Synovial Fluid From Patients With Early Osteoarthritis Modulates Fibroblast-like Synoviocyte Responses to Toll-like Receptor 4 and Toll-like Receptor 2 Ligands via Soluble CD14

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Objective. Synovial inflammation, a feature of both osteoarthritis (OA) and meniscal injury, is hypothesized to be triggered in part via stimulation of Toll-like receptors (TLRs). We undertook this study to test whether a TLR-2– or TLR-4–stimulating factor in synovial fluid (SF) from patients with early knee OA with meniscal injury could lead to inflammatory activation of synoviocytes.

Methods. SF was obtained from patients with early OA cartilage damage undergoing arthroscopic meniscal procedures. SF was used to stimulate primary cultures of fibroblast-like synoviocytes (FLS) and cell lines transfected with TLR-2 or TLR-4. SF was used either alone or in combination with a TLR-2 stimulus (palmitoyl-3-cysteine-serine-lysine-4 [Pam$_3$CSK$_4$]) or a

TLR-4 stimulus (lipopolysaccharide [LPS]). In blocking experiments, SF was preincubated with anti-CD14 antibody.

Results. SF from these patients did not stimulate interleukin-8 (IL-8) release from TLR transfectants. Compared with SF on its own, SF (at concentrations of 0.09–25%) in combination with TLR-2 or TLR-4 ligands resulted in significant augmentation of IL-8 release from both transfectants and primary FLS. Soluble CD14 (sCD14), a coreceptor for TLRs, was measured in SF from patients with early OA at levels comparable to those in patients with advanced OA and patients with rheumatoid arthritis. Blockade with anti-CD14 antibody abolished the ability of SF to augment IL-8 production in response to LPS, and diminished Pam$_3$CSK$_4$ responses.

Conclusion. SF augments FLS responses to TLR-2 and TLR-4 ligands. This effect was largely due to sCD14. Our results demonstrate that sCD14 in the setting of OA and meniscal injury sensitizes FLS to respond to inflammatory stimuli such as TLR ligands.

Many patients with osteoarthritis (OA) or joint injury develop low-grade synovitis, and there is growing evidence that synovitis affects symptoms (1–3) and rate of cartilage degeneration (4) in patients with OA. Synovial infiltration by macrophages and lymphocytes (5,6) has been demonstrated in both early- and advanced-stage OA (7,8). Moreover, infiltrates occur in patients with a history of joint injury undergoing arthroscopic surgery for meniscal tears, even in the absence of radiographic evidence of OA (9). However, the molecular stimulus for synovitis in the setting of OA or meniscal injury is as yet unclear.

Initial inflammatory responses to infection are mediated by receptors for pathogen-associated molecular patterns, including Toll-like receptors (TLRs). Cell-surface TLRs include TLR-2 and TLR-4; TLR-2 binds lipopeptides from prokaryotic cell walls, and TLR-4 recognizes lipopolysaccharide (LPS) from gram-negative bacteria. Binding of ligands to TLRs initiates intracellular signaling that results in inflammatory gene transcription. Recent studies in various models of tissue injury and repair (10), including ischemia-reperfusion (11), noninfectious lung injury (12), and femoral fracture (13), have implicated TLRs in inflammatory responses after noninfectious injury. In these settings, TLRs may be triggered by endogenous danger signals (damage-associated molecular patterns [DAMPs]) produced by tissue injury and cellular stress.

DAMPs that interact with TLRs, particularly TLR-2 and TLR-4, include extracellular matrix components such as low molecular weight fragments of hyaluronan (HA) (14), tenascin-C (TN-C) (15), and the cellular products high mobility group box chromosomal protein 1 (HMGB-1) (16) and Hsp96 (17). TN-C and Hsp96 activate synovial fibroblasts via TLRs in the setting of rheumatoid arthritis (RA) (15,17). Both DAMPs and TLRs are expressed in OA joint tissues, including synovial membrane and cartilage (18). Murine chondrocyte nitric oxide and matrix metalloproteinase 13 production were diminished in response to low molecular weight HA and HMGB-1 in TLR-2/TLR-4–double-knockout mice (19), suggesting a role for TLRs in chondrocyte catabolic responses. The importance of TLR responses in fibroblast-like synoviocytes (FLS) is less clear in OA and joint injury, as most studies have focused on FLS responses in RA. However, synovial fibroblasts from both OA and RA patients respond to TLR ligands with increased chemokine production (20), an activity that has obvious implications for development of synovitis in both inflammatory and "noninflammatory" arthritis.

Given the strong association between joint injury and risk of OA development and progression (21), we hypothesized that endogenous activation of TLRs stimulates inflammatory responses in patients with OA or joint injuries (22). We anticipated that endogenous TLR ligand(s) present in synovial fluid (SF) from patients with OA could lead to inflammatory activation of FLS. We collected SF samples from patients with early-stage OA cartilage damage undergoing arthroscopic surgery for meniscal tears. We then tested the ability of SF to induce inflammatory cytokine production in 2 in vitro systems: 1) TLR-negative cell lines transfected with TLR-2 or TLR-4, and 2) primary FLS cultures.

PATIENTS AND METHODS

Reagents. Culture media, antibiotics, and additives were from Invitrogen. LPS from *Escherichia coli 0111:B4* (a TLR-4 ligand) and palmitoyl-3-cysteine-serine-lysine-4 (Pam_3CSK_4; a TLR-2 ligand) were purchased from Invivogen, and recombinant CD14 was purchased from PeproTech.

SF and serum samples. Patients were recruited from orthopedic practices at Rush University Medical Center (Chicago, IL) and the Hospital for Special Surgery (New York, NY) under Institutional Review Board (IRB)–approved protocols at both institutions; all patients gave informed consent. There were 3 patient groups and 1 asymptomatic donor group. Their characteristics are shown in Table 1.

The early-OA (meniscectomy) group consisted of patients undergoing arthroscopic meniscectomy for treatment of meniscal tears. These patients were recruited from the practices of 2 orthopedic surgeons (CB-J, NV) at Rush University Medical Center. SF and serum were obtained at the time of surgery and banked for the Rush Knee Arthritis and Meniscal Injury Repository. Early-stage OA cartilage change was confirmed in these patients by intraoperative inspection, and the degree of degradation was scored using the Outerbridge system (23).

The advanced-OA group consisted of patients undergoing total knee replacement (TKR) who were recruited at the Hospital for Special Surgery. Diagnosis of OA was made by the operating surgeon. All patients with advanced OA had intraoperative evidence of full-thickness diffuse cartilage loss (24).

The third group was the RA group. These were patients with a clinical diagnosis of RA who were undergoing TKR at the Hospital for Special Surgery. SF from these patients was included for comparison of soluble CD14 (sCD14) levels.

The fourth group was the asymptomatic donor group. This consisted of 10 asymptomatic organ donors with no documented history of joint disease. SF from this group was obtained via the Gift of Hope Organ & Tissue Donor Network (Elmhurst, IL). Specimens were collected with IRB approval within 24 hours of death.

Synovial tissues for FLS culture. Synovial membranes were obtained from 5 asymptomatic organ donors via the Gift of Hope Organ & Tissue Donor Network. Cartilage degeneration in these donors was assessed and graded by an experienced reader using the modified Collins score (from grade 0 = normal cartilage to grade 4 = full-thickness cartilage erosion of >30%) (25). Characteristics of these donors are presented in Table 2.

Cell culture. Transfected HEK 293 cells expressing TLR constructs were a gift from Dr. R. W. Finberg (University of Massachusetts Medical School, Worcester) (26). HEK 293 cells transfected with TLR-4 alone served as negative control for cells cotransfected with TLR-4 and myeloid differentiation factor 2 (MyD2); MyD2 is required for LPS signaling via TLR-4. HEK 293 cells transfected with CD14 alone served as negative control for cells cotransfected with TLR-2 and the coreceptor CD14. Cells were maintained in Dulbecco's modi-

Table 1. Characteristics of the patient groups included in the analysis of SF soluble CD14*

	Patients with early OA (undergoing meniscectomy) (n = 30)	Patients with advanced OA (n = 7)	RA patients (n = 6)	Asymptomatic donors (n = 10)
Soluble CD14 in SF, μg/ml	2.10 (1.54–2.36)	2.29 (1.48–3.36)	1.70 (1.55–1.94)	0.72 (0.55–1.43)
Age, years	56 (47–62)	69 (55–77)	67 (51–72)	64 (52–74)
BMI, kg/m^2	29.5 (27–35)	26.6 (24.3–35.5)	NR	NR
Male, %	50	71	16	40
Caucasian, %	76	100	50	60
Outerbridge grade, no.			NR	NR
1	1	–		
2	11	–		
3	10	–		
4	8†	7‡		
K/L grade, no.		NR		NR
0	1		0	
1	6		0	
2	10		1	
3	4		3	
4	0		1	
Missing	9		1	

* Except where indicated otherwise, values are the median (interquartile range). SF = synovial fluid; RA = rheumatoid arthritis; BMI = body mass index; NR = not reported; K/L = Kellgren/Lawrence.
† All grade 4 lesions in this group were focal cartilage defects.
‡ Patients who did not have diffuse grade 4 (full-thickness) cartilage wear were excluded from the advanced osteoarthritis (OA) group.

fied Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Hyclone) and 0.5 μg/ml puromycin (TLR-2 transfectants) or 0.5 mg/ml Geneticin (TLR-4 transfectants). Control experiments confirmed the specificity of this system, as LPS only stimulated TLR-4/MyD2–transfected cells while Pam$_3$CSK$_4$ only stimulated TLR-2/CD14–transfected cells.

FLS cultures were established from synovial biopsy samples by enzymatic digestion (27). Tissues were treated for 90 minutes with 1 mg/ml of Pronase (Calbiochem) followed by overnight digestion with 1 mg/ml of type I collagenase (Worthington). Dissociated cells were resuspended in DMEM supplemented with 10% FBS, antibiotics, nonessential amino acids, sodium pyruvate, sodium bicarbonate, L-glutamine, and 0.5 μM β-mercaptoethanol. Select FLS at passage 4 were immunostained with fluorescein isothiocyanate–conjugated anti-human CD14 antibody (clone 61D3; eBioscience) or an isotype control, and analyzed on a FACSCalibur flow cytometer (BD Biosciences). Cultures were negative for CD14 at

Table 2. Characteristics of the tissue donors from whom fibroblast-like synoviocyte cultures were established

Donor/age/sex	Cartilage score, tibia/femur*
1/65/F	2/3
2/19/M	0/0
3/59/F	2/1
4/55/M	2/3
5/38/F	1/1

* Cartilage degeneration and structural joint integrity were scored according to the modified Collins grading system (25) (from grade 0 = normal cartilage to grade 4 = full-thickness cartilage erosion of >30%).

passage 4, indicating depletion of CD14+ synovial macrophages. FLS were used between passages 4 and 9.

In vitro stimulation with SF and TLR ligands. Ten SF specimens were tested for endotoxin contamination (Pyrochrome endotoxin assay; Associates of Cape Cod). Only 1 specimen contained measurable endotoxin activity, and this specimen was excluded from further experiments. HEK 293 transfectants (2 × 10^4 cells/well) or FLS (3 × 10^3 cells/well) were seeded in 96-well tissue culture plates. After 24 hours, cells were stimulated with indicated concentrations of patient SF, LPS, Pam$_3$CSK$_4$, or recombinant CD14 alone or in combination for 6 or 18 hours. Supernatants were collected for interleukin-8 (IL-8) and IL-6 measurement by enzyme-linked immunosorbent assay (R&D Systems). These cytokines are known products of FLS and are induced by TLR stimulation in monocytes. Total RNA was isolated from cell lysates after 6-hour stimulations using the RNeasy Mini RNA Isolation Kit (Qiagen). In blockade experiments, anti-human CD14 (monoclonal antibody [mAb] clone MEM-18; Abcam) was added to SF at 37°C and incubated for 1 hour prior to use as stimuli.

Real-time quantitative polymerase chain reaction (qPCR) measurement of IL-8, TLR-2, and TLR-4. Messenger RNA (mRNA) levels of IL-8, TLR-2, and TLR-4 were determined by real-time PCR. Total RNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad). iQ SYBR Green Supermix (Bio-Rad) was used for PCR, and reactions were carried out on a Bio-Rad CFX 96 real-time system. The following human primers were used: for IL-8, 5'-ATGACTTCCAAGCTGGCCGTGGCT-3' (forward) and 5'-TCTCAGCCCTCTTCAAAAACTTCTC-3' (reverse); for TLR-2, 5'-TGGATGGTGTGGGTCTTGG-3' (forward) and 5'-AGGTCACTGTTGCTAATGTAGG-3' (reverse); for TLR-4, 5'-GCCCTGCGTGGAGGTGGTTC-3' (forward) and 5'- TCCAGAAAAGGCTCCCAGGGCT-3' (reverse); for

GAPDH, 5′-CAACGGATTTGGTCGTATT-3′ (forward) and 5′-GATGGCAACAATATCCACTT-3′ (reverse). After normalizing C_t values to GAPDH, expression levels were calculated relative to media controls (28).

Flow cytometry and immunostaining of TLR expression in FLS. For flow cytometric analysis, FLS were nonenzymatically dissociated, resuspended in phosphate buffered saline containing 1% bovine serum albumin and 0.05% sodium azide, and immunostained with phycoerythrin (PE)–conjugated mAb against human TLR-2 (clone TL2.1), PE-conjugated mAb against TLR-4 (clone HTA125), or a PE-conjugated isotype control mAb (mouse IgG2a) (all mAb from eBioscience). Analysis was performed on a FACSCanto II flow cytometer (BD Flow Cytometry Systems). For visualization of immunostaining, FLS plated in 6-well tissue culture plates were grown to 80–90% confluence and stained with biotinylated anti–TLR-4 mAb (clone HTA 125; Abcam) followed by Alexa Fluor 488–labeled streptavidin (Invitrogen). Cells were visualized under a 40× water-immersion objective. A 2-photon laser (Chameleon Ultra; Coherent) was used for excitation. Images of representative fields were acquired with PrairieView software (Prairie Technologies) and fluorescence densities were analyzed with Imaris software (Bitplane).

Measurement of sCD14 in SF and serum. SF was centrifuged at 500g to remove cell debris prior to storage at −80°C. SF and serum specimens were thawed and diluted 1:200, and sCD14 was measured using the Human CD14 DuoSet (R&D Systems).

Statistical analysis. Data were analyzed using GraphPad Prism software, version 5.0. Cytokine levels from in vitro experiments were compared between groups using one-way analysis of variance (ANOVA). If a P value less than 0.05 was obtained, Tukey's post-test was applied. For comparison of sCD14 levels between patient groups, Kruskal-Wallis ANOVA with Dunn's multiple comparison post-test was applied. Association between sCD14 levels and FLS IL-8 release was tested with Spearman's rho.

RESULTS

Most SF samples from patients with early OA do not directly stimulate HEK 293 cells transfected with TLR-2 or TLR-4. To test whether a TLR-4– or TLR-2–activating factor could be detected in SF specimens, we used HEK 293 cells transfected with TLR-4 and the cofactor MyD2, TLR-4 alone, TLR-2 and the cofactor CD14, or CD14 alone. In total, we screened 31 SF specimens at 25% concentration both from patients with early OA (n = 23) and from patients with advanced OA (n = 8; undergoing TKR) for the ability to stimulate IL-8 production from transfectants. None of the SF specimens stimulated CD14- or TLR-2/CD14–transfected cell lines, and only 1 reproducibly stimulated cells transfected with TLR-4 (data not shown). As this specimen was in limited supply, its stimulatory capacity could not be further characterized. SF concentrations

>25% caused cell death indicated by trypan blue staining and could not be analyzed.

Effects of OA SF on IL-8 production from HEK 293 transfectants in response to TLR-2 and TLR-4 ligands. We next stimulated HEK 293 transfectants with the TLR-4 ligand LPS or with the TLR-2 ligand Pam$_3$CSK$_4$, alone or in combination with SF. LPS and Pam$_3$CSK$_4$ stimulated cotransfected cells as expected (Figures 1A and B). A 25% concentration of SF alone did not stimulate IL-8 production from either set of transfectants, but addition of SF to control ligands significantly increased levels of IL-8 compared to either Pam$_3$CSK$_4$ alone (Figure 1A) or LPS alone (Figure 1B).

OA SF augments IL-8 and IL-6 production by FLS in response to both TLR-2 and TLR-4 ligands. To determine whether SF affects cells within the joint, we established primary FLS cultures. Similar to responses of HEK 293 cells, a 25% concentration of SF from OA patients significantly increased production of IL-8 in response to both Pam$_3$CSK$_4$ (Figure 1C) and LPS (Figure 1D). SF also augmented IL-6 production in response to these ligands (Figures 1E and F). In total, 23 separate OA SF specimens (22 from patients with early OA and 1 from a patient with advanced OA) were tested in combination with LPS and 6 in combination with Pam$_3$CSK$_4$. All SF specimens augmented LPS responses and 5 of 6 augmented responses to Pam$_3$CSK$_4$.

OA SF may contain factors that can directly induce IL-8 production, such as IL-1β or tumor necrosis factor α (TNFα) (24). In control experiments (not shown), we tested whether FLS were responsive to these 2 cytokines. Exposure to IL-1β (1 or 10 ng/ml), but not to TNFα, induced release of IL-8 from FLS. However, we previously reported that SF IL-1β was only detected in 1 of 18 patients with early knee OA (24) and measured at a concentration of <1 pg/ml. Although OA SF may contain other unknown factors that can directly induce IL-8 production, of 23 samples tested, only 4 (no. 61 [Figure 1C], no. 52 and no. 564 [Figure 1F], no. 83 [not shown]) induced IL-8 or IL-6 without addition of LPS or Pam$_3$CSK$_4$. Two of these 4 samples (no. 61 and no. 83) could be attributed to IL-8 in SF itself. Sufficient SF from sample no. 564 was not available for cytokine analysis, while the fourth specimen (no. 52) induced twice the IL-6 in SF alone. With the exception of this 1 sample, augmentation of cytokine release from FLS in response to SF plus TLR ligands could not be attributed to a direct effect of SF in the majority of cases.

To determine whether cells remaining in SF could explain the effect, 3 control assays were performed (data not shown). First, we treated SF with 250 units/ml
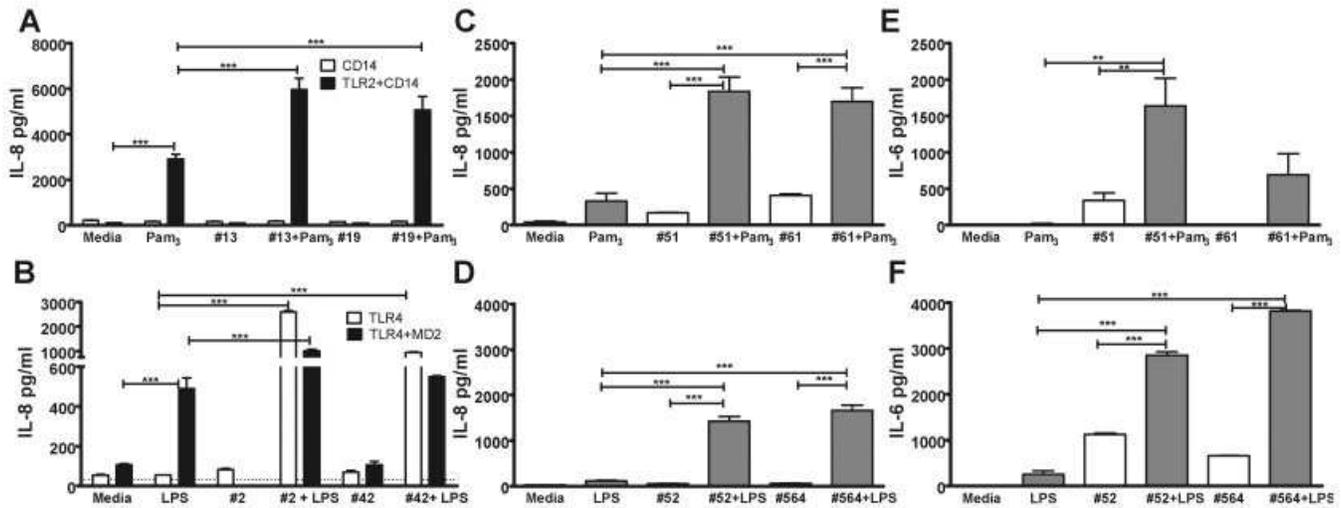
Figure 1. Synovial fluid (SF) augments responses to the Toll-like receptor 2 (TLR-2) ligand palmitoyl-3-cysteine-serine-lysine-4 (Pam$_3$CSK$_4$; Pam$_3$) (**A, C,** and **E**) and to the TLR-4 ligand lipopolysaccharide (LPS) (**B, D,** and **F**) both in HEK 293 transfected cell lines (**A** and **B**) and in primary fibroblast-like synoviocyte (FLS) cultures (**C–F**). Cells were stimulated as described in duplicate or triplicate wells with 25% SF (listed by specimen no.) with 100 or 500 ng/ml Pam$_3$CSK$_4$ (**A, C,** and **E**), with 100 ng/ml LPS (**B, D,** and **F**), with SF plus Pam$_3$CSK$_4$ (**A, C,** and **E**), or with SF plus LPS (**B, D,** and **F**). In **A,** HEK 293 cells were transfected with CD14 or with both TLR-2 and CD14. In **B,** HEK 293 cells were transfected with TLR-4 or with both TLR-4 and myeloid differentiation factor 2 (MyD2; MD2). After 18 hours, interleukin-8 (IL-8) was measured by enzyme-linked immunosorbent assay in culture supernatants. In **C–F,** FLS were stimulated similarly to HEK 293 cells. Shown are FLS IL-8 production (**C** and **D**) and IL-6 production (**E** and **F**) after 18 hours. Results shown are representative of 2–3 separate experiments. Values are the mean ± SD. ** = $P < 0.01$; *** = $P < 0.001$.

hyaluronidase (Sigma) for 10 minutes to reduce viscosity prior to centrifugation to remove cells. Second, we filtered SF through a 0.45 μm filter prior to use. These 2 treatments had no effect on our results. Finally, we incubated unfiltered SF with LPS alone for 18 hours in the absence of FLS. No IL-8 production above baseline was observed. Therefore, effects could not be attributed to cells supplied by SF.

Titration of SF in combination with LPS. FLS were stimulated with 2-fold dilutions of SF from 25% down to <1%, in combination with LPS (100 ng/ml). There was a dose-related decrease in IL-8 production in response to LPS, but concentrations as low as 0.09% were still effective (Figure 2A). Next, 10-fold dilutions of LPS from 1 μg/ml to 0.01 ng/ml were used alone or with 3% SF. LPS alone induced IL-8 release at higher concentrations (Figure 2B), and addition of SF augmented this response. At low LPS concentrations (1 ng/ml), IL-8 production was only observed with addition of SF. This potentiating effect of SF was also observed at the transcriptional level (Figure 2C), measured by real-time qPCR.

SF does not up-regulate TLR expression in FLS. Unknown SF factors could up-regulate TLR expression, thereby augmenting responses to ligands. We compared

TLR-2 and TLR-4 mRNA levels in unstimulated and SF-stimulated FLS. Exposure to SF (3%) did not increase but rather tended to decrease TLR-4 mRNA levels and had no effect on TLR-2 mRNA levels (Figure 2D). After correcting for background staining with isotype control, cell surface staining analyzed by flow cytometry was consistent with the mRNA expression pattern (for TLR-2, mean fluorescence intensity [MFI] 1,098 untreated, 1,054 treated; for TLR-4, MFI 788 untreated, 584 treated). Given the high background of flow cytometry samples, we visualized immunostaining of TLR-4 using 2-photon microscopy. As demonstrated in Figure 2E, the mean ± SD MFI of TLR-4 staining in cells exposed to 3% SF (179.7 ± 21.9 arbitrary units [AU]) was comparable to that in unstimulated cells (173.0 ± 25.8 AU).

Soluble CD14 levels in SF from patients with OA. Heat-treating SF at 98°C for 10 minutes prior to use destroyed its ability to augment LPS responses in FLS (data not shown), indicating that a heat-denaturable factor was responsible. One soluble protein found in SF from RA patients (29) that can interact with both TLR-2 and TLR-4 is sCD14 (30,31). We measured sCD14 in SF from 30 patients with early OA; characteristics of these patients are shown in Table 1. Levels of sCD14 in these
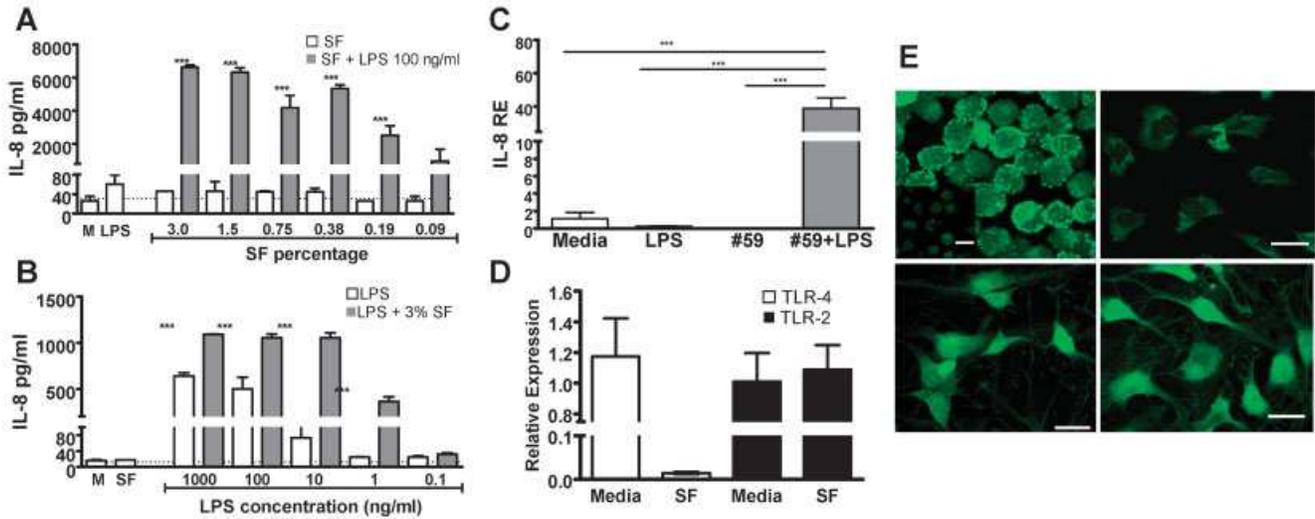
Figure 2. A and **B,** Cells were stimulated as described in Patients and Methods with SF in decreasing percentages alone or with 100 ng/ml LPS (**A**) or with LPS in decreasing concentrations alone or with 3% SF (**B**), all in duplicate. Dotted line shows the enzyme-linked immunosorbent assay limit of detection. *** = $P < 0.001$ versus SF or LPS alone. **C,** Shown are IL-8 mRNA levels measured by quantitative polymerase chain reaction after 6 hours of stimulation with 3% SF from patient 59 with or without 100 ng/ml LPS. *** = $P < 0.001$. **D,** Shown are mRNA levels of TLR-4 and TLR-2 after 6 hours of stimulation with or without 3% SF from patient 59. Expression levels were calculated relative to media control (M). Results in **A–D** are representative of 2 separate experiments. Values are the mean ± SD. **E,** Shown are 2-photon images of TLR-4 staining. Top left, Positive control, TLR-4 staining of HEK 293 cells transfected with TLR-4 (isotype control staining shown in **inset**). Top right, Unstimulated FLS culture stained with isotype control monoclonal antibody (mean ± SD mean fluorescence intensity [MFI] 61.4 ± 30.0 arbitrary units [AU]). Bottom left, TLR-4 staining of unstimulated FLS (mean ± SD MFI 173.0 ± 25.8 AU). Bottom right, TLR-4 staining of FLS stimulated with 3% SF (mean ± SD MFI 179.7 ± 21.9 AU). Bars = 20 μm. RE = relative expression (see Figure 1 for other definitions).

patients ranged from 1 to 5 μg/ml (Figure 3A), comparable to levels in advanced OA and RA. Median levels in SF from 10 asymptomatic postmortem donors without a history of joint disease (Figure 3A) were 0.72 μg/ml (interquartile range 0.55–1.4), lower than those in the OA patient groups ($P < 0.05$). Soluble CD14 was then
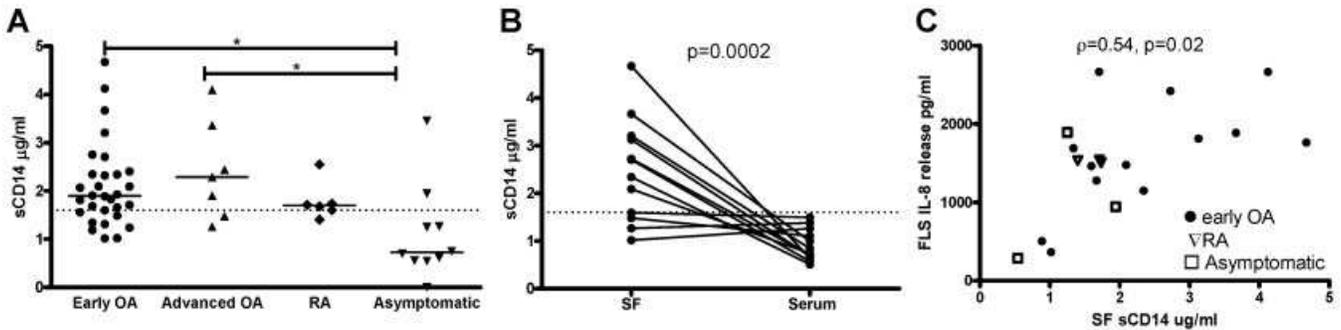
Figure 3. Levels of soluble CD14 (sCD14) in SF in patients with early osteoarthritis (OA) are comparable to levels observed in patients with advanced OA and in patients with rheumatoid arthritis (RA), elevated in SF compared with serum, and correlate with SF augmentation of FLS LPS response. **A,** SF levels of sCD14 in patients with early OA undergoing meniscectomy (n = 30), in patients with advanced OA undergoing total knee replacement (n = 7), and in RA patients undergoing total knee replacement (n = 6), as well as in asymptomatic organ donors without known joint disease (n = 10) were measured by enzyme-linked immunosorbent assay (see Table 1 for patient and donor characteristics). Horizontal lines indicate the median. * = $P < 0.05$ by Kruskal-Wallis test. **B,** Soluble CD14 levels were measured in paired SF and serum samples from patients with early OA undergoing meniscectomy (n = 12) (see Patients and Methods). Levels of sCD14 were higher in SF than in paired serum specimens ($P = 0.0002$ by Mann-Whitney U test). Dotted line = level in pooled, normal human serum. **C,** FLS were stimulated for 18 hours, as described in Patients and Methods, with 100 ng/ml LPS plus 0.2% SF from patients with early OA (n = 12), RA patients (n = 3), and asymptomatic control donors (n = 3). IL-8 levels measured in supernatants after stimulation correlated with sCD14 levels in the SF. See Figure 1 for other definitions.
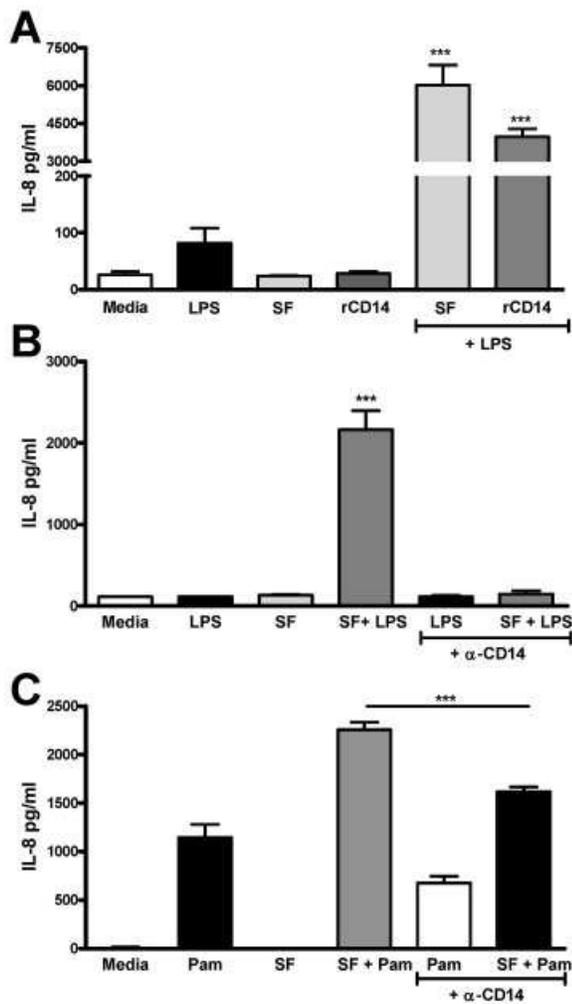
Figure 4. Addition of recombinant CD14 (rCD14) augments response of FLS to LPS (**A**), and incubation with anti-CD14 abrogates SF augmentation of both the LPS response (**B**) and the Pam₃CSK₄ response (**C**). In **A,** primary FLS were stimulated with 100 ng/ml LPS, 25% SF from patient 21, 0.5 μg/ml recombinant CD14 (expected concentration in 25% SF), SF plus LPS, or recombinant CD14 plus LPS. Results are representative of 2 experiments. Values are the mean ± SD. *** = $P < 0.001$ versus all other groups. In **B** and **C,** 1.5% SF from patient 59, either with 100 ng/ml LPS (**B**) or with Pam₃CSK₄ (**C**), was preincubated with monoclonal anti-CD14 (clone MEM-18) prior to incubation with FLS. The anti-CD14 by itself had no effect on IL-8 (data not shown). Values are the mean ± SD. In **B,** *** = $P < 0.001$ versus all other groups. In **C,** *** = $P < 0.001$. See Figure 1 for other definitions.

measured in paired SF and serum samples from 12 patients with early OA. In 8 of 12 patients, SF levels were higher than serum levels (Figure 3B). The sCD14 concentration in an age-matched, pooled normal human serum specimen was 1.6 μg/ml.

FLS response to SF plus LPS correlates with sCD14 concentration in SF. To determine if the SF sCD14 concentration correlated with the magnitude of IL-8 release in vitro, we tested 18 SF specimens (12 from patients with early OA, 3 from RA patients, and 3 from asymptomatic control donors) in a single experiment. We used an SF concentration of 0.2%, as titration experiments indicated that low concentrations of SF were sufficient. IL-8 release from FLS exposed to SF plus LPS correlated strongly (Spearman's $\rho = 0.54$, $P = 0.02$) with SF sCD14 concentrations (Figure 3C) and was not disease specific. The lowest FLS response and the lowest sCD14 concentration were measured in the only specimen (from an asymptomatic control donor) with no structural joint or cartilage damage (Collins grade 0).

Recombinant CD14 reproduces, and anti-CD14 blocks, the potentiating effect of SF on LPS responses in FLS cultures. To test whether CD14 augments LPS responses in FLS, recombinant CD14 was used in place of SF, at levels comparable to 25% SF (0.5 μg/ml). Recombinant CD14 in combination with 100 ng/ml LPS induced 40-fold higher IL-8 release than LPS alone, and approached levels stimulated by SF plus LPS (Figure 4A). Next, SF (3%) was preincubated with a 10-fold greater molar equivalent of anti-CD14 mAb prior to use. This treatment abolished SF augmentation of LPS responses and diminished SF augmentation of Pam₃CSK₄ responses in FLS cultures (Figures 4B and C).

DISCUSSION

OA leads to chronic joint symptoms and disability, and low-grade inflammation within the synovial membrane is associated with pain (2) and contributes to progression of cartilage loss (4). We found synovitis associated with symptoms in patients undergoing arthroscopic surgery for meniscal tears, even in the absence of radiographic OA (9). The majority of these patients had degenerative-type meniscal tears and intraoperative evidence of early cartilage damage, consistent with the view that meniscal tears may be a sign of early-stage "preradiographic" disease in many patients (32). Close to 1 million arthroscopic meniscal procedures are performed every year in the US (33). Therefore, patients for whom these procedures are indicated represent a population in which mediators involved in early stages of OA can be explored. We hypothesized that soluble TLR ligands present in SF of these patients are responsible for activating FLS. To test this hypothesis, we screened SF using in vitro stimulation of TLR-2– and TLR-4–expressing cells (HEK 293 transfectants). Although we

did not detect a TLR agonist in these patients, SF consistently enhanced TLR transfectant and primary FLS responses to classic TLR-2 and TLR-4 ligands (Figure 1).

Very low concentrations of SF ($<0.1\%$) enhanced IL-8 in response to LPS in FLS cultures, and SF allowed primary FLS to respond to LPS at concentrations 100-fold lower (1 ng/ml) than in the absence of SF (100 ng/ml) (Figures 2A and B). This was not due to a direct effect of SF on cytokine production, nor to up-regulation of TLR expression in FLS (Figures 2D and E). As SF augmented responses to both TLR-2 and TLR-4 ligands (Pam_3CSK_4 and LPS), we assessed sCD14 levels in SF. CD14 is a coreceptor for TLR-2 and TLR-4 on various cell types, particularly macrophage lineage cells. It transfers LPS to the TLR-4 complex and enhances LPS-mediated signaling in macrophages (34). Similarly, CD14 facilitates TLR-2 ligand recognition by receptor complexes (31). It is expressed on monocytes as a glycosyl phosphatidylinositol–anchored membrane protein (35), but it is also found in soluble form. Whether SF sCD14 levels correlate with fluid monocyte counts could not be answered in this study, as fluid specimens were centrifuged and frozen prior to analysis. Future work is necessary to determine cellular sources of sCD14 in patients with early OA, but higher levels in SF compared to serum suggests local production in the joint.

Three lines of evidence support a role for SF sCD14 in mediating FLS responses to TLR-2 or TLR-4 ligands in our assays. First, SF sCD14 concentrations correlated with IL-8 release in response to SF plus LPS (Figure 3C). Second, substitution of recombinant CD14 in place of SF recapitulated the SF effects (Figure 4A). Addition of recombinant CD14 to LPS did not fully restore FLS IL-8 release to levels seen with SF, so we cannot rule out a role for additional factors supplied by SF such as endogenous TLR ligands or other cytokines in a few samples. Finally, anti-CD14 mAb abolished the effect of SF on LPS responses and diminished Pam_3CSK_4 responses. Similar to membrane-anchored CD14, sCD14 facilitates LPS-induced leukocyte activation in vitro (30). In endothelial cells (36), sCD14 increased sensitivity to LPS; high levels of LPS stimulated cells, but low levels required the presence of sCD14, similar to our observations in FLS cultures exposed to SF (Figure 2). In endothelial cells, expression of membrane-anchored CD14 was still required to respond to low levels of LPS even in the presence of sCD14 (36). In our study, FLS were negative for membrane-anchored CD14 by flow cytometry prior to

use in stimulation assays, although we cannot rule out the possibility of low-level expression.

Soluble CD14 is found in RA SF, and recombinant CD14 added to LPS augments intercellular adhesion molecule 1 expression by RA FLS cultures (29). The present study demonstrates that SF sCD14 is not disease specific, as it was also found in patients with early and advanced OA, RA patients, and asymptomatic donors (Figure 3A). Regardless of diagnosis, the sCD14 concentration correlated with SF augmentation of FLS IL-8 production induced by LPS. Although immunosuppressive agents used by RA patients may have lowered sCD14 levels in this group, sCD14 levels measured in our RA patients were comparable to those previously reported (29). Our asymptomatic organ donors are not ideal controls given the variable presence of cartilage degeneration and potential degradation postmortem. Still, sCD14 levels detected in SF from 10 donors collected within 24 hours of death were significantly lower than in patients. SF from 3 of these donors were tested in FLS assays, and were also able to augment LPS responses. Interestingly, the lowest response (and lowest sCD14 concentration) was measured in SF from the only control with normal cartilage (Collins grade 0). It is possible that structural joint damage, irrespective of mechanism of damage, influences the level of sCD14 found in SF.

Definitive proof that sCD14 plays an important role in OA pathogenesis will need future experimentation using disease models. In the current investigation, sCD14 in SF allowed FLS to respond to lower levels of TLR-2 and TLR-4 ligands, but did not by itself stimulate cytokine production. We speculate that sCD14 in SF may play a role in sensitizing cell populations within the joint to TLR stimuli, not only in inflammatory arthritis, but also in OA. If the threshold for synovial inflammatory response to TLR-2 and TLR-4 ligands is lowered by increasing amounts of sCD14 in SF, then inflammation may be triggered more easily when TLR ligands are produced. This might be expected in 3 scenarios. First, increased production of endogenous ligands is expected during periods of increased matrix turnover episodically in chronic arthritis, or after a joint injury (a meniscal tear or ligament injury). In this scenario, SF sCD14 might affect development of effusion, synovitis, and symptomatic flares in both OA and RA patients. Second, the association of crystal deposition and OA has been recently reviewed (37). Both uric acid and calcium pyrophosphate have been demonstrated to activate TLR pathways. "Sensitizing" of synoviocyte TLR responses by increased levels of sCD14 in OA may increase the

inflammatory response to these crystals. Finally, both OA and RA are reported risk factors for septic arthritis (38). Although immunosuppressive medications likely affect risk in RA patients, sCD14 may be an additional risk factor in both RA and OA. Soluble CD14 may lower the threshold for, or increase severity of, TLR responses to infectious agents.

FLS responses described in this study were observed in vitro, so it is not clear whether effects of SF sCD14 are functional in vivo. SF concentrations $>25\%$ induced cell death, so the effect of 100% SF could not be determined. The viscosity of SF likely limits diffusion or availability of oxygen and media components important for cell survival in monolayer cultures. This also limits our ability to detect TLR agonist activity which might be in low concentrations. Still, our culture system was an attempt to partially recreate the joint environment in vitro, and we found that SF exerted an augmenting effect on TLR responses at least in part via sCD14. More work is needed to characterize additional SF factors that may influence FLS responses to TLR ligands, and to determine effects of SF sCD14 on other cell types that respond to TLR stimuli in vivo.

In summary, the current investigation supports a role for sCD14 in potentiating FLS responses to TLR-2 and TLR-4 ligands in OA patients with early and advanced disease. As a similar role for CD14 has been suggested in RA, our work suggests similarity in TLR activation requirements of FLS in these 2 different arthritides. Our findings do not specifically address initiation of disease, but are consistent with reports supporting a role for local inflammation even in early stages of OA. We suspect that differences between extent and severity of inflammation in OA and RA may be due to differences in adaptive inflammatory responses, as more pronounced autoantibody production and systemic inflammation are more characteristic of RA. CD14 has recently been demonstrated to participate in signaling and intracellular trafficking of other TLRs including TLR-3, TLR-7, and TLR-9 (39,40); therefore, the effect of SF on other TLR ligands including candidate endogenous ligands needs to be explored.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Scanzello had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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