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MicroRNAs Targeting Programmed Cell Death Protein 1 (PD-1) Promote Natural Killer Cell Exhaustion in Rheumatoid Arthritis

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ABSTRACT

Natural killer (NK) cells play a role in the pathogenesis of rheumatoid arthritis (RA). Upregulated levels of programmed cell death protein 1 (PD-1) is a sign of exhausted NK cells that could be regulated by microRNAs (miRNAs). In this investigation, we determined PD-1 expression on NK cells (as a representation of NK cell exhaustion) in RA patients and evaluated if miRNAs are involved in the modulation of PD-1 expression in NK cells.

Peripheral blood specimens were obtained from 40 RA patients and 20 healthy subjects. NK cells were isolated by negative selection from a pool of peripheral blood mononuclear cells. The frequency of PD-1–expressing NK cells and the expression of PD-1 on NK cells were analyzed by flow cytometry. Real-time PCR was used to measure the expression levels of PD-1 mRNA and miRNAs in the NK cells.

The percentage of the PD-1–expressing NK cells and Mean fluorescence intensity (MFI) of PD-1 expression on the NK cells were significantly higher in the RA cases compared to the controls. The mRNA expression of PD-1 was significantly upregulated in NK cells from RA patients compared to healthy subjects. The expression levels of miR-28, miR-138, and miR-4717 were significantly downregulated in the NK cells from RA patients compared to the healthy group.

In RA, miRNAs probably regulate the NK cell exhaustion process through driving PD-1 expression.

Keywords: MicroRNA; Natural killer cell; NK cell exhaustion; Programmed cell death protein 1; Rheumatoid arthritis

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INTRODUCTION

Rheumatoid arthritis (RA) is considered a chronic inflammatory and autoimmune disease. About 1% of people worldwide suffer from this disease.[1,2] Pathoetiological mechanisms of RA underlie the interactivity among various contributing factors like genetic, epigenetic, and environment.[3] Additionally, the similarity between the human leukocyte antigen (HLA)-DRB1 molecules of individuals and antigens from various germ sources, like bacteria from the digestive system and a number of viruses, is regarded as a partial explanation for immune tolerance breaks and autoimmune responses in RA. Immune system players, like dendritic cells, natural killer (NK) cells, macrophages, and different subtypes of lymphocytes, have been reported to infiltrate the synovial membrane in RA subjects. The inflammatory milieu developed by immune cells leads to osteoclast differentiation, fibroblast activation, and synovial remodeling.[4-6]

Immune cell exhaustion illustrates a dysfunctional state of immune cells, generally under chronic stimulation of immune cells in cancer and chronic inflammatory diseases.[7] In autoimmune disorders, the development of tumor cells, chronic infections, and inflammatory conditions generally lead to the exhaustion of innate immune cells like NK cells, thus limiting the antitumor and inflammatory capacity of NK cells.[8] Several mechanisms have been suggested for NK cell exhaustion. Programmed cell death protein 1 (PD-1 [CD279]) is an inhibitory immune checkpoint receptor induced on activated T and B cells. PD-1 engagement by the PD-L1 (B7-H1) and PD-L2 (B7-DC), which are expressed on antigen-presenting cells (APCs), infected cells, and cancerous cells, controls peripheral tolerance.[9] Other than T and B cells, PD-1 is also expressed in innate immune cells, including natural killer T (NKT) cells, NK cells, and type 2 innate lymphoid cells.[10] The functional and phenotypic features of PD-1–expressing NK cells have not been fully described. Regardless of their phenotype, higher PD-1 expression in NK cells leads to functional exhaustion, showing a decreased ability to proliferate, impaired cytotoxicity, and poor cytokine generation compared to the PD-1–negative NK cells.[11,12] Therefore, when their cytotoxic function and cytokine-producing capacity are repressed, the phenotypically exhausted NK cells (expressing exhaustion markers) might also be functionally exhausted.

There are controversial roles for NK cells during RA pathogenesis; while some studies attribute a protective role to NK cells during RA, others have reported the pathogenic function of these cells during inflammatory arthritis. Via releasing interferon (IFN)-γ, NK cells have been able to hinder the differentiation of $CD4^+$ T cells into the pathogenic helper T (Th) 17 cells and subsequent osteoclastogenesis in patients with RA.[13-15] On the other hand, research shows that synovial NK cells of RA patients can induce macrophages by IFN-γ to produce tumor necrosis factor (TNF)-α,[16, 17] which in turn might stimulate macrophages and synovial fibroblasts to generate proinflammatory mediators and induce osteoclastogenesis.[18] Altered expression of immune regulatory molecules of NK cells has been observed in RA patients that affect NK cell function in promoting or suppressing inflammation and other disease complications.[19] However, the functionally exhausted NK cells have not been investigated in depth in RA.

MicroRNAs (miRNAs) are endogenous, small, noncoding RNAs that bind to the complementary sequences in the 3′-untranslate regions (3′-UTRs) of target mRNAs that regulate gene expression posttranscriptionally.[20,21] Their altered expression and function have been linked to various diseases, such as cancer and systemic autoimmune disorders, including RA.[22,23] They can modulate different processes in RA and might be associated with disease outcomes.[24] Emerging data has revealed that some miRNAs can regulate the surface expression of immune checkpoint receptors like PD-1 on NK cells or corresponding ligands on cancer cells.[25] With respect to miRNAs modulating PD-1 expression, it has been reported that miR-28,[26] miR138,[27] and miR-4717[28] target PD-1 mRNA, leading to the exhaustion of immune cells. However, there is no evidence of PD-1 expression regulation by miRNAs in NK cells from RA patients.

The gap remains regarding the involvement of NK cell exhaustion in RA pathogenesis and the pathways of PD-1 expression regulation by miRNAs in NK cells. Regarding the evidence mentioned above, we evaluated the expression of PD-1 (as a marker of NK cell exhaustion) in NK cells of RA patients. Additionally, the expression levels of miR-28, miR138, and miR-4717 in NK cells and their potential regulatory roles in modulating the expression of PD-1 in NK cells were assessed to decipher their role in RA pathogenesis.

MATERIALS AND METHODS

Human Subjects

According to the criteria described by the American College of Rheumatology (ACR) 2010,[29] a total of 40 RA patients referred to the Rheumatology Clinics of Emam Ali and Shahid Rajaei Hospitals (affiliated with Alborz University of Medical Sciences) and 20 sex- and age-matched healthy individuals were included in this study. RA subjects were all new cases, most of whom were under routine treatment with corticosteroids or nonsteroidal anti-inflammatory drugs (NSAIDs). None of the subjects were was treated with disease-modifying antirheumatic drugs (DMARDs) or biologics (like adalimumab and etanercept). The disease activity of RA subjects was measured by different indices, including disease activity score-28 (DAS28), simplified disease activity index (SDAI), 28 tender joint count, and 28 swollen joint count. The included patients had mild disease severity. RA patients with a history of cancer and other autoimmune diseases were ruled out from this study. Informed consent forms were signed by all participants. The demographic specifications and laboratory findings of the study subjects are summarized in Table 1.

Blood Sampling and PBMC Isolation

Fresh intravenous blood (10 mL) was taken from each person, poured into tubes containing heparin, and mixed with an equal volume of phosphate-buffered saline (PBS). Peripheral blood mononuclear cells (PBMCs) were separated using a Ficoll-Hypaque density gradient medium (Lymphodex, inno-train, Germany) in a ratio of 2:1 in a falcon (2 blood:1 Ficoll) and centrifuged at 1500 rpm for 15 minutes at 20°C. The isolated PBMCs were then washed twice with PBS. Being assessed by trypan blue staining and observed by light microscopy (Nikon E100 Binocular Microscope, Japan), it was observed that the viability of isolated PBMCs was>98%.

Table 1. Baseline data and clinical presentations of the study subjects.

Characteristics	RA patients (n=40)	Controls (n=20)
Gender, Female/Male; n (%)	26 (65%)/14 (35%)	13 (65%)/7 (35%)
Smoker/ Non-smoker; n (%)	17 (42.5%)/23 (57.5%)	8 (40%)/12 (60%)
Age in Years (Mean±SD)	45.81±12.93	42.25±12.32
Disease Duration in Years (Mean±SD)	10.7±4.9	-
DAS28	3.63±1.37	-
SDAI	52.23±24.56	-
28 Tender Joint Count	3.31±2.27	-
28 Swollen Joint Count	2.96±2.28	-
ESR (mm/h)	23.59±16.78	-
CRP (mg/L)	6.53±3.46	-
RF (IU/mL)	34.13±9.31	-
Anti-CCP (IU/mL)	39.43±13.68	-
C3 Complement*; n (%)	35 (87.5%)	-
C4 Complement*; n (%)	34 (85%)	-
Corticosteroid Use; n (%)	27 (67.5%)	-
NSAID use; n (%)	29 (72.5%)	-

RA, rheumatoid arthritis; DAS28, disease activity score; SDAI, simplified disease activity index; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; RF, rheumatoid factor; Anti-CCP, anticyclic citrullinated peptide; SD, standard deviation; NSAID; nonsteroidal anti-inflammatory drug

* The number of subjects with higher levels of C3 and C4 complement levels from the normal range were included.

NK Cell Isolation

Human NK cells were separated from PBMCs by negative (depletion of non-NK cells) selection of $CD16^{+}CD56^{+}$ NK cells via magnetic-activated cell sorter (MACS) columns (Miltenyi Biotec, San Diego, CA, UAS) based on the company's guidelines. Afterward, NK cells were stained with anti-CD56 (BioLegend, San Diego, CA, USA). The purity of NK

cells was determined by flow cytometry, which was>92% (Supplementary Figure 1). The viability of NK cells was>90%, as determined by trypan blue staining and direct observation by light microscopy (Nikon E100 Binocular Microscope, Japan).

Flow Cytometry Analysis

Cell staining was performed with fluorochrome-labeled monoclonal antibodies. For this purpose, 500 000 cells were incubated with anti-PD-1-APC, anti-CD-3-FITC, and anti-CD-56 PerCP/Cy5.5 (BioLegend, San Diego, CA, USA) at room temperature for 20 minutes. Stained cells were assessed by flow cytometry using CyFlow Space flow cytometer (Sysmex, Kobe, Japan). The lymphocyte region was first gated for NK cells, and then the frequency of PD-1$^+$ cells was determined. Data analysis was conducted by FlowJo Software (v10). The mean fluorescence intensity (MFI) of PD-1 expression in NK cells was analyzed.

RNA Extraction, cDNA Synthesis, and qRT-PCR

Using the TRIzol reagent (Invitrogen, Carlsbad, CA, UAS), total cellular RNA was isolated from NK cells. The number of RNA samples and their purity were measured by NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA). Synthesis of the first strand complementary DNA (cDNA) was performed using miScript II RT Kit (Qiagen, Germany) and Transcriptor first strand cDNA Synthesis Kit (Roche, Germany) for miRNA and PD-1 mRNA expression analysis, respectively.

Real-time PCR measurement of PD-1 mRNA levels in NK cells from study participants was conducted through StepOnePlus real-time PCR device (Applied Biosystems, Foster City, CA, USA) and RealQ Plus SYBR Green Master Mix (Ampliqon, Denmark). The primer sequence for real-time PCR mRNA expression evaluation of PD-1 was forward: 5′-CGTGGCCTATCCACTCCTCA-3′ and reverse: 5′-ATCCCTTGTCCCAGCCACTC-3′. The ingredients of the reaction mixture in each PCR tube were 12 µL master mix, 10 µL cDNA, 0.5 µL primers (each forward and reverse), and RNase-free water for a final volume of 30 µl. The PCR thermocycling was followed through the holding step of 95°C for 15 minutes, 40 cycles of amplification at 90°C for 20 seconds, 63°C for 45 seconds, and then 70°C for 60 seconds.

Quantification of the expression levels of miR-28, miR-138, and miR-4717 was performed by StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the miScript SYBR Green PCR Kit (Qiagen, Germany). The reaction mixtures comprised 10 µL master mix, 8 µL cDNA, 1 µl primers (each forward and reverse), and RNase-free water to a final volume of 30 µL. The real-time PCR thermocycling regulations were as follows: initial 95°C for 15 minutes; 45 cycles of 95°C for 15 seconds; 60°C for 35 seconds; and 65°C for 45 seconds.

In order to compute the relative transcript levels of PD-1 and miRNA, the C_t values were normalized to the corresponding housekeeping genes, namely, β2-microglobulin and RUN6, respectively. The comparative C_t approach was used to measure relative miRNA and PD-1 expression levels developed by Livak and Schmittgen before.[30]

Statistical Analysis

Statistical analysis and graphing were performed using GraphPad Prism Version 7 (GraphPad Software Inc. San Diego, CA, UAS). Normal distribution of scale data was analyzed by the Shapiro-Wilk test. A two-tailed unpaired Student's *t*-test was used to compare means between patients and controls. To calculate the correlation coefficients, we used the Pearson correlation coefficient test. The threshold for statistical significance was set at p values<0.05.

RESULTS

Baseline Data

The baseline data and demographic characterization of study subjects are listed in Table 1. The patient group was comprised of 26 (65%) females and 14 (35%) males, while the healthy control group included 13 (65%) females and 7 (35%) males. The frequency of smoking subjects in the RA and control groups were 17 (42.5%) and 8 (40%), respectively. Mean age of the RA and control groups were 45.81±12.93 and 42.25±12.32 years, respectively. The disease duration of the RA patients was 10.7±4.9 years. Regarding disease severity, scores for disease activity score of 28 joints (DAS28), simplified disease activity index (SDAI), 28 tender joint count, and 28 swollen joint count were 3.63±1.37, 52.23±24.56, 3.31±2.27, and 2.96±2.28, respectively. As for inflammatory components, levels of erythrocyte sedimentation rate, C-reactive protein (CRP), rheumatoid factor, and anti-cyclic citrullinated peptide were 23.59±16.78 mm/h, 6.53±3.46 mg/L, 34.13±9.31

IU/mL, and 39.43±13.68 IU/mL, respectively. Higher levels of C3 and C4 complement components were detected in 35 (87.5%) and 34 (85%) RA cases. With respect to the therapeutic regimen, 27 (67.5%) RA cases were using corticosteroids, while 29 (72.5%) subjects were using NSAIDs. None of the RA subjects were under treatment with biologics.

NK Cell Frequency

The results indicated that the total number of NK cells (among PBMCs) was significantly higher in the circulation of RA cases compared to the control group (p=0.004, Figure 1A). Figure 1B shows the percentage of PD-1–expressing NK cells among the PBMCs isolated from study subjects. Analysis indicated that the percentage of PD-1–expressing NK cells was significantly higher in RA cases compared to controls (p=0.017). Additionally, the MFI of PD-1 expression on NK cells from RA subjects was significantly higher compared to the control group (p=0.008, Figure 1C). Figure 1D demonstrates the histograms of flow cytometric analysis of PD-1–expressing NK cells and the MFI of PD-1 expression on the NK cells from an RA case and a healthy control as a sample.

Expression Level of PD-1 and miRNAs

Real-time PCR results demonstrated that the mRNA expression of PD-1 was significantly upregulated in the NK cells from RA cases compared to healthy subjects (fold change=1.90, p=0.0004, Figure 2A). Additionally, the expression levels of miR-28 (fold change=0.77, p=0.001, Figure 2B), miR-138 (fold change=0.79, p=0.022, Figure 2C), and miR-4717 (fold change=0.80, p=0.048, Figure 2D) were significantly downregulated in NK cells of RA patients in comparison to the healthy group.

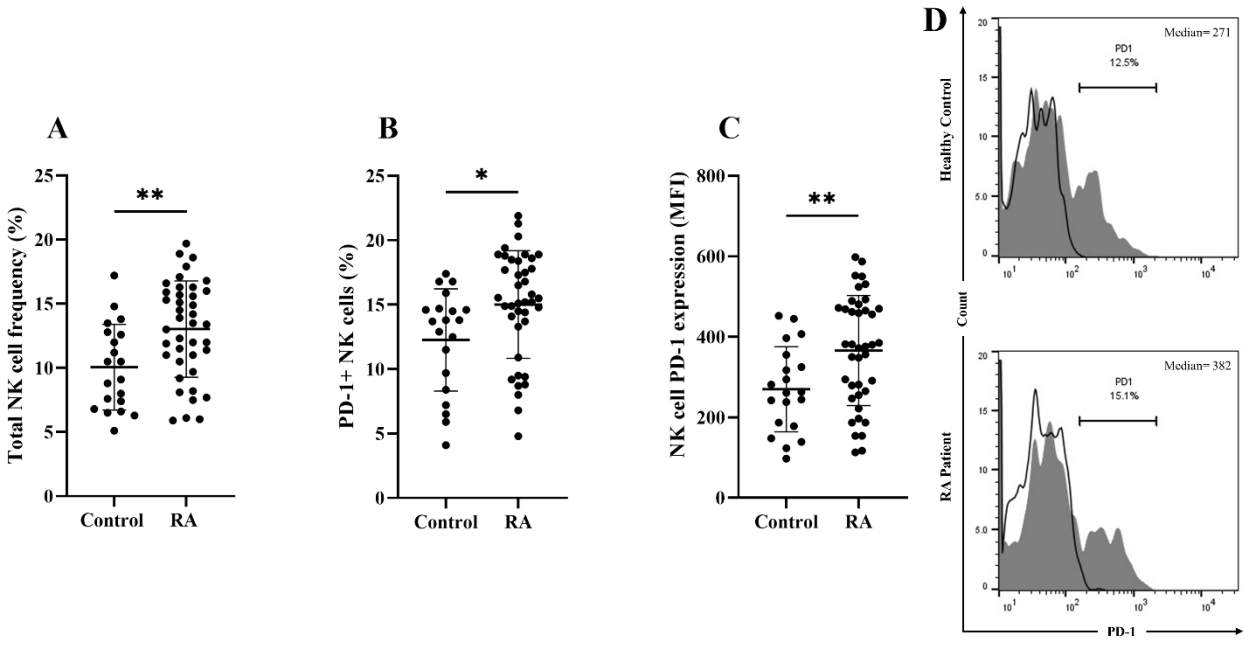

Figure 1. Peripheral blood mononuclear cells (PBMCs) were isolated from the whole blood samples of Rheumatoid arthritis (RA) patients and healthy controls and evaluated for NK cells by flow cytometry. Bar graphs show the total frequency of Natural killer (NK) cells (A), percentage of PD-1–expressing NK cells (B), and Mean fluorescence intensity (MFI) of programmed cell death protein 1 (PD-1) expression on Natural killer (NK) cells in the Peripheral blood mononuclear cells (PBMCs) from RA cases and healthy controls (C). The histograms of flow cytometric analysis of PD-1 expressing NK cells and Mean fluorescence intensity (MFI) of PD-1 expression on the NK cells from a RA case and a healthy control as a sample are shown (D). Independent sample t-test was used to compare the groups (*p<0.05, **p<0.01).

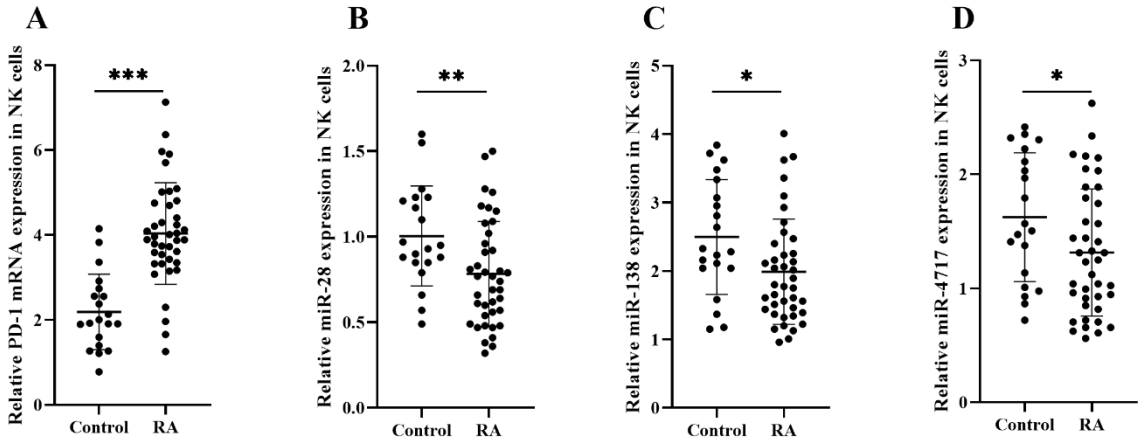

Figure 2. Natural killer (NK) cells were isolated from the Peripheral blood mononuclear cells (PBMC) pool of blood samples. Real-time PCR was used to determine the mRNA expression of programmed cell death protein 1 (PD-1) and the expression levels of microRNAs (miRNAs) in NK cells from RA patients and healthy controls. The graphs show the mRNA expression of PD-1 (A) and expression levels of miR-28 (B), miR-138 (C), and miR-4717 (D) in the PD-1–expressing NK cells from RA cases and healthy controls. Independent sample t-test was used to compare the groups (*p<0.05, **p<0.01, ***p<0.001).

Correlation Analysis

PD-1 mRNA expression in NK cells was significantly correlated with the expression levels of miR-28 (r=−0.53, p=0.0004, Figure 3A), miR-138 (r=−0.365, p=0.02, Figure 3B), and miR-4717 (r=−0.409, p=0.008, Figure 3C).

It was observed that the MFI of PD-1 expression on NK cells had a significantly negative correlation with the expression levels of miR-28 (r=−0.431, p=0.005, Figure 3D), miR-138 (r=−0.334, p=0.034, Figure 3E), and miR-4717 (r=−0.376, p=0.016, Figure 3F).

Correlation analysis of molecular data with the inflammatory and clinical presentations of RA cases also indicated a statistically positive correlation (r=0.32, p=0.04) between MFI of PD-1 expression on NK cells and serum CRP levels in RA subjects (Table 2).

Table 2. Correlation analysis between the molecular and clinical data of RA patients.

Clinical data Molecular data	CRP *r* (*p*)	ESR *r* (*p*)	RF *r* (*p*)	Anti-CCP *r* (*p*)	TJC28 *r* (*p*)	SJC28 *r* (*p*)	DAS28 *r* (*p*)	SDAI *r* (*p*)
PD-1 mRNA	0.19(0.23)	0.21(0.18)	0.16(0.3)	0.14(0.39)	0.08(0.64)	0.09(0.6)	0.16(0.34)	0.14(0.38)
PD-1 MFI	**0.32(0.04)**	0.27(0.09)	0.19(0.23)	0.2(0.22)	0.18(0.26)	-0.19(0.24)	0.29(0.07)	-0.06(0.71)
miR-28	-0.07(0.68)	-0.03(0.87)	-0.1(0.55)	0.02(0.89)	-0.19(0.23)	-0.16(0.32)	-0.26(0.1)	-0.04(0.78)
miR-138	-0.16(0.32)	-0.08(0.62)	-0.09(0.58)	-0.04(0.8)	0.03(0.85)	0.05(0.78)	-0.21(0.2)	0.03(0.83)
miR-4717	-0.06(0.7)	-0.05(0.72)	0.02(0.88)	-0.11(0.49)	-0.03(0.87)	0.09(0.59)	-0.08(0.63)	-0.07(0.66)

CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; RF, rheumatoid factor; Anti-CCP, anti-cyclic citrullinated peptide; TJC28, 28 tender joint count; SJC28, 28 swollen joint count; DAS28, disease activity score; SDAI, simplified disease activity index; MFI, Mean fluorescence intensity

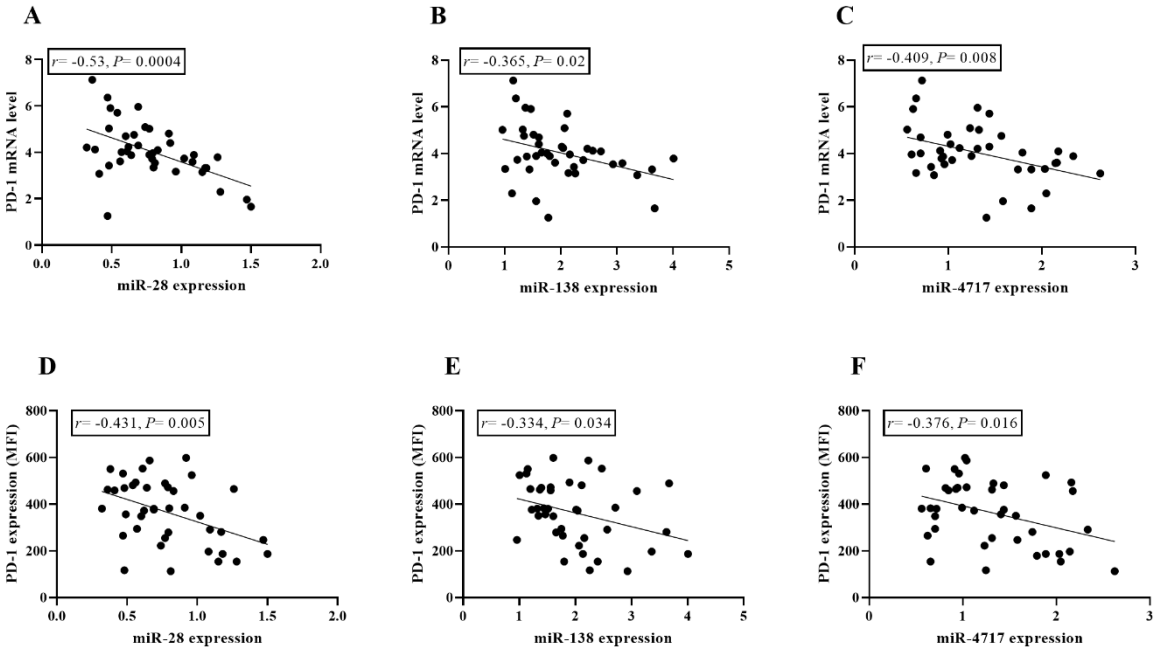

Figure 3. Correlation analysis of the expression of miR-28 (A), miR-138 (B), and miR-4717 (C) and PD-1 mRNA expression in Natural killer (NK) cells, as well as the expression of miR-28 (D), miR-138 (E), and miR-4717 (F) and Mean fluorescence intensity (MFI) of programmed cell death protein 1 (PD-1) expression on the PD-1–expressing NK cells from Rheumatoid arthritis (RA) patients. Correlation analysis was performed by calculating the Pearson correlation coefficient.

DISCUSSION

Infiltration of NK cells has been demonstrated in the synovial tissues of RA cases. NK cells can stimulate B cells, induce maturation or death of dendritic cells, trigger osteoclast differentiation, and promote cytokine production by fibroblasts and monocytes in RA.[4] As a result, NK cells play paramount roles in RA pathogenesis. In this study, we tried to evaluate whether NK cells are functionally exhausted in RA patients. We also assessed the miRNA function in orchestrating PD-1 expression on NK cells in these patients. Our findings confirmed NK cell exhaustion in RA patients, typified by the upregulation of PD-1 expression on NK cells. Furthermore, miRNAs targeting PD-1 were detected to be downmodulated in NK cells of RA subjects and inversely correlated with PD-1 levels in mRNA level and surface expression on NK cells.

The total number of NK cells is higher in the circulation of RA patients.[31,32] Despite this, NK cells in the circulation of RA subjects demonstrate impaired effector functions, illustrated by decreased IFN-γ secretion and diminished cytotoxic properties. This might stem from the downmodulation of activating receptors like CD16[33] or overexpression of inhibitory receptors like CD56[34] and NKG2A.[35] Furthermore, a study indicated that the number of perforin$^+$ NK cells was reduced in the circulation of RA cases compared with controls, implying an impaired effector function of NK cells through reduced cytolytic activity.[36] Our study indicated a higher number of NK cells in the circulation of RA cases than in healthy subjects. On the other hand, the number of PD-1–expressing NK cells was higher in the RA cases, which coincided with upregulated levels of PD-1 expression in NK cells from RA cases. Therefore, NK cells from RA cases are functionally impaired due to NK cell exhaustion through upregulated levels of PD-1 expression in these cells.

A number of reports have shown that synovial NK cells play a pathogenic function during the RA disease course. Coculturing synovial NK cells from RA cases with monocytes led to the differentiation of monocytes into pathogenic osteoclasts.[37] Additionally, studies show that the number of NK cells in the synovium of RA patients is higher.[32,38] Synovial NK cells present an upregulated level of chemokine receptors that facilitates

their recruitment to the involved joints.[31] NK cells from RA synovial tissue are mostly $CD69^{+}NKp44^{+}$, demonstrating an activated phenotype while expressing lower amounts of perforin.[32,38] Such NK cells present upregulated surface expression of CD94-NKG2A[39] (as inhibitory molecules), which suppress the production of IFN-γ and TNF-α cytokines by NK cells.[35,38] Considering decreased cytotoxic function and a suppressed ability to produce IFN-γ by synovial NK cells in RA patients,[32] these NK cells might be involved in the escalation of the joint inflammation, as reduced IFN-γ permits the differentiation of $CD4^{+}$ T cells to pathogenic Th17 subset.[40] Which is invariably blamed as a major culprit of RA pathogenesis. Even though the mechanism of NK cell exhaustion development is not clear in RA, exhausted NK cells contribute to inflammation and exacerbate disease outcomes. Our study sheds further light on the pathogenic role of functionally exhausted NK cells in RA cases. We observed that NK cells expressed higher amounts of PD-1 molecule, probably leading to the suppression of NK cell function.

Additionally, there was a significant correlation between PD-1 levels in NK cells and CRP. Although not statistically significant, there was a positive correlation between PD-1 expression on NK cells and disease severity in RA patients. All these demonstrate that functionally impaired or exhausted NK cells in RA patients are associated with the perpetuation of inflammation and promote disease outcomes.

Another goal of this research was to evaluate the mechanisms of NK cell exhaustion by regulating PD-1 expression in these cells in RA patients. Li et al. reported that miR-28 could target PD-1 in T cells, resulting in a functionally exhausted T cell. Furthermore, miR-28 inhibition was associated with the upregulation of PD1, TIM3, and BTLA expression in the exhausted T cells, which were collectively involved in T cell suppression. miR-28–mediated exhaustion of T cells was associated with impaired function of these cells, as illustrated by impaired generation of IL-2 and TNF-α by these exhausted T cells.[41] miR-138 was also observed to target both PD-1 and CTLA-4 in $CD4^{+}$ T cells from glioma patients.[27] By interacting with the 3′-UTR of PD-1 mRNA, it was revealed that miR-4717 could modulate the expression of PD-1.[42] Here, we measured the expression of miR-28, miR-138, and miR-4717 in NK cells from RA cases and healthy controls. It was observed that transcript levels of all these miRNAs were significantly downregulated in NK cells from RA patients compared to healthy subjects. Furthermore, there was a significant inverse correlation between transcript levels of miRNAs and both the MFI of PD-1 in NK cells and mRNA expression of PD-1 in NK cells. This can imply other regulatory mechanisms of PD-1 expression on NK cells that collectively modulate NK-cell behavior and the disease outcome of RA patients.

There are a number of limitations in this study. First, the number of study subjects were relatively low. Second, we did not analyze other molecules involved in NK cell exhaustion, such as NKG2A and TIM-3. Third, other molecular pathways involved in regulating the expression of PD-1 on NK cells were not assessed in this study. Fourth, we were unable to investigate NK cells in the synovial fluid of RA cases, otherwise it could yield a comprehensive understanding of NK-cell exhaustion regarding PD-1 overexpression in RA. Fifth, even though we isolated $CD16^{+}CD56^{+}$ cells, NK cells were not assessed based on the CD56 expression pattern. $CD56^{dim}CD16^{+}$ NK cells constitute approximately 90% of the mature NK cell population in circulation. However, $CD56^{bright}CD16^{+}$ NK cells, which show more cytotoxic function, can stimulate fibroblast-like synoviocytes (FSL) in the synovium of RA patients and promote inflammatory pathogenic conditions.[19] Therefore, exhausted NK cells, along with altered expression of inhibitory and activating receptors, might be modulated by the upregulation of the inhibitory CD56 receptor. Hence, we suggest that future investigations concentrate on the NK-cell subset (based on the CD56 expression pattern) and checkpoint molecules like PD-1.

In conclusion, this study asserts that the functionally exhausted NK cells in the circulation of RA subjects are mirrored through the upregulation of PD-1 expression on these cells. Exhaustive NK cells might be developed due to continuous stimulation of NK cells by an unknown self-antigen in RA subjects or because of a negative feedback response by the immune system to promote a self-tolerance state to diminish the inflammatory state and, hence, the disease burden. However, the functionally exhausted NK cells might contribute to the progression of inflammation, as these cells produce little IFN-γ, which prompts the development of pathogenic Th17 cells and inflammatory conditions in RA disease. Functionally exhausted NK cells in our study were associated with higher CRP levels (a general marker of inflammation) and higher disease severity scores in RA cases. Our investigations

also suggest that miR-28, miR-138, and miR-4717 constitute a part of the pathway toward regulating PD-1 in NK cells and the exhaustion of these cells. Above all, further investigations are warranted in order to unveil the pathologic and therapeutic implications of NK-cell exhaustion over the etiology and pathogenesis of RA.

STATEMENT OF ETHICS

This study was approved by the Ethics Committee of Tabriz University of Medical Sciences (IR.TBZMED.REC.1398.1298). All individuals voluntarily signed a written informed consent form. All methods were performed in accordance with relevant guidelines and regulations of Tabriz University of Medical Sciences.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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