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Microglial Derived Tumor Necrosis Factor- α Drives Alzheimer's Disease-Related Neuronal Cell Cycle Events

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Abstract

Massive neuronal loss is a key pathological hallmark of Alzheimer's disease (AD). However, the mechanisms are still unclear. Here we demonstrate that neuroinflammation, cell autonomous to microglia, is capable of inducing neuronal cell cycle events (CCEs), which are toxic for terminally differentiated neurons. First, oligomeric amyloid-beta peptide (A β O)-mediated microglial activation induced neuronal CCEs via the tumor-necrosis factor- α (TNF α) and the c-Jun Kinase (JNK) signaling pathway. Second, adoptive transfer of CD11b+ microglia from AD transgenic mice (R1.40) induced neuronal cyclin D1 expression via TNF α signaling pathway. Third, genetic deficiency of TNF α in R1.40 mice (R1.40-*Tnf α* ^{-/-}) led to induce neuronal CCEs. Finally, the mitotically active neurons spatially co-exist with F4/80+ activated microglia in the human AD brain and that a portion of these neurons are apoptotic. Together our data suggest a cell-autonomous role of microglia, and identify TNF α as the responsible cytokine, in promoting neuronal CCEs in the pathogenesis of AD.

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Keywords

Alzheimer's disease; microglia; neuronal cell cycle; tumor necrosis factor- α (TNF α); neuroinflammation; adoptive transfer; c-Jun Kinase (JNK)

Introduction

The cell cycle comprises a series of highly coordinated events that take place when a cell is ready for division (reviewed in (Byrnes and Faden, 2007)). In most tissues, an aberrantly induced cell cycle may initiate cancer. However, in highly differentiated cells, where exit from the cell cycle is permanent and the cells enter a G₀ phase, aberrant induction of the cell cycle leads to altered function and sometimes cell death. Mature neurons in the adult central nervous system represent a well-characterized population of terminally differentiated cells that permanently exit the mitotic state after leaving the ventricular or sub-ventricular zone during differentiation (Herrup and Yang, 2007). In Alzheimer's disease (AD), neurons susceptible to degeneration re-enter an aberrant cell cycle, fail to complete the cell cycle and eventually perish (Herrup and Yang, 2007; Yang and Herrup, 2007).

Neuronal cell cycle events (CCEs) may constitute an intrinsic response to cellular stress as they are observed in a variety of neurodegenerative diseases including AD (Busser et al., 1998; McShea et al., 2007; Nagy et al., 1997; Vincent et al., 1996), Parkinson's disease (Hoglinger et al., 2007; Jordan-Sciutto et al., 2003) and others (Jordan-Sciutto et al., 2002a; Jordan-Sciutto et al., 2002b; Osuga et al., 2000). Aberrant neuronal CCEs have also been found in mouse models of neurodegeneration, including the retinoblastoma knockout (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992), degeneration of retinal photoreceptors or Purkinje cells in SV40 T-antigen transgenic mice (al-Ubaidi et al., 1992; Feddersen et al., 1992) and granule cells of staggerer and lurcher mice (Herrup and Busser, 1995). Furthermore, neuronal DNA synthesis without cell proliferation has also been observed in apoptotic neurons in a rodent model of hypoxia and ischemia (Kuan et al., 2004).

While the factor(s) responsible for inducing neuronal CCEs in each of these individual disease conditions are likely distinct, our studies have focused on identifying the underlying mechanism(s) for inducing neuronal CCEs specifically in AD. The present report utilizes R1.40 mice, which express a complete genomic copy of the human *APP* gene carrying a familial AD mutation (K670N/M671L) (Lamb et al., 1997). In R1.40 mice neuronal CCEs begin at 6 months of age and progress with age. By 18 months, most of the neuronal populations subject to degeneration in AD are marked by CCEs. R1.40 mice also exhibit A β plaques beginning around 6–7 months after the first neuronal CCEs. Tellingly, the microglial activation also co-insides with the first appearance of neuronal CCEs and the latter is hAPP- and β -secretase (BACE1)-dependent (Varvel et al., 2009). These studies suggest that the generation of soluble A β is critical for both the onset of neuronal CCEs as well as altered microglial activation.

There is also an intimate correlation between CCEs and microglial immune activation. Induction of systemic inflammation with lipopolysaccharide (LPS)-induced microglial activation and neuronal CCEs in the cortex of young R1.40 mice (Varvel et al., 2009). Treating R1.40 mice with non-steroidal anti-inflammatory drugs (NSAIDs) before the appearance of neuronal CCEs blocked microglial activation and prevented neuronal CCEs (Varvel et al., 2009).

In the current study, we provide direct evidence that neuronal CCEs lie downstream of microglial activation, production of TNF α , activation of c-Jun N-terminal Kinase (JNK) signaling. Our data carry implications for therapeutic strategies to block neuronal CCEs, which we propose will be neuroprotective in AD.

Materials and Methods

Animals

R1.40 (or R/R) (Lamb et al., 1997), *Tnfa*^{-/-} (Pasparakis et al., 1996) and *Cx3cr1^{gfp/gfp}* (Jung et al., 2000) were in C57BL/6J background (mixed gender) and obtained from Drs. Bruce Trapp (Cleveland Clinic) and Dan Littman (HHMI, New York University School of Medicine). Animals were housed at the Cleveland Clinic Biological Resources Unit, a facility fully accredited by the AAALAC. Experimental protocols were performed in accordance with US National Institutes of Health guidelines on animal care and were approved by the Cleveland Clinic Animal Care and Use Committee.

Antibodies

The antibodies utilized in the present study are listed in Table 1.

Cell cultures and treatments

Neuronal and microglial cultures were prepared as described previously (Bhaskar et al., 2009; Saura et al, 2003). Primary microglia was incubated with oligomeric A β ₁₋₄₂ peptide (rPeptide, Cat # A-1 163-1; A β O; 4.0 μ g/ml or 1 μ M; described in (Stine et al., 2003)) for 24 h at 37°C. The microglial-conditioned media (CM) was removed and mixed with BrdU (10 μ M) and one fourth (25%) or one sixteenth (6.25%) of the media present in the primary neurons at 21 DIV was replaced with equal volume of microglial CM containing BrdU. To remove A β O in the CM, 6E10 antibody was used to immunoprecipitate A β O from CM prior to neuronal treatment. For TNF α studies, prior to neuronal treatment, the A β O-activated microglial CM (with 10 μ M BrdU) was mixed with purified anti-TNF α antibody (eBioscience, Cat # 14-7349-85; Clone: MAb11) or non-specific mouse IgG (Sigma-Aldrich; final concentration of 125 ng/ml) and incubated for 24 h at 37°C. Neurons were also treated directly with mouse IgG (125 ng/ml), recombinant TNF α (Sigma-Aldrich, Cat # T7539) or IL-6 (PeproTech, Cat # 216-16) (both at 250 pg/ml) or vehicle in the presence of BrdU (10 μ M). For the analysis of specific JNK inhibitor, neurons were treated with SP600125 (Bennett et al., 2001) (Sigma-Aldrich, Cat # S5567; 15 μ M (Bennett et al., 2001; Han et al., 2001); 30 min preincubation) prior to recombinant TNF α (250 pg/ml + 10 μ M BrdU) treatment. A β O treated microglia was also fixed at 4% paraformaldehyde (PFA) and processed for double immunofluorescence with oligomer-specific antibodies NU1 (Lambert et al., 2007) and A11 (Kayed et al., 2007). All experiments were done in triplicates or more with neurons and microglia derived from 3 independent litters.

Isolation and adoptive transfer of microglial cells

Mononuclear cells were isolated from a pool of 2–3 brains per group as previously described (Bergmann et al., 1999). Briefly, the mice were anaesthetized; transcardially perfused with phosphate buffer, brains removed and dissociated in 0.25% trypsin/RPMI media. Mononuclear cells were separated via 30% and 70% isotonic percoll gradient followed by magnetic assisted cell sorting (Dynabead FlowComp™ Flexi kit, Cat # 110-61D; Life Technologies; DSB-X™ Biotin Protein Labeling Kit, Life Technologies; Cat # D-20655) using a CD11b antibody (Millipore) and elution method per manufacturer's protocol. Purified microglia (1×10^6 cells/ml; in 50 μ l) from donor mice with described genotypes were injected with or without anti-TNF α antibody (Abcam, Cat # ab1793; 2 μ g/ml) or

mouse IgG (Sigma-Aldrich; 2 µg/ml) into the layer VI of the cortex of six-month old C57BL/6J non-transgenic recipient mice with the following stereotaxic coordinates: from Bregma: -1.94 mm posterior; 1.5 mm lateral; 1.5 mm dorsoventral (and as previously described (Cardona et al., 2006)). After 48 h, the recipient mice were perfused with 4% PFA and the brains were processed for immunofluorescence analysis for cyclin D1/NeuN and quantitative morphometry.

Multiplex cytokine array

To detect the levels of most common pro-inflammatory cytokines (IL-1 β , IL-6, TNF α and INF γ) released from A β O-activated primary microglial cells, we have utilized the SearchLight® Infrared 4-Plex Cytokine array kit (Pierce Endogen, Cat# 84504). Briefly, primary microglia were stimulated with different concentrations of A β O or vehicle for 24 h at 37°C. After the incubation, 100 µl of A β O-stimulated microglial CM was processed for detection of cytokines as per manufacturer's instructions. The absolute levels of secreted cytokines (in pg/ml) microglial conditioned media were determined via the SearchLight® Array Analyst (Version 2.2.0.0; AxioCor Inc.) software. For the correlational analysis, we compared the percentage of BrdU immunoreactive neurons following treatment with 0, 6.25 or 25% of A β O-stimulated microglial CM against concentration of secreted cytokines from microglia when stimulated with 0, 0.2 or 0.4 µg/ml of A β O.

Immunohistochemistry and immunofluorescence

CD45 immunohistochemistry—30 µm free floating sections were incubated in 10 mM sodium citrate buffer (pH 6.0) for 10 min at 95°C. The sections were washed in PBS with 0.1% Tween (PBST), quenched with 0.3% H₂O₂ in PBST for 15 min, washed three times in PBST and then blocked in 5% Normal Goat Serum (NGS) with 0.3% Triton X-100 in PBS for 1 h at room temperature (RT). After blocking, the sections were incubated with primary antibody against CD45 (1:250) overnight at 4°C. After washing, sections were incubated with biotinylated goat anti-rat secondary antibody (1:500; Vector Laboratories, Burlingame, CA), washed and incubated with an avidin-biotin peroxidase complex (ABC Elite kit; Vector Laboratories) and developed in peroxidase substrate kit (SK-4100; Vector Laboratories) as per manufacturer's instructions. Sections incubated without primary antibody served as control and did not display any specific staining. Sections were counter stained with Haematoxylin, dehydrated through graded ethanol, cleared in xylene and coverslipped with Permount® mounting media.

Triple immunofluorescence for the human AD brain—4% PFA-fixed human AD brain autopsy tissue (36 h post-mortem interval; Braak and Braak Stage V/IV) was obtained from Division of Pathology and Laboratory Medicine, Cleveland Clinic Foundation. After cryo-protecting the tissue in 0.1 M sodium phosphate buffer (Sorenson's buffer; pH 7.6) with 20% glycerol, 30 µm free-floating sections were either stored in cryo-storage glycol solution (0.2 M sodium phosphate buffer, pH 7.4, 1% polyvinylpyrrolidone or PVP-40, 30% sucrose and 30% ethylene glycol) or processed for free floating immunofluorescence staining. First for cyclin D1, NeuN and F4/80 triple staining, the sections were incubated with 2N HCl for 30 min at 37°C to allow for nuclear permeabilization followed by neutralization with 0.1 M sodium borate buffer (pH 8.6) for 10 min at RT. After washing multiple times with PBS, the sections were incubated in 10 mM sodium citrate buffer (pH 6.0) for 10 min at 95°C for antigen retrieval, then washed and blocked in 10% NGS with 0.4% Triton X-100 (blocking buffer) for 1 h at RT. The sections were incubated with rabbit monoclonal antibody against cyclin D1 (1:250 in blocking buffer) for 72 h at 4°C, washed in PBS and incubated with Alexa-546® conjugated goat anti-rabbit secondary antibody (1:500 in blocking buffer) for 1 h at RT. After several washes in PBS, the sections were blocked with blocking buffer for 2 h at RT followed by incubation with primary antibody cocktail

(mouse monoclonal antibody against NeuN at 1:500 + rat monoclonal antibody against F4/80 at 1:500) in blocking buffer overnight at 4°C. After several washes in PBS, the sections were incubated with cocktail of secondary antibodies (Alexa-488® conjugated goat anti-rat antibody at 1:500 + Alexa-647® conjugated goat anti-mouse antibody at 1:500) for 1 h at RT. The sections were washed in PBS for five times. To block the auto-fluorescence due to lipofuscin, the sections were then incubated with freshly prepared, filtered 1% solution of Sudan Black for 5 min at RT followed by a quick incubation for 1 min in 70% ethanol. The sections were then washed five times in PBS and mounted on slides for confocal microscopy.

For triple labeling with Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), Cyclin D1 and NeuN, first the 30 µm sections were processed for nuclear permeabilization and antigen retrieval as described above. Then the sections were blocked in blocking buffer and processed for TUNEL staining as per manufacturer's instructions (Roche; *In Situ* Cell Death Detection Kit, TMR Red; Cat # 12 156 792 910). After washing several times, the sections were processed for double immunofluorescence with mouse monoclonal antibody against NeuN and rabbit monoclonal antibody against Cyclin D1 followed by respective secondary antibodies as described above.

Immunofluorescence

Double immunofluorescence—For the double immunofluorescence of mouse brain sections, 30 µm free-floating mouse brain sections were processed for nuclear permeabilization and antigen retrieval as described above. After washing several times in PBS, the sections were blocked in blocking buffer for 1 h at RT. The sections were incubated with a mixture of primary antibodies against NeuN and Cyclin D1 in blocking buffer with specific dilutions as mentioned above. After washes, the sections were incubated with a mixture of Alexa-488® conjugated goat anti-mouse secondary antibody (for NeuN at 1:500 dilution) and biotinylated goat anti-rabbit secondary antibody (for Cyclin D1 at 1:500 dilution), both in blocking buffer, for 2 h at RT. Following several washes, the sections were incubated with Streptavidin-Alexa-546® (1:250 in blocking buffer) for 1 h at RT, washed in PBS and cover-slipped with Dako fluorescent mounting media (Cat # S3023).

For visualization of BrdU incorporation in primary neurons, we have performed double immunofluorescence with BrdU and MAP2 antibodies as previously described (Bhaskar et al., 2009). Neurons positive for MAP2 alone or for both MAP2 and BrdU were quantified by scoring five random fields per treatment as described previously (Bhaskar et al., 2009; Varvel et al., 2008). For MAP2, BrdU and TUNEL triple labeling, first the coverslips were processed for TUNEL staining, then followed by double immunofluorescence with BrdU and MAP2. Double (BrdU and MAP2) or triple (BrdU, TUNEL and MAP2) positive cells were normalized against total number of MAP2 positive cells in each field, which was considered 100%. For each *in vitro* experimental condition, the neurons derived from at least 2–3 different litters and cultured in 6–12 independent culture wells were utilized for treatments and quantification. Statistical methods utilized for *in vitro* analysis are described in the figure legends for the respective data sets.

Quantitative morphometry

Quantification of NeuN and Cyclin D1 immunoreactive neurons from the six-month-old WT, R/R and R/R-*Tnfr*^{-/-} mice were performed as previously described (Varvel et al., 2008) with minor modifications. Briefly, n=4 animals were utilized per genotype. Prior to neuropathological analysis, the mice were anesthetized and perfused with phosphate buffer, brains were removed, and left half was microdissected into cortex, hippocampus and rest of the brain, weighed and stored at -80°C for biochemical analyses. Right half was immerse

fixed in 4% paraformaldehyde and serial brain sections were collected. A total of five evenly spaced sections at sagittal plane from midline towards lateral end of the hemi-brain covering fronto-temporal cortices spread across multiple planes were stained for NeuN and Cyclin D1. We scored NeuN positive cells within cortical layers II and III for the presence or absence of Cyclin D1 immunoreactivity in six random fields per section. Only cells with a discernable portion of their nucleus in the section were scored. In each field, the percentage of double positive (NeuN and Cyclin D1) cells was calculated by normalizing against the total number of NeuN positive neurons in the same field, which was considered 100%. The percentage of Cyclin D1 positive neurons in each section (from 6 random fields) from $n=4$ animals per genotype was averaged and expressed as mean $\pm$ SEM. All counts were performed in a blinded manner and the data were analyzed via one-way ANOVA followed by a Tukey-multiple comparison post-hoc test.

For the quantification of Cyclin D1 and NeuN positive neurons in the adoptive transfer experiments, 30 μ m serial free-floating coronal brain sections from the recipient mouse brain encompassing the needle track were stained for Cyclin D1 and NeuN. A total of five sections per recipient mouse brain were utilized for quantification. The section with widest area of needle track was considered as the medial section (or "N"; Fig. 4C). Four adjacent sections, two anterior to this medial section (N+1 and N+2; Fig. 4C) and two posterior to the medial section (N-1 and N-2; Fig. 4C) were scored per animal. 1–2 mm area around the needle track in the ipsilateral cortex (dashed line in Fig. 4C) was defined as region of interest (ROI) and the percentage of double positive cells (NeuN and Cyclin D1) were counted and normalized against total number of NeuN positive neurons in the same field, which was considered as 100% and quantified as described (see previous paragraph). Note, because of very small brain region that encompass needle track, we used consecutive sections for the quantification, which may introduce an inherent bias in the quantifications performed. Identical region on the contralateral side of the section was also defined as ROI and scored for comparison. A total of $n=3$ recipient mice per treatment group were utilized for the quantification. All counts were performed in a blinded manner and data were analyzed with one-way ANOVA followed by a Tukey post-hoc test.

SDS-PAGE and Western Immunoblotting

Detergent soluble cortical lysates were prepared via homogenizing in Tissue-Protein Extraction Reagent (T-PER, 78510; Pierce) with protease (P8340 Sigma-Aldrich) and phosphatase (P5726; Sigma-Aldrich) inhibitor cocktails. Primary neurons were directly lysed in 1x LDS buffer containing reducing agent. Proteins were separated by 4–12% Bis-Tris Novex® NuPAGE gels, Western-blotted with PVDF membrane and probed for described antibodies (Table 1) (used at 1:1000 dilution for TNF α , phospho-STAT3, total STAT3, phospho-JNK, total JNK, phospho-p38 MAPK, total p38 MAPK, phospho-IKK α/β , total IKK α/β , phospho I κ B α , total I κ B α , phospho-p65, total p65, PI3K α , phospho-Akt and total Akt. The GAPDH was used at 1:10,000) followed by respective secondary antibodies. Membranes were developed using ECL reagent (NEL101001EA; Perkin Elmer) and immunoreactive bands (obtaining integrated density value or IDV for each band) were quantified in AlphaEaseFC™ Software (Alpha Innotech Corporation).

Immunoprecipitation of A β

Immunoprecipitation of A β O from microglial CM was performed as previously described (Lee et al., 1998) with minor modifications. Briefly, 1 ml of CM derived from microglia treated with either 4 μ g/ml A β O or vehicle was pre-cleared with 50 μ l of 10% Protein A-Sepharose (PAS) 4B beads (Amersham Biosciences, Piscataway, NJ), which was pre-blocked with 0.2% gelatin for 1 h at 4°C. Following 1000 $\times$ g spin for 5 min, the supernatant was mixed with 18 μ l of 6E10 anti-A β antibody, incubated overnight at 4°C and mixed with

50 μ l of 10% PAS (pre-blocked) for 1 h incubation at 4°C. After 1000 $\times$ g spin for 5 min, proteins in the supernatant and those bound to PAS beads as well as the A β O treated CM were resolved by 8–12 % Bis-Tris gradient gels. Transfer to polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore, Bedford, MA) and visualization using ECL detection was according to manufacturer's instructions (PerkinElmer Life Sciences, Boston, MA). The blots were probed with 6E10 primary antibody followed by horseradish peroxidase conjugated secondary antibodies. Membranes were developed using ECL reagent (NEL101001EA; Perkin Elmer).

Results

Conditioned Media From A β O-Stimulated Microglia Induces Neuronal CCEs

Primary microglia were stimulated with synthetic A β O at 4 μ g/ml for 24 hours. As has been reported in previous studies (Maezawa et al., 2011; Sondag et al., 2009; Wu et al., 2000), exposure of microglia to A β O induced morphological alterations consistent with enhanced activation. Indeed, a dose dependent alteration in cellular morphology was observed (Fig. 1A–E). Immunofluorescence labeling with Iba1 and the anti-oligomer antibodies A11 (Kayed et al., 2007) (Fig. 1F–H), and NU1 (Lambert et al., 2007) (Fig. 1I–K) as well as A β -specific antibody 6E10 (Fig. 1L–N) suggested presence of A β inside the microglial cells (Fig. 1H, K, N).

Conditioned media (CM) from the A β O-stimulated microglia was collected and transferred to primary cortical neurons at various concentrations (0, 6.25 or 25%) for 24 hours in the presence of BrdU in order to assess the induction of DNA replication (Fig. 2A). Whereas there was little to no BrdU incorporation in primary neurons exposed to CM from vehicle-treated microglial cells (Fig. 2B–D and 2L), exposure of primary neurons to A β O-stimulated microglial CM promoted a dose-dependent increase in BrdU incorporation in about 40–60% of MAP2 positive neurons (Fig. 2E–G and 2L). Since we previously demonstrated that A β O can directly induce neuronal CCEs at around 2 μ g/ml concentration (Bhaskar et al., 2009), it remained possible that small amounts of A β O carried over in the microglial CM was inducing DNA replication in these studies. To control for this possibility, the CM was immunoprecipitated (IP'd) with the 6E10 antibody prior to incubation with the neurons. Western blot analysis of the CM following IP with 6E10 revealed that no detectable A β O remained (Fig. 2K); the dose-response effect of IP'd CM was identical to that of the untreated CM (Fig. 2H–K). Quantification of the percentage of BrdU and MAP2 double positive neurons following treatment with different concentrations (0, 6.25% and 25%) of vehicle- or A β O-activated microglial CM revealed a statistically significant increase (~40% for 6.25% CM treatment and ~60–80% for 25% of CM treatment) in the percentage of BrdU positive neurons, which was dependent upon the concentration of the CM (Fig. 2L). Notably, treatment of neurons with 6E10 IP'd CM also displayed a concentration dependent increase of the percentage of BrdU positive neurons (Fig. 2L). Finally, as we have previously demonstrated (Bhaskar et al., 2009), neurons that are not treated with CM show a basal level (~5–10%) of the MAP2 positive neurons display BrdU incorporation. It is unclear whether this baseline induction of BrdU incorporation is due to continued division of a small number of neuronal stem cells or possible de-differentiation of a low number of non-neuronal cells in the culture. Together, these results suggest that a soluble factor(s) present in the microglial CM are capable of inducing DNA replication in primary neurons.

A β O-stimulated microglia release of TNF α promotes neuronal CCEs

Activation of microglia results in increased secretion of a large number of proinflammatory cytokines and chemokines, including IL-6, TNF α , IL-1 β and INF γ amongst others (Aloisi, 2001). To gain insight into which soluble factor(s) released into the A β O-stimulated

microglial CM promotes neuronal CCEs, we used an infrared-based four-plex cytokine array to assess the levels of IL-6, TNF α , IL-1 β and INF γ in the CM following dose-dependent exposures of primary microglia to A β Os (Fig. 3A). Among the four cytokines measured, TNF α exhibited the most robust increase nearly an order of magnitude (Fig. 3B) – although IL-6 also exhibited a modest, yet significant increase. By Spearman's rank correlation there was a close correlation between the A β O-mediated secretion of TNF α and percentage of BrdU+ neurons induced by different concentrations of CM ($r = 0.9863$) (Fig. S1D) and a trend towards positive correlation between A β Os and IL-6 ($r = 0.5959$) (Fig. S1B). No statistically significant correlations were found for either IL-1 β or INF γ (Fig. S1A and S1C).

TNF α levels exhibited the most significant correlation with exposure to A β Os (Fig. 3B and Fig. S1D) and previous studies (Wu et al., 2000) suggest that the induction of neuronal CCEs is dependent upon the concentration of TNF α in the CM. To further confirm the effects of TNF α on the neuronal CCE phenotype, primary cortical neurons were pre-incubated with an anti-TNF α antibody (or a non-specific mouse IgG) prior to exposure to A β O-stimulated microglial CM. Notably, pre-incubation with the TNF α antibody greatly reduced the levels of neuronal BrdU incorporation (Fig. 3F–H) when compared to the IgG control (Fig. 3C–E). In addition, neurons pre-incubated with the TNF α antibody exhibited longer processes and arborization when compared to the IgG control (Fig. 3C and 3F). As an additional control, pre-incubation of primary neurons with mouse IgG alone also did not induce BrdU incorporation (Fig. 3I–K). We next examined if TNF α can directly induce neuronal CCEs. Exposure of primary cortical neurons to recombinant human TNF α (250 pg/ml) for 24 h, significantly induced BrdU incorporation in MAP2 positive neurons (Fig. 3L–N). Notably, recombinant TNF α also increased protein levels of cyclin D1 and PCNA (an S-phase marker (Prosperi, 1997)) in primary neurons with maximum induction reached at the 1 ng concentration of human recombinant TNF α (Fig. 3O–3Q). Quantification of the percentage of BrdU incorporation induced upon exposure of primary neurons to CM revealed that treatment with either A β O-stimulated microglial CM or recombinant TNF α increased the number of BrdU/MAP2 double-positive cells to approximately 40–60% (Fig. 3R). By contrast, only a basal level of about 5–10% of double-labeled cells was observed after exposure to vehicle-treated microglial CM (Fig. 3R). The percentage of BrdU positive neurons dropped to basal levels when the neurons were pretreated with the anti-TNF α antibody, but not with mouse IgG (Fig. 3R). Finally, unlike recombinant TNF α , direct incubation of primary cortical neurons with recombinant human IL-6 (250 pg/ml) for 24 h failed to induce BrdU incorporation in MAP2 positive neurons (data not shown). Taken together, these results provide evidence that TNF α is up-regulated upon exposure of microglia to A β Os and is a major factor promoting the induction of neuronal CCEs in cultured neurons.

Microglia induce neuronal CCEs

Previous studies suggested a direct link between neuroinflammation and induction of neuronal CCEs (Varvel et al., 2009). Notably, promotion of neuroinflammation could induce neuronal CCEs at an earlier age in the R1.40 mice and reduction of neuroinflammation through treatment with NSAIDs could block the appearance of neuronal CCEs (if NSAID treatment was initiated early) (Varvel et al., 2009). To determine whether the signal that drives neuronal CCEs was cell-autonomous to the microglia, a series of adoptive transfer experiments were performed. Microglia from six-month-old WT and R/R mice were purified using an established protocol (Bergmann et al., 1999) with minor modifications that included CD11b antibody-mediated enrichment of microglia/monocytes (Fig. 4A–B). Purified microglia from the WT or R/R mice were microinjected (50,000 cells/injection) into cortical layer VI of 6-month old non-transgenic (isogenic) recipient mice (Fig. 4A). Forty-eight hours following adoptive transfer, recipient brains were analyzed for the presence of

donor microglia within 1–2 mm radius of the needle track. The section with the widest area of needle track was considered as the center of the track ('N'; Fig. 4C); two serial sections anterior (+2) and two posterior (–2) to 'N' were also quantified. Injected microglia survived in large numbers as demonstrated in control transplants using microglia from R/R-*Cx3cr1^{gfp/+}* mice, in which all of the microglia are GFP-positive (Cardona et al., 2006) (Fig. 4D–4E, inset in 4E), appeared activated with swollen cell bodies and short processes (Fig. 4E inset). Notably, not only did the donor microglia appear activated within the recipient brain, but some of the Iba1+ resident microglia present within close proximity to the donor microglia also displayed a activated phenotype (Fig. S2B). In contrast, Iba1+ resident microglia on the contralateral side appeared to be in a resting (non-activated) state (Fig. S2A). When purified microglia from R1.40 mice were injected into non-transgenic hosts, the transplant induced cyclin D1 (Baldin et al., 1993) expression in NeuN positive neurons existed within a 100–200 μ m radius around the needle track in the ipsilateral cortex of the host animal (Fig. 4O–Q); cyclin D1 expression was also slightly increased at the homologous location in the contralateral cortex (Fig. 4L–N), but the increase was not significant.

Most importantly, injection of the vehicle alone, or the adoptive transfer of microglia purified from age-matched non-transgenic controls, did not induce neuronal cyclin D1 expression in either the ipsilateral or contralateral regions of the recipient brain (Fig. 4F–H and Fig. 4I–K, respectively). Quantification of the cyclin D1/NeuN double positive neurons also revealed a statistically significant increase (Fig. 4R, $p < 0.05$) in neuronal CCEs in the recipients that received R/R microglia (50% cyclinD1 positive neurons in the ipsilateral cortex within ~200 μ m radius around the needle track; Fig. 4R) when compared to recipients that received either non-transgenic microglia or vehicle alone (10% cyclinD1 positive neurons in ipsilateral cortex; Fig. 4R). These results suggest that microglia derived from the A β -enriched pro-inflammatory milieu within the R1.40 brain can directly promote cyclin D1 expression in the WT host brain.

Role of TNF α signaling in promoting neuronal CCEs *in vivo*

Adoptive transfer studies in which purified microglia from R/R mice were transferred into non-transgenic recipients were performed in the presence of either an anti-TNF α antibody or a non-specific mouse IgG control. As expected, the ipsilateral cortex of recipient mice receiving purified R1.40 microglia with mouse IgG in the inoculum displayed numerous cyclin D1/NeuN double-positive neurons in layer VI near the injection site (Fig. 5A–C) and these results were very similar to those observed with injection of R/R microglia alone (Fig. 4R). By contrast, the percentage of double-positive cells in animals injected with R/R microglia together with the anti-TNF α antibody was reduced (Fig. 5D–F) to the level observed in animals injected with vehicle or non-transgenic microglia (Fig. 4G and 4J, respectively). Quantification revealed this decrease to be highly statistically significant ($p < 0.0001$ – Fig. 5G).

Since neuronal CCEs and altered microglial morphologies initially appear at six months of age in the R1.40 mice, if TNF α was the relevant factor we would expect its levels to be elevated in R/R mice at this age. To examine this possibility, both TNF α mRNA and protein levels were assessed within the cortex of six-month-old non-transgenic and R/R mice by RT-PCR, and Western blotting, respectively. While we did not detect any significant difference in the levels of TNF α mRNA, there was a significant increase in the levels of TNF α protein in cortical lysates from R/R mice compared to age-matched non-transgenic mice (Fig. 5H–I) – a nearly two-fold increase as compared to controls. To further validate the importance of TNF α for the induction of neuronal CCEs, R/R mice were mated to animals carrying a null mutation in the *Tnfa* gene (Pasparakis et al., 1996). At six months of

age neuronal CCEs first appear in the R/R mice. Following this, non-transgenic, R/R and TNF α deficient R/R mice (R/R-TNF α ^{-/-}) were aged six months. As expected, and based on previous studies, six-month-old R/R mice, but not age-matched non-transgenics, exhibited a significant number of cyclin D1 positive neurons in layers II/III of the cortex (Fig. 5M–O). In striking contrast, the number of cyclin D1 positive neurons in the identical regions of the cortex of R/R-TNF α ^{-/-} mice was significantly reduced (Fig. 5P–R) to a level that appeared similar to age-matched non-transgenic controls (Fig. 5J–L). Quantitation revealed that less than 10% of neurons were positive for cyclin D1 in non-transgenic mice, while this number increased to ~35% in R/R mice (Fig. 5S). TNF α deficiency in the R/R mice significantly ($p < 0.001$) reduced the numbers of cyclin D1 positive neurons to a level that was indistinguishable from non-transgenic controls (Fig. 5S – red bar). Six-month-old TNF α deficient mice did not display any alterations in the expression of cyclin D1 in neurons (data not shown). We used cyclin D1 expression as a read-out to assess mitotically active neurons in *in vivo* studies, while BrdU was utilized in cell culture studies. Entry of neurons into the mitotic state is a relatively slow process, especially in a chronic condition like AD. It is unclear when “*at risk*” neurons really enter the cell cycle. It is still unknown when this occurs during the course of the disease and how long it may take them to replicate their DNA. Hence, in order to use BrdU to mark all of the neurons *in vivo*, it requires a continuous supply of BrdU *ad libitum* in water for several months. Because of this, we utilized cyclin D1 as a marker to identify ‘cycling’ neurons *in vivo*.

TNF α is a potent inflammatory cytokine (McCoy and Tansey, 2008), which activates a number of intracellular pathways (reviewed in (Croft et al., 2013; Ware, 2005)). To address which signaling pathways were involved in neuronal CCEs, we examined the levels of active components of these major pathways (MAPK, PI3K–Akt, STAT3 and NF κ B) via Western blot analysis of cortical lysates from six-month-old R/R mice and non-transgenic controls. Notably, levels of activated JNK (phosphorylated at T183/Y185), but not the components of other pathways, were elevated, in 6-month-old R/R cortex (Fig. S3A–I) compared to age-matched WT, indicating that the JNK pathway is activated when neuronal CCEs first appear in the R/R mice.

To examine whether JNK signaling could be responsible for the effects of TNF on induction of neuronal CCEs, we utilized a specific JNK inhibitor (SP600125) in our *in vitro* model of neuronal CCE induction. 30 min pre-incubation of primary neurons with SP600125 prior to incubation with TNF α , resulted in a dramatic decrease in neuronal BrdU incorporation (Fig. 6A–C). Taken together, these results suggest that TNF α -induced neuronal CCEs are mediated via activation of neuronal JNK signaling pathway. To determine if induction of neuronal DNA replication by TNF α precedes neuronal apoptosis, we treated 21 DIV neurons with recombinant human TNF α (250 pg) for 24 h in the presence of BrdU and performed triple immunofluorescence for MAP2, BrdU and TUNEL. While vehicle treatment did not induce any BrdU incorporation in neurons (Fig. 6D–G), rTNF α significantly induced DNA replication (Fig. 6H–K) and approximately 20% of MAP2 positive neurons were also positive for BrdU and TUNEL (Fig. 6L). Together, these results suggest that rTNF α induces neuronal DNA replication, which eventually leads to neurodegeneration via apoptosis.

Microglial activation and neuronal CCEs in R1.40 mice and human AD

R/R mice displayed neuronal CCEs and microglial activation (Iba1 staining) beginning at six months of age –six to seven months before fibrillar A β aggregates (Varvel et al., 2009). At 6 months of age in the R/R transgenic mice, the number of activated microglia (CD45 positive cells with thickened process and rounded cell bodies) were more abundant, primarily in frontal cortex layers II/III (where neuronal CCEs first appear) compared to

controls (Fig. 7A–B). The CD45 immunoreactivity increased progressively and was more pronounced in 20 month old R1.40 mice (Fig. 7D). While it is unclear whether CD45+ microglia are resident or peripherally derived monocytes, a previous study demonstrated that resident microglia do express CD45 in response to inflammatory stimuli, such as HIV-1 encephalitis (Cosenza et al., 2002).

To address the relevance of a spatio-temporal relationship between microglial activation and neuronal CCEs for human disease, we performed triple immunofluorescence labeling with antibodies against NeuN, cyclin D1, and F4/80 (a marker of activated microglia (Austyn and Gordon, 1981)) in human brain sections from subjects who died with AD. F4/80 positive microglia were observed in the superior and middle temporal gyri (Fig. 7G), where numerous NeuN positive neurons displayed expression of cyclin D1 (Fig. 7F). NeuN and cyclin D1 double immunoreactive cells were found near F4/80 positive microglial cells (Fig. 7G). Notably, certain cyclin D1 positive cells were observed to be negative for NeuN, suggesting the possibility of astrocyte proliferation and microgliosis, which is often expected in the advanced stages of AD. Interestingly, some cyclin D1 positive neurons were also TUNEL positive (Fig. 7J), suggesting that cell-cycle entry is associated with neuronal apoptosis in human AD brain. In brain regions that show very low incidence or total lack of neuronal cyclin D1 expression (for example deeper cortical areas of temporal gyri) were TUNEL negative (Fig. 7M–N). While these results qualitatively established a spatio-temporal correlation between neuronal CCEs and microglial activation both in R/R mice and tissue sections from AD, additional systematic studies involving quantitative morphometry are warranted in human AD tissue sections with consideration of various co-morbid conditions at the time of death.

In summary, our study suggests that microglia-derived TNF α induces neuronal CCEs following A β O-mediated activation in a manner dependent upon the activation of TNF receptor (TNFR) and intracellular JNK signaling pathway (Fig. 7Q).

Discussion

In the current studies, we demonstrated that neuronal CCEs, which are one of the earliest, but least understood, cellular pathologies observed in AD, could be induced by microglia, the innate immune cell of the brain. In part, our evidence derives from experiments based on an adoptive transfer protocol (Cardona et al., 2006). In particular, A β O-activated microglial CM induced DNA replication within primary cortical neurons *in vitro* and microglia from R1.40 mice but not from non-transgenic controls, induced neuronal cyclin D1 expression within a wild-type recipient mouse brain. Furthermore, our studies point to the microglial TNF α and neuronal JNK pathway as a key mediator for the induction of microglial-mediated neuronal CCEs. Finally, the onset of CCEs in neurons is also associated with neuronal cell death (TUNEL-positivity) in the AD cortex.

Neuronal CCEs in aging and disease

The induction of neuronal CCEs has been proposed to be an indication of neuronal distress (Chen et al., 2010; Yang et al., 2006). Neuronal CCEs have been observed in human AD, mouse models of AD (Chen et al., 2010; Yang et al., 2006), traumatic brain injury (Cernak et al., 2005; Di Giovanni et al., 2005) and hypoxia/Ischemia (Kuan et al., 2004). The concept is that neuronal distress, irrespective of the brain region, has the potential to lead to the activation of mitotic events in neurons (Herrup and Yang, 2007). In spinal cord injury, expression of cyclin D1, cdk4, PCNA, cyclin G, retinoblastoma, E2F5 transcription factor ensues 4–24 h following injury in rats (Di Giovanni et al., 2003). Notably, expression of numerous genes, associated with inflammation (COX2, NOS, HO1, IL-1R, IL-1 β), observed contemporaneously (Di Giovanni et al., 2003). These studies suggest that neuronal CCEs are

an early and unique neuropathological alteration observed in neurons “at risk” for neurodegeneration. Furthermore, in a mouse model of accelerated aging (Senescence-accelerated mice 8 or SAMP8), neuronal CCEs are also observed; suggesting that genetically accelerating the aging process can also accelerate neuronal CCEs (Casadesus et al., 2012), although the mechanism(s) remains unclear.

There is now a persuasive body of evidence favoring a significant inflammatory component in AD (reviewed in (Wyss-Coray, 2006)). First, inflammatory cells (microglia and astrocytes) as well as cytokines, chemokines, and complement components are present at elevated levels in AD brains (Akiyama et al., 2000; McGeer and McGeer, 2001). Second, the inflammatory changes occur prior to the deposition of A β in several different mouse models of AD (Dudal et al., 2004; Kitazawa et al., 2005; Yoshiyama et al., 2007). Third, patient populations receiving sustained treatment with NSAIDs during mid-life exhibited a >50% decreased risk of AD in retrospective studies (McGeer et al., 1996). Although prospective studies with NSAIDs have been unsuccessful – reviewed in (McGeer and McGeer, 2007) – this may simply reflect that NSAIDs offer pre-morbid but not therapeutic protection. Fourth, studies implicate inflammatory pathways in modulating A β pathology including cyclooxygenase, complement factors, transforming growth factor β , the chemokine CCL2 (MCP1) and its receptor CCR2, IL-1 β and CD40 (Eikelenboom and Veerhuis, 1996; Shafte et al., 2008). Fifth, a recent genome-wide association study (GWAS) identified a single-nucleotide polymorphism within the CR1 locus, encoding the complement component (3b/4b), on chromosome 1, to be associated with sporadic AD (Lambert et al., 2009). Notably, CD33 and MS4A4/MS4A6E immune system genes have shown significant association for AD in separate GWAS studies in humans (Hollingsworth et al., 2011; Naj et al., 2011). Finally, an arginine-to-histidine substitution at amino acid 47 (R47H) in the triggering receptor expressed on myeloid cells 2 (TREM2) gene, an important regulator of inflammatory processes, increased the risk of developing late-onset AD by three fold (Guerreiro et al., 2013; Jonsson et al., 2013). Taken together, these studies suggest that immune/inflammatory pathways directly contribute to the development and progression of AD.

Specifically, recent studies have demonstrated a possible link between the above mentioned late-onset AD (LOAD) genes and alterations in TNF α signaling. For example, in a recent study, a single vaccination with A β resulted in the clearance of plaques via upregulation of TREM2 and signal regulatory protein- β 1 (SIRP- β 1) in the brain. This concurrently caused down-regulation of TNF α and IL-6 in transgenic mouse models of AD (Fisher et al., 2010). A similar inverse relationship between surface expression of CD33 membrane receptor on monocytes and levels of TNF α was observed. When levels of CD33 were reduced, there was an increase in secretion of IL-1 β , IL-8 and TNF α from the monocytes (Lajaunias et al., 2005). Together, these results suggest that the functional alterations in some of these LOAD genes may contribute to disease pathogenesis via loss-of-function and lead to up-regulation of pro-inflammatory cytokines, including TNF α .

TNF α and induction of neuronal CCEs

Our studies implicate TNF α as the cytokine most likely to be responsible for the effects of the A β O-activated microglia. The evidence for this included enhanced levels of TNF α in six-month old R1.40 mouse brain, increased BrdU incorporation after incubation of primary cortical neurons with exogenous TNF α , neutralization of the effects of CM with an anti-TNF α antibody, and the *in vivo* demonstration that a genetic deficiency of *Tnfa* blocked the normal increase in neuronal cyclin D1 expression in the R1.40 mouse brain. Several previous studies have implicated TNF α as a mitogen. It induces DNA replication in hepatic cells (Kirillova et al., 1999) and promotes proliferation of adult neural stem cells (Widera et

al., 2006). Furthermore, exogenous TNF α can lead to the degradation of retinoblastoma (a negative regulator of cell proliferation) leading to apoptosis of locus coeruleus neurons *in vitro* (Dey and Snow, 2007).

There is also evidence for a role of TNF α in AD. Increased levels of TNF α are present in both the brains and plasma of AD patients (Bruunsgaard et al., 1999; Fillit et al., 1991). A β -dependent microglial release (Combs et al., 2001; Galimberti et al., 1999; Yates et al., 2000) and neurotoxic effects (Heneka et al., 1998; Ogura et al., 1997) of TNF α has been previously demonstrated. A β O-mediated release of TNF α inhibits LTP *in vitro* (Rowan et al., 2007; Wang et al., 2005) and impairs recognition memory in mice (Alkam et al., 2008). A GWAS found an association between single-nucleotide polymorphisms in *TNFA* and/or its receptor and sporadic AD (Collins et al., 2000; Perry et al., 2001). In recent studies, A β O significantly activate microglia and lead to an enhanced secretion of TNF α (Dhawan et al., 2012; He et al., 2012) when compared to microglia stimulated with A β F (He et al., 2012). Notably, infusion of A β O, but not A β F, into the ventricles of rats impaired learning and memory function and lead to neurodegeneration in a manner dependent upon the activation of the TNF α and the NF- κ B pathway (He et al., 2012).

TNF α , JNK activation and neuronal CCEs

JNK is essential for cell proliferation and survival (Lopez-Bergami et al., 2007; Ventura et al., 2006; Xia et al., 2006). CEP-1347, a specific JNK inhibitor, promoted the survival of cortical neurons via blocking A β -induced activation of JNK as well as other downstream events associated with JNK pathway activation. In these studies, CEP-1347 also blocked A β -mediated expression of cyclin D1, DP5, cytoplasmic cytochrome c, caspase 3-like activity, calpain activation and apoptosis (Bozyczko-Coyne et al., 2001). Taken together, these results suggest that TNFR-mediated activation of the JNK pathway may directly induce neuronal CCEs, although additional experiments are required to further define neuronal pathways that link microglial-produced TNF α to neuronal pathways that promote CCEs.

Conclusions

In summary, we provide evidence that microglial production of TNF α plays a critical role in the induction of neuronal CCEs in AD. As these neuronal CCEs lead to neurodegeneration, it is plausible that this pathway explains at least some of the cognitive decline seen in neurodegenerative diseases such as AD. These studies suggest novel targets for blocking neuronal CCEs that could ultimately be utilized for therapeutic intervention in AD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AD	Alzheimer's disease
CCE	cell cycle events
TNFα	Tumor Necrosis Factor- α
JNK	c-Jun Kinase
AβO	oligomeric amyloid-beta peptide
IL-6	interleukin-6
LPS	lipopolysaccharide
ROI	region of interest
STAT3	Signal transducer and activator of transcription 3
IKKα/β	I κ B kinase α/β
p38 MAPK	p38 mitogen activated protein kinase
PI3K	Phosphatidylinositol 3-kinase
TNFR	TNF receptor
BrdU	Bromodeoxyuridine
PFA	paraformaldehyde
CM	conditioned media
PBS	phosphate buffered saline
RT	room temperature
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
MAP2	microtubule associated protein-2
AβF	fibrillar amyloid-beta peptide
COX2	cyclooxygenase-2
NOS	nitric oxide synthase
IDV	integrated density value
PAS	protein-A-sepharose
INFγ	interferon- γ
IL-1β	interleukin-1 β
PCNA	proliferating cell nuclear antigen
NSAID	non-steroidal antiinflammatory drug
GFP	green fluorescent protein

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Highlights

- Microglial-derived TNF α induces neuronal CCEs via the JNK signaling pathway
- Adoptive transfer of R1.40 microglia drives neuronal CCEs in the host mouse brain
- Genetic deficiency of *Tnfa* in R1.40 mice blocks neuroinflammation and neuronal CCEs
- Activated microglia co-exist with mitotically-active and apoptotic neurons in AD

Fig. 1. Soluble factor(s) from AβO-activated microglia induce neuronal DNA replication
 (A–D) Treatment of primary microglia with different concentrations AβO (0.2, 2 and 20 μg/ml), but not vehicle (Veh), for 24 h alters microglial morphology from resting (rod-like) to an activated (macrophage-like) phenotype. Scale bar 20 μm. (E) Schematic showing the gradation of microglial activation with increasing concentrations of AβO treatment. (F–H) Confocal image with orthogonal view (in H) showing AβO (4.0 μg/ml for 24 h)-activated, Iba1 positive microglia (green) containing AβO inclusions immunoreactive for A11 oligomer-specific antibody (red; merge appears yellow in H). (I–N) AβO-activated microglia (green) contains Aβ inclusions that are immunoreactive for an AβO-specific monoclonal antibody NU1 (red in J) or Aβ-specific antibody 6E10 (red in M). Merged images are shown in K and N, DAPI is shown in blue in K. In I–K and L–N, AβO concentration was 4 μg/ml and 0.2 μg/ml respectively. Scale bars 10 μm.

Fig. 2. Soluble factor(s) secreted from AβO-activated microglia induce neuronal DNA replication (A) Schematic showing primary microglia derived from postnatal day 3 (P3) pups were incubated with 4 μg/ml AβO for 24 h, prior to collecting CM for neuronal treatment. Primary cortical neurons were incubated with 25% of the AβO-activated microglial CM containing 10 μM BrdU for 24 h prior to immunofluorescence analysis. (B–J) Double immunofluorescence revealing no DNA replication (BrdU incorporation) in the MAP2 + neurons treated with vehicle (Veh)-activated microglial CM (B, C, D). AβO-activated microglial CM induces neuronal DNA replication (E, F, G). Scale bar 30 μm. (H–K) Treatment of primary neurons with AβO-immunoprecipitated (IP'd) microglial CM showing numerous BrdU/MAP2 double positive neurons (H, I, J). In (K), AβO from microglial CM was IP'd utilizing 6E10 antibody prior to neuronal treatment. Lane 1: AβO-treated CM prior to IP; lane 2: vehicle treated microglial CM prior to IP; lane 3 and 4: AβO-treated CM after IP with 6E10; lane 5: Protein A Sepharose (PAS) beads containing 6E10-AβO complex after IP; lane 6: recombinant Aβ showing different AβOs and monomeric Aβ (AβM). (L) Quantification of double positive (BrdU/MAP2) neurons revealing a statistically significant, dose-dependent increase (vehicle versus 6.25% CM or 25% CM treatment with or without IP of AβO; n=3 independent cultures; run in triplicates/culture; ***p<0.001; two-way ANOVA followed by a Bonferroni post hoc test; mean ± SEM) in the percentage of BrdU + neurons following microglial CM treatment.

Fig. 3. TNF α mediates microglia-mediated neuronal cell cycle events *in vitro*

(A) A representative image showing a four-plex mouse cytokine infrared (IR) array spotted with antibodies against IL-6, TNF α , IL-1 β and INF γ (spots are identified in the schematic on the right) display increasing intensity in the IR signal when hybridized with increasing concentrations of a mixture of these four recombinant cytokines (top panel). A representative image of an IR array probed with microglial CM that are treated with either vehicle (Veh) or different concentrations of A β O. Note a concentration dependent increase IR signal for IL-6 and TNF α , but not for IL-1 β or INF γ (bottom panel). (B) Quantification of IR signal intensities for each of the four cytokines in microglial CM reveal a dose-dependent increase in the levels of secreted TNF α , to less extent for IL-6, but not for IL-1 β or INF γ following treatment with different concentrations of A β O. (C–N) Double immunofluorescence of 21DIV primary cortical neurons for MAP2 and BrdU reveal that 30 min pre-treatment of neurons with anti-TNF α antibody (F–H), but not mouse IgG (C–E), prior to 24 h of incubation with 25% of A β O-activated microglial CM (A β O-CM) reduced the number of double (BrdU and MAP2) positive neurons. Direct incubation of 21DIV primary cortical neurons with recombinant mouse TNF α (250 pg/ml; 24 h) induced the

number of double (BrdU and MAP2) positive neurons (L-N) compared to those treated with mouse IgG alone (I-K). Scale bar 30 μ m. **(O-Q)** Western blot analysis of 21 DIV primary cortical neurons revealing significantly elevated (* $p < 0.05$ with unpaired t test; graphs shown in P and Q) levels of cyclin D1 and PCNA following rTNF α , but not vehicle, treatment. **(R)** Quantification of double (BrdU and MAP2) positive cells from different conditions described in C-N reveal statistically significant (mean $\pm$ SEM; * $p < 0.05$ versus Veh – clear white bar) increase in double positive cells following A β O-CM treatment, a significant decrease (mean $\pm$ SEM; * $p < 0.05$) following pre-incubation of neurons with anti-TNF α antibody, but not by mouse IgG (mean $\pm$ SEM; ** $p < 0.01$). Treatment with mouse IgG alone (without A β O-CM) is similar to vehicle treatment with no difference in the percentage of BrdU+ neurons. Direct treatment of neurons with recombinant 1 ng of TNF α (rTNF α) significantly (mean $\pm$ SEM; *** $p < 0.001$ versus Veh or mouse IgG alone) increased the number of double positive neurons. For the cytokine array experiments; samples were ran in quadruplicates per treatment; all other data sets derived from triplicates for each treatment and from at least two independent neuron and microglial cultures. One-way ANOVA followed by a Newman-Keuls multiple comparison test were used.

Fig. 4. Purified microglia from R1.40 mice induces neuronal cell cycle events *in vivo*
(A–B) Schematic showing the magnetic-based isolation of CD11b+ microglia and intracerebral injections of purified microglia into recipient mouse brain. Microglia purified from R/R-*Cx₃cr1^{tgfp/+}* mouse brain are viable and show robust GFP expression *in vitro* prior to adoptive transfer (in B; Scale bar 10 μ m). **(C)** Five serial sections (30 μ m thick), two anterior (N+1 and N+2) and two posterior (N–1 and N–2) were analyzed for quantification and the 1–2 mm area marked in the hashed circle defined as ROI for quantification. **(D–E)** Representative images captured under low power (D) and high power (E) of a medial section showing GFP + microglia from R/R-*Cx₃cr1^{tgfp/+}* donor within recipient mouse brain, showing activated phenotype after 48 h (inset in E). Scale bars (30 μ m in E; 10 μ m for inset in E). **(F–Q)** 6-month-old R/R microglia, but not WT, was capable of inducing cyclin D1 expression (red in G, J, M, P) with NeuN+ neurons (green in F, I, L, O) in the ipsilateral, but not contralateral, cortex of 6-month-old WT recipient mouse brain. Recipient mice receiving vehicle (RPMI) did not exhibit neuronal cyclin D1 expression (F–H). Merged images are in H, K, N and Q. Scale bar 30 μ m. **(R)** Quantification of cyclin D1 and NeuN + neurons revealed a statistically significant (n=3 recipients per treatment; five sections per mouse; six random fields per section were scored; mean $\pm$ SEM; *p<0.05; one-way ANOVA with a Tukey post hoc test) increase in the percentage of double + (cyclin D1 and NeuN) neurons in the ipsilateral cortex of recipient mouse brain that received microglia from 6 month old R/R donors compared to other recipients receiving either microglia from WT or vehicle.

Fig. 5. Microglia-derived TNFα induces neuronal cyclin D1 expression *in vivo*

(A–F) Double immunofluorescence for NeuN (green), cyclin D1 (red) show reduced number of double (cyclin D1 and NeuN) positive cells (arrows) in the layer VI of the cortex when purified R/R microglia was transferred with anti-TNFα antibody, but not with mouse IgG, into two-month-old non-transgenic (WT) recipient mouse brain. (G) Percentage of cyclin D1 + neurons are significantly (**** $p < 0.0001$; unpaired t test; $n = 3$ recipient mouse brains) reduced in the recipient mouse brain that received purified R/R microglia with anti-TNFα antibody than compared to those that received R/R microglia with mouse IgG. (H) Western blot analysis of detergent soluble cortical lysates revealed an increase in the levels of TNFα in the six-month-old R/R mouse brain compared to age-matched WT. (I) Quantification of Western blots for TNFα revealed a statistically significant increase (mean $\pm$ SEM; ** $p < 0.01$; $n = 4$ per group; unpaired t test) in the integrated density value (IDV) ratio for TNFα/GAPDH in the six-month-old R/R compared to age-matched WT. (J–R) Layer II/III of six month old R/R showing significantly higher number of NeuN + neurons expressing cyclin D1 (arrows) compared to age-matched non-transgenics (WT) and R/R-Tnfα^{-/-} mice in the identical brain region. Scale bar 20 μm. (S) Percentage of cyclin D + neurons are significantly (mean $\pm$ SEM; *** $p < 0.001$; one-way ANOVA with Tukey post hoc test; $n = 4$ animals per group) higher in layer II/III of the cortex in 6 month old R/R mice compared to age-matched WT or R/R-Tnfα^{-/-} mice.

Fig. 6. Activation of TNF α induces neuronal DNA replication and apoptosis

(A–B) Double immunofluorescence for MAP2 (green) and BrdU (red) reveal reduction in the double (MAP2 and BrdU) positive neurons following 30 min pre-incubation of 21DIV neurons with SP600125 (a JNK inhibitor), but not vehicle, prior to 24 h incubation with 250 pg/ml rTNF α . Scale bar 20 μ m. (C) Quantification reveal statistically significant (mean $\pm$ SEM; ***p < 0.001; unpaired *t* test; n=3 replicates in two independent cultures) decrease in the percentage of BrdU + neurons following SP600125 pretreatment. (D–K) Triple immunofluorescence of 21DIV primary cortical neurons for MAP2 (blue), BrdU (green), TUNEL (red) following 24 h treatment with 250 pg of rTNF α reveal a neuron positive for both BrdU and TUNEL. Scale bar 10 μ m. (L) Quantification of the triple positive (MAP2+BrdU+TUNEL) neurons reveal that about 20% (*p < 0.05; unpaired *t* test; mean $\pm$ SEM; n=3) of MAP2 positive neurons are also positive for both BrdU and TUNEL in response to rTNF α .

Fig. 7. Relationship between neuroinflammation, neuronal cell-cycle events and neurodegeneration in the R1.40 transgenic mouse model and in human AD brain
(A–B) Immunohistochemical analysis revealing the presence of CD45 immunoreactivity in layer II and III of the cortex of 6 month old R/R mouse brain (B) but not in age-matched non-transgenics (A). **(C–D)** CD45 immunoreactivity is significantly enhanced in 20-month-old R/R mouse brain (D) compared to age-matched non-transgenics (C). **(E–H)** Triple immunofluorescence labeling reveals cyclin D1 expression (red in F) within NeuN + neurons (purple in E; arrows in H) in the temporal cortex of a human AD brain where numerous F4/80 + (green in G) microglia/macrophage also co-exist (merged image in H). **I–L)** Triple immunofluorescence labeling reveals that a portion of NeuN + neurons (purple in

I) expressing cyclin D1 (green in K) is also Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) positive (red in J; arrows in L), merged image in L). Scale bars 20 μ m. **(M–P)** As an unaffected regional control, deeper layers of the temporal gyri show no cyclin D1 expression and are also devoid of TUNEL reactivity in NeuN positive neurons. Scale bar 20 μ m. **(Q)** Schematic showing microglial activation by A β O results in the release of TNF α that binds to TNFR present on susceptible neurons. Interaction between TNF α and TNFR recruits several adaptor proteins including Act1 and TNF Receptor Associated Factor (TRAF) and induces activation of JNK directly or via Mitogen Activated Protein Kinase Kinase (MKK). Activation of JNK induces the expression of cyclin D1, and thus renders “*at risk*” neurons to acquire the identity of a mitotic cell.

Table 1

The antibodies utilized in the present study

Antibodies	Species/Clone	Source
<i>Cell cycle antibodies</i>		
Cyclin D1	Rabbit monoclonal	Abcam (ab16663)
PCNA	Rabbit polyclonal	Abcam (ab15497)
BrdU	Rat monoclonal	Abcam (ab6326)
<i>Antibodies for immune molecules</i>		
CD45	Rat monoclonal	AbD Serotec (MCA 1388)
F4/80	Rat monoclonal	AbD Serotec (MCA497G)
CD11b	Mouse monoclonal	Millipore (MAB1387Z)
TNF α	Mouse monoclonal	Abcam (ab1793)
<i>Other antibodies</i>		
NeuN	Mouse monoclonal	Millipore (MAB377)
MAP2	Mouse monoclonal	Sigma (M9942)
6E10	Mouse monoclonal	Covance (SIG-39300)
GAPDH	Mouse monoclonal	Sigma (G8795)
NU1	Mouse monoclonal	Lambert et al., 2007.
Phospho-STAT3 (Y705)	Rabbit polyclonal	Cell Signaling (#9131)
Total-STAT3	Rabbit polyclonal	Cell Signaling (#9132)
Phospho-p38 MAPK (T180/Y182)	Rabbit polyclonal	Invitrogen (36–8500)
Total-p38 MAPK	Rabbit polyclonal	Cell Signaling (#9212)
Phospho-JNK (T183/Y185)	Rabbit polyclonal	Cell Signaling (#9251S)
Total-JNK	Rabbit polyclonal	Cell Signaling (#9252)
Phospho-IKK α/β (S176/180)	Rabbit monoclonal	Cell Signaling (#2697S)
Total- IKK α/β	Rabbit polyclonal	Santa Cruz Biotech (sc-7607)
Phospho-I κ B α (S32/36)	Mouse monoclonal	Cell Signaling (#9246S)
Total-I κ B α	Rabbit polyclonal	Santa Cruz Biotech (sc-371)
Phospho-p65 (S536)	Rabbit monoclonal	Cell Signaling (#3033S)
Total-p65	Rabbit polyclonal	Santa Cruz Biotech (sc-109)
Phospho-Akt (S473)	Rabbit polyclonal	Cell Signaling (#9271)
Total-Akt	Rabbit polyclonal	Cell Signaling (#9272)
PI3K α	Rabbit polyclonal	Santa Cruz Biotech (sc-7174)