Expanded CD1c+CD163+ DC3s population in synovial tissues is associated with disease progression of osteoarthritis

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Research Article

Keywords: Osteoarthritis, Dendritic Cells, DC3, Synovium Fluid

Posted Date: January 24th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1250384/v1

Expanded CD1c+CD163+ DC3s population in synovial tissues is associated with disease progression of osteoarthritis

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Conflict of Interest: The authors declare that they have no conflict of interest.

Abstract

Objective: The mechanisms underlying osteoarthritis (OA) have recently been hypothesized to involve a dysfunctional immune system. This study aimed to evaluate the landscape of immune cells infiltrating synovial tissue and to determine their function in OA progression.

Design: Synovial tissue, synovium fluid (SF) and peripheral blood were collected from 21 patients with OA. Mononuclear cells were isolated and characterized using flow cytometry. H&E staining and multiplex immunohistochemistry (mIHC) histological assessment of synovial samples were performed. Cytokine levels in the SF were measured using ELISA.

Results: We observed similar frequencies of immune cells in the synovium and SF, which were enriched in macrophages, T cells, and DCs. Notably, DC3s, $CD1c^{+}DC163^{+}$ DCs, an inflammation-induced subpopulation of DCs, was significantly expanded in the synovium and SF. Furthermore, we found that DC3s were primarily located within the ectopic lymphoid-like structure (ELLS) in close proximity to $CD8^{+}$ T cells. Finally, the level of TNF-α and IL12p70 in the SF correlated with the severity of OA, suggesting a possible link between DC3s and OA progression.

Conclusion: These data suggest that OA is an immune system-related disease and that DC3s may play an active role in OA progression by promoting ELLS formation and inflammatory responses.

Keywords: Osteoarthritis, Dendritic Cells, DC3, Synovium Fluid

Introduction

Osteoarthritis (OA) is the most prevalent arthritic condition in the elderly (over 60 years worldwide), and thus has a high impact on patient activity, and is associated with heavy economic burden[1]. Pain is the most common sign of disease and the leading cause of disability[2]. Although OA is mainly considered a degradative condition of the articular cartilage, there is increasing evidence demonstrating that OA is a low-grade inflammatory disease that affects all tissues of the joint, characterized by profound changes in intracellular mechanisms, and decreased efficiency of the immune system with ageing[3].

After T cells, macrophages, and other immune cells infiltrate into joint tissues, cytokines and chemokines such as TNF-α and IFN-γ are secreted, the complement system is activated, and cartilage-degrading factors such as matrix metalloproteinases are released, causing damage to the articular cartilage[4]. There has been considerable success in the treatment of rheumatoid arthritis using anti-cytokine therapies[5]. However, these therapies did not show much effect in OA, highlighting the more complex nature of OA pathogenesis. Thus, a better understanding of the pathogenic mechanisms of chronic immune activation and the development of novel therapeutic strategies for OA are urgently required.

Dendritic cells (DCs) are a class of bone marrow-derived cells arising from lymphomyeloid hematopoiesis. DCs form an essential interface between the innate sensing of pathogens and the activation of adaptive immunity[6]. The initiation and control of immune responses depends on three major subsets: plasmacytoid DCs (pDCs), myeloid/conventional DC1 (cDC1s), and DC2 (cDC2s). Previously, we

demonstrated that both macrophages and DCs were enriched in the joint synovium, suggesting that the type, density, and location of immune cells within the local milieu may strongly influence OA pathogenesis. Here, we aimed to evaluate and explore the full spectrum of immune cell types and DC subsets using high-dimensional flow cytometry and multiplex immunohistochemistry (mIHC). Specifically, cDC2s can be further separated into two subpopulations according to BTLA and CD163 expression: $BTLA^{+}CD163^{-}$ cDC2s and $BTLA^{-}$ $CD163^{+}$ DC3s, with the latter primarily induced by inflammation[7 8]. In the current study, significantly increased levels of inflammatory DC3s were observed in the synovium compared with PBMCs and synovial fluid (SF). In addition, DC3s and $CD8^{+}$ T cells co-localized within ectopic lymphoid-like structures (ELLS), suggesting a role for DC3s in organizing ELLS by attracting and activating $CD8^{+}$T cells. Moreover, the number of ELLS is proportional to disease severity. Together, this study revealed that DC3s were highly enriched in the joints of OA patients, with potentially pathogenic roles in disease progression.

Method

1. Study Population

From March 2021 to June 2021, patients with OA in the Orthopedic Surgery Department of Guanghua Hospital, Shanghai University of Traditional Chinese Medicine (21 patients) were enrolled. The diagnostic criteria for OA conformed to those of the American College of Rheumatology. We used the Kellgren and Lawrence (K&L) score to assess disease severity in patients with OA through imaging. Blood testing of

each patient revealed that there was no inflammatory reaction in the body before the operation. The clinical characteristics of the study population are shown in Figure 1A. This study was approved by the Ethics Committee of Guanghua Hospital and all study participants provided written informed consent.

2. Sample Preparation for Flow Cytometry Analysis

Before surgery, 5 mL of venous whole blood from was collected OA patients, placed in a sodium heparin anticoagulation tube and transported at room temperature. During total knee arthroplasty (TKA) surgery, synovial tissue was collected, and the joint fluid was placed in EDTA tube, and transported on ice at 4°C. Peripheral blood mononuclear cells (PBMCs) were separated by density centrifugation on Lymphoprep (Axis-Shield, Norway). For the flow cytometry assay, 100 μl fluorescently labeled antibody mixture was added to each sample (containing CD16-BV510, CD33-BV711, CD56-PE, CD19-BV650, CD14-A700, CD15/CD66b-PerCP-Cy5.5, CD1c-BB515, CD45-APC-Cy7, CD3-PE-Cy5.5, BTLA-PE-Cy5, CD88-PC594, CD141-APC, FceRIα-Biotin/Streptavidin -BUV395, CD123-BV786, CD163-BV605, and HLADR-BV421). Meanwhile, mononuclear cells (MNs) were obtained from tissues as follows: after rinsing tissue with 1×PBS three times, weighing, and first cutting into 1 cm × 1 cm pieces with ophthalmological scissors for the mIHC assay, the remaining tissue was cut into small pieces. SF were frozen in aliquots of 100 μl at -80 °C for subsequent cytokine detection; digestive enzymes (type II collagenase 10 mg/mL+DNaseI100 U/mL) was then separately added to synovial tissue and SF, digested for 1 h in a 37 ℃ shaker, and filtered through a 70 μm mesh. Five ml of MACS buffer containing 1% FBS was then

added, and the sample was centrifuged at 500×g for 10 min. The supernatant was discarded, 100 μL of MACS buffer was added to resuspend the cells, the cells were transferred into a sterile EP tube, Zombie Yellow-BV570 was added for staining (RT, 30 min), and 100 μl fluorescently labeled antibody mixture was added for cell surface staining (room temperature, 15 min). The cells were washed with 1 mL of MACS buffer (800×g, 2 min) and the supernatant was discarded. Cells were then fixed in fixation buffer (eBioscience, Intracellular Fixation Buffer) for 10 min at 4 °C, washed with 1 mL MACS buffer and resuspended in 500 μL MACS buffer. A BD Fortessa flow analyzer and FlowJo software (V10.7.2) was used for data analysis.

3. Histological assessment of the synovium samples

Synovial tissue samples were retrieved for histological analysis after paraffin embedding. Following hematoxylin and eosin (H&E) staining of 4 μm-thick paraffin-embedded sections, multiplex immunohistochemistry (mIHC) was performed according to the manufacturer's instructions (Thermo Fisher Scientific Logo, Opal® Kit). This process was performed using the following antibodies and fluorescent dyes in the following order: CD1c/Opal570, CD8/Opal520, and CD163/Opal650. Detailed procedures for mIHC and quantitative analysis were performed as previously reported[9]. Slides were scanned and imaged using the PerkinElmer Vectra3® platform and analyzed in batches using PerkinElmer inform and R script for the quantification of positively stained cells.

4. Cytokine detection

The joint fluid was thawed at room temperature for 10 min. Before use, the joint fluid

was centrifuged at 10,000×g for 10 min and the supernatant was collected. ELISA assays for human TNF-α, IL12p70, or IL23 were performed according to the manufacturer's instructions (Absin Bioscience Inc., abs510012/abs510006/abs510013). The corresponding cytokine concentrations in each sample were obtained by referring to the standard curve.

5. Statistical analysis

Continuous variables were compared using the t-test or non-parametric Mann–Whitney U test, as appropriate. The Kruskal-Wallis test was used for the analysis of immune cell frequencies between the study groups. In the latter case, variations in statistical significance were further subjected to post-hoc pairwise analyses. The Mann-Whitney U test was performed to assess differences in cytokine expression in SF samples. All the reported p-values were two-tailed. A p-value <0.05 was considered to show a statistically significant difference. Statistical analyses were performed using GraphPad Prism 8.3.0 software and SPSS 25.0. The number of nearest neighbors was calculated on the assigned coordinates of each cell using R software with the spatstat package.

Results

1. Differences in immune-cell patterns between synovium tissue, SF and PBMCs in OA patients.

OA patients undergoing total knee arthroplasty (TKA) were enrolled in our study. Classification of 10 KL3 and 11 KL4 OA patients was performed on the basis of clinical

158 signs. Of the 21 patients identified, the mean age was 71 years, with no symptoms of
159 inflammation (Figure 1A). A total of 17/21 patients with OA were female, consistent
160 with the fact that autoimmune disease patients are most commonly female[10]. For each
161 patient, mononuclear cells from the tissues (MNs) and peripheral blood (PBMCs) were
162 isolated, as shown in Figure 1B.

163 To characterize the immune status of the synovium, we used 18-marker high-
164 dimensional flow cytometry to study the major immune cell populations compared to
165 paired PBMCs and SFs. Nine immune cell subsets were annotated: neutrophils (SSC-
166 $H^{hi}CD15^{+}CD16^{+}$), eosinophils (SSC-$H^{hi}CD15^{+}$), basophils ($FceRI\alpha^{+}HLADR^{-}$), T cells
167 ($CD3^{+}$), B cells ($CD19^{+}$), NK cells ($CD3^{-}CD56^{+}$), NKT cells ($CD3^{+}CD56^{+}$),
168 macrophages ($CD88^{+}CD14^{+}$), and DCs ($CD123^{+}HLADR^{+}$ pDCs, $CD1c^{+}$ mDCs, and
169 $CD141^{+}$ mDCs). Fig. 1 shows the conventional manual gating strategy used to define
170 these populations.

171 A comparative analysis was performed on the synovium, SF, and PBMCs. A
172 similar pattern of immune cells was observed in the synovium and SF, which was
173 completely different from that observed in PBMCs (Figure 1C-E). Although the number
174 of each immune cell population varied greatly among patients, the frequency of
175 macrophages showed major differences in synovium (50.77% ± 29.65% of $CD45^{+}$ cells;
176 P = .0001) compared to PBMCs (10.96% ± 6.38) (Figure 1D, SFigure 2). The
177 frequencies of total DCs in the SF and synovium were also significantly higher than
178 those in the PBMCs, whereas fewer neutrophils and B cells were found. NK cells and
179 NK T cells did not exhibit any major differences. However, a decreased percentage of

T cells was observed in both the synovium and SF (~25% $CD45^+$ cells). Taken together, these data showed that macrophages, T cells, and NK cells were enriched, whereas granulocytes were decreased in the synovium compared with PBMCs.

2. High infiltration of $CD1c^+CD163^+$ DC3s in synovium.

DCs comprise various subsets and exhibit distinct functions in autoimmune diseases[11]**.** The DC subgroups pDCs ($CD123^+$), DC1s ($CD123^-CD141^+$), DC2s ($CD1c^+BTLA^+CD163^-$), and DC3s ($CD1c^+BTLA^-CD163^+$) were further investigated in patients with OA. Each DC subgroup was gated as shown in Figure 2A. In line with earlier reports, the percentage of DC1s or total DCs was significantly higher in the synovium and SF than in the PBMC[12] group, whereas the percentage of pDCs did not differ between the three groups (Figure 2B). A similar proportion of DC2s was observed in SF and PBMCs. Intriguingly, DCs in the synovium were composed of almost 100% DC3s. Among $CD45^+$ immune cells, ~0.5% of DC3s were enriched in the synovium, while DC3 levels were found to be 10-fold higher in the SF. These results indicate that an increased number of cDCs accumulates during OA progression.

3. DC3s organize into an ectopic lymphoid -like structure (ELLS) associated with disease severity.

To better explore the extent of synovitis and the contribution of cells to the degree of inflammation, we performed histopathological analysis of synovial tissue samples collected from KL3 and KL4 OA patients. As shown in the left H&E image in Figure

202 3A, the lining cells formed a single layer, and the synovial stroma showed normal
203 cellularity with no inflammatory infiltrates. However, the presence of synovial lesions
204 consistent with low-grade synovitis demonstrated an increase in the thickness of the
205 lining layer and stromal cellularity, with the presence of a few, mostly perivascular
206 lymphocytes. Comparatively, high-grade synovitis (Figure 3A, right picture) was
207 distinguished by the presence of a greatly thickened lining, increased infiltration of
208 numerous lymphocytes, and typical ELLS.

209 Next, we applied mIHC to further investigate cellular infiltration in the OA
210 synovium by simultaneously gating for CD1c, CD8, and CD163. $CD1c^{+}CD163^{+}$ DC3s
211 and $CD8^{+}$ T cells were readily detected (Figure 3B). Recently, DC3s was proved *in vivo*
212 to efficiently induce differentiation of $CD8^{+}$T cells, while DC3 infiltration was found
213 to correlate with $CD8^{+}$ T cells accumulation in breast tumors[7]. Consistent with this, we
214 observed that DC3s and $CD8^{+}$T cells were highly enriched in ELLS. We hypothesized
215 that ELLS might be organized by DC3s, together with $CD8^{+}$T cells. To confirm this,
216 we applied cellular phenotyping of the fluorescence image and depicted the spatial
217 location of DC3s and $CD8^{+}$ T cells *in situ* (SFigure 3). We divided the DC3s-$CD8^{+}$ T
218 cell distance as < 20 μm and > 20 μm [9]. The relative number of DC3s was calculated
219 and significantly higher numbers of DC3s were recovered within 20 μm than beyond
220 (Figure 3C). In particular, ELLS were only found in < 20 μm positive samples including
221 OA18, OA38, and OA39. These data suggest that the aggregation of DC3s was
222 positively related to the formation of the ELLS structure.

223 DC3s are an important source of pro-inflammatory cytokines, including TNF-α,

L12p70, and IL23[7]. As shown in Figure 3D-F, the levels of TNF-α and IL12p70 in the SF of KL4 patients were significantly higher than those in KL3. Together, these results suggest that the increased infiltration of DC3s in joint infiltration, together with the elevated proinflammatory cytokines in SF, may play a critical role in the progression of OA.

Discussion

OA was initially defined as a disease induced by mechanical stress in the form of cartilage destruction, with minimal, if any, involvement of immune responses. Thus, OA, in contrast to rheumatoid arthritis (RA), has long been regarded as a non-inflammatory disease[13-15]. However, recent studies have demonstrated that, at least in certain patients, OA is an inflammatory disease, with patients frequently found to exhibit inflammatory infiltration of synovial membranes[16,17]. Recent studies have shown that the number of inflammatory cells in synovial tissue is higher than that in peripheral blood[18,19]. Both macrophages and T cells play important roles in OA pathogenesis[20-23]. Consistent with previous studies, we also found higher percentages of macrophages in synovial tissue and SF than in PBMCs, whereas a decreased number of T cells was measured in synovial tissue and SF[16,24].

Surprisingly, among $CD45^+$ cells, DCs were redundant in synovial tissue and SF. DCs have the broadest range of antigen presentation. The activation of innate immunity plays a critical role in the development and progression of OA. Innate immunity, including inflammasome activation, is triggered by small endogenous molecules called damage-associated molecular patterns, which are released in the extracellular media

after cell stress or damage, bind to pathogen-recognition receptors, including distinct Toll-like receptors, and activate the secretion of pro-inflammatory cytokines, resulting in joint inflammation. Moreover, the functional role of Toll-like receptors expressed on DCs in OA development has also been reported[25].

DCs are necessary for the activation of naïve T-cells. In this study, we found that DC3s, defined as $CD1c^{+}CD163^{+}$ cells, were significantly enriched in the synovium and could prime naïve $CD8^{+}$ T cells. In synovial tissue, $CD8^{+}$T cells are often located at the periphery of the aggregation[24]. As shown by our mIHC results, a substantial number of DC3s and $CD8^{+}$T cells were observed within the synovium. Consistent with this, higher levels of TNF-α and IL12p70, which are mainly secreted by DC3, were measured in the SF of patients with OA. Finally, DC3s-$CD8^{+}$T cells formed a tight structure named ELLS. Similar lymphoid-like structures, such as tertiary lymphoid organs in tumors, have been previously noted[26,27]. These structures are composed of various immune cell types, including dendritic cells and antigen-specific B-and T-lymphocytes. In contrast to secondary lymphoid organs, TLSs are not imprinted during embryogenesis, but are formed in non-lymphoid tissues in response to local inflammation. Before surgery, all enrolled patients with OA experienced pain with severe joint deformation. This may be related to the increased infiltration of DC3s into the synovium, which would prime more $CD8^{+}$T cells, causing more serious damage to the joint. Indeed, increased levels of TNF-α and IL12p70, of which DC3s are a major source, were observed in the SF of KL4 patients compared to KL3 patients. Although whether DC3s could predict poor OA prognosis needs to be further studied, our results strongly suggest that DC3s could

play a critical role in OA pathogenesis, and that targeting DC3s might be a novel therapeutic target to treat OA in both the early and late phases.

STATEMENTS & DECLARATIONS

Acknowledgments

This study was supported by Shanghai 13th Five-Year Key Specialized College — Department of Osteoarthritis and Arthritis of Integrated Traditional Chinese and Western Medicine (shslczdzk04801).

Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [Lu Meng], [Guowei Qiu] and [Chenxin Gao]. The first draft of the manuscript was written by [Guowei Qiu] and [Lu Meng]. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Role of the funding source

This study was supported by Shanghai 13th Five-Year Key Specialized College — Department of Osteoarthritis and Arthritis of Integrated Traditional Chinese and Western Medicine (shslczdzk04801).

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Competing interests

The authors declare that they have no conflict of interest.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval

This is an observational study. The Ethics Committee of Guanghua Hospital has confirmed that no ethical approval is required.

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Figure Legends

Figure 1. Landscape of immune cells in synovium, SF and paired PBMC.

(A) 21 OA patients undergoing TKA were studied. (B) PBMC cells and tissue MNs were isolated separately from 21 OA donors. (C) Identification of 9 major immune cell subsets by tSNE of flow cytometry data of synovium, SF and PBMC using the FlowSOM algorithm. (D) Proportions of monocytes/macrophages, DCs, T cells and NK cells to $CD45^+$ cells in PBMC, synovium and SF. Significance was assessed by Wilcoxon matched-pairs signed rank test. ***, $P < 0.001$. ****, $P < 0.0001$.

Figure 2. DC3s significantly infiltrated in synovium. (A) Gating strategy for identification of DC2s and DC3s in Flow Cytometry. (B) 4 DCs subsets were defined in PBMC, synovium and SF. (C) Histograms showing the proportion of DCs of $CD45^+$ cells in synovium comparing with PBMC or SF. Significance was assessed by Wilcoxon

matched-pairs signed rank test. **, $P < 0.01$. ***, $P < 0.001$. ****, $P < 0.0001$.

Figure 3. DC3s infiltrated in synovium is correlated with $CD8^+$T cells. (A) H&E stain. (B) Representative mIHC image shows the staining for CD1c (yellow), CD163 (red), CD8 (green) in the OA synovium. In left picture, white arrow indicates DC3s ($CD1c^+CD163^+$). A typical ELLS consist plenty of DC3s and $CD8^+$T cells was showed in right image. Magnification ×200. (C) Association of DC3s infiltration with $CD8^+$ T cells. Increased number of DC3 were measure when $CD8^+$T cells located close to DC3s (<20um). Each dot represents a single data point; blue dots represent ELLS negative samples including OA37 and OA40, and violet dots represent ELLS positive ones. Histograms showing higher level of (D) IL12p70 and (E) TNF-α in severer OA patients. (F) No significant differences of IL23 were observed. Significance was assessed by Wilcoxon matched-pairs signed rank test. *, $P < 0.05$. **, $P < 0.01$.

Figure S1. Gating strategy used to define immune cell types by flow cytometry.

Figure S2. Proportions of each immune cell subsets to $CD45^+$ cells in PBMC, synovium and SF. Red line present 10%$CD45^+$.

Figure S3. Distance analysis of DC3s and $CD8^+$T cell. Cellular phenotype of the fluorescence image depicted the spatial location of CD1c+CD163+ (red dots), CD163+(green dots) and CD8+(blue dots) in the synovium. Solid plots connected the nearest cells within 20 μm from the CD1c+CD163+ and CD163+ to CD8+ respectively.

Figure 1.

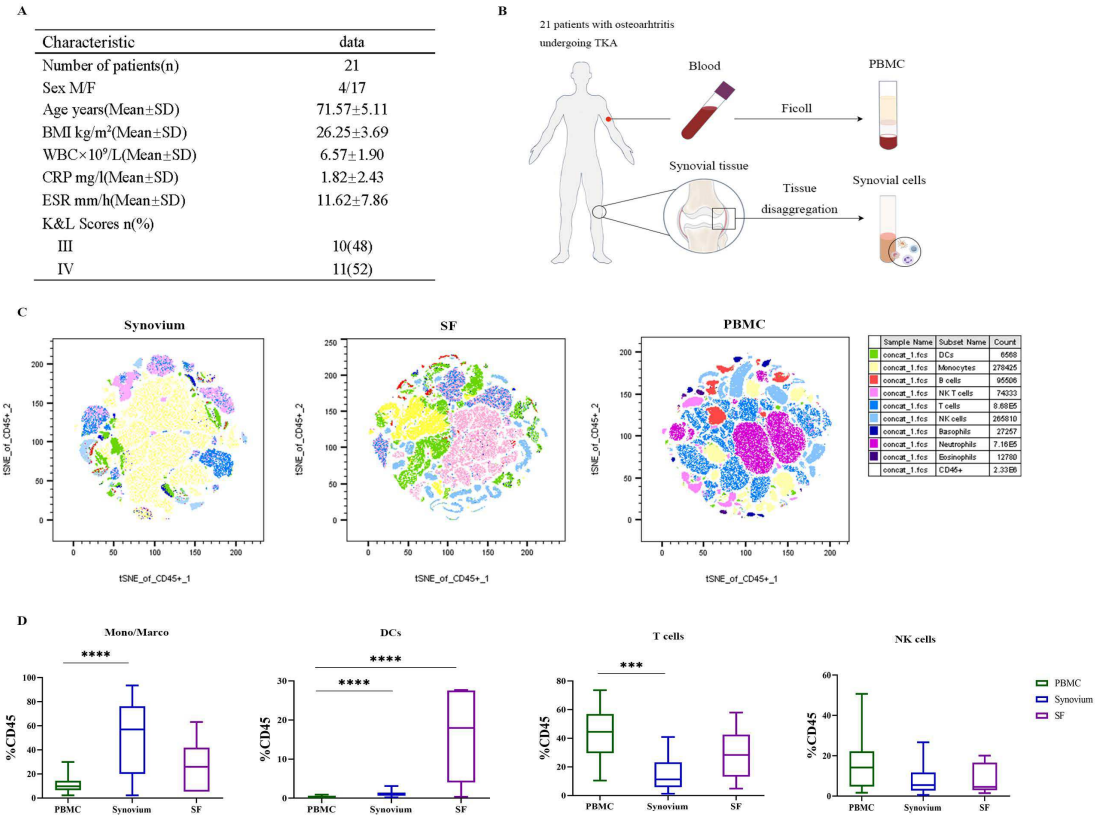

A
Characteristic | data
Number of patients(n) | 21
Sex M/F | 4/17
Age years(Mean±SD) | 71.57±5.11
BMI kg/m²(Mean±SD) | 26.25±3.69
WBC×10⁹/L(Mean±SD) | 6.57±1.90
CRP mg/l(Mean±SD) | 1.82±2.43
ESR mm/h(Mean±SD) | 11.62±7.86
K&L Scores n(%)
III | 10(48)
IV | 11(52)
B
21 patients with osteoarhtritis undergoing TKA
Blood
PBMC
Ficoll
Synovial tissue
Tissue disaggregation
Synovial cells
C
Synovium
SF
PBMC
tSNE_of_CD45+_2
tSNE_of_CD45+_1
Sample Name | Subset Name | Count
concat_1.fcs | DCs | 6568
concat_1.fcs | Monocytes | 278425
concat_1.fcs | B cells | 95506
concat_1.fcs | NK T cells | 74033
concat_1.fcs | T cells | 8.68E5
concat_1.fcs | NK cells | 265810
concat_1.fcs | Basophils | 27257
concat_1.fcs | Neutrophils | 7.16E5
concat_1.fcs | Eosinophils | 12780
concat_1.fcs | CD45+ | 2.33E6
D
Mono/Marco
DCs
T cells
NK cells
%CD45
****
***
PBMC
Synovium
SF

Figure 2.

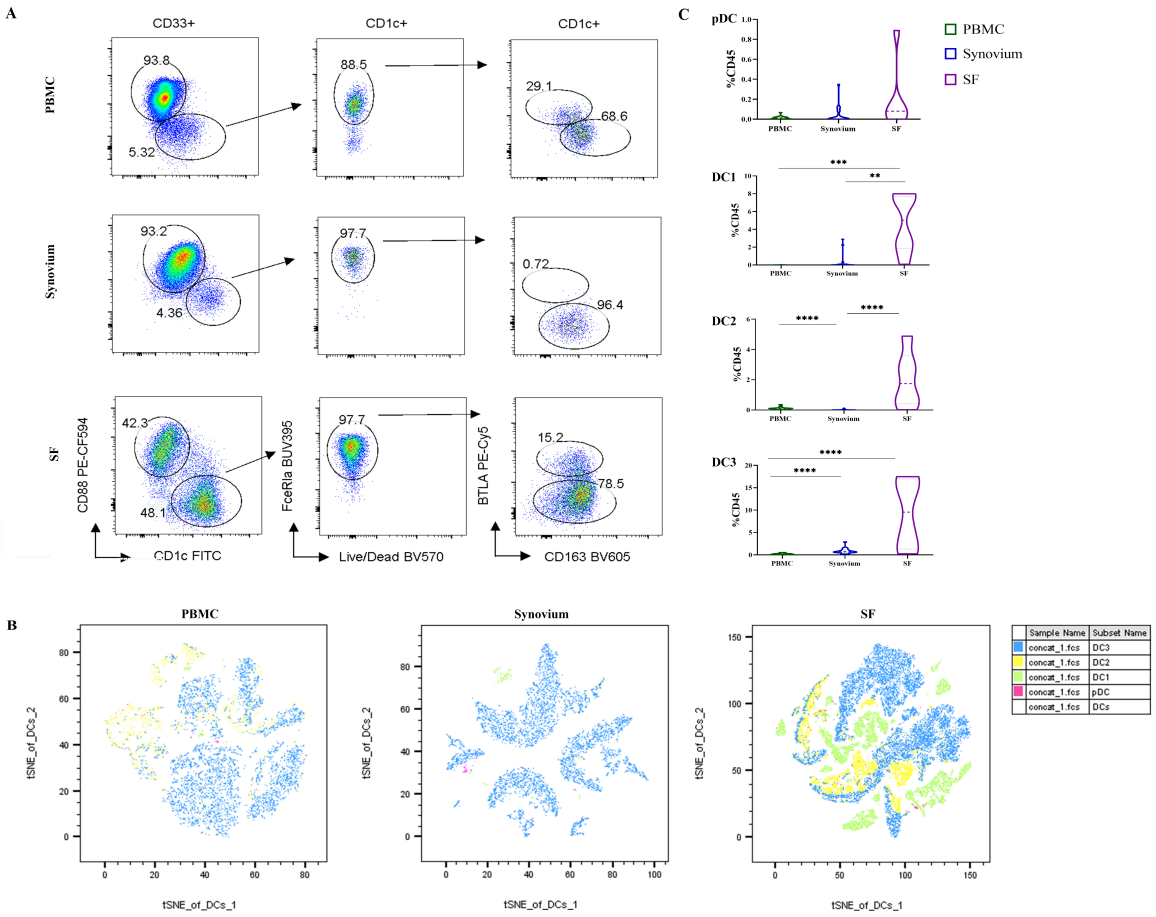
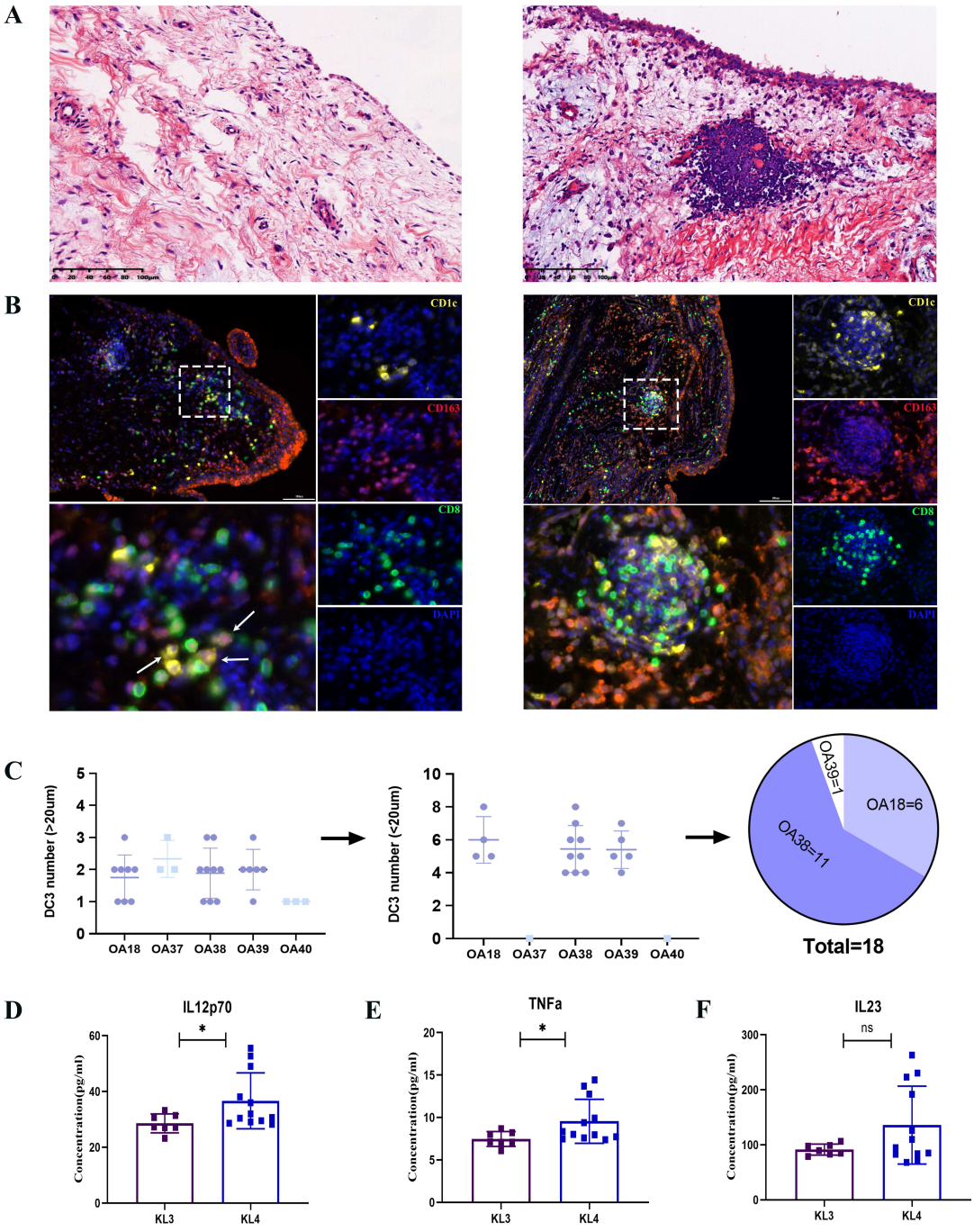

A
CD33+
CD1c+
CD1c+
PBMC
93.8
5.32
88.5
29.1
68.6
Synovium
93.2
4.36
97.7
0.72
96.4
SF
42.3
48.1
97.7
15.2
78.5
CD88 PE-CF594
CD1c FITC
FcεRIa BUV395
Live/Dead BV570
BTLA PE-Cy5
CD163 BV605
C
pDC
DC1
DC2
DC3
%CD45
PBMC
Synovium
SF
B
PBMC
Synovium
SF
tSNE_of_DCs_1
tSNE_of_DCs_2
Sample Name
Subset Name
concat_1.fcs
DC3
DC2
DC1
pDC
DCs

Figure 3.


A
B
CD1c
CD163
CD8
DAPI
C
DC3 number (>20um)
OA18 OA37 OA38 OA39 OA40
DC3 number (<20um)
OA18 OA37 OA38 OA39 OA40
OA39=1
OA18=6
OA38=11
Total=18
D
IL12p70
Concentration(pg/ml)
KL3 KL4
E
TNFa
Concentration(pg/ml)
KL3 KL4
F
IL23
ns
Concentration(pg/ml)
KL3 KL4

Supplementary Files

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- SupplementalFigures.pdf