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ORIGINAL ARTICLE

Major depressive disorder patients on antidepressant treatments display higher number of regulatory T cells

Mohd Ashari NOOR SURYANI[1], Mohamed Sanusi SITI NOR FAIRUS[1], Che Hussin CHE MARAINA[1], Kah Keng WONG[1], Shafei MOHD NAZRI[3], Mohd Yasin MOHD AZHAR[2]

[1]Department of Immunology, [2]Department of Psychiatry and [3]Department of Community Medicine, School of Medical Sciences, Health Campus, Universiti Sains Malaysia, 16150, Kubang Kerian, Kelantan.

Abstract

Introduction: Regulatory T cell (Treg) is a subtype of T lymphocyte that plays a crucial role in establishing immunologic self-tolerance and maintaining immune homeostasis. In this study, we set out to investigate the percentage and absolute count of Tregs in major depressive disorder (MDD) patients and their correlation with disease severity. *Materials & Methods:* This is a case-control study consisting of 47 MDD patients and 47 healthy controls. MDD patients were treated with antidepressant drugs according to their physician's choice. The severity of MDD was assessed using Beck Depression Inventory (BDI) and Montgomery-Asberg Depression Rating Scale (MADRS) at the time of recruitment. Healthy controls completed the Depression Anxiety Scoring System (DASS21) questionnaire to ensure they were in good mental health without history of MDD. The percentage and absolute count of $CD4^+$ $CD25^+$ Tregs and $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs were identified by multiparameter flow cytometry. *Results:* The percentage and absolute count of $CD4^+$ $CD25^+$ Treg cells were significantly higher in MDD patients than in healthy controls ($P<0.001$, in both cases). Likewise, the percentage and absolute count of $CD4^+$ $CD25^+$ $FOXP3^+$ Treg cells were also significantly higher in MDD patients compared to healthy controls ($P=0.003$ and $P=0.002$, respectively). However, there was no significant correlation between the percentage and absolute count of $CD4^+$ $CD25^+$ Treg and $CD4^+$ $CD25^+$ $FOXP3^+$ Treg cells with BDI or MADRS score. *Conclusions:* Our results suggest that antidepressant treatments contributed to an upregulation of Tregs in MDD patients.

Keywords: Major depressive disorder, regulatory T cell, percentage, absolute count, severity

INTRODUCTION

Major depressive disorder (MDD) is not only recognised as a mental disorder, but also as an immune system disorder.[1,2] MDD patients have been found to exhibit dysregulation of the immune system manifested by increased inflammatory cytokines, acute phase proteins, chemokines and adhesion molecules in the peripheral blood and cerebrospinal fluid.[2,3] Meta-analyses showed that MDD patients had significantly higher levels of pro-inflammatory cytokines including interleukin (IL)-6, IL-1, TNF-α and acute phase C-reactive protein (CRP) in comparison with controls.[4-6] MDD is also associated with decrease lymphocytes,[7] NK cells,[8] and elevated number of B cells compared to controls.[9]

$CD4^+$ $CD25^+$ regulatory T (Treg) cells are highly specialised T cell subpopulation pivotal in the suppression of immune response and mediate immune tolerance,[10,11] whose generation requires the transcription factor FOXP3.[12] Tregs are also specialised in cytokine release to maintain tolerance to self-antigens.[13] Both *in vitro* and *in vivo* analyses suggest that Tregs could suppress the proliferation and cytokine production of T effector cells.[14,15] Its dysfunction is involved in the pathogenesis of several autoimmune diseases such as systemic lupus erythematosus, multiple sclerosis, asthma, and rheumatoid arthritis.[16, 17]

Previous studies suggested that $CD4^+$ $CD25^+$ Tregs could inhibit the inflammatory responses

Address for correspondence: Dr Mohd Ashari Noor Suryani, Department of Immunology, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia. Tel: 000, Fax: 000, Email: *suryani@usm.my*

in MDD patients by expressing and releasing anti-inflammatory cytokines such as IL-10 and transforming growth factor beta (TGF-β).[10,18,19] Li *et al*.[15] reported that the level of $CD4^+$ $CD25^+$ Tregs was significantly decreased in MDD patients compared to the level in healthy controls. Miller[7] suggested that Tregs may contribute to the severity of depression through downregulation of chronic inflammatory response.

To date, the association of Treg levels with MDD remains unclear and previous studies reported $CD4^+$ $CD25^+$ Treg cells only. In this study, we measured both $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^+$ $FOXP3^+$ Treg cells in MDD patients (n=47) and healthy controls (n=47). The percentage and absolute count of $CD4^+$ $CD25^+$ Tregs and $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs between MDD patients treated with antidepressants and healthy controls were compared, and the associations between the percentage and absolute count of $CD4^+$ $CD25^+$ Tregs and $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs with the severity of MDD were examined.

MATERIALS AND METHODS

Study subjects and research tools

This case-control study was conducted in Kelantan, Malaysia, from February 2015 until March 2016. A total of 47 MDD patients and 47 healthy controls were included. MDD patients were recruited from Psychiatry Clinic, Hospital Universiti Sains Malaysia, Kelantan, Malaysia. All MDD patients were treated with antidepressant drugs according to their physician's choice. Majority of the patients (n=29/47; 61.7%) were on selective serotonin reuptake inhibitors antidepressants (escitalopram or fluoxetine or fluoxemine) while the remaining patients were either on mirtazapine (noradrenergic and specific serotonin antidepressants) or duloxefine (serotonin-norepinephrine reuptake inhibitors) or quetiapine fumarate (antipsychotic drug). Healthy controls were recruited from hospital employees and students with good general health and had no history of mental disorder diagnosis. MDD patients were diagnosed according to the Diagnostic and Statistical Manual of Mental Disorder (DSM-5) criteria. Patients with additional psychiatric diagnosis, autoimmune diseases, allergic diseases, immunocompromised or immunosuppressed diseases and current pregnancy were excluded from this study.

The severity of MDD patients was assessed using patient-rated questionnaire according to Beck Depression Inventory (BDI) and clinician-rated questionnaire according to Montgomery-Asberg Depression Rating Scale (MADRS). Healthy controls were required to complete a health questionnaire according to Depression Anxiety Scoring System (DASS 21) to ensure they were in good mental health and had no history of MDD. The study was approved by the Research and Ethics Committee, Universiti Sains Malaysia (USM/JEPeM/1403122). written informed consent was obtained from all participants. About 10 ml of peripheral blood were collected from each subject by venipuncture and stored in an ethylenediamine tetraacetic acid (EDTA) tube (Becton Dickinson, Plymouth, UK). The blood samples were processed within 4 hours of blood collection. Full blood count analysis was conducted on 500 µl of the blood using Sysmex XS-800i automated hematology analyzer (SYSMEX Corporation, Kobe, Japan).

Peripheral blood mononuclear cells (PBMCs) isolation

PBMCs were isolated from peripheral blood by density gradient centrifugation technique. In brief, 3 ml of lymphoprep (Axis-Shield, Oslo, Norway) was added into a falcon tube. A total of 9 ml fresh blood was layered carefully over the lymphoprep using Pasteur pipette. The sample was centrifuged at 500x g for 30 minutes at 20°C in a swinging-bucket rotor without brake. After centrifugation, the second layer (buffy coat) was aspirated and transferred into new falcon tube using a pipette. Around 8-10 ml PBS (GE Healthcare, UK) was added into the falcon tube and the mixture was centrifuged at 500x g for 10 minutes at 20°C. The supernatant was removed completely. For removal of platelets, the cell pellet was resuspended in 10 ml PBS and centrifuged at 300x g for 10 minutes at 20°C. The supernatant was removed and the washing step was repeated again. The cell pellet was then resuspended in 1 ml of PBS. The number of cells was counted using hemocytometer and its concentration was adjusted to $1x10^7$ cells/ml.

Immunophenotyping of Tregs

20 µl of FITC Mouse Anti-Human CD4 and 20 µl of APC Mouse Anti-Human CD25 (BD Biosciences, USA) were pipetted into the bottom of each 12x75 mm tube. PBMC (100 µl) was added into the tube. The tube was vortexed and incubated on ice for 20 minutes, protected from light. At the end of incubation, 2 ml of washing buffer (PBS) was added. The cell suspension was centrifuged at 250x g for 10 minutes. The

supernatant was discarded and the cell pellet was dislodged by tapping the tube. A total of 2 ml 1x Human FOXP3 buffer A (BD Biosciences, USA) was added drop by drop. The mixture was mixed well and incubated on ice for 10 minutes, protected from light. Then, the cell suspension was centrifuged at 500x g for 5 minutes. The supernatant was removed using pipette and the cell pellet was dislodged by tapping the tube. FOXP3 Buffer C (500 μl; BD Biosciences, USA) was added into the tube to permeabilise the cells. The mixture was mixed well and incubated on ice for 30 minutes. To wash the cell, 2 ml of PBS was added into the tube and centrifuged at 500x g for 5 minutes. The washing step was repeated twice, and 20 μl of PE Mouse Anti-Human FOXP3 (BD Biosciences, USA) was added into the tube. The mixture was mixed well and incubated on ice for another 30 minutes before repeating the washing step. The cells were then resuspended in 300 μl of PBS and analysed immediately using flow cytometer.

Flow Cytometric Analysis

The number of cells binding to antibodies was analysed using BD FACSCanto II flow cytometer and BD FACSDiva™ software (BD Biosciences, San Jose, CA, USA) by using an appropriate setting. The acquisition event was 20,000 cells per second. Data were analysed using the FlowJo v10 software (TreeStar, Ashland, OR, USA). Two types of Treg cells were measured in this study: $CD4^+$ $CD25^+$ Tregs and $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs (Fig. 1). The absolute count of Tregs was calculated by multiplying the percentage of Tregs with total number of lymphocytes obtained from complete blood count analysis.

Statistical Analysis

Data were analyzed using SPSS statistical

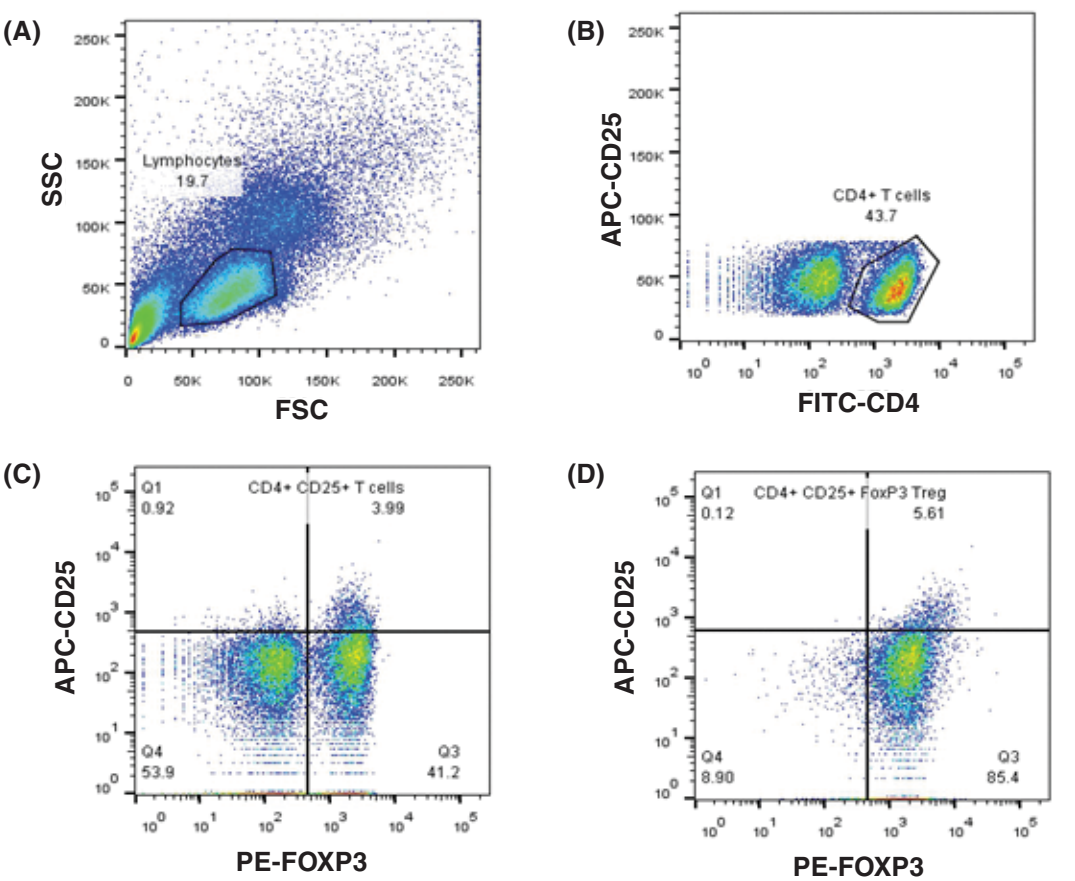

FIG. 1: Multiparameter flow cytometry to identify $CD4^+$ $CD25^+$ Tregs and $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs. **(A)** Lymphocytes were analysed using forward scatter (FSC) and side scatter (SSC) gating strategy. **(B)** The lymphocytes were subgated and plotted in CD4 versus CD25 dot plots to measure $CD4^+$ $CD25^+$ Tregs. **(C)** $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs were selected by further subgating and plotted CD25 versus FOXP3 dot plots. **(D)** $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs were identified in the right upper quadrant.

package program v22 (IBM Corporation, USA) and GraphPad Prism v6 (GraphPad Software Inc, La Jolla, CA, USA) software. As the data were not normally distributed, non-parametric test was applied for all statistical analysis. All data were presented using median and interquartile range (median ± IQR). Mann-Whitney test was used to compare the percentage and absolute count of Tregs between MDD patients and healthy controls. The correlation between percentage and absolute count of Tregs with the severity of MDD were tested using Spearman's rank correlation. Results with $P<0.05$ were considered as statistically significant.

RESULTS

The mean age ± SD of MDD patients was 39.7 ± 13.07 years old with 42.5% of them were within 45 to 65 years old. The mean age of healthy controls was 28.0 ± 8.69 years old and 46.8% of them were between 18 to 24 years old. The mean age of MDD onset was 36.26 ± 11.88 years old.

In MDD group, 29 (61.7%) were females and 18 (38.3%) were males while in healthy controls, 32 (68.1%) were females and 15 (31.9%) were males. Majority of MDD patients and healthy controls were Malays which accounted for 95.7% of study population, while the other 4.3% were Chinese. Sociodemographic characteristics were summarized in Table 1.

Percentage and absolute count of $CD4^+$ $CD25^+$ Tregs

The median ± IQR percentage of $CD4^+$ $CD25^+$ Tregs in MDD patients (3.70 ± 3.30) was significantly higher than in healthy controls (2.30 ± 2.50) with $P<0.001$ (Fig. 2A). The absolute count of $CD4^+$ $CD25^+$ Tregs was also significantly higher in MDD patients (9.50 ± 9.00 x 10^4/ml) compared to healthy controls (5.30 ± 4.40 x 10^4/ml) with $P<0.001$ (Fig. 2B).

Percentage and absolute count of $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs

The median ± IQR percentage of $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs in MDD patients and healthy controls were 1.55 ± 2.00% and 1.10 ± 1.00% respectively. The percentage was significantly higher in MDD patients compared to healthy controls ($P=0.003$) (Fig. 3A). Likewise, the absolute count of $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs was also significantly higher in MDD patients (3.90 ± 5.90 x 10^4/ml) compared to healthy controls (2.80 ± 2.50 x 10^4/ml) ($P=0.002$) (Fig. 3B).

The Correlation of Treg Levels with Severity of MDD

The BDI and MADRS scores of the MDD patients were summarised in Table 2. At the time of recruitment, the mean score for BDI and MADRS were 20.28 ± 9.28 and 28.21 ± 8.51, respectively.

TABLE 1: Sociodemographic characteristics of healthy controls and MDD patients

	Healthy controls (n=47)	MDD (n=47)
Age		
Mean (SD)	28.0 (8.69)	39.7 (13.07)
Age group, n (%)		
18-24	22 (46.8)	9 (19.2)
25-44	21 (44.7)	18 (38.3)
45-65	4 (8.5)	20 (42.5)
Age of MDD onset		
Mean (SD)	-	36.26 (11.88)
Gender, n (%)		
Male	15 (31.9)	18 (38.3)
Female	32 (68.1)	29 (61.7)
Race, n (%)		
Malay	45 (95.7)	45 (95.7)
Chinese	2 (4.3)	2 (4.3)

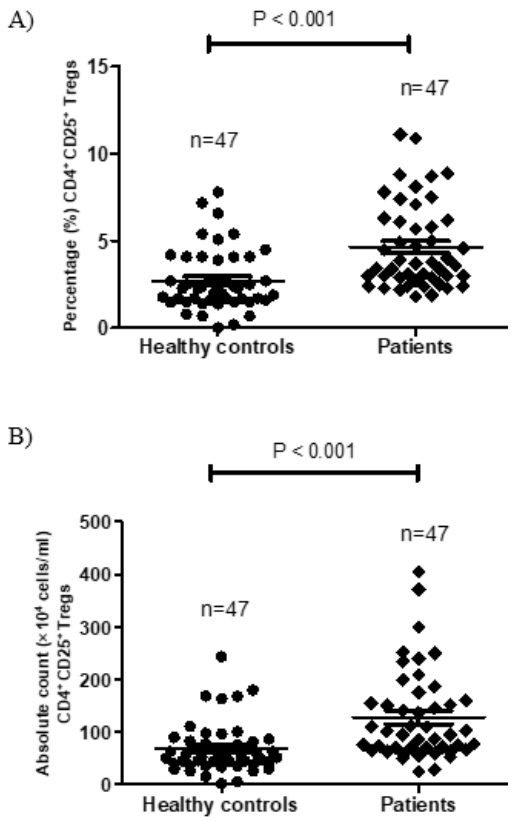

FIG. 2: The percentage (A) and absolute count (B) of $CD4^+$ $CD25^+$ Tregs in healthy controls and MDD patients.

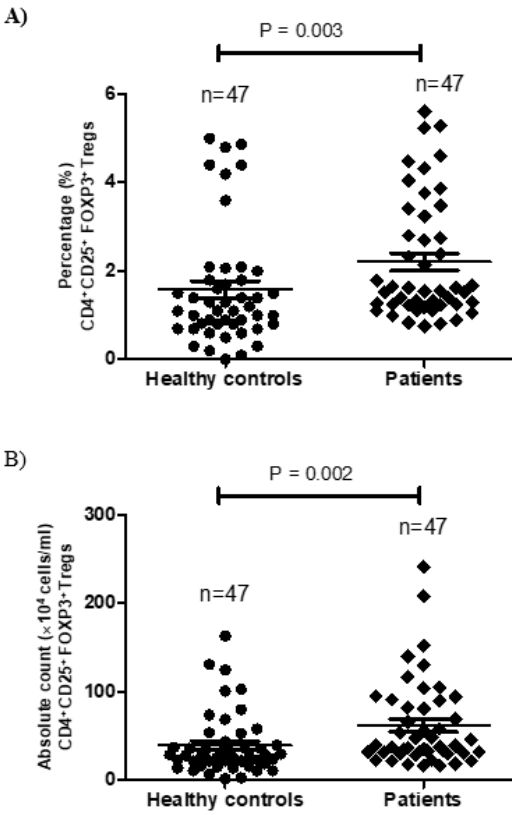

FIG. 3: The percentage (A) and absolute count (B) of $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs in healthy controls and MDD patients.

Correlation of Treg cells percentage and absolute count between different severity of MDD based on MADRS scale is shown in Table 3. Our study showed that the BDI score was not significantly correlated with the percentage (r=-0.060, P=0.689) and absolute count (r=-0.128, P=0.392) of $CD4^+$ $CD25^+$ Tregs. Likewise, there was no significant correlation between the percentage (r =-0.012, P=0.938) and absolute count (r =-0.038, P=0.798) of $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs with the BDI score.

This study also found that there was no significant correlation between the percentage (r=-0.073, P=0.626) and absolute count (r =-0.002, P=0.990) of $CD4^+$ $CD25^+$ Tregs with MADRS score. Moreover, MADRS score was also not significantly correlated with the percentage (r =0.132, P=0.375) and absolute count (r =0.068, P=0.650) of $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs.

DISCUSSION

MDD has been associated with several alterations in cellular immunity. The percentage and absolute count of $CD4^+$ $CD25^+$ Tregs have been shown to be decreased in the peripheral blood of MDD patients before treatment.[15] Our study showed that the percentage and absolute count of $CD4^+$ $CD25^+$ Tregs were significantly increased in MDD patients with antidepressant treatment

TABLE 2: BDI and MADRS scores of MDD patients (n=47)

	Min	Max	Mean	SD
BDI Score	11	47	20.28	9.28
MADRS Score	15	49	28.21	8.51

Independent-t test
†Min: minimum score, Max: maximum score, SD: standard deviation

TABLE 3: Correlation of Treg cells percentage and absolute count between different severity of MDD based on MADRS scale.

	Severity of MDD	Median (IQR)	χ^2 stat[a] (df)	*p*-value
$CD4^+$ $CD25^+$ Tregs				
Percentage (%)	Mild	3.20 (1.18)	1.180 (2)	0.554
	Moderate	3.65 (4.48)		
	Severe	4.50 (4.85)		
Absolute counts (cells/μl)	Mild	89 (42)	0.472 (2)	0.790
	Moderate	91 (88)		
	Severe	104 (130)		
$CD4^+$ $CD25^+$ $FOXP3^+$ Tregs				
Percentage (%)	Mild	1.33 (0.77)	2.859 (2)	0.239
	Moderate	1.65 (2.65)		
	Severe	1.65 (2.19)		
Absolute counts (cells/μl)	Mild	33 (25)	1.831 (2)	0.400
	Moderate	47 (56)		
	Severe	39 (63)		

* Kruskal Wallis test

compared to healthy controls. This finding was in accordance with Himmerich *et al*.[13] who also demonstrated a significant increase of $CD4^+$ $CD25^+$ Treg cells in MDD patients during antidepressant therapy.

Several studies addressing the number of Treg in MDD reported $CD4^+$ $CD25^+$ Treg only.[13,15] Some studies suggested that measuring $CD4^+$ $CD25^+$ Treg cells only may not provide a precise assessment of Treg numbers.[20] CD25 (also known as IL-2 receptor) is expressed by various types of cells and not only unique to Tregs.[21] For example, conventional T cells express CD25 when activated by T cell receptor (TCR) ligation.[10] It has also been found that FOXP3 is the transcription factor required for the development and function of natural $CD4^+$ $CD25^+$ Tregs.[12]. Continued FOXP3 expression in mature Tregs is needed to maintain the transcriptional and functional program established during Treg cells development.[22] According to Zhou *et al*.[23], when Tregs lose this transcription factor they form 'exTregs' which are effector-like T cells that no longer serve to regulate immune functions. Hence, measuring $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs is essential for the measurement of functional Tregs as implemented in our current study in MDD patients.

Consistent with $CD4^+$ $CD25^+$ Tregs, we also found a significantly higher percentage and absolute count of $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs in the peripheral blood of MDD patients compared with healthy controls. Our results are in accordance with Grosse *et al*. [24] who also found significantly higher $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs in MDD patients after antidepressant treatment with either venlafaxine or imipramine. In another study, Zhang *et al*.[25] found that antidepressant desipramine upregulated $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs cells in allergic rhinitis (AR) patients. Past studies showed that MDD was associated with chronic inflammation as manifested by increased inflammatory cytokines including tumour necrosis factor alpha (TNF-α), IL-1 and IL-6 in the peripheral blood.[6,7] As Tregs are a potent suppressor of chronic inflammation, lower Tregs was found in MDD patients before treatment,[15] and antidepressant medications might cause an upregulation of $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs to counter the exaggerated inflammatory response.

Miller[7] hypothesised that Tregs may be associated with the severity of MDD through downregulation of chronic inflammatory responses. However, our study showed that neither BDI nor MADRS scores were correlated significantly with the percentage or absolute count of $CD4^+$ $CD25^+$ Tregs and $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs. This indicated that the increase in $CD4^+$ $CD25^+$ Tregs and $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs were not associated with the severity of MDD. Our finding was comparable with those by Himmerich *et al*.[13] which showed that the relative changes in HAMD-21 scores (severity scale) during antidepressant therapy

did not correlate significantly with the baseline proportion or relative changes of $CD4^+$ $CD25^+$ Tregs. Himmerich *et al*.[13] also found that patients with moderate to severe depression did not differ from those with milder depression in terms of the baseline level of $CD4^+$ $CD25^+$ Tregs. The similar Tregs proportion in mild and severe MDD in this study suggested that higher number of Tregs in MDD patients was caused by antidepressant drugs and less likely due to the disease severity.

We acknowledge the limitations of this study as follows: (i) Samples recruitment in this study involved MDD patients who had received prior treatments with antidepressant drugs. Studies have been conducted on newly diagnosed, un-medicated MDD patients and found that Treg cells were reduced in these patients [15, 26]; (ii) MDD patients were treated with different types of antidepressant drugs according to physician's choice. The specific effects of different antidepressants cannot be excluded, and we had examined the potential effects of specific antidepressant on the Treg populations in our cohort of patients but we did not find any significant influence (data not shown); (iii) The number of newly diagnosed patients without concurrent chronic diseases (who were excluded in this study) was very small in our local hospital. However, the prevalence of MDD was higher among adults with chronic diseases compared to those without any chronic illnesses [27]

CONCLUSION

Our results suggest that the number of $CD4^+$ $CD25^+$ Treg and $CD4^+$ $CD25^+$ $FOXP3^+$ Treg cells were increased by antidepressant treatments in MDD patients independent of the severity of the disease.

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Declaration: All authors have disclosed no conflicts of interest.

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