Published in final edited form as:
Neurochem Res. 2013 January ; 38(1): 218–225. doi:10.1007/s11064-012-0913-z.

Enhanced expression of glia maturation factor correlates with glial activation in the brain of triple transgenic Alzheimer's disease mice

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Abstract

We previously demonstrated that glia maturation factor (GMF), a brain specific protein, isolated, sequenced and cloned in our laboratory, induce expression of proinflammatory cytokines and chemokines in the central nervous system (CNS). We also reported that the up-regulation of GMF in astrocytes leads to the destruction of neurons suggesting a novel pathway of GMF-mediated cytotoxicity of brain cells, and implicated its involvement in the pathogenesis of inflammatory neurodegenerative diseases. In the present study, we examined the expressions of GMF in triple-transgenic Alzheimer's disease (3xTg-AD) mice. Our results show a 13-fold up-regulation of GMF and 8–12-fold up-regulation of pro inflammatory cytokines tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), IL-1β, interferon gamma (IFN-γ), and chemokine (C-C motif) ligand 2 (CCL2) and C-X-C motif chemokine 10 (CXCL10/IP-10) mRNA as determined by quantitative real-time RT-PCR in the brain of 3xTg-AD mice as compared to non-transgenic (Non-Tg) mice. In conclusion, the increase in GMF and cytokine/chemokine expression was correlated with reactive glial fibrillary acidic protein (GFAP)-positive astrocytes and ionized calcium binding adaptor molecule 1 (Iba-1)-positive microglia in 3xTg-AD mice.

Keywords

Glia maturation factor (GMF); neuro inflammation; reactive astrocytes; cytokine/chemokine; transgenic mouse; Alzheimer's disease (AD)

Introduction

Alzheimer's disease (AD) is the most common cause of dementia among older people and characterized by neuritic plaques containing aggregates of β-amyloid peptide, neurofibrillary tangles and degenerating neurons (1, 2). Alzheimer's disease is a neurodegenerative disease, resulting in progressive neuronal death with progressive cognitive decline and memory loss, affecting 5 million Americans and over 35 million individuals worldwide. Diagnosis of AD is routinely made based on the presence of amyloid plaques (APs) and deficits in memory and learning. Mutations in the amyloid precursor protein (APP) resulting in its increased production are linked with early onset of AD.

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Several transgenic mice with AD-like neuropathology are developed by overexpressing different mutant human APP (3). For example, a transgenic mouse that overexpresses mutant human APP in which amino acid valine at position 717 is replaced with phenylalanine, show many of the AD neuropathology, including APs formation and astrogliosis (4) but no significant loss of memory or deficit learning (5, 6). Another transgenic mouse model overexpressing wild-type APP751 did show deficient spatial learning at old age but no substantial APs observed (7). However, a transgenic mouse (8) overexpressing human APP with mutations at amino acid positions 670 and 671 in which lysine 670 changed to asparagine while methionine 671 to leucine did show both pathological and behavioral hallmarks of AD, dense APs and impaired learning and memory at ages 9–10 months (8).

Glia maturation factor (GMF) is an inflammatory protein, isolated, sequenced and cloned in our laboratory (9–11), plays a major role in the pathogenesis of central nervous system (CNS) inflammatory and neurodegenerative diseases. In the current studies we have examined GMF expression in relation with glial activation in the CNS using a triple-transgenic mouse model of AD (3xTg-AD).These triple-transgenic mice express mutant APP, PS1 and tau ($APP_{Swe}PS1_{M146V}tau_{P301L}$), and develop both plaque and neurofibrillary tangle pathology in the cerebral cortex, hippocampus, and amygdala regions (8). These mice also exhibit deficits in synaptic plasticity, including long-term potentiation (LTP) and develop progressive cognitive and neuropathological deficits (8, 12). In this study, we have examined the expression of GMF in the hippocampus, frontal cortex and entorhinal cortex of 3xTg-AD mice and report that these mice also express GMF in association with APs and NFTs.

Material and Methods

Reagents

GMF CT-11 monoclonal antibody (IgG1) against a synthetic peptide corresponding to the C-terminal 11 amino acid residues of GMF was affinity-purified with protein-A (38). PCR primers were synthesized at Integrated DNA Technologies (Coralville, IA). Trizol® reagent and ThermoScript™ RT-PCR system for first-strand cDNA synthesis were purchased from Invitrogen Corporation, (Grand Island, NY). Anti-ionized calcium binding adaptor molecule 1(Iba-1, Wako chemicals, Richmond, VA), rabbit polyclonal-glial fibrillary acidic protein (GFAP, Chemicon, Temecula, CA), Beta Amyloid, 1–16, 6E10 monoclonal antibody (Covance, Dedham, MA) were purchased.

Experimental procedure

Triple transgene mouse model of Alzheimer's disease (3xTg AD) harboring $PS1_{M146v}APP_{Swe}$ and Tau_{P30IL} transgenes were used in this study. Mice in each group (transgene and control group were anaesthetized and perfused with saline. One hemi brain was frozen for neurochemical analysis, and the other hemi brain was used for histopathological studies. Brain tissue homogenates were prepared from these mice for GMF Western blotting. GMF protein level was measured by ELISA in the tissue lysates of hippocampus, frontal cortex and entorhinal cortex regions of 3xTg-AD mice and non-transgenic control mice. GMF expression was measured in advanced aged (16-months old) 3xTg-AD mice that show extensive (APs) and neurofibrillary tangles (NFTs) as well as from age-matched non transgenic (Non-Tg) control mice. The frozen sections were used for immunofluorescence and immunohistochemistry.

Western blotting for GMF

Tissues were extracted with a lysis buffer (1% Triton X-100, 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 50 mM NaF, 0.1 mM sodium vanadate, 1 mM benzamidine, 1 mM PMSF, and 10 g/ml each of aprotinin, leupeptin, chymostatin, pepstatin A and antipain). Equal amount of protein samples were separated on 4–20% gradient gels by SDS-polyacrylamide gel electrophoresis, and electroblotted onto nitrocellulose membranes. Protein blots were probed with specific GMF primary antibody (1:1000 dilutions) and developed by using the appropriate secondary antibody conjugated to horseradish peroxidase (HRP). The membrane were stripped and reprobed with β-actin as a loading control.

Enzyme-linked immunosorbent assay (ELISA

GMF protein concentration was measured by sandwich ELISA procedure in the tissue lysates as described previously (11, 13). The lower detection limits of this GMF ELISA is 10–15 pg/ml. ELISA results were presented as mean ± standard deviations of more than three independent experiments.

Immunofluorescence and immunohistochemistry

The sections were processed for immunohistochemistry as previously described (14–16). Free-floating brain tissue sections were incubated with primary antibodies (rabbit polyclonal GFAP antibody, mouse monoclonal 6E10 antibody, rabbit polyclonal Iba-1 antibody, CT-11 monoclonal GMF antibody) and then with appropriate secondary antibodies. Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 568 goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) for fluorescence labeling and goat anti-rabbit IgG biotinylated antibody for Iba-1, goat-anti mouse IgG alexa 488 for GMF were used. Immunohistochemical detection of microglial activation was done by Iba-1 immunoreactivity using Avidin-biotin complex (ABC) staining procedure with DAB substrate (Vector Laboratories, Burlingame, CA) as described previously (1, 14–16).

Quantitative estimation of mRNA by real-time PCR

Real-time PCR was carried out as described earlier (17–20). Briefly, total RNA was isolated from the tissues by the acid guanidinium thiocyanate-phenol-chloroform method (21), using the Trizol reagent. The primer pairs were selected to yield a single amplicon based on dissociation curves and analysis by acrylamide gel electrophoresis. The following oligonucleotide primers were used: GMF, 5’-ACG CTG GGA GTA AGA ACA AGC T-3’ and 5’-GGT CCT CGG TGT TTC TTA TTT CAA A-3’; TNF-α, 5’-CAT CTT CTC AAA ATT CGA GTG ACA A-3’ and 5’-TGG GAG TAG ACA AGG TAC AAC CC-3’; IL-1β, 5’-CAA CCA ACA AGT GAT ATT CTC CAT G-3’ and 5’-GAT CCA CAC TCT CCA GCT GCA-3’; IL-6, 5’-GAGGATACCACTCCCAACAGACC-3’ and 5’-AAGTGCATCATCGTTGTTCATACA-3’; IFN-γ, TCAAGTGGCATAGATGTGGAAGAA-3’ and 5’-TGGCTCTGCAGGATTTTCATG-3’; IP-10, 5’-GCC GTC ATT TTC TGC CTC AT-3’ and 5’-GCT TCC CTA TGG CCC TCA TT-3’; 18S, 5’-TAA GTC CCT GCC CTT TGT ACA CA-3’ and 5’-GAT CCG AGG GCC TCA CTA AAC-3’. Real-time quantitative PCR was performed using 7300 real-time PCR system (Applied Biosystem, Foster City, CA). The thermal cycler parameters were as follows: hold for 2 min at 50°C and 10 min at 95°C for one cycle followed by amplification of cDNA for 40 cycles with melting for 15 s at 95°C and annealing and extension for 1 min at 60°C. The values were normalized using 18S rRNA as an endogenous internal standard (22).

Statistical analysis

Results were presented as mean ± S.D. Statistical significance was assessed with one-way analysis of variance (ANOVA) followed by Tukey's procedure using SigmaStat software (SPP, Chicago, IL). A value of $p< 0.05$ was considered statistically significant.

Results

Increased GMF expression in the brain of triple transgenic Alzheimer's disease mice

GMF is an inflammatory protein, isolated, sequenced and cloned in our laboratory, plays a major role in the pathogenesis of CNS inflammatory diseases. In the present study we demonstrate, for the first time, a significant up regulation of GMF mRNA and protein expression in an experimental mouse model of AD, 3xTg-AD harboring PS1M146V, APPSwe, and tauP301L transgenes. We determined the levels of GMF in the brain of advanced aged (16–months) 3xTg AD mice that show extensive amyloid plaque and neurofibrillary tangles. Homogenates from the whole brain tissue were prepared from 3xTg-AD and age-matched non transgenic (NonTg) control mice and analyzed by Western blotting. We found a significant increase in GMF protein levels in 3xTg-AD mice compared to the NonTg controls (Fig. 1). GMF protein levels are determined by ELISA from microdissected brain regions of 3xTg-AD and non-transgenic control mice. GMF protein levels are significantly ($p<0.001$) elevated in the hippocampus, frontal cortex and entorhinal cortex of 3xTg-AD mice (Fig. 2). Age-matched non-transgenic mouse samples derived from identical regions were employed as controls. The hippocampus, frontal cortex and entorhinal cortex were the regions chosen because these regions are implicated in the earliest stages of AD disease.

Co-localization of activated glial cells and APs in the brain of triple transgenic Alzheimer's disease mice

Neuro inflammation is a hallmark of AD brain and is characterized by the presence of activated astrocytes and microglia surrounding amyloid plaques. Therefore, we used immunohistochemistry to demonstrate co-localization of strongly stained GMF-positive glial cells with amyloid plaques in 3xTg-AD brains. Immunostaining for glial activation markers GFAP (for astrocytes) and IAb1 (for microglia) revealed numerous activated astrocytes (Fig. 3A) and microglial cells (Fig. 4A) in 3xTg AD brains, particularly in close proximity to thioflavin S-positive and 6E10-positive amyloid plaques (Figs. 3C, 4C). Double immunofluorescence labeling for GMF (Fig. 5A) and amyloid plaques (Fig. 5B) show strongly GMF-positive cells with glial cell morphology are co-localized with amyloid plaques (Fig. 5C). The astrocytes adjacent to the amyloid plaque display ramified processes and express GMF and GFAP, indicative of an activated phenotype associated with inflammation. We used a well characterized and a highly specific anti-GMF (G2-09) antibody prepared in our laboratory, and an anti-beta amyloid (Aβ) antibody 6E10 (Figs. 3B and 4B).

Increased GMF mRNA and proinflammatory cytokine and chemokine mRNA expression in the brain of triple transgenic Alzheimer's disease mice

Consistent with our immunochemical results, we detected a significant, 13-fold increase ($p < 0.001$) in GMF mRNA in the brain of 3xTg-AD mice compared to age-matched NonTg controls (Fig. 6). We also detected significantly increased levels of proinflammatory cytokines (TNF-α, IL-6, IL-1β, IFN-γ) and chemokines (MCP-1, IP-10) in 3xTg-AD mice compared to age-matched control mice. We measured mRNA levels by quantitative real time RT-PCR.

Discussion

Although the main cause of neuronal death in Alzheimer's disease is commonly attributed to the amyloid plaques and the neurofibrillary tangles, it has become clear that other biological response modifiers including the local chronic inflammatory responses mediated by the glia (microglia and astrocytes) contribute to the severity of the pathologic process(23–26). For example, gliosis and microglial activation associated with amyloid plaques is a common feature of AD. Activated glia are known to secrete pro-inflammatory cytokines and other toxic substances capable of causing neuronal death (26–28).

Glia maturation factor is a protein discovered, isolated and cloned in our laboratory (9, 10, 29, 30). Recent data from our laboratory suggest that it performs important intracellular function of regulating the stress-activated signal transduction pathways, such as p38 MAP kinase and NFκB activation (31–34). While we believe that the stress-related function of GMF normally plays an adaptive role in helping the nervous system cope with adverse situations such as infection or injury, it could under certain conditions also aggravate an on-going pathologic process (35–37). Of particular relevance is our recent observation that over-expressing GMF in astrocytes promotes the production and secretion of granulocyte macrophage colony stimulating factor which in turn activates microglia(18). Our hypothesis is that the pro-inflammatory tendency of GMF could adversely augment the course of neurodegenerative diseases (11, 38). In the present study, we demonstrate, for the first time, a significant up-regulation of GMF mRNA and GMF protein expression by immunoblotting, ELISA and quantitative PCR in an experimental mouse model of AD, 3xTg-AD harboring PS1M146V, APPSwe, and tauP301L transgenes. This increased GMF level observed in the present study is in the brain of advanced aged 3xTg-AD mice that showed extensive APs and neurofibrillary tangles, the pathological hallmarks of AD. The hippocampus, frontal cortex and entorhinal cortex regions were chosen for the present study since these regions are affected in the earliest stages of human AD pathogenesis with severe neuronal death. The 3xTg-AD mice develop an age-related and progressive neuropathological characteristics of APs and tangle and this is the first mouse model known to develop both plaque and tangle (8). These characteristics closely mimic the human AD pathology and therefore we have investigated the GMF expression in this particular 3xTg-AD mouse model in the current study.

It's well known that activated glia and T cells produced a number of proinflammatory cytokines including TNF-α, IL-6, IL-1β, and IFN-γ in the CNS. Several proinflammatory cytokines were detected and implicated in the pathogenesis AD by promoting inflammatory responses. In the present study, we have also investigated the expression of inflammatory molecules GMF, TNF-α, IL-6, IL-β, IFN-γ, CCL2 (MCP-1) and CXCL10 in 3xTg-AD mice and in age-matched non-Tg control mice and demonstrate that these inflammatory molecule mRNAs were significantly elevated in the brain of 3xTg-AD mice. These results further support the close similarities of 3xTg-AD mice with human AD characteristics especially with regard to GMF, the main focus of our current study. The GMF expression is higher (13 fold over control) when compared to the increase of TNF-α, IL-6, IL-β, IFN-γ, CCL2 (MCP-1) and CXCL10 indicating GMF's critical inflammatory role in the AD pathogenesis. Increased GMF expression may augment or synergize with other cytokines or chemokines in chemoattraction, proliferation, activation and release of other inflammatory mediators from glial cells and inflammatory cells leading to the AD symptoms. The overexpression of GMF along with other inflammatory molecules in 3xTg-AD mice suggests a critical role in contributing to in situ inflammation leading to deleterious microenvironment. Factors triggering the overproduction of GMF in CNS remain unknown but may include APs, neurofibrillary tangles, inflammatory cells and inflammatory molecules in an autocrine and/or paracrine signaling manner. In this regard, we have

reported earlier that GMF can be induced by the administration of Aβ (38) and as well as myelin oligodendrocyte glycoprotein (MOG) peptide (39) in mice.

In accord with our previous results (11, 40), our present study shows a tight correspondence between GMF and APs in the hippocampus, frontal cortex and entorhinal cortex regions of AD and the GMF expression is more in hippocampus and entorhinal cortex than in frontal cortex in 3xTG-AD mice. Among the areas studied both entorhinal and hippocampus showed more AD pathology as evidenced by the dense presence of APs in 3xTg-AD mice.

The current data showing presence of GMF in activated glia in the close vicinity of APs and NFTs support the hypothesis that GMF overexpression is closely involved in the pathogenesis of AD. The etiology of AD remains unknown and there is no definite treatment yet available. In conclusion, our present results show that GMF is significantly upregulated and that the augmented expression of GMF is associated with AD pathology.

Acknowledgments

We thank Scott Knight, Ashna Zaheer, and Marcus Ahrens for excellent technical help. 3xTg-AD mice were from Dr. Frank M. LaFerla, University of California, Irvine, obtained from Dr. Gloria Lee, University of Iowa. This work was supported by the Department of Veterans Affairs Merit Review award (to A.Z.) and by the National Institute of Neurological Disorders and Stroke grant NS-47145 (to A.Z.).

Abbreviations used

CCL2	Chemokine (C-C motif) ligand 2
CXCL10	C-X-C motif chemokine 10
CNS	central nervous system
ELISA	enzyme-linked immunosorbent assay
GFAP	glial fibriallary acidic protein
IFN-γ	interferon-gamma
IL-1	interleukin-1
Non-Tg	non-transgenic mice
GMF	glia maturation factor
Real-time PCR	Real-time polymerase chain reaction
3xTg-AD mice	triple transgenic Alzheimer’s disease
TNF-α	tumor necrosis factor-alpha

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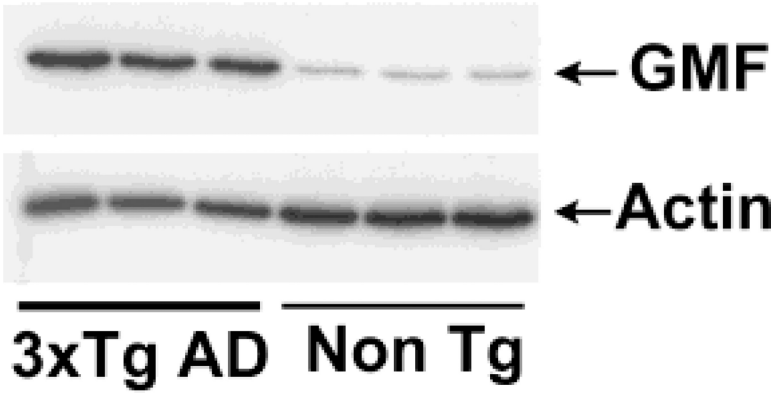

Fig. 1. Up-regulation of GMF expression in the brain of advanced aged triple transgene mice

Homogenates from the whole brain tissue from 3xTg-AD transgene mice and non-Tg control mice were prepared and the expression of GMF was determined by immunoblotting procedure using anti-GMF monoclonal antibody (CT-11). β-actin was used as a loading control. Three mice in each group were analyzed.

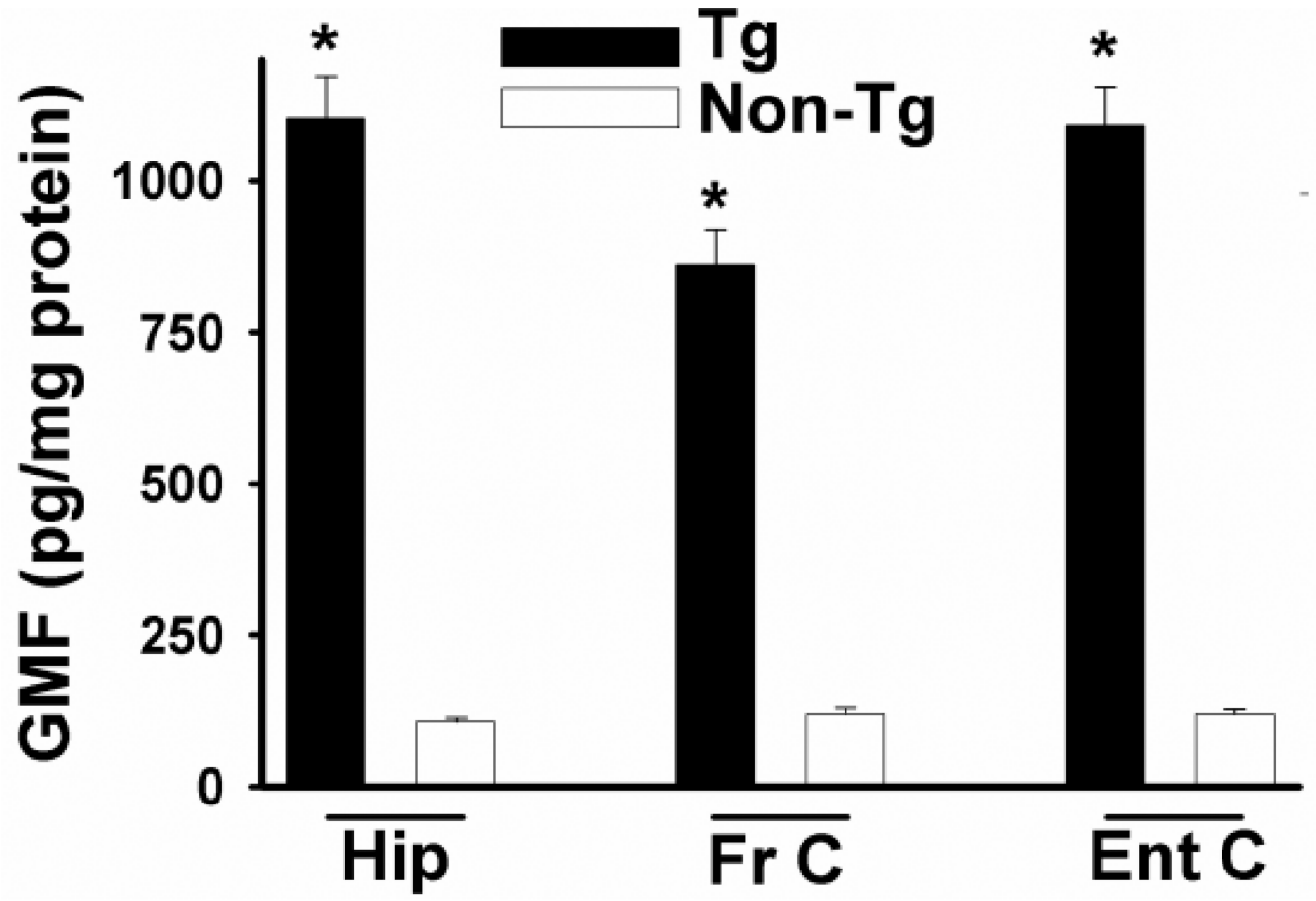

Fig. 2. Up-regulation of GMF protein in the brain regions of triple transgene mice

Tissue lysates of hippocampus, frontal cortex and entorhinal cortex were prepared from 3xTg-AD transgene mice and non-transgenic control mice. GMF protein level was measured in these lysate samples by ELISA procedure. The results were presented as Mean±SD. * = vs. control, p=<0.001. Hip, hippocampus; Fr C, frotal cortex and Ent C, entorhinal cortex.

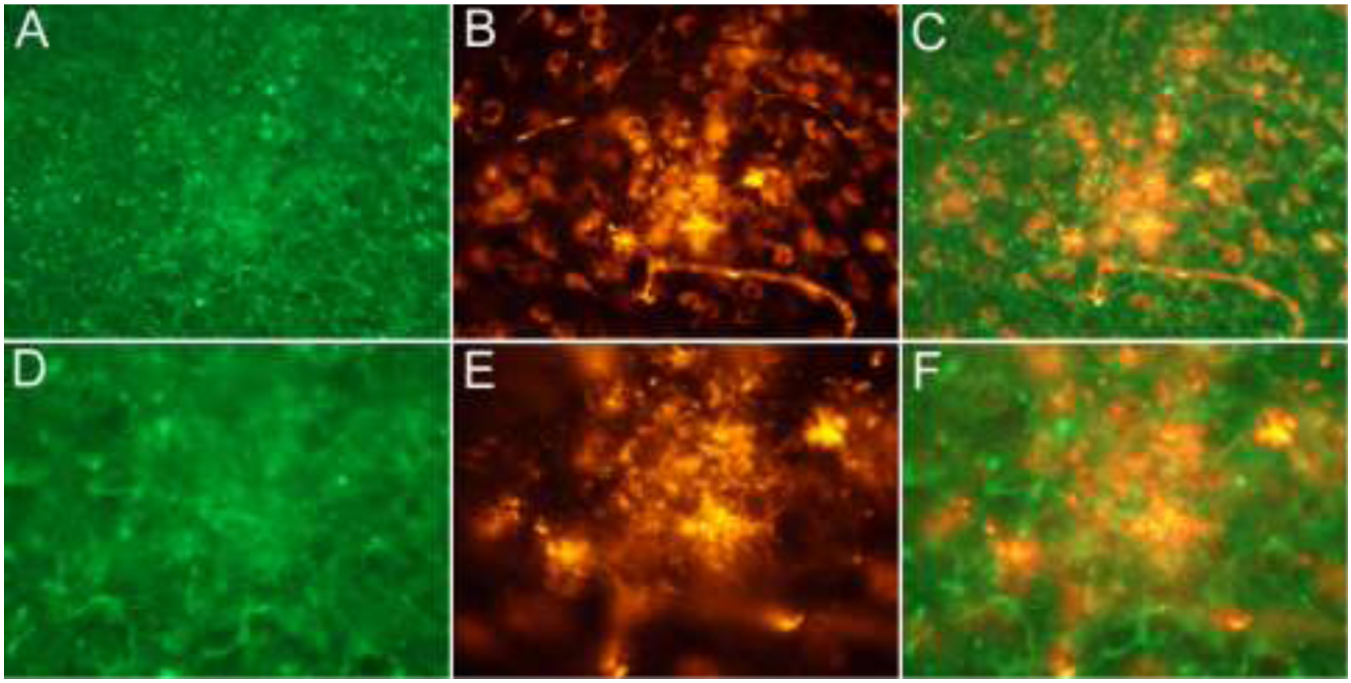

Fig. 3. Co-localization of GMF-positive glial cells with APs in triple transgene mice brain

Double immunofluorescence labeling for GMF (A,D green color, antibody CT-11) and amyloid plaques (B,E antibody 6E10, red color) was performed in the brain of advanced aged 3xTg-AD mice. Merged image (C,F yellow color) shows co-localization of glial cells and APs. Original magnification A,B,C = ×200; D,E,F = ×400).

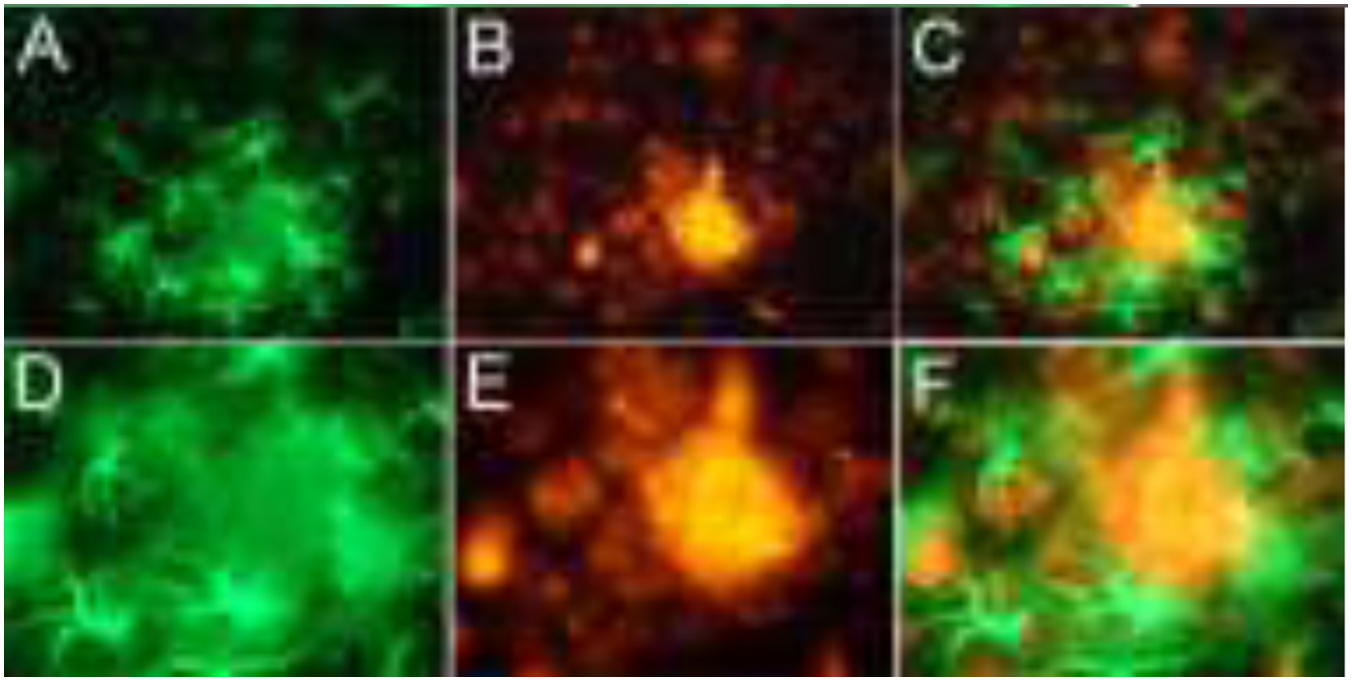

Fig. 4. Co-localization of astrocytes and APs in triple transgene mice brain

Double immunofluorescent labeling of GFAP-positive astrocytes (A,D green color) and amyloid plaques by 6E10 (B,E red color) was performed in the brain of advanced aged 3xTg-AD mice. Merged image (C,F yellow color) shows co-localization of activated astrocytes with APs. Original magnification A,B,C = ×200; D,E,F = ×400).

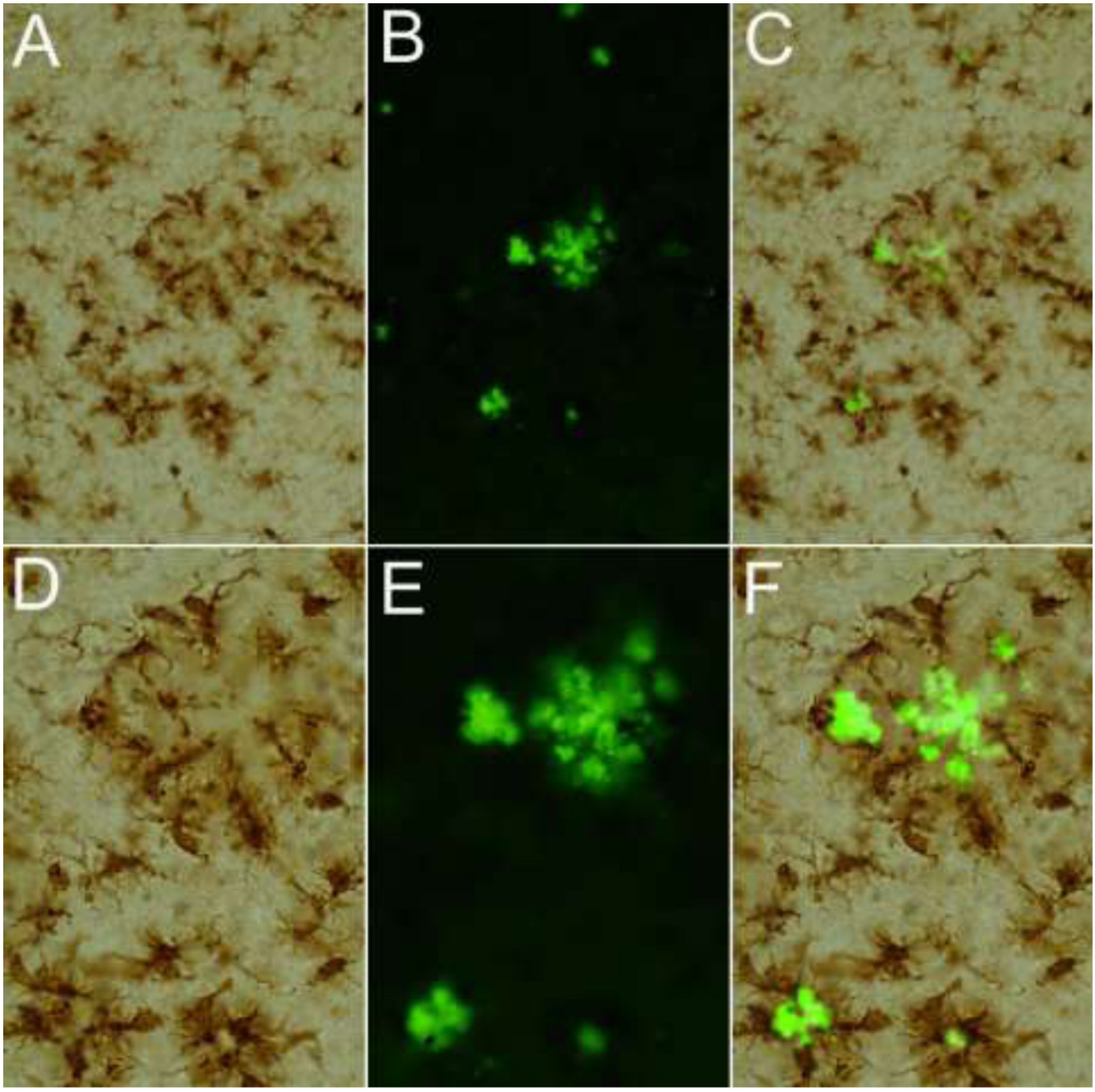

Fig. 5. Co-localization of microglia with thioflavin-S positive plaques in triple transgene mice brain

Immunostaining of microglia (A,D brown color, antibody Iba-1) and APs (B,E green color, thioflavin S). Activated microglia and their association with APs (**C**,F brown color and green color) were shown in 3xTg-AD mice brain. Original magnification A,B,C = ×200; D,E,F = ×400).

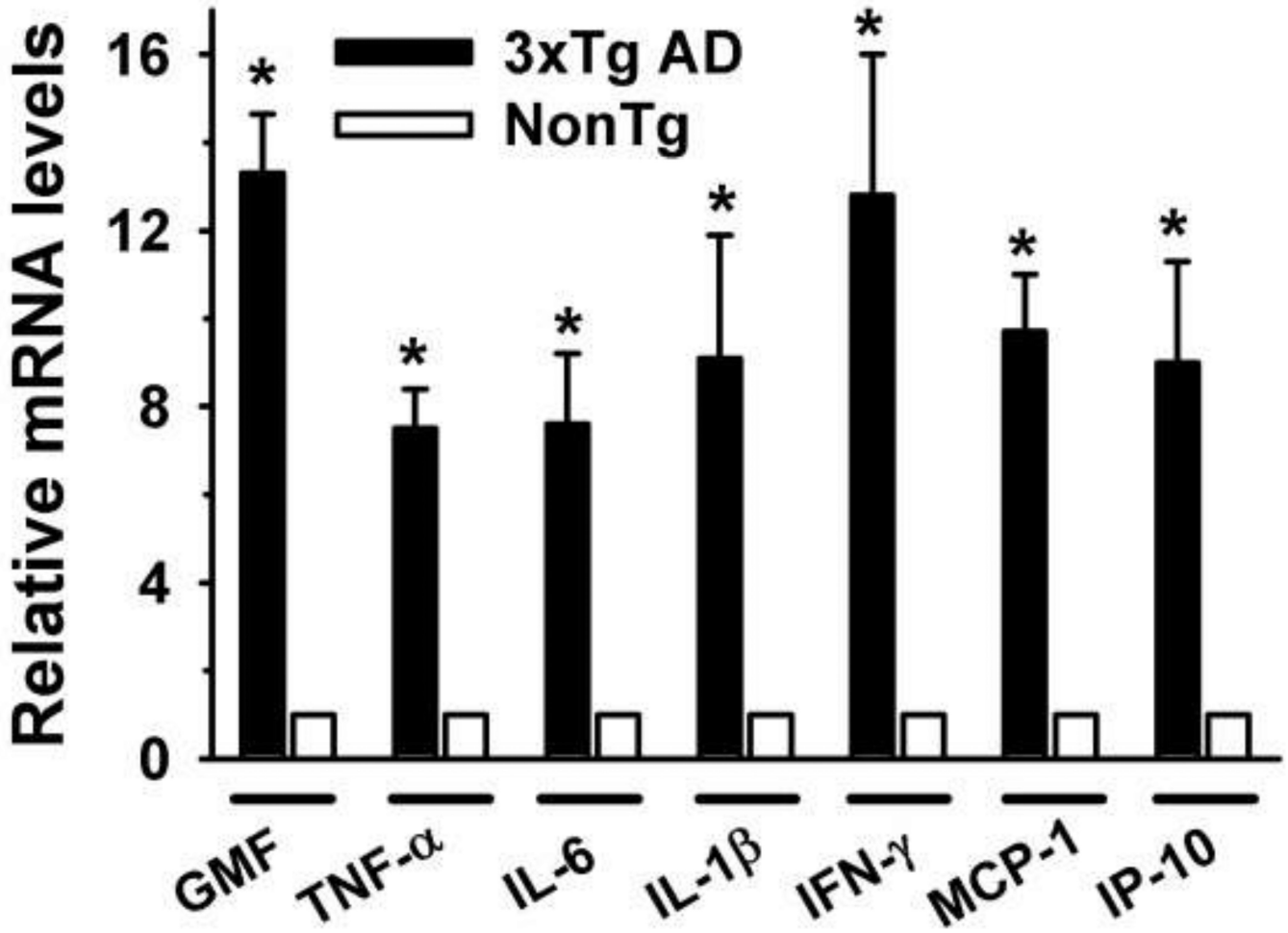

Fig. 6. Increased expression of GMF and proinflammatory molecules mRNA expression in triple transgene mice brain

mRNA levels of GMF as well as proinflammatory cytokines TNF-α, IL-6, IL-1β, IFN-γ and chemokines CCL2 and CXCL10/IP-10 were studied by quantitative PCR in 3xTg-AD mice and non-Tg control mice brain. 18S rRNA was used as an endogenous internal standard. The results were expressed as relative mRNA. Significantly different expressions between 3xTg AD and non-Tg mice were labelled with asterisks (*$P < 0.001$).