










## CD14<sup>+</sup> monocytes and soluble CD14 of synovial fluid are associated with osteoarthritis progression

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### ABSTRACT

**Objectives:** This study aims to investigate the role of cluster of differentiation 14 (CD14) expressed monocytes and soluble CD14-mediated pathway in the synovial inflammation of knee osteoarthritis (OA).

**Patients and methods:** Between May 2012 and July 2013, a total of 35 patients with knee OA (9 males, 26 females; mean age: 66.3±8.8 years; range, 52 to 79 years) were included in this cross-sectional study. Synovial fluid was obtained from knee joints of 35 OA patients. The CD14<sup>+</sup> monocytes from synovial fluid mononuclear cells (SFMCs) were isolated using the MACS. The fibroblast-like synoviocytes (FLSs) isolated from knee joint tissue were incubated with recombinant CD14 and lipopolysaccharide (LPS) for 24 h. Cytokine profiling was performed with the Luminex® Performance Assay or magnetic bead panel kit. The expression of CD14 and CD16 was analyzed by immunohistochemistry and flow cytometry.

**Results:** The concentration of sCD14 in synovial fluid was correlated with the interleukin-6 (IL-6) level (n=35) ( $\rho=0.654$ ,  $p<0.001$ ). The culture supernatants of CD14<sup>+</sup> monocytes isolated from SFMC (n=15) showed a correlation between sCD14 and IL-6 ( $\rho=0.784$ ,  $p=0.001$ ), along with complement component 3 ( $\rho=0.756$ ,  $p=0.010$ ), IL-1 $\beta$  ( $\rho=0.652$ ,  $p=0.012$ ), and tumor necrosis factor-alpha ( $\rho=0.806$ ,  $p=0.001$ ). Following recombinant CD14 and LPS treatment, OA FLS synergistically enhanced the secretion of IL-6, IL-8, and matrix metalloproteinase 3 (n=3,  $p<0.05$ ). In five paired-samples from identical patients, the proportions of CD14<sup>+</sup> monocytes were significantly elevated in recurrent synovial fluid compared to those in initial synovial fluid ( $p=0.043$ ). When monocyte subsets were analyzed in SFMC (n=26), CD14<sup>+</sup>CD16<sup>+</sup> monocytes were abundant ( $p=0.019$ ) and had higher toll-like receptor 4 expression than CD14<sup>+</sup>CD16<sup>-</sup> ( $p<0.001$ ).

**Conclusion:** Our study results suggest that CD14<sup>+</sup> monocytes and the sCD14-mediated pathway play an important role in OA aggravation through inflammatory cytokine secretion.

**Keywords:** Cytokines, monocytes, osteoarthritis, synovial fluid.

Osteoarthritis (OA) is mainly considered to be a degenerative condition of the articular cartilage.<sup>1</sup> The pathogenesis of OA has been known as cartilage damage, abnormal bone remodeling, and inflammation of the synovium.<sup>2</sup> Although OA is usually regarded as a non-inflammatory disease, low-grade inflammation has been reported and is directly related to disease severity.<sup>3</sup> Synovitis in OA consistently stimulates cartilage degradation,

fibroblast-like synoviocytes (FLSs) activation, and joint injury.<sup>4</sup>

Due to the secreted inflammatory cytokines/chemokines, OA synovium has elevated immune cells (T cells, B cells, and monocytes/macrophages) that is less than that in rheumatoid arthritis, but higher than that in a healthy individual.<sup>5-8</sup> Among the immune cells,

**Received:** July 12, 2021 **Accepted:** October 02, 2021 **Published online:** March 03, 2022

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### Citation:

Lee HR, Lee S, Yoo IS, Yoo SJ, Kwon MH, Joung CI, et al. CD14<sup>+</sup> monocytes and soluble CD14 of synovial fluid are associated with osteoarthritis progression. Arch Rheumatol 2022;37(3):335-343.

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monocytes play a critical role in OA synovitis through their own phagocytic activity and secretion of inflammatory mediators.<sup>9,10</sup> Synovial monocytes lead to inflammatory edema, synovium lining cell thickening, and chondrocyte apoptosis via synovial fluid.<sup>11</sup> The excessive transmigration and aberrant activation of monocytes could cause cartilage destruction and joint inflammation.<sup>12</sup> The major immune cells of synovial fluid in OA are also monocytes/macrophages.<sup>13</sup> Monocytes can be subdivided based on the expression of cluster of differentiation 14 (CD14) and CD16; that is, CD14<sup>+</sup>CD16<sup>+</sup> (intermediate, 5 to 10% of monocytes) and CD14<sup>+</sup>CD16<sup>-</sup> (classical, 90 to 95% of monocytes).<sup>14</sup> The CD14<sup>+</sup>CD16<sup>-</sup> monocyte is most abundant in healthy human peripheral blood, while the CD14<sup>+</sup>CD16<sup>+</sup> monocyte is dramatically increased in inflammatory disease.<sup>15</sup> In OA synovial fluid, CD14<sup>+</sup>CD16<sup>+</sup> monocytes are elevated compared to peripheral blood.<sup>16</sup>

The CD14 acts as a co-receptor for toll-like receptor 4 (TLR4) and TLR2, stimulating the TLR signaling pathway.<sup>17,18</sup> Soluble CD14 (sCD14) is mainly produced by activated monocytes/macrophages and directly binds to lipopolysaccharide (LPS).<sup>19</sup> The CD14/LPS combines with the TLR4/MD2 complex, which induces secretion of inflammatory molecules such as reactive oxygen species (ROS), interleukin-1 beta (IL-1 $\beta$ ), and IL-6.<sup>20</sup> In the present study, we aimed to investigate the role of CD14 expressed monocytes and soluble CD14-mediated pathway in the inflammation of knee OA. To understand the correlation between sCD14 and OA inflammation, the level of inflammatory cytokines in synovial

fluid were determined in this study. The role of CD14<sup>+</sup> monocytes in disease progression was also studied.

## PATIENTS AND METHODS

This single-center study was conducted at Chungnam National University Hospital, Department of Internal Medicine, Division of Rheumatology between May 2012 and July 2013. A total of 35 patients with knee OA (9 males, 26 females; mean age: 66.3 $\pm$ 8.8 years; range, 52 to 79 years) were included (Table 1). Inclusion and exclusion criteria were in accordance with the American College of Rheumatology (ACR) criteria for the classification OA of the knee.<sup>21</sup> Patients were excluded if any of the following disorders: inflammatory diseases such as septic arthritis and rheumatoid arthritis, history of knee surgery, infectious diseases, cancer, multiple sclerosis, stroke, and immune system disorder.

Cartilage and synovium were obtained from the patients who underwent total knee arthroplasty. Synovium was incubated with 0.1% collagenase (Sigma-Aldrich, MO, USA) in Dulbecco's modified Eagle's medium (DMEM; Gibco, MA, USA) for 2 h at 37°C. After washing, isolated FLS were cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin, and 100 mg/mL streptomycin and, then, maintained in a 5% CO<sub>2</sub> incubator at 37°C. The FLS was used for experiments after four to six passages.

**Table 1.** Baseline characteristics of all patients

Variables	Subjects with knee OA (n=35)		
	n	%	Mean $\pm$ SD
Age (year)			66.3 $\pm$ 8.8
Sex			
Female	26	74.3	
Kellgren-Lawrence score (grade)			2.9 $\pm$ 0.8
Corticosteroid injection during arthrocentesis	24	68.6	
Treatment			
Steroid	4	11.4	
NSAID	17	48.6	
Acetaminophen	9	25.7	

SD: Standard deviation; NSAID: Non-steroidal anti-inflammatory drug.

Synovial fluid samples were obtained from knee arthrocentesis, which was performed for symptom relief or prior to knee injection. Whole synovial fluid was centrifuged, and the supernatant was stored at  $-80^{\circ}\text{C}$  until use.

### **Immunohistochemistry**

Synovial tissue specimens were fixed in 4% paraformaldehyde for 24 h and, then, embedded in paraffin. For immunohistochemistry analyses, serial sections of synovium were obtained and mounted on charged glass slides (Fisher Scientific, NH, USA). The sections were deparaffinized and, then, treated with 3% hydrogen peroxide in methanol to quench the endogenous peroxidase activity. Samples were, then, incubated with 1% bovine serum albumin (Gibco) to block non-specific binding. After blocking, sections were incubated with primary mouse anti-CD14 (Cat #ab181470, Abcam, Cambridge, UK; 1:100 dilution) antibody, rabbit anti-CD16 (Cat #ab203883, Abcam; 1:100 dilution) antibody, or mouse anti-CD56 (Cat #ab9018, Abcam; 1:100 dilution) antibody at  $4^{\circ}\text{C}$  overnight. After washing, the slides were incubated in a 1:250 dilution of secondary antibody at room temperature for 15 min. Images were observed under light microscopy (Olympus, Tokyo, Japan).

### **Measurement of soluble CD14 and cytokines**

The concentration of soluble CD14 was measured using an enzyme-linked immunosorbent assay (ELISA) kit for human CD14 (Cat #DC140, R&D Systems, MN, USA) according to the manufacturer's instructions. Cytokine levels were estimated by interpolation from a standard curve generated using an ELISA reader (Molecular Devices, CA, USA) at 450 nm. Cytokine and chemokine profiling was performed with the Luminex<sup>®</sup> Performance Assay (Cat #LUHM000 as control, Cat #LUHM 200 for IL-1- $\alpha$ , Cat #LUHM201 for IL-1 $\beta$ , Cat #LUHM206 for IL-6, Cat #LUHM208 for IL-8, Cat #LUHM210 for TNF- $\alpha$ , Cat #LUHM217 for IL-10, Cat #LUHM285 for IFN- $\gamma$ , R&D Systems) or human cytokine/chemokine magnetic bead panel kit (Cat #HMMP1MAG-55K for MMP-3, Cat #HCMP2MAG-19K for C3, Millipore Sigma, MA, USA) according to the manufacturers' instructions.

### **CD14 positive cell isolation and culture**

Synovial fluid mononuclear cells (SFMCs) were obtained from whole synovial fluid using Ficoll-Paque<sup>™</sup> Plus (GE Healthcare, IL, USA) density gradient centrifugation. The CD14<sup>+</sup> cells were labeled with CD14 MicroBeads (Cat #130-050-201, Miltenyi Biotec, Bergisch Gladbach, Germany) and isolated using the MACS system according to the manufacturer's instructions. The purity of isolated CD14<sup>+</sup> cells was determined to be  $>90\%$  (data not shown). The CD14<sup>+</sup> cells were maintained in Roswell Park Memorial Institute culture medium (RPMI; Gibco) supplemented with 2 mM L-glutamate and 10% FBS (Gibco) at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$ .

Isolated FLSs ( $3 \times 10^3$ ) were seeded onto 96-well plates and were incubated in complete media with recombinant CD14 (0.5  $\mu\text{g}/\text{mL}$ ; Peprotech, NJ, USA) and LPS (100  $\text{ng}/\text{mL}$ ; Sigma-Aldrich) for 24 h. After centrifugation, the FLS supernatants were analyzed.

### **Flow cytometric analysis**

The cells were stained using allophycocyanin (APC)-conjugated anti-CD3 (Cat #555335, BD, NJ, USA), PerCP-Cy5.5-conjugated anti CD4 (Cat #560650, BD), fluorescein isothiocyanate (FITC)-conjugated anti-CD14 (Cat #11-0149-42, eBioscience, CA, USA), PE-Cy7-conjugated anti-CD16 (Cat #555408, BD), and phycoerythrin (PE)-conjugated anti-TLR4 (Cat #12-9917-41, eBioscience). Flow cytometry was performed with a FACSCanto<sup>™</sup> II flow cytometer (Becton, Dickinson & Company, NJ, USA), and data were processed with the FlowJo<sup>™</sup> software (version 10.7.1; Becton, Dickinson & Company, NJ, USA).

### **Statistical analysis**

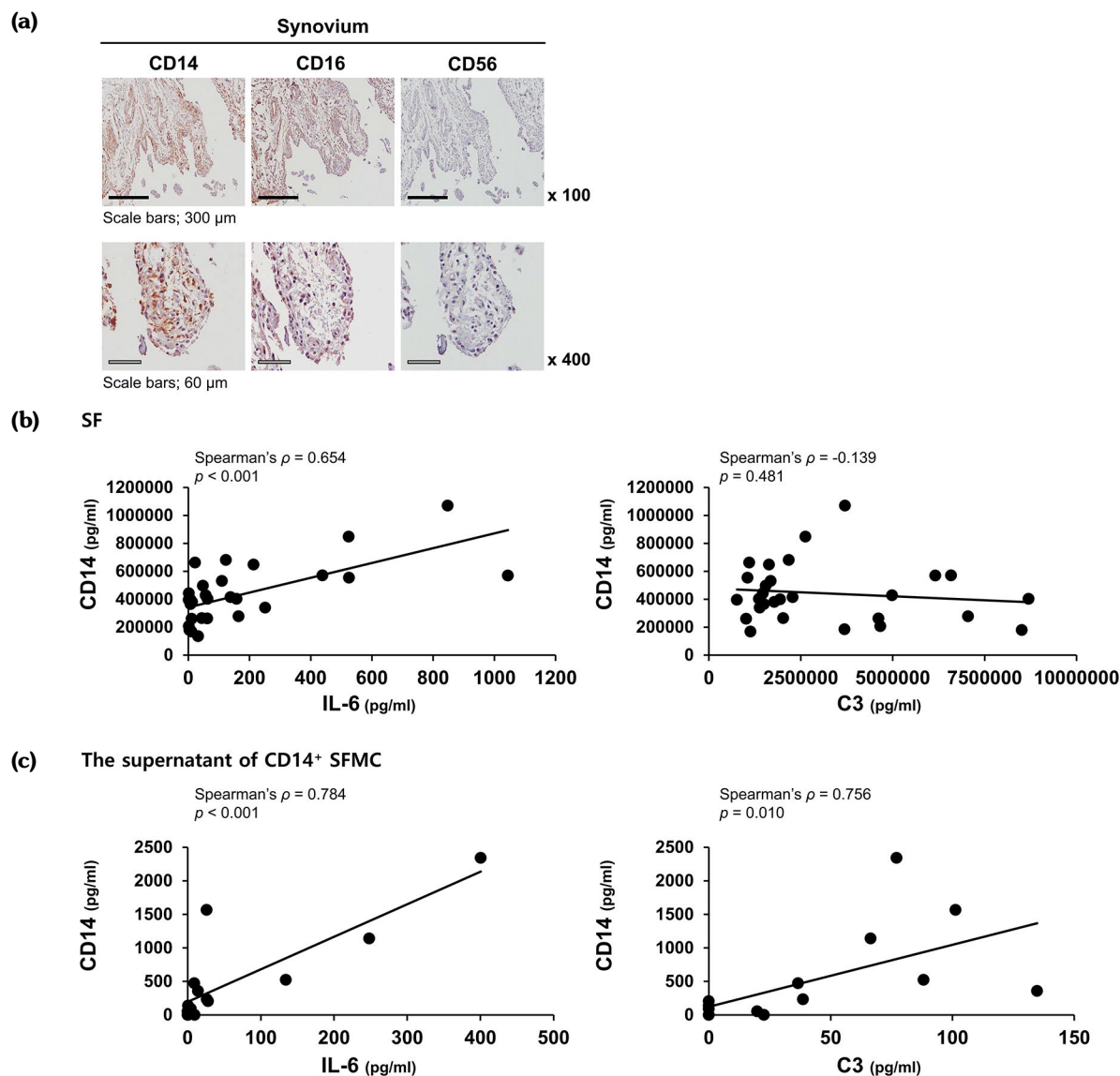
Statistical analysis was performed using the SPSS program version 22.0 software (IBM Corp., Armonk, NY, USA). Descriptive data were expressed in mean  $\pm$  standard deviation (SD), median (min-max) or number and frequency. The associations between CD14 and cytokines levels were determined by Spearman's correlation coefficient. Group comparisons were performed using One-way analysis of variance (ANOVA), followed by the Duncan's *post hoc* test. Different letters (a, b, c) indicated values having significant difference at  $p < 0.05$ . The same letter represented groups that are not statistically significant. Direct

comparisons between two groups were performed using a paired t-test. The Wilcoxon signed-rank test was used for non-parametric comparisons between the results of initial and recurrent paired samples. A  $p$  value of  $<0.05$  was considered statistically significant.

## RESULTS

### sCD14 was correlated with IL-6 in OA synovial fluid

Monocytes/macrophages are present in abundance in OA synovium and are regarded



**Figure 1.** Positive correlation between sCD14 and IL-6 on OA patients. **(a)** Expression of CD14, CD16, and CD56 in the synovium from OA patients was analyzed using immunohistochemistry. Representative pictures are shown. Magnification is  $\times 100$  or  $\times 400$ . Black scale bar is 300  $\mu$ m and gray scale bar is 60  $\mu$ m. **(b)** The levels of inflammatory cytokines and sCD14 were examined in OA synovial fluids according to the manufacturer's instructions. Scatterplot shows the correlation between sCD14 and IL-6 concentration of synovial fluid from OA patients ( $n=35$ ). **(c)** CD14<sup>+</sup> cells were isolated from OA SFMC ( $n=15$ ) and were incubated in FBS containing RPMI at 37°C in 5% CO<sub>2</sub>. After 24 h, the supernatants were analyzed according to the manufacturer's instructions. Each symbol represents an individual donor. The resulting Spearman's correlation coefficient ( $\rho$ ) and corresponding  $p$  value are reported.

CD: Cluster of differentiation; SF: Synovial fluid; OA: Osteoarthritis; RPMI: Roswell Park Memorial Institute; SFMC: Synovial fluid mononuclear cells; FBS: Fetal bovine serum; IL-6: Interleukin-6; C3: Complement component 3.

as major inflammatory cytokine providers in synovial inflammation.<sup>22</sup> Thus, OA synovium was stained with anti-CD14 or anti-CD16 antibodies in this study. The CD14 and CD16 are commonly considered to be markers for monocytes, whereas CD16 and CD56 are considered to be markers of natural killer cells. Expression of both CD14 and CD16 was highly detected in OA synovium, but CD56 was not expressed (Figure 1a). We confirmed that CD14<sup>+</sup>CD16<sup>+</sup> monocytes were abundant in the OA synovium.

The CD14 acts as the receptor for LPS and assists signal activation, resulting in the production of inflammatory cytokines.<sup>19</sup> The correlation between CD14 and inflammatory cytokines was analyzed in OA synovial fluid (n=35). The sCD14 levels were significantly related to IL-6 concentration ( $\rho=0.654$ ,  $p<0.001$ ) (Figure 1b and Table 2). These data suggest that sCD14 may induce IL-6 secretion from monocytes and cause an inflammatory response in OA SFMC. To test this hypothesis, the CD14<sup>+</sup> monocytes were isolated from SFMC and incubated in FBS containing RPMI. After 24 h, the supernatants were collected (n=15). The IL-6 show expectably significant correlative levels with sCD14 in CD14<sup>+</sup> monocytes ( $\rho=0.784$ ,  $p=0.001$ ) (Figure 1c and Table 3). Complement component 3 (C3) ( $\rho=0.756$ ,  $p=0.010$ ), IL-1 $\beta$  ( $\rho=0.652$ ,  $p=0.012$ ), and tumor necrosis factor-alpha (TNF- $\alpha$ ) ( $\rho=0.806$ ,  $p=0.001$ ) levels revealed a positive correlation in the supernatant of CD14<sup>+</sup> SFMC.

**sCD14 induced FLS inflammation synergistically with LPS treatment**

Next, we investigated whether sCD14 in synovial fluid would have an effect on FLS inflammation. Following treatment with recombinant CD14 and LPS for 24 h, the concentrations of IL-6, IL-8, and matrix metalloproteinase 3 (MMP-3) in the supernatant of FLS from OA patients (n=3) were measured by Luminex<sup>®</sup> assay or magnetic bead panel kit. The production of IL-6, IL-8, and MMP-3 were synergistically elevated in CD14/LPS-treated cells compared to CD14 or LPS alone ( $p<0.05$ ) (Figure 2). Thus, sCD14 of synovial fluid could assist the activation of OA FLS and eventually led to production of pro-inflammatory cytokines, thereby probably further contributing to cartilage damage and inflammation.

**CD14<sup>+</sup>CD16<sup>+</sup> monocytes were enriched in OA SFMC compared to CD14<sup>+</sup>CD16<sup>-</sup>**

To determine whether CD14<sup>+</sup> monocytes are relevant to OA disease activity, CD14<sup>+</sup> monocytes were isolated from OA SFMC (n=5). Among the total number of study patients who underwent arthrocentesis, five patients underwent repeat arthrocentesis due to recurrence of swelling in the same knee (time interval: 1-11 months). Four of these patients received triamcinolone injection after initial arthrocentesis, and one patient did not. As shown in Figures 3a and b, the proportion of CD14<sup>+</sup> monocytes was enormously increased in recurred synovial fluid ( $58.6\pm35.0\%$ ), compared to initial synovial fluid ( $8.7\pm8.6\%$ ,  $p=0.043$ ). The abundant CD14<sup>+</sup> monocytes in synovial fluid could reflect OA inflammation.

**Table 2.** Correlation between sCD14 and cytokines of synovial fluid in OA patients

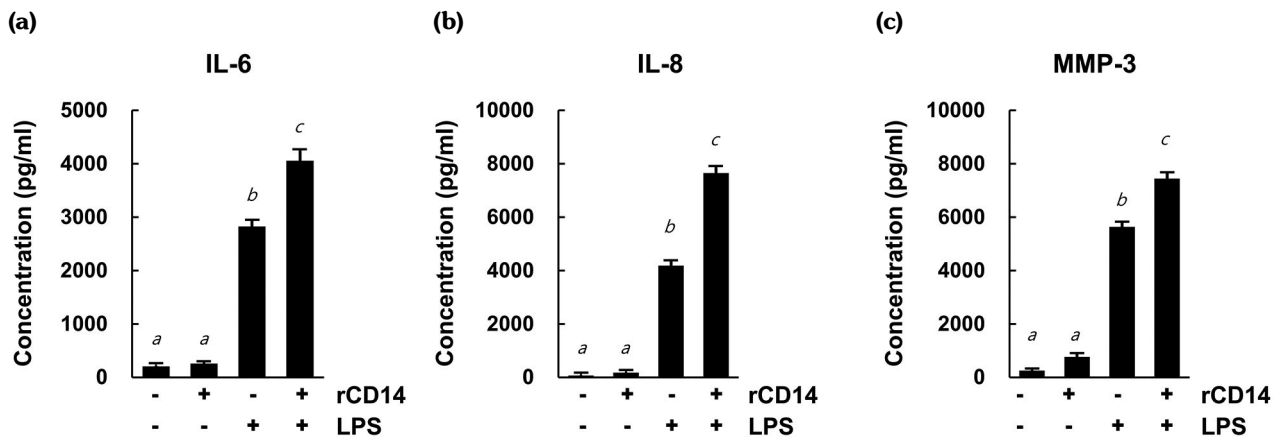
Cytokine name	Spearman's $\rho$	$p$
IL-6	0.654	<0.001
C3	-0.139	0.481
IL-1 $\alpha$	-0.080	0.680
IL-1 $\beta$	-0.134	0.489
IL-8	-0.182	0.343
IL-10	0.104	0.592
IFN- $\gamma$	0.050	0.796
TNF- $\alpha$	-0.160	0.409

sCD14: Soluble cluster of differentiation 14; IL: Interleukin; C3: Complement component 3; IFN- $\gamma$ : Interferon gamma; TNF- $\alpha$ : Tumor necrosis factor-alpha.

**Table 3.** Correlation between sCD14 and cytokines in the supernatant of CD14<sup>+</sup> SFMC

Cytokine name	Spearman's $\rho$	$p$
IL-6	0.784	0.001
C3	0.756	0.010
IL-1 $\beta$	0.652	0.012
IL-8	0.501	0.068
TNF- $\alpha$	0.806	0.001

sCD14: Soluble cluster of differentiation 14; SFMC: Synovial fluid mononuclear cells; IL: Interleukin; C3: Complement component 3; TNF- $\alpha$ : Tumor necrosis factor-alpha.



**Figure 2.** Synergistic increase of inflammatory mediators with sCD14/LPS treatment in FLS. Isolated FLS from patients (n=3) with OA were stimulated with recombinant CD14 (0.5  $\mu\text{g}/\text{mL}$ ) and LPS (100 ng/mL) in RPMI containing 10% FBS at 37°C in 5% CO<sub>2</sub>. After 24 h, levels of secreted IL-6, IL-8, and MMP-3 in stimulated and unstimulated OA FLS were measured according to the manufacturer's instructions. The bar represents the mean. Statistical analysis was performed by one-way ANOVA, followed by Duncan's post hoc test. Different letters (**a-c**) show values having significant difference at  $p < 0.05$ . The same letter represents groups that are not statistically significant.

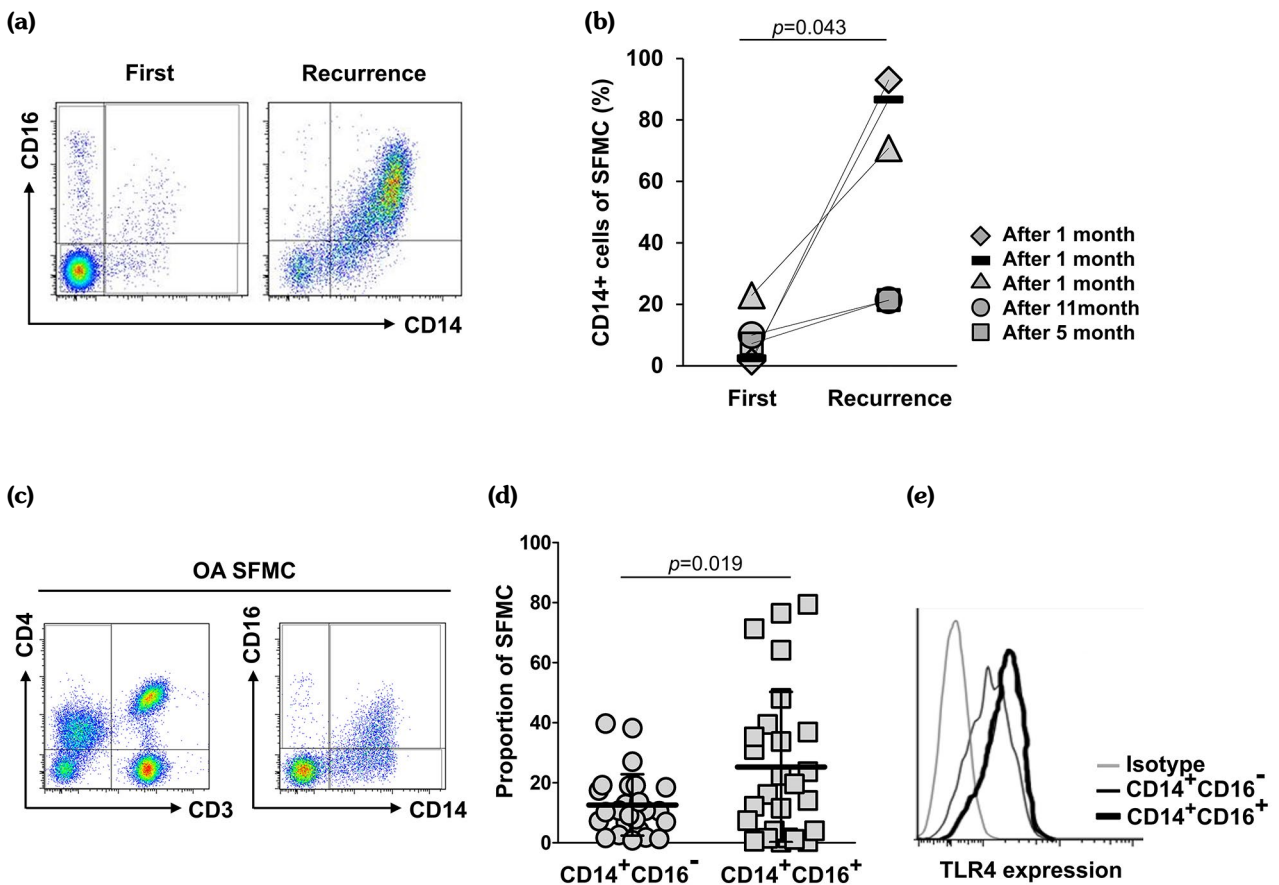
IL: Interleukin; MMP-3: Matrix metalloproteinase-3; rCD14: Recombinant CD14; LPS: Lipopolysaccharide; RPMI: Roswell Park Memorial Institute; FBS: Fetal bovine serum; FLS: Fibroblast-like synoviocytes.

The expressions of CD3, CD4, CD14, and CD16 in the isolated SFMC were determined using flow cytometry (Figure 3c). There was an abundance of CD3<sup>+</sup>CD4<sup>-</sup> cells (20.7±12.8%), CD3<sup>+</sup>CD4<sup>+</sup> cells (16.7±12.9%), and CD14<sup>+</sup> cells (36.6±25.2%) in OA SFMC (data not shown). Among SFMC (n=26), the proportion of CD14<sup>+</sup>CD16<sup>+</sup> monocytes (21.2±21.8%) was increased compared to that of CD14<sup>+</sup>CD16<sup>-</sup> (10.9±10.0%) ( $p=0.019$ ) (Figure 3d). These results are consistent with the CD14 and CD16 staining of synovium. TLR4 expression was higher in CD14<sup>+</sup>CD16<sup>+</sup> monocytes (307±272 M.F.I.) than in CD14<sup>+</sup>CD16<sup>-</sup> (159±128 M.F.I.) ( $p < 0.001$ ) (Figure 3e). The TLR2 expression was also increased more in CD14<sup>+</sup>CD16<sup>+</sup> than CD14<sup>+</sup>CD16<sup>-</sup> monocytes (data not shown). Accordingly, inflammation in OA might be more dependent on CD14<sup>+</sup>CD16<sup>+</sup> than CD14<sup>+</sup>CD16<sup>-</sup> monocytes. Owing to the nominal proportion of CD16<sup>+</sup> cells in CD14<sup>-</sup> cells, the CD14<sup>-</sup>CD16<sup>+</sup> subpopulation was not included in this study.

## DISCUSSION

Synovium from OA patients shows the abnormal infiltration of mononuclear cells in both early and late stages of OA.<sup>12</sup> As CD16 is

also expressed in natural killer cells, we stained the synovium with the specific antibody for the CD56 natural killer cell marker. We verified a high expression of CD14 and CD16, but not CD56, in OA synovium. As CD14 is involved in host defense mechanism of monocytes, inflammatory-related molecules were screened. The whole synovial fluid from OA patients revealed a positive correlation between the levels of sCD14 and IL-6. As IL-6 is a representative inflammatory cytokine and secreted by activated monocyte,<sup>23</sup> monocytes were isolated and analyzed. The CD14 was associated with inflammatory molecules (i.e., IL-6, C3, IL-1 $\beta$ , and TNF- $\alpha$ ) in the supernatant of CD14<sup>+</sup> SFMC. In a study by Daghestani et al.,<sup>24</sup> sCD14 in synovial fluid was shown to have a strong correlation with the severity of joint space narrowing and OA pain. The activated TLR signaling on monocytes induces production of pro-inflammatory cytokines/chemokines and the delivery of pain signal.<sup>25</sup> Also, OA is known to exhibit increased expression and activation of C3 in synovial joints, and C3 is regarded as a critical factor for OA development.<sup>26</sup> Released C3 has been shown to contribute to accelerate enzymatic activity, cartilage degradation, and inflammation in joint inflammation.<sup>27</sup> These findings suggest that CD14<sup>+</sup> monocytes may play an important



**Figure 3.** Percentage repartition of CD14<sup>+</sup> monocytes on SFMC from OA patients. **(a)** SFMCs were isolated from OA patients and stained with specific markers. The single cells were gated in the isolation SFMC using FSC-A and FSC-H, and monocytes were identified by CD14 and CD16 expressions. Representative data are shown. **(b)** The population of CD14<sup>+</sup> was examined on SFMC from five OA patients who underwent arthrocentesis at both baseline (first) and follow-up (recurrence) times. Each symbol corresponds to one pair from the same individual donor. **(c)** To study monocyte population, SFMC were isolated and analyzed with a specific marker as schemed. The single cells were gated in the isolation SFMC using FSC-A and FSC-H, and T cells were identified by CD3 and CD4 expressions. To detect monocytes, CD14 and CD16 expressions were gated in singlets. Representative data are shown. **(d)** SFMC from OA patients (n=26) were divided by CD14 and CD16 expressions and analyzed by flow cytometry. Each symbol represents an individual donor, and the bar represents the mean. p=0.019 **(e)** The surface expression of TLR4 was detected in a monocyte subset of OA SFMC. Isotype control is represented by the grey line, CD14<sup>+</sup>CD16<sup>-</sup> expressed TLR4 is indicated by the black line, and CD14<sup>+</sup>CD16<sup>+</sup> expressed TLR4 is represented by the bold line. Data represent results of one experiment, which was performed in triplicate with similar results.

CD: Cluster of differentiation; SFMC: Synovial fluid mononuclear cells; TLR4: Toll-like receptor 4; OA: Osteoarthritis; FSC-H: Forward scatter height; FSC-A: Forward scatter area.

role in inflammatory response in OA synovium and sCD14 may be also an essential component in that response.

Joint inflammation can be influenced by crosstalk of monocytes and FLS via synovial fluid. Activated monocytes secrete abundant inflammatory cytokines/chemokines in response to TLR pathway activation. With LPS stimulation, IL-6 and IL-8 are significantly elevated in OA

FLS.<sup>28</sup> In this study, FLSs from patients with OA were incubated with recombinant CD14 and LPS and, then, inflammatory cytokines were screened. As expected, CD14-stimulated OA FLS demonstrated a synergistic increase of IL-6, IL-8, and MMP-3 with LPS treatment. The MMP-3 has been reported to be secreted by FLS and cause cartilage degradation of joint.<sup>29,30</sup> Furthermore, when CD14<sup>+</sup>

macrophages are depleted in OA synovial cells *in vitro*, production of IL-6, IL-8, and MMP-3 is attenuated in FLS.<sup>31</sup> Therefore, it is likely that CD14-stimulated FLS secretes these cytokines, which could contribute to worsening OA progression. Next, we investigated whether CD14 affected inflammatory activity in OA. The CD14<sup>+</sup> monocytes were considerably increased in recurred synovial fluid compared to initial synovial fluid. The short period of recurrence was related to the high proportion of CD14<sup>+</sup> monocytes in SFMC. Although the reason for the increased population in synovial fluid is not clear, CD14<sup>+</sup> monocytes and sCD14 may have a crucial role in OA inflammation.

Among monocytes, the CD14<sup>+</sup>CD16<sup>+</sup> intermediate monocytes are reported to respond to inflammation and have more phagocytic activity in peripheral blood.<sup>32</sup> In the current study, we showed CD16<sup>+</sup> cell infiltration in OA synovium, and an abundance of CD14<sup>+</sup>CD16<sup>+</sup> monocytes were found in OA SFMC compared to CD14<sup>+</sup>CD16<sup>-</sup>. Interestingly, another study also showed that CD14<sup>+</sup>CD16<sup>+</sup> monocytes were the major subpopulation of monocytes in the synovial fluid from a knee with OA and are therefore associated with quality of life.<sup>16</sup> The CD14<sup>+</sup>CD16<sup>+</sup> monocyte population also upregulated TLR2/4 expression. As sCD14 is the co-receptor for TLR2 and TLR4 and enhances pro-inflammatory responses,<sup>28</sup> the high expression of TLRs indicates that monocytes are activated and participate in CD14-mediated inflammatory response. Nevertheless, further studies are needed to reveal the comparison between CD14<sup>+</sup>CD16<sup>+</sup> and CD14<sup>+</sup>CD16<sup>-</sup> in OA severity.

This study has some limitations. This is mainly cross-sectional study, although there are five patients with following arthrocentesis. Thus, it is difficult to conclude the long-term progression of OA. We did not have samples of normal control or disease control to compare the results. In addition, this study has a relatively small sample size. There is no available information to calculate an appropriate sample size. A more detailed, large-scale investigation of CD14 in the joint of OA would confirm the critical role of CD14 in triggering cartilage degeneration.

In conclusion, our study findings suggest that CD14 and CD14<sup>+</sup> monocytes can be suggested as

important mediators for OA inflammation, which implies that they can act as therapeutic targets for OA.

**Ethics Committee Approval:** The study protocol was approved by the Chungnam National University Hospital Institutional Review Board (2012-04-008 and 2018-05-061). The study was conducted in accordance with the principles of the Declaration of Helsinki.

**Patient Consent for Publication:** A written informed consent was obtained from each patient.

**Data Sharing Statement:** The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Author Contributions:** Validation and visualization, project administration and writing: H.R.L.; Data curation, investigation, resources, validation and visualization: S.L.; Data curation, investigation, and resources: I.S.Y., S.J.Y., M.H.K., C.I.J., J.A.P.; Conceptualization and methodology: S.W.K.; Conceptualization and methodology, project administration, writing and supervision: J.K.

**Conflict of Interest:** The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

**Funding:** This work was supported by the Chungnam National University Hospital Research Fund 2013.

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