# Sensitization of IFN-γ Jak-STAT signaling during macrophage activation

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**A general paradigm in signal transduction is ligand-induced feedback inhibition and the desensitization of signaling. We found that subthreshold concentrations of interferon-γ (IFN-γ), which did not activate macrophages, increased their sensitivity to subsequent IFN-γ stimulation; this resulted in increased signal transducer and activator of transcription 1 (STAT1) activation and increased IFN-γ–dependent gene activation. Sensitization of IFN-γ signaling was mediated by the induction of STAT1 expression by low doses of IFN-γ that did not effectively induce feedback inhibition. IFN-γ signaling was sensitized *in vivo* after IFN-γ injection, and STAT1 expression was increased after injection of lipopolysaccharide and in rheumatoid arthritis synovial cells. These results identify a mechanism that sensitizes macrophages to low concentrations of IFN-γ and regulates IFN-γ responses in acute and chronic inflammation.**

Interferon-γ (IFN-γ), or type II IFN, is a pleiotropic cytokine widely involved in the regulation of both innate and adaptive immune responses. The major biological activities of IFN-γ include antiviral and antiproliferative properties, macrophage activation, control of apoptosis and promotion of antigen processing, presentation and T helper type 1 ($T_H1$) differentiation[1]. Given the key role played by IFN-γ in modulating immune responses, tight control of IFN-γ action is important for maintaining homeostasis as well as eliciting competent immune responses. A lack of IFN-γ responses, as occurs in IFN-γ and IFN-γ receptor (IFN-γR)–deficient mice, as well as in patients with mutations in the IFN-γR, results in impaired immunity to a variety of microbial pathogens[2–4]. On the other hand, uncontrolled or excessive IFN-γ action is deleterious as well. Selective overexpression of IFN-γ in the liver, pancreas or epidermis of transgenic mice results in chronic active hepatitis[5], insulin-dependent diabetes mellitus[6,7] and lupus nephritis[8], respectively. Hypersensitivity to IFN-γ, as is seen in suppressor of cytokine signaling 1 (SOCS1)-deficient mice, leads to a complex fatal neonatal disease characterized by fatty degeneration of liver, monocytic infiltration of pancreas, heart and lung, abnormal cellularity in thymus, spleen and bone marrow and severe lymphopenia[9–12].

IFN-γ action is regulated in two distinct ways: control of IFN-γ production and modulation of IFN-γ signaling. IFN-γ production can be up-regulated by T cell receptor engagement in $T_H1$ cells and by interleukin 12 (IL-12) and IL-18 in natural killer cells and $CD8^+$ T cells. Once IFN-γ is secreted and binds to its cell-surface receptor, the receptor-associated tyrosine kinases Janus kinase1 (Jak1) and Jak2 become activated, leading to the activation of signal transducer and activator of transcription 1 (STAT1) by phosphorylation of a conserved tyrosine residue[13,14]. STAT1 plays a major role in mediating the immune and pro-inflammatory actions of IFN-γ[15,16]. Several mechanisms negatively regulate IFN-γ signaling, including loss of expression of IFN-γR2 in T cells[17,18], attenuation of signaling by tyrosine phosphatases[19–22] and inhibition of IFN-γR–associated Jak1 and Jak2 by SOCS proteins, especially SOCS1[23]. In addition, STAT1 DNA binding and transcriptional activity are suppressed by protein inhibitor of activated STAT1 (PIAS1) and PIASy[24,25]. Several of these inhibitory mechanisms, for example, loss of IFN-γR2 expression and induction of SOCS expression, are activated by IFN-γ itself. Thus, similar to most cytokines, IFN-γ induces feedback inhibition to limit its own activity. Also similar to most cytokines that use the Jak-STAT signaling pathway, expression of STAT proteins is currently not thought to play a key role in the regulation of IFN-γ signaling.

The molecular mechanisms of feedback inhibition and negative regulation of cytokine signal transduction pathways have been the subjects of extensive investigation[23], but the sensitization of cytokine signaling is not well understood. IFN-γ signaling is positively modulated by previous exposure to low, subthreshold, concentrations of the type I IFNs IFN-α and IFN-β (IFN-α/β)[26]. The mechanism of sensitization of IFN-γ signaling by type I IFNs has not been completely defined, but depends upon low amounts IFN-α/β signaling. This signaling leads to an association between the two non-ligand–binding receptor subunits IFN-αR1 and IFN-γR2 and increased dimerization of tyrosine-phosphorylated STAT1[26]. We show here that low, subthreshold, concentrations of IFN-γ induce autosensitization of IFN-γ signaling by a distinct mechanism that involves increased expression of STAT1.

## Results

### IFN-γ signaling is sensitized by soluble factors

We investigated whether the responsiveness of macrophages to IFN-γ is regulated during cell differentiation *via* signal transduction. IFN-γ

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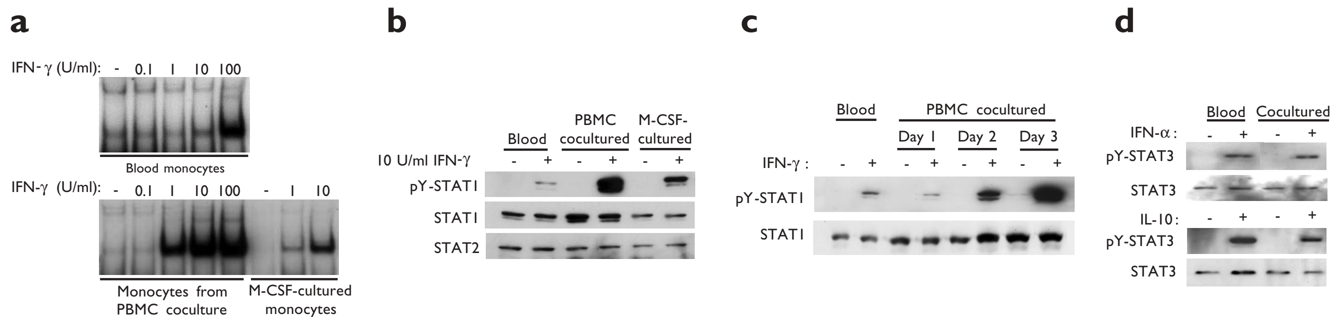


**Figure 1. Monocyte sensitization of IFN-γ signaling during PBMC cocultures.** Blood monocytes were isolated from fresh PBMCs and used immediately. Cocultured monocytes corresponded to CD14+ cells purified from a 3-day PBMC coculture. M-CSF–cultured monocytes were obtained by culturing pure monocytes for 3 days with 20 ng/ml of M-CSF. All three cell types were activated with IFN-γ for 10 min and total cell extracts were prepared. (**a**) STAT activation was measured by EMSA with the hSIE oligonucleotide. One representative of six experiments is shown. (**b**) Immunoblot of cell extracts analyzed with anti–tyrosine-phosphorylated STAT1 (pY-STAT1). The same filter was reprobed with anti-STAT1 or anti-STAT2. (**c**) Monocytes were isolated from PBMC cocultures and treated with 10 U/ml of IFN-γ for 10 min. Cell extracts were analyzed for pY-STAT1 and STAT1 protein by immunoblotting. (**d**) Blood monocytes and PBMC cocultured monocytes were stimulated with 5 ng/ml of IFN-α or 10 ng/ml of IL-10. Cell extracts were analyzed for pY-STAT3 and STAT3 by immunoblotting.

signaling was assessed by measuring STAT1 DNA binding by electrophoretic mobility shift assay (EMSA) and tyrosine phosphorylation by immunoblotting. Three populations of human monocytes or macrophages at distinct stages of cellular differentiation or activation were studied: freshly isolated peripheral blood monocytes, CD14+ cells derived from 3-day peripheral blood mononuclear cell (PBMC) cocultures and macrophages cultured for 3 days with macrophage colony-stimulating factor (M-CSF). In a dose-response experiment, IFN-γ did not induce detectable STAT1 DNA binding in fresh blood monocytes until the dose reached 100 U/ml (**Fig. 1a**, upper panel). Monocytes derived from PBMC cocultures responded strongly to 1 U/ml of IFN-γ (**Fig. 1a**, lower panel). This indicated that IFN-γ signaling became sensitized during culture, as defined by increased STAT1 activation in response to low doses of IFN-γ. In contrast to the PBMC coculture system, where monocytes were mixed with other blood cell types during the course of culture, IFN-γ responses were minimally increased when monocytes were purified before culture (**Fig. 1a**, lower panel); the slight increase in IFN-γ responsiveness detected in cells cultured with M-CSF was not consistently observed and has not been further investigated.

Phosphorylation of STAT1 Tyr$^{701}$ is a prerequisite for its dimerization and activation of DNA binding. IFN-γ–induced STAT1 tyrosine phosphorylation was markedly increased in monocytes derived from PBMC cocultures (**Fig. 1b**). Monocytes that were rendered more sensitive to IFN-γ will be referred to as primed monocytes hereafter. Compared to blood monocytes, STAT1 was modestly increased in cocultured monocytes (**Fig. 1b**). Reprobing the same filter for STAT2 demonstrated comparable STAT2 amounts in all lanes (**Fig. 1b**). IFN-γ signaling sensitization did not become apparent until the second day of PBMC coculture and increased in a time-dependent manner (**Fig. 1c**). STAT1 increased in parallel with the sensitization of IFN-γ signaling (**Fig. 1c**), which was highly reproducible among >40 different blood donors. In ~50% of experiments, induction of STAT1 expression was marked and correlated with the increase in tyrosine phosphorylation (data not shown). The increase in STAT1 appeared insufficient to explain increased STAT1 tyrosine phosphorylation in the other ~50% of experiments (such as those in **Fig. 1b**,**c**). Jak-STAT signaling was not globally sensitized in PBMC coculture–primed monocytes, as STAT3 activation by IFN-α and IL-10 remained unchanged during the course of culture (**Fig. 1d**). These results demonstrated that IFN-γ signaling in monocytes became sensitized during PBMC coculture and suggested that factors derived from lymphocytes or natural killer cells that were present in these cultures may induce sensitization.

Sensitization of IFN-γ signaling could potentially be mediated by cell-cell contact between monocytes and other cell types and by soluble factor(s) secreted during PBMC coculture. To discriminate between these two possibilities, we used a culture system in which monocytes and CD14− blood cells were spatially separated by a cell-impermeable membrane in transwell plates. In transwell-cultured monocytes, IFN-γ signaling was sensitized to a similar extent as in monocytes from PBMC cocultures (**Fig. 2a**), with a concomitant increase in STAT1 protein (**Fig. 2a**). In addition, sensitization could be induced by PBMC coculture supernatants (**Fig. 2b**). Both transwell and supernatant-transfer experiments implied the involvement of one or more soluble factors in the process of IFN-γ signaling sensitization. Supernatant-induced sensitization occurred with faster kinetics than sensitization in transwell-cultured monocytes (**Fig. 2b**), which was consistent with the idea that the supernatants contained factors that were produced *de novo* during transwell or PBMC cocultures.

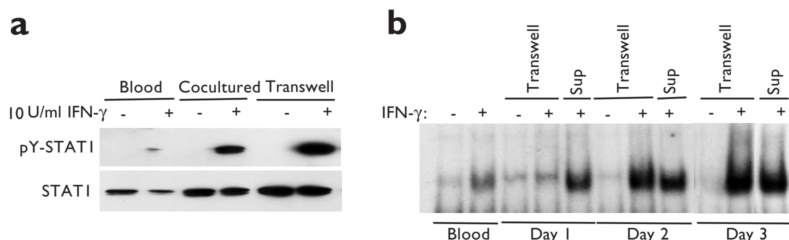


**Figure 2. Soluble factors produced in PBMC cocultures mediate IFN-γ signaling sensitization.** (**a**) Blood and cocultured monocytes were obtained as in Fig. 1. Monocytes were collected from the bottom chambers of transwells after 3 days of culture. Cells were subsequently activated with 10 U/ml of IFN-γ for 10 min and cell extracts analyzed by immunoblotting for pY-STAT1 and total STAT1. (**b**) Monocytes were cultured in transwell chambers or with PBMC coculture supernatants before stimulation with 10 U/ml of IFN-γ for 10 min. Cell extracts were subjected to EMSA with the hSIE oligonucleotide.

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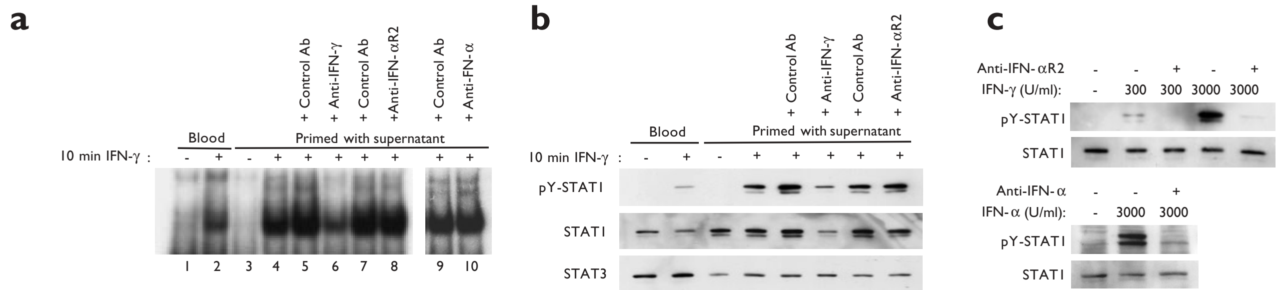


d

IFN-γ IFN-α

pg/ml

Exp. 1 Exp. 2 Exp. 3 Exp. 4 Exp. 5

**Figure 3. Monocyte IFN-γ signaling sensitization is mediated by IFN-γ, but not by IFN-α, in PBMC cocultures.** (**a**) Monocytes were cultured with PBMC coculture supernatants for 3 days with saturating concentrations of neutralizing antibodies (0.5 μg/ml of anti–IFN-γ, 2 μg/ml of anti–IFN-αR2 and 2 μg/ml of anti–IFN-α). Isotype-matched control antibodies were used at the same concentrations. At the end of culture, cells were extensively washed and treated with 10 U/ml of IFN-γ for 10 min. STAT DNA binding in cell extracts was analyzed by EMSA as before. Primed with supernatant, cultured for 3 days with PBMC coculture supernatants. (**b**) Immunoblotting of the same extracts used in **a**. (**c**) Monocytes were incubated with 2 μg/ml of mAbs to human IFN-αR2 or IFN-α, this was followed by 10-min activation with IFN-α. Cell extracts were analyzed for both pY-STAT1 and total STAT1 protein by immunoblotting. (**d**) Supernatants were collected from five independent 3-day transwell cultures and the amounts of IFN-γ and IFN-α present in culture supernatants were determined with ELISA.

## IFN-γ itself mediates signaling sensitization

IFN-γ signaling is sensitized through cross-talk between type I and type II IFN signal transduction pathways[26]. To assess the role played by IFNs in IFN-γ signaling sensitization, monocytes were primed with supernatants derived from PBMC cocultures in the presence of neutralizing antibodies to IFNs or IFNRs. As expected and as assessed by EMSA, PBMC coculture supernatants induced IFN-γ signaling sensitization (**Fig. 3a**, lane 4 *versus* 2). Antibodies directed against IFN-αR2, the ligand binding chain of IFN-α/βR, or against IFN-α did not prevent sensitization of IFN-γ signaling (**Fig. 3a**, lanes 8 and 10). In contrast, the addition of IFN-γ antibodies during the priming culture blocked sensitization of signaling. STAT1 activation was essentially the same as observed in fresh blood monocytes (**Fig. 3a**, compare lane 6 to lanes 2 and 5). Consistent with the DNA-binding results, IFN-γ antibodies also blocked the increase in STAT1 tyrosine phosphorylation, whereas IFN-αR2 blocking antibody had no effect (**Fig. 3b**). The increase in STAT1 protein during priming was blocked in parallel to suppression of IFN-γ signaling (**Fig. 3b**). Similar results were obtained when soluble IFN-γRs were used to block IFN-γ activity during priming (data not shown). Antibodies to IFN-αR2 and IFN-α were capable of completely inhibiting IFN-α–induced STAT1 activation, demonstrating that these reagents completely blocked type I IFN signaling (**Fig. 3c**).

To determine whether the lack of any apparent sensitizing effect of type I IFNs was secondary to a lack of production of type I IFNs during these cultures, the amounts of IFN-α in PBMC coculture supernatants were determined. When a sensitive enzyme-linked immunosorbent assay

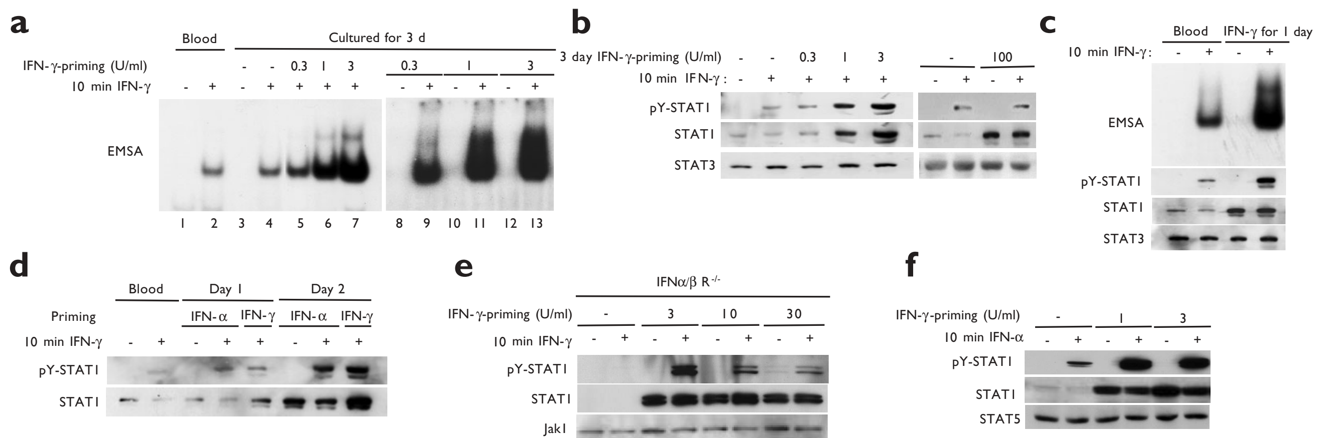


**Figure 4. Subthreshold concentrations of exogenous IFN-γ can sensitize IFN-γ signaling in primary human monocytes and in macrophages from IFN-α/βR–deficient mice.** (**a**) Monocytes were treated with increasing concentrations of IFN-γ for 3 days (0.3, 1 and 3 U/ml correspond to 15, 50 and 150 pg/ml, respectively). Cells were then washed and stimulated with 10 U/ml of IFN-γ for 10 min. STAT activation was measured by EMSA. Autoradiographs were exposed for 16 h (lanes 1–7) and 3 days (lanes 8–13) in order to visualize any baseline STAT1 activity after priming. (**b**) Cells were cultured for 3 days with low or high doses of IFN-γ, and extracts were analyzed for pY-STAT1 and total STAT1 and STAT3 protein by immunoblotting. (**c**) Monocytes were primed for 1 day with 3 U/ml of IFN-γ, washed and activated with 10 U/ml of IFN-γ for 10 min. STAT activation was analyzed by EMSA and immunoblotting. (**d**) Monocytes were primed with 30 U/ml (150 pg/ml) of IFN-α or 3 U/ml (150 pg/ml) of IFN-γ, washed and stimulated with 10 U/ml of IFN-γ for 10 min. Cell extracts were analyzed for pY-STAT1 and total STAT1 by immunoblotting. (**e**) Bone marrow–derived macrophages from type I IFNR–deficient mice[27] were cultured for 1 day with IFN-γ, washed and restimulated for 10 min with 30 U/ ml of IFN-γ. (**f**) Monocytes primed for 2 days with 1 or 3 U/ml of IFN-γ were washed and stimulated with 5 ng/ml of IFN-α. Cell extracts were analyzed for pY-STAT1 and total STAT1 and STAT5 by immunoblotting.

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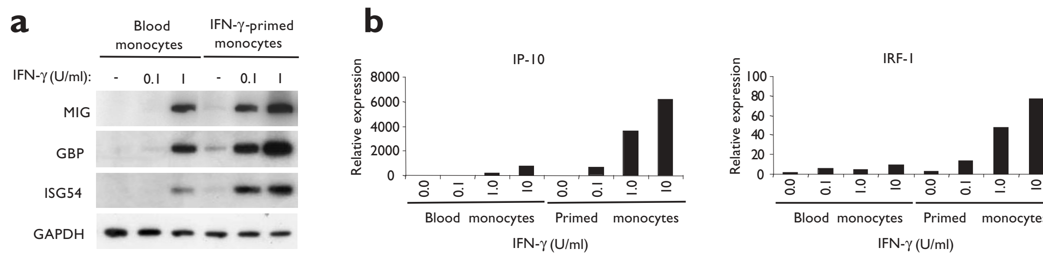


**Figure 5. Priming with low doses of IFN-γ sensitizes activation of IFN-γ–inducible genes.** Blood monocytes or monocytes primed with 3 U/ml of IFN-γ for 3 days were treated with IFN-γ for 3 h. (**a**) Steady-state MIG, GBP, ISG54 and GAPDH mRNA were measured with semi-quantitative RT-PCR. (**b**) IP-10, IRF-1 and GAPDH mRNA were measured with quantitative real-time PCR. Amounts of IP-10 and IRF-1 mRNA, normalized relative to GAPDH mRNA, are shown.

(ELISA)—capable of detecting as little as 3 pg/ml of IFN-α—was used, no IFN-α was detected in any of the supernatants tested (**Fig. 3d**). Thus, these results do not conflict with a previous report[26], which showed that type I IFNs can sensitize IFN-γ signaling, because IFN-α was absent in our culture systems. In contrast to IFN-α, low amounts of IFN-γ were detected in all culture supernatants that were tested (**Fig. 3d**). Taken together, our data suggested a role for IFN-γ in the sensitization of its own signaling pathway in human monocytes.

Purified exogenous IFN-γ was used to determine whether this cytokine alone is sufficient to sensitize its own signaling. Purified blood monocytes were incubated for 3 days with low doses of IFN-γ, which corresponded to the concentrations detected in PBMC cocultures. As expected, culturing monocytes without exogenous IFN-γ did not promote IFN-γ–induced STAT1 DNA-binding activity (**Fig. 4a**, lane 4). Culturing monocytes with low concentrations of IFN-γ sensitized IFN-γ signaling in a dose-dependent manner (**Fig. 4a,b**, top panel). No baseline STAT1 activity was detected after priming cultures and before restimulation with IFN-γ (**Fig. 4a**, lanes 8, 10 and 12). In contrast, when a saturating dose of IFN-γ (100 U/ml) was used during the priming period, sensitization of IFN-γ signaling was not observed, consistent with the development of feedback inhibition (**Fig. 4b**, right panel). When 100 U/ml of IFN-γ was used, low baseline STAT1 tyrosine phosphorylation was inconsistently detected at the end of the priming period, but sensitization of IFN-γ signaling was never observed (data not shown). Expression of STAT1 was increased in IFN-γ–primed cells. In contrast to PBMC cocultures, when used at low concentrations, purified IFN-γ consistently induced increased STAT1 protein expression that correlated with the increase in STAT1 tyrosine phosphorylation (**Fig. 4b–d**). IFN-γ priming did not alter the amounts of STAT3 or other components of Jak-STAT pathway (**Figs. 4b**, and data not shown).

Because signaling sensitization occurred rapidly in monocytes primed with PBMC coculture supernatants (**Fig. 2b**), we tested whether IFN-γ–mediated sensitization also occurred at early time points. In monocytes primed with IFN-γ for 1 day, IFN-γ signaling was already sensitized, as shown by increased IFN-γ–induced STAT1 DNA binding and tyrosine phosphorylation (**Fig. 4c**). Low doses of IFN-α were also able to sensitize IFN-γ signaling (**Fig. 4d**). To determine whether constitutively low amounts of type I IFNs were necessary for priming to occur, experiments were done with bone marrow–derived macrophages from mice deficient in the type I IFNR[27]. Priming with low doses of IFN-γ effectively sensitized IFN-γ signaling, with a concomitant increase in STAT1 protein, even in the absence of type I IFN signaling (**Fig. 4e**). Feedback inhibition became apparent as the priming dose of IFN-γ was increased (**Fig. 4e**), similar to that seen with human macrophages (**Fig. 4b**). Because IFN-α also activates STAT1, the effects of priming with IFN-γ on IFN-α signaling were tested. Low dose IFN-γ effectively sensitized activation of STAT1 by IFN-α in macrophages (**Fig. 4f**). These results demonstrated that in macrophages, IFN-γ signaling could be sensitized by both type I and type II IFNs and that sensitization by IFN-γ was not dependent upon type I IFNs.

## Sensitization of IFN-γ–dependent gene activation

Macrophage activation by IFN-γ is mediated by the activation of expression of genes important for macrophage effector functions. The regulation of several of these genes—including those encoding the chemokines monokine induced by IFN-γ (MIG) and inducible protein-10 (IP-10), the transcription factor IFN regulatory factor 1 (IRF-1) as well as guanylate-binding protein (GBP) and IFN-stimulated gene 54 (ISG54)—is STAT1-dependent[13,14]. We assessed the physiological role played by sensitization of IFN-γ signaling by analyzing IFN-γ induction of gene expression in monocytes primed with low doses of IFN-γ. One prediction from the signaling studies is that gene expression would be induced by lower doses of IFN-γ. Indeed in primed macrophages relative to blood monocytes, expression of MIG, GBP and ISG54 mRNA was induced by lower doses of IFN-γ (**Fig. 5a**). Stimulation of primed monocytes with IFN-γ also resulted in substantially higher expression of IP-10 and IRF-1 mRNA than in control monocytes (**Fig. 5b**). Thus, priming with low doses of IFN-γ also sensitized IFN-γ–dependent gene activation.

## STAT1 and SOCS expression in primed monocytes

To determine the mechanism of IFN-γ signaling sensitization, the effects of priming on the components of the IFN-γ–Jak-STAT pathway were examined. Priming of macrophages with low dose IFN-γ had no discernable effect on cell surface IFN-γR expression (**Fig. 6a**), and no changes in IFN-γR protein or mRNA expression were detected by immunoblotting or reverse transcription polymerase chain reaction (RT-PCR) (data not shown). Consistent with comparable IFN-γR expression, activation of Jak1 and Jak2 did not differ between M-CSF–cultured and IFN-γ–primed cells (**Fig. 6b**). These results indicated that priming does not alter proximal steps in IFN-γ signaling upstream of STAT1 activation and prompted us to examine the kinetics of STAT1 deactivation.

Similar to established protocols[19,28], IFN-γ signaling input was terminated by removing IFN-γ or adding genistein (to inhibit Jak kinase activity) (**Fig. 6c**). In both cases, STAT1 activity was terminated rapidly and with comparable kinetics (**Fig. 6c** and data not shown), indicating that rates of STAT1 deactivation did not differ between M-CSF–cultured and IFN-γ–primed cells and excluding a role for phosphatases or SOCS proteins. We addressed the role of increased STAT1 expression in sensitization of IFN-γ signaling using two approaches. First, forced overexpression of STAT1 in the absence of any priming

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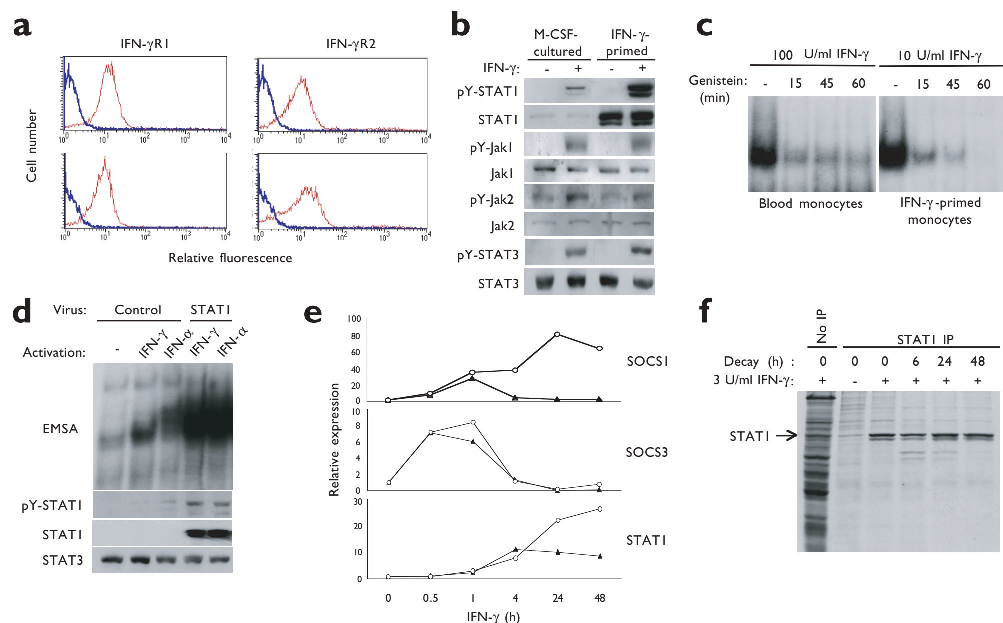


**Figure 6. IFN-γ signaling sensitization mediated by increased STAT1 protein expression.** (**a**) Flow cytometric analysis of surface expression of IFN-γR1 and IFN-γR2 (thin lines). Thick lines indicate staining with isotype-matched control antibodies. (Upper panels) M-CSF–cultured monocytes; (lower panels) IFN-γ–primed monocytes. (**b**) M-CSF–cultured and IFN-γ–primed monocytes were treated with 10 U/ml of IFN-γ for 10 min. IFN-γ–induced tyrosine phosphorylation of STAT1, Jak1, Jak2 and STAT3 was assessed by immunoblotting. (**c**) Monocytes were stimulated with IFN-γ for 10 min, followed by the addition of 100 μM of genistein. STAT activation was assessed by EMSA with the hSIE oligonucleotide. (**d**) HeLa cells were infected with control GFP- or STAT1-encoding adenoviruses at a multiplicity of infection of 1000; 2 days after infection, cells were activated with 400 U/ml of IFN-γ or 3000 U/ml of IFN-α. STAT DNA binding was assessed by EMSA with the hSIE oligonucleotide. pY-STAT1 and total STAT1 and STAT3 protein were analyzed by immunoblotting. (**e**) Monocytes were cultured with 3 U/ml (filled triangles) or 100 U/ml (open circles) of IFN-γ, and SOCS1, SOCS3 and STAT1 mRNA were measured with real-time PCR. (**f**) Monocytes were pulsed with [$^{35}$S]methionine (see Methods) and STAT1 was immunoprecipitated from cell extracts obtained after an additional 0, 6, 24, and 48 h. IP, immunoprecipitation.

stimulus resulted in sensitization of STAT1 signaling (**Fig. 6d**), thus indicating that increased STAT1 expression was sufficient to sensitize IFN-γ signaling. Second, the effects of priming on IFN-γ activation of STAT1 and STAT3 were compared. IFN-γ weakly activated STAT3 in human monocytes (**Fig. 6b**), and STAT3 activation and STAT3 protein were minimally affected by priming. Comparable activation of STAT3 in M-CSF–cultured and IFN-γ–primed cells was consistent with comparable IFN-γR expression and Jak activation (**Fig. 6a,b**). In contrast to STAT3, STAT1 expression greatly increased, as did STAT1 activation, when the same cells were treated with IFN-γ (**Fig. 6b**). These results, taken together, implied a role for increased STAT1 expression in the sensitization of IFN-γ signaling. When primed monocytes were stimulated with IFN-α, STAT1, but not STAT3, activation was sensitized (**Fig. 4f** and data not shown), suggesting that increased STAT1 expression can affect signaling by at least two receptors.

IFN-γ induces SOCS1 expression, which feeds back and inhibits IFN-γ signaling. The kinetics of SOCS1 mRNA expression were analyzed when monocytes were treated with either low (3 U/ml) or high (100 U/ml) concentrations of IFN-γ that induce, respectively, priming or feedback inhibition. Low doses of IFN-γ induced transient increases in SOCS1 mRNA that returned to baseline amounts after 4 h and remained low during the remainder of the priming period (**Fig. 6e**). In contrast, when high concentrations of IFN-γ were used, SOCS1 mRNA continued to increase for 24 h and remained elevated for the duration of the priming period. STAT1 mRNA was elevated in a sustained manner with both priming and activating concentrations of IFN-γ (**Fig. 6e**). Thus, priming of macrophages with low doses of IFN-γ resulted in high expression of STAT1 in the absence of induction of feedback inhibition by SOCS1. The sustained increase in STAT1 mRNA indicated that activation of STAT1 gene expression contributed to the increase in amounts

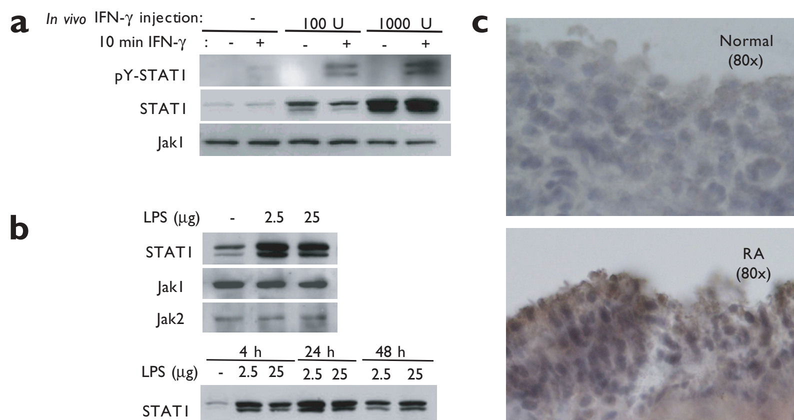


**Figure 7. Regulation of IFN-γ signaling and STAT1 expression *in vivo*.** (**a**) Six-week-old C57BL/6J mice were injected with IFN-γ; resident peritoneal macrophages were collected 1 day later and stimulated for 10 min with 100 U/ml of IFN-γ. pY-STAT1 and total STAT1 and Jak1 protein were analyzed by immunoblotting. (**b**) Mice were injected with saline or LPS. (Upper panel) Immunoblotting of STAT1, Jak1 and Jak2 protein in cell extracts from splenic macrophages (CD11b$^{+}$ populations) isolated 1 day after injection. (Lower panel) Immunoblotting of STAT1 protein in cell extracts from splenic macrophages isolated after injection. One representative of three experiments is shown. (**c**) Normal and RA synovial tissues were stained with anti-STAT1. Photomicrographs are representative of ten RA and nine normal specimens.

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of STAT1. In addition, pulse-chase experiments showed that newly synthesized STAT1 protein was stable (**Fig. 6f**). The combination of sustained expression of STAT1 mRNA and the stability of STAT1 protein contributed to the increase in STAT1 protein observed during priming. In contrast, SOCS1 protein is extremely labile[23], and we were not able to detect SOCS1 protein expression by immunoprecipitation or immunoblot.

### IFN-γ signaling and STAT1 expression *in vivo*

The physiological role of increased STAT1 expression was further investigated by analyzing its expression *in vivo* during conditions of acute and chronic inflammation. Preinjection of mice with a low dose of IFN-γ (100 U) resulted in increased STAT1 protein and autosensitization of IFN-γ signaling *in vivo* in resident peritoneal macrophages (**Fig. 7a**). When mice were injected with tenfold more IFN-γ (1000 U), STAT1 protein increased further; in contrast, after restimulation with IFN-γ, STAT1 tyrosine phosphorylation remained high but showed minimal additional increase. This indicated the induction of feedback inhibition that blunted the effects of increased STAT1 expression and showed that IFN-γ–mediated sensitization and feedback inhibition of IFN-γ signaling *in vivo* was similar to regulation *in vitro*.

Injection of lipopolysaccharide (LPS) serves as a model of septicemia and results in rapid IFN-γ expression, which is thought to play an important role in LPS toxicity. Injection of mice with LPS induced STAT1 expression in splenic macrophages within 4 h, and increased STAT1 expression was sustained for at least 48 h (**Fig. 7b**). Rheumatoid arthritis (RA) is a chronic inflammatory condition primarily involving joints that is believed to represent a $T_H1$ response. RA joint macrophages are strongly activated and express high amounts of IFN-γ–inducible genes, despite low concentrations of extracellular IFN-γ[29]. We investigated the expression of STAT1 in RA using immunohistochemistry to analyze inflammatory synovial tissue obtained during joint-replacement surgery. RA synovial tissue cells expressed high amounts of STAT1 relative to normal controls, especially in the macrophage-containing lining layer where cells are most highly activated (**Fig. 7c**). The differences in expression of STAT1 were statistically significant (RA *versus* normal, $P < 0.05$). These results demonstrated high STAT1 amounts *in vivo* in two inflammatory settings where strong IFN-γ responses lead to tissue pathology and suggested that high STAT1 expression contributed to these inflammatory processes.

## Discussion

We found that low doses of IFN-γ that did not themselves activate macrophages sensitized signaling in response to restimulation with IFN-γ. The mechanism of IFN-γ signaling sensitization involved increased STAT1 expression in the absence of feedback inhibition by SOCS1. This finding is reminiscent of a report demonstrating sensitization of IFN-γ signaling by low constitutive amounts of type I IFNs that were subthreshold for detectable signaling events[26]. However, the mechanisms of sensitization appear to differ, as type I IFNs did not affect STAT1 tyrosine phosphorylation, but enhanced dimerization of phosphorylated STAT1[26]. In contrast, we found that exposure to low doses of IFN-γ markedly enhanced subsequent STAT1 tyrosine phosphorylation. Sensitization by IFN-γ was independent of concomitant type I IFN signaling, as sensitization occurred in macrophages from mice deficient in type I IFNRs. Another difference appears to be that sensitization of IFN-γ signaling by type I IFNs was constitutive in several cell types, whereas autosensitization of IFN-γ signaling occurred preferentially in macrophages relative to lymphocytes and was regulated by glucocorticoids (unpublished data). IFN-γ is a major activator of macrophages, and sensitization of IFN-γ signaling may be particularly important to achieve full macrophage activation early in immune responses when IFN-γ concentrations are low. Autoregulation of IFN-γ signaling by IFN-γ appears to be complex and cell-type specific, as IFN-γ actually suppresses IFN-γ signaling in lymphocytes by down-regulating expression of the IFN-γR[17,18].

A key paradigm in signal transduction is ligand-mediated desensitization and feedback inhibition of signaling, which limits the intensity and duration of signal transduction events and thus prevents the potentially deleterious consequences of excessive cellular responses. Desensitization can be mediated by a variety of mechanisms, including modification and endocytosis of receptors[30], suppression of receptor expression[17,18] or induction of negative regulators of signaling, such as SOCS or PIAS[23]. In contrast, autoamplification of signal transduction, which would be helpful in the early phases of immune responses where rapid and strong activation of cells is required, is not well understood. The only previous example of which we are aware is the amplification of IL-2 signaling by IL-2 itself. This mechanism induces prolonged expression of high-affinity IL-2Rs, thus allowing for a burst of proliferation that is sustained over several days in response to low doses of IL-2[31]. In contrast to IL-2, autosensitization of IFN-γ signaling did not involve increased expression of ligand-binding receptor subunits. Instead, sensitization was mediated, at least in part, by increased expression of STAT1. STAT1 protein increased throughout the priming period, which could be explained by the sustained expression of STAT1 mRNA that leveled-off ~4 h after addition of IFN-γ, combined with the stability of STAT1 protein. Thus, receptor-STAT interactions can be increased either by increasing receptor or STAT expression.

The differences between these two mechanisms are that increasing receptor expression will increase activation of multiple signaling pathways downstream of one receptor, whereas increasing STAT1 expression will selectively activate only one pathway downstream of Jaks and will have the potential to regulate activation of many different receptors that contain docking sites for STAT1. Selective activation of STAT1-dependent signaling will not only sensitize Jak-STAT signaling, but will alter the balance between STAT1-dependent and opposing signaling pathways downstream of the IFN-γR[32,33], and thus has the potential to qualitatively alter the outcomes of IFN-γ signaling.

The Jak-STAT signaling pathway is typically regulated by modulation of the function of pre-existing Jak and STAT proteins, either by post-translational modification or by protein-protein interactions. Important post-translational modifications include tyrosine and serine phosphorylation of Jaks and STATs[34], methylation of STATs[35] and degradation by proteosomes[23]. Key protein-protein interactions include inhibition of Jak catalytic activity by SOCS proteins[36] and of STAT DNA-binding activity by PIAS proteins[37]. STATs are broadly and constitutively expressed and, although forced overexpression of STATs affects signaling[38], a role for physiologic modulation of STAT protein in regulating intensity of cytokine signaling has not previously been reported.

Here, we have provided several lines of evidence that support a role for increased STAT1 expression in IFN-γ signaling sensitization. First, sensitization was not accompanied by any changes in expression of IFN-γRs or in activation of Jak1, Jak2 or STAT3 by IFN-γ. These results indicated that IFN-γ delivered a comparable proximal signal to both nonprimed and primed macrophages. Second, the rate of STAT1 deactivation was comparable in nonprimed and primed cells, indicating that priming did not inactivate a STAT1 phosphatase or suppress degradation by proteosomes. Third, sensitization of signaling was specific for STAT1 relative to STAT3 when either IFN-γ or IFN-α were

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used to stimulate primed cells; this was consistent with increased expression of STAT1 but not STAT3. Finally, forced expression of STAT1 led to increased STAT1 activation. These data argue for a model in which increased intracellular STAT1 leads to more efficient docking onto the activated IFN-γR complex. A key component of this model is that low priming doses of IFN-γ capable of activating sustained STAT1 expression did not effectively activate feedback inhibition by SOCS1. In contrast, high activating doses of IFN-γ induced sustained expression of SOCS1, and thus engaged feedback inhibition.

In contrast to experiments in which purified IFN-γ was used, in the PBMC coculture system the induction of STAT1 expression was variable and, in many experiments, the increase in STAT1 protein was insufficient to explain the increase in STAT1 tyrosine phosphorylation. Thus, other factors may also contribute to sensitization of IFN-γ signaling. One possibility would be basal production of type I IFNs. Future experiments should aim to identify factors other than IFN-γ present in PBMC cocultures that regulate IFN-γ signaling.

A key question is under what conditions does the sensitization of IFN-γ signaling we have described here occur *in vivo*. Macrophage responsiveness to IFN-γ *in vivo* can vary[39]. We have shown that autosensitization of IFN-γ signaling occurs *in vivo* after injection of low doses of IFN-γ that mimic transient or low IFN-γ production, such as may occur early in an immune response or with low amounts of antigens or pathogens. In addition, we have investigated the regulation of STAT1 expression and IFN-γ signaling *in vivo* after LPS injection and in RA, which represent examples of, respectively, acute and chronic inflammation in which IFNs are expressed.

STAT1 protein expression rapidly increased after injection of LPS and was elevated in RA synovium, especially in the lining layer that contains the most highly activated macrophages. These results suggested that elevated STAT1 expression may play a role in regulating the intensity of IFN-γ signaling in these inflammatory states. LPS injection, at the doses used, resulted in basal STAT1 activity and the induction of feedback inhibition, consistent with high expression of SOCS1 and SOCS3[40] (unpublished data). IFN-γ signaling in RA macrophages was also partially suppressed by high expression of SOCS proteins[41] (unpublished data). Thus, injection with high doses of LPS or chronic inflammation in RA appear to induce a state in which increased STAT1 expression is opposed by the induction of feedback inhibition, similar to that detected when macrophages are activated with high doses of IFN-γ. SOCS proteins compete with STATs for docking to cytokine receptors[23], including IFN-γR[38]; thus, high expression of STAT1 would serve the function of partially overcoming or balancing inhibition by SOCS proteins. This suggests that the sensitivity of macrophages to IFN-γ is regulated by the opposition of STAT1 and SOCS proteins that are expressed at different relative amounts, depending upon the intensity or duration of an activating stimulus.

## Methods

**Reagents and cell culture.** Experiments with animals were approved by the Hospital for Special Surgery animal care and use committee. Recombinant human IFN-γ was from Roche Molecular Biochemicals (Indianapolis, IN), IFN-αA was from Biosource International (Camarillo, CA), and IL-10 and M-CSF were from R&D Systems (Minneapolis, MN). IFN-γ neutralizing antibody was from R&D Systems, and mAbs to human IFN-α (MMHA2) and human IFN-αR2 (MMHAR2) were from PBL Biomedical Laboratories (New Brunswick, NJ). PBMCs were obtained from whole blood from disease-free volunteers using a protocol approved by the Hospital for Special Surgery institutional review board by density gradient centrifugation with Ficoll (Gibco-BRL, Gaithersburg, MD) and cultured in RPMI-1640 medium (Gibco-BRL) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). Human monocytes were purified from PBMCs immediately after isolation or after 1–3 days of culture by positive selection with anti-CD14 magnetic beads, as recommended by the manufacturer (Miltenyi Biotec, Auburn, CA). In some experiments, negative selection was used to purify monocytes. Similar results were obtained regardless of the method of monocyte purification. Purity of monocytes was >97%, as verified by flow cytometric analysis. In transwell cultures, transwell inserts with membranes of 0.4-μm pore size (Becton Dickinson, Franklin Lake, NJ) were used to separate $CD14^+$ and $CD14^-$ cells. The ratio of $CD14^+$ to $CD14^-$ cells in transwell cultures was 1:4, the typical composition of PBMCs. Bone marrow–derived macrophages were from type I IFNR–deficient mice[27], obtained by culturing bone marrow with 10 ng/ml of M-CSF as described[42].

**EMSA, immunoblotting and metabolic labeling of STAT1.** Total cell extracts were obtained, and protein amounts quantified with the Bradford assay (BioRad, Hercules, CA), as described[43]. Cell extracts (5 μg) were incubated for 15 min at room temperature with 0.5 ng of $^{32}$P-labeled double-stranded high-affinity sis-inducible element (hSIE) oligonucleotide[43], 5′-GTCGACATTTCCCGTAAATC-3′, in a 15-μl binding reaction with 40 mM NaCl and 2 μg of poly(dI)•poly(dC) (Pharmacia, Piscataway, NJ); complexes were resolved on nondenaturing 4.5% polyacrylamide gels. For immunoblotting, cell lysates (5 μg) were fractionated on 7.5% polyacrylamide gels with SDS-PAGE, transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA) and incubated with specific antibodies; enhanced chemiluminescence was used for detection. For pulse-chase experiments, cells were treated with IFN-γ for 8 h followed by overnight labeling in medium that consisted of a 90/10 mixture of methionine-free and regular RPMI-1640 with 5% fetal bovine serum and 100 μCi/ml of [$^{35}$S]methionine, as described[44]. Cells were then extensively washed to remove unincorporated [$^{35}$S]methionine, and STAT1 immunoprecipitations were done at various times. Monoclonal antibodies (mAbs) to STAT1 (clone 1), STAT2 (clone 22), STAT3 (clone 84) and Jak1 (clone 73) were from BD Transduction Laboratories (Lexington, KY); polyclonal Jak2 antibody was from Upstate Biotechnology (Lake Placid, NY). The polyclonal antibody used for STAT1 immunoprecipitation was from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphorylation-specific ($Tyr^{701}$) STAT1 antibody (number 9171) and phosphorylation-specific ($Tyr^{705}$) STAT3 antibody (number 9131) were from Cell Signaling Technology (Beverly, MA). Phosphorylation-specific Jak1 ($Tyr^{1022/1023}$) antibody (number 44-442) and phosphorylation-specific Jak2 ($Tyr^{1007/1008}$) antibody (number 44-426) were from Biosource International.

**Analysis of mRNA.** For semi-quantitative RT-PCR, total cellular RNA was isolated with Trizol (Gibco-BRL) and treated with RNase-free DNase. cDNA was obtained with Moloney murine leukemia virus reverse transcriptase (MMLV-RT) (Gibco-BRL). Each cDNA (2.5%) was subjected to 22–25 PCR cycles with conditions that resulted in a single specific amplification product of the correct size: 30 s denaturation at 94 °C, 1 min annealing at 55 °C and 30 s extension at 72 °C in a GeneAmp 9600 thermal cycler (Perkin Elmer, Norwalk, CT). dNTPs were used at 100 μM and 1 μCi of [$^{32}$P]α-dATP was added to each reaction. No amplification products were obtained when reverse transcriptase was omitted, which indicated the absence of contaminating genomic DNA. Amplification was empirically determined to be in the linear range. For real-time quantitative PCR, DNA-free RNA was obtained with the RNeasy MiniKit (Qiagen, Valencia, CA) with DNase treatment, and 1 μg of total RNA was reverse-transcribed with random hexamers and MMLV-RT. Real-time PCR was done in triplicate with the iCycler iQ thermal cycler and detection system (BioRad) and the PCR Core Reagents kit (Applied Biosystems, Foster City, CA) with 500 nM primers; the final $Mg^{2+}$ concentration was adjusted to 4 mM, as described[45]. Fourfold serial dilutions of cDNAs were used to generate curves of log input amount *versus* threshold cycle, and comparable slopes, for a given primer set, were obtained for the group of cDNAs being tested (signifying comparable efficiencies of amplification). mRNA amounts were normalized relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. When RT was omitted, threshold cycle number increased by at least ten, signifying lack of genomic DNA contamination or nonspecific amplification; the generation of only the correct size amplification products was confirmed with agarose gel electrophoresis. Oligonucleotide primers used were as follows: MIG: 5′-TTGGGCATCATCTTGCTGGTTCT-3′ and 5′-TGGCTGACCTGTTTCTCCCACTT-3′; GBP: 5′-TGAGCAGCACCTTCGTGTACAAT-3′ and 5′-TAGGAACAGAAGTCTGCTACTTG-3′; ISG54: 5′-GTGATAGTAGACCCAGGCATAGT-3′ and 5′-CCCTTGTTATTCCTCACC-3′; IP-10: 5′-TTGCTGCCTTATCTTTCTGACTC-3′ and 5′-ATGGCCTTCGATTCTGGATT-3′; IRF-1: 5′-ATGAGACCCTGGCTAGAG-3′ and 5′-AAGCATCCGGTACACTCG-3′; GAPDH: 5′-GTGAAGGTCGGAGTCAAC-3′ and 5′-TGGAATTTGCCATGGGTG-3′; SOCS1: 5′-TGTTGTAGCAGCTTAACTGTATC-3′ and 5′-AGAGGTAGGAGGTGCGAGT-3′; SOCS3: 5′-CACTCTTCAGCATCTCTGTCGGAAG-3′ and 5′-CATAGGAGTCCAGGTGGCCGTTGAC-3′; STAT1: 5′-TGGGTTTGACAAGGTTCTT-3′ and 5′-TATGCAGTGCCACGGAAAG-3′.

**STAT1 overexpression.** Cells were infected with recombinant adenoviruses encoding both STAT1 and green fluorescence protein (GFP) or control viruses encoding GFP alone. Production of adenoviruses and cellular infection were as described[46]. The infection rate was >95%, as assessed by flow cytometry, and cell extracts were prepared 2 days after infection.

**ELISA.** ELISAs were done with paired antibody sets, as recommended by the manufacturers (BD Pharmingen, San Diego, CA and Endogen, Woburn, MA).

**Immunohistochemistry.** Synovial tissue was obtained from patients undergoing arthroplasty or total joint replacement who met the American College of Rheumatology criteria for RA[47]. Normal synovial tissue was obtained fresh from patients undergoing amputation for diabetes or other etiologies. The protocol was approved by the institutional review board of Northwestern University Medical School and informed consent was obtained. Fresh synovial tissues were frozen and 4-μm sections were cut and immunoperoxidase

stained with an avidin biotin technique (Vector Laboratories, Burlingame, CA) as described[48–51]. Slides, air-dried for 2–16 h, were fixed in cold acetone for 20 min. All subsequent incubations were done for 15 min at 37 °C in a moist chamber. Synovial tissues were pretreated with 50 µl of diluted normal horse serum, incubated with anti-STAT1 (clone 1, BD Transduction Laboratories) or isotype-matched control antibody. Slides were washed and then incubated with biotinylated anti-mouse and subsequently incubated with avidin-conjugated horseradish peroxidase complex. Slides were washed and then stained with diaminobenzidine tetrahydrochloride substrate, rinsed, counterstained with Harris's Hematoxylin and dipped in saturated lithium carbonate for bluing. Serial sections were examined, cell types identified by morphology and the percentages of cells expressing the target antigens determined. All sections were analyzed by a pathologist blind to the identity of the samples and each section was reviewed by two additional observers. Data were analyzed with Student's *t*-tests.

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Competing interests statement

The authors declare that they have no competing financial interests.

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