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FEATURED ARTICLE

Reactive or transgenic increase in microglial TYROBP reveals a TREM2-independent TYROBP–APOE link in wild-type and Alzheimer's-related mice

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Abstract

Introduction: Microglial TYROBP (DAP12) is a network hub and driver in sporadic late-onset Alzheimer's disease (AD). TYROBP is a cytoplasmic adaptor for TREM2 and other receptors, but little is known about its roles and actions in AD. Herein, we demonstrate that endogenous *Tyrobp* transcription is specifically increased in recruited microglia.

Methods: Using a novel transgenic mouse overexpressing TYROBP in microglia, we observed a decrease of the amyloid burden and an increase of TAU phosphorylation stoichiometry when crossed with *APP/PSEN1* or *MAPT*^{P301S} mice, respectively. Characterization of these mice revealed *Tyrobp*-related modulation of apolipoprotein E (*Apoe*) transcription. We also showed that *Tyrobp* and *Apoe* mRNAs were increased in *Trem2*-null microglia recruited around either amyloid beta deposits or a cortical stab injury. Conversely, microglial *Apoe* transcription was dramatically diminished when *Tyrobp* was absent.

Conclusions: Our results provide evidence that TYROBP-APOE signaling does not require TREM2 and could be an initiating step in establishment of the disease-associated microglia (DAM) phenotype.

KEYWORDS
Alzheimer's disease, amyloid, apolipoprotein E, APP/PSEN1, DAM, Dap12, microglia, PS19, RNAscope, tauopathy, Trem2, Tyrobp

1 | NARRATIVE

1.1 | Contextual background

Microglia play a sentinel role in the brain, capable of detecting a wide variety of environmental stimuli, including microbial pathogens, aggregated proteins (such as amyloid beta [Aβ]), and cellular debris (such as membrane fragments). This sensing activity is an essential part of the host response and is broad in scope, sometimes triggering homeostatic adjustment, while, at other times, activating a host defense response. Microglia are also of interest in neurodegenerative diseases due to

proteinopathies, eg, Alzheimer's disease (AD), in which large genetic studies have reported increased disease risk linked to many loci associated with microglial genes implicated in clearance of Aβ peptides.[1–9] More recently, transcriptomic analyses have revealed distinct profiles and signatures for microglia associated with aging and aging-related diseases, indicating that a wide range of specific proteins in microglia underlie sensing, activation, and/or other cellular responses. Using RNA sequencing, Hickman et al. identified 100 transcripts highly enriched in microglia and coined the term "sensome" to describe this class of microglial transcripts.[10] Network analysis of this list identified a *TYROBP* (for tyrosine kinase binding protein; also known as *DAP12*, for DNAX activating protein-12)-centered pathway with 24 of these 100 genes interacting directly with *TYROBP* and 20 interacting indirectly with *TYROBP*. Concurrently, members of our team used an integrative network-based approach and identified *TYROBP* as a key network driver in sporadic late-onset AD.[11] More recently, Keren-Shaul et al.[12] used single-cell RNA sequencing in mice to define a specific microglial phenotype that they termed "disease-associated microglia" (DAM). *Tyrobp* was one of the genes most robustly upregulated in the proposed earliest stage of transition of microglia from the basal "homeostatic state" into the DAM phenotype.

TYROBP is a transmembrane signaling polypeptide that contains *an immunoreceptor phosphotyrosine-based activation motif* (ITAM) in its cytoplasmic domain. TYROBP is expressed in microglia in the brain and serves as an adaptor for a variety of immune receptors, including two molecules closely linked to AD pathogenesis: TREM2 (triggering receptor expressed on myeloid cells 2) and CR3 (complement receptor 3). TREM2 is expressed at the plasma membrane of microglia in the brain and some mutations and polymorphisms of *TREM2* are linked to autosomal dominant AD or sporadic late-onset AD.[13] Other *TREM2* mutations can cause a polycystic leukoencephalopathy osteodystrophy also known by the eponym Nasu-Hakola disease.[14] Most *TYROBP* mutations represent loss-of-function mutations and also result in Nasu-Hakola disease.[15] Similarly, *TYROBP* genetic variants have been identified in early-onset AD.[16] TREM2 is (among other things) a microglial Aβ receptor promoting microglial phagocytosis and proliferation and is required for microglia to limit growth of Aβ deposits.[17,18] In addition to the influences of environmental factors on TREM2,[19] this molecule is also essential for a full transition of homeostatic microglia to a DAM state. Keren-Shaul et al.[12] described a two-stage program for DAM transition with a *Trem2*-independent step (stage 1) during which *Tyrobp* and other genes are upregulated, followed by a *Trem2*-dependent step during which both *Tyrobp* and *Trem2* are upregulated (stage 2). Krasemann et al.[20] described a very similar microglial signature associated with neurodegenerative diseases, designated the "MGnD" phenotype, and showed that the transition from homeostatic to MGnD microglia was both TREM2- and apolipoprotein E (APOE)-dependent with a TREM2–APOE signaling pathway driving the transition from homeostatic microglia to MGnD. *APOE* ε4, one of the *APOE* polymorphisms, is a major risk factor for late-onset AD, and emerging evidence suggests that APOE (mostly produced by astrocytes in a normal brain) can also bind to TREM2.[21,22] This event defines an interesting and potentially disease-relevant pathway wherein extracellular APOE, as a ligand for TREM2, triggers upregulation of APOE in microglia.

RESEARCH IN CONTEXT

1. **Systematic review**: The authors review the literature on the role(s) played by microglial signal transduction molecules in the sensation of changes in the extracellular environment, such as the accumulation of amyloid beta (Aβ) deposits. This environmental sensing involves a receptor complex formed by intramembranous oligomerization of the ectodomain and transmembrane domain of TREM2 (triggering receptor expressed on myeloid cells 2) with the transmembrane and cytoplasmic domains of TYROBP (tyrosine kinase binding protein, also known as DAP12). This complex is linked to transcription of the microglial APOE (apolipoprotein E) gene, causing accumulation of cytoplasmic *Apoe* mRNA, and, concurrently, with a switching from a homeostatic microglia phenotype to a disease-associated microglia (DAM) phenotype. The role of TREM2 in this sensing and signaling has been reported, but, until now, less has been known about the role of TYROBP. Because TYROBP (but not TREM2) has been implicated by computational approaches to act as a "hub" or "driver" underlying late onset sporadic Alzheimer's disease (AD), the authors propose that TYROBP may also play important roles either (1) in sensing the accumulation of Aβ deposits, (2) in driving phenotypic switching, or (3) both. The relevant citations supporting this formulation and hypothesis are presented.
2. **Interpretation**: Our results provide compelling evidence that TYROBP–APOE signaling in the microglial sensome does not require TREM2. We propose that activation of TREM2-independent TYROBP–APOE signaling could be an early or even initiating step in the transformation of microglia from the homeostatic phenotype to the DAM phenotype.
3. **Future directions**: Other microglial signal transduction events are known (involving, e.g., transcription of miR155, *Axl*, *Ccl12*, *TGFβ*, *Il6*) as are events involving complement components interacting with each other and with their receptors. There are also important post-translational cytoplasmic protein phosphorylation events (especially protein tyrosine phosphorylation and dephosphorylation on TYROBP). Future efforts will focus on (1) identification of the underlying cause-and-effect relationships and (2) definition of the temporal sequences of these transcriptional and post-translational events as well as on (3) an elucidation of how these signals integrate with one of the fundamental physiological functions of microglia, ie, phagocytosis. Additionally, because the ε4 allele of APOE is both the most common and most potent genetic risk factor for common, late-onset, sporadic AD, we plan to determine whether and how TYROBP-related transcription of various APOE isotypes (ε2, ε3, ε4, in homozygous or heterozygous pairs) might differentially modulate the signal transduction events leading to the DAM phenotype.

When DAM and MGnD are compared, Keren-Shaul et al.[12] also observed by single-cell RNA sequencing an apparent sequence of events whereby *Tyrobp* was upregulated prior to the upregulation of *Trem2*. For clarity, because DAM and MGnD microglia appear to share key features of the phenomena described here, we will refer only to DAM for the remainder of this report. However, insofar as we are aware, principles established here underpin both DAM and MGnD.

In light of the central role of TYROBP in the microglial sensome,[10] its key role as adaptor for multiple microglial receptors,[23] its upregulation in the early *Trem2*-independent DAM stage 1[12] and its upregulation in AD,[11] we hypothesized that the upregulation of *Tyrobp* might be an early event that begins during the initial microglial response to the accumulation of Aβ deposits. Further, we propose that chronic sustained sensation of Aβ deposits by microglia might generate ongoing intracellular signals that influence the progression and pathogenesis of AD.

1.2 | Study design and main results

We used a number of strategies aimed at interrogation of the causes and consequences of *TYROBP* upregulation in microglia. Using dual RNA *in situ* hybridization and immunohistochemistry, we found that *Tyrobp* mRNA level is significantly increased when microglia are recruited, including in wild-type (WT) mice, in an *APP/PSEN1* transgenic mouse model of cerebral Aβ amyloidosis, and in a $MAPT^{P301S}$ transgenic mouse model of tauopathy. To determine whether elevated TYROBP can modify microglial phenotype and AD pathogenesis, we generated a novel transgenic mouse, designated the $Iba1^{Tyrobp}$ mouse, wherein the *Iba1* promoter was used to drive overexpression of a mouse *Tyrobp* transgene in microglia. We observed a reduced density of amyloid plaques and an apparent increase in the stoichiometry of TAU phosphorylation when $Iba1^{Tyrobp}$ mice were crossed with either *APP/PSEN1* or $MAPT^{P301S}$ mice, respectively. In addition to the alteration of both *APP/PSEN1* and $MAPT^{P301S}$ phenotypes, we observed that a constitutive increase in TYROBP influenced the transcription of *Apoe* and some associated genes. Finally, using two mouse models of cerebral Aβ amyloidosis and a mouse model of penetrating cortical stab injury, we showed that upregulation of *Tyrobp* and *Apoe* does not require *Trem2*, but that upregulation of microglial *Apoe* requires *Tyrobp* to reach normal levels.

1.3 | *Trem2–Tyrobp–Apoe* choreography

TREM2, *TYROBP*, and *APOE* are three microglial genes linked in a pathway contributing to the pathogenesis of AD and in the transition to DAM.[12] *TYROBP* was identified as a key driver in sporadic late-onset AD.[11] TREM2 binds to TYROBP, its intracellular adaptor, to initiate its signal transduction pathway, and naturally occurring loss-of-function mutations of either TYROBP or TREM2 can lead to Nasu-Hakola disease.[15,24] There is a general assumption among investigators in this research area that genetic deletion or overexpression of either TREM2 or TYROBP would result in identical phenotypes in disease models, but, until now, this has not been tested directly. We previously demonstrated amelioration of behavioral and electrophysiological deficits in *APP/PSEN1* and $MAPT^{P301S}$ mice on a *Tyrobp*-null background, despite a concurrent absence of effect on amyloid pathology and an apparent increase in the stoichiometry of phosphorylated TAU versus total TAU.[25–27] Homozygous deletion of *Trem2* can also lead to amelioration of both amyloidosis and tauopathy,[28,29] but those effects vary according to the mouse model, and the age and level of deficiency at sacrifice.[27,30,31] Lee et al.[32] used bacterial artificial chromosome (BAC)-mediated transgenesis to overexpress the human *TREM2* in the mouse genome and showed that TREM2 overexpression reduces amyloid accumulation in 5xFAD mice. Using a similar BAC system, Gratuze et al.[33] assessed the impact of $TREM2^{R47H}$ in $MAPT^{P301S}$ mice but no TREM2 overexpression was reported in that study.

The possible existence of an early TREM2-independent phase in conversion of microglia to DAM was described by Keren-Shaul et al.[12] but was not evident in studies by either Krasemann et al.[20] or Zhou et al.[34] *Apoe* has also been described as a participant in stage 1 of DAM with *Tyrobp*,[12] and it has been suggested that APOE drives the DAM transition through a TREM2–APOE pathway.[20] Moreover, *Apoe* has been reported to influence both amyloidosis and tauopathy histological phenotypes in mouse models.[35,36] A complete elucidation of the choreography of the regulatory interactions among these genes and their cognate proteins therefore remains an area of intense interest. We would suggest that the discrepancies across the various analyses might be explained in part by the fact that DAM microglia are located in the immediate proximity of the plaques, and that neither bulk- nor single-cell–RNA sequencing can distinguish homeostatic versus DAM phenotypes because both techniques generate an average transcriptomic analysis from all microglia within a particular tissue sample. This formulation played a major role in prompting us to use dual RNA *in situ* hybridization and immunohistochemistry in the current study in which we sought to determine (1) the effects of transgenic overexpression of *Tyrobp* on amyloid and TAU pathologies and (2) the relationship of the induction of *Tyrobp* to these pathologies and to the induction of *Trem2* and *Apoe*.

While this article was in preparation, Chen et al.[37] reported spatial transcriptomics and *in situ* sequencing in App^{NL-G-F} mice, using this protocol due to considerations identical to those motivating our studies herein; i.e., to avoid any potential confound derived from averaging the transcriptomes in a tissue sample without providing for spatial resolution of isolated resident microglia versus plaque-associated recruited microglia. These investigators proposed the formulation of a plaque-induced gene (PIG) network within the microglia and astrocytes in immediate proximity to amyloid plaques; this PIG network was defined by differential expression of 57 genes.[37] Top genes highly upregulated in the proximity of the plaques and as early as 3 months of age were *Tyrobp*, *Apoe*, and several complement-related genes. Notably, *Tyrobp* is a key regulator of the complement subnetwork,[11] and C1q is downregulated in *APP/PSEN1* and $MAPT^{P301S}$ mice in the absence of *Tyrobp*.[26,27] The authors performed the same type of experiment in human AD

brain slices and confirmed the enrichment of *Tyrobp* and several complement components (*C1qA*, *C1qB*, *C1qC*, and *Clu*). Of particular relevance to our data herein, *Trem2* was not included in the human PIG network.

1.4 | Study conclusions and perspectives

In addition to confirming that TYROBP overexpression in microglia is sufficient to alter both amyloidosis and tauopathy phenotypes, our data indicate that *Tyrobp* upregulation is an early marker of recruited microglia and can occur even in the brains of *Trem2*-deficient mice. Similarly, we observed that the increased *Apoe* mRNA level in microglia is *Trem2*-independent, whether in injury or AD-related mouse models. Finally, we observed that microglial *Apoe* mRNA level was greatly attenuated in plaque-associated microglia in *Tyrobp*-deficient mice. These data confirm the model proposed by Keren-Shaul et al.[12] in which *Tyrobp* and *Apoe* transcripts are increased first, and neither transcription event requires the presence of *Trem2*. Moreover, Meilandt et al.[38] recently reported that microglial APOE expression was not reduced, but, on the contrary, was increased in *PS2APP;Trem2*$^{-/-}$ mice compared to microglial APOE expression in *PS2APP;Trem2*$^{+/+}$ mice. In that same study, Meilandt et al. also analyzed the expression profiles of fluorescence-activated cell sorting (FACS)-purified microglia from *5xFAD* mice that expressed TREM2 normally and from FACS-purified microglia from *5xFAD* mice deficient in *Trem2*. These investigators observed a two-fold reduction in *Apoe* in one dataset (GSE132508)[12,38] but no reduction at all in the other (GSE65067).[38,39] However, Parhizkar et al.[40] reported that the absence of functional TREM2 reduces plaque-associated APOE. This is in line with what Krasemann et al.[20] proposed when they showed that genetic targeting of *Trem2* suppresses the APOE pathway. Our observations and conclusions herein apparently differ from those of Parhizkar et al.[40] to the extent that, in our hands, microglial amyloid plaque sensing followed by upregulation of *Tyrobp* and *Apoe* are preserved in the absence of *Trem2* and, as a consequence of the *Trem2* deficiency, microglia recruitment into the proximity of amyloid plaques is reduced. This relationship points to the fact that the absence of functional TREM2 will block appearance of the full DAM phenotype and therefore the associated clearance of Aβ is reduced. Nevertheless, we propose a model wherein the sensing of amyloid plaques—which takes place upstream of amyloid plaque clearance—involves *Tyrobp* and *Apoe* but not necessarily *Trem2*. Because APOE isoforms differentially influence AD age at onset and progression,[41] future investigations will be aimed at elucidating whether and how various homozygous or heterozygous APOE isotype combinations affect TYROBP–APOE signaling, and/or microglial sensing and phenotype switching.[42]

TYROBP is a 113 amino acid polypeptide with a minimal extracellular region,[43,44] making it unlikely that TYROBP is the sole player in a signal transduction pathway involving both the perception of the environment and the triggering of the switch from homeostatic phenotype to DAM. However, TYROBP is the adaptor for many receptors other than TREM2,[23] and therefore, it is plausible and perhaps likely that other TYROBP receptors could play key roles in sensing the deposition of amyloid. For example, numerous SIGLEC (sialic acid-binding immunoglobulin-type lectins) proteins carry a positively charged residue in their transmembrane domain that participates in oligomerization of the SIGLEC with TYROBP. The primary SIGLEC ligand is a sialic acid that accumulates in many pathological conditions including cerebral Aβ amyloidosis.[45,46] Moreover, Siglec-H interacts with TYROBP, and its expression has been reported to be elevated in *5xFAD* mice.[32] CD33 (SIGLEC-3) is also one of the most abundant SIGLECs in the human brain, and genome-wide association studies (GWAS) implicated a polymorphism near *CD33* as a genetic risk factor for AD.[2,4,47] CD33 and TREM2 both interact with TYROBP, either directly (TREM2) or via common intracellular signaling factors (CD33). Griciuc et al.[48] recently investigated crosstalk between CD33 and TREM2 and proposed that CD33 acts upstream of TREM2. They also showed that *Cd33* and *Tyrobp* expression levels did not change in *Trem2*$^{-/-}$ versus WT microglia. This formulation provides evidence that CD33–TYROBP signaling could occur upstream of the recruitment and upregulation of TREM2.

Rather than the somewhat exclusively "*Trem2*-centric" view of DAM proposed in the existing AD microglia literature,[20,34,40] we propose that *Tyrobp* can play a central role in an alternative and early pathway in the microglial sensome,[10] even in the absence of any change in *Trem2* levels. The data that we present here document the robust consequences of TYROBP overexpression in both *APP/PSEN1* and *MAPT*^{P301S} mice. We confirm here that upregulation in microglia of both *Tyrobp* and *Apoe* constitute interconnected events in microglia sensing of amyloid deposits, and that these events take place independently of *Trem2*.

2 | CONSOLIDATED RESULTS AND STUDY DESIGN

With the inclusion of *Tyrobp* as a PIG gene[37] and because of our prior validation of its actions as a driver of AD,[11,25–27] we hypothesized that constitutive overexpression of microglial *Tyrobp* via transgenesis would alter both amyloid and TAU pathologies. In 4-month-old *APP/PSEN1* mice, TYROBP overexpression was associated with a 50% decrease in the amyloid burden, similar to what occurs with the upregulation of *Trem2*[32]. We confirmed this decrease by measuring levels of human Aβ42 and Aβ40 by enzyme-linked immunosorbent assay (ELISA) in the cerebral cortices of these mice. An evaluation of plaque burden in 8-month-old mice in three different brain regions confirmed the magnitude and statistical significance associated with the reduction of amyloid burden. In *MAPT*^{P301S} mice, we previously reported that deficiency of *Tyrobp* increased TAU phosphorylation and spread,[27] and we were puzzled, therefore, to observe a similar increase in TAU phosphorylation in the *MAPT*^{P301S} mice with overexpression of TYROBP. *MAPT*^{P301S};*Iba1*^{Tyrobp} microglia are also more reactive compared to *MAPT*^{P301S} microglia, and reactive microglia have been reported to drive TAU pathology, so these two observations are compatible.[49,50] This exacerbation of pathology under conditions of either down- or

upregulation of *Tyrobp* in *MAPT*^{P301S} mice reveals the complexity of the microglial events underpinning tauopathy. Our formulation is that, for any particular microglial activation status, there exists some optimum level of *Tyrobp* expression, and that either elevation or deficiency of *Tyrobp* levels can be detrimental. These data also support a key role for microglial TYROBP in AD pathology progression, as we proposed in our previous reports on mice deficient in *Tyrobp*.[25–27]

Despite the obvious differences across *APP/PSEN1* and *MAPT*^{P301S} mouse models and the diverse consequences of *Tyrobp* upregulation in each of these mice, there are shared changes in *Axl*, *Ccl2*, *Tgfβ*, and *Il6* mRNAs in both *APP/PSEN1* or *MAPT*^{P301S} mice overexpressing TYROBP. These genes have been recently associated with *Apoe* in microglia, macrophages, and mononuclear phagocytes. AXL has been identified as a regulator of APOE,[51] and accumulation of IL6 and CCL2 have been associated with APOE overexpression.[52,53] Similarly, reciprocal suppression of TGFβ and induction of APOE have been described in DAM microglia.[20] *Apoe* mRNA levels are indeed upregulated in *MAPT*^{P301S};*Iba1*^{Tyrobp} mice compared to *MAPT*^{P301S} mice, but *Apoe* levels are unchanged in *APP/PSEN1;Iba1*^{Tyrobp} mice compared to *APP/PSEN1* mice. However, in bulk RNA sequencing performed on hippocampi from male *APP/PSEN1;Iba1*^{Tyrobp} versus *APP/PSEN1* mice, we identified *Apoe* as a potential (activation z-score: 2.44; p-value overlap: 0.224) upstream regulator, suggesting the possible existence of a relationship between *Tyrobp* upregulation and *Apoe*. While a TREM2–APOE pathway has been described,[20] it is interesting to note that our data indicate that the TYROBP–APOE relationship is detectable even in the absence of *Trem2* upregulation.

To investigate further the interactions among *Trem2*, *Tyrobp*, and *Apoe* in microglia, we used a penetrating cortical stab injury paradigm by introducing a small lesion via stereotactic surgery into one hemisphere of the mouse brain to induce a recruitment of microglia around the injury site. Using this experimental paradigm in WT, *Trem2*$^{-/-}$, or *Tyrobp*$^{-/-}$ mice, we confirmed that microglia upregulate both *Tyrobp* mRNA and *Apoe* mRNA when recruited in close proximity to the injury, and these events are readily detectable even in the absence of *Trem2*. Interestingly, we failed to observe an upregulation of *Apoe* mRNA in microglia from *Tyrobp*$^{-/-}$ mice subjected to penetrating cortical stab injury. Because microglia are also recruited around Aβ plaques, we used two different mouse models of human cerebral Aβ-amyloidosis (ie, *TgCRND8* and *APP/PSEN1* mouse lines) that were either WT or gene-targeted for *Trem2* or *Tyrobp*, respectively. Similarly, we observed that both *Tyrobp* and *Apoe* mRNAs were upregulated in amyloid plaque-associated microglia even in the absence of *Trem2*. Moreover, and as predicted with the stab-injury paradigm, we observed a substantial decrease in the induction of *Apoe* mRNA in plaque-associated microglia when *Tyrobp* was absent.

3 | DETAILED METHODS AND RESULTS

3.1 | Methods

See supporting information for full details.

3.2 | Results

3.2.1 | *Tyrobp* transcription is increased in recruited microglia

Microglia continuously sense changes in the brain environment and are recruited to sites of injury, microbial invasion, or where abnormal folding or modification of cellular constituents are detected, as with the accumulation of aggregated Aβ. We performed dual RNA *in situ* hybridization (RNAscope) and immunohistochemistry for *Tyrobp* and IBA1, respectively, in WT mice and observed increased levels of *Tyrobp* mRNA in areas exhibiting recruited microglia (Figure 1A). Using the same experimental approach in two independent mouse models of cerebral amyloidosis (*APP/PSEN1*[54] and *5xFAD*[55]), we observed a similar pattern in that the *Tyrobp* mRNA level was extensively and selectively increased in microglia recruited in close proximity to amyloid plaques compared to microglia that are more distant from the plaques (Figure 1B-C). We similarly assayed *Tyrobp* mRNA and IBA1 protein in the *MAPT*^{P301S} mice[56] (also known as PS19), a mutant tauopathy mouse model. We previously described an elevated number of anti-phosphorylated-TAU immunostained neurons in the piriform cortex of this mouse model (Figure 1D).[27] The changes that we observed in these mice were analogous to the changes observed around amyloid plaques in that we detected increased amounts of *Tyrobp* mRNA in microglia surrounding areas of aggregated protein pathology (Figure 1D). We confirmed the increase of TYROBP at the protein level in microglia around amyloid plaques (Figure 1E) as previously reported.[57] To discriminate between the role of TYROBP in activated versus recruited microglia, we isolated primary microglia from WT mice and exposed them to the gram-negative bacterial endotoxin lipopolysaccharide (LPS) to induce microglial activation,[58] the status of which we established by quantifying the robust increase of *Tnfα* mRNA after LPS treatment. Interestingly, *Tyrobp* mRNA level was unchanged, suggesting that *Tyrobp* transcription may be increased only when microglia are *both* recruited *and* activated but not in resident microglia, despite evidence that these residents are also activated (Figure 1F).

3.2.2 | Microglia are normal in *Iba1*^{Tyrobp} mice

To determine whether constitutive elevation of TYROBP via transgenesis may influence microglial phenotype and progression of AD pathology, we generated a novel transgenic mouse overexpressing *Tyrobp* in microglia in the central nervous system. We used the mouse *Tyrobp* and *enhanced green fluorescent protein* (EGFP) sequences separated by an internal ribosome entry site (IRES) under the control of the mouse *Iba1* regulatory sequences (Figure S1[59] in supporting information). Microinjections were performed in C57BL/6J mice and one line (B6.Cg-Tg[*Iba1-Tyrobp*-IRES-*Egfp*]34Mee/J) was selected for further use based on expression level of the transgene, now referred to *Iba1*^{Tyrobp}. We first assessed the overexpression of *Tyrobp* mRNA by real-time quantitative polymerase chain reaction (RT-qPCR) and measured a ≈2.5-fold increase (Figure 2A). Despite this elevated mRNA level, western blot

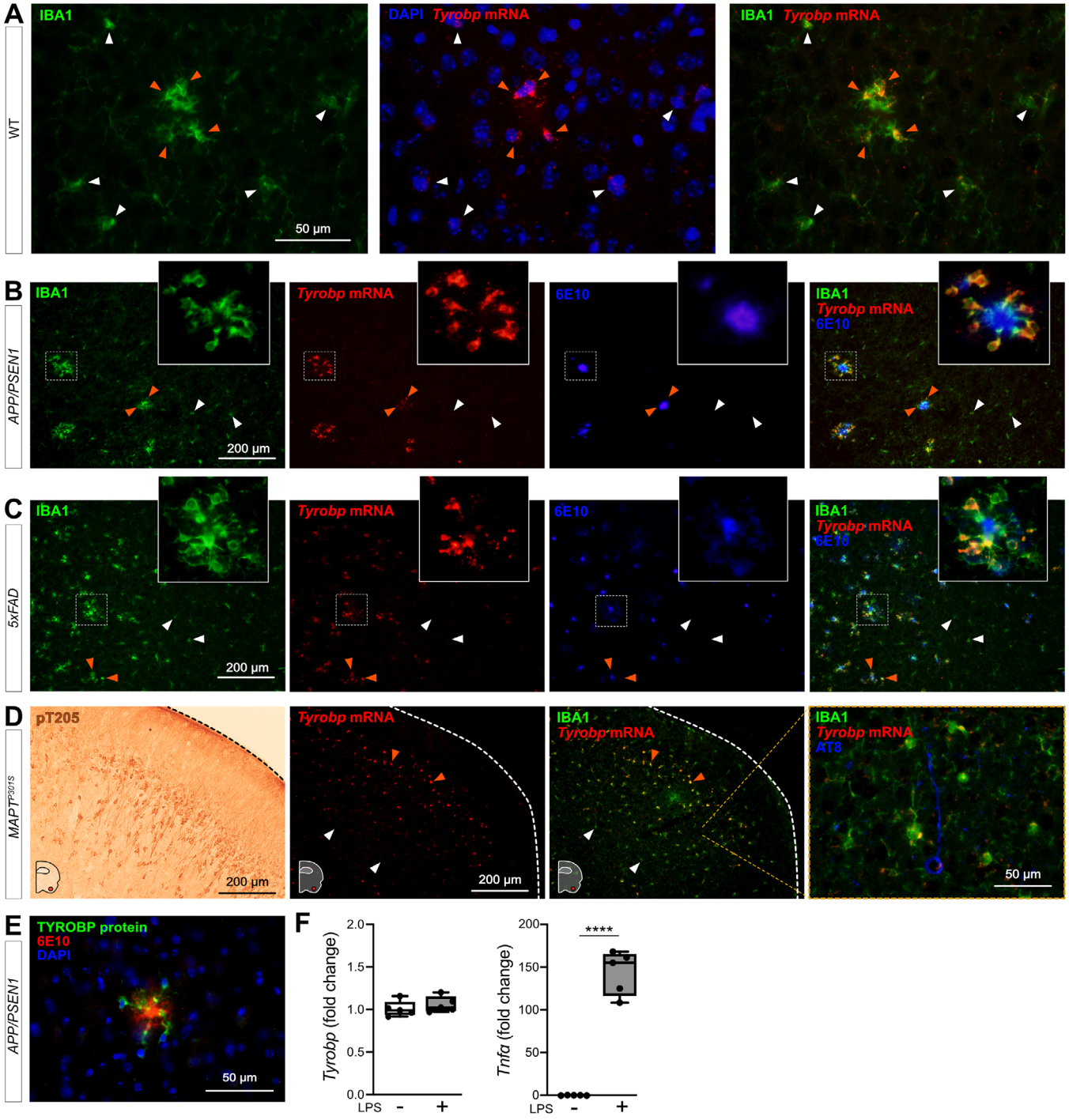

FIGURE 1 *Tyrobp* mRNA is increased in recruited microglia. A, Dual RNA fluorescent *in situ* hybridization (RNAscope) and immunohistochemistry for *Tyrobp* mRNA (red) and IBA1 protein (green), respectively, in wild-type (WT) mice (DAPI in blue). Scale bar = 50 μm. B-C, Dual RNA *in situ* hybridization and immunohistochemistry for *Tyrobp* (red), IBA1 (green), and amyloid beta (Aβ; antibody 6E10; blue) in *APP/PSEN1* (B) and *5xFAD* (C) mice. Scale bar = 200 μm. D, Left panel: representative image of immunohistochemistry with antibody pT205 in the piriform cortex of $MAPT^{P301S}$ (PS19) mice. Scale bar = 200 μm. Right panels: dual RNA *in situ* hybridization and immunohistochemistry for *Tyrobp* (red), IBA1 (green), and p-TAU (antibody AT8; blue) in the piriform cortex of $MAPT^{P301S}$ mice. Scale bars = 200 and 50 μm. E, Co-immunohistochemistry for TYROBP (green) and human Aβ (antibody 6E10; red) in *APP/PSEN1* mice (DAPI in blue). Scale bar = 50 μm. F, Real-time quantitative polymerase chain reaction analyses of *Tyrobp* and *TNF*α mRNAs in WT primary microglia with and without lipopolysaccharide. Mice were either 4 (A) or 8 (B-E) months of age and were all WT for *Tyrobp*. White and orange arrows indicate examples of non-recruited and recruited microglia, respectively. Slice thickness = 10 μm

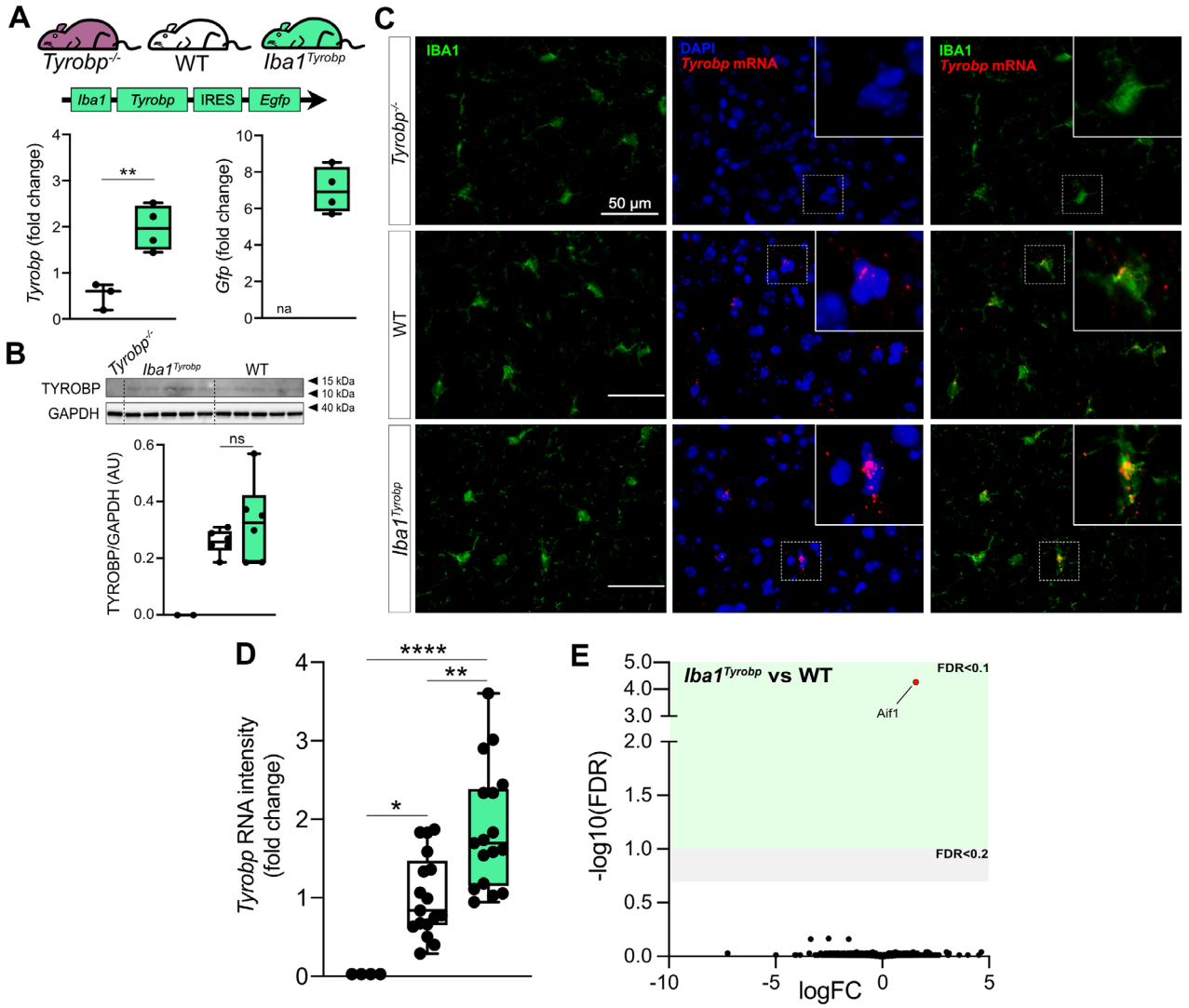

FIGURE 2 Generation of *Iba1*^{Tyrobp} mice. A, Hippocampi from 4-month-old *Tyrobp*$^{-/-}$, wild-type (WT), and *Iba1*^{Tyrobp} mice were assayed for *Tyrobp* and *Gfp* mRNAs by real-time quantitative polymerase chain reaction (n = 3–4 mice per group). B, Representative western blot and quantification of TYROBP and GAPDH in the cortex of the same groups used in (A) (n = 2–6 mice per group). C, Dual RNA fluorescent *in situ* hybridization and immunohistochemistry for *Tyrobp* mRNA (red) and IBA1 (green), respectively, (DAPI in blue) in *Tyrobp*$^{-/-}$, WT, and *Iba1*^{Tyrobp} mice. Scale bar = 50 μm and slice thickness = 10 μm. D, Quantification of *Tyrobp* mRNA intensity from the experiment described in (C). n = 4, 17, and 17 slices per group (from N = 1 mouse per genotype) for *Tyrobp*$^{-/-}$, WT, and *Iba1*^{Tyrobp} mice, respectively. E, Volcano plot representation of the whole hippocampal DEGs in *Iba1*^{Tyrobp} versus WT mice (n = four 4-month-old males per genotype). Error bars represent means ± standard error of the mean. Statistical analyses were performed using a Student t-test (A) or a one-way analysis of variance followed by a Tukey's post hoc test (B, D), $^{*}P < .05$, $^{**}P < .01$, $^{****}P < .0001$. na, not applicable; ns, non-significant

analyses of protein extracts from the cortex did not reveal a significant overexpression at the protein level (Figure 2B). Using combined RNA *in situ* hybridization and immunohistochemistry for *Tyrobp* and IBA1, respectively, in mice deficient in TYROBP (*Tyrobp*$^{-/-}$), WT or overexpressing *Tyrobp* on the *Iba1* promoter (*Iba1*^{Tyrobp}), we confirmed the 2-fold increase in *Tyrobp* mRNA in *Iba1*^{Tyrobp} mice compared to WT (Figure 2C-D). We observed that only a subset of microglia was overexpressing *Tyrobp* mRNA in *Iba1*^{Tyrobp} mice. This selectivity is likely due to the use of the *Iba1* promoter in a WT background without extensive microglia activation, thereby also accounting for the lack of a significant increase of TYROBP at the protein level in the resting state. RNA sequencing of hippocampi from *Iba1*^{Tyrobp} mice did not reveal any differentially expressed genes (DEGs) other than *Aif1* (= *Iba1*), which is increased due to the inclusion of the first two exons in the transgenic vector (Figure S1). These data indicate that *Iba1*^{Tyrobp} microglia do not display molecular and phenotypic changes in mice that are lacking in certain backgrounds of aggregated protein pathology.

3.2.3 | TYROBP overexpression in microglia decreases amyloid plaque load in *APP/PSEN1* mice

To assess whether TYROBP overexpression in microglia modulates Aβ deposition in *APP/PSEN1* mice, double-heterozygous

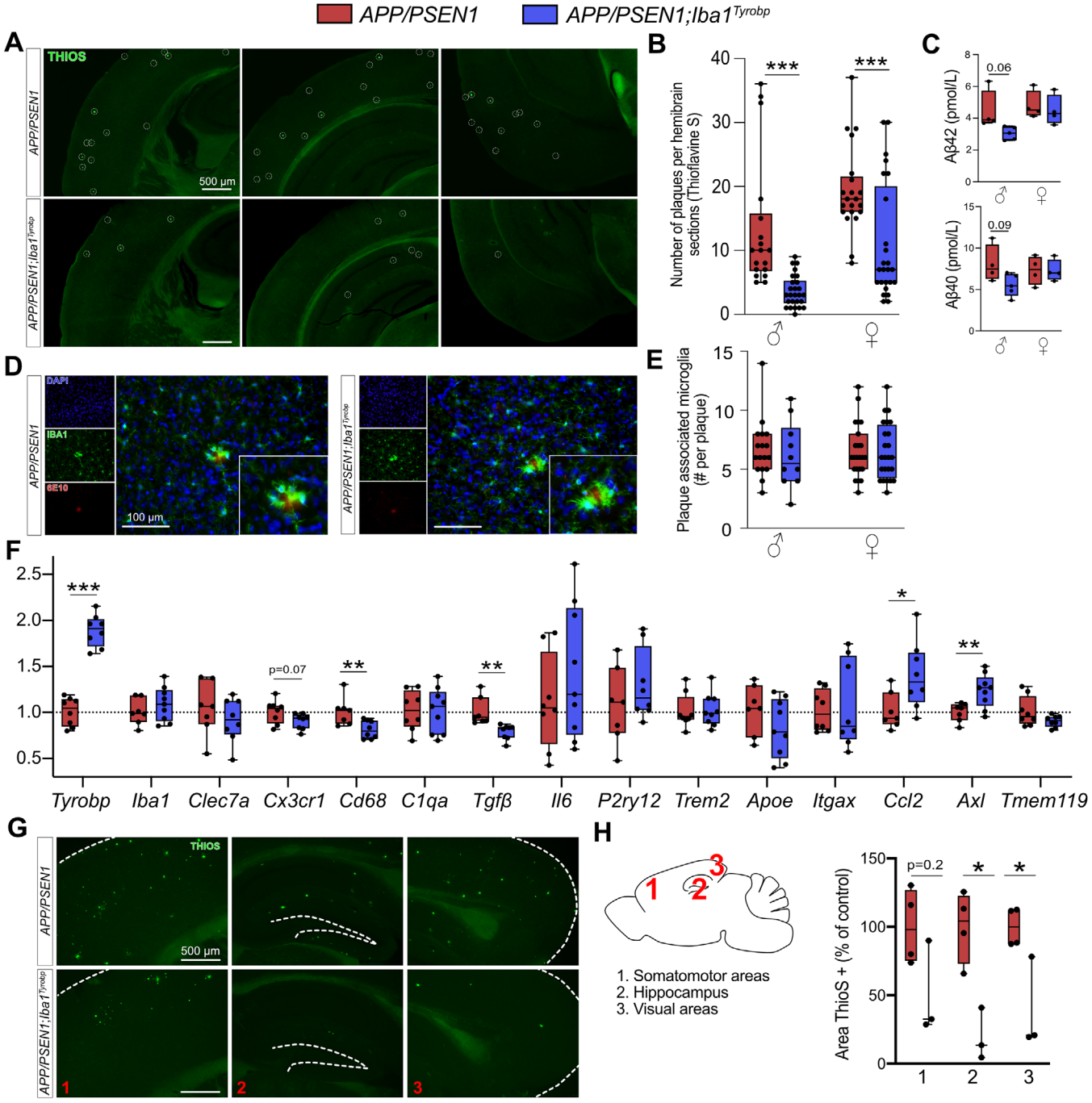

FIGURE 3 Transgene-derived *Tyrobp* upregulation decreases amyloid plaque load in *APP/PSEN1* mice. A, Representative images of thioflavine-S (ThioS) staining in *APP/PSEN1* and *APP/PSEN1;Iba1*^{Tyrobp} mice at 4 months of age. Scale bar = 500 μm. B, Quantification of the number of ThioS-positive plaques per hemibrain in *APP/PSEN1* and *APP/PSEN1;Iba1*^{Tyrobp} mice at 4 months of age. N = 4–5 mice per genotype and sex with three slices per animal. C, Human amyloid beta (Aβ)42 and Aβ40 concentrations measured by enzyme-linked immunosorbent assay in the cortices of the same groups described in (B). D, Representative images of double-label immunohistochemistry with anti-IBA1 and anti-6E10 antibodies in *APP/PSEN1* and *APP/PSEN1;Iba1*^{Tyrobp} mice at 4 months of age. Scale bar = 100 μm. E, Quantification of the number of plaque-associated microglia in the four groups described in (B). N = 10–24 plaques from 4–5 mice per group. F, Real-time quantitative polymerase chain reaction analyses of microglial gene mRNAs in the hippocampi of *APP/PSEN1* and *APP/PSEN1;Iba1*^{Tyrobp} mice at 4 months of age. N = 7–9 mice per group, females and males were pooled. G, Representative images of ThioS staining in male *APP/PSEN1* and *APP/PSEN1;Iba1*^{Tyrobp} mice at 8 months of age. Scale bar = 500 μm. H, Quantification of the ThioS immunoreactive area in male *APP/PSEN1* and *APP/PSEN1;Iba1*^{Tyrobp} mice at 8 months of age (somatomotor and visual areas of the cortex, and hippocampus were quantified). N = 3–4 mice per group. Error bars represent means ± standard error of the mean. Statistical analyses were performed using a two-way analysis of variance followed by a Sidak post hoc test for (B and E), a Kruskal-Wallis test for (C), a Student t-test for (F) and a Mann-Whitney test for (H), $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$

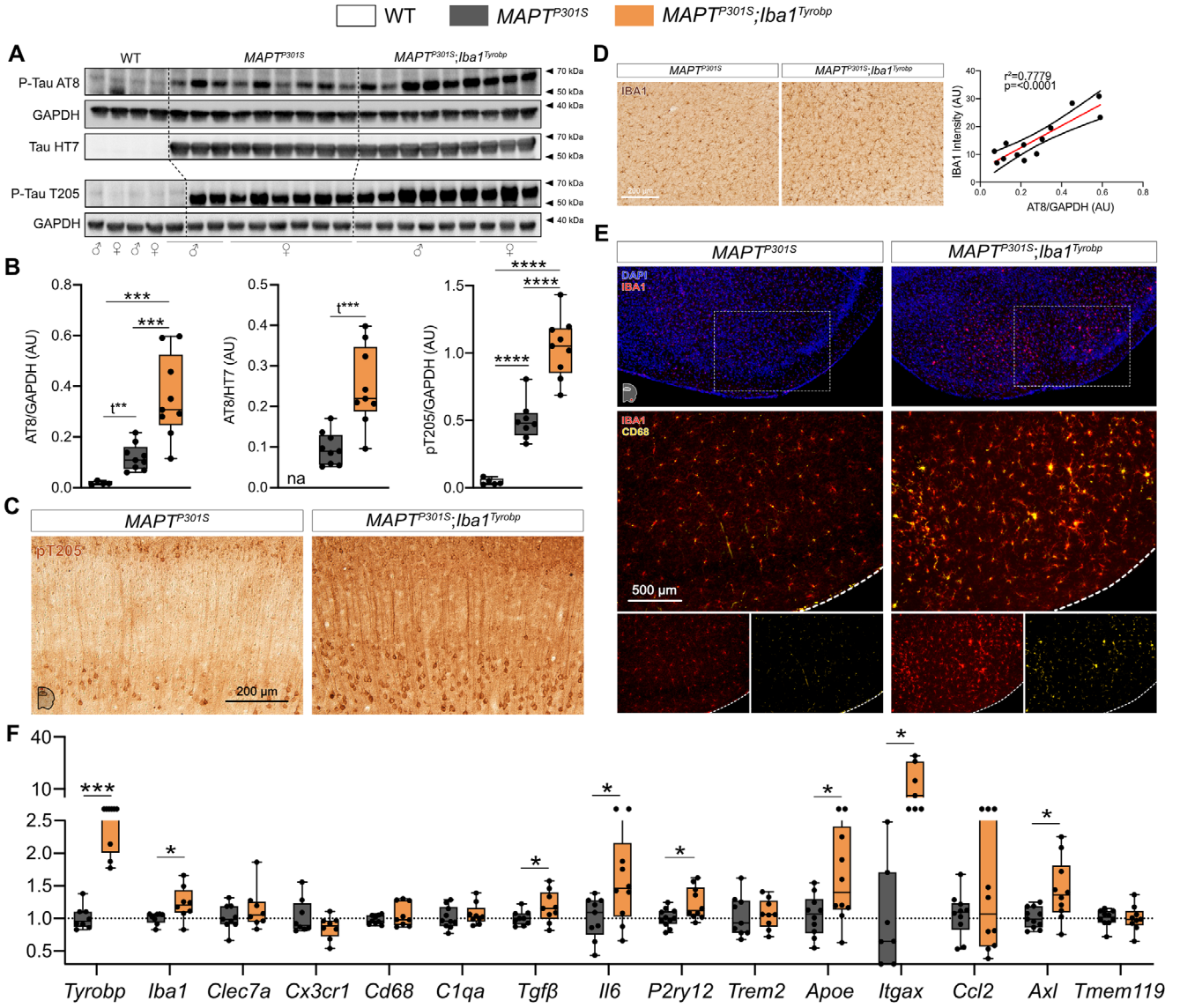

FIGURE 4 Transgene-induced *Tyrobp* upregulation increases apparent stoichiometry of TAU phosphorylation and microglial activation in 4-month-old *MAPT*^{P301S} mice. A, Western blot analyses of phosphorylated TAU on S202 or T205 epitopes (AT8 and pT205 antibodies) and total human TAU (HT7 antibody) in cortical homogenates from wild-type, *MAPT*^{P301S} (PS19), and *MAPT*^{P301S};*Iba1*^{Tyrobp} mice at 4 months-old. n = 4–9 mice per group. B, Densitometric analyses of western blots presented in (A) standardized to GAPDH or HT7. C, Representative images of DAB-immunohistochemistry with antibody pT205 in 4-month-old *MAPT*^{P301S} and *MAPT*^{P301S};*Iba1*^{Tyrobp} mice. Scale bar = 200 μm. D, Left panel: representative images of anti-IBA1 immunohistochemistry on the same groups described in (C). Scale bar = 200 μm. Additional representative pictures are presented in Figure S2 in supporting information. Right panel: western blot-AT8/GAPDH quantification plotted against anti-IBA1 immunoreactivity in the cortex. Linear regression with trend line (red line) and 95% confidence intervals (black lines) are indicated. E, Representative images of double-label immunofluorescence with anti-IBA1 and anti-CD68 antibodies in the piriform cortex on the same groups described in (C). Scale bar = 500 μm. F, Real-time quantitative polymerase chain reaction analyses of microglial gene mRNAs in the hippocampus of *MAPT*^{P301S} and *MAPT*^{P301S};*Iba1*^{Tyrobp} mice at 4 months of age. N = 7–11 per group. Error bars represent means ± standard error of the mean. Statistical analyses were performed using a one-way analysis of variance followed by a Tukey's post hoc test for (B) or a Student t-test for (B) when *t is indicated and (F), $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$

APP/PSEN1;*Iba1*^{Tyrobp} mice were generated and studied at 4 months of age. We measured a ≈50% decrease of the plaque density in the cerebral cortices of both male and female *APP/PSEN1*;*Iba1*^{Tyrobp} mice compared to sex-matched *APP/PSEN1* mice (Figure 3A-B) in sections stained for amyloid plaques using thioflavin S (ThioS). This observation was supported by measuring levels of human Aβ42 and Aβ40 by ELISA, both of which were apparently associated with a trend toward decrease in the cortex with TYROBP overexpression, mostly among male *APP/PSEN1*;*Iba1*^{Tyrobp} mice (Figure 3C). There was no genotype-dependent difference in the number of plaque-associated microglia (Figure 3D-E), unlike what has been reported in *5xFAD* mice in the presence of a transgenic increase in TREM2.[32] To evaluate

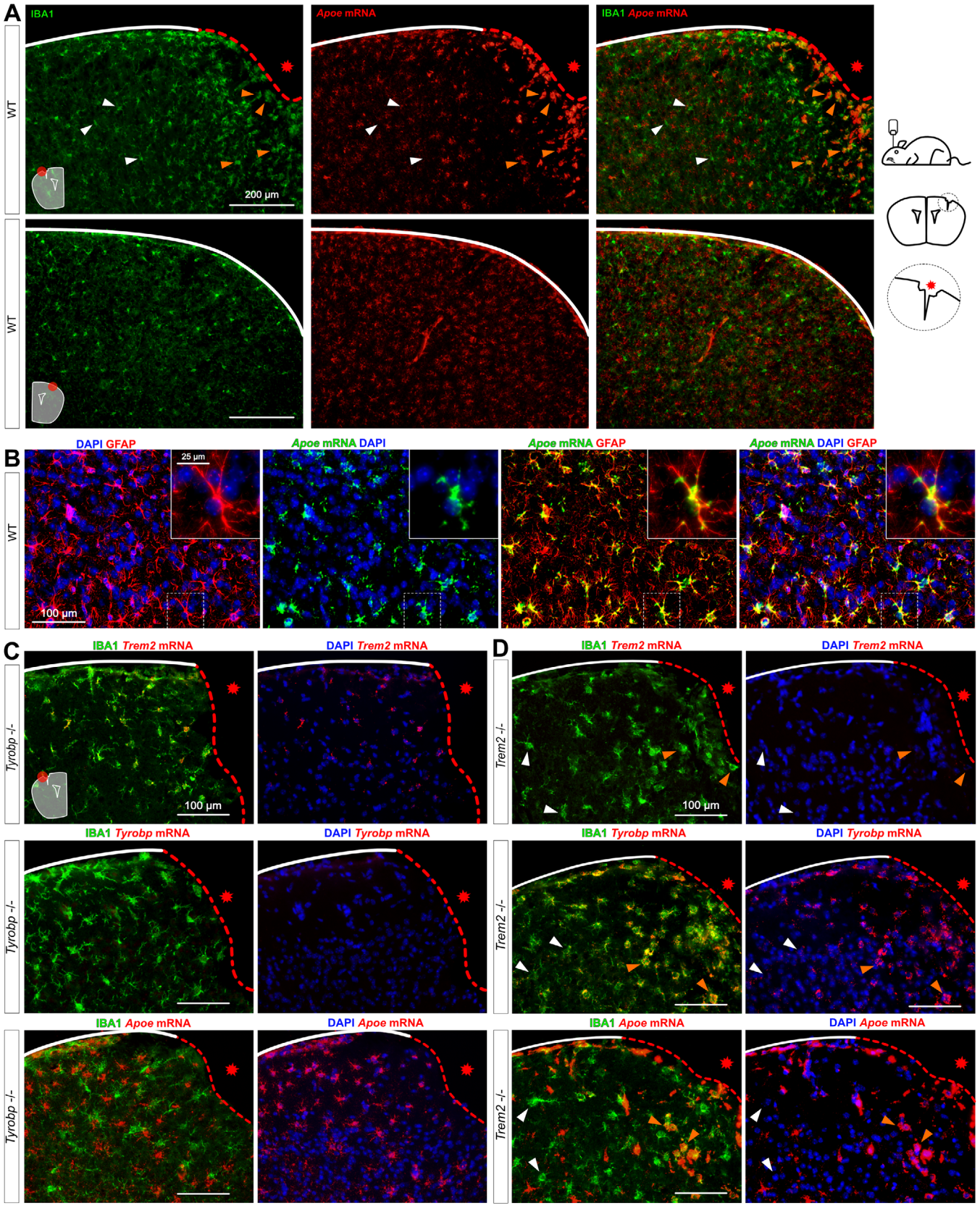

FIGURE 5 Increases of *Tyrobp* and *Apoe* mRNAs in microglia recruited to a site of stab injury are *Trem2*-independent. A, Stab-injured wild-type (WT) mice were sacrificed 3 days after injury and dual RNA fluorescent *in situ* hybridization and immunohistochemistry for *Apoe* mRNA (red) and anti-IBA1 (green), respectively, was performed. The injured ipsilateral area (red dotted line) is shown on the top row and the uninjured contralateral area is shown on the bottom row. Scale bar = 200 μm. B, Dual RNA fluorescent *in situ* hybridization and immunohistochemistry for

microglial activation, we probed both groups with anti-IBA1 antibody and observed a weaker staining in *APP/PSEN1;Iba1*^{Tyrobp} mice (Figure S2A). We next performed RT-qPCR on a group of microglial genes previously described in homeostatic or activated microglia. There was a significant increase of *Axl* and *Ccl2* and a decrease of *Cd68* and *Tgfβ* in brains of *APP/PSEN1;Iba1*^{Tyrobp} mice (Figure 3F). Finally, we observed that the decrease of amyloid plaques persisted in 8-month-old *APP/PSEN1;Iba1*^{Tyrobp} mice as shown by the decreased percentage of ThioS positive areas in somatomotor, hippocampus, and visual areas (Figure 3G-H).

3.2.4 | TYROBP overexpression in *MAPT*^{P301S} mice increases TAU phosphorylation and microglial activation

We previously reported that deletion of *Tyrobp* altered both mouse amyloidosis and tauopathy phenotypes and the microglial response to these pathologies.[25–27] In *MAPT*^{P301S};*Iba1*^{Tyrobp} double heterozygous mice, western blot analyses using AT8 and T205 antibodies revealed increased levels of phosphorylated-TAU (p-TAU) in the cortex of both male and female mice compared to *MAPT*^{P301S} mice at 4 months of age, whereas total human TAU levels detected with the HT7 antibody were unchanged (Figure 4A-B). Increased p-TAU within brains from *MAPT*^{P301S};*Iba1*^{Tyrobp} mice was further confirmed immunohistochemically (Figure 4C). We also observed increased IBA1 intensity in *MAPT*^{P301S};*Iba1*^{Tyrobp} compared to *MAPT*^{P301S} mice and this IBA1 increase was correlated with the increased p-TAU (Figure 4D, Figure S2B). We confirmed an increased microglial activation state by double-label immunohistochemistry with anti-IBA1 and anti-CD68 in the piriform cortex (Figure 4E). Using RT-qPCR, we measured increases of *Tyrobp*, *P2ry12*, *Apoe*, *Axl*, *Itgax*, *Iba1*, *Tgfβ*, and *Il6* mRNAs in *MAPT*^{P301S};*Iba1*^{Tyrobp} mice compared to *MAPT*^{P301S} mice (Figure 4F).

3.2.5 | Induction of microglial *Tyrobp* and *Apoe* is *Trem2*-independent in a model of cortical stab injury

To assess the interactions among *Trem2*, *Tyrobp*, and *Apoe* in microglia, we used an injury paradigm by introducing a small penetrating cortical stab injury via stereotactic surgery into one hemisphere of the mouse brain to induce a recruitment of microglia around the injury site.[60] We first used injured WT mice and combined RNA *in situ* hybridization and immunohistochemistry for *Apoe* and IBA1, respectively. In the intact hemisphere, most *Apoe* mRNA was not located in microglia but rather in astrocytes, the source of most APOE in the brain. However, *Apoe* mRNA was dramatically increased in microglia recruited on the lesioned side (Figure 5A-B). Following the same procedure in *Tyrobp*$^{-/-}$ mice, *Apoe* mRNA was not induced in microglia on either side (Figure 5C), but strikingly, mRNA levels of *Tyrobp* and *Apoe* were highly upregulated in the recruited microglia of injured *Trem2*$^{-/-}$ mice (Figure 5D, Figure S4 in supporting information). Taken together, these data indicate that *Tyrobp* upregulation in recruited microglia around the traumatic lesion is *Trem2*-independent. Moreover, the increase of *Apoe* transcripts in recruited microglia in the same mouse model of injury appears to be *Tyrobp*-dependent but *Trem2*-independent.

3.2.6 | Induction of microglial *Tyrobp* and *Apoe* around amyloid plaques is *Trem2*-independent, and *Apoe* upregulation is dramatically decreased when *Tyrobp* is absent

To investigate further these interactions among *Trem2*, *Tyrobp*, and *Apoe* in microglia in the presence of mutant human *APP*, we first performed dual RNA *in situ* hybridization and immunohistochemistry for *Tyrobp*, IBA1, and 6E10 in *TgCRND8* mice[61] on either a WT or *Trem2*-null background. Despite reduced recruitment of microglia around plaques when *Trem2* was deleted,[57] *Tyrobp* mRNA was still increased in plaque-associated microglia (Figure 6A) as was *Apoe* mRNA in plaque-associated microglia in the same *TgCRND8;Trem2*$^{-/-}$ mice (Figure 6B). We confirmed that the plaques-associated microglia upregulating *Tyrobp* were the ones upregulating *Apoe* (Figure 6C). We then assayed *APP/PSEN1* mice that were either WT or deficient in *Tyrobp* and, while the expression of *Apoe* was not completely abolished by deletion of *Tyrobp*, we confirmed a substantial decrease in the induction of *Apoe* mRNA in plaque-associated microglia when *Tyrobp* was absent (Figure 6D).

In summary, our results provide compelling evidence that: (1) upregulation of *Tyrobp* mRNA level is an early event occurring in recruited microglia and (2) TYROBP–APOE signaling in the microglial sensome is readily detectable even in the absence of *Trem2*. We propose that activation of the TYROBP–APOE pathway could be an early or even initiating step in the transformation of microglia from the homeostatic phenotype to the DAM phenotype (Figure 7).

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Apoe (green) and GFAP (red) in non-injured WT mice. C-D, The same stab injury protocol was used in *Tyrobp*$^{-/-}$ (C) and *Trem2*$^{-/-}$ (D) mice. Anti-IBA1 staining and DAPI staining are shown in green and blue, respectively. Top row: *Trem2* mRNA (red); middle row: *Tyrobp* mRNA (red); bottom row: *Apoe* mRNA (red). Mice were 4 months of age, and slice thickness = 10 μm. The red asterisk indicates the injured side. White and orange arrows indicate examples of non-recruited and recruited microglia, respectively

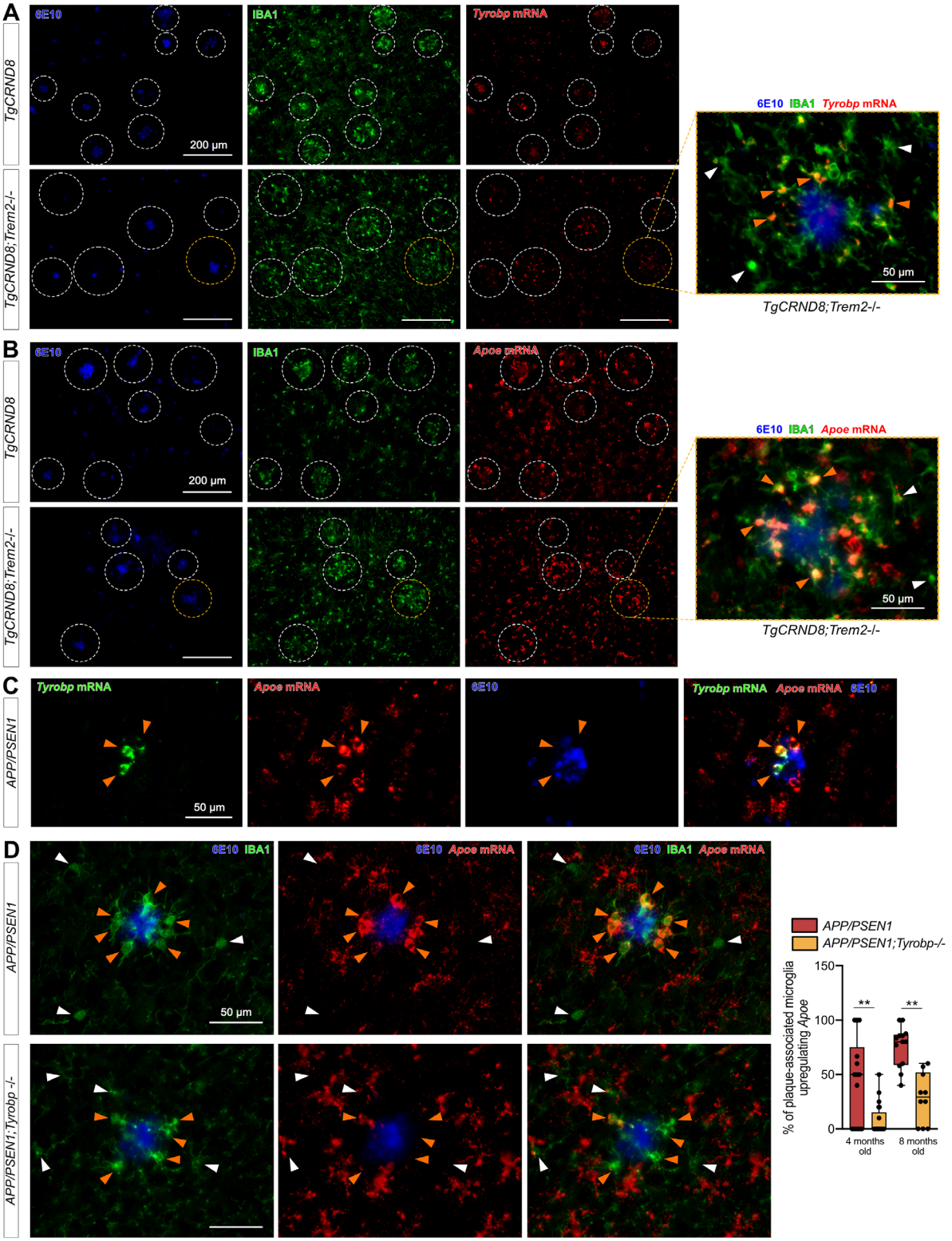

FIGURE 6 Increases in *Tyrobp* and *Apoe* mRNAs in amyloid plaque-associated microglia are *Trem2*-independent. A, Dual RNA fluorescent *in situ* hybridization and immunohistochemistry for *Tyrobp* mRNA (red), anti-IBA1 (green), and human amyloid beta (Aβ; 6E10 antibody; blue) in *TgCRND8* mice on wild-type (WT; top row) or *Trem2*$^{-/-}$ (bottom row) background. Scale bar = 200 or 50 μm. B, Dual RNA fluorescent *in situ* hybridization and immunohistochemistry for *Apoe* mRNA (red), anti-IBA1 (green), and human amyloid (6E10 antibody; blue) in the same mice as in (A). Scale bar = 200 or 50 μm. C, Dual RNA fluorescent *in situ* hybridization and immunohistochemistry for *Tyrobp* mRNA (green), *Apoe* mRNA (red), and 6E10 (blue) in *APP/PSEN1* mice. Scale bar = 50 μm. D, Dual RNA fluorescent *in situ* hybridization and immunohistochemistry for *Apoe* mRNA (red), anti-IBA1 (green), and human Aβ (6E10 antibody; blue) in *APP/PSEN1* mice on a WT (top row) or *Tyrobp*-null (bottom row) background. Scale bar = 50 μm. Right panel: quantification of the number of plaque-associated microglia with upregulated *Apoe* mRNA in the same mice as in (D). N = 2–3 mice per group (A-D)

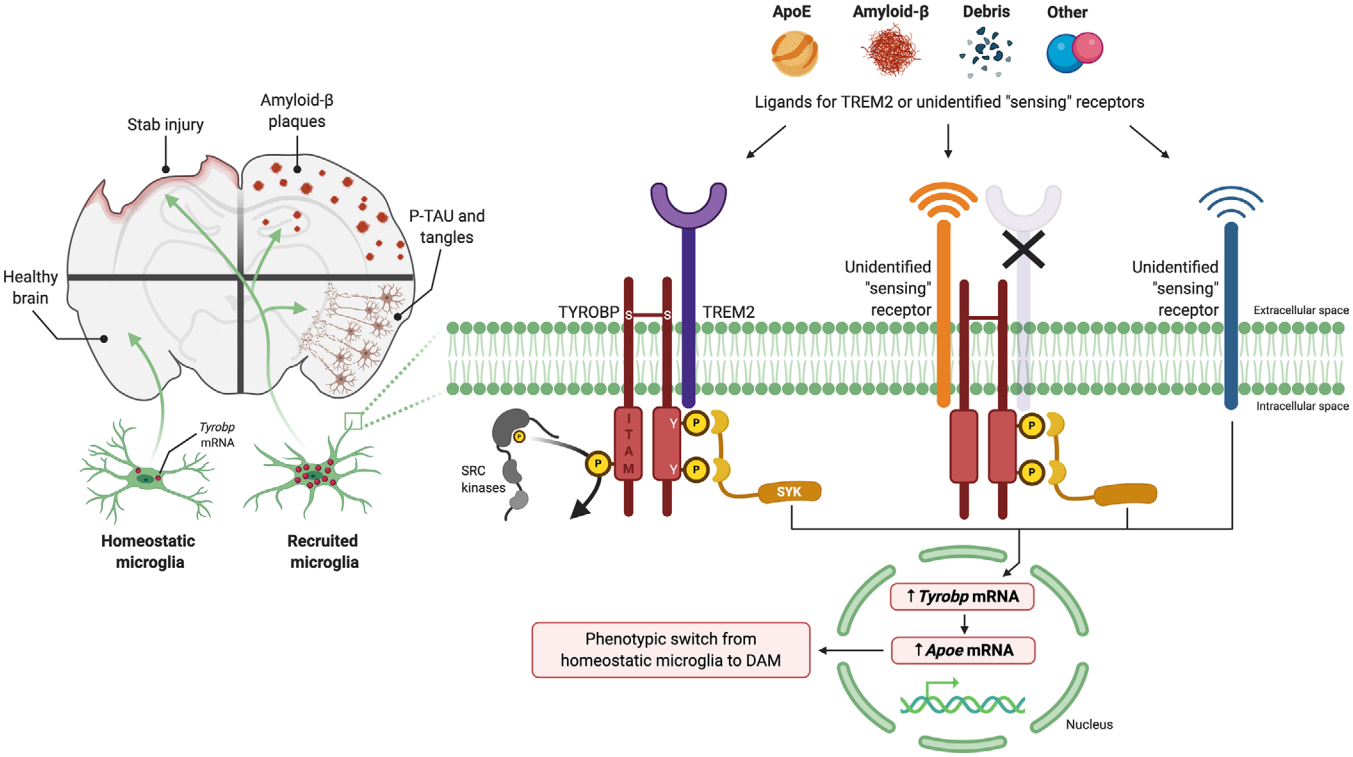

FIGURE 7 Proposed ligand-induced *Tyrobp* signaling in recruited microglia. Left panel, in response to penetrating stab injury or accumulation of amyloid beta (Aβ) deposits or misfolded TAU, *Tyrobp* transcription is upregulated in microglia, thereby marking these cells as "recruited microglia." Right panel, we observed that both microglial recruitment and *Tyrobp* upregulation occur in the absence of TREM2, indicating the existence of "sensing" receptors. Multiple alternative signaling pathways can be considered: Ligand signaling is initiated by APOE, Aβ, debris, or other ligands at sensing receptors and leads to phosphorylation of the tyrosine residues in the cytoplasmic ITAM of TYROBP by SRC kinases and the recruitment of SYK. In turn, SYK signaling leads to upregulated transcription of *Tyrobp* and *Apoe*. This series of events forms the basis for the phenotypic switch from homeostatic microglia to DAM. In mice lacking TREM2, microglial recruitment is retained, and transcription of both *Tyrobp* and *Apoe* is induced. Because these are constitutive TREM2 knockout mice, we are unable to exclude the possibility that some unknown sensor developed as compensation for the absence of TREM2. Another possibility is the existence of unidentified sensing receptor(s) that can upregulate TYROBP and APOE through a mechanism that does not require formation of complexes with TYROBP itself. APOE, apolipoprotein E; DAM, disease-associated microglia; ITAM, immunoreceptor tyrosine-based activation motif; SYK, spleen tyrosine kinase; TREM2, triggering receptor expressed on myeloid cells-2; TYROBP, tyrosine kinase binding protein

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

Mickael Audrain, Jean-Vianney Haure-Mirande, Sam Gandy, and Michelle E. Ehrlich designed the study. Mickael Audrain performed the experiments and analyzed the data. Justyna Mleczko contributed to the RNA *in situ* hybridization-related experiments. Minghui Wang and Bin Zhang contributed to the RNA sequencing analysis. Jennifer K. Griffin, Peter H. St George-Hyslop, and Paul Fraser provided the *TgCRND8* mice. Mickael Audrain, Sam Gandy, and Michelle E. Ehrlich wrote the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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