Activation of Monocyte Effector Genes and STAT Family Transcription Factors by Inflammatory Synovial Fluid Is Independent of Interferon γ

By Tapas K. Sengupta,* Amy Chen,* Zhong Zhong,§ James E. Darnell, Jr.,§ and Lionel B. Ivashkiv*‡

*From the *Department of Medicine, Hospital for Special Surgery, ‡Graduate Program in Immunology, Cornell University Graduate School of Medical Sciences, New York 10021; and §Laboratory of Molecular Cell Biology, The Rockefeller University, New York 10021*

Summary

Activated monocytes play an important role in the pathogenesis of inflammatory arthritis. Blood monocytes which enter the inflamed joint become activated upon adherence to extracellular matrix and exposure to a complex inflammatory environment. We have analyzed the mechanism of monocyte activation by soluble factors present in inflammatory synovial fluid (SF). Greater than 75% of inflammatory SFs tested (a total of 22 fluids to date) increased cell surface expression and dramatically increased mRNA levels of monocyte activation markers FcγRI, FcγRIII, and HLA-DRA. This induction was not triggered by adherence, a known activating stimulus, and several lines of evidence showed that induction was not dependent upon interferon γ (IFN-γ). Induction was not prevented by neutralizing anti-IFN-γ antibodies and IFN-γ was not detected in the SFs using a sensitive enzyme-linked immunosorbent assay. The SFs also were not able to activate the IFN-γ–activated transcription factor Stat1, thus providing further support for the absence of IFN-γ. SFs did activate a related signal transducer and activator of transcription (STAT) family factor, termed Stat-SF, which bound specifically to the IFN-γ response region (GRR), a well-characterized transcription element in the FcγRI promoter. Based upon DNA-binding specificity and mobilities in gel shift assays, and reactivity with specific antisera, Stat-SF likely contains Stat3, or a closely related STAT family member. Neutralization of interleukin 6, a cytokine present in SFs which is known to activate Stat3, abolished the activation of Stat-SF and inhibited the induction of FcγRI expression by SFs. These results demonstrate the activation of monocytes by inflammatory SF and suggest that monocyte activation at an inflammatory site may occur in the absence of IFN-γ through the triggering of signal transduction pathways that activate STAT transcription factors.

Activated monocytes are found in the inflamed synovium of patients with inflammatory arthritis, such as rheumatoid arthritis (RA)[1]. These monocytes express high levels of immune effector molecules such as HLA class II antigens, Fc receptors (FcR), proteases, cytokines, and chemotactic factors (1–15 and references therein). Activated monocytes may contribute to the pathogenesis of inflammatory arthritis through several mechanisms, including increased (auto)antigen presentation by class II molecules, increased cytotoxicity and release of toxic reactive oxygen intermediates mediated by FcR, degradation of connective tissue by proteases, stimulation of inflammation by secretion of proinflammatory cytokines, and recruitment of new inflammatory cells to the joint by secreted chemotaxins.

Multiple pro-inflammatory mediators have been measured in the inflamed joint (1–15), but the identity of the major monocyte activator(s) in vivo has not been conclusively demonstrated. IFN-γ, a potent monocyte activator which can activate many complex effector functions, has been proposed to activate monocytes in inflammatory arthritis. The role of IFN-γ, however, is controversial because it has been difficult to detect IFN-γ in synovial fluid (SF) specimens (1–4, 6, 9, 13, 14), and indeed, one group has suggested that IFN-γ synthesis is actively suppressed during synovitis (9). Other factors which have been detected in the inflamed joints of patients, such as IL-1, IL-6, IL-8, TNF-α, platelet-derived growth factor (PDGF), GM-CSF, and complement breakdown products, have been postulated to be important in

[1] *Abbreviations used in this paper:* EU, endotoxin unit; GRR, IFN-γ response region; OA, osteoarthritis; PDGF, platelet-derived growth factor; RA, rheumatoid arthritis; SF, synovial fluid; STAT, signal transducer and activator of transcription.

driving monocyte activation and synovial inflammation. However, it is unclear whether these factors are present in a bioactive form (9, 16). Furthermore, since the biological effect of a cytokine can be altered or even switched to have the completely opposite effect in the presence of other stimuli (16–18), it is difficult to predict the role of a cytokine in vivo based upon studies with individual purified cytokines in vitro.

Monocyte activation is a complex process that can be triggered by multiple stimuli, which often act in concert (19). Since different stimuli can trigger different effector functions, the functional characteristics of the activated monocyte can vary. IFN-γ and bacterial endotoxin are the most potent monocyte-activating factors, and are capable of activating complex effector functions such as antigen presentation and cytotoxicity. Other stimuli, such as the cytokines IL-1, IL-6, TNF-α, and GM-CSF, or adherence to extracellular matrix, have been shown to induce monocyte "early genes" or other cytokine genes. The effect of these stimuli upon monocyte phenotype or effector functions has not been as well characterized as the effect of IFN-γ, but they appear to trigger a more limited cellular response (19, 20). Thus, activation of monocyte effector functions in the absence of IFN-γ or endotoxin may depend upon the concerted action of several activating stimuli.

A large number of cytokines, growth factors, and the IFNs have been shown to activate the JAK family of tyrosine kinases, which phosphorylate and activate the STAT (signal transducers and activators of transcription) transcription factors (21). To date, five distinct but homologous members of the STAT family have been identified and designated Stat1 through Stat5. STAT proteins are localized to the cytoplasm or the plasma membrane in resting cells. After activation, the STATs dimerize, translocate to the nucleus, and acquire DNA-binding activity. An individual STAT protein may be activated by multiple ligands, but certain ligands preferentially activate particular STATs (21). For example, IFN-γ preferentially activates Stat1 (previously designated IFN-γ activated factor [GAF], p91, or Stat91), and IL-6 preferentially activates Stat3. Stat3, which is contained in complexes that have been termed serum induced factor A (SIF-A) or acute phase response factor (APRF) (22–24), is activated by several stimuli which have been detected in inflammatory SFs, including IL-6, IL-10, IFN-α, PDGF, and leukemia inhibitory factor (LIF) (21–24).

We have chosen to study the response of monocytes to a complex inflammatory environment that attempts to reproduce in vivo conditions, rather than the response to purified cytokines. In our experiments, we have modeled the in vivo process of monocyte migration into an inflamed joint by simultaneously adhering purified human blood monocytes to a substrate and exposing them directly to SF. A majority of the SF samples induced several monocyte activation markers such as FcR and HLA DR antigens. This induction appears to be independent of IFN-γ but does involve the triggering of a signal transduction pathway that activates a STAT transcription factor, termed Stat-SF, closely related to Stat3. IL-6 in SFs is important for Stat-SF activation, and is necessary, but not sufficient, for induction of FcγRI expression. This in vitro cell system can be used to further characterize the activated phenotype and to explore the molecular mechanism of monocyte activation by a complex inflammatory stimulus.

Materials and Methods

Monocyte Isolation and Culture. Mononuclear cells from disease-free volunteers were obtained from whole blood or buffy coats (New York Blood Center, New York) by density gradient centrifugation using Ficoll metrizoate (Lymphoprep; GIBCO BRL, Gaithersburg, MD; endotoxin <0.25 endotoxin units [EU]/ml). Monocytes were further purified utilizing an aggregation method (25) that minimizes activation. Briefly, monocytes were aggregated for 45 min at 4°C at a monocyte density of 3.5–4.5 $\times$ 10^6/ml (estimated by myeloperoxidase staining) and purified by unit gravity sedimentation through fetal bovine serum (FBS) (Hyclone; endotoxin <0.06 EU/ml). Cell aggregates were disrupted and platelets completely removed by two washes with HBSS/1 mM EDTA and cells were cultured adherently on plastic dishes (Corning Inc., Corning, NY) in complete medium (AIM V medium; [GIBCO BRL; endotoxin <0.6 EU/ml] supplemented with 10% heat-inactivated human serum). IFN-γ (Genzyme Corp., Cambridge, MA; endotoxin <0.1 ng/μg) was added to purified monocytes immediately before plating. Neutralizing goat anti-IFN-γ, anti-IL-6, anti-TGF-β, anti-IL-10 IgG, control goat IgG, and a mAb against IL-6 were purchased from R&D Systems Inc., Minneapolis, MN. Pilot experiments demonstrated that these antisera neutralized exogenously added cytokines both in the absence or presence of SF.

Serum sources included autologous serum, a serum bank set up locally, and AB serum (GIBCO BRL) and gave similar results. Monocytes were >90% pure as assayed by cell surface expression of CD14 or the CD32 epitope recognized by mAb IV.3 (Medarex, New Lebanon, NH). In some experiments, monocytes were further purified to >97% purity (no CD3-positive cells detectable by flow cytometry) by removing additional T and B cells using magnetic beads (Dynal, Inc., Great Neck, NY) and gave similar results. Lack of monocyte activation during isolation was confirmed by lack of induction of IL-1 mRNA as measured by Northern analysis and RT-PCR. The total number of monocyte donors tested was greater than 40. The results obtained with different donors were qualitatively similar, even though the magnitude of induction varied. Throughout all experimental procedures, a scrupulous effort was made to avoid endotoxin contamination, including purchasing reagents that had been tested for endotoxin (GIBCO BRL; Hyclone Labs, Logan, UT; Genzyme Corp.), minimizing the use of glassware (and baking all glassware used at 190°C for 4 h), and by use of a water source (Millipore Corp., Bedford, MA) that contained <0.06 EU/ml of endotoxin as measured by the E-TOXATE *Limulus* amebocyte lysate assay (Sigma Chemical Co., St. Louis, MO).

SFs. SFs were obtained from patients with definite and classic RA, as defined by the American College of Rheumatology criteria, by the patients' physicians for medically indicated reasons. Fluids were handled using sterile technique, centrifuged for 10 min at 10,000 *g* to remove cells and particulate debris, aliquoted, and stored at −80°C. In some experiments, SFs filtered through a 22-μm filter were used and gave similar results. SFs were added to purified monocytes before plating.

Flow Cytometric Analysis. Flow cytometric analysis was performed using an Ortho Diagnostics Systems Cytofluorograph IIs (Westwood, MA). The following murine mAbs were used: Leu-M3 (anti-CD14; Becton Dickinson & Co., Mountain View, CA), 4B5 (anti-CD3; Boehringer Mannheim, Indianapolis, IN), IV.3 (anti-CD32), 32.2 (anti-FcγRI), 3G8 (anti-CD16), and TAL.1B5

(anti-HLA-DR; Dako Corp., Carpinteria, CA). IgG1 (MOPC 21) and IgG2a (UPC10) isotype controls were obtained from Sigma Chemical Co. Cells were washed with FACS media (HBSS with 0.1% BSA) and incubated for 30 min at 4°C with a saturating amount of mAb. Cells were then washed twice and incubated with PE-conjugated goat $F(ab')_2$ fragment anti-mouse Ig (Biosource International, Camarillo, CA/Tago, Inc., Burlingame, CA). Cells were analyzed after two additional washes.

Preparation of Cellular RNA and Northern Hybridization Analysis. Total cellular RNA was isolated using RNAzol (TM Cinna Scientific, Friendswood, TX) according to the instructions of the manufacturer. 2–5 μg of RNA was fractionated on 1.2% formaldehyde agarose gels, transferred to Hybond-N membranes, and hybridized with random primer–labeled (Boehringer Mannheim) DNA probes using standard techniques (26). DNA probes used were: FcγRI 1.3-kb XhoI fragment (27), FcγRIII (CD16) 0.8-kb HindIII-BamH1 fragment (28), HLA-DRA 0.5-kb PstI fragment (29), IL-1 1.0-kb HindIII fragment (30), and GAPDH 1.2-kb EcoRI fragment (31). Laser densitometry was performed using a model 300 Series Computing Densitometer with ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA).

ELISA. The IFN-γ–specific ELISA was used according to the instructions of the manufacturer (Biosource International). SF samples were reduced and alkylated as described (15, 32) to inactivate RF. Pilot experiments showed that pretreatment of SFs with hyaluronidase (4) did not significantly affect detection of IFN-γ. The standard curves of IFN-γ were generated using serial dilutions of IFN-γ (Genzyme Corp; sp act, 2.5×10^7 U/mg) diluted in SF or 10% normal human serum (15) and were similar. The standard samples were reduced and alkylated in the same manner as the SF samples. Reduction and alkylation did not significantly affect the measurement of IFN-γ.

Nuclear and Cellular Extract Preparation. A nuclear mini-extract procedure (33) was used with modifications. 7×10^6 monocytes were washed in HBSS, resuspended, and incubated in buffer A (10 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM Pefablock SC (Boehringer Mannheim) and 20 μg/ml of aprotinin, leupeptin, antipain, and pepstatin A) for 10 min at 4°C. NP-40 was added to a final concentration of 1% and after a 1-min incubation, nuclei were pelleted in a microcentrifuge, washed in buffer A, and lysed in 20 μl of buffer C (10 mM Hepes, 420 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, and the same protease inhibitors as buffer A). After 20 min of incubation at 4°C, insoluble chromatin was pelleted by centrifugation, and the supernatant was aliquoted and stored at −80°C. Total cell extracts were prepared by lysis in buffer containing 20 mM Hepes (pH 7.0), 300 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, 0.1% Triton X-100, 0.5 mM DTT, 200 μM PMSF, and 20% glycerol as described (24). The protein concentration of extracts was determined using the Bradford assay.

DNA Binding and Gel Shift Assays. 3 μg of nuclear extract or 8 μg of cell extract was incubated with 0.2 or 0.5 ng of ^{32}P-labeled, double-stranded oligonucleotide probe as described (34), except that incubation was for 15 min on ice in a 10-μl reaction containing 80 mM KCl and 2 μg of poly-dI-dC (Pharmacia, Piscataway, NJ) (nuclear extracts) and a 15-μl reaction containing 100 mM NaCl and 1 μg of poly-dI-dC (cell extracts). In competition experiments, an excess of unlabeled competitor oligonucleotide was incubated for 5 min with the nuclear extract before addition of labeled probe. Oligonucleotide sequences are: Ly-6E GAS, 5′ CATGTTATGCATATTCCTGTAAGTG (35); hSIE, 5′ GTCGACATTTCCCGTAAATCGTCGA (24); GRR, 5′ AGCATGTTTCAAGGATTTGAGATGTATTTCCCAGAAAAG (36); mutant GRR, 5′ AGCATGTTTCAAGGATTTGAGATGTAT*GGACCAGACA*AG (STAT binding sites are underlined; mutated bases are in italics). In supershift experiments, 1 μl of a 1:10 dilution of specific antiserum (24) was added to extracts for 15 min before adding radiolabeled probe. Samples were resolved on 4 or 5% polyacrylamide gels in 0.25× TBE buffer at 11 V/cm at room temperature. Gels were dried and subjected to autoradiography.

Results

SF Increase Cell Surface Expression of FcγRI, HLA DR, and FcγRIII (CD16). We screened a panel of SFs for the ability to induce cell surface expression of the monocyte activation markers FcγRI, HLA DR, and FcγRIII (CD16). The results of a representative flow cytometry experiment are shown in Fig. 1. SF1, derived from an RA patient, significantly increased cell surface expression of all three activation makers. The induction of FcγRI and HLA DR expression was comparable with that seen with 100 U/ml (4 ng/ml) of IFN-γ. A neutralizing anti-IFN-γ antibody prevented induction by IFN-γ (Fig. 1, *A* and *C*), but not by SF1 (Fig. 1, *B* and *D*). The slight inhibition of SF induction of FcγRI (Fig. 1 *B*) by anti-IFN-γ antibody was not detected consistently. These results suggest SF-triggered induction of FcγRI and HLA DR expression is mediated by a factor(s) other than IFN-γ. SF1 triggered a dramatic induction of FcγRIII expression (Fig. 1 *E*), consistent with previous work which has attributed this effect to the presence of TGF-β (11).

In these experiments, both control and SF- and cytokine-treated monocytes were plated and allowed to adhere simultaneously with exposure to the soluble stimuli. This approach mimics the in vivo situation, where monocytes migrate into an inflamed joint and adhere to a connective tissue substrate as they become exposed to the inflammatory environment. Although, consistent with other reports (19, 20), adherence induced expression of fos, IL-1, and activation of NF-κB, it did not affect expression of FcγRI, FcγRIII, or HLA DR when compared to freshly isolated monocytes, or monocytes cultured nonadherently in TEFLON dishes (data not shown).

Table 1 summarizes the results of screening 22 RA SFs. We chose to initially focus upon RA SFs because RA is a relatively specific diagnosis and joint inflammation in RA has been studied extensively (1–15). 17 out of the 22 RA SFs tested induced expression of FcγRIII, confirming results obtained in a different system that used nonadherent culture of monocytes (11). Greater than 80% of the RA SFs tested induced FcγRI and HLA DR expression, and most SFs induced all three activation markers. The major known inducer of both FcγRI and HLA DR is IFN-γ. However, since experiments with neutralizing antibodies against IFN-γ did not prevent induction by SFs (Fig. 1; data not shown), other agents present in the fluid may be responsible. The induction of the markers we have examined does not appear to be specific to RA, since our limited survey of SFs obtained from patients with osteoarthritis (OA) or chronic traumatic effusions (Table 1), and seronegative arthritis (data not shown), showed in-

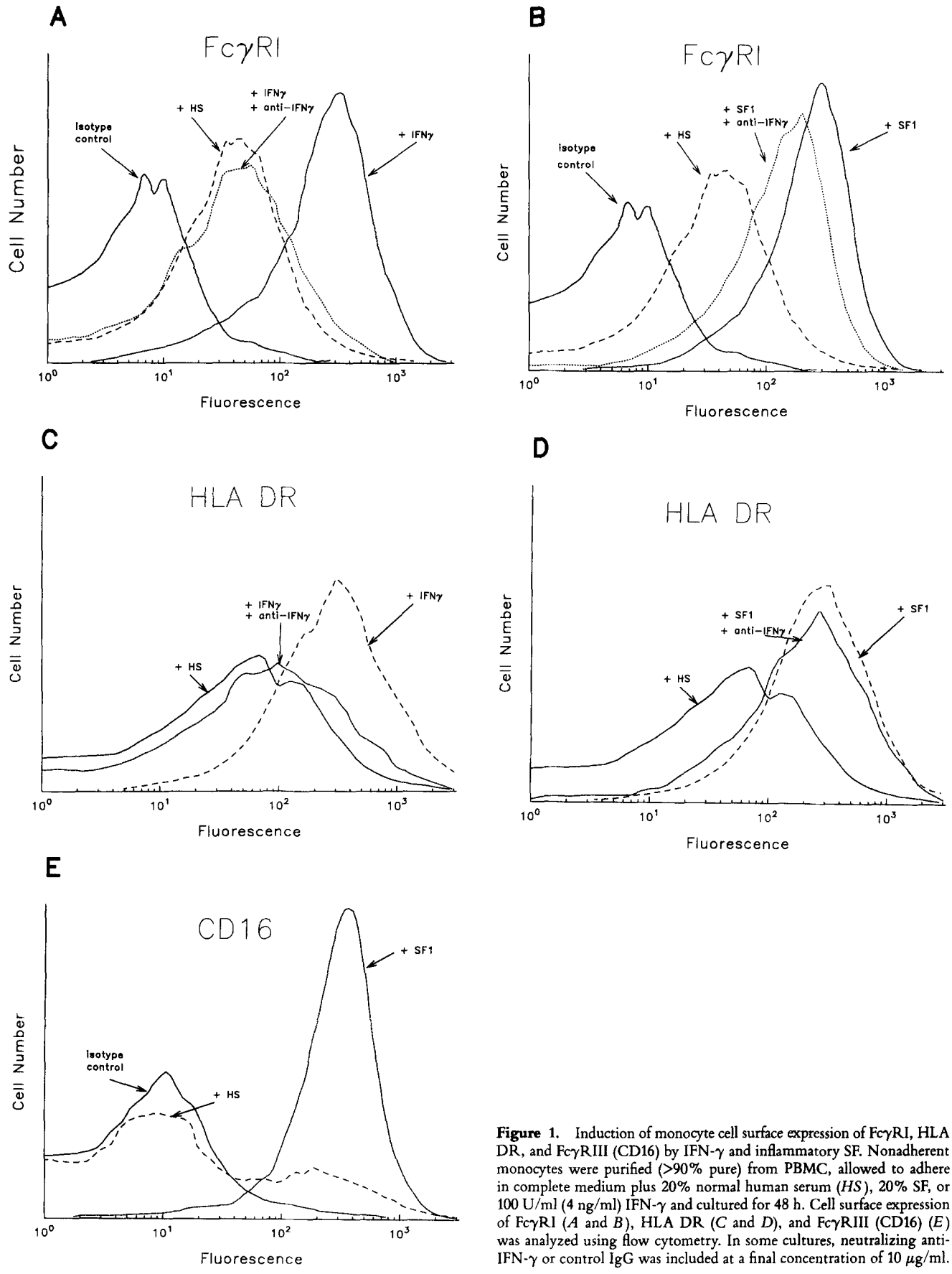

Figure 1. Induction of monocyte cell surface expression of FcγRI, HLA DR, and FcγRIII (CD16) by IFN-γ and inflammatory SF. Nonadherent monocytes were purified (>90% pure) from PBMC, allowed to adhere in complete medium plus 20% normal human serum (*HS*), 20% SF, or 100 U/ml (4 ng/ml) IFN-γ and cultured for 48 h. Cell surface expression of FcγRI (*A* and *B*), HLA DR (*C* and *D*), and FcγRIII (CD16) (*E*) was analyzed using flow cytometry. In some cultures, neutralizing anti-IFN-γ or control IgG was included at a final concentration of 10 μg/ml.

Table 1. *Induction of Monocyte Cell Surface Markers by SFs*

	FcγRI	FcγRIII	HLA DR
RA	21/22	17/22	13/16
OA	1/3	2/3	1/3
Trauma*	1/2	0/2	ND

Fluids obtained from patients with RA, OA, and traumatic effusions were tested using flow cytometry for induction of monocyte cell surface expression of FcγRI, FcγRIII, and HLA DR. The numerator depicts the number of fluids that induced expression; the denominator, the total number of fluids tested. Positive induction was defined as a greater than twofold increase in mean cell surface fluorescence (FcγRI and FcγRIII) and an increase comparable with that induced by 100 U/ml (4 ng/ml) IFN-γ (HLA DR).

* Chronic effusions secondary to mechanical injury which required arthroscopy.

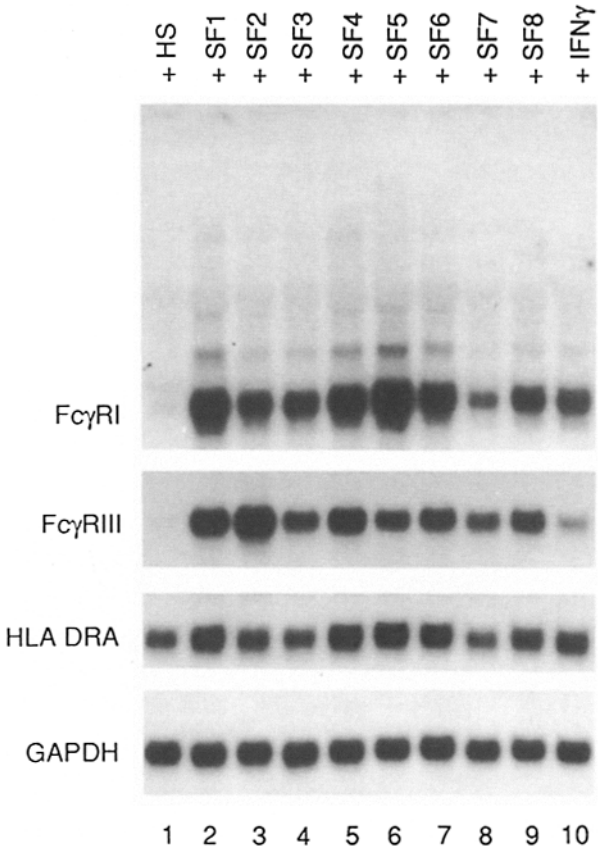

Figure 2. Induction of FcγRI, FcγRIII (CD16), HLA DRA mRNA levels by inflammatory SF. Nonadherent purified blood monocytes were allowed to adhere in complete medium plus 20% normal human serum (HS; lane *1*), 20% SF obtained from eight different RA patients (lanes *2–9*), or 100 U/ml (4 ng/ml) IFN-γ (lane *10*) and cultured for 24 h. 5 μg of total cellular RNA was fractionated on an agarose formaldehyde gel, transferred to a nylon membrane, and hybridized with radiolabeled cDNA probes.

duction of these markers. Both OA (37) and trauma such as meniscal tears may result in a disease process which has an inflammatory component which may contribute to the development of a joint effusion. Our data show that a majority of SFs obtained from RA patients who are felt to have active disease by clinical criteria are capable of inducing several monocyte activation markers suggestive of the triggering of a complex activation program.

Increased Cell Surface Expression of FcγRI, FcγRIII, and HLA DR Is Associated with Increased Steady State Levels of mRNA. We determined the effect of SF treatment on FcγRI, FcγRIII, and HLA DRA mRNA levels using Northern hybridization analysis (Fig. 2). All eight SFs selected on the basis of induction of cell surface expression of FcγRI also induced FcγRI mRNA levels, and all eight fluid induced mRNA levels of the CD16 subunit of FcγRIII. The induction of HLA DRA mRNA expression was much less dramatic (Fig. 2), consistent with a high baseline level of expression and the lower induction detected by flow cytometry. However, laser densitometry and comparison to levels of the GAPDH housekeeping gene showed that six out of the eight fluids tested increased HLA DRA mRNA levels to a degree comparable to the twofold induction seen with 100 U/ml (4 ng/ml) IFN-γ (Fig. 2, lane *10*). Fig. 3 demonstrates that SF1 increased mRNA levels of FcγRI and FcγRIII, but not GAPDH, in a dose-dependent manner. The induction of HLA DRA mRNA by RA SF1 was near maximal with 10% SF, was fivefold in this experiment, and varied between two- and fivefold, depending upon the donor (monocytes from five different donors have been tested; data not shown).

SF Levels of IFN-γ Are Low or Undetectable. IFN-γ is a major monocyte activator and is a strong inducer of FcγRI and HLA DR. However, it has been difficult to detect IFN-γ in inflamed joints, and the role of IFN-γ in synovial inflammation is controversial (1–4, 6, 9, 13, 14). The inability of neutralizing anti-IFN-γ antibody to prevent SF induction of FcγRI or HLA DRA surface expression (Fig. 1 and data not

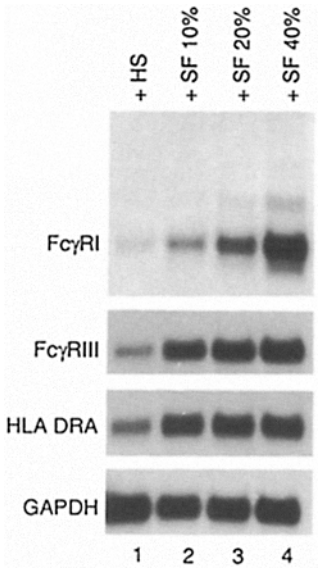

Figure 3. Dose response of induction of mRNA levels by inflammatory SF. Purified blood monocytes were allowed to adhere in complete medium plus 20% normal human serum (*HS*, lane *1*), 10% SF (lane *2*), 20% SF (lane *3*), and 40% SF (lane *4*) obtained from an RA patient and cultured for 24 h. 5 μg of total cellular RNA was fractionated on an agarose formaldehyde gel, transferred to a nylon membrane, and hybridized with radiolabeled cDNA probes.

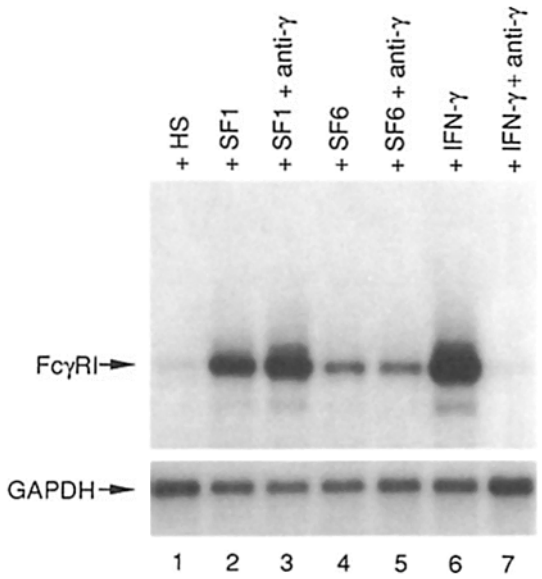

Figure 4. Neutralizing anti-IFN-γ antibody does not inhibit induction of FcγRI mRNA by SFs. Purified blood monocytes were cultured for 24 h in complete medium plus 20% normal human serum (*HS*) or SF. IFN-γ (200 U/ml = 8 ng/ml) and neutralizing anti-IFN-γ antibody (10 μg/ml) were added at the beginning of the culture. 2 μg of total cellular RNA was fractionated on an agarose formaldehyde gel, transferred to a nylon membrane, and hybridized with radiolabeled cDNA probes.

shown) suggested that this induction was not triggered by IFN-γ. This conclusion is supported by results showing that the neutralizing anti-IFN-γ antibody also inhibited the induction of FcγRI mRNA levels by IFN-γ, but not by SF1 or SF6 (Fig. 4), or by two other SFs that were tested (data not shown).

To address the issue of the presence of IFN-γ in the SFs more directly, we assayed IFN-γ levels using both an ELISA and a sensitive bioassay. Table 2 shows that IFN-γ was not detectable in 21 out of 22 RA SFs using an ELISA that can detect 200 pg/ml IFN-γ, and the remaining one fluid had a low level of IFN-γ, between 200 and 400 pg/ml. This would correspond to an IFN-γ concentration of 40–80 pg/ml final concentration in our cultures, where we used 20% SF, and is much lower than the levels of IFN-γ (4 ng/ml), that were needed for induction of FcγRI, comparable with that seen with the SFs. We also used a bioassay that measures the activation of DNA-binding by transcription factor Stat1 by IFN-γ. Within minutes of treatment of cells with IFN-γ, Stat1 becomes phosphorylated and acquires specific DNA-binding activity (38). Monocytes were cultured with IFN-γ or SFs, nuclear extracts were prepared, and the activation of Stat1 was assayed using gel shift assays with a radiolabeled Ly-6E promoter oligonucleotide probe that preferentially binds Stat1 compared with other STAT family members (21, 35). Fig. 5, lane *3*, shows that 10 U/ml (400 pg/ml) IFN-γ induced Stat1 DNA-binding activity. This DNA-binding activity was specific, since the shifted band was not detected in the presence of a 50-fold excess of unlabeled competitor oligonucleotide (Figs. 5, lane *2*). None of the eight SFs tested induced Stat1 DNA-binding activity (Fig. 5, lanes *4–11*). Since recent experiments with Stat1 mutant cell lines have demonstrated that Stat1 activation is necessary for IFN-γ responses of a wide spectrum of genes (39), our results strongly suggest that SF-triggered gene induction does not employ the IFN-γ signal transduction pathway.

Inflammatory SFs Activate a Stat3-related Transcription Factor that Binds Specifically to an FcγRI Promoter Sequence. We performed gel shift assays using a radiolabeled high affinity serum inducible element (hSIE) oligonucleotide probe, which binds several STAT proteins and complexes (24, 40). This approach allowed us to determine if SFs activated DNA-binding of STAT transcription factors other than Stat1. Fig. 6 *A* shows that SFs induced an hSIE DNA-binding complex, which we have provisionally designated Stat-SF (lanes *3–10*), and which has a distinct mobility from the Stat1 homodimer (41) induced by IFN-γ (lane *2*). In this experiment, we tested four of the SFs used in Figs. 2–5, as well as four additional SFs that induced FcγRI expression. To date, all 12 SFs tested have in-

Table 2. *IFN-γ Levels in SFs*

IFN-γ	<200 pg/ml	200–400 pg/ml	>400 pg/ml
RA	21/22	1/22	0/22
OA	3/3	0/3	0/3
Trauma	2/2	0/2	0/2

IFN-γ levels were measured using an ELISA with a sensitivity of 200 pg/ml (equivalent to 5 U/ml).

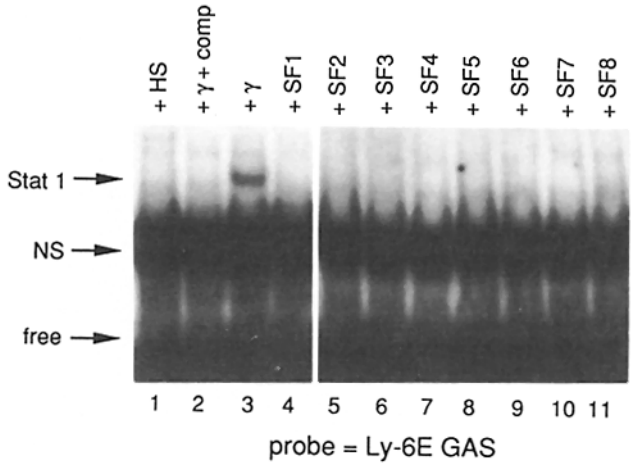

Figure 5. SFs do not activate Stat1-binding to the Ly-6E promoter STAT (*GAS*) site. Purified monocytes were incubated for 20 min in complete medium plus 20% normal human serum (lane *1*), 10 U/ml (400 pg/ml) IFN-γ (lanes *2* and *3*), or 20% SF obtained from eight different patients (lanes *4–11*), and nuclear extracts were prepared. 3 μg of nuclear extract was incubated with 0.2 ng of radiolabeled oligonucleotide containing the binding site for Stat1 and DNA-protein complexes were resolved on 4% nondenaturating polyacrylamide gels. (*comp*) The extract in lane *2* was preincubated with a 50-fold molar excess of unlabeled oligonucleotide; (*free*) free unbound oligonucleotide; (*NS*) nonspecific binding.

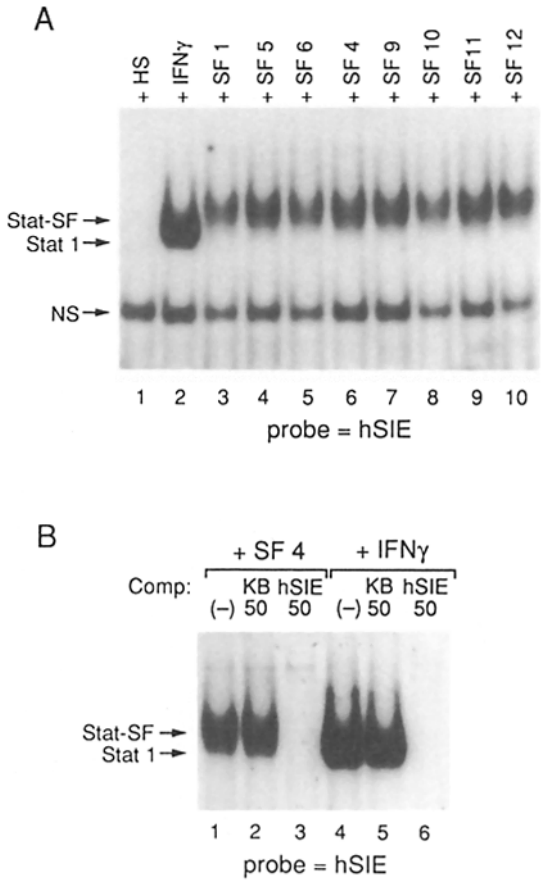

Figure 6. Activation of specific binding to the hSIE STAT site by SFs. Purified monocytes were incubated for 15 min, cellular extracts were prepared, and 8 μg of extract was incubated with 0.5 ng of radiolabeled hSIE oligonucleotide containing a binding site for several STAT proteins. DNA–protein complexes were resolved on 5% nondenaturing polyacrylamide gels. (*A*) SF-induction of a complex (*Stat-SF*) with a mobility different from Stat1. Monocytes were treated with 20% normal human serum (lane *1*), 100 U/ml (4 ng/ml) IFN-γ (lane *2*), or 20% SF obtained from eight different patients (lanes *4–10*). (*B*) SF-induced complex binds specifically to the hSIE. A 50-fold molar excess of unlabeled NF-κB or hSIE oligonucleotide was preincubated with the extracts for 5 min before adding radiolabeled probe. (*comp*) Competitor oligonucleotide. An experiment using a representative SF is shown.

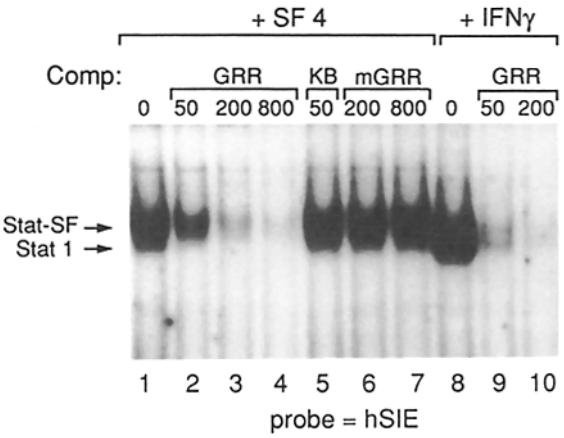

Figure 7. Binding specificity of Stat-SF. Cellular extracts (8 μg) from monocytes treated for 15 min with SF4 or IFN-γ were incubated with a 50–800-fold molar excess of unlabeled competitor oligonucleotides for 5 min before adding radiolabeled hSIE probe and subsequent analysis by gel shift assay. The GRR is an inducible transcription element from the FcγRI promoter which contains a STAT site, and the mGRR contains four base substitutions within this STAT site. (*KB*) NF-κB site. An experiment using a representative SF is shown.

duced formation of a DNA–protein complex that migrated similar to the migration of the Stat–SF complex in Fig. 6 (data not shown). The binding of Stat–SF proteins to the hSIE was specific, because it was not affected by a 50-fold excess of unlabeled NF-κB oligonucleotide, but was abolished by a 50-fold excess of unlabeled hSIE (Fig. 6 *B*). Fig. 6 *B* shows a representative experiment; the specificity of DNA-binding of the Stat–SF complex induced by five different SFs has been tested.

The FcγRI promoter contains a sequence, termed the IFN-γ response region (GRR), that mediates induction by IFN-γ, contains a STAT binding site TTCCCAGAA, and is known to bind Stat1 (36, 42, 43). We used a 39-bp GRR oligonucleotide (36) as a competitor in gel shift assays to compare the binding of Stat1 and Stat-SF to the GRR (Fig. 7). A 50-fold excess of the GRR effectively competed for Stat1 binding (Fig. 7, lanes *8–10*), but a 200–800-fold excess of the GRR was required to achieve a comparable level of competition for Stat-SF binding (Fig. 7, lanes *1–4*). A mutant GRR, mGRR, containing four base substitutions within the GRR STAT site, did not compete for Stat-SF binding even when present in 800-fold excess (Fig. 7, lanes *6* and 7). Taken together, these results show that Stat-SF binds specifically to the GRR STAT site, but with a lower affinity than Stat1.

We used specific antisera generated against Stat1 or Stat3 (24) to investigate whether Stat-SF was antigenically related

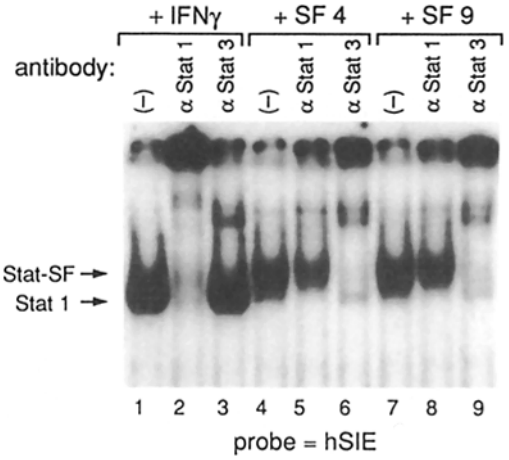

Figure 8. Reactivity of Stat-SF with specific anti-STAT antisera. Cellular extracts (8 μg) from monocytes treated for 15 min with SFs or IFN-γ were incubated with 1 μl of a 1:10 dilution of specific antisera generated against Stat1 or Stat3 (24) for 15 min before adding radiolabeled hSIE probe. DNA–protein complexes were resolved on 5% nondenaturating polyacrylamide gels.

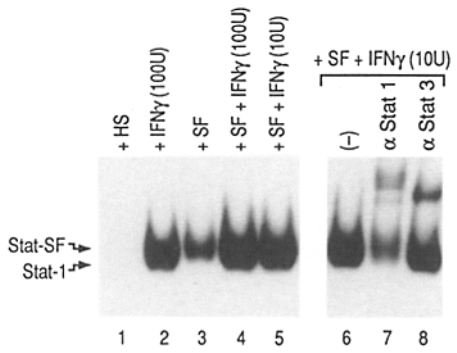

Figure 9. Stat1 activation by IFN-γ in the presence of SFs. Purified monocytes were incubated for 15 min, and 8 μg of cellular extract was incubated with 0.5 ng of radiolabeled hSIE oligonucleotide. IFN-γ was used at 100 or 10 U/ml. Lanes *5–8* contained the same extract, which was preincubated with specific antisera against Stat1 or Stat3.

to these proteins. Stat1 homodimer binding to the hSIE was completely abolished by the Stat1, but not the Stat3, antiserum (Fig. 8, lanes *1–3*). Stat-SF binding, in contrast, was abolished by Stat3 antiserum, and was affected only slightly by the Stat1 antiserum (Fig. 8, lanes *4–9*; results from a representative experiment are shown). These results show that the predominant STAT protein induced by SFs is distinct from Stat1, which is induced by IFN-γ. The combination of Stat-SF DNA-binding specificity and mobility in gel shift assays, and its reactivity with the specific antisera, suggest strongly that Stat-SF contains Stat3 or a very closely related STAT family member.

Role for IL-6, but not IFN-γ, in Activation of Stat-SF and FcγRI. The difference in mobility between Stat-SF and IFN-γ-induced Stat1 (Fig. 6 *A*) allowed us to address directly whether we could detect IFN-γ bioactivity in the presence of SFs. Fig. 9 shows that addition of exogenous IFN-γ to SF, at concentrations of 100 or 10 U/ml, resulted in the activation of an additional DNA-binding complex with the mobility of Stat1. Supershift experiments with anti-Stat1 and anti-Stat3 antibodies showed that the predominant DNA-binding complex in cells treated with both SF and IFN-γ contained Stat1 (Fig. 9, lanes *6–8*). Thus, SF did not mask IFN-γ bioactivity, and we would readily have detected the presence of IFN-γ in the SFs using this assay.

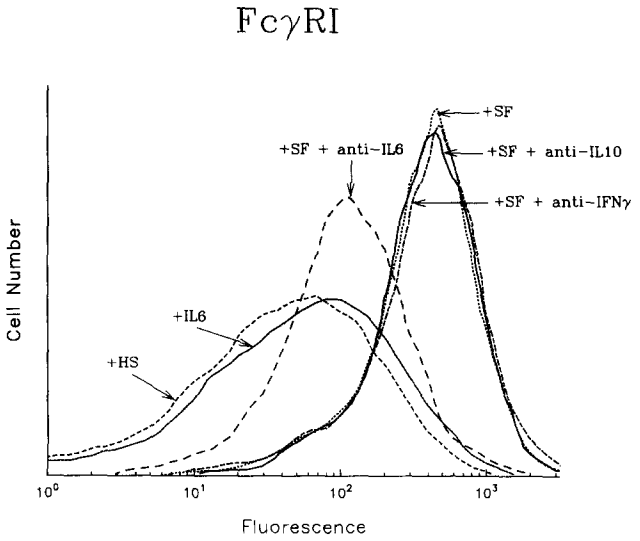

Figure 11. Neutralization of IL-6 inhibits induction of FcγRI expression by SFs. Purified monocytes were cultured for 48 h in complete medium plus 20% human serum (*HS*) or synovial fluid (*SF*). Neutralizing or control antibodies were included at 10 μg/ml and cell surface expression of FcγRI was detected using flow cytometry. A representative experiment is shown.

A major activator of Stat3 is IL-6, a cytokine that is present in most SFs (1, 5). Therefore, we tested whether neutralization of IL-6 in SFs would affect the activation of Stat-SF and the expression of FcγRI. Fig. 10 shows a representative experiment demonstrating that neutralization of IL-6, but not TGF-β, IL-10, or IFN-γ, resulted in the complete inhibition of Stat-SF activation. The effect of neutralization was observed with both a specific anti-IL-6 antiserum and a mAb that has been tested extensively for lack of cross-reactivity with other cytokines. These antibodies effectively neutralized exogenous IL-6, as determined by the prevention of the activation of SIF-A, an IL-6–activated DNA-binding complex which contains Stat3 (24), and which has a mobility similar to Stat-SF (Fig. 10, lanes *10–12*). These results show that IL-6 plays a major role in SF activation of Stat-SF, most likely through the activation of Stat3.

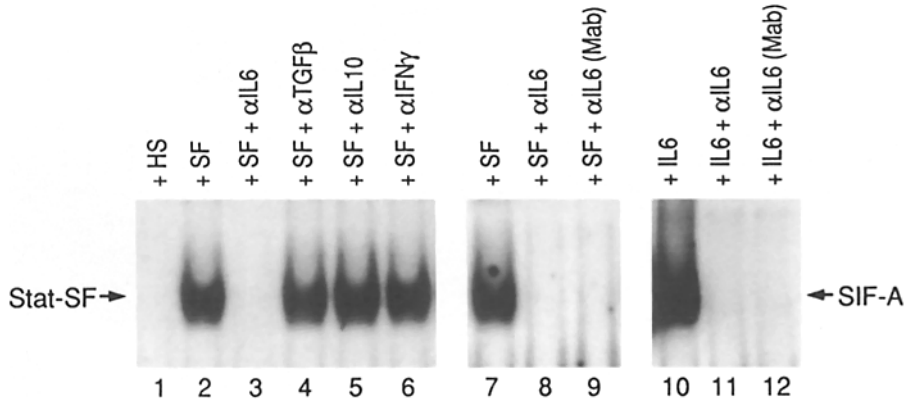

Figure 10. Neutralization of IL-6 prevents Stat-SF activation by SFs. Purified monocytes were incubated for 15 min with HS, SF, or 20 ng/ml IL-6 in the presence or absence of 10 μg/ml of neutralizing goat antibodies or a mAb specific for IL-6 (lanes *9* and *12*); control preimmune antibodies were used to keep IgG concentrations constant. 8 μg of cellular extract was analyzed for binding to a radiolabeled hSIE probe. A representative experiment from the testing of eight SFs is shown. (*SIF-A*) The Stat3-containing complex induced by IL-6 (lane *10*) (24).

We used flow cytometry to determine whether inhibition of Stat-SF activation correlated with inhibition of FcγRI expression. Fig. 11 shows that neutralization of IL-6, which inhibited Stat-SF activation (Fig. 10), also dramatically inhibited the induction of FcγRI expression. This inhibition also occurred at the level of FcγRI mRNA (data not shown). On the other hand, neutralizing antibody to IFN-γ or IL-10 had no effect upon SF induction of FcγRI expression (Fig. 11). Interestingly, addition of IL-6 alone had minimal effect upon FcγRI expression, consistent with previous reports (44). Our data show that IL-6 and Stat-SF activation are necessary but not sufficient for SF induction of FcγRI, and suggest that a second signal, in addition to IL-6, is required.

Discussion

Monocytes make an important contribution to synovial inflammation in rheumatoid arthritis. Understanding how monocytes are activated in the inflamed joint would provide useful information about the pathogenesis of disease and also about the monocytic cell activation program. We have modeled the activation of monocytes which enter the inflamed joint by adhering resting monocytes in the presence of inflammatory SF. This approach makes possible an analysis of monocyte activation by a complex inflammatory stimulus, similar to activation in vivo. We have used this model to demonstrate SF induction of monocyte effector genes, address the issue of whether IFN-γ plays an important role in monocyte activation in the inflamed joint, and show that SFs activate a signal transduction pathway that uses specific members of the STAT family of transcription factors.

FcγRI, FcγRIII, and HLA DR are expressed at high levels in activated monocytes and in inflamed SF cells and synovial tissue (1–15). Monocytes which enter an inflamed joint encounter numerous potentially activating stimuli, including (*a*) adherence to the extracellular matrix; (*b*) cell–cell interactions with synoviocytes and lymphocytes; and (*c*) exposure to soluble pro-inflammatory stimuli. Our results show that adherence plus the soluble factors in SF are sufficient to activate monocyte expression of FcγRI, FcγRIII, and HLA DR, and likely activate a complex monocyte activation program. Adherence alone has been shown to activate NF-κB/rel family transcription factors and induce expression of cytokines such as IL-1 and TNF-α (20), findings which we have reproduced in our system (Ivashkiv, L., unpublished data). Adherence alone did not activate expression of FcγRI, FcγRIII, and HLA DR (Ivashkiv, L., unpublished data), but activation signals triggered by adherence likely affect the functional phenotype of the monocytes. Therefore, we carried out our analysis of the mechanism of monocyte activation by soluble pro-inflammatory stimuli in a system where both control and SF- and cytokine-stimulated monocytes were allowed to adhere as they were exposed to the soluble stimulus. This approach has the advantage of reproducing the simultaneous exposure to an inflammatory environment and adherence that occurs in vivo.

The issue of whether IFN-γ plays a role in activating monocytes in inflammatory synovitis has been controversial (1–4, 6, 9, 13, 14). Our experiments provide three independent lines of evidence that the induction of FcγRI and HLA DR in our system is not dependent upon IFN-γ. SF levels of IFN-γ were very low or undetectable when measured by ELISA, consistent with the findings of other investigators (4). In addition, neutralizing antibodies to IFN-γ did not inhibit the ability of SFs to induce cell surface and mRNA expression of FcγRI and HLA DR, and SFs did not activate transcription factor Stat1, which is necessary for gene activation by IFN-γ (39). Furthermore, SFs did not mask the presence of IFN-γ, since we readily detected IFN-γ that was added to SFs both by ELISA (Ivashkiv, L., unpublished data), and by activation of Stat1 (Fig. 9). Taken together, these findings demonstrate that monocyte activation by SFs does not depend upon IFN-γ, and strongly argue that monocyte activation in inflammatory disease can occur in the absence of IFN-γ.

Treatment of monocytes with SFs induced a specific DNA-binding activity, which we have provisionally designated Stat-SF, within 15 min of stimulation. This DNA-binding protein bound to oligonucleotides containing a STAT binding site, but not to an irrelevant NF-κB oligonucleotide or a mutant oligonucleotide with four base substitutions within the STAT site. In contrast to IFN-γ–induced Stat1, Stat-SF binding activity was neutralized by a specific antiserum directed against Stat3, but not by an antiserum against Stat1. Stat-SF also had a mobility in a gel shift assay which was distinct from that of Stat1 (Fig. 6). A tyrosine-phosphorylated protein which migrated with the mobility of Stat3 was immunoprecipitated from SF-treated monocytes by the Stat3 antiserum (Sengupta, T., and Z. Zhong, unpublished data). Based upon these criteria, we believe that Stat-SF contains Stat3 or a very closely related member of the STAT family.

We have determined that the SF factor which plays a predominant role in activation of Stat-SF is IL-6. IL-6 is a pro-inflammatory cytokine present in a majority of SFs (1, 5), and activates DNA-binding of the SIF-A complex, which contains Stat3. SIF-A has been proposed to be a homodimer of Stat3, although the presence of additional proteins has not been ruled out (21, 24). The similarity of the mobility of SIF-A and Stat-SF (Fig. 10), and their common activation by IL-6 suggest that these complexes may be closely related or identical. However, different DNA-binding complexes often comigrate on the nondenaturing gels used in these assays. Thus, differences in the identity or posttranslational modification of proteins which associate with Stat3 to form SIF-A or Stat-SF could have escaped detection in our experiments. The relationship between Stat-SF and SIF-A can be investigated by a biochemical analysis of purified proteins.

Our data with neutralizing antibodies showed that TGF-β, IL-10, and IFN-γ, are not necessary for Stat-SF activation. Other cytokines, however, may play an important role, possibly by regulating proteins that associate with Stat3 in the Stat-SF complex. An interesting issue is why we did not detect activation of STATs by other cytokines present in SFs, especially cytokines, such as LIF, oncostatin M (OsM), IL-10, and PDGF, which activate Stat3 (24, 40, 45–47). This lack of cytokine activity cannot be completely explained by

the absence of the cytokine from a specific SF, limitations of the gel shift assays, or lack of monocyte receptors for a factor such as PDGF (Sengupta, T., unpublished data). It will be interesting to explore whether SFs can suppress monocyte responses to particular cytokines, possibly by uncoupling signal transduction pathways.

Interestingly, neutralization of IL-6 and inhibition of Stat-SF activation resulted in the inhibition of the induction of FcγRI expression. Stat-SF binds specifically, albeit with relatively low affinity, to the GRR, an inducible transcription element present in the FcγRI promoter (36). Thus, Stat-SF may regulate FcγRI expression directly through binding to the GRR, but an indirect mechanism of regulation is also possible. The presence of IL-6 in SFs is necessary, but not sufficient to activate FcγRI expression, since treatment of monocytes with purified IL-6 had minimal effect upon FcγRI expression. Treatment with IL-6, however, did activate the SIF-A complex, which has a similar mobility to Stat-SF. Our data suggest that the SFs are providing a second signal that synergizes with the IL-6 signal. An interesting possibility is that the second signal modifies the SIF-A complex, possibly by changing the identity of the proteins that make up the complex, or by altering posttranslational modifications.

STAT proteins are triggered by numerous cytokines to activate transcription of many cellular genes leading to major changes in cell phenotype. Our system provides an opportunity to systematically study the activation of STAT proteins and physiologically relevant target effector genes which are activated by inflammatory SF, and to determine which factors present in SF are important for activation. This will yield a greater understanding of monocyte activation by complex inflammatory stimuli in vivo, and should provide insight into pathogenetic mechanisms in inflammatory arthritis.

The authors thank Drs. Peggy Crow, Jeff Edberg, and Patricia Finn for critical reading of the manuscript, and Drs. Jeff Edberg and Bob Kimberly for helpful suggestions and discussions. We thank Drs. Charles Christian for his support, the rheumatologists at the Hospital for Special Surgery for providing SFs, Dr. Jeff Edberg for providing mAbs, and Drs. Lydia Pan and Brian Seed for providing plasmids. We are grateful to Carl Triscari and the Flow Cytometry Core Facility for assistance with flow cytometry and David Dow and the Molecular Biology Core Facility for oligonucleotide synthesis.

This work was supported by National Institutes of Health grant MAMDC 2 P60 AR38520-06 DF4.

Address correspondence to Dr. Lionel B. Ivashkiv, Department of Medicine, Hospital for Special Surgery, 535 East 70th Street, New York, NY 10021.

Received for publication 25 July 1994 and in revised form 21 October 1994.

References

1. Harris, E.D. Jr. 1993. Etiology and pathogenesis of rheumatoid arthritis. *In* Textbook of Rheumatology. W.N. Kelly, E.D. Harris, S. Ruddy, and C.B. Sledge, editors. W.B. Saunders, Philadelphia. 833–873.
2. Degre, M., O.J. Mellbye, and O. Clark-Jenssen. 1983. Immune interferon in serum and synovial fluid in rheumatoid arthritis and related disorders. *Ann. Rheum. Dis.* 42:672–676.
3. Husby, C., and R.C. Williams, Jr. 1985. Immunohistochemical studies of interleukin-2 and γ-interferon in rheumatoid arthritis. *Arthritis Rheum.* 28:174–181.
4. Firestein, G., and N. Zvaifler. 1987. 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.* 30:864–871.
5. Guerne, P.-A., B.L. Zuraw, J.H. Vaughan, D.A. Carson, and M. Lotz. 1989. Synovium as a source of interleukin-6 in vitro. *J. Clin. Invest.* 83:585–592.
6. Firestein, G.S., J.M. Alvaro-Garcia, and R. Maki. 1990. Quantitative analysis of cytokine gene expression in rheumatoid arthritis. *J. Immunol.* 144:3347–3353.
7. Broker, B.M., J.C.W. Edwards, M.W. Fanger, and P.M. Lydyard. 1990. The prevalence and distribution of macrophages bearing FcγRI, FcγRII, and FcγRIII in synovium. *Scand. J. Rheum.* 19:123–135.
8. Bucala, R., C. Ritchlin, R. Winchester, and A. Cerami. 1991. Constitutive production of inflammatory and mitogenic cytokines by rheumatoid synovial fibroblasts. *J. Exp. Med.* 173: 569–574.
9. Feldmann, M., F.M. Brennan, D. Chantry, C. Haworth, M. Turner, P. Katsikis, M. Londei, E. Abney, G. Buchan, K. Barret, et al. 1991. Cytokine assays: role in evaluation of the pathogenesis of autoimmunity. *Immunol. Rev.* 119:105–123.
10. Koch, A.E., S.L. Kunkel, J.C. Burrows, H.L. Evanoff, G.K. Haines, R.M. Pope, and R.M. Strieter. 1991. Synovial tissue macrophage as a source of chemotactic cytokine IL-8. *J. Immunol.* 147:2187–2195.
11. Wahl, S.M., J.B. Allen, G.R. Welch, and H.L. Wong. 1992. Transforming growth factor-β in synovial fluid modulates FcγRIII (CD16) expression on mononuclear phagocytes. *J. Immunol.* 148:485–490.
12. Lotz, M., T. Moats, and P.M. Villiger. 1992. Leukemia inhibitory factor is expressed in cartilage and synovium and can contribute to the pathogenesis of arthritis. *J. Clin. Invest.* 90: 888–896.
13. Chen, E., E.C. Keystone, and E.N. Fish. 1993. Restricted cytokine expression in rheumatoid arthritis. *Arthritis. Rheum.* 36:901–910.
14. Miyake, S., H. Yagita, T. Maruyama, H. Hashimoto, N. Miyasaka, and K. Okumura. 1993. β1 integrin-mediated interaction with extracellular matrix proteins regulates cytokine gene expression in synovial fluid cells of rheumatoid arthritis patients. *J. Exp. Med.* 177:863–868.
15. Malyak, M., R.E. Swaney, and W.P. Arend. 1993. Levels of synovial fluid interleukin-1 receptor antagonist in rheumatoid arthritis and other arthropathies: potential contribution from

synovial fluid neutrophils. *Arthritis Rheum.* 36:781–789.
16. Cope, A.P., and F.M. Brennan. 1992. Cytokine measurements in biological fluids. *Brit. J. Rheumatol.* 31:721–724.
17. Kishimoto, T., T. Taga, and S. Akira. 1994. Cytokine signal transduction. *Cell.* 76:253–262.
18. Zimmer, T., and P.P. Jones. 1990. Combined effects of tumor necrosis factor-α, prostaglandin E_2, and corticosterone on induced Ia expression on murine macrophages. *J. Immunol.* 145:1167–1175.
19. Adams, D.O., and T.A. Hamilton. 1992. Molecular basis of macrophage activation: diversity and its origins. *In* The Macropahge. C.E. Lewis and J. O'D. McGee, editors. Oxford University Press, New York. 75–114.
20. Haskill, S., C. Johnson, D. Eierman, S. Becker, and K. Warren. 1988. Adherence induces selective mRNA expression of monocyte mediators and proto-oncogenes. *J. Immunol.* 140:1690–1694.
21. Darnell, J.E., Jr., I.M. Kerr, and G.R. Stark. 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science (Wash. DC).* 264:1415–1421.
22. Akira, S., Y. Nishio, M. Inoue, X.-J. Wang, S. Wei, T. Matsusaka, K. Yoshida, T. Sudo, M. Naruto, and T. Kishimoto. 1994. Molecular cloning of APRF, a novel IFN-stimulated gene factor 3 p91-related transcription factor involved in the gp130-mediated signaling pathway. *Cell.* 77:63–71.
23. Wegenka, U.M., C. Lutticken, J. Buschmann, J. Yuan, F. Lottspeich, W. Muller-Esterl, C. Schindler, E. Roeb, P.C. Heinrich, and F. Horn. 1994. 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.* 14:3186–3196.
24. Zhong, Z., Z. Wen, and J.E. Darnell, Jr. 1994. Stat3: A STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science (Wash. DC).* 264:95–98.
25. Mentzer, S.J., P.M. Guyre, S.J. Burakoff, and D.V. Faller. 1986. Spontaneous aggregation as a mechanism for human monocyte purification. *Cell. Immunol.* 101:312–319.
26. Maniatis, T., E.F. Fritch, and J. Sambrook. 1989. Molecular Cloning, a Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
27. Allen, J.M., and B. Seed. 1989. Isolation and expression of functional high-affinity Fc receptor complementary DNAs. *Science (Wash. DC).* 243:378–381.
28. Romeo, C., M. Amiot, and B. Seed. 1992. Sequence requirements for induction of cytolysis by the T cell antigen/Fc receptor ζ chain. *Cell.* 68:889–897.
29. Wake, C.T., E.O. Long, M. Strabin, N. Gross, R. Accolla, S. Carrel, and B. Mach. 1982. Isolation of cDNA clones encoding HLA DR α chains. *Proc. Natl. Acad. Sci. USA.* 79:6979–6983.
30. Auron, P.E., A.C. Webb, L.J. Rosenwasser, S.F. Mucci, A. Rich, S.M. Wolff, and C.A. Dinarello. 1984. Nucleotide sequence of human monocyte interleukin 1 cDNA. *Proc. Natl. Acad. Sci. USA.* 81:7907–7911.
31. Tso, J.Y., X.-H. Sun, T. Kao, K.S. Reece, and R. Wu. 1985. Isolation and characterization of rat and human glyceraldehyde-3-phosphate dehydrogenase cDNAs: genomic complexity and molecular evolution of the gene. *Nucleic Acids Res.* 13:2485–2502.
32. Malyak, M., F.G. Joslin, E.L. Verderber, S.P. Eisenberg, and W.P. Arend. 1991. IL-1ra ELISA: reduction and alkylation of synovial fluid eliminates interference by IgM rheumatoid factors. *J. Immunol. Methods.* 140:281–288.
33. Schreiber, E., P. Matthias, M.M. Muller, and W. Schaffner. 1989. Rapid detection of octamer binding proteins with "mini-extracts" prepared from a small number of cells. *Nucleic Acids Res.* 17:6419.
34. Ivashkiv, L.B., H.-C. Liou, C.J. Kara, W.W. Lamph, J.M. Verma, and L.H. Glimcher. 1990. mXBP/CRE-BP2 and c-Jun form a complex which binds to the cyclic AMP, but not to the 12-0-tetradecanoylphorbol-13 acetate, response element. *Mol. Cell. Biol.* 10:1609–1621.
35. Khan, K.D., K. Shuai, G. Lindwall, S.E. Maher, J.E. Darnell, Jr., and A.L.M. Bothwell. 1993. Induction of the Ly-6A/E gene by interferon α/β and γ requires a DNA element to which a tyrosine-phosphorylated 91-kDa protein binds. *Proc. Natl. Acad. Sci. USA.* 90:6806–6810.
36. Pearse, R.N., R. Feinman, K. Shuai, J.E. Darnell, Jr., and J.V. Ravetch. 1993. Interferon γ-induced transcription of the high-affinity Fc receptor IgG requires assembly of a complex that includes the 91-kDa subunit of transcription factor ISGF3. *Proc. Natl. Acad. Sci. USA.* 90:4314–4318.
37. Pelletier, J.-P., P.J. Roughly, J.A. DiBattista, R. McCollum, and J. Martel-Pelletier. 1991. Are cytokines involved in osteoarthritic pathophysiology? *Sem. Arthritis Rheum.* 20 (Suppl.): 2:12–25.
38. Shuai, K., G.R. Stark, I.M. Kerr, and J.E. Darnell, Jr. 1993. A single phosphotyrosine residue of Stat91 required for gene activation by interferon-γ. *Science (Wash. DC).* 261:1744–1746.
39. Muller, M., C. Laxton, J. Briscoe, C. Schindler, T. Improta, J.E. Darnell, Jr., G.R. Stark, and I.M. Kerr. 1993. Complementation of a mutant cell line: central role of the 91 kDa polypeptide of ISGF3 in the interferon-α and -γ signal transduction pathways. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:4221–4228.
40. Sadowski, H.B., K. Shuai, J.E. Darnell, Jr., and M.Z. Gilmam. 1993. A common nuclear signal transduction pathway activated by growth factor and cytokine receptors. *Science (Wash. DC).* 261:1739–1744.
41. Shuai, K., C.M. Horvath, L.H. Tsai Huang, S.A. Qureshi, D. Cowburn, and J.E. Darnell, Jr. 1994. Interferon activation of the transcription factor Stat91 involves dimerization through SH2-phosphotyrosyl peptide interactions. *Cell.* 76:821–828.
42. Eichbaum, Q.G., R. Iyer, D.P. Raveh, C. Mathieu, and R.A.B. Ezekowitz. 1994. Restriction of interferon γ responsiveness and basal expression of the myeloid human FcγR1b gene is mediated by a functional PU.1 site and a transcriptional initiator consensus. *J. Exp. Med.* 179:1985–1996.
43. Larner, A.C., M. David, G.M. Feldman, K. Igarashi, R.H. Hackett, D.S.A. Webb, S.M. Sweitzer, E.F. Petricoin III, and D.S. Finbloom. 1993. Tyrosine phosphorylation of DNA binding proteins by multiple cytokines. *Science (Wash. DC).* 261:1730–1733.
44. Ravetch, J.V., and J.-P. Kinet. 1991. Fc Receptors. *Annu. Rev. Immunol.* 9:457–492.
45. Lehmann, J., D. Seegert, I. Strehlow, C. Schindler, M.-L. Lohmann-Matthes, and T. Decker. 1994. IL-10–induced factors belonging to the p91 family of proteins bind to IFN-γ-responsive promoter elements. *J. Immunol.* 153:165–172.
46. Yuan, J., U.M. Wegenka, C. Lutticken, J. Buschmann, T. Decker, C. Schindler, P.C. Heinrich, and F. Horn. 1994. The signaling pathways of interleukin-6 and gamma interferon coverage by the activation of different transcription factors which bind to common responsive DNA elements. *Mol. Cell. Biol.* 14:1657–1668.
47. Silvennoinen, O., C. Schindler, J. Schlessinger, and D.E. Levy. 1993. Ras-independent growth factor signaling by transcription factor phosphorylation. *Science (Wash. DC).* 261:1736–1739.