Glucocorticoids potentiate IL-6-induced SP-B expression in H441 cells by enhancing the JAK-STAT signaling pathway

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Ladenburger A, Seehase M, Kramer BW, Thomas W, Wirbelauer J, Speer CP, Kunzmann S. Glucocorticoids potentiate IL-6-induced SP-B expression in H441 cells by enhancing the JAK-STAT signaling pathway. Am J Physiol Lung Cell Mol Physiol 299: L578–L584, 2010. First published August 6, 2010; doi:10.1152/ajplung.00055.2010.—The respiratory distress syndrome (RDS) contributes to perinatal morbidity and mortality associated with preterm birth. Surfactant protein B (SP-B) is decreased in RDS. Both maternal antenatal steroid administration and chorioamnionitis reduce the incidence and severity of RDS. An important mediator in chorioamnionitis is IL-6 using the JAK-STAT signaling pathway for signal transduction. We hypothesized that the steroids, betamethasone (BTM) and dexamethasone (DXM), and IL-6 had synergistic effects on SP-B gene expression and STAT3 phosphorylation in H441 cells. DXM and BTM increased SP-B mRNA levels by 16.5 (13.3)-fold and IL-6 alone by 2.3-fold. After 48-h exposure of cells to DXM or BTM, IL-6 caused a significantly greater increase in SP-B mRNA levels (28.1-fold) than IL-6 or glucocorticoids alone. Whereas IL-6 stimulated tyrosine phosphorylation of STAT3 in a time- and dose-dependent way, DXM and BTM had no effect on STAT3 phosphorylation. Both DXM and BTM could potentiate IL-6-induced phosphorylation of STAT3. The synergy of glucocorticoids and IL-6 on SP-B gene expression and the effect of glucocorticoids on IL-6-induced STAT3 phosphorylation could be blocked by a JAK inhibitor. Expression level analysis showed that glucocorticoids increased the expression of the IL-6-binding subunit receptor (IL-6R) on mRNA and protein level. Our findings could represent an example of a pulmonary regulation system in which one role of glucocorticoids is to increase the effect of a cytokine by upregulation of its receptor. The described in vitro interaction of IL-6 and glucocorticoids could help explain the clinical observation that prenatal inflammation in preterm babies with antenatal steroid administration can attenuate severity of RDS. 

dxm; betamethasone; surfactant protein B; interleukin-6 receptor; STAT3; Janus kinase; respiratory distress syndrome

NEONATAL RESPIRATORY DISTRESS syndrome (RDS), which is characterized by surfactant deficiency, accounts for significant neonatal morbidity and mortality in preterm infants (32). Surfactant is a mixture of lipids and proteins and maintains alveolar integrity by reducing surface tension at the alveolar air-tissue interface (10). Furthermore, surfactant plays an important role in the control of host defense and inflammation in the lung (17). In surfactant metabolism, the surfactant protein B (SP-B), an 8-kDa hydrophobic protein, plays a key role and is essential for the biophysical properties and physiological function of surfactant (40). SP-B promotes the formation and stability of the surfactant monolayer on the alveolar surface through its interactions with dipalmitoyl phosphatidylcholine, the principal surface-active phospholipid of surfactant (38). SP-B is mainly synthesized in alveolar type II cells and Clara cells (38).

Antenatal administration of betamethasone (BTM) or dexamethasone (DXM) effectively reduces the incidence and severity of RDS in premature infants (3). However, the molecular mechanisms by which antenatal corticosteroids induce cell and organ maturation have not yet been fully understood (25a).

In addition to the antenatal steroids, chorioamnionitis also reduces the incidence and severity of RDS (36). Recent studies reported an incidence of chorioamnionitis of up to 60% in extremely preterm infants (18). In animal models of preterm birth, chorioamnionitis has been shown to increase the surfactant pool size and to improve lung compliance on the one hand, but to simplify the lung structure on the other hand (2). The current knowledge about these inflammation-induced effects on lung function and maturation is scarce (16).

In clinical studies, decreased incidence of RDS after exposure to chorioamnionitis was associated with increased IL-6 concentrations in cord blood and bronchoalveolar lavage fluid (29). The expression of the proinflammatory IL-6 can be induced by several stimuli and is under complex regulatory control (30). Given its pleiotropic capacity, IL-6 modulates proliferation, differentiation, and maturation of different cell lines (30).

For signal transduction, IL-6 and steroid hormones use different signaling pathways. The biological activities of IL-6 are mediated by the IL-6 receptor (IL-6R) system, which comprises two membrane proteins, the IL-6-binding subunit receptor and the signal transducing β-subunit receptor, gp130, which is required for high-affinity binding and signal transduction (30). Binding of IL-6 to IL-6R is followed by the association and subsequent homodimerization of gp130. Homodimerization of gp130 induces phosphorylation of a protein tyrosine kinase belonging to the Janus kinase (JAK1/2) family, which in turn activates cytoplasmic proteins, including STAT proteins. STAT proteins translocate to the nucleus, where they activate NF-κB or acute-phase response factor, which then binds to an acute-phase response element, thus initiating transcription of acute-phase proteins (30). In contrast, glucocorticoids bind to intracellular glucocorticoid receptors (GR). The glucocorticoid-GR-complex migrates to the nucleus and binds to specific DNA regions called glucocorticoid response elements, which stimulate or inhibit the expression of specific genes (1).

In this study, we hypothesized that glucocorticoids DXM and BTM and IL-6 interacted on the SP-B expression via the
JAK-STAT pathway. We studied the effects in H441 cells, a cell line with characteristics of bronchiolar Clara cells.

**MATERIALS AND METHODS**

**Reagents.** Recombinant human IL-6 was obtained from R&D Systems (Abingdon, United Kingdom), DXM and BTM from Sigma-Aldrich (St. Louis, MO), and the JAK Inhibitor I from Calbiochem (cat. no. 420099; La Jolla, CA).

**Cell culture.** NCI-H441 cells [American Type Culture Collection (ATCC), Rockville, MD], a human lung adenocarcinoma cell line with characteristics of bronchiolar Clara epithelial cells, were grown on plastic tissue culture dishes in RPMI medium containing 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml). On 6-cm dishes, cells were allowed to grow until 80% confluence before addition of the investigated agents. Incubation was carried out at 37°C in a humidified atmosphere of 95% room air and 5% CO₂. H441 cells were used in experiments between passages 3 and 20.

**Viability detection.** H441 cell viability after exposure to IL-6 (100 ng/ml), DXM (10⁻⁷ to 10⁻⁵ M), BTM (10⁻⁷ to 10⁻⁵ M), or JAK Inhibitor I (0.1–1 μM) was evaluated after 1–3 days using flow cytometry-based quantification of ethidium bromide (1 mmol/l; Sigma-Aldrich) uptake as previously described (34). No significant difference on cell viability of H441 cells could be detected after incubation with IL-6, DXM, BTM, and JAK inhibitor compared with nontreated cells (data not shown).

**RNA extraction and reverse transcription.** Total RNA was isolated from H441 cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. The RNA was eluted in 40-μl water, and purity and yield were photometrically determined. Reverse transcription of 5 μg of total RNA was induced by addition of 500 μg/ml oligo(dT)₁₂ primers (Roche, Basel, Switzerland), RNase inhibitor (10 U/μl; Roche), dNTP (5 mmol/l each dNTP; Qiagen), and Omniscript transcriptase (0.2 U/μl; Qiagen) for 1 h at 37°C. The cDNA was denatured at 90°C for 5 min and used for PCR amplification.

**Real-time PCR analysis.** Human SP-B (Hs00167036), human IL-6R (Hs00169842), and human β-actin (Hs99999903) quantitative real-time PCR (TaqMan) primers and probes were obtained from Applied Biosystems (Weiterstadt, Germany). All PCRs were performed using 1 μg/μl cDNA per reaction in duplicates of 30-μl volume on an ABI Prism 7300 Sequence Detection System (TaqMan) using a 2-step PCR protocol after initial denaturing of the DNA (10 min at 95°C) with 40 cycles of 95°C for 15 s and 60°C for 1 min. Universal PCR Master Mix as obtained from Applied Biosystems included all reagents with Taq polymerase apart from specific primers and probes. The amplification batches did not include template controls. Neither negative controls nor mRNA resulted in elevated fluorescence signals after PCR. Dilution experiments were performed to ensure similar efficiency of the PCRs, and standard curves were calculated referring the threshold cycle (CT) to the log of each cDNA dilution step. Results of SP-B were normalized referring to the threshold cycle (CT) to the log of each cDNA dilution step. Results of SP-B were normalized to β-actin, and mean fold changes in mRNA expression were calculated by the ∆∆CT method (20).

**Western blot analysis.** H441 cells were rinsed with ice-cold Tris-buffered saline (TBS) and incubated in 100-μl lysis buffer (Cell Lysis Buffer; Cell Signaling Technology), 0.1 mM PMSF, and Complete Mini Protease Inhibitor Cocktail and PhosSTOP phosphatase inhibitor cocktail (Roche) for 10 min on ice. The lysate was cleared by centrifugation at 14,000 rpm for 10 min, and the supernatant was used for Western immunoblotting analysis. Protein concentrations were determined for each sample using the Bradford assay (Bio-Rad, Richmond, CA), and equal amounts of cellular protein were loaded and separated by SDS-PAGE on 10% Bis-Tris gels and electrophoretically transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were blocked in 5% BSA for 1 h at room temperature and successively incubated with primary antibodies overnight at 4°C. Western Blots were probed with primary antibodies to β-actin, STAT3, phospho-STAT3 (Tyr705), phospho-STAT3 (Ser727), JAK1, JAK2, TYK2 (Cell Signaling Technology), IL-6Rα (Santa Cruz Biotechnology), and gp130 (Santa Cruz Biotechnology), followed by the corresponding horseradish peroxidase-conjugated secondary antibody (Pierce, Bonn, Germany) for 1 h at room temperature. Specific protein bands were visualized using enhanced chemiluminescence (SuperSignal West Dura; Pierce) and detected using the LAS-3000 computer-based luminescent image analyzer (Fujiﬁlm, Tokyo, Japan). Accumulated signals were analyzed using AIDA software (Raytest).

**Statistical analysis.** All results are shown as representative of three separate experiments. Results are given as means ± SE. Data were analyzed by the Mann-Whitney-Wilcoxon test. A P value <0.05 was considered significant. All statistical analyses were performed using the statistical software GraphPad Prism 5.0 (GraphPad Software, San Diego, CA).

**RESULTS**

**IL-6 and glucocorticoids synergistically activated SP-B mRNA expression in H441 cells.** We studied a possible synergistic effect of steroids and the proinflammatory cytokine IL-6 on SP-B mRNA expression in H441 cells. The effect of DXM, BTM, IL-6, and DXM or BTM combined with IL-6 on SP-B mRNA levels in H441 cells were measured by real-time PCR. PCR results, which are expressed as SP-B mRNA transcripts normalized to β-actin, showed that DXM and BTM caused a 16.5-fold (DXM; Fig. 1) and 13.3-fold (BTM; data not shown) increase in SP-B mRNA compared with untreated cells. IL-6 alone had only a moderate effect on SP-B mRNA level (2.8-fold increase) compared with the effect of glucocorticoids. Incubation of H441 cells with IL-6 in combination with either DXM or BTM for 48 h led to a synergistic effect with a
28.1-fold (DXM; Fig. 1) and 28.0-fold (BTM; data not shown) increase of SP-B mRNA expression compared with untreated cells. There was no significant difference between the effects of the 2 used glucocorticoids, DXM and BTM.

**IL-6 induced a dose- and time-dependent STAT3 phosphorylation.** To characterize the involved signaling pathway of the observed synergistic effect of glucocorticoids and IL-6 on SP-B expression in H441 cells, we studied the effect of IL-6 alone on STAT3 phosphorylation using an antibody specific for phosphorylation of Tyr705, the amino acid residue necessary for activation of STAT3. Western blot analysis revealed that STAT3 was phosphorylated by IL-6 in a dose-dependent manner between 10 and 100 ng/ml (Fig. 2A). Phosphorylation was time-dependent after stimulation with 50 ng/ml IL-6 for a period of 7–30 min (Fig. 2B). These experiments showed that IL-6 activated STAT3 phosphorylation in a dose- and time-dependent manner in H441 cells.

**Glucocorticoids enhanced IL-6-induced STAT3 phosphorylation.** We tested whether glucocorticoids also influenced the IL-6-activated STAT signaling in H441 cells. Preincubation of H441 cells with DXM or BTM for 48 h enhanced the effect of IL-6 on STAT3 phosphorylation (20.2-fold increase) compared with the effect of IL-6 on STAT3 phosphorylation without DXM preincubation (8.4-fold increase). Figure 3 shows the results for DXM. No significant difference was obtained when BTM was used instead of DXM (data not shown). Taken together, in addition to the synergistic effect of glucocorticoids and IL-6 on SP-B mRNA expression, glucocorticoids enhanced IL-6-induced STAT3 phosphorylation in H441 cells.

**Inhibition of the glucocorticoid effect on IL-6-induced STAT3 phosphorylation by JAK inhibition.** We tested the effect of JAK inhibition on IL-6-induced STAT3 phosphorylation and the glucocorticoid effect on IL-6-induced STAT3 phosphorylation because STAT3 was phosphorylated by JAK. A JAK inhibitor efficiently inhibited phosphorylation of STAT3 by IL-6 alone as well as by IL-6 in combination with either DXM (Fig. 4) or BTM (data not shown). These results showed that the effect of glucocorticoids on IL-6-induced STAT3 phosphorylation in H441 cells was mediated by JAK.

**Inhibition of the synergistic effect of glucocorticoids and IL-6 on SP-B mRNA expression by JAK inhibition.** We then analyzed whether the synergistic effect of glucocorticoids and IL-6 on SP-B mRNA level could also be antagonized by the JAK inhibitor. Whereas the JAK inhibitor only moderately attenuated the effect of either DXM (Fig. 5) or BTM (data not shown) alone, it could efficiently antagonize the effect of IL-6 (Fig. 5) and the synergistic effect of DXM (Fig. 5) or BTM (data not shown) combined with IL-6 on SP-B mRNA expression. The same effect as shown for DXM in Fig. 5 was obtained for BTM without a significant difference between DXM and BTM (data not shown). In summary, these experiments demonstrated that the synergistic effect of steroids on IL-6-induced SP-B expression was mediated by JAK.

**Glucocorticoids did not alter JAK1, JAK2, TYK2, and STAT3 protein level and did not change phosphorylation of STAT3.** We tested the influence of glucocorticoids on protein concentrations of JAK1, JAK2, TYK2, and STAT3 and the phosphorylation level of STAT3 in H441 cells to rule out whether glucocorticoids had direct influence on the expression level of members of the JAK-STAT signaling pathway or the phosphorylation level of STAT3. Neither the protein level of JAK1, JAK2, TYK2, and STAT3 (Fig. 6A) nor the phosphorylation level of STAT3 (Tyr705 and Ser727; Fig. 6B) were affected by DXM or BTM (data not shown).

Glucocorticoids induced IL-6R expression on mRNA and protein level in H441 cells. The findings suggested that the influence of glucocorticoids on IL-6 effects in H441 cells could be due to an increased expression of either IL-6R or gp130. DXM induced IL-6R mRNA in a time- and dose-dependent manner.
DISCUSSION

The prevention and care of RDS includes the use of prenatal glucocorticoids. However, the beneficial effects of steroids on the neonatal lung is accompanied by adverse effects, such as inhibition of type II cell proliferation (24) and impairment of pulmonary growth (22). It is therefore of great interest to design treatments allowing to take advantage of the maturational effect of glucocorticoids while avoiding their side effects. In clinical practice, glucocorticoids are often administered in presence of chorioamnionitis. Thus the molecular mechanisms of chorioamnionitis-induced lung maturation in presence of glucocorticoids should be defined more in detail.

In this study, we showed that glucocorticoids and IL-6 synergistically induced SP-B mRNA expression and glucocorticoids enhanced IL-6-induced STAT3 phosphorylation in H441 cells. Both effects could be antagonized by JAK inhibition, underlining the involvement of the JAK-STAT signaling pathway in this process. We demonstrated that glucocorticoids had no effect on STAT3, JAK1/2, and TYK2 protein expression, STAT3 phosphorylation, and gp130 expression, but glucocorticoids could induce IL-6R expression in H441 cells. The latter finding could explain the synergistic effect of glucocorticoids and IL-6 on SP-B mRNA expression and the positive effect of glucocorticoids on IL-6-induced STAT3 phosphorylation.

We studied the gene expression of SP-B because SP-B is considered to be essential for surfactant processing, storage, secretion, and normal function in vivo (37). H441 cells were used because they have been a useful approach to the investigation of the regulation of surfactant components (35). The H441 cell line is morphologically similar to Clara cells. The synthesis and processing of SP-B in this cell line are similar to that in explant cultures of fetal lung or cultured isolated type II cells (26). H441 cells respond to regulatory factors that influence SP-B expression in a manner similar to that observed in human type II pneumocytes, which justifies the use of this human adenocarcinoma cell line to study cellular maturational effects of corticosteroid at a manner in H441 cells (Fig. 7A). A maximal increase of 4.0-fold was seen with a DXM concentration of 10^{-7} M after an incubation time of 12 h. Also, BTM induced IL-6R mRNA expression with a maximal increase of 3.8-fold in the same concentration and incubation time as used for DXM (data not shown). IL-6 alone had no effect on IL-6R mRNA expression, and IL-6 also did not influence the effect of DXM or BTM (data not shown). In addition, on protein level, DXM (Fig. 7B) and BTM (data not shown) increased IL-6R protein expression above controls. DXM (Fig. 7B) and BTM (data not shown) had no detectable effect on gp130 protein expression. The effect of glucocorticoids on IL-6R mRNA and protein expression could be shown as constant after an incubation of 12-48 h. No significant difference on IL-6R mRNA and protein induction was obtained between DXM and BTM.

Fig. 3. Effect of glucocorticoids on IL-6-induced STAT3 phosphorylation. H441 cells were stimulated with DXM or IL-6 alone for 15 min or preincubated with DXM for 48 h before stimulation with IL-6 for 15 min. STAT3 phosphorylation was detected by immunoblotting with anti-phospho-STAT3 (Tyr705) antibodies (top), and β-actin-normalized quantification of pSTAT3 expression compared with untreated control is shown at the bottom. A representation of 3 independent experiments is shown. Results are given as means ± SE. Data were analyzed by the Mann-Whitney-Wilcoxon test. n = 4.

*Significant differences compared with untreated cells (P < 0.05).
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molecular level (28, 42). Our results are consistent with previous studies that reported the stimulatory effect of glucocorticoids on surfactant protein gene transcription in H441 (9, 26, 28) and lung explants (19) and the effect of intrauterine IL-6 on the expression of SP-B in the fetal lung (13). However, our findings of glucocorticoid-enhanced IL-6 effects on SP-B expression have not previously been described. A 48-h exposure interval for steroids was selected because of the similarity to clinical practice. Data from the Cochrane database indicate similar efficacy of the 2 preparations on reducing the odds ratios for RDS (51% BTM and 45% DXM), however, only BTM and not DXM significantly reduces mortality in premature infants (48 vs. 11%) (4). Although these clinical data suggest a therapeutic advantage for BTM over DXM (4), in the present in vitro study, BTM and DXM achieved similar effects. This is consistent with previous reports that found no significant differences between BTM and DXM when the nuclear receptor-dependent genomic effect was measured as indicator of relative potency (14). Likewise, DXM and BTM showed similar dose-response patterns of SP-B expression in H441 cells and in human type II pneumocytes (28).

As well as the effect of glucocorticoids on IL-6-induced SP-B expression, the effect of glucocorticoids on IL-6-induced STAT3 phosphorylation in lung epithelial cells could influence the lung homeostasis. Pathway analysis indicated that STAT3 regulated cellular homeostasis through a complex regulatory network that likely enhanced alveolar epithelial cell survival and influenced maintenance of alveolar structures and of surfactant homeostasis during pulmonary remodeling processes, necessary for the protection of the lung during injury (41). The cytoprotective function of STAT3 signaling in respiratory epithelial cells could be demonstrated in different lung models, as shown for oxygen injury (12) and adenoviral infection (21). Consistent with these results, increased STAT3 activity was associated with wound healing in chronically inflamed mouse lungs (5).

Steroid receptors and STAT transcription factors are different sets of signaling molecules and activate gene transcription by binding to their respective response elements (1). These signal transduction pathways have been regarded as unrelated. However, besides the known effect of glucocorticoids on surfactant synthesis, the IL-6-STAT3 signaling axis plays an important role in surfactant protein homeostasis and respiratory inflammation in the lung (44). Transcription of the SP-B gene is mediated by cytokines belonging to the IL-6 family and STAT3 in respiratory epithelial cells (43). Our findings that the effect of IL-6 on SP-B expression can be antagonized by JAK inhibition underlines the role of STAT3 phosphorylation in IL-6-induced SP-B expression. Although the signaling pathways of glucocorticoids and IL-6 seemed to be unrelated, a cross talk between the IL-6-JAK-STAT and the glucocorticoid-nuclear receptor pathway was reported (33). IL-6 and glucocorticoids synergistically activated the IL-6 response element on the rat α2-macroglobulin promotor (APRE), whereas no synergisms could be found for two other IL-6 response elements, the JunB promoter (JRE-IL-6) and the interferon regulatory factor-1 (IRF-1) promotor (IRF-GAS). Before, synergy...
between STAT5 and glucocorticoids was reported in the activation of the β-casein gene transcription (31). As explanation for the synergistic effect of glucocorticoids and IL-6 on SP-B mRNA expression and STAT3 phosphorylation in H441 cells, we found an upregulation of IL-6R by glucocorticoids, whereas BTM and DXM did not augment STAT3 protein expression or STAT3 phosphorylation in H441 cells. Although initial studies focused on the role of tyrosine kinases in STAT activation, more recent studies have identified other positive regulators. These include serine kinases (15). Wen et al. (39) showed that phosphorylation of a single serine (residue 727) in STAT3 was also required for maximal transcriptional activity. We could not detect any effect of glucocorticoids on STAT phosphorylation on either Tyr705 or Ser727. Although we studied possible phosphorylation of STAT3 by glucocorticoids, we cannot exclude additional nongenomic signaling cascades contributing to the synergistic effect of IL-6 and DXM/BTM on SP-B expression and STAT3 activation (27).

The capability of glucocorticoids to upregulate IL-6R has already been demonstrated in animal models (8) and different human cell types (7, 23). Duan and Simpson-Haidaris (6) showed that DXM potentiated IL-6 induction of the fibrinogen in the hepatic cell line HepG2 and in lung epithelial cell line A549. DXM potentiation was also due to the induction of IL-6R expression (6).

A similar synergistic activity as described in our study between IL-6 and glucocorticoids for SP-B expression in H441 cells was found for expression of SP-A and SP-B between DXM and keratinocyte growth factor (KGF, or fibroblast growth factor 7) in alveolar epithelial cells (25). However, the molecular mechanism of the KGF-DXM synergy remained unknown.

Our findings potentially represent a pulmonary regulatory system in which one role of glucocorticoids is to increase the effect of a cytokine by upregulation of its receptor. Endogenous and/or exogenous glucocorticoids could increase the expression of IL-6R in lung epithelial cells leading to a stronger activation of the STAT3 signaling pathway by IL-6 in lung epithelial cells. The subsequent increase of STAT3 activation and SP-B synthesis could result in a better lung homeostasis during an inflammatory reaction.

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Fig. 7. Effect of glucocorticoids on IL-6 receptor (IL-6R) and glycoprotein 130 (gp130) expression. A: H441 cells were incubated with DXM for different time periods (2, 6, 12, 24, and 48 h; top) or different concentrations (10⁻⁶ to 10⁻¹⁰ M; bottom), and mRNA expression of IL-6R normalized for β-actin were measured by real-time PCR. Relative increase of IL-6R mRNA of treated (DXM) compared with untreated H441 cells is summarized in A. Results are given as means ± SE. Data were analyzed by the Mann-Whitney-Wilcoxon test. n = 6. *Significant differences compared with untreated cells (P < 0.05, respectively). B: H441 cells were incubated with DXM (10⁻⁷ M) for different time periods (12, 24, and 48 h), and IL-6R, gp130, and β-actin protein expression were determined by immunoblotting (top). β-Actin-normalized quantification of IL-6R or gp130 expression compared with control is shown at the bottom. A representation of 3 independent experiments is shown. Results are given as means ± SE. Data were analyzed by the Mann-Whitney-Wilcoxon test. n = 3. *Significant differences compared with untreated cells (P < 0.05).
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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