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Local cytokine profiles in knee osteoarthritis: elevated synovial fluid interleukin-15 differentiates early from end-stage disease

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Summary

Objective: Much of what is known about the inflammatory response in the synovial membrane (SM) of patients with osteoarthritis (OA) comes from studies of synovial tissues from end-stage disease. In this study, we sought to better characterize the inflammatory infiltrate in symptomatic patients with early signs of knee OA, and to determine how inflammatory cell populations relate to the pattern of cytokine and degradative enzyme production.

Methods: Study populations comprised patients with degenerative meniscal tears and early cartilage thinning undergoing arthroscopic procedures (early OA) and patients undergoing total knee replacement for end-stage OA. Quantitative real-time polymerase chain reaction (PCR) was used to measure expression of SM cytokines and enzymes implicated in the pathogenesis of inflammatory arthritis and OA, as well as cell lineage-specific markers. We quantified synovial fluid (SF) cytokines and enzymes by enzyme-linked immunosorbent assay (ELISA) and SM cell populations by immunohistochemistry.

Results: We found increased levels of SF interleukin-15 (IL-15) protein in the early knee OA patients when compared to end-stage OA. Both SF IL-15 protein and numbers of CD8 cells within SM correlated with matrix metalloproteinase-1 (MMP-1) and three levels. TNF-α, IL-6 and IL-21 were also detectable in the SF of the majority of patients, and IL-15 levels were associated with IL-6 levels.

Conclusion: IL-15 is elevated in early knee OA, suggesting activation of an innate immune response in the SM. The association of IL-15 expression with CD8 transcripts and MMPs implicates this cytokine in OA pathogenesis and as a candidate therapeutic target.

Key words: Osteoarthritis, Inflammation, Synovium, Synovitis, Interleukin-15.

Introduction

There is growing interest in defining the role inflammation plays in osteoarthritis (OA), which is often associated with low-grade synovitis[1]. Synovitis has been associated with symptoms and progression of cartilage degeneration[2,3]. At the cellular level, infiltration of macrophages and perivascular T and B lymphocytes is observed, and these infiltrates have been demonstrated in both early and advanced disease[4–7]. However, the stimuli and consequences of this infiltration remain unclear.

Cytokines produced in innate immune responses, such as interleukin-1β (IL-1β), IL-6, tumor necrosis factor-α (TNF-α) and IL-15, are found routinely in rheumatoid arthritis (RA) joint tissues. Blockade of each of these cytokines has met with therapeutic success in RA patients[8–11]. Evidence of lymphocytic activation, such as autoantibody and TH1 cytokine production, and association of the Major Histocompatibility Complex (MHC) class II "shared epitope" with aggressive disease suggest that the adaptive immune response also plays a role[12–14]. In OA, the cellular constituents of the infiltrates are similar to RA, but infiltration is usually less robust[15]. Synovial fluid (SF) levels of IL-1β, IL-6 and TNF-α are detectable in some but not all OA patients, and are generally lower than in inflammatory arthritis[16]. Furthermore, evidence of lymphocyte activation and MHC association are more variable than in RA, suggesting inconsistent activation of adaptive immunity[17–19]. Despite these differences, inflammation is characteristic of many advanced OA patients but early disease has been insufficiently studied, due to the complexity of defining early OA and difficulty obtaining sufficient tissue for study. As a result,

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we know little about inflammatory pathways activated early on, when therapeutic intervention may be most desirable.

We sought to better characterize the synovial inflammatory milieu in patients with early signs of OA compared to end-stage disease. Furthermore, we sought to determine how inflammatory cells and cytokine expression relate to proteolytic enzyme production in knee OA. Expression of T-lymphocyte and macrophage lineage markers was examined in synovial tissue, and cytokine (IL-1β, IL-6, TNF-α, IL-15, IL-21, and IL-2) and matrix metalloproteinase (MMP-1, MMP-3, MMP-9, and MMP-13) levels were measured. The proteases were chosen based on their documented up-regulation in patients with OA, while the cytokines were chosen based on their known roles in inflammatory arthritis. IL-1β, IL-6, and TNF-α have been demonstrated in SF or tissue of some (mainly advanced) OA patients, but levels of common-γ chain cytokines (IL-15, 21 and 2) in the local synovial environment have not been previously reported.

Methods

PATIENTS

Early knee OA

Given the association between meniscal pathology and progressive cartilage damage, we focused on patients presenting for arthroscopic meniscal repair[20–22]. Patients ($n = 19$) with degenerative meniscal tears scheduled for arthroscopic surgery were recruited from the practices of two orthopedic surgeons at Hospital for Special Surgery (HSS). Inclusion criteria included age $\geq$40, knee pain $\geq$3 months, cartilage abnormality and degenerative meniscal tear documented on pre-operative magnetic resonance imaging (MRI) or by intra-operative assessment. We excluded patients with Kellgren–Lawrence (K–L) grade >2 on pre-operative knee films, a history of traumatic or sports-related knee injury, inflammatory arthritis, previous knee surgery, and intra-articular corticosteroid or hyaluronan within 3 months of surgery.

End-stage knee OA

Patients with advanced knee OA ($n = 15$) meeting ACR criteria (clinical + radiographic) who were undergoing total knee replacement surgery were recruited. Patients were required to have pre-operative K–L score $\geq$2 and intra-operative evidence of full-thickness chondral loss. Exclusion criteria and histologic analyses of synovial membrane (SM) from these patients were reported previously[6]. Briefly, patients with significant comorbidities, avascular necrosis, or inflammatory arthropathy were excluded.

The study was approved by the Institutional Review Board at HSS, and informed consent was obtained from all patients.

CLINICAL, RADIOGRAPHIC, AND MRI DATA

Demographic data were obtained by chart review. Pre-operative knee films were assessed using the K–L score in a blinded fashion by a musculoskeletal radiologist. Pre-operative MRI was available for review on eleven early OA patients, and was analyzed by a single MR radiologist. MR imaging was performed on a 1.5 or 3.0 Tesla imaging system (General Electric Healthcare, Milwaukee, WI). Fast spin echo images were obtained to assess articular cartilage using a previously validated cartilage-sensitive pulse sequence[23]. Meniscal integrity was scored as follows: 0 = normal, 1 = intrasubstance degeneration, 2 = degenerative/horizontal tear, 3 = complex tear with radial component or displaced fragment. Cartilage integrity was evaluated according to the Modified Outerbridge scale[23].

SYNOVIAL TISSUE AND JOINT FLUID SAMPLES

SM was obtained from the suprapatellar region, abutting the trochlear cartilage. Previous investigation demonstrated lymphocytic perivascular infiltrates in this location in 58% of end-stage OA patients[6]; the end-stage specimens included here represent a randomly sampled subset of these patients. Tissue specimens were frozen and stored at −70°C. SF was obtained by aspiration just prior to surgical intervention. After centrifugation to remove cells and debris, aliquots were frozen and stored at −70°C.

QUANTITATIVE PCR ANALYSIS

Total RNA was extracted from homogenized SM using a purification kit (Gentra Systems, Inc., Minneapolis, MN). All RNA was DNAse-treated, oligo-dT primed, and cDNA synthesized. mRNA levels of cytokines (IL-1β, IL-6, TNF-α, IL-15, IL-21 and IL-2), cell lineage-specific molecules (CD3δ, CD4, CD8, CD14, and CD68) and metalloproteinases (MMP-1, MMP-3, MMP-9 and MMP-13) were measured by real-time PCR (RT-PCR) using specific primers (sequences available upon request) and iQ Sybr-Green Supermix (BioRad, Hercules, CA). After normalizing to GAPDH, expression levels were calculated relative to the mean value for each gene product in the end-stage OA group.

MEASUREMENT OF CYTOKINE, MMP-1 AND MMP-3 LEVELS

SF IL-15 and IL-6 levels were measured using R & D systems Quantikine™ ELISA kits (Minneapolis, MN; lower limit of detection = 2 for IL-15 and 0.7 for IL-6). IL-1β, TNFα, IL-2 and IL-21 were measured using Invitrogen Biosource™ ELISA kits (Camarillo, CA; lower limits of detection = 0.06, 0.09, 4, and 15 pg/ml, respectively). MMP-1 and MMP-3 levels were measured using the Amersham Biotrak Activity Assay Systems (GE Healthcare, Piscataway, NJ). These assays incorporate an activation step to convert pro- to active MMP and a modified urokinase detection system to detect total MMP concentrations[24]. Omission of the activation step allows detection of active enzyme only. Limits of detection are 0.1 ng/ml (MMP-1) and 0.25 ng/ml (MMP-3).

To determine whether ELISA results could be false positives from interfering rheumatoid factor (RF) or other heterotopic antibodies, the IL-15 ELISA was repeated with additional SF specimens including three of the higher expressors from the early OA group. Heteroblock™, a reagent which blocks crosslinking from heterotopic antibodies (Omega Biologicals Inc., Bozeman MT), was added to ELISA plate wells at 500 ng/ml prior to fluid specimens[25].

IMMUNOHISTOCHEMICAL STAINING

Additional SM was available from seven end-stage and four early knee OA patients. Six micron sections of paraffin-embedded, formalin fixed tissue were stained using standard immunoperoxidase technique and monoclonal antibodies (mAbs) against CD3ε (clone PS1, T cells), CD8 (clone 1A5, cytotoxic T cells), and CD68 (clone KP-1, macrophages) (all from Ventana Inc., Tucson, AZ). Isotype matched irrelevant mAbs were used as controls. Positively stained cells were quantified in fields containing synovial lining layer with underlying vascularized subintima. Results are expressed as mean number of positive cells per high power field (hpf). To localize cells expressing the IL-15 receptor α-chain, immunoperoxidase staining with polyclonal goat-anti-human IL-15 Rα (N-19, Santa Cruz Biotechnology, Inc) was perfomed.

STATISTICAL ANALYSIS

Given small sample size and some irregularly distributed variables, nonparametric tests were applied throughout. The Mann–Whitney *t*-test was performed to evaluate differences between group medians, and Spearman's correlation coefficients were calculated using Prism 5.0 software (GraphPad, Inc., San Diego, CA). General linear modeling (GLM) was performed using SAS 9.1 to investigate the influence of BMI, age, and gender on mRNA levels (CD8, IL-15, MMP-1 and MMP-3). Only CD8 relative expression (RE) did not fit a normal distribution (Kolmogorov–Smirnov test) but was distributed exponentially, and so was log-transformed prior to inclusion in GLM. Protein levels were measured in a subset of patients (due to SF availability), therefore SF IL-15 and MMP-1 levels were stratified by gender, BMI (obese/non-obese) and age (<65/65+); between group differences were analyzed by the Mann–Whitney test.

Results

PATIENT CHARACTERISTICS

Demographic characteristics of all patients are shown in Table I. Early OA patients tended to be younger (Mann–Whitney $P = 0.133$), more predominantly male (Chi-square $P = 0.21$), and have lower BMI (Mann–Whitney $P = 0.144$), though these differences were not statistically significant. Of eleven early OA patients with MRI available, all had at least a grade 2 (ulceration/fibrillation/fissuring of articular cartilage involving less than 50% thickness) diffuse cartilage lesion in one or more locations. All meniscal lesions were $\geq$grade 2: eight medial, two lateral, and one bilateral. Review of operative reports from these 11 patients revealed no significant discrepancies in MRI detection of meniscal tears, and correlation between MRI and

Table I
Characteristics of patients included in the study

	Early OA	End-stage OA
N	18	15
Median age (interquartile range)	60.5 (52.5–70)	68.0 (65.5–71.5)
Median BMI (interquartile range)	27.46 (23.27–33.05)	32.74 (27.79–35.08)
Male/Female	10/9	6/9
Median K–L score (interquartile range)	1 (0–1)	3 (3–3)

intra-operative grading of femoral cartilage abnormality (Spearman *r*'s: medial = 0.58, lateral = 0.71). The eight remaining patients without available MRI had chart documentation (outside MRI report and/or operative report) of degenerative meniscal tear and diffuse cartilage loss or fibrillation.

CELL-SPECIFIC TRANSCRIPT LEVELS IN EARLY VS END-STAGE OA

Cell-specific genes were measured in SM, focusing on T-lymphocyte (CD3δ, CD4 and CD8) and macrophage (CD14, CD68) markers. Levels of these transcripts were similar in both OA groups (data not shown).

RELATIONSHIP BETWEEN CELL NUMBERS AND GENE EXPRESSION IN SYNOVIUM

To determine whether mRNA levels of cell-specific markers reflected cellular density, immunohistochemical staining was carried out for CD3, CD8 and CD68. Paraffin-embedded tissue from seven end-stage and four early knee OA patients were analyzed. Typical staining patterns using anti-CD3ϵ, anti-CD8, and anti-CD68 mAbs are shown in Fig. 1. Antibodies against CD3δ and CD8 stained cells with morphological features of lymphocytes. These cells were mainly in perivascular aggregates. As expected, macrophages were seen in two distributions: within the lining, and scattered throughout sublining areas and lymphocytic accumulations. These patterns were similar in both patient groups, but lymphocytic infiltrates were identified in all four early OA vs four of seven (57%) end-stage specimens.

There were correlations between mRNA levels of these markers and numbers of positively stained cells per hpf. Spearman correlation coefficients were 0.460 for CD3δ mRNA vs number of CD3ϵ+ cells/hpf; 0.636 for CD8 mRNA vs CD8+ cells/hpf; and 0.454 for CD68 mRNA vs CD68+ cells/hpf. Only the relationship between CD8 mRNA and cell density achieved statistical significance (95% CI: 0.04–0.90, $P = 0.035$). Similarly, a previous report indicated that RT-PCR analysis of CD8 transcripts reflects cell numbers in peripheral blood[26]. These results support use of transcript levels of CD8 as an approximation of CD8+ lymphocyte abundance in SM when tissue is limiting.

SYNOVIAL IL-1β, IL-6 AND TNF-α TRANSCRIPT AND PROTEIN ABUNDANCE IN EARLY AND END-STAGE OA PATIENTS

SM transcript levels of IL-1β, IL-6, and TNF-α were first measured using quantitative RT-PCR. Expression levels of each transcript, relative to the mean of the end-stage OA group, were similar in end-stage and early OA (Fig. 2a). SF collected on the day of surgery was available from 15 early and 10 end-stage OA patients. SF protein levels of these cytokines (Fig. 2b) were not significantly different in the two patient groups. SF IL-6 levels were most variable and appeared to trend higher in some of the early patients (ns). In comparison, TNF-α levels [Fig. 3(b)] were comparable in all patients, while IL-1β was detectable in only four end-stage and one early OA specimen.

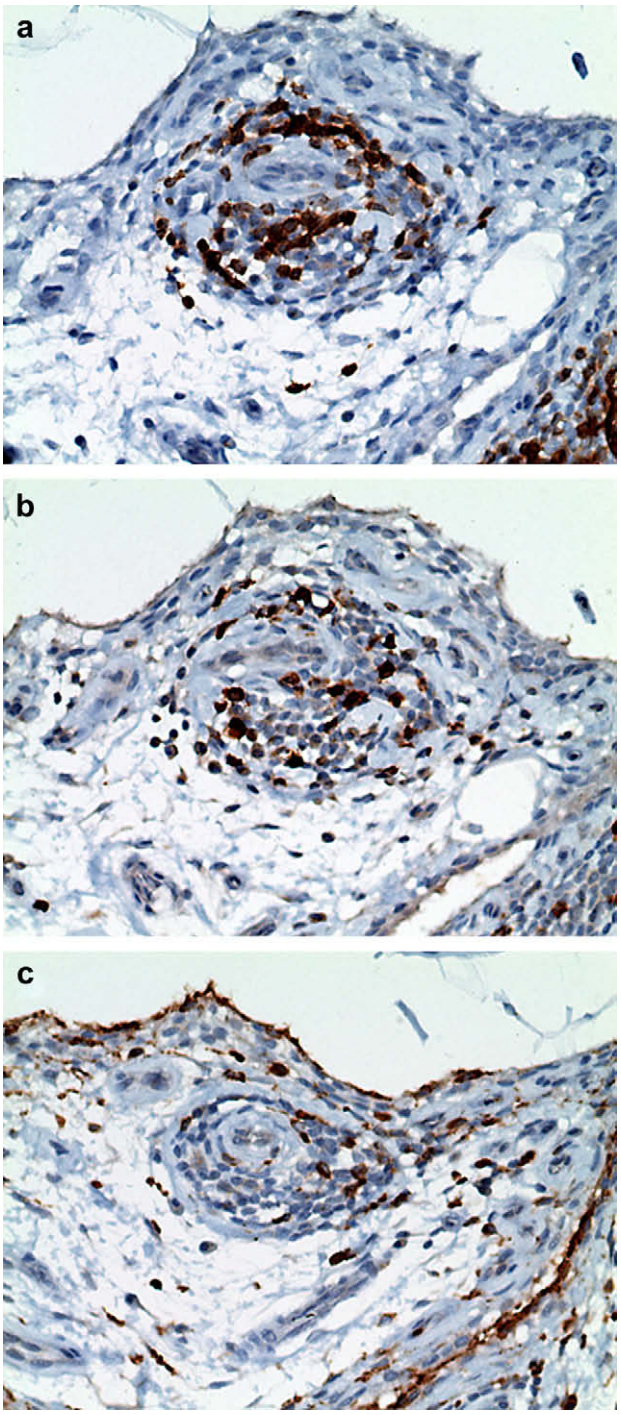

Fig. 1. Immunohistochemical staining of a representative perivascular lymphoid aggregate in serial synovial sections from an early knee OA patient. Standard immunoperoxidase technique was used as described in the text. Staining with the (a) anti-CD3ϵ mAb (b) anti-CD8 mAb and (c) anti-CD68 mAb is shown. Positive staining appears brown.

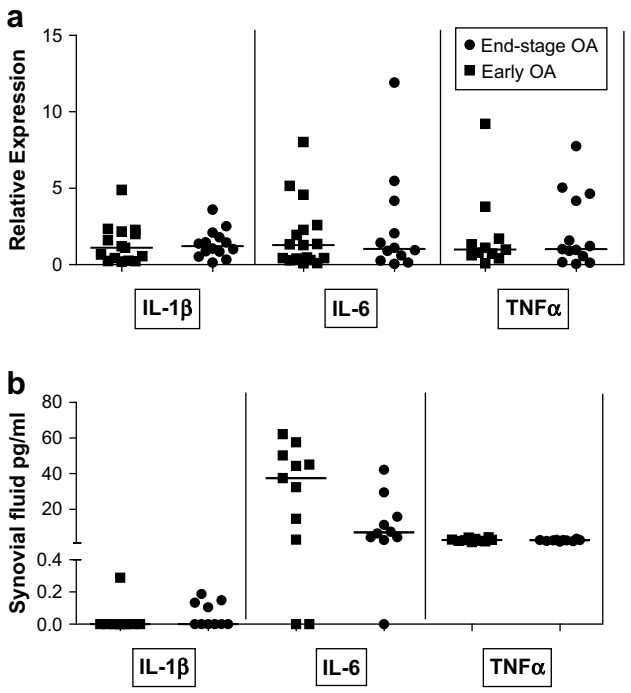

Fig. 2. IL-1β, IL-6 and TNF-α levels in the SM and SF of patients with early (■) and end-stage knee OA (●). Transcript levels in SM were measured by qRT-PCR and expressed relative to the mean value in the end-stage OA patients (Relative expression = 1). (a) IL-1β, IL-6 and TNF-α transcript levels in SM and (b) SF protein levels did not differ significantly between early and end-stage knee OA patients, although SF IL-6 levels trended higher in the early patients (Mann–Whitney P = ns).

IL-15 TRANSCRIPT AND PROTEIN LEVELS IN OA SM AND SF

Synovial transcript and protein levels of IL-15 were examined next. Transcript levels were comparable in the two groups, but SF protein levels were significantly higher in early OA patients [Fig. 3(a and b) , left panels] and were detectable in all early and most end-stage patients (9/10). Levels of IL-15 protein in end-stage patients were consistent with one previous report comparing active RA to a small number of end-stage OA controls, but no reports in early disease patients were found[27]. The IL-15 ELISA was repeated in five specimens, in the presence of Heteroblock™ to eliminate interference from RF. Minimal effect on results was observed (Table II).

A significant relationship between CD8 and IL-15 transcript levels in the combined OA patients was found (Spearman's $r = 0.415$, 95% CI: 0.02–0.70) which was observed when analyzing the early OA patient group alone ($r = 0.508$, 0.01–0.80). This relationship is consistent with the known role of IL-15 in recruitment and survival of CD8+ lymphocytes.

OTHER COMMON-γ CHAIN CYTOKINES IN THE SYNOVIAL ENVIRONMENT OF PATIENTS WITH EARLY AND END-STAGE OA

Given the IL-15 findings, we decided to measure two other common-γ chain cytokines, IL-21 and IL-2. IL-21 relative mRNA levels were statistically higher in SM from the end-stage patients [Fig. 3(a), middle panel], but differences were not seen in protein levels [Fig. 3(b)]. SM IL-2 mRNA was detectable in over half of patients, but protein was only detectable in SF from four end-stage patients.

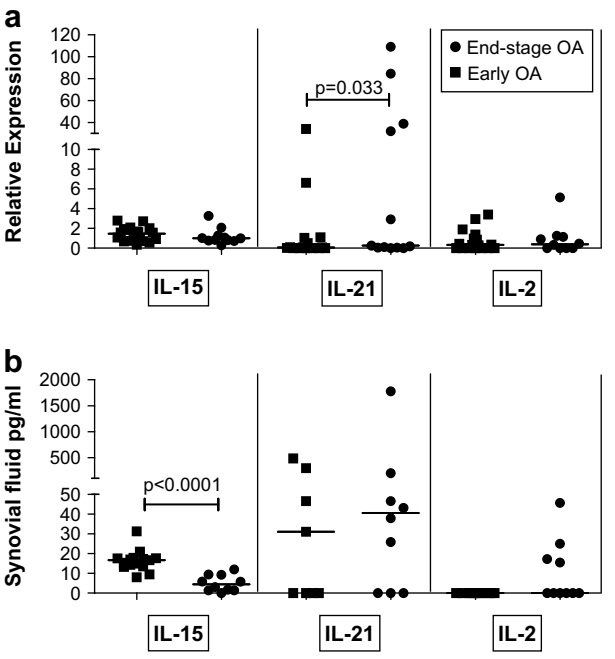

Fig. 3. IL-15, IL-21 and IL-2 levels in the SM and SF of patients with early (■) and end-stage knee OA (●). (a) mRNA levels (expressed as in Fig. 2) of IL-15 and IL-2 were similar in early and end-stage knee OA patients, while IL-21 transcript levels were significantly higher in the end-stage knee OA patients. (c) IL-15 protein levels in SF were significantly higher in early OA than in end-stage disease ($P < 0.001$), but fluid levels of L-21 and IL-2 were not. IL-2 was only detectable by ELISA in 4 end-stage patients.

RELATIONSHIPS BETWEEN IL-15 AND OTHER SYNOVIAL CYTOKINES

An association between IL-15 and IL-6 mRNA (Spearman $r = 0.38$, 95% CI: 0.001–0.669) and protein levels (Spearman $r = 0.58$, 0.20–0.80) was observed. IL-15 mRNA levels correlated with both TNF (Spearman $r = 0.51$, 0.09–0.77) and IL-21 (Spearman $r = 0.41$, 0.03–0.69) transcript levels, but not protein.

LOCALIZATION OF IL-15 RECEPTOR α-CHAIN IN SYNOVIUM

Both secretion and recognition of IL-15 is dependent on expression of the α-chain (IL-15 Rα) of the trimeric IL-15 receptor[28]. We performed immunocytochemical staining for IL-15 Rα, to localize the potential cellular sources and targets of IL-15 in SM. Figure 4 depicts results in three representative end-stage OA patients. IL-15 Rα+ cells were primarily observed in the synovial lining layer and endothelium.

Table II
Measurement of SF IL-15 (pg/ml) in the presence or absence of RF blockade

Specimen	+Blockade	−Blockade	Δ pg/ml	% Difference*
578 (early)	26.6	28.0	−1.4	−5.0
581 (early)	14.8	15.9	−1.1	−6.6
602 (early)	19.1	18.5	0.6	3.20
3 (end-stage)	17.3	17.5	−0.2	−1.1
654 (end-stage)	11.1	10.7	0.4	3.7

*% Difference = change in concentration with addition of Heteroblock™/concentration measured without Heteroblock™ × 100.

MMP-1, -3, -9 AND -13 TRANSCRIPT ABUNDANCE IN SYNOVIAL TISSUE FROM EARLY AND END-STAGE OA

To investigate whether synovial expression of MMPs implicated in OA pathogenesis were related to synovial inflammation, we measured a panel of MMP genes. MMP-1 (interstitial collagenase), MMP-3 (stromelysin), MMP-9 (gelatinase), and MMP-13 (collagenase-3) have all been implicated in cartilage matrix remodeling. Levels of enzyme transcripts were similar in end-stage and early OA SM (data not shown). Univariate analyses of enzyme and cell-specific gene expression in SM of all OA patients revealed positive associations between CD3δ and MMP-1 and -3, and CD68 and MMP-3 transcripts (Table III). Similar correlation coefficients were obtained when analyzing the patient groups separately.

The trend seen between CD8 mRNA with MMP-3 transcript levels (Table III) was confirmed after quantification of cellular infiltration by immunohistochemical staining. Numbers of CD8+ cells were associated with both MMP-1 ($r = 0.70$, $P = 0.016$) and MMP-3 ($r = 0.77$, $P = 0.005$) transcript levels (Fig. 5).

MEASUREMENT OF MMP-1 AND -3 IN SF

Production of active MMP proteins is dependent on post-translational processes such as proteolytic activation and inhibitor production. Therefore, levels of both total and active MMP-1 and MMP-3 were measured in SF from early OA patients (see Methods). We found statistically significant ($P < 0.05$) associations between levels of SM mRNA and both total (MMP-1 $r = 0.718$, MMP-3 $r = 0.790$) and active (MMP-1 $r = 0.909$, MMP-3 $r = 0.650$) SF enzymes.

RELATIONSHIP BETWEEN SF LEVELS OF IL-15 AND MMP-1 AND -3 EXPRESSION IN SYNOVIAL TISSUE

To explore a potential functional role for IL-15, we investigated whether SF IL-15 protein levels measured in patients with early ($n = 11$) and end-stage ($n = 10$) OA were associated with synovial MMP transcripts or protein levels. A relationship was identified between IL-15 and total MMP-1 and -3 levels at the mRNA [Fig 6 (a and b), early + end-stage patients] and protein level [Fig 6 (c and d), mostly early OA patients due to limiting quantity of fluid]. SF IL-15 also correlated with levels of active MMP-1 ($r = 0.576$, $P = 0.025$).

INFLUENCE OF GENDER, BMI AND AGE

As known risk factors for OA were not equally distributed among the two patient groups, (Table I), we ran generalized linear models to control for these variables. CD8 (log-transformed), IL-15, MMP-1 and MMP-3 mRNA levels were all independent of age, gender, and BMI (data not shown). In addition, we analyzed SF IL-15 and MMP-1 protein levels after stratifying on gender, BMI (obese vs non-obese) and age (<65 vs 65+). There were no significant differences identified based on these risk factors, whether analyzing the early and end-stage groups separately or combined.

Discussion

Most studies of synovial inflammation in OA patients focus on end-stage disease and utilize ACR criteria for diagnosis[29]. However, these criteria may not be ideal for identifying patients with earlier stage disease as they largely

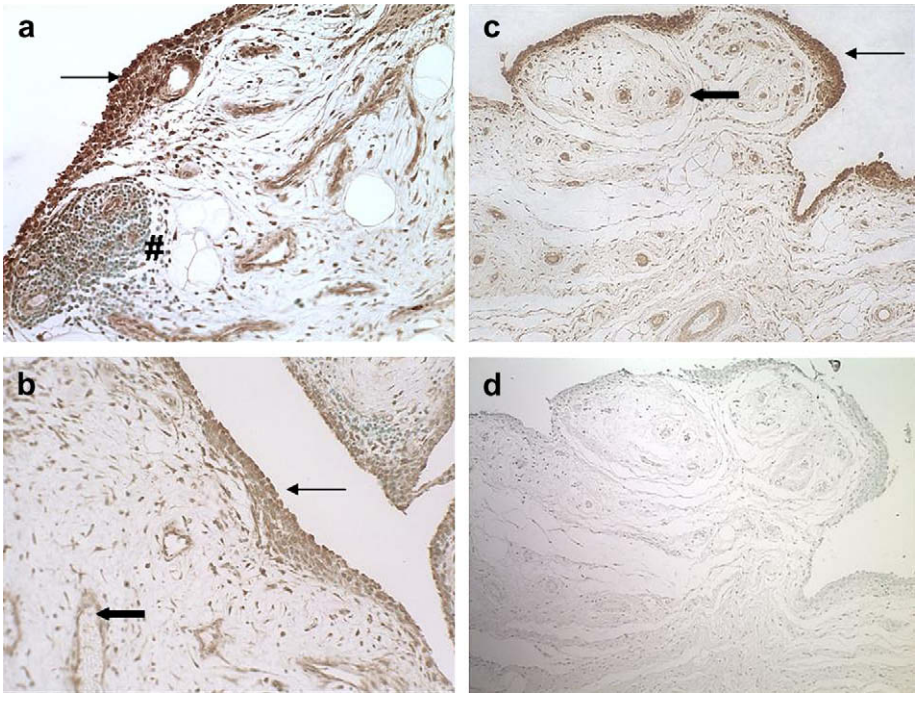

Fig. 4. Immunohistochemical localization of IL-15 Rα in representative patients with end-stage knee OA. (a–c): SM thin sections from three different patients were stained using standard immunoperoxidase technique with the polyclonal goat-anti-human IL-15 Rα (N-19, Santa Cruz Biotechnology, Inc), and counter-stained with methyl green. Positive staining appears brown, and was seen primarily in the lining cells (thin arrow), endothelium (block arrow) and in scattered cells within the subintima and perivascular mononuclear aggregates (panel a, #). (d): Negative control: non-specific goat-IgG was applied in place of the specific primary antibody.

Table III
Relationships between cell-specific genes and MMP transcript levels (RE) in the synovium of OA patients (early + end-stage)

	MMP-1 RE	MMP-3 RE	MMP-9 RE	MMP-13 RE
CD3δ RE	***r* = 0.431*** **(0.079–0.687)**	***r* = 0.471 (0.122–0.716)**	*r* = 0.072 (−0.300–0.425)	*r* = −0.210 (−0.537–0.174)
CD8 RE	*r* = 0.253 (−0.152–0.585)	*r* = 0.362 (−0.042–0.664)	*r* = 0.176 (−0.230–0.529)	*r* = −0.376 (−0.309–0.466)
CD68 RE	*r* = 0.273 (−0.115–0.589)	***r* = 0.390 (0.016–0.668)**	*r* = −0.292 (−0.602–0.095)	*r* = 0.066 (−0.326–0.438)

*All *r* values represent Spearman correlation coefficients (95% CI in brackets). Bolded values were statistically significant ($P < 0.05$).

exclude patients under 50 years of age, at which point osteophytosis on radiographs or bony overgrowth exam may already be present. More importantly, these criteria exclude patients with any joint warmth, effectively eliminating patients with clinical signs of inflammation. Therefore we sought to define a symptomatic patient population with early signs of cartilage degeneration in whom we could study the inflammatory response. We recruited patients with degenerative meniscal tears presenting for arthroscopy, who had documented diffuse cartilage abnormality.

Degenerative menisci can be detected quite commonly in an aging population, and their contribution to OA incidence and progression has been documented more recently. Two independent groups have demonstrated that MRI defined meniscal degeneration in patients with pre-existing OA is associated with progressive cartilage loss, and is associated with development of incident OA in a community population[30–32]. All early OA patients included in our current study had documented degenerative meniscal tears. The trajectory of this population needs to be defined prospectively, but this group is at risk for progression of OA despite not yet meeting current ACR criteria.

The most interesting and novel finding in this report is that levels of IL-15 were increased in early knee OA compared with end-stage patients [Fig. 3(b)]. IL-15 levels in SF were significantly higher in early OA patients, and were detectable in all early patients. This is the first report of SF IL-15 levels in a well-defined knee OA population at any disease stage. A recent case-control study lends support to our findings[33]. Utilizing a proteomic approach to serum biomarker discovery, IL-15 was one of four serum proteins found to be associated with both established disease and new development of knee and hand OA. Although patients in our study were not matched for known OA risk factors, SF IL-15 levels appeared independent of age, gender or BMI.

IL-15 elevation in the early OA group points to a potential role for innate immune system activation in the pathogenesis of OA. IL-15 is induced in response to activation of Toll like receptor (TLR)- 4[34]. Its presence in the SF of these patients suggests innate stimuli within the joint at an early stage of disease. However, IL-15 often acts as a membrane bound mediator. Soluble cytokine detectable in these patients may be produced in excess and not bound by available membrane receptors, or there may be soluble forms specific to the synovial environment.

IL-15 shares many activities with IL-2, which we only detected in four of the end-stage SF specimens. In contrast to IL-2, IL-15 is not primarily a product of activated lymphocytes. Instead, it is produced by many cell types including fibroblasts, phagocytes, dendritic cells, skeletal muscle and bone marrow stromal cells (reviewed in[35]). This makes it an attractive candidate for early immunostimulatory events within the synovium. Also in contrast to IL-2, IL-15 preferentially recruits and activates the NK cell and CD8+T-lymphocyte subset and supports the survival of CD8+ memory cells[36–38]. Although the specific impact of IL-15 on disease pathogenesis remains to be tested, our additional observations implicate its activity within OA joints. We found a positive association between IL-15 and CD8 transcript levels suggesting that IL-15 may contribute to recruitment or survival of CD8 lymphocytes within the joint in OA patients consistent with the known role of IL-15. Alternatively, these gene products may share common transcriptional regulation or cellular sources. We also observed a significant association between SF IL-15 and IL-6 protein levels. Of interest, these two cytokines together can influence NK cell activities in tumor environments, IL-15 directly and IL-6 indirectly through counteraction of TGF-β mediated inhibition[39]. In the knee, the significance of the IL-15/IL-6 association is unclear, but likely represents a common cellular source for these two cytokines. Both cytokines can be produced by synovial fibroblasts, macrophages, and potentially adipocytes as well, abundant cell types in the knee joint[40,41].

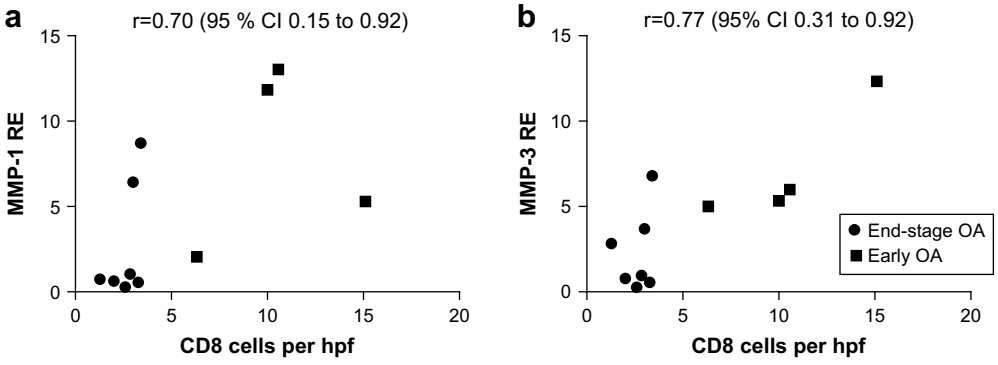

Fig. 5. Relationship between protease expression and CD8+ cells in synovium. Positively staining cells per hpf were quantitated as described in the text ($n = 11$, four early and seven end-stage OA). Numbers of infiltrating CD8+ cells/hpf correlated with (a) MMP-1 and (b) MMP-3 transcript levels (RE) within the tissue.

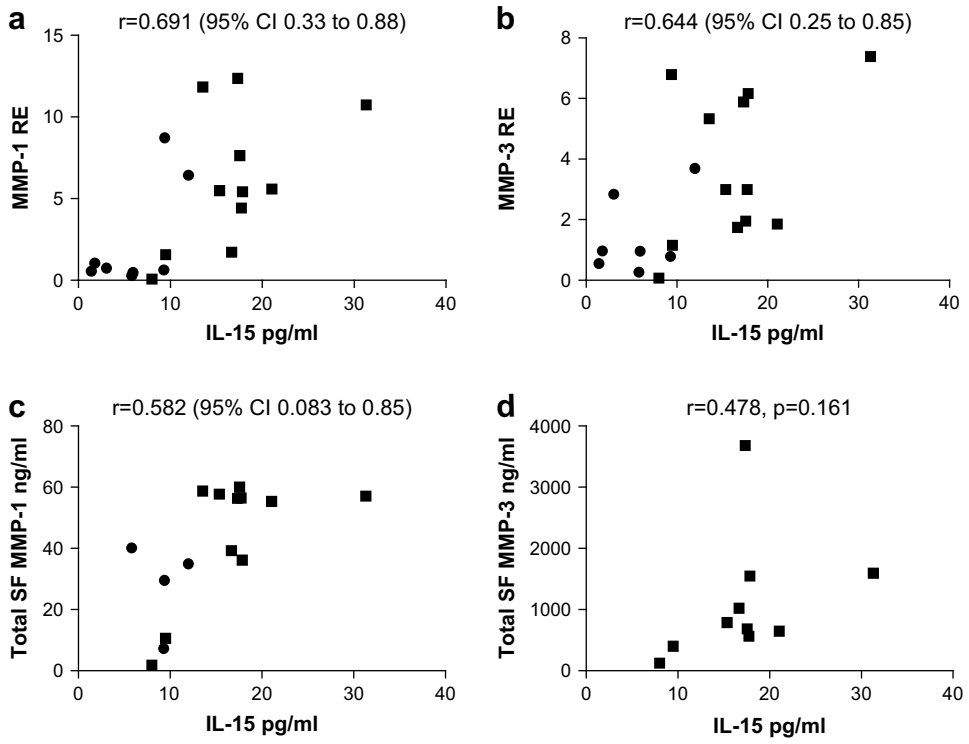

Fig. 6. Relationship between SF IL-15 and MMP-1/3 levels in patients with knee OA. SF levels of IL-15, MMP-1 ($n = 15$, 11 early and four end-stage OA) and MMP-3 ($n = 10$, all early OA) were measured by ELISA. SF IL-15 pg/ml correlated with (a) MMP-1 and (b) MMP-3 RE levels. In addition, SF IL-15 levels were associated with (c) total SF MMP-1 and (d) total SF MMP-3 protein levels. $r =$ spearman correlation coefficients (95% confidence interval). In panel (d), p value is reported rather than confidence interval given smaller number of measurements. Early OA (■), end-stage knee OA (●).

IL-15 has been shown to induce MMP production by fibroblasts and other cells *in vitro*, specifically MMP-9 and MMP-1[42,43]. In our patients, we observed associations between SF IL-15 protein levels, and both MMP-1 and MMP-3 expression (Fig. 6). These observations suggest a potential contribution of IL-15 to generation of mediators of matrix remodeling in this disease. We have demonstrated expression of IL-15 receptor α-chain in synovial lining cells and endothelium (Fig. 4) in OA patients; other groups have localized MMP-1 and -3 to these areas of the OA SM as well[44,45].

MMP-3 may reflect the pattern of synovial inflammation in RA, and is being investigated as a prognostic biomarker in OA[46,47]. The association we observed between MMP-3 and CD8+ lymphocytes suggests that this enzyme may reflect levels of inflammation even in OA patients. Although CD8 cells can produce MMPs *in vitro*, it is unlikely that numbers of these cells within synovial tissue are sufficient to produce the high levels of these enzymes seen in the fluid[48]. Instead, it is more likely that lymphocytes act indirectly *via* effects on fibroblasts or macrophages to induce enzyme production[49,50].

Like IL-15, other cytokines measured in this study (IL-1β, IL-6 and TNF-α) can be induced by TLR stimulation[51]. However, only IL-15 levels were differentially expressed in early and end-stage OA patients, suggesting that this cytokine, its stimuli, or its downstream targets may be potential candidates for molecular biomarkers of early disease development. Longitudinal studies are warranted to evaluate the utility of SF IL-15 in this capacity.

There are reports of T-lymphocyte activation marker expression in SM of end-stage OA patients[17], but it is still controversial whether these cells contribute substantially to the inflammatory response in OA. We measured two other common-γ chain cytokines in this study which are products of activated T-lymphocyte subsets, namely IL-21 and IL-2. Substantial levels of IL-21 protein were detectable in both early and end-stage patients, arguing that these lymphocytic populations are activated. IL-21, like IL-15, can act on CD8+ and NK cells, but also has effects on B-cell maturation[52]. Clearly, further work is necessary to confirm the cellular source and specific role that this lymphocyte product plays in the synovial reaction.

There are limitations inherent to this study. In particular, our early OA patient population does not fit currently accepted criteria for diagnosis of knee OA. But given limitations of the accepted criteria, these patients were chosen based on presence of early cartilage damage, symptoms, and increased risk for progressive OA. Despite similar enzyme and cell-specific mRNA levels in the early and end-stage patients, we can't rule out differences in SF enzyme levels or tissue cellular infiltration densities in the two patient groups as SF and tissue quantities were limited. For instance, CD8 mRNA levels were similar in early and end-stage patients, but specimens with the highest numbers of infiltrating CD8+ cells were all from the early group (Fig. 5). And although CD8 transcript levels approximated cellular infiltration in our analysis, this was evaluated in a small subset of specimens. Finally, this cross-sectional study cannot implicate IL-15 or inflammation in disease progression. However, future longitudinal studies can now be planned to document disease evolution and define the role of IL-15. The development of effective therapies to prevent OA progression has

been hindered by our inability to adequately define this prevalent joint disease at an early stage, so description of an appropriate patient population is important. The novel finding of increased IL-15 levels in this patient group provides further evidence of activation of innate immunity within SM. Future work may delineate molecular pathways responsible for IL-15 production in early OA. And given the central role that IL-15 plays as an early mediator of immune responses, this cytokine or its downstream mediators may represent future targets of therapy in early OA.

Conflict of interest

No potential conflicts of interest on behalf of any of the authors have been declared.

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