

Dual induction of TREM2 and tolerance-related transcript, Tmem176b, in amyloid transgenic mice: implications for vaccine-based therapies for Alzheimer's disease

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ABSTRACT

Vaccine-based autoimmune (anti-amyloid) treatments are currently being examined for their therapeutic potential in Alzheimer's disease. In the present study we examined, in a transgenic model of amyloid pathology, the expression of two molecules previously implicated in decreasing the severity of autoimmune responses: TREM2 (triggering receptor expressed on myeloid cells 2) and the intracellular tolerance-associated transcript, Tmem176b (transmembrane domain protein 176b). *In situ* hybridization analysis revealed that both molecules were highly expressed in plaque-associated microglia, but their expression defined two different zones of plaque-associated activation. Tmem176b expression was highest in the inner zone of amyloid plaques, whereas TREM2 expression was highest in the outer zone. Induced expression of TREM2 occurred coincident with detection of thioflavine-S-positive amyloid deposits. Transfection studies revealed that expression of TREM2 correlated negatively with motility, but correlated positively with the ability of microglia to stimulate CD4⁺ T-cell proliferation, TNF (tumour necrosis factor) and CCL2 (chemokine ligand 2) production, but not IFN γ (interferon γ) production. TREM2 expression also showed a positive correlation with amyloid phagocytosis in unactivated cells.

However, activating cells with LPS (lipopolysaccharide), but not IFN γ , reduced the correlation between TREM2 expression and phagocytosis. Transfection of Tmem176b into both microglial and macrophage cell lines increased apoptosis. Taken together, these data suggest that, *in vivo*, Tmem176b⁺ cells in closest apposition to amyloid may be the least able to clear amyloid. Conversely, the phagocytic TREM2⁺ microglia on the plaque outer zones are positioned to capture and present self-antigens to CNS (central nervous system)-infiltrating lymphocytes without promoting pro-inflammatory lymphocyte responses. Instead, plaque-associated TREM2⁺ microglia have the potential to evoke neuroprotective immune responses that may serve to support CNS function during pro-inflammatory anti-amyloid immune therapies.

Key words: antigen presentation, autoimmunity, Clast1, neuroinflammation, Torid.

INTRODUCTION

Microglia are the resident tissue macrophage of the CNS (central nervous system) (Carson et al., 2007; Graeber and

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Abbreviations: A β , amyloid β peptide; CCL2, chemokine ligand 2; CFSE, carboxyfluorescein succinimidyl ester; CNS, central nervous system; DAMP, danger-associated molecular pattern; DMEM, Dulbecco's modified Eagle's medium; EAE, experimentally induced autoimmune encephalomyelitis; FBS, fetal bovine serum; GFP, green fluorescent protein; HPRT, hypoxanthine phosphoribosyl transferase; IFN γ , interferon γ ; IL, interleukin; KO, knockout; LPS, lipopolysaccharide; PFA, paraformaldehyde; qPCR, quantitative PCR; Thio-S, thioflavine-S; Tmem176b, transmembrane domain protein 176b; TNF, tumour necrosis factor; TREM2, triggering receptor expressed on myeloid cells 2; WT, wild-type.

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Streit, 2010). Their activation is now a well-recognized feature of Alzheimer's disease in humans and amyloid pathogenesis in transgenic animal models (Schwab et al., 2009; Cameron and Landreth, 2010; Graeber and Streit, 2010). However, the net beneficial compared with detrimental consequences of microglial activation during amyloid pathogenesis is still unclear. In part, the inability to resolve this debate is due to the highly plastic phenotype of microglia.

Like all macrophages, CNS-resident microglia are able to acquire a broad array of both cytotoxic and cytoprotective functional states (Carson et al., 2007; Graeber and Streit, 2010). Perhaps most relevant to Alzheimer's disease, microglia can be activated to produce pro-inflammatory cytokines and ROS (reactive oxygen species), as well as to phagocytose cell debris, amyloid and produce neuroprotective growth factors (Ziv et al., 2006; Butovsky et al., 2007; Takahashi et al., 2007; Schwab et al., 2009; Cameron and Landreth, 2010). In addition, microglia can differentiate into antigen-presenting cells able to direct T-cell proliferation and activation (Carson et al., 2006). Multiple *in vivo* and *in vitro* studies have demonstrated that microglia are relatively inefficient in driving pro-inflammatory CD4⁺ T-cell responses as compared with mature dendritic cells or even with other macrophage populations (reviewed in Carson et al., 2006). Thus studies focused on harnessing T-cell-driven anti-amyloid therapies for Alzheimer's disease have for the most part ignored whether microglia have the potential to regulate anti-amyloid T-cell responses and whether amyloid pathogenesis alters microglial antigen-presenting cell function (Webster et al., 2001; Monsonogo and Weiner, 2003; Lemere et al., 2006; Wilcock and Colton, 2009; Cameron and Landreth, 2010; Graeber and Streit, 2010).

Previous studies now illustrate that microglia can play physiologically significant roles as antigen-presenting cells that are distinct from the roles played by peripheral 'professional' antigen-presenting cells (Byram et al., 2004; Carson et al., 2006). For example, neuroprotective CD4⁺ T-cell responses have been shown to lower the rate of neuronal cell death in the facial motoneuron nucleus following facial axotomy (Serpe et al., 1999; Jones et al., 2005). Consistent with other models of CNS autoimmunity, peripheral immune cells outside the CNS were absolutely required to initiate CD4⁺ T-cell responses following facial axotomy (Hickey and Kimura, 1988; Byram et al., 2004; Greter et al., 2005). Conversely, while microglia were unable to initiate these T-cell responses, development of neuroprotective T-cell function was absolutely dependent on subsequent antigen-presentation by microglia within the injured CNS (Byram et al., 2004).

The acquisition of specific microglial phenotypes is a consequence of multiple regulatory inputs provided by interactions with neurons, glia and CNS-infiltrating immune cells (Carson et al., 2007; Tian et al., 2009). For example, healthy neurons express ligands for inhibitory receptors such as CD200 receptor, CX3CR1 and CD45 (Mott et al., 2004; Cardona et al., 2006; Koning et al., 2009). Damaged and/or dying neurons also express and/or release molecules recognized by a wide array

microglial-expressed receptors specific for DAMPs (danger-associated molecular patterns). For example, DAMP receptors recognize the presence of free ATP, phosphatidylserine on the external plasma membrane and expression of heat-shock proteins (Grommes et al., 2008; Stefano et al., 2009; Clark et al., 2010; Skaper et al., 2010; Toulme et al., 2010). Thus the net microglial response to any specific tissue damage and/or pathogen is determined by the summation of all of their local environmental cues. Regulation by summated environmental cues suggests that microglial activation can be heterogeneous and localized if activation/inhibition signals are also localized in their availability. Indeed, many studies have defined region-specific responses of microglia to inflammatory stimuli (Melchior et al., 2006).

Microglial heterogeneity can also precede pathology. Simply as a function of normal development and aging, the expression of TREM2 (triggering receptor expressed on myeloid cells 2) becomes increasingly heterogeneous (Schmid et al., 2002; Carson et al., 2006; Schmid et al., 2009; Thrash et al., 2009). Early in post-natal development all microglia express similar levels of TREM2 that are readily detected by *in situ* hybridization analysis (Thrash et al., 2009). By young adulthood, only subsets of microglia expressed levels of TREM2 that were readily detectable by *in situ* hybridization (Schmid et al., 2002; Carson et al., 2006). Notably, the highest levels of TREM2 expression per microglia and the greatest percentage of TREM2 expression were found in brain regions that develop amyloid pathology in human Alzheimer's disease and in transgenic models of amyloid pathology (Schmid et al., 2002; Carson et al., 2006). Although the disease mechanism is unknown, humans lacking a functional TREM2 pathway develop early-onset cognitive dementia that is apparent by the third decade of life and which is distinct from Alzheimer's disease-associated dementia (Bianchin et al., 2004; Klünemann et al., 2005; Montalbetti et al., 2005; Chouery et al., 2008).

In our present study, we focus on the role of TREM2 as modulating microglial function in amyloid pathology for two reasons. First, although TREM2 expression is not unique to microglia, TREM2 is enriched ~10-fold in microglia as compared with macrophages that acutely infiltrate the CNS (Schmid et al., 2009). Furthermore, Frank et al. (2008) reported TREM2 expression in plaque-associated microglia in aged amyloid transgenic mice. Secondly, functional studies suggest that TREM2 is primarily an anti-inflammatory receptor associated with repair mechanisms. Although TREM2-mediated activation of macrophages increases nitric oxide production, it also promotes phagocytosis of apoptotic cell debris and select subsets of pathogens (Takahashi et al., 2005; Hsieh et al., 2009; N'Diaye et al., 2009). TREM2 KO (knockout) mice reveal that TREM2 functions to attenuate macrophage production of pro-inflammatory cytokines in response to pro-inflammatory stimulation (Takahashi et al., 2005; Hamerman et al., 2006; Turnbull et al., 2006; Neumann and Takahashi, 2007; Takahashi et al., 2007). In a model of colonic mucosal injury, macrophage expression of TREM2 was required to promote complete and

efficient repair of injured tissue (Seno et al., 2009). With specific relevance to CNS disease, overexpressing TREM2 in peripheral antigen-presenting cells did decrease the severity and speeded the recovery from EAE (experimentally induced autoimmune encephalomyelitis) (Takahashi et al., 2007). Conversely, blocking TREM2 activity increased the severity of EAE (Piccio et al., 2007).

Influx of macrophages into the CNS is also implicated in playing roles in amyloid pathogenesis. We have previously reported that in a spontaneously resolving LPS (lipopolysaccharide)-induced model of CNS neuroinflammation, macrophages infiltrating the CNS are induced to express similar levels of the intracellular tetraspan molecule, called Tmem176b (transmembrane domain protein 176b) as expressed by CNS-resident microglia (Schmid et al., 2009). As yet, little is known concerning Tmem176b expression and function. However, Louvet et al. (2005) have demonstrated that transfection of Tmem176b into immature dendritic cells prevents activation-induced increases in MHC class II, co-stimulatory molecules and cytokines required for pro-inflammatory activation of CD4⁺ T-cells. In addition, increased Tmem176b expression was found to correlate with increased allograft acceptance in a rat cardiac transplant model (Louvet et al., 2005). Based on their data, Louvet and colleagues termed this molecule Torid (tolerance-related and induced transcript). In the present study, we have chosen to use the NCBI unigene designation, Tmem176b, for this molecule. Taken together the published data suggest that Tmem176b may function to limit pro-inflammatory T-cell responses and/or to promote resolution of pro-inflammatory T-cell responses.

In our present study, we find that both TREM2 and Tmem176b are induced co-incident with amyloid plaque development in APP23 transgenic mice. Our *in vitro* assays suggest that, *in vivo*, amyloid-induced expression of these molecules has the potential to regulate antigen-presenting function in plaque-associated microglia.

MATERIALS AND METHODS

In situ hybridization analysis

All animal husbandry and experimental protocols were IACUC approved. APP23 transgenic mice express the human familial AD mutant APP751 with Swedish double mutations at positions 670/671 (APPK670N, M671L) (Sturchler-Pierrat et al., 1997). The expression of human APPsw is driven by the murine Thy-1 promoter and is restricted to neurons. Unmanipulated C57Bl/6 APP23 transgenic and C57Bl/6 wild-type mice were anaesthetized by halothane inhalation and subjected to intracardiac perfusion with a 4% PFA (paraformaldehyde) saline solution at the ages indicated in the Results section and the Figure legends. Brains were immediately harvested, post-fixed in a series of 4% PFA sucrose solutions as described

previously prior to cryosectioning (Carson et al., 1993). *In situ* hybridization was performed on free-floating cryosections as described previously (Thrash et al., 2009). Briefly, coronal sections (25 µm thick) were hybridized at 55°C for 16 h with a ³⁵S-labelled riboprobe (10⁷ c.p.m./ml). Excess probe was removed by washing at room temperature (23°C) for 30 min in 0.03 M NaCl, 0.003 M sodium citrate (2 × SSC) containing 10 µM 2-mercaptoethanol, followed by a 1 h incubation with 4 µg/ml ribonuclease, 0.5 M NaCl, 0.5 M EDTA, 0.05 M Tris/HCl (pH 7.5), at 37°C. Sections were then washed under high-stringency conditions for 1.5 h at 55°C in 0.5 × SSC, 50% formamide and 10 µM 2-mercaptoethanol, followed by a 1 h incubation at 68°C in 0.1 × SSC, 5 µM 2-mercaptoethanol and 0.1% *N*-lauryl sarcosine. Myeloid cells and blood vessels were identified by their ability to bind biotinylated tomato lectin (Sigma), whereas neuronal nuclei were identified by labelling with biotinylated NeuN (Sigma). Bound biotinylated tomato lectin or NeuN was visualized by standard streptavidin-horseradish peroxidase methodology. To co-localize TREM2 expression with compact parenchymal Aβ (amyloid β-peptide) deposits and cerebral amyloid angiopathy, some brain sections were stained with Thio-S (thioflavine-S). Sections were mounted on to FisherBrand SuperFrost/plus slides (Fischer Scientific) and dehydrated with ethanol and chloroform. Slides were exposed for 3 days to Kodak X-AR film and dipped in Ilford K-5 emulsion (Polysciences). After 3 weeks, slides were developed with Kodak D19 developer (Fischer Scientific), fixed and counterstained with Mayer's haematoxylin.

Preparation of mixed glial cultures

Mixed glial cultures were prepared as described previously (Carson et al., 1998, 1999). Briefly, brains from post-natal day 1–3 C57Bl/6 mice were stripped of meninges, and the cortices mechanically dissociated, seeded into six-well plates and maintained in DMEM (Dulbecco's modified Eagle's medium), supplemented with 10% FBS (fetal bovine serum) and insulin (5 µg/ml). After 2 weeks *in vitro*, cultures were maintained in serum-free DMEM-based OM-7 medium for 2 days (Carson et al., 1998). After 2 days in serum-free medium, cultures were treated with TNF (tumour necrosis factor) (50 ng/ml), Aβ-(1–42) (25 µM) or TNF and Aβ for 5 days. Cultures were then dissociated into single-cell suspensions and microglial activation was assayed by flow cytometry as described previously (Carson et al., 1998, 1999).

Generation of TREM2 cell lines

Murine C57Bl/6 BV-2 microglial cells were transformed with various amounts of WT-TREM-2 (WT is wild-type) or sh-TREM2 (sh is short hairpin) lentiviral vectors in DMEM plus Polybrene (2 µg/ml) and gently spun for 1.5 h at 4°C. At 2 days post-infection, GFP⁺ (green fluorescent protein) cells were purified using a FACSAria cell sorter (BD Biosciences). GFP⁺ cell lines were derived from single-cell cultures. The relative surface level of TREM2 expression in each cell line was

determined using flow cytometry using a monoclonal anti-TREM2 antibody (R&D Systems) and compared with TREM2 levels in untreated BV-2 cells.

qPCR (quantitative PCR) analysis of TREM2 expression levels

To quantify TREM2 mRNA expression, cDNA templates were prepared from each cloned TREM2 BV-2 cell line as described previously (Schmid et al., 2009). qPCR analysis of TREM2 expression was performed as described previously with primer sets previously detailed in Schmid et al. (2009). In brief, a constant amount of 200 ng of cDNA from each RT (reverse transcription) or each dilution of the appropriate standard, was amplified in 25 μ l of TaqMan PCR Core Reagent (Applied Biosystems) according to the manufacturer's instructions. The reaction mixture consisted of 0.5 unit of AmpliTaq Gold polymerase, each of the four dNTPs (0.2 mM), with dUTP replacing dTTP, each pair of primers (300 nM) and MgCl₂ (3 mM final concentration) in the above-described Tris buffer. Amplifications were performed in an ABI Prism 7700 Sequence Detector System (Applied Biosystems). Each sample was analysed in duplicate. At the end of each experiment, amplification products were fractionated by gel electrophoresis to verify that they migrated as a single band with the expected size. Data were normalized to the endogenous reference HPRT (hypoxanthine phosphoribosyl transferase); then, using the C_t (comparative cycle threshold) method, the amount of RNA transcripts were expressed as a reference to a standardized unstimulated microglia sample. CT was defined as low as transcripts were abundant. For validation of the $\Delta\Delta C_t$ method, we also used standards for calibration of HPRT and TREM2 cDNA as described previously (Schmid et al., 2009). Using the standard curve, it was verified that the absolute copy number of HPRT transcripts was of the same order of magnitude in all samples analysed by qPCR.

Amyloid phagocytosis assay

HiLyteFluor™ 647 (Anaspec)-A β -(1–40) was resuspended in Tris/EDTA (pH 8.2) at 20 μ M and then incubated in the dark for 3 days at 37°C to promote aggregation. Microglial cells were pretreated in low serum (0.5% FBS supplemented with insulin) \pm LPS (50 ng/ml), \pm IFN γ (100 units/ml) for 24 h prior to the addition of aggregated fluorescently labelled A β . Amyloid phagocytosis and surface expression of TREM2 were determined by flow-cytometric analysis 5 h post-addition of 100 nM aggregated HiLyteFluor™ 647-A β -(1–40).

Mixed lymphocyte proliferation assays

Naïve CD4⁺ T-cells were isolated from lymph nodes harvested from 6–8-week-old BALB/c mice and C57Bl/6 mice bred at the UC Riverside vivarium as described previously (Carson et al., 1998, 1999). In brief, CD3⁺ T-cells were positively enriched from single-cell lymph node suspensions using

antibody (anti-CD3)-conjugated magnetic beads. CD8⁺ T-cells were subsequently depleted from these suspensions using anti-CD8-conjugated magnetic beads. Flow-cytometric analysis indicated >98% purity of the CD4⁺ T-cell preparations. T-cells were labelled with CFSE (carboxyfluorescein succinimidyl ester) as described previously (Ploix et al., 2001). The cloned GFP⁺ BV-2 microglial cells lines were used as allogeneic antigen-presenting cells for BALB/C CD4⁺ T-cells and as syngeneic antigen-presenting cells for C57Bl/6 CD4⁺ T-cells. Following plating into 96-well tissue-culture plates, BV-2⁺ cells were irradiated with 1500 rads. CFSE-labelled CD4⁺ T-cells were plated at a 1:4 ratio of antigen-presenting cells to CD4⁺ T-cells. Following 5 days of co-culture, CD4⁺ T-cells were harvested and their CFSE levels were measured using a FACS Calibur equipped with BD Cell Quest software (version 5.2.1). CFSE data was quantified using FlowJo software (version 8.8.4; Treestar).

Detection of secreted cytokines using flow cytometric bead arrays

Protein concentrations of IL (interleukin)-6, IL-10, CCL2 [chemokine C ligand 2; also called MCP1 (monocyte chemoattractant protein-1)], IFN- γ , TNF and IL-12p70 in the supernatants of serum-free microglia/T cell co-cultures were measured using the BD™ CBA (cytokine bead array) mouse inflammation kit according to the manufacturer's protocol (BD Biosciences). In brief, 500 μ l of collected supernatants were incubated for 2 h with 50 μ l of mixed capture beads and 50 μ l of the PE-detection reagent. The PE-detection reagent is a mixture of PE-conjugated anti-mouse IL-6, IL-10, CCL2, IFN- γ , TNF and IL-12p70 antibodies. The samples were washed and resuspended in 300 μ l of wash buffer and analysed immediately by flow cytometry using a BD FACSCalibur and BD Cell Quest software (version 5.2.1). Cytokine protein concentrations were determined using BD cytometric Bead Array Software according to the instructions in the kit.

Scratch assay

The previously described cloned BV-2 cell lines were plated and grown to confluency in serum-free medium. A linear scratch was made in each confluent culture with a 0.6 cm spatula tip and the cultures were returned to 37°C for 6 h. Quantification of the number of cells entering the scratch zone was determined based on blinded analysis of digital photographs. TREM2 mRNA expression was determined for each culture analysed in each scratch assay.

Annexin V apoptosis assay of Tmem176b-transfected cells

RAW 264.7 or BV2 cells were transfected using the cell line nucleofector kit V according to the manufacturer's instructions (Lonza Group). In brief, 1×10^6 cells were incubated with 0.46 pmol of plasmid encoding CMV (cytomegalovirus)-driven

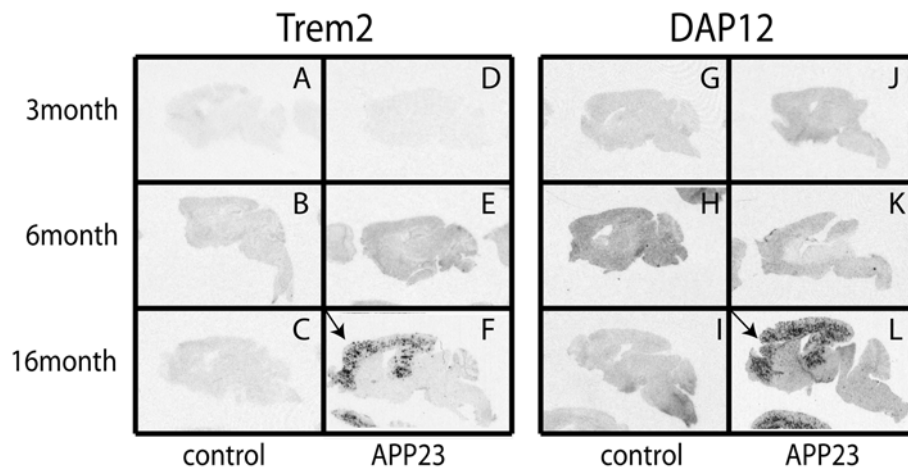


Figure 1 TREM2 and DAP12 expression is induced co-ordinate with amyloid plaque deposition. Autoradiograms of sagittal brain sections from non-transgenic (A–C and G–I) and APP23 transgenic (D–F and J–L) age-matched siblings. (A–F) Brain sections hybridized with ^{33}P -labelled TREM2 riboprobes. (G–L) Brain sections hybridized with ^{33}P -labelled DAP12 riboprobes. Arrows indicated amyloid-associated induction of TREM2 and DAP12 expression.

expression of GFP (AcGFP) or GFP–Tmem176b fusion protein (AcGFP–Tmem176b) and transfected using Amaxa program D-032. Following transfection, cells were incubated for 24 or 48 h and then harvested using commercially prepared cell dissociation buffer (Invitrogen). Cells were labelled with annexin V according to the manufacturer's instructions (BD Biosciences) and levels of annexin V labelling on GFP⁺ cells was quantified using a BD FACSCalibur Flow equipped with BD Cell Quest software (version 5.2.1).

RESULTS

Molecules associated with modulation of antigen presentation are robustly up-regulated in response to amyloid deposition

Previous reports have correlated increased expression of TREM2 and Tmem176b with decreased autoimmune responses (Louvet et al., 2005; Takahashi et al., 2005). In addition, we previously reported that microglial expression of TREM2 was highest in regions that are predisposed to develop amyloid pathology (Schmid et al., 2002, 2009). Furthermore, Frank et al. (2008) have reported that TREM2 immunoreactivity was elevated in plaque-associated microglia in transgenic mice with fully developed amyloid pathology. As yet the spatial organization and kinetics of TREM2 expression in relationship to aging, amyloid plaque formation and induction of additional markers of microglia/macrophage activation are unexamined. TREM2 is an orphan receptor that lacks an intracellular signalling tail and is absolutely dependent on the co-expression of DAP12 to mediate its intracellular signalling.

Therefore, in the present study, we examined whether increased TREM2 expression paralleled plaque formation and was coincident with increased expression of DAP12 as well as Tmem176b in APP23 transgenic mice (Figures 1–4). Finally, we also compared the expression of these three molecules to the induced expression of C1qA, a sensitive marker of general microglial activation (Färber et al., 2009). C1qA has also been identified as being induced in plaque-associated microglia/macrophages, as well as neurons (Korotzer et al., 1995; Afagh et al., 1996; Lue et al., 2001; Fan and Tenner, 2004).

TREM2 can be expressed as a full-length receptor or a splice variant lacking the transmembrane and intracellular domains (Schmid et al., 2002; Piccio et al., 2008). Cell-associated TREM2 immunoreactivity has the potential to reflect either cell-expressed TREM2 or the binding of soluble TREM2 to ligand-expressing cells. Therefore, in the present study, we measured mRNA expression. Figures 1 and 3 depict autoradiograms of sagittal brain sections from age-matched WT and APP23 C57Bl/6 mice hybridized with ^{33}P -labelled riboprobes. WT levels of TREM2 and DAP12 are not detected by autoradiogram analysis at all

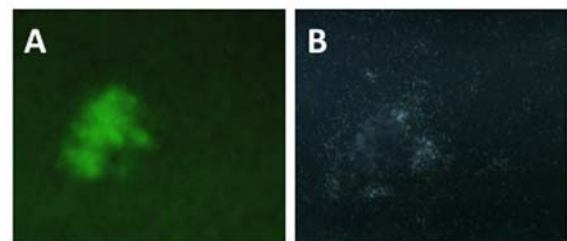


Figure 2 Induced TREM2 expression surrounds Thio-S-positive plaques (A and B) A brain section from a 10-month-old APP23 transgenic mouse. A compact parenchymal plaque is visualized in green with Thio-S (A). TREM2 expression is visualized with a ^{33}P -labelled riboprobe (white grains in film emulsion) (B).

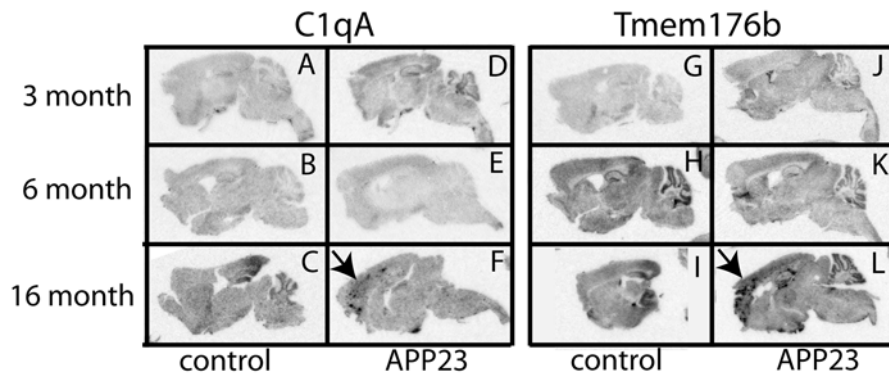


Figure 3 C1qA and Tmem176b expression increases in WT mice with age and in association with amyloid plaque deposition in APP23 transgenic mice

Autoradiograms of sagittal brain sections from non-transgenic (A–C and G–I) and APP23 transgenic (D–F and J–L) age-matched siblings. (A–F) Brain sections hybridized with ³³P-labelled C1qA riboprobes. (G–L) Brain sections hybridized with ³³P-labelled Tmem176b riboprobes. Arrows indicated amyloid-associated induction of C1qA and Tmem176b expression.

ages examined (3, 6 and 16 months of age; Figures 1A–1C and 1G–1I) consistent with our previous reports (Schmid et al., 2002; Thrash et al., 2009). In contrast, robust induction of both TREM2 and DAP12 is readily detected in a punctate pattern of expression in the cortex and hippocampus of APP23 mice. The kinetics and spatial pattern of induction were identical for both molecules (Figures 1D and 1E and 1J–1L). The appearance of this pattern of expression was co-incident with the deposition of amyloid plaques (Figure 2). Specifically, the detection of Thio-S-positive plaques (Figure 2A) was always associated with induced expression of TREM2 surrounding the plaque (Figure 2B). The transmembrane adaptor molecule DAP12 has the potential to mediate intracellular signalling for most of the classic TREM family members. Therefore we tested whether

altered or increased expression of TREM1, TREM3 or TREM4 could be detected in APP23 transgenic mice as compared with non-transgenic siblings. However, amyloid-associated induction of all other TREM family members was not detected (data not shown).

C1q expression in the murine brain is known to increase with age (Reichwald et al., 2009). Using *in situ* hybridization analysis, we were also able to detect a reproducible global increase in C1qA expression in murine brain sections that was apparent by 16 months of age (Figures 3A–3C). As previously reported, amyloid deposition was associated with increased C1qA expression (Figures 3D–3E) (Pisalyaput and Tenner, 2008; Reichwald et al. 2009). We observed a similar global age-associated increase in Tmem176b expression in

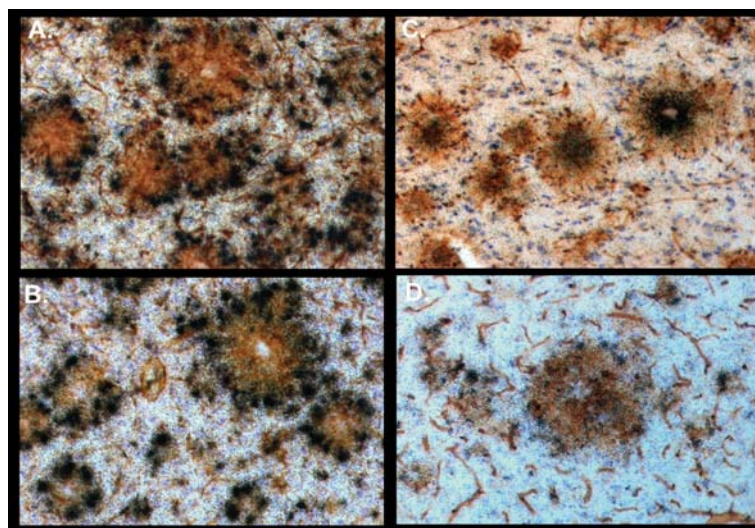


Figure 4 Different zones of amyloid-associated microglial activation are defined by regional gene expression

(A–D) Brain sections from 16-month-old APP23 transgenic mice. Microglia, macrophages and blood vessels are visualized in brown with tomato lectin. Nuclei are visualized in blue with haematoxylin. Expression of TREM2 (A), DAP12 (B), Tmem176b (C) and C1qA (D) are visualized by ³³P-labelled riboprobes (black grains in film emulsion).

non-transgenic WT mice that was apparent by 6 months of age (Figures 3G–3L). Expression of Tmem176b in Purkinje cells and cerebellar granule cells has also previously been reported (Maeda et al., 2006). In the present study, we observed an age-associated increase in Tmem176b expression in Purkinje and cerebellar granule cells that was independent of APP transgene expression. Tmem176 expression was also elevated in a punctate pattern of expression similar to that seen with C1qA riboprobes in the cortex and hippocampus of APP23 transgenic mice (Figures 3J–3L).

Expression patterns of TREM2/DAP12, Tmem176b and C1qA distinguish between zone-specific and global amyloid-associated activation of microglia

At the level of autoradiogram analysis all four molecules (TREM2, DAP12, Tmem176b and C1qA) showed similar amyloid-associated increases in expression (Figures 1 and 3). However, differences in their patterns of expression were readily detected when examined at the level of cell-type-specific expression (Figures 4 and 5). In Figure 4, activated microglia and macrophages can be detected surrounding amyloid plaques. Strikingly, whereas expression of TREM2 (Figure 4A) and DAP12 (Figure 4B) were robustly induced in subsets of

microglia on the outer zone of the plaques, Tmem176b (Figure 4C) was induced within the inner zone of amyloid plaques. C1qA (Figure 4D) served as a positive control demonstrating that all microglia in the region of amyloid plaques were activated and displayed elevated expression of C1qA. In Figure 5, right-pointing arrows indicate plaque-associated microglia that express either TREM2 (Figures 5A and 5B) or Tmem176b (Figures 5C and 5D), whereas left-pointing arrows indicate plaque-associated TREM2⁻ or Tmem176b⁻ microglia. Although amyloid plaque deposition was always associated with increased TREM2 expression (Figure 2), the ratio of TREM2⁺ to TREM2⁻ microglia in the outer zone of the plaque varied widely between animals. This same pattern of amyloid-associated TREM2, DAP12 and Tmem176b expression was also observed in all other APP and APP/PS1 transgenic mouse models examined (data not shown).

TREM2 ligand-binding activity is up-regulated in areas with amyloid plaque deposition

Several potential ligands have been reported for TREM2 that differ dramatically in their structures (Klesney-Tait et al., 2006). In addition, it is still unclear how many types of ligands can trigger TREM2-mediated activation and what is the identity of endogenous ligands expressed in the CNS. The unknown

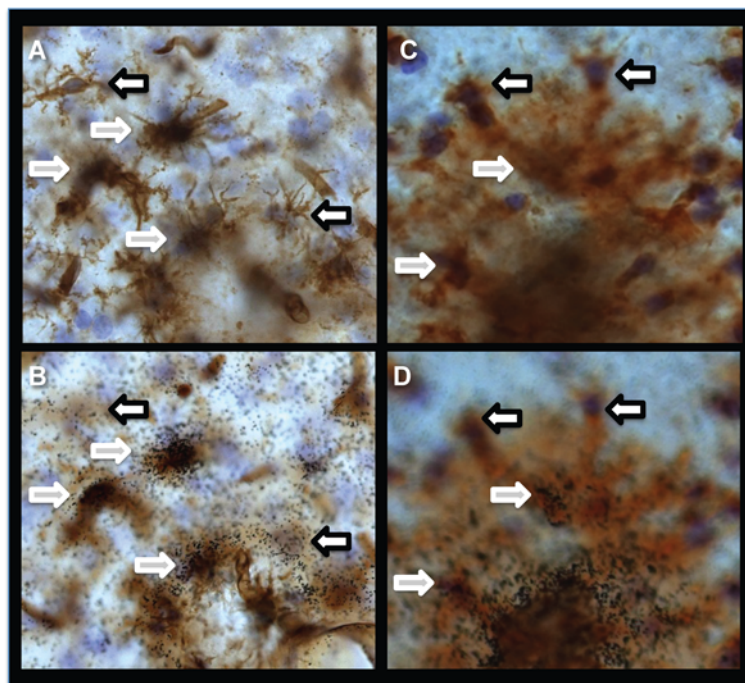


Figure 5 TREM2 and Tmem176b are expressed by plaque-associated microglia in the outer and inner zones respectively of the amyloid plaque

(A–D) Brain sections from 16-month-old APP23 transgenic mice. Microglia, macrophages and blood vessels are visualized in brown with tomato lectin. Nuclei are visualized in blue with haematoxylin. Expression of TREM2 (A and B) and Tmem176b (C and D) are visualized by ³³P-labelled riboprobes (black grains in film emulsion). (A and C) The focus at the level of the tomato lectin labelling. (B and D) The focus at the level of the film emulsion. Left-pointing arrows indicate examples of tomato-lectin-positive cells negative for riboprobe labelling. Right-pointing arrows indicate examples of tomato-lectin-positive cells positive for riboprobe labelling.

identity of CNS-expressed TREM2 ligand(s) precludes direct analysis of co-ordinate induction of TREM2 and its ligand(s). However, we and others have used a fusion protein composed of the extracellular domain of TREM2 fused to the Fc region of human IgG to detect TREM2 binding activity as an assay of putative TREM2 ligand(s) expression (Hamerman et al., 2006). TREM2 binding activity was detected in multiple cells in the region immediately surrounding amyloid plaques (Figures 6A and 6B). Although TREM2 binding activity can be detected on macrophages (Hamerman et al., 2006) and microglia (DS Davis and MJ Carson, unpublished data) by flow cytometry, TREM2 binding activity by Iba1⁺ microglia was only rarely detected by immunofluorescence in tissue sections (Figures 6C and 6E). However, the robust induction of TREM2 binding activity in the same regions expressing high levels of TREM2 suggests that TREM2 may regulate activation of microglia surrounding amyloid plaques (Figures 4 and 6D).

To test whether A β stimulated TREM2 expression directly, isolated primary microglia and BV-2 microglial cells were treated with aggregated amyloid for 24 h or 5 days. Flow-cytometric and qPCR analysis failed to detect A β -induced increases in microglial expression of TREM2 (data not shown). However, addition of aggregated amyloid for 5 days to mixed

glial cultures comprised of astrocytes, oligodendrocytes and microglia did result in increased expression of TREM2 that was comparable with the induction caused by TNF after 5 days of treatment (Figure 7A). Treating mixed glial cultures with both amyloid and TNF did not increase microglial expression of TREM2 more highly than that seen in cultures treated with amyloid only (Figure 7A).

Increasing TREM2 expression promotes amyloid phagocytosis in unstimulated cells

To test the consequences of increasing TREM2 expression, we used previously described overexpression and knock-down vectors to modulate TREM2 expression in BV-2 microglial cells (Takahashi et al., 2005). BV-2 cells were used because homogeneous cloned cell lines derived from single cells could be generated and maintained following transduction with over-expression and knock-down vectors. TREM2 has previously been reported to promote phagocytosis of apoptotic cell debris, selected classes of pathogenic bacteria, but not zymosan particles derived from the walls of *Saccharomyces cerevisiae*, *Candida albicans* or other types of particulate beads (Takahashi et al., 2005; N'Diaye et al., 2009). Therefore, using these cloned

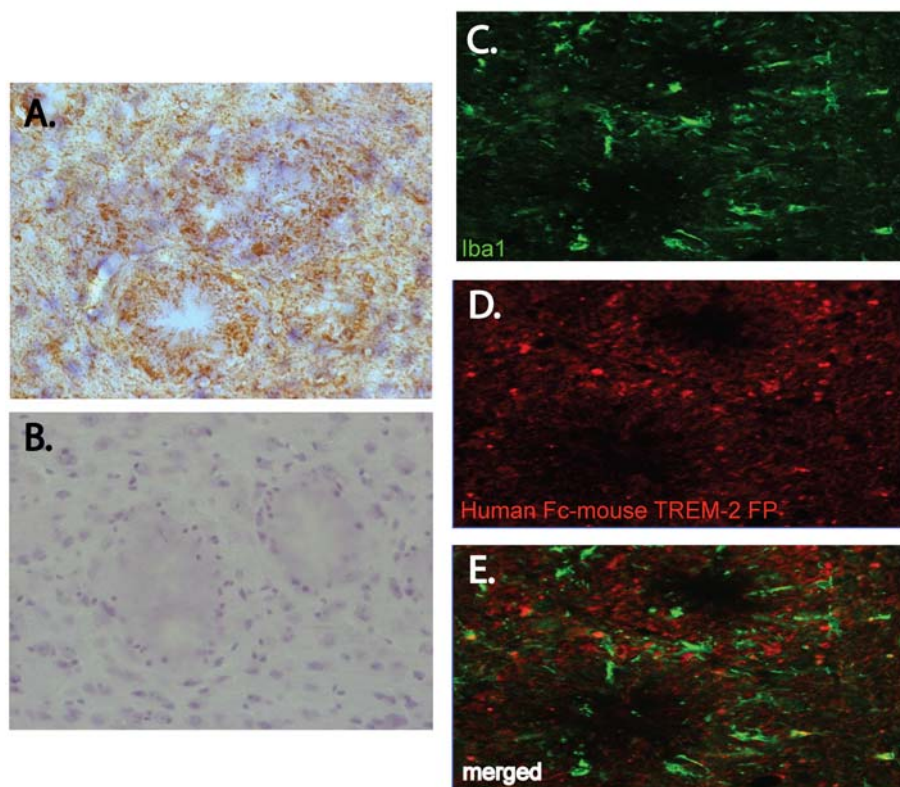


Figure 6 TREM2 binding activity is detected in the region of amyloid plaque deposition TREM2 expression is induced by aggregated amyloid and promotes amyloid phagocytosis

TREM2-IgG fusion protein labelling of APP23 brain sections is visualized in brown by DAB (diaminobenzidine) immunohistochemistry (A) and by immunofluorescence in red (D and E). Microglia and macrophages are visualized by immunofluorescence with Iba1 in green (C and E). In (A and B) nuclei are visualized in blue with haematoxylin. (B) The absence of immunolabelling with secondary antibodies directed against human IgG. (E) The merged images of (C and D).

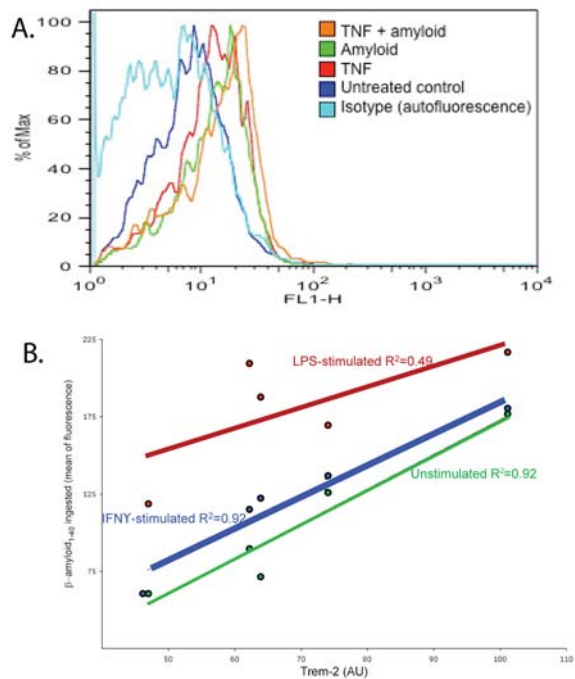


Figure 7 TREM2 expression is induced by aggregated amyloid and promotes amyloid phagocytosis

(A) Flow-cytometric analysis of TREM2 expression of the surface of CD11b⁺ microglia in primary mixed glial cultures treated for 5 days with TNF only, amyloid only or both TNF and amyloid. (B) Flow-cytometric analysis of cloned BV-2 microglial cell lines phagocytosis of fluorescently labelled AB1-40 (mean fluorescence intensity relative to WT BV-2 cells compared with TREM2 surface expression) in untreated (green line), IFN γ -treated (blue line) and LPS-treated (red) cell lines. APC (allophycocyanin)-labelled TREM2 antibodies were used to quantify the level of TREM2 expression in amyloid-treated BV-2 cell lines.

BV-2 microglial cell lines, we tested whether the level of TREM2 expression would correlate positively with amyloid phagocytosis (Figure 7B). We found that increasing TREM2 expression was tightly correlated ($r^2=0.92$) with phagocytosis of A β -(1-40) in a 5 h phagocytosis assay using unstimulated BV-2 cell lines (green line, Figure 7B). Previous studies examining TREM2-triggered phagocytosis had not examined whether prior cell activation altered the propensity of TREM2 to regulate microglial phagocytosis. Therefore we also examined A β phagocytosis following a 24 h stimulation with LPS (red line, Figure 7B) or IFN γ (blue line, Figure 7B). Strikingly, whereas IFN γ did not alter the correlation between increasing TREM2 expression and increasing A β phagocytosis ($r^2=0.92$), treating cells with LPS did. LPS alone is a potent stimulant of amyloid phagocytosis. Adding aggregated A β after 24 h of LPS pre-treatment dramatically decreased the correlation between TREM2 expression and phagocytosis ($r^2=0.49$).

TREM2 expression is associated with decreased motility

TREM2 deficiency in humans is associated with alterations in the expression of multiple cytoskeletal molecules (Kiialainen

et al., 2007). We therefore tested whether modulating TREM2 expression would alter cell motility in standard scratch assays (Figure 8). In this assay, a uniform scratch is made in confluent cultures of BV-2 microglial cells. Within 6 h, WT BV-2 cells can be detected migrating into the 'scratch wound'. Figure 8 represent examples of migration observed in the BV-2 cells with the highest and lowest TREM2 levels. Using cloned BV-2 microglial cell lines, we found that increasing TREM2 levels was associated with a decrease in the number of cells found within the scratch zone 6 h post-scratch (Figures 8A and 8B). By contrast decreasing TREM2 expression correlated with increased numbers of BV-2 cells within in the scratch zone (Figures 8A and 8B).

TREM2 expression correlates with increased antigen-presenting cell function as assayed by T-cell proliferation and cytokine production

TREM2-mediated activation was initially described to increase the expression of molecules required for antigen-presentation to CD4⁺ T-cells (Bouchon et al., 2001). However, as yet, no direct examination of TREM2-regulated antigen-presenting cell activity has been reported. In the present study we compared the ability of two cloned BV-2 cell lines that differed ~5-fold in TREM2 expression to act as antigen-presenting cells to allogeneic CFSE-labelled BALB/c CD4⁺ T-cells. We used two measures of microglial antigen presentation: stimulation of T-cell proliferation and stimulation of T-cell cytokine production. T-cell proliferation was quantified by flow-cytometric analysis of CFSE level per cell. In Figures 9(A) and 9(B), representative data from a single experiment is shown. In Figure 9(C) data from six experimental replicates is quantified, demonstrating the reproducibility of TREM2 modulation of microglial antigen-presenting cell function. After 5 days, only 3.7% of CD4⁺ T-cells completed six or more rounds of cell division when stimulated by allogeneic TREM2lo BV-2 cells (Figure 9A). By contrast, nearly 37% of CD4⁺ T-cells underwent six or more rounds of cell division when stimulated by allogeneic TREM2hi BV-2 microglial cells. When assaying cytokine and chemokine production in the supernatants of BV-2 microglial-CD4⁺ T-cell cultures, we could not detect expression of IL-12, IFN γ , IL-6 or the anti-inflammatory cytokine IL-10 in T-cell cultures stimulated with either TREM2lo or TREM2hi BV-2 microglial cells. By contrast, both TNF and CCL2 were readily detected in T-cells stimulated with either allogeneic TREM2lo and TREM2hi BV-2 microglial cells. However, ~4-fold higher levels of TNF and CCL2 were detected in T-cell cultures stimulated with allogeneic TREM2hi BV-2 microglial cells. Taken together these data demonstrate that higher levels of TREM2 correspond with greater antigen-presenting cell function in microglia without triggering T-cell production of IFN γ . Increased TREM2 expression and increased antigen-presenting cell function only loosely correlated with increased MHC class II expression. TREM2hi cell lines displayed ~1.5-fold higher levels of MHC class II as quantified by flow-cytometric analysis (data not shown).

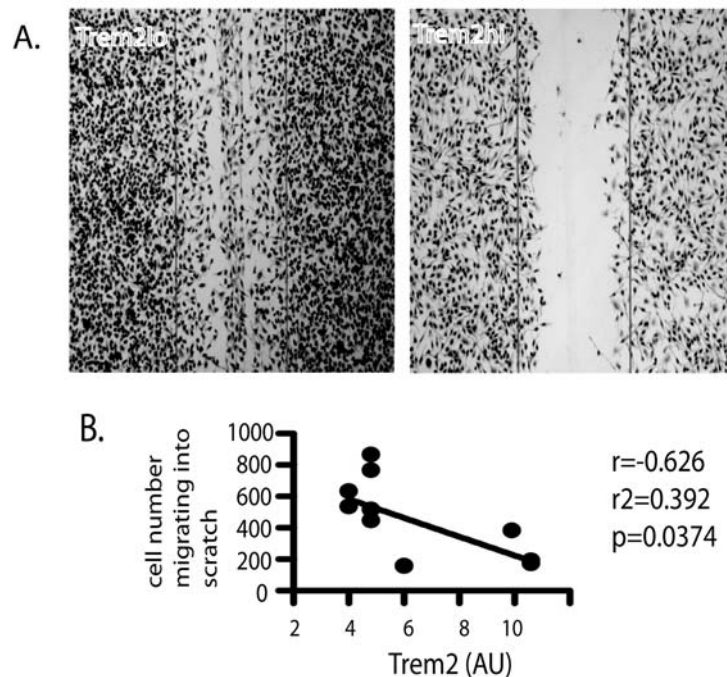


Figure 8 TREM2 expression negatively correlates with cell motility

The number of cells within the scratch zone was quantified as a function of TREM2 expression per culture 6 h post-injury. (A) Representative migration in scratched confluent TREM2lo (left-hand side) and TREM2hi (right-hand side) cultures. (B) Number of cells per scratch zone as a function of TREM2 mRNA of each scratch-tested culture. The Pearson correlation co-efficient was used to determine significance.

Tmem176b overexpression promotes cell death

Transfection of Tmem176b into immature antigen-presenting cells prevents their differentiation into mature potent antigen-presenting cells. Therefore we wished to repeat the T-cell proliferation assays with Tmem176b-overexpression cloned cell lines. However, we were unable to generate Tmem176b cell lines with stable Tmem176b overexpression. We therefore analysed the effect of Tmem176b on cell death using annexin V labelling. We observed a nearly 40% increased rate of cell death as judged by increased annexin V labelling 48 h following transfection of either RAW macrophage (Figure 10A) and BV-2 microglial (Figure 10B) cells with Tmem176b overexpression vectors as compared with cells transfected with GFP-only overexpression vectors. Transfecting BV-2 microglial and RAW macrophage cells with a Tmem176b-overexpression vector had no effect on amyloid phagocytosis (data not shown).

DISCUSSION

Microglial activation associated with amyloid plaque deposition is a well-recognized feature of Alzheimer's disease and of amyloid pathogenesis in transgenic mouse models (Schwab et al., 2009; Cameron and Landreth, 2010; Graeber and Streit,

2010). Repetitive two-photon imaging reveals that microglia quickly recognize and accumulate around $A\beta$ aggregates. However, the consequences of amyloid-associated microglial activation are under substantial debate. Notably, the accumulation of $A\beta$ is associated with insufficient or dysregulated microglial phagocytic activity (Schwab et al., 2009; Cameron and Landreth, 2010; Graeber and Streit, 2010). In addition, microglial production of pro-inflammatory cytotoxic molecules is suggested to play at least a partial role in neuronal dysfunction. Indeed, two-photon imaging of amyloid transgenic mice has revealed active microglial interactions with neurons in areas with detectable neuronal loss (Fuhrmann et al., 2010). Although microglial expression of CX3CR1 has been linked with limiting microglial neurotoxicity in a wide array of neurodegenerative models, deletion of CX3CR1 reduced neuronal loss in amyloid transgenic mice (Cardona et al., 2006; Fuhrmann et al., 2010). These studies suggest that microglia detrimentally contribute to amyloid pathogenesis. By contrast, other studies have raised doubts as to the functional significance of microglial activation in the disease process. For example, no changes in amyloid pathogenesis were detected following acute genetic [HSVTK (herpes simplex virus thymidine kinase)] triggered ablation of large portions of proliferating microglia (Grathwohl et al., 2009).

In many studies, all microglia/macrophages are analysed as a single population. However, studies using bone marrow chimaeric mice to genetically manipulate peripheral macrophages

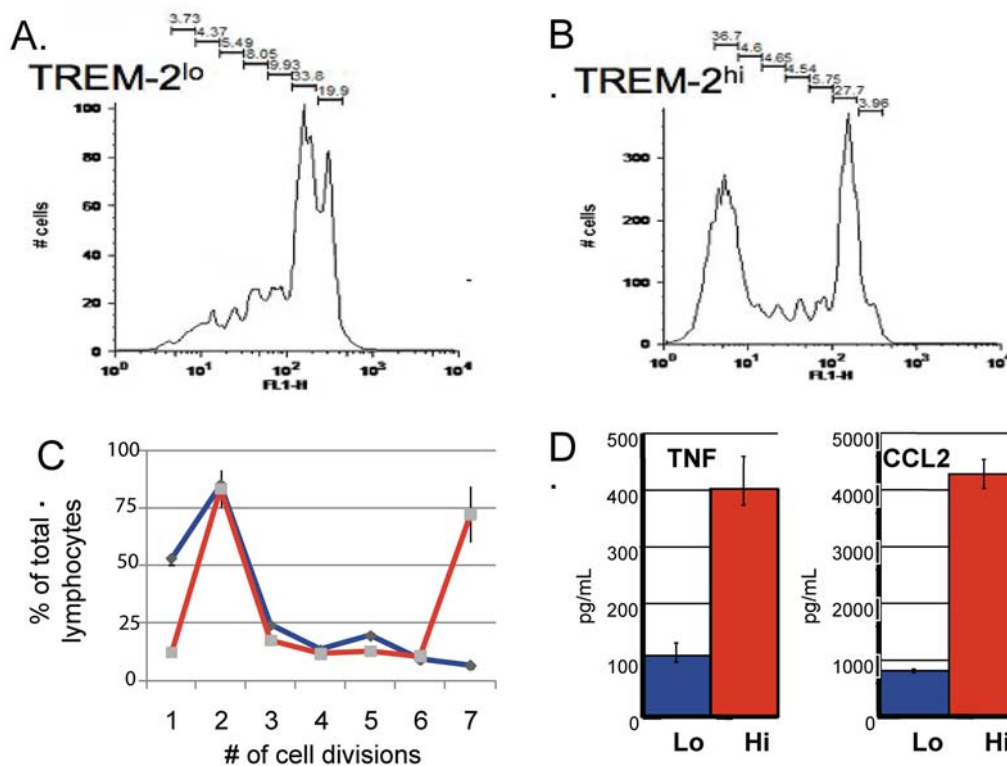


Figure 9 TREM2 expression positively correlates with antigen presentation

Cloned BV-2 microglial cell lines with ~10-fold difference in TREM2 RNA expression were used to stimulate the proliferation of allogeneic (BALB/c) CFSE-labelled CD4⁺ T-cells. Representative flow-cytometric analysis of T-cell proliferation using TREM2^{lo} BV-2 cells or when using TREM2^{hi} are depicted in (A) and (B) respectively. (C) The mean number of cells \pm S.E.M. undergoing each round of cell division in T-cells stimulated with TREM2^{lo} (blue line) or TREM2^{hi} (red line) BV-2 cells. (D) The levels of TNF and CCL2 found in the supernatants of T-cell cultures stimulated with TREM2^{lo} (blue bars) or TREM2^{hi} (red bars).

compared with CNS-resident microglia have demonstrated that these two types of macrophages are not functionally identical (reviewed in Carson et al., 2006). Specifically, several groups have reported that CCR2⁺ CNS-infiltrating macrophages have greater A β phagocytic ability than CNS-resident microglia (El Khoury et al., 2007; Yong and Rivest, 2009). Our present study reveals additional heterogeneity in the macrophage populations that define specific activation zones associated with amyloid plaques in APP23 mice. While C1qA expression was detected in all plaque-associated myeloid cells, Tmem176b expression was most prominent in cells in the inner zone of plaques. By contrast, TREM2 was most prominently induced in cells on the outer zones of the plaques.

The mechanisms driving this highly localized expression of TREM2 and Tmem176b are as yet undefined. However, our data implicate glia and neurons as playing critical regulatory roles. Addition of aggregated A β to primary microglia and BV-2 cells failed to increase expression of TREM2 or Tmem176b (data not shown). However, microglial expression of TREM2 was increased when microglia were exposed to A β in the presence of the multiple cell types present in standard mixed glial cultures. By contrast, microglial expression of Tmem176b did not change with this treatment, perhaps because microglial expression of

Tmem176b is already very high in mixed glial culture microglia. We speculate that in mixed glial cultures, the high levels of Tmem176b expression may play a role in maintaining the constant percentage of microglia observed in mature mixed glial cultures (Carson et al., 1998). High Tmem176b expression may limit microglia from increasing as a percentage of cells in culture, even though these cells are continually proliferating by promoting microglial cell death.

Expression of a receptor does not necessarily indicate that a cell is constitutively activated by that receptor. However, in our transgenic model, the expression of DAP12, the adaptor molecule mediating TREM2 intracellular signalling, and TREM2 binding activity were both detected in the same plaque-associated regions as TREM2 expression. In addition, flow-cytometric analysis reveal that macrophages and microglia constitutively express TREM2 binding activity (Hamerman et al., 2006). Therefore TREM2 expression probably leads to TREM2-mediated activation.

Our *in vitro* assays suggest that this differential expression will have functional consequences for processes relevant to disease progression. For example, our data suggest that TREM2/DAP12^{hi} cells are less likely to migrate away from plaques owing to lower motility. The robust phagocytic-inducing properties of LPS by-passes or reduces the ability of

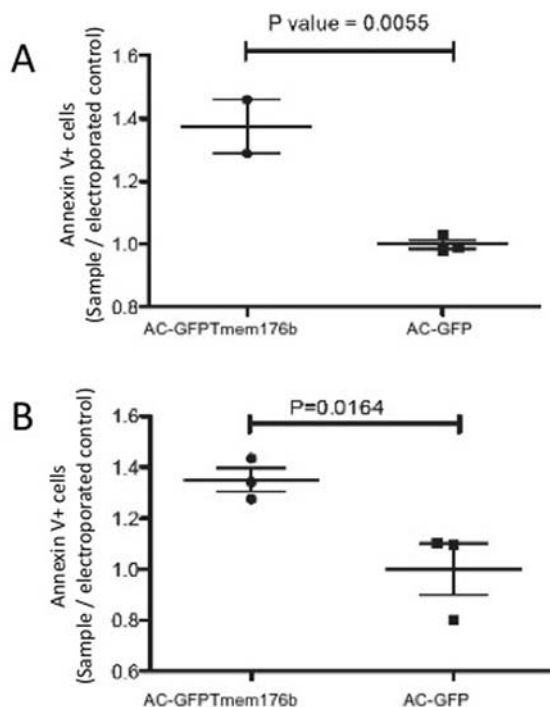


Figure 10 Tmem176b overexpression promotes annexin V labelling

RAW macrophage (A) and BV-2 microglial (B) cell lines were transiently transfected with Tmem176b-GFP overexpression or GFP-only overexpression plasmids. Annexin V labelling of GFP⁺ cells was determined 48 h post-transfection using flow-cytometric analysis (means \pm S.E.M. for three replicate experiments). Between replicate experiments, the final total cell death ranged from ~35 to ~50% of cultured cells. Significance was determined using the Student's *t* test.

TREM2 to modulate A β phagocytosis. Thus TREM2/DAP12hi cells may only be more phagocytic than TREM2/DAP12lo cells when in environments lacking other strong phagocytic stimuli, such as those mediated by TLR4 (Toll-like receptor 4; the LPS receptor). These data suggest that the relative importance of TREM2-triggered phagocytosis may be less important for amyloid clearance and more important for antigen capture prior to antigen-presentation to CD4⁺ T-cells. It is also possible that other microglial-expressed DAP12-associated receptors, such as SIRPB1, may play more prominent roles in regulating A β phagocytosis (Gaikwad et al., 2009). Our data would also suggest that these other DAP12-associated functions would also be limited to the subset of DAP12-expressing microglia surrounding the outer zone of amyloid plaques.

T-cells are not generally hypothesized to play critical roles in Alzheimer's disease. However, the function of microglial antigen-presentation has been hypothesized to differ from that of the professional immune system (Byram et al., 2004; Carson et al., 2006). Specifically, microglia are inefficient in promoting or sustaining pro-inflammatory T-cell responses (Hickey and Kimura, 1988; Carson et al., 1998, 1999). Thus microglia are unlikely to play biologically significant roles in the initiation of T-cell activation. Rather, after initial T-cell activation, T-cell

function is dependent on antigen-presentation within a tissue to localize and direct T-cell responses. As demonstrated in a model of facial axotomy, microglia are not only more effective in driving neuroprotective T-cell responses than non-CNS antigen-presenting cells, antigen presentation by microglia can play absolutely essential roles in eliciting neuroprotection, even in the presence of CNS-infiltrating peripheral antigen-presenting cells (Davis and Carson, 2009).

The role of TREM2 in promoting neuroprotective responses is suggested by three observations. First, in ongoing studies, we have also observed induction of microglial expression of both TREM2 and Tmem176b in the facial axotomy model at times co-incident with the generation of neuroprotective T-cell responses (data not shown and DS Davis and MJ Carson, unpublished data). Secondly, blocking TREM2 function during EAE increased the severity of EAE. Thirdly, introduction of TREM2 into peripheral antigen-presenting cells promoted more complete remission in a relapsing remitting model of EAE.

Neuroprotective T-cell responses may also play roles in more than neuronal survival. T-cells can limit TNF-induced influx of neurotoxic macrophages into the CNS (Stalder et al., 1998). More recently, several studies have demonstrated that mice lacking T-cells have dramatically reduced performance in learning and memory tasks (Brynskikh et al., 2008; Derecki et al., 2010). Supplementation of lymphocytes to T-cell-deficient mice improves performance in these assays. *In vivo*, both TREM2 and Tmem176b expression have been correlated with reduced pathology following autoimmune responses (Louvet et al., 2005; Takahashi et al., 2007). Taken together with the data presented in our present study, it is tempting to speculate that, in amyloid pathology, Tmem176b may promote death of activated macrophages, whereas TREM2-triggered microglial antigen-presentation to CD4⁺ T-cells may provide neuroprotection, albeit insufficiently. However, this same type of microglial activation may interfere with the effectiveness of active immunization therapies designed to elicit pro-inflammatory CD4⁺ T-helper responses against plaque-associated molecules. Alternatively, plaque-associated activation of microglia may elicit T-cell-mediated neuroprotection and 'wound repair responses' that help maintain optimal CNS function during immune therapies with pro-inflammatory consequences (Fisher et al., 2010). Relevant to this last speculation is the observation that epithelial repair in a stem-cell-dependent model of colonic injury/repair was incomplete and associated with an increase in pro-inflammatory macrophages in TREM2-KO mice (Seno et al., 2009). Future studies using TREM2 and Tmem176b KO and overexpression models should help define whether specific forms of plaque-associated microglial activation should be promoted to provide neuroprotection during immune therapies.

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