** nestin+) in the infected group ($p<0.0001$). Immature glia (**Fig. 4D,** A2B5+) showed a significant increase in the MV infected group compared to the PBS group (*$p=0.0433$). We did not observe any differences in immature neurons, and astrocytes. Mature neurons (**Fig. 4C,** β-III tubulin+) showed a trend towards increase in the infected compared to the uninfected group ($p=0.0542$). Our studies indicate that at 9 dpi in the pediatric age, there is a significant contraction in NSPC pool in both the hippocampus and the SVZ. Since only neurons are infected in this model, we propose that the decline in the NSPCs is due to the anti-viral immune response, as opposed to direct infection by the virus [24].

Figure 3. Hippocampus- Neural cells at 9 dpi

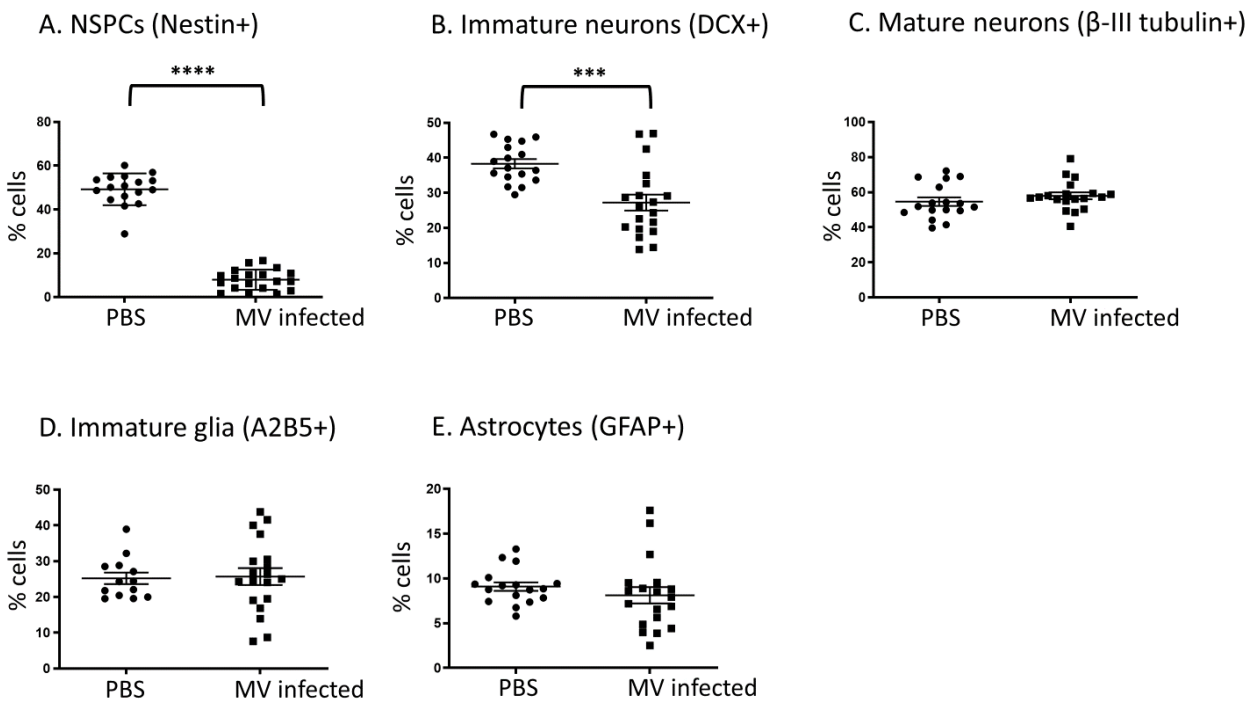

Figure 3. Hippocampal NSPC pool decreases during pediatric CNS infection: At 9 dpi, the hippocampi from the brains of the infected and uninfected pups were processed for flow cytometry for various neural cell markers. A significant decrease in Nestin+ cells (NSPCs- 3A) and in DCX+ cells (immature neurons- 3B) was seen in the infected group compared to the uninfected group (****$p<0.0001$ and ***$p=0.0002$). No differences were seen in β-III tubulin+ (mature neurons- 3C), A2B5+ (immature glia- 3D), and GFAP+ (astrocytes- 3E). Student t-test was used. For these experiments n=13-19 and both male and female and sick and non-sick animals were included.

Figure 4. SVZ- Neural cells at 9 dpi

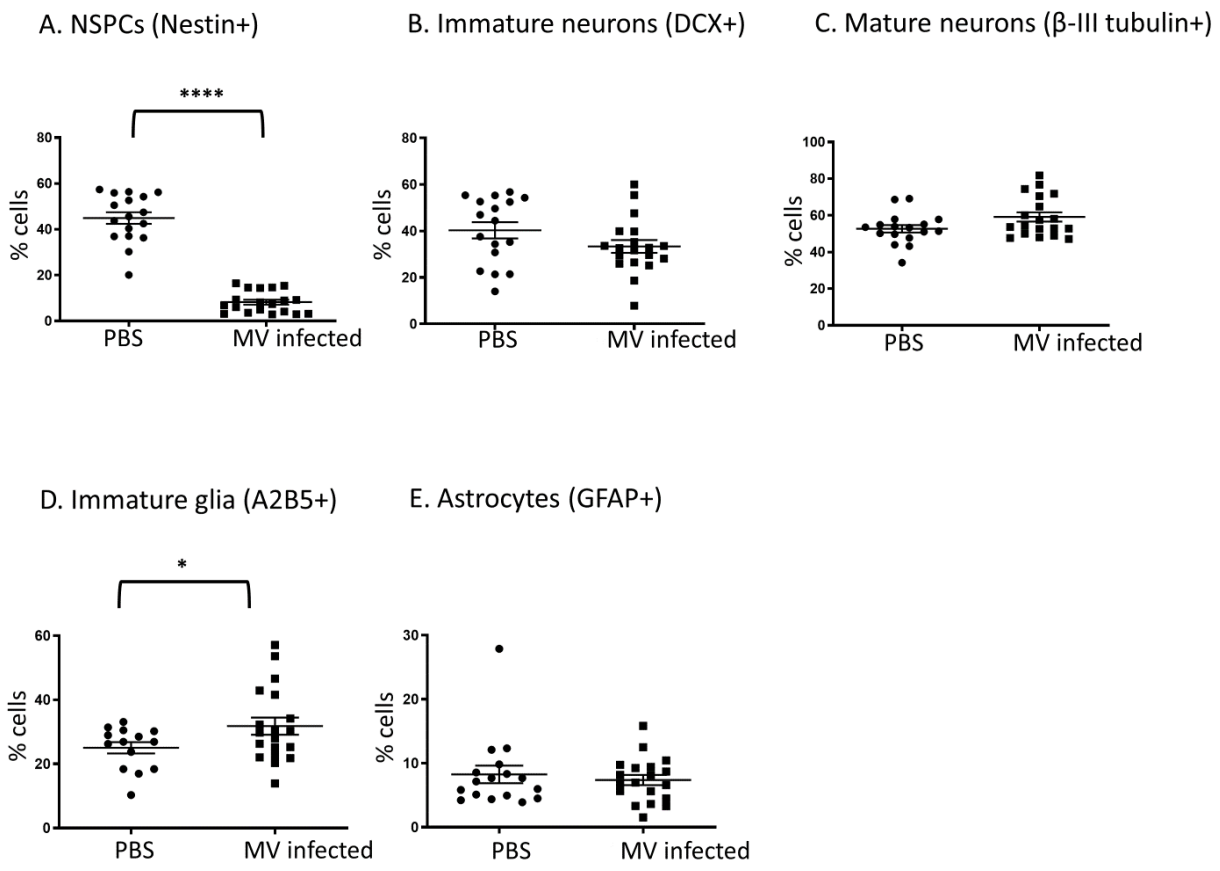

Figure 4. SVZ NSPC pool decreases during pediatric CNS infection: At 9 dpi the SVZ from the brains of the infected and uninfected pups were processed for flow cytometry for various neural cell markers. A significant decrease in Nestin+ cells (NSPCs- 4A) was seen in the infected compared to the uninfected group (****p<0.0001). A2B5+ cells (immature glia- 4D) also showed a significant increase in the infected group (*p=0.0433). β-III tubulin+ cells (mature neurons- 4C) showed an increase in the MV infected group which was trending towards significance (p=0.0542). No differences were seen in DCX+ cells (immature neurons- 4B) and GFAP+ (astrocytes- 4E). Student t-test was used. For these experiments n=13-19 and both male and female and sick and non-sick animals were included.

3.4 The decrease in NSPC pool correlates with the infiltration of immune cells at 9dpi

As we observed an active immune response and a decrease in NSPC pool at the same time, we wanted to understand if the decrease in NSPC pool correlates with the infiltration of specific immune cells in the brain at 9 dpi. A Pearson's correlation test was used for this purpose. The hippocampus showed (**Fig. 5**), a strong negative correlation between NSPCs and macrophage/microglia (R^2=0.5453, p<0.0001), neutrophils (R^2= 0.3013, p=0.0055), CD4+ T cells (R^2= 0.5691, p<0.0001), CD8+ T cells

(R^2= 0.3293, p=0.0065), and B cells (R^2= 0.5811, p<0.0001). We saw a similar negative correlation between NSPC pool and immune cells in the SVZ (**Fig. 6**). Specifically, Macrophage/microglia (R^2= 0.6132, p<0.0001), neutrophils (R^2=0.2617, p=0.0106), CD4+ T cells (R^2= 0.5230, p<0.0001), CD8+ T cells (R^2= 0.4653, p=0.0007) and B cells (R^2= 0.5607, p<0.0001). Thus, we observed that with the increasing infiltration of immune cells, the NSPC pool decreases in both the hippocampus and the SVZ at this age.

Figure 5. Hippocampus- correlation between NSPCs and immune cells

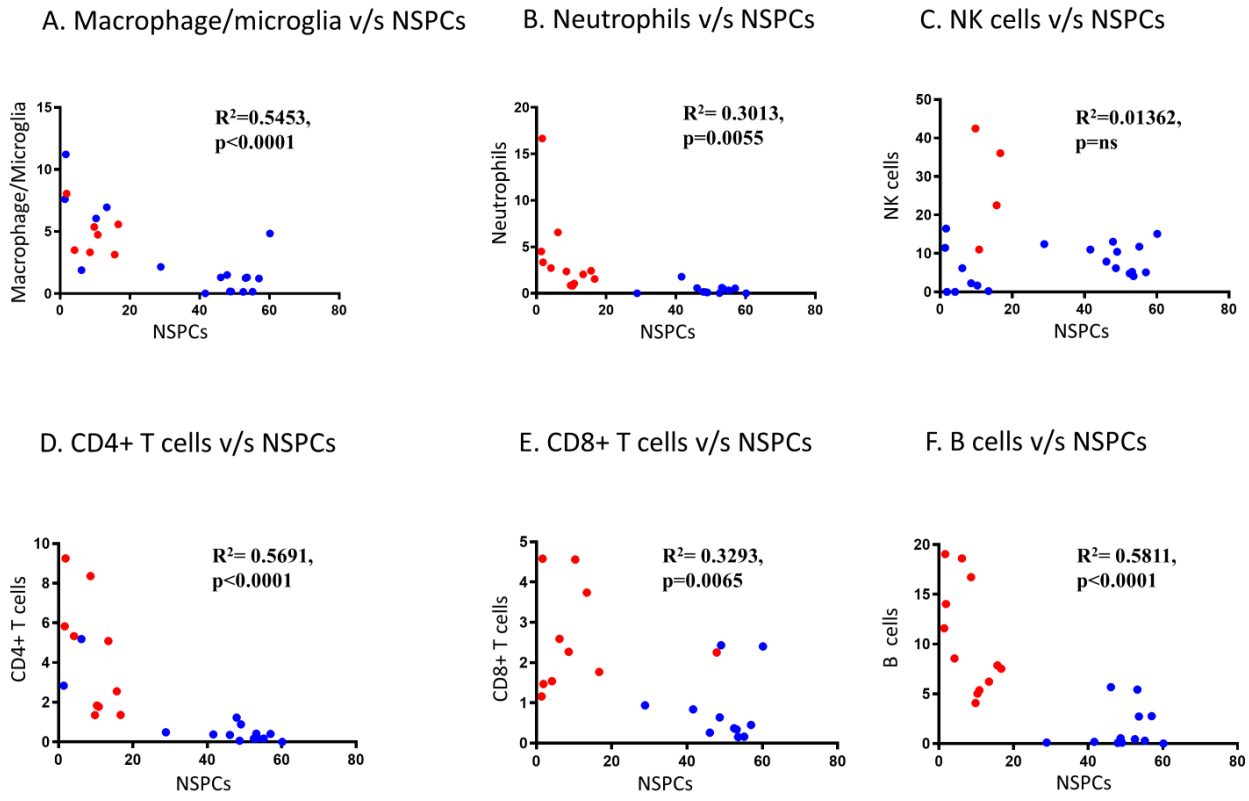

Figure 5. Hippocampus shows negative correlation of NSPCs and immune cells: Pearson's correlation between NSPCs and immune cells in the hippocampus of uninfected (blue) and MV-infected (red) pediatric mice. Negative correlations between NSPCs and immune cells were found with macrophage/microglia (R^2=0.5453, p<0.0001- 5A), neutrophils (R^2= 0.3013, p=0.0055- 5B), CD4+ T cells (R^2= 0.5691, p<0.0001- 5D), CD8+ T cells (R^2= 0.3293, p=0.0065- 5E) and B cells (R^2= 0.5811, p<0.0001- 5F).

Figure 6. SVZ - correlation between NSPCs and immune cells

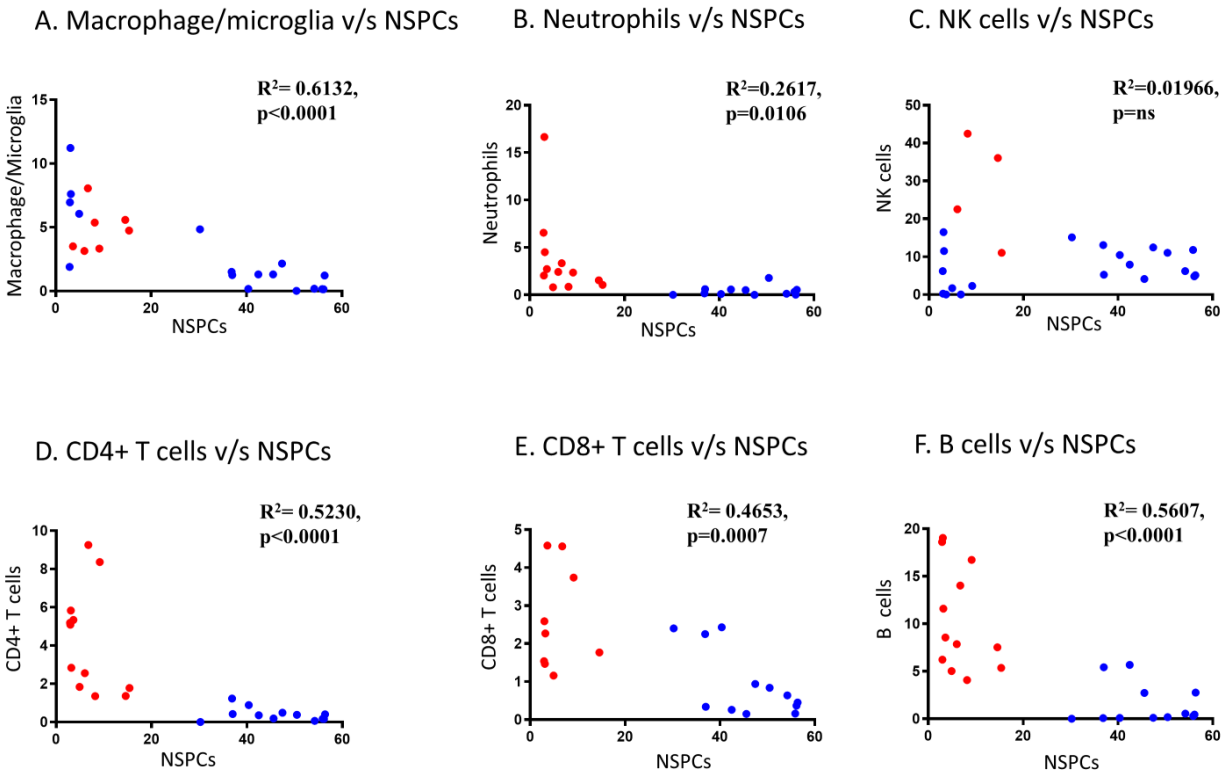

Figure 6. SVZ shows negative correlation of NSPCs and immune cells: Pearson's correlation between NSPCs and immune cells in the SVZ of uninfected (blue) and MV-infected (red) pediatric mice. Negative correlations between NSPCs and immune cells were found with macrophage/microglia (R^2= 0.6132, p<0.0001- 6A), neutrophils (R^2=0.2617, p=0.0106- 6B), CD4+ T cells (R^2= 0.5230, p<0.0001- 6D), CD8+ T cells (R^2= 0.4653, p=0.0007- 6E) and B cells (R^2= 0.5607, p<0.0001- 6F).

4. DISCUSSION:

In this study, we have characterized an *in* vivo model of neurotrophic pediatric infection, where we observe neuropathology along with NSPC loss. This model will allow us to follow survivors of pediatric infections into adulthood in order to carry out long-term studies. We have previously seen that neonates succumb to MV infection while adults show 100% survival [5]. Here, we show that the pediatric mice have a survival rate that is intermediate between the neonates and adults. This reflects that the age of the host has a crucial influence on the outcomes of a CNS viral infection, and that potential development

of both the brain and immune response in childhood can confer partial protection against viral infection. We showed that the pediatric anti-viral immune response leads to a decrease in NSPC pool in the hippocampus and the SVZ. We hypothesize that this might be due to cell death, inhibition of proliferation, or a change in NSPC phenotype where they no longer express nestin. In adulthood, the hippocampus and the SVZ are the neurogenic niches of the brain that are responsible for adult neurogenesis. In the hippocampus, NSPCs are localized in the sub-granular zone (SGZ) where their function is to give rise to new neurons that migrate and integrate into the existing circuitry of the dentate gyrus (DG). Hippocampal neurogenesis in the adulthood is mainly responsible for learning, social behavior, and memory processes [19, 38, 39]. The decrease in the hippocampal NSPC pool at 9 dpi was maintained even at 90 dpi (data not shown). Therefore, a decrease in the hippocampal NSPCs at a young age could not only alter total cell population in the adult brain but also lead to deficits in memory and cognition in adulthood. At 9 dpi, we also saw a reduction in the immature neuron (DCX+) population, which could reflect a decrease in neurogenesis and potential memory impairment in adulthood.

In the pediatric SVZ, we saw a decrease in NSPC pool at 9 dpi (Figure 4) and at 90 dpi (data not shown). The NSPCs in the SVZ are different functionally from that in the hippocampus. From the SVZ, newly formed neurons migrate via the rostral migratory stream (RMS) to the olfactory bulb (OB), which is responsible for olfaction and olfactory memory [19, 20]. A depletion in the NSPC pool here might lead to impairment of olfaction. In the SVZ, we also saw an increase in immature glia (A2B5+), which implies an increase in gliogenesis early in infection. As the neurons in our model are being infected, this increase could be a protective mechanism to prevent more neurons from getting infected and thus contain viral infection.

Apart from the changes in the neural cell populations, we also saw significant increases in the infiltration of innate (NK cells, neutrophils, and macrophage/microglia) and adaptive (CD4, CD8, and B cells) immune cells. Previous studies in our model have seen that CD4+ T cells are imperative for controlling viral spread in conjunction with B cells or CD8+ cells [29]. Thus, we wanted to understand if

immune cell infiltration correlate with NSPC numbers. We saw a negative correlation between NSPC numbers and immune cells in the pediatric CNS in early infection. One potential explanation for the decrease in the NSPC pool during increased immune cell infiltration could be the inflammatory milieu. Although anti-viral cytokines (*e.g.* IFNγ) are critical to control the viral spread, they also have been shown to inhibit NSPC proliferation, which might decrease NSPC pool [40-42]. Since the NSPCs are not infected by MV, our findings suggest that the pediatric anti-viral immune response reduces the NSPC pool for an extended period of time. This contraction in NSPC numbers could be a protective or pathological. A potential protective response could be to keep NSPC damage to a minimum during active infection, or that the loss of NSPCs is purely a pathological outcome with no protective consequences for the host.

Our future studies aim at answering the outstanding question about the long-term impact of pediatric neurotropic infection on neural cells and behavior.

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