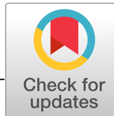
Received: 10 February 2020 | Revised: 12 June 2020 | Accepted: 23 June 2020
DOI: 10.1111/joor.13041



ORIGINAL ARTICLE

JOURNAL OF ORAL REHABILITATION
WILEY

Increased chemokine RANTES in synovial fluid and its role in early-stage degenerative temporomandibular joint disease

Shi-Yang Feng[1,2] | Jie Lei[1,2] | Hui-Min Chen[2,3] | Yi-Xiang Wang[2,4] | Adrian U-Jin Yap[1,5,6,7] | Kai-Yuan Fu[1,2]

[1]Center for TMD & Orofacial Pain, Peking University School & Hospital of Stomatology, Beijing, China
[2]National Clinical Research Center for Oral Diseases, Beijing, China
[3]Department of General Dentistry II, Peking University School & Hospital of Stomatology, Beijing, China
[4]Central Laboratory, Peking University School and Hospital of Stomatology, Beijing, China
[5]Department of Dentistry, Ng Teng Fong General Hospital, National University Health System, Singapore
[6]Faculty of Dentistry, National University of Singapore, Singapore
[7]Duke-NUS Medical School and National Dental Research Institute Singapore, National Dental Centre, SingHealth, Singapore

Correspondence
Kai-Yuan Fu and Jie Lei, Center for TMD & Orofacial Pain, Peking University School & Hospital of Stomatology, No. 22 Zhong Guan Cun South Ave, Beijing 100081, China.
Emails: kqkyfu@bjmu.edu.cn and leijierebecca1986@163.com

Funding information
National Natural Science Foundation of China, Grant/Award Number: 81870788; Research Foundation of Peking University School and Hospital of Stomatology, Grant/Award Number: PKUSS20140204

Abstract

Background: Degenerative joint disease (DJD) of the temporomandibular joints (TMJs) in adolescents and young adults is closely associated with disc displacement without reduction (DDw/oR).

Objective: This study aimed to determine the pathogenesis of early-stage TMJ DJD induced by DDw/oR.

Methods: 31 female subjects aged 12-30 years were enrolled, comprising 12 patients with DDw/oR without DJD, 13 with DDw/oR and early-stage DJD, and 6 healthy volunteers. The synovial fluid samples of the subjects were screened for 27 inflammatory-related cytokines using multiple cytokine array. Significantly increased cytokines and a key regulator of osteoclastogenesis "receptor activator of nuclear factor-κB ligand" (RANKL) were further determined by sandwich immunoassay. These factors were also assessed for the possible pathophysiologic actions on RAW264.7 cell proliferation, migration, osteoclastogenesis and bone-resorbing activity using Cell Counting Kit-8, Transwell system, tartrate-resistant acid phosphatase staining and osteo assay plates.

Results: Macrophage-derived inflammatory protein-1 beta (MIP-1β) and regulated upon activation normal T cell expressed and secreted (RANTES) were found to vary significantly in relation to the controls. In contrast to an unchanged concentration of RANKL, a strong increase in the level of RANTES was detected in subjects with DDw/oR and early-stage DJD. MIP-1β concentrations were only elevated in subjects with DDw/oR without DJD. Functionally, both MIP-1β and RANTES could enhance macrophage migration in a concentration-dependent manner, while only RANTES exhibited a promoting effect on osteoclast formation and bone-resorbing activity.

Conclusions: Chemokine RANTES was significantly upregulated and might be a key regulator of osteoclastogenesis contributing to DDw/oR-induced early-stage TMJ DJD.

KEYWORDS
adolescent, arthritis, bone, inflammatory processes/inflammatory markers, remodelling, temporomandibular disorders

© 2020 John Wiley & Sons Ltd

1 | INTRODUCTION

Degenerative joint disease (DJD) of the temporomandibular joints (TMJs) is a significant subtype of TMJ disorders with prevalence ranging from 18% to 77% among patients with temporomandibular disorders (TMDs).[1] It is characterised by deterioration of articular tissue with concomitant osseous changes in the condyle and/or articular eminence.[2] Clinical manifestations of TMJ DJD include oro-facial pain, joint sounds and limited jaw movements.[3] TMJ DJD may interfere with normal condylar development, leading to mandibular asymmetry, retrognathia and anterior open bite if it occurs during adolescence.[4] The aetiology of TMJ DJD is complex and multifactorial in nature involving trauma, parafunction, occlusal instability, functional overloading and increased joint friction.[5,6] The TMJ disc offers lubrication and distributes functional as well as parafunctional loads over the condyle surface.[7] TMJ disc displacements, especially disc displacement without reduction (DDw/oR), appear to play an essential role in the onset and progression of DJD.[8] Displaced discs might interfere with condylar mobility and lead to overloading of the anterior surfaces of condyle.[9] Articular cartilage and subarticular bone destruction gradually occur over time.[10] Based on high-resolution cone-beam computed tomography (CBCT), our earlier study showed a close association between recent-onset DDw/oR and early-stage degenerative TMJ changes.[10] However, the molecular mechanisms underlying this phenomenon remain unclear.

Soluble mediators, for example cytokines, chemokines and growth factors, are thought to play significant roles in the pathogenesis of degenerative joint changes.[11] Previous studies showed that several inflammatory and immune mediators of TMJs can be found in synovial fluid.[12,13] Specifically, the levels of pro-inflammatory mediators including interleukin (IL)-1β, IL-6, IL-8, IL-17, IL-22, tumour necrosis factor (TNF)-α and interferon (IFN)-γ, which are associated with synovitis and connective tissue destruction, were raised in the synovial fluid of patients with TMDs.[13-16] Furthermore, IL-10, osteoprotegerin (OPG) and vascular endothelial growth factor (VEGF) found in the synovial fluid of TMJs could have an anti-inflammatory effect.[13] Although these findings indicate that several mediators may be involved in the pathogenesis of degenerative joint changes, the characterisation of mediators involved in the initiation and/or progression of TMJ DJD has not been widely performed.

As such, the aim of this study was to establish the key molecular mediators involved in early-stage TMJ DJD associated with DDw/oR via multiple cytokine array, enzyme-linked immunosorbent assay (ELISA) and electrochemiluminescence technique. More specifically, we verified and quantified C-C family-derived chemokines "macrophage-derived inflammatory protein-1 beta" (MIP-1β) and "regulated upon activation normal T cell expressed and secreted" (RANTES) in the synovial fluid of subjects with DDw/oR and early-stage TMJ DJD, and that of normal controls. The possible effects of the two chemokines on joint inflammation and bone resorption were also investigated.

2 | MATERIALS AND METHODS

2.1 | TMJ synovial fluid analysis

2.1.1 | Subject selection

The approval for the study was obtained from the Biomedical Institutional Review Board of the Peking University School and Hospital of Stomatology (PKUSSIRB-201947100), and written informed consents were attained from all participants.

Subject inclusion criteria were as follows: (i) female adolescents and young adults aged 12-30 years old; (ii) patients diagnosed with TMJ DDw/oR, in accordance with the Diagnostic Criteria for Temporomandibular Disorders (DC/TMD)[2] and confirmed by magnetic resonance imaging (MRI), who had current or prior TMJ locking with limited opening; (iii) onset of less than 12 months; and (iv) indication for arthrocentesis due to limited mouth opening and/or pain. Subject exclusion criteria were as follows: (i) history of prior TMD therapy; (ii) presence of TMD signs/symptoms in the contralateral or bilateral TMJs; (iii) presence of systematic joint diseases (eg rheumatoid arthritis); and (iv) manifestation of late-stage DJD based on CBCT such as deviation in form, extensive sclerosis, osteophyte formation and cyst-like lesions.[10,17-20] Clinical evaluation and data collection were carried out by a single TMD specialist, who was trained and calibrated in accordance with the DC/TMD training and calibration guidelines.

A total of 30 patients were screened in 3 months. Ultimately, 25 samples from 25 eligible patients were analysed, and the remaining 5 samples were rejected due to insufficient volume (less than 0.9 mL) or blood contamination. The patients were further divided into two subgroups: DDw/oR without DJD (n = 12) and DDw/oR with early-stage DJD (n = 13), based on CBCT assessment. Radiographic appearances of discontinuity of the articular cortex and/or surface erosion/destruction of the condyle were considered as early-stage degenerative changes (Figure 1).[10,18-20] Six healthy volunteers with no history of TMD, regular jaw movements/functioning and normal TMJ appearance on CBCT were recruited as normal controls.

2.1.2 | Sample collection

Synovial fluid samples were collected during arthrocentesis of the TMJs. Local anaesthetic (0.5-1.0 mL 2% lidocaine) was first injected into the preauricular area using a 21-gauge needle and syringe. The needle was then adjusted and inserted anteriorly, superiorly and medially into the superior joint space. 1.0 mL of saline solution was injected into the upper compartment of the TMJ and mixed with synovial fluid three times by repeated aspiration and reinjection. Almost all lavage fluid (1.0 mL) was retrieved (estimated recovery rate of 90%-100%), and the few samples that had less than 0.9 mL of collected fluid or were contaminated with blood were rejected. As for healthy volunteers, arthrocentesis was not performed after synovial fluid sample collection. All samples

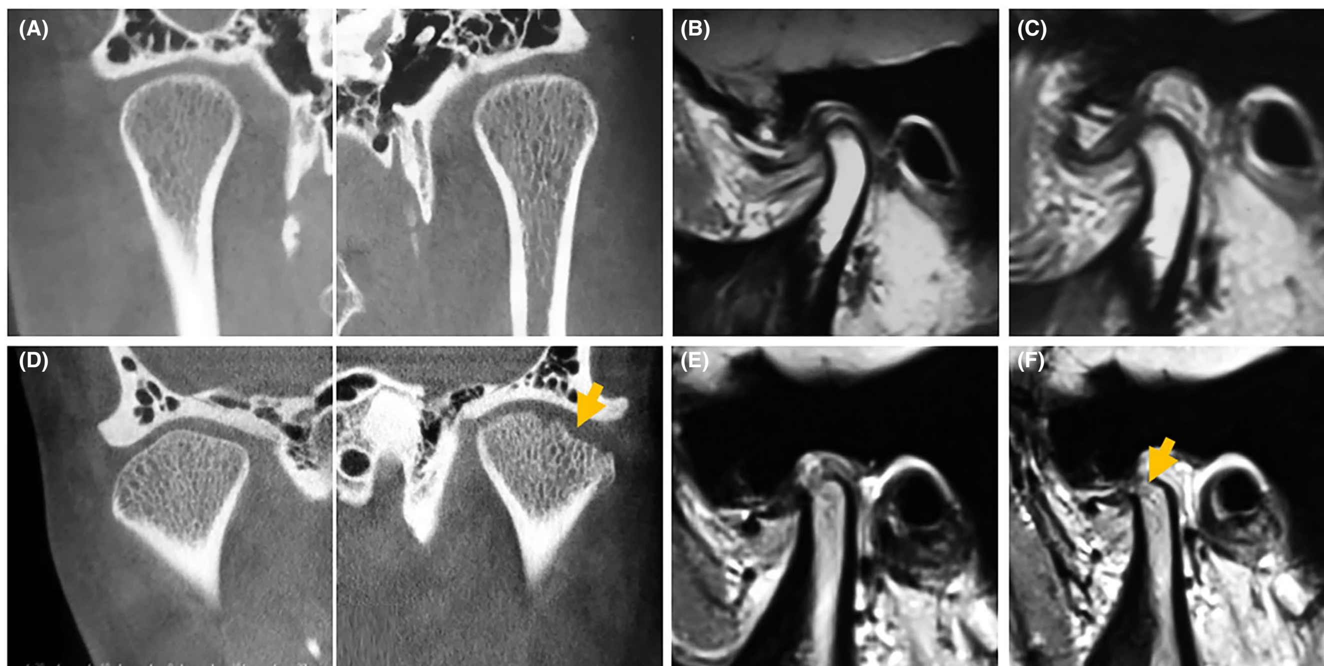


FIGURE 1 CBCT and MRI images of TMJs with DDw/oR without DJD (A-C) and DDw/oR with early-stage DJD (D-F). (A) Bilateral condyles demonstrating continuity of the articular cortex (without DJD). (B) Displaced disc in closed-mouth position. (C) Displaced disc in open mouth position. (D) Left condyle with early-stage DJD showing subchondral bone destruction (arrowhead). (E) Displaced disc in closed-mouth position. (F) Displaced disc in open mouth position with the absent cortical bone and a focal abnormal signal area in the subarticular region (arrow head) [Colour figure can be viewed at wileyonlinelibrary.com]

were centrifuged at 1500 *g* for 10 min at 4°C to remove cells and debris, stored in aliquots at −80°C and analysed with the following assays.

2.1.3 | Protein quantification

Total protein concentration in the synovial fluid was utilised as the standard for normalising cytokine levels in the different samples. Protein quantification was performed using the bicinchoninic acid (BCA) assay method (Pierce, Thermo Scientific, Rockford, IL, USA).

2.1.4 | Cytokine screening

To determine multiple cytokine levels in the synovial fluid, 18 samples from the three different groups (6 samples/group) were randomly selected, which included 6 "without DJD" samples from 12 patients with DDw/oR without DJD, 6 "with DJD" samples from 13 patients with DDw/oR with DJD and another 6 "control" samples from the 6 healthy volunteers.

The 27 cytokines screened by the Bio-Plex Human Cytokine 27-Plex Panel (Bio-Rad Laboratories, Hercules, CA, USA) were as follows: IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17A, IFN-γ, TNF-α, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), PDGF-BB, fibroblast growth factor (FGF)-2, VEGF, IL-8, interferon-inducible protein (IP)-10, eotaxin, monocyte chemotactic protein (MCP)-1, MIP-1α, MIP-1β and RANTES.

All samples were assayed according to the manufacturer's protocol, which allows for the determination of cytokines with coefficients of variation <15%. Each assay was performed in duplicate with eight standards. The levels of cytokines in the samples were expressed as pg/mg.

2.1.5 | Cytokine determination

Electrochemiluminescence technique and enzyme-linked immunosorbent assay were used to analyse all 31 synovial fluid samples from 12 patients with DDw/oR without DJD, 13 patients with DDw/oR with DJD and 6 healthy volunteers. Inflammatory mediators MIP-1β, RANTES and a key regulator of osteoclastogenesis RANKL were evaluated using the V-PLEX Human MIP-1β Kit (Meso Scale Discovery, Rockville, MD, USA), the Human RANTES ELISA Kit (R&D Systems, MN, USA) and the Magnetic Luminex Human RANKL Assay Kit (R&D Systems, MN, USA), respectively. The sensitivity of the assays was 0.37 pg/mL for MIP-1β, 2.0 pg/mL for RANTES and 4.7 pg/mL for RANKL. The measurements were carried out using freshly thawed aliquots of synovial fluid in duplicates.

2.2 | Cytological studies

2.2.1 | Cell culture

The RAW264.7 murine macrophage cell line was purchased from Peking Union Medical College (Beijing, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) and 1% penicillin-streptomycin (Gibco, Carlsbad, CA, USA) at 37°C in a humidified 5% CO_2 atmosphere. The medium was changed every 2 days.

2.2.2 | Cell proliferation assay

To evaluate the effects of MIP-1β and RANTES on macrophage proliferation, cell proliferation assay was performed using Cell Counting Kit-8 (CCK-8; Dojindo, Tokyo, Japan) according to the manufacturer's instructions. Briefly, the cells were seeded at a density of 1×10^3 per well in 96-well plates in 100 μL per well of DMEM/10% FBS supplemented with different concentrations of recombinant mouse MIP-1β (0, 5, 50 and 100 ng/mL) (R&D Systems, MN, USA) or RANTES (0, 5, 50 and 100 ng/mL) (R&D Systems, MN, USA) for 6, 24, 48, 72 and 96 hours, respectively. After incubating the cells with CCK-8 solution for 2 hours, optical density (OD) was read at 450 nm using an absorbance microplate reader (ELx808; BioTek, Winooski, VT, USA).

2.2.3 | Cell migration assay

The Transwell migration assay was performed to investigate macrophage migration after treatment with different concentrations of MIP-1β or RANTES. The system consisted of 24-well Transwell plate and 8.0-μm pore polyethylene terephthalate membrane inserts (Falcon, Corning, NY, USA). After starvation with serum-free DMEM for 24 hours, the cells were seeded at a density of 1×10^5 per well in 100 μL serum-free DMEM in the upper chamber of the Transwell system. The lower chamber was filled with 650 μL serum-free DMEM containing different concentrations of MIP-1β or RANTES (0-100 ng/mL). After incubation for 20 hours at 37°C, cells that migrated to the bottom side of the Transwell membrane were fixed with 95% ethanol and stained with 0.1% crystal violet. For each well, five random fields were selected and counted under a light microscope (×200 magnification; Olympus, Tokyo, Japan).

2.2.4 | Osteoclast differentiation assay and tartrate-resistant acid phosphatase (TRAP) staining

To determine whether MIP-1β and RANTES could directly induce osteoclast differentiation from macrophages, RAW264.7 cells were plated at a density of 1×10^4 per well in 24-well plates in 500 μL per well of DMEM/10% FBS supplemented with different concentrations of MIP-1β or RANTES (0-100 ng/mL) in the absence or presence of 5 ng/mL "receptor activator of nuclear factor kappa-B ligand" (RANKL; R&D Systems, MN, USA). After 4 days, cells were fixed with 4% paraformaldehyde for 15 minutes and then subjected to TRAP (Sigma-Aldrich, St. Louis, MO, USA) staining. Osteoclasts were determined to be TRAP-positive multinuclear (three or more nuclei) cells. For each well, nine random fields of vision were examined and counted under a light microscope (×100 magnification).

2.2.5 | Bone resorption pit assay

The bone resorption function of osteoclasts derived from RAW264.7 cells induced by RANKL with or without RANTES was analysed using osteo assay plates (Corning Inc, Corning, NY, USA). Briefly, cells were plated at a density of 1×10^4 per well in 24-well osteo assay plates in 500 μL per well of DMEM/10% FBS supplemented with 5 ng/mL RANKL with or without 50 ng/mL RANTES. After 7 days, each well was washed with bleach solution to remove cells completely. For each well, nine random fields of vision containing bone resorption pits were observed under a light microscope (×100 magnification). The percentage of resorption area relative to total area of each field was quantified using Image-Pro Plus version 6.0 (Media Cybernetics Inc, Rockville, MD, USA).

2.3 | Statistical analysis

Demographic and clinical characteristics of the subjects were presented as frequency and mean ± SD. As cytokine levels were not normally distributed, data of cytokine assay were presented as median and interquartile range and analysed using the Kruskal-Wallis with post hoc Mann-Whitney *U* tests. Correlations between data sets were evaluated by Spearman's rank correlation coefficient. For cytological studies, data were expressed as mean ± SEM of 6-8 independent experiments with duplicate wells. One-way ANOVA with post hoc Bonferroni's multiple comparison test or unpaired *t* test was employed to analyse these data sets. All statistical analyses were performed using Prism version 7.0 (GraphPad Software, La Jolla, CA, USA) with significance level set at $P < .05$.

3 | RESULTS

3.1 | Clinical findings

Demographic information and clinical information of the three groups including gender, age, maximum unassisted mouth opening (MMO), TMJ pain visual analogue scale (VAS) score and duration of illness were collected and listed in Table 1. Only "duration of illness" was significantly different between the three groups ($P < .001$).

3.2 | Protein quantification

No significant difference in total protein concentration was observed among the three groups (P = .405) (Figure 2A). As the total protein concentration of samples varied considerably (260-2140 μg/mL), cytokine levels were calibrated to protein concentrations.

TABLE 1 Demographic and clinical characteristics of the study subjects

	Control	DDw/oR without DJD	DDw/oR with DJD
No. of subjects	6	12	13
Age (years)	23.2 ± 1.9 (21-26)	20.9 ± 4.4 (16-29)	19.4 ± 5.0 (12-26)
Gender (F/M)	6/0	12/0	13/0
Maximal mouth opening (mm)	NA	30.8 ± 5.7 (22-42)	35.2 ± 8.5 (24-52)
Pain VAS score	0	1.7 ± 1.6 (0-4)	2.8 ± 1.8 (0-5)
Duration of disease (months)	NA	1.3 ± 1.2 (0.2-4.0)	4.8 ± 3.6 (0.3-12.0)*

Note: VAS, visual analogue scale, from 0 (no pain) to 10 (intolerable pain); NA, not available. Values were mean ± SD (range) or the number of subjects.
*$P < .05$.

3.3 | Screened cytokines in synovial fluid

Out of the 27 inflammatory-related cytokines assessed, a total of 26 were detectable in the synovial fluid. Table 2 shows the median cytokine concentrations for the three groups. Concentrations of MIP-1β and RANTES were observed to differ significantly among the control, "without DJD" as well as "with DJD" groups (P = .005 and P = .011, respectively). For the concentrations of other cytokines, no significant difference was found among the three groups. Based on the cytokine array, RANTES levels were found to be strongly and positively correlated with IL-9 ($r_s = 0.810$, $P < .001$) and MIP-1β ($r_s = 0.833$, $P < .001$). The correlation between RANTES and MIP-1α was moderately strong (rs = 0.419, P = .084). There was, however, no significant correlation between these cytokine levels and pain.

3.4 | Identification of MIP-1β, RANTES and RANKL

To further assess the variances of inflammatory mediators MIP-1β and RANTES among the three groups, sandwich immunoassay technology was used. MIP-1β was detected in all samples with median values of 1.28, 3.80 and 2.33 pg/mg in the control, "without DJD" and "with DJD" groups. MIP-1β content was statistically different among the groups (P = .013). Moreover, it was significantly higher in the "without DJD" group than in the control group (P = .010). MIP-1β

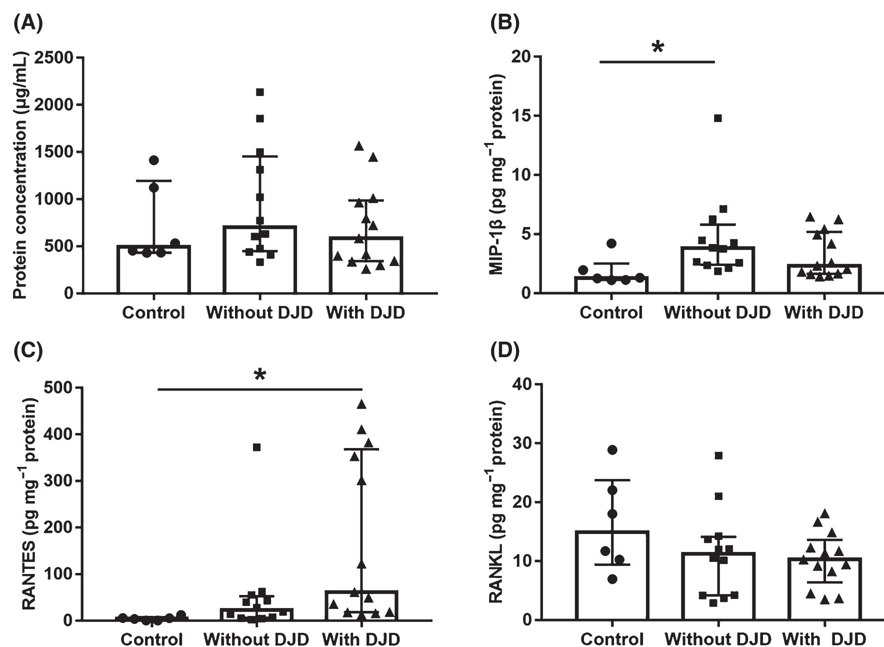


FIGURE 2 Measurement of molecular mediators in the synovial fluid. (A) Total protein concentration of synovial fluid samples (median with 25th and 75th percentiles) for the control (n = 6), DDw/oR without DJD (n = 12) and DDw/oR with DJD (n = 13) groups. No significant difference was observed among the three groups. (B-D) Concentrations of MIP-1β, RANTES and RANKL (median with 25th and 75th percentiles) for the control (n = 6), DDw/oR without DJD (n = 12) and DDw/oR with DJD (n = 13) groups. (B) The levels of MIP-1β for the "without DJD" group were significantly higher than the control group. (C) The levels of RANTES for the "with DJD" group were significantly higher than the control group. (D) The levels of RANKL were not exhibited significant difference in the different three groups. Concentration was calculated per 1 mg of synovial fluid sample total protein. * indicates significant difference at $P < .05$

TABLE 2 Cytokine concentrations (median with 25th and 75th percentiles) in synovial fluid samples

Cytokine	Control (n = 6)	DDw/oR without DJD (n = 6)	DDw/oR with DJD (n = 6)	*P*-value
IL-1β	0.03 (0.00; 0.11)	0.02 (0.00; 0.16)	0.04 (0.00; 0.11)	.993
IL-1ra	20.55 (0.00; 32.92)	16.72 (0.00; 44.75)	14.13 (0.00; 23.48)	.891
IL-2	0.20 (0.00; 1.01)	0.30 (0.00; 0.48)	0.12 (0.00; 0.53)	.939
IL-4	0.00 (0.00; 0.20)	0.00 (0.00; 0.05)	0.00 (0.00; 0.02)	.657
IL-5	13.68 (4.57; 24.77)	14.17 (0.11; 18.77)	12.19 (3.43; 20.11)	.823
IL-6	0.55 (0.15; 1.02)	0.35 (0.00; 3.19)	0.78 (0.44; 2.62)	.419
IL-7	0.00 (0.00; 0.43)	0.00 (0.00; 1.19)	0.00 (0.00; 1.54)	.804
IL-8	1.01 (0.00; 3.27)	1.14 (0.31; 3.22)	0.52 (0.00; 1.08)	.456
IL-9	0.69 (0.00; 2.30)	3.13 (2.24; 7.15)	3.52 (1.07; 14.60)	.061
IL-10	1.73 (1.19; 2.18)	1.02 (0.29; 1.89)	1.16 (0.69; 1.91)	.353
IL-12	0.66 (0.00; 1.42)	0.53 (0.00; 0.71)	0.49 (0.00; 1.46)	.785
IL-13	0.38 (0.00; 1.37)	0.00 (0.00; 0.00)	0.00 (0.00; 1.43)	.194
IL-15	206.77 (76.64; 459.39)	113.83 (0.00; 192.72)	128.92 (14.70; 265.94)	.460
IL-17A	1.65 (0.00; 4.16)	0.81 (0.00; 1.82)	1.47 (0.00; 3.10)	.589
Eotaxin	3.20 (1.64; 4.92)	2.15 (1.03; 4.07)	3.06 (1.64; 7.18)	.470
FGF basic	5.09 (1.51; 11.36)	6.16 (1.23; 9.13)	8.96 (1.80; 16.19)	.778
G-CSF	312.76 (16.89; 995.84)	179.54 (5.08; 501.08)	275.04 (11.37; 615.91)	.548
GM-CSF	0.00 (0.00; 2.09)	0.00 (0.00; 0.66)	0.00 (0.00; 1.34)	.957
IFN-γ	1.45 (0.31; 1.99)	0.78 (0.00; 1.54)	0.91 (0.00; 2.49)	.581
IP-10	184.02 (77.96; 394.16)	148.47 (117.88; 539.08)	146.62 (98.38; 281.23)	.949
MCP-1	11.21 (4.56; 18.81)	9.89 (6.06; 18.78)	7.78 (5.08; 10.61)	.641
MIP-1α	0.03 (0.00; 0.15)	0.20 (0.11; 0.54)	0.08 (0.00; 0.26)	.058
PDGF-BB	0.00 (0.00; 0.00)	0.00 (0.00; 0.00)	0.00 (0.00; 0.00)	1.000
MIP-1β	0.95 (0.38; 1.74)	3.48 (2.27; 9.80)	3.08 (1.90; 11.25)	.011*
RANTES	4.39 (2.85; 9.57)	64.23 (44.21; 133.27)	90.94 (20.41; 364.96)	.005*
TNF-α	0.84 (0.00; 2.43)	0.92 (0.23; 2.20)	0.52 (0.00; 3.35)	.855
VEGF	279.00 (159.98; 369.66)	175.32 (36.20; 258.89)	191.16 (88.83; 314.07)	.343

Note: Each cytokine concentration (pg/mg) was calculated per 1 mg of synovial fluid sample total protein.
*$P < .05$.

in the "without DJD" group tends to be higher than the "with DJD" group, and that of the "with DJD" group was higher than control group, but differences were not statistically significant (Figure 2B). As for RANTES, it was detected in 29 synovial fluid samples (29/31) and undetectable in 2 healthy controls (2/31). The median values were 4.38, 23.09 and 61.31 pg/mg for the control, "without DJD" and "with DJD" groups. Statistically significant difference in RANTES concentration was noted among the three groups ($P < .001$). It was significantly higher in the "with DJD" group than in the control group ($P < .001$). While RANTES concentration for the "with DJD" group tends to be higher than the "without DJD" group, which in turn was higher than control group, variations were not statistically significant (Figure 2C).

As a key regulator of osteoclastogenesis, RANKL was also determined in all samples. Although RANKL was detectable (around 2.8-22.0 pg/mg protein), the levels of RANKL in the synovial fluid of DDw/oR patients with or without DJD showed no significant difference from those in the controls ($P = .365$) (Figure 2D).

3.5 | Effects of MIP-1β and RANTES on RAW264.7 cell proliferation and cell migration

Neither MIP-1β nor RANTES caused significant changes in RAW264.7 cell proliferation at concentrations up to 100 ng/mL. When compared to the control group, the number of migrated RAW264.7 cells treated with MIP-1β (Figure 3A and C) or RANTES (Figure 3B and D) was significantly enhanced in a dose-dependent manner ($P = .013$ and $P = .001$, respectively). When the concentration of chemokines rose, the number of migrated cells increased. The results showed that both MIP-1β and RANTES could promote RAW264.7 cell migration.

3.6 | Effects of MIP-1β and RANTES on osteoclast differentiation in RAW264.7 cells

RAW264.7 cells were treated with MIP-1β or RANTES for 4 days, in the absence or presence of RANKL (5 ng/mL). MIP-1β plus RANKL

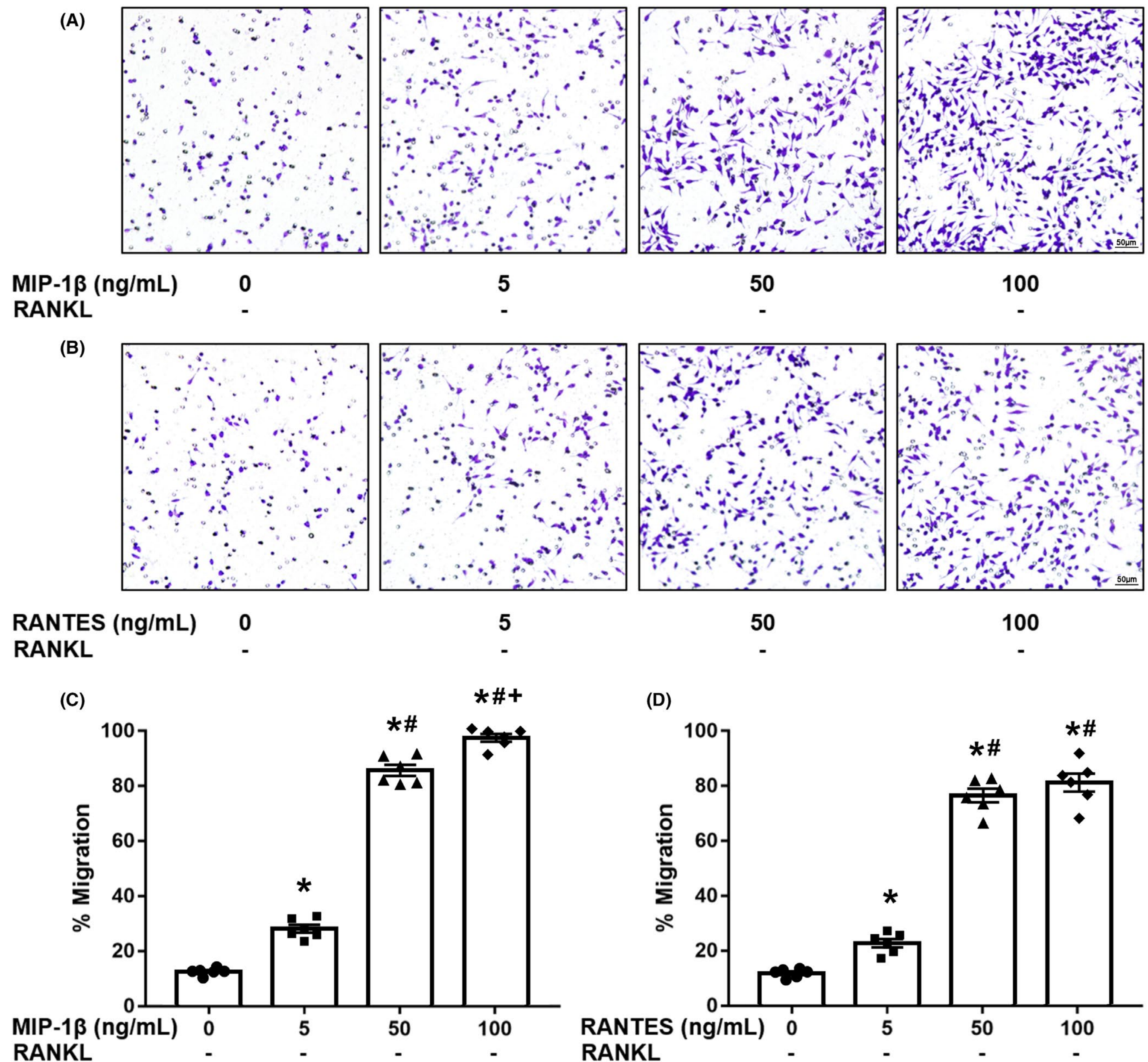


FIGURE 3 Effects of MIP-1β and RANTES on RAW264.7 cell migration. (A) MIP-1β enhanced RAW264.7 cell migration in a concentration-dependent manner. (B) RANTES enhanced RAW264.7 cell migration in a concentration-dependent manner. (C) Percentage of cell migration with different concentrations of MIP-1β. (D) Percentage of cell migration with different concentrations of RANTES. Data were presented as mean ± SEM (n = 6 wells/group). * indicates significant difference compared with 0 ng/mL chemokine-treated RAW264.7 cells at $P < .05$. # indicates significant difference compared with 5 ng/mL chemokine-treated RAW264.7 cells at $P < .05$. + indicates significant difference compared with 50 ng/mL chemokine-treated RAW264.7 cells at $P < .05$. Scale bar: 50 μm [Colour figure can be viewed at wileyonlinelibrary.com]

did not show any advantage in promoting osteoclast formation compared with RANKL single stimulation ($P = .730$) (Figure 4A and C). Interestingly, RANTES promoted osteoclast formation in RANKL-induced RAW264.7 cells ($P < .001$) (Figure 4B and D). When the concentration of RANTES reached 50 ng/mL, the number of TRAP-positive multinucleated cells was significantly increased compared with the control and “5 ng/mL RANTES” group, which in turn was similar to the “100 ng/mL RANTES” group ($P = 1.000$). MIP-1β or RANTES alone did not induce osteoclast differentiation from RAW264.7 cells in the absence of RANKL. Therefore, we selected 5 ng/mL RANKL with or without 50 ng/mL RANTES for bone resorption pit assay.

3.7 | Effect of RANTES on bone-resorbing activity in RANKL-induced RAW264.7 cells

RAW264.7 cells were cultured in 24-well osteo assay plates in the presence of RANKL with or without RANTES. After culturing for

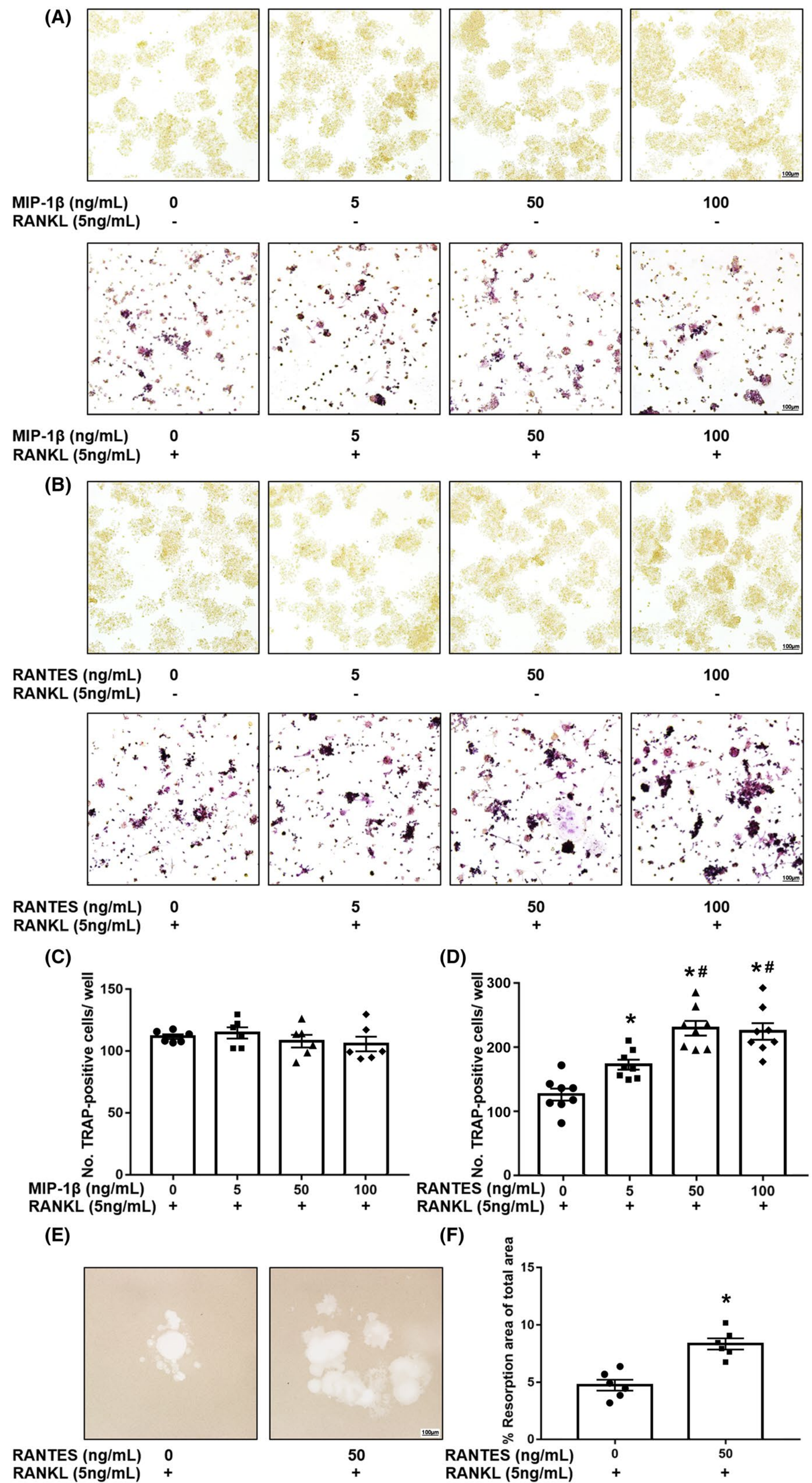


FIGURE 4 Effects of MIP-1β and RANTES on RAW264.7 cell osteoclastogenesis and bone-resorbing activity. (A) MIP-1β did not substantially affect osteoclastogenesis in RAW264.7 cells. (B) RANTES stimulated osteoclast formation in RANKL-treated RAW264.7 cells, but did not induce osteoclast differentiation from RAW264.7 cells without RANKL. (C) No. TRAP-positive cells per well with different concentrations of MIP-1β and 5 ng/mL RANKL. (D) No. TRAP-positive cells per well with different concentrations of RANTES and 5 ng/mL RANKL. (E) RANTES enhanced the resorptive activity of RANKL-induced osteoclasts. (F) The percentage of resorption area relative to total area. Data were presented as mean ± SEM (n = 6-8 wells/group). * indicates significant difference compared with 5 ng/mL RANKL plus 0 ng/mL chemokine-treated RAW264.7 cells at $P < .05$. # indicates significant difference compared with 5 ng/mL RANKL plus 5 ng/mL chemokine-treated RAW264.7 cells at $P < .05$. Scale bar: 100 μm

7 days, more resorption pits on the bottom of plates were observed in the RANTES group ($P < .001$) (Figure 4E and F), suggesting that RANTES could enhance the resorptive activity of RANKL-induced osteoclasts.

4 | DISCUSSION

In this study, we screened the synovial fluid of DDw/oR subjects without and with early-stage DJD and healthy controls for 27 inflammatory mediators. Results of the multiple cytokine array performed indicated that the concentrations of MIP-1β and RANTES were significantly different between subjects with DDw/oR and healthy controls. In addition, through the sandwich immunoassay technique, RANTES was found to be the only mediator discriminating DDw/oR subjects with early-stage DJD from controls. Although RANKL as a key osteoclastogenesis regulator was detectable in all synovial fluid samples, there was no significant difference between the three groups. When investigating the effects of MIP-1β and RANTES on macrophage proliferation, migration and osteoclastogenesis, both MIP-1β and RANTES enhanced macrophage migration, while only RANTES could promote RANKL-induced osteoclast formation. Collectively, the findings indicated that RANTES may contribute to the pathogenesis of DDw/oR-induced early-stage TMJ DJD and provided new insights for the early diagnosis and management of TMJ degeneration.

TMJ DJD is generally considered a chronic disease characterised by cartilage lesions. However, other joint structures, including subchondral bone and synovial tissue, also play crucial roles in the pathogenesis of DJD. Emerging studies have implicated that remodelling in subchondral bone may precede cartilage degradation and contribute to the initiation and/or progression of DJD.[21,22] Our previous study found that about 60% of young patients (10-30 years old) with recent-onset DDw/oR (within a year) presented with radiographically visible early-stage TMJ DJD.[10] Osteoarthritic condylar changes would eventually reach a stable end stage in 1-4 years in most cases.[23-26] In the present study, we selected subjects with DDw/oR onset of less than a year and divided them into two subgroups, that is without or with DJD. Only early-stage degenerative changes that presented radiographically as discontinuity of the articular cortex and/or surface erosion/destruction of the condyle were involved. The average duration of DDw/oR without and with DJD was 1.3 and 4.8 months correspondingly. As this was the active phase of subchondral bone resorption, the occurrence of molecular changes associated with early-stage DJD was anticipated.

The initiation and progression of DJD have been found to be associated with inflammatory factors. Inflammatory mediators are responsible for the loss of metabolic homeostasis of the tissues forming joints by promoting catabolic and destructive processes.[27] The type of inflammatory mediators varies depending on the stage of DJD.[28] Considering the importance of inflammatory mediators in the pathogenesis of DJD, we selected a high-sensitivity multiple cytokine array method to screen key inflammatory mediators associated with early-stage DJD. Consequently, the levels of two inflammatory mediators, namely MIP-1β and RANTES, were found to vary significantly in relation to the controls. Supplementary studies confirmed that the levels of RANTES were increased in the synovial fluid of DDw/oR subjects with early-stage DJD, while MIP-1β concentrations were elevated in those with DDw/oR without DJD. Although previous studies as well as ours published in 1990s showed that the synovial fluid levels of IL-1β, IL-6 and TNF-α were significantly increased in TMJ DJD,[13,29,30] the level of these cytokines was not found to be significantly different among groups in the present study. In earlier studies, Schüllar's projection and transpharyngeal radiographs rather than CBCT were used, and the radiographic changes in both early- and late-stage DJD were included. However, late-stage DJD, such as deviation in form, extensive sclerosis, osteophyte formation and cyst-like lesions, was not included in the present study. Moreover, CBCT can detect very early bony changes which could not be detected with regular imaging.[19] We recruited patients with DDw/oR with and without early-stage DJD and not specifically those with DJD. Therefore, the disparity could be attributed to the stage of TMJ DJD as the subjects in our study only had early-stage DJD of less than a year. The increased level of RANTES suggests that RANTES is a possible candidate of key molecules involved in the initial stage of bone resorption. Conversely, key osteoclastogenesis regulator RANKL, which plays an important role in the activation of osteoclast precursor cells through interaction with RANK,[31,32] did not differ significantly in the synovial fluid of the three groups. Similarly, RANKL level was reported to be weak and unchanged in both DJD patients and healthy participants.[33-35] The above findings suggest that increased levels of MIP-1β and RANTES could be involved in the pathogenesis of DDw/oR-induced early-stage degenerative joint disease.

MIP-1β and RANTES are C-C motif chemokines, which could be released by chondrocytes, synovial fibroblasts and inflammatory cells.[36,37] MIP-1β is characterised by its inflammatory and chemokinetic properties.[38] It has been reported that elevated MIP-1β levels in synovial fluid and peripheral blood can predict the risk of rheumatoid arthritis (RA), but not osteoarthritis.[39,40] RANTES contributes to the migration of blood monocytes, T lymphocytes, natural killer cells and eosinophils, and is highly expressed in tissues, synovial fluid and peripheral blood of patients with RA or osteoarthritis.[41,42] Previous studies had focused mainly on the role of RANTES in synovial inflammatory immune response and cartilage extracellular matrix degradation. Specifically, RANTES can trigger and aggravate inflammatory immune response by facilitating immunocompetent cells infiltration and activating synovial fibroblasts to produce inflammatory mediators.[11] Besides, RANTES can also induce matrix metalloproteinase (MMP)-1, MMP-3, MMP-13 and inducible nitric oxide synthase release from synovial fibroblasts and chondrocytes, which contribute to cartilage degradation.[43,44] In the present study, the migration assay identified MIP-1β and RANTES as potent chemotactic factors for RAW264.7 cells. This corroborated earlier studies and suggests that these cytokines might be responsible for attracting macrophages to inflammatory sites. Moreover, RANTES exhibited a

direct promoting effect on osteoclast formation and bone-resorbing activity, which signified that RANTES may act as a key mediator in the pathogenesis of early-stage DJD. To the best of our knowledge, this is the first study to show increased RANTES in the synovial fluid of subjects with DDw/oR and early-stage DJD and that RANTES had a positive effect on osteoclast differentiation from macrophages. Collectively, our results indicate that increased RANTES might be closely related to the initiation and/or progression of DDw/oR-induced early-stage DJD.

Despite the positive findings, there were some limitations with our study. First, the subjects only involved females aged 12-30 years that does not represent the entire population with early-stage DJD. The female gender was selected due to their higher TMD prevalence, odds of disc displacements/TMJ DJD and to limit the possible impact of hormones on findings.[45] Second, the samples were mixtures of TMJ synovial fluid and saline solution, not native synovial fluid. This might also explain the low concentrations of some common inflammatory cytokines (eg IL-1β, TNF-α) that did not achieve statistical significance. Finally, RAW264.7 cells cannot be considered equal to macrophages of early-stage DJD. For the above reasons, additional studies incorporating male youths, larger patient cohorts, more sensitive assays and macrophages from patients are needed to validate the role of RANTES in the pathogenesis of early-stage DJD.

5 | CONCLUSIONS

This study determined that chemokine RANTES was significantly upregulated and might be a key regulator of osteoclastogenesis contributing to DDw/oR-induced early-stage TMJ DJD. The mechanisms of RANTES in TMJ DJD warrant further investigation. The latter has bearing on the early diagnosis of TMJ DJD and clinical use of targeted drugs for modifying subchondral bone resorption.

ACKNOWLEDGEMENTS

This work was supported by the grants from the National Natural Science Foundation of China (81870788) and Research Foundation of Peking University School and Hospital of Stomatology (PKUSS20140204).

CONFLICTS OF INTEREST

All authors declare no conflict of interests.

AUTHOR CONTRIBUTIONS

SY Feng contributed to conception and design, data acquisition, analysis and interpretation, and drafted and critically revised the manuscript; J. Lei, K. Y. Fu contributed to conception and design, data acquisition, analysis and interpretation, and critically revised the manuscript; H. M. Chen contributed to design, data acquisition and analysis, and critically revised the manuscript; Y. X. Wang contributed to conception and design, data analysis and interpretation, and critically revised the manuscript; and AU Yap contributed to conception and interpretation, and drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/joor.13041.

ORCID

Shi-Yang Feng https://orcid.org/0000-0003-4730-9761
Jie Lei https://orcid.org/0000-0002-3429-4436
Yi-Xiang Wang https://orcid.org/0000-0001-5291-9826
Adrian U-Jin Yap https://orcid.org/0000-0003-0361-6209
Kai-Yuan Fu https://orcid.org/0000-0001-7049-9888

REFERENCES

1. Pantoja LLQ, de Toledo IP, Pupo YM, et al. Prevalence of degenerative joint disease of the temporomandibular joint: a systematic review. *Clin Oral Invest*. 2019;23(5):2475-2488.
2. Schiffman E, Ohrbach R, Truelove E, et al. Diagnostic criteria for temporomandibular disorders (DC/TMD) for clinical and research applications: recommendations of the International RDC/TMD Consortium Network* and Orofacial Pain Special Interest Group dagger. *J Oral Facial Pain Headache*. 2014;28(1):6-27.
3. Takatsuka S, Yoshida K, Ueki K, Marukawa K, Nakagawa K, Yamamoto E. Disc and condyle translation in patients with temporomandibular disorder. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 2005;99(5):614-621.
4. Krisjane Z, Urtane I, Krumina G, Neimane L, Ragovska I. The prevalence of TMJ osteoarthritis in asymptomatic patients with dentofacial deformities: a cone-beam CT study. *Int J Oral Maxillofac Surg*. 2012;41(6):690-695.
5. Tanaka E, Detamore MS, Mercuri LG. Degenerative disorders of the temporomandibular joint: etiology, diagnosis, and treatment. *J Dent Res*. 2008;87(4):296-307.
6. Asakawa-Tanne Y, Su S, Kunimatsu R, et al. Effects of enzymatic degradation after loading in temporomandibular joint. *J Dent Res*. 2015;94(2):337-343.
7. Nickel JC, Iwasaki LR, Gonzalez YM, Gallo LM, Yao H. Mechanobehavior and ontogenesis of the temporomandibular joint. *J Dent Res*. 2018;97(11):1185-1192.
8. de Melo DP, Sousa Melo SL, de Andrade Freitas Oliveira LS, Ramos-Perez FMdM, Campos PSF. Evaluation of temporomandibular joint disk displacement and its correlation with pain and osseous abnormalities in symptomatic young patients with magnetic resonance imaging. *Oral Surg Oral Med Oral Pathol Oral Radiol*. 2015;119(1):107-112.
9. Dias IM, Cordeiro PC, Devito KL, Tavares ML, Leite IC, Tesch RS. Evaluation of temporomandibular joint disc displacement as a risk factor for osteoarthrosis. *Int J Oral Maxillofac Surg*. 2016;45(3):313-317.
10. Lei J, Han J, Liu M, Zhang Y, Yap AU, Fu KY. Degenerative temporomandibular joint changes associated with recent-onset disc displacement without reduction in adolescents and young adults. *J Craniomaxillofac Surg*. 2017;45(3):408-413.
11. Beekhuizen M, Gierman LM, van Spil WE, et al. An explorative study comparing levels of soluble mediators in control and osteoarthritic synovial fluid. *Osteoarthritis Cartilage*. 2013;21(7):918-922.
12. Vos LM, Kuijer R, Huddleston Slater JJ, Stegenga B. Alteration of cartilage degeneration and inflammation markers in temporomandibular joint osteoarthritis occurs proportionally. *J Oral Maxillofac Surg*. 2013;71(10):1659-1664.

13. Kellesarian SV, Al-Kheraif AA, Vohra F, et al. Cytokine profile in the synovial fluid of patients with temporomandibular joint disorders: a systematic review. *Cytokine*. 2016;77:98-106.
14. de Alcântara Camejo F, Azevedo M, Ambros V, et al. Interleukin-6 expression in disc derangement of human temporomandibular joint and association with osteoarthrosis. *J Craniomaxillofac Surg*. 2017;45(5):768-774.
15. Monasterio G, Castillo F, Rojas L, et al. Th1/Th17/Th22 immune response and their association with joint pain, imagenological bone loss, RANKL expression and osteoclast activity in temporomandibular joint osteoarthritis: A preliminary report. *J Oral Rehabil*. 2018;45(8):589-597.
16. Guven O, Tekin U, Salmanoglu B, Kaymak E. Tumor necrosis factor-alpha levels in the synovial fluid of patients with temporomandibular joint internal derangement. *J Craniomaxillofac Surg*. 2015;43(1):102-105.
17. Zhao YP, Zhang ZY, Wu YT, Zhang WL, Ma XC. Investigation of the clinical and radiographic features of osteoarthrosis of the temporomandibular joints in adolescents and young adults. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 2011;111(2):e27-e34.
18. Comert Kilic S, Kilic N, Sumbullu MA. Temporomandibular joint osteoarthritis: cone beam computed tomography findings, clinical features, and correlations. *Int J Oral Maxillofac Surg*. 2015;44(10):1268-1274.
19. Fu KY, Zhang WL, Liu DG, Chen HM, Ma XC. Cone beam computed tomography in the diagnosis of temporomandibular joint osteoarthrosis. *Zhonghua kou qiang yi xue za zhi*. 2007;42(7):417-420.
20. Wiberg B, Wänman A. Signs of osteoarthrosis of the temporomandibular joints in young patients: a clinical and radiographic study. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 1998;86(2):158-164.
21. Kisand K, Tamm AE, Lintrop M, Tamm AO. New insights into the natural course of knee osteoarthritis: early regulation of cytokines and growth factors, with emphasis on sex-dependent angiogenesis and tissue remodeling. A pilot study. *Osteoarthritis Cartilage*. 2018;26(8):1045-1054.
22. Klose-Jensen R, Hartlev LB, Boel L, et al. Subchondral bone turnover, but not bone volume, is increased in early stage osteoarthritic lesions in the human hip joint. *Osteoarthritis Cartilage*. 2015;23(12):2167-2173.
23. Lee JY, Kim DJ, Lee SG, Chung JW. A longitudinal study on the osteoarthritic change of the temporomandibular joint based on 1-year follow-up computed tomography. *J Craniomaxillofac Surg*. 2012;40(8):e223-e228.
24. Manfredini D, Favero L, Gregorini G, Cocilovo F, Guarda-Nardini L. Natural course of temporomandibular disorders with low pain-related impairment: a 2-to-3-year follow-up study. *J Oral Rehabil*. 2013;40(6):436-442.
25. Kumm J, Tamm A, Lintrop M, Tamm A. Diagnostic and prognostic value of bone biomarkers in progressive knee osteoarthritis: a 6-year follow-up study in middle-aged subjects. *Osteoarthritis Cartilage*. 2013;21(6):815-822.
26. Schiffman EL, Ahmad M, Hollender L, et al. Longitudinal stability of common TMJ structural disorders. *J Dent Res*. 2017;96(3):270-276.
27. Wojdasiewicz P, Poniatowski LA, Szukiewicz D. The role of inflammatory and anti-inflammatory cytokines in the pathogenesis of osteoarthritis. *Mediators Inflamm*. 2014;2014:561459.
28. Vangsness CT Jr, Burke WS, Narvy SJ, MacPhee RD, Fedenko AN. Human knee synovial fluid cytokines correlated with grade of knee osteoarthritis–a pilot study. *Bull NYU Hosp Jt Dis*. 2011;69(2):122-127.
29. Fu K, Ma X, Zhang Z, Pang X, Chen W. Interleukin-6 in synovial fluid and HLA-DR expression in synovium from patients with temporomandibular disorders. *J Orofac Pain*. 1995;9(2):131-137.
30. Fu K, Ma X, Zhang Z, Chen W. Tumor necrosis factor in synovial fluid of patients with temporomandibular disorders. *J Oral Maxillofac Surg*. 1995;53(4):424-426.
31. Anesi A, Generali L, Sandoni L, Pozzi S, From GA. Osteoclast differentiation to osteonecrosis of the jaw: molecular and clinical insights. *Int J Mol Sci*. 2019;20(19):4925.
32. Raje NS, Bhatta S, Terpos E. Role of the RANK/RANKL Pathway in Multiple Myeloma. *Clin Cancer Res*. 2019;25(1):12-20.
33. Wakita T, Mogi M, Kurita K, Kuzushima M, Togari A. Increase in RANKL: OPG ratio in synovia of patients with temporomandibular joint disorder. *J Dent Res*. 2006;85(7):627-632.
34. Zhao R, Chen N-N, Zhou X-W, et al. Exogenous IFN-beta regulates the RANKL-c-Fos-IFN-beta signaling pathway in the collagen antibody-induced arthritis model. *J Transl Med*. 2014;12:330.
35. Agarwal S, Misra R, Aggarwal A. Synovial fluid RANKL and matrix metalloproteinase levels in enthesitis related arthritis subtype of juvenile idiopathic arthritis. *Rheumatol Int*. 2009;29(8):907-911.
36. Yang DW, Qian GB, Jiang MJ, Wang P, Wang KZ. Inhibition of microRNA-495 suppresses chondrocyte apoptosis through activation of the NF-kappaB signaling pathway by regulating CCL4 in osteoarthritis. *Gene Ther*. 2019;26(6):217-229.
37. Ikić Matijašević M, Flegar D, Kovačić N, et al. Increased chemotaxis and activity of circulatory myeloid progenitor cells may contribute to enhanced osteoclastogenesis and bone loss in the C57BL/6 mouse model of collagen-induced arthritis. *Clin Exp Immunol*. 2016;186(3):321-335.
38. Lee D, Shin K-J, Kim DW, et al. CCL4 enhances preosteoclast migration and its receptor CCR5 downregulation by RANKL promotes osteoclastogenesis. *Cell Death Dis*. 2018;9(5):495.
39. Kuo SJ, Huang CC, Tsai CH, Hsu HC, Su CM, Tang CH. Chemokine C-C motif ligand 4 gene polymorphisms associated with susceptibility to rheumatoid arthritis. *Biomed Res Int*. 2018;2018:9181647.
40. Sucur A, Jajic Z, Artukovic M, et al. Chemokine signals are crucial for enhanced homing and differentiation of circulating osteoclast progenitor cells. *Arthritis Res Ther*. 2017;19(1):142.
41. Hampel U, Sesselmann S, Iserovich P, Sel S, Paulsen F, Sack R. Chemokine and cytokine levels in osteoarthritis and rheumatoid arthritis synovial fluid. *J Immunol Methods*. 2013;396(1–2):134-139.
42. Pierzchala AW, Kusz DJ, Hajduk G. CXCL8 and CCL5 expression in synovial fluid and blood serum in patients with osteoarthritis of the knee. *Arch Immunol Ther Exp*. 2011;59(2):151-155.
43. Agere SA, Akhtar N, Watson JM, Ahmed S. RANTES/CCL5 induces collagen degradation by activating MMP-1 and MMP-13 expression in human rheumatoid arthritis synovial fibroblasts. *Front Immunol*. 2017;8:1341.
44. Nakamura H, Tanaka M, Masuko-Hongo K, et al. Enhanced production of MMP-1, MMP-3, MMP-13, and RANTES by interaction of chondrocytes with autologous T cells. *Rheumatol Int*. 2006;26(11):984-990.
45. Bueno CH, Pereira DD, Pattussi MP, Grossi PK, Grossi ML. Gender differences in temporomandibular disorders in adult populational studies: a systematic review and meta-analysis. *J Oral Rehabil*. 2018;45(9):720-729.

How to cite this article: Feng S-Y, Lei J, Chen H-M, Wang Y-X, Yap AU-J, Fu K-Y. Increased chemokine RANTES in synovial fluid and its role in early-stage degenerative temporomandibular joint disease. *J Oral Rehabil*. 2020;47:1150–1160. https://doi.org/10.1111/joor.13041