Synoviocytes-derived Interleukin 35 Potentiates B Cell Response in Patients with Osteoarthritis and Rheumatoid Arthritis

Ngar-Woon Kam, Dehua Liu, Zhe Cai, Wah-Yan Mak, Chun-Kwok Wong, Kwok-Hing Chiu, Kam-Yiu Wong, Wai-Leuk Tsang, and Lai-Shan Tam

ABSTRACT. ***Objective.*** Elevated expression of interleukin 35 (IL-35) is associated with autoimmune disease, including rheumatoid arthritis (RA). The present study was undertaken to determine the functional interaction among IL-35, B cells, and stromal cells residing in the synovium of patients with RA and osteoarthritis (OA).

Methods. IL-35 (EBI-3/p35) expression was investigated in RA and OA synovium using quantitative real-time PCR (qRT-PCR) and immunohistochemistry. IL-35 receptor (IL-35R) expression on B cells dissociated from synovium and periphery of patients with RA, OA, and healthy donor controls (HC) was determined by flow cytometry. The degree of B cells activation after IL-4 and/or IL-35 stimulation was measured by flow cytometry and qRT-PCR. Synovial fibroblasts (SF) purified from RA and OA synovium were cocultured with peripheral HC B cells in the presence/absence of tumor necrosis factor-α (TNF-α) and with/without anti-IL-35–blocking antibodies.

Results. EBI-3/p35 transcripts were expressed in close proximity to B cells residing in RA and OA synovium. IL-35R subunits, gp130 and IL-27Rα, but not IL-12Rβ2, were expressed in B cells extracted from the synovium and periphery of patients with RA/OA. Notably, RA synovium expressed the highest level of IL-27Rα on their cell surface. IL-35 induced proliferation and IgG production in HC B cells. Cocultures of HC B cells with RASF, but not OASF, exhibited significantly elevated B cells activation. TNF-α–induced, RASF-dependent secretion of IgG in B cells is partly IL-35–dependent.

Conclusion. To our knowledge, for the first time we demonstrated that synovial/peripheral B cells expressed IL-35R and were responsive to IL-35 stimulation. SF residing in RA synovium can be linked to B cell activation and maintenance in RA synovium through IL-35. (J Rheumatol First Release December 15 2017; doi:10.3899/jrheum.161363)

Key Indexing Terms:
INTERLEUKIN 35 B CELLS RHEUMATOID ARTHRITIS
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Rheumatoid arthritis (RA) is a chronic inflammatory disease largely orchestrated by the interaction between activated resident synovial fibroblast (SF) and immune cells, leading to immune-mediated cartilage destruction and bone erosion[1]. RA exhibits a high degree of molecular and cellular heterogeneity with variable histological features[2,3], which have been subdivided into the fibroblast- (pauciimmune), diffuse-, or lymphoid-rich subtypes. The latter subtype is characterized by synovitis with follicular structures consisting of T/B cells aggregates or clusters of CD21+ stromal follicular dendritic cells — features normally found in germinal centers (GC) of lymphoid tissue[1,3]. This ectopic structure has been shown to support T cells priming and autoantibodies production within the synovial tissue[4,5,6].

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Interleukin 35 (IL-35) is a heterodimeric cytokine belonging to the IL-12 family, and composed of an α chain (p35) and a β chain [Epstein-Barr virus induced gene 3 (EBI-3)]. Given that IL-35 is a member of the IL-12 cytokine family, the receptor and signaling pathway of IL-35 overlap with IL-12, by using IL-12Rβ2 and gp130, which are shared with IL-12 and IL-27 receptor complexes, respectively. Accordingly, IL-35 signaling has been found to be mediated through the unique pairing of receptor chains: heterodimer or homodimer of receptor chains IL-12Rβ and gp130 on T cells[7] and heterodimer of IL-12Rβ2:IL-27Rα on B cells[8]. Studies using multiple *in vivo* disease models have identified IL-35 as an antiinflammatory product of Foxp3+ regulatory T cell (Treg), which restricts the activation and proliferation of effector T cells and expansion of IL-17–secreting T helper 17 (Th17) cells[9,10,11,12,13]. Lately, this cytokine has been revealed to induce regulatory B cells for IL-10 and IL-35 production[8]. While these data suggest an immunosuppressive role of IL-35, its functional involvement in animal disease models and human diseases remains controversial. For example, IL-35 has been found to be a proinflammatory mediator in Lyme arthritis or collagen-induced arthritis mice model[14,15], and it can promote the release of proinflammatory mediators in peripheral blood mononuclear cells (PBMC)[16]. Interestingly, while *EBI-3* is a Foxp3 target gene in mouse Treg, induction of FoxP3 activity does not enhance EBI-3 or p35 in humans[17]. Moreover, human Treg fails to express IL-35[18]. Altogether, these findings raise the possibility that IL-35 may not exclusively be an antiinflammatory cytokine and that it can exert different biological functions in rodents versus humans. In relation to RA, elevated synovial and serum IL-35 levels have been documented[19]; however, its precise role in synovial histopathology and humoral responses remains elusive. To our knowledge, only 1 study has analyzed the expression of IL-35 transcripts on RASF[16] and shown that they demonstrated the expression and localization of IL-35 subunits on synovial cell infiltrates. To date, there is little knowledge of the distribution of IL-35 expression in relation to the common histopathologic features shared by most of the chronic inflammatory arthritis, such as the local organization of infiltrating mononuclear cells into aggregative structures. Here we particularly focused on B cells and assayed the localization between IL-35–expressing cells and intrasynovial B cells within RA synovitis, and further examined the effect of IL-35 directly on B cells or indirectly through the presence of resident stromal cells.

MATERIALS AND METHODS

Patients and samples. Our study was approved by the Clinical Research Ethics Committee KWC-REC [KW/EX-14-214(82-21)], HKEC (HKEC 2014-114), and CUHK-NTEC (2014.167), and informed consent was obtained from all participants. A total of 14 patients with diagnosed RA based on the revised 1987 American College of Rheumatology criteria for RA were recruited. Synovial tissue were collected from 9 of the 14 patients who underwent arthroplastic joint surgery and synovectomy. Eleven cases with osteoarthritis (OA) were recruited and synovial tissue from 7 of the 11 cases with OA patients undergoing total joint prosthesis were included as controls. EDTA anticoagulated blood samples were obtained from the remaining recruited patients with RA (n = 5) and OA (n = 4). PBMC isolated from buffy coat of anonymous donors drawn from Hong Kong Red Cross Blood Transfusion Service was used as HC. Full details are in the Supplementary Methods (available with the online version of this article).

Immunohistochemistry and immunofluorescence staining. Formaldehyde-fixed paraffin sections of RA and OA synovium were stained with standard H&E to evaluate the presence of diffuse or aggregate synovitis[2], and then assessed for the presence of IL-35 by immunohistochemistry (Supplementary Methods).

Isolation of human B cells from blood and synovia. To isolate synovial mononuclear cells (syn-MN), synovium were gently minced and incubated (30 min, 37°C) with collagenase D and DNAse solution (400 U/ml, Roche). Syn-MN were separated by gradient centrifugation using Histopaque (1.083 g/ml, Sigma). To isolate peripheral cells, PBMC were isolated by Ficoll-Paque gradient (GE Healthcare). Synovial and peripheral B cells were isolated using magnetic bead–based protocol (Miltenyi Biotec; Supplementary Methods, available with the online version of this article).

Flow cytometric analysis. Isolated CD19+ B cells were blocked and stained with PerCP-anti-IL-12Rβ2, ALEXA700-gp130, and PE-IL-27Rα (R&D Systems) for 30 min and analyzed by flow cytometry (Beckman Navios, Beckman Coulter Inc.). In addition, combination of IgD-FITC and CD27-APC was also used (BD Pharmingen; Supplementary Methods).

Cell cultures, activation, and carboxyfluorescein succinimidyl ester (CFSE) labeling. RASF/OASF were obtained as previously described[20]. B cells (1×10^6 cells/well) were incubated with 100 ng/ml of recombinant IL-4 (R&D), IL-35:Fc recombinant protein (Enzo Life Sciences), or a combination of both for 72 h. For differentiation experiment, B cells were prestimulated with recombinant 1 μg/ml CD40L for 24 h before addition of the above-mentioned stimulation for 6 days. For proliferation experiment, PBMC (1×10^6/well) were labeled with 5 μM CFSE (Biolegend) in phosphate buffered saline/0.1% bovine serum albumin for 8 min at 37°C, pretreated with 1 μg/ml CD40L for 1 h, followed by stimulation of IL-4 and/or IL-35 for 4 days. Cells were collected and stained with APC-conjugated anti-CD19 (BD Biosciences). To detect apoptotic cell death, cultured B cells were stained with FITC-conjugated Annexin V (BD PharMingen). B cell proliferation and apoptosis were determined by flow cytometry analysis.

Cocultures of B cells with RASF and OASF. Fibroblasts were seeded into 24 well plates (4×10^4 cells/1 ml/well) and used for coculture experiments at 90% confluence as previously reported[21]. CD19+ B cells (1×10^6 cells) were added onto pretreated [with/without of 10 ng/ml tumor necrosis factor-α (TNF-α)] fibroblast layer directly or through microporous PET cell inserts (1-μm pore, Becton Dickinson). Control cocultures received no fibroblasts. Where indicated, neutralizing anti-IL-35 (V1.4F5.25, Shenandoah Biotechnology) or isotype-matched control antibody (10 μg/ml, R&D) was added to the fibroblast layer 4 h before coculture and throughout the experiment for 72 h.

ELISA and cytometric bead array (CBA). IL-6, IL-8, and IL-10 were measured using BD CBA Human Inflammation Kit (BD Biosciences). Data were acquired with BD FACSCalibur and analyzed with BD CBA software. Concentrations of IL-35 were measured by ELISA (USCN Life Science) and total IgA/IgG/IgM were detected using Human IgA/IgG/IgM ELISA quantitation set (Bethyl Laboratories), according to manufacturer's instruction.

Quantitative real-time PCR (qRT-PCR). Total RNA was extracted from synovia using Trizol (Life Technologies) as previously reported[6,22]. RNA was isolated from cells by using RNeasy Mini kit (Qiagen). The RNA was reversed transcribed (Takara) into cDNA, which served as a template for the amplification by qRT-PCR using SYBR Green gene expression assay (QuantStudio 12K Flex detection system). Relative quantification was measured using the comparative Ct (threshold cycle) and normalized the Ct values to GAPDH. Primers used are shown in Table 1.

Statistical analysis. Differences in quantitative variables were analyzed by the Mann–Whitney U test when comparing 2 groups and by the Kruskal–Wallis with Dunn's posttest when comparing > 2 groups. All analyses were performed using GraphPad Prism software. A p value < 0.05 was considered statistically significant.

RESULTS

EBI-3 and p35 transcripts are preferentially expressed in close proximity to CD20+ cells in RA inflammatory synovium. A previous study quantified IL-35 (p35 and EBI-3) expression in RA synovium by semiquantitative immunohistochemistry[16]. Here we evaluated IL-35 levels quantitatively by qRT-PCR in parallel to CXCL13/CXCR5 expression, which are master regulators of ectopic lymphoid neogenesis. A trend toward higher IL-35 mRNA expression in RA as compared to OA synovium was observed (Figure 1A). Moreover, a positive correlation (Pearson r = 0.7, p = 0.02) between EBI-3 and CXCR5 mRNA in RA, but not OA, synovium was detected (Supplementary Figure 1A, available with the online version of this article). When comparing to other B cell activation cytokines, APRIL and B cell activating factor belonging to the TNF family (BAFF), IL-35 transcripts were more diversely detected and expressed at substantially lower amounts throughout the synovium (Figure 1A). Altogether, these observations suggested a distinct mechanism of transcriptional regulation on IL-35 expression and its expression might be associated with different features of histopathological subtypes within arthritis disease.

Although none of our recruited patients showed GC-like structures, mononuclear cell infiltrates consisting of aggregate structures were visible in 7 out of 9 patients (Figure 1B; Supplementary Figure 1B, available with the online version of this article). In addition, 2 patients revealed diffuse infiltration. Accordingly, by co-staining synovial tissues with antibodies recognizing B cells and IL-35 (EBI-3 and p35), we found that IL-35 had a tendency to accumulate around CD20+ B cells in aggregated RA synovium (Figure 1C, panels A–E). In addition, p35 (panels A–B) and EBI-3 (panels C–D) expressions were stronger in aggregated synovium as compared with diffuse synovitis (panels G–I). EBI-3+ cells were also observed in OA synovium (panels N–O), while p35+ cells (panels L–M) were barely detectable. Notably, CD20+EBI-3+p35+ cells were found in the RA synovium, indicating the presence of IL-35–producing B cells, as previously reported in mice[23].

Table 1. List of primers used for SYBR Green–based quantitative real-time PCR.

Gene	Primers	Sequences (5′-3′)
APRIL	APRIL-F	CCC CGT TCC TCA CTT TTC C
	APRIL-R	CGC CCC TCA TCT ACA CAC AC
BAFF	BAFF-F	TGA GTC TGG TGA CTT TGT TTC GAT
	BAFF-R	CTT GGT ATT GCA AGT TGG AGT TCA T
PRDM-1 (BLIMP-1)	PRDM-1-F	TTC AAA TGT CAG ACT TGC AAC AAG G
	PRDM-1-R	TCT TGA GAT TGC TGG TGC TGC TA
BCL-6	BCL-6-F	CTC CGT GCC CAT GTG CTT A
	BCL-6-R	TTT GTG ACG GAA ATG CAG GTT A
EBI-3	EBI-3-F	TCC TTC ATT GCC ACG TAC AG
	EBI-3-R	GCT CTG TTA TGA AAG GCA CG
GAPDH	GAPDH-F	ATG GGG AAG GTG AAG GTC G
	GAPDH-R	AGG CAG CAC TAC TCA GAC ATC TTC A
p35	p35-F	AGG AAT GTT CCC ATG CCT TCA
	p35-R	CCA ATG GTA AAC AGG CCT CCA C
PAX-5	PAX-5-F	AGG CAG CAC TAC TCA GAC ATC TTC A
	PAX-5-R	CTG GCC AGA TTG GCC TTC A
XBP-1	XBP-1-F	GTG AGC TGG AAC AGC AAG TGG TA
	XBP-1-R	TCT TCA GCA ACC AGG GCA TC

BAFF: B cell activating factor belonging to the tumor necrosis factor family.

Identification of IL-35R on B cells. To determine whether IL-35 contributed to the activation of autoreactive B cells, we analyzed whether B cells express receptor for interacting with IL-35. Flow cytometric analysis revealed that gp130 and IL-27Rα chains were present in all B cells isolated from peripheral blood (PB, PB-B; Figure 2A and C), where their expressions were lower in patients with OA compared with the RA and HC groups. We also isolated synovial B cells (syn-B) from RA and OA synovium, where both gp130 and IL-27Rα showed significantly higher surface levels in RA compared with OA (Figure 2B and C). Notably, although the differences in expression failed to be statistically significant, gp130 and IL-27Rα expression were higher in syn-B as compared with PB-B. In contrast, the IL-12Rβ2 receptor chain, which expressed on CD4+ cells (positive control; Supplementary Figure 2A, available with the online version of this article), as previously reported[24], was barely detectable in all disease groups, regardless of whether B cells were isolated from PB-B or syn-B.

IL-35 induces secretion of cytokines and IgG in B cells. These findings raised the question of whether treatment of IL-35 influences the activity of IL-35R–bearing B cells. A previous report[16] demonstrated that recombinant IL-35 (100 ng/ml) effectively induced cytokines production in healthy PBMC. Here we applied the same dosage on PB-B isolated from RA and HC. PB-B cells from patients with RA constitutively secreted low levels of IL-6 and IL-8, and both cytokines were upregulated upon stimulation with IL-35 or IL-4 (our comparative standard; Figure 3A). Similar effects were observed in HC, but at substantially lower amounts. Interestingly, IL-35–induced IL-8 and IL-6 secretion in PB-B from patients with RA were found to be downregulated in the presence of IL-4. Additionally, we found that IL-10 levels from both resting and IL-4–stimulated PB-B (both RA and HC) levels were below the detection threshold of the test used, while weak expression of 1.9 pg/ml and 2.4 pg/ml were detected in IL-35 and IL-4 plus IL-35–stimulated B cells, respectively (data not shown). Increased IL-10 production was observed, albeit to a very low level (mean 4.86 pg/ml vs 11.8 pg/ml; data not shown) in IL-35–stimulated PBMC

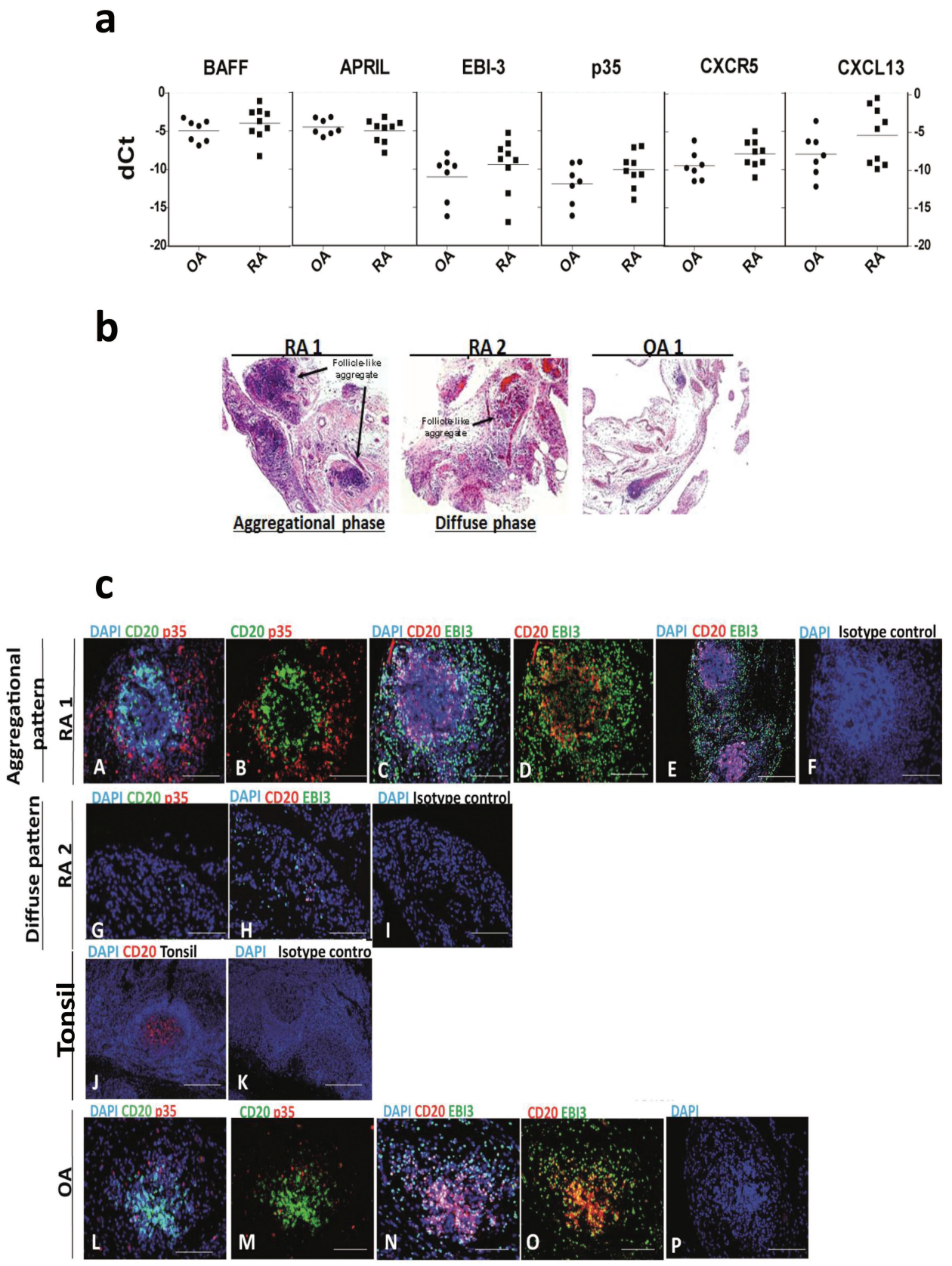

Figure 1. Localization and distribution of IL-35 subunits in RA and OA synovium with synovial histomorphological pattern. (A) Basal expression of indicated genes *IL-35* subunits (EBI-3 and p35), *BAFF*, and *APRIL* in synovia (error bars, SEM; n = 9 RA, n = 7 OA). Transcript expression was evaluated by SYBR Green–based quantitative real-time PCR. Results are expressed as the difference in threshold cycle (ΔCt), normalized to GAPDH. (B) H&E staining shows representative examples of cellular infiltration in RA and OA synovium (diffuse and aggregational pattern). (C) Two representative examples of sequential paraffin-embedded sections of RA synovial membrane, demonstrating that B cells are surrounded by IL-35–positive cells. RA patient 1 (aggregated A–F) double immunofluorescence staining for B cells CD20 (red)/EBI-3 (green) and CD20 (green)/p35 (red); merged double staining for EBI-3 (green) and CD20 (red) on RA sections confirmed that part of the EBI-3+ cells were of B cell origin (C–E, double-stained cells are identified in yellow); absence of p35+ cells (G) and scattered EBI-3+ cells (H) and B cells (G–H) were occasionally found in RA patient 3 (diffused, G–I). Lymphoid organization in RA synovium closely resembled secondary lymphoid organs, tonsil (J–K). Bars: 200 μm in (E, G–K); 100 μm in (A–D). Representative sequential sections of OA synovium (L–P) double-stained for CD20 (green)/p35 (red), CD20 (red)/EBI-3 (green). Bars: 100 μm in (L–P). Nuclei are counterstained in blue (DAPI). Appropriate isotype control was used to confirm specificity. IL-35: interleukin 35; RA: rheumatoid arthritis; OA: osteoarthritis; SEM: standard error of the mean; *BAFF*: B cell activating factor belonging to the tumor necrosis factor family.

isolated from HC and patients with RA, suggesting that external sources of cytokines/factors produced from immune cells are necessary for the full effect of IL-10 production in IL-35–activated B cells[23].

In relation to humoral responses, IgG production was significantly promoted in PB-B of patients with RA upon IL-35 treatment as compared to resting and IL-4 treatment (Figure 3B). The induced IgG production in IL-35–stimulated RA B cells was significantly upregulated as compared to HC, suggesting that RA PB-B cells are more responsive to IL-35 stimulation. In contrast, total IgM production was barely affected upon treatment with IL-35, while IgA production was only modestly modulated in HC PB-B.

Effect of IL-35 on B cell differentiation and proliferation. We analyzed whether IL-35 was also regulating the transcriptional level of genes required for B cell expansion and differentiation. Unexpectedly, IL-35 treatment failed to induce *BCL-6*, *XBP-1*, and *PAX-5* gene expression in B cells, whereas these transcription factors were all considerably enhanced by IL-4 (Figure 3C). BLIMP-1 transcripts remained relatively constant among different treatments. We next assessed phenotypical changes of B cells through the surface expression of IgD (naive/non-switched) and CD27 (class-switched memory). Whereas IgD and CD27 expression were barely modulated upon treatment with IL-35 in CD40L pre-treated B cells, an activated but partially differentiated phenotype (mildly elevated CD27 expression with maintained expression of IgD; Figure 3D; Supplementary Figure 2B) was observed in CD40L prestimulated B cells treated with IL-4 plus IL-35. Lastly, treatment with IL-4 plus IL-35 remarkably promoted the proliferation of B cells (Figure 3E).

IL-35 expression is increased by TNF-α–activated RASF and OASF. We next hypothesized that resident stromal cells might prime B cells depending on the capacity to produce IL-35. We saw that qRT-PCR showed that EBI-3 and p35 were constitutively expressed in RASF and OASF, whereas EBI-3 expression was highly inducible, and p35 was consistently expressed regardless of TNF-α stimulation (Figure 4A). We confirmed this observation by immunofluorescent staining (Figure 4B), expanding recent evidence of overexpression of these factors at mRNA level in RASF only[16]. Unfortunately, consistent with previous reports[25,26], our ELISA could not detect any secretory EBI-3/p35 in the culture supernatant even in TNF-α–activated RASF/OASF.

Blockade of IL-35 abrogates TNF-α–stimulated, RASF-dependent IgG secretion. We next assessed whether synoviocytes-derived IL-35 would affect B cells' ability in producing IgG and whether this ability depends upon direct cell-cell interactions. Upon coculture RASF with CD20+ HC PB-B, IgG production was remarkably upregulated, which was further enhanced upon TNF-α stimulation (Figure 4C). A neutralizing IL-35 antibody was added to cocultures [the used anti-EBI-3 neutralized IL-35, but not IL-27 (a heterodimer of EBI-3-p28)[27]]. This analysis resulted in the substantial inhibition of IgG but not IgA production (data not shown). Blocking IL-35 in cocultures with a transwell support also resulted in partial inhibition of IgG production, suggesting that TNF-α–induced, RASF-mediated B cell activation is at least partly dependent on elevated IL-35 released from TNF-α–stimulated RASF. Although OASF displayed similar behavior to RASF, this change did not reach statistical significance. Notably, the reduction of IgG production was unlikely due to apoptotic cell death because only a maximum of ~6% of B cells that were treated with the neutralizing antibodies bound more annexin V than cells from control (Supplementary Figure 1E, available with the online version of this article).

DISCUSSION

In our report, we showed that IL-35 expression is closely associated with chronically inflamed tissue with substantial lymphoid aggregates. These cell aggregates contained CD20+ B cells that expressed IL-35R. The expression of IL-35R has been reported in mature tonsillar B cells[28], but to our knowledge, ours is the first report of IL-35R expressed by syn-B cells isolated from patients with RA. By extending the previous report of IL-35 biological functions on normal PBMC[16], our study characterizes a previously undefined role of IL-35 in B cells, which can be identified as a proinflammatory cytokine. In support of this, we demonstrated that a direct activating capacity of RASF/OASF in B cells for IgG secretion was dependent on IL-35, produced by resident stromal synoviocytes.

IL-12 family members have been shown to correlate

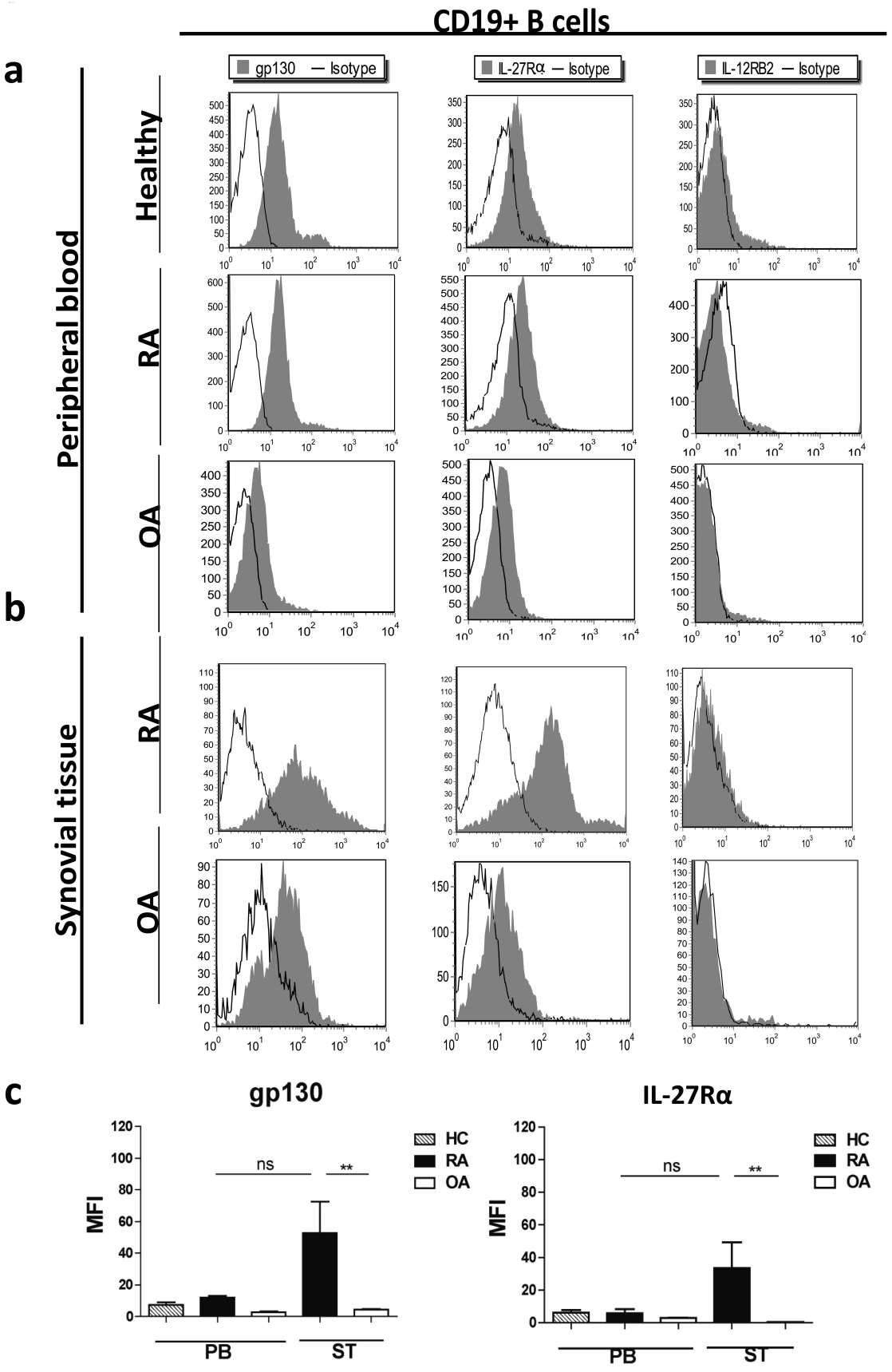

Figure 2. Identification of IL-35 receptor chains in the CD19+ B cells isolated from periphery and synovia in patients with RA and OA, and HC. (A) Freshly isolated CD19+ B cells from PB (top 3 panels) of donors with RA (n = 5), OA (n = 5), and HC (n = 3). (B) Synovial tissues (ST; RA = 4, OA = 4) are used to determine the protein expression for IL-35 receptor chains: gp130 (left), IL-27Rα (middle), and IL-12β2 (right) and analyzed by flow cytometry. The shaded histograms represent binding of anti-gp130, anti-IL-27Rα, and anti-IL-12β2 antibodies, and the open histograms show binding of the respective isotype controls. (C) IL-27Rα and gp130 are expressed at comparable low levels in both compartments of OA (OA PB and OA ST), while level of IL-27Rα and gp130 in RA ST is much higher than in RA PB. Expression of IL-27Rα and gp130 is similar in the RA PB to those in HC PB. Results are presented as mean ± SEM. **p < 0.01 compared with unstimulated. IL-35: interleukin 35; RA: rheumatoid arthritis; OA: osteoarthritis; HC: healthy controls; PB: peripheral blood; SEM: standard error of the mean; MFI: mean fluorescence intensity; ns: not significant.

significantly with RA disease activity and total leukocyte count[29,30,31]. Accordingly, previous works indicated that serum levels of IL-35 are higher in early RA than in HC[16], OA, and established RA[19], and thus the low IL-35 expression observed in our RA synovium may have been due to the selection of patients with endstage disease. Additionally, we observed a positive correlation between the CXCR5 and EBI-3 expression in the RA but not OA synovial microenvironment, suggesting that EBI-3+ cells might closely interact with CXCR5-bearing cells within RA synovial tissue, and we hypothesize that syn-B cells are candidates for CXCR5-bearing cells. Therefore, we demonstrated that IL-35 is preferably expressed in close proximity with B cell aggregate infiltrates within synovium. Here, we classified patients based upon cellular aggregates[2], but not in the context of synovial histopathology; in particular, presence or absence of functional ectopic lymphoid structures (ELS). Because ELS are capable of sustaining autoreactive B cell activation and differentiation[6], future studies are required to correlate IL-35 expression with the acquisition of functional features involved in ELS.

Although IL-12Rβ1 and β2 were previously found to be expressed in human tonsillar[28] cells, results from our study showed that gp130 and IL-27Rα, but not IL-12Rβ2, are constitutively expressed on human CD19+ B lymphocytes isolated from periphery and synovium. These inconsistent observations may be related to different states of activation (PB vs tonsillar B cells). Moreover, Collison, *et al*[7] previously reported that IL-35 could be signaled through homodimers of either IL-12Rβ2 or gp130. Given the present understanding of IL-35R complexity and our data reported here, IL-35 seems to mediate signaling through various forms of cell surface receptor chains in different types of cells. Because RA is thought to be a heterogeneous disease, we cannot exclude the possibility that different forms of IL-35R may be related to the disease status. Whether signaling induced by IL-35 can be productively mediated through the homodimer/heterodimer of IL-35R chains on B cells and that they are physiologically relevant for distinct function in different disease scenarios warrants further investigation.

The specialized role of IL-35 in the help of B cells was supported by the observation that B cells enhanced the protein levels of IL-6 and IL-8 PB-B of patients with RA. Interestingly, in the presence of IL-4, IL-35–induced secretion of IL-8 (a potent chemokine for neutrophils) in PB-B cells from patients with RA was remarkably inhibited. Although IL-4 has been previously shown to inhibit IL-8 synthesis in stimulated monocytes[32], our data appear to be the first to document the inhibitory effect of IL-4 on IL-8 expression in IL-35–activated B cells. At sites of inflammation (synoviocytes-derived cytokines, such as IL-35) and in the presence of T cell–derived cytokines (such as IL-4), this effect would be predicted to contribute to the transition from acute to chronic inflammation through preventing further neutrophil recruitment. Further studies will be required to validate this hypothesis. Kochetkova, *et al*[33] reported that an antiinflammatory involvement of IL-35 was exhibited in RA; we hypothesized that the discrepancy lies in the difference between humans and mice (such as EBI-3 expression[18]). In addition, differential roles of IL-35 have been demonstrated to be dependent on culture conditions[13], in particular, antiinflammatory functions of IL-35 are highly dependent on IL-10 production[8,33].

Although IL-35 treatment directly promoted IgG secretion, we could not demonstrate any significant changes in the expression of genes involved in plasma cells differentiation unless in the presence of IL-4. These findings indicate that proinflammatory potentials of IL-35 might be predominately involved in active IgG biosynthesis. Although the mechanism(s) involved in IL-35–mediated IgG production is not well documented, we speculate that autocrine action of IL-6, as well as endogenous IL-35 (Shen, *et al*'s study[23]; Supplementary Figure 1C and 1D), may be involved in inducing/sustaining IgG production.

Constitutively expressed IL-35 in RASF and OASF was identified to be upregulated upon TNF-α activation; however, quantification of soluble IL-35 production by ELISA was technically difficult. One explanation would be that IL-35 exhibits poor secretory process as compared to IL-12 and IL-27[25]. Alternatively, IL-35 is produced, but additional, yet unidentified proteins are needed for efficient secretion[26]. Also, the functional secreted form of IL-35 may not be a heterodimer; it can be expressed as a higher ordered multimer or in a form of exosomes, in which they may modify their structure according to the synovial microenvironment.

Blocking IL-35 in the cocultures of RASF with healthy PB-B cells could remarkably downregulate RASF-dependent IgG production in B cells, suggesting that resident synoviocytes contain a potential source of IL-35 for inducing B cell activation. However, in line with our previous finding[21], RASF-derived IL-35 in B cell activation is dependent on epigenetic abnormalities related to the anatomical local-

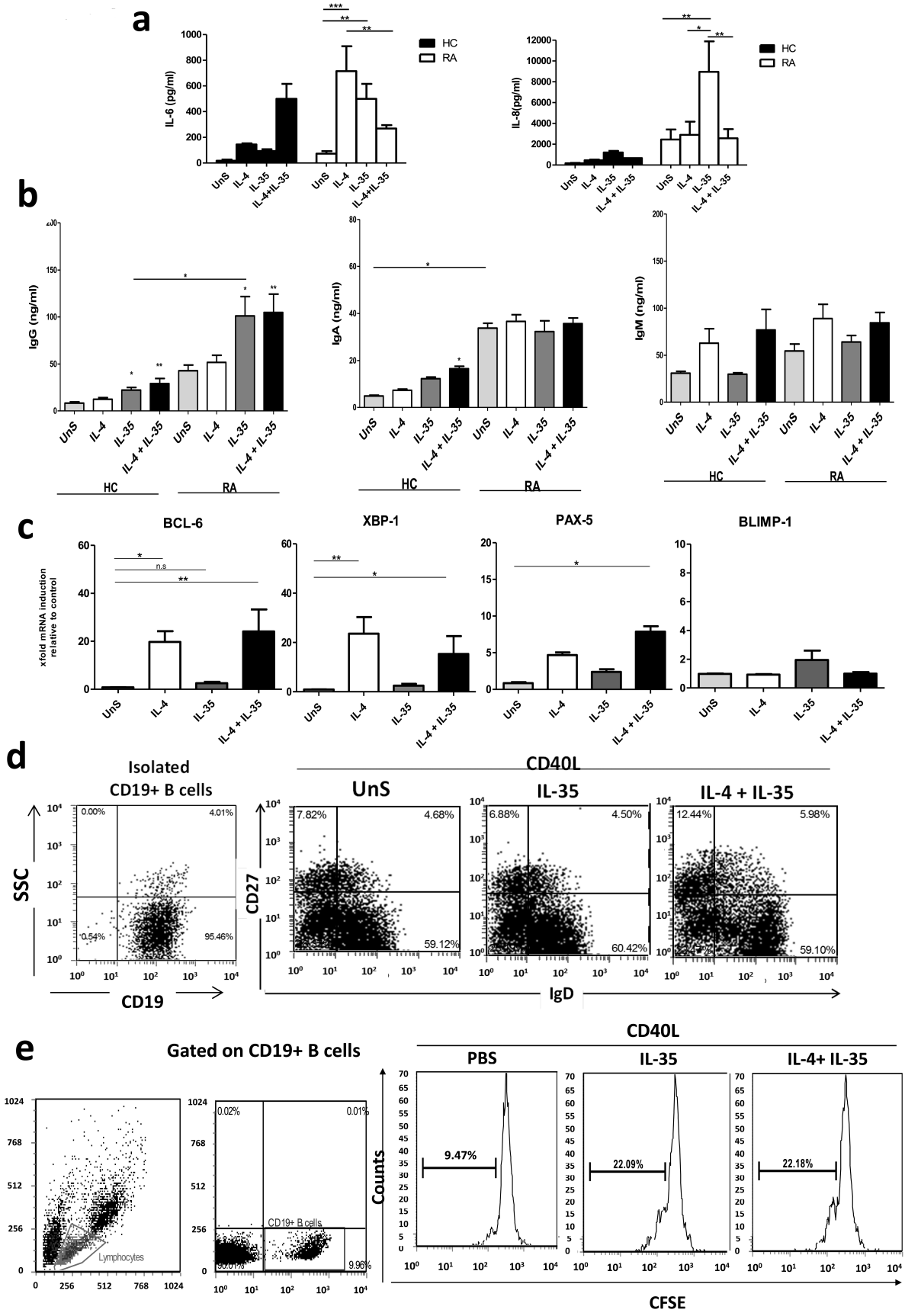

Figure 3. Effect of IL-35 on B cell activation, differentiation, and proliferation in patient with RA. (A–B) Purified B cells were cultured with IL-4 (100 ng/ml), IL-35 (100 ng/ml), combination of both (IL-4 + IL-35), or left untreated (unstimulated; UnS). Following 72 h of culture, levels of IL-6 and IL-8 in B cells isolated from HC and RA were measured by CBA assay (A) and total IgA/IgG/IgM were measured by ELISA (B). Results from a representative experiment of 6 similar experiments are shown; (C) SYBR Green–based quantitative real-time PCR analysis of BCL-6, XBP-1, PAX-5, and BLIMP-1 mRNA expression in response to IL-4, IL-35, combination of both, or medium alone (UnS) in purified B cells for 72 h. Results are expressed as mean ± SEM of the mRNA fold increase calculated at indicated timepoint by setting the baseline UnS expression as 1. Data were normalized using mammalian GAPDH as an endogenous control gene. (D) Pretreatment of purified B cells with CD40L for 24 h, followed by stimulation with IL-35 alone (100 ng/ml) or in the presence of IL-4 (100 ng/ml) or left untreated (UnS) for 5 days. After 6 days of culture, the cells were stained and assessed for IgD and CD27 expression. Expression of the cell surface molecules on 10,000 viable cells, and as a result the density of the dot plots reveals cell numbers. (E) PBMC isolated from HC (n = 3) were labeled with CFSE and prestimulated with recombinant CD40L (1 μg/ml) for 1 h. After that, PBMC were stimulated with IL-4 (100 ng/ml) in the presence or absence of IL-35 (100 ng/ml). After 96 h of culture, B cell proliferation was determined by gating on CD19+ cells. Results are representative of result from 3 similar experiments. *p < 0.05, **p < 0.01 compared to UnS. IL-35: interleukin 35; RA: rheumatoid arthritis; HC: healthy controls; CBA: cytometric bead array; SEM: standard error of the mean; ns: not significant; PBMC: peripheral blood mononuclear cells; CFSE: carboxyfluorescein succinimidyl ester.

ization (synovial vs dermal) rather than disease specificity (RA vs OA), because OASF exerts similar activities. Moreover, treatment with anti-IL-35–neutralizing antibody in cocultures system (transwell and cell-cell contact) displayed a significant, but partial inhibition of IgG production, suggesting that additional factors might account for the observed B cell activation either through cell-dependent [adhesion molecules, e.g., vascular cell adhesion molecule (VCAM-1)] or cell-independent mechanisms (chemokines, e.g., CXCL12, or cytokines, e.g., BAFF[34,35]). Accordingly, previous work demonstrated that IL-35 could modulate VCAM-1 expression[36], a well-known molecule to be expressed on SF for B cell activation[37]. Further studies are required to elucidate whether IL-35–induced VCAM-1 expression could mediate the effect of SF-release IL-35 on B cells. In addition, TNF-α stimulation could enhance IL-35 production in RASF, leading us to hypothesize that IL-35 level in synovial fluid/serum could be modulated by anti-TNF-α therapy. We have demonstrated the importance of IL-35 in promoting intrasynovial humoral immunity, and characterize a direct effect on RA therapy by inhibiting inflammatory arthritis through IL-35 production in RASF.

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ONLINE SUPPLEMENT

Supplementary material accompanies the online version of this article.

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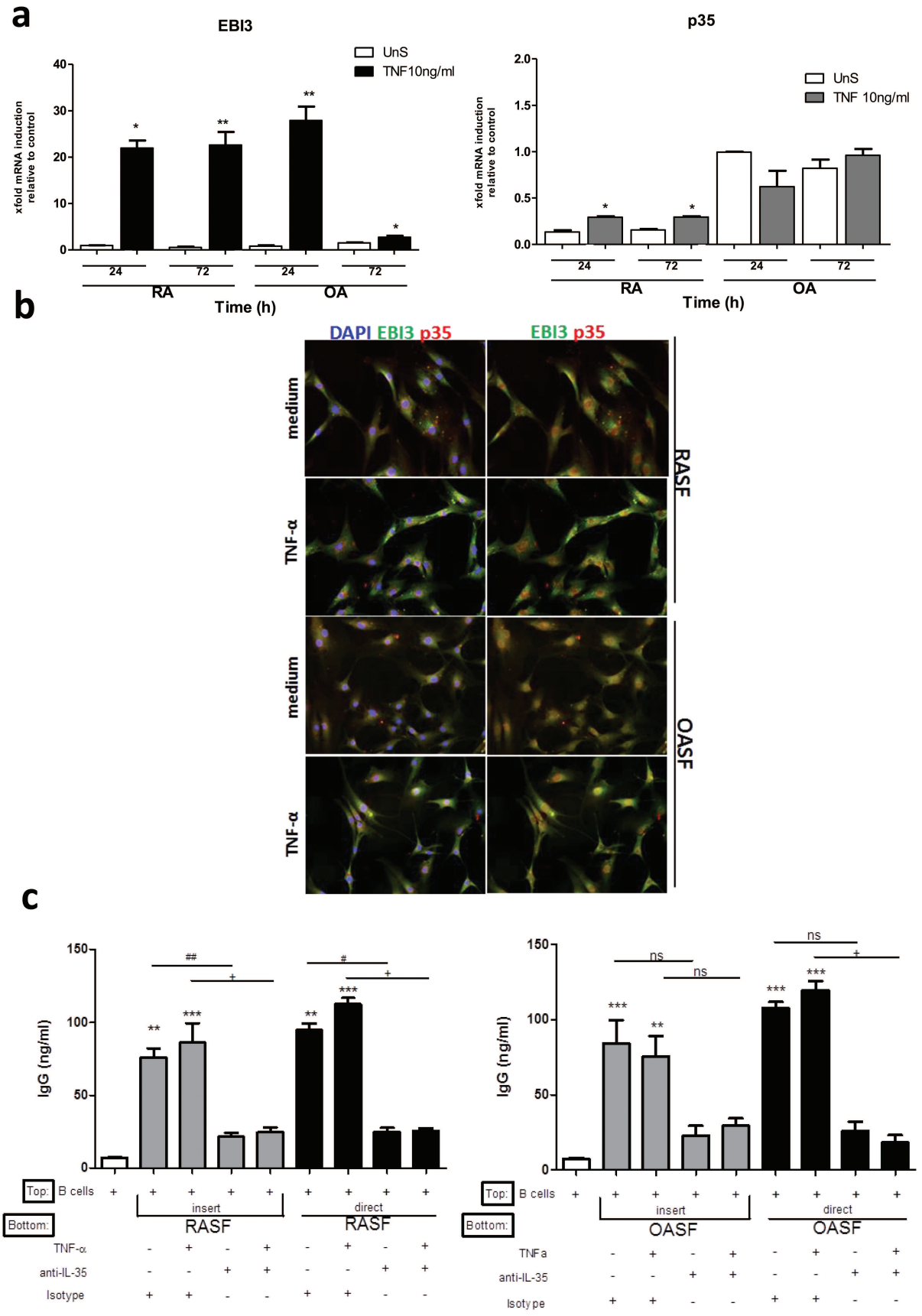

Figure 4. Induction of IL-35 mRNA and protein in RASF and OASF in response to TNF-α and the capacity of RASF-dependent IgG production in B cells by the involvement of IL-35. (A) Induction of EBI-3 and p35 mRNA in RASF and OASF in response to TNF-α stimulation. Quantitative SYBR Green–based real-time PCR analysis of EBI-3 and p35 mRNA expression. RASF and OASF were stimulated with 10 ng/ml TNF-α or left untreated (UnS) for 24 h and 72 h. EBI-3 (black) and p35 (grey) mRNA expression was evaluated and results are expressed as mean ± SEM of the mRNA fold increase calculated at each timepoint by setting the baseline unstimulated expression as 1. Data were normalized using mammalian GAPDH as an endogenous control gene. Results are expressed as the difference in threshold cycle (ΔCt), normalized to GAPDH. (B) Fibroblasts were seeded at density of 5 × 104 cells/200 μl onto cover slides a day before treatment. Representative microphotographs of IL-35 subunits immunostaining on RASF and OASF in resting conditions and upon TNF-α treatment. RASF/OASF were grown on coverslips and processed for immunofluorescence as described under Materials and Methods. Nuclei are counterstained in blue (DAPI), EBI-3 is stained in green (Alexa-488), p35 is stained in red (Alexa-555). Images were taken at 20× magnification and with the same exposure time for both conditions. (C) Purified CD19+ B cells were cultured with RASF (left) or OASF (right) for 72 h. B cells were cultured alone (open bars) or in coculture fibroblasts in direct physical contact (black bars) or a transwell system (grey bar). All cultures were incubated with (+) or without (–) 10 μg/ml anti-IL-35 or isotype-matched antibodies. IgG levels in supernatants were measured by ELISA. Results are presented as mean ± SEM of at least 3 experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with B cells culture alone. $^{\#}p < 0.05$ RASF/OASF-B direct cell-contact cocultures compared with isotype-matched antibodies. $^{\#\#}p < 0.01$ RASF/OASF-B transwell cocultures compared with isotype-matched antibodies. $^{+}p < 0.05$, TNF-α–stimulated RASF/OASF-B cocultures compared with isotype-matched antibodies. IL-35: interleukin 35; RA: rheumatoid arthritis; OA: osteoarthritis; SF: synovial fibroblasts; TNF-α: tumor necrosis factor-α; SEM: standard error of the mean; ns: not significant.

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