ARTHRITIS & RHEUMATISM
Vol. 54, No. 7, July 2006, pp 2084–2095
DOI 10.1002/art.21942

Regulation of Pre–B Cell Colony-Enhancing Factor by STAT-3–Dependent Interleukin-6 *Trans*-Signaling

Implications in the Pathogenesis of Rheumatoid Arthritis

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Objective. **To determine whether interleukin-6 (IL-6) *trans*-signaling directs the expression of pre–B cell colony-enhancing factor (PBEF) in vitro and in vivo.**

Methods. **Complementary DNA from rheumatoid arthritis (RA) synovial fibroblasts treated with IL-6 and soluble IL-6 receptor (sIL-6R) was used to probe a cytokine microarray. PBEF regulation by the IL-6–related cytokines, IL-6, sIL-6R, oncostatin M (OSM), IL-11, and leukemia inhibitory factor (LIF) was determined by reverse transcription–polymerase chain reaction analysis. IL-6–mediated STAT-3 regulation of PBEF was determined using a cell-permeable STAT-3 inhibitor peptide. Antigen-induced arthritis (AIA) was induced in wild-type ($IL\text{-}6^{+/+}$) and IL-6–deficient ($IL\text{-}6^{-/-}$) mice. PBEF and STAT were detected by immunohistochemistry, immunoblotting, and electrophoretic mobility shift assay. Synovial levels of PBEF were quantified by enzyme immunoassay.**

Results. **IL-6 *trans*-signaling regulated PBEF in a STAT-3–dependent manner. In addition, PBEF was regulated by the IL-6–related cytokine OSM, but not IL-11 or LIF. Flow cytometric analysis of the IL-6–related cognate receptors suggested that OSM regulates PBEF via its OSM receptor β and not its LIF receptor. The involvement of PBEF in arthritis progression was confirmed in vivo, where induction of AIA resulted in a 4-fold increase in the synovial expression of PBEF. In contrast, little or no change was observed in $IL\text{-}6^{-/-}$ mice, in which the inflammatory infiltrate was markedly reduced and synovial STAT-1/3 activity was also impaired. Analysis of human RA synovial tissue confirmed that PBEF immunolocalized in apical synovial membrane cells, endothelial cells, adipocytes, and lymphoid aggregates. Synovial fluid levels of PBEF were significantly higher in RA patients than in osteoarthritis patients.**

Conclusion. **Experiments presented herein demonstrate that PBEF is regulated via IL-6 *trans*-signaling and the IL-6–related cytokine OSM. PBEF is also actively expressed during arthritis. Although these data confirm an involvement of PBEF in disease progression, the consequence of its action remains to be determined.**

Rheumatoid Arthritis (RA) is a polyarticular synovitis disease characterized by the activation and proliferation of synovial tissue with associated degradation of articular cartilage. Fibroblast-like cells appear to be hyperplastic, hyperproductive, and phenotypically distinct from their "normal" counterparts and, as a result, are believed to play a major role in disease pathogenesis, altering the synovial environment from a site of acute inflammation to one of chronic inflammation (1). One of the major factors thought to contribute to the chronic milieu is interleukin-6 (IL-6), exerting its effects through 2 membrane-bound receptors, namely, IL-6R and gp130 (2).

IL-6–related cytokine receptors are divided into nonsignaling α-receptors (IL-6R, IL-11R, ciliary neuro-

Supported by Arthritis Research Campaign, project grants 14002 and 14006.

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Submitted for publication October 11, 2005; accepted in revised form March 24, 2006.

trophic factor receptor [CNTFR]) and signal-transducing membrane receptors (gp130, leukemia inhibitory factor receptor [LIFR], and oncostatin M receptor β [OSMRβ]). In addition, all IL-6–related cytokines require at least 1 molecule of gp130 for signaling. IL-6, IL-11, and CNTF initially bind to their α-receptors and bind to gp130 homodimer (IL-6 and IL-11) or gp130/LIFR heterodimer (CNTF). In the case of LIF and OSM, signals are elicited by the interaction of these cytokines with gp130 and LIFR (LIF and OSM) or OSMRβ (OSM).

Expression of the universal signal transducing receptor gp130 is ubiquitous; however, the cellular distribution of the α-receptors is more limited (2). Cells lacking the cognate IL-6R can be made responsive to IL-6 via a soluble IL-6R (sIL-6R) through a process known as IL-6 *trans*-signaling (3). Patients with RA have high synovial fluid levels of IL-6 and sIL-6R (4). Because structural cells in the synovial joint lack the cognate receptor, *trans*-signaling is believed to be the main pathway that governs IL-6 activity in RA (4). Interestingly, clinical trials involving antibodies to IL-6R showed a reduction in both RA disease activity and markers of inflammation, such as C-reactive protein and the erythrocyte sedimentation rate (5).

Activation of RA synovial fibroblast-like cells by IL-6 *trans*-signaling regulates the expression of a monocyte chemoattractant protein (CCL2), thereby exerting an influence on leukocyte trafficking into the joint (4). With this in mind, we used microarray analysis to explore additional sIL-6R–inducible factors that may be contributing to chronic inflammation in the stromal environment. Using a complementary DNA (cDNA) cytokine microarray, we identified the novel protein pre–B cell colony-enhancing factor (PBEF) as being significantly up-regulated in RA synovial fibroblasts following IL-6 *trans*-signaling.

Functional analysis of PBEF has confirmed its role as a soluble secreted protein (6–11) that can regulate both apoptosis (10) and gene transcription (8). We report the regulation of PBEF both in vitro and in vivo and discuss the potential contribution of PBEF to the pathology associated with RA.

PATIENTS AND METHODS

Synovial tissue and fluid. Ethical approval was obtained from the Bro-Taf Regional Health Authority of Wales prior to beginning the study. All patients were diagnosed as having RA for at least 3 months. Patients were taking nonsteroidal antiinflammatory drugs (NSAIDs), corticosteroids, and disease-modifying antirheumatic drugs (DMARDs) (with the exception of tumor necrosis factor inhibitors), either alone or in combination. These regimens are consistent with conventional rheumatology clinical practice. Synovial tissue was collected from RA patients during routine joint synovectomy at the time of total knee joint replacement. Synovial fluid was collected from 12 RA patients and 10 osteoarthritis patients during routine joint aspiration. Synovial fluid samples were rendered cell-free by centrifugation, and the supernatants were stored at –80°C.

Human synovial fibroblasts (HSFs). HSFs were isolated from RA synovial tissue. Briefly, HSFs were digested for 1 hour at 37°C with 1 mg/ml of collagenase (Sigma-Aldrich, Poole, UK). Cells were cultured in Dulbecco's modified Eagle's medium containing 100 units/ml of penicillin/streptomycin; 1 μM hydrocortisone, 1× insulin-transferrin-selenium-X, and 20% heat-inactivated fetal calf serum (Gibco Invitrogen, Paisley, UK). All stimulations were performed on growth-arrested HSFs, and cells were exclusively used between the third and seventh passages. Cells were stimulated with 30 ng/ml of IL-6, sIL-6R, OSM, IL-11, or LIF (R&D Systems, Oxford, UK) as indicated in the figure legends.

Growth-arrested HSFs were subjected to flow cytometric analysis of membrane receptors using phycoerythrin (PE)–conjugated anti-human gp130 (R&D Systems), PE-conjugated anti-human CD126 (anti–IL-6R; BD PharMingen, Oxford, UK), PE-conjugated anti-human gp190 (anti-LIFR; BD PharMingen). OSMRβ was detected using anti-human OSMRβ (N-20) (Santa Cruz Biotechnology, Santa Cruz, CA), with PE-conjugated $F(ab')_2$ fragment of goat anti-mouse IgG (Dako, Cambridge, UK).

To control for the activation of STAT-3, HSFs were preincubated for 1 hour with 100 μM STAT-3 inhibitor peptide (STAT-3iP; Calbiochem, Abingdon, UK), which acts as a cell-permeable inhibitory analog of the STAT-3–SH2 domain–binding phosphopeptide (12). Cells were subsequently challenged for 2 hours with 30 ng/ml of IL-6 and sIL-6R, and total RNA was prepared as described below.

Microarray of stimulated HSFs. RNA was extracted from HSFs with 500 μl of TRI reagent (Sigma-Aldrich) and processed according to the manufacturer's protocol. Total RNA (2 μg) was reverse transcribed using human cytokine-specific primers (R&D Systems), 3,000 Ci/mole of α^{33}P-dCTP (Amersham, Buckinghamshire, UK), and reverse transcriptase (R&D Systems) according to the R&D Systems human cytokine expression array protocol. Labeled cDNA was purified and hybridized (overnight at 65°C) to the human cytokine expression array according to the manufacturer's protocol. The arrays were exposed to a phosphor screen, and the results were analyzed with QuantityOne software (Bio-Rad, Hertfordshire, UK). The pixel intensity in each spot of the array was corrected against background readings. The normalized signal was calculated by representing the average gene spot intensity as a percentage of the signal from a housekeeping gene. Data generated from 4 housekeeping genes (β_2-microglobulin, β-actin, cyclophilin A, and transferrin R) were averaged to give the final value. Genes that were up-regulated 2–3-fold were chosen for further analysis.

Reverse transcription–polymerase chain reaction (RT-PCR) analysis of up-regulated genes. Total RNA (0.5 μg) was reverse transcribed using random hexanucleotide primers and Superscript II reverse transcriptase (Gibco Invitrogen). Oligo-

nucleotide primers for β-actin were used as a normalization control. Oligonucleotide primer sets for RT-PCR analysis of CCL2, PBEF, and β-actin were those described previously (13,14). The primer sequences for gp130 were: 5′-GGATACTGGAGTGACTGGAGTGAAG-3′ (coding strand) and 5′-CCATCTTGTGAGAGTCACTTCATAATC-3′ (noncoding strand). All primers were annealed at 55°C, and 10-μl aliquots were removed from the PCR reaction at 20 cycles (β-actin) 25 cycles (gp130), and 28 cycles (PBEF and CCL2).

In silico analysis of STAT binding sites in the PBEF promoter. The PBEF promoter sequence was retrieved from the genomic bacterial artificial chromosome (BAC) clone RP11-22N19 submitted to the National Center for Biotechnology Information database (accession no. AC007032). Analysis included the proximal and distal promoter regions, as described previously (7).

Induction of murine antigen-induced arthritis (AIA). Experiments were undertaken in 7–8-week-old male inbred C57BL/6J wild-type (IL-$6^{+/+}$) and IL-$6^{-/-}$ mice. Procedures were performed in accordance with UK Home Office–approved project license PPL-30/1820. Briefly, male mice were immunized subcutaneously with 1 mg/ml of methylated BSA (mBSA) emulsified with an equal volume of Freund's complete adjuvant (CFA) and injected intraperitoneally with 100 μl of heat-inactivated *Bordetella pertussis* toxin (all reagents from Sigma-Aldrich). The immune response was boosted 1 week later. Twenty-one days after the initial immunization, AIA was induced by intraarticular administration of 10 μl of mBSA (10 mg/ml) into the right knee joint. Three days after the intraarticular injection, mice were killed for biochemical (n = 3) and immunohistochemical (n = 3) analyses.

Immunohistochemistry of AIA mouse joints. Joints were fixed in neutral buffered formalin, decalcified with formic acid at 4°C, and then embedded in paraffin. For immunohistochemical analysis of PBEF, mid-sagittal serial sections (7 μm in thickness) were first incubated in peroxidase-blocking solution (Dako) to eliminate endogenous peroxidase activity. To inhibit endogenous biotin, sections were incubated in a biotin-blocking system (Dako). PBEF was detected with 1 μg/ml of rabbit IgG–purified polyclonal antibody (Amgen, Thousand Oaks, CA). For assessment of phosphorylated STAT-1 and STAT-3, sections were incubated in 10 m*M* citrate buffer (pH 6) high-temperature antigen-retrieval solution using the microwave method. Phosphorylated STAT was detected using pSTAT-1 (Tyr^{701}) and pSTAT-3 (Tyr^{705}) (clone 58E12) rabbit antibodies (Cell Signaling Technology, Beverly, MA). All sections were probed using a biotinylated $F(ab')_2$ fragment of swine anti-rabbit IgG (Dako) and StreptABComplex/HRP (Dako) and developed using diaminobenzidine chromogen, with hematoxylin counterstaining. Control slides were probed with 1 μg/ml of naive rabbit IgG and processed as above.

Preparation of mouse joint extracts. Joints of the right hind leg from IL-$6^{+/+}$ and IL-$6^{-/-}$ experimental animals harvested on day 0 (i.e., control) and day 3 after arthritis induction were trimmed of skin, muscle, and excess bone around the articular joint and immediately frozen on dry ice. Tissue was ground under liquid nitrogen, and resuspended in 1 ml of 10 m*M* HEPES–KOH (pH 7.9) containing, 1.5 m*M* $MgCl_2$, 10 m*M* KCl, 0.5 m*M* dithiothreitol (DTT), and 0.2 m*M* phenylmethylsulfonyl fluoride (PMSF), 50 m*M* NaF, 1 m*M* Na_3VO_4, and protease inhibitors (Sigma-Aldrich) diluted 1:1,000. This suspension was incubated on ice for 30 minutes, with continuous agitation. Following centrifugation, the supernatant was removed and used as the cytosolic extract for PBEF protein analysis. The cell pellet was resuspended in 0.5 ml of 20 m*M* HEPES–KOH (pH 7.9) containing 420 m*M* NaCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* EDTA, 25% glycerol, 0.3 m*M* DTT, 0.2 m*M* PMSF, 50 m*M* NaF, 1 m*M* Na_3VO_4, and protease inhibitors (Sigma-Aldrich) diluted 1:1,000. Extracts were incubated on ice for 30 minutes, with continuous agitation. Following centrifugation, the supernatant was removed and used as the nuclear extract for analysis of STAT transcription factor activation. Total protein concentration was determined using a modified Bradford assay (Bio-Rad).

Analysis of PBEF in cytosolic extracts. Mouse knee cytosolic extract (10 μg) was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, followed by Western blotting. PBEF expression was analyzed using 0.2 μg/ml of rabbit IgG–purified polyclonal antibody (Amgen), and detected using biotinylated $F(ab')_2$ fragment of swine anti-rabbit IgG (Dako), streptavidin–horseradish peroxidase (Amersham) and SuperSignal West Pico chemiluminescent substrate (Pierce, Chester, UK). Specificity was confirmed with recombinant murine PBEF (Amgen), and blots were probed with 0.2 μg/ml rabbit IgG.

Analysis of STAT in nuclear extracts. Electrophoretic mobility shift assays (EMSAs) were performed as previously described (15). Briefly, 10 μg of mouse knee nuclear extracts was incubated with α^{32}P-dTTP–labeled double-stranded oligonucleotide containing a STAT-binding consensus sequence (SIE-m67). The oligonucleotide sequences for the SIE-m67 probe were 5′-CGACATTTCCCGTAAATCG-3′ (sense) and 3′-GTAAAGGGCATTTAGCAGC-5′ (antisense). DNA protein complexes were resolved by nondenaturing PAGE through 6% polyacrylamide gels in 0.5× Tris–borate–EDTA buffer. The gel was then dried and exposed to x-ray film at –70°C, with intensifying screens.

Densitometric analysis of bands. Gel images were analyzed for peak intensity using ImageJ imaging software (NIH Image, National Institutes of Health, Bethesda, MD; online at: http://rsb.info.nih.gov/ij/). The data from the densitometry of the Western blots and EMSAs were normalized to a baseline of 1. Statistical analysis of the differences in peak intensities was performed by the homoscedastic Wilcoxon-Mann-Whitney U test. *P* values less than 0.05 were considered statistically significant.

Immunolocalization of PBEF in RA tissue. RA synovial tissue was processed for immunohistochemical analyses by fixation in 4% neutral buffered formalin and embedded in paraffin. Serial sections (7 μm) were incubated in a peroxidase-blocking solution (Dako), and PBEF was detected using human visfatin rabbit polyclonal antibodies (AdipoGen, Seoul, South Korea). Control slides were probed with 1 μg/ml of naive rabbit IgG and processed as above. Sections were probed using biotinylated $F(ab')_2$ fragment of swine anti-rabbit IgG (Dako) and StreptABComplex/HRP (Dako) and developed using diaminobenzidine chromogen, with hematoxylin counterstaining.

EIA of PBEF in synovial fluid. Quantification of synovial fluid levels of PBEF was assessed using an enzyme immunoassay kit (Phoenix Pharmaceuticals, Karlsruhe, Germany). Individual samples were measured in duplicate. Statis-

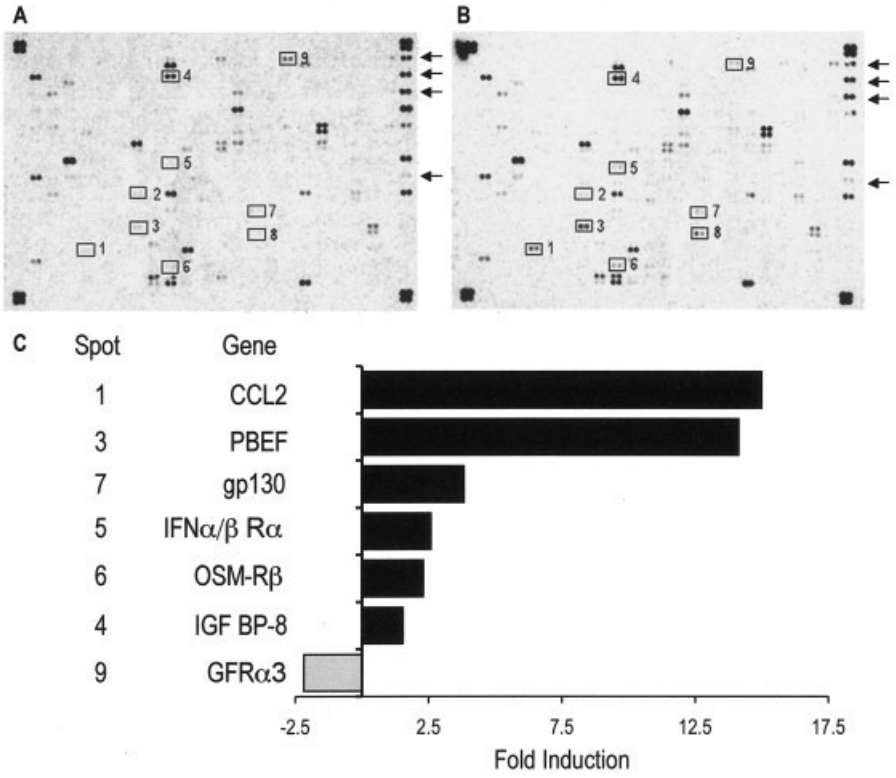

Figure 1. Cytokine expression array of growth-arrested human synovial fibroblasts (HSFs) obtained from patients with rheumatoid arthritis. This array comprises 375 cloned cDNA fragments, printed in duplicate, of cytokines, chemokines, immunomodulatory factors, and their receptors. Control genes include genomic DNA–positive controls (the tetrads at the 4 corners), 9 housekeeping genes (far right column, top), and 4 negative controls (far right column, bottom). The array was probed with ^{33}P-labeled cDNA that was prepared using cytokine-specific primers. **A,** Cytokine expression of unstimulated HSFs. **B,** HSFs stimulated for 2 hours with 50 ng/ml of interleukin-6 (IL-6) and soluble IL-6 receptor. Numbered boxed areas in **A** and **B** indicate the following signals, which correspond to the numbered spots shown in **C**: 1 = CCL2, 2 = macrophage migration inhibitory factor, 3 = pre–B cell colony-enhancing factor (PBEF), 4 = insulin-like growth factor binding protein 8 (IGFBP-8), 5 = interferon-α/β receptor α-subunit (IFNα/βRα), 6 = oncostatin M receptor β-subunit (OSMRβ), 7 = gp130, 8 = IL-1 receptor type I, and 9 = glial cell line–derived neurotrophic factor receptor α3-subunit (GFRα3). The expression of 4 housekeeping genes, β_2-microglobulin, β-actin, cyclophilin A, and transferrin R, was consistent in each sample (arrows). **C,** Comparison of cytokine expression signals in the microarray between control and stimulated HSFs. The normalized signal was calculated by representing the average gene spot intensity as a percentage of the signal from a housekeeping gene. Data generated from 4 housekeeping genes (β_2-microglobulin, β-actin, cyclophilin A, and transferrin R) were averaged to give the final value.

tical analysis of PBEF concentrations in synovial fluid from RA and OA patients was compared by the homoscedastic Wilcoxon-Mann-Whitney U test. *P* values less than 0.05 were considered statistically significant.

RESULTS

Identification of genes regulated by IL-6 *trans*-signaling. The human cytokine expression array from R&D Systems was used to analyze changes in the expression of cytokine, chemokine, and inflammatory mediator genes in HSFs following stimulation with IL-6 and sIL-6R (Figure 1). No significant changes in the relative expression of the housekeeping genes β_2-microglobulin, β-actin, cyclophilin A, and transferrin R were observed between samples, and these genes were subsequently used for normalization. Normalized genes that were regulated more than 2-fold are shown graphically in Figure 1C. Regulation of CCL2 by IL-6 *trans*-

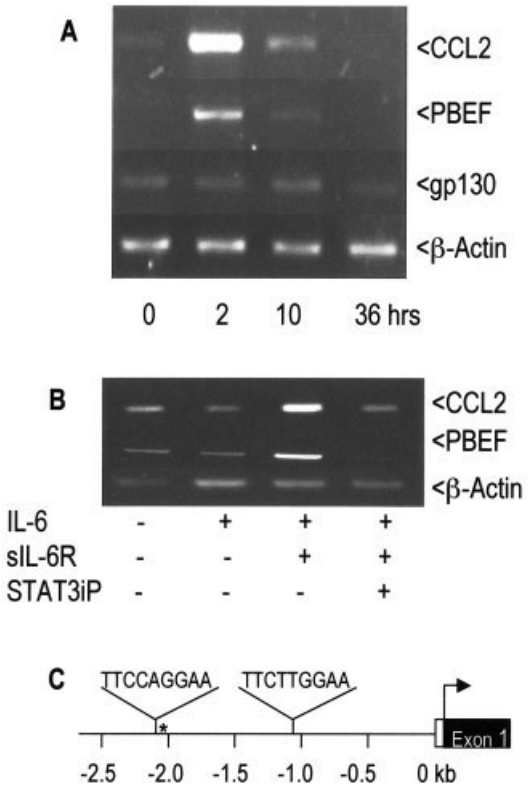

Figure 2. Semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) analysis of growth-arrested human synovial fibroblasts (HSFs) obtained from patients with rheumatoid arthritis. HSFs were serum starved for 48 hours prior to stimulation. At set time intervals, mRNA was extracted for RT-PCR analysis. Conditions were optimized, and β-actin was used as a control gene. **A,** HSFs were unstimulated or were stimulated for 2, 10, and 36 hours with 30 ng/ml each of interleukin-6 (IL-6) in combination with soluble IL-6 receptor (sIL-6R). Results of RT-PCR analysis for CCL2, pre–B cell colony-enhancing factor (PBEF), gp130, and β-actin mRNA are shown. **B,** HSFs were unstimulated or were stimulated for 2 hours with either 30 ng/ml of IL-6, 30 ng/ml each of IL-6 in combination with sIL-6R, or 30 ng/ml each of IL-6 in combination with sIL-6R following preincubation for 1 hour with 100 μM STAT-3 inhibitor peptide (STAT-3iP). Results of RT-PCR analysis (2 independent experiments) for CCL2, PBEF, and β-actin mRNA are shown. **C,** Schematic representation of elements in the PBEF promoter region that correspond to the palindromic STAT DNA-binding sequence $TT(N)_5AA$. Exon 1 is shown with a noncoding region (open box) and a coding region (solid box). The distance from the transcription-initiation site (arrow) is marked (in kb). The asterisk represents one of the TATA boxes (based on reference 7).

signaling in HSFs has previously been reported (4) and was used as an internal positive control.

Several induced genes not previously affiliated with IL-6 *trans*-signaling, including PBEF, gp130, interferon-α/β receptor α-subunit (IFNα/βRα), OSMR β-subunit (OSMRβ), and insulin-like growth factor binding protein 8 (IGFBP-8), were shown to be up-regulated, whereas expression of glial cell line–derived neurotrophic factor receptor α3-subunit was down-regulated in this array. The apparent induction of IL-1R type I was eliminated from further analysis because of the high degree of variability observed between the labeling of the duplicate spots.

Regulation of a novel cytokine (PBEF) by IL-6 *trans*-signaling. Verification of the array data by RT-PCR and flow cytometric analyses confirmed that IL-6 *trans*-signaling promoted strong induction of CCL2 and PBEF, but not gp130 (Figure 2A). Of particular interest was the regulation of PBEF, which showed a level of induction (~15-fold) comparable to that of CCL2. Subsequent RT-PCR analysis demonstrated that CCL2 and PBEF share similar expression profiles and that their regulation by IL-6 *trans*-signaling could be blocked by the inclusion of STAT-3iP (Figure 2B). In silico analysis of the 5′-promoter region of the genomic BAC clone RP11-22N19 containing the PBEF gene, revealed 2 putative STAT binding sites (TTCCAGGAA and TTCTTGGAA), as predicted from the consensus STAT-binding sequence $TT(N)_5AA$ (16). These were present in both the proximal (1 kb upstream) and distal (2 kb upstream) promoter regions, respectively (Figure 2C)

Regulation of PBEF by IL-6–related cytokines. To determine whether other IL-6–related cytokines also regulate PBEF, growth-arrested HSFs were stimulated with OSM, LIF, and IL-11. OSM up-regulated PBEF messenger RNA (mRNA) expression after 2 hours. However, no stimulatory effects were observed with either LIF or IL-11 (Figure 3A). Since OSM elicits a response via a heterodimer complex consisting of either gp130 and LIFR or gp130 and OSMRβ, these data suggest that OSM mediates the control of PBEF through its selective cognate receptor OSMRβ. This was confirmed by flow cytometry, which showed that HSFs express gp130 and OSMRβ, but not IL-6R or LIFR (Figure 3B). Indeed, consistent with the data presented in Figure 1, IL-6 *trans*-signaling was found to enhance OSMRβ expression on HSFs (Figure 3B), suggesting a close relationship between IL-6 *trans*-signaling, OSM activity, and the regulation of PBEF. Expression of the cognate IL-11R could not be ascertained due to the lack of a suitable antibody.

Regulation of PBEF by IL-6 in vivo. Based on these in vitro observations, we tested whether IL-6 governs PBEF expression during experimental arthritis. Immunohistochemistry and Western blot analysis using antibodies against murine PBEF were performed on joint sections and mouse knee cytosolic extracts

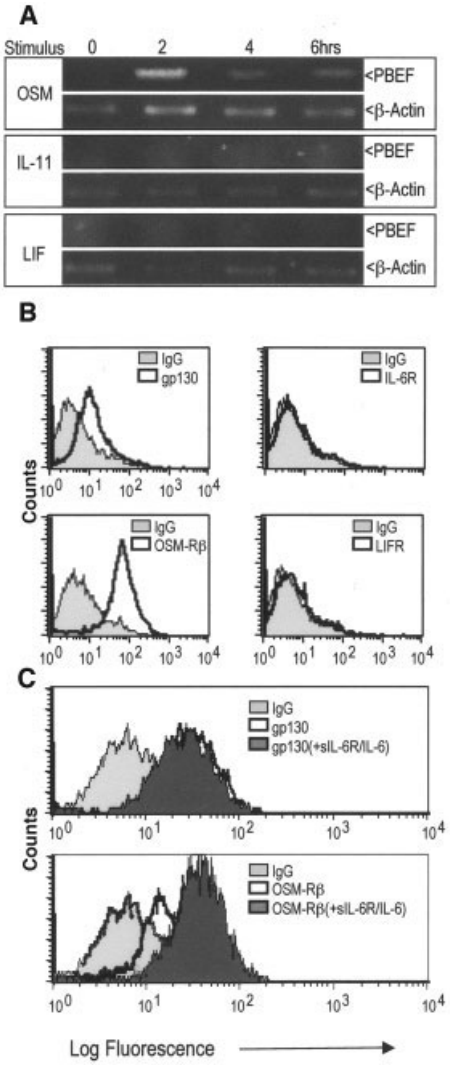

Figure 3. Semiquantitative reverse transcription–polymerase chain reaction of pre–B cell colony-enhancing factor (PBEF) following interleukin-6 (IL-6)–related cytokine stimulation of growth-arrested human synovial fibroblasts (HSFs) obtained from patients with rheumatoid arthritis. **A,** HSFs were stimulated for 0, 2, 4, and 6 hours with 30 ng/ml of IL-6–related cytokines oncostatin M (OSM), IL-11, and leukemia inhibitory factor (LIF), and PBEF and β-actin mRNA were measured. **B,** Expression of IL-6–related cytokine receptors on HSFs. HSFs were analyzed by flow cytometry for surface expression of gp130, IL-6 receptor (IL-6R), OSM receptor β-subunit (OSMRβ), and LIF receptor (LIFR). **C,** Flow cytometric analysis of gp130 and OSMRβ following IL-6 *trans*-signaling. Following 16 hours of stimulation with 30 ng/ml of IL-6 and soluble IL-6R (sIL-6R), the expression of gp130 remained unchanged and cell surface OSMRβ was up-regulated 2.5-fold.

prepared from IL-$6^{+/+}$ and IL-$6^{-/-}$ mice 3 days after arthritis induction (i.e., during the acute inflammatory phase of the model) (4). PBEF was present in low amounts in the joints of nonarthritic IL-$6^{+/+}$ mice, with occasional staining in the cells of the synovium and articular cartilage chondrocytes (Figure 4A). There was an increase in staining for PBEF in the joint sections of IL-$6^{+/+}$ mice, and this coincided with a large inflammatory infiltrate in the synovial tissue and a marked activation of STAT-1/3 (Figure 4). PBEF staining was diffuse in the tissue sections, suggesting that it was secreted into the surrounding area.

Fibroblast-like synoviocytes in the synovial membrane and subintimal synovium showed both cytoplasmic and nuclear staining for PBEF (Figure 4E inset). In addition, cells of the inflammatory infiltrate also appeared to be positive for PBEF (Figure 4E). In contrast, synovial infiltration in IL-$6^{-/-}$ mice was markedly reduced, and this was accompanied by less PBEF staining. (Figure 4C). There was very little staining for PBEF in the synovial membrane and subintimal lining.

Joint sections were probed for phosphorylated STAT-1 and STAT-3. Both pSTAT-1 and pSTAT-3 were detected throughout the synovial tissue of IL-$6^{+/+}$ mice with AIA (Figures 4E and H). Although pSTAT-1 and pSTAT-3 were detected in IL-$6^{-/-}$ mice (Figures 4F and I), levels were markedly reduced and approached those in normal, nonarthritic mice (Figures 4D and G).

These results were supported by the findings of Western blotting for PBEF in cytoplasmic extracts (Figure 5A) and EMSA for STAT activity in nuclear extracts prepared from the corresponding mouse knee joints (Figure 5B). Densitometry showed a significant mean fold increase in PBEF expression in cytosolic extracts from the knee joints of IL-$6^{+/+}$ mice with AIA (mean ± SEM 4.42 ± 1.19, n = 3), as compared with their normal, nonarthritic counterparts ($P < 0.05$) (Figure 5C). Both normal IL-$6^{-/-}$ mice (1.39 ± 0.26, n = 3) and IL-$6^{-/-}$ mice with AIA (1.50 ± 0.25, n = 3) showed no significant difference in PBEF levels as compared with normal IL-$6^{+/+}$ mice ($P > 0.05$) (Figure 5C). However, IL-$6^{-/-}$ mice showed no evidence of PBEF induction following AIA ($P > 0.05$). Densitometry also showed a significant increase in STAT DNA binding activity in IL-$6^{+/+}$ mice following arthritis induction (1.94 ± 0.19, n = 3; $P < 0.05$) (Figure 5D). In contrast, IL-6 deficiency led to impaired STAT activation (0.57 ± 0.18, n = 3), which showed no evidence of induction following AIA (0.64 ± 0.36, n = 3) (Figure 5C).

Expression of PBEF in RA synovial tissue. Immunolocalization of PBEF in RA synovium indicated positive cytoplasmic and nuclear staining in the apical

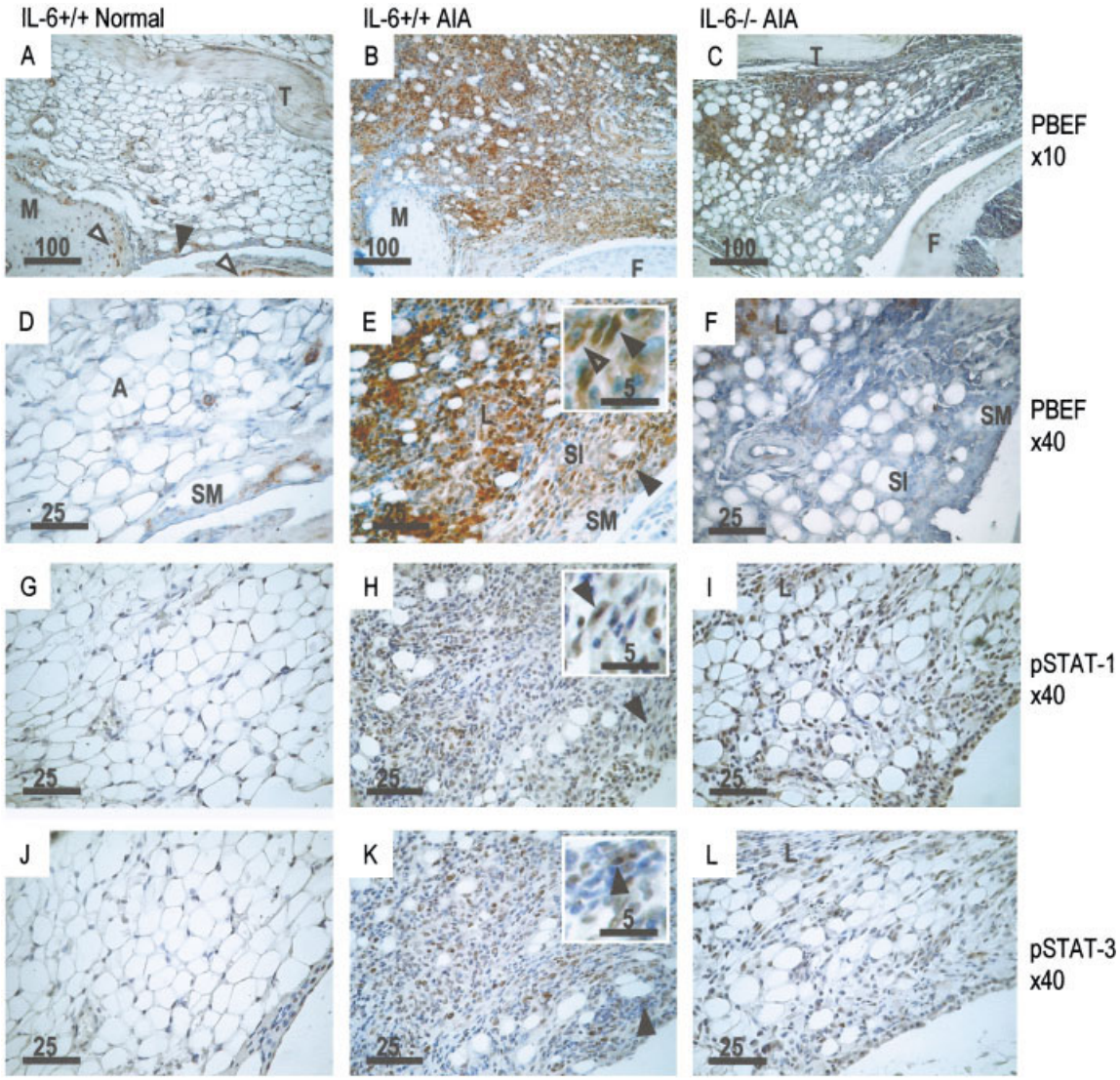

Figure 4. Immunohistochemical analysis of pre–B cell colony-enhancing factor (PBEF) and STAT activation in mice with antigen-induced arthritis (AIA). IL-$6^{+/+}$ and IL-$6^{-/-}$ mice were primed with methylated bovine serum albumin BSA (mBSA), then given a single intraarticular injection of mBSA into the right knee joint. After 3 days, histology samples were obtained, and serial sections of the joints were prepared for immunohistochemistry. **A,** Control IL-$6^{+/+}$ mouse, showing PBEF immunostaining in cells of the synovial lining and subsynovium (**solid arrowhead**) and in chondrocytes of the meniscus (**M**) and articular cartilage (**open arrowheads**). **T** = patellar tendon. **B,** IL-$6^{+/+}$ mouse with AIA, showing PBEF immunostaining and extensive leukocyte infiltration in the synovium. **F** = femur. **C,** IL-$6^{-/-}$ mouse with AIA, showing PBEF immunostaining. **D,** High-power view of PBEF immunostaining in a control IL-$6^{+/+}$ mouse. **SM** = synovial membrane; **A** = adipose tissue. **E,** High-power view of PBEF immunostaining in an IL-$6^{+/+}$ mouse with AIA, showing PBEF in the synovial membrane, subintimal (**SI**) synovium, and adipose tissue. A large inflammatory infiltrate (**L**) in the synovium also stained positive for PBEF. **Inset,** Fibroblast-like synoviocytes in the synovial membrane showed both nuclear (**solid arrowhead**) and cytoplasmic (**open arrowhead**) staining for PBEF. **F,** High-power view of PBEF immunostaining in an IL-$6^{-/-}$ mouse with AIA, showing very little staining in the synovial membrane and subintimal synovium. PBEF was associated with the (reduced) leukocyte infiltrate. **G,** Control IL-$6^{+/+}$ mouse, showing no immunostaining for pSTAT-1. **H,** IL-$6^{+/+}$ mouse with AIA, showing nuclear staining for pSTAT-1 throughout the synovium. **Inset,** Fibroblast-like synoviocytes in the synovial membrane showed nuclear staining for pSTAT-1 (**arrowhead**). **I,** IL-$6^{-/-}$ mouse with AIA, showing reduced nuclear staining for pSTAT-1, with strongest staining in areas with leukocyte infiltrates. **J,** Control IL-$6^{+/+}$ mouse, showing no immunostaining for pSTAT-3. **K,** IL-$6^{+/+}$ mouse with AIA, showing nuclear staining for pSTAT-1 throughout the synovium. **Inset,** Fibroblast-like synoviocytes in the synovial membrane showed nuclear staining for pSTAT-3 (**arrowhead**). **L,** IL-$6^{-/-}$ mouse with AIA, showing pSTAT-3 staining similar to that of pSTAT-1. **Arrowheads** in **E, H,** and **K** indicate the regions shown in the respective insets. Scale bars represent μm.

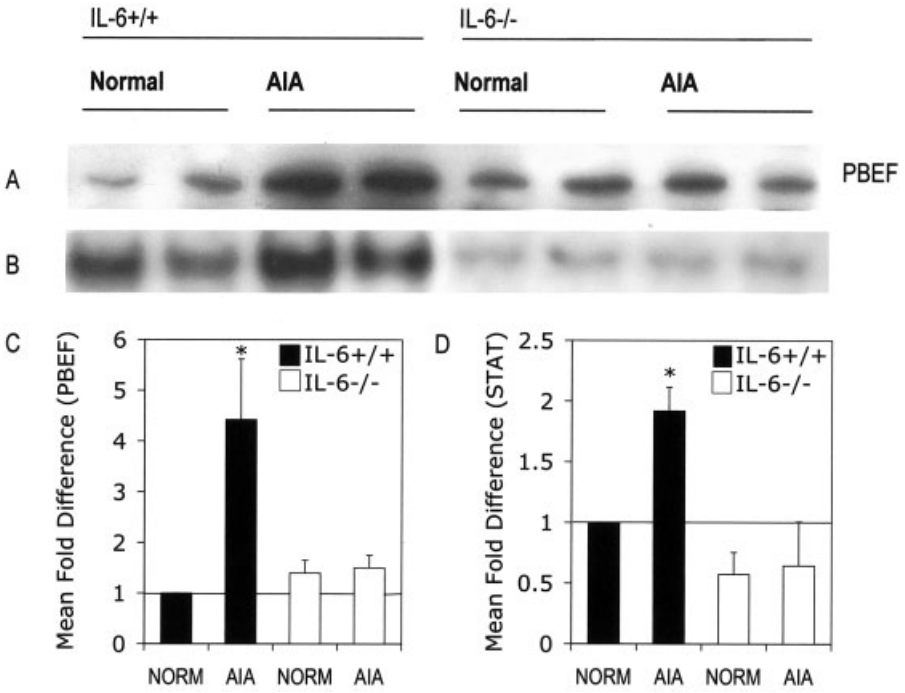

Figure 5. Biochemical analysis of pre–B cell colony-enhancing factor (PBEF) and STAT activation in knee joint extracts prepared from normal (nonarthritic) and arthritic IL-$6^{+/+}$ IL-$6^{+/+}$ and IL-$6^{-/-}$ mice. Samples were obtained on day 3 after induction of antigen-induced arthritis (AIA). **A,** Western blot analysis of PBEF protein in cytoplasmic extracts from normal and arthritic IL-$6^{+/+}$ and IL-$6^{-/-}$ mice. PBEF was resolved at 50 kd. Shown are 2 representative samples from each group (n = 3 mice per group). **B,** Corresponding electrophoretic mobility shift assay of nuclear extracts of the samples in **A**, showing DNA protein complexes of STAT transcription factors. **C** and **D,** Densitometry of PBEF (**C**) and STAT (**D**) in mouse knee homogenates was performed. Peak intensities were normalized to a baseline value of 1 (nonarthritic IL-$6^{+/+}$ basal levels). Values are the mean and SEM fold difference in peak intensity in normal and arthritic IL-$6^{+/+}$ and IL-$6^{-/-}$ mice, as compared with basal levels (horizontal line) ($* = P < 0.05$, by Wilcoxon-Mann-Whitney U test).

synovial membrane cells, which are made up of fibroblast-like and macrophage-like cells (Figures 6A and B). Evidence of PBEF expression was also observed in endothelial cells lining the capillaries and within tissue lymphoid aggregates (Figure 6C). In both cases, the staining pattern revealed cytoplasmic and nuclear localization. In addition, there was some evidence of nuclear PBEF staining in cells identified within adipose tissue (Figure 6D). Rabbit IgG used as negative controls on corresponding serial sections showed no nonspecific staining (data not shown).

Expression of PBEF in synovial fluid. RA patients had a significantly higher synovial fluid concentration of PBEF (mean ± SEM 150.0 ± 39.07 ng/ml) than did osteoarthritis patients (64.1 ± 21.58 ng/ml) (Figure 6E).

DISCUSSION

IL-6 and its soluble receptor are expressed in large quantities in synovial tissue and synovial fluid from patients with RA and are believed to be important in instigating and maintaining the pathology of RA (4). Indeed, monoclonal antibodies that block IL-6R–mediated signaling have proven to be clinically beneficial in patients with RA (17). STAT-3 is the principal STAT transcription factor activated by IL-6 (18), and inappropriate STAT-3 activity is closely associated with experimental arthritis, where its activities have been linked with the regulation of the expression of cytokines and chemokines and the induction of antiapoptotic genes that prevent HSF and T cell apoptosis (4,19–22).

In experimental models of inflammation, IL-6–

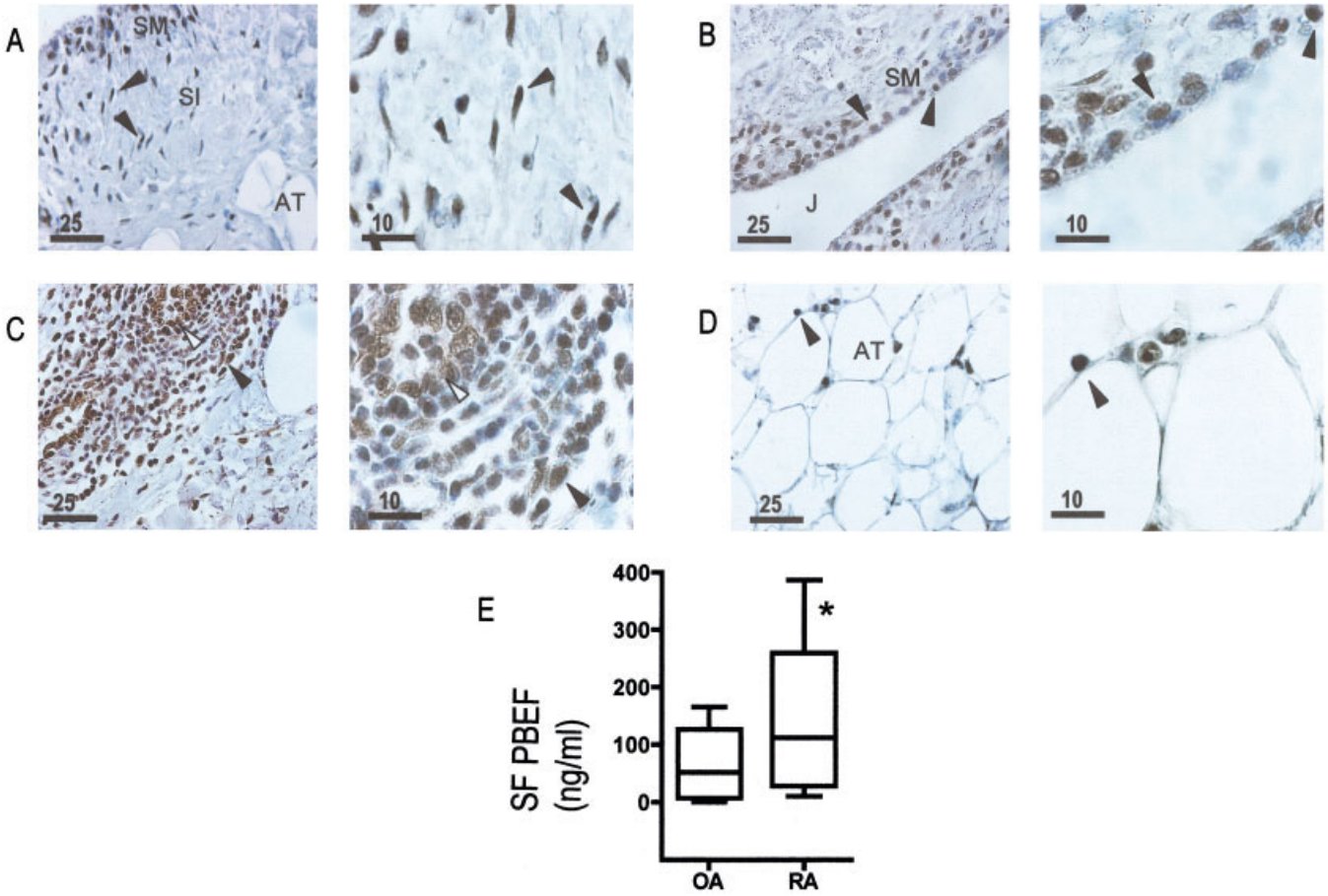

Figure 6. A–D, Immunohistochemical staining for pre–B cell colony-enhancing factor (PBEF) in rheumatoid arthritis (RA) synovial tissue at 2 different magnifications (in μm), and **B,** PBEF concentrations in synovial fluid from patients with osteoarthritis (OA) and RA. **A,** Synovial tissue containing positively stained spindle-shaped fibroblast-like cells within the thickened synovial membrane (**SM**) and in the loose adipose connective tissue (**AT**) of the subintimal (**SI**) synovial lining (**arrowheads**). **B,** Synovial membrane containing positively stained nuclei of rounded macrophage-like cells on the apical surface (**arrowheads**). **J** = joint. **C,** Lymphoid aggregate in the subintimal synovial lining showing staining for PBEF. Both the cytoplasm and nuclei appear to be stained (**solid arrowhead**). Endothelial cells of the blood vessels also stained positive (**open arrowhead**). **D,** Adipose tissue situated below the subintimal synovial lining showing adipocytes with nuclear staining (**arrowhead**). **E,** Box and whiskers plot of PBEF concentrations in synovial fluid (SF) from 10 OA and 12 RA patients ($* = P < 0.05$, by Wilcoxon-Mann-Whitney U test). Boxes show the 25% and 75% percentiles, horizontal lines within the boxes show the median, and whiskers show the upper and lower extremes.

deficient mice display reduced leukocyte infiltration accompanied by altered chemokine expression (4,23). Such a response can, however, be reversed through reconstitution of IL-6 *trans*-signaling (4,23). During the acute phase of arthritis in the AIA model (day 3 after intraarticular administration of mBSA), the synovial tissue becomes infiltrated with large numbers of mononuclear cells that invade the subintimal synovium, underlying adipose tissue, smooth muscle layer, and joint tendon. As a consequence, these mononuclear cells contribute not only to the local production of chemokines, but also to the local levels of sIL-6R through shedding of their membrane-bound IL-6 receptor (Nowell MA, et al: unpublished observations). IL-6 *trans*-signaling promotes mononuclear leukocyte recruitment both in vitro and in vivo via the production of CCL2 (4).

We have demonstrated that IL-6 *trans*-signaling also regulates the expression of the novel cytokine-like factor PBEF in human synovial fibroblasts and that this expression appears to be governed by STAT-3. STAT regulation of PBEF was confirmed by in silico analysis of the promoter region of PBEF, where we identified the presence of 2 putative STAT-binding sites. We have also shown that PBEF is up-regulated in the inflamed synovium of mice with AIA and is associated with an increase in STAT activation. In the absence of IL-6, the degree of

leukocyte infiltration following arthritis induction is markedly reduced, along with diminished levels of PBEF and STAT. Although in vitro appraisal of synovial fibroblasts highlights these cells as one source of PBEF, it is evident from histologic staining of mouse and human tissue that other stromal cells and infiltrating leukocytes may contribute to synovial PBEF levels. Since IL-6 *trans*-signaling also regulates leukocyte recruitment, PBEF levels may also be impaired through the reduced influx of leukocytes that is seen in IL-$6^{-/-}$ mice.

Of the other IL-6–related cytokines known to activate gp130, only OSM regulated PBEF expression in vitro. Levels of both LIF and OSM are elevated in synovial fluid and serum from RA patients (24,25) and share many activities due to their utilization of a common receptor complex (26). However, OSM is unique in its ability to bind with both the LIFR and an OSMRβ (27). Both receptors activate STAT-1 and STAT-3, but OSMRβ can also recruit STAT-5, which accounts for some of the different bioactivities observed with OSM (28). Flow cytometry, however, confirmed that OSM directs PBEF expression through OSMRβ, since LIFR was not detected in HSFs. Although not directly tested in these experiments, it is expected that neither cardiotropin 1 (CT-1) nor CNTF would induce PBEF in HSFs since both cytokines require LIFR for signaling. However, it remains to be determined whether IL-31 promotes PBEF expression in HSFs, since IL-31 requires OSMRβ and a selective IL-31 binding receptor (29). The unresponsiveness of HSFs to IL-11 also suggests that HSFs do not express IL-11R. The mechanism by which IL-11 transmits its signal is very similar to that of IL-6 (i.e., the requirement of an α-receptor and gp130 homodimerization for signal transduction); however, IL-11 and IL-6 have opposing effects, and IL-11 is reported to suppress arthritis activity (30,31). Further insight into the activity of IL-11 may therefore provide valuable clues to the potential pathologic involvement of PBEF in arthritis progression.

Our study has demonstrated elevated levels of PBEF in synovial fluid from RA patients as compared with OA patients. These levels are consistent with serum concentrations of PBEF described recently (32). Furthermore, PBEF expression has been immunolocalized to several cells of the synovium, including fibroblast-like and macrophage-like synoviocytes, endothelial cells, and adipocyte-like cells. Of particular interest is the expression of PBEF within synovial lymphoid aggregates. In RA, these aggregates are believed to consist of antigen-specific lymphocytes that have been trapped in the synovium by proretentive and antiapoptotic signals elicited by synovial fibroblasts (1). Aberrant apoptosis and inappropriate chemokine activity of synovial fibroblasts and inflammatory cells in the RA joint are believed to be major contributory factors to the pathogenesis of arthritis (33–36). Such activities may relate to the involvement of PBEF. Specifically, PBEF has been shown to prevent fibroblasts and neutrophils from entering apoptotic cell death (10,37) and can activate protein kinase B (Akt) following engagement of the insulin receptor (11). PBEF has also been shown to regulate both IL-6 and CXCL8 gene transcription in epithelial cells (8). It was recently suggested that dysregulated PBEF expression plays a significant role in a number of other inflammatory disorders, including infection-induced preterm birth, acute lung injury, sepsis, colorectal cancer, and metabolic syndrome (7,9–11,15).

Overexpression of PBEF in disease is thought to be more than simply a biomarker of inflammation (9); as its name suggests, pre–B cell colony-enhancing factor has been described as a novel factor in B cell development (6,38,39). PBEF is also known as visfatin, a cytokine-like factor associated with visceral fat, and increased serum levels of visfatin have been linked with obesity and type 2 diabetes mellitus (11,40). Sequence and functional analysis of the PBEF protein also suggest that this protein may act as an enzyme that is involved in energy metabolism, a nicotinamide phosphoribosyltransferase (NAmPRTase) (41–43). In addition to controlling intracellular NAD+, the NAmPRTase activity of PBEF has the ability to modulate a number of biologic processes, including apoptosis, by directly controlling the transcriptional regulatory activity of a NAD-dependent protein deacetylase, the silent information regulator SIR-2 (43). However, attempts to assay NAmPRTase in human peripheral blood cells have been unsuccessful, suggesting that this enzyme is tightly governed and may require further processing or regulation before orchestrating enzymatic activities (44). The involvement of an NAmPRTase activity in disease progression is highlighted by the specific noncompetitive inhibitor FK866, which is currently under investigation in phase I clinical trials for the treatment of prostate melanoma (45,46).

PBEF is likely to have multipotency, acting as both an intracellular enzyme and a secreted cytokine. This would incorporate it into a family of proteins that show dual activities including neuroleukin, cyclophylin, CXCL7, connective tissue activation peptide III, and macrophage migration inhibitory factor (MIF) (47–50). PBEF has properties very similar to MIF, although it should be noted that IL-6 *trans*-signaling did not upregulate MIF in the mRNA cytokine array (Figure 1).

Like MIF, PBEF lacks a signal sequence (6,51), shows both nuclear and cytoplasmic immunolocalization (52,53), and bears structural and functional resemblance to previously characterized microbial enzymes (41–43,50). It has also been suggested that either a cofactor or protein modification may be required for activation of the catalytic activities of both PBEF (43) and MIF (50). Consequently, PBEF is associated with a number of factors known to act as mediators of inflammation during disease (11,54).

In conclusion, this study has demonstrated that IL-6 *trans*-signaling and the IL-6–related cytokine OSM regulate PBEF. The regulation of this protein in inflammatory arthritis suggests that PBEF actively contributes to the pathology observed; however, it remains to be determined how the high levels of PBEF affect disease progression clinically.

ACKNOWLEDGMENTS

We wish to acknowledge the rheumatologists and nursing staff in the South Wales area for facilitating the collection of clinical samples.

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