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NFATc2 Modulates Microglial Activation in the AβPP/PS1 Mouse Model of Alzheimer's Disease

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Abstract

Alzheimer's disease (AD) brains are characterized by fibrillar amyloid-β (Aβ) peptide containing plaques and associated reactive microglia. The proinflammatory phenotype of the microglia suggests that they may negatively affect disease course and contribute to behavioral decline. This hypothesis predicts that attenuating microglial activation may provide benefit against disease. Prior work from our laboratory and others has characterized a role for the transcription factor, nuclear factor of activated T cells (NFAT), in regulating microglial phenotype in response to different stimuli, including Aβ peptide. We observed that the NFATc2 isoform was the most highly expressed in murine microglia cultures, and inhibition or deletion of NFATc2 was sufficient to attenuate the ability of the microglia to secrete cytokines. In order to determine whether the NFATc2 isoform, in particular, was a valid immunomodulatory target *in vivo,* we crossed an NFATc2$^{-/-}$ line to a well-known AD mouse model, an AβPP/PS1 mouse line. As expected, the AβPP/PS1 × NFATc2$^{-/-}$ mice had attenuated cytokine levels compared to AβPP/PS1 mice as well as reduced microgliosis and astrogliosis with no effect on plaque load. Although some species differences in relative isoform expression may exist between murine and human microglia, it appears that microglial NFAT activity is a viable target for modulating the proinflammatory changes that occur during AD.

Keywords

Alzheimer's disease; amyloid; cytokine; microglia; neuroinflammation; NFAT; transcription factor

INTRODUCTION

The transcription factor, nuclear factor of activated T cells (NFAT), has a classically described role in regulating immune cell phenotype [1–11]. Through calcineurin dependent

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dephosphorylation, it is able to interact with additional transcription factors to regulate transcription of numerous genes in diverse immune cell types [2, 4, 5, 7, 8, 10, 12–19]. In addition, there are several isoforms of NFAT (NFATc1-c4 and NFAT5) that demonstrate both cooperative and separate effects on regulating cellular phenotype depending upon the context [20–26].

Significant work in the field has shown that NFAT proteins are not restricted to immune cells. In the brain, NFAT isoforms now have well described roles in regulating the phenotypes of neurons [27–32], astrocytes [33–42], and microglia [38, 43–47] during both normal physiology and disease. Although several extensive studies have characterized the ability of NFAT isoforms to regulate astrocyte phenotype during AD [33, 36, 39, 41, 48, 49], our prior work has focused on understanding the role of NFAT in regulating microglial phenotype during disease [46]. Comparing both *in vitro* and *in vivo* inhibitory strategies using pharmacologic and peptide-based inhibitors, we have observed a critical role of NFAT in regulating microglial activation state in response to amyloid-β (Aβ) stimulation [46].

Based upon importance of NFAT in regulating microglial proinflammatory activation and the fact that robust microgliosis is associated with Aβ plaque deposition during disease, we hypothesized that inhibition of microglial NFAT activity may be an attractive target for attenuating disease. We identified NFATc2 (NFAT1) as the most abundantly expressed isoform in primary murine microglia. Based upon this isoform predominance, we tested whether loss of NFATc2, in particular, was sufficient to attenuate the gliosis and proinflammatory changes associated with disease. Although NFAT isoforms may have redundant functions, it is feasible that NFATc2 may be more selectively targeted for future drug design compared to other isoforms [50]. To assess the relative importance of this isoform during disease, we examined the response of murine wild type and NFATc2 knockout (NFATc2$^{-/-}$) microglia and NFATc2 knockdown using siRNA to Aβ stimulation. We also crossed the NFATc2$^{-/-}$ mice to a well-known transgenic mouse model of AD, an AβPP/PS1 line. Although human NFAT isoform relative expression levels may differ from mice, our findings demonstrate that NFATc2 has a positive role in regulating microglial cytokine secretion *in vitro* and microglial activation phenotype *in vivo* and supports the idea that inhibiting NFAT activity is a valid approach for limiting microgliosis during AD.

MATERIALS AND METHODS

Animals

C57BL/6 and AβPP/PS1 (strain 005864 B6.Cg-Tg(APPswe,PSEN1dE9)85Dbo) transgenic mice were purchased from Jackson Laboratory (Bar Harbor, ME). The transgenic animals express the human *AβPP*, amyloid beta (A4) precursor protein and the human *PSEN1,* presenilin 1. NFATc2$^{-/-}$ (strain 000197-UNC B6; 129-*Nfatc2*^{tm1Rao}/Mmnc) mice were purchased from Mutant Mice Regional Resource Center (MMRRC). These mice have a complete loss of NFATc2 (NFAT1) protein due to disrupted gene. NFATc2$^{-/-}$ mice were bred with AβPP/PS1 mice to produce F1 offspring. The heterozygous F1 generation of AβPP/PS1xNFATc2$^{+/-}$ and NFAT$^{+/-}$ mice were then bred to each other to produce F2 offspring consisting of AβPP/PS1, wild type (WT), NFATc2$^{-/-}$, NFATc2$^{+/-}$, AβPP/PS1xNFATc2$^{-/-}$, and AβPP/PS1xNFATc2$^{+/-}$. AβPP/PS1, wild type (WT), NFATc2$^{-/-}$, and

AβPP/PS1xNFATc2$^{-/-}$ mice with the same genetic background were then maintained at the University of North Dakota's Center for Biomedical Research. This breeding strategy also insured that littermates were used for comparison. The AβPP/PS1xNFATc2$^{-/-}$ mice are now available from MMRRC (B6.Cg-Nfatc2^{tm1Rao} Tg(APPswe,PSEN1dE9)85Dbo/Mmmh). Only male mice were used at 8 months of age with an n = 8–10 per genotype. All animals were maintained with standard housing conditions in a 12-h light/dark cycle with *ad libitum* food and water until use. All animal experiments were approved by the UND Institutional Animal Care and Use Committee (IACUC). The investigation conforms to the National Research Council of the National Academics Guide for the Care and Use of Laboratory Animals (8th edition).

Primary cultures

Microglia were grown from the brains of C57BL/6 and NFATc2$^{-/-}$ mice at postnatal day 1–3 according to our prior protocols [51]. Microglia were isolated from mixed glial cultures at 14 days *in vitro* for use. The remaining astrocytes were passaged and plated for use according to our prior work [52]. Microglia and astrocyte cultures were maintained in DMEM/F12 (Life Technologies, Carlsbad, CA) media supplemented with 10% heat inactivated fetal bovine serum, 5% heat inactivated horse serum and antibiotics (penicillin, streptomycin, neomycin) at 37°C and 5% CO_2. Primary cortical neurons were grown from E16 mouse brains in Neurobasal media with B27supplements (Life Technologies, Carlsbad, CA) according to our prior protocol [53].

Microglial cell lines, BV2 and HMC3

Immortalized murine microglial BV2 cells were obtained from Dr. Gary E. Landreth, Cleveland, Ohio. Human microglial cell line, HMC3, obtained from the ATCC (Manassas, VA), were maintained in DMEM/F12 (Gibco RBL, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 5% horse serum, and 1% antibiotics (penicillin, streptomycin, neomycin).

NFAT knockdown using siRNA

Murine microglial cells, BV2, were transfected with an eGFP expression plasmid, control siRNA, or mouse siGENOME SMARTpool siRNA for NFATc1, NFATc2, and NFATc4 (GE Dharmacon, Lafayette, CO) using the appropriate Nucleofector program as described by the manufacturer (Amaxa Inc., Gaithersburg, MD). Human microglial cells, HMC3, were transfected with an eGFP plasmid or human siGENOME SMARTpool NFATc4 siRNA using Lipofectamine 3000 according to the manufacturer's protocol. pCMV-eGFP plasmid was a gift from Dr. Saobo Lei and Nicholas Cilz and was used as transfection control in all knockdown experiments. Cells were lysed using RIPA buffer (20mM Tris, pH 7.4, 150mM NaCl, 1mM Na3VO4 10mM NaF, 1mM EDTA, 1mM EGTA, 0.2mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 0.1% SDS, and 0.5% deoxycholate) 48-h post-transfection and lysates used for western blot analyses.

Cell stimulation

Primary murine microglia were stimulated with or without lipopolysaccharide (LPS, Sigma-Aldrich, St. Louis, MO) or human $A\beta_{1–42}$ (rPeptide, Bogart, GA) prepared in distilled water and used immediately as a mixture of conformations. Cells were preincubated 30 min with or without 1 μM FK506 (Alexis Biochemicals, Enzo Life Sciences, Farmingdale, NY) or vehicle control (DMSO) prior to Aβ or LPS stimulations. BV2 cells were transfected with control, NFATc1, or NFATc2 siRNA using nucleofection. Cells were stimulated with either 10 μM scrambled $A\beta_{1–42}$ or different concentrations of monomeric $A\beta_{1–42}$ or fibrillar $A\beta_{1–42}$ at 48-h post transfection. Media from stimulated cells was collected 24-h post-treatment and TNF-α levels quantified using commercial ELISA (R&D Systems, Minneapolis, MN).

Western blotting

Hippocampi of male 8-month-old WT, NFATc2$^{-/-}$, AβPP/PS1, and AβPP/PS1xNFATc2$^{-/-}$ mice (n = 8) were collected from the left hemispheres, flash frozen, lysed in RIPA buffer, and resolved using 10% SDS-PAGE. Western blot analyses were performed with antibodies specific for different NFAT isoforms (NFAT c1, c3, c4, Santa Cruz Biotechnology, Dallas, TX; NFATc2, Cell Signaling Technology, Danvers, MA), AβPP (Y188, Abcam, Cambridge, MA), PSD95 (Cell Signaling Technologies), synaptophysin (EMD Millipore, Billerica, MA), and TNFα (Abcam, Cambridge, MA). Western blots were also performed from culture lysates of primary mouse microglia, neuron, and astrocytes using the same anti-NFAT antibodies. In all cases, loading control antibodies recognizing either α-tubulin or actin were used for densitometric quantitation using Adobe Photoshop software (Adobe Systems, San Jose, CA).

NFAT c1 and c2 transcription factor activation assay

NFATc1 and c2 transcription factor activity assays were performed using nuclear lysates prepared from primary mouse microglia cultures. Cells were plated in serum free DMEM/F12 media overnight before stimulation with 10 μM$A\beta_{1–42}$ for 1 h at 37°C. After the stimulation was over, the plates were washed with PBS and nuclear lysates were prepared following the manufacturer protocol (Active Motif, Carlsbad, CA). The amount of protein in the nuclear lysates was determined by the Bradford assay [54] then used to perform activity assessments using the TransAm NFATc1 kit (Active Motif, Carlsbad, CA) with slight modification of the manufacturer protocol to substitute with an anti-NFATc2 detection antibody for quantifying NFATc2 activity as well. Activities of both NFATc1 and c2 were measured with a plate reader (Biotek, Winooski, VT) at 450 nm with a reference filter at 630 nm.

Enzyme-linked immunosorbent assay (ELISA)

Commercial cytokine ELISAs were performed using media collected from murine microglia or BV2 cells stimulated with or without LPS, $A\beta_{1–42}$, or FK506 for 24 h according to the manufacturer's protocols (R&D Biosystems, Minneapolis, MN).

Tissue cytokine mRNA Real-time PCR

Flash frozen spleens from 8-month-old WT, NFATc2$^{-/-}$, AβPP/PS1, and AβPP/PS1xNFATc2$^{-/-}$ mice were lysed using Qiazol RNA lysis buffer and RNA was isolated using Qiagen RNeasy Lipid Tissue Kit (Qiagen, Germantown, MD) according to the manufacturer's protocol. For amplification of mRNA for TNF-α expression, 150 ng of RNA was used as a template for performing real-time PCR using the iTaq Universal SYBR Green One-Step kit (Bio-Rad, Hercules, CA). Fold change in the level of mRNA expression for each group was calculated as 2^-ΔΔ*C*t with respect to average mRNA expression of the WT group. The values obtained for 5 animals per group were averaged and plotted (±SD; *$p < 0.05$).

Immunohistochemistry

For mouse brain histology, right hemispheres from brains of 8-month-old WT, NFATc2$^{-/-}$, AβPP/PS1, and AβPP/PS1xNFATc2$^{-/-}$ mice ($n = 8$) were collected, fixed in 4% paraformaldehyde for 48 h, then changed to 30% sucrose in PBS for 14 days changing the sucrose every 4 days. The fixed tissue was then embedded in a matrix for 40 μM serial sectioning (Neuroscience Associates, Knoxville, TN). Serial sections per well were immunostained using anti-Iba-1, CD68, GFAP (Cell Signaling technology, Danvers, MA), and Aβ (4G8, Biolegend, San Diego, CA) antibodies. NFATc2 immunostaining was performed with anti-NFATc2 (Cell Signaling Technology, Danvers, MA) on brain sections from AβPP/PS1 mice. Antibody binding was visualized using Vector VIP as the chromogen. A set of serial sections were also stained using the Campbell-Switzer stain (Neuroscience Associates, Knoxville, TN). In order to quantitate immunostaining, 1.25X pictures of 3 consecutive sections (960 μm apart) throughout the temporal cortex region were obtained. Images were taken using an upright Leica DM1000 microscope and Leica DF320 digital camera system. Optical densities from similar temporal cortex regions for each brain section were measured using Adobe Photoshop software. The optical density values per condition (4 conditions), per brain (10 brains per condition) and per section (3 sections per brain) were obtained, averaged and plotted. Representative images using 20X magnification are shown.

Statistical analysis

Data are shown as mean ± standard deviation (SD) and representative of three independent experiments unless noted otherwise. Values statistically different from controls were determined using Student's *t test,* one-way ANOVA, or two-way ANOVA where appropriate. The Tukey-Kramer *post hoc* test or Holm-Sidak multiple pair-wise comparisons were used to determine *p*-values where applicable.

RESULTS

NFATc2 was activated by Aβ stimulation in murine microglia cultures

In order to assess the relative abundance of NFAT isoform expression across cell types in the brain, we compared protein levels of NFAT isoforms in cultured murine microglia, astrocytes, and neurons by western blot analysis. As expected, multiple molecular weight bands were detected for each isoform across cell types consistent with the many splice

variants that exist for the proteins [55]. Although detectable levels of NFATc1, c2, and c4 were observed in microglia, NFATc2 appeared the most abundant isoform (Fig. 1A). To validate that the isoforms were behaving as expected, microglia were stimulated with Aβ and activity of NFATc1 and c2 were quantified. Surprisingly, Aβ stimulated increased activity of only NFATc2 and not NFATc1 perhaps reflecting some specificity of response (Fig. 1B). Although the banding pattern of anti-NFAT antibodies on a western blot varied depending upon their isoforms, the antibodies used for this study were validated using siRNA against their respective NFAT proteins. The murine microglial cell line, BV2, was transfected with siRNA for NFATc1, NFATc2, NFATc3, and NFATc4. Western blot analysis demonstrated decreased protein in the NFATc1 and NFATc2 knock down samples with no difference in NFATc3 blots since they were negative regardless (Fig. 1C). NFATc4 knockdown did not attenuate band detection of the NFATc4 western blots suggesting that this antibody was not specific for murine NFATc4 (Fig. 1C). However, NFATc4 siRNA knockdown using the human microglial cell line, HMC3, demonstrated a clear attenuation of western blot detection demonstrating the specificity of the NFATc4 antibody for human protein and validating expression of NFATc4 in human microglia (Fig. 1D). This data suggests a possible difference in abundance of particular NFAT isoform(s) across species.

NFATc2 knockdown had an attenuated proinflammatory phenotype following Aβ or LPS stimulation

Based upon the observed increase in NFATc2 activity, we hypothesized that it would be required for the subsequent proinflammatory phenotype changes that occurred in microglia upon Aβ stimulation. To test this idea, cultures of NFATc2$^{-/-}$ and C57BL/6 microglia were compared following Aβ and bacterial endotoxin LPS stimulation. Morphologically, NFATc2$^{-/-}$ microglia appeared similar to C57BL/6 cells with robust immunoreactivity for the microglial marker, CD68 (Fig. 2A). Moreover, the absence of NFATc2 expression did not appear to reflect in any development compensatory increase in relative levels of other isoforms in these cells (Fig. 2B). However, stimulation of either cell type with Aβ or LPS demonstrated significantly attenuated secretion of both IL-6 and TNFα in the NFATc2$^{-/-}$ cells compared to C57BL/6 (Fig. 2C-F). More importantly, although pretreatment of C57BL/6 microglia with the calcineurin/NFAT inhibitor, FK506 [56, 57], was sufficient to attenuate cytokine secretion, it had no inhibitory ability on the NFATc2$^{-/-}$ cells (Fig. 2E,F). This demonstrated that the drug inhibitory effect in microglia required NFATc2 activity. This was further validated using siRNA knockdown of NFATc2 in the BV2 cells. Cells transfected with NFATc2 siRNA were treated with either scrambled Aβ or varying concentrations of monomeric or fibrillar Aβ. Monomeric and fibrillar Aβ dose-dependently stimulated TNFα secretion from the BV2 cells (Fig. 2G). As expected, siRNA knockdown of NFATc2 attenuated cytokine secretion (Fig. 2G). However, siRNA knock down of NFATc1 also attenuated Aβ-stimulated TNFα secretion although less effectively than NFATc2 knockdown (Fig. 2G). Nevertheless, this data suggested that NFATc1 may also have some role in regulating the Aβ-stimulated phenotype in microglia.

AβPP/PS1xNFATc2$^{-/-}$ mice had attenuated brain cytokine levels

Based upon the relative importance of NFATc2 in regulating microglial activation observed *in vitro,* we hypothesized that it would have a similar importance *in vivo*. In order to test this

idea, we crossed the NFATc2$^{-/-}$ mice to a common AD mouse model of disease, AβPP/PS1 mice. Consistent with the *in vitro* observation, plaque associated microglia in 8-month-old mice demonstrated robust NFATc2 immunoreactivity in the AβPP/PS1 mice brains (Fig. 3A). More importantly, hippocampi from the AβPP/PS1xNFATc2$^{-/-}$ mice had attenuated membrane-associated TNFα levels compared to the AβPP/PS1 brains demonstrating the relative importance of NFATc2 in regulating microglial phenotype *in vivo* (Fig. 3B,F). To assess how significant the effect of NFATc2 deletion was on peripheral immune phenotype, TNFα mRNA levels were quantified from spleens. Surprisingly, TNFα mRNA levels were elevated in the AβPP/PS1 mice compared to WT mice demonstrating an overall systemic immune activation in these transgenic mice (Fig. 3G). However, TNFα mRNA was also significantly elevated in AβPP/PS1xNFATc2$^{-/-}$ spleens compared to WT mice (Fig. 3G). This is consistent with broader isoform expression in peripheral immune cells and likely redundancy of function. Additionally, an increase in protein levels of PSD95 but not synaptophysin that was observed in the AβPP/PS1 mouse hippocampi was attenuated in the AβPP/PS1xNFATc2$^{-/-}$ brains (Fig. 3D,E). AβPP/PS1xNFATc2$^{-/-}$ mice also had a slightly attenuated overexpression of AβPP compared to AβPP/PS1 mice bringing protein levels down to no significance from WT mice (Fig. 3C). These data demonstrated the importance of NFATc2 in regulating several changes in the AβPP/PS1 brains including proinflammatory state.

Aβ plaque load was not significantly different between AβPP/PS1 and AβPP/PS1xNFATc2$^{-/-}$ brains

In order to test whether the potential reduction in AβPP overexpression or an attenuated microglial activation phenotype, we observed in the AβPP/PS1xNFATc2$^{-/-}$ mice resulted in altered levels of Aβ accumulation in the brain, we next quantified plaque load in the 8-month-old mice. However, plaque load assessed by both anti-Aβ immunoreactivity and the Campbell-Switzer stain revealed no differences between the AβPP/PS1 and AβPP/PS1xNFATc2$^{-/-}$ mice (Fig. 4). This demonstrated that attenuated NFATc2 expression had no adverse effects on plaque accumulation.

Microgliosis and astrogliosis were attenuated in the AβPP/PS1xNFATc2$^{-/-}$ versus AβPP/PS1 mouse brains

Based upon the attenuated TNFα levels we observed in the AβPP/PS1xNFATc2$^{-/-}$ mice, we predicted attenuated activation of microglia, in particular. Quantitation of microgliosis via both CD68 and Iba-1 immunoreactivity demonstrated a significant reduction in AβPP/PS1xNFATc2$^{-/-}$ brains compared to AβPP/PS1 (Fig. 5). Interestingly, the AβPP/PS1xNFATc2$^{-/-}$ mouse brains also demonstrated a significantly lower level of astrogliosis, assessed by GFAP immunoreactivity, when compared to the AβPP/PS1 mouse brains (Fig. 5). These findings supported a role for NFATc2 in regulating proinflammatory changes in the brain particularly with respect to AD.

DISCUSSION

From our work as well as others it is clear that microglia express NFAT isoforms and these have a role in regulating cellular phenotype [38, 43–47]. In this study, we found that

NFATc2 is the most abundantly expressed isoform in murine microglia and its activity was selectively increased by Aβ stimulation. Moreover, NFATc2$^{-/-}$ microglia had significantly decreased TNFα and IL-6 secretion following Aβ and LPS activation. Consistent with the high level of NFATc2 expression, we observed in microglia *in vitro,* deletion of NFATc2 resulted in significant attenuation of proinflammatory TNFα levels and microgliosis in the AβPP/PS1xNFATc2$^{-/-}$ mice. Although NFATc2 was critical in regulating Aβ-stimulated cytokine secretion in murine cells, relative NFAT isoform expression level murine compared to human microglia is still needed. Nevertheless, the collective data support the idea that NFAT is an attractive target for attenuating gliosis in AD.

The possibility of selectively targeting NFATc2 compared to other isoforms remains appealing given the fact that it has a c-Jun N-terminal kinase (JNK) phosphorylation activation epitope unique from other NFAT isoforms [50]. This suggests it may be feasible to design small molecules for isoform selective inhibition. However, NFATc2$^{-/-}$ mice have been reported to have an enhanced TH_2 immune response [58], reduced muscle growth [59], dysfunctional chondrogenesis [60], and osteoarthritis [61]. In addition, it appears that aged NFATc2$^{-/-}$ mice can develop anemia and lymphocytosis as well [62]. Therefore, it is possible that long term complete inhibition of this NFAT isoform may have unwanted adverse effects. Nevertheless, the clinical utility of the widely used indirect NFAT inhibitors cyclosporine A (CsA) and FK506 [56, 57, 63] validate that NFAT inhibition, in principle, is a valid approach for long-term immunomodulation. Nevertheless, selective NFAT inhibition strategies are still required since adverse side effects of both CsA and FK-506 exist due to their indirect inhibitory mechanism that relies on inhibition of the phosphatase, calcineurin [63]. For example, small molecule [64, 65] as well as peptide based inhibitors [33, 36, 39, 41, 45, 66–70] of NFAT but not calcineurin have been described. It is likely that continued preclinical development in this area will produce agents with the immunomodulatory action of NFAT inhibition without the adverse consequences of nephrotoxicity associated with FK-506 and CsA [71, 72].

We have focused our study on primarily NFATc2 in microglia but appreciate that other isoforms are expressed as well. Indeed, our data showed potential differences in NFATc4 expression between human and mouse microglia. Although our Aβ stimulation showed no increase in NFATc1 activity, it is likely that these other isoforms may also be involved in regulating microglial activation particularly *in vivo* and future work with isoform selective inhibition or knockdown may better elucidate this. Defining the relative contribution of isoforms in regulating microglial phenotype is particularly important. Future work assessing particularly the role of individual isoforms in regulating human and murine microglial phenotype will be of interest.

Although we remain interested in microglial NFAT, it is clear that this transcription factor has an important role in regulating astrocyte activation as well. Indeed, since our animals have NFATc2 deleted from all cells we cannot exclude the possibility that the decrease in gliosis and cytokine levels observed in the AβPP/PS1xNFATc2$^{-/-}$ was not also due to loss of astrocyte activation. In fact, we did observe a significant decrease in GFAP positive astrocytic staining in these mice. This is entirely consistent with prior work demonstrating that astrocytic NFAT is important in regulating cellular phenotype during AD [33, 36, 39,

41]. Consistent with this pleiotropic role of the transcription factor, our data showed decreased gliosis, both microgliosis and astrogliosis, in the brains of AβPP/PS1xNFATc2$^{-/-}$ mice supporting the idea that NFATc2 is a relevant immunomodulatory transcription factor in the brain. However, based upon the heterogeneity of isoform expression, it is clear that dissection of isoform and cell selective function is needed to better determine the utility of targeting these transcription factors for therapeutic discovery.

It was somewhat surprising to find that protein levels of the post-synaptic marker, PSD95, were increased in the AβPP/PS1 brains and this was attenuated by NFATc2 deletion. However, in the same lysates we did not observe any difference in the pre-synaptic marker protein, synaptophysin. It is interesting to speculate that expression of the human mutant AβPP or PS1 was sufficient to produce a dysregulation of the post-synaptic compartment that was NFATc2 dependent. It is clear that neurons also express the NFATc2 isoform and it has a critical role in regulating trophic factor stimulated axon growth [29]. More importantly, prior work has shown that oligomeric Aβ stimulates increased neuronal calcineurin activity and spine loss that is NFAT dependent supporting the notion that NFAT has some role in regulating the post-synaptic compartment and Aβ toxicity [30, 32, 73].

Another interesting consideration is the role of Aβ as a putative transcription factor regarding its ability to regulate both AβPP and BACE expression via binding to an AβID in their promoters [74, 75]. Moreover, NFAT has a reported role in regulating BACE1 expression as well. NFATc4 has been demonstrated to regulate BACE1 expression and bind to a region in the BACE1 promoter [76]. Similarly, NFATc2 has a reported binding site in the BACE1 promoter [77]. This suggests that Aβ might stimulate increased NFAT activity to drive acquisition of a proinflammatory phenotype in the microglia but also increased Aβ production which might feed-forward to stimulate additional NFAT activity and Aβ production.

Based upon our observations that the decreased microgliosis and astrogliosis did not correlate with altered plaque load in the AβPP/PS1xNFATc2$^{-/-}$ mice, these mice may not only provide a model of assessing whether or not NFATc2 is involved in the proinflammatory changes of disease but also whether reducing inflammatory changes independent of Aβ plaque load is sufficient to offer protection against memory decline. Prior work, although directed at astrocytic NFAT, has shown that attenuating NFAT activity is sufficient to improve memory performance in an AD mouse model suggesting that similar findings would occur in these mice [36]. Future work with behavioral outcomes and microglial selective NFATc2 inhibition will better resolve the question of which cell type, neurons, astrocytes, or microglia, is the most reasonable target for AD. Collectively, our findings support the ongoing interest in shifting study of NFAT biology from the peripheral immune system to brain for the purpose of understanding its ability, perhaps in an isoform selective fashion, to regulate the proinflammatory changes that occur during injury and disease.

In conclusion, these data demonstrate both *in vitro* and *in vivo* in a mouse model of AD that the transcription factor NFATc2 has a role in regulating microglial activation. This suggests the possibility of isoform selective inhibitor design to limit inflammation in AD.

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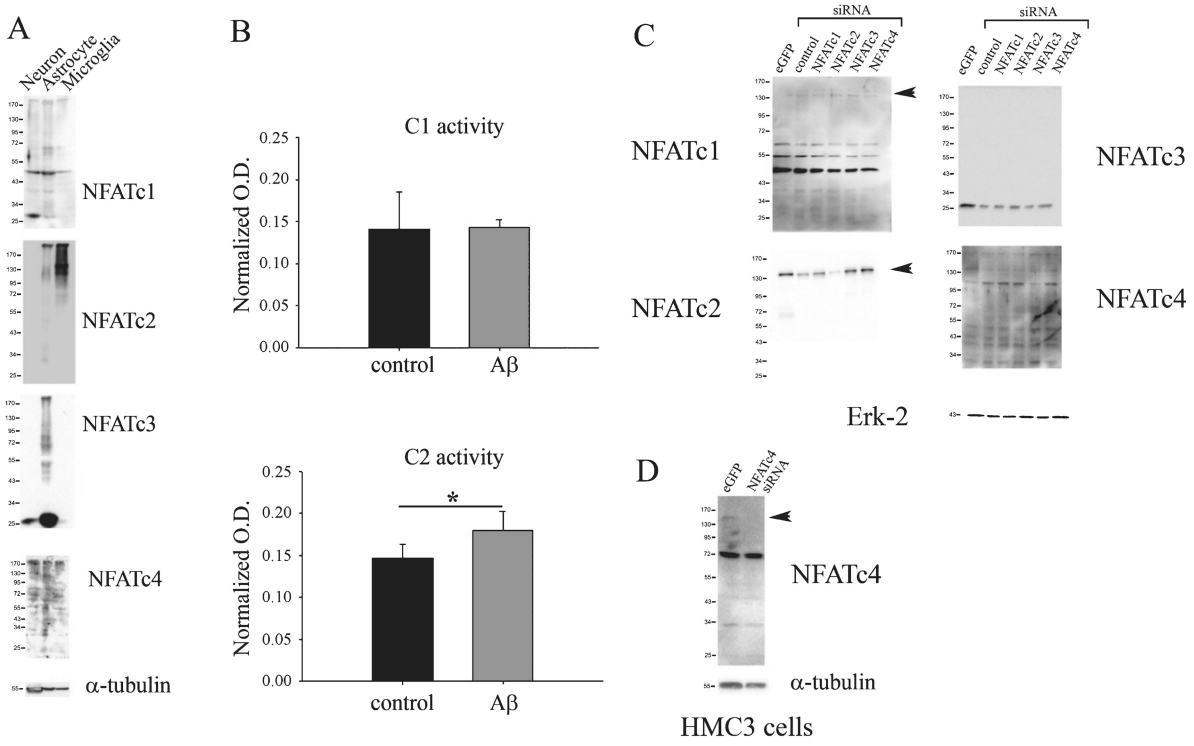

Fig. 1.

$A\beta_{1–42}$ stimulation increased NFATc2 activity in mouse microglia. Primary microglia, astrocyte, and cortical neuron cultures were grown from embryonic day 16 (neurons) or postnatal day 1–3 (astrocytes/microglia) C57BL/6 mouse brains. A) Cultures were lysed in RIPA buffer, quantified, separated by 10% SDS-PAGE, and western blotted using antibodies that recognized NFATc1, c2, c3, and c4 with an anti-α-tubulin antibody as a loading control. B) Microglia cultures were stimulated with 10 μM $A\beta_{1–42}$ for 1 h followed by nuclear isolation and a colorimetric transactivation quantitation assay for both NFATc1 and NFATc2 using a commercial plate-based assay. The graph shown is the average ± SD optical density normalized by nuclear protein content (Normalized O.D.) of 3 independent experiments, $*p < 0.05$. C) BV2 cells transfected with transfection control (eGFP expressing plasmid), control siRNA, siRNA for NFATc1, NFATc2, NFATc3, or NFATc4 were lysed in RIPA buffer and lysates western blotted using anti-NFATc1, anti-NFATc2, anti-NFATc3, and anti-NFATc4 antibodies. D) Human microglia cell line, HMC3, was transfected with an eGFP expressing plasmid or siRNA for NFATc4 and lysates western blotted using an anti-NFATc4 antibody.

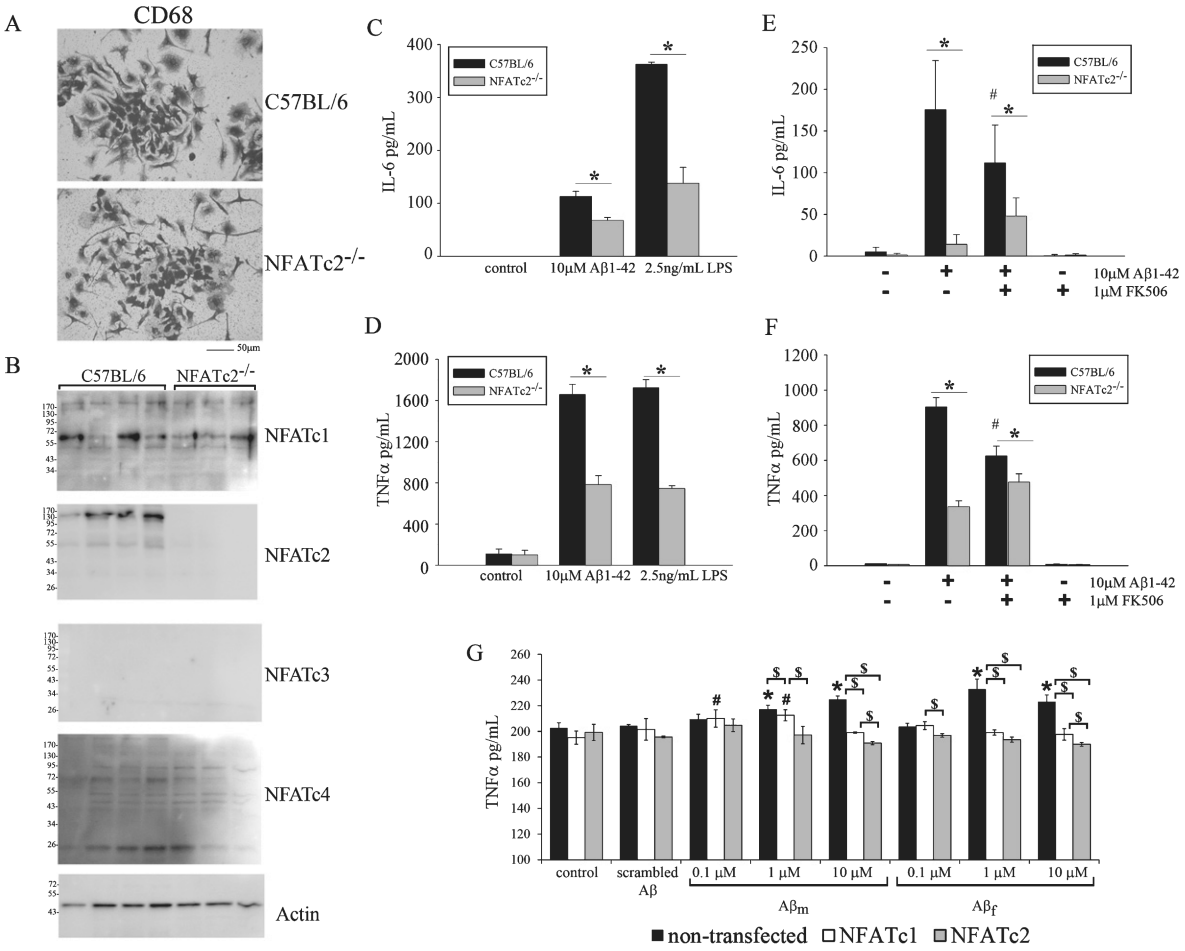

Fig. 2.

NFATc2 knock out microglia had attenuated cytokine secretion following both $A\beta_{1–42}$ and LPS stimulation. Microglia cultures were grown from NFATc2$^{-/-}$ and C57BL/6 postnatal day 1–3 mouse brains. A) Microglia were plated overnight in serum free media then fixed in 4% paraformaldehyde for immunostaining using anti-CD68 antibodies and Vector VIP as the chromogen. Immunostains are representative of 3-4 independent cultures. B) Alternatively, microglia were lysed in RIPA buffer, quantified, separated by 10% SDS-PAGE and western blotted using anti-NFATcl, NFATc2, NFATc3, and NFATc4 antibodies with anti-actin antibodies used as the loading control. Blots are from 3-4 independent cultures. Microglia cultures were also stimulated overnight in serum free media in the absence or presence of 10 μM $A\beta_{1–42}$ or 2.5 ng/mL LPS. Media was collected to quantify secretion of IL-6 (C) and TNFα (D) by commercial ELISA. Graphs are the average ± SD of 3 independent experiments, $^{*}p < 0.05$. Microglia cultures were stimulated overnight with or without 10 μM $A\beta_{1–42}$ or 1 μM FK506 and the media was collected to quantify IL-6 (E) and TNFα (F) levels by commercial ELISA. Graphs are the average ± SD of 3 independent experiments, $^{*}p < 0.05$, $^{\#}p < 0.05$ from respective control. (G) Murine microglia cells, BV2, were either non-transfected or transfected with siRNA for NFATc1 and NFATc2. Cells were stimulated with either scrambled Aβ, monomeric Aβ, or fibrillar Aβ 48-h post-transfection and the media used for quantifying TNFα levels using ELISA. TNFα concentrations were

averaged and graphed ± SD from 3 independent experiments, $^{*}p < 0.05$ and $^{\#}p < 0.05$ from their respective non-transfected controls, $^{\$}p < 0.05$ from respective Aβ treatment.

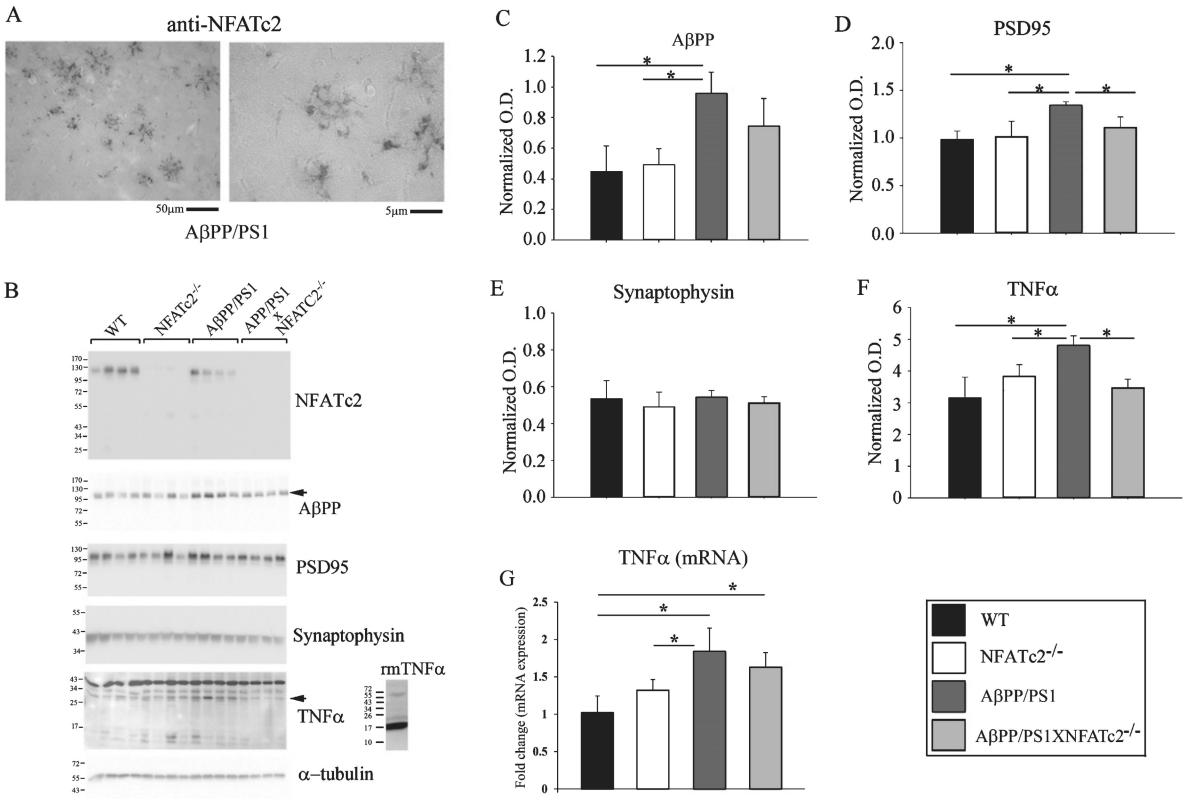

Fig. 3.

Brains of AβPP/PS1xNFATc2$^{-/-}$ mice had attenuated TNFα levels compared to AβPP/PS1 mice correlating with loss of plaque-associated NFATc2 immunoreactivity. A) Right brain hemispheres from male AβPP/PS1 mice were fixed, sectioned and immunostained using anti-NFATc2 antibody. Images shown are representative from temporal cortex at 20X and 63X magnification. B) Male WT, NFATc2$^{-/-}$, AβPP/PS1, and AβPP/PS1xNFATc2$^{-/-}$ mice at 8 months of age (n = 8–10) were collected for western blot analysis. Hippocampi were collected from left hemispheres and lysed, quantitated, and separated by 10% SDS-PAGE along with positive control mouse recombinant TNFα (rmTNFα) for western blot analysis using anti-NFATc2, AβPP (C), PSD95 (D), Synaptophysin (E), and TNFα antibodies (F). Antibodies recognizing α-tubulin were used as loading controls. Blots shown are representative of 8 animals per genotype. Graphs are the average ± SD of optical densities of individual antibodies normalized to their respective loading control (Normalized O.D.) from 8 animals per condition (genotype), *$p < 0.05$. G) Levels of TNF-α mRNA from spleens were determined using real time PCR. Fold change in the level of mRNA expression for each group was calculated as 2^-ΔΔC_t with respect to average mRNA expression of the WT group. The values obtained for 5 animals per group were averaged and plotted ± SD, *$p <$ 0.05.

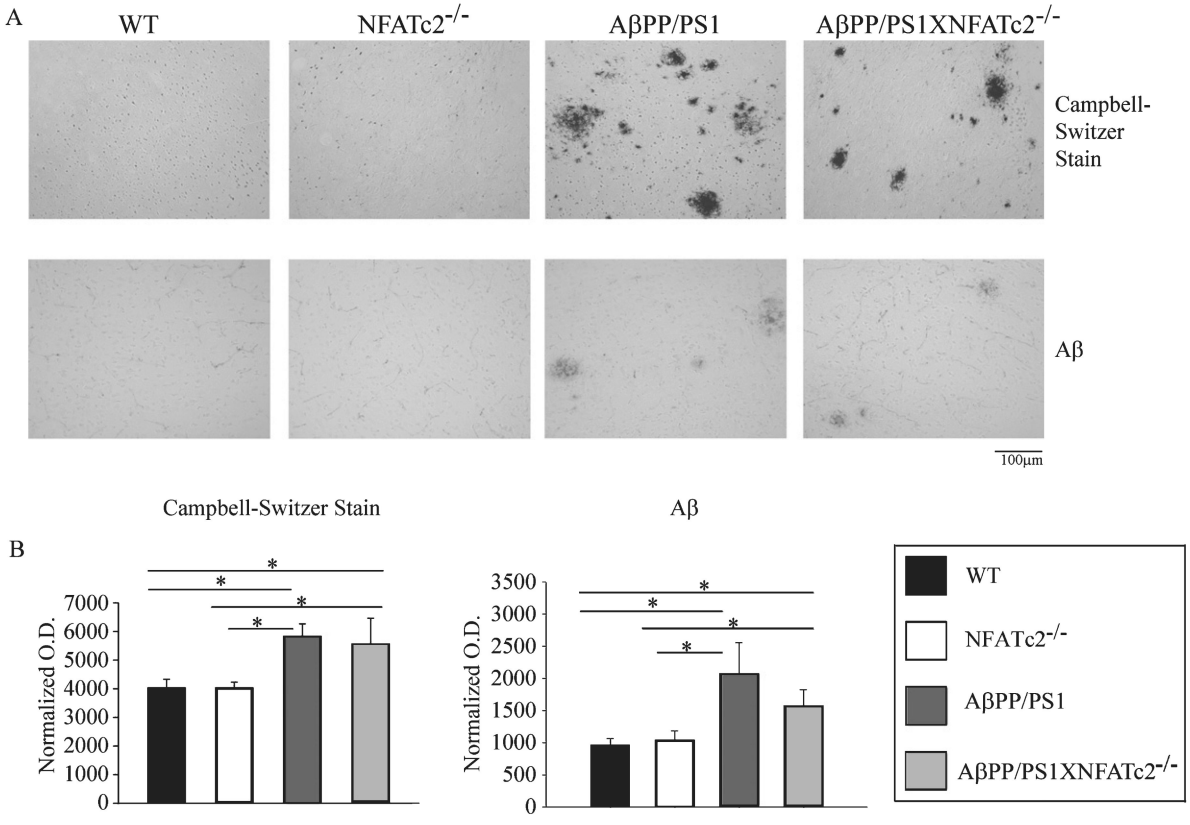

Fig. 4.

Brains of AβPP/PS1xNFATc2$^{-/-}$ mice had no differences in plaque load compared to AβPP/PS1 mice. Male WT, NFATc2$^{-/-}$, AβPP/PS1, and AβPP/PS1xNFATc2$^{-/-}$ mice at 8 months of age (n = 10) were collected for immunohistochemical analysis. Right hemispheres were fixed, serially sectioned and (A) plaques visualized using either the Campbell-Switzer histologic stain or anti-Aβ (4G8) immunostaining. Images shown are representative from the temporal cortex of 10 animals per genotype. B) Immunostaining intensity was quantified as described in the Materials and Methods. Graphs are the average ± SD of normalized optical densities (Normalized O.D.) of immunostaining from the temporal cortex of 10 animals per condition (genotype), *$p < 0.05$.

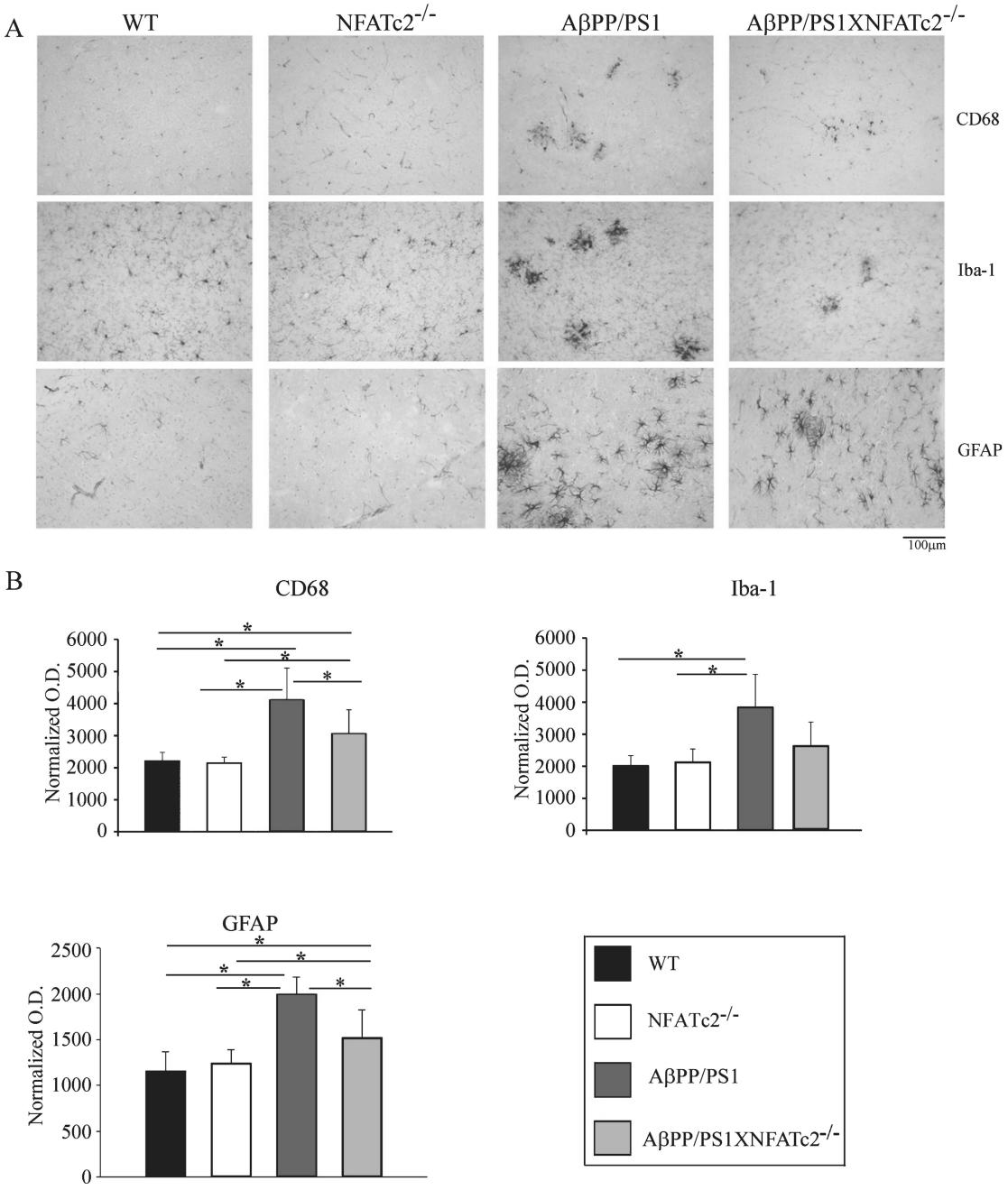

Fig. 5.

Brains of AβPP/PS1xNFATc2$^{-/-}$ mice had attenuated gliosis compared to AβPP/PS1 mice. Male WT, NFATc2$^{-/-}$, AβPP/PS1, and AβPP/PS1xNFATc2$^{-/-}$ mice at 8 months of age (n =10) were collected for immunohistochemical analysis. Right hemispheres were fixed, serially sectioned and gliosis was visualized using (A) anti-CD68, Iba-1, or anti-GFAP immunostaining to visualize microglia and astrocytes, respectively. Images shown are representative from the temporal cortex of 10 animals per genotype. B) Immunostaining intensity was quantified as described in the Materials and Methods. Graphs are the average ±

SD of normalized optical densities (Normalized O.D.) of immunostaining from the temporal cortex of 10 animals per condition (genotype), $*p < 0.05$.