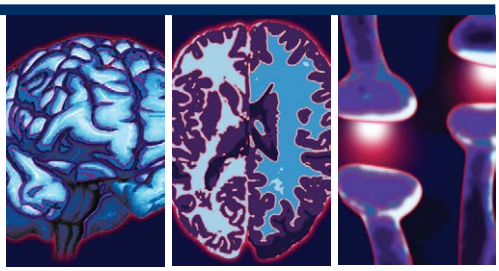

Sex Differences of NOD2 and TOLLIP in Patients with Major Depressive Disorder and Human Volunteers: Role of Androgen and Androgen Receptor

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Abstract

Background

Toll-like receptor (TLR) -4 mRNA expression is higher in the acute stage of major depressive disorder (MDD) compared with healthy controls. Some previous studies showed that higher TLR-4 expression in male than in female. However, the result is opposite to the higher prevalence of MDD in female. Here, we sought to explore the biological sex difference in mRNA expression levels of negative regulators of the TLR-4 pathway to provide some reasonable explanations.

Methods

We obtained peripheral blood mononuclear cells (PBMCs) from 100 patients with MDD and 53 healthy controls and used quantitative reverse transcription-polymerase chain reaction analysis for negative regulators of TLR 4 signaling studies. For androgen effect on the TLR regulator expression, we used the embryonic mouse hippocampal cell line (mHippoE-14), dihydrotestosterone as androgen receptor agonist, and flutamide as an antagonist.

Results

Among healthy controls, PBMCs from males had significantly higher TOLLIP and NOD2 mRNA levels than those from females. By contrast, mRNA for TOLLIP, but not NOD2, was higher in males than females in MDD. In addition, NOD2 and AR mRNAs were found to be lower in male MDD patients than in male healthy controls. Experiments using mHippoE-14 showed that inhibition of AR signaling with the antagonist flutamide suppressed NOD2 expression, whereas treatment AR signaling with the agonist dihydrotestosterone and antagonist flutamide could not increase NOD2 expression.

Conclusions

TOLLIP and NOD2, negative regulators for TLR-4 pathway, are significantly higher in male than female in health control. The difference in NOD2 disappeared in patients with MDD. The

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decrease of NOD2 mRNA expression is associated with androgen receptor.

Keywords

Negative regulation, Toll-like receptor, Innate immunity, Major depressive disorder, Androgen, Receptor

Abbreviations

ANCOVA: Analysis Of Covariance; AR: Androgen Receptor; ARIP3: AR-Interacting Protein 3; BMI: Body Mass Index; CS-FBS: Charcoal-Stripped Serum; CRP: C-Reactive Protein; DHT: Dihydrotestosterone; DMEM: Dulbecco's Modified Eagle Medium; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; HAMD: Hamilton Depression Rating Scale; IL: Interlukin IFNAR1: Interferon-α And -β Receptor Subunit 1; IRAK3: IL-1 Receptor-Associated Kinase 3; JAK2: Janus Kinase 2; LPS: Lipopolysaccharide; MCP-1: Monocyte Chemoattractant Protein-1; MDD: Major Depressive Disorder; Myd88: Myeloid Differentiation Primary Response Gene 88; Myd88s: Myeloid Differentiation 88 Short; NOD2: Nucleotide Binding Oligomerization Domain Containing Protein 2; NWD1: NACHT and WD Repeat Domain-Containing Protein 1; Pbmcs: Peripheral Blood Mononuclear Cells; Qrt-PCR: Quantitative Reverse Transcription-Polymerase Chain Reaction; SPSS: Statistical Product And Service Solutions; STAT3: Signal Transducer And Activator of Transcription 3; ST2L: Suppressor of Tumorigenicity 2; Full-Length Form; TLR: Toll-Like Receptor; TNF: Tumor Necrosis Factor;TNFAIP3: TNFα–Induced Protein 3; TOLLIP: Toll-Interacting Protein; SIGIRR: Single Immunoglobulin IL-1R–Related Receptor; SOCS1: Suppressor of Cytokine Signaling 1;VISA: Virus-induced Signaling Adapter

Introduction

Major depressive disorder (MDD) is a leading cause of disability in developed countries and is responsible for 7.4% of total disability-adjusted life years [1,2]. However, some fundamental questions remain unanswered. One of the most striking unexplained observations is the sex-based difference in the prevalence of MDD, which is twice as common in women as men [3-5]. Sex differences in exposure to stressful life events, coping styles, and reactivity to stress have been previously invoked to explain this difference [6]. However, whether the immune system plays a role in this relationship is still unknown.

Recent accumulating evidence indicates that activation of the inflammatory immune system influences neurochemical reactions and contributes to MDD [7-9]. Interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α, C-reactive protein (CRP), monocyte chemoattractant protein-1 (MCP-1) [9-12], and other inflammation-related proteins have been found in the plasma and cerebrospinal fluid of MDD patients as well as in postmortem samples [13]. Sex-based differences in cytokine expression have been recently reviewed [14]. Virus challenge in adult rats results in higher expression of genes encoding MYD88 (myeloid differentiation primary response gene 88), STAT3 (signal transducer and activator of transcription), JAK2 (Janus kinase 2), VISA (virus-induced signaling adaptor), JUN, IFNAR1 (interferon-α [IFN-α] and -β receptor subunit 1) and the interferon-induced GTP-binding protein, MX2, in female rats than in male rats [15]. Moreover, the production of IFN-α after exposure of peripheral blood mononuclear cells (PBMCs) to Toll-like receptor (TLR)-7 ligands is higher in females than in males [16]. A vaccination study also showed that the expression of TNF and other pro-inflammatory genes in PBMCs is also higher in women than in men [17]. From the perspective of inflammation, cytokines can partially explain why the prevalence of MDD is higher in females than in males.

Recent studies have demonstrated a significant association between innate immune, especially TLR-4–mediated signaling, and depression [18-21]. In contrast to sex differences in cytokines, sex differences in innate immune responses in MDD have received less research attention [22]. It has been reported that male mice exhibit greater morbidity after lipopolysaccharide (LPS) injection than female mice [23]. However, there is no difference in the severity of sickness symptoms after LPS injection in humans, even though women exhibited a greater pro-inflammatory response than males [24]. Patients with MDD show higher TLR-4 expression in the prefrontal cortex [19] and higher TLR-4

signaling in PBMCs [21]. There are significant associations between MDD severity and anxiety, body weight loss, and TLR-4 mRNA levels [25]. However, TLR-4 expression levels in adults were reported to be higher in males than in females [22], which argue against data relating to prevalence. In addition, our previous investigation of negative regulators of TLR signaling, including IRAK3 (IL-1 receptor-associated kinase 3), SOCS1 (suppressor of cytokine signaling 1), MyD88s (myeloid differentiation 88 short), TOLLIP (Toll-interacting protein), TNFAIP3 (TNFα–induced protein 3), ST2L (suppressor of tumorigenicity 2, full-length form), and SIGIRR (single immunoglobulin IL-1R–related receptor) showed that TNFAIP3 pathways play an important role in MDD [26]. However, no previous studies have reported sex differences in these regulators or their influence on sex-specific diseases. Accordingly, we here sought to investigate sex differences in negative regulators of TLR signal pathways in patients with MDD and human volunteers and assess the effects of androgen and androgen receptor (AR) signaling on these regulators.

Materials and Methods

■ Aims

Our aim was to examine sex-specific differences in negative regulators of TLR signaling in healthy controls and patients with MDD.

■ Design, setting, and participants

This study was embedded in our previous work, an observational study that investigated negative regulators of TLR signaling in MDD [26]. Institutional Review Board approval was obtained from the hospital ethics committee (101-5012A3, 103-5114B, and 103-6984A3). Patients and healthy controls were enrolled in the study after receiving verbal and written information about the study and providing written consent. Patients with MDD were screened by two psychiatrists before entering the study. A Structured Clinical Interview for DSM-IV Axis I Disorders as well as a detailed evaluation of current psychiatric symptoms and previous medical treatments were performed during screening. Patients with other major psychotic disorders, substance dependence (including alcohol), severe systemic physical illness, including metabolic syndrome, obesity (body mass index [BMI] > 34 kg/m^2) or inflammatory disease, or those who received immune-modulating drugs, were excluded from the study. All patients were examined for blood pressure and received chest X-rays, electrocardiographic examinations, and routine blood tests to exclude possible chronic systemic physical illness. None reported taking any antidepressants for at least1 week before entering the study. Healthy controls with neither a personal history nor a first-degree relative with a psychiatric disorder were recruited from the community. The same psychiatrist who performed screens of MDD patients assessed the healthy control group using the Diagnostic and Statistical Manual of Mental Disorders (Fourth Edition). MDD patients were free from antidepressant treatment at the time of the study. After screening, blood samples were collected.

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

Venous blood (10 mL) samples were taken after fasting for 9 hours. PBMCs were isolated from venous blood samples by Ficoll-Paque (GE, #17-5442-02) density gradient centrifugation. Immediately after collection, samples were stored at 80°C until they were assayed. The protocol used for mRNA analyses was the same as that used in our previous study (Hung et al, 2016). qRT-PCR was performed using primers listed in Table 1. Expression levels of target genes in each sample relative to that of glyceraldehyde-

Table 1: Primers use for qPCR.

human SOCS1	5′-GAC CCC TTC TCA CCT CTT GA-3′ (sense) 5′-GTA GGA GGT GCG AGT TCA GG-3′ (antisense)
human TOLLIP	5′-GAC AAC TGT CTC CGT CGC A -3′ (sense) 5′-CGG GAG CTC ACC GAT GTA-3′ (antisense)
human SIGIRR	5′-CCC AGC TCT TGG ATC AGT CT-3′ (sense) 5′-AGT CAG GGG CCC TAT CAC AG-3′ (antisense)
human MyD88s	5′-TCA TCG AAA AGA GGT TGG CT-3′(sense) 5′-GAT GGG GAT CAG TCG CTT CT -3′ (antisense)
human NOD2	5′- CGG CGT TCC TCA GGA AGT AC-3′ (sense) 5′-ACC CCG GGC TCA TGA TG-3′ (antisense)
human TNFAIP3	5′-GGA CTT TGC GAA AGG ATC G-3′ (sense) 5′-TCA CAG CTT TCC GCA TAT TG-3′ (antisense)
human ST2L	5′-CCC ACT CAG GAA AGA AAT CG-3′ (sense) 5′-TTC GCA TAT CCA GTC CTA TTG A-3′ (antisense)
human IRAK3	5′-CTC GGT CAT CTG TGG CAG TA -3′ (sense) 5′-TTC TAG GTG GGA CCG GAA GT-3′ (antisense)
human GAPDH	5′-TGC ACC ACC AAC TGC TTA GC-3′ (sense) 5′-GGC ATG GAC TGT GGT CAT GAG-3′ (antisense)
mouse TOLLIP	5′-GCGGGTCTCTGTGCAGTT-3′ (sense) 5′-TGTGGGTGTTATACGGAGGAA-3′ (antisense)
mouse SIGIRR	5′-GGATGACAAAGATCCCATGC-3′ (sense) 5′-ATGCAGATCCTGGTTTCCTG-3′ (antisense)
mouse NOD2	5′-CCTGGTACGTGCCCAAAGTAG-3′ (sense) 5′ –GCCAAGTAGAAAGCGGCAAA-3′ (antisense)
mouse GAPDH	5′-GCA CAG TCA AGG CCG AGA AT-3′ (sense) 5′-GCC TTC TCC ATG GTG GTG AA-3′ (antisense)

3-phosphate dehydrogenase (GAPDH), used as an endogenous control, were calculated based on the threshold cycle, CT, where the difference in CT (-ΔCT) representing relative expression in 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.

Cell culture

The mHippoE-14 embryonic mouse hippocampus cell line was obtained from CEDARLANE and maintained in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies/GIBCO, Cat# 12100-046) containing 10% charcoal-stripped serum (CS-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 dihydrotestosterone (DHT) experiments, cells were seeded in 6-well plates and treated with 10 nM DHT for 24 hours. In flutamide experiments, cells were seeded in 6-well plates and treated with 10 uM flutamide for 48 hours.

Statistical analysis

All results are presented as means ± standard deviation. mRNA levels shown in **Tables 1-4** and **Figure 1a** are presented as -ΔCT; mRNA levels in **Figure 1b** and c are presented as $2^{-\Delta\Delta CT}$. Independent t-tests were used to compare differences in age and BMI in **Table 2, Figure 1a and 1b**. Analysis of covariance (ANCOVA) with age and BMI adjustment was used for all factors in **Tables 3 and 4 and Figure 1c** and 1d. Two-tailed t-test was used to compare differences in cell line models **(Figure 1e)**. All statistical analyses were performed using Statistical Product and Service Solutions (SPSS), version 22. For each test, *P*-values < 0.05 were considered significant.

Table 2: Demographic data for MDD patients and healthy controls.

	MDD		
	Males (n = 21)	Females (n = 79)	*P*-value
Age (years)	44.86 ± 8.66	45.91 ± 10.33	0.67[1]
BMI (kg/m²)	22.63 ± 3.76	24.39 ± 4.76	0.08[1]
HAMD	28.55 ± 6.65	26.65 ± 4.63	0.24
	Healthy controls		
	Males (n = 15)	Females (n = 38)	*P*-value
Age (years)	43.13 ± 14.44	38.42 ± 9.40	0.26[1]
BMI (kg/m²)	23.72 ± 3.35	22.43 ± 2.88	0.17[1]
[1]Student's t-test			

Results

Demographic data

A total of 153 subjects were included in the study, of which 100 were patients with MDD (79 females, 21 males) and 53 were healthy controls (38 females, 15 males). As shown in **Table 2**, there was no difference in age between males and females (male vs. female: 44.86 ± 8.66 vs. 45.91 ± 10.33 years [$P = 0.67$] for MDD, and 39.80 ± 15.73 vs. 37.54 ± 10.48 years [$P = 0.26$] for healthy controls), BMI (male vs. female: 22.63 ± 3.76 vs. 24.39 ± 4.76 [$P = 0.08$] for MDD and 23.72 ± 3.35 vs. 22.43 ± 2.88 [$P = 0.17$] for healthy controls), or HAMD score among MDD patients (male vs. female: 28.55 ± 6.65 vs. 26.65 ± 4.63 [$P = 0.24$]).

NOD2 mRNA levels are higher in males than in females in healthy controls but not in MDD patients

To investigate sex-related differences in negative regulators of TLR signaling, we evaluated the mRNA expression of SOCS1, TOLLIP, SIGIRR, MyD88s, NOD2, TNFAIP3, ST2L, and IRAK3. There were no differences in SOCS1, SIGIRR, MyD88s, TNFAIP3, ST2L, or IRAK3 mRNA levels between males and females in either group. TOLLIP mRNA levels, expressed as -ΔCT, were higher in males than in females in both the MDD group (male vs. female: -9.57 ± 2.06 vs. -10.85 ± 1.84 [$F = 6.203$, $p = 0.015$]) and healthy control group (male vs. female: -8.66 ± 1.75 vs. -10.45 ± 2.44, [$F = 7.175$, $p = 0.010$]). By contrast, NOD2 mRNA levels were higher in males than in females in the healthy control group (male vs. female: -5.21 ± 1.27 vs. -6.16 ± 1.77 [$F = 4.099$, $p = 0.048$]), but not in the MDD group (male vs. female: -5.96 ± 1.02 vs. -6.43 ± 1.41 [$F = 2.337$, $p = 0.13$])**(Tables 3 and 4)**.

NOD2 and AR mRNA levels are lower in male MDD patients than in healthy controls

To investigate the association between negative regulators of the TLR pathway and MDD in males, we compared mRNA levels of IL-6, AR, and negative regulators in male healthy controls and MDD patients. This analysis showed that IL-6 mRNA expression was significantly higher in male MDD patients than in healthy controls (-9.35 ± 2.08 vs. -7.35 ± 2.76 [$F = 5.352$, $p = 0.027$]), whereas MYyD88s, NOD2, and AR mRNA expression were lower in the MDD group than in healthy group (healthy controls vs.

MDD: MYyD88s, -5.98 ± 0.91 vs. -6.60 ± 0.76 [F =6.115, p = 0.019]; NOD2, -5.21 ± 1.27 vs. -5.96 ± 1.02 [F =5.256, p = 0.029]; AR, - 10.81 ± 1.04 vs. -11.69 ± 1.37 [F = 5.449, p = 0.026]) **(Figure 1a)**. These results suggest an important role of the AR and NOD2 in depression.

■ NOD2 mRNA expression is regulated by androgen/AR signalings

To further clarify the effect of androgen/AR signaling on TOLLIP and NOD2, we treated the mHippoE-14 hippocampal cell line with the AR agonist dihydrotestosterone (DHT) or antagonist flutamide for 48 hours. Blocking AR signaling with flutamide for 48 hours significantly inhibited both TOLLIP and NOD2 mRNA expression. However, DHT could not increase NOD2 level when AR was inhibited by flutamide. These results suggest an important role of the AR in regulating NOD2 expression.

Table 3: Sex differences in negative regulators of TLR4 signaling in healthy controls.

	Males (n = 15)	Females (n = 38)	F and p-value[1]
IL-6	-9.35 ± 2.08	-8.06 ± 3.25	F = 1.954 p = 0.169
SOCS1	-5.63 ± 0.93	-5.07 ± 1.13	F = 2.896 p = 0.095
TOLLIP	-8.66 ± 1.75	-10.45 ± 2.44	F = 7.175 p = 0.01*
SIGIRR	-4.72 ± 1.50	-4.09 ± 1.61	F = 1.984 p = 0.165
MyD88s	-5.98 ± 0.91	-6.23 ± 0.88	F = 0.580 p = 0.450
NOD2	-5.21 ± 1.27	-6.16 ± 1.77	F = 4.099 p = 0.048*
TNFAIP3	-5.65 ± 0.73	-5.60 ± 0.69	F = 0.024 p = 0.877
ST2L	-9.20 ± 1.47	-9.22 ± 2.15	F = 0.048 p = 0.828
IRAK3	-5.67 ± 0.93	-5.64 ± 0.62	F = 0.056 p = 0.814

[1]Analysis of covariance with age and BMI adjustment; *P < 0.05

Table 4: Sex differences in negative regulators of TLR4 signaling in MDD patients.

	Males (n = 21)	Females (n = 79)	F and p-value[1]
IL-6	-7.35 ± 2.76	-6.94 ± 2.98	F = 0.339 p = 0.562
SOCS1	-5.60 ± 1.34	-5.19 ± 1.59	F = 0.774 p = 0.381
TOLLIP	-9.57 ± 2.06	-10.85 ± 1.84	F = 6.203 p = 0.015*
SIGIRR	-4.87 ± 1.57	-4.17 ± 1.69	F = 2.343 p = 0.129
MyD88s	-6.60 ± 0.76	-6.45 ± 0.94	F = 0.452 p = 0.503
NOD2	-5.96 ± 1.02	-6.43 ± 1.41	F = 2.337 p = 0.130
TNFAIP3	-6.39 ± 1.12	-5.96 ± 1.44	F = 2.288 p = 0.134
ST2L	-9.08 ± 1.57	-9.95 ± 2.66	F = 1.773 p = 0.186
IRAK3	-5.45 ± 0.87	-5.98 ± 1.78	F = 1.207 p = 0.275

[1]Analysis of covariance with age and BMI adjustment; *P < 0.05

Discussion

In this work, the findings of higher IL-6 mRNA level in patients with MDD compared with healthy control in both gender correspond to previous clinical studies in male [27] and in female [28]. The lower AR mRNA level in male MDD patients is also consistent with previous post mortem study which reported that the amount of AR mRNA in the paraventricular nucleus of the hypothalamus was decreased by ~2.7-fold in the depressed patients as compared to the controls [29].

A number of studies have investigated sex differences in immune and psychiatric diseases. Women have stronger immune responses than men in terms of the percentage of total lymphocytes mobilized and cytotoxic lymphocytes [30], and are more likely to experience feelings of social isolation and depression following LPS injection than males, an association that is correlated with cytokine levels [31]. Changes in Th1 cytokines following antidepressant treatment of MDD patients show a trend towards differences according to sex [32]. However, sex-related differences in negative regulators and their association with MDD have not been previously reported. In the current study, we found that TOLLIP and NOD2 mRNA levels were higher in males than in females in healthy controls, whereas only TOLLIP mRNA levels were higher in males in the MDD group. Considering that previous studies have shown that the association between an enhanced immune response and the prevalence of depression is stronger in females than in males [3-5,7-9], the reported elevated expression and activity of TLR in males represents a contradictory outcome [33]. Our data is the first that suggest some possible explanations to resolve this conflict.

Dual roles of NOD2 in TLR4-mediated signal transduction [34] reflect its different effects on behavior. The association of NOD2 with major depression was the subject of a recent investigation, which showed that anxiety levels,

a.

Male	Health controls n = 15	MDD n = 21	*p* value
Age (year)	43.13 ± 14.44	44.86 ± 8.66	0.66
BMI (kg/m²)	23.72 ± 3.35	22.63 ± 3.76	0.38

b.

Female	Health controls n = 38	MDD n = 79	*p* value
Age (year)	37.54 ± 10.48	45.91 ± 10.33	0.000*
BMI (kg/m²)	22.43 ± 2.88	24.39 ± 4.76	0.007*

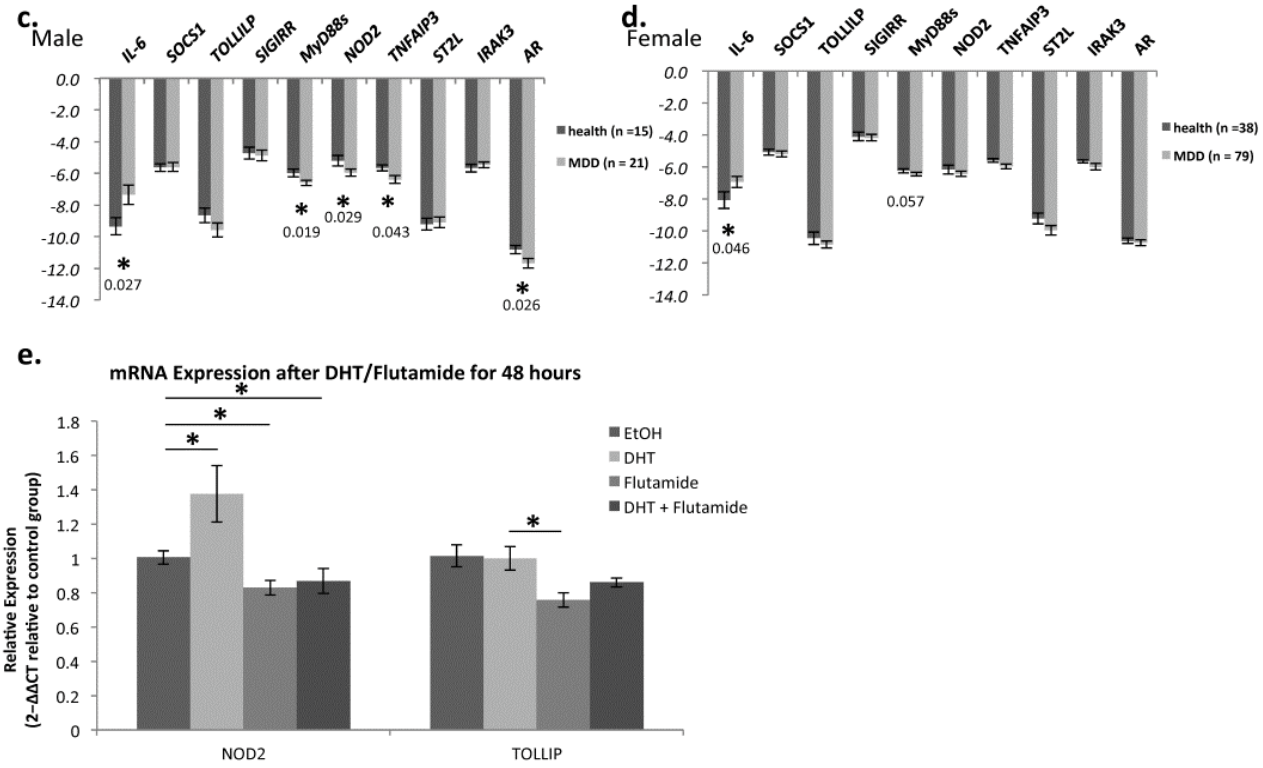

Figure 1: Androgen/AR signaling regulates the expression of NOD2. (a-d) The expression of IL-6 is higher in male and female MDD patients, whereas NOD2, MyD88s and AR expression are lower in male MDD patients. (e) DHT significantly increased NOD2 mRNA expression. Flutamide significantly decreased TOLLIP and NOD2 mRNA expression in the mHippoE-14 cell line. DHT and Flutamide treatment could not increase NOD2 mRNA expression
*$P < 0.05$.

Water avoidance stress-induced recognition memory deficits, as well as corticosterone levels were elevated in Nod1/2-double-knockout mice [35]. In contrast, a NOD2 agonist and LPS were shown to synergize with each other to worsen mouse sickness behavior [36]. Few studies have reported sex differences in NOD2 expression level. However, a mutation in the *NOD2* gene has been implicated as a possible cause of Crohn's disease, which is more prevalent in females than males [37]. There is also virtually no consideration of the relationship between androgen and NOD2 in the literature, with only a single report noting that NWD1 (NACHT and WD repeat domain-containing protein 1), another member of the NLR family, modulates AR signaling in prostate tumorigenesis [38]. These results are the first to point out the interaction.

Differences in TOLLIP expression between males and females seem controversy. Female rats have been reported to express higher levels of TOLLIP mRNA in colonizing microglia than males [39]. In prostate cancer cells, both estrogen and testosterone can cause DNA methylation in the *TOLLIP* gene [40]. TOLLIP has previously been shown to interact with ARIP3 (AR-interacting protein 3) [41].

Although it is not clear why these differences were observed, especially on TOLLIP and NOD2 between male and female, one of the possible explanations is the effect of androgen. In our cell line, androgen could increase mRNA expression of TOLLIP and NOD2 both but only NOD2 could be decreased in the presence of flutamide. The change of NOD2 is associated with AR but the interaction of TOLLIP and androgen/AR is not simply through direct interaction. Therefore,

the specific mechanism responsible for the higher expression of TOLLIP in males compared with females needs further clarification.

There are several limitations to this study. First, numerous confounding factors, including other hormones, lifestyle and environment, may influence the observed sex differences. Thus, interactions among antidepressants, cytokines, and negative regulators will require further controlled studies. Second, increases in mRNA expression were not reflected in increases in protein expression. Third, the sample size was relatively low, especially for males. Further analyses using larger sample sizes and well-designed experiments are needed to confirm these results. Besides, a more comprehensive approach using an animal model can confirm these findings.

Conclusion

In conclusion, we provide a possible explanation for the conflict between the concept that depression is more prevalent in females and the observed higher expression level of TLR in males than in females. Our data further suggest that, in conjunction with current therapeutic regimens, modulating the expression of NOD2 in males or TOLLIP in females to rebalance TLR-mediated inflammatory signaling may provide a potential approach for MDD management.

Declarations

■ Ethics approval and consent to participation

All protocols were approved by Kaohsiung Chang Gung Memorial Hospital ethics committee (101-5012A3, 103-5114B and 103-6984A3). Patients and healthy controls were enrolled in the study after receiving verbal and written information about the study and providing written consent.

Consent for Publication

Not applicable.

Availability of Data and Materials

The dataset supporting the conclusions of this article is included within the article.

Competing Interests

The authors declare that they have no competing interests.

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Authors' Contributions

YY Hung conceived and designed the study experiments. HY Kang contributes to design the study experiments. TL Huang recruited patients. YL Huang performed the qRT-PCR, semi-quantitative RT-PCR. YN Lee wrote the paper and all authors reviewed the manuscript.

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