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# Resistin, an Adipokine with Potent Proinflammatory Properties<sup>1</sup>

Maria Bokarewa,<sup>2\*</sup> Ivan Nagaev,<sup>†</sup> Leif Dahlberg,<sup>‡</sup> Ulf Smith,<sup>†</sup> and Andrej Tarkowski\*

The adipokine resistin is suggested to be an important link between obesity and insulin resistance. In the present study, we assessed the impact of resistin as inflammatory cytokine in the setting of arthritis. In vitro experiments on human PBMC were performed to assess cytokine response and transcription pathways of resistin-induced inflammation. Proinflammatory properties of resistin were evaluated in animal model by intra-articular injection of resistin followed by histological evaluation of the joint. Levels of resistin were assessed by ELISA in 74 paired blood and synovial fluid samples of patients with rheumatoid arthritis. Results were compared with the control group comprised blood samples from 34 healthy individuals and 21 synovial fluids from patients with noninflammatory joint diseases. We now show that resistin displays potent proinflammatory properties by 1) strongly up-regulating IL-6 and TNF- $\alpha$ , 2) responding to TNF- $\alpha$  challenge, 3) enhancing its own activity by a positive feedback, and finally 4) inducing arthritis when injected into healthy mouse joints. Proinflammatory properties of resistin were abrogated by NF- $\kappa$ B inhibitor indicating the importance of NF- $\kappa$ B signaling pathway for resistin-induced inflammation. Resistin is also shown to specifically accumulate in the inflamed joints of patients with rheumatoid arthritis and its levels correlate with other markers of inflammation. Our results indicate that resistin is a new and important member of the cytokine family with potent regulatory functions. Importantly, the identified properties of resistin make it a novel and interesting therapeutic target in chronic inflammatory diseases such as rheumatoid arthritis. *The Journal of Immunology*, 2005, 174: 5789–5795.

Resistin is a newly described 12.5-kDa adipokine that is a member of a cysteine-rich secretory protein family (1). cDNA sequence analyses revealed the existence of a family of resistin-related molecules (RELM)<sup>3</sup> or “found in the inflammatory zone” (FIZZ) proteins. These proteins were identified independently, and each has distinct tissue distribution. FIZZ1/RELMa has been found in allergic pulmonary inflammation in mice, but no human analog to FIZZ1 is identified so far. FIZZ2/RELMb is expressed predominantly in the small intestine and mucosal epithelial cells, whereas FIZZ3/resistin is expressed in white adipose tissue in rodents.

Resistin was originally described as an adipocyte-derived polypeptide that provided the link between obesity and insulin resistance (2, 3). Resistin is expressed at very low levels, if at all, in human adipose cells, whereas high levels are expressed in mononuclear leukocytes, macrophages, spleen, and bone marrow cells (4–6). Low levels of resistin are also expressed in lung tissue,

resting endothelial cells, and in placenta (4). However, no difference in resistin expression in adipocytes and myocytes was found between nondiabetic vs type 2 diabetic subjects (5, 7, 8), although circulating levels of resistin in these groups were different.

Expression of resistin is modulated by a variety of endocrine factors. In rodent adipose cells, resistin expression is induced by corticosteroids, prolactin, testosterone, and growth hormone, whereas insulin, epinephrine, and somatotrophin have an inhibitory effect (9). Many of these substances interact with the nuclear hormone receptor family. Resistin gene expression is induced by C/EBP $\alpha$  (10) whereas it is repressed by peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ) (4) through the direct binding of these transcription factors to the resistin promoter.

Investigations reported so far have been focused on the role of resistin as an inducer of insulin resistance. However, the fact that resistin is abundantly expressed in bone marrow cells and, in particular, in leukocytes and macrophages, and that molecules of the RELM family are found in inflamed tissues suggests that resistin can play a role in the inflammatory process. However, very little and contradictory information is currently available on this. It has been shown in subjects with type 2 diabetes that increased C-reactive protein levels are related to higher circulating levels of resistin but also other cytokines are elevated (11, 12). Proinflammatory cytokines (IL-1, IL-6, and TNF- $\alpha$ ) increase the expression of resistin in human PBMC (13), whereas TNF- $\alpha$  is a negative regulator of resistin expression in mouse adipose cells (14). Studies examining resistin expression as part of the endotoxin response have shown that resistin mRNA expression is detectable in the early phase (6), but undetectable following 24 h of endotoxin exposure (15).

In the present study, we show that resistin accumulates locally in the inflamed joints of patients with rheumatoid arthritis (RA) and that its levels correlate with the intensity of inflammation as defined by the intra-articular white blood cell count and IL-6 levels. Our studies on the role of resistin in the inflammatory process reveal that it exhibits potent proinflammatory properties and that

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<sup>3</sup> Abbreviations used in this paper: RELM, resistin-related molecule; FIZZ, found in the inflammatory zone; PPAR- $\gamma$ , peroxisome proliferator-activated receptor- $\gamma$ ; RA, rheumatoid arthritis.

Table I. Sequences of probes and primers used for determination of gene expression

Gene	Probe	Forward Primer	Reverse Primer
Resistin	TGCCAGAGCGTCACCTCCAGGG	GTCGCCGGCTCCCTAATATTT	GTGACGGCGAAGCCTCG
TNF- $\alpha$	CCCAGGTGACAAGCCTGTAGCCC	GCCAGGCAGTCAGATCA	GCTTGAGGGTTTGTACAAC
IL-6	TGAAAGCAGCAAAGAGGCACTGGC	GCCTTGAGAAAGGAGACATGTA	CACCAGGCAAGTCTCCTCAT
IL-1 $\beta$	CCTGTGGCCTTGGGCCTCAA	GCAACAAGTGGTGTCTCCAT	CACTCTCCAGCTGTAGAGTGGG
IL-8	TTCTCCACAACCCTTGCACCCAG	GCGCAACACAGAAATATTGTGA	TGAATTCTCAGCCCTCTTCAAA

resistin is able to induce arthritis when injected into healthy mouse joints. In addition, we show that it is an important regulatory cytokine triggering the release of other proinflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6. Finally, we demonstrate that the proinflammatory effects of resistin are mediated through the NF- $\kappa$ B signaling pathway.

## Materials and Methods

### Patients with RA and control individuals

Plasma and synovial fluid samples were collected from 74 patients with RA, fulfilling American College of Rheumatology criteria (16), who attended the Rheumatology clinic at Sahlgrenska University Hospital. The loss of cortical definition on recent radiographs was defined as an erosive disease. Presence of rheumatoid factor of any of the Ig isotypes was considered as positive. Control blood samples were obtained from 34 healthy blood donors, and synovial fluids were obtained from 21 patients with non-inflammatory joint diseases (osteoarthritis  $n = 8$ ; chondrocalcinosis  $n = 2$ ; villonodular synovitis  $n = 1$ ; knee contusion  $n = 4$ ; meniscus rupture  $n = 4$ ; cruciate ligament rupture  $n = 2$ ). Synovial fluid was ob-

tained by arthrocentesis of the knee joint and blood samples were drawn on the same occasion from the cubital vein into tubes containing sodium citrate. Collected samples were centrifuged at  $800 \times g$  for 15 min, aliquoted, and stored frozen at  $-20^\circ\text{C}$  until use.

### Cell stimulation

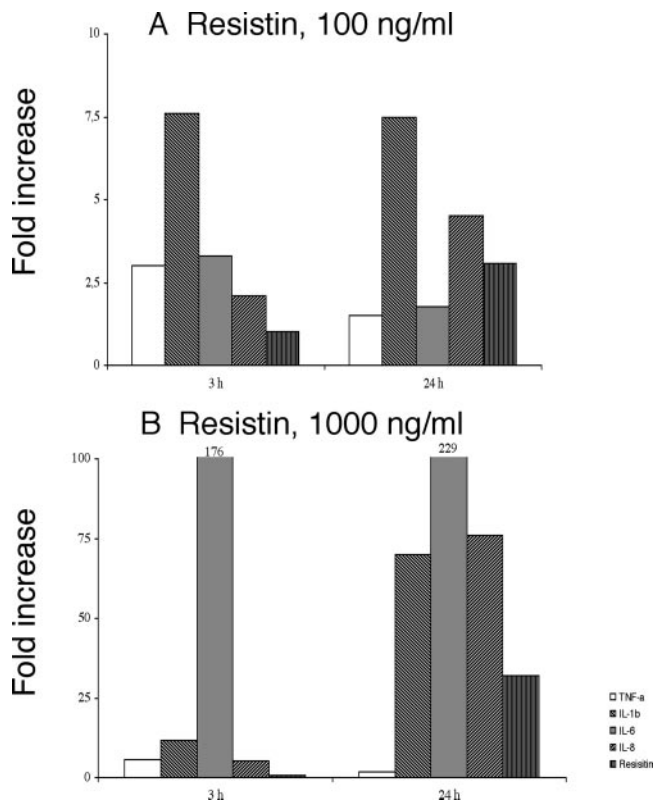
PBMC were prepared from heparinized blood of healthy individuals by separation on a Lymphoprep density gradient. The monocytic cell line THP-1 was originally obtained from American Type Culture Collection. Freshly isolated PBMC were resuspended to  $2 \times 10^6/\text{ml}$  and cultured with recombinant human resistin (endotoxin concentration below  $0.1 \text{ ng}/\mu\text{g}$ ) at final concentrations of 10–5000 ng/ml. To assess specificity of resistin-induced effects, additional experiments were performed with monoclonal anti-resistin Abs (R&D Systems) or parthenolide (Sigma-Aldrich), a specific NF- $\kappa$ B inhibitor, in the cell culture before stimulation. LPS (10 ng/ml) was used as a positive control in all experiments. The cell stimulation period differed depending on the read-out system used. For extracellular release of cytokines and resistin, supernatants were collected following 48 h of stimulation. For assessment of gene expression, total mRNA was prepared 0, 3, and 24 h after stimulation. The stimulation period for the analysis of NF- $\kappa$ B was 2 h.

### RNA isolation and RT-PCR assays

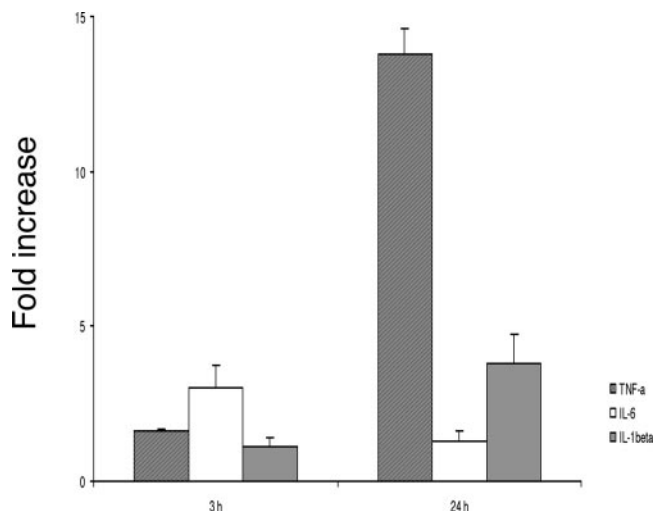
Total RNA from harvested cells was extracted by an RNeasy kit (QIAGEN), and concentration was assessed spectrophotometrically at 260 nm. The gene expression was measured with TaqMan real-time PCR (Applied Biosystems) as previously described (5). Table I shows the sequences for the probes and primers used.

### EMSA

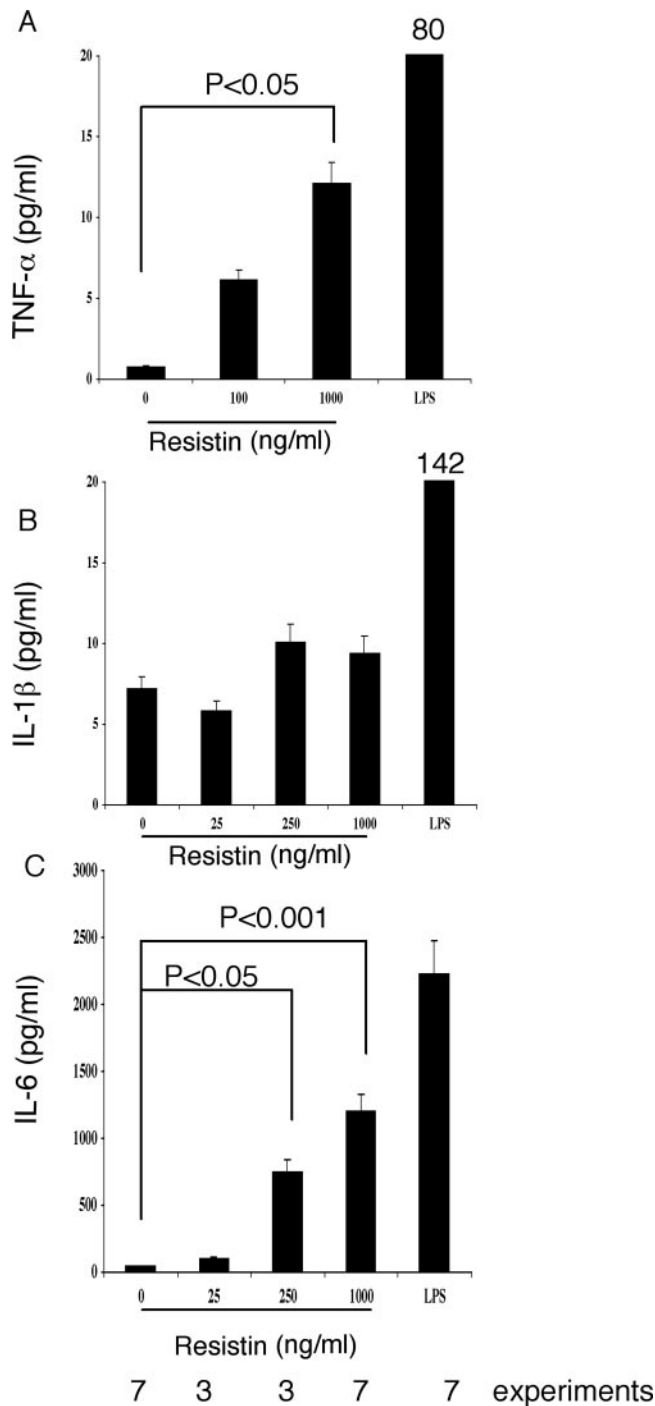
Human PBMC ( $10^7$ ) were stimulated with recombinant resistin (10–500 ng/ml). After 2 h, the stimulation was stopped with ice-cold PBS, cells were washed, and nuclear extracts were prepared as previously described (17). The sequences for oligonucleotides used for the assay were as follows: NF- $\kappa$ B: sense, 5'-GGCTCAAACAGGGGGCTTTCCCTCCTC AATAT-3', antisense 5'-GGATATTGAGGAGGGAAAGCCCCCTGTT



**FIGURE 1.** Up-regulation of cytokine mRNA expression ( $n = 3$ –8 subjects) by human PBMC following stimulation with resistin at a concentration of 100 ng/ml (A) and 1000 ng/ml (B). Human PBMC ( $2 \times 10^6/\text{ml}$ ) were incubated with a defined concentration of recombinant human resistin, and cell pellets were collected following 3 h of stimulation. Results are presented as fold increase of a specific mRNA over the level of respective mRNA expression in nonstimulated cell cultures. PBMC stimulated with LPS (10 ng/ml) were used as a positive control.

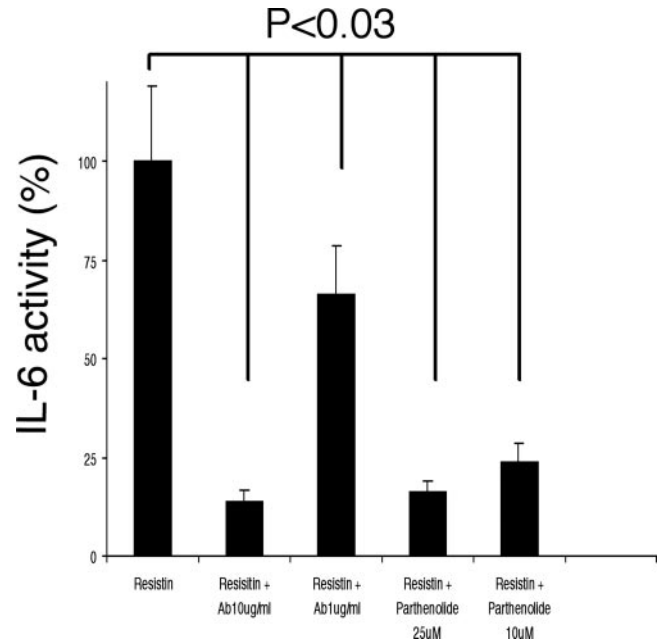


**FIGURE 2.** Increase of resistin mRNA expression following stimulation with IL-1 $\beta$ , IL-6, and TNF- $\alpha$ . Results are presented as fold increase of a specific mRNA over the level of respective mRNA expression in nonstimulated cell cultures.



**FIGURE 3.** Increase of IL-6 (A), TNF- $\alpha$  (B), but not of IL-1 $\beta$  (C) in supernatants following stimulation with resistin. Human PBMC ( $2 \times 10^6$ /ml) were incubated with a defined concentration of recombinant human resistin, and supernatants were collected following 48 h of stimulation. LPS (10 ng/ml) is provided as a positive control.

TGAG-3'. Oligonucleotides were annealed at 56°C. The double-stranded product was purified by elution from the electrophoretic gel. Double-stranded oligonucleotides were labeled with [ $\alpha$ - $^{32}$ P]deoxynucleotide (Amersham Biosciences) using Klenow polymerase (5 U/ml; Roche). Binding reactions were performed by incubation of nuclear extract protein (5  $\mu$ g) in binding buffer (pH 7.9, 20 mM Tris-HCl, 30 mM NaCl, 5  $\mu$ M EGTA, 5% glycerol) containing 0.2  $\mu$ g/ml BSA, 5  $\mu$ g of poly(dI-dC), 1 mM DTT, and 1  $\mu$ l of  $^{32}$ P-labeled double-stranded oligonucleotides (0.1 pmol), at room temperature for 20 min. For competition studies, a 100 M excess of unlabeled double-stranded oligonucleotides was added to the reaction mixture and incubated for 20 min before the introduction of the  $^{32}$ P-labeled probe.



**FIGURE 4.** Inhibition of IL-6 expression in resistin-stimulated PBMC following treatment with monoclonal anti-resistin Ab (1–10  $\mu$ g/ml), and parthenolide (10–25  $\mu$ M), a specific NF- $\kappa$ B inhibitor. Results are calculated as a residual activity of IL-6 compared with non-treated PBMC stimulated with recombinant resistin (200 ng/ml). The figure represents the cumulative results of four independent experiments.

For supershift assays, antiserum to p65 and p50 subunits of NF- $\kappa$ B (Santa Cruz Biotechnology) was incubated with nuclear extracts for 15 min at room temperature. Samples containing an equal amount of protein were loaded directly onto 2.5% polyacrylamide gel prepared in Tris-borate-EDTA buffer (0.25 $\times$ ), and electrophoresis was performed at 200 V at room temperature. The gel was vacuum-dried and exposed to x-ray film for 48 h at  $-70^\circ\text{C}$ .

#### Determination of cytokine levels

The level of IL-6 in synovial fluid and supernatants was determined by the proliferation of the IL-6-dependent cell line, B13.29. Amount of IL-6 was calculated using standard dilutions of rIL-6 (Genzyme). Levels of TNF- $\alpha$  and IL-1 $\beta$  were assessed with an ELISA kit (R&D Systems) according to the manufacturer's recommendations. OD of the tested samples was compared with the values obtained from serial dilution of respective recombinant human cytokine.

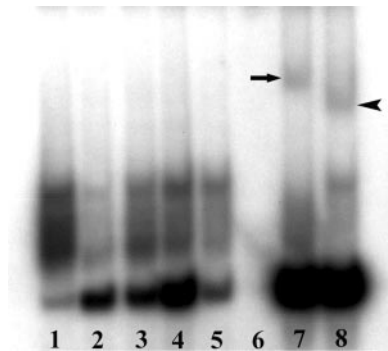
#### Resistin levels

Resistin levels in patient samples and cell supernatants were determined by a sandwich ELISA (BioVendor). Briefly, matched samples of plasma and synovial fluid were diluted 1/10 in BSA-PBS and introduced into the parallel strips coated with capture polyclonal anti-resistin Abs. Biotin-labeled anti-resistin Abs, streptavidin-HRP conjugate, and corresponding substrate were used for color development. The obtained absorbance values were compared with serial dilution of recombinant human resistin. Lowest detectable level was 1 ng/ml.

#### Animal model of resistin-triggered arthritis

Female NMRI mice, 6–8 wk old, weighing 25–30 g, were purchased from ALAB. The mice were bred and housed in the animal facility of the Department of Rheumatology under standard conditions of temperature and light and were fed laboratory chow and water ad libitum. Mouse recombinant resistin (PeproTech) was diluted in PBS and injected intraarticularly into the right knee joint (0.1–100 ng/knee) in a total volume of 20  $\mu$ l. Control mice received mouse albumin (100 ng/knee) (Sigma-Aldrich) in 20  $\mu$ l of PBS buffer. The mice were sacrificed by cervical dislocation 3 days after the intraarticular injection, and the injected knee was removed for histological examination after routine fixation, decalcification, and paraffin embedding of the tissue. Tissue sections of the knee joints were cut and stained with H&E. All the slides were coded and evaluated blindly by two





**FIGURE 5.** Binding of nuclear extracts from PBMC stimulated with resistin to NF- $\kappa$ B specific binding sites, assessed by EMSA. *Lane 1*, Positive control, binding of nuclear extracts stimulated with LPS (10 ng/ml). *Lanes 2–5*, Binding of nuclear extracts from PBMC stimulated with recombinant human resistin 0, 25, 250, and 1000 ng/ml, respectively. *Lane 6*, Nuclear extract as in *lane 5* after preincubation with an excess of competitive binding unlabeled NF- $\kappa$ B oligonucleotides. *Lane 7*, Nuclear extract as in *lane 5*. Shows a decreased mobility of the NF- $\kappa$ B complexed with anti-p65 Abs (marked with an arrow). *Lane 8*, Nuclear extract as in *lane 5*. Shows a decreased mobility of the NF- $\kappa$ B complexed with anti-p50 Abs (marked with an arrowhead).

investigators with respect to synovial hypertrophy, the inflammatory cell infiltration of synovia, pannus formation, and cartilage and subchondral bone destruction. Synovial hypertrophy was defined as synovial membrane thickness of more than two cell layers. Intensity of inflammatory cell infiltration of synovia was graded arbitrarily from 0 to 3.

In one experiment, intra-articular resistin-injected NMRI mice were treated i.p. with the soluble TNF- $\alpha$  receptor analog (p75-IgG fusion protein, Etanercept; Wyeth Pharmaceuticals), 100 mg/mouse/day, as previously described (18).

To assess the role of PPAR- $\gamma$ -dependent mechanisms on the development of arthritis following intra-articular injection of resistin, naive NMRI mice were treated with PPAR- $\gamma$  agonist (rosiglitazone, Avandia; Glaxo-SmithKline AB) 0.3 mg/mouse/day, i.p., 4 days before resistin injection and during 4 days following the injection.

#### Statistical analysis

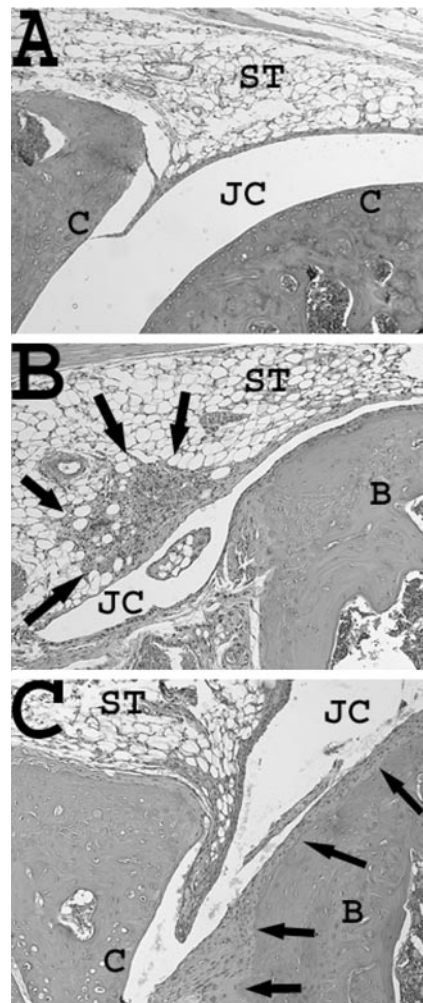
The level of continuous variables was expressed as mean  $\pm$  SEM. Difference between the groups was calculated using the Mann-Whitney *U* test. Interrelation between parameters studied was calculated with the Spearman correlation coefficient. For all statistical evaluation of the results, *p* values  $<0.05$  were considered significant.

## Results

### Resistin is a proinflammatory cytokine

To assess the role of resistin in inflammation, human PBMC ( $n = 7$ ) were stimulated with increasing concentrations of resistin (0–1000 ng/ml). This led to a marked induction of the genes for the proinflammatory cytokines TNF- $\alpha$ , IL-6, and IL-1 $\beta$  (Fig. 1, A and B). Increased mRNA for TNF- $\alpha$  and IL-6 were already seen 30 min after the exposure to resistin. IL-6 mRNA levels continued to increase following 24 h of resistin stimulation, whereas TNF- $\alpha$  mRNA levels declined after 3 h. Importantly, stimulation with resistin also led to the up-regulation of resistin mRNA itself, showing that it induces a positive feedback mechanism. Increased resistin mRNA was detectable after 3 h, and it was maintained throughout the 24 h of the experiment.

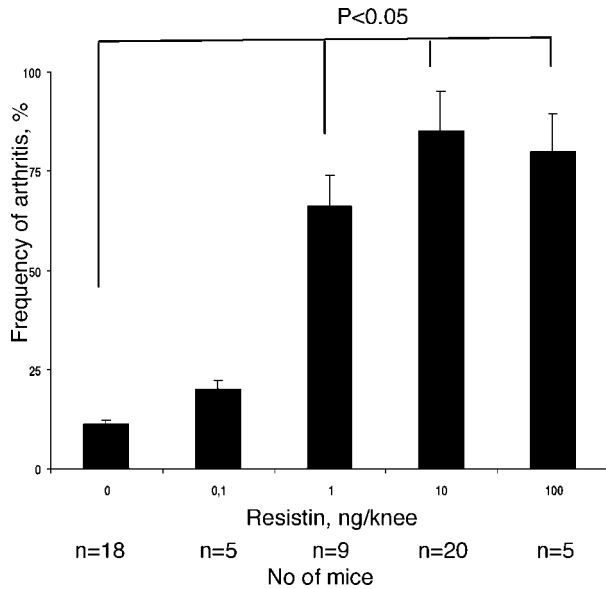
The ability of resistin to induce inflammatory response in synoviocytes was evaluated by *in vitro* stimulation of synovial fluid cells from patients with acute synovitis ( $n = 4$ ) with resistin (10–1000 ng/ml). PCR analysis showed an increased expression of TNF- $\alpha$  and IL-6 mRNA similar to that found with human PBMC (data not shown).



**FIGURE 6.** Intraarticular injection of mouse resistin into knee joints of healthy mice induces arthritis. Histopathology of knee joint 4 days after injection of control vehicle (mouse albumin) revealed no signs of arthritis (A), recombinant mouse resistin (10 ng/joint) induces within 4 days synovitis (arrows indicate inflammatory cells in the synovium) (B), and pannus formation with cartilage destruction (arrowheads) (b) knee joint 4 days after injection of control vehicle (mouse albumin) (C). Original magnification  $\times 10$ . JC, joint cavity; C, cartilage; ST, synovial tissue; B, bone.

To assess whether the increased mRNA levels were also accompanied by an increased cytokine release into the medium, the supernatants of human PBMC ( $n = 7$ ) stimulated with resistin (0–5000 ng/ml) were assessed for the levels of synthesized proinflammatory cytokines. A concentration-dependent increase of IL-6 activity as well as TNF- $\alpha$  and IL-1 $\beta$  levels were found in all PBMC cultures (Fig. 2, A–C). To evaluate the intensity of resistin-induced cytokine response by human PBMC, the cell cultures were stimulated with LPS (10 ng/ml), PHA (1.5  $\mu$ g/ml) and Con A (0.6  $\mu$ g/ml). The levels of cytokines induced by the highest resistin concentration tested (5000 ng/ml) were similar to those induced by LPS (IL-6 level in supernatants, resistin,  $280 \pm 80$  pg/ml, vs LPS,  $350 \pm 100$  pg/ml). PHA and Con A induced significantly lower levels of proinflammatory cytokines by human PBMC compared with human resistin (IL-6 levels in supernatants, PHA,  $120 \pm 75$  pg/ml, vs ConA,  $25 \pm 40$  pg/ml, vs resistin,  $280 \pm 80$  pg/ml).

To assess whether the proinflammatory cytokines used at physiological concentrations could induce the expression of resistin, human PBMC ( $n = 5$ ) were stimulated with recombinant TNF- $\alpha$ , IL-6, and IL-1 $\beta$  (concentrations, 1–5 ng/ml). We observed that



**FIGURE 7.** Recombinant resistin (doses, 0–100 ng/knee joint) injected intra-articularly gives dose-dependent rise to arthritis in healthy mouse recipients. n, indicates number of mice used for each resistin dose. Control mice ( $n = 18$ ) injected with mouse albumin (100 ng/knee joint) are indicated as resistin 0.

only TNF- $\alpha$  induced resistin mRNA, and this was maintained throughout the whole experimental period (Fig. 3). In contrast, IL-6 and IL-1 $\beta$  did not increase resistin mRNA to a significant extent. In comparison, stimulation of human PBMC with LPS (10 ng/ml) resulted in an up-regulation of resistin mRNA and in increased resistin levels in the supernatants following 48 h of stimulation. The human monocytic cell line THP-1 responded to resistin stimulation with a cytokine production pattern similar to PBMC (data not shown), whereas neither stimulation with TNF- $\alpha$  nor with LPS gave rise to the expression of resistin mRNA.

#### Intracellular signaling of resistin

To assess the possible mechanism for the cytokine induction, we studied the ability of resistin to activate the NF- $\kappa$ B signaling pathway. Nuclear extracts of PBMC stimulated with resistin (10–500 ng/ml) were subjected to EMSA. As shown in Fig. 4, resistin-induced dose-dependent NF- $\kappa$ B activity resulted in the translocation of NF- $\kappa$ B from the cytoplasm to the nucleus. Importantly, both p65 and p50 subunits containing complexes of NF- $\kappa$ B were detected in the nuclear extracts of the PBMC stimulated with resistin.

To further evaluate the potential role of NF- $\kappa$ B in resistin-induced cytokine activation, parthenolide, a specific NF- $\kappa$ B inhibitor, was added to the PBMC culture before the addition of resistin. Supernatants from these cells following 48 h of resistin showed a marked suppression of IL-6 activity (range, 84–88%) in the medium of the cultures treated with parthenolide (10 and 25  $\mu$ M) compared with nontreated cultures (Fig. 5). The *in vitro* effect achieved by inhibition of NF- $\kappa$ B signaling pathway was very comparable to a direct neutralization of resistin. Preincubation of resistin with monoclonal anti-resistin Abs (final concentration, 1–10  $\mu$ g/ml) before the addition of resistin to the PBMC cultures resulted in a concentration-dependent down-regulation of IL-6 release. Reduction of IL-6 activity by 34% was observed already with the lowest concentration of the Abs, and increased to 77% (range, 68–86%) with the highest concentration used (10  $\mu$ g/ml) (Fig. 5).

#### Resistin induces inflammation *in vivo* when introduced intraarticularly

Recombinant mouse resistin was injected intra-articularly in the knee joints of healthy NMRI mice at amounts ranging from 0 to 100 ng/knee. Control animals obtained mouse albumin (100 ng/knee). Whereas intra-articular injection of the mouse albumin did not give rise to a localized inflammatory response (Figs. 6A and 7), injection of resistin caused arthritis (Figs. 6, B and C, and 7). Histological analyses of the joints injected with resistin revealed infiltration of synovial tissue with leukocytes (Fig. 6B), in several cases associated with hypertrophy of synovial lining layer and pannus formation (Fig. 6C). The frequency of arthritis increased in a dose-dependent manner and occurred in up to 80% of the joints injected with resistin (Fig. 7). The knee joints injected with control vehicle (mouse albumin, marked as resistin 0) only revealed signs of mild synovitis in 2 of 18 cases (11%). The endotoxin concentration of the recombinant murine resistin injected intra-articularly was below 0.1 ng/ $\mu$ g and, thus, below the level that could trigger arthritis (19).

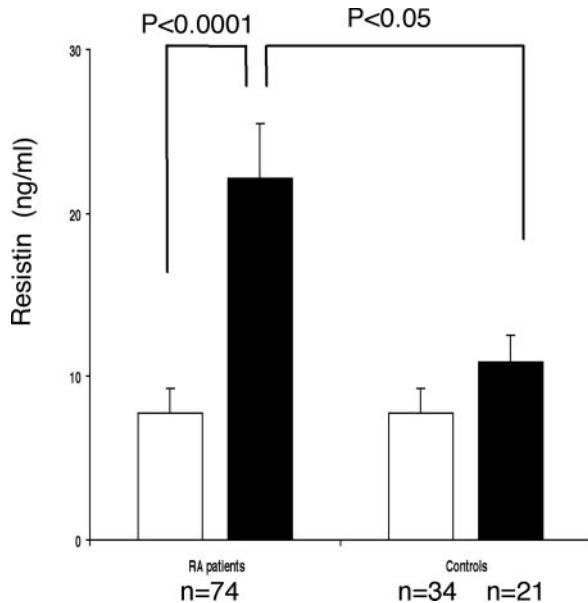
Recombinant resistin (10 ng/knee) was injected intraarticularly to 20 female NMRI mice and to 7 male NMRI mice. The frequency of arthritis in these groups was comparable and independent of the gender (14 of 20 vs 5 of 7, respectively). Recombinant mouse resistin (10 ng/knee) was also injected in the knee joints of C57BL/6 male mice ( $n = 7$ ). Histological signs of arthritis were observed in 5 of 7 mice (71%).

To evaluate the role of TNF- $\alpha$  in the resistin-induced arthritis, the mice intra-articularly injected with resistin (10 ng/knee), were *i.p.* administered with TNF- $\alpha$  receptor analog (etanercept, 100 mg/

Table II. Clinical and demographic characteristics of patients with rheumatoid arthritis, individuals with degenerative and traumatic joint diseases (synovial fluid), and healthy blood donors (blood)

	RA Patients $n = 74$	Controls	
		Blood $n = 34$	Synovial fluid $n = 21$
Age, years (range)	61 $\pm$ 2 (24–84)	42 $\pm$ 7 (18–67)	64 $\pm$ 2 (33–88)
Sex, male/female	22/52	12/22	11/10
Disease duration, years (range)	10 $\pm$ 1 (1–34)		
Radiological changes, erosive/non-erosive	41/33	NA	NA
Rheumatoid factor, +/-	44/30	NA	NA
Treatment with DMARD			
Methotrexate	24		
Other DMARD	15		
None	34		

NA, Not analyzed; DMARD, disease-modifying anti-rheumatic drugs.



**FIGURE 8.** Resistin accumulates in the inflamed joints of patients with RA. Empty bars represent resistin levels in blood whereas filled bars represent synovial fluid levels of resistin.

mouse/day) during the whole experiment starting 1 h before intra-articular instillation of resistin. Control group of mice was treated with daily i.p. injections of PBS. Histological analyses of the resistin-injected joints revealed no difference in the frequency of arthritis between the groups treated with etanercept and the controls (7 of 10 vs 6 of 10, not significant).

Activation of PPAR- $\gamma$  signaling pathway has been shown to efficiently down-regulate resistin expression in mice (4). To evaluate the ability of PPAR- $\gamma$  agonist (rosiglitazone) to prevent the development of arthritis following intraarticular injection of resistin (10 ng/knee), naive NMRI mice ( $n = 15$ ) were treated with rosiglitazone (0.3 mg/mouse/day) 4 days before and 4 days after resistin injection. Histological analysis of the injected joints showed no difference in the frequency or intensity of resistin-induced arthritis in the mice treated with rosiglitazone compared with the controls (8 of 15 vs 10 of 15; NS).

#### Increased resistin levels in the inflamed joints of patients with RA

Resistin levels were assessed in the paired blood and synovial fluid samples of 74 patients with RA. Clinical and demographic data of the patient population and the control group are presented in Table II. Synovial fluid samples from RA patients showed significantly higher levels of extracellular resistin compared with the matched blood samples ( $22.1 \pm 3.2$  vs  $5.7 \pm 0.5$  ng/ml,  $p < 0.0001$ ; Fig. 8). In addition, resistin levels in synovial fluid originating from patients with RA were clearly higher than in the synovial fluid of control patients having degenerative/traumatic joint diseases ( $22.1 \pm 3.2$  vs  $10.9 \pm 0.7$ ,  $p < 0.05$ ). Furthermore, the resistin levels in RA synovial fluid showed a significant correlation to synovial leukocyte count ( $r = 0.66$ ,  $p = 0.003$ ) and IL-6 levels ( $r = 0.36$ ,  $p = 0.014$ ). In contrast, resistin levels in blood were neither related to the duration of RA, age of the patients, nor to circulating C-reactive protein levels or white blood cell counts.

## Discussion

Previous studies concerning the adipokine resistin have focused on its putative role in insulin resistance. In the present investigation, we studied the potential role of resistin in the inflammatory pro-

cess. We show that human PBMC and synovial leukocytes respond to extracellular resistin by producing a broad spectrum of proinflammatory cytokines. This spectrum of cytokines includes TNF- $\alpha$ , which is considered to have a dominant regulatory role in the cytokine cascade, as well as IL-6, and IL-1 $\beta$ . Interestingly, resistin induced its own production in human PBMC thus providing a positive feedback circuit. Furthermore, exposure of PBMC to TNF- $\alpha$ , but not IL-6 or IL-1 $\beta$ , induced the expression of resistin. Taken together, these data clearly show that resistin exerts a tight control of the cytokine inflammatory cascade, a property very comparable with that of TNF- $\alpha$  and, thereby, may provide an alternative pathway for activation of cytokine release even in the absence of TNF- $\alpha$ . The presence of an alternative pathway for cytokine activation provides an explanation for the only partial effect of TNF- $\alpha$  inhibitors in a sizeable group of RA patients (20–22) and for the lack of efficacy following intra-articular injection of TNF- $\alpha$  inhibitors (23). Interestingly, the THP-1 monocytic cell line that lacks resistin gene expression was still capable of responding to resistin stimulation with cytokine release. This observation clearly indicates that monocytes express the, so far, unidentified receptor for resistin.

Endothelial cells have recently been reported as resistin-sensitive cells, responding to resistin with up-regulation of endothelin 1 and vascular cell adhesion molecules (24), indicating that resistin-sensitive cells may be broadly distributed throughout the body. The present study shows that the NF- $\kappa$ B transcription pathway mediates the stimulatory effect of resistin on cytokine release. Resistin induced, directly or indirectly, the translocation of NF- $\kappa$ B from the cytoplasm to the nucleus as detected by EMSA. Moreover, a significant abrogation of the distal effects of resistin were seen when PBMC were treated with parthenolide, a selective NF- $\kappa$ B inhibitor. These data strongly support the proinflammatory regulatory properties of resistin.

By injecting recombinant resistin into healthy knee joints in mice, we demonstrated that intraarticular exposure to resistin had *in vivo* proinflammatory properties. A single injection of recombinant mouse resistin (10 ng/knee) was sufficient to induce leukocyte infiltration and hyperplasia of the synovia. This amount is comparable with the concentration of resistin found in the synovial fluid during acute joint effusion in RA patients. Importantly, we demonstrated that treatment of resistin-injected mice with TNF- $\alpha$  receptor analog did not prevent the development of resistin-mediated arthritis. This observation suggests TNF- $\alpha$ -independent *in vivo* mechanisms in mediation of joint inflammation. We also tried to prevent the development of resistin-mediated arthritis by activating PPAR- $\gamma$  signaling and thereby inhibiting positive feedback through the endogenous resistin expression (4). High frequency of arthritis despite the treatment of mice with PPAR- $\gamma$  agonist indicated that resistin exerts inflammation by a direct stimulation of cells in the joint tissues.

Our study also demonstrates that 1) resistin accumulates in the synovial fluid of patients with RA, and 2) stimulation of human synovial fluid leukocytes with resistin *in vitro* also resulted in increased IL-6 and IL-1 $\beta$  mRNA levels. The levels of resistin were significantly higher in inflammatory joint effusion (i.e., in RA) than in patients with primarily noninflammatory joint disease such as osteoarthritis or joint trauma. Interestingly, circulating resistin levels were low in RA patients suggesting an increased local production and/or a preferential accumulation of this molecule at the site of inflammation. The latter findings are in agreement with recently published observation of increased resistin levels in synovial fluid (25). There are several possible mechanisms for the



different resistin levels in blood and joint cavity such as: 1) different leukocyte populations are present in these two compartments; 2) the intra-articular presence of resistin-inducing substances; and/or 3) prompt inactivation and elimination of resistin from the circulation. Previous reports about high resistin expression in human bone marrow cells (4), known to be the important source of inflammatory cells infiltrating synovium during arthritis, are consistent with the present findings. Furthermore, resistin levels correlated with intraarticular leukocyte counts and IL-6 levels, supporting a link to other mediators of inflammation.

In conclusion, our study demonstrates that resistin is a molecule accumulating at the site of inflammation and, once there, supports the inflammatory process by triggering cytokine production and NF- $\kappa$ B activation while simultaneously up-regulating its own expression. Finally these properties are readily inhibited by either neutralization of resistin using specific mAbs, or by the use of a specific NF- $\kappa$ B inhibitor, all these pathways are of potential interest in the treatment of arthritis.

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## Disclosures

The authors have no financial conflict of interest.

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