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Inhibition of IFN-γ Signaling by Glucocorticoids[1]

Xiaoyu Hu,* Wai-Ping Li,† Charis Meng,† and Lionel B. Ivashkiv[2]*†

Recent reports suggest that a novel mechanism of glucocorticoid (GC) immunosuppressive action is inhibition of signaling by IL-2 and IL-12, cytokines that use the Janus kinase-STAT signaling pathway. We investigated whether GCs could also block activation of Janus kinase-STAT signaling by IFN-γ, a potent proinflammatory cytokine. Addition of dexamethasone to PBMC cultures resulted in a dramatic inhibition of IFN-γ activation of STAT1. Several days of exposure to GCs were required for inhibition of IFN-γ signaling to become apparent, and the underlying mechanism was down-regulation of STAT1 expression. GCs suppressed the expression of STAT1 mRNA, but did not affect STAT1 protein stability. STAT1 expression and IFN-γ signaling were preferentially suppressed in macrophages. GCs did not act directly on macrophages, but worked indirectly by regulating macrophage-lymphocyte interactions that control STAT1 expression. GCs inhibited IFN-γ-inducible gene expression, thus demonstrating the physiological significance of inhibition of signal transduction. Our results identify a novel level of regulation of IFN-γ signaling, whereby GCs control the amplitude of IFN-γ signaling by regulating STAT1 expression. These results suggest that inhibition of IFN-γ signaling contributes to the immunosuppressive action of GCs. ***The Journal of Immunology*****, 2003, 170: 4833–4839.**

Interferon-γ is a strong activator of inflammatory responses and cellular immunity, and is a major activator of macrophages (1). IFN-γ is produced predominantly by NK, Th1, and $CD8^+$ cells and binds to a heterodimeric cell surface receptor that is ubiquitously expressed. Ligation of the IFN-γR results in activation of the receptor-associated tyrosine kinases Janus kinase 1 (Jak1)[3] and Jak2, leading to the tyrosine phosphorylation and activation of the transcription factor STAT1 (1, 2). Experiments using mice and cell lines deficient for STAT1 have demonstrated that STAT1 mediates many, but not all, of the immune and proinflammatory effects of IFN-γ (3). Consistent with the potent cell-activating properties of IFN-γ, overproduction and hyper-responsiveness to this cytokine are associated with deleterious effects, including autoimmunity and tissue damage secondary to excessive inflammation (4–11). Much attention has been focused on understanding the mechanisms that restrain or attenuate IFN-γ action, thus protecting the host from excessive IFN-γ action. IFN-γ signaling upstream of STAT1 is subject to down-regulation by suppressors of cytokine signaling (SOCS) that inactivate Jaks and tyrosine phosphatases and by decreased expression of the IFN-γR2 subunit (12–14). Once STAT1 is activated, its activity can be suppressed by protein inhibitor of activated STAT1 that suppresses DNA binding and by tyrosine phosphatases (15–18).

Glucocorticoids (GCs) are potent anti-inflammatory and immunosuppressive agents and are used in the treatment of numerous autoimmune and inflammatory diseases (19). Endogenous GCs play a physiologic role in feedback inhibition of immune/inflammatory responses and in maintaining homeostasis. GCs inhibit the NF-κB and AP-1 families of transcription factors (20) and thus suppress the production of multiple cytokines and chemokines, including IL-2, IL-6, IFN-γ, and IL-8 (21). In addition to the well-established inhibition of NF-κB and AP-1 pathways, the interaction of GCs with the Jak-STAT signaling pathway is being increasingly appreciated. Such interactions may have either a positive or a negative impact on cytokine signaling. One example of a positive interaction is synergistic activation of β-casein gene expression in the mammary gland that is mediated by a direct physical interaction between prolactin-activated STAT5 and the GC receptor (GCR) that is activated by GCs (22). In T lymphocytes, the synthetic GC dexamethasone (dex) reduces cellular responsiveness to IL-12 (23) and inhibits IL-12 induced STAT4 activation without altering the phosphorylation of Jaks (24). Dex also inhibits IL-2-induced STAT5 activation in primary human T cells (25). However, the mechanisms for inhibiting IL-12 and IL-2 signaling seem to differ, as inhibition of IL-2 signaling by GCs involves the suppression of IL-2R β-chain and Jak3 expression with concomitant decreased activation of Jaks (25). Thus, the outcomes and mechanisms of cross-talk between Jak-STAT and GC pathways are cell type and cytokine specific.

Although interaction of GCs with cytokine signaling pathways represents an area of increasing interest, relatively little is known about the interplay between GCs and IFN-γ signaling. GCs have been shown to potentiate IFN-γ induction of FcγRI gene expression in myeloid cells (26), and a recent report showed that the underlying mechanism is potentiation of STAT1 transcriptional activity, probably by functional interaction among GCR, STAT1, and PU.1 transcription factors (27). In contrast, GCs inhibited IFN-γ induction of MHC class II expression (28, 29), indicating that GCs can also suppress IFN-γ activity. In this report we demonstrate that GCs negatively regulate IFN-γ signaling, but that several days of exposure to GCs are required for this inhibition to become apparent. One mechanism underlying the suppression of IFN-γ signaling by GCs is down-regulation of STAT1 expression.

*Graduate Program in Immunology, Weill Graduate School of Medical Sciences of Cornell University, and †Department of Medicine, Hospital for Special Surgery, New York, NY 10021

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[2] Address correspondence and reprint requests to Dr. Lionel B. Ivashkiv, Department of Medicine, Hospital for Special Surgery, 535 East 70th Street, New York, NY 10021. E-mail address: ivashkivl@hss.edu

[3] Abbreviations used in this paper: Jak, Janus kinase; CHX, cycloheximide; dex, dexamethasone; GC, glucocorticoid; GCR, glucocorticoid receptor; hSIE, high affinity Sis-inducible element; IP-10, inducible protein-10; IRF-1, IFN regulatory factor-1; SOCS, suppressor of cytokine signaling.

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These results identify a novel mechanism for down-regulation of IFN-γ signaling and suggest that inhibition of IFN-γ signaling contributes to the immunosuppressive action of GCs.

Materials and Methods

Reagents and cell culture

Recombinant human IFN-γ was purchased from Roche (Indianapolis, IN), and water-soluble dex was obtained from Sigma-Aldrich (St. Louis, MO). PBMCs were obtained from whole blood from disease-free volunteers by density gradient centrifugation using Ficoll (Invitrogen, Carlsbad, CA) and were cultured in RPMI 1640 medium (Invitrogen, San Diego, CA) supplemented with 10% charcoal-stripped FBS (HyClone, Logan, UT). Monocytes were purified from PBMCs immediately after isolation or after 2–3 days of culture by positive selection using 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. The purity of monocytes was >97% as verified by flow cytometric analysis using a FACScan flow cytometer with CellQuest software (BD Biosciences, San Jose, CA), as previously described (30). Staining for cell surface IFN-γ-R1 was performed using mAb GIR-94 (BD PharMingen, San Diego, CA).

EMSAs, immunoprecipitation, and immunoblotting

Cell extracts were obtained, and protein levels were quantitated using the Bradford assay (Bio-Rad, Hercules, CA) as previously described (30). 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 in a 15-μl binding reaction containing 40 mM NaCl and 2 μg of poly(dI-dC) (Pharmacia Biotech, Piscataway, NJ), as previously described (31), and complexes were resolved on nondenaturing 4.5% polyacrylamide gels. For immunoblotting, 10 μg of cell lysates or immunoprecipitates (see below) were fractionated on 7.5% polyacrylamide gels using SDS-PAGE, transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA), and incubated with specific Abs, and ECL was used for detection. mAbs against STAT1, STAT3, and Jak1 were obtained from Transduction Laboratories (Lexington, KY), and polyclonal Jak2 Ab was purchased from Upstate Biotechnology (Lake Placid, NY). Phosphorylation-specific (tyrosine 701) STAT1 Ab was obtained from Cell Signaling Technology (Beverly, MA), and phosphorylation-specific Jak1 (tyrosine 1022/1023) Ab was purchased from BioSource International (Camarillo, CA). For Jak immunoprecipitations, whole-cell extracts corresponding to 10–20 × 10^6 cells were incubated with anti-Jak1 polyclonal Ab (Santa Cruz Biotechnologies, Santa Cruz, CA), and immunoprecipitates were collected and washed as previously described (31).

Analysis of apoptosis and cell viability

Detection of apoptotic cells using annexin V staining, propidium iodide exclusion, and detection of cells with subdiploid cellular DNA content was performed as previously described (30).

Metabolic labeling of STAT1

For pulse-chase experiments, cellular proteins were radiolabeled by culturing cells overnight in medium that consisted of a 90/10 mixture of methionine-free and regular RPMI with 5% FBS and 100 μCi/ml of [^{35}S]methionine as previously described (32). Cells were then extensively washed to remove unincorporated [^{35}S]methionine and cultured in medium containing cold methionine, and whole-cell lysates were collected at various times. STAT1 protein was immunoprecipitated from cell lysates using anti-STAT1 polyclonal Ab (Santa Cruz Biotechnologies) as previously described (32).

Analysis of mRNA levels

Total cellular RNA was isolated using TRIzol (Life Technologies, Gaithersburg, MD) according to the instructions of the manufacturer. For RT-PCR, RNA was treated with RNase-free DNase (Life Technologies), and cDNA was obtained using Moloney murine leukemia virus reverse transcriptase (Life Technologies). A small part (2.5%) of each cDNA was subjected to 20–25 cycles of PCR using conditions that result 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). The dNTPs were used at 100 μM, and 1 μCi of [α-^{32}P]dCTP was added to each reaction. No amplification products were obtained when reverse transcriptase was omitted, indicating the absence of contaminating genomic DNA. Amplification was empirically determined to be in the linear range, and accuracy of quantitation was verified using serial dilutions of cDNA at a constant cycle number. For real-time quantitative PCR, 10 ng of cDNA was used for each reaction. PCR was performed in triplicate using the iCycler iQ thermal cycler and detection system (Bio-Rad) and the PCR Core Reagents kit (PE Applied Biosystems, Foster City, CA) with 500 nM primers. mRNA levels were normalized relative to GAPDH mRNA. When reverse transcriptase was omitted, threshold cycle number increased by at least 10, signifying the lack of genomic DNA contamination or nonspecific amplification, and generation of only the correct size amplification products was confirmed using agarose gel electrophoresis. The oligonucleotide primers used are: Stat5, 5′-CCC GGA ACG CAA CCT GTG GAA CC and 5′-GGG GCG AGA GGC GGG AGT CAA GA; inducible protein-10 (IP-10), 5′-TTG CTG CCT TAT CTT TCT GAC TC and 5′-ATG GCC TTC GAT TCT GGA TT; IFN regulatory factor-1 (IRF-1), 5′-ATG AGA CCC TGG CTA GAG and 5′-AAG CAT CCG GTA CAC TCG; GAPDH, 5′-GTG AAG GTC GGA GTC AAC and 5′-TGG AAT TTG CCA TGG GTG; and Stat1, 5′-TGG GTT TGA CAA GGT TCT T and 5′-TAT GCA GTG CCA CGG AAA G.

Results

Inhibition of IFN-γ signaling and STAT1 expression in PBMCs requires prolonged incubation with dex

Simultaneous addition of dex and IFN-γ was previously shown not to affect proximal events in IFN-γ signaling (27). However, if GCs regulate IFN-γ signaling by inducing de novo expression of SOCS proteins or by decreasing the expression of IFN-γR-Jak-STAT signaling proteins, we would predict that preincubation with dex would be necessary to detect any effect of dex on IFN-γ signaling. Therefore, we investigated the effects of preincubation with dex on IFN-γ signal transduction in human PBMCs. Freshly isolated cells were cultured with dex for up to 4 h and stimulated with IFN-γ for 10 min, and cell extracts were subjected to EMSA. IFN-γ treatment resulted in the rapid induction of specific STAT1 DNA binding (Fig. 1*A*, *lane 2*) as previously described (31). Activation of STAT1 DNA binding by IFN-γ was not altered by up to 4 h of preincubation with dex (Fig. 1*A*, *lanes 3–5*), consistent with the results reported by Aitomaki et al. (27). IFN-γ-induced phosphorylation of tyrosine 701 on STAT1 is a prerequisite for STAT1 dimerization and activation. STAT1 tyrosine phosphorylation was induced by IFN-γ stimulation and was not inhibited by dex in a manner that correlated with STAT1 DNA-binding activity (Fig. 1*A*, *middle panel*). Reprobing of a replicate filter showed that treatment with dex for up to 4 h did not alter cellular STAT1 protein expression (Fig. 1*A*, *bottom panel*). These results suggest that dex does not directly induce the expression of inhibitors such as SOCS that are rapidly induced and whose inhibitory effects are typically apparent within 1–2 h (12).

Given that long periods of treatment with GCs are required for effective repression of IFN-γ-induced MHC class II gene expression (28, 29), we assessed the effect of prolonged dex treatment on IFN-γ signaling. When the period of culture with dex was extended to 24 and 72 h, the induction of STAT1 DNA binding by IFN-γ was strongly suppressed (Fig. 1*B*). In addition to inhibition of signaling, incubation with dex for 24 or 72 h resulted in a dramatic decrease in STAT1 protein expression to nearly undetectable levels (Fig. 1*B*). Reprobing of the same filter revealed that STAT3, Jak1, and Jak2 levels were comparable in all lanes (Fig. 1*B* and data not shown), demonstrating that STAT1 expression was specifically suppressed by dex. Inhibition of IFN-γ-induced STAT1 activation and suppression of STAT1 protein levels by dex have been observed in >30 independent experiments using different blood donors (data not shown). Although, in contrast to effects on thymocytes and activated cells, dex does not cause apoptosis of human PBMCs such as those used in these experiments (33), we carefully investigated whether cell death may contribute to the

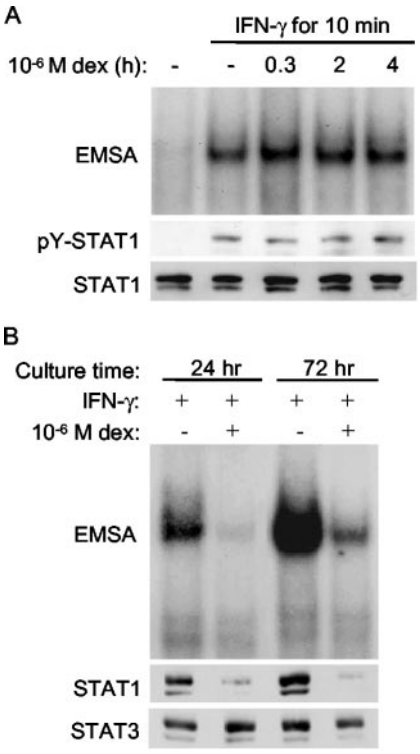

FIGURE 1. Prolonged dex treatment inhibits IFN-γ signaling and STAT1 protein expression. *A*, PBMCs were treated with 10^{-6} M dex for the indicated periods of time, followed by a 10-min stimulation with 10 U/ml IFN-γ. Whole-cell extracts were used in the EMSA with the hSIE STAT-binding oligonucleotide (*top panel*). The same extracts were analyzed using immunoblotting with Ab against tyrosine-phosphorylated STAT1 (pY-STAT1), followed by probing a replicate filter with Ab against STAT1 (*middle* and *bottom panels*). *B*, PBMCs were cultured for 24 and 72 h in the presence or the absence of 10^{-6} M dex and subsequently activated with IFN-γ for 10 min. Cell extracts (5 μg) were subjected to EMSA (*top panel*). The same cell extracts were assayed for both STAT1 and STAT3 levels by immunoblotting (*middle* and *bottom panels*).

decreased STAT1 activation or expression. Trypan blue and propidium iodide exclusion were used to quantify the numbers of viable cells, annexin V staining was used to assess the numbers of cells in the early phases of apoptosis, and cells in the later stages of apoptosis were measured using flow cytometry to identify cells with subdiploid DNA content (Table I). All four approaches showed that treatment with dex resulted in a <10% decrease in the number of viable cells, indicating that the nearly complete block in STAT1 DNA binding and the lower STAT1 levels (Fig. 1*B*) could not be explained on the basis of dex-induced apoptosis and lower cell numbers.

Table I. *Percentage of viable cells assessed by four different assays following 10^6 M dex treatment of human PBMC*

	Culture Duration					
	24 h		48 h		72 h	
	Control	dex	Control	dex	Control	dex
Trypan blue negative	86	79	90	79	82	75
PI negative	ND	ND	ND	ND	90	81
Annexin V negative	ND	ND	ND	ND	82	78
% cells with 2n DNA content	90	87	86	81	84	80

The dose dependence of inhibition of IFN-γ signaling by dex was investigated (Fig. 2*A*). Importantly, dex inhibited STAT1 activation at concentrations that correspond to physiologic (10^{-8}–10^{-10} M), stress (10^{-7} M), and therapeutic (10^{-6} M) corticosteroid concentrations (19). Suppression of STAT1 expression by dex was dose dependent as well and correlated with the level of suppression of STAT1 activation (Fig. 2*A*, *panel 2*). Levels of STAT3 and Jak1 were comparable in all conditions (Fig. 2*A*). These results suggest that inhibition of STAT1 expression not only may play a role in mediating the therapeutic immunosuppressive effects of GCs, but may also be important in homeostasis and the physiologic regulation of the intensity of cellular responses to IFN-γ. Dex inhibited IFN-γ activation of STAT1 tyrosine phosphorylation (Fig. 2*B*, *lanes 2* and *3*), consistent with the inhibition of DNA binding. RU486 is a competitive antagonist of the GCR that is activated by dex. Coincubation of cells with dex and a 10-fold molar excess of RU486 completely reversed the inhibitory effects of dex on both STAT1 tyrosine phosphorylation and STAT1 expression (Fig. 2*B*). These results indicate that inhibition of IFN-γ signaling is mediated by binding of dex to GC receptors.

Dex suppresses STAT1 mRNA expression, but does not alter STAT1 protein stability

We investigated the level at which dex regulated STAT1 expression. First, the effect of dex on STAT1 mRNA expression was assessed using real-time PCR. Four hours of dex treatment did not alter STAT1 mRNA levels (Fig. 3*A*, bars 2 and 3), consistent with the finding that 4 h of dex treatment did not suppress STAT1 protein expression (Fig. 1*A*). In contrast, after 24 or 48 h of culture, STAT1 mRNA levels were significantly lower in cells treated with dex than in control cells (Fig. 3*A*). The lower STAT1 mRNA levels in dex-treated relative to control cells paralleled the lower STAT1 protein levels in dex-treated cells (Figs. 1*B* and 2). Dex

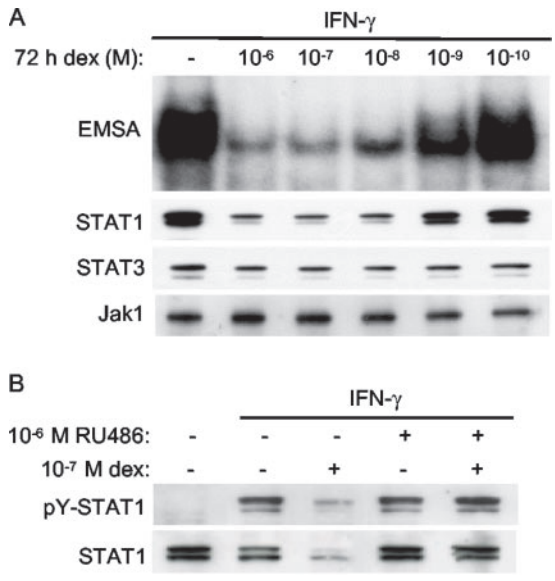

FIGURE 2. Dex inhibits IFN-γ signaling and STAT1 expression in a dose-dependent fashion, and this inhibition is mediated by the GCR. *A*, PBMCs were treated with different doses of dex for 72 h before a 10-min stimulation with IFN-γ (10 U/ml). Cell extracts were assayed for binding to the hSIE oligonucleotide using EMSA. The same extracts were analyzed for STAT1, STAT3, and Jak1 protein levels by immunoblotting. *B*, PBMCs were cultured for 48 h with 10^{-7} M dex, 10^{-6} M RU486, or both, and subsequently treated with 10 U/ml of IFN-γ. Whole-cell extracts were assayed for both tyrosine-phosphorylated and total STAT1 protein levels by immunoblotting.

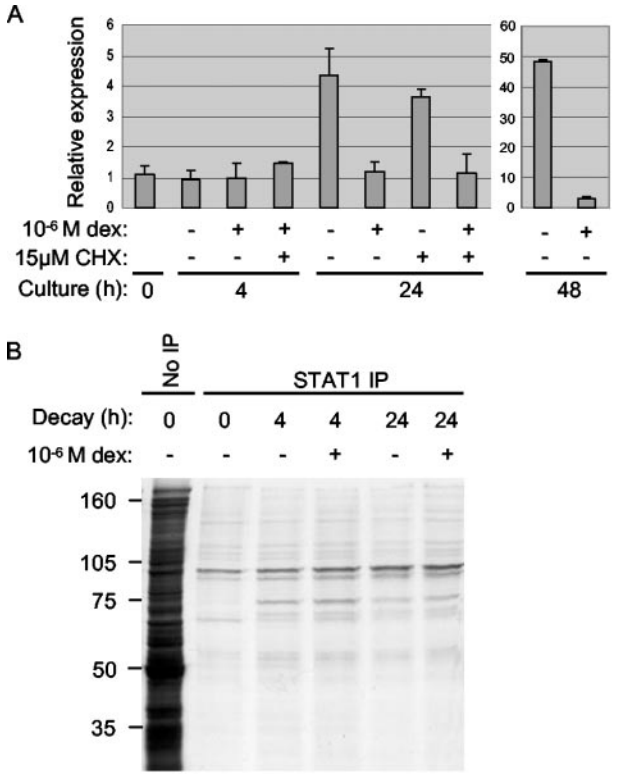

FIGURE 3. Dex inhibits STAT1 mRNA expression but does not affect STAT1 protein stability. *A*, PBMCs were either left untreated (bar 1) or were treated with dex and/or CHX for the indicated time periods. STAT1 mRNA was measured using real-time PCR as described in *Materials and Methods*. A representative experiment of three performed is shown. *B*, PBMCs were pulsed with [^{35}S]methionine overnight, washed, and cultured in complete medium in the presence or the absence of dex. STAT1 was immunoprecipitated from cell extracts obtained after an additional 0, 4, and 24 h. IP, immunoprecipitation.

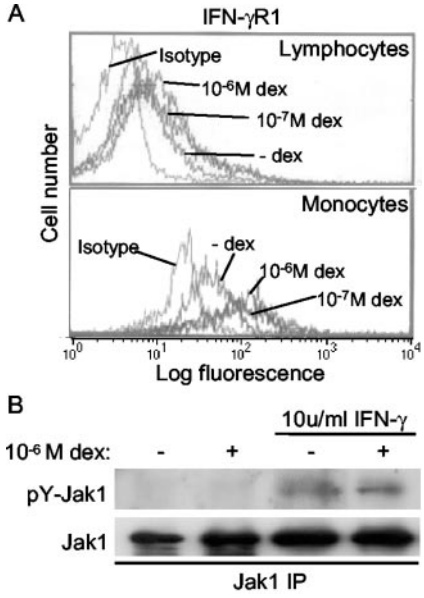

FIGURE 4. Dex does not inhibit proximal steps in the IFN-γ signal transduction pathway. *A*, PBMCs were incubated with the indicated doses of dex for 48 h and surface expression of IFN-γR1 was analyzed by flow cytometry. Staining with isotype-matched control Ab and with anti-IFN-γR1 Ab on lymphocytes (*upper panel*) and monocytes (*lower panel*) is shown. *B*, PBMCs were cultured with or without 10^{-6} M dex for 48 h before a 10-min stimulation with IFN-γ. Anti-Jak1 immunoprecipitates were subjected to immunoblotting using tyrosine phosphorylation-specific Jak1 Ab.

blocked the increase in STAT1 mRNA that occurred during culture (Fig. 3), which was also observed at the protein level (data not shown; see also Fig. 5). It was possible that dex suppressed STAT1 mRNA expression by inducing a repressor of STAT1 expression. This possibility was investigated using cycloheximide (CHX) to block de novo protein synthesis. Dex suppressed STAT1 mRNA expression even in the presence of CHX during 24 h of culture (Fig. 3*A*, bars 7 and 8); it was not possible to test the effects after 48 h of culture secondary to cell death in CHX-treated wells. These results indicate that dex suppressed STAT1 mRNA expression, and this suppression was not dependent on de novo protein synthesis. Next, pulse-chase experiments were performed to examine the effect of dex on STAT1 protein stability. As previously reported, STAT1 was stable, with no clear degradation over 24 h (Fig. 3*B*). The addition of dex did not have any apparent effect on the stability of STAT1 protein. These results, taken together, indicate that one mechanism by which dex suppresses STAT1 expression is inhibition of STAT1 gene expression.

Dex does not affect IFN-γ signaling upstream of STAT1

We investigated whether, in addition to suppressing STAT1 expression, dex affected the expression or activation of other components of the IFN-γR-Jak-STAT signaling pathway. Cell surface expression of IFN-γR1 was analyzed using flow cytometry. Concentrations of dex (10^{-7} and 10^{-6} M) that strongly inhibited IFN-γ activation of STAT1 minimally affected cell surface IFN-γR1 expression on lymphocytes (Fig. 4*A*, *upper panel*). Consistent with previous reports (34), dex actually increased the expression of IFN-γR1 on monocytes (Fig. 4*A*, *lower panel*). Ligation of the IFN-γR results in activation of receptor-associated Jak1 and Jak2. Dex did not alter the levels of these kinases (Fig. 2*A* and data not shown), and we investigated whether dex affected the activation of Jak1 by tyrosine phosphorylation. Induction of tyrosine phosphorylation of Jak1 by IFN-γ was not inhibited by dex (Fig. 4*B*). These results indicate that, consistent with the literature (27), dex did not inhibit proximal steps in IFN-γ signal transduction. Taken together with the dramatic decrease in STAT1 expression after treatment with dex (Fig. 1) and with the evidence that STAT1 levels modulate IFN-γ signaling (35, 36), these results strongly suggest that the decrease in STAT1 expression is the major mechanism by which dex inhibits IFN-γ signaling.

Dex inhibits IFN-γ signaling in monocytes, but not in CD14$^-$ blood cells

To identify the cell types in which dex inhibited IFN-γ signaling, PBMCs were cultured for 3 days with or without dex, separated into CD14$^+$ monocytes and CD14$^-$ cells (mostly lymphocytes), and stimulated with IFN-γ. STAT1 DNA binding was strongly induced by IFN-γ in CD14$^+$ monocytes, where inhibition of STAT1 activation by dex was prominent (Fig. 5*A*, *lanes 3* and *4*). In contrast, the same dose of IFN-γ only weakly activated STAT1 in CD14$^-$ cells, and an inhibitory effect of dex was not apparent (*lanes 7* and *8*). The levels of STAT1 protein were higher in monocytes than in CD14$^-$ cells, and the high level of STAT1 expression in CD14$^+$ monocytes was suppressed by dex, whereas dex did not affect STAT1 protein levels in CD14$^-$ cells (Fig. 5*B*, *top panel*). Immunoblotting of the same extracts with Ab against STAT3 showed comparable levels of STAT3 protein (Fig. 5*B*, *bottom panel*). These results indicate that dex inhibited IFN-γ signaling in monocytes preferentially relative to CD14$^-$ cells.

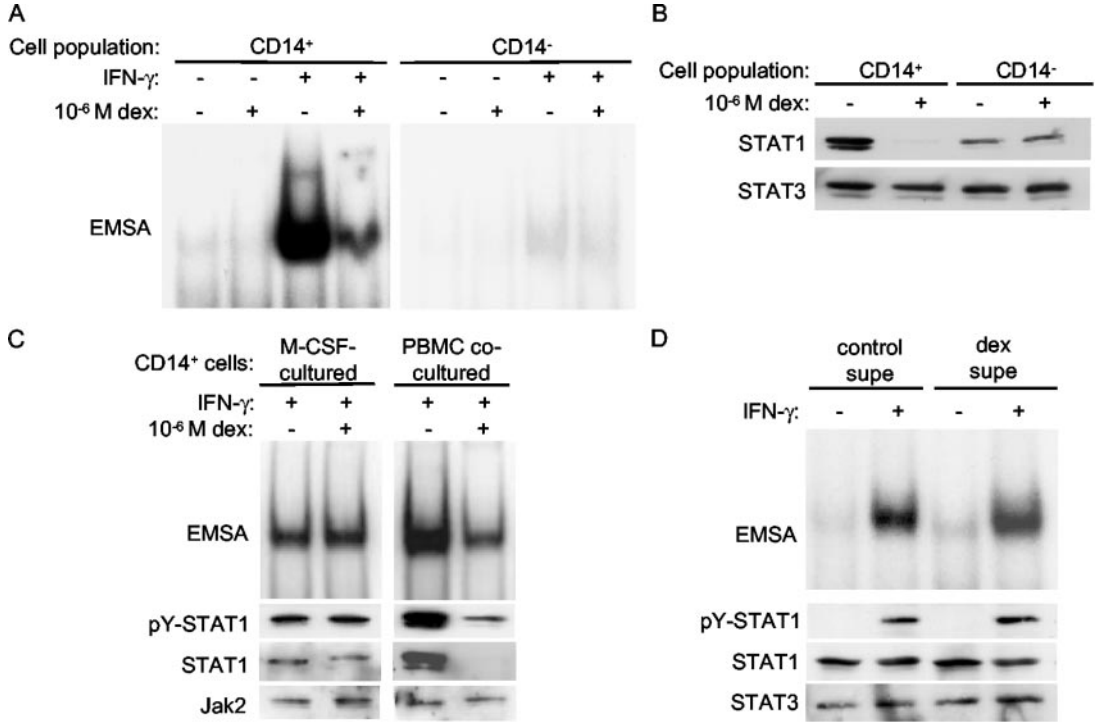

FIGURE 5. Inhibition of IFN-γ-induced STAT1 activation by dex occurs in monocytes that are derived from PBMC cultures. *A*, PBMCs were cultured in the presence or the absence of 10^{-6} M dex for 3 days, and then $CD14^+$ and $CD14^-$ populations were isolated and activated with IFN-γ for 10 min. Equal amounts of cell extracts were loaded onto each lane and subjected to EMSA. *B*, The extracts corresponding to lanes 1, 2, 5, and 6 in *A* were used to determine protein levels of STAT1 and STAT3 by immunoblotting. *C*, M-CSF-cultured monocytes were treated with 10^{-6} M dex for 3 days, followed by a 10-min IFN-γ stimulation. Dex (10^{-6} M) was added at the initiation of a parallel PBMC culture from the same donor. $CD14^+$ cells were selected from the 3-day culture and subsequently treated with IFN-γ for 10 min. Cell extracts were analyzed using EMSA and immunoblotting. A representative experiment of five performed is shown. *D*, Monocytes were cultured for 2 days with supernatants obtained from cultures of control or dex-treated $CD14^-$ cells. At the end of culture, monocytes were stimulated with 10 U/ml of IFN-γ for 10 min. Cell extracts were analyzed by EMSA and immunoblotting.

Having demonstrated that IFN-γ signaling was inhibited in monocytes from dex-treated PBMC cultures, we investigated whether dex could inhibit IFN-γ signal transduction in purified monocytes. PBMCs were isolated and cultured with M-CSF, a survival factor, for 3 days in the presence or the absence of dex. In M-CSF-cultured monocytes, IFN-γ-induced STAT1 activation was not affected by dex treatment, as manifested by both STAT1 DNA binding and STAT1 tyrosine phosphorylation (Fig. 5*C*). In contrast, dex effectively blocked IFN-γ-induced STAT1 DNA binding and STAT1 tyrosine phosphorylation in PBMC culture-derived monocytes (Fig. 5*C*). Similar results were obtained when M-CSF was added during PBMC cultures, indicating that M-CSF did not block dex-mediated inhibition (data not shown). Dex had no effect on STAT1 protein levels in M-CSF-cultured monocytes, whereas STAT1 levels were elevated in PBMC culture-derived monocytes and were suppressed to below the level of detection by dex (Fig. 5*C*, *panel 3*). Immunoblotting of the same filter with anti-Jak2 Abs demonstrated the equivalence of Jak2 levels in all cell extracts, confirming comparable protein loading (Fig. 5*C*, *bottom panel*). These results indicate that dex does not directly regulate STAT1 levels or IFN-γ signaling in monocytes, but suggest that dex alters lymphocyte-monocyte interactions during coculture such that STAT1 expression is suppressed, with a concomitant decrease in levels of STAT1 activation by IFN-γ. It was possible that dex suppressed IFN-γ signaling and STAT1 expression by inducing lymphocytes to secrete an inhibitory factor. This possibility was addressed using supernatants from control lymphocyte cultures and from lymphocytes treated with dex, which would contain any putative inhibitory factors. Supernatants from dex-treated lymphocytes did not alter IFN-γ-induced STAT1 activation or protein levels (Fig. 5*D*). These results suggest that dex did not induce the expression of inhibitory factors by lymphocytes and are consistent with the finding that suppression of STAT1 expression by dex did not require de novo protein synthesis (Fig. 3*A*).

Physiological significance of inhibition of IFN-γ signaling and STAT1 expression by dex

The significance of the dex-mediated inhibition of IFN-γ signaling was assessed by determining whether suppression of STAT1 activation resulted in decreased expression of IFN-γ-inducible STAT1 target genes. Stimulation with IFN-γ led to the induction of expression of the STAT5, IP-10, and IRF-1 genes (Fig. 6); IRF-1 and IP-10 have been previously shown to be induced in a STAT1-dependent fashion (37, 38). Dex treatment resulted in the

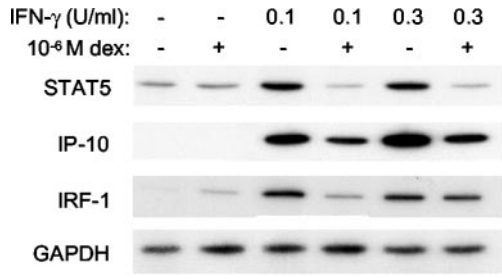

FIGURE 6. GCs suppress activation of IFN-γ-inducible genes. PBMCs were treated with 10^{-6} M dex for 3 days and IFN-γ for 3 h. Steady state mRNA levels were determined using semiquantitative RT-PCR as described in *Materials and Methods*.

suppression of induction of STAT5, IP-10, and IRF-1 mRNA levels (Fig. 6). These results are consistent with results obtained with STAT1-deficient mice (39, 40) and provide a functional correlate for the dex-mediated inhibition of IFN-γ signaling.

Discussion

GCs are major immunosuppressive and anti-inflammatory factors. It is well appreciated that an important molecular mechanism of GC action is inhibition of NF-κB and AP-1 transcription factors and thus of proinflammatory cytokines that activate cells via NF-κB and AP-1, such as IL-1 and TNF. It is becoming increasingly apparent that GCs also affect signaling by cytokines that use the Jak-STAT pathway. Somewhat paradoxically, at least in terms of inflammation and immunity, GCs can potentiate the transcriptional activity of STAT5 and STAT1 by direct interactions of GCR with STAT5 (22) or by potentiating STAT1 transcriptional activity (27). In contrast, GCs can inhibit IL-2 and IL-12 signaling indirectly by inhibiting receptor or Jak3 expression (23, 25). We have now identified inhibition of STAT1 expression as a mechanism by which GCs inhibit IFN-γ signaling.

Jaks and STATs are broadly and constitutively expressed, and the Jak-STAT signal transduction pathway is typically regulated by post-translational modifications. Transfection studies expressing STATs in deficient cell lines or overexpressing STATs in cell lines have shown that the level of STAT activation by a cytokine receptor correlates with the expression level of STATs (35, 41, 42), probably secondary to concentration-dependent interaction of STATs with docking sites on their respective receptors. We have previously demonstrated a physiological role for increased STAT1 expression in amplifying IFN-γ signaling after macrophages are exposed to low, physiological doses of IFN-α or IFN-γ (36). This report describes the opposite effect, inhibition of IFN-γ signaling when STAT1 expression is suppressed by physiologic and therapeutic concentrations of GCs. These studies demonstrate that dynamic regulation of STAT1 expression constitutes an important mechanism for fine-tuning the amplitude of IFN-γ responses.

Suppression of STAT1 protein expression by dex occurred only when monocytes were cocultured with lymphocytes and was not apparent until after several days of culture. STAT1 protein levels increased in monocytes during coculture with lymphocytes, and this increase was blocked by dex (Fig. 5*C*). Diminished STAT1 protein levels can be explained at least in part by dex-mediated suppression of STAT1 mRNA expression, which increased over the course of control PBMC cocultures (Fig. 3*A*). Dex did not destabilize STAT1 protein and did not act directly on monocytes. Instead, dex worked indirectly by regulating macrophage-lymphocyte interactions (Fig. 5). The requirements for monocyte-lymphocyte coculture to observe dex effects on IFN-γ signaling and for several days of culture for the dex effect to become apparent explain why suppression of IFN-γ signaling by dex was not observed by other investigators, who used purified monocytes and added dex and IFN-γ simultaneously (26, 27). STAT1 is constitutively expressed in many cell types, and its expression is increased by type I and type II IFNs and probably by additional factors (43). We have shown that low levels of IFN-γ produced during PBMC culture contribute to increased STAT1 expression (36). Thus, dex suppression of monocyte-lymphocyte interactions that lead to low level IFN-γ production during PBMC cocultures probably is one mechanism by which dex suppresses STAT1 expression. However, inhibition of IFN-γ signaling and STAT1 expression by dex in PBMC cocultures could not be explained solely on the basis of inhibition of IFN-γ production (X. Hu, unpublished observation), and thus dex also regulates the expression of additional secreted factors or cell surface molecules important in macrophage-lymphocyte interactions.

The suppressive effect of GCs on STAT1 expression and activation is reminiscent of the action of the immunosuppressant fludarabine, which also decreased STAT1 expression and thus activation (44). It is interesting that two chemically unrelated immunosuppressive agents share one molecular mechanism of action. A major difference between fludarabine and GCs is that GCs are endogenous molecules, and low amounts of GCs are constitutively present under physiological conditions, while fludarabine can only be given as therapeutic agent exogenously. GC production is increased under conditions of stress or elevated production of cytokines, such as IL-1 and TNF, and GCs play an important role in maintaining homeostasis within the immune system (19). The importance of such an endogenous counter-regulatory feedback loop is revealed in mice rendered GC deficient by adrenalectomy (45), which developed increased mortality due to uncontrolled cytokine production after virus infection. Recently, it has been suggested that IFNs and STATs may perform homeostatic functions as well, such as regulating baseline levels of MHC molecules (46). GCs effectively suppressed IFN-γ signaling even at physiological concentrations (Fig. 2*A*), suggesting that GCs regulate the amplitude of IFN-γ signaling under homeostatic conditions. During an inflammatory reaction, endogenous GCs may be insufficient to block IFN-γ signaling, but therapy with pharmacological doses of GCs may be able to suppress STAT1 expression and IFN-γ signaling. In pilot experiments we found a trend toward suppression of STAT1 expression in patients with severe inflammatory diseases who were treated with high dose i.v. methylprednisolone (C. Meng, unpublished observations). Future studies with larger numbers of patients and additional controls will be required to address the regulation of STAT1 expression and IFN-γ signaling during therapy with GCs.

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