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Detection of Bacterial Antigens and Alzheimer's Disease-like Pathology in the Central Nervous System of BALB/c Mice Following Intranasal Infection with a Laboratory Isolate of Chlamydia pneumoniae

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Detection of bacterial antigens and Alzheimer's disease-like pathology in the central nervous system of BALB/c mice following intranasal infection with a laboratory isolate of *Chlamydia pneumoniae*

Running Title: AD-like Pathology following Cpn Infection

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ABSTRACT

Pathology consistent with that observed in Alzheimer's disease (AD) has previously been documented following intranasal infection of normal wild-type mice with *Chlamydia pneumoniae* (Cpn) isolated from an AD brain (96-41). In the current study, BALB/c mice were intranasally infected with a laboratory strain of Cpn, AR-39, and brain and olfactory bulbs were obtained at 1-4 months post-infection (pi). Immunohistochemistry for amyloid beta or Cpn antigens was performed on sections from brains of infected or mock-infected mice. *Chlamydia*-specific immunolabeling was identified in olfactory bulb tissues and in cerebrum of AR-39 infected mice. The Cpn specific labeling was most prominent at 1 month pi and the greatest burden of amyloid deposition was noted at 2 months pi, whereas both decreased at 3 and 4 months. Viable Cpn was recovered from olfactory bulbs of 3 of 3 experimentally infected mice at 1 and 3 months pi, and in 2 of 3 mice at 4 months pi. In contrast, in cortical tissues of infected mice at 1 and 4 months pi no viable organism was obtained. At 3 months pi, only 1 of 3 mice had a measurable burden of viable Cpn from the cortical tissues. Mock-infected mice (0 of 3) had no detectable Cpn in either olfactory bulbs or cortical tissues. These data indicate that the AR-39 isolate of Cpn establishes a limited infection predominantly in the olfactory bulbs of BALB/c mice. Although infection with the laboratory strain of Cpn promotes deposition of amyloid beta, this appears to resolve following reduction of the Cpn antigen burden over time. Our data suggest that infection with the AR-39 laboratory isolate of Cpn results in a different course of amyloid beta deposition and ultimate resolution than that observed following infection with the human AD-brain Cpn isolate, 96-41. These data further support that there may be differences, possibly in virulence factors, between Cpn isolates in the generation of sustainable AD pathology.

INTRODUCTION

Alzheimer's disease (AD) is the most common dementia in the US, accounting for 50 to 70 percent of cases. More than 5 million Americans are living with a diagnosis of AD as of 2013 with 90-95% of cases in the 65 and older segment of the population. Early stage of disease involves memory impairment (Fargo and Bleiler. 2014). In the advanced stages of AD, individuals require assistance with daily activities and, ultimately, in the final stage become bed-bound and are reliant on around-the-clock care (Hebert, *et al*. 2003). AD is a fatal disorder with the progression from the earliest symptoms to total functional dependency and death in an untreated person often occurring within 8-10 years post diagnosis (Fargo and Bleiler. 2014) .

Although much is known about the disease process and progression of AD, the initiating factors or cause(s) of the disease still remain a mystery. AD has an early onset familial form that is primarily driven by autosomal dominant genetic alterations in genes encoding the beta amyloid precursor protein, as well as the loci encoding presenilins 1 and 2 (Goate, *et al*. 1991; Levy-Lahad, *et al*. 1995; Rogaev, *et al*. 1995; Wolfe. 2007). Transgenic mouse models have been developed to induce enhanced β-amyloid production and subsequent deposition of β-amyloid (Hall and Roberson. 2012; Wisniewski and Sigurdsson. 2010), and serve as models for early onset AD, which accounts for ~3-5 % of all reported cases. One important issue that cannot be addressed using these model systems is how to target the early initiating events in sporadic late-onset AD and not just the "tombstone" lesions that are the result of a long chain of pathological processes (Wisniewski and Sigurdsson.

2010). In this regard, animal models that mimic the sporadic late-onset form of AD have been developed, but these are hampered by the lack of understanding of the primary factors that promote the deposition of β-amyloid. Currently, models that experimentally induce AD-like pathology use bacterial toxins such as streptozotocin (Labak, *et al*. 2010), chronic stress (Alkadhi, *et al*. 2010), or colchicine to chemically induce damage (Kumar, *et al*. 2007) to the CNS to initiate pathology. As several infectious agents, including *Chlamydia pneumoniae* (Cpn), have been proposed to enhance risk or play a causal role in AD (Gerard, *et al*. 2006; Balin, *et al*. 1998), animal models have been developed to study the effects of this infection (Little, *et al*. 2004; Little, *et al*. 2005) with regards to AD-like pathology. However, there remains a dearth of experimental animal systems that accurately model sporadic late-onset AD, leaving the scientific community with few options to address key questions related to the initiation/ progression of late-onset disease.

The identification of Cpn in AD brain tissue (Balin, *et al*. 1998) was a stimulus to investigate the potential role that this organism plays in the induction and progression of late-onset AD and led to the establishment of a mouse model to investigate this occurrence (Little, *et al*. 2004). In the original experimental system, BALB/c mice were infected with Cpn isolated from human AD brain autopsy tissue. The isolate of Cpn, 96-41, was propagated in HEp-2 cells and then introduced into 3 month old BALB/c mice via intranasal inoculation; brain tissue was analyzed at monthly time points up through 3 months pi following intranasal delivery.

Our first study utilized the human AD-brain isolate of Cpn to induce AD-like pathology in non-transgenic mice (Little, *et al*. 2004), and was designed to address Koch's postulates. The first postulate requires that the infectious organism be isolated from tissues of an affected individual. In this particular case, the first postulate is satisfied, but for other cases of the disease this issue is still debate (Itzhaki, *et al*. 2004). To satisfy Koch's second postulate, the pathogen must be isolated from a diseased organism and grown in pure culture. Cpn was isolated post-mortem from human AD-brain tissue and grown in culture (although culture required a eukaryotic cell as this is an obligate intracellular bacterium). Third, the organism was introduced into a mouse, and induced pathology consistent with AD, while uninfected mice did not display the same pathology. Fourth, the organism was identified in the tissues of affected mice, but was not re-isolated from the tissue. Thus, Koch's postulates were used as a general guide, and although difficult to use in their purest sense when addressing any intracellular infection, our findings support the hypothesis that Cpn infection can induce β-amyloid deposition in the brain and contribute directly to pathogenesis.

In mice infected with Cpn in our first report, β-amyloid deposits were identified as early as two months pi, with the greatest number of deposits identified at three months pi. The number and size of amyloid deposits increased over time, thus the development of AD-like pathology appeared to be progressive. The experimental induction of mouse derived β-amyloid deposits in inbred BALB/c mice (not genetically modified) at 5 and 6 months of age (2 and 3 months pi) indicates that infection can trigger the production and deposition of β-amyloid in the mouse brain. In contrast, in transgenic mouse models used to study AD, 6 months of age is very early to observe substantial amyloid deposits, yet we observed

substantial pathology 2 months after introduction of the infectious agent into non-transgenic animals. Cpn is a respiratory pathogen and was introduced into mice via an intranasal inoculation. This is the natural route of infection and the organism can be responsible for an acute respiratory illness. The respiratory infection appears to precede dissemination to other organ systems (Little, *et al*. 2005) and age is an important factor in the host's ability to control the dissemination, with even greater spread with the advent of immunosenescence in older animals.

In contrast with the initial report associating Cpn with the induction of AD-like pathology in the brains of BALB/c mice (Little, *et al*. 2004), the current study was performed with a respiratory isolate and common laboratory strain of Cpn, AR-39. The purpose was to determine if this well-studied laboratory isolate of Cpn would induce pathology in a similar manner and to the same degree over a similar time course, as that observed for the human CNS isolate used previously. This approach will inform potential differences in outcomes when infecting mice with Cpn originally isolated from lung tissues and used as a laboratory isolate as compared to that from human AD brain.

MATERIALS AND METHODS

HEp-2 cell line: The human epithelial, HEp-2, cell line (ATCC, Rockville MD) was maintained in MEM supplemented with 10% fetal bovine serum (FBS) (Cell Gro Mediatech, Inc, Manassas, VA), 5mM L-Glutamine (Thermo Fisher Scientific, Pittsburgh, PA) at 37^{o}C and 5% CO_2. 1-2 x 10^5 cells were plated in a T25 tissue culture flask (Thermo Fisher Scientific, Pittsburgh, PA) and passaged as needed prior to collection for the propagation of Cpn.

Propagation and purification of *Chlamydia pneumoniae*: *Chlamydia pneumoniae* (Cpn), AR-39 isolate, was obtained from the ATCC (ATCC, Rockville, MD) and propagated in the HEp-2 cell line similar to the technique described for the Cpn brain isolate, 96-41(Little, *et al*. 2004; Campbell, *et al*. 1991). Prior to infection of BALB/c mice, homogenates of 72 h culture supernatants and Cpn infected HEp-2 cells were sonicated for 30 seconds and passed through a series of filter membranes with decreasing pore size to collect the elementary bodies. The organism was resuspended in Hanks Balanced Salt Solution (HBSS), aliquoted, and stored at -80 C. The quantitation of inclusion forming units subsequently was determined following infection of HEp-2 epithelial cells with a series of 10 fold serial dilutions of the concentrated organism. The inclusions were identified by immunofluorescence using a Chlamydia-specific antibody directly conjugated to FITC (Imagen^{TM}; DAKO, Carpenteria, CA). Aliquots were diluted in HBSS to a working concentration for the intranasal infection of mice.

Mice: Six week old female BALB/cJ mice were purchased from Jackson Laboratories (Bar Harbor, ME) and acclimated for 2 weeks prior to use. Mice were housed in groups of 2-3 in HEPA-filter caged racks, with infected mice housed separately from uninfected mice, within the containment facility at Philadelphia College of Osteopathic Medicine. All animal husbandry was performed using Biosafety Level 2 precautions and in a Class II biosafety cabinet. Mice were fed food and water ad libitum. All animal protocols were approved by the IACUC at PCOM.

Infection of mice with *Chlamydia pneumoniae*: Under manual restraint, 8 week old, female BALB/cJ mice were inoculated intranasally with 5 x 10^5 inclusion forming units of the AR-39 isolate of Cpn diluted in 50 µl of HBSS. Six mice were inoculated at 8 weeks of age for each time point and the brains were analyzed at 1, 2, 3 and 4 months post infection. Four age and sex matched mice were mock-infected with vehicle alone, HBSS, as a control for each time point. At each time point, 3 experimentally-infected and 2 mock-infected control mice were anesthetized, cardiac-perfused and organs were collected and immersion fixed in 4 % paraformaldehyde for embedding, sectioning and immunohistochemical analysis. The remaining 3 experimentally-infected and 2 mock-infected control mice at each time point other than for 2 month animals for which frozen tissue was not available were euthanized and organs were collected and snap-frozen in liquid nitrogen and then stored at -80 C until analysis for detection and quantification of viable organism.

Recovery and quantification of *Chlamydia pneumoniae*: Quantification of viable Cpn was performed in an identical manner to our previous report (Little et al 2005) (Little, *et al*. 2005) in the following manner. Frozen tissue was thawed and a 10% weight to volume homogenate was prepared in serum-free minimal essential medium (MEM) (Thermo Fisher Scientific, Pittsburgh, PA) supplemented with 2mM Glutamine. Serial ten-fold dilutions (in 200µL) were added to 4 well Lab Tech chamber slides (Naperville, IL) on which HEp-2 cells were previously plated. Negative control wells contained cells mock-infected with medium alone. The chamber slides were incubated at 37^{o}C in 5% CO_2 for 2.5 hrs, washed with HBSS and refilled with fresh complete medium supplemented with 2µg/ml cycloheximide (Sigma-Aldrich, St. Louis, MO) followed by incubation for 48 hrs at 37^{o}C. After incubation, slides were washed with HBSS, fixed in 50% methanol at RT for 20 min, washed twice in HBSS, and labeled with a 1:10 dilution of FITC-conjugated Chlamydia-specific antibody (Imagen^{TM}; DAKO, Carpenteria, CA) for 90 min in the dark at 37°C. Slides were washed in phosphate buffered saline (PBS) and counterstained with a 2 µg/ml of bisBenzamide (Sigma-Aldrich, St. Louis, MO) in PBS for 1 min, washed in PBS and coverslipped with aqueous mounting medium (Imagen^{TM}; DAKO, Carpenteria, CA). All titers are calculated as inclusion forming units (IFU)/ml of 10% weight to volume tissue homogenate.

Antibodies: The following *Chlamydia*-specific antibodies were generated in mice: RDI-PROAC1p (Research Diagnostics Incorporated, Flanders, NJ) (AC1P) (monoclonal I_gG) specific for *Chlamydia* lipopolysaccharide used at a dilution of 1:10 (5µg/ml), M6600 (DakoCytomation, Carpinteria, CA) (monoclonal I_gG) specific for Cpn major outer membrane protein used at a dilution of 1:10 (10µg/ml), and 10C-27 (Fitzgerald, Concord, MA) (monoclonal I_gG) specific for Cpn used at a dilution of 1:100 (1µg/ml). Additionally, B65256R (Biodesign International, Saco, ME) (B56R) specific for *Chlamydia* purified elementary bodies was generated in rabbit and used at a dilution of 1:200 (2µg/ml). Both secondary antibodies specific for either mouse, AP-Goat anti-mouse IgG conjugate (Zymed Laboratories, San Francisco, CA), or rabbit, AP-Goat anti-rabbit IgG conjugate (Zymed Laboratories, San Francisco, CA), were used at a concentration of 2 µg/ml. All antibodies were diluted to working concentration in 2% FBS/PBS blocking buffer (Thermo Fisher Scientific, Pittsburgh, PA). For the detection of Aβ amyloid, the following

antibodies were used at a recommended concentration of 2 μg/ml: a rabbit polyclonal antibody specific for the carboxyl-terminal fragment of Aβ amyloid 1-42 (catalogue: A1976 Oncogene Research Products, Boston, MA), and a mouse monoclonal antibody (4G8) to the 17-24 amino acid peptide of human Aβ amyloid 1-42 (catalogue:9220-05 Signet Laboratories Inc., Dedham, MA). For all amyloid specific immunolabeling, secondary antibodies consisted of HRP conjugated sheep anti-Mouse IgG (H + L) or donkey anti-rabbit IgG (H + L). Antibodies were used at a dilution of 1:300 as recommended by the supplier (Amersham Biosciences, Piscataway, NJ and Life Technologies, Inc, Grand Island, NY).

Immunohistochemistry: Brain sections from experimental and control mice were immunolabeled for Aβ amyloid or Cpn antigen at 1, 2, 3, and 4 months post infection using the aforementioned antibodies. Coronal sections were deparaffinized with xylene (Thermo Fisher Scientific, Pittsburgh PA) rehydrated in a series of graded alcohol solutions (Electron Microscopy Sciences, Fort Washington, PA), followed by de-ionized (DI) H_2O. Slides were then placed in Citra antigen retrieval buffer (BioGenex, San Ramon, CA) and steamed in a 2100 Retriever (Pick Cell Laboratories, Amsterdam, Netherlands) for 20 min at high pressure and temperature (120° C). Slides were then rinsed with PBS pH 7.4 (Sigma-Aldrich, St Louis, MO) 3 x 5 minutes. Endogenous peroxidase activity was quenched utilizing a 3% solution of H_2O_2/PBS (Thermo Fisher Scientific, Pittsburgh, PA) for 5 min at RT. Sections were rinsed 1 x 5 min in PBS and blocked 3 x in 2% heat inactivated fetal bovine serum (FBS)/PBS. A total of 30 coronal brain sections, 10 sets of 3 sections (1 per antibody), were immunolabeled per mouse. The sections were spaced equally (approximately every 70-100 microns in brain tissue) from rostral (bregma + 2.22mm) to caudal (bregma -5.88mm) in order to provide samples representative of the regions spanning the entire brain of each mouse. Slides receiving Chlamydia-specific primary antibodies B56R or a cocktail of 10C-27, AC1P, M6600 were applied to tissue sections and placed in a humidified chamber at 37 °C for 90 min. The sections were rinsed 3 x 5 min each and then blocked 3 x 15 min each in 2% FBS/PBS, and incubated with appropriate secondary antibodies in a humidified chamber for 1 hour at 37 °C. Following incubation, sections were rinsed with DI H_2O 3 x 5 min and developed using alkaline phosphatase new magenta for 15 min (BioFX, Owings Mills, MD) at RT. Sections were rinsed in DI H_2O 3 x 5 min followed by one PBS rinse for 5 min. Acidified Harris's Hematoxylin (Thermo Fisher Scientific, Pittsburgh, PA) was applied to sections for 1 min. One DI H_2O rinse followed counterstaining and the sections were contrasted in PBS for 5 min. Finally, the sections were rinsed with DI H_2O 3 x 5 min, air dried, and crystal mounted (BioMeda, Foster City, CA). Once dry, the sections were permounted and coverslipped.

Slides receiving mouse primary antibodies were blocked in mouse on mouse (M.O.M.) IgG blocking reagent (Vector M.O.M. kit, Vector Laboratories, Burlingame, CA) for 1 hr at RT, rinsed, and incubated for 5 min in the M.O.M. blocking buffer. For all sections, the primary antibodies were incubated overnight at 4^0C. The sections were rinsed in PBS 3 x for 5 min each, blocked 3 x for 15 min each in 2% FBS/PBS, and incubated with appropriate secondary antibodies in a humidified chamber for 2 h at RT. The sections labeled with anti-amyloid antibodies were rinsed with PBS 3 x for 10 min each and

visualized with 3, 3'-Diaminobenzidine (DAB) (Sigma *FAST*™, Sigma-Aldrich, St. Louis, MO). Sections were rinsed with dH_2O, counterstained with Harris' Alum Hematoxylin (EM Sciences Harleco®, EM Industries, Inc., Hawthorne, NY), and permounted.

Microscopic Analysis: Digital images were captured using Image-Pro Plus Phase 3 Imaging System software (Media Cybernetics, Silver Spring, MD) on a Nikon Eclipse E800 microscope using a Spot RT Camera (Diagnostic Instruments, Sterling Heights, MI).

Statistical Analysis:
Statistical analysis was performed using the student t-test followed by pair-wise testing of uninfected (n=8) relative to each experimental infected timepoint (n=3) using Microsoft excel statistical analysis software and P values of < 0.05 which were considered significant.

RESULTS

Recovery of infectious *Chlamydia pneumoniae* from olfactory bulbs and cerebrum: Olfactory bulbs and cerebral tissues were dissected from BALB/c mice following euthanization, snap frozen, and homogenized prior to incubation with HEp-2 cells in culture to determine if detectable levels of viable Cpn could be recovered from the central nervous system. Ten-fold serial dilutions of the homogenized tissues were incubated with HEp-2 cells to determine the amount of viable infectious Cpn present in the tissues at 1, 3 and 4 months pi. Tissue from the 2 month animal was not available. Infectious Cpn was recovered and quantified from 3 of 3 olfactory bulbs at 1 month pi, ranging from 3×10^3 to 3×10^5 IFU/ml of tissue homogenate (Fig 1a). At 3 months pi, Cpn was detected in 3 of 3 olfactory bulbs with a range of 2×10^5 to 3×10^6 IFU/ml of tissue homogenate (Fig 1a). At 4 months, Cpn was detected in 2 of 3 olfactory bulbs with a range of 0 to ~2×10^6 IFU/ml tissue homogenate (Fig. 1a). Of the 3 olfactory bulbs tested from the mock infected animals, no Cpn was recovered. In contrast to the olfactory bulbs, Cpn was not recovered from the brain tissue (cerebrum) at 1 and 4 months pi, although at 3 months, Cpn was recovered and quantified at 3×10^4 IFU/ml of tissue from 1 of 3 brains (Fig. 1b). This same mouse had 3×10^5 IFU/ml in the olfactory bulb as noted above. With regards to brain tissues analyzed from the 3 control animals, no Cpn was detected.

Identification and distribution of *Chlamydia pneumoniae* antigen in the central nervous system: Cpn antigen was detected in olfactory bulb tissues at 1 and 3 months pi using antibodies specific for Cpn LPS and outer membrane proteins. Representative immunolabeling for Cpn in these tissues at 1 month pi was principally intracellular (Fig.2). The labeling profiles consisted of large intracellular vacuoles, often perinuclear with prominent well-defined inclusions. Furthermore, Cpn antigen labeling (LPS and outer membrane proteins) was documented within the cerebrum with a quantitative analysis of 10 total slides per animal distributed rostral to caudal with distances measured from Bregma (Table 1). Intracellular immunolabeling was observed to be both perinuclear and diffuse in the cytoplasm with very few clearly documentable intracellular inclusions (Fig. 3). However, upon close examination, punctate immunolabeling was observed in numerous cells (Fig. 3c,e).

Table 1: Location of *Chlamydia pneumoniae* immunoreactivity and Aβ 1-42 amyloid deposits over 4 months post infection within brains of Cpn-infected mice. (A) The location and number of immunoreactive amyloid deposits or Cpn antigen is designated in millimeters (section location in mm) rostral or caudal to the mouse bregma. (B) Statistical analysis of Cpn-specific immunoreactivity and Aβ 1-42 immunoreactive depostis from infected and uninfected mouse brains. For each time point, N = animals analyzed, * indicate statistical significance.

Table 1

A

	Bregma									Total	
	2.22	**1.7**	**0.38**	**-1.28**	**-2.75**	**-3.8**	**-4.92**	**-5.46**	**-5.88**	**Inf**	**Un**
1 Mo Cpn	**22**	**10**	**22**	**18**	**23**	**42**	**9**	**6**	**2**	**154**	**24**
Amyloid	**0**	**1**	**0**	**3**	**3**	**0**	**2**	**1**	**0**	**10**	**0**
2 Mo Cpn	**18**	**15**	**19**	**5**	**26**	**21**	**20**	**10**	**0**	**134**	**14**
Amyloid	**8**	**30**	**11**	**16**	**31**	**43**	**35**	**6**	**0**	**180**	**12**
3 Mo Cpn	**11**	**11**	**13**	**22**	**5**	**9**	**9**	**4**	**7**	**91**	**10**
Amyloid	**0**	**9**	**14**	**7**	**4**	**3**	**3**	**11**	**2**	**53**	**10**
4 Mo Cpn	**0**	**11**	**13**	**15**	**13**	**10**	**10**	**1**	**3**	**76**	**9**
Amyloid	**0**	**0**	**3**	**6**	**3**	**1**	**5**	**1**	**0**	**19**	**5**
Cpn	**51**	**47**	**67**	**60**	**67**	**82**	**48**	**21**	**12**	**455**	**57**
Amyloid	**8**	**33**	**12**	**5**	**32**	**19**	**33**	**1**	**0**	**143**	**27**

B

Chlamydia-specific immunoreactivity					Amyloid deposits				
	uninfected n=2		infected n=3			uninfected n=2		infected n=3	
time p.i.	mean	s.d.	mean	s.d.	time p.i.	mean	s.d.	mean	s.d.
1	12	5.65	51.33*	32.01	1	0	0	3.33	1.167
2	7	2.83	44.67*	33.56	2	6	1	60*	8
3	5	1.41	30.33*	12.06	3	5	1	17.67*	0.67
4	4.50	0.71	25.33	24.21	4	2.5	1.5	6.33	2.167
ALL	7.13	4.02	37.92	21.22	ALL	3.38	2.28	21.83	19.08

Quantitative analysis of Cpn antigen in the brain at 1 through 4 months post-intranasal inoculation revealed peak Cpn antigen burden (154 immunoreactive profiles) at 1 month (Table 1A). Cpn-specific immunoreactivity demonstrated a step-wise decrease at 2, 3, and 4 months pi with 134, 91, and 76 immunoreactive profiles, respectively. With regards to specific coordinates in the brain, the greatest Cpn antigen burden (ie, 42 Cpn immunoreactive profiles) was documented at 1 month in multiple sections 3.8 mm caudal

to bregma. These sections contain the entorhinal cortex, perirhinal cortex, hippocampus, and amygdala, all regions affected in Alzheimer's disease. A low but detectable number of non-specific Cpn immunoreactive sites were detected within mock-infected control mouse brain tissue with an average number of 0.355/section analyzed. The mean number of immunoreactive sites identified was 7.125/ mouse +/- 4.01 and based upon the results of the student t-test a statistically significant difference ($p < 0.05$) was observed between experimentally infected tissue and mock-infected control mouse tissue at all timepoints analyzed; 1 month p.i. (51.33 +/- 32.01), 2 months p.i. (44.67 +/- 33.56), and 3 months p.i.(30.33 +/- 12.06). No statistically significant difference was observed in the 4 month p.i. (25.33 +/- 24.21) experimental group relative to uninfected control tissue (Table 1 B).

Identification and distribution of amyloid antigen in the central nervous system

Antibodies specific for amyloid beta 1-40 (Aβ1-40) and amyloid beta 1-42 (Aβ1-42) were used to determine if immunoreactive deposits could be detected in mock infected controls and experimentally infected BALB/c mice. A limited number of Aβ1-40 immunoreactive deposits were observed exclusively in the brains of experimentally infected mice at 2 months post-infection (data not shown). No Aβ1-40 deposits were detected in the brains of any control mice at any timepoint nor in experimentally infected mice at 1, 3 and 4 months post infection.

Quantiative analysis of amyloid burden revealed the highest number of Aβ 1-42 immunoreactive deposits (43) at 2 months pi 3.8 mm caudal to bregma, similar to Cpn immunoreactivity at 1 month pi (Table 1A). Overall Aβ 1-42 immunoreactivity was greatest at 2 month pi, having been minimal at 1 month pi and decreasing at 3 and 4 months pi. As noted above, these sections contain the entorhinal cortex (Ect), perirhinal cortex (Prh), cerebral peduncle (Cp), hippocampus, and amygdala, all regions affected in Alzheimer's disease (see Fig 4 for Aβ 1-42 immunoreactive deposits). A low but detectable number of amyloid-specific immunoreactive sites were detected within mock-infected control mouse brain tissue at 2, 3, and 4 months p.i. with an average number of 0.17/section analyzed. The mean number of immunoreactive sites identified was 3.38/ mouse +/- 2.28 and based upon the results of the student t-test a statistically significant difference ($p < 0.05$) was observed between experimentally infected tissue and mock-infected control mouse tissue at 2 months p.i. (60/mouse +/- 8) and 3 months p.i. (17.67 +/-0.67). No statistically significant difference was detected at 1 month p.i. (3.33 +/- 1.17) or 4 months p.i. (21.83 +/- 19.08) (Table 1 B).

DISCUSSION

This study was designed as a follow-up investigation to the initial report of experimental induction of AD-like pathology in BALB/c mice following intranasal inoculation with *C pneumoniae* (Little, *et al*. 2004). The key difference in the current study as compared to that by Little et al 2004 was that the AR39 respiratory lab strain was used to evaluate the effects in the brain as compared to the 96-41 brain strain used in the initial report. *Chlamydia*- specific immunolabeling was identified in olfactory bulb tissues and in brains (cortical tissues) of AR-39-infected mice. The Cpn-specific labeling was most prominent at 1 month post-infection (pi) and the greatest burden of amyloid deposition was noted at 2 months pi, whereas both decreased at 3 and 4 months pi. The majority of amyloid deposits

at these times were immunoreactive for Aβ 1-42. Interestingly, a limited number of Aβ 1-40 immunoreactive deposits also was identified (data not shown), but only at the 2 month time point, the time of peak amyloid burden. Viable Cpn was recovered from the olfactory bulb tissues of 3 of 3 experimentally infected mice at 1 and 3 months pi, and 2 of 3 at 4 months pi. In contrast, in cerebral cortical tissues of experimentally infected mice, only at 3 months pi did 1 of 3 mice have a measurable burden of viable Cpn. Mock-infected control mice had no detectable Cpn in either olfactory bulbs (0 of 3) or cortical tissues (0 of 3). These data indicate that, following intranasal infection, the AR-39 respiratory isolate of Cpn establishes a limited infection predominantly in the olfactory bulbs of BALB/c mice. Furthermore, although infection with the laboratory strain of Cpn promotes deposition of Aβ amyloid, this appears to resolve following reduction of the Cpn antigen burden over time.

In our current study, brains were analyzed at 1 through 4 months pi by immunohisto-chemistry with antibodies specific for Chlamydia antigen and antibodies specific for Aβ-amyloid 1-42. Similar to the initial report utilizing the 96-41 human AD-brain isolate, no substantial amyloid deposits were observed at 1 month pi and a limited degree of AD-like pathology was identified at 2 months pi with AR-39. In contrast to the original study utilizing the brain isolate, at 4 months pi AD-like pathology was comparable to that observed in mock infected mice and infected mice at 1 month pi, suggesting that the degree of pathology had decreased between 2 through 4 months pi. Identification and quantitative analysis of Chlamydia antigen burden indicated that peak Chlamydia antigen burden preceded peak amyloid deposition. The greatest Chlamydia antigen burden in brains of infected BALB/c mice was noted at 1 month pi, and decreased at 2 through 4 months pi, whereas peak amyloid burden was at 2 months pi, and decreased thereafter. Taken together, the burden of Chlamydia antigen and number of amyloid deposits suggests that Cpn infection serves as a primary stimulus for Aβ-amyloid processing and deposition in brain tissues. While consistent co-localization of amyloid with Chlamydia antigen was not apparent, both were present in the same regions at times consistent with AD-like pathology. As the course of infection preceded the course of pathology development, infection may serve as a stimulus for inflammation as well as for beta amyloid production and deposition. Precedence for infection in exacerbating AD-like pathology has been reported for other types of infections in different animal models (McManus, *et al*. 2014; Wang, *et al*. 2014). Once the infection has been controlled or resolved completely, levels of soluble amyloid apparently decrease presumably following internalization by glial cells (Hawkes, *et al*. 2012) and/or washout into the blood, thereby resulting in fewer deposits documented at the 3 and 4 month timepoints. These findings support our contention that laboratory strains of Cpn from respiratory infections as compared to Cpn brain isolates are less capable of creating long-standing damage in the CNS. In this regard, at the present time, we do not know what inoculum of Cpn is sufficient to not only initiate but to promote chronic human disease, nor do we understand potentially different virulence factors of Cpn isolated from different tissue sites. Our animal studies do support our contention that infection (even in modest titers - 10^5 organisms), specifically through an intranasal route, can initiate changes in the brain consistent with early AD-like pathology.

In mice infected with the 96-41 Cpn brain isolate, Aβ-amyloid deposits were identified as

early as two months pi, with the greatest number of deposits identified at three months pi. As the number and size of amyloid deposits increased over time, the development of AD-like pathology appeared to be progressive. This is an important issue as early initiating events resulting in sporadic late-onset AD have not been addressed using genetically modified transgenic models that principally emulate familial AD, not the more common late-onset form of disease. Furthermore, animal models that mimic the sporadic late-onset form of AD have been hampered by the lack of understanding of the primary factors that promote the early deposition of Aβ-amyloid, however numerous experimentally induced animal models utilizing direct injection of microbial products have been shown to induce transient amyloid production and deposition (Krstic, *et al*. 2012; Erickson, *et al*. 2012). Our current study with a respiratory isolate of Cpn supports the induction of transient amyloid deposition and contrasts with our previous work suggesting that a brain isolate of Cpn results in progressive amyloid accumulation.

Interestingly, a previous study did not identify substantial AD-like pathology in the brain following infection with a respiratory isolate/laboratory strain of Cpn (TWAR 2043) (Boelen, *et al*. 2007). Boelen and co-workers infected BALB/c mice, via intranasal inoculation, and examined brain tissue at one and three months pi based upon the assumption that TWAR 2043 and the human AD brain isolate 96-41 used by Little et al, 2004 would both induce a progressive pathology following infection. The number of amyloid beta deposits was reported as 1 or 2 aggregates per section without a preference for a certain brain region in the Boelen et al study, and the researchers indicated that Cpn was not detected in the CNS at 1 or 3 months pi. In addition, both mock-infected and Cpn infected mice displayed no difference in amyloid deposits. The clear difference noted from the Little et al 2004 study of number and size of deposits was notably different from the Boelen et al report. Boelen et al noted that these discrepancies could be due to the fact that the TWAR 2043 Cpn strain used may have different virulence properties than the human AD-brain isolate, 96-41. TWAR 2043 and 96-41 display different phenotypes with respect to the ability to establish a persistent infection and the subsequent induction of pathology within the brains of BALB/c mice.

Our current findings support the contention that isolates of Cpn may differ in their ability to establish chronic or persistent infection and promote progressive pathology. Numerous questions remain as to the nature of the organisms that typically infect the human population. Pertinent issues, just to name a few, include: risk factors promoting infection at specific sites in the body, spread into different tissues and organs following initial infection, virulence factors expressed by the organism and/or host response, and age at which infection occurs. With regards to age, a prior study of Cpn infection in older animals suggests that older age at time of infection promotes the establishment of a brain infection (Little, *et al*. 2005). Further, to address our current study that a modest inoculum of a respiratory isolate of Cpn initiated specific but non-sustainable change in the brain following intranasal inoculation, we preliminarily inoculated a small group of animals with Cpn AR-39, either twice (days 0 and 30), or three times (days 0, 30 and 60) and sacrificed at day 90, and found that individual BALB/c mice inoculated twice displayed 68 amyloid deposits and those inoculated 3 times had 177 amyloid deposits (unpublished observations). In comparison, mice receiving only a single intranasal inoculation as

observed in the current study at 3 months pi had an average of ~17 - 18 deposits (53 deposits/3 mice) (see table 1A). These preliminary observations would suggest that multiple inocula of Cpn may exacerbate pathology in the brain, but further experiments are required to clarify this. Furthermore, respiratory or blood-borne organisms may become altered after invading different tissue sites including the brain and this may reflect biovar and serovar differences with Cpn, although this remains to be determined. Future sequencing analyses and specific characterization of different tissue and organ isolates may help to resolve these issues.

In summary, host immune responses that limit or reduce Cpn replication and antigen burden may effectively decrease Cpn as a primary stimulus for long-term production of Aβ-amyloid in our experimental system. We propose that the difference in progressive versus non-progressive AD-like pathology is due to as yet uncharacterized differences between human AD-brain adapted isolates such as 96-41, and the respiratory isolates/ laboratory strains TWAR 2043 and AR-39. This implies that there are different virulence factors including tissue tropism for different isolates of Cpn. Thus, the ability of the organism to enter and persist in the central nervous system and potentiate a chronic inflammatory response may be critical to its role in the initiation and maintenance of AD pathogenesis.

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AUTHOR AND CONTRIBUTORS

CSL contributed to the conception and design of the work as well as the acquisition, analysis and interpretation of the data and drafting the manuscript, **TAJ** contributed to the acquisition, analysis and interpretation of the data, **CJH** contributed to design of the work as well as the acquisition, analysis and interpretation of the data and editing the manuscript, **HM** contributed to the acquisition and interpretation of the data, **DMA** contributed to the conception of the work as well the interpretation of the data and editing the manuscript, **BJB** contributed to the conception and design of the work as well as the analysis and interpretation of the data and drafting the manuscript.

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Figure 1. Recovery of viable *Chlamydia pneumoniae* from olfactory bulb and brain tissues following intranasal infection. (**A**) At 1, 3 and 4 months post-infection, viable Cpn was recovered from olfactory bulb tissue homogenates of 8 BALB/c mice, 3 of 3 mice at one month pi, 3 of 3 mice at 3 months pi, and 2 of 3 mice at 4 months pi. (**B**) In contrast, only 1 mouse demonstrated viable Cpn from cerebral cortical tissue at any time; that being 1 mouse at 3 months pi. Viable Cpn was quantified as infectious forming units /ml of tissue homogenate.

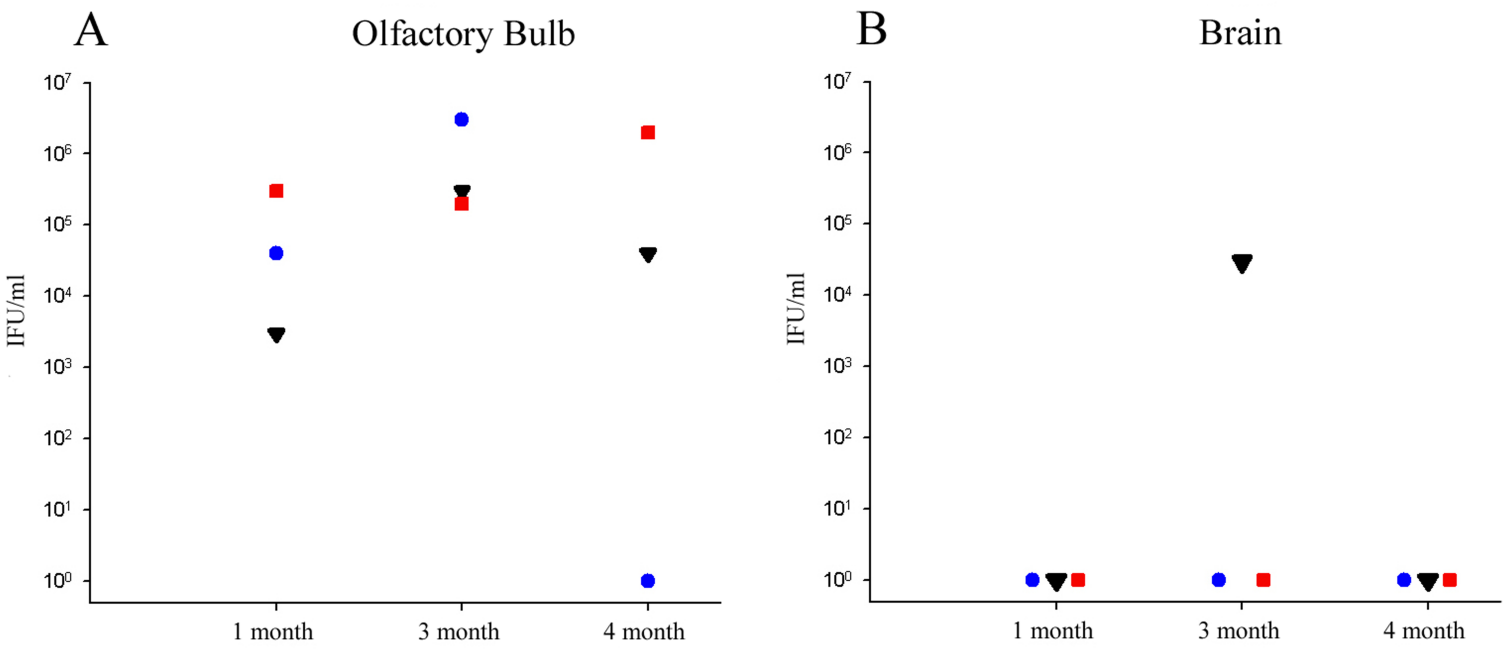
Figure 2. *Chlamydia pneumoniae* specific immunoreactivity in olfactory bulbs. Cpn (AR-39) antigens were detected in olfactory bulb tissues at 1 month post-infection following intranasal inoculation (arrows). **A**, **B** and **C**, **D** are representative images from 2 different mice infected with Cpn and labeled with a cocktail of anti-Cpn antibodies (RDI-PROAC1p, M6600, 10C-27). E, F are representative images from mock infected mice comparably immunolabeled. Mag bars **A-F** = 50μm.

Figure 3. *Chlamydia pneumoniae* specific immunoreactivity in the central nervous system. Representative images of Cpn-specific antigen labeling in the brains of infected mice at 1 month (panels **A** and **B**), 3 months (panels **C** and **D**), and 4 months (panels **E** and

F) post infection. The upper right corner of each image is a higher magnification image of Cpn-specific antigen labeling as designated by the low magnification arrow. Mag bars = 50μm.

Figure 4. Beta amyloid Aβ 1-42 deposits in the CNS at 2 months pi following intranasal infection with *Chlamydia pneumoniae* AR-39. Brains were examined by light microscopy for the presence of Aβ 1-42 using a specific anti-Aβ 1-42 antibody. (**A**-**E**) Representative images of Aβ 1-42-specific labeling (arrowheads) are shown within different regions of this brain section. Ect (Entorhinal cortex), L Ent (Lateral entorhinal cortex), Th (Thalamus), Prh (Perirhinal cortex), Cp (Cerebral peduncle). Mag bars (**A**) 100 μm (**B**-**E**) 20 μm.

Figure 1.TIF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IFU/ml
10^7
10^6
10^5
10^4
10^3
10^2
10^1
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1 month
3 month
4 month
B
Brain
IFU/ml
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10^5
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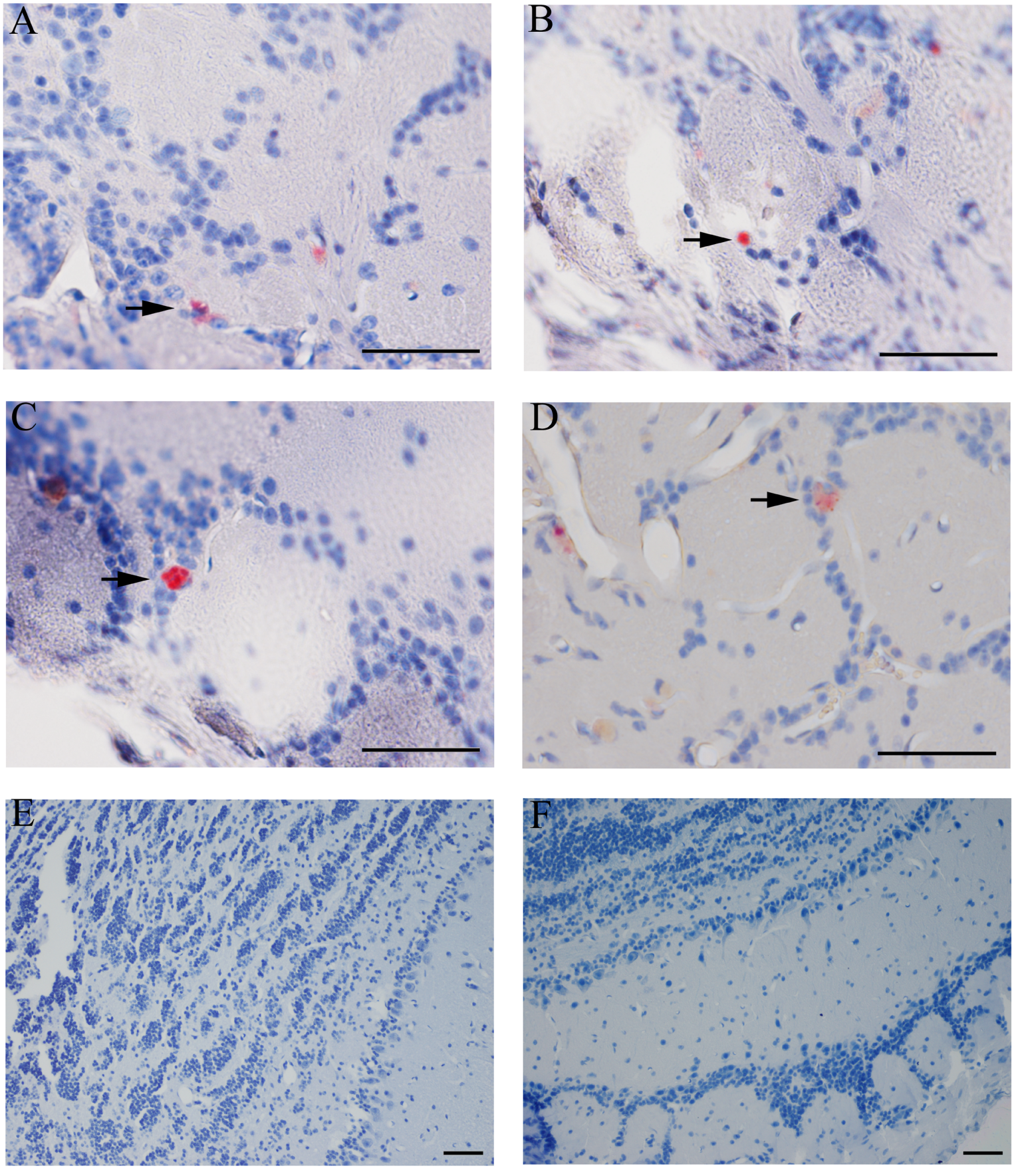
1 month
3 month
4 month

Figure 2.TIF



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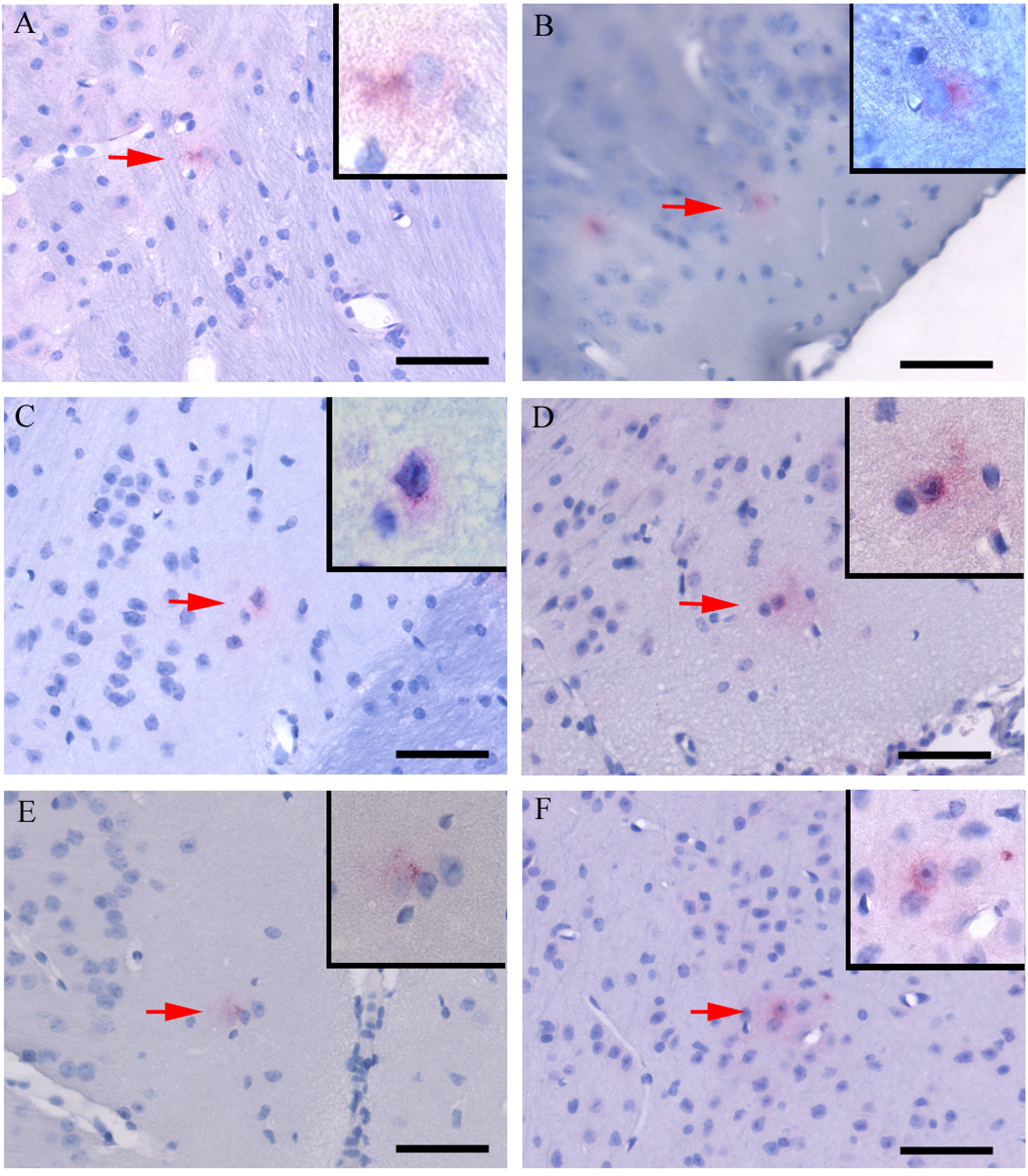

Figure 4.TIF