Preferential and Persistent Activation of the STAT1 Pathway in Rheumatoid Synovial Fluid Cells

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ABSTRACT. ***Objective.*** Inflammatory cytokines such as interleukin 1 (IL-1), IL-6, and tumor necrosis factor-α are produced in great quantities in inflamed rheumatoid joints. However, little is known about the pathogenic significance of each cytokine in the proliferative synovitis and destruction of bone and joint. We investigated the role of cytokine receptor signals transduced into cells at the foci of rheumatoid inflammation.

Methods. Synovial fluid (SF) cells from patients with rheumatoid arthritis (RA) and osteoarthritis (OA) were examined for the activation of a group of cytokine receptor signaling molecules, signal transducers and activators of transcription (STAT).

Results. DNA binding of STAT1 in SF cells was observed in 8 out of 14 patients with RA, but in none of the 10 patients with OA studied, and this was prevented by preincubation of these cells with neutralizing anti-IL-6 antibody. IL-6 activated both STAT1 and STAT3 in normal peripheral blood (PB) leukocytes, and preferentially STAT1 in rheumatoid SF cells. Moreover, STAT1 activation in rheumatoid SF cells appeared to be continuous, in contrast to the transient activation in normal PB leukocytes.

Conclusion. STAT1 and STAT3 are differentially regulated in response to IL-6 in different cell types. The continuous STAT1 activation may be of pathogenic significance in the progression and persistence of RA. (J Rheumatol 2001;28:1952–9)

Key Indexing Terms:
RHEUMATOID ARTHRITIS OSTEOARTHRITIS SYNOVIAL FLUID
INTERLEUKIN 6 SIGNAL TRANSDUCERS AND ACTIVATORS OF TRANSCRIPTION (STAT)

An increase in the number of proliferated synovial cells and activated leukocytes can be seen in the synovial membrane and synovial fluid (SF) of patients with rheumatoid arthritis (RA)[1-4]. Activation of cells and the subsequent secretion of cytokines play important roles in autoimmune and inflammatory diseases[5,6]. After interaction with specific receptors, cytokines initiate signals directed at both the cytoplasmic and the nuclear compartments, with many of the nuclear signals culminating in the induction of new genes. In this process, cytokines activate specific members of the signal transducers and activators of transcription (STAT) family of transcription factors[7-9]. The STAT proteins usually reside in the cytoplasm and become activated and migrate to the nucleus when phosphorylated on specific tyrosine residues, presumably as a direct result of JAK protein tyrosine kinase action. Different STAT become activated by different cytokine receptors. For example, interferon-γ (IFN-γ) has been reported to activate STAT1, and interleukin 6 (IL-6) to activate STAT1 and STAT3[7]. The pattern of STAT that becomes activated determines, at least in part, the patterns of gene expression and the phenotype of activated cells.

Progressive destruction of cartilage and bone is believed to be mainly mediated by cytokine induction of osteoclasts and destructive enzymes[10]. Cytokines such as IL-1, IL-6, and tumor necrosis factor-α (TNF-α) are actively produced in the inflamed synovium[11-14]. IL-1 and TNF-α have been implicated to be important in cartilage damage because they induce destructive matrix metalloproteinases, such as matrix metalloproteinase 1 and 3, as well as bone resorption in *in vitro* cultures[15-18]. There is increasing evidence that IL-6 may inhibit bone formation and induce bone resorption through its stimulatory effect on osteoclasts[19]. In spite of these findings, some groups have claimed that high cytokine levels in RA samples are not associated with any particular disease type, differences in duration, severity of disease, or therapy[5,10,20]. Therefore, it is unclear which of these cytokines is important for the rapid RA progression in the disease environment. In contrast to cytokine production and degradation, phosphorylation and dephosphorylation of STAT is a rapid process completed over a time scale of minutes, implying the possibility that cytokine

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receptor signaling rather than cytokine concentration might more accurately reflect the course of the disease.

We examined DNA binding of STAT in SF cells from patients with synovitis, and identified continuous cytokine receptor signaling through STAT1 that was selectively observed in RA.

MATERIALS AND METHODS

Subjects. Fourteen patients with RA (Table 1) according to American College of Rheumatology criteria[21] and 10 patients with osteoarthritis (OA) (Table 2) as controls were studied.

Measurement of cytokine concentrations in SF. SF was passed through a 0.45 μm pore filter (Millipore, Bedford, MA, USA). For measurement of IFN-γ concentration, a sandwich-type human IFN-γ ELISA kit (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) was used according to manufacturer's instructions. IL-6 concentrations were measured by chemiluminescent enzyme immunoassay using Human IL-6 CLEIA Fujirebio (Fujirebio Inc., Tokyo, Japan) according to manufacturer's instructions[22]. SF samples were pretreated with IgG coated beads before these assays so as not to cross-detect rheumatoid factor.

Cell preparation and incubation. Peripheral blood (PB) leukocytes were isolated from the whole blood of healthy volunteers and patients with RA by dextran sedimentation at room temperature, and were suspended in RPMI 1640 medium (Nikken Biomedical Co., Kyoto, Japan) supplemented with 2 mM L-glutamine, 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 mM streptomycin. Hep3B hepatoma cells were grown in high glucose Dulbecco's modified Eagle's medium (Nikken Biomedical) supplemented with 2 mM L-glutamine, 10% FCS, 100 U/ml penicillin, and 100 mM streptomycin. These cells were incubated in the presence or absence of 10 ng/ml of human recombinant IL-6 (rIL-6; provided by Ajinomoto Co., Kawasaki, Japan) or 100 IU/ml of human rIFN-γ (provided by Hayashibara Biochemical Laboratory, Okayama, Japan) for the time indicated in a 5% CO_2 incubator at 37°C before nuclear extract and cell lysate preparation.

In some experiments, SF from patients with RA was incubated in the presence or absence of neutralizing goat anti-IFN-γ, anti-IL-6, or control goat IgG at a final concentration of 20 μg/ml for 2 days in a 5% CO_2 incubator before nuclear extract preparation. These antibodies were purchased from R&D Systems Inc., Minneapolis, MN, USA.

Nuclear extract preparation. SF samples were treated with 5 U/ml of hyaluronidase (Mochida Pharmaceutical Co., Tokyo, Japan) for 15 min at 37°C, and were washed twice with phosphate buffered saline (PBS). PB leukocytes and Hep3B cells were collected and washed twice with PBS.

A nuclear mini-extract procedure was used[23,24]. In this procedure, 3–10 × 10^6 cells were resuspended in 450 μl of ice cold buffer A (10 mM HEPES, pH 7.6, 10 mM KCl, 1.5 mM $MgCl_2$ supplemented with 0.5 mM sodium vanadate, 1 mM DTT, 1 mM Pefabloc SC, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml antipain, 2 μg/ml pepstatin A, 1 mM PMSF) by gentle pipetting in a blue tip. The cells were allowed to swell on ice for 10 min, after which 50 μl of a 10% solution of Nonidet P-40 (NP-40) was added, and the cells were vigorously vortexed. After a 10 min incubation on ice, nuclei were pelleted in a microcentrifuge, washed in buffer A, lysed in 50 μl of ice cold buffer C with the same composition as buffer A except for the use of 420 mM instead of 10 mM KCl, and vigorously vortexed. After a further 20 min incubation on ice, insoluble precipitates were pelleted by centrifugation, and the supernatant was stored at –80°C. The protein concentration of the nuclear extract was determined by using the bicinchoninic acid assay (Pierce, Rockford, IL, USA).

Gel shift assay. Oligonucleotide probe sequences used in the gel shift assays were: c-fos high affinity serum inducible element (hSIE), 5'-GTCGACATTTCCCGTAAATCGTCGA[24-26]; mutant hSIE, 5'-GTCGACAT<u>CCA</u>C-

Table 2. Characteristics of 10 patients with OA.

Patient	Age/Sex	Disease Duration, yrs	CRP, mg/dl	IFN-γ, pg/ml	IL-6, pg/ml
15	47 F	3	0.2	< 5.0	349.4
16	66 M	0.1	0.2	21.6	5287.0
17	58 F	5	0.2	< 5.0	84.9
18	46 M	0.1	ND	< 5.0	4003.0
19	62 F	2	ND	< 5.0	51440.0
20	53 M	0.5	ND	< 5.0	6617.0
21	41 F	0.25	ND	< 5.0	3668.0
22	61 F	4	ND	ND	ND
23	63 M	5	ND	< 5.0	320.0
24	64 F	0.2	ND	ND	ND

ND: not done.

Table 1. Characteristics of 14 patients with RA.

Patient	Age/Sex	Disease Duration, yrs	RF*	CRP, mg/dl	Disease Classification,† Class/Stage	Medication	IFN-γ, pg/ml	IL-6, pg/ml
1	68 F	42	+	4.49	2 IV	S	5.4	18040.0
2	64 F	19	+	4.20	3 IV	N, D	115.0	25680.0
3	49 F	14	–	0.96	2 IV	—	< 5.0	4853.0
4	56 F	13	–	1.4	2 III	N, D	< 5.0	2102.0
5	64 M	3	–	12.4	2 II	N, D, S	14.3	12280.0
6	44 F	11	+	1.0	2 III	—	7.5	7544.0
7	48 F	8	+	3.0	2 III	N, D	16.7	3469.0
8	43 F	20	–	2.7	2 IV	N, D	5.8	8030.0
9	46 F	0.1	–	1.56	2 I	N	87.9	30120.0
10	42 F	19	–	0.2	2 IV	N, D	10.0	3570.0
11	52 F	14	+	0.7	2 IV	N, D, S	183.0	146.3
12	66 F	7	+	5.3	3 IV	N, D, S	< 5.0	265.2
13	40 F	6	–	1.6	2 IV	N, D, S	11.2	991.4
14	71 F	25	+	1.6	2 IV	N	62.4	3890.0

* RF assessed by latex fixation text. † Steinbrocker classification. N: nonsteroidal antiinflammatory drugs; D: disease modifying antirheumatic drugs; S: low dose (< 7.5 mg/day) prednisone.

CGTAAATCGTCGA (mutant bases are underlined). Five micrograms of nuclear extract was mixed with 4 μg of poly(dI-dC) and 0.2–0.4 ng of radiolabeled double-stranded oligonucleotide in a binding buffer (10 mM HEPES, pH 7.8, 100 mM KCl, 1 mM EDTA, 5 mM $MgCl_2$, 10% glycerol, 5 mM DTT). Mouse antisera for supershift assays were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA. Each of the antibodies was added to the nuclear extract at room temperature 30 min before adding the radiolabeled probe. Oligonucleotide competitors were also added 30 min before adding the radiolabeled probe. After 30 min of binding reaction at room temperature, the DNA-protein complexes were separated by electrophoresis on a 4% polyacrylamide gel containing 2.7% glycerol in TGE buffer. The gel was then dried and autoradiographed.

Immunoblot analysis. Cells were solubilized with NP-40 lysis buffer (buffer A supplemented with 1% NP-40 described in nuclear extract preparation). Clear lysates were obtained by centrifugation, and the protein concentration of the cell lysate was determined using the BCA assay. Five micrograms of cell lysate was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and subsequent immunoblotting with mouse anti-STAT1 and anti-STAT3 monoclonal antibodies (purchased from Transduction Laboratories, Inc., Lexington, KY, USA) using an enhanced chemiluminescence detection system (Amersham Life Science, Buckinghamshire, England) according to manufacturer's instructions.

RESULTS

DNA binding of STAT in SF cells and the responsible cytokines. SF cell components freshly isolated from patients with synovitis were assessed by FACS analysis (data not shown). SF from patients with RA contained 5 to 35% CD3+ T cells, 0.5 to 2% CD20+ B cells, 20 to 73% CD15+ granulocytes, 5 to 12% CD14+ cells, with the remainder made up of CD3–CD20–CD14–CD15– cells. SF from patients with OA contained 8 to 28% CD3+ T cells, 0.1 to 1% CD20+ B cells, 1 to 5% CD15+ granulocytes, 5 to 31% CD14+ cells, with the remainder CD3–CD20–CD14–CD15– cells. The CD3–CD20–CD14–CD15– cells were confirmed to be fibroblastic synovial cells by histologic analysis.

To determine whether some cytokines are in fact acting on cells at the foci of inflammation, we investigated the DNA binding of STAT, which is known as one of the varieties of signal transducing molecules downstream of the cytokine receptors. SF cells from 14 patients with RA (Patients 1–14) and 10 patients with OA (Patients 15–24) were examined using gel shift assays (Figure 1). We used a double-stranded radiolabeled c-fos hSIE oligonucleotide probe, which is known to bind several activated STAT[24-26]. The formation of a DNA-STAT complex was observed in 8 out of 14 patients with RA (Patients 1–8), but in none of the 10 patients with OA. The results for representative RA cases with DNA-STAT formation (Patients 1–5) are shown in Figure 1. The specificity of the DNA-STAT formation was confirmed by the finding that this formation (Figure 1, lane 1) was eliminated by the addition of a 160-fold molar excess of unlabeled hSIE (Figure 1, lane 2), but was not affected by a 160-fold molar excess of unlabeled mutant hSIE, containing 3 base substitutions within the STAT binding site (Figure 1, lane 3). Supershift assays using mouse antisera against each of the STAT molecules were performed to identify which STAT was activated. The specific band almost disappeared and was supershifted by the addition of anti-STAT1 (Figure 1, lane 4). No supershift, however, was observed by the addition of anti-STAT2, anti-STAT3, anti-STAT4, or anti-STAT6 (Figure 1, lanes 5–8). These data show that STAT1 is preferentially activated.

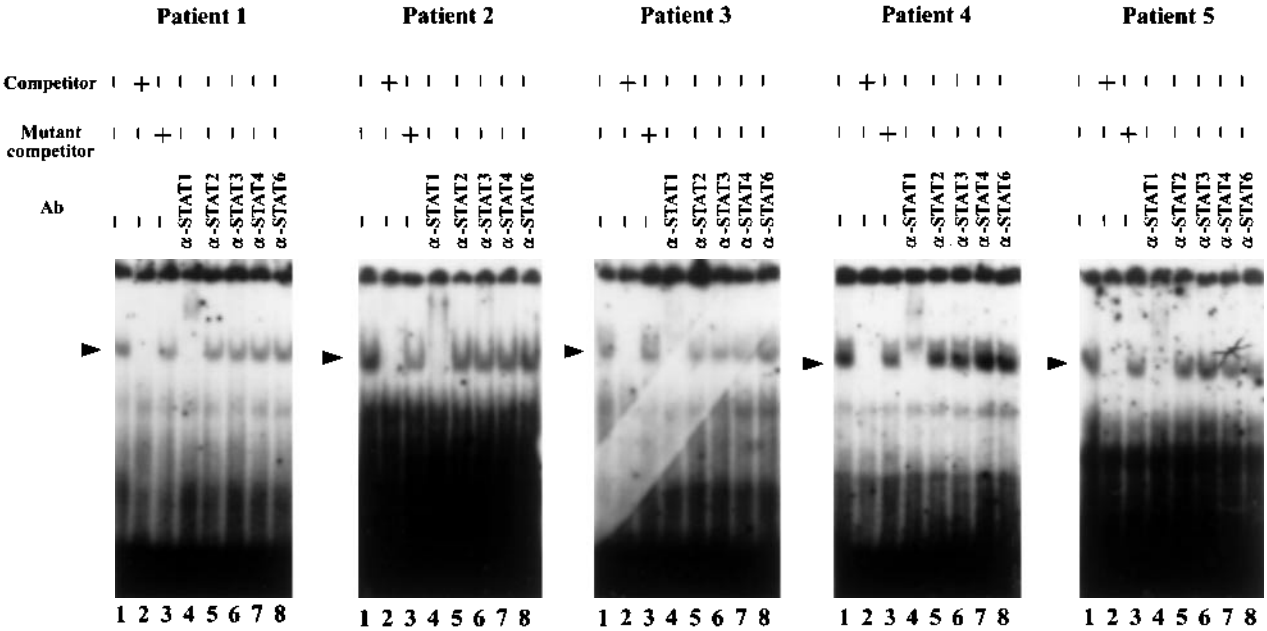

Figure 1. STAT-DNA formation in SF cells from patients with RA. Five micrograms of SF cell nuclear extract from RA patients (Patients 1–5) were assayed for binding to a double-stranded radiolabeled hSIE oligonucleotide using gel shift assays. STAT-DNA complexes were separated by electrophoresis in the absence (lane 1) or presence of 160-fold molar excess of unlabeled hSIE (lane 2), 160-fold molar excess of unlabeled mutant hSIE (lane 3), anti-STAT1 antibody (lane 4), anti-STAT2 antibody (lane 5), anti-STAT3 antibody (lane 6), anti-STAT4 antibody (lane 7), or anti-STAT6 antibody (lane 8). Arrows indicate specific complexes.

The expression level of STAT1 and STAT3 proteins was next examined in SF cells from these patients by immunoblot analysis using specific antisera against STAT1 and STAT3 in Figure 2. Although STAT protein levels are quite variable, both 91 kDa STAT1-specific and 92 kDa STAT3-specific bands were detected in both the STAT1 activation positive (Patients 1–4) and negative (Patients 9 and 10) patients with RA, and STAT protein levels were higher in STAT1 activation negative RA patients (Patients 9 and 10) than in some STAT1 activation positive RA patients (Patients 2 and 3), suggesting that DNA binding activity of STAT1 is not regulated by the protein expression level.

STAT1 can be activated by various kinds of cytokines, including IFN-γ, IFN-α, IL-2, IL-6, platelet derived growth factor and epidermal growth factor[7,9]. To identify which cytokine directly activates STAT1, SF was preincubated in the presence of neutralizing anti-IFN-γ or anti-IL-6 antibody for 2 days before nuclear extract preparation. Figure 3 shows the result for an RA case (Patient 6). The DNA-STAT formation (Figure 3, lane 2) was eliminated by the addition of 160-fold molar excess of unlabeled hSIE (Figure 3, lane 1). The band almost disappeared and was supershifted by the addition of anti-STAT1 (Figure 3, lane 6), but not by the addition of anti-STAT3 (Figure 3, lane 10), showing that STAT1 was preferentially activated as in other RA cases (Patients 1–5) shown in Figure 1. The STAT1-DNA formation was mostly prevented by preincubation of SF cells with neutralizing anti-IL-6 antibodies for 2 days before nuclear extract preparation (Figure 3, lane 4), but not by preincubation with anti-IFN-γ antibodies (Figure 3, lane 5) or the control goat antibody (Figure 3, lane 3). As less than 10% of granulocytes and none of the other cells died over 2 days of culture (data not shown), differences in DNA binding cannot be explained by alterations in cell type. The STAT1-DNA formation in other RA cases (Patients 1–5, 7, 8) was also prevented only by the anti-IL-6 antibody as well (data not shown). The absence of STAT1 activity in the presence of neutralizing anti-IL-6 suggests that IL-6 may be the cytokine responsible for STAT1 activation in RA.

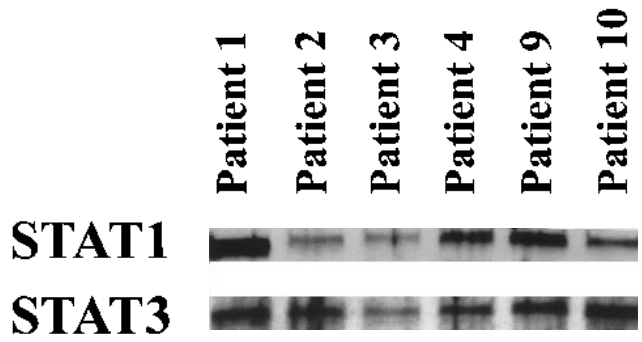

Figure 2. Expression of STAT proteins in SF cells from patients with RA. SF cells from STAT1 activation positive (Patients 1–4) and negative RA patients (patients 9, 10) were solubilized with 1% NP40 lysis buffer, and 5 μg of the cell lysate were analyzed by SDS-PAGE under reducing condition and subsequent immunoblotting with anti-STAT1 and -STAT3 antibodies.

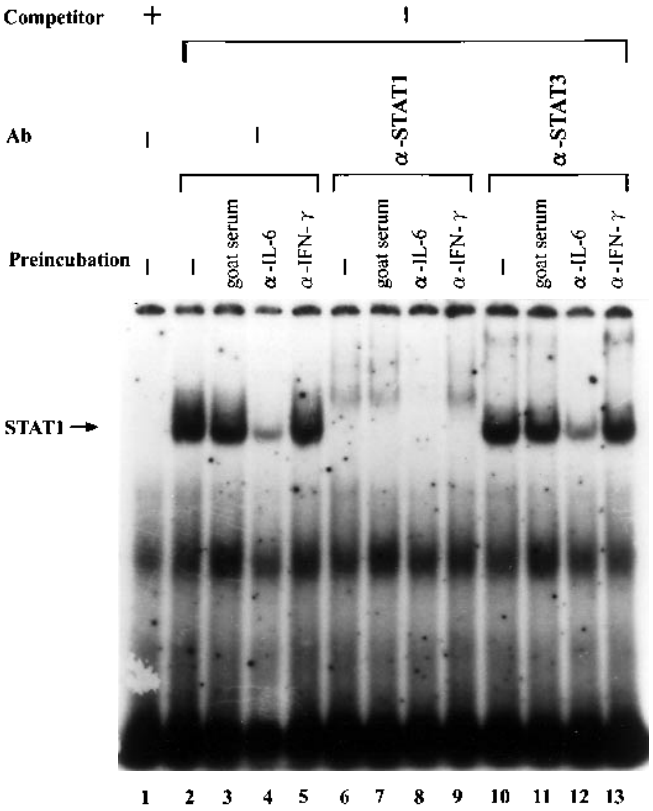

Figure 3. STAT-DNA formation after preincubation of rheumatoid SF cells with neutralizing antibodies against cytokines. SF cells from an RA patient (Patient 6) were cultured in the absence (lanes 1, 2, 6, 10) or presence of control goat serum (lanes 3, 7, 11), 20 μg/ml of goat antibody against IL-6 (lanes 4, 8, 12), or 20 μg/ml goat antibody against IFN-γ (lanes 5, 9, 13) for 2 days before nuclear extract preparation. Five micrograms of the nuclear extract were assayed for binding to a double-stranded radiolabeled hSIE oligonucleotide using gel shift assays. STAT-DNA complexes were analyzed in the absence (lanes 2–5) or presence of 160-fold molar excess of unlabeled hSIE (lane 1), anti-STAT1 antibody (lanes 6–9), or anti-STAT3 antibody (lanes 10–13). Arrow indicates specific complexes.

Cytokine production. We assayed IL-6 and IFN-γ levels in SF from patients with RA and OA using a chemiluminescent enzyme immunoassay and an ELISA, respectively. The results are shown in Tables 1 and 2.

IL-6 concentration was only weakly associated with STAT1 activation. Although IL-6 concentrations in RA patients with STAT1 activation (Patients 1–8) were more than 2102 pg/ml, some RA patients without STAT1 activation (Patients 9, 10, 14) and even some OA patients (Patients 16, 18–21) showed comparable IL-6 concentrations. STAT1 activation rather than IL-6 concentration appeared to be more closely associated with disease activity.

IFN-γ was detected only in trace amounts. This is consistent with the difficulty of several other groups to detect IFN-γ protein in inflamed joints[27,28], although a recent report by

Quayle, *et al* claimed that SF granulocytes are activated by IFN-γ[29]. As it is known from gene targeting experiments that STAT1 plays an essential and dedicated role in mediating IFN-dependent biologic responses[30,31], the lack of STAT1 activation in patients with detectable IFN-γ levels (87.9 pg/ml in Patient 9, 183.0 pg/ml in Patient 11, and 62.4 pg/ml in Patient 14) suggests that IFN-γ did not act on cells in these patients. The STAT1 activation in one patient with an IFN-γ concentration of 115.0 pg/ml (Patient 2) was not affected by neutralizing anti-IFN-γ antibody (Figure 3), suggesting that IFN-γ signaling was unlikely also in this patient.

IL-6 induction of STAT1 and STAT3 in different cell types. Although IL-6 was at first reported to activate STAT3 in hepatocytes[32,33], recent reports show that STAT1 can also be activated in response to IL-6 in other cell types such as fibroblasts, fibrosarcoma cells, and lymphocytes[34-37]. To determine the pathogenic significance of this preferential STAT1 usage in response to IL-6, we next examined which STAT is activated in IL-6 stimulated PB leukocytes, since they have cellular components similar to those of SF. STAT binding activity in IL-6 stimulated PB leukocytes from a healthy volunteer was examined (Figure 4). Two specific bands were detected 5 min after IL-6 stimulation (Figure 4A, lane 1). Both bands were eliminated by the addition of an excess of unlabeled probe (Figure 4A, lane 2), but not by that of a mutant probe containing 3 base substitutions within the STAT binding site (Figure 4A, lane 3). A lower nonspecific band was also observed, which was eliminated by the addition of either of the competitors (Figure 4A, lanes 2 and 3). Supershift assays revealed that these 2 specific bands represented activated STAT1 (Figure 4A, lane 4) and STAT3 (Figure 4A, lane 6). This finding contrasts to the preferential activation of STAT1 in the case of rheumatoid SF cells. Moreover, as shown in Figure 4B, DNA binding capacity disappeared at 3 h, suggesting that STAT activation in normal PB leukocytes is transient in contrast to the continuous activation in rheumatoid SF cells. As shown in Table 3, IL-6 concentration in the culture medium of normal PB leukocytes did not decrease after 3 h of IL-6 stimulation, implying that the decrease of STAT1 activation 3 h after IL-6 stimulation is not due to IL-6 degradation, but to induction of unresponsiveness to IL-6. Similarly, IL-6 level did not change in the SF of STAT1 activation positive rheumatoid SF cells cultured for 48 h without neutralizing antibodies. As continuous STAT1 activation in the rheumatoid SF cells was observed, it appears that unresponsiveness to IL-6 is lacking in these rheumatoid cells in contrast to normal PB leukocytes. In Figure 4C, immunoblot analysis revealed that both STAT1 and STAT3 proteins were constitutively expressed regardless of the DNA binding activity of these proteins. The same result was obtained by using PB leukocytes from RA patients whose SF cells contained activated STAT1 (data not shown).

Next, specific reactivity of anti-STAT1 and anti-STAT3 antibodies used in our assays was confirmed. As shown in Figure 5, nuclear extracts prepared from IL-6 stimulated

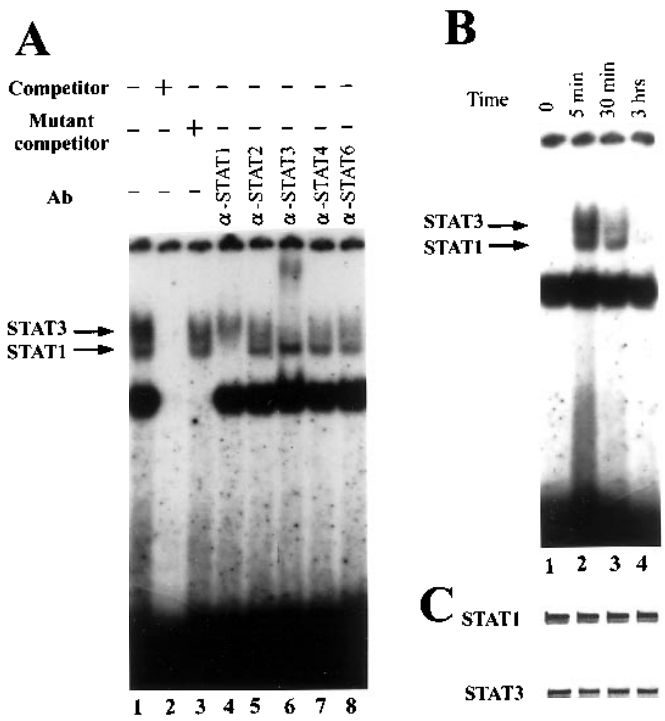

Figure 4. STAT binding activity and protein expression level in IL-6 stimulated PB leukocytes. PB leukocytes from healthy volunteers were cultured with 10 ng/ml recombinant human IL-6 before nuclear extract and cell lysate preparation. A. Five micrograms of the nuclear extract were assayed for binding to a double-stranded radiolabeled hSIE oligonucleotide using gel shift assays. The specific complexes are shown by arrows. Nuclear extracts obtained 5 min after IL-6 stimulation were assayed for DNA binding in the absence (lane 1) or presence of 160-fold molar excess of unlabeled hSIE (lane 2), 160-fold molar excess of unlabeled mutant hSIE (lane 3), or each of the anti-STAT antibodies indicated (lanes 4–8). B. Nuclear extracts obtained from IL-6 stimulated PB leukocytes for the time indicated were analyzed for DNA binding as in (A). C. PB leukocytes stimulated with IL-6 for the time indicated were solubilized with 1% NP40 lysis buffer, and the cell lysates were analyzed by SDS/PAGE under reducing condition and subsequent immunoblotting with anti- STAT1 and STAT3 antibodies.

Table 3. Levels of IL-6 remain constant in the culture medium of IL-6 stimulated normal PB leukocytes and in the rheumatoid SF cell culture without neutralizing antibodies.

Cell Type	IL-6 Concentration, pg/ml 0	3 h	48 h
PB leukocytes	9820.0	9754.0	ND
SF cells			
Patient 1	18040.0	ND	17210.0
Patient 2	25680.0	ND	28330.0
Patient 3	4853.0	ND	4751.0
Patient 4	2102.0	ND	2211.0
Patient 5	12280.0	ND	10320.0
Patient 6	7544.0	ND	7249.0
Patient 7	3469.0	ND	3528.0
Patient 8	8030.0	ND	7220.0

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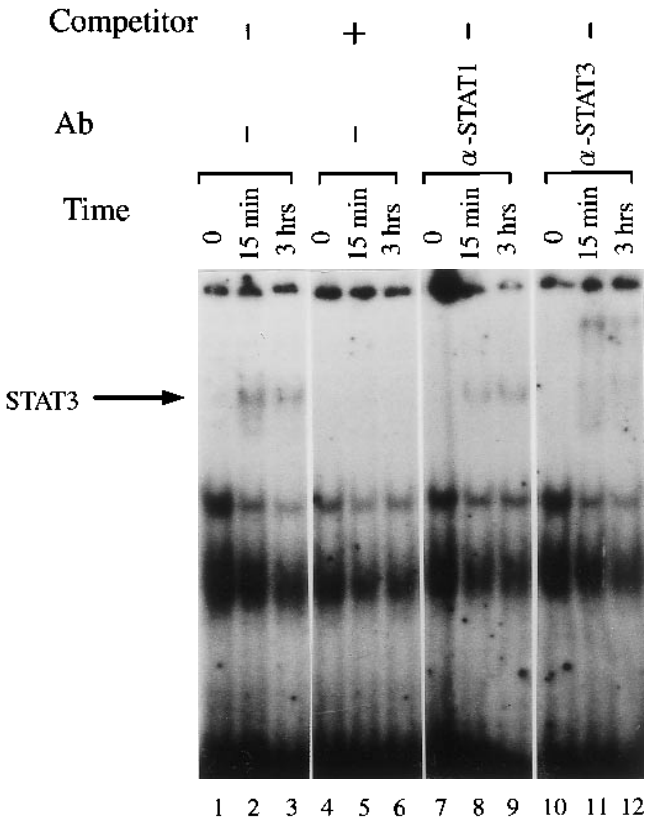

Figure 5. STAT binding activity in IL-6 stimulated Hep3B hepatocyte cells. Hep3B cells were cultured with 10 ng/ml of recombinant human IL-6 for the time indicated before nuclear extract preparation. Five micrograms of the nuclear extract were assayed for binding to a double-stranded radiolabeled hSIE oligonucleotide using gel shift assays. STAT-DNA complexes were analyzed in the absence (lanes 1–3) or presence of 160-fold molar excess of unlabeled hSIE (lanes 4–6), anti-STAT1 antibody (lanes 7–9), or anti-STAT3 antibody (lanes 10–12). Arrow indicates specific complexes.

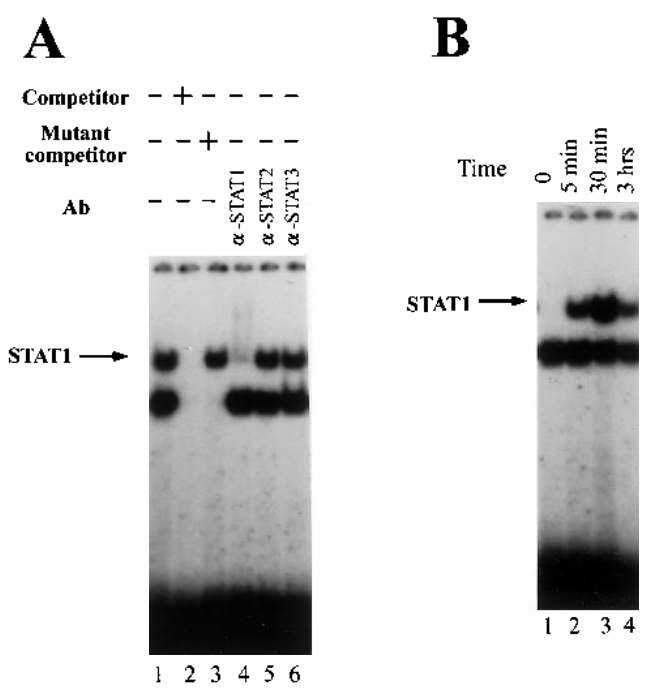

Figure 6. STAT binding activity in IFN-γ stimulated PB leukocytes. PB leukocytes from healthy volunteers were cultured with 100 IU/ml of recombinant human IFN-γ before nuclear extract preparation. Five micrograms of the nuclear extract were assayed for binding to a double-stranded radiolabeled hSIE oligonucleotide using gel shift assays. Arrows indicate specific complexes. A. Nuclear extracts obtained 5 min after IFN-γ stimulation were analyzed for DNA binding in the absence (lanes 1) or presence of 160-fold molar excess of unlabeled hSIE (lane 2), 160-fold molar excess of unlabeled mutant hSIE (lane 3), or each of the anti-STAT antibodies indicated (lanes 4–6). B. Nuclear extracts obtained from IFN-γ stimulated PB leukocytes for the time indicated were analyzed for DNA binding as in (A).

Hep3B hepatocyte cells at various time points were analyzed by gel shift assays. DNA binding of STAT was observed 15 min after IL-6 stimulation (Figure 5, lane 2), and then it decreased by 3 h (Figure 5, lane 3). DNA binding of STAT was eliminated by the addition of an excess of unlabeled probe (Figure 5, lanes 5 and 6). Supershift bands were observed by the addition of anti-STAT3 (Figure 5, lanes 11 and 12), but not by addition of anti-STAT1 (Figure 5, lanes 8 and 9), showing that STAT3 is selectively activated in response to IL-6 in hepatocytes, which is in agreement with previous reports[32,33]. In Figure 6, nuclear extracts prepared from IFN-γ stimulated PB leukocytes were analyzed by gel shift assays. DNA binding of STAT was observed 5 min after IFN-γ stimulation and then declined by 3 h (Figure 6B). This DNA-STAT formation was eliminated by the addition of an excess of unlabeled probe (Figure 6A, lane 2), but not by the addition of an excess of unlabeled mutant probe (Figure 6A, lane 3). Unlike in the case of Hep3B cells, the DNA-STAT complex disappeared and was supershifted by the addition of anti-STAT1 (Figure 6A, lane 4), but not by the addition of anti-STAT3 (Figure 6A, lane 6), showing that STAT1 is selectively activated in response to IFN-γ in PB leukocytes, which is in agreement with previous reports that IFN-γ activates only STAT1[7,30,31]. STAT1 and STAT3 were shown to be selectively activated in IFN-γ stimulated PB leukocytes and IL-6 stimulated hepatocytes, respectively, demonstrating the specific reactivity of anti-STAT1 and anti-STAT3 antibodies used in our assays.

DISCUSSION

As cytokines are important mediators of the immune and inflammatory systems, establishing their role in autoimmune diseases such as RA should provide useful information about the pathogenesis of this disease. We used gel shift assays to analyze the cytokine receptor signaling in SF cells from patients with synovitis. This approach makes possible the assessment of the cytokine action transduced into cells for various disease types. DNA binding of STAT was observed in none of the 10 OA patients versus 8 of the 14 patients with RA we studied. Supershift assays revealed

that, of several known STAT, STAT1 was preferentially activated. The elimination of STAT1 activity in the presence of neutralizing anti-IL-6 antibody suggested that IL-6 was the responsible cytokine. In addition, IL-6 concentrations in the SF were measured. Cytokines are known to be produced continuously in the rheumatoid joint regardless of disease type, duration, activity, or therapy[5,10,20]. This was also the case with the RA patients we studied, although IL-6 levels were generally higher in RA patients than in OA patients. It was noteworthy that rheumatoid disease activity appeared to be more closely related to STAT1 activation rather than to IL-6 levels.

The pattern of STAT activation in response to IL-6 described in reports to date shows activation of 2 of the STAT, STAT1 and STAT3[7,32-36]. STAT1 was preferentially activated in SF cells from 8 out of 14 patients with RA. IL-6 stimulated PB leukocytes from a healthy donor, whose cellular components were similar to those of SF cells, showed activation of both STAT1 and STAT3. As IL-6 activated STAT1 in both normal and rheumatoid leukocytes, it is unlikely that STAT1 activation in response to IL-6 is of specific importance in rheumatoid pathogenesis. The significance of the preferential STAT1 activation remains to be investigated. Also in contrast to our result, a recent report by Wang, *et al* identified STAT3, but not STAT1, activation in freshly isolated SF cells from patients with inflammatory arthritis, in spite of the use of an oligonucleotide probe with a sequence identical to the one we used (c-fos hSIE)[27]. The lack of STAT3 activity in our cases cannot be attributed to the use of an unsuitable anti-STAT3 antibody, as this antibody was shown to detect STAT3 activation in IL-6 stimulated Hep3B cells and PB leukocytes. We observed preferential activation of STAT1 in SF cells from 8 out of 14 RA cases. However, it might be necessary to examine additional cases, as there is such a case reported by Wang, *et al*.

IL-6 activated STAT1 in both normal and rheumatoid leukocytes. However, unlike the case of normal PB leukocytes stimulated with IL-6 *in vitro*, where STAT activation is transient — as shown by the disappearance of the STAT-DNA complexes 3 h after IL-6 stimulation, IL-6 receptor signaling through STAT1 in rheumatoid SF cells appears to be continuous. This constant action of cytokines might have pathogenic significance.

Differential activation of IL-6 dependent STAT1, especially between activated and resting T cells, has been described[37]. Therefore examining which SF cell types responded to IL-6 gives important information about the pathogenesis of this disease. However, we could not determine which SF cell types contained the activated STAT1, probably for technical reasons. We separated granulocytes, mononuclear cells, and fibroblastic synovial cells from SF cells, and analyzed STAT1 activation in each cell population in comparison with unseparated SF cells. DNA-STAT1 binding was observed in unseparated SF cells, but not in each separated population (data not shown). STAT1 activation appears to decline during the process of cell separation. The synovial environment may be necessary for continuous STAT1 activation.

STAT1 activation was observed in 8 out of 14 patients with RA we studied. Thus RA patients could be divided into 2 groups in terms of STAT1 activation. The IL-6 in RA patients without STAT activation ranged from trace levels (146.3 pg/ml) to abundant levels (30120.0 pg/ml). The observation that some RA patients showed high concentrations of IL-6 in spite of the lack of STAT activation (Patients 9, 10, 14) may suggest that the unresponsiveness to cytokines observed in physiological settings is retained in this group of RA patients. These patients showed low levels of C-reactive protein (CRP), and were apparently in a stable stage of the disease. Thus cytokine receptor signaling rather than cytokine level seems to be more useful for assessment of disease activity. This finding also suggests that progression of rheumatoid inflammation may be associated with an absence of unresponsiveness to cytokines. One patient (Patient 12) without STAT activation was exceptional in terms of disease activity. This patient showed rapid destruction of the joint accompanied by elevation of CRP. However, a very low level of IL-6 (265.2 pg/ml) was detected in this patient. As proinflammatory cytokines are linked in a network or cascade where IL-6 is induced by TNF-α and IL-1 in RA synovial cell cultures[38,39], other cytokines outside of this cascade may perform a pathogenic function in this patient. Thus, heterogeneous groups of disorders may be included in the disease of RA in terms of types of active cytokines and responsiveness to cytokines.

It is believed that the immunologic activity of synovial infiltrating T lymphocytes responding to an unknown antigen may initiate a cascade of events that accounts for many of the characteristic features of RA, such as persistent cell growth and activation, and cytokine production and its action. However, considerable uncertainty remains regarding the identification of the molecule of central importance in the pathogenesis. Our data showing continued IL-6 receptor signaling through STAT1 implies that the IL-6-STAT1 pathway is one of the key components in the pathogenesis of this disease, and that the lack or dysfunction of antiinflammatory molecules that downregulate cytokine signaling, such as the recently identified STAT-induced STAT inhibitor, might be responsible for the continued rheumatoid inflammation[40].

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REFERENCES

1. Cush JJ, Lipsky PE. Phenotypic analysis of synovial tissue and peripheral blood lymphocytes isolated from patients with rheumatoid arthritis. Arthritis Rheum 1988;31:1230-8.
2. Hemler ME, Glass D, Coblyn JS, Jacobson JG. Very late activation antigens on rheumatoid synovial fluid T lymphocytes. Association with stages of T cell activation. J Clin Invest 1986;78:696-702.
3. Burmester GR, Yu DTY, Irani A-M, Kunkel HG, Winchester RJ. Ia+ T

cells in synovial fluid and tissues of patients with rheumatoid arthritis. Arthritis Rheum 1981;24:1370-6.
4. Yokota A, Murata N, Saiki O, Shimiz M, Springer TA, Kishimoto T. High avidity state of leukocyte function-associated antigen-1 on rheumatoid synovial fluid T lymphocytes. J Immunol 1995; 155:4118-24.
5. Feldmann M, Brennan FM, Maini RN. Role of cytokines in rheumatoid arthritis. Annu Rev Immunol 1996;14:397-440.
6. Feldmann M, Brennan FM, Maini RN. Rheumatoid arthritis. Cell 1996;85:307-10.
7. Darnell JE Jr, Kerr IM, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 1994;264:1415-21.
8. Kishimoto T, Taga T, Akira S. Cytokine signal transduction. Cell 1994;76:253-62.
9. Taniguchi T. Cytokine signaling through nonreceptor protein tyrosine kinases. Science 1995;268:251-5.
10. Arend WP, Dayer J-M. Inhibition of the production and effects of interleukin-1 and tumor necrosis factor α in rheumatoid arthritis. Arthritis Rheum 1995;38:151-60.
11. Chu CQ, Field M, Feldmann M, Maini RN. Localization of tumor necrosis factor-α in synovial tissues and the cartilage-pannus junction in patients with rheumatoid arthritis. Arthritis Rheum 1991; 34:1125-32.
12. Miyasaka N, Sato K, Goto M, et al. Augmented interleukin 1 production and HLA-DR expression in the synovium of rheumatoid arthritis patients: possible involvement in joint destruction. Arthritis Rheum 1988;31:480-6.
13. Firestein GS, Alvaro-Gracia JM, Maki R. Quantitative analysis of cytokine gene expression in rheumatoid arthritis. J Immunol 1990;144:3347-53.
14. Hirano T, Matsuda T, Turner M, et al. Excessive production of interleukin 6/B cell stimulatory factor-2 in rheumatoid arthritis. Eur J Immunol 1988;18:1797-801.
15. Saklatvala J, Sarsfield SJ, Townsend Y. Purification of two immunologically different leukocyte proteins that cause cartilage resorption, lymphocyte activation and fever. J Exp Med 1985;162:1208-15.
16. Gowen M, Wood DD, Ihrie EJ, McGuire MKB, Russell RG. An interleukin-1 like factor stimulates bone resorption in vitro. Nature 1983;306:378-80.
17. Dayer J-M, Beutler B, Cerami A. Cachectin/tumor necrosis factor stimulates collagenase and prostaglandin E2 production by human synovial cells and dermal fibroblasts. J Exp Med 1985;162:2163-8.
18. Dayer J-M, de Rochemonteix B, Burrus B, Semczuk S, Dinarello CA. Human recombinant interleukin 1 stimulates collagenase and prostaglandin E2 production by human synovial cells and dermal fibroblasts. J Exp Med 1986;77:645-8.
19. Roodman GD. Interleukin 6: an osteotrophic factor? J Bone Miner Res 1992;7:475-8.
20. di Giovine FS, Nuki G, Duff GW. Tumor necrosis factor in synovial exudates. Ann Rheum Dis 1988;47:768-72.
21. Arnett FC, Edworthy SM, Bloch DA, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315-24.
22. Bronstein I, Edwards B, Voyta JC. 1,2-Dioxetanes: Novel chemiluminescent enzyme substrates. Applications to immunoassays. J Biolumin Chemilumin 1989;4:99-111.
23. Schreiber E, Matthias P, Muller MM, Schaffner W. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. Nucleic Acids Res 1989;17:6419.
24. Sengupta TK, Chen A, Zhong Z, Darnell JE Jr, Ivashkiv LB. Activation of monocyte effector genes and STAT family transcription factors by inflammatory synovial fluid is independent of interferon γ. J Exp Med 1995;181:1015-25.
25. Sadowski HB, Shuai K, Darnell JE Jr, Gilman MZ. A common nuclear signal transduction pathway activated by growth factor and cytokine receptors. Science 1993;261:1739-44.
26. Zhong Z, Wen Z, Darnell JE Jr. Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. Science 1994;264:95-8.
27. Wang F, Sengupta TK, Zhong Z, Ivashkiv LB. Regulation of the balance of cytokine production and the signal transducer and activator of transcription (STAT) transcription factor activity by cytokines and inflammatory synovial fluids. J Exp Med 1995;182:1825-31.
28. Firestein G, Zvaifler N. Peripheral blood and synovial fluid monocyte activation in inflammatory arthritis. II. Low levels of synovial fluid and synovial tissue interferon suggest that γ-interferon is not the primary macrophage activating factor. Arthritis Rheum 1987; 30:864-71.
29. Quayle JA, Watson F, Bucknall RC, Edwards SW. Neutropils from the synovial fluid of patients with rheumatoid arthritis express the high affinity immunoglobulin G receptor, FcgRI (CD64): role of immune complexes and cytokines in induction of receptor expression. Immunology 1997;91:266-73.
30. Meraz MA, White JM, Sheehan KCF, et al. Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. Cell 1996;84:431-42.
31. Durbin JE, Hackenmiller R, Simon MC, Levy DE. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. Cell 1996;84:443-50.
32. Akira S, Nishio Y, Inoue M, et al. Molecular cloning of APRF, a novel IFN-stimulated gene factor 3 p91-related transcription factor involved in the gp130-mediated signaling pathway. Cell 1994;77:63-71.
33. Wegenka UM, Lutticken C, Buschmann J, et al. The interleukin-6-activated acute-phase response factor is antigenically and functionally related to members of the signal transducer and activator of transcription (STAT) family. Mol Cell Biol 1994;14:3186-96.
34. Schindler C, Darnell JE Jr. Transcriptional responses to polypeptide ligands: The JAK-STAT pathway. Annu Rev Biochem 1995; 64:621-51.
35. Guschin D, Rogers N, Briscoe J, et al. A major role for the protein tyrosine kinase JAK1 in the JAK/STAT signal transduction pathway in response to interleukin-6. EMBO J 1995;14:1421-9.
36. Gerhartz C, Heesel B, Sasse J, et al. Differential activation of acute phase response factor/STAT3 and STAT1 via the cytoplasmic domain of the interleukin 6 signal transducer gp130. J Biol Chem 1996;271:12991-8.
37. Teague TK, Schaefer BC, Hildeman D, et al. Activation-induced inhibition of interleukin 6-mediated T cell survival and signal transducer and activator of transcription 1 signaling. J Exp Med 2000;191:915-25.
38. Brennan FM, Chantry D, Jackson A, Maini R, Feldmann M. Inhibitory effect of TNF-α antibodies on synovial cell interleukin-1 production in rheumatoid arthritis. Lancet 1989;2:244-7.
39. Butler D, Maini RN, Feldmann M, Brennan FM. Blockade of TNFα with chimeric anti-TNFα monoclonal antibody, cA2 reduces IL-6 and IL-8 release in RA MNC cultures: A comparison with IL-1 ra. Eur Cytokine Netwk 1995;6:225-30.
40. Naka T, Narazaki M, Hirata M, et al. Structure and function of a new STAT-induced STAT inhibitor. Nature 1997;387:924-9.