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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

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Summary

It is well accepted that the presence of cytokines belonging to the Th1/Th17/Th22 axis of immuno-inflammatory response in the joint environment, such as IL-1β, IL-17 and IL-22, respectively, are associated with pathogenesis of several synovial joint degenerative disorders. During temporomandibular joint osteoarthritis (TMJ-OA), IL-1β and IL-17 have been implicated in the inflammation and resorption of sub-chondral bone; however, the role of Th22 response in the TMJ-OA pathophysiology has not been established. This study aimed to compare the expression of Th1/Th17/Th22-type cytokines, chemokines and chemokine receptors in synovial fluid samples obtained from TMJ-OA or disk displacement with reduction (DDWR) patients. In addition, it aimed to associate these levels with joint pain, imagenological signs of bone degeneration, RANKL production, osteoclastogenesis and osteoclast-induced bone resorption. Higher levels of IL-1β, IL-17 and IL-22 were expressed in TMJ-OA compared with DDWR subjects, and these increased levels significantly correlated with RANKL expression, joint pain and articular bone degeneration. Higher levels of CCR5, CCR6 and CCR7, as well as their respective ligands CCL5 and CCL20, responsible for recruitment of IL-1β, IL-17 and IL-22-producing cells, were over-expressed in TMJ-OA compared with DDWR subjects. Osteoclastogenesis and osteoclast-induced bone resorption were significantly greater in presence of synovial fluid from TMJ-OA compared with DDWR subjects. These data demonstrate that cytokines, CCLs and CCRs associated with the Th1/Th17/Th22 axis of immuno-inflammatory response are involved in TMJ-OA pathogenesis. These findings suggest that IL-22 is involved in the RANKL expression in TMJ-OA, which in turn induces differentiation of osteoclasts and subsequent resorption of sub-chondral bone.

KEYWORDS

bone resorption, chemokines, cytokines, interleukin-22, RANKL, temporomandibular osteoarthritis

GM and FC contributed equally to this work and should be considered as joint first authors.

1 | INTRODUCTION

Osteoarthritis (OA) is a chronic degenerative disease of synovial joints characterized by progressive articular cartilage deterioration, abnormal sub-chondral bone resorption and synovial inflammation.[1] Temporomandibular joint osteoarthritis (TMJ-OA) is one of the most common forms of temporomandibular disorder (TMD), which frequently associates with condylar erosion and flattening, disk perforation, pain during functional activities and TMJ palpation, joint sounds, mouth opening deviation, jaw hypomobility, and worsened mastication.[2] Despite being defined as a local inflammatory disease, its development could be due to either local factors, including microtrauma or macrotrauma secondary to disk displacement,[3] or systemic factors, including systemic inflammatory disorders such as rheumatoid arthritis, psoriatic arthritis or reactive arthritis.[4] Actually, many factors have been proposed as responsible for the TMJ-OA development, such as genetic factors, overloading, unilateral chewing, bruxism and internal derangements; however, the molecular basis of the TMJ-OA aetiopathogenesis remains unclear.[2,5]

TMJ-OA is described as a low-inflammatory arthritic condition, compared with rheumatoid arthritis which is described as a high-inflammatory disease.[6] Nonetheless, inflammation during TMJ-OA plays a pivotal role in the onset and progression of the disease, as well as in pain intensity.[4] Although some inflammatory mediators are common among local TMJ-OA and systemic inflammatory diseases,[7] local levels of these mediators as well as their specific composition may change. In fact, different cytokines, chemokines, chemokine receptors, enzymes and pro-bone-resorptive factors have been proposed as markers of active TMJ-OA.[8,9] Particularly, higher levels of interleukin (IL)-1β, IL-6, IL-17, interferon (IFN)-γ, tumour necrosis factor (TNF)-α, prostaglandin E_2 (PGE_2), matrix metalloproteinase (MMP)-2, MMP-9, aggrecanase-1, superoxide dismutase and receptor-activator of nuclear factor-κB ligand (RANKL) have been detected in synovial fluid from TMJ-OA patients, compared with disk displacement with reduction (DDWR), disk displacement without reduction (DDWOR) or healthy subjects.[5,10-16]

The host's immuno-inflammatory response plays a central role in the pathogenesis of several chronic inflammatory diseases characterized by bone resorption, such as rheumatoid arthritis, osteoporosis, Paget's disease, facial osteolytic lesions and periodontitis.[17,18] In rheumatoid arthritis, the Th1/Th17/Th22 axis of immuno-inflammatory response has been associated with the onset, progression and severity of the disease, and the Th2/Th9/Treg axis with disease remission and healing.[19-21] In fact, Th1-, Th17- and Th22-related cytokines and chemokines have been identified as responsible for the bone damage observed in rheumatoid arthritis, and an increased detection of IL-1β (Th1), IL-17 (Th17) and IL-22 (Th22) in the synovial fluid from rheumatoid arthritis patients has been associated with enhanced expression of RANKL and subsequent articular bone resorption.[18,20,22]

Similar to rheumatoid arthritis, increased levels of IL-1β, IL-17 and RANKL have been reported in synovial fluid from TMJ-OA patients;[14] therefore, this pilot study aimed to analyse whether increased levels of Th1-, Th17- and Th22-related cytokines, CC-chemokines (CCLs) and CC-chemokine receptors (CCRs) are associated with, joint pain, signs of articular bone degeneration, RANKL production and osteoclast-induced bone resorption during TMJ-OA.

2 | MATERIALS AND METHODS

2.1 | Subjects

In this pilot investigation, the study population comprised four patients attending to the evaluation and management of TMJ-OA at the Prosthetics Clinic, Faculty of Dentistry, Universidad de Chile. As controls, two DDWR patients were selected, which is a low-inflammatory and non-bone resorptive TMJ disease. The diagnosis of TMJ-OA and DDWR was based on clinical and imagenological features, in accordance with the principles of Diagnostic Criteria for TMD (DC/TMD) for degenerative joint disease (DJD) and DDWR, respectively.[23] TMJ-OA diagnosis was established in joints with DJD—according to DC/TMD—and joint pain (ie arthralgia), to distinguish from other degenerative joint diseases (eg osteoarthrosis). TMJ-OA patients fulfilled the following inclusion criteria: (a) They had any TMJ noise during jaw movement or function in the last 30 days or reported any noise during the clinic exam; (b) they had complained of chronic moderate-to-severe arthralgia, aggravated by jaw movement; (c) they had crepitus detected with palpation during mouth opening, closing, lateral, and/or protrusive movements and (d) they had at least one of the following degenerative changes detected by cone-beam computed tomography (CBCT): sub-chondral cyst, erosion, generalized sclerosis or osteophytes. DDWR patients fulfilled the following inclusion criteria: (a) They had any TMJ noise during jaw movement or function in the last 30 days or reported any noise during the clinic exam; (b) they had clicking, popping and/or snapping noise during both opening and closing mouth movements, detected with palpation during at least one of three repetitions of mouth movements and (c) they had evident disk displacement detected by magnetic resonance imaging (MRI): In the maximum intercuspal position, the posterior band of the disk was located anterior to the 11:30 position and the intermediate zone of the disk was anterior to the condylar head; and on full opening, the intermediate zone of the disk was located between the condylar head and the articular eminence. These diagnoses were made on each TMJ and only unilaterally affected patients were included in this study. In case of double diagnosis (eg TMJ-OA and DDWR in the same joint, or TMJ-OA in one side and DDWR in the other side), the patient was excluded. Patients with previous TMD therapy, jaw trauma, TMJ pain associated with another TMD, pregnancy, multiple OA, systemic rheumatic, neurological, endocrine, immune or inflammatory disease were also excluded from this study, based on their medical history and related laboratory examinations. Evaluation and data registration were performed by one TMD specialist (GF), who had been formally trained and calibrated in accordance with the DC/TMD Training and Calibration Guidelines.[24] The reliability test showed that the examiner had substantial to almost perfect reliability (Kappa > 0.9) for the tested diagnoses. All

the participants voluntarily agreed to participate in this study after receiving a clear explanation of the protocol and by signing an IRB-approved informed consent form (Protocol #012/2016). This study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2000. Due to ethical restrictions, this study did not include samples obtained from healthy individuals.

2.2 | Joint pain

In each patient, arthralgia was recorded during mandibular movements using the visual analogue scale (VAS), ranging from 0 (no pain) to 10 (intolerable pain), and determining the duration of illness in months. VAS scores were recorded during maximum unassisted mouth opening and ipsilateral/contralateral laterotrusive and protrusive movements, and duration of illness was considered as the number of months since the pain began in every single TMJ. Evaluation and data registration were performed by one calibrated TMD specialist (GF) during DC/TMD examination protocol.

2.3 | Joint imaging

Multiple-layered X-ray CBCT or MRI of both TMJs were carried out in TMJ-OA and DDWR patients, respectively. The CBCT images were taken with the teeth in occlusion and with a standardized head posture using a cone-beam computed tomography X-ray unit (Planmeca ProMax® 3D; Helsinki, Finland). The exposure settings were as follows: standard scan mode with an imaging volume of 5 × 3 cm, 90 kV and 14 mA, and an exposure time of 12.3 seconds. On the multiple-layered tomograms, articular bone degenerative changes were quantified using an imagenological scale ranging from 1 to 4, in a dichotomic way, depending on the presence or absence of each of the following signs: Sub-chondral cysts, erosions, generalized sclerosis or osteophytes; being 1 when just one bone degenerative sign was detected and successively to 4 when all four bone degenerative signs were detected. The MRI examinations were performed in the maximum intercuspal position and on full opening using a 1.5-Tesla MRI system (1.5-T Philips Intera®; Hoffman-LaRoche Ltd., Best, The Netherlands) with proton density and T_2-weighted imaging sequence for sagittal and T_1 for parasagittal. Two of the authors (GM and WD) analysed the images individually without any information on the clinical findings. The registrations of the two observers were compared, and in case of discrepancy, the final diagnosis was reached by consensus and dubious findings were not reported.

2.4 | Demographic, clinic and imagenological characteristics

Table 1 shows the comparison of the demographic data, joint pain measured as VAS score, duration of illness in months, and the frequency of detection of imagenological signs of bone degeneration between the TMJ-OA and DDWR patients. Gender was matched between the groups; however, DDWR patients were younger than the TMJ-OA patients. In TMJ-OA patients, the most frequently detected bone degenerative signs were erosion and osteophytes. Generalized sclerosis was undetectable in all studied individuals.

2.5 | Synovial fluid sample collection

Synovial fluid was collected during arthrocentesis by puncture with a 21-gauge needle inserted infero-laterally into the superior joint space under extra-capsular local anaesthesia. After injection of 2 mL of sterile saline solution and 1 minute mixing by repeated opening and closing of the patient's mouth, samples of synovial fluid and saline solution mixture devoid of blood contamination were aspirated. Total cells were recovered by centrifugation at 15 000 *g* for 30 minutes, washed twice with PBS and subsequently stored at 4°C in 300 μL RNA-Safer Stabilizer Reagent (Omega Biotek Inc., GA, USA) until further analysis. Cell counting was performed with a haemocytometer using a phase contrast microscopy (Axiovert 100; Zeiss Co., Göttingen, Germany) and cell viability was calculated by exclusion of Trypan blue dye. Cells obtained from the synovial fluid were used for qPCR analysis and synovial fluid samples devoid of cells were used for osteoclast differentiation and bone resorption assays. Patients were given no medication for at least 2 weeks before the sample collection. To attain a consistent surgical procedure and treatment protocol, surgical interventions were performed by the same surgeon (CN). For each individual, the experiments were performed separately.

2.6 | Isolation of cytoplasmic RNA and synthesis of first-strand cDNA

Total cytoplasmic RNA was isolated from obtained cells using 400 μL of ice-cold lysis buffer containing 0.5% Igepal® CA-630

TABLE 1 Demographic data, joint pain and signs of articular bone degeneration

	Gender	Age	Joint pain		Signs of articular bone degeneration			
	Female	Years	VAS	Months	Erosion	Sclerosis	Osteophytes	Cyst
TMJ-OA								
n = 4	50%	53.6 ± 26.6	6.6 ± 1.5	17.3 ± 16.5	40%	0%	40%	20%
DDWR								
n = 2	50%	24.5 ± 2.1	1.5 ± 0.5	3.5 ± 0.5	0%	0%	0%	0%

Data are expressed as mean ± SD. DDWR, disk displacement with reduction; TMJ-OA, temporomandibular joint osteoarthritis; VAS, visual analogue scale.

(Sigma-Aldrich, Saint Louis, MO, USA) as described previously.[14] Isolated RNA was then quantified using a spectrophotometer (Synergy HT; Bio-Tek Instrument Inc., Winooski, VT, USA) and the first-strand cDNA was synthesized from 5 μg of total RNA using a reverse transcription kit following the manufacturer's instructions (SuperScrip™; Invitrogen, Grand Island, NY, USA).

2.7 | Expression of cytokines, chemokines, chemokine receptors and RANKL

To assess the pattern of immuno-inflammatory response detected in TMJ-OA and DDWR patients, the mRNA expression levels for the cytokines IL-1β, IL-4, IL-9, IL-17, IL-22 and TGF-β1; the CC-chemokines CCL5, CCL17 and CCL20; the CC-chemokine receptors CCR4, CCR5, CCR6 and CCR7, as well as the pro-bone-resorptive factor RANKL, were quantified by qPCR. The 18S rRNA expression levels were quantified as endogenous control. In brief, 100 ng of cDNA was amplified using the appropriate primers (Table S1) and a KAPA™ SYBR® Fast qPCR reagent (KAPA Biosystems, Woburn, MA, USA) in a StepOnePlus® equipment (Applied Biosystems, Singapore) using the following protocol: 95°C for 3 minutes and 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. To detect non-specific product formation and false-positive amplification, a melt curve of 95°C for 15 seconds, 60°C for 1 minute and 95°C for 15 seconds was performed.

2.8 | Differentiation and activation of $TRAP^+$ osteoclasts

Osteoclastogenesis assays were carried out by determining the number of cells expressing the osteoclast-specific marker tartrate-resistant acid phosphatase (TRAP), to analyse the osteoclast differentiation and activation in response to synovial fluid samples obtained from TMJ-OA and DDWR patients. In brief, mouse macrophage-monocyte RAW 264.7 cells (ATCC® TIB-71™) were cultured in 24-well culture plates at 5×10^3 cells/well in DMEM supplemented with 10% foetal bovine serum, 50 U/mL penicillin, 50 μg/mL streptomycin (Gibco Invitrogen Corp.) and 35 ng/mL rhRANKL (R&D Systems Inc.) for 3 days. Cells were then washed twice with fresh DMEM devoid of rhRANKL and cultured in the presence of synovial fluid samples at different dilutions (1:1 to 1:8) for 7 days. For comparison, negative control wells received fresh DMEM and positive control wells received fresh DMEM containing rhRANKL. Previous to cell fixation with 37% formaldehyde-citrate-acetone, TRAP staining was determined using a leukocyte acid phosphatase kit following the manufacturer's instructions (Sigma-Aldrich). $TRAP^+$ cells with three or more nuclei were considered osteoclasts and quantified using a visible light microscopy (AxiostarPlus; Carl Zeiss Co., Germany).

2.9 | Bone resorption-pit assay

Additionally, the osteoclast activity was analysed by quantifying the bone resorption-pit areas produced in response to synovial fluid samples obtained from TMJ-OA and DDWR patients. In brief, RAW 264.7 cells were cultured at 1×10^3 cells/well in the same conditions as described above using an osteo-assay 96-well plate (Corning® Osteo Assay Surface; Tewksbury, MA, USA), following the manufacturer's protocol. After 2 weeks, the supernatant was removed, cells were brushed away and the plate wells were washed once with 100 μL of 5% bleach solution for 5 minutes and twice with 200 μL of distilled water, allowed to dry at room temperature for 4 hours, and finally stained with 0.5% toluidine blue. Individual pits or multiple pit clusters were observed using a visible light microscopy and the area of resorption was measured using an image analysis software (AxioStarPlus; Carl Zeiss Co., Germany).

2.10 | Statistical analysis

The qPCR data were analysed using the StepOne Software v2.2.2 (Applied Biosystems) and the relative quantification, expressed as mRNA fold-change mean ± SD, was obtained using the $2^{-\Delta\Delta Ct}$ method. The TMJ-OA data were calculated by normalizing the cytokine, CCL, CCR or RANKL mRNA expression to 18S rRNA expression and using the DDWR normalized data as reference. Osteoclast differentiation was expressed as $TRAP^+$ cells/well mean ± SD and bone resorption was expressed as resorption-pits μm^2/well mean ± SD. The statistical analysis was performed using the SPSS 22.0 software (IBM Corp., Armonk, NY, USA). The normality of data distribution was determined using the Kolmogorov-Smirnov test and differences were determined using the unpaired Student's *t* test or ANOVA and Tukey post-hoc tests. Correlation coefficients between RANKL, cytokines and joint pain duration in months were obtained using the Pearson's test, and correlation coefficients between RANKL, cytokines, joint pain measured as VAS score and articular bone degeneration scale were obtained using the Spearman's test. *P* values <.05 were considered significant.

3 | RESULTS

3.1 | Expression of cytokines and RANKL

The mRNA expression for IL-1β, IL-4, IL-17, IL-22 and RANKL was detected in all samples obtained from TMJ-OA or DDWR patients. IL-9 and TGF-β1 were expressed in all samples obtained from DDWR patients; however, 60% and 80% of the TMJ-OA samples expressed IL-9 and TGF-β1 mRNAs, respectively. IL-17 was the highest over-expressed cytokine detected within TMJ-OA, with a fold-change of 13.8 ($P < .001$) (Figure 1A). Similarly, IL-1β (8.3-fold, $P < .001$), IL-22 (3.6-fold, $P = .006$) and RANKL (5.6-fold, $P = .004$) were also significantly over-expressed in TMJ-OA compared with DDWR. Conversely, IL-4, IL-9 and TGF-β1 were down-regulated in TMJ-OA. When the mRNA relative expression was re-calculated considering the adjusted IL-4, IL-9 and TGF-β1 expression in TMJ-OA as 1 as a reference for fold-change, the IL-4 and TGF-β1 expression in DDWR was 6.4-fold ($P = .012$) and 3.4-fold

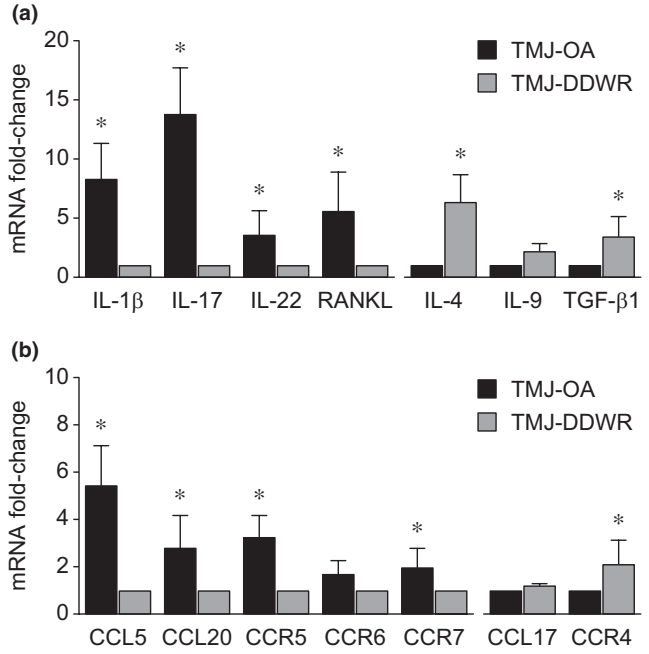

FIGURE 1 Cytokine, RANKL, chemokine and chemokine receptor expression. Quantification of (A) cytokines IL-1β, IL-4, IL-9, IL-17, IL-22, and TGF-β1 and pro-bone resorptive factor RANKL as well as (B) chemokines CCL5, CCL17, and CCL20 and chemokine receptors CCR4, CCR5, CCR6, and CCR7 is represented as mRNA fold-change in total cells obtained from temporomandibular joints affected by osteoarthritis (TMJ-OA) or disk displacement with reduction (DDWR). For quantification of IL-1β, IL-17, IL-22, RANKL, CCL5, CCL20, CCR5, CCR6, and CCR7 mRNA expression in cells obtained from DDWRs was considered as 1 as a reference for fold-change. For quantification of IL-4, IL-9, TGF-β1, CCL17, and CCR4 mRNA expression in cells obtained from TMJ-OAs was considered as 1 as a reference for fold-change. Data from 10 independent experiments are shown as mean ± standard deviation. Each experiment was performed in duplicate. *$P < .05$. IL, interleukin; CCL, CC-chemokine; CCR, CC-chemokine receptor; TGF, transforming growth factor; RANKL, receptor activator of nuclear factor-κB ligand

($P = .041$) higher, respectively, than the levels detected in TMJ-OA (Figure 1A). No significant differences were detected in the IL-9 expression between TMJ-OA and DDWR.

3.2 | Expression of chemokines and chemokine receptors

The mRNA expression for CCL5, CCL17, CCL20, CCR4, CCR5, CCR6 and CCR7 was detected in all samples obtained from TMJ-OA or DDWR patients. In TMJ-OA, CCL5 (5.4-fold, $P = .001$), CCL20 (2.8-fold, $P = .018$), CCR5 (3.2-fold, $P = .001$) and CCR7 (2.1-fold, $P = .026$) were significantly over-expressed compared with DDWR (Figure 1B). Conversely, CCR4 was down-regulated in TMJ-OA. When the mRNA relative expression was re-calculated, considering the adjusted CCR4 expression in TMJ-OA as 1 as a reference for fold-change, the CCR4 expression in DDWR was 2.1-fold ($P = .011$) higher than the levels detected in TMJ-OA (Figure 1B). No significant differences were detected in the CCR6 and CCL17 expression between TMJ-OA and DDWR.

3.3 | Correlation analysis between RANKL, cytokines, joint pain and imagenological scale of articular bone degeneration

The analysis of correlations between the mRNA expression of RANKL and cytokines, and joint pain and the imagenological scale of signs of articular bone degeneration yielded a significant positive correlation between RANKL and bone degeneration ($P < .001$) and between IL-1β, IL-17 and IL-22 and bone degeneration ($P = .025$, $P = .027$ and $P < .001$, respectively) in samples obtained from TMJ-OA patients (Table 2). Conversely, there was no significant correlation between RANKL, IL-1β, IL-17 and IL-22 and joint pain, measured as VAS score or duration of illness in months.

3.4 | Correlation analysis between RANKL and cytokines

Table 3 shows the correlation analysis between the mRNA expression for RANKL and the cytokines IL-1β, IL-17 and IL-22 in samples obtained from TMJ-OA patients. There was a significant positive correlation between RANKL and IL-1β ($P = .018$), IL-17 ($P = .024$) and IL-22 ($P = .001$). Conversely, there was no correlation between RANKL and the cytokines IL-4 and IL-9; the chemokines CCL5, CCL17 and CCL20; and the chemokine receptors CCR4, CCR5, CCR6 and CCR7.

TABLE 2 Correlation analysis between RANKL, cytokines, joint pain and imagenological scale of articular bone degeneration in TMJ-OA patients

	RANKL	IL-1β	IL-17	IL-22	
Joint pain (VAS)	0.778	0.332	0.319	0.677	*r*-Spearman
	$P = .068$	$P = .521$	$P = .537$	$P = .140$	*P* value
Joint pain (months)	−0.718	−0.245	−0.232	−0.607	*r*-Pearson
	$P = .108$	$P = .640$	$P = .658$	$P = .201$	*P* value
Bone degeneration scale	0.999	0.868	0.862	0.994	*r*-Spearman
	$P < .001$	$P = .025$	$P = .027$	$P < .001$	*P* value

The Pearson's and Spearman's correlation coefficients (*r*-Pearson and *r*-Spearman) between RANKL, IL-1β, IL-17 or IL-22 mRNA expression and joint pain and imagenological scale of signs of articular bone degeneration was calculated in TMJ-OA patients. For the VAS scores, joint pain was analysed during mandibular movements. IL, interleukin; RANKL, receptor activator of nuclear factor-κB ligand; VAS, visual analogue scale.

3.5 | Osteoclast formation and activation

The number of induced TRAP$^+$ osteoclasts was significantly higher in response to synovial fluid samples obtained from TMJ-OA as compared with samples from DDWR patients (Figure 2A,B), being 60.0 vs 21.2 TRAP$^+$ cells/well upon undiluted synovial fluid ($P < .001$) and 31.4 vs 10.2 TRAP$^+$ cells/well upon 1:2 synovial fluid dilution ($P = .008$). Interestingly, when RAW 264.7 cells were induced in the presence of synovial fluid obtained from TMJ-OA, higher number of TRAP$^+$ osteoclasts were observed as compared with the same cells cultured in presence of rhRANKL ($P > .05$), used as positive control.

3.6 | Bone resorption-pit production

Similarly, the resorptive activity was significantly higher when osteoclasts were exposed to synovial fluid samples obtained from TMJ-OA as compared with samples from DDWR patients (Figure 3A,B), being 118.6 vs 41.0 μm^2/well upon undiluted synovial fluid ($P < .001$) and 56.0 vs 23.2 μm^2/well upon 1:2 synovial fluid dilution ($P = .016$), reaching bone resorption levels higher than those detected when osteoclasts were induced in presence of rhRANKL ($P > .05$), used as positive control.

4 | DISCUSSION

TMJ-OA is a prevalent degenerative TMD characterized by inflammation and abnormal resorption of articular cartilage and sub-chondral bone.[2] Pro-inflammatory cytokines play a key role in the TMJ-OA inflammation, and an increment in the recruitment of cytokine-producing immuno-inflammatory cells infiltrating the TMJ synovium has been associated with internal joint derangements and degenerative deterioration of disk and articular surfaces.[5,14] The results of the present pilot study revealed that higher levels of IL-1β, IL-17 and IL-22, associated with the Th1, Th17 and Th22-pattern of immuno-inflammatory response, were detected in TMJ-OA as compared with DDWR, and these increased cytokine levels significantly correlated with the enhanced RANKL expression and the detection of signs of articular bone degeneration. In addition, increased levels of CCL5, CCL20, CCR5 and CCR7, responsible for the Th1, Th17 and Th22-type cell recruitment, were detected in TMJ-OA as compared with DDWR. The small sample size and the lack of generalizability, however, could be a clear limitation of the present study; consequently, the results must be interpreted with caution.

In different studies, pro-inflammatory cytokines including IL-1β, IL-17 and TNF-α have been identified and associated with synovial inflammation, connective tissue destruction, articular cartilage deterioration and sub-chondral bone resorption.[5,11,14,25,26] In fact, IL-1β, IL-17 and TNF-α may exert osteoclastogenic and pro-bone-resorptive activities by inducing the production of RANKL by osteoblasts and Th17 lymphocytes.[22,27] Moreover, IL-17 may facilitate local inflammation by recruiting and activating immuno-inflammatory cells, which result in an abundance of inflammatory cytokines, such as IL-1β and TNF-α, which subsequently enhance the production of RANKL by osteoblasts and Th17 lymphocytes.[28] Meanwhile, Th17 lymphocytes have the capacity to induce differentiation and activation of osteoclasts by

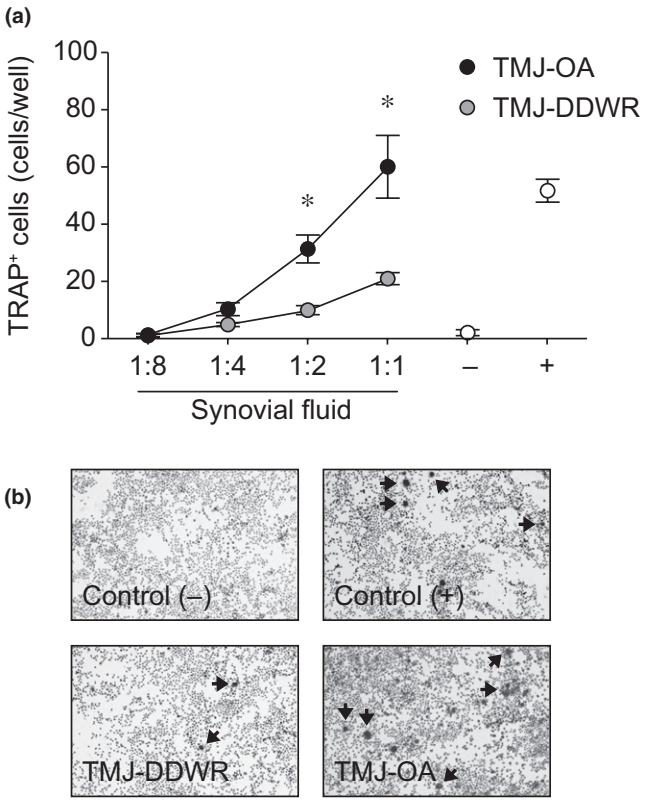

FIGURE 2 Osteoclast activation. A, TRAP$^+$ osteoclasts induced in response to synovial fluid obtained from temporomandibular joints affected by osteoarthritis (TMJ-OA) or disk displacement with reduction (DDWR). Non-induced RAW 264.7 cells were used as negative control (–) and RAW 264.7 cells maintained in DMEM supplemented with rhRANKL were used as positive control (+). Quantification of TRAP$^+$ osteoclast is represented as cells/well. Data from seven independent experiments are shown as mean ± standard deviation. Each experiment was performed in duplicate. B, Representative experiment quantified in (A) (magnification ×100). *$P < .05$. TRAP, tartrate-resistant acid phosphatase

TABLE 3 Correlation analysis between RANKL and cytokines in TMJ-OA patients

	IL-1β	IL-17	IL-22	
RANKL	0.795	0.774	0.923	*r*-Pearson
	$P = .018$	$P = .024$	$P = .001$	*P* value

The Pearson's correlation coefficient (*r*-Pearson) between RANKL and cytokines IL-1β, IL-17 or IL-22 mRNA expression was calculated in TMJ-OA patients. IL, interleukin; RANKL, receptor activator of nuclear factor-κB ligand.

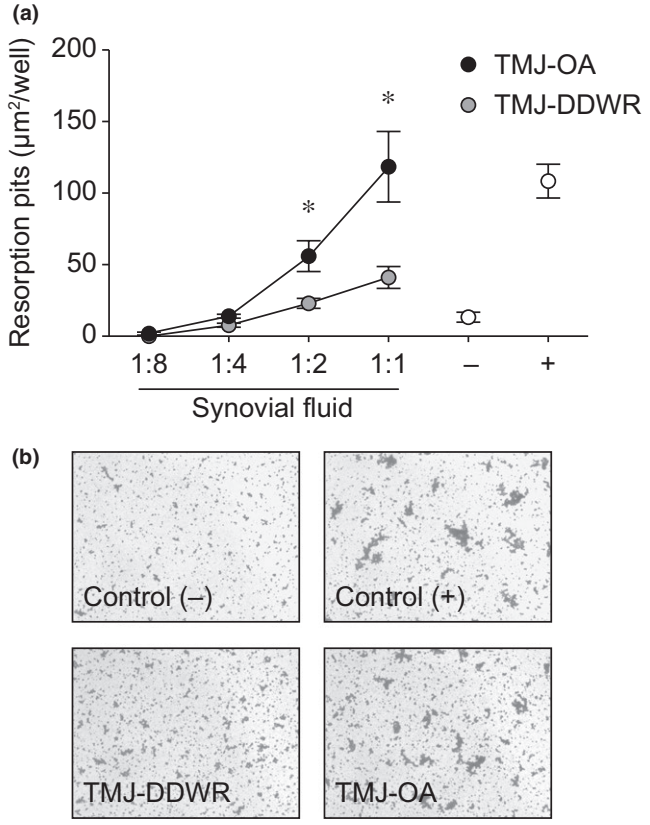

FIGURE 3 Bone resorption-pit assay. A, Bone resorption-pits induced in response to synovial fluid obtained from temporomandibular joints affected by osteoarthritis (TMJ-OA) or disk displacement with reduction (DDWR). Synovial fluid dilutions from 1:1 to 1:8 were used for comparisons. Non-induced RAW 264.7 cells were used as negative control (–) and RAW 264.7 cells maintained in DMEM supplemented with rhRANKL were used as positive control (+). Quantification of resorption-pit area is represented as μm^2/well. Data from seven independent experiments are shown as mean ± standard deviation. Each experiment was performed in duplicate. B, Representative experiment quantified in (A) (magnification ×200). *$P < .05$

directly acting in their precursors and mature osteoclasts through RANKL expression.[22,29] In this study, when RAW 264.7 osteoclast precursor cells were induced in presence of synovial fluid obtained from TMJ-OA, higher number of mature osteoclasts and greater areas of bone resorption were detected, as compared with the same cells cultured in presence of synovial fluid obtained from DDWR. Similarly, an increment in the number of mature osteoclasts and areas of bone resorption were detected when cells were induced in presence of synovial fluid obtained from TMJ-OA, as compared with cells stimulated with rhRANKL, used as positive control. These data suggest that RANKL, together with other pro-bone-resorptive mediators present within TMJ-OA synovial fluid, such as IL-1β, IL-17 and IL-22, play a role in the osteoclast differentiation and activation.

This is the first report proposing the role of IL-22 in the pathogenesis of TMJ-OA. The data presented in this study demonstrate the over-expression of IL-22 in TMJ-OAs and reveal for the first time that the increased levels of IL-22 detected in TMJ-OAs are associated with enhanced expression of the pro-bone-resorptive factor RANKL and increased detection of signs of articular bone degeneration. Further support of this hypothesis comes from the fact that IL-22 plays a pro-inflammatory role through the synergistic activity with IL-1β and TNF-α.[20,30] In addition, IL-22 can induce indirectly osteoclastogenic and bone resorption by induction of Th17 lymphocyte activity and IL-17 production.[31] In fact, previous reports have detected over-expressed levels of IL-22 in rheumatoid arthritis synovial fibroblasts, demonstrating a pathogenic role of IL-22 in the rheumatic joint inflammation and destruction through the modulation of the IL-1β and IL-17R expression.[32,33]

CCLs and CCRs play a pivotal role in the recruitment of lymphocytes towards inflamed tissues.[34] Indeed, CCLs and CCRs have been proposed as therapeutic targets in rheumatoid arthritis due to its strong capacity to chemoattract T lymphocytes towards affected joints.[34] In the present study, increased levels of Th1-, Th17- and Th22-related CCLs and CCRs were found in TMJ-OA as compared with DDWR. Thus, it could be hypothesized that the increased detection of Th1, Th17 and Th22 type of cytokines in synovial fluid of OA-ATM patients could be a consequence not only of the differentiation and activation of these T-cell phenotypes locally in inflamed tissues, in a site-specific manner, but also of the increased recruitment of differentiated Th1, Th17 and Th22 lymphocytes from regional lymph nodes that drain the ATMs towards the affected joints, following a specific chemoattractive gradient.

In this study, the role of the Th1/Th17/Th22 immuno-inflammatory cell pathways in the pathogenesis of the TMJ-OA was examined, by analysing the production of IL-1β, IL-17 and IL-22. Similarly, it was analysed the role of the Th2/Th9/Tregulatory cell pathways, responsible for the production of IL-4, IL-9 and TGF-β1, respectively. These data demonstrated that the expression of IL-4 and TGF-β1 was down-regulated in TMJ-OA. In fact, IL-4 and TGF-β1 levels in DDWR were higher than the levels detected in TMJ-OA. This may be related to the role of IL-4 and TGF-β1 in inhibiting expression of pro-inflammatory cytokines, minimizing the TMJ inflammation and destruction. In fact, although the Th1/Th17/Th22 axis has been associated with the onset, progression, and severity of the joint inflammation and destruction in rheumatoid arthritis, the Th2/Th9/Tregulatory axis has been related to disease healing.[19-21]

5 | CONCLUSION

Overall, these preliminary results lead us to propose that the pro-inflammatory and destructive activity of IL-1β, IL-17 and IL-22 during the pathogenesis of TMJ-OA might be through up-regulation of RANKL, which in turn could induce the differentiation and activation of osteoclasts and the subsequent resorption of sub-chondral bone characteristic of the disease.

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CONFLICT OF INTEREST

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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