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

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Summary

The balance between type 1 and 2 T helper cell cytokine production plays an important role in several animal models of autoimmunity, and skewed patterns of cytokine expression have been described in human inflammatory diseases. Many cytokines activate signal transducer and activation of transcription (STAT) transcription factors, which, in turn, activate transcription of inflammatory effector genes. We used mononuclear cell priming cultures and inflammatory synovial fluids (SFs) derived from arthritis patients to examine the regulation of cytokine production and STAT activity by an inflammatory synovial microenvironment. Exposure to SFs during priming resulted in an 81% inhibition of interferon (IFN)-γ, but not interleukin (IL) 4, production by effector cells generated in priming cultures. SF suppression was mediated by IL-4 and IL-10 and inhibition of IL-12 expression, and it was reversed in a dominant fashion by exogenous IL-12. SFs blocked the sustained activity of transcription factor Stat1, but not Stat3, during the priming period, and Stat1 activity was differentially regulated by cytokines in parallel with their positive or negative regulation of IFN-γ production. Active Stat3, but not Stat1, was detected in cells from inflamed joints. These results suggest a role for altered balance of Stat1 and Stat3 transcriptional activity in the regulation of T cell differentiation and in the pathogenesis of inflammatory synovitis.

Activation of cells and subsequent secretion of cytokines play an important role in autoimmune and inflammatory diseases. Predominant expression of either Th1 (IFN-γ and lymphotoxin) or Th2 (IL-4, IL-5, IL-6, and IL-10) cytokines has been associated with active inflammation (1). For example, Th1 cytokines contribute to the pathogenesis of experimental allergic encephalitis (2) and collagen-induced arthritis (3), whereas elevated Th2 cytokine production has been associated with inflammatory bowel disease (4), autoantibody production (5), and chronic arthritis associated with the caprine arthritis–encephalitis virus (CAEV)[1] (6). Patterns of cytokine expression during an immune response are controlled by a complex regulatory network that uses cytokines and factors derived from accessory cells and T cells (7). IL-12 and IFN-γ are the primary stimulators of Th1 responses, whereas Th2 cytokine production is stimulated by IL-4, IL-10, and E series prostaglandins (PGEs). There is little information available concerning which of these factors is important in regulating cytokine expression in inflammatory diseases.

Many cytokines that regulate immune responses activate specific members of the STAT (signal transducers and activators of transcription) family of transcription factors (8, 9). STATs exhibit differential binding to target sequences present in gene promoters, and the pattern of STATs that become activated determines, at least in part, patterns of gene expression and the phenotype of activated cells. In experiments using cell lines, purified cytokines preferentially activate particular STATs by inducing tyrosine phosphorylation of STATs by cytokine receptor–associated Jak kinases. For example, IFN-γ preferentially activates Stat1, and IL-6 preferentially activates Stat3 (8). However, regulation of STATs in a more physiological setting is more complex and may involve regulation of STAT dephosphorylation (10) and serine phosphorylation (11) as well as regulation of Jak kinase activity by additional ligands (12). Thus, the pattern of activated STATs may reveal information about integrated cellular responses to multiple ligands.

Chronic inflammatory arthritis (synovitis) in humans rep-

[1] *Abbreviations used in this paper:* CAEV, caprine arthritis–encephalitis virus; CM, complete medium; MNC, mononuclear cell; mRNA, messenger RNA; PGE, E series prostaglandin; SF, synovial fluid; SIF, serum-inducible factor; STAT, signal transductors and activators of transcription.

resents an intensively studied process in which multiple cytokines are expressed and are likely to contribute to pathogenesis (13). The presence of high levels of Th2 cytokines and regulators, such as IL-10, PGEs, and TGF-β, coupled with the low levels of IFN-γ protein in actively inflamed joints (13–17), suggests that down-regulation of Th1 cytokines may occur during synovitis (16). We studied the regulation of cytokine production and STAT activity using an in vitro priming system in which mononuclear cells (MNC) from peripheral blood are exposed to synovial fluids (SFs) from patients with inflammatory arthritis. The aim is to reproduce existing conditions when blood mononuclear cells enter an inflamed joint and are exposed to a complex inflammatory microenvironment. Culture with SFs resulted in the inhibition of Stat1 activity, with concomitant polarization of cytokine production toward a Th2 phenotype.

Materials and Methods

Cell Culture and Flow Cytometry. Ficoll density gradient–purified peripheral blood MNC obtained from disease-free volunteers was stimulated with 1.3 μg/ml PHA in complete medium (CM; RPMI 1640 supplemented with glutamine and 5% fetal bovine serum). SFs or plasma controls were added at a final concentration of 30% at the initiation of culture. After 7 d, effector cells were harvested and washed, and 1.8×10^6 cells were restimulated in CM with 10 μg/ml PHA. After culture, >90% of cells were viable, as assessed by trypan blue and propidium iodide staining, and cell surface phenotype was analyzed using flow cytometry. The following purified mAbs were used. T cell markers: 4B5 (anti-CD3), 13B8.2 (anti-CD4), and B9.11 (anti-CD8); B cells: J4.119 (anti-CD19) and BLy.1 (anti-CD20); monocytes: IV.3 (anti–CD32 monocyte-specific epitope) and RM052 (anti-CD14); NK cells: 3G8 (anti-CD16) and T1.99 (anti-CD56). mAbs were purchased from Amac, Inc. (Westbrook, ME), except for 4B5 (Boehringer Mannheim Corp., Indianapolis, IN) and IV.3 (Medarex, New Lebanon, NH). IgG1 (MOPC 21) and IgG2a (UPC10) isotype controls were obtained from Sigma Chemical Co. (St. Louis, MO).

SFs. SFs were obtained, after receiving informed consent, from patients with seropositive definite or classic rheumatoid arthritis (11 SFs), or with chronic seronegative inflammatory arthritis (6 SFs), by the patients' physicians for medically indicated reasons. The protocol for obtaining research samples has been approved by the Institutional Review Board. SFs were handled using sterile technique and centrifuged for 10 min at 10,000 *g* to remove cells and particulate debris; aliquots were stored at −80°C.

ELISA. Indirect ELISA was performed using specific monoclonal capture antibodies anti–IFN-γ (Genzyme, Cambridge, MA) and MP4-25D2 (anti–IL-4; PharMingen, San Diego, CA) and secondary antibodies anti–IFN-γ (R&D Systems, Minneapolis, MN) and 8D4.8 (anti–IL-4; PharMingen), using the protocol provided by PharMingen. All samples were tested in duplicate. The values in Table 1 represent cytokine secretion per 10^6 $CD3^+$ cells, which was calculated using the following formula: (measured cytokine secretion per 10^6 MNC ÷ percentage of $CD3^+$ cells) × 100 = cytokine secretion per 10^6 $CD3^+$ cells.

cDNA Preparation and PCR. RNA was prepared using RNAzol (TM Cinna Scientific, Friendswood, TX), and cDNA was obtained using 1 μg of total cellular RNA and avian myeloblastosis virus reverse transcriptase (Invitrogen, San Diego, CA). 1 or 5% of each cDNA was subjected to 25 (glyceraldehyde-3-phosphate dehydrogenase), 30 (IL-2), or 35 (IL-12 p40) cycles of PCR amplification, with 1 min denaturation at 94°C, 1 min annealing at 60°C, and 1.5 min extension at 72°C. The GAPDH and IL-2 primers span introns and corresponds to primers provided by Stratagene (La Jolla, CA), and the IL-12 p40 primer sequences are 5′ GAAGATGGTATCACCTGGAC and 5′ GAAGAAGCTGCTGGTGTAGT. PCR resulted in single detectable amplification products of the correct size containing appropriate restriction sites. Amplification was determined to be in the linear range by using serial dilutions of cDNA and different cycle numbers.

DNA-binding Assays. Cell extracts were prepared, and 8 μg of extract was assayed for binding to a radiolabeled hSIE oligonucleotide (containing a STAT binding site; reference 18) or to a control Sp1 oligonucleotide using gel shift assays, as previously described (17, 18).

Results

SFs Skew Cytokine Production toward a Th2 Pattern. Priming of lymphocytes or MNC results in the generation of effector cells that exhibit an enhanced capacity to produce both Th1 and Th2 cytokines. Addition of regulatory factors during the priming period results in skewing of cytokine production toward a Th1 or Th2 pattern (7). Table 1 shows the MNC cell surface phenotype and cytokine production, in our system, before and after 7 d of priming culture. Priming was effective, because restimulation of effector cells, which were predominantly lymphocytes, on day 7 resulted in sevenfold more IFN-γ production and 11-fold

Table 1. *Cytokine Secretion and Phenotype of Primed Blood Mononuclear Cells*

	IFN-γ	IL-4	$CD3^+$	$CD4^+$	$CD19^+$	$CD3^+$ or $CD19^{+*}$
	pg/ml				%	
Fresh MNC	420 ± 40	30 ± 10	54 ± 5	40 ± 6	11 ± 2	68 ± 6
Primed MNC (day 7)	2,900 ± 840	340 ± 130	78 ± 3	58 ± 4	14 ± 2	94 ± 4

Freshly isolated MNC or day 7 effector cells were analyzed for cell surface marker expression using flow cytometry. 1.8×10^6 cells were stimulated in CM with 10 μg/ml PHA, culture supernatants were collected after 24 h, and cytokine concentrations were measured by ELISA; values shown have been normalized according to numbers of $CD3^+$ cells (see Materials and Methods). The results shown are derived from at least seven independent experiments.

*Non-$CD3^+$ or -$CD19^+$ day 7 cells expressed monocyte (<5%) and NK cell (<3%) markers.

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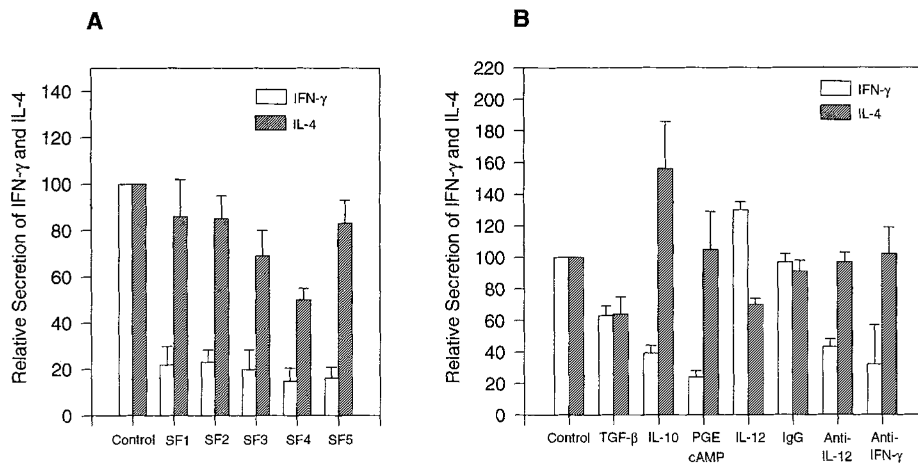

Figure 1. Regulation of IFN-γ and IL-4 secretion by SFs and cytokines. Priming cultures were carried out (*A*) in CM plus 30% plasma (control) or 30% FS. In (*B*), CM was supplemented with 30% plasma and TGF-β (20 ng/ml), IL-10 (20 ng/ml), PGE_2 (10^{-6} M) and 8-Br-cAMP (1 mM), IL-12 (20 ng/ml), control IgG (20 μg/ml), anti–IL-12 IgG (20 μg/ml), or anti–IFN-γ IgG (20 μg/ml). On day 7, cells were washed and analyzed by flow cytometry; 1.8×10^6 effector cells, containing comparable numbers of $CD3^+$ and $CD4^+$ cells, were restimulated in CM with 10 μg/ml PHA. Culture supernatants were harvested after 24 h, and cytokine levels were assayed by ELISA. Cell viability (>90%) and cell surface phenotype (the percentage of cells that were $CD3^+$, $CD4^+$, $CD8^+$, and $CD19^+$) were comparable; SFs decreased cell yield by 20%. Results obtained with the five most extensively tested SFs are shown; SFs 1 and 4 were obtained from patients seropositive for rheumatoid factor, whereas SFs 2, 3, and 5 were obtained from seronegative patients. 16 out of 17 SFs (11 patients seropositive for rheumatoid factor) screened inhibited IFN-γ production by >60%. Data are shown as mean relative secretion of cytokines ± SD compared with the control culture in each experiment. The values are based on a minimum of (*A*) five or (*B*) three experiments with different donors.

more IL-4 production than stimulation of freshly isolated cells. The effect of SFs, obtained from patients with active inflammatory arthritis, on the balance of cytokine production was tested next. Treatment with SFs resulted in a mean 81% inhibition of IFN-γ production (Fig. 1 *A*; five representative SFs out of 17 tested are shown) and a parallel inhibition of IFN-γ messenger RNA (mRNA) levels (data not shown). In contrast, priming for IL-4 synthesis was largely unaffected, because SF-treated cells secreted 49–86% (mean 74%) as much IL-4 as control cells. Thus, treatment with SFs resulted in the preferential inhibition of IFN-γ production, which is typical of a Th2 response.

Inflammatory SFs typically contain high levels of factors that can promote Th2 responses, such as IL-10, PGEs, and TGF-β (13, 14) and low levels of IFN-γ (15–17); many contain no detectable IL-12 (Ivashkiv, L.-B., unpublished data). Next, to test whether any of these agents play an important or dominant role in regulating IFN-γ production in our system, we added individual agents or neutralizing antibodies to the priming cultures (Fig. 1 *B*). The inhibitory effect of SFs on IFN-γ production was mimicked by adding IL-10 or cAMP, or by neutralizing either IFN-γ or IL-12. Thus, no one regulatory factor was dominant, and several different factors or mechanisms could mediate the SF effect.

Role of IL-4, IL-10, and IL-12 in Regulation of IFN-γ Production by SFs. SFs contain a complex mixture of cytokines that may have synergistic or antagonistic effects. Furthermore, cytokines detected in SFs by immunological methods may be denatured or complexed to inhibitors and have no bioactivity (16, 19). Therefore, rather than measuring cytokine levels or fractionating SFs to attempt to identify active molecules, we used neutralizing antibodies to test the effect of blocking cytokine activity during the priming period. Neutralization of both IL-10 and IL-4 during priming was required to reverse the SF inhibition of IFN-γ production (Fig. 2 *A*). As expected (7), priming for IL-4 production in both control and SF-treated cultures depended on the presence of IL-4 during the priming period (Fig. 2 *B*).

IL-10 is expressed in inflamed synovium (14) and inhibits production of several accessory cell-derived cytokines, including the Th1 stimulatory cytokine IL-12 (20). We investigated whether SF treatment resulted in a deficit in endogenous IL-12 activity in priming cultures. SFs specifically suppressed induction of IL-12 mRNA during initiation of priming cultures, because treatment with SFs resulted in significantly lower levels of IL-12 but not IL-2 mRNA 3 h after stimulation (Fig. 3). Addition of exogenous IL-12 to control cultures resulted in a modest stimulation of IFN-γ production (Figs. 1 *B* and 2 *A*), probably because endogenously produced IL-12 is present at near saturating levels in our culture system. In contrast, addition of exogenous IL-12 to SF-treated cultures resulted in a dramatic ninefold induction of IFN-γ production and a reversal of SF inhibition (Fig. 2, *C* and *D*). Taken together, these results show that SF treatment resulted in a relative deficit in IL-12 activity. Our results demonstrate that SFs regulated priming by modulating expression of several regulatory cytokines during the priming period.

Parallel Regulation of Stat1 Activity and Priming for IFN-γ Production. Because many of the cytokines that regulate T cell differentiation activate STATs, we investigated the activation of STAT DNA binding activity during priming cultures by using gel shift assays. Binding of three complexes of similar mobility to the hSIE oligonucleotide (18) was induced in control cultures, where priming for both IFN-γ and IL-4 production occurs (Fig. 4, *A*, lane *4*, and *C*, lane *3*). DNA binding was specific for the STAT sequence within the hSIE, derived predominantly from T cells, and was dependent on tyrosine phosphorylation (data not shown). STAT DNA binding activity was not detected until ~1 d after stimulation; it peaked at 1–2 d and persisted over at least 3 d of priming culture (Fig. 4, *A*, lanes

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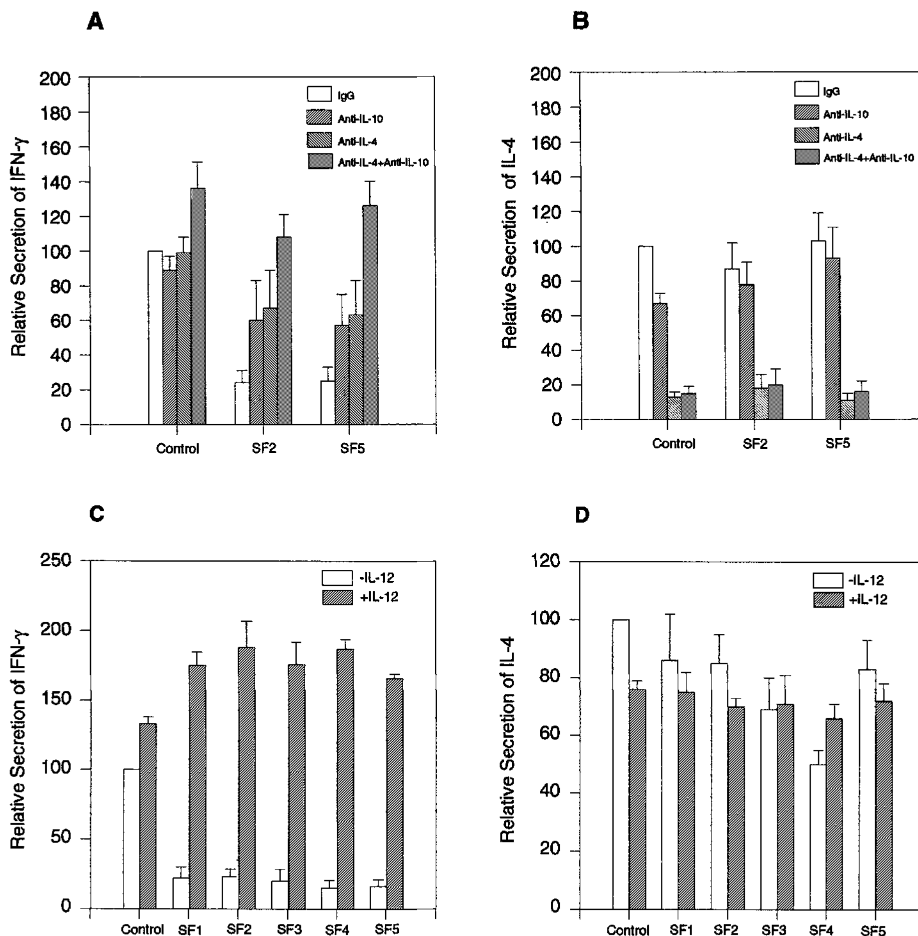

Figure 2. Role of IL-4, IL-10, and IL-12 in regulation of cytokine secretion by SFs. (*A* and *B*) Cells were primed and cultured with SFs and neutralizing IgG to IL-10 (20 μg/ml) or IL-4 (20 μg/ml). Control IgG was used to achieve a total IgG concentration of 40 μg/ml in each culture. Results obtained with two representative SFs are shown. (*C* and *D*) IL-12 (20 ng/ml) was added at initiation of cultures. Cell surface phenotype, viability, and yield were comparable in all cultures. The values for relative cytokine secretion are based on three independent experiments.

1–4, and *C*, lanes *1–4*). This result contrasts with the previously described rapid activation of STATs after treatment with high concentrations of purified cytokines (8, 9), which we could reproduce in our system (Fig. 4 *A*, lanes *5–7*). The most straightforward interpretation of these data is that the initial priming stimulation with PHA does not activate STATs, but subsequent production of cytokines during culture results in a delayed activation.

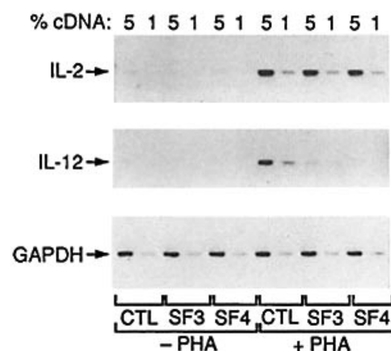

Figure 3. Regulation of IL-2 and IL-12 mRNA levels by SFs. Peripheral blood MNCs were stimulated with 1.3 μg/ml PHA, and cells were harvested after 3 h, RNA extracted, and reverse transcribed into cDNA. IL-2, IL-12 p40 subunit, and mRNA levels were compared using semiquantitative PCR, as described in Materials and Methods. For each sample, two different quantities of cDNA (5 and 1% of cDNA obtained using 1 μg of RNA) were subjected to PCR.

The hSIE oligonucleotide used in our studies preferentially binds protein complexes that contain Stat1 and Stat3, termed serum-inducible factor (SIF) A (upper complex, which contains Stat3), SIF-B (middle complex, which contains both Stat3 and Stat1), and SIF-C (lower complex, which is a homodimer of Stat1) (18). Because the STAT complexes induced during priming had an identical mobility to SIF-A, -B, and -C (data not shown), we tested whether these complexes reacted with specific antisera against Stat1 or Stat3 (18) in supershift experiments (Fig. 4 *B*). The lower two complexes reacted with the Stat1 antiserum, whereas the upper two complexes reacted with the Stat3 antiserum. Thus, priming resulted in the activation of Stat1 and Stat3, and the complexes we have detected probably correspond to SIF-A, -B, and -C. Interestingly, treatment with SFs, which suppressed priming for IFN-γ but not IL-4 production (Fig. 1 *A*), resulted in the preferential suppression of the lower two complexes, which contain Stat1, throughout the time course of the experiment (Figs. 4 *C* and 5 *A*). The inhibition of Stat1 activity was not secondary to down-regulation of Stat1 protein levels (data not shown) and thus reflected regulation of DNA binding.

We next investigated the relationship between successful priming for IFN-γ production and the activity of Stat1

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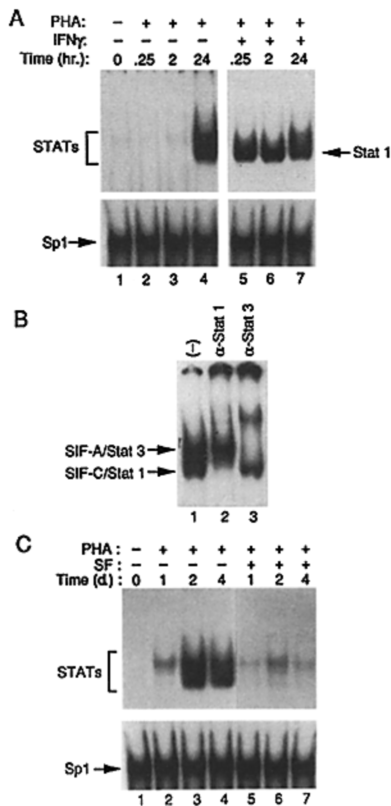

Figure 4. Regulation of STAT DNA binding activity during priming. Cells were harvested at the indicated time points, and 8 μg of cell extract was assayed for binding to a radiolabeled hSIE oligonucleotide (containing a STAT binding site; reference 18) or to a control Sp1 oligonucleotide using gel shift assays. Representative experiments are shown. (*A*) IFN-γ (100 U/ml) was added in lanes *5–7*. (*B*) 1 μl of a 1:10 dilution of specific anti-Stat1 or anti-Stat3 antiserum (18) was incubated with extracts (obtained 24 h after PHA stimulation) for 15 min before adding radiolabeled probe. (*C*) SF was used at 30% final concentration.

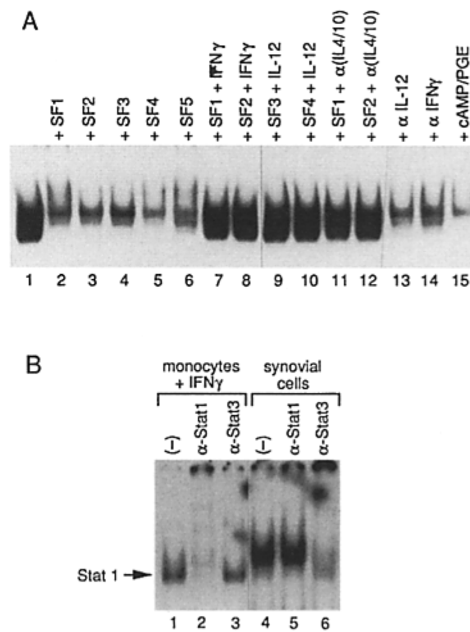

Figure 5. Modulation of STAT DNA binding activity by SFs and inflammatory stimuli. (*A*) Extracts from cells primed for 2 d in the presence of the indicated stimuli were assayed for binding to a radiolabeled hSIE oligonucleotide (18). The SFs, cytokines, and neutralizing antibodies were used as described in the legends to Figs. 1 and 2. (*B*) hSIE binding by control extracts from IFN-γ–stimulated monocytes (which contain Stat1; reference 17) was compared with binding by extracts from synovial cells freshly isolated from inflamed joint effusions. A representative extract from the six extracts tested is shown. 1 μl of a 1:10 dilution of each specific antiserum (18) was used.

during the priming period. IL-12, IFN-γ, and antibodies to IL-4 and IL-10, which blocked SF inhibition of IFN-γ production (Fig. 2 and data not shown), also blocked SF inhibition of Stat1 activity (Fig. 5 *A*, lanes *1–12*). Furthermore, treatments that mimicked SF inhibition of IFN-γ production, such as neutralization of IFN-γ or IL-12, or addition of cAMP (Fig. 1 *B*), also inhibited Stat1 activity (Fig. 5 *A*, lanes *13–15*) but did not affect Stat1 protein levels (data not shown). These results demonstrate that several factors differentially regulated the delayed phase of Stat1 activity in parallel with the differential regulation of Th1 and Th2 responses.

Pattern of STAT Activity during Synovitis. We investigated whether the pattern of STAT DNA binding activity detected in SF-treated cultures reflected the pattern of STAT activity in vivo during synovial inflammation. Extracts prepared from cells freshly isolated from joint effusions of patients with inflammatory arthritis contained predominantly Stat3 but not Stat1 DNA binding activity (Fig. 5 *B*). This is consistent with the inability of several groups, including ours, to detect IFN-γ protein in inflammatory arthritis (15–17). In contrast, in delayed-type hypersensitivity, a Th1 response, active Stat1 is present in skin lesions (21).

Discussion

Relatively little is known about the mechanisms that regulate the balance of Th1 versus Th2 cytokine production during chronic human inflammatory diseases such as synovitis. T cells that enter inflamed synovium are exposed to multiple agonists and antagonists that preexist in the joint or are produced by cells as they become activated. We have investigated mechanisms regulating cytokine production using a culture system that attempts to mimic the complexity of regulation in vivo. Our results show that soluble synovial inflammatory mediators specifically inhibit transcription factor Stat1 in PHA-activated T cells. A functional correlate of Stat1 suppression is the inhibition of development of effector T cells that produce high levels of IFN-γ. Stat1 activity and IFN-γ production were regulated in parallel by a cytokine regulatory network that involved IL-4, IL-10, and IL-12 and was modulated by SFs.

IL-4, IL-10, and IL-12 have previously been shown to regulate Th1 versus Th2 cytokine production (7). In many systems, IL-4 alone is sufficient to induce a Th2 response and is dominant over IL-12 (7, 22). SF suppression of IFN-γ production required IL-10 (and inhibition of IL-12) in ad-

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dition to IL-4, and exogenous IL-12 restored IFN-γ production in a dominant fashion. These results suggest that accessory cell–derived factors, such as IL-10 and IL-12, may play an important or dominant role in the regulation of T cell cytokine production during synovial inflammation. The imbalance in cytokine production we have described reflects the pattern of cytokine expression in chronic human synovitis and CAEV-associated arthritis (6, 13). Th2 cytokines can contribute to pathogenesis by driving synovial production of antibodies and formation of immune complexes, or by inducing expression of metalloproteases (23) and inflammatory mediators, such as soluble CD23 (24). Alternatively, Th2 cytokines may contribute to pathogenesis by suppressing Th1-mediated clearance of (auto)antigens or inappropriately activated cells (25).

Stat1 activity in priming cultures was differentially regulated by SFs, cAMP, IL-4, IFN-γ, and IL-12 (Figs. 4 and 5; Wang, F., unpublished data). This antagonistic regulation of a STAT factor by several opposing cytokines represents a novel mechanism for modulating STAT signaling and allows Stat1 to function as a cellular "node" that integrates multiple signals into a transcriptional response. The block of Stat1 activity could be achieved by preventing production of the cytokine, which directly activates Stat1, interruption of intracellular signals upstream of Stat1 activation (12) or activation of Stat1-specific tyrosine phosphatases. At this time, it is not clear which cytokine directly activates Stat1 in priming cultures. Stat1 can be activated by many cytokines, including IFN-γ, IFN-α, IL-2, IL-6, platelet-derived growth factor, and epidermal growth factor (8, 9). The absence of Stat1 activity in the presence of neutralizing anti–IFN-γ antibodies (Fig. 5 *A*) suggests that IFN-γ may be the responsible cytokine. However, persistence of Stat1 activity over several days would be atypical after IFN-γ activation, and culture supernatants contained very low levels of IFN-γ immuno- and bioactivity (Ivashkiv, L. B., unpublished data). This suggests that IFN-γ may act indirectly or that alternative mechanisms, such as regulation of Stat1 dephosphorylation, may be important.

Our previous work showed that SFs directly activate, in monocytes, a DNA-binding complex that contains Stat3 but not Stat1 (17). The new results demonstrate that SFs use a different mechanism, namely, inhibition of Stat1, to achieve a similar skewing of the balance of transcriptional activity in activated T cells. Stat3 binds weakly to the Stat1 target sequences in several promoters and does not activate transcription of promoters containing these sites in cotransfection assays (reference 26; Zhong, Z., unpublished data). Thus, SF-treated lymphocytes will probably express Stat3 target genes in the absence of expression of genes whose transcription depends on Stat1. The altered balance of Stat3 and Stat1 transcriptional activity correlates well with the altered balance of cytokine production and was also detected in cells from inflamed joints (Fig. 5 *B*). These results suggest a role for Stat1 and Stat3 in the regulation of the development of Th phenotype and in the pathogenesis of inflammatory synovitis.

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