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Full-length Article

TNFAIP3, a negative regulator of the TLR signaling pathway, is a potential predictive biomarker of response to antidepressant treatment in major depressive disorder

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ABSTRACT

Inflammation and abnormalities in Toll-like receptor (TLR) expression and activation have been linked to major depressive disorder (MDD). However, negative regulators of TLR pathways have not been previously investigated in this context. Here, we sought to investigate the association of depression severity, measured by the 17-item Hamilton Depression Rating Scale (HAMD-17), with mRNA expression levels of negative regulators of the TLR pathway, including SOCS1, TOLLIP, SIGIRR, MyD88s, NOD2 and TNFAIP3, in peripheral blood mononuclear cells (PBMCs) from 100 patients with MDD and 53 healthy controls, before and after treatment with antidepressants. Positive regulators of the TLR4 pathway, including Pellino 1, TRAF6 and IRAK1, were also investigated. Among all patients, MyD88s, and TNFAIP3 mRNAs were expressed at lower levels in PBMCs from patients with MDD. Multiple linear regression analyses revealed that TNFAIP3 mRNA expression before treatment was inversely correlated with severity of depression and effectively predicted improvement in HAMD-17 scores. Among 79 treatment-completers, only TNFAIP3 mRNA was significantly increased by treatment with antidepressants for 4 weeks. Treatment of human monocytes (THP-1) and mouse microglia (SIM-A9) cell lines with fluoxetine significantly increased TNFAIP3 mRNA expression and suppressed IL-6 levels. The suppressive effect of fluoxetine on IL-6 was attenuated by knockdown of TNFAIP3 expression. These findings suggest that both dysfunction of the negative regulatory system in patients with MDD and antidepressant treatment exert anti-inflammatory effects, at least in part through increased expression of the *TNFAIP3* gene. They also indicate that modulating expression of the *TNFAIP3* gene to rebalance TLR-mediated inflammatory signaling may be potential therapeutic strategy for treating MDD.

1. Introduction

Major depressive disorder (MDD) represents a combination of mood, anxiety, cognition, sleep, and appetite symptoms that last for more than 2 weeks. MDD, which is responsible for 7.4% of total disability-adjusted life years (DALYs) worldwide (Whiteford et al., 2013), is highly associated with inflammation (Maes et al., 2015; Miller and Raison, 2016; Vogelzangs et al., 2014). One meta-analysis reported elevated levels of pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α and interleukin (IL)-6, in patents with MDD (Dowlati et al., 2010). Studies have also noted that MDD is associated with increased monocyte numbers (Seidel et al., 1996) and prostaglandin E2 secretion (Nishino et al., 1989). The currently favored hypothesis is that pro-inflammatory cytokines penetrate into the central nervous system and alter the activity of indoleamine 2,3-dioxygenase (IDO), which activates the kynurenine pathway and decreases the level of serotonin (Haroon et al., 2012; Myint and Kim, 2014).

It has been reported that treatments for depression are associated with a reduction in inflammation. Selective serotonin reuptake inhibitors (SSRIs) have been shown to diminish the output of interferon-γ (IFN-γ) in whole blood stimulated with

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lipopolysaccharide (LPS) (Maes et al., 1999). Fluoxetine, an antidepressant of the SSRI class, have been shown to suppress LPS-stimulated expression of IFN-γ–inducible protein 10, also known as C-X-C motif chemokine ligand 10 (CXCL10), in human monocytes (Tsai et al., 2014). Fluoxetine was also demonstrated to reduce LPS-induced pro-inflammatory IL-6 and TNF-α in human peripheral blood mononuclear cells (PBMCs) (Waiskopf et al., 2014). A variety of pathways, including the 5-HTT (5-hydroxytryptamine transporter), nuclear factor-kappB (NF-κB), IL-10, and cAMP have been investigated for their role in mediating the actions of SSRIs (Walker, 2013). Fluoxetine was also shown to inhibit LPS-induced decreases in intracellular acetylcholinesterase (AChE-S), which interacts with NF-κB–activating intracellular RACK1 (receptor for activated C kinase 1) (Waiskopf et al., 2014). However, the specific molecular mechanisms by which antidepressants of the SSRI class reduce inflammation have not yet been fully elucidated.

In terms of innate immune responses to MDD, recent studies have demonstrated an association between activation of Toll-like receptor (TLR)-4–mediated signaling and depression (Henry et al., 2016; Hung et al., 2014). Clinical studies have shown that TLR4 expression in the prefrontal cortex is enhanced in patients with MDD (Garate et al., 2014). Altered peripheral expression of TLRs appears to be associated with a heightened inflammatory state and depression (Crupi and Cuzzocrea, 2016). In addition, clinical studies have reported up-regulated TLR4 signaling in PBMCs in patients with MDD (Hung et al., 2014). Notably, TLR4 was found to be an independent risk factor for MDD severity and shown to be associated with symptoms of depression, including body weight loss and anxiety (Breese et al., 2008; Wu et al., 2015). We recently reported that antidepressant treatment attenuated increases in TLR4 mRNA associated with MDD (Hung et al., 2016), suggesting a possible interaction between antidepressants and TLR4 signaling pathways through an as yet unclear molecular mechanism(s).

Negative regulators of TLR4 signaling have been shown to intersect almost every step of the TLR signaling pathway, serving to protect against the potential harm of a prolonged, TLR-induced cytokine storm by controlling the magnitude of the peak response and/or duration of the response. IRAK3 (IL1 receptor-associated kinase 3), SOCS1 (suppressor of cytokine signaling 1), MyD88s (myeloid differentiation 88 short), TOLLIP (Toll-interacting protein), TNFAIP3 (TNF α-induced protein 3), ST2L (suppressor of tumorigenicity 2, full-length form), and SIGIRR (single immunoglobulin IL-1R–related receptor) are among the negative regulators of TLR-mediated immune responses (Liew et al., 2005). One of these important negative regulators, TNFAIP3 (also known as A20), acts as a deubiquitinase with specificity for lysine 63 (K63)-linked ubiquitin chains on TRAF6 (TNF receptor-associated factor 6) to suppress NF-κB activation and inflammatory responses (Shembade and Harhaj, 2012). While TNFAIP3 expression was reported to be associated with bipolar disorder (Barzman et al., 2014; Padmos et al., 2008), whether regulation of this negative regulator of TLR-mediated signaling occurred in MDD patients treated with antidepressants remains unknown.

Here, we investigated differences in the gene expression profile of positive and negative regulators of TLR signaling in patients with MDD and sought to determine possible anti-inflammatory mechanisms underlying the actions of the antidepressant, fluoxetine.

2. Materials and methods

2.1. Experimental design

Inpatients with MDD were recruited from the psychiatric ward of Kaohsiung Chang Gung Memorial Hospital, Taiwan, from August 2013 to May 2016. Blood samples for mRNA analysis were obtained from patients before and after antidepressant treatment, and from 53 healthy controls at baseline. Institutional Review Board approval was obtained from the hospital ethics committee (101-5012A3, 103-5114B and 103-6984A3). After receiving verbal and written information about the study, patients and healthy controls provided written consent to participate.

2.2. Participants

Patients with MDD were screened by two psychiatrists before entering the study. The screening steps, which were similar to those described in our previous work (Hung et al., 2016), included a Structured Clinical Interview for DSM-IV Axis I Disorders as well as a detailed assessment of current psychiatric symptoms and previous treatment. The 17-item Hamilton Depression Rating Scale (HAMD-17) was used by the same psychiatrists to assess the severity of depression. Patients with psychotic disorders, substance dependence (including alcohol), severe metabolic syndrome, severe obesity (body mass index [BMI] > 34 kg/m^2) or systemic inflammatory disease, or those who received antibiotics, anti-inflammatory or immune-modulating drugs, were excluded from the study. All patients were tested for blood pressure and received chest X-rays, electrocardiographic examinations, and routine blood tests after hospitalization to exclude possible chronic systemic physical illness. Enrolled patients reported no antidepressant use for at least 1 week before entering the study. Healthy controls, recruited from the community, had neither a personal history nor a first-degree relative with a psychiatric disorder. The same psychiatrist who performed screens of MDD patients assessed the healthy control group using Diagnostic and Statistical Manual of Mental Disorders (Fourth Edition) criteria to rule out psychiatric disease.

After a clinical examination, blood samples were taken at two time points: at inclusion (baseline) and 4 weeks after initiating antidepressant treatment. Between these two time points, patients were hospitalized in the psychiatric ward of Kaohsiung Chang Gung Memorial Hospital with good drug adherence, regular sleep-wake cycles, a well-controlled diet, and limited smoking.

2.3. Treatment

Treatment was administered as dictated by medical considerations, meaning that the choice of treatment was not influenced by the study and was chosen based on clinical judgment. After screening at baseline, chosen antidepressants were administered and recorded. The antidepressants included escitalopram (10–20 mg/d; n = 14), fluoxetine (40–80 mg/d; n = 12), paroxetine (20–40 mg/d; n = 12), sertraline (75 mg/d; n = 1) duloxetine (60–120 mg/d; n = 25), venlafaxine (37.5–225 mg/d; n = 7), bupropion (150–300 mg/d; n = 3), and agomelatine (25–50 mg/d; n = 5). All patients were administered benzodiazepines as anxiolytics or hypnotics, and had not received their medication for at least 8 h prior to blood sampling. Supportive psychotherapy sessions were provided one to two times, and regular activities were suggested during hospitalization.

2.4. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis

Venous blood (5 mL) samples were drawn between 8:00 am and 10:00 am, after patients had fasted for 9 h. PBMCs were isolated from venous blood samples by Ficoll-Paque (GE, #17-5442-02) density gradient centrifugation. The isolated PBMCs were labeled with BD IMag anti-human CD14 Magnetic Particles – DM (BD Biosciences, #557769) according to the Magnetic Labeling Protocol. The labeled fraction containing CD14(+) monocytes from 24 completers was collected for further analysis. The tubes were

placed in a 80 °C freezer immediately after collection and were stored there until they were assayed. The protocol used for mRNA analyses was the same as that used in our previous study (Hung et al., 2016, 2014).

qRT-PCR was performed using the following sets of primers: SOCS1 5′-GAC CCC TTC TCA CCT CTT GA-3′ (sense) and 5′-GTA GGA GGT GCG AGT TCA GG-3′ (antisense); TOLLIP, 5′-GAC AAC TGT CTC CGT CGC A -3′ (sense) and 5′-CGG GAG CTC ACC GAT GTA-3′ (antisense); SIGIRR, 5′-CCC AGC TCT TGG ATC AGT CT-3′ (sense) and 5′-AGT CAG GGG CCC TAT CAC AG-3′ (antisense); MyD88s, 5′-TCA TCG AAA AGA GGT TGG CT-3′(sense) and 5′-GAT GGG GAT CAG TCG CTT CT -3′ (antisense); NOD2, 5′- CGG CGT TCC TCA GGA AGT AC-3′ (sense) and 5′-ACC CCG GGC TCA TGA TG-3′ (antisense); human TNFAIP3, 5′-GGA CTT TGC GAA AGG ATC G-3′ (sense) and 5′-TCA CAG CTT TCC GCA TAT TG-3′ (antisense); mouse TNFAIP3, 5′-AAA CCA ATG GTG ATG GAA ACT G-3′ (sense) and 5′-GTT GTC CCA TTC GTC ATT CC-3′ (antisense); ST2L, 5′-CCC ACT CAG GAA AGA AAT CG-3′ (sense) and 5′-TTC GCA TAT CCA GTC CTA TTG A-3′ (antisense); IRAK3, 5′-CTC GGT CAT CTG TGG CAG TA -3′ (sense) and 5′-TTC TAG GTG GGA CCG GAA GT-3′ (antisense); IRAK1, 5′-TGC TAG AGA CCT TGG CTG GT-3′ (sense) and 5′-GTG CTT CTC AAA GCC ACT CC-3′ (antisense); TRAF6, 5′-CTG TCC TTT GGC AAA TGT CA-3′ (sense) and 5′-TCA TGG CAA CCA AAA GTA CTG-3′ (antisense); Pellino1, 5′-CCA AGC CTG GAA TAT GGA GA-3′ (sense) and 5′-TGC TTC ACG GTA GGA GTG TG-3′ (antisense); human IL-6, 5′-GTA GCC GCC CCA CAC AGA-3′ (sense) and 5′-CAT GTC TCC TTT CTC AGG GCT G-3′ (antisense); mouse IL-6, 5′-TCC AGA AAC CGC TAT GAA GTT C-3′ (sense) and 5′-CAC CAG CAT CAG TCC CAA GA-3′ (antisense); human GAPDH, 5′-TGC ACC ACC AAC TGC TTA GC-3′ (sense) and 5′-GGC ATG GAC TGT GGT CAT GAG-3′ (antisense); and mouse GAPDH, 5′-GCA CAG TCA AGG CCG AGA AT-3′ (sense) and 5′-GCC TTC TCC ATG GTG GTG AA-3′ (antisense). Relative expression levels of target genes in each sample were calculated based on the threshold cycle (CT), where the difference in CT ($-\Delta CT$) used to represent relative expression of clinical samples was defined as $CT_{GAPDH} - CT_{sample}$. The $2^{-\Delta\Delta CT}$ method was used to calculate relative changes in expression of target genes for cell assays, where $\Delta\Delta CT = \Delta CT_{treatment\ group} - \Delta CT_{control\ group}$.

2.5. Cell culture

The THP-1 cell line was obtained from BCRC (Food Industry Research and Development Institute, Taiwan) and maintained at 2×10^5 cells/mL in RPMI 1640 medium supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 10% fetal bovine serum (FBS). The SIM-A9 cell line was obtained from the American Type Culture Collection (ATCC) and maintained in DMEM/F12 (Life Technologies/GIBCO, Cat# 12400-016) containing 5% horse serum and 10% FBS. All media contained 1.5 μg/mL penicillin/streptomycin/neomycin, and cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere. In fluoxetine experiments, both cell types were seeded in 6-well plate for 24 h, and then treated with

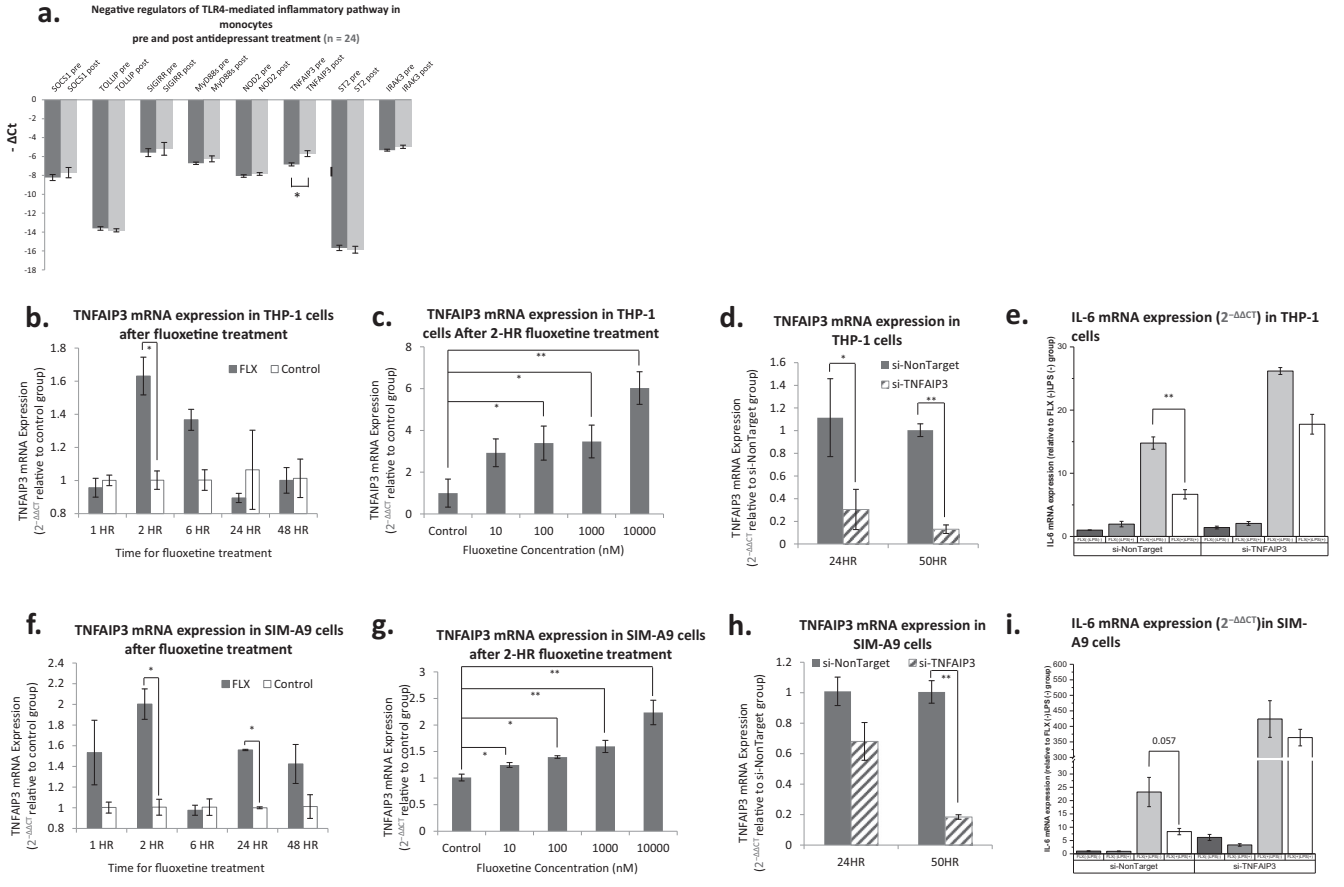

Fig. 1. Antidepressants directly increase the expression of TNFAIP3. (a) In monocytes isolated from PBMCs collected from patients before and after antidepressant treatment (n = 24), only the amount of TNFAIP3 was increased among all tested negative regulators. (b, c) Fluoxetine significantly increased TNFAIP3 mRNA expression in the THP-1 cell line (b), an effect that was concentration dependent (c). (d, e) Fluoxetine significantly attenuated the LPS-induced increase in IL-6 mRNA expression. siRNA-mediated knockdown of TNFAIP3 partially blocked the inhibitory effect of fluoxetine on IL-6 mRNA (e). (f, g) Fluoxetine significantly increased TNFAIP3 mRNA expression in the SIM-A9 cell line (b), an effect that was concentration dependent (c). (h, i) Fluoxetine has marginal effects in attenuating the LPS-induced increase in IL-6 mRNA expression. siRNA-mediated knockdown of TNFAIP3 partially blocked the inhibitory effect of fluoxetine on IL-6 mRNA (i). *p-value < 0.05, **p-value < 0.01.

different concentrations (10 nM, 100 nM, 1 μM and 10 μM) of fluoxetine. After 1, 2, 6, 24 or 48 h, cells were harvested for further analysis.

2.6. Transient transfection with siRNA

ON-TARGET plus SMARTpool small interfering RNA (siRNA) against human TNFAIP3 (L-009919-00-0005) and ON-TARGETplus non-targeting siRNA pool (D-001810-01) were purchased from Dharmacon. The *Trans*IT-X2 Dynamic Delivery System (Mirus, #MIR 6000) was used for siRNA transfection, as described by the manufacturer. In brief, THP-1 and SIM-A9 cells grown overnight in 6-well plates were transfected with 30 nM TNFAIP3 siRNA or control siRNA. Twenty-four hours after transfection, cells were treated with 0.1 μM fluoxetine (Sigma, #F132) or control buffer (Sigma, #F132) for an additional 24 h, and then incubated with 1 μg/mL LPS or phosphate-buffered saline (PBS) for 2 h and harvested for further analysis.

2.7. Statistical analysis

All results are presented as means ± standard deviation. mRNA levels shown in tables and Fig. 1a are presented as $-\Delta CT$; mRNA levels in Fig. 1b–i are presented as $2^{-\Delta\Delta CT}$. The chi-square test was used to compare differences in demographic data (e.g., sex), and Student's *t*-test was used to compare differences in age and BMI. A multivariate analysis of covariance (MANCOVA), adjusted for age, sex, and body mass index (BMI), was used to compare pre-treatment expression levels of negative regulators in patients with MDD with those in healthy controls (Table 2, I vs. III). A paired *t*-test was used to compare differences in expression levels of negative regulators before and after antidepressant treatment (Table 2, I vs. II). A linear regression model with forward selection was used to establish the relationship between baseline mRNA levels of negative regulators and baseline HAMD-17 scores (Table 3). A linear regression model was also used to establish the relationship between baseline mRNA levels of negative regulators and decreases in HAMD-17 scores (Table 4). Student's *t*-test was used to compare differences in all cell line models. All statistical analyses were performed using Statistical Product and Service Solutions (SPSS), version 22. For each test, p-values <0.05 were considered significant.

3. Results

3.1. Demographic and clinical characteristics of the study population

A total of 100 MDD patients were recruited (21 males and 79 females). Seventy-nine patients successfully completed a 4-week antidepressant treatment. Among demographic variables, age was significantly lower in the control group (39.75 ± 11.12 years) than in MDD patients before (45.69 ± 9.96 years) and after (45.65 ± 9.92 years) treatment (Table 1). There was no significant difference in BMI between healthy controls and MDD patients before treatment, but the BMI in patients after treatment (24.29 ± 4.82 kg/m^2) was higher than that in the control group (22.80 ± 3.05 kg/m^2). HAMD-17 scores after treatment were significantly reduced (8.52 ± 5.75) compared with baseline (26.87 ± 5.24).

Gene expression profiling revealed a decrease in negative regulators of TLR signaling in PBMCs from MDD patients that is associated with severity of depression

To explore differences in the expression profile of positive and negative regulators of TLR signaling between controls and MDD patients, we first isolated PBMCs from enrolled individuals and analyzed the mRNA expression of negative and positive regulators of TLR signaling, including TNFAIP3, MyD88s, NOD2, SOCS1, TOLLIP, SIGIRR, ST2L, IRAK3, IRAK1, TRAF6, TRAF6 and Pellino1. Among all regulators, TNFAIP3 and MyD88s were significantly decreased in PBMCs isolated from MDD patients at baseline compared with healthy controls, based on age-, sex-, and BMI-adjusted MANCOVAs. No significant differences were found for NOD2, SOCS1, TOLLIP, SIGIRR, ST2L or IRAK3, or for the positive regulators of TLR signaling, Pellino 1, TRAF6, and IRAK1. Comparisons of baseline expression of negative regulators between MDD patients and healthy controls are summarized in Table 2 (I vs. III). The clinical association of the expression of negative regulators with severity of depression was assessed using a linear regression analysis, which showed that only TNFAIP3 levels were negatively correlated with HAMD-17 scores (r = −0.207, p = 0.046; Table 3).

3.2. TNFAIP3 expression is increased in MDD patients after antidepressant treatment and serves as a predictive factor for antidepressant response

To evaluate the association between antidepressant treatment and negative regulators of TLR signaling, we reassessed the 79 patients who completed the 4 weeks of therapy. Only TNFAIP3 mRNA expression in PBMCs was significantly increased after 4 weeks of therapy compared with baseline (Table 2, I vs. II). To further investigate the association between antidepressant treatment and negative regulators of TLR signaling, we performed a linear regression analysis, which showed that TNFAIP3 (r = −0.269, p = 0.024) and ST2L (r = 0.248, p = 0.036) levels were predictive of decreases in HAMD-17 scores among the 79 completers (Table 4).

3.3. TNFAIP3 expression is significantly increased in MDD patients treated with SSRI antidepressants

Among the 79 patients who received 4-week antidepressant treatment, 39 received SSRIs, whereas 31 received a serotonin–nor epinephrine reuptake inhibitor (SNRI). The other nine patients

Table 1
Demographic findings and clinical data for healthy controls and MDD patients.

	(1) MDD Pre-treatment (n = 100)	(2) MDD Post-treatment (n = 79)	(3) Healthy controls (n = 53)	p-value (1) vs. (3)	p-value (2) vs. (3)
Age (years)	45.69 ± 9.96	45.65 ± 9.92	39.75 ± 11.12	0.001**	0.002**
Sex (M/F)	21/79	16/63	15/38	0.313	0.287
BMI (kg/m^2)	24.02 ± 4.60	24.29 ± 4.82	22.80 ± 3.05	0.055	0.033*
Smoking	22	17	6	0.105	0.131
HAMD-17	26.87 ± 5.24	8.52 ± 5.75	–	–	–

Age, BMI: Student's *t*-test.
Sex, Smoking: chi-square test.
* p-value < 0.05.
** p-value < 0.01.

Table 2
mRNA expression levels (−ΔCt) of negative regulators in healthy controls and patients with MDD.

	MDD		III. Healthy controls	*F-, T- and p*-values	
	I. Pre-treatment	II. Post-treatment		*I* vs. *III*	*I* vs. *II*
SOCS1	−5.28 ± 1.55	−5.31 ± 1.26	−5.23 ± 1.10	F = 0.690 p = 0.408	T = −1.163 p = 0.247
TOLLIP	−10.58 ± 1.95	−10.90 ± 2.12	−9.94 ± 2.39	F = 1.826 p = 0.179	T = −1.078 p = 0.283
SIGIRR	−4.32 ± 1.68	−4.43 ± 1.59	−4.27 ± 1.59	F = 0.927 p = 0.337	T = −1.139 p = 0.257
MyD88s	−6.48 ± 0.90	−6.43 ± 0.80	−6.10 ± 0.77	F = 8.584 p = 0.004**	T = 0.668 p = 0.505
NOD2	−6.33 ± 1.35	−6.44 ± 1.50	−5.89 ± 1.69	F = 2.673 p = 0.104	T = 0.576 p = 0.566
TNFAIP3	−6.05 ± 1.39	−5.32 ± 0.89	−5.61 ± 0.69	F = 5.752 p = 0.018*	T = 4.173 p = 0.000**
ST2L	−9.76 ± 2.49	−10.06 ± 1.96	−9.38 ± 1.59	F = 1.425 p = 0.235	T = 0.496 p = 0.620
IRAK3	−5.87 ± 1.64	−5.65 ± 0.89	−5.57 ± 0.61	F = 0.010 p = 0.920	T = 1.039 p = 0.301
IRAK1	−6.27 ± 1.01	−6.33 ± 0.81	−6.23 ± 0.62	F = 0.010 p = 0.921	T = 0.469 p = 0.640
TRAF6	−7.02 ± 1.27	−7.24 ± 1.36	−7.08 ± 0.87	F = 0.573 p = 0.450	T = −0.530 p = 0.598
Pellino1	−4.37 ± 1.68	−4.65 ± 1.34	−4.53 ± 1.83	F = 1.416 p = 0.236	T = 0.099 p = 0.921
IL−6	−8.49 ± 1.84	−8.71 ± 1.87	−9.24 ± 1.64	F = 4.365 p = 0.038*	T = −2.927 p = 0.004**

I vs. II: Paired *t*-test.
I vs. III: age, sex, BMI-adjusted multivariate analysis of covariance.
GAPDH was used as a housekeeping gene to assess the relative abundance of mRNA.
* p-value < 0.05.
** p-value < 0.01

Table 3
Relationship between baseline mRNA levels of negative regulators and baseline HAMD-17 scores.

Independent factors	HAMD-17 score		
	Standardized coefficients	t	*p*-value
SOCS1	−0.022	−0.215	0.830
TOLLIP	0.128	0.841	0.403
SIGIRR	−0.025	−0.226	0.821
MyD88s	−0.073	−0.641	0.523
NOD2	0.199	1.946	0.055
TNFAIP3	−0.207	−2.024	0.046*
ST2	0.046	0.841	0.403
IRAK3	−0.108	−1.108	0.283

Linear regression with forward-to-remove was used to analyze the association between negative regulators and severity of depression.
* p-value < 0.05.

Table 4
Relationship between baseline mRNA levels of negative regulators and decreases in HAMD-17 scores.

Independent factors	HAMD-17 score change		
	Standardized coefficients	T	*p*-value
SOCS1	−0.078	−0.661	0.511
TOLLIP	0.099	0.806	0.423
SIGIRR	−0.072	−0.581	0.563
MyD88s	−0.045	−0.329	0.743
NOD2	0.050	0.412	0.681
TNFAIP3	−0.269	−2.312	0.024*
ST2L	0.248	2.131	0.036*
IRAK3	0.044	0.374	0.709

Linear regression with forward-to-remove was used to analyze the association between negative regulators and severity of depression.
* p-value < 0.05.

were treated with agomelatine or bupropion. SSRI treatment significantly increased TNFAIP3 mRNA levels in PBMCs, whereas SNRIs treatment was associated with a trend towards increased TNFAIP3 expression that did not reach statistical significance. SSRIs also increased MyD88s and NOD2 mRNA expression, whereas SNRIs decreased TOLLIP expression (Table 5).

3.4. Fluoxetine induces TNFAIP3 expression in monocytes and microglia cells

We next isolated monocytes from PBMCs and examined mRNA expression profiles of negative regulators. Similar to the results obtained from PBMCs, TNFAIP3 mRNA levels were significantly increased in monocytes from MDD patients after antidepressant treatment (Fig. 1a). To further examine the potential effects of fluoxetine, an antidepressant of the SSRI class, on the expression of TNFAIP3 mRNA in monocytes and microglia, we pretreated THP-1 cells, a monocyte cell line, and SIM-A9 cells, a microglia cell line, for varying durations with different concentrations (10^{-7} to 10^{-5} M) of fluoxetine. Treatment with fluoxetine for 2 h induced an increase in TNFAIP3 mRNA expression in THP-1 and SIM-A9 cells ($p < 0.05$, Fig. 1b and f), an effect that was concentration dependent (Fig. 1c and g).

3.5. TNFAIP3 is required for suppression of IL-6 expression by fluoxetine

To examine the mechanism by which fluoxetine suppresses the expression of IL-6, we first transfected THP-1 and SIM-A9 cells with siRNA targeting TNFAIP3 for 24 h and then treated cells with fluoxetine (10^{-5} M) for 24 h to induce TNFAIP3. After pretreatment, THP-1 and SIM-A9 cells were treated with LPS to stimulate the production of IL-6. The suppressive effect of fluoxetine on LPS-induced

Table 5
Comparison of mRNA expression levels (-ΔCt) of negative regulators in MDD patients before and after two different antidepressant treatments.

	SSRIs (n = 39)		*p*-value	SNRIs (n = 32)		*p*-value
	Pre-treatment	Post-treatment		Pre-treatment	Post-treatment	
SOCS1	−4.79 ± 1.54	−4.80 ± 1.30	p = 0.967	−5.70 ± 1.47	−5.86 ± 0.95	p = 0.239
TOLLIP	−10.27 ± 1.64	−10.08 ± 1.65	p = 0.530	−11.56 ± 1.75	−11.93 ± 2.23	p = 0.042*
SIGIRR	−4.11 ± 1.59	−4.09 ± 1.43	p = 0.665	−4.55 ± 1.93	−4.81 ± 1.70	p = 0.112
MyD88s	−6.85 ± 1.00	−6.57 ± 0.73	p = 0.028*	−6.04 ± 0.70	−6.20 ± 0.72	p = 0.304
NOD2	−6.37 ± 1.05	−6.00 ± 1.36	p = 0.004*	−6.75 ± 1.43	−7.03 ± 1.53	p = 0.140
TNFAIP3	−6.33 ± 1.01	−5.32 ± 0.82	p = 0.000**	−5.96 ± 1.91	−5.20 ± 0.95	p = 0.052
ST2L	−10.53 ± 1.86	−10.09 ± 2.45	p = 0.081	−9.76 ± 2.80	−9.94 ± 1.09	p = 0.640
IRAK3	−5.91 ± 1.54	−5.80 ± 1.15	p = 0.521	−5.70 ± 1.81	−5.47 ± 0.44	p = 0.736

A paired sample *t*-test was used to compare differences between pre- and post-treatment.
GAPDH was used as a housekeeping gene to assess the relative abundance of mRNA.
SSRIs, selective serotonin reuptake inhibitors; SNRIs, Serotonin–norepinephrine reuptake inhibitors.
* p-value < 0.05.
** p-value < 0.01.

IL-6 production in THP-1 and SIM-A9 cells was partially blocked by siRNA-mediated knockdown of TNFAIP3 (Fig. 1e and i).

4. Discussion

In this study, we demonstrated significant changes in negative regulators of the TLR-mediated inflammatory pathway in acute MDD patients, showing that, prior to antidepressant treatment, TNFAIP3 and MyD88s expression were lower in PBMCs from MDD patients than in healthy controls. These results suggest an imbalance in the TLR-mediated inflammatory pathway in MDD. Moreover, we found that antidepressant treatment significantly increased TNFAIP3 expression both *in vivo* and *in vitro*, and this is the first report to suggest the involvement of TNFAIP3 in the mechanism by which antidepressants may reduce inflammation in MDD patients.

SSRIs have long been reported to exert anti-inflammatory effects (Ohgi et al., 2013; Tynan et al., 2012) and effectively modulate inflammatory diseases (Branco-de-Almeida et al., 2012; Koh et al., 2011). SSRIs, including paroxetine and fluoxetine, were recently reported to reduce IL-6 secretion through inhibition of LPS-induced IκBα phosphorylation rather than through actions at the 5-HT receptor (Durairaj et al., 2015). Our results support these finding, showing that TNFAIP3 is required for suppression of LPS-mediated IL-6 expression by fluoxetine. In addition, both SNRIs and SSRIs exerted similar effects on the serotonin transporter, but only SSRIs significantly increased TNFAIP3 expression (Table 5), further highlighting the fact that neither 5-HT receptors nor serotonin transporters are involved in the anti-inflammatory response to fluoxetine. Together, our study may provide novel evidence of unique peripheral mechanisms by which fluoxetine may reduce inflammation, which may be more associated with response than its effects on serotonin, which should be equally changed by SNRIs. Furthermore, our analysis of clinical outcomes revealed that lower expression of TNFAIP3 in patients before antidepressant treatment predicted greater improvement in HAMD scores after antidepressant treatment (Table 4), suggesting that clinical responses are associated with antidepressants and TNFAIP3.

Interestingly, the current study found that low TNFAIP3 levels were passively correlated with pretreatment HAMD scores. To our knowledge, this is the first study to report an association between clinical severity in MDD and expression of the *TNFAIP3* gene. Reducing TNFAIP3 expression would cause excessive production of pro-inflammatory cytokines, including TNF-α and IL-6 (Guedes et al., 2014). IL-6, in turn, has been shown to reduce 5-HT levels through activation of the tryptophan-metabolizing enzyme, IDO (Myint and Kim, 2014). Our findings strengthen the concept that dysregulation of TLR signaling is causally linked to major depression.

TNFAIP3 has attracted considerable attention by virtue of its role in autoimmune diseases, including systemic lupus erythematosus (SLE) (Han et al., 2016) and rheumatic arthritis (RA) (Ruiz-Larranaga et al., 2016). A case report noted depression as an initial feature of SLE (Marian et al., 2010). The prevalence of depression is also significantly higher in SLE patients compared with the general population, but the severity of depression is independent of SLE disease characteristics (van Exel et al., 2013). In addition, increased levels of TNFAIP3 have been shown to ameliorate disease progression in an animal model of RA (Vande Walle et al., 2014). Interestingly, SSRIs have been shown to significantly inhibit the spontaneous production of TNF, IL-6, and CXCL10 in human RA synovial membrane cultures (Sacre et al., 2010) and was reported to be as effective as methotrexate, the first-line treatment for RA patients (Baharav et al., 2012). The efficacy of methotrexate monotherapy for arthritis is associated with TNFAIP3 (Plant et al., 2012). In light of the involvement of SSRI effectiveness in MDD and RA, the use of methotrexate in MDD patients with low TNFAIP3 status warrants further investigation.

The mechanisms underlying the effects of negative regulators of TLR-mediated inflammatory signaling, especially TNFAIP3, on the psychopathology of depression are largely unknown. There is some evidence linking TNFAIP3-mediated inhibition of the activation of the NLRP3 inflammasome, which mediates innate immune system activation, with the susceptibility to depression-like behavior (Alcocer-Gomez et al., 2016; Cheng et al., 2016). A TNFAIP3 deficiency in macrophages significantly enhances NLRP3 inflammasome-mediated caspase-1 activation, pyroptosis, and interleukin-1β secretion (Vande Walle et al., 2014) by restricting ubiquitination of pro-interleukin-1β protein complexes (Duong et al., 2015). On the other hand, TNFAIP3 overexpression significantly alleviates inflammatory responses and reduces activation of NF-κB and the NLRP3 inflammasome (Li et al., 2015). Taken together, these results support our concept that TNFAIP3 is a potentially important target in the treatment of MDD. In addition, there is also some evidence suggesting that TNFAIP3 is associated with neurodegeneration. TNFAIP3 targets the TNF apoptotic pathway by inhibiting proteolytic cleavage of caspase-8 and -3; it can also effectively protect the SH-SY5Y neuronal cell line from apoptosis (Yu et al., 2006). RNF11 (RING finger protein 11), in association with the TNFAIP3 ubiquitin-editing complex, is a key negative regulator of NF-κB and has been suggested as a negative regulator of neurodegeneration in a microglia cell line (Dalal et al., 2012). TNFAIP3-knockout mice display a remarkable degree of reactive microgliosis and astrogliosis (Guedes et al., 2014), and mice with selective deletion of TNFAIP3 in astrocytes exhibit

demyelination owing to NF-κB and STAT1 hyperactivation (Wang et al., 2013). These data support an important role for TNFAIP3 in neuronal homeostasis (Pranski et al., 2012).

This study has several limitations. One limitation is the absence of a healthy control group exposed to the same environment. The interactions among antidepressants, cytokines, and negative regulators will require further controlled studies. Moreover, treatment was not randomized, so it is possible that clinical factors that determined treatment might have influenced results. Finally, the sample size of the control group in the current study is relatively small, and washout periods of antidepressants before collecting baseline samples may be insufficient. Further analyses using larger sample sizes and longer washout period are needed to confirm these results.

Although the analysis conducted in our current study may be limited, this report is nonetheless the first to link negative regulators of the TLR pathway, in particular TNFAIP3, with MDD. The therapeutic implications of our findings are considerable. In conjunction with current therapeutic regimens, modulating expression of the *TNFAIP3* gene to rebalance TLR-mediated inflammatory signaling may provide a potential approach for MDD management.

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