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Dysregulation of TLR5 and TAM Ligands in the Alzheimer's Brain as Contributors to Disease Progression

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Abstract

The hypothesis that accumulation of beta-amyloid (Aβ) species in the brain represents a major event in Alzheimer's disease (AD) pathogenesis still prevails; nevertheless, an array of additional pathological processes contributes to clinical presentation and disease progression. We sought to identify novel targets for AD within genes related to amyloid precursor protein (APP) processing, innate immune responses, and the catecholamine system. Through a series of bioinformatics analyses, we identified *TLR5* and other genes involved in toll-like receptor (TLR) signaling as potential AD targets. It is believed that Aβ species induce activation of microglia and astrocytes in AD, with a negative impact on disease progression. The TAM (Tyro3, Axl, Mer) family of receptor tyrosine kinases plays pivotal roles in limiting inflammatory responses upon TLR stimulation, for which we further studied their implication in the *TLR5* alterations observed in AD. We validated the up-regulation of *TLR5* in the frontal cortex of moderate AD cases. In addition, we observed up-regulation of the TAM ligands protein S (*PROS1*), galectin-3 (*LGALS3*), and Tulp-1. Furthermore, we identified an association of the TAM ligand *GAS6* with AD progression. In THP-1 cells, co-stimulation with Aβ and flagellin for 24 h induced up-regulation of *TYRO3* and *GAS6*, which could be prevented by neutralization of TLR5. Our results underscore the role of TLR dysregulations in AD, suggesting the presence of an immunosuppressive response during moderate disease stages, and implicate TAM signaling in AD immune dysregulation.

Keywords Alzheimer's disease · Toll-like receptors · TAM receptor tyrosine kinases · Gene expression · TLR5 · GAS6

Introduction

Abnormalities of β-amyloid (Aβ) metabolism are still believed to function as central elements in Alzheimer's disease (AD) pathogenesis, triggering a cascade of events that ultimately lead to neuronal cell damage and death. Nevertheless, current evidence points to a scenario where amyloid pathology is likely to be the result of a different initiating pathological event, such as a response to certain stressors [1]. Dysregulation of amyloid processing protein (APP)-interacting proteins may affect Aβ production. Among the various functions of APP, it has been suggested to have a role in stress responses mediated by catecholamines. Moreover, prolonged inflammatory responses in the brain can lead to a loss of catecholaminergic neurons, influenced by age and duration of the pro-inflammatory stimulus [2]. The role of inflammatory processes in a number of psychiatric and neurodegenerative disorders has gained much attention in recent years [3, 4]. Neuroinflammation is likely to contribute to the pathogenesis of AD by the activation of microglia in response to Aβ accumulation through sensing molecules, including complement receptors, toll-like receptors (TLRs), and NOD-like receptors (NLRs) [5–7]. Moreover, the APOEε4 allele, the major genetic risk factor identified to date for AD [8], has recently been implicated in the innate immune response to TLR ligands in humans [9]. Similarly, several other genes conferring AD susceptibility seem to play roles in innate immune responses [10, 11].

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TLRs can activate microglia and astrocytes, and mediate an array of different pathways resulting in both neuroprotective/anti-inflammatory and neurotoxic/pro-inflammatory phenotypes [12]. Consecutively, even though TLR activation triggers or amplifies immune responses in the central nervous system (CNS), evidence suggests that TLR signaling also plays critical roles in neuroprotective and restorative processes in response to various insults [13]. Because prolonged immune responses are detrimental to normal brain functions, mechanisms that restrict neuroinflammation exist. One such mechanism involves the TAM receptors (Tyro3, Axl and Mer), a family of widely expressed receptor tyrosine kinases that inhibit the propagation of pro-inflammatory signals resulting from TLR stimulation, by dampening the production of pro-inflammatory and inducing that of anti-inflammatory mediators [14]. Many factors define the downstream effects of TAM activation, including cell type-specific activation by the main (Protein S and Gas6) or additional (Tubby, Tulp-1, and Galectin-3) ligands, and patterns of co-expression [15–19]. In the CNS, the biological activities of the members of the TAM system have not yet been widely studied. Moreover, even though a number of abnormalities observed in TAM-deficient mice and microglia are also neuropathological features of AD [20–22], the TAM system has been scarcely studied in the context of this disorder.

In the present study, we sought to investigate the expression changes implicating APP processing, and the catecholamine and innate immune systems in AD, and performed a series of computational analyses to identify novel molecular targets. We further focused on our resulting top target, *TLR5*, and investigated the potential implication of a TLR5-TAM system interaction in the pathobiology and clinical progression of AD using human frontal cortex and a cellular model of microglia-like cells.

Short Methods

Bioinformatics Analyses for Target Identification Briefly, we used microarray expression data corresponding to entorhinal cortex (EC), hippocampus (HIP), and superior frontal gyrus (SFG) of AD and control brains from a publicly available data set (EMBL-EBI ArrayExpress ID: E-GEOD-48350 [23]) to analyze the region-specific expression changes and interactions, non-attributable to aging, in four gene sets (color within figures): APP-related (dark red), GWAS-identified AD candidates (lilac), catecholamine system-involved (brown), and innate immunity genes (gray blue). For this, we performed statistical comparisons, correlation tests, and classification tasks. We further assessed dysregulations of the differentially expressed (DE) genes in peripheral blood of mild cognitive impairment (MCI), AD, and control individuals from the AddNeuroMed cohort (Gene Expression Omnibus (GEO) accessions: GSE63060 and GSE63061 [24]). By a point-based system, including results from the different analyses, we assigned scores to each gene and ranked them to identify the strongest AD targets. Finally, we built a protein-protein interaction (PPI) network with the top 10 targets, and included APP and microtubule-associated protein tau (MAPT), as the pivotal AD players, to shed some light into potential molecular links with, or mechanisms implicating the identified genes in, AD pathophysiology. Detailed methods for all bioinformatics analyses can be found in the Supplementary Methods.

Biological Material Total RNA extracts from frontal cortex homogenates of 35 cognitively healthy controls (CTRL), 35 MCI, and 34 AD (13 in moderate and 21 in advanced disease stages) cases were used for validation of *TLR5* up-regulation and further experiments on the potential involvement of the TAM system. Approval to work with human tissues is granted to Michael T. Heneka by the Ethics Committee of the University Hospital Bonn. The THP-1 cell line was used for the in vitro assessment of the effects of Aβ exposure, and co-stimulation with the natural ligand for TLR5, flagellin (FliC). Basic sample description and detailed methods for all experiments can be found in the Supplementary Methods.

RT-qPCR Gene expression levels were analyzed by the comparative ΔΔCt method through one-step RT-PCR using the QuantiTect SYBR green RT-PCR Kit (QIAGEN), following manufacturer's instructions. Pre-validated QuantiTect Primer Assays (QIAGEN) were used to amplify the following: *TYRO3* (QT00055482), *AXL* (QT00067725), *MERTK* (QT00031017), *PROS1* (QT00011746), *GAS6* (QT00049126), *TULP1* (QT00080528), *TUB* (QT00037149), *LGALS3* (QT00094052), *CX3CR1* (QT00203434), *AIF1* (QT00013279), *TREM2* (QT00063868), *GFAP* (QT00081151), *NEFL* (QT00096369), *TLR5* (QT01682079), *EGR1* (QT00218505), and *GAPDH* (QT00079247). Ct values obtained for target genes were normalized using *GAPDH* as the reference gene.

ELISA TLR5 activation was assessed by measuring interleukin (IL)-8 and tumor necrosis factor (TNF)-α in cell culture supernatants by ELISA (DuoSet ELISA Development Systems, R&D Systems), following manufacturer's instructions. Optical density was determined at 450 nm, with 570 nm wavelength correction.

In Vitro $A\beta_{42}$ Uptake Proteins from THP-1 cells were extracted by cell lysis using 2× RIPA buffer. Replicates from the same experiment were pooled and proteins were extracted and precipitated. The uptake of Aβ was assessed by its intracellular relative contents, detected by western blot with the 6E10 antibody (#SIG-39320, Covance, 1:1000), normalized to β-actin.

Statistical Analysis Significance levels were set to $p < 0.05$. For all analyses, we used STATISTICA 7 (StatSoft), GraphPad Prism 7, and MeV software. Detailed methods for all performed analyses can be found in the Supplementary Methods.

Flagellin–β-Amyloid Sequence Alignment and TLR5–β-Amyloid Docking Simulations The possibility of sequence similarities between FliC and $A\beta_{42}$ was investigated by performing an alignment of their sequences using Jalview 2.9.0b2 [25]. Docking simulations to explore the ability of Aβ species to interact with TLR5 were performed through the GRAMM-X (Global RAnge Molecular Matching) Protein-Protein Docking Web Server v.1.2.0 [26] and images were created with Swiss-PdbViewer v4.1 [27]. FASTA sequences used for alignment and PDB files used for docking simulations were obtained from the RCSB Protein Data Bank [28]. Detailed methods can be found in the Supplementary Methods.

Results

Identification of TLR5 as a Relevant Target Gene for AD

Expression changes between AD and control brains were obtained controlling for the effects of age and gender. In total, over 130 genes were investigated: 11 related to, and including, APP, 46 involved in the development and function of the catecholamine system, 28 AD candidates identified through large GWA studies, and over 50 genes participating specifically in innate immune responses. We identified 43 of these genes showing non-age-related altered expression levels in AD (Fig. 1a), in at least one brain region: 19 DE in EC, 17 in HIP, and 15 in SFG, with *CLEC7A* ($p = 0.00013$, adjusted $p = 0.0009$), *CHGB* ($p = 0.00026$, adjusted $p = 0.0015$), and *TLR5* ($p = 9.9 \times 10^{-7}$, adjusted $p = 2.1 \times 10^{-5}$) presenting the highest significance, respectively. Analysis results of all significant genes for the comparisons between AD and controls, and between young and aged controls, can be found in Table S1. From these 43 DE genes, 9 were also found to be up-regulated in peripheral blood in AD (Fig. S1), compared to controls, where *TLR5* ($p = 0.000303$, adjusted $p = 0.003032$) and *APBB3* ($p = 0.000315$, adjusted $p = 0.002836$) were the most significantly changed in AD that remained unchanged in MCI.

Partial correlation coefficients for each brain region were calculated, while controlling for age and gender, to observe associations of APP and its related genes with the GWAS candidates and those genes involved in the catecholamine and innate immune systems, and to identify relations with clinicopathological variables in AD cases, namely *APOE* genotype, Braak stage, and Mini-Mental State Examination (MMSE) test score. Broadly, the larger number of correlations was observed in EC, particularly for genes of the APBA family. However, more correlations with *APP* were observed in HIP and SFG (Fig. 1b). Outside the APBA and APBB family members, *CHGB* and *TLR5* were the genes correlating with the larger number of APP-related gene set members. *TLR5* and *IFIH1* expression showed correlations with disease status in all brain regions, particularly in SFG (Table S2). Moreover, immune-related genes negatively correlated with *APOE* genotype, while three genes (*MOXD1*, *APLP2*, and *FCGR1A/C*) positively correlated with MMSE score. No gene's expression correlated with Braak staging of AD cases (Fig. S2).

We further used classification and regression trees (C&RTs) as a means to study the relative importance of regional changes in gene expression for AD. In all brain regions, good classification accuracy was observed, although control membership was predicted more accurately than AD membership (sensitivity<specificity), particularly in EC and SFG, where most genes used in the classifier were immune-related and included *TLR5* (Fig. S3). However, for predictions of the clinicopathological features, it was the catecholamine system set the one contributing in the largest proportion (Table S3). We speculate that these observations might reflect, on one hand, that the innate immune system activates in response to the occurrence of neuropathology early in the disease while, on the other hand, the catecholamine system might contribute to the clinical progression of AD.

By integration of these results, we found our 43 targets were grouped in 19 subsets of 1 to 6 genes (Table S4), where *TLR5* showed the highest ranking. We took the top 10 genes (*TLR5*, *SORL1*, *TLR1*, *TBK1*, *IFIH1*, *C3*, *APLP2*, *ECSIT*, *APBB1IP*, and *APBB3*) to build a PPI network that allowed the visualization of the relationships between these top targets with *APP* and *MAPT* (Fig. 2a). We discuss these genes in all detail in the Supplementary material. Network nodes were found grouped in four clusters corresponding to APP processing-related proteins and MAPT (yellow), complement system proteins (green), TLR signaling proteins (red), and mitochondrial proteins (blue). We observed that the complement response is linked in our network to APP processing and tau by a connection between C3 and APP and that, furthermore, the complement links the TLR signaling with these AD molecules through a connection between C3 and TLR1/TLR5. More information on our top targets can be found in the complementary discussion (Supplementary Material).

To follow-up on our top target, we validated the dysregulation of *TLR5* using RT-qPCR in a subset of our independent sample (Fig. 2b), where we observed increased levels (Bonferroni post-hoc $p < 0.001$ for all comparisons) of *TLR5* mRNA in the frontal cortex of moderate AD cases ($n = 4$), but not MCI ($n = 10$) or advanced AD ($n = 5$). Moreover, there was a modest correlation of *TLR5* mRNA levels with the

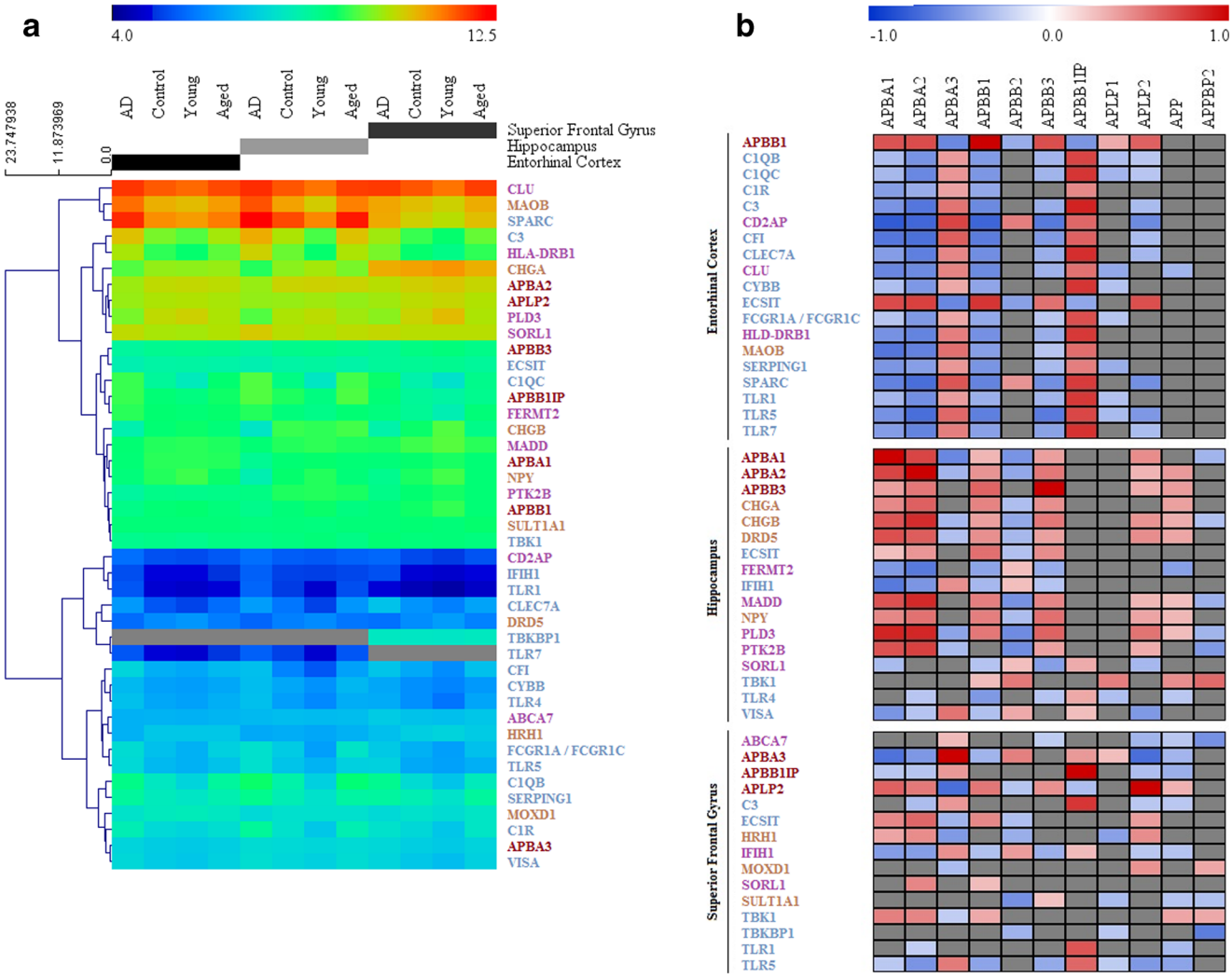

Fig. 1 Identification of AD target genes in the E-GEOD-48350 dataset. **a** Mean expression levels in Alzheimer's disease, all cognitively normal controls, young controls, and aged controls, in entorhinal cortex, hippocampus, and superior frontal gyrus. Genes were considered differentially expressed in a given region when the Benjamini-Hochberg-adjusted *p*-values for the comparison between AD and all controls were significant, while those corresponding to the comparison between young and aged controls were not significant. All comparisons were adjusted for age and gender. Hierarchical clustering was performed by Euclidean distance with complete linkage. **b** Region-specific correlation coefficients of the corresponding regional significant genes with APP-related genes in AD and control brains. Partial correlations were obtained while controlling for the effects of age and gender. Gray squares represent non-significant correlations. Gene sets: APP-related (dark red), GWAS-identified AD candidates (lilac), catecholamine system-involved (brown), and innate immunity genes (gray blue)

expression of the constitutive microglial cell marker *AIF1* (Iba1, $p = 0.023$, $r = 0.5324$, Fig. 2b), but not with markers for activated microglia, neuronal, or astroglial cells. *TLR5* also correlated with the mRNA levels of the neuronal transcription factor *EGR1* ($p = 0.013$, $r = 0.4439$). This suggests that *TLR5* was likely expressed by microglial cells. However, the regulation of *TLR5* by *EGR1*, although possible, is not clear from our data.

Characterization of TAM System Alterations in AD

To investigate whether the TAM system, an important limiting mechanism of TLR signaling, is involved in AD pathobiology, in general, and in the alterations we observed of *TLR5* in AD, in particular, we first characterized the gene expression patterns of the TAM receptors and ligands in the AD frontal cortex. We observed no change in gene expression of the TAM receptors. Nevertheless, mRNA levels showed a slight tendency towards increases during moderate and/or advanced AD, depending on the receptor (Fig. 3a), which may reflect their different functionalities. We included in our study all five reported TAM ligands and found that *PROS1* (protein S) and *LGALS3* (galectin-3) showed a similar pattern of expression, with increases during moderate stages of AD but not in advanced stages of the disease (Fig. 3b). On the other hand, *GAS6* presented progressive slight increases in gene expression from normal cognition through AD-type pathology, to finally reach significance, when compared to control and MCI brains, at the advanced AD stage.

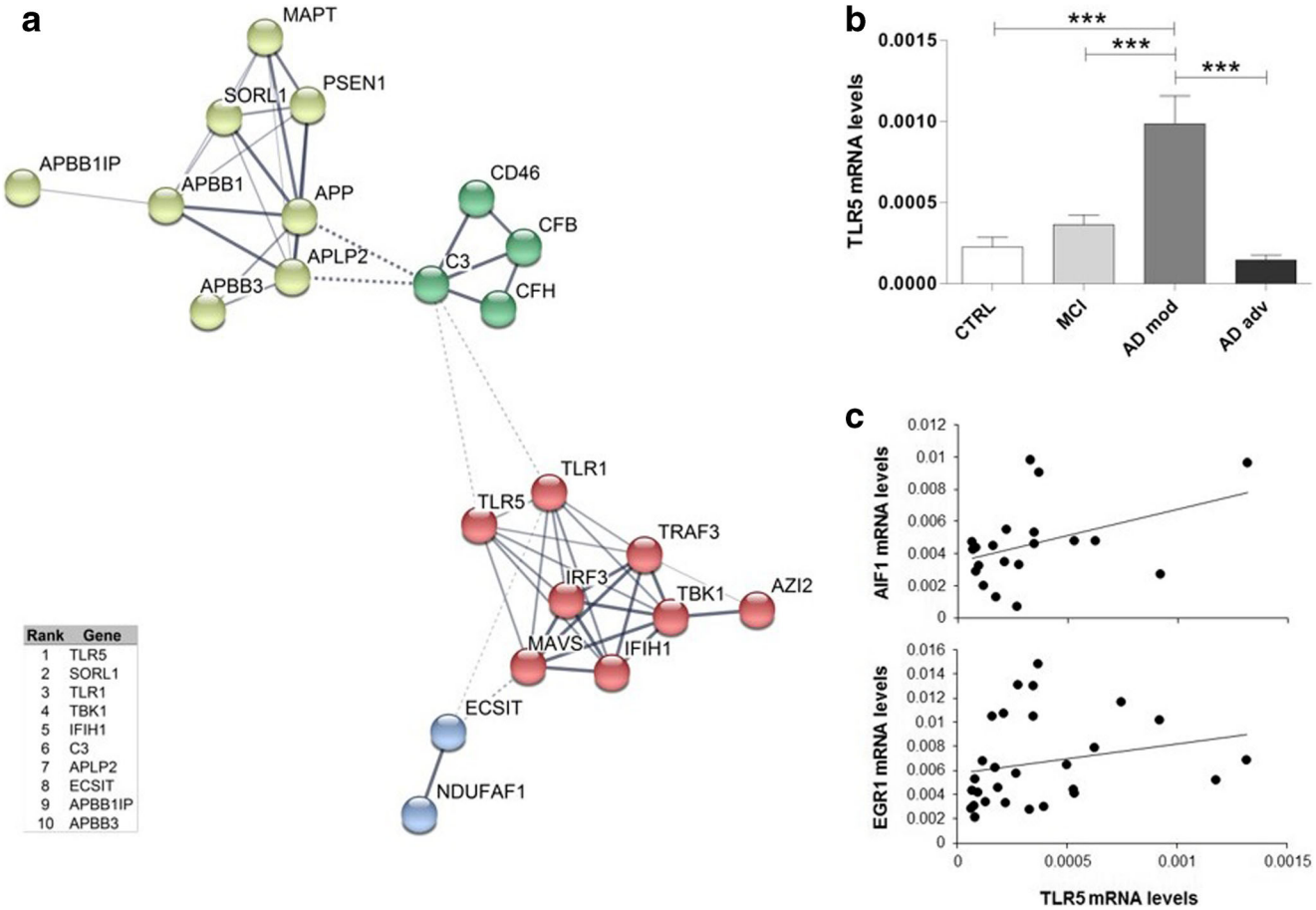

Fig. 2 **a** Protein-protein interaction network of the top 10 targets identified in our study, after systematic integration of results, with incorporation of APP and MAPT. The network was created using STRING v.11, expanded by 10 interactions in the Confidence view, and clustered with the MCL algorithm. **b** Validation of *TLR5* dysregulation in AD. The gene expression of *TLR5* was up-regulated in frontal cortex of moderate AD cases in an independent sample ($N = 28$). ***$p < 0.001$. **c** *TLR5* mRNA levels positively correlated with those of *AIF1* (when adjusted for age, gender, and PMI) and *EGR1*. Gene expression levels measured by RT-qPCR are presented as the mean values of the $2^{-\Delta Ct}$ transformation

We explored the potential relationships between TAM system elements using correlation matrices and hierarchical clustering (Fig. 3c, d), both of which revealed that *AXL* may more likely relate to *GAS6*, and *MERTK* (Mer) with *TUB* (Tubby); however, both pairs could be seen as what we called a "late subsystem," affected only at late AD stages. On the other hand, *TYRO3* appeared to form an "early subsystem" with the potential to affect moderate stages of AD. This latter would also comprise *PROS1*, *LGALS3*, and *TULP1*.

Because gene expression was measured from brain tissue homogenates, which include all cell types present in the tissue, our approach to try to elucidate the cell type-specific expression patterns of the TAM system elements was to measure the mRNA levels of markers for microglia (*AIF1*, *CX3CR1*, *TREM2*), astrocytes (*GFAP*), and neurons (*NEFL*), and test how these correlated with the gene expression of TAM system elements. We found that not all three microglial markers we used showed a common expression pattern: the levels of *AIF1* remained stable over the different disease stages, while levels of *CX3CR1* showed a non-significant tendency to increase, in a progressive manner, along with disease progression. *TREM2* was, however, increased in advanced AD cases (Fig. 4a). Similarly, *GFAP* expression showed increases only in those cases with advanced AD. This might suggest that micro- and astrogliosis are present in the frontal cortex in AD only at late disease stages.

Considering the results from our correlation tests, we suggest that, in our study, the TAM system elements might have been expressed by activated microglia and astrocytes in differential patterns (Fig. 4b). Furthermore, the ratios between *TYRO3* ($p < 0.01$) and *AXL* ($p < 0.001$) to *TREM2* were increased in moderate AD cases. Although the ratios of all three TAM receptors to *GFAP* showed significant changes in the ANCOVA results ($p < 0.0001$), pairwise comparisons were not significant by the Bonferroni post-hoc tests (Fig. S4). However, as the interactions between microglia and astroglia are complex and thus difficult to interpret by our simplistic approach to try to resolve the cell type-specificity issue, we should not draw strong conclusions out of this analysis.

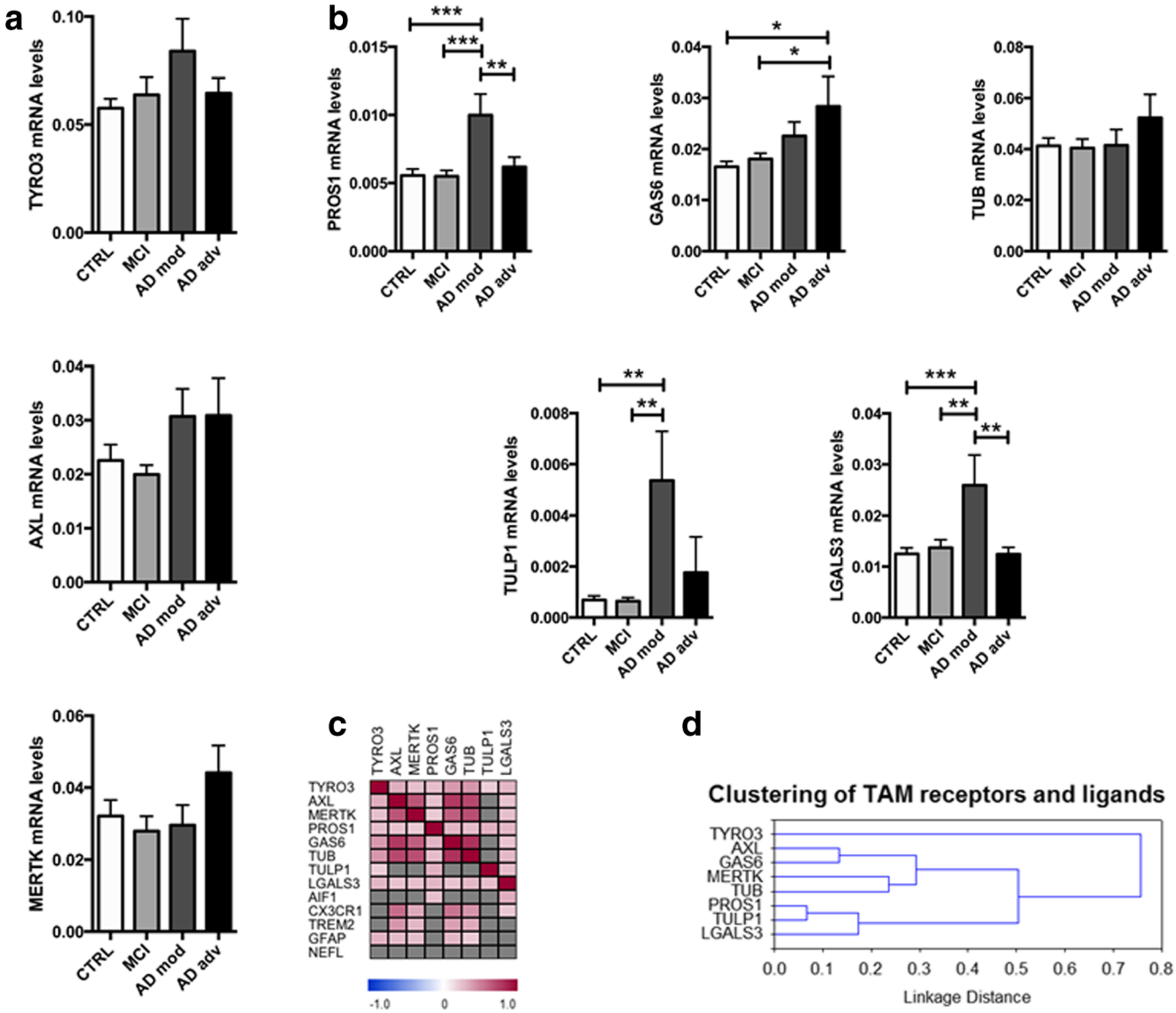

Fig. 3 Expression of the TAM system elements in frontal cortex. Gene expression of the TAM receptors (**a**) and the reported TAM ligands (**b**) was measured by RT-qPCR in frontal cortex of 104 frozen brain samples. Correlation tests (**c**) and gene hierarchical clustering (**d**) were applied to explore the associations between the system elements

Next, we attempted to elucidate the potential clinical relevance of the TAM system through correlation and linear regression tests. First, to corroborate the relationships between the clinicopathological parameters we had available, we showed that all parameters were highly correlated to each other, that the MMSE test scores were highly associated with the neuropathological findings, and that cognitive decline, as measured by the MMSE scores, was slightly more associated with tau pathology than with the amount and density of amyloid plaques. Interestingly, however, the *APOE* genotype showed no relationship with these parameters in our sample (Fig. S5-A/C), which can be likely attributed to the small sample size. Although some negative correlations were observed between TAM ligands and the MMSE scores, the only targets whose expression appeared to importantly correlate with the neuropathological features were *GAS6* and the cell markers *TREM2* and *GFAP* (Fig. S5-B/C). Nevertheless, only *GAS6* ($p = 0.014$, Beta = 0.47) and *GFAP* ($p = 0.037$, Beta = 0.26) were associated with disease progression, considered as the continuous coding of "disease status," by linear regression, suggesting a detrimental effect of *GAS6* and *GFAP* in AD.

Investigation of the Relationship Between TLR5 Signaling and the TAM System in AD

In our independent sample, the expression of *TLR5* correlated with that of the TAM ligand *PROS1* ($p = 0.025$, $r = 0.5266$) in AD brains, but not with that of the other TAM elements part of the "early subsystem." This might be partly explained by the observation that *TLR5* and *PROS1* were both associated with

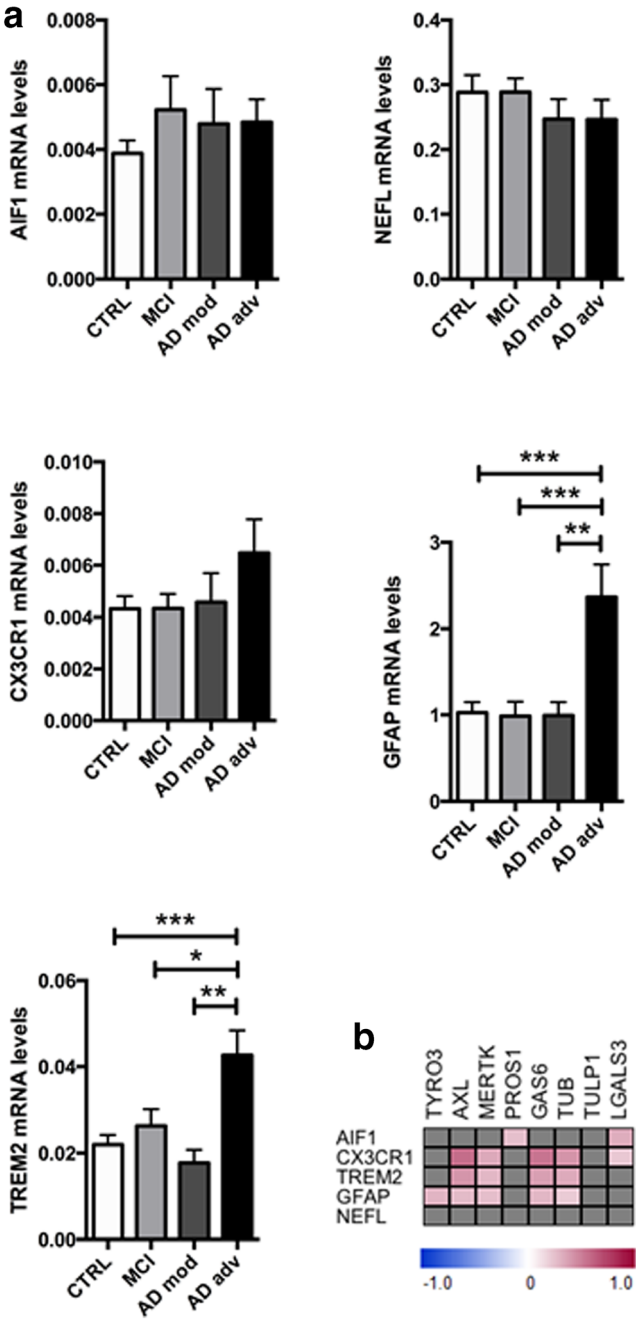

Fig. 4 Relationships between the TAM system elements and cell markers. Gene expression of cell markers for microglia (*AIF1*, *CX3CR1*, *TREM2*), astrocytes (*GFAP*), and neurons (*NEFL*) was measured by RT-qPCR in frontal cortex (**a**) and correlated with the expression of elements of the TAM system (**b**)

the expression of *AIF1*, but no other cell type-specific marker in this sample.

In an attempt to investigate whether Aβ species can directly stimulate TLR5 receptors and its potential implications for the expression of TAM system elements, we used PMA-differentiated THP-1 cells as a model of macrophage/microglia-like cells. TLR5 involvement was assessed through treatment with human anti-TLR5 neutralizing antibody, prior to main stimulations. Cells were stimulated for 24 h with either FliC, the natural ligand for TLR5, a preparation of oligomeric Aβ (which may also contain amyloid fibrils), or both, to assess the delayed response to these compounds. Treatments did not affect cell viability/metabolism, as assessed by the XTT assay. To determine cell activation in response to treatments, we measured IL-8 and TNFα secreted in the culture supernatants by ELISA (Fig. 5a, b).

As expected, stimulation with FliC caused an increase in the concentration of both inflammatory molecules, prevented by the neutralization of TLR5. However, surprisingly, we found that exposure to our Aβ preparation showed anti-inflammatory effects after 24 h, significantly reducing the levels of IL-8 and TNFα secreted by THP-1 cells. This effect appeared to be inhibited by neutralization of TLR5 in the case of IL-8, but not TNFα, suggesting that IL-8 secretion may be more dependent on TLR5 than that of TNFα. Furthermore, the presence of Aβ in the culture medium inhibited IL-8 increases in response to FliC in a manner similar to that observed for the TLR5 neutralizing antibody, while the effect on TNFα was the same as that observed for the stimulation with Aβ alone. Neutralization of TLR5 did not appear to further potentiate the effects of Aβ (Fig. 5b).

Gene expression of *TLR5* was not affected by the different treatments in THP-1 cells. However, we observed a non-significant tendency for *TLR5* mRNA levels to decrease in response to FliC, which was not observed upon TLR5 neutralization, while the opposite was observed for the treatment with Aβ alone. The presence of Aβ also prevented the slight decrease in *TLR5* expression observed for FliC when cells were co-stimulated (Fig. 5c). Moreover, *TLR5* expression at 24 h was inversely correlated to the levels of secreted IL-8 and TNFα (Fig. 5d). Together, these observations suggest that, contrary to our initial beliefs, increased levels of *TLR5* mRNA are actually indicative of an impaired response to immune stimuli, and that prolonged stimulation of TLR5 by Aβ species might result in deficient immune responses.

Gene expression of *TYRO3* and *GAS6* at 24 h was not affected by Aβ treatment alone, but their expression was induced when FliC and Aβ species were present together in the culture medium. This effect was completely inhibited by TLR5 neutralization in the case of *TYRO3* while, for *GAS6*, the induction did not reach significant levels when TLR5 was neutralized (Fig. 6a). Although showing non-significant changes, *PROS1* had a tendency to increase in the presence of both stimuli when TLR5 was neutralized, while Aβ slightly increased *LGALS3*, effect inhibited by TLR5 neutralization; when both stimuli were present in the medium and TLR5 was neutralized, *LGALS3* also presented a tendency towards increases and thus, to some extent, *LGALS3* expression may be dependent on TLR5 activity. *TULP1* appeared to not be expressed in THP-1 cells. Together, these observations suggest that Aβ stimulation or TLR5 activation alone does not

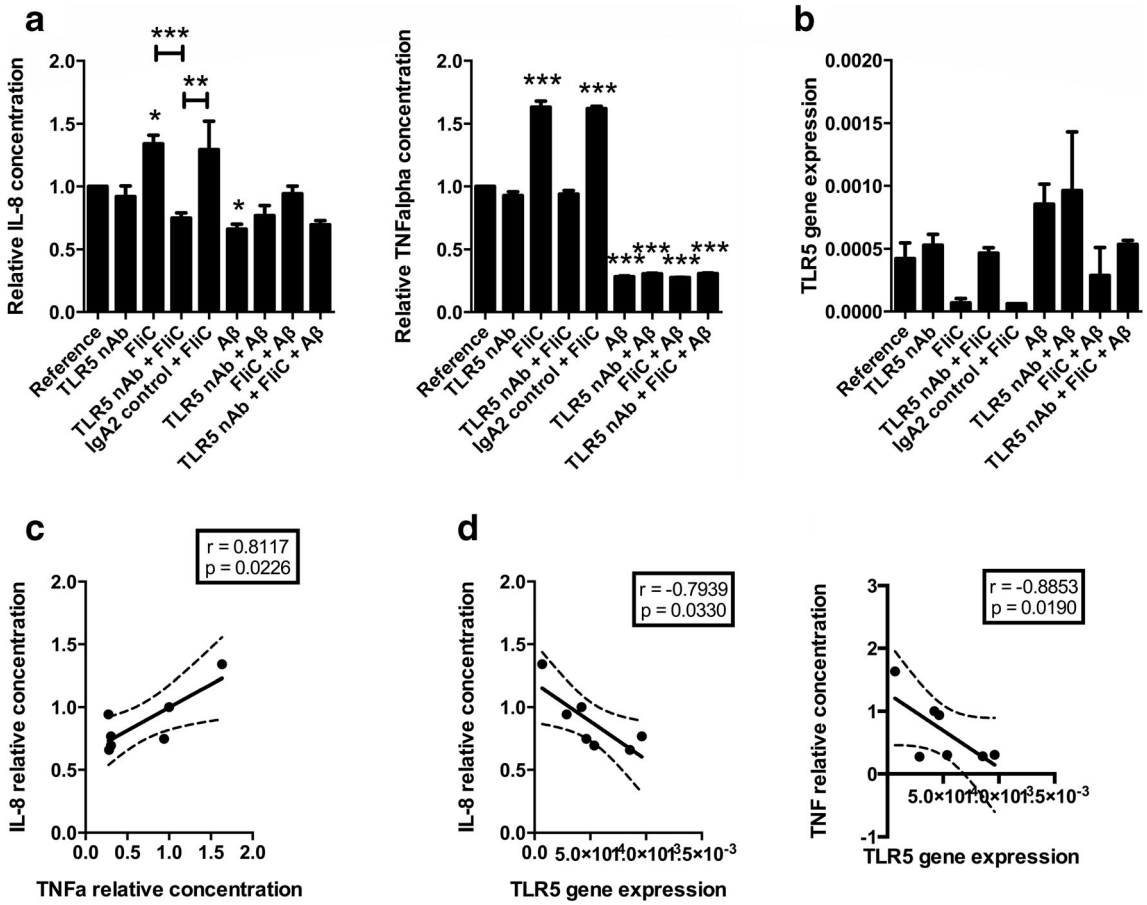

Fig. 5 Effects of amyloid stimulation on *TLR5* gene expression. Differentiated THP-1 cells were stimulated with FliC and/or an oligomeric Aβ preparation for 24 h. Concentrations of IL-8 and TNFα were measured in culture supernatants by ELISA (**a**) and *TLR5* gene expression was measured by RT-qPCR (**b**). IL-8 and TNFα concentrations correlated positively (**c**), while *TLR5* expression correlated negatively with the concentrations of secreted IL-8 and TNFα (**d**)

affect the expression of TAM system elements; nevertheless, when a chronic immune stimulus exists and Aβ species are present in the environment, there may be an up-regulation of certain components of the system that might abolish the response to the immune stimulus and potentially impact the uptake of Aβ (Fig. 6b, c). However, further experiments are required to see if the non-significant tendencies we observed in the present study, such as for the expression of *TLR5*, *PROS1*, and *LGALS3*, as well as for Aβ uptake, can reach significant levels.

That Aβ was able to prevent the inflammatory response to FliC made us wonder about the possibility of a TLR5–Aβ interaction given in such a way that, instead of activating the receptor, Aβ could be blocking its ligand binding capacity. In an initial attempt to explore this idea, we made use of sequence alignment to identify any similarities between FliC and Aβ that might be recognized by TLR5 (Supplementary Methods). We found some alignment within residues 333-373 of FliC with $A\beta_{42}$ (Fig. S6) that, even if not falling within the TLR5 recognition domain, may open the possibility for a TLR5–Aβ interaction. We further created protein-protein docking simulations between the human TLR5 model structure in its biological assembly, and three different Aβ species: Aβ 1-40 and Aβ 1-42 monomeric structures, an Aβ fibril-like structure that we termed "oligomer," and fibrillar Aβ. Moreover, to simplify the receptor's structure, we also created docking simulations for the zebrafish TLR5 with monomeric and fibrillar Aβ (Fig. S7). From the docking simulations with human TLR5, we did not observe great potential for Aβ monomers to bind to TLR5 in a manner relevant for the inhibition of ligand binding. However, we believe the possibility may exist of interference for the interactions with adapters (outside of the FliC recognition domain) to initiate signaling. Moreover, for the zebrafish TLR5 structure, we observed several possibilities for Aβ monomers to interact with the receptor and potentially block FliC recognition. Aβ aggregates, on the other hand, showed a clearer potential to interact with TLR5 in a manner that would obstruct ligand recognition.

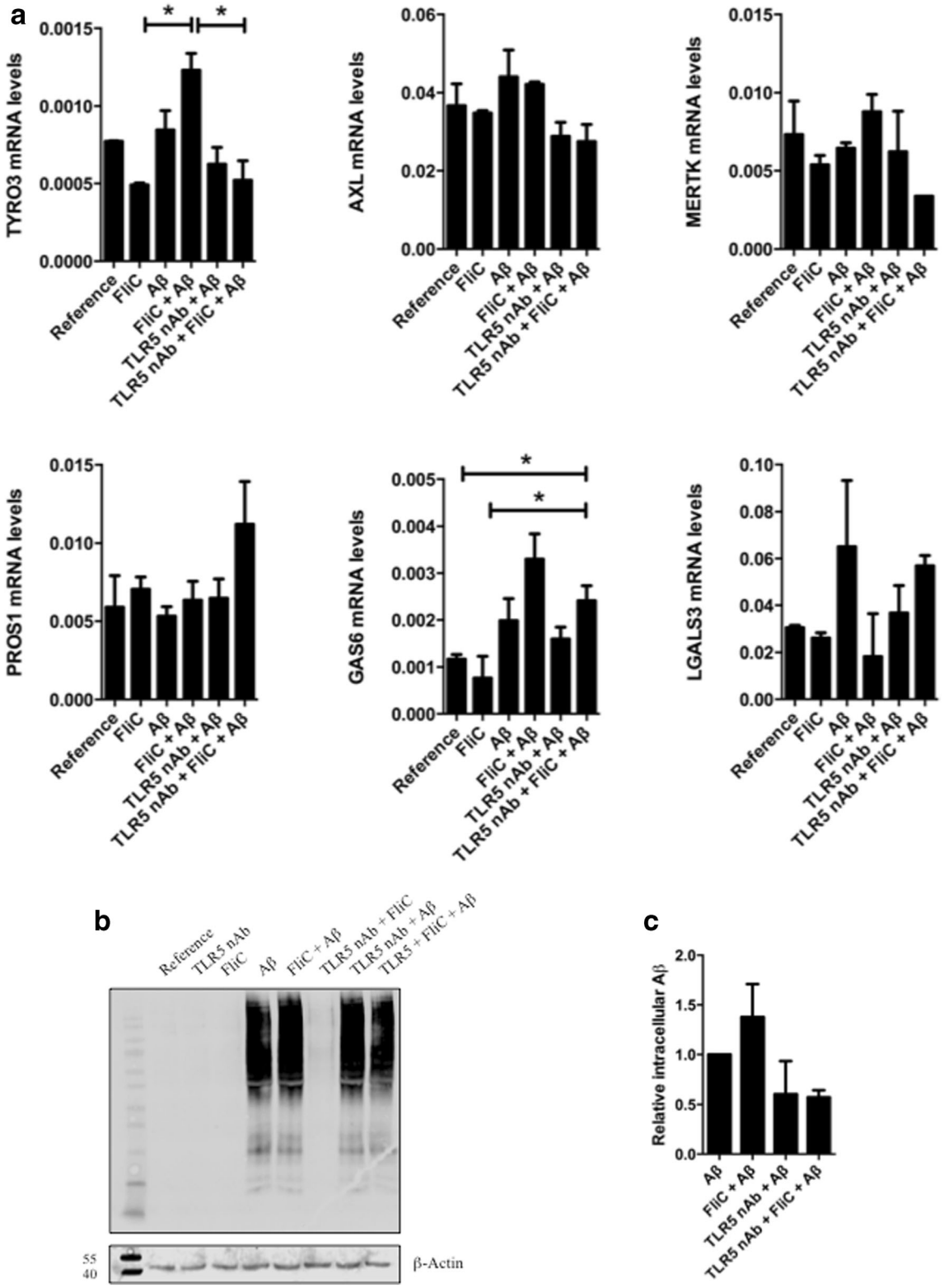

Fig. 6 Effects of amyloid stimulation on the expression of TAM system elements. Differentiated THP-1 cells were treated as before, and the expression of the TAM receptors and ligands was measured by RT-qPCR (**a**). The functional implications of TAM system dysregulation were assessed for the uptake of Aβ from the culture medium through western blot using the 6E10 antibody (**b**). Relative intracellular concentrations of Aβ (**c**) were normalized to the reference band (β-actin). The different treatments from each gel were normalized to the reference condition (Aβ treatment alone)

Discussion

Our study provides evidence supporting that dysregulations in the catecholamine and innate immune systems contribute to AD neuropathology with a regional specificity that may reflect the propagation of the disorder from EC to HIP and SFG, and underscores the involvement of abnormalities in TLR signaling and complement system molecules, such as *TLR5*, *TBK1, ECSIT*, and *C3*. We propose these and other genes as relevant AD targets. Moreover, we chose to focus on our top target and, by characterizing TLR5 and the TLR signaling-limiting TAM system in AD frontal cortex and a cellular model, we showed in this study evidence of the presence of an immunosuppressive response in moderate AD cases, arguably mediated through the TAM system, and the potential implication of TLR5 signaling, upon prolonged immune stimulation in the presence of Aβ. Furthermore, we showed that the latter finding may drive the up-regulation of *GAS6* we observed in AD brains, which proved to exert a negative impact on disease progression (worsening of the neuropathology and cognitive decline) in our study.

TLRs have been proposed to contribute to AD pathology by different mechanisms activated by Aβ in microglial cells [29], and increases in mRNA levels have been observed in tissue associated to plaques in mice [30]. However, even when dysregulations in TLR signaling have been widely implicated in AD, most studies have focused on TLRs 2 and 4 [30–35]. TLR5 recognizes FliC from Gram-positive and Gram-negative bacteria in a specific manner, and bacterial infection has been associated with increased occurrence of AD [36–38]. Moreover, TLR5-deficient mice develop hyperlipidemia, hypertension, insulin resistance, and increased adiposity, among other metabolic features [39], all of which have been extensively proposed as factors increasing the risk to develop AD. Consistent reports of increased *TLR5* expression in animal models of not only AD but also Parkinson's disease and dementia with Lewy bodies [30, 40, 41] have been made. Herein, we showed that *TLR5* is up-regulated in the frontal cortex of moderate AD cases, with evidence of its expression by microglial cells.

In our study, we found that FliC and Aβ can exert opposite effects on the TLR5 delayed response, which leads to an inhibition of TLR5's response to FliC by Aβ species. Our docking simulations suggest that Aβ is unlikely to bind to TLR5 in its FliC recognition site, but we do not discard the possibility of some sort or TLR5-Aβ interaction that might block TLR5 signaling, which is of interest for future studies. Furthermore, our experiments suggest a transcriptional induction of *TLR5* indicates an anti-inflammatory or impaired immune response that can result from prolonged Aβ exposure, which might then point to an immunosuppression in frontal cortex of moderate AD cases, according to our findings. Consistent with our observations, Chakrabarty et al. [42] have recently reported an interaction of the TLR5 ectodomain with Aβ species that modulates the activation of TLR5 signaling and ameliorates Aβ toxicity, further suggesting a TLR5-based therapeutical approach to target Aβ aggregation. From our study, we believe that overproduction of Aβ might initially serve as a defense mechanism against brain insult. For example, most DE genes in EC were innate immune genes and showed stronger correlations with APP-related genes in this brain region, while dysregulation of APP-related genes became more prominent in brain regions known to be affected at later AD stages. However, the chronic co-occurrence of the insult and Aβ species may lead to immune impairments that result detrimental for disease progression. In this context, we also add to the mounting evidence that infection could represent an important risk factor in the pathogenesis of AD [38, 43], although the brain insult we refer to here might or not be of an infectious nature.

Our initial observations on the TAM system in AD reflected disease-dependent changes in associations between different elements of the system, without actual gene expression dysregulations, that implicated subtle changes in the expression patterns and interactions between TAM receptors and their ligands early in AD neuropathology (unpublished data [44]). Based on these and our current observations, we propose the existence of two TAM subsystems in the human AD brain, with each of them differentially participating in its neuropathology. We speculate that the first of such subsystems, possibly conformed by protein S, galectin-3 and Tulp-1, through activation of Tyro3, could be involved in the generation of an enhanced immunosuppressive response to the pro-inflammatory activity triggered by ongoing neuropathological processes in moderate stages of AD and, perhaps, participate as well in the transition to chronic inflammation, while the second subsystem, probably conformed by Gas6 and Tubby acting through Axl and Mer, respectively, might be involved in chronic inflammatory processes and the phagocytosis of an increasing number of apoptotic neurons as disease progresses. We found evidence to suggest that TLR5 may be partially involved in these processes and contribute to the regulation of the expression of TAM system elements.

Further evidence implicating the TAM receptors in AD comes from studies showing that Axl levels associate with the concentrations of $A\beta_{42}$ in the cerebrospinal fluid (CSF) of cognitively healthy individuals [45], and its plasma levels with brain amyloid burden—measured by positron emission tomography (PET)—in the Alzheimer's disease Neuroimaging Initiative (ADNI) cohort [46]. Moreover, our observations from both human brain and THP-1 cells highlight a potential implication of Gas6 in the pathophysiology of AD, which should be investigated in more depth. In humans, Gas6 levels were previously found increased in the CSF of AD patients and correlating with disease duration and MMSE scores [47]. Experimentally, it has been shown that activation of Tyro3 by

Gas6 protects cortical neurons in vitro from the apoptosis induced by Aβ, and reduces Aβ production and the ratio $A\beta_{40}$/$A\beta_{42}$, as well as affects Aβ deposition, plaque formation patterns, and astrogliosis in a positive manner in animal models of AD [20]. However, increasing concentrations of Gas6 showed the ability to inhibit these effects, which might be in agreement with our observations of increases in *GAS6* mRNA levels in human frontal cortex as AD progresses. Whether alterations in *TLR5* expression and signaling actually affect Aβ uptake through the TAM system, contributing to Aβ aggregation and plaque formation, remains unclear from our study due to important sample size limitations and is a target of future exploration.

Even when the small sample sizes from our study are an important limitation for the interpretability of our observations and further experiments would be required to validate these, we believe our work provides valuable evidence in support of less-explored genes associated with AD pathology that will open interesting avenues for research in this field.

Authors' Contributions MHR: Conceived and designed the study, performed the bioinformatics analyses and lab experiments, analyzed and interpreted the data, and prepared the manuscript.

FS: Performed the cell culture experiments.

FB: Helped with the design of cell culture experiments.

MPK: Provided support and advice for the cell culture experiments, and contributed with critical revisions to the manuscript.

MTH: Conceived and designed the study, helped with manuscript preparation, and approved the final version of the manuscript.

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Compliance with Ethical Standards

Conflict of Interests The authors declare that they have no conflicts of interest.

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