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ORIGINAL ARTICLE

Tim-3-immune Checkpoint Receptor Expression on CD4+ and CD8+ T cells and Rheumatoid Arthritis Disease Activity

[1]Tamer M. Soliman, [2]Esam M. Abu Al Fadl, [2]Ahmed R. Radwan, [3]Yasmine M. Amrosy, [4]Sherif M.H. El-kannishy [5]Eman Elmansoury, [1]Heba A. Ahmed, [6]Alaa A. Badran

[1]Department of Clinical Pathology, Sohag Faculty of Medicine, Sohag University, Sohag, Egypt
[2]Department of Rheumatology and Rehabilitation, Sohag Faculty of Medicine, Sohag University, Sohag, Egypt
[3]Department of Clinical Pathology, Helwan Faculty of Medicine, Helwan, Egypt
[4]Department of Toxicology, Mansoura Hospital, Faculty of Medicine, Mansoura University, Mansoura, Egypt
[5]Department of Microbiology and Immunology, Faculty of Medicine, Mansoura University, Mansoura, Egypt
[6]Department of Clinical Pathology, Faculty of Medicine, Mansoura University, Mansoura, Egypt

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Corresponding Author:
Alaa Aboelnour Badran
Department of Clinical Pathology, Faculty of Medicine, Mansoura University, Mansoura, Egypt.
Tel.: 01117576066
alaaaboelnoour@mans.edu.eg
aalaa_abo@yahoo.com

ABSTRACT

Background: *Rheumatoid arthritis (RA), is a chronic inflammatory autoimmune disease, characterized by dysregulated T cell immune response..* ***Objective:*** *To investigate the Tim-3 surface expression on peripheral blood CD4$^+$ and CD8$^+$ T cells in RA patients concerning disease activity.* ***Methods:*** *A cross-sectional case-controlled study involving 157 RA patients who were categorized by disease activity score 28 (DAS28) into 4 groups; patients with remission and low, moderate, and high RA activity groups. The assessment of Tim-3 expression on peripheral CD4 and CD8 T cells of patients and controls using flow cytometry was done.* ***Results:*** *The peripheral expression of Tim-3 on CD4+ and CD8+ T cells was significantly higher in RA patients as compared to controls (for CD4+ T cells, 3.55 ± 1.12 % in remission group vs. 1.21 ± 0.52 in control group, p <0.001; for CD8+ T cells, 5.95 ± 1.49 % in remission group vs. 1.80 ± 0.73 % in control group, p <0.001). There was an inverse correlation between percentages of both peripheral Tim-3+CD4+ and Tim-3+CD8+ T cell and RA DAS28, (r = –0.425, p = 0.001) and (r= –0.597, p = <0.001) respectively.* ***Conclusion:*** *The upregulated Tim-3 expression on peripheral CD4+ and CD8+ T-cells suggests a potential role of this immune checkpoint receptor in T cell immune dysregulation in RA. Tim-3 expression was negatively correlated with disease progression. Tim-3 could be a useful biomarker in determining the rheumatoid disease activity, progression, and represents an important target for inhibitors intervention in RA.*

INTRODUCTION

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease characterized by recurrent synovitis caused by inflammatory cells infiltration, angiogenesis, and synovial fibroblasts proliferation [1]

To classify a patient as a definite RA patient, or not a history of symptom duration, an entire joint evaluation, and at least one serologic test and one acute-phase response measure must be obtained[2] The anti-cyclic citrullinated peptide (anti-CCP) is a serologic test of choice in RA diagnosis, assessment of disease activity, follow-up, and even the prediction of diseases occurrence[3].

T lymphocytes have a central role in the pathogenesis of RA [4]as the activation of these cells and their secreted cytokines such as interleukin (IL-6), interferon (IFN-γ), and tumor necrosis factor (TNF-α) are involved in RA cellular immune responses, [5,6]

Immune checkpoint inhibitor proteins expressed on immune cells have an important physiologic intrinsic inhibitory of the immune system that is essential for normal modulation of the duration and amplitude of immune responses. These proteins can either attenuate or augment the immune response. Increased expression of the immune checkpoint inhibitor proteins has been associated with various disease conditions, including cancer, autoimmunity, and sepsis [7].

T cell immunoglobulin- and mucin-domain-molecule-3 (Tim-3), is a transmembrane coinhibitory receptor protein molecule and is expressed on the surface of T cells [8]. Tim-3 negatively regulates both innate and adaptive immune responses [9-11]through the engagement with Galactin-9 which triggers the apoptosis of T helper (Th1) cells causing selective loss of these IFN-γ producing T cells, thus modulating the balance of Th1 and Th2 cytokines. Furthermore, it exerts a suppressive effect on Th17 cells and promotes the induction of regulatory T (Treg) cells, that are

involved in T cell tolerance induction, hence limiting tissue damage from excessive immune activation '

It was proposed that Tim-3, like other members of T cell inhibitory molecules such as programmed death receptor 1 (PD-1) and co-stimulatory receptor cytotoxic T- lymphocyte antigen-4 (CTLA-4), has an inhibitory effect as the preclinical data support the therapeutic modulation of TIM3 in multiple disease contexts, including autoimmunity [9-12].

The present study aimed to estimate the expression pattern of Tim-3 on peripheral T cells in RA patients and address its relation to disease activity.

METHODOLOGY

This study is a hospital-based cross-sectional case-controlled study. A total of 157 RA patients had attended the Rheumatology outpatient Clinic of Sohag University Hospitals. In addition to 49 age-, sex-matched healthy subjects served as normal controls. The study protocol was approved by the Ethical Committee of Medical Research Ethics Committee of Sohag Faculty of Medicine **IRB: Soh-Med-21-11-31**. In adherence to the Helsinki Principles, informed consent was obtained from all participants.

Diagnosis of RA was based on the criteria established in 2010 by the American College of Rheumatology/European League Against Rheumatism Collaborative Initiative [2]. RA patients with different degrees of disease activity were included. Patients suffering from associated other inflammatory or autoimmune diseases were excluded from the study.

Cases with RA were defined based on the physician's decision, the type and side of articular involvement, rheumatoid factor (RF) and anti-CCP positivity, symptoms duration, radiologic findings, and acute phase reactants.

At the time of venipuncture, assessment of RA disease activity was done using the disease activity score (DAS28) based on joint tenderness counts (0-28), joint swelling counts (0-28), and erythrocyte sedimentation rate (ESR) and calculated from the website (www.das-score.nl) [13]. According to DAS28, patients were divided into 4 groups: remission (DAS28 ≤2.6), low (DAS28 >2.6 and ≤3.2), moderate (DAS28 >3.2 and ≤5.1), and high RA disease activity (DAS28 >5.1) groups [14].

Sample Collection

For each subject, a 10 ml venous blood sample was collected and divided into 3 tubes. The first tube contains EDTA (Ethylenediaminetetraacetic acid) for complete blood count and ESR testing using the conventional Westergren method. The second tube also contains EDTA for flow cytometric assay. A third plain tube was used where blood was allowed to clot and centrifuged at 3000 RPM for 10 min to obtain serum for determination of anti-CCP antibody test.

Flow cytometry

Specimen processing:

The collected EDTA blood was layered in a 20 ml sterile tube on the top of an equal volume of density gradient LymphoprepTM (Ficoll-Hypaque) (Biosera, Nile, France) for isolation of peripheral blood mononuclear cells (PBMCs) by centrifugation at 400×g for 30 minutes at 20 ºC without brake, to ensure separation of the PBMCs from the denser Ficoll/erythrocyte layer below, and the less dense dilute plasma layer above. After centrifugation, the isolated PBMCs were washed 2 times and resuspended in phosphate-buffered saline (PBS) containing 2% fetal bovine serum.

Labeled monoclonal antibodies:

Antihuman monoclonal antibodies (mAbs) labeled with fluorochrome were used, including phycoerythrin/Cyanine 5 (PE/Cy5) anti-CD3 (BD), fluorescein isothiocyanate (FITC)-labeled anti-CD4 (BD), peridinin-chlorophyll-protein (PerCP)-labeled anti-CD8 (BD), and phycoerythrin (PE)-labeled anti-Tim-3 (Abcam-Cambridge, MA-USA). Isotype-matched control antibodies included Cy5-, FITC-, PerCP-, and PE-conjugated rat antibodies were used to subtract the background staining control.

Procedure:

Briefly, 100 µL PBMC suspension to Eppendorf tubes was placed on ice, and 5 µL of each monoclonal antibody was added to each tube, placed at 4°C, kept away from light for 30 minutes. After 2 times of PBS washing, 0.4 ml PBS containing 1% serum was added to re-suspend cells and transferred to the flow tube. Data acquisition and analysis were performed by Becton Dickinson FACScalibur™ flow cytometry with (BD Biosciences, San Jose, CA, USA). The labeled cells were analyzed, a maximum of 30,000 events was acquired, and CellQuest software (v5.2) was used for data acquisition and analysis (BD Biosciences, San Diego, CA, USA). For compensation analysis, the spillover amount of each fluorochrome into other channels was determined and subtracted.

Flow cytometric analysis (figure 1):

After the acquisition, cells were first gated on forward (FSC) and side scatter (SSC) properties, i.e., size and granularity; to select lymphocyte population, then the expression of CD4 and CD8 on the gated cells was tested to characterize CD4+CD3+ and CD8+CD3+ cell populations. Then for each cell population, the percentage of Tim-3+ expression was assessed.

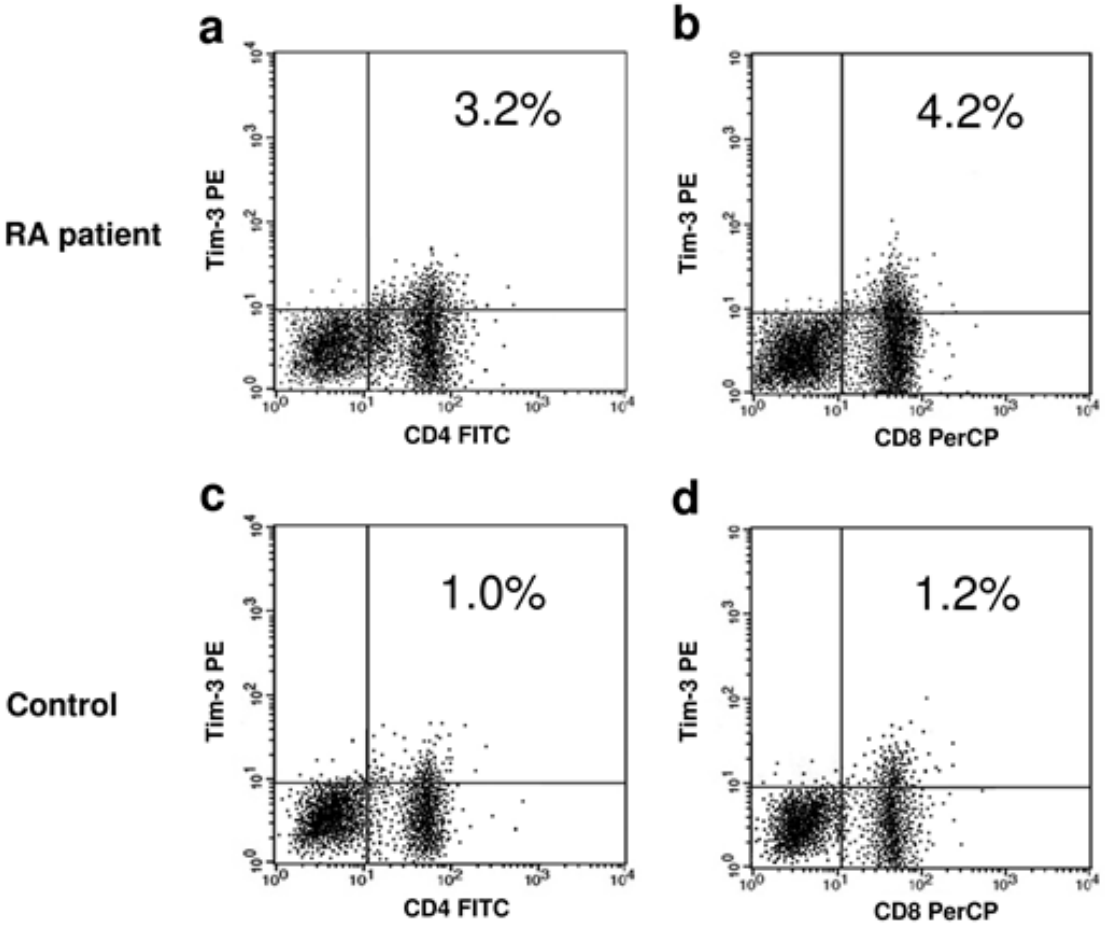

Fig. 1: Dot plot histograms show the percentage of Tim-3 expression on peripheral blood CD4+CD3+ and CD8+CD3+ cells of a RA patient (a,b) and a normal control (c,d).

Statistical analysis

Statistical analysis was performed using SPSS (version 24.0, IBM, New York, USA). After testing the normality of the data by the Kolmogorov-Smirnov test, data were statistically described in terms of mean and SD for quantitative data. One-way ANOVA test was appropriately used for comparison between the studied groups. The Pearson correlation was used to determine the correlations between Tim-3+CD4+CD3+ and Tim-3+CD8+CD3+ cells, Tim-3+CD4+CD3+ cells and DAS28, Tim-3+CD8+CD3+ cells and DAS28, Tim-3+CD4+CD3+ cells and serum-anti-CCP levels, and finally, Tim-3+CD8+CD3+ cells and serum-anti-CCP levels. A *p*-value of < 0.05 was considered significant.

RESULTS

The study included 157 RA patients (49 males and 108 females) and 49 age- and sex-matched controls (18 males and 32 females). Table (1) shows the number, gender, age, and the percentage of Tim-3 expression for both peripheral blood CD4+CD3+ and CD8+CD3+ cells for all study groups including the control group.

Table 1: Collected study data

		RA patients' disease activity				Control	ANOVA (*p*-value)
		Remission	Low	Moderate	High		
DAS28		≤ 2.6	2.6 to ≤ 3.2	3.2 to ≤ 5.1	> 5.1		
Number		31	39	46	41	49	
Gender n (%)	**Male**	10 (32.3)	11 (28.2)	15 (32.6)	13 (31.7)	18 (36.7)	
	Females	21 (67.7)	28 (71.8)	31 (67.4)	28 (68.3)	32 (65.3)	
Age (years)		45.42 ± 11.13	41.36 ± 12.22	47.24 ± 10.98	46.51 ± 12.24	43.14 ± 12.09	0.129
Anti-CCP (U/mL)		16.26 ± 13.74	23.16 ± 20.87	51.96 ± 41.81	79.14 ± 67.13	N/A	< 0.001
Tim-3+CD4+CD3+ cells (%)		3.55 ± 1.12	3.18 ± 1.18	2.58 ± 1.00	2.62 ± 1.19	1.21 ± 0.52	< 0.001
Tim-3+CD8+CD3+ cells (%)		5.95 ± 1.49	5.15 ± 1.32	4.37 ± 1.23	3.91 ± 1.32	1.80 ± 0.73	< 0.001

Level of significance: *p*-value <0.05

Using the ANOVA test, a highly significant statistical difference was found among all groups as regards Tim-3 expression on both CD4+CD3+ and CD8+CD3+ cells in peripheral blood (table 1, figure 2). A strong positive correlation was found between Tim-3+CD4+CD3+ and Tim-3+CD8+CD3+ cells percentages (figure 3). Paired Tim-3 expression percentages of peripheral blood CD4+CD3+ and CD8+CD3+ cells are shown in figure 4.

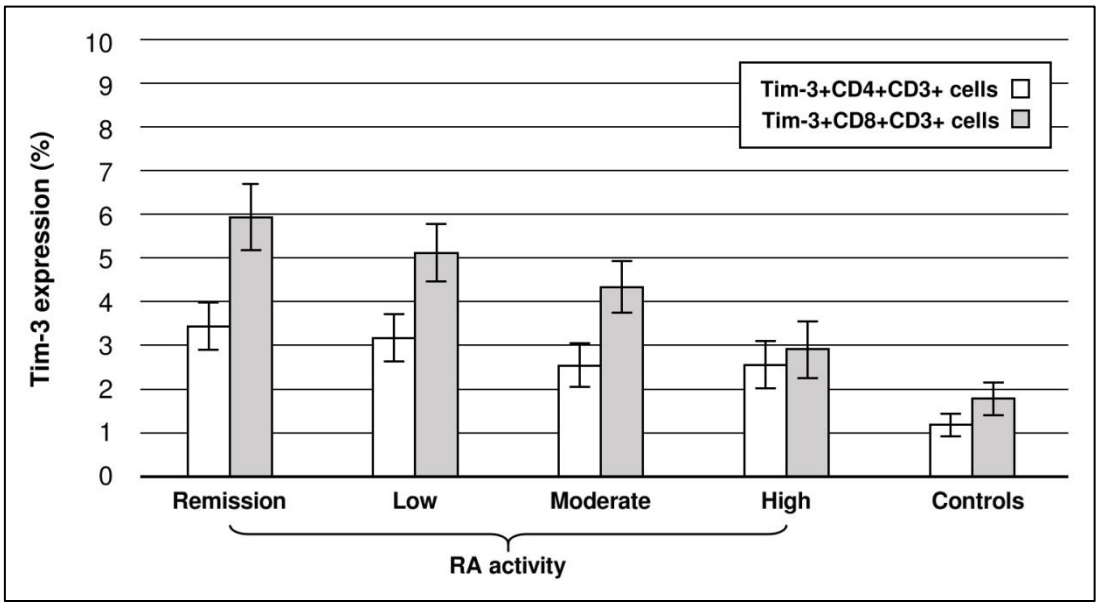

Fig. 2: Tim-3 percentage expression on peripheral blood CD4+CD3+ and CD8+CD3+ cells among all studied groups.

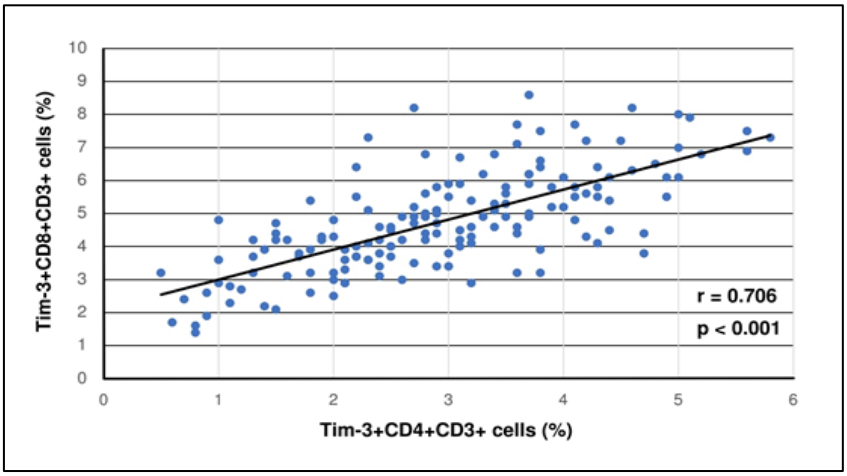

Fig. 3: Scatter plot shows the correlation between the percentages of patients' peripheral blood Tim-3+CD4+CD3+ and Tim-3+CD8+ CD3+ cells.

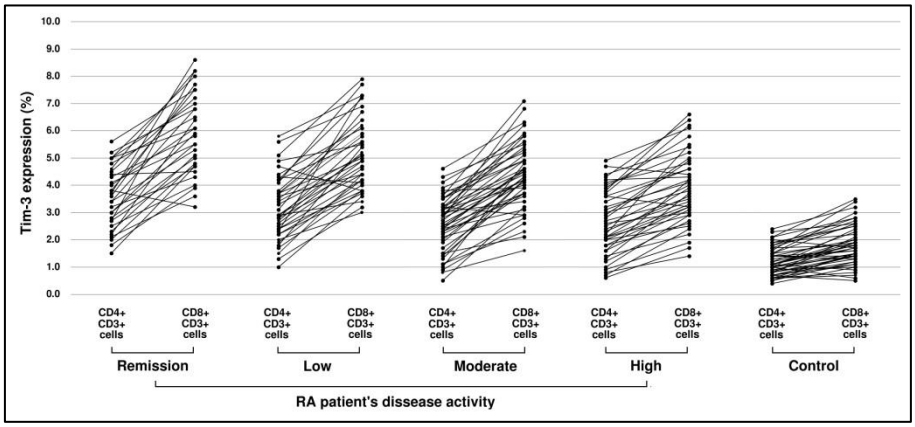

Fig. 4: Paired Tim-3 expression percentage on peripheral blood CD4+CD3+ and CD8+CD3+ cells among all studied groups.

As shown in figure 5, moderate correlations were found between DAS28 and both peripheral Tim-3+CD4+CD3+ cells (r = –0.425, p < 0.001; Fig. 5A) and peripheral Tim-3+CD8+CD3+ cells (r = –0.597, p < 0.001; Figure 5B) percentages.

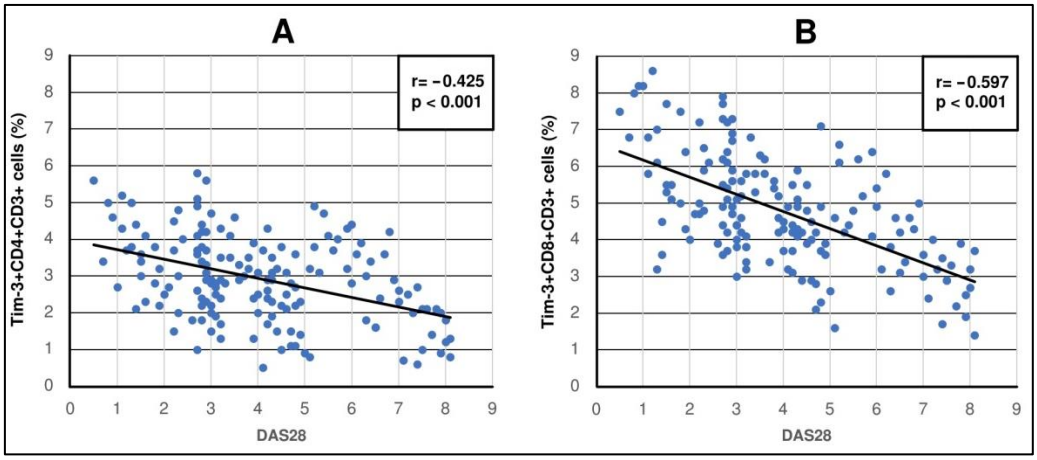

Fig. 5: Two scatter plots show the correlation between DAS28 and Tim-3+CD4+CD3+ cells (A) and between DAS28 and Tim-3+CD8+CD3+ cells (B).

Table (1) also show the serum anti-CCP levels for all disease activity groups. Using the ANOVA test, a highly significant statistical difference in serum anti-CCP levels was found among these groups. Serum anti-CCP levels showed a moderate negative correlation with Tim-3+CD4+CD3+ cells percentage (r = –0.565, p < 0.001; Figure 6A) and a strong negative correlation with Tim-3+CD8+CD3+ cells percentage (r = –0.677, p < 0.001; Figure 6B).

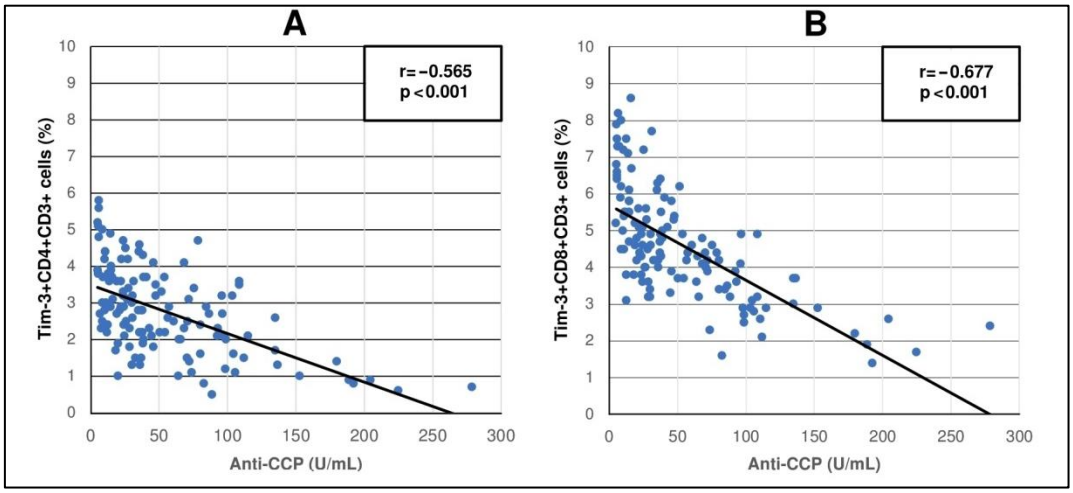

Fig. 6: Two scatter plots show the correlation between serum anti-CCP levels and Tim-3+CD4+CD3+ cells percentage (A) and between serum anti-CCP levels and Tim-3+CD8+CD3+ cells percentage (B) in anti-CCP positive patients.

DISCUSSION

RA is a chronic systemic inflammatory disease of unclear etiology. Managing RA includes the assessment of disease activity. Several compound indices were developed to assess clinical disease activity like DAS28 [15] and only a few biomarkers are used in the diagnosis and follow-up of RA [16]

The regulation of immune responses comprises several mechanisms whose dysregulation may lead to the development of autoimmune diseases [17]. Signaling via immune checkpoint or inhibitory receptors is one of these mechanisms and is responsible for the regulation of immune response and tolerance [18]. Tim-3 plays a critical role in downregulating immune responses and therefore may affect the development of RA [19] In this study, we investigated the expression of Tim-3 in peripheral blood and found a significant increase in Tim-3 expression on CD4+ and CD8+ T cells in RA cases. The upregulation of Tim-3 was negatively correlated with the severity of RA. This finding is consistent with the finding of Koohini et al. [20] and Skejo et al. [21] as they reported a significantly higher Tim-3+/PD-1+/CD4+ T cells in patients with RA compared to that in controls, and similarly, the percentage of Tim-3+/PD-1+/CD4+ T cells were also revealed an inverse correlation with DAS28. Moreover, Skejø et al. reported that Tim-3 was upregulated on memory-prone T cells and that soluble Tim-3 plasma levels are elevated and correlate with DAS28, CRP, and radiographic progression.

Also, our findings were consistent with Li et al. who demonstrated an increased expression of Tim-3 on the peripheral CD4+, CD8+, NK T cells, and monocytes from RA patients compared to those of healthy controls. They also found an inverse correlation with (DAS28). Moreover, they found a higher expression of Tim-3 on $CD4^+$ and $CD8^+$ T cells in synovial fluid than in peripheral blood that directly reflects the inflammatory status of RA and ensures the critical roles in both systemic and local immune systems in RA. He also concluded that the increased Tim-3 expression on CD4+ T and CD8+ T cells might be due to the activation of T-cells during the process of RA, which induces Tim-3 upregulation, which in turn reduces immune reaction, to a certain extent, and protects tissue injury from autoimmune responses. This further illustrates that Tim-3 might be strongly correlated with disease activity and might be engaged in the RA prognosis [22].

Also, our findings were consistent with Matsumoto al. who studied TIM-3 by enzyme-linked immunosorbent assay methods and found TIM-3 elevated in RA patients compared with those in healthy subjects. [23]

In contrast, Lee and colleagues found insignificant differences in Tim-3 mRNA expression between PBMCs from RA patients and those from healthy controls. They also found that Tim-3 mRNA expression

in the synovial tissue of RA patients was significantly higher than in osteoarthritis patients. Besides, Tim-3 mRNA expression in peripheral blood PBMCs of RA patients was negatively correlated with the DAS28 [22]. Moreover, Lee et al. reported that Tim-3 mRNA expression was correlated with higher Gal-9 mRNA levels in PBMCs of RA patients and this was significantly higher in patients with low disease activity compared to those with moderate to high disease activity. Besides, Gal-9 mRNA expression in PBMCs of RA patients was positively correlated with forkhead box P3 (FoxP3) mRNA expression [24].

The increased expression of Tim-3 may be due to the autoregulation mechanisms to control the immunopathological events, as the upregulation of Tim-3 expression was associated with the proliferation, apoptosis, and functional failure of the lymphocytes, which may further initiate the decrease of lymphocytes .[11]The observation of an increase in Tim-3 expression in CD4+ and CD8+ cells provides evidence for an increased T cell exhausted phenotype in RA patients that modulated regulation of the immune response to avoid Treg control [20] and this may, therefore, be both a therapeutic target and prognostic biomarker for the disease activity.

CONCLUSION

Tim-3 upregulated expression on peripheral blood CD4+ and CD8+ T Cells was negatively correlated with disease progression. So, this immune checkpoint receptor; Tim-3 could play a crucial role in regulating T cells' immune responses. Tim-3 is also a useful biomarker in determining disease activity, progression, and represents an important target for immune checkpoint inhibitors intervention in RA.

Conflict of interest

The authors have no conflicts of interest with the content of this paper.

Data availability statement

The authors confirm that all data supporting the findings of this study are available within the article, its supplementary material, and upon reasonable request.

Funding statement

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