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13. ABSTRACT ***(Maximum 200 Words)***

Systemic inflammation has been hypothesized to induce or exacerbate the onset and progression of autism spectrum disorders. Dendritic spines receive the majority of excitatory synapses in the brain and play a critical role in cognitive development and learning. Defects in dendritic spine formation have been found in the brains of patients with some autistic spectrum disorders. Here we explored the effects of systemic inflammation on secondary neuroinflammation and dendritic spine development. We found that systemic inflammation triggered a much greater influx of macrophages into the young developing brain than into the mature brain without causing changes in dendritic spine formation or maturation. Our data suggest that this may be due to developmentally regulated changes in CNS-intrinsic immunity. Specifically, the expression of high levels of anti-inflammatory, neuroprotective receptors by CNS-resident microglia may be required to sustain optimal brain function in infancy and childhood when individuals are likely to be exposed to common inflammatory insults. Our completed studies provide a de facto screen to identify genetic and/or environmental factor that alter the normal developmental progression of CNS-intrinsic immunity and/or CNS intrinsic responses to systemic inflammation reported in our studies.

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Appendix 1: Abstracts of oral and poster

Project Title: Microglia as biosensors and effectors of neurodysfunction
PI: Carson, Monica J
Project period: 4/1/09-4/30/10

INTRODUCTION:
Autism occurs during the post-natal period that neurons form new experience-dependent synaptic connections. Dendritic spines are small protrusions on the surface of the dendrite that receive the majority of excitatory synapses in the brain and play a critical role in cognitive development and learning (Segal, 2005, Ethell et al. 2005). Defects in dendritic spine formation have been found in the brains of patients with some autistic spectrum disorders, such as Rett Syndrome and Fragile X Syndrome (Fiala et al., 2002). Therefore, defects in the formation of dendritic spines may represent an anatomical and physiological basis for the cognitive deficits in these disorders. However, the mechanisms of the abnormal dendritic spine development remain unknown in these autism spectrum disorders. Recently, several studies have hypothesized that overactivation of the peripheral immune system via perinatal/early postnatal infection or immunization may contribute to susceptibility and onset of autism. Robust peripheral inflammatory responses are associated with long lasting activation of central nervous system (CNS) microglia and astrocytes, a phenomenom termed gliosis. Seminal studies by Pardo and colleagues have documented the presence of microgliosis within the CNS of autistic individuals (Pardo et al. 2005). However, these studies do not indicate whether the observed gliosis is a cause of neuronal dysfunction or a response to dysfunctional neurons. Indeed, activated microglia are now recognized to have the potential ***for both*** neuroprotective and neurodestructive functions (reviewed in Carson et al. 2007, Melchior et al. 2010 and Streit et al. 2005).
Additional Misc. Background information: For our studies, it is important to realize that microglia are the resident tissue macrophage of the brain. They seed the developing brain and spinal cord early in prenatal development. They differ from nearly all other macrophages in the body because they are long-A lived and largely self-renewing. All other monocyte and macrophage populations in the body are short-lived and replaced by bone marrow derived cells every few weeks. Using flow cytometry, we can distinguish these two populations by their differential expression of CD45: microglia express an order of magnitude lower levels of CD45 than macrophages and other peripheral immune cells that acutely infiltrate the brain during neuroinflammation (Carson et al. 1998). Using flow cytometry, we can quantify the different activation states of CNS-resident microglia.

Project Objective: To determine whether and the extent that systemic inflammation and/or inflammatory signals delivered in maternal milk lead to microglia activation and/or defects in dendritic spine maturation in the developing post-natal brain.

BODY:
In brief, we successfully completed all 4 tasks detailed in our statement of work (SOW) in the 12 month time period of our project. The data derived from this study have revealed previously undescribed developmental regulation of CNS-intrinsic immunity that likely:

a) actively protects the developing CNS from the destructive effects of an activated peripheral immune system
b) facilitates activated peripheral immune system to enter and effectively defend the developing brain against pathogenic insults

From these observations, we hypothesize that disruption of CNS-intrinsic immunity by genetic and/or environmental toxins would increase an individual's susceptibility to develop dendritic spine defects previously reported in the brains of individuals with autism spectrum disorders. Furthermore, because our study has revealed a reproducible and developmentally regulated maturation of CNS-intrinsic immunity, our studies have had one additional unanticipated benefit.

We have defined an experimental screen using standard commercially available antibody markers of immune activation (please see figures 3,4,9). This screen can be used to quantify in animal models systems whether environmental insults (toxins, pathogens) alone or in conjunction with genetic defects alter CNS-intrinsic immunity and subsequent synaptic maturation.

***Task 1:* To obtain USAMRMC ACURO approval for animal studies.**

This task was accomplished prior to any other tasks being attempted because all of our proposed studies required the use of animal models.

***Task 2:* Using flow cytometry, contrast two methods of inducing CNS inflammation and/or gliosis in murine pups.**

In this task, we defined the type and extent of brain inflammation caused by a primary systemic inflammation triggered in young pups or in the dams of nursing pups. Specifically, mice were injected intraperitoneally (IP) with LPS (a component of gram negative bacteria) to mimic peripheral infections and to provide a standardized acute inflammatory event. This is a standard and well-characterized method for the induction of systemic inflammation in all ages of mice. Here, we injected young nursing pups at postnatal 3 (P3), P7, P14, P21. P21 was the age of weaning. We also injected young adult mice (3 months old: never pregnant mice and nursing dams) 1) IP LPS into pups only

2) IP LPS into nursing dams (pups exposed to cytokines from maternal milk)

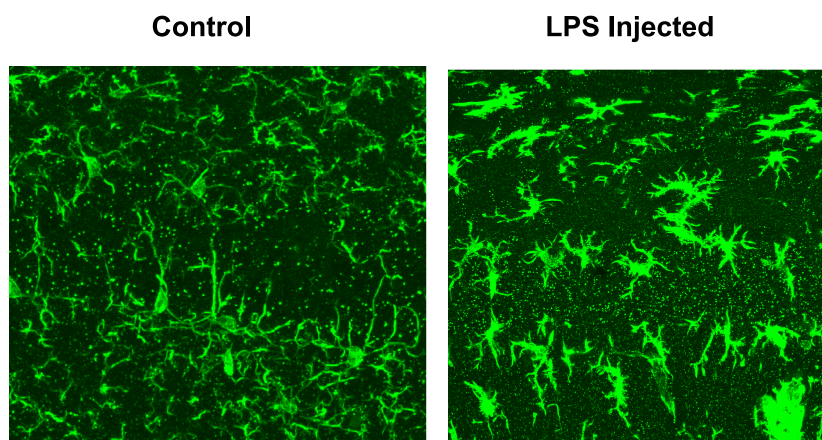
3) IP LPS into nursing dams AND IP LPS into pups

Brain inflammation was detected by quantifying microglial activation and influx of peripheral immune cells into the CNS histologically and by flow cytometry.

At all ages, intraperitoneal (IP) injection of LPS induced microglial activation within 24 hours. Figure 1 (below) shows a representative histological detection of microglial activation with the CA1 region of the hippocampus at post-natal day 15 (P15) control mice (fig 1, left panel) or LPS-injected mice (24 hours post-injection; fig 1 right panel). Although CNS-resident microglia can be distinguished from acutely infiltrating/blood derived macrophages by flow cytometry, as yet there are no markers that distinguish these populations in histologic sections. Therefore in figure 1, the Iba-1 antibody labels both CNS-resident microglia and macrophages (green labeling). However, the histologic data do reveal that

Figure 1: The antibody Iba-1 labels microglia and macrophages in tissue sections (green).

Iba1 Staining in P15 CA1 Reveals Activated Microglia Following LPS Injections

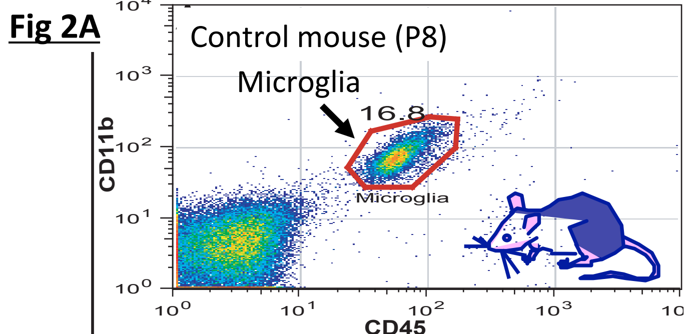


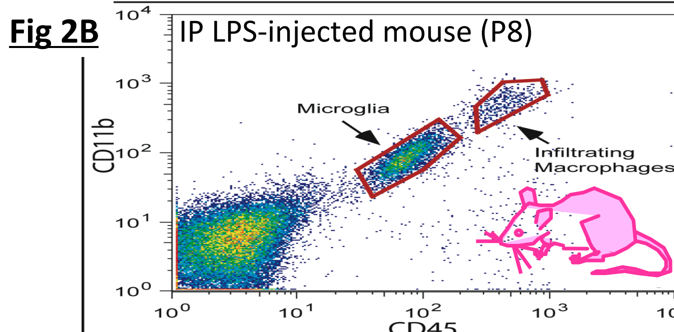
systemic inflammation resulting from a single acute IP injection of LPS results in readily detectable overt changes in microglial/macrophage morphology within 24 hours. The observed changes in morphology are indicative of microglial activation but do not define the type of microglial activation (neuroprotective/anti-inflammatory versus neurotoxic/pro-inflammatory).

Therefore, we have used standard flow cytometric methodologies to quantify the activation states of microglia in brain cell suspensions (methods described in detail in Carson et al. 1998). In brief, CNS-resident microglia and CNS-infiltrating macrophages can be distinguished from all other cells in brain cell suspensions by their expression of myeloid markers such as CD11b. CNS-resident microglia can be distinguished from CNS-infiltrating macrophages by their much log-order lower expression levels of CD45

(figure 2). Figure 2 depicts representative data from analysis of post-natal day 8 brain cell suspensions. Each dot in figure 2A and 2B represents the fluorescence level of each cell in brain cell suspensions prepared from uninjected post-natal day 8 mice (Figure 2A) or from post-natal day 8 mice 24 hours post-IP LPS injection (figure 2B). Please note that in this figure, both the X- and Y-axes are on log-scale. In both figures, the large population of cells in the bottom left quadrant represents cells that express neither CD11b nor CD45. These are neurons, astrocytes and oligodendrocytes present in the brain cell suspensions. In figure 2A (unmanipulated healthy mice), there is a single large population of CD11b+ cells that express low levels of CD45. These are CNS-resident microglia. In figure 2B (IP LPS injected mice), two CD11b+ populations can be detected. In response to systemic inflammation, a CD11b+ population that expresses high levels of CD45 can now be detected. These are the blood-derived CNS-infiltrating macrophages.

Figure 2: Flow cytometric analysis of brain cell suspensions from post-natal day 8 (P8) mice. Unmanipulated mice (fig. 2A) and mice 24 hours post IP LPS injection (fig. 2B)

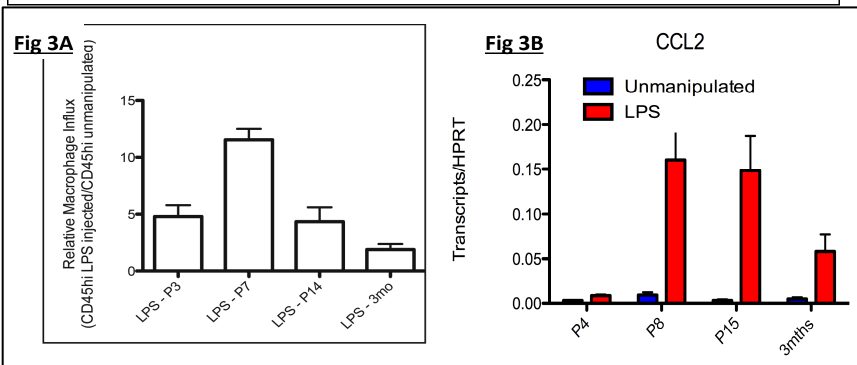




Upon close examination of figure 2A, a small number of CD45hi, CD11b+ cells can be detected. This indicates that small numbers of peripheral blood-derived macrophages could be detected in the healthy CNS. This was found to be true in all ages of mice. However, the total number of blood-derived macrophages was always less than 5% of the total CNS-resident population at all ages examined. IP-injection of LPS also triggered the influx of macrophages into the brain at all ages examined such as that depicted in figure 2b. The magnitude of macrophage influx is readily quantified by flow cytometric analysis (see figure 3).

One of the most surprising and significant findings of our study was that the magnitude of macrophage influx (assayed 24 hours post-LPS injection) was dramatically regulated as a function of development (figure 3). LPS injection at post-natal day 7 triggered the greatest influx of proinflammatory macrophages (figure 3A). This was initially surprising because this is a time of active synaptogenesis during which hippocampal dendritic spines are beginning to mature. Strikingly, with the exception of post-natal day 3 injections (post-natal day 4 harvest) when CCL2 expression is not detected, this age-dependent influx was associated with LPS-induced expression of the macrophage chemoattractant, CCL2 (assayed by quantitative real-time PCR [qPCR] analysis of total brain RNA from unmanipulated and LPS-injected mice: figure 3b). These data suggest that the CNS actively recruits macrophage infiltration into the CNS in response to systemic inflammation via CNS expression of chemokines.

Figure 3: LPS-induced macrophage influx is dependent on age IP injection occurs. Fig 3A is the quantitation of flow cytometric analysis of macrophage influx (ages indicate injection times, influx quantified 24 hours post-injection). Fig 3B is the qPCR analysis of chemokine expression per brain normalized to expression of the HPRT housekeeping gene (ages indicate harvest times, 24 hours post-injection). Fig 3b: Uninjected mice (blue bars), IP LPS injection mice (red bars). Data in panels A and B represent 3 replicate experiments (mean +/- SEM).



Using flow cytometry, we can also analyze the expression levels of an additional activation marker on specific cell populations (micro-glia versus macrophages). This is

accomplished by “gating” on the microglia versus the macrophage populations detected in figure 2 (see red circles on figures 2A and 2B) and then by analyzing the expression levels of that specific activation marker on a third fluorescent channel. For example, figure 4 depicts flow cytometric analysis of basal and LPS-induced expression of two neuroprotective, alternative activation markers (the anti-inflammatory receptors TREM2 and TLT2) by CNS resident microglia at 4 different ages (for review of microglia/macrophage activation states, please see Carson et al. 2007).

In figure 4, each histogram represents the distribution of fluorescence in this third fluorescence channel on all (CD11b+, CD45low) microglia from unmanipulated and LPS-injected mice labeled either with isotype control antibodies (orange and green histograms) to define the level of non-specific autofluoresence or with specific antibodies against TREM2 or TLT2 (to define the level of expression above autofluorescence: blue and red histograms). In addition, the Y-axis is percent maximum of total cell population analyzed (linear scale 0-100%), while the X-axis is the level of fluorescence (log scale) per the standard format for flow cytometric analyses. At all ages, we detected at least a 2-fold increase in the mean expression levels of the anti-inflammatory receptors, TREM2 and TLT2 on microglia from IP LPS injected mice (red histograms) as compared to microglia from uninjected mice (blue unfilled histograms).

In addition to quantifying anti-inflammatory markers of activation, we also characterized expression of pro-inflammatory markers of activation (figure 5). In contrast to the data presented in figure 4, LPS did not induce any statistically significant increase in microglial expression of pro-inflammatory molecules such as CD40 (a receptor that promotes pro-inflammatory cytokine production) or molecules that allow microglia to evoke and sustain pro-inflammatory T cell responses such as the co-stimulatory molecule, B7.2.

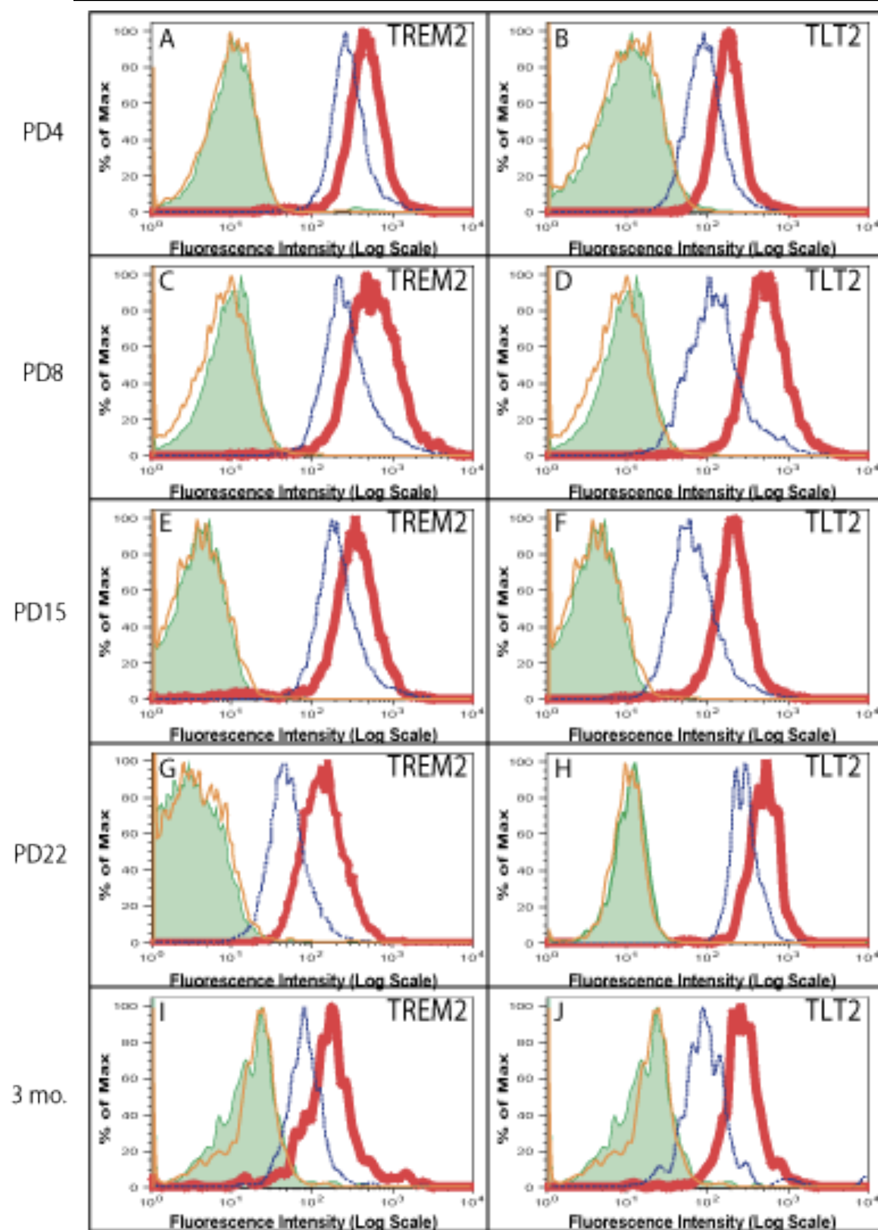


Figure 4: Microglia increase expression of neuroprotective anti-inflammatory receptors TREM2 and TLT2 24 hours post-IP LPS injections at all ages examined: postnatal day (PD) 4 through 3 months. Microglia were identified by gating on CD11b+, CD45low cells in brain suspensions per Figure 2. Fluorescence levels of microglia labeled with non-specific isotype control antibodies from uninjected mice (orange unfilled histograms) and from LPS-injected mice (green filled histograms). Fluorescence of microglia labeled with antibodies against TREM2 (panels A, C, E,G, I) or TLT2 (panels B,D,F,H,J) from unmanipulated mice (blue unfilled histograms) or LPS mice (red unfilled histograms at ages PD 4 (panels A, B), PD8 (panels C,D), PD15 (panels G,H), 3 months (panels I, J)

Considered together, the data from figures 4 and 5 indicate that systemic inflammation preferentially directs microglial activation toward alternative (neuroprotective) instead of classical pro-inflammatory (cyto-toxic) activation states at all ages.

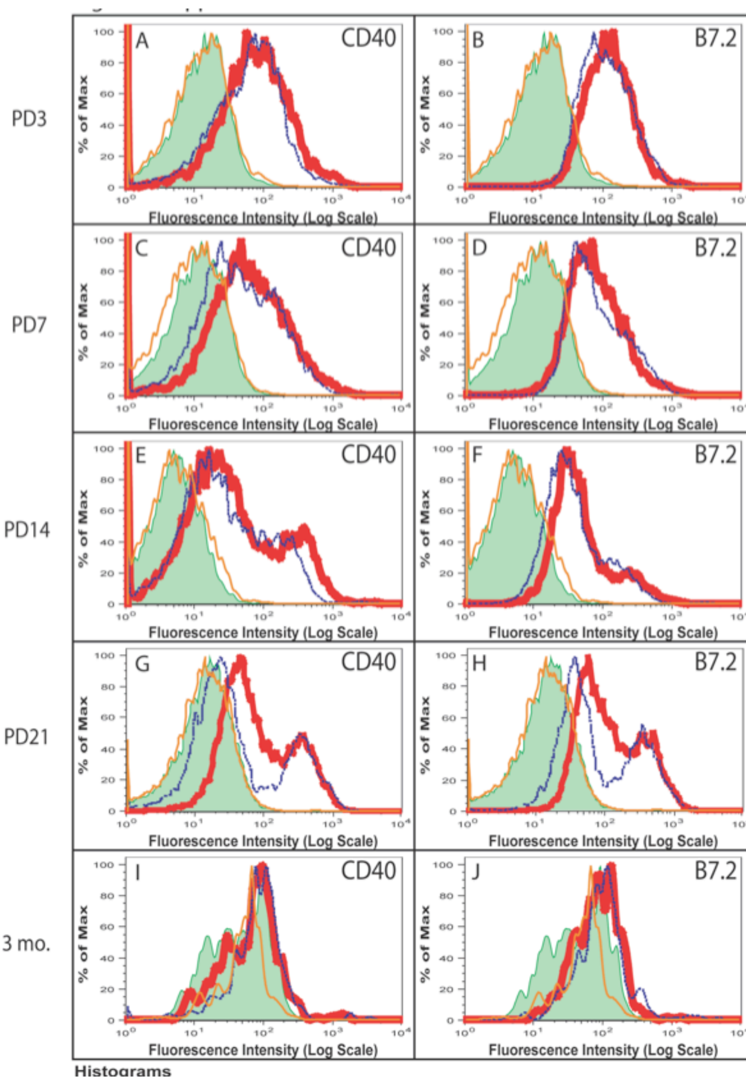


Figure 5 (right): IP LPS injection does not increase microglial expression of pro-inflammatory molecules at all ages examined. Microglia were identified by gating on CD11b+, CD45low cells in brain suspensions per Figure 2. Fluorescence levels of microglia labeled with non-specific isotype control antibodies from uninjected mice (orange unfilled histograms) and from LPS-injected mice (green filled histograms). Fluorescence of microglia labeled with antibodies against CD40 (panels A, C, E, G, I) or B7.2 (panels B,D,F,H,J) from unmanipulated mice (blue unfilled histograms) or LPS mice (red unfilled histograms at ages PD 4 (panels A, B), PD8 (panels C,D), PD15 (panels G,H), 3 months (panels I, J)

Prior to our studies, it was unexamined whether the same dose of LPS injected by the same IP injection route would trigger the same magnitude and type of microglial activation and macrophage influx in 3 month old virgin mice versus in 3 month old nursing dams. We found that pregnancy history and nursing status had no effect on the magnitude of neuroinflammation triggered by IP LPS injection (figure 6). Figure 6A depicts the typical fluorescence profile of brain cell suspensions of young adult mice (3 months), 24 hours post-IP LPS injection. Figure 6b depicts the CD45 frequency profile of all CD11b+ cells (gating on all cells in upper right panel of figure 6A) in brain cell suspensions from LPS-injected 3 month virgin females (red filled histogram) as compared to LPS-injected nursing dams (blue unfilled histograms). Note that there are no differences in the numbers of CD45lo microglia or in the numbers of CD45hi macrophages in virgin females (red) as compared to nursing dams (blue). We also failed to detect any change in the

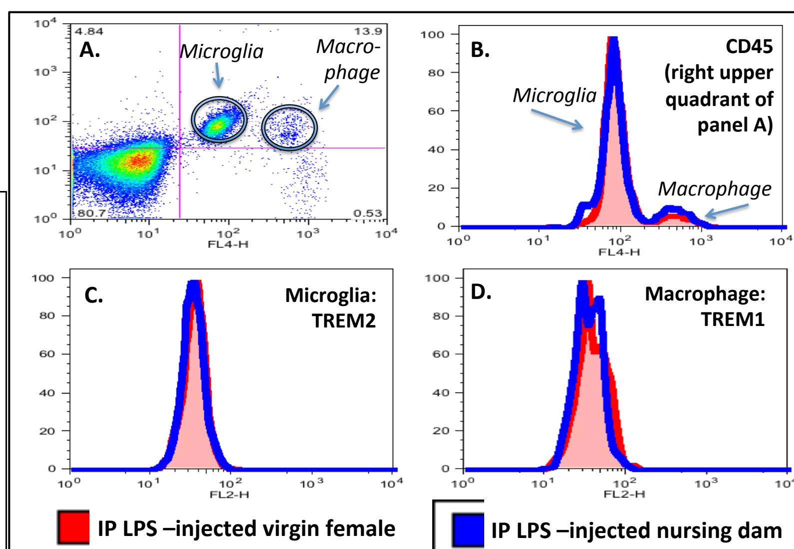


Figure 6: LPS-induced microglial activation and macrophage influx & activation do not differ between virgin female mice (3 months) and nursing dams (3 months). Panel A depicts CD45 levels (X-Axis, FL-4) and CD11b levels (Y-axis) in brain suspensions from virgin female, 24 hours post-IP LPS injection. In panels B-D, red filled histograms depicts data from LPS-injected females, blue histograms from LPS-injected nursing dams. Panel B represents CD45 levels in all CD11b+ cells (gate=right upper quadrant of panel 6A). Panel C depicts TREM2 levels on microglia (see gate, panel 6A). Panel D depicts TREM1 levels on macrophages (see gate, panel 6A)

activation states of microglia (note identical microglial expression levels of the anti-inflammatory receptor, TREM2 in cells isolated from non-injected and injected dams, figure 6C) or of macrophages (note identical macrophage expression levels of the pro-inflammatory receptor, TREM1 in cells isolated from non-injected and injected dams: figure 6D).

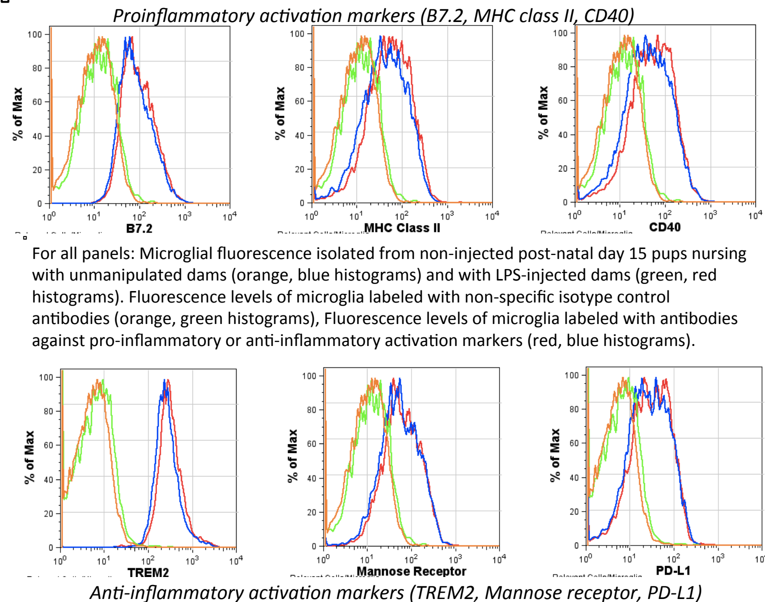


Figure 7 (right): Basal activation levels of microglia isolated from unmanipulated post-natal day 15 mice nursing with unmanipulated dams (blue histograms) and with LPS-injected dams (red histograms) were identical. Orange and green histograms depict background fluorescence

The major question posed and answered in task 3 of our project was whether systemic inflammation sufficient to cause neuro-inflammation in the nursing dam would cause or amplify neuroinflammation in the nursing pup (figures 7 and 8). We found that that systemic inflammation in the nursing dam induced by IP LPS injection had no effect on the basal microglial activation (figure 7) or on subsequent IP LPS-induced inflammation in injected, nursing pups (figure 8).

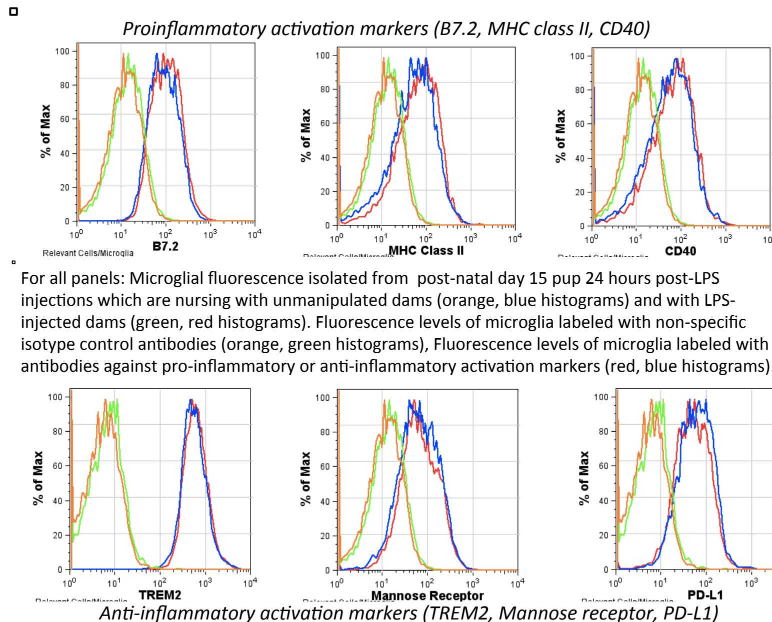


Figure 8 (left): Microglial activation in LPS-injected pups (postnatal day 15), 24 hours post-injection did not differ between pups nursing with unmanipulated dams (blue histograms) versus with LPS-injected dams (red histograms). Orange and green histograms depict background fluorescence

Summary for Task 2:
IP LPS injection causes neuroinflammation characterized by microglial activation and macrophage influx into the CNS of developing pups. However neither basal nor LPS-induced microglial activation in nursing post-natal pups is altered by the acute systemic inflammation sufficient to cause microglial activation in the nursing dams.

Task 3: To define spatial localization and molecular signature of microgliosis associated with each time point and treatment paradigm.

Prior to our studies, there has not been a quantitative analysis of microglial phenotype during development in the healthy and inflamed CNS. By both histologic and flow cytometric analysis, we found that microglia display a non-polarized but highly activated phenotype at post-natal time points associated with dendritic spine development and myelination.

Figure 9: Microglial phenotype changes with post-natal development. Panel A depicts CD45 and CD11b levels in microglia isolated from unmanipulated mice. Panels B-J depict the expression of individual activation markers by the CD45low, CD11b+ microglial population identified in panel A. The histograms in each panel, represent analysis of microglia isolated in the same experiment from different aged mice. Panel 9K: qPCR analysis of Arginase I expression.

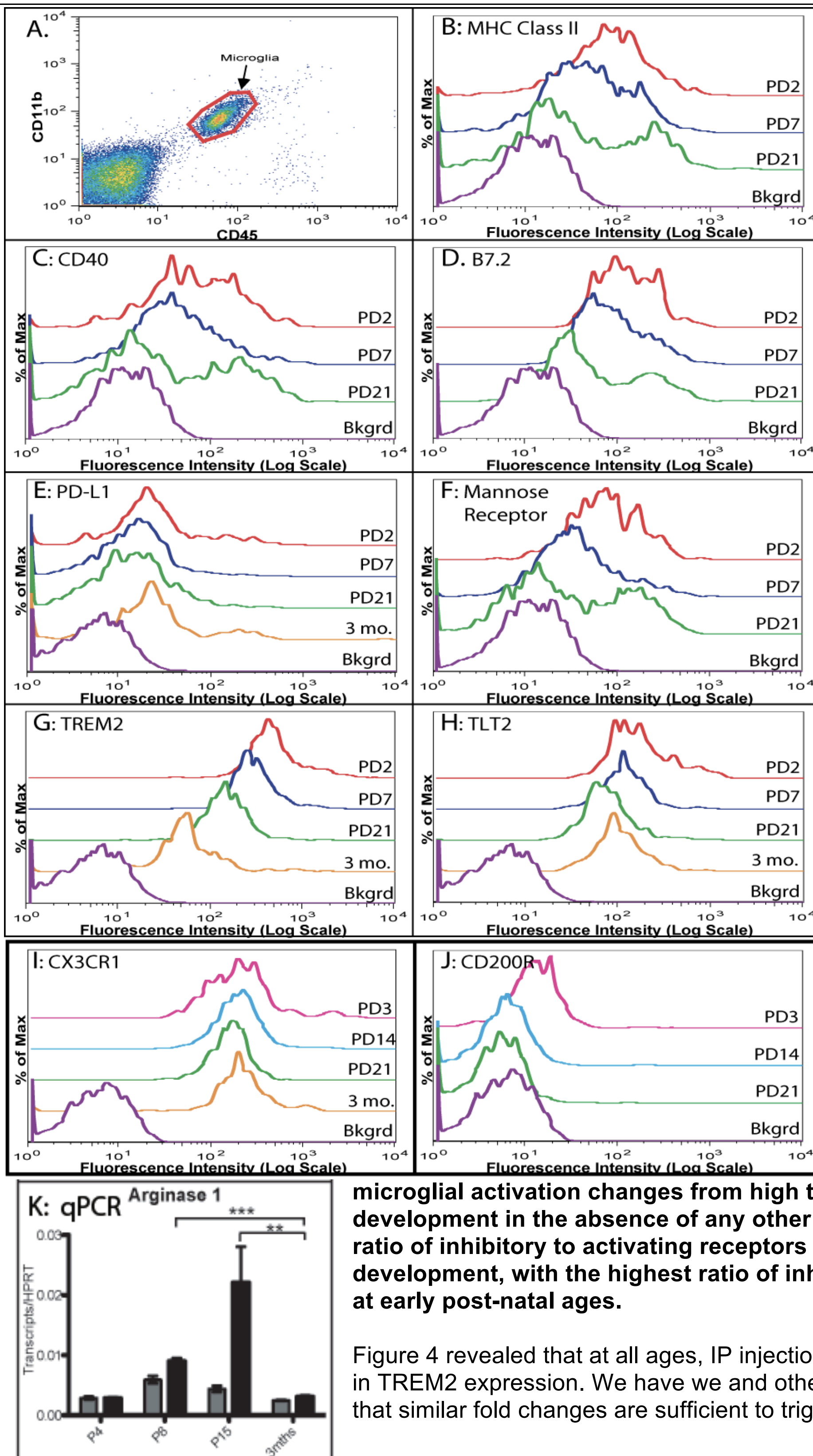


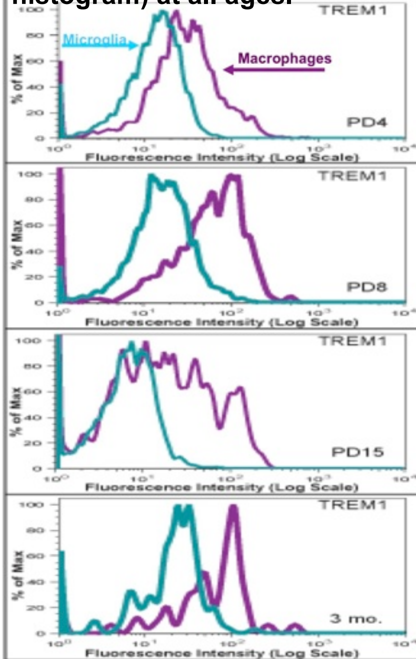
Figure 9 depicts the development associated changes in microglial phenotype. Microglia from all ages listed in panels A-H were isolated and analyzed on the same day as each other. Microglia from all ages of mice listed in panels I and J were isolated and analyzed on the same day as each other. At early ages microglia expressed high levels of general activation markers such as MHC class II, of pro-inflammatory (classic) activation markers such as CD40 and B7.2. However, microglia also expressed high levels of anti-inflammatory (alternative) activation markers such as mannose receptor, TREM2 and TLT2.

We observed relatively constant expression of CX3Cr1 and CD200R, two receptors that are well described to inhibit microglial and macrophage activation in a wide variety of neurodegenerative models (panels I and J). In addition, we also demonstrated in similar experiments that microglia expressed high levels of the polyamine synthetic enzyme characteristic of alternative activation states, Arginase I (Arg1) in the healthy CNS of developing post-natal but not mature mice (figure 9K). Thus, our studies demonstrate two points. _First,_ **the basal level of microglial activation changes from high to low as a function of normal development in the absence of any other pathogenic signals**_. Second._ **the ratio of inhibitory to activating receptors change as a function of normal development, with the highest ratio of inhibitory receptors being detected at early post-natal ages.**

Figure 4 revealed that at all ages, IP injection of LPS caused a ~2-fold increase in TREM2 expression. We have we and other have previously demonstrated that similar fold changes are sufficient to trigger and promote microglial wound-

healing and anti-inflammatory responses within the CNS (Carson et al. 2007, Melchior et al. 2010). Furthermore, the data in figure 9 clarifies that the final level of LPS-induced TREM2 expression is much higher (up to a log order higher expression) in the young developing CNS than in the mature CNS due to the higher basal level of expression in the developing CNS. By contrast, IP-LPS injection caused no increase of pro-inflammatory molecules in the developing CNS. This means that while the levels of pro-inflammatory molecules is high in the LPS injected developing CNS (due to the very high basal levels of expression), the ratio of anti- to pro-inflammatory molecules expressed by microglia is increased by systemic inflammation during early post-natal development (figure 5).

Figure 10: CNS-infiltrating (CD45hi) macrophages (purple histogram) express higher levels of pro-inflammatory molecules than CNS-resident (CD45low) microglia (blue histogram) at all ages.



Flow cytometric analysis allows us to quantify the activation states of CNS-infiltrating macrophages separately from CNS-resident microglia by specifically gating on each population (see figure 2). Phenotypic analysis of the acutely infiltrating macrophages demonstrated that macrophages expressed levels of pro-inflammatory molecules such as TREM1, MHC class II, CD40, B7.2) much higher than activated microglia. In contrast to TREM2, TREM1 serves to amplify pro-inflammatory responses of macrophages to LPS (figure 10). Taken together with the data in figure 5, these data suggest that the increase in proinflammatory macrophages visualized in histologic sections from our studies and those of others groups are not activated microglia. Instead they are likely blood-derived macrophages recently infiltrating the CNS. These data have important implications for therapeutic interventions aimed at reducing CNS neuroinflammation because the vascular compartment is much more therapeutically accessible than the CNS.

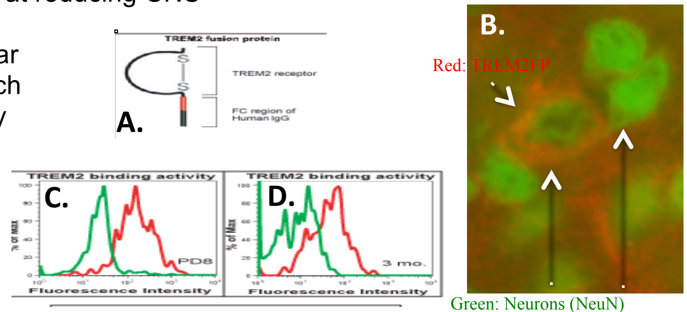
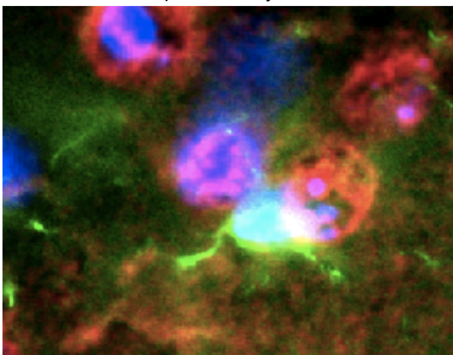


Figure 11: Putative ligands for the anti-inflammatory receptor TREM2 are detected on CNS neurons and on CNS-infiltrating macrophages following LPS-induced systemic inflammation. Panel A depicts TREM2 fusion protein used to detect putative TREM2 ligands. Panel B: histologic section from CNS of post-natal day 15 mouse 24 hours post-LPS injection. Red= TREM2Fusion protein binding activity (sites of putative TREM2 ligand expression), Green=NeuN+ cells (neurons). Panels C and D depict TREM2 fusion protein binding activity (red histograms) on cd45hi macrophages infiltrating CNS of post-natal day 8 (panel C) and 3 month old mice 24 hours post-LPS injection. Background autofluorescence=green histograms

Ligands for TREM2 are still being identified and are as yet primarily defined as TREM2 binding activities. Although the identities of TREM2 ligands have not been fully identified, putative TREM2 ligand expression in tissue sections and on cells can be detected and quantified using a TREM2-human IgG fusion protein (figure 11, and Melchior et al. 2010). This fusion protein consists of the extracellular ligand binding domain of TREM2 (figure 11A) fused to the Fc region of human IgG. Using the fusion protein, we discovered that in histologic sections of LPS-injected mice (all ages), a subset of neurons (green NeuN+ cells, figure 11B) expressed detectable TREM2fusion protein binding activity (red+ cells, figure 11B). We also detected neuronal expression of TLT2 ligands in the hippocampus in close apposition

Figure 12: Activated microglia labeled in green with Iba1 are found in close apposition to neurons labeled in red by TLT2 binding activity (neurons expressing putative TLT2 ligand). Brain section is from postnatal day 8 mouse, 24 hours post-LPS injection.

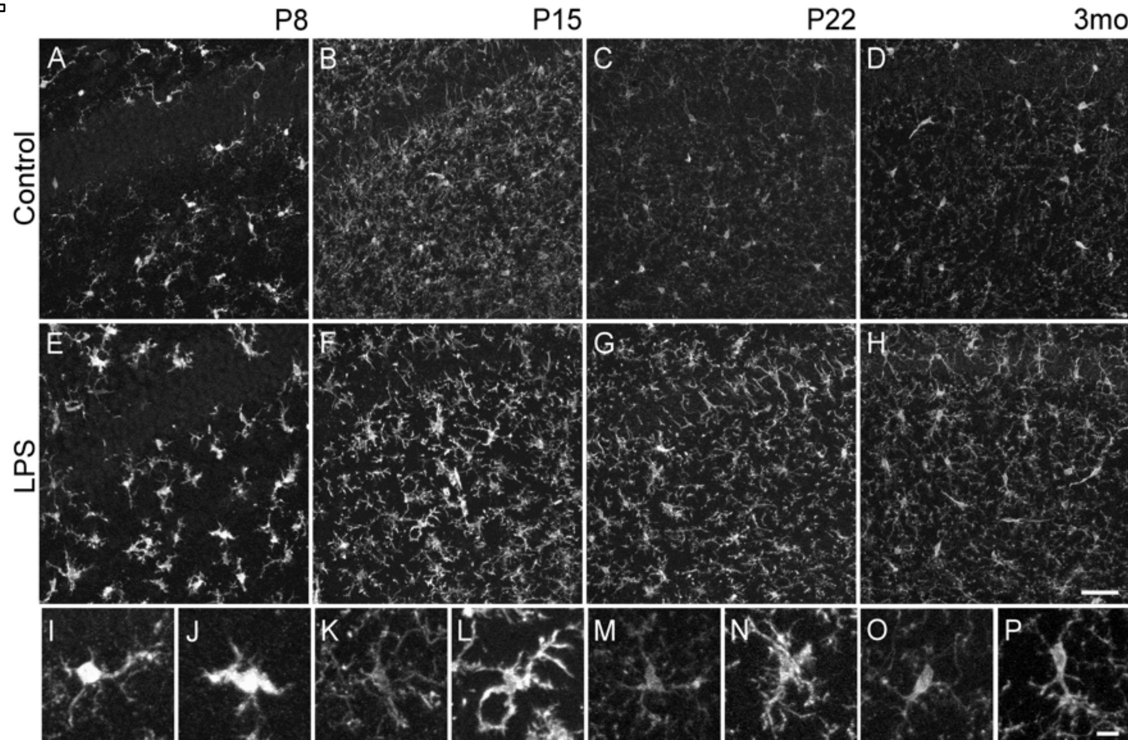


to activated microglia (These data suggest that under normal conditions, CNS neurons prime anti-inflammatory repair responses.
We also found that CNS-infiltrating macrophages also expressed high levels of the putative TREM2 ligand at all ages examined (figure 11C and 11D). Thus, pro-inflammatory macrophages infiltrating the CNS also carry the signals likely to activate anti-inflammatory functions by CNS-resident microglia.

Histological analysis demonstrated that LPS caused similar microglial activation and macrophage influx throughout the CN (figure 13). However, consistent with the flow cytometry data discussed, age-dependent variations in basal morphology and LPS-induced morphology were readily detected. In general, basal and LPS-induced morophological signs of activation were most apparent at post-natal day 8 (P8) and post-natal day 15 (P15).

With three critical exceptions, all histologic and flow cytometric measures of microglial activation and

Figure 13. LPS-induced changes in the distribution and morphology of microglia and macrophages in developing mouse hippocampus. (A-H) Confocal images showing the distribution of Iba1-positive cells (microglia and macrophages) in P8, P15, P22 and adult hippocampi of control (top panels) and LPS-treated mice (24h after an intraperitoneal injection of LPS, middle panels). (I-P) High magnification images of Iba1-positive cells in P8 (I,J), P15 (K,L), P22 (M,N) and adult (O,P) hippocampi of control (I,K,M,O) and LPS-treated (J,L,N,P) mice.



macrophage influx had returned to normal age-appropriate levels within 72 hours post-LPS injection at all ages examined. Strikingly, sustained expression of two anti-inflammatory receptors, TREM2 and TLT2 was observed for 7 days post-LPS injection of post-natal day 14 pups (figure 14). Sustained expression of pro-inflammatory molecules was not observed. Furthermore, neuronal labeling by TREM2 fusion proteins was also sustained in this same period of time, suggesting TREM2 mediated activation of microglia is sustained in young but not mature CNS following systemic inflammation. Because TREM2 triggers microglial anti-inflammatory, wound healing functions, these three data suggest that acute bouts of systemic inflammation are associated with polarizing the immune response of CNS-resident microglia toward resolution of inflammation and repair, especially in young developing mice.

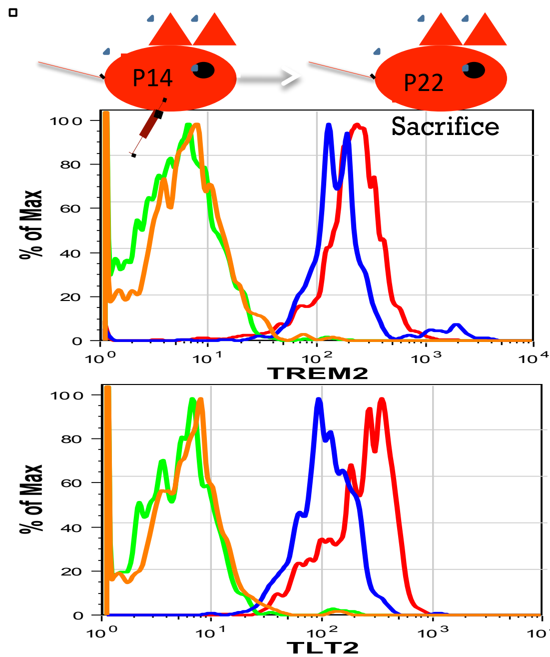


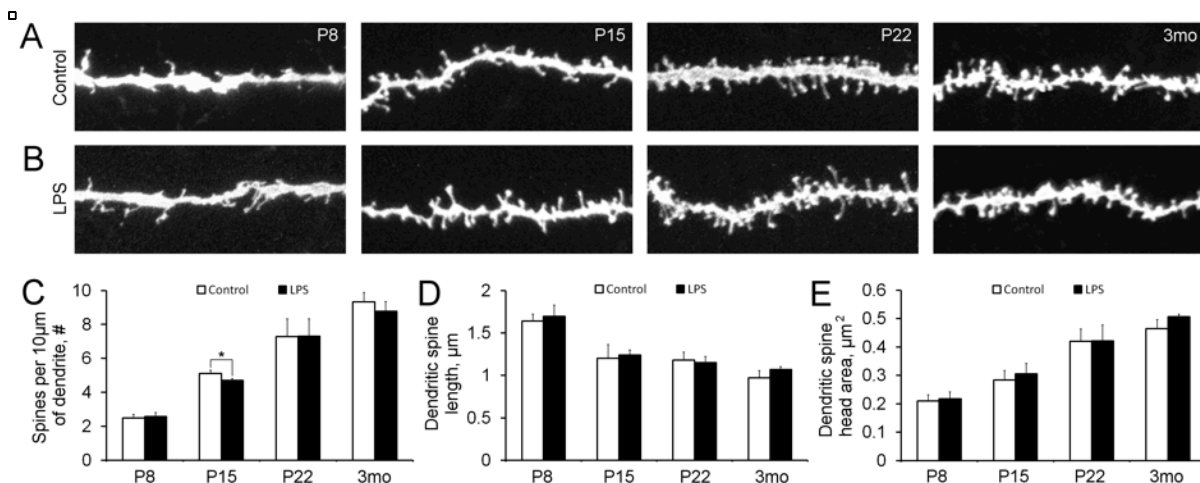
Figure 14: Microglial expression of TREM2 and TLT2 remains elevated 7 days post-LPS injection when injection occurs at post-natal day 14. Orange: Background fluorescence (Untreated); Green: Background fluorescence (LPS-injected); Blue-TREM2 expression (Untreated); Red-TREM2 expression (LPS-injected)

In sum, we speculate that in the young developing CNS, the peripheral immune system (blood-derived pro-inflammatory macrophages) are able to enter and defend the entire CNS in response to systemic insults and pathogens. By contrast, while microglia are poised to respond with dual anti-inflammatory wound healing and pro-inflammatory pathogen defense functions, their functions are biased toward anti-inflammatory functions when insults sufficient to trigger peripheral immune mediated neuroinflammation are encountered.

Summary for Task 3: Basal and LPS-induced microglial activation is not constant but changes with development. In early post-natal development, net microglial activation is toward an anti-inflammatory, wound healing phenotype.

Task 4: To define the correlation between forms of microgliosis and inflammation quantified in tasks 2 and 3 with synapse and dendritic spine maturation in CNS tissue derived from treated and untreated pups.

Figure 15. The effects of LPS-induced systemic inflamation on dendritic spine number and morphology in early postnatal and adult mouse hippocampus (A, B). Confocal images of DiI labeled CA1 dendrites in stratum radiatum of P8, P15, P22 and adult mouse brain from (A) control and (B) LPS-treated mice. (C-E) Quantitative analysis of (C) dendritic spine density, (D) dendritic spine length and (E) dendritic spine head area in CA1 hippocampal neurons of P8, P15, P22 and adult control and LPS-treated mice. Error bars indicate SD (n = 1,000-5,000 spines per group).

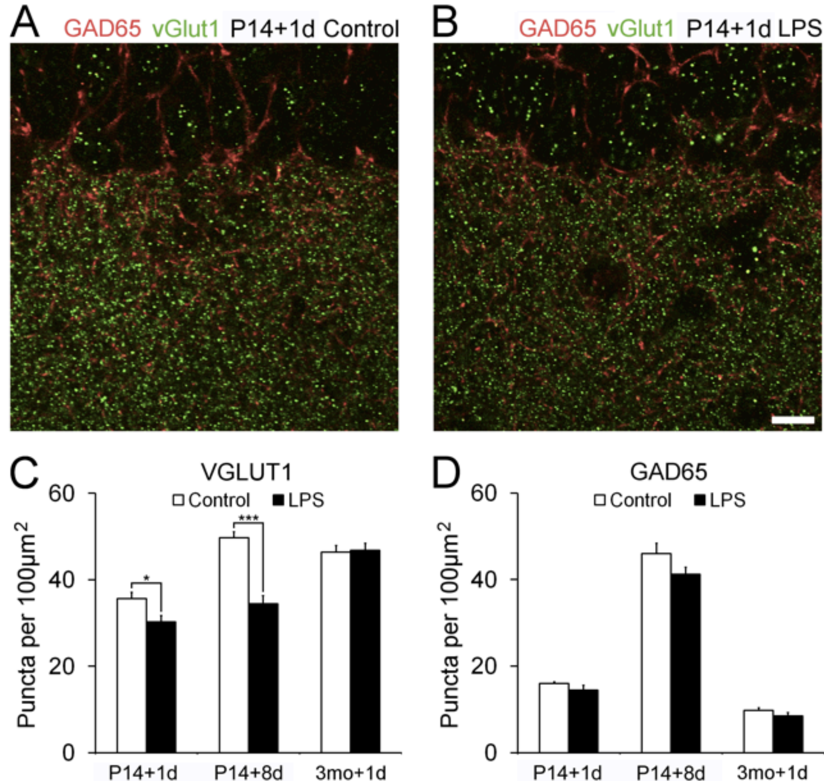


We initially assumed that the influx of highly activated pro-inflammatory macrophages 24 hours post-LPS injection of P7 and P14 mice would alter the normal maturation of synaptogeneis ongoing during these time frames. We dye-injected tissue sections from unmanipulated and LPS injected mice to visualize neuronal dendritic spines. Figure 15A depicts representative labeling of dendritic spines in tissue sections from unmanipulated (figure 15A) and LPS-injected (figure 15B) mice. Tissue sections were blinded and analyzed for dendritic spine length and head area (all variables associated with normal synapse maturation). Figure 15C-E depict quantitation of the numbers of dendritic

spines (fig 15C), dendritic spine length (fig 15D) and dendritic spine head size (fig 15E) in the CA1 region of the hippocampus. We could readily detect normal age associated increases in dendritic spine head size as well as age associated decreases in dendritic spine length. However, with one exception, there were no significant differences in unmanipulated and LPS-treated mice in the multiple cohorts analyzed. These data suggest that CNS-intrinisic immunity changes as documented in prior sections of this report to maintain CNS function during the many systemic inflammatory insults sustained during infancy and childhood.

The one exception where we did observe a very small but statistically significant difference in dendritic spine numbers was 24 hours post-injection of post-natal day 14 mice (figure 15C). Therefore, we further investigated the effects of LPS-induced inflammation on the development and maturation of inhibitory (GAD65+) and excitatory (Vglut+) synapses with the hippocampus (figure 16). As with the dendritic spine analysis, we observed a significant inhibition of the normal developmental formation of Vglut+ synapses at 24 hours post-injection when injection occurred at post-natal day 14. Strikingly, this inhibition was sustained and even more pronounced one week following LPS injection at post-natal day 14. We did not observe this LPS-induced decrease in excitatory synapses in LPS-injected adult mice. We speculate that this developmental phenomenon is likely to have neuroprotective outcomes for neurons during the critical period of hippocampal maturation. In adult mice, it has been reported that conditions that decrease the numbers of excitatory synapses decrease excitotoxic cell death and thus are associated with neuroprotection and neuronal survival.

Figure 16. The analysis of synaptic innervation of CA1 neurons in developing and adult mouse hippocampi in response to LPS-induced systemic inflammation. (A-B) Confocal images showing the distribution of (A) vGlut1-positive excitatory pre-synaptic boutons (green), and (B) GAD65-positive inhibitory (red) presynaptic sites in P15 and adult hippocampi of control (A) and LPS-treated mice (24h after an intraperitoneal injection of LPS, B). (C-D) Quantitative analysis of number of vGlut1-positive pre-synaptic boutons and GAD65-positive puncta per 100 μm^2 area in stratum radiatum of P15 (C) and adult (D) hippocampi of control and LPS-treated mice. Data represent average values. Error bars indicate SD (n = 1,000 per group)



Summary for Task 4: CNS-intrinsic immunity appears to compensate and prevent neuronal damage when systemic inflammation occurs during critical windows of postnatal brain development. In addition, we have identified post-natal day 14 as a critical window in which systemic inflammation triggers sustained acquisition of anti-inflammatory phenotypes by microglia coupled with sustained decreases in excitatory synapse formation. We speculate that the latter phenomenon is neuroprotective due to the resultant increased threshold for excitotoxic cell death.

KEY RESEARCH ACCOMPLISHMENTS:

- New revelation that CNS-intrinsic immunity changes both qualitatively and quantitatively as a function of normal development
- Demonstration that acute bouts of systemic inflammation during early post-natal periods equivalent to infancy and childhood in humans polarizes CNS-resident microglia to acquire anti-inflammatory/wound healing phenotypes
- Demonstration that systemic inflammation triggers robust macrophage influx during critical stages of synaptogenesis and during developmental windows associated with onset of autism spectrum disorders in humans
- Demonstration that CNS neurons express putative ligands (TREM2 binding activity, TLT2 binding activity) for the anti-inflammatory receptors TREM2 and TLT2 expressed by CNS-resident microglia.
    - These data suggest that defects in neurons that alter their expression of these putative ligands will lead to prolonged CNS inflammation and perhaps poorer repair following resolution of CNS inflammation
- Demonstration that CNS-infiltrating pro-inflammatory macrophages also express putative ligands (TREM2 binding activity) for the anti-inflammatory microglial receptor TREM2.
    - These data are consistent with other published observations that activated pro-inflammatory immune cells frequently express molecules which help resolve inflammation.
- Demonstration that in otherwise healthy developing pups, systemic inflammation does not cause alterations in normal developmental process of synaptogenesis at all developmental ages except post-natal day 14. Post-natal day 14 is a critical developmental window for hippocampal maturation and is a time point when LPS-induced inflammation leads to inhibition of excitatory synapse formation in the hippocampus.
    - Based on the bulk of published literature in adult models, we speculate that this may be a neuroprotective mechanisms limiting the susceptibility of critical hippocampal neurons to excitotoxic cell death.
- Demonstration that comparable systemic inflammation in nursing dam (mother) does not induce or exacerbate inflammation in nursing pups

REPORTABLE OUTCOMES:

The data listed in the research accomplishments section above are sufficient to be assembled into three separate manuscripts concerning normal development of microglia, development associated changes in inflammation, the consequences for synaptogenesis and the potential neuronal regulation of CNS-intrinsic immunity. However, we have chosen to assemble these observations into a much more comprehensive manuscript with the goal of publishing in a high impact journal. We are currently in the submission and review process. We will forward accepted manuscripts generated from this project to this funding agency as soon as our studies are published.

The following are the completed reportable outcomes.

- The consequence of our observations is the de facto development of murine screen to detect if genetic and/or environmental factors alter normal development of CNS-intrinsic immunity and/or responses to systemic inflammatory challenges
- We have presented the data presented in previous sections of this report at national scientific conferences and published the following abstracts:
    - **D. S. DAVIS**, S. S. PUNTAMBEKAR, B. ALMOLDA ARDID, S. SLONIOWSKI, I. M. ETHELL, *M. J. CARSON. 2009 Age related changes in CNS-immune system interactions: Implications for age-specific risk and treatment of neurologic disorders. Abstracts of the 39th Annual Meeting for the Society for Neuroscience. Nanosymposium 11: Neuroinflammation: accentuating the positive 11.1.
    - ***D. S. DAVIS**, S. SLONIOWSKI, T. BILOUSOVA, S. S. PUNTAMBEKAR, I. M. ETHELL, M. J. CARSON. 2010 Post-natal regulation of tolerated CNS inflammation in response to systemic immune challenge. Abstracts of the 40th Annual Meeting for the Society for Neuroscience 561.19.

- o ***S. P. SLONIOWSKI**[1], D. S. DAVIS[2], T. V. BILOUSOVA[3], S. S. PUNTAMBEKAR[2], M. J. CARSON[2], I. M. ETHELL[2]. 2010 Effects of systemic inflammation on developing hippocampal neurons: Dissecting neuron-microglial interactions. Abstracts of the 40th Annual Meeting for the Society for Neuroscience 561.9.
- o Sloniowski, S., Davis, D.S., Launderdale, K., Rumi, R., Carson, M.J., Ethell, I.M. 2010. **EFFECTS OF SYSTEMIC INFLAMMATION ON DENDRITIC SPINE DEVELOPMENT IN THE HIPPOCAMPUS OF NEONATAL MICE.** *2010 Transactions of the American Society for Neurochemistry.* PTW03-19
- o Davis, D.S., Sloniowski, S., Carter, W., Ethell, I.M., Carson, M.J. 2010 **DEVELOPMENTALLY REGULATED CNS IMMUNE-SYSTEM INTERACTIONS: IMPLICATIONS FOR THE ONSET & PROGRESSION OF NEURODEVELOPMENTAL DISORDERS.** *2010 Transactions of the American Society for Neurochemistry* PTW04-02
- o Carson, M.J., Ethell, I.M. 2010 **FROM BIRTH TO OLD AGE, MICROGLIA AS BIOSENSORS AND EFFECTORS OF NEUROPROTECTION AND NEUROTOXICITY** *2010 Transactions of the American Society for Neurochemistry* C08-03.
- o Carson M, 2010 **CNS-IMMUNE SYSTEM INTERACTIONS: A DANCE THAT CHANGES WITH DEVELOPMENT, AGE AND EXPERIENCE. Plenary Symposium Lecture: no abstract available. 10th international congress on neuroimmunology**

- UNCF-Merck **Graduate Science Research Dissertation Fellowship** was awarded to Deirdre S. Davis, a graduate student and under-represented minority supported by this project (only 12 pre-doctoral fellowships awarded per year) based on training received and data gathered while completing SOW
  - o fellowship term: September 1, 2010-August 30, 2012,
  - o Direct costs provided to Deirdre Davis over 24 month term of fellowship: $52,000.

CONCLUSION:

This was a highly productive and successful research project that led to potentially paradigm shaping observations. We initiated the project with the hypothesis that acute inflammation would be detrimental for a very sensitive measure of neuronal function and synaptogenesis: dendritic spine formation. We also hypothesized also initially assumed that inflammation would be muted as compared to that observed in adult mice. Our studies proved these initial hypotheses (shared by many in the research community) to be incorrect.

Somewhat surprisingly, we demonstrated that systemic inflammation not only triggered a much greater influx of macrophages into the young developing brain than into the mature brain. We also demonstrated that this macrophage influx caused no detectable damage to dendritic spine formation or maturation when systemic inflammation during most of post-natal development. The one exception was the observation that systemic inflammation occurring during a very sensitive period of hippocampal maturation (post-natal day 14) led to an inhibition in formation of excitatory synapses (but not inhibitory synapses). The net result of this inhibition of excitatory synapse formation would cause the neurons in the inflamed hippocampus to be less sensitive to excitotoxic cell death at a critical period in their developmental maturation.

We now hypothesize the general benign and perhaps beneficial responses to systemic inflammation may be in part a consequence of the very high levels of TREM2 expressed and the high levels of TLT2 induced following systemic inflammation in the developing brain. Thus in the healthy individual, CNS-intrinsic immunity changes as a function of normal development to sustain optimal brain function during time periods that individuals are likely to be exposed to common inflammatory insults. Our completed studies, de facto provide a screen to identify genetic and/or environmental factor that alter the normal developmental progression of CNS-intrinsic immunity and/or CNS-intrinsic responses to systemic inflammation.

Future experiments using TREM2 KO and TLT2 KO mice will test if either or both of these anti-inflammatory (and microglial-enriched) receptors are required to maintain dendritic spine

maturation during acute bouts of systemic inflammation. As part of future high risk/high impact studies, we will also test whether introduction of TREM2 and TLT2 into the peripheral immune system can ameliorate dendritic spine defects observed in two genetic models of autism spectrum disorders (fragile X and Rett syndrome).

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Appendix 1: Abstracts from oral and poster presentations of data generated from this project

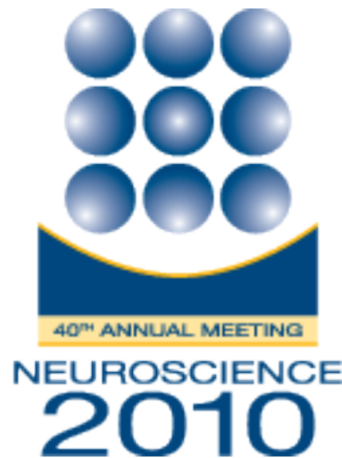
2009 Annual Meeting of the Society for Neuroscience

Graduate Student, Deirdre S. Davis' abstract was selected for oral presentation and to be the first lead-off presentation in the Nanosymposium entitled "Neuroinflammation: Accentuating the Positive".
Saturday, Oct 17, 2009, 1:00 PM - 3:30 PM Room S404

Chair: Katrin Andreasson , Stanford University School of Medicine, Palo Alto, CA, United States

Program#/Poster#: 11.1 Title: Age related changes in CNS-immune system interactions: Implications for age-specific risk and treatment of neurologic disorders
Location: Room S404 Presentation Time: Saturday, Oct 17, 2009, 1:00 PM - 1:15 PM

Authors: **D. S. DAVIS**, S. S. PUNTAMBEKAR, B. ALMOLDA ARDID, S. SLONIOWSKI, I. M. ETHELL, *M. J. CARSON;
Div. of Biomed. Sci., Univ. of California Riverside, Riverside, CA Abstract: Microglia are the tissue macrophage of the CNS able to rapidly respond to insults occurring within the CNS. Systemic inflammation induced outside the CNS can also lead to microglial activation. Here we find that systemic inflammation induced by intraperitoneal LPS injection leads to widespread microglial activation accompanied by a small transient influx of peripheral macrophages. Unlike, microglial activation induced by injection of LPS directly into the CNS, intraperitoneal injection of LPS did not lead to robust pro-inflammatory activation of microglia. Rather, we observed induced expression of selected members of the TREM family of immunomodulatory receptors that may be indicative of alternative anti-inflammatory activation responses. Because microglial phenotype is highly plastic and readily modulated by environmental cues, we also examined microglial phenotype and responses to systemic inflammation as a function of normal development and extreme aging. We, and others have hypothesized that during early childhood, the brain has a unique profile of susceptibility to systemic inflammation because this timeframe corresponds to the period of active brain development and experience driven synapse formation. In addition, within the CNS, age-related changes in the phenotype and function of microglia have been hypothesized to contribute to the onset and progression of age-linked neurodegenerative disorders such as Alzheimer's disease. Here, using flow cytometry and dual immunohistochemical/in situ hybridization, we demonstrate that (1) basal microglial phenotype displays increasing regional diversity as a function of healthy aging (2) macrophage influx into the CNS caused by LPS-induced systemic inflammation decreases with age (3) microglial responses to systemic inflammation are more robust during early post-natal development than after CNS maturation is complete.
Taken together, these data contribute to the growing literature demonstrating differential age-linked CNS susceptibility to insults associated with neurodevelopmental disorders, neurodegeneration and responses to therapeutic intervention. These studies have been supported by NIH, DOD, Dana Foundation and UCR Biomed PIC Grant program



Presentation Abstract for the 2010 Annual Meeting

Program#/Poster#: 561.19/T6

Title: Post-natal regulation of tolerated CNS inflammation in response to systemic immune challenge

Authors: ***D. S. DAVIS**, S. SLONIOWSKI, T. BILOUSOVA, S. S. PUNTAMBEKAR, I. M. ETHELL, M. J. CARSON;
Univ. of California, Riverside, Univ. of California, Riverside, Riverside, CA

Abstract: Neuroinflammation triggered by systemic inflammation and occurring during critical periods of CNS development is hypothesized to contribute to the development and/or pathogenesis of many neurodevelopmental disorders including cerebral palsy, schizophrenia and autism. In the adult CNS, the type and magnitude of microglial activation and macrophage influx triggered by systemic inflammation are well characterized. As yet, little is known about if and how microglial phenotype changes during critical periods of CNS development associated with synaptogenesis and oligodendrocyte development. Here, using flow cytometry and dual in situ hybridization/immunohistochemistry we show that in the early postnatal murine brain, microglia display an activated phenotype that is not polarized toward either "classic" proinflammatory or "alternative" anti-inflammatory activation states. Furthermore, we find that at all ages examined, systemic inflammation induced by an intraperitoneal injection of LPS leads to widespread activation of microglia and a transient influx of peripheral macrophages into the murine CNS. However, the magnitude of macrophage influx into the CNS is developmentally modulated. Systemic inflammation triggers the highest level of macrophage influx into the CNS between days 7 and 14. In addition, CNS-infiltrating macrophages display a highly polarized pro-inflammatory phenotype. During this same developmental period, microglial activation is associated with high level expression of multiple alternative activation markers and induction of TREM2. We speculate that these microglial-specific forms of activation in part serve to compensate for the higher levels of pro-inflammatory macrophages that infiltrate the CNS during early development. Altogether our data contribute to the growing literature demonstrating that the brain may have age-specific susceptibilities to insults associated with the development and treatment of neurodevelopmental disorders.

Poster Presentation: 2010 annual meeting of the Society for Neuroscience
Program#/Poster#: 561.9/S8
Title: Effects of systemic inflammation on developing hippocampal neurons: Dissecting neuron-microglial interactions Location: Halls B-H Presentation Time: Tuesday, Nov 16, 2010, 8:00 AM - 9:00 AM Authors: ***S. P. SLONIOWSKI**[1], D. S. DAVIS[2], T. V. BILOUSOVA[3], S. S. PUNTAMBEKAR[2], M. J. CARSON[2], I. M. ETHELL[2]; [1]Neurosci. Grad. Program, [2]Div. of Biomed. Sci., Univ. of California, Riverside, Riverside, CA; [3]Dept. of Mol. and Med. Pharmacol., UCLA, Los Angeles, CA Abstract: Dendritic spines are small protrusions on the surface of dendrite where postsynaptic densities of the majority of CNS excitatory synapses are located. Spine number, shape and size directly influence synaptic function and abnormalities in spine morphology have been associated with a range of cognitive and behavioral pathologies. Pathological stimuli can adversely affect dendritic spine shape and number, and this is seen in neurodegenerative disorders and some forms of mental retardation and autism. Moreover, neuroinflammation has been suggested to contribute to the etiology of autism and schizophrenia. Therefore, we investigate the effects of systemic inflammation on dendritic spine development in the hippocampi of P7-P21 mice and compare them to the responses in adult mouse brain. To induce a systemic inflammation, the mice were IP injected with LPS. Acute effects of systemic inflammation were evaluated 24h following the LPS injection. Our results indicate that LPS injections resulted in higher Iba1 immunoreactivity as well as changes in the morphology of Iba1 positive cells indicative of microglial activation in postnatal day 8 (P8), P15 and P22 mouse hippocampus. However, no detectable changes in dendritic spine density, length or morphology were found in CA1 hippocampal neurons 24h following injections of LPS at P15, P22 or in adult. In order to dissect the effects of activated microglia and infiltrating macrophages on developing neurons, we tested whether activated microglia were cytotoxic to cultured hippocampal neurons and whether their toxicity differed from CNS-infiltrating macrophages. No differences in neuronal numbers were detected between untreated neuronal cultures or those co-cultured for 2-3 days with CD45lo microglia from adult brain of LPS-injected mice. By contrast, significant neuronal loss and reduced dendritic length were seen after 2-3 days in cultures receiving CD45hi macrophages isolated from the same brain of LPS-injected mice. Moreover, the addition of activated microglia to hippocampal cultures at 2 weeks *in vitro* for 2 days has not changed the density or lengths of dendritic spines, which accommodate postsynaptic sites of excitatory synapses. Furthermore, microglia cultured with immature but not synaptically active hippocampal neurons maintained a classic amoeboid shape usually associated with inflammatory responses and produced detectable levels of TNFα. These data suggest that the maturation state and activity of neurons can modulate microglia phenotype and function. Future studies will further elucidate neuron-microglial interactions in developing hippocampal neurons in healthy and diseased brain.

PTW03-19

EFFECTS OF SYSTEMIC INFLAMMATION ON DENDRITIC SPINE DEVELOPMENT IN THE HIPPOCAMPUS OF NEONATAL MICE

Sloniowski, S., Davis, D.S., Launderdale, K., Rumi, R., Carson, M.J., Ethell, I.M.
Division of Biomedical Sciences, UC Riverside, Riverside, USA

Dendritic spines are small protrusions on the surface of dendrite where post-synaptic densities of the majority of excitatory synapses are located in the brain. Pathological stimuli can adversely affect dendritic spine shape and number, as seen in neurodegenerative disorders, some forms of mental retardation and autism. Moreover, neuroinflammation has been suggested to contribute to the etiology of autism and schizophrenia. Therefore, it is important to assess the effects of inflammation on the developing brain. Here, we investigate the effects of systemic inflammation on dendritic spine development in the hippocampi of P3-P21 mice and compare them to the responses in adult mouse brain. To induce a systemic inflammation, the mice were injected intraperitoneally with lipopolysaccharide (LPS). To visualize dendritic spines, fixed mouse brains were sectioned and labeled with DiI using DiOlistic approach. Acute effects of systemic inflammation were evaluated 24h following the LPS injection. Our preliminary results indicate a higher proportion of spines with smaller heads (<0.4 "m2) in CA1 hippocampus of LPS-treated mice as compared to controls in both postnatal day 22 and the adult brains (p=0.01), while average spine length remained unchanged. A significant increase in spine density in the CA1 hippocampus of LPS-treated mice as compared to controls was also noted in P22 brain (p=0.02), but not in the adult brain. Future studies will further elucidate the acute effects of systemic inflammation on the dendritic spine number and morphology in early postnatal mouse brain (P3-P14) and long-term effects on the dendritic spine development.

PTW04-02

DEVELOPMENTALLY REGULATED CNS IMMUNE-SYSTEM INTERACTIONS: IMPLICATIONS FOR THE ONSET & PROGRESSION OF NEURODEVELOPMENTAL DISORDERS

Davis, D.S., Sloniowski, S., Carter, W., Ethell, I.M., Carson, M.J.
University of California, Riverside, Riverside, USA

Neuroinflammation triggered by systemic inflammation and occurring during critical periods of CNS development is hypothesized to contribute to the development and/or pathogenesis of many neurodevelopmental disorders including cerebral palsy, schizophrenia and autism. In the adult CNS, the type and magnitude of microglial activation and macrophage influx triggered by systemic inflammation are well characterized. As yet, little is known about if and how microglial phenotype changes during critical periods of CNS development associated with synaptogenesis and oligodendrocyte development. Here, using flow cytometry and dual in situ hybridization/immunohistochemistry we show that in the early postnatal murine brain, microglia display an activated phenotype that is not polarized toward either “classic“ proinflammatory or “alternative“ anti-inflammatory activation states.
We also find that at all ages examined, systemic inflammation induced by an intraperitoneal injection of LPS leads to widespread activation of microglia and a transient influx of peripheral macrophages into the murine CNS. However, the magnitude of macrophage influx into the CNS is developmentally modulated. In addition, CNS-infiltrating macrophages display a highly polarized pro-inflammatory phenotype. During this same developmental period, microglial activation is associated with high level expression of multiple alternative activation markers and induction of TREM2. Altogether our data contribute to the growing literature demonstrating age-specific CNS susceptibilities to insults associated with the development and treatment of neurodevelopmental disorders.

2010 Transactions of the American Society for Neurochemistry
Invited Colloquia Presentation: Microglial Heterogeneity and the Protection of Brain Function

C08-03

FROM BIRTH TO OLD AGE, MICROGLIA AS BIOSENSORS AND EFFECTORS OF NEUROPROTECTION AND NEUROTOXICITY

Carson, M.J., Ethell, I.M.
University of California , Riverside, USA

Microglia are the tissue macrophage of the CNS and respond not only to direct insults to the CNS, but also to primary systemic inflammation. Systemic inflammation occurring during perinatal periods has been implicated in exacerbating and/or triggering the onset of a variety of pandevelomental neurologic disorders. Systemic inflammation in aged Alzheimer's disease patients has also been correlated with an increased decline in cognitive performance. We, and others have hypothesized that during early childhood, the brain has a unique profile of susceptibility to systemic inflammation because this timeframe corresponds to the period of active brain development and experience driven synapse formation. In addition, age-related changes in the phenotype and function of microglia have been hypothesized to contribute to the onset and progression of age-linked neurodegenerative disorders such as Alzheimer's disease. Here, using flow cytometry and dual immunohistochemical/in situ hybridization, we demonstrate that (1) microglia display an activated but non-polarized mixed classic/alternative activation state during early post-natal development (2) the influx of polarized pro-inflammatory macrophages into the CNS caused by LPS-induced systemic inflammation is agedependent and is most robust between post-natal days 7 and 14 (3) systemic inflammation triggered changes in neuronal dendritic spines in both the young, developing and mature CNS, but type of changes triggered were age specific. Taken together, these data contribute to the growing literature demonstrating differential agelinked CNS susceptibility to insults associated with neurodevelopmental disorders, neurodegeneration and responses to therapeutic intervention.

10th International Congress on Neuroimmunology

Plenary Symposium 3..Auditorium

Modulating the immune response into the brain

Chairs: C. Whitacre and M. Carson

633 – CNS-IMMUNE SYSTEM INTERACTIONS: A DANCE THAT CHANGES WITH DEVELOPMENT, AGE AND EXPERIENCE

Carson Monica 11.30

University of California Riverside ~ Riverside ~ United States

No abstract available