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Pre–B Cell Colony-Enhancing Factor/Visfatin, a New Marker of Inflammation in Rheumatoid Arthritis With Proinflammatory and Matrix-Degrading Activities

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***Objective.* To study possible mechanisms that mediate induction of the recently described adipocytokine pre–B cell colony-enhancing factor (PBEF) in joints of patients with rheumatoid arthritis (RA), and to analyze whether levels of PBEF correlate with disease severity and whether PBEF itself has the potential to act as a proinflammatory and destructive mediator in RA.**

***Methods.* RA synovial fibroblasts (RASFs) and monocytes were stimulated with Toll-like receptor (TLR) ligands, cytokines, and recombinant human PBEF or were transfected with PBEF expression constructs or with PBEF-specific small interfering RNA. Production of interleukin-6 (IL-6), IL-8, and tumor necrosis factor α (TNFα) was measured by enzyme-linked immunosorbent assay, and expression of matrix metalloproteinases (MMPs) was assessed by real-time polymerase chain reaction. PBEF expression in synovial tissue, synovial fluid, serum, and SFs was assessed by immunohistochemistry, in situ hybridization, Western blotting, and enzyme immunoassays.**

***Results.* In RASFs, PBEF was up-regulated by TLR ligands and cytokines that are characteristically present in the joints of patients with RA. In synovial tissue, RASFs were the major PBEF-expressing cells. A predominance of PBEF was found in the synovial lining layer and at sites of invasion into cartilage. Levels of PBEF in serum and synovial fluid correlated with the degree of inflammation and clinical disease activity. Moreover, PBEF itself activated the transcription factors NF-kB and activator protein 1 and induced IL-6, IL-8, MMP-1, and MMP-3 in RASFs as well as IL-6 and TNFα in monocytes. PBEF knockdown in RASFs significantly inhibited basal and TLR ligand–induced production of IL-6, IL-8, MMP-1, and MMP-3.**

***Conclusion.* Our findings establish PBEF as a proinflammatory and destructive mediator of joint inflammation in RA and identify PBEF as a potential therapeutic target.**

Rheumatoid arthritis (RA) is a chronic inflammatory disease that ultimately leads to the progressive destruction of joint cartilage and bone. Synovial fibroblasts (SFs) and inflammatory cells such as macrophages and T cells play key roles in this process. There is mounting evidence for an important function of innate immunity in the pathogenesis of RA (1). Activation of cells by microbial components and also by endogenous molecules via pattern recognition receptors, such as Toll-like receptors (TLRs), results in the production of a variety of cytokines, chemokines, and matrix metalloproteinases (MMPs), some of which are characteristically observed in patients with RA (2). We previously reported induction in SFs of the proinflammatory cytokine interleukin-6 (IL-6) and the chemokines IL-8, granulocyte chemotactic protein 2, macrophage chemotactic protein 2, and RANTES by the TLR-2 ligand bacterial peptidoglycan (3,4). In a subsequent study, we demonstrated overexpression of TLR-3 in RA synovial tissue and established that RNA released by necrotic synovial

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fluid cells can act as endogenous ligand for TLR-3 on cultured RASFs (5). In the current study, we performed subtractive hybridization experiments with untreated and poly(I-C)–treated RASFs to investigate novel TLR-3–dependent gene regulation. We observed that pre–B cell colony-enhancing factor (PBEF) was up-regulated by the TLR-3 ligand poly(I-C).

PBEF was originally cloned from a complementary DNA (cDNA) library of activated human peripheral blood mononuclear cells (PBMCs) and identified as a secreted protein that enhances the effect of stem cell factor and IL-7 on pre–B cell colony formation (6). Later, it became evident that PBEF is a multifunctional protein, having nicotinamide phosphoribosyltransferase, adipokine, and cytokine activities. In smooth muscle cells (SMCs), intracellularly located PBEF regulates NAD+-dependent reactions and promotes the acquisition of a mature SMC phenotype (7,8). Fukuhara et al reported the expression of PBEF in visceral fat adipocytes and noted that its insulin-like effect is dependent on binding to the insulin receptor (9). Because of its presence in visceral fat, PBEF is also referred to as visfatin. Furthermore, antiapoptotic effects of PBEF are documented in neutrophils (10). Regarding cytokine activities, PBEF induces IL-6 and IL-8 in amniotic cells (11), whereas down-regulation of PBEF results in inhibition of the thrombin-stimulated increase of IL-8 secretion in pulmonary artery endothelial cells (12).

Levels of PBEF in serum and synovial fluid are elevated in patients with RA (13,14). Furthermore, Nowell et al (13) demonstrated increased synovial expression of PBEF in antigen-induced arthritis in mice. The up-regulation of PBEF was shown to be regulated by IL-6 trans-signaling via STAT-3. However, the role of PBEF in joint inflammation remains to be determined.

We investigated whether PBEF is involved in the inflammatory and destructive processes in the rheumatoid joint. The expression of PBEF was examined in synovial tissue, serum, and synovial fluid obtained from patients with RA. We showed that levels of PBEF correlate with the degree of inflammation and clinical disease activity in patients with RA. Stimulation of RASFs and primary monocytes with recombinant human PBEF (rHuPBEF), as well as PBEF overexpression and knockdown experiments, revealed that PBEF acts as a proinflammatory mediator by triggering the release of cytokines, chemokines, and destructive enzymes that are characteristically observed in the inflamed joints of patients with RA. Thus, our findings indicate that PBEF is a marker of inflammation, and that PBEF itself promotes inflammatory and destructive processes in the joints of patients with RA.

PATIENTS AND METHODS

Patients and tissue preparation. Synovial tissue specimens were obtained during synovectomy or joint replacement surgery from patients with RA and patients with osteoarthritis (OA), after informed consent had been obtained (Department of Orthopedic Surgery, Schulthess Clinic, Zurich, Switzerland). RASFs and OASFs were isolated from synovial tissue, digested by collagenase, and used after passages 4–8, as previously described (5). To obtain tissue sections, synovial specimens were fixed in paraformaldehyde and embedded in paraffin. Sera and synovial fluid from patients with RA and patients with OA were collected, centrifuged, and stored at −80°C until analyzed. Before analysis, synovial fluid samples were pretreated for 1 hour at 37°C with 1 mg/ml of hyaluronidase (Fluka, Buchs, Switzerland). All patients with RA fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) criteria for the classification of RA (15).

Stimulation assays. RASFs and OASFs were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Basel, Switzerland) supplemented with 10% fetal calf serum (FCS) and stimulated with the following agents: poly(I-C) (20 μg/ml; Invitrogen, San Diego, CA), lipopolysaccharide (LPS) from *Escherichia coli* (100 ng/ml; List Biological Laboratories, Campbell, CA), palmitoyl-3-cysteine-serine-lysine-4 (bacterial lipoprotein [bLP]) (300 ng/ml; Invitrogen), IL-1β (1 ng/ml; R&D Systems, Abington, UK,) and TNFα (10 ng/ml; R&D Systems).

PBMCs were isolated by standard Ficoll density-gradient centrifugation from blood samples from healthy donors. CD14+ monocytes were separated using CD14 MACS MicroBeads according to the manufacturer's protocol (Miltenyi Biotec, Gladbach, Germany). The purity of the CD14+ cell fraction, as assessed by flow cytometry, was consistently >90%. CD14+ cells were cultured in RPMI 1640 supplemented with 5% FCS. RASFs and CD14+ cells were stimulated with rHuPBEF (Phoenix Pharmaceuticals, Belmont, CA) in the presence of polymyxin B sulfate (5 μg/ml) (Sigma, Basel, Switzerland).

Real-time polymerase chain reaction (PCR). Quantification of specific PBEF and MMP messenger RNA (mRNA) was performed by SYBR Green and TaqMan real-time PCR, respectively. The primer sequences used are as follows: for PBEF, forward 5′-AATACCCACCCAACACAAGC-3′, reverse 5′-TCACGGCATTCAAAGTAGGA-3′; for MMP-1, forward 5′-TGTGGACCATGCCATTGAGA-3′, reverse 5′-TCTGCTTGACCCTCAGAGACC-3′, probe 5′-AGCCTTCAAACTCTGGAGTAATGTCACACC-3′; for MMP-3, forward 5′-GGGCCATCAGAGGAAATGAG-3′, reverse 5′-CACGGTTGGAGGGAAACCTA-3′, probe 5′-AGCTGGATACCCAAGAGGCATCCACAC-3′. The endogenous control 18S cDNA was used for correcting the results with the comparative threshold cycle method for relative quantification, as described by the manufacturer.

In situ hybridization. PBEF sense and PBEF antisense probes for in situ hybridization were prepared according to

methods previously described (16). In situ hybridization was performed using the method described by Kriegsmann et al (17).

Immunohistochemical analysis. Synovial tissue sections were deparaffinized and pretreated with trypsin (1 mg/ml; Sigma). After blocking endogenous peroxidase and nonspecific binding, slides were incubated overnight at 4°C with anti-human PBEF antibodies (5 μg/ml; Bethyl Laboratories, Montgomery, TX). Sections were then incubated with biotinylated goat anti-rabbit IgG (Jackson ImmunoResearch, Soham, UK) in Tris buffered saline with 3% bovine serum albumin for 30 minutes at room temperature, followed by incubation for 30 minutes with horseradish peroxidase (HRP)–conjugated streptavidin complex at room temperature (ABC kit; Vector, Peterborough, UK). HRP-labeled cells were visualized using aminoethylcarbazole substrate–chromogen (Dako, Glostrup, Denmark). Nuclei were counterstained with hematoxylin. To identify subsets of synovial cells expressing PBEF, slides were additionally incubated with monoclonal mouse anti-human CD68 or anti-human vimentin antibodies (2 μg/ml; Dako), respectively. Bound mouse primary antibodies were detected using alkaline phosphatase–conjugated goat anti-mouse IgG antibodies (Jackson ImmunoResearch). Alkaline phosphatase–labeled cells were visualized using Fast Blue B reagent. In control experiments, rabbit IgG and isotype-matched mouse IgG were used instead of the primary antibodies.

Western blotting. *Protein preparation from supernatants.* Cultured SFs were grown in T75 culture flasks (7×10^5 cells/flask) in DMEM supplemented with 0.5% FCS and subsequently were stimulated for 24 hours with poly(I-C) or were left untreated. Supernatants were collected and concentrated using an Amicon Ultra-15 centrifugal filter device (Millipore, Billerica, MA).

Protein preparation from tissue. Protein was extracted by resuspending the crushed snap-frozen tissue in extraction buffer, with subsequent aceton precipitation overnight at −20°C. Proteins were separated on 10% sodium dodecyl sulfate–polyacrylamide gels and blotted on Protran nitrocellulose transfer membranes (Schleicher & Schuell, Dassel, Germany). Membranes were probed with anti-PBEF antibodies (0.5 μg/ml; Bethyl Laboratories) and detected with HRP-conjugated secondary antibodies, using an enhanced chemiluminescence Western blotting detection system (Amersham Pharmacia Biotech, Little Chalfont, UK). Blots were stripped and reprobed with monoclonal mouse anti-human α-tubulin antibodies (Sigma) to confirm similar loading of the gels.

Construction of PBEF plasmids. Total RNA from RASFs was extracted, and 1 μg RNA was transcribed to first-strand cDNA using the Moloney murine leukemia virus reverse transcriptase system (Invitrogen). The cDNA was amplified with the upstream primer, 5′-GCGGGATCCGCGATGATGTGCTGCTTCCAGTTC-3′, containing a *Bam* H1 restriction site and a Kozak sequence for eukaryotic translation. The downstream primer was 5′-CCGCTCGAGCGGCCCGAGATGAATCCTGCG-3′, containing an *Xho* I restriction site. Full-length PBEF fragments, including the signal sequence and fragments encoding the mature PBEF peptide sequence, were cloned into pcDNA3.1/myc-His vector. The recombinant plasmids were transfected into DH5α-competent cells (Invitrogen), and colonies were identified by restriction enzyme digestion and sequencing.

Transfection of PBEF small interfering RNA (siRNA) into RASFs. PBEF stealth siRNAs were designed based on the human PBEF cDNA reference sequence (NM_005746.1), using the BLOCK-iT RNAi Designer (Invitrogen). Stealth106 siRNA PBEF 5′-AAUAAACUUUGCUUGUGUUGGGUGG-3′ and stealth 106 scrambled PBEF 5′-CCACAACAACAAACGUUGAUCCAUU-3′ were used. One day before transfection, RASFs were plated in DMEM/10% FCS without antibiotics, in a 24-well plate (30,000 RASFs per well). For each transfection, 50 n*M* PBEF stealth siRNA was diluted in 50 μl Opti-MEM I (Invitrogen) without serum and mixed with 1 μl Lipofectamine 2000 in 50 μl Opti-MEM I. After incubation for 20 minutes at room temperature, PBEF stealth siRNA–Lipofectamine 2000 complexes were added to each well. Transfected cells were further incubated at 37°C for 48 hours before culture medium was replaced and RASFs were used for further experiments.

Enzyme-linked immunoabsorbant assay (ELISA) and enzyme immunoassay (EIA). IL-6 and TNFα proteins were detected by ELISA with the OptEIA Kit (BD PharMingen, San Diego, CA), and PBEF/visfatin protein was detected using a human EIA kit (Phoenix Pharmaceuticals), according to the manufacturer's instructions. Absorption was measured at 450 nm, and data were analyzed using Revelation version 4.22 software (Dynex Technologies, Denkendorf, Germany).

Electrophoretic mobility shift assay (EMSA). For EMSA, RASFs were cultured to 80–90% confluency in culture flasks (75 cm^2) and incubated with 100 ng/ml of rHuPBEF. Cells were collected by scratching in ice-cold phosphate buffered saline, at different time points (0, 10, 30, and 90 minutes after stimulation). DNA-binding proteins were extracted from RASFs according to the method described by Andrews and Faller, which utilizes hypotonic lysis followed by high-salt extraction of nuclei (18). The binding EMSA was carried out using a Panomics EMSA Gel Shift kit, according to the manufacturer's instructions (Panomics, Redwood City, CA).

Statistical analysis. The Mann-Whitney U test, Wilcoxon's test, and the nonparametric Spearman's correlation were used, as appropriate, for statistical evaluation of the data by SPSS software (SPSS, Chicago, IL). *P* values less than 0.05 were considered significant.

RESULTS

Induction of PBEF in synovial fibroblasts by TLR ligands and inflammatory cytokines. In order to investigate novel TLR-3–dependent gene regulation, we performed subtractive hybridization between cDNA prepared from poly(I-C)–stimulated and unstimulated RASFs. PBEF was one of the transcripts shown to be up-regulated by TLR-3 activation. The induction of PBEF mRNA in RASFs was validated at various time points after poly(I-C) treatment, by real-time PCR (Fig-

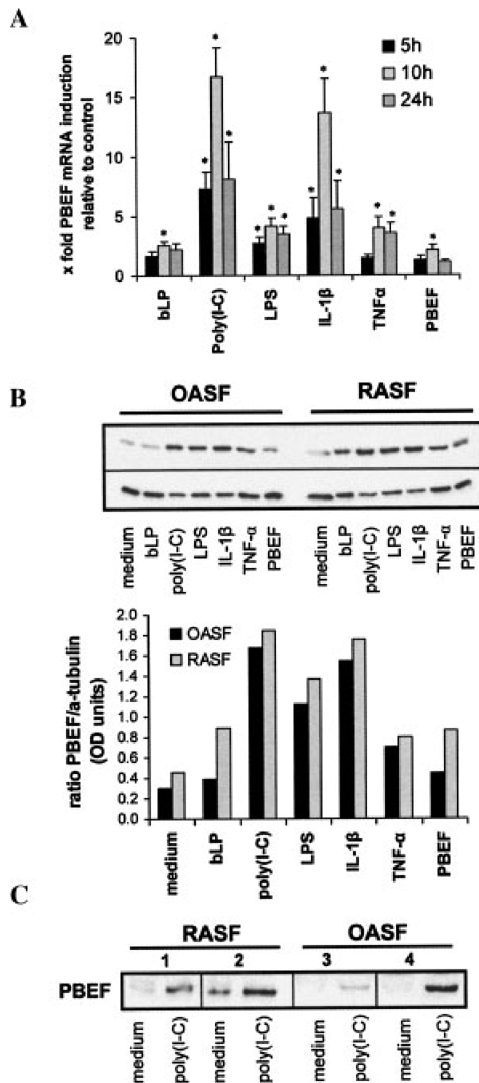

Figure 1. Pre–B cell colony-enhancing factor (PBEF) induction in rheumatoid arthritis synovial fibroblasts (RASFs) by Toll-like receptor (TLR) ligands and cytokines. **A,** RASFs (n = 5) were treated for 5, 10, and 24 hours with the indicated stimuli or were left untreated. Bars show the mean and SEM. $* = P < 0.05$, treated versus untreated cultures. **B,** Western blot analysis of PBEF expression in RASF and osteoarthritis SF (OASF) cell lysates, 24 hours following stimulation with the indicated TLR ligands and cytokines (α-tubulin served as a loading control). Protein levels were evaluated using densitometry. Results are representative of 3 individual experiments. **C,** Western blot analysis of secreted PBEF protein in supernatants of RASFs (n = 2) and OASFs (n = 2) stimulated for 24 hours with poly(I-C). bLP = bacterial lipoprotein; LPS = lipopolysaccharide; IL-1β = interleukin-1β; TNFα = tumor necrosis factor α; OD = optical density.

ure 1A). The expression of PBEF was significantly up-regulated after 5, 10, and 24 hours of poly(I-C) stimulation, with a peak at 10 hours after stimulation. To investigate the regulation of PBEF expression by other TLR ligands and proinflammatory cytokines present in RA synovial fluid, SFs were additionally treated with the TLR-2 ligand bLP, the TLR-4 ligand LPS, IL-1β, TNFα at optimal concentrations, and PBEF itself. IL-1β potently up-regulated PBEF mRNA, to levels similar to those reached with poly(I-C) stimulation. The stimulatory effects of TNFα, LPS, bLP, and PBEF on the expression of PBEF mRNA were less prominent but were significant 10 hours following stimulation.

To analyze the expression of PBEF at the protein level, cell lysates from RASFs and OASFs treated with the indicated stimuli were subjected to Western blot analysis (Figure 1B). PBEF was found to be constitutively expressed by SFs, and its expression was further increased following stimulation with bLP, poly(I-C), LPS, TNF, IL-1β, or rHuPBEF. Additionally, we observed a tendency toward higher PBEF levels in RASFs compared with OASFs.

In previous studies, PBEF was shown to be a secreted protein, despite the lack of the typical signal peptide that is common to other secreted proteins (10). To analyze whether SFs have the potential to secrete PBEF protein as a cytokine, we performed Western blot analyses for PBEF using supernatants of unstimulated and poly(I-C)–stimulated RASFs and OASFs. Cultured RASFs released PBEF protein constitutively, as shown in the supernatants of unstimulated RASFs (Figure 1C). In the supernatants of unstimulated OASFs, PBEF protein was not detectable. However, in response to stimulation with poly(I-C), both RASFs and OASFs clearly showed up-regulated secretion of PBEF. Collectively, the results indicate that SFs secrete PBEF protein, and that the production of PBEF is up-regulated by TLR ligands, most notably by poly(I-C), as well as by the cytokines IL-1β and TNFα.

Predominant expression of PBEF in RASFs in the synovial lining and at sites of invasion. Next, we analyzed the expression of PBEF in synovial tissue from patients with RA. In situ hybridization revealed pronounced expression of PBEF mRNA predominantly in the synovial lining and at sites of attachment and invasion of RASFs into cartilage or bone (Figure 2A). The expression of PBEF protein in RA synovia was also confirmed by immunohistochemistry, documenting abundant expression of PBEF in RA synovium (Figure 2B). PBEF expression was found to be highest at sites of invasion and in the synovial lining layer, and it was detected to a lesser extent in the sublining and perivascular regions. Double-labeling revealed that PBEF

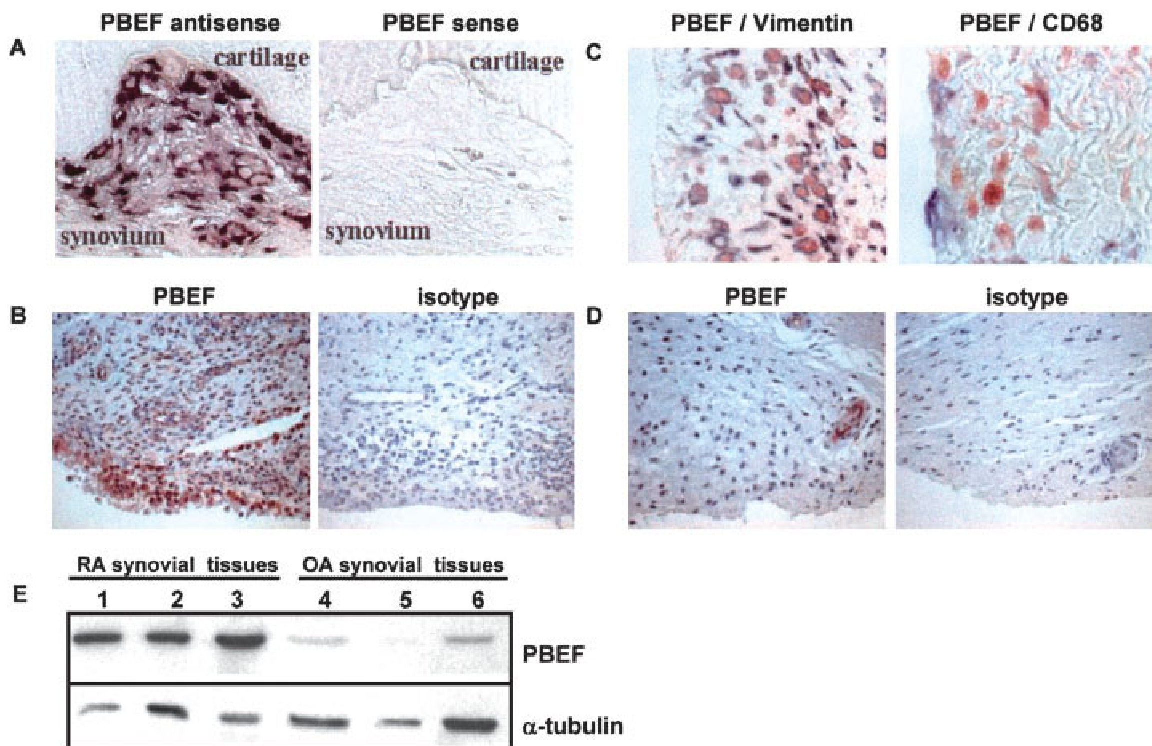

Figure 2. PBEF overexpression in synovial tissue samples from patients with RA. **A,** Representative section of RA synovial tissue specimens (n = 5) hybridized in situ with specific antisense RNA probes for PBEF mRNA. As negative control, tissue sections were hybridized with the sense probes. Cells expressing PBEF mRNA appear as dark blue. **B,** Representative section of RA synovial tissue (n = 10) stained for PBEF protein, with the corresponding tissue sections stained with isotype antibodies. PBEF protein appears as red. Nuclei were stained with hematoxylin. **C,** Double-labeling in RA synovial tissue. PBEF appears as red, and vimentin and CD68 appear as blue. **D,** Representative section of OA synovial tissue stained for PBEF (n = 5). Corresponding tissue sections were stained with isotype control antibodies. PBEF protein appears as red. Nuclei were stained with hematoxylin. **E,** Western blot showing the expression of PBEF protein in synovial tissue samples obtained from 3 patients with RA and 3 patients with OA; α-tubulin served as a loading control. (Original magnification $\times$ 400 in **A** and **C**; $\times$ 100 in **B** and **D**.) See Figure 1 for definitions.

was more frequently expressed in vimentin-positive SFs than in CD68-positive monocyte/macrophages (Figure 2C).

To study the association of PBEF levels with chronic joint inflammation, we compared the expression of PBEF protein in RA and noninflammatory OA synovial tissue. Analysis of OA synovial tissue sections revealed reduced expression of PBEF protein compared with that in RA synovial tissue, with PBEF being mainly expressed around small vessels (Figure 2D). Western blot analysis of total protein extracted from synovial tissue from 3 individual patients with RA and 3 patients with OA confirmed increased expression of PBEF protein in synovial tissue from the joints of patients with RA compared with patients with OA (Figure 2E).

Positive correlation of PBEF with the C-reactive protein (CRP) level and clinical disease activity in patients with RA. Because of the abundant expression of PBEF in the joints of patients with RA compared with that in the joints of patients with OA, we investigated whether serum and synovial fluid levels of PBEF might also reflect the severity of inflammation. Levels of PBEF were significantly higher in serum and synovial fluid samples from patients with RA compared with those in samples from patients with OA (Figure 3A). However, high variability in PBEF levels was observed among individual patients with RA. Using correlation analysis with the CRP level and PBEF, we analyzed whether these varying levels of PBEF might be associated with the degree of inflammation (Figure 3B). A significant

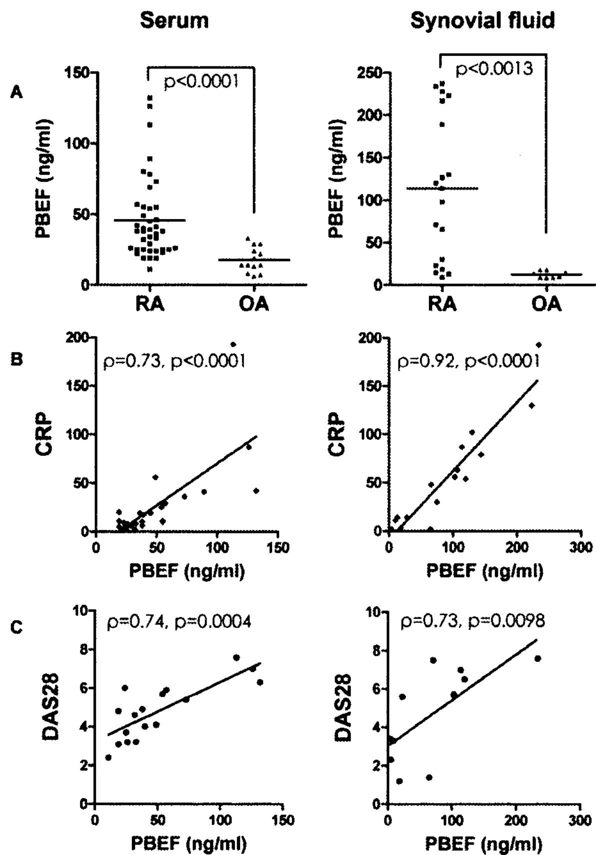

Figure 3. Correlation of elevated PBEF levels and disease severity in patients with RA. **A,** Serum and synovial fluid levels of PBEF in patients with RA and patients with OA, as measured by enzyme immunoassay. Horizontal bars show the means. **B,** Significant correlation of C-reactive protein (CRP) levels and PBEF concentrations in serum and synovial fluid from patients with RA. **C,** Significant correlation of the Disease Activity Score in 28 joints (DAS28) and PBEF concentrations in serum and synovial fluid from patients with RA. See Figure 1 for other definitions.

positive correlation between the CRP level and PBEF in serum and in synovial fluid samples was observed. Additionally, the levels of PBEF in serum and synovial fluid and the Disease Activity Score in 28 joints (19) in individual patients with RA also showed a significant positive correlation (Figure 3C). Therefore, our results demonstrated that PBEF is associated with serum markers of inflammation as well as clinical disease activity in RA.

PBEF-induced production of IL-6, MMP-1, and MMP-3 in RASFs. To investigate the functional role of PBEF secreted in joints of patients with RA, RASFs were stimulated with increasing amounts of rHuPBEF for 24 hours. Recombinant human PBEF induced a dose-dependent increase in the levels of IL-6, MMP-1, and MMP-3. Already after stimulation with physiologic concentrations of rHuPBEF (50–200 ng/ml), IL-6 production was significantly up-regulated (Figure 4A). Similarly, RASFs treated with rHuPBEF showed significantly up-regulated expression of MMP-1 and MMP-3 mRNA upon incubation with 100 and 200 ng/ml of rHuPBEF (Figure 4B). To ascertain that the stimulatory effect was not attributable to contamination with endotoxin, RASFs were cultured in the presence of polymyxin. Polymyxin neutralized the stimulatory effect of 10 ng/ml of LPS, whereas the effect of PBEF on IL-6, MMP-1, and MMP-3 production remained unchanged (data not shown).

We additionally analyzed whether comparable results could be obtained by overexpressing PBEF in RASFs, using a eukaryotic expression vector. When a pcDNA3.1/PBEF/His expression construct was transfected into RASFs, the resulting protein could be demonstrated in the cell lysate by Western blotting (Figure 4C). Supernatants from RASFs obtained 48 hours after transfection with the PBEF/His construct showed significantly higher levels of IL-6 compared with that in RASFs transfected with the empty pcDNA3.1/His vector (Figure 4D). Additionally, levels of MMP-1 and MMP-3 were significantly higher in PBEF/His vector–transfected RASFs compared with those in controls (Figure 4E). These results demonstrated that PBEF induces proinflammatory cytokines and destructive enzymes in RASFs.

Reduction of basal and TLR ligand–induced IL-6, MMP-1, and MMP-3 levels by PBEF knockdown. PBEF is expressed in unstimulated RASFs, and we demonstrated that PBEF regulates IL-6, MMP-1, and MMP-3 production. Therefore, we investigated whether PBEF knockdown inhibits the characteristically high basal production of IL-6, MMP-1, or MMP-3 in RASFs. For this purpose, RASFs were transfected with PBEF-specific siRNA (siPBEF) or with nonspecific scrambled siRNA (scrambled) as a control. To ensure that responses to siPBEF transfection reflected PBEF knockdown, PBEF protein in cell lysates was quantified 72 hours after transfection. In 3 different RASF cultures analyzed, the inhibition of PBEF by specific siRNA was 69%, 59%, and 74%, respectively, as compared with that in controls (Figure 5A). Basal secretion of IL-6 was measured in the culture supernatants of siRNA-transfected RASFs (n = 6), 4, 6, and 24 hours after medium replacement. The basal production of IL-6 was

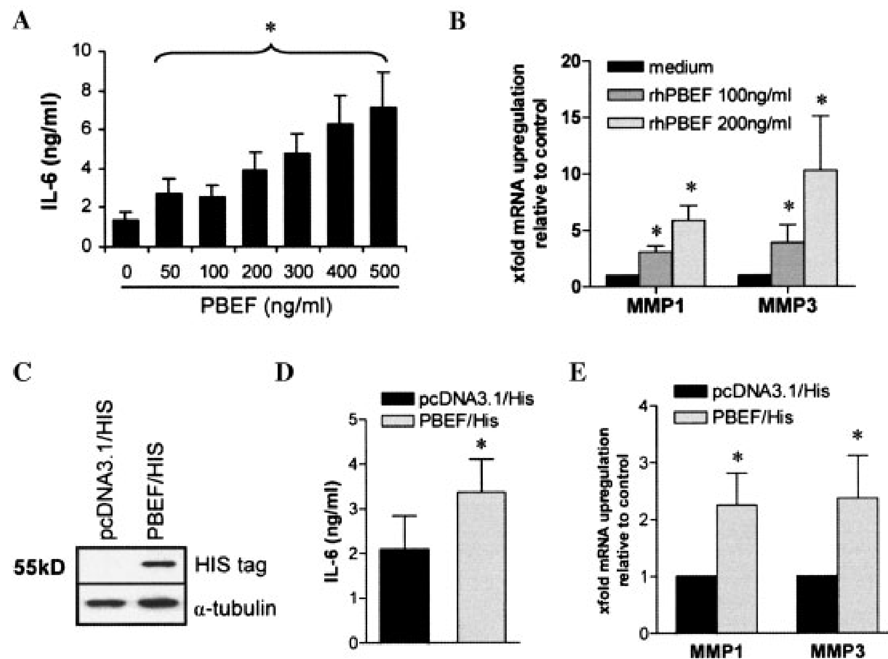

Figure 4. Up-regulation of proinflammatory cytokines and matrix-degrading enzymes in RASFs by recombinant human PBEF (rhPBEF) and PBEF overexpression. **A,** RASF cultures (n = 5) were incubated with the indicated concentrations of rhPBEF. Twenty-four hours following stimulation, levels of IL-6 were measured by enzyme-linked immunosorbent assay (ELISA). **B,** RASF cultures (n = 5) were incubated with 100 ng/ml and 200 ng/ml of rhPBEF. Up-regulation of matrix metalloproteinase 1 (MMP-1) and MMP-3 mRNA was determined by real-time polymerase chain reaction. **C,** Western blot showing detection of PBEF with anti-His antibody at 55 kd in cell lysates from PBEF/His–transfected RASFs, but not from RASFs transfected with plasmid containing His alone (pcDNA3.1/His). **D,** Levels of IL-6 in supernatants (as measured by ELISA), following 24-hour incubation of RASF cultures overexpressing PBEF (n = 5). **E,** Up-regulation of MMP in RASF cultures overexpressing PBEF (n = 5) compared with that in mock-transfected RASFs (as measured by real-time polymerase chain reaction), following incubation for 24 hours. Bars show the mean and SEM. * = $P < 0.05$ versus control cultures. See Figure 1 for other definitions.

significantly lower in RASFs with down-regulated PBEF compared with controls at all time points analyzed. The most pronounced effect was seen 6 hours following medium replacement, with a mean ± SEM inhibition of 48.8 ± 7.2% (Figure 5B). Additionally, basal levels of MMP-1 and MMP-3 mRNA were down-regulated 4.7 ± 1.6–fold and 2.8 ± 0.6–fold, respectively, 48 hours following siPBEF transfection (Figure 5C).

In previous studies, we demonstrated that TLR-2, TLR-3, and TLR-4 ligands induce high amounts of cytokines and matrix-degrading enzymes in RASFs (3,5,20). In order to investigate whether PBEF is involved in the up-regulation of these effector molecules, we treated siPBEF-transfected RASFs with bLP, poly(I-C), and LPS, and analyzed the induction of IL-6, IL-8, MMP-1, and MMP-3, 24 hours after TLR ligand stimulation. PBEF knockdown significantly inhibited the up-regulation of all measured effector molecules (Figures 5D and E). These data indicated that PBEF is implicated in basal as well as TLR ligand–induced production of proinflammatory cytokines and matrix-degrading enzymes.

PBEF induction of TNFα and IL-6 in primary human monocytes. It has been demonstrated that primary blood monocytes express PBEF (10). However, it has not been determined whether monocytes are responsive to PBEF. Because we observed high levels of PBEF in serum obtained from patients with RA, we analyzed whether human primary blood monocytes are activated either by rHuPBEF or by overexpression of PBEF. Monocyte cultures treated with 50 ng/ml of rHuPBEF, a concentration that corresponds to the level of PBEF in

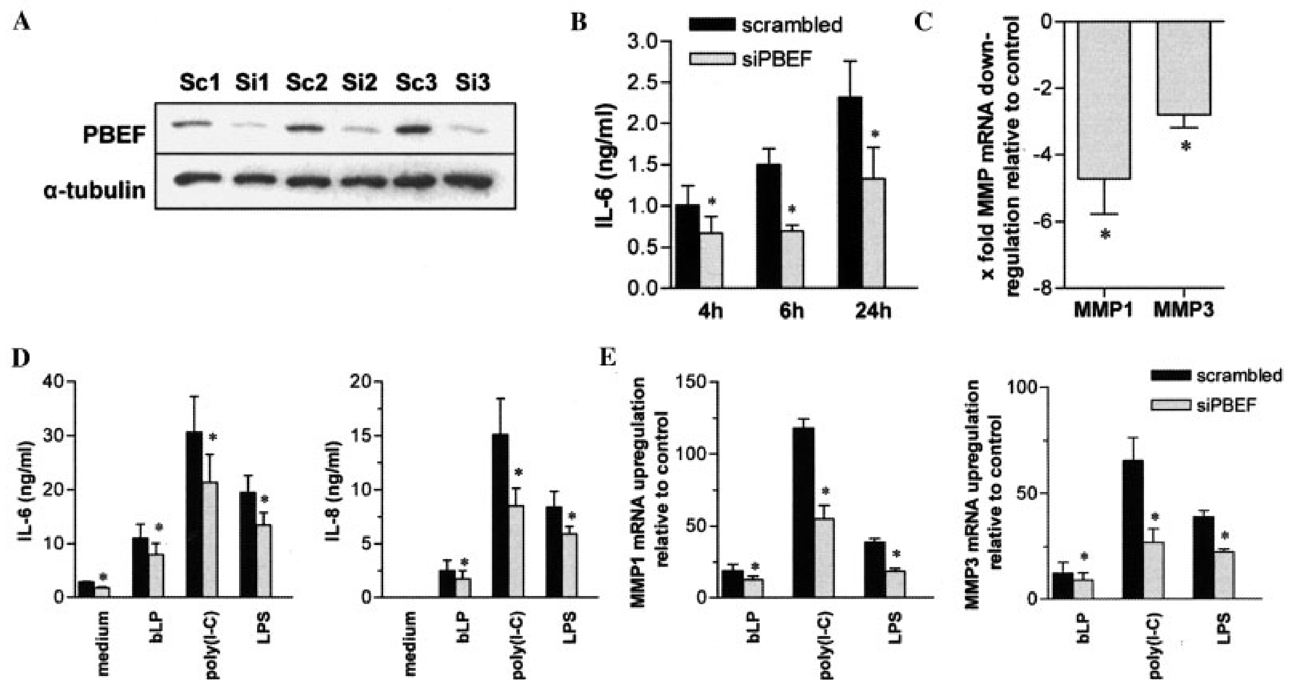

Figure 5. Inhibition of basal and TLR ligand–induced cytokine and matrix metalloproteinase (MMP) production by PBEF knockdown. **A,** RASFs transfected with PBEF-specific small interfering RNA (siRNA; Si1–3) and nonspecific scrambled siRNA (Sc1–3) were subjected to Western blot analysis for PBEF; α-tubulin served as a loading control. **B,** RASF cultures (n = 5) with down-regulated PBEF and corresponding control cultures were incubated for 4, 6, and 24 hours, followed by measurement of IL-6 in the culture supernatant. **C,** MMP-1 and MMP-3 mRNA up-regulation in siPBEF-transfected RASFs compared with scrambled siRNA–transfected RASFs was measured by real-time polymerase chain reaction (n = 5 cultures for 5 different patients). **D,** RASFs with down-regulated PBEF and corresponding controls were stimulated with the indicated TLR ligands for 24 hours, and levels of IL-6 and IL-8 were determined in the culture supernatants. **E,** RASFs with down-regulated PBEF and corresponding controls were stimulated with the indicated TLR ligands for 24 hours. MMP-1 and MMP-3 mRNA up-regulation in siPBEF- and scrambled siRNA–transfected RASFs compared with untreated RASFs is shown. Bars show the mean and SEM. * = $P < 0.05$ versus control-transfected RASFs. See Figure 1 for other definitions.

serum from patients with RA, showed significantly higher production of IL-6 and TNFα as compared with that in untreated cultures. The stimulatory effect of rHuPBEF on monocytes was dose dependent (Figures 6A and B). Similarly, overexpression of PBEF in monocytes resulted in a 5.0 ± 1.5–fold increase in TNFα secretion (Figure 6C). Thus, PBEF is a potent activator of human monocytes, inducing the production of key proinflammatory cytokines such as IL-6 and TNFα.

PBEF stimulation of RASFs via NF-κB and AP-1. Activation of the transcription factors NF-κB and AP-1 is a principal step in the initiation and maintenance of inflammatory responses. Therefore, we studied whether rHuPBEF activates NF-κB and AP-1 signaling pathways in RASFs. Nuclear extracts of RASFs stimulated with rHuPBEF (100 ng/ml) were subjected to EMSA. Ninety minutes following stimulation, the activation of RASFs resulted in translocation of NF-κB and AP-1 from the cytoplasm to the nucleus (data not shown). Therefore, PBEF has the capacity to activate 2 major transcription factors, NF-κB and AP-1.

DISCUSSION

In the present study, we demonstrated induction of the adipocytokine PBEF in RASFs via TLR stimulation as well as by stimulation with IL-1β and TNFα. Furthermore, we observed potent proinflammatory and matrix-degrading activities of PBEF and showed that in patients with RA, levels of PBEF correlate with the severity of inflammation.

Using a subtractive hybridization assay of RASFs stimulated with poly(I-C), we found PBEF to be up-regulated. However, PBEF induction in RASFs is not restricted to TLR-3 activation, as shown by stimulation experiments with other TLR ligands and proinflamma-

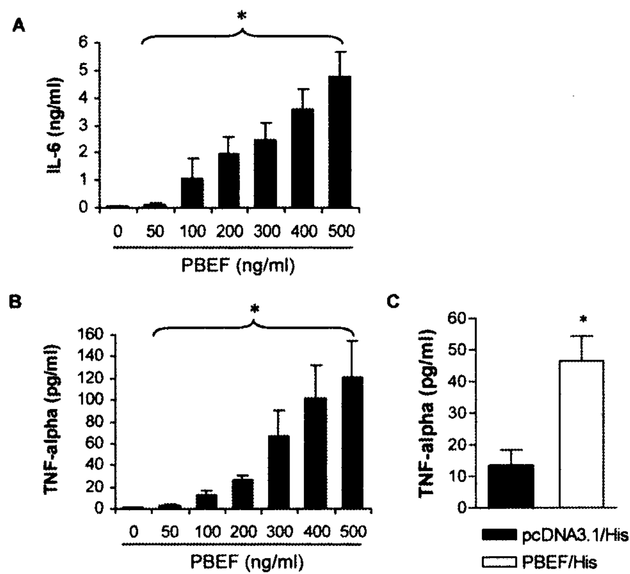

Figure 6. Up-regulation of key proinflammatory cytokines in human primary monocytes by recombinant human pre–B cell colony-enhancing factor (rHuPBEF) and PBEF overexpression. Monocytes were incubated with the indicated concentrations of exogenously added rHuPBEF (n = 5 cultures for 5 different patients). **A** and **B,** Twenty-four hours following stimulation, levels of interleukin-6 (IL-6) and tumor necrosis factor α (TNFα) were measured in the culture supernatants. **C,** Monocytes transfected with PBEF/His expression constructs and monocytes transfected with plasmids containing His alone (pcDNA3.1/His) were incubated for 24 hours. Levels of TNFα were measured in the culture supernatants (n = 5). Bars show the mean and SEM. $* = P < 0.05$ versus control cultures.

tory cytokines that can be found in the joints of patients with RA (1). Interestingly, PBEF itself induces its own production, indicating the existence of a positive feedback-regulating mechanism. Expression and up-regulation of human PBEF has previously been documented in neutrophils by IL-1β and LPS, in amniotic cells by TNFα, in monocytic cells by nitric oxide, in adipocytes by hypoxia, and in RASFs by IL-6 trans-signaling (10,11,13,21–23). Taken together, these data show that signaling pathways of the innate immune system have a strong regulatory effect on the expression of PBEF in a variety of cell types.

Our study demonstrates accumulation of PBEF in the joints of patients with RA and identifies SFs as the major PBEF-producing cells in the rheumatoid synovium. PBEF expression is predominantly localized at the site of invasion into cartilage and in the synovial lining. This staining pattern may reflect the local availability of PBEF-stimulating agents such as cytokines and TLR ligands in the lining layer. Additionally, hypoxic conditions that are most marked at the site of invasion into cartilage may drive local PBEF expression, as has been shown previously in adipocytes (22).

It is known that IL-6 exerts stimulatory effects on T cells and B cells, thus favoring chronic inflammatory responses, whereas MMPs have been closely linked to the progressive destruction of articular cartilage in rheumatoid joints. A hallmark of RASFs is the high basal production of IL-6 and MMPs (24). We showed that PBEF has an important role in the regulation of these key proinflammatory and matrix-degrading molecules. Recombinant human PBEF induces the expression of IL-6 and MMPs in RASFs, suggesting that secreted PBEF contributes to the local inflammatory and destructive processes in arthritic joints. Moreover, in our experiments, down-regulation of PBEF in RASFs by siRNA not only decreased basal IL-6, MMP-1, and MMP-3 levels but also significantly inhibited TLR ligand–induced production of cytokines and destructive enzymes. TLRs were shown to be key players in inflammatory and destructive processes in RA (3,4,20,25). TLR ligands of microbial origin as well as endogenous TLR ligands were demonstrated to be present in RA synovial fluid as possible drivers of inflammatory processes (1,26). Our findings suggest that the stimulation of RASFs by TLR ligands is at least partially dependent on PBEF expression. Therefore, targeting PBEF not only might counteract its direct stimulatory effect on RASFs but also reduces TLR-driven proinflammatory and destructive responses.

Peripheral blood monocytes have been shown to be a source of PBEF in the blood circulation. Our study shows that primary human monocytes are responsive to PBEF, suggesting that PBEF acts in an autocrine manner to increase serum levels. Notably, activation of monocytes by PBEF results in the production TNFα, a key cytokine in the pathogenesis of RA (27). Moreover, PBEF present in synovial fluid might trigger chronic inflammation not only via induction of proinflammatory cytokine production by RASFs but also by a direct antiapoptotic effect on neutrophils (10). Neutrophils are abundant in the synovial fluid of patients with RA and are less susceptible to TNFα-induced apoptosis than are blood neutrophils. In this regard, it has been shown that synovial fluid from patients with RA exhibits an antiapoptotic effect on neutrophils (28).

The possible mechanisms by which PBEF exerts its proinflammatory effects in the arthritic joint are incompletely understood. The identification of PBEF in

the visceral fat added PBEF to a growing list of adipocytokines with potent effects on immunity and inflammation in addition to their metabolic activity (9). Leptin, adiponectin, resistin, and most recently PBEF have been shown to be up-regulated in patients with RA compared with healthy control subjects (14,29,30). PBEF mimics insulin signaling by binding to the insulin receptor with an affinity similar to that of insulin but does not share the binding site with insulin on the insulin receptor. In contrast, previous studies have shown intracellular expression of PBEF and have demonstrated that PBEF is a nicotinamide phosphoribosyltransferase (7,8). Overexpression of PBEF in human vascular SMCs induced enhanced survival by its regulatory effect on NAD-dependent deacetylase activity. Moreover, it has been shown that subcellular localization of PBEF is dependent on the cell cycle, suggesting a role for PBEF in cell cycle regulation (31). Using immunohistochemical analysis, we observed nuclear and cytoplasmic expression of PBEF. Whether the effects provoked by PBEF are dependent on binding to the insulin receptor or an as yet unknown receptor, or alternatively by its enzymatic activity, needs to be determined in further studies.

Elevated levels of PBEF in serum and synovial fluid from patients with RA have been observed in this and previous studies (13,14). Our new finding of a strong correlation of PBEF with markers of inflammation such as the CRP level provides support for an important role of this cytokine in inflammatory reactions. This is further underscored by the correlation of PBEF concentrations with scores for clinical disease activity (comprising the tender and swollen joint counts and the erythrocyte sedimentation rate) (32). These findings suggest that PBEF is a marker of the severity of inflammation in patients with RA.

Taken together, our results suggest that PBEF plays a key role as a mediator of innate immune pathways in chronic synovial inflammation and joint destruction, and identify this adipocytokine as a possible therapeutic target for the treatment of RA.

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AUTHOR CONTRIBUTIONS

Dr. Kyburz had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Brentano, Kyburz.
Acquisition of data. Brentano, Schorr, Ospelt.
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REFERENCES

1. Brentano F, Kyburz D, Schorr O, Gay R, Gay S. The role of Toll-like receptor signalling in the pathogenesis of arthritis. Cell Immunol 2005;233:90–6.
2. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nat Immunol 2004;5:987–95.
3. Kyburz D, Rethage J, Seibl R, Lauener R, Gay RE, Carson DA, et al. Bacterial peptidoglycans but not CpG oligodeoxynucleotides activate synovial fibroblasts by Toll-like receptor signaling. Arthritis Rheum 2003;48:642–50.
4. Pierer M, Rethage J, Seibl R, Lauener R, Brentano F, Wagner U, et al. Chemokine secretion of rheumatoid arthritis synovial fibroblasts stimulated by Toll-like receptor 2 ligands. J Immunol 2004;172:1256–65.
5. Brentano F, Schorr O, Gay RE, Gay S, Kyburz D. RNA released from necrotic synovial fluid cells activates rheumatoid arthritis synovial fibroblasts via Toll-like receptor 3. Arthritis Rheum 2005;52:2656–65.
6. Samal B, Sun Y, Stearns G, Xie C, Suggs S, McNiece I. Cloning and characterization of the cDNA encoding a novel human pre-B-cell colony-enhancing factor. Mol Cell Biol 1994;14:1431–7.
7. Rongvaux A, Shea RJ, Mulks MH, Gigot D, Urbain J, Leo O, et al. Pre-B-cell colony-enhancing factor, whose expression is up-regulated in activated lymphocytes, is a nicotinamide phosphoribosyltransferase, a cytosolic enzyme involved in NAD biosynthesis. Eur J Immunol 2002;32:3225–34.
8. Van der Veer E, Nong Z, O'Neil C, Urquhart B, Freeman D, Pickering JG. Pre-B-cell colony-enhancing factor regulates NAD+-dependent protein deacetylase activity and promotes vascular smooth muscle cell maturation. Circ Res 2005;97:25–34.
9. Fukuhara A, Matsuda M, Nishizawa M, Segawa K, Tanaka M, Kishimoto K, et al. Visfatin: a protein secreted by visceral fat that mimics the effects of insulin. Science 2005;307:426–30.
10. Jia SH, Li Y, Parodo J, Kapus A, Fan L, Rotstein OD, et al. Pre-B cell colony-enhancing factor inhibits neutrophil apoptosis in experimental inflammation and clinical sepsis. J Clin Invest 2004; 113:1318–27.
11. Ognjanovic S, Bryant-Greenwood GD. Pre-B-cell colony-enhancing factor, a novel cytokine of human fetal membranes. Am J Obstet Gynecol 2002;187:1051–8.
12. Ye SQ, Zhang LQ, Adyshev D, Usatyuk PV, Garcia AN, Lavoie TL, et al. Pre-B-cell-colony-enhancing factor is critically involved in thrombin-induced lung endothelial cell barrier dysregulation. Microvasc Res 2005;70:142–51.
13. Nowell MA, Richards PJ, Fielding CA, Ognjanovic S, Topley N, Williams AS, et al. Regulation of pre–B cell colony-enhancing factor by STAT-3–dependent interleukin-6 trans-signaling: implications in the pathogenesis of rheumatoid arthritis. Arthritis Rheum 2006;54:2084–95.
14. Otero M, Lago R, Gomez R, Lago F, Dieguez C, Gomez-Reino JJ, et al. Changes in plasma levels of fat-derived hormones adiponectin, leptin, resistin and visfatin in patients with rheumatoid arthritis. Ann Rheum Dis 2006;65:1198–201.

15. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315–24.
16. Pap T, Franz JK, Hummel KM, Jeisy E, Gay R, Gay S. Activation of synovial fibroblasts in rheumatoid arthritis: lack of expression of the tumour suppressor PTEN at sites of invasive growth and destruction. Arthritis Res 2000;2:59–64.
17. Kriegsmann J, Keyszer GM, Geiler T, Brauer R, Gay RE, Gay S. Expression of vascular cell adhesion molecule-1 mRNA and protein in rheumatoid synovium demonstrated by in situ hybridization and immunohistochemistry. Lab Invest 1995;72:209–14.
18. Andrews NC, Faller DV. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. Nucleic Acids Res 1991;19:2499.
19. Prevoo ML, van 't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LB, van Riel PL. Modified disease activity scores that include twenty-eight–joint counts: development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. Arthritis Rheum 1995;38:44–8.
20. Seibl R, Birchler T, Loeliger S, Hossle JP, Gay RE, Saurenmann T, et al. Expression and regulation of Toll-like receptor 2 in rheumatoid arthritis synovium. Am J Pathol 2003;162:1221–7.
21. Turpaev K, Bouton C, Diet A, Glatigny A, Drapier JC. Analysis of differentially expressed genes in nitric oxide-exposed human monocytic cells. Free Radic Biol Med 2005;38:1392–400.
22. Segawa K, Fukuhara A, Hosogai N, Morita K, Okuno Y, Tanaka M, et al. Visfatin in adipocytes is upregulated by hypoxia through HIF1α-dependent mechanism. Biochem Biophys Res Commun 2006;349:875–82.
23. Bae SK, Kim SR, Kim JG, Kim JY, Koo TH, Jang HO, et al. Hypoxic induction of human visfatin gene is directly mediated by hypoxia-inducible factor-1. FEBS Lett 2006;580:4105–13.
24. Ospelt C, Neidhart M, Gay RE, Gay S. Synovial activation in rheumatoid arthritis. Front Biosci 2004;9:2323–34.
25. Roelofs MF, Joosten LA, Abdollahi-Roodsaz S, van Lieshout AW, Sprong T, van den Hoogen FH, et al. The expression of Toll-like receptors 3 and 7 in rheumatoid arthritis synovium is increased and costimulation of Toll-like receptors 3, 4, and 7/8 results in synergistic cytokine production by dendritic cells. Arthritis Rheum 2005;52:2313–22.
26. Marshak-Rothstein A. Toll-like receptors in systemic autoimmune disease. Nat Rev Immunol 2006;6:823–35.
27. Feldmann M, Brennan FM, Foxwell BM, Taylor PC, Williams RO, Maini RN. Anti-TNF therapy: where have we got to in 2005? J Autoimmun 2005;25 Suppl:26–8.
28. Ottonello L, Frumento G, Arduino N, Bertolotto M, Mancini M, Sottofattori E, et al. Delayed neutrophil apoptosis induced by synovial fluid in rheumatoid arthritis: role of cytokines, estrogens, and adenosine. Ann N Y Acad Sci 2002;966:226–31.
29. Schaffler A, Ehling A, Neumann E, Herfarth H, Tarner I, Scholmerich J, et al. Adipocytokines in synovial fluid. JAMA 2003;290:1709–10.
30. Bokarewa M, Nagaev I, Dahlberg L, Smith U, Tarkowski A. Resistin, an adipokine with potent proinflammatory properties. J Immunol 2005;174:5789–95.
31. Kitani T, Okuno S, Fujisawa H. Growth phase-dependent changes in the subcellular localization of pre-B-cell colony-enhancing factor. FEBS Lett 2003;544:74–8.
32. Van Gestel AM, Haagsma CJ, van Riel PL. Validation of rheumatoid arthritis improvement criteria that include simplified joint counts. Arthritis Rheum 1998;41:1845–50.