Online supplement to: Synoviocytes-derived Interleukin 35 Potentiates B Cell Response in Patients with Osteoarthritis and Rheumatoid Arthritis. *The Journal of Rheumatology*. doi:10.3899/jrheum.161363

ONLINE SUPPLEMENTARY MATERIAL

Supplementary Methods

RA patients and healthy subject group

The group of RA patients consisted of 10 women and 4 men (n=14), mean age 56 years (range 47–68), mean disease duration 0.5 years (range 0.2–21), median C-reactive protein (CRP), 7.95 mg/l (range 0.6–23.9)], mean erythrocyte sedimentation rate (ESR) 41.5 (range 14-121), fulfilling the revised 1987 American College of Rheumatology criteria were recruited from the Division of Rheumatology and the department of Orthopaedics & Traumatology at the Prince Wales Hospital, Princess Margaret Hospital and Pamela Youde Nethersole Eastern Hospital. 92.3% with RA were positive for anti-CCP antibodies and RF, whereas only one patient with RA was negative for both anti-CCP antibodies and RF. Recruited patients were treated with combination of synthetic disease modifying anti-rheumatic drugs (DMARDs) with methotrexate and one patient was treated with DMARD and tocilizumab. Synovial tissue from nine out of the total fourteen patients who underwent arthroplastic joint surgery and synovectomy were collected. Eleven cases with OA (8 women and 3 men; mean age 65 years, range 56–85) were recruited. Synovial tissue from seven out of the total eleven cases with OA patients undergoing total joint prosthesis were included as controls. PB from remaining recruited RA (n=5) and OA (n=4) patients was obtained for flow cytometry analysis. PBMC were isolated from buffy coat of anonymous donors drawn from Hong Kong Red Cross Blood Transfusion Service was used as HC.

Heamatoxylin-eosin staining (H&E)

Formaldehyde-fixed, paraffin-embedded sections (4 μm) were deparaffinized and stained with standard haematoxylin and eosin (H&E). Aggregates size were evaluated and graded by counting the number of cells in a radius starting from an estimated centre of the aggregates. Aggregate size was then categorised as grade 1, an inflamed synovium expressed with follicle-like lymphocytic aggregate (2-5 cells - radial cell count surrounding the vessel); grade 2, an inflamed synovium expressed with follicle-like lymphocytic aggregate (6-10 cells in the radus); grade 3, an inflamed synovium expressed with follicle-like lymphocytic aggregate (>10 cells in the radius). Tissue with

hypertrophy of the cell lining layer and scarcely infiltrated lymphocytes is defined as diffused pattern.

Immunofluorescence staining

To analyse T/B cell segregation and IL-35 expression, sequential sections undergone antigen retrieval (DAKO), non-specific binding was blocked/avoided (DAKO, X0909), followed by incubation with either mouse anti-human CD20 (DAKO), or goat anti-human p35 (Santa cruz) or rabbit anti-human EBI-3 (Millipore) and species-matched isotype control. Slides were then washed, incubated 30min with secondary antibodies donkey anti-mouse-ALEXA568 or anti-rabbit-ALEXA488 in the case of double stain for CD20/EBI-3. For CD20/p35, we used donkey anti goat-ALEXA555, or goat anti-mouse-ALEXA488. Freshly isolated B cells cytospin and RA and OA synovial fibroblast (RASF/OASF) were fixed in methanol/acetone 1:1 followed by blocking and antibody incubation as mentioned above. For RASF and OASF, cells were stimulated with/without 10ng/ml TNF-α for 24h before fixation. All slides were counterstained with DAPI and examined under Leica microscope, DM6000 B. All antibodies were diluted in the TBS with 0.1% BSA.

Isolation of CD19+ human B cells

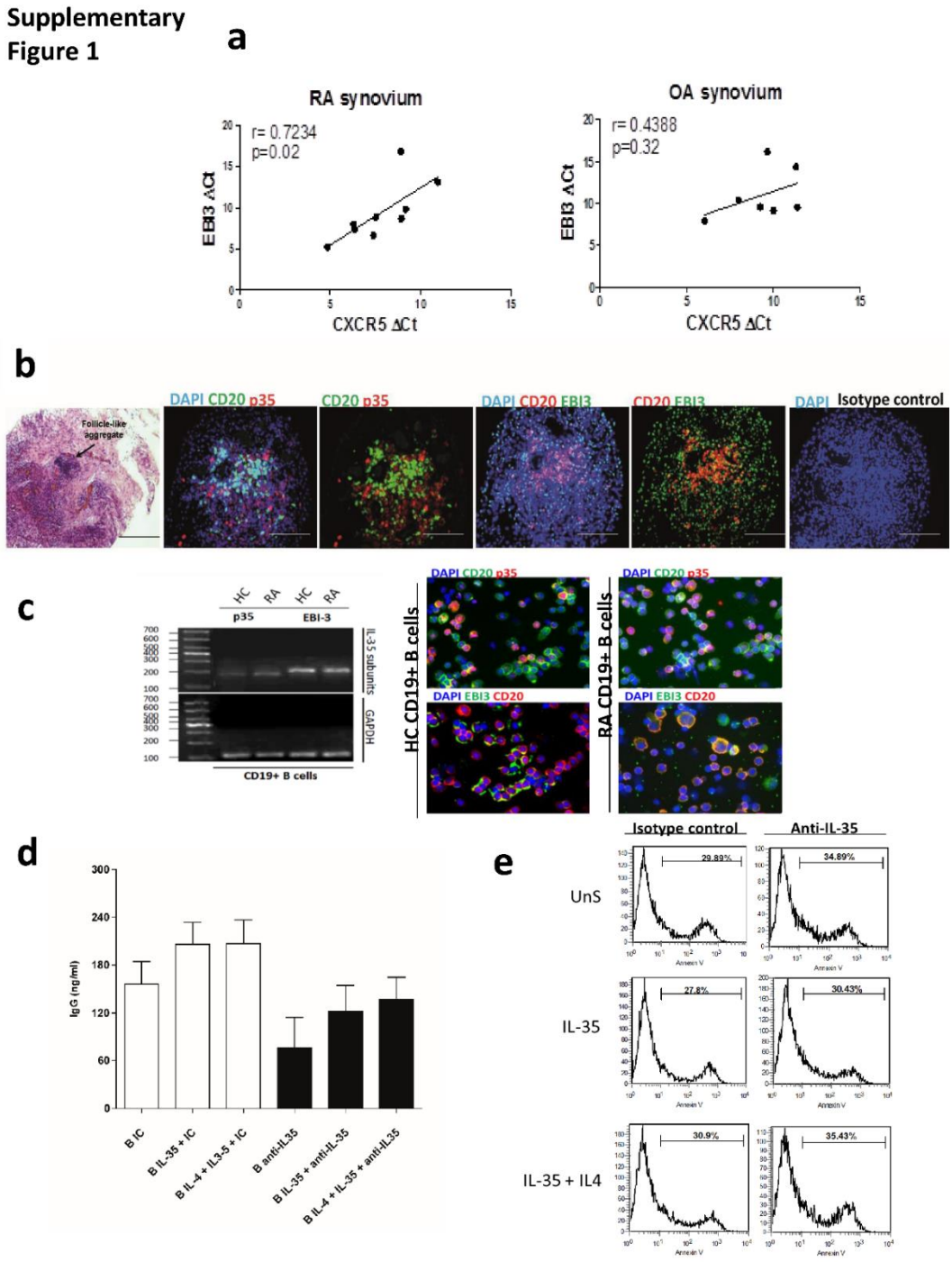
PBMC and syn-MN were then resuspended in chilled MACS buffer [PBS, 0.5% FCS and 2mM EDTA], washed and incubated with CD19 microbeads (130-050-301; Miltenyi Biotec) for 15 min at 4^{o}C and subsequently passed through magnetic separation columns separation (LS; Miltenyl Biotech). The bead-bound cells were collected as enriched, positively selected CD19+ B cells. Purified CD19+ B cells were either re-suspended in FACS buffer for flow cytometry or in DMEM supplemented with 10% FCS, 50 IU/mL penicillin-streptomycin, 10 mM HEPES buffer (Gibco/Invitrogen) for subsequent culture works. Preparation were typically >95% pure.

Flow cytometric analysis

Isolated CD19+ B cells at a density of 1x10^{6} cells per 100µl cold buffer (PBS supplemented with 0.5% (w/v)mBSA and 2mM EDTA) were washed, blocked by PBS/0.1%BSA. Cells were surface stained with PerCP-anti-IL-12Rβ2, ALEXA700-gp130 and PE-IL-27RA (R&D Systems) for 30mins at 4^{o}C and analyzed by flow cytometry (Beckman Navios, Beckman Coulter Inc). In

addition, the combination of IgD-FITC and CD27-APC was also used (BD Pharmingen) Initial gating was set according to forward and side light scatter to exclude dead cells from the analysis and at least 10,000 events were counted for each sample. Spectral overlap between the fluorochromes was compensated electronically by single colour control samples. The following controls were also used: unstained cells, cells stained with FITC-conjugated or APC-conjugated secondary antibody only, cells treated with PerCP/ALEXA700/PE-conjugated mouse or rabbit IgG1 as an isotype control.

Supplementary Figure 1

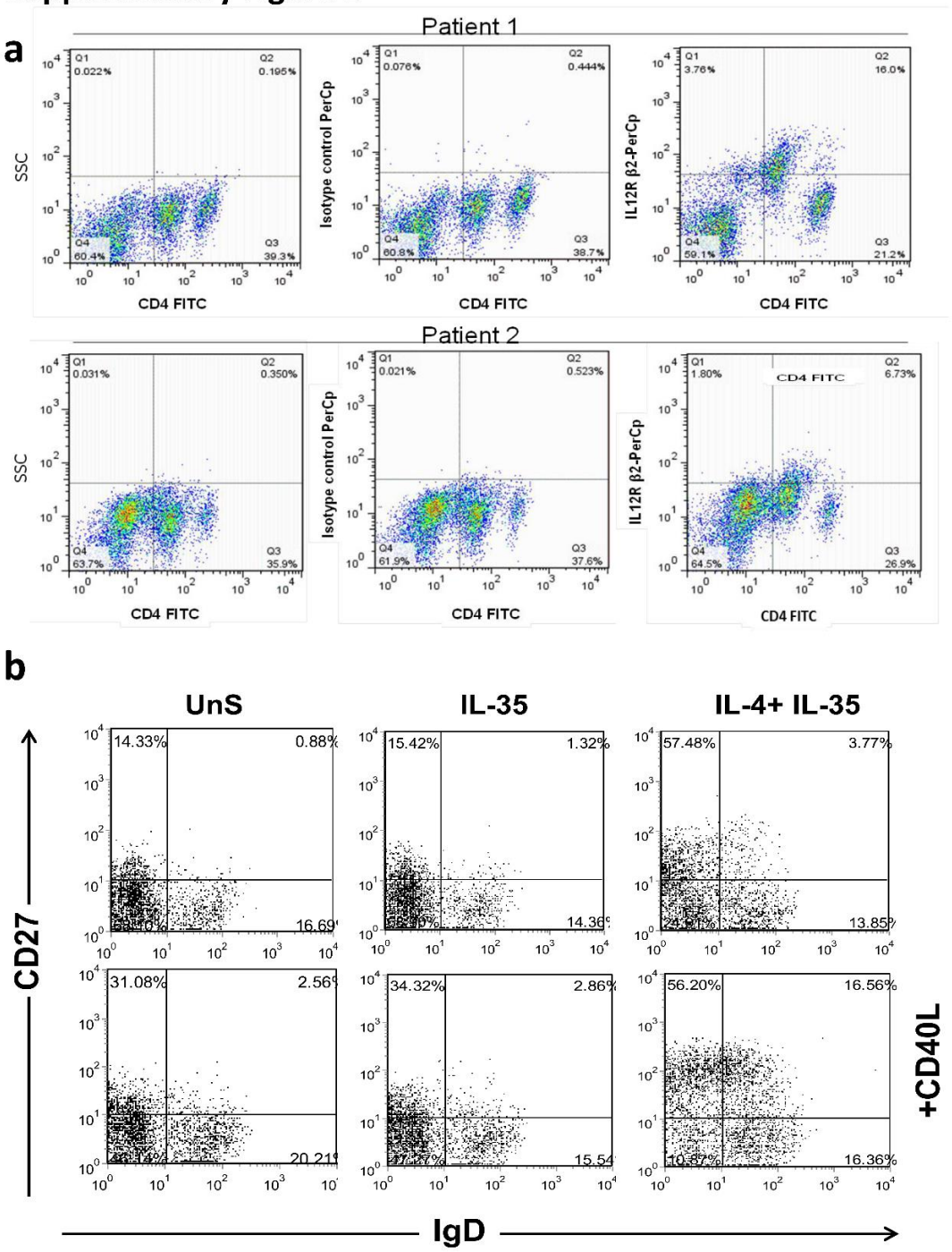


Supplementary Figure 1. Expression of IL-35 in RA synovium and blockade of endogenous IL-35 expression in B cells

a) Positive correlation between EBI-3 and CXCR5 gene expression in RA synovium. Pearson's rank correlation: r^2=0.72, p=0.02, n= 9; No correlation between EBI-3 and CXCR5 in OA

synovium. Pearson's rank correlation: r^2=0.44, p=0.32, n= 7. **b**) Representative staining of RA patient: double stained for CD20 (green)/p35 (red) and CD20 (red)/EBI-3 (green); Bars: 100 µm. Nuclei are counterstained in blue (DAPI). Appropriate isotype control was used to confirm specificity. **c**) Basal transcript expression was evaluated by SYBR-Green based qRT-PCR and amplified products EBI-3 (193bp) and p35 (170bp) of purified B cells are run on the 3% agarose gel. GAPDH (108bp) was used as endogenous control to confirm equal loading. Double immunofluorescence staining from cytospun purified B cells from HC PB demonstrating the constitutive protein expression of EBI-3 (green)/CD20 (red) and p35 (red)/CD20 (green); We observed constitutive comparable co-expression of EBI-3 and p35 transcripts in the isolated $CD19^+$ B cells in RA and healthy control (HC), further confirmed at protein level in HC by immunostaining of cytospin samples. Bars: 100 µm; **d**) B cells isolated from HC were stimulated with IL-35 (100ng/ml), IL-35 + IL-4 (100ng/ml each) or left untreated in the presence blocking antibodies anti-IL-35 (filled, 10µg/ml). Isotype-matched antibodies act as controls (IC, 100ng/ml). After 72 h of culture, secretion of IgG in culture supernatant was assessed by ELISA; **e**) Quantification of apoptosis through Annexin V staining and flow cytometric analysis. Flow cytometry histograms for the fluorescence intensity measured in FL-1 (Annexin V-FITC) channel. Purified B cells were incubated for 72h with 100ng/ml IL-35, IL-35+IL-4 (100ng/ml) or left untreated (UnS). Numbers in histograms are percent of annexin V-positive cells after treatment with the stimuli. Data are representative of triplicate cultures and the figure is representative of three independent experiments.

Supplementary Figure 2



Supplementary Figure 2. Expression of IL-12Rβ2 on peripheral CD4+ T cells and impact of IL-35 on B cells differentiation patient with RA

a) Freshly PBMC isolated from peripheral blood of healthy donors (n=2) are used to determine the expression of IL-12β2 on $CD4^+$ T cells and analysed by flow cytometry. Dot-plots show the

results of single staining on PBMC with anti-CD4-FITC antibody (left panel); double staining on PBMC with anti-CD4-FITC and isotype-PerCp antibodies (middle panel) and double staining on PBMC with anti-CD4-FITC and anti- IL-12β2-PerCp antibodies (right panel). **b**) Purified $CD19^+$ B cells from RA patients were pre-treated with CD40L (lower panel) for 24h. The day after, cells were stimulated with IL-35 alone (100ng/ml, middle panel) or in the presence IL-4 (100ng/ml, right panel) or left untreated (UnS, left panel) for 5 days. At the end of the culture, cells were collected, stained and assessed for the B cell phenotypical markers (IgD and CD27) by flow cytometry. Dot plots reveal the changes of IgD and CD27 surface expression on B cells upon different drug treatment.