

SOCS3 Modulates Interleukin-6R Signaling Preference in Dermal Fibroblasts

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Aims: This study aims to investigate the mechanisms in the apparent preference for mitogen-activated protein kinase /ERK signaling through interleukin (IL)-6R in dermal fibroblasts.

Methods: Dermal fibroblasts isolated from IL-6KO mice were pretreated with specific ERK or STAT3 chemical inhibitors or SOCS3 specific siRNA and treated with rmIL-6. Phosphorylation was monitored via enzyme-linked immunosorbent assay or immunohistology. SOCS3 interaction with p120Ras-Gap was examined by co-immunoprecipitation and Western blot. Expression of *MMP2* mRNA was assessed via real-time quantitative polymerase chain reaction.

Results: A dose response phosphorylation of ERK1/2 occurred while no STAT3 activation (p-Tyr705) was induced after IL-6 treatment, despite an increase in Ser727 phosphorylation. Inhibition of STAT3 in fibroblasts potentiated IL-6R induced ERK phosphorylation and vice versa. Phosphorylated SOCS3 and p120 RasGAP co-immunoprecipitated in response to IL-6 treatment. SOCS3 siRNA knockdown allowed STAT3 phosphorylation after rmIL-6 treatment. Chemical inhibition of IL-6R signaling altered the IL-6 modulated mRNA expression of *MMP-2*.

Conclusions: SOCS3 interaction with p120 Ras-Gap plays a role in determining the preference for IL-6R signaling through ERK in dermal fibroblasts. This study provides insight into the pleiotropic nature of IL-6 and the selective signaling mechanism elicited by the IL-6R system in dermal fibroblasts. It may further indicate a method for manipulation of IL-6R function.

Introduction

INTERLEUKIN-6 (IL-6) is a multifunctional cytokine involved with numerous cellular processes (Kamimura and others 2003). Originally discovered as a B-cell growth and differentiation factor, IL-6 was later implicated in the regulation of hematopoiesis and liver-specific process such as regeneration and the acute phase response (Kopf and others 1995), and facilitating the transition between innate and acquired immunity (Jones and Rose-John 2002; Kaplanski and others 2003). Additionally, IL-6 plays a governing role in inflammation, acting as a pro- and anti-inflammatory regulator. IL-6 is released by numerous immune cells, and muscle cells, endothelial cells, keratinocytes, and fibroblasts (Sehgal 1990). Control of IL-6 expression is carefully maintained by necessity as altered levels can contribute to various pathologies such as autoimmune diseases, carcinogenesis, and impaired wound healing (Gallucci and others 2000; Paschoud and others 2006).

IL-6 exerts its effects by signaling through a multi-subunit receptor complex composed of a ligand-binding 80 kDa IL-6

receptor alpha subunit (IL-6R- α) and a 130 kDa signal transducing subunit (gp130) (Heinrich and others 2003). The alpha subunit primarily exists in the transmembrane form, but a soluble form can be produced that allows for so called "trans-signaling" where the IL-6:sIL-6R- α complex can have an effect on any cell expressing gp130 (Kamimura and others 2003). The gp130 subunit is ubiquitously expressed and exists in both a membrane bound and soluble (sgp130) form (Hibi and others 1990), however unlike the sIL-6R- α , sgp130 acts as an IL-6R antagonist (Narazaki and others 1993). The gp130 subunit has no intrinsic kinase activity but instead contains regions required for its association with nonreceptor Janus tyrosine kinases (JAK). Dimerization of the receptor complex allows receptor bound Jaks to phosphorylate and activate one another, triggering three distinct signaling pathways; JAK/STAT, MAPK/ERK, or AKT/PKB (Heinrich and others 2003; Kamimura and others 2003). Each pathway can function independent of one another; however, cross talk between them also occurs (Kamimura and others 2003). This complex interaction that exists between pathways associated with IL-6R signaling has been termed the "Signal

Orchestration Model" (Kamimura and others 2003). This model may explain the very pleiotropic nature of IL-6 and suggests that the overall balance of distinct signals could determine the final biological outputs elicited by its receptor (Kamimura and others 2003).

The control of IL-6 activity is maintained through several negative regulators including protein tyrosine phosphatases, protein inhibitors of activated STAT (PIAS), and members of the suppressor of cytokine signaling (SOCS) family (Fischer and others 2004). In particular, SOCS3 can be rapidly induced by IL-6 (Larsen and Ropke 2002) where it usually acts as classical feedback inhibitor by physically interfering with JAK kinase activity (Fischer and others 2004) and STAT3 nuclear transport (Larsen and Ropke 2002). SOCS3 can also be phosphorylated on two key residues (Y204 and Y221) allowing it to modulate the MAPK/ERK pathway by interacting with Ras GTPase activating protein 120 (p120 RasGAP) (Cacalano and others 2001) causing sustained MAPK/ERK signaling (Pamonsinlapatham and others 2009).

It is well known that IL-6 plays a key role in the healing of various organs including liver (Cressman and others 1995) and skin (Gallucci and others 2000). While dermal fibroblasts represent a minor source of IL-6 within the skin as compared with inflammatory cells, they play a central role in the proper healing of wounds. Previous studies investigating the IL-6R signaling mechanisms involved in the modulation of wound healing found that dermal fibroblasts in IL-6 deficient mice (IL-6KO) signal primarily through the ERK pathway (Gallucci and others 2006). Since the JAK/STAT pathway has been reported as a major signaling cascade in response to IL-6R activation, this lack of signaling is perplexing. The current study sought to investigate the mechanisms that are involved in the preference for ERK1/2 signaling in dermal fibroblasts in response to IL-6. Herein it was found that SOCS3 phosphorylated after IL-6 treatment and interacted with the p120 RasGAP. Serine phosphorylation of STAT3 (S727) occurred after IL-6 treatment in these cells but did not result in increased STAT3 function. Knockdown of SOCS3 expression via siRNA, or inhibition of ERK signaling utilizing chemical inhibitors potentiated JAK/STAT signaling in these cells. The functional consequences of selective chemical manipulation of IL-6R signaling were also demonstrated, as IL-6 induced transcriptional modulation of *MMP2* was profoundly altered in fibroblasts. These data may partially explain the signaling preference toward the MAPK/ERK pathway in dermal fibroblasts, provide further insight into the pleiotropic nature of IL-6 and its association with cross talk of IL-6R signaling pathways, and indicate the possibility of pharmaceutical manipulation of IL-6R function.

Materials and Methods

Fibroblast isolation

Dermis was collected and separated from epidermis as previously described (Gallucci and others 2004a). Briefly, skin was collected from mouse pups (0–48 h old), rinsed in 70% ethanol. Skins were incubated dermis down in culture dishes containing phosphate-buffered saline (PBS)+3.6% dispase (Sigma, St Louis, MO) overnight at 4°C. The dermis was separated from the epidermis and placed in a collagenase solution (1 mg/mL in PBS), incubated at 37°C, vortexed, and sterile filtered. Cells were adjusted to a concentration of

5×10^6 and frozen in CryoStore media (VWR, West Chester, PA). Fibroblasts were resuspended in dulbecco's modified Eagle's medium (DMEM)+5% fetal bovine serum (Invitrogen, Carlsbad, CA), plated at 8×10^3 cells cm^{-2} in 60 or 100 mm culture dishes, and incubated at 37°C and 5% CO_2 and 95% room air. The primary C57/BL6 and IL-6KO fibroblasts were cryopreserved at -140°C until further culture.

Fibroblast culture and treatment

Fibroblasts were isolated and cultured as described. Before treatment with rmIL-6, fibroblasts were washed in PBS, and culture media was replaced with serum free DMEM containing 60 IU/mL penicillin, 100 UI/mL streptomycin, and 1 mg/mL Bovine Serum Albumin for 1 h. For wild type (WT) fibroblasts, cells were incubated for 2 h in serum free media, and media was replaced every 30 min. If cultures were exposed to signal transduction inhibitors, LLL-3 (a gift from Dr. Pui-Kai Li, Ohio State University) and PD98059 (EMD Biosciences, San Diego, CA) were used at a concentration of 20 μM (Song and others 2005) and 30 μM , respectively. Each inhibitor or dimethyl sulfoxide (DMSO) (vehicle) was added to respective culture wells. The fibroblasts were incubated with the inhibitor or vehicle for 30 min, and various concentrations of IL-6 were then added to the plates for a 1-h incubation or followed a time course. After treatment, fibroblasts were collected for mRNA or protein analysis as described below.

Immunoprecipitation

IL-6KO dermal fibroblasts were prepared as described above and cultured to 90% confluency in 100 mm culture plates. Before treatment, fibroblasts were cultured in serum free DMEM for 2 h. IL-6 KO fibroblasts were then treated for 2 h with the proteosomal inhibitor MG132 (10 μM) to facilitate the detection of phosphorylated SOCS3 (Haan and others 2003). The inhibitors PD98059 and LLL3 were then applied at 30 μM and 20 μM respectively for 30 min, then treated with various rmIL-6 concentrations. At indicated time points, culture plates were rinsed once with ice-cold phosphate buffered saline, and co-immunoprecipitation was carried out essentially as previously described (Kamat and others 2007). Isolated total protein was determined and 100.0 μg of protein from each sample was incubated with the appropriate antibody while rocking at 4°C overnight. Protein-G Sepharose beads (40 μL , Sigma) were precleared with cell lysis buffer containing 1% milk for 1 h before immunoprecipitation. After preclearing, 20 μL of beads were incubated on a rocker with protein sample at 4°C for 1.5 h. Samples were then spun down, and an additional 20 μL of beads were added to the sample for 1.5 h at 4°C. After final incubation, samples were then spun down and washed two times with completed cell lysis buffer (containing protease inhibitors and PMSF). After final wash, beads were resuspended in 25 μL of cell lysis buffer plus 25 μL of 2 \times Laemmli denaturing sample buffer (Sigma). Samples were electrophoresed on 4%–20% Precise Protein Gradient gels (Thermo Scientific, Rockford, IL). Western blot was performed as previously described (Gallucci and others 2006). Monoclonal anti-phospho-tyrosine (Cell Signaling, Boston, MA), anti-p120 RasGAP (Santa Cruz Biotechnology, Santa Cruz, CA), anti-ERK (Cell Signaling), and anti-phospho-ERK (Cell Signaling) were applied to the blots at a 1:1,000 dilution

and fluorescently labeled secondary anti-species polyclonal antibody(s) (Rockland, Gilbertsville, PA) at a 1:10,000 dilution. Blots were imaged via the Molecular Imager ProPlus imaging system (BioRad, Hercules, CA).

Real-time quantitative polymerase chain reaction

Total RNA from mouse skin or dermal IL-6KO fibroblasts was prepared and cDNA synthesized as previously described (Gallucci and others 2004a). Primers for mouse genes were synthesized by Invitrogen. Quantitative polymerase chain reaction was performed on an ABI PRISM 7000 SDS, utilizing SYBR Green Mastermix (Anaspec, Fremont, CA) according to the manufacturer's instructions. Quantitative values of genes of interest were normalized based on 28s rRNA content using the ddCt method (Livak and Schmittgen 2001).

Enzyme-linked immunosorbent assay analysis

Total and phosphorylated ERK or STAT protein from IL-6KO fibroblast culture was measured using cell-based enzyme-linked immunosorbent assay (ELISA). (Raybiotech, Norcross, GA) IL-6KO fibroblast cells (6×10^4 /well) were seeded in a 96-well plate and incubated until cells were confluent in each well. Upon confluency, cells were serum starved for 1 h and then treated with inhibitors and IL-6 as previously described. The ELISA was performed according to the manufacturer's instructions and analyzed using a 96-well plate reader (Molecular Devices, Sunnyvale, CA).

Statistical analysis

All experiments were replicated, and representative findings are shown. Statistical significance was determined by one-way analysis of variance. When the *F* value was significant,

the means were compared using Fisher *post-hoc* analysis. In all statistical comparisons, a *P* value of <0.05 was used to indicate a significant difference.

Results

ERK and STAT3 pathway interact in dermal fibroblasts during IL-6R signaling

While the JAK/STAT pathway is the most dogmatic of the IL-6R signaling cascades, a previous time course investigation by this laboratory revealed that upon IL-6 treatment, ERK phosphorylation occurred within 1 min, reaching a maximum by 10 min, while minimal alteration in STAT3 phosphorylation was seen in IL-6KO fibroblasts (Gallucci and others 2006). To examine ERK phosphorylation in a dose response to IL-6 treatment, IL-6KO fibroblasts were treated with various concentrations of rmIL-6 for 10 min and phorbol 12-myristate 13-acetate (PMA) as a positive control. After treatment, total and phosphorylated ERK protein were assessed via ELISA. Similar to the previous report, IL-6 induced phosphorylation of ERK after treatment with 5 ng/mL rmIL-6, with maximal phosphorylation at 20 ng/mL with both expression levels similar to that seen with PMA treatment (Fig. 1a).

To examine cross talk that may occur between IL-6R signaling pathways, IL-6KO fibroblast cultures were treated with signal transduction inhibitors 30 min before being treated with various concentrations of rmIL-6. Fibroblasts were pretreated with the inhibitors LLL-3 (a novel STAT3 inhibitor (Song and others 2005)) or PD98059 (a MAPK/ERK inhibitor) and total and phosphorylated STAT3 or ERK1/2 was analyzed by ELISA. IL-6 treatment alone either significantly decreased (5 ng/mL) or did not affect (20 ng/mL) STAT3 phosphorylation in these cells, while pretreatment

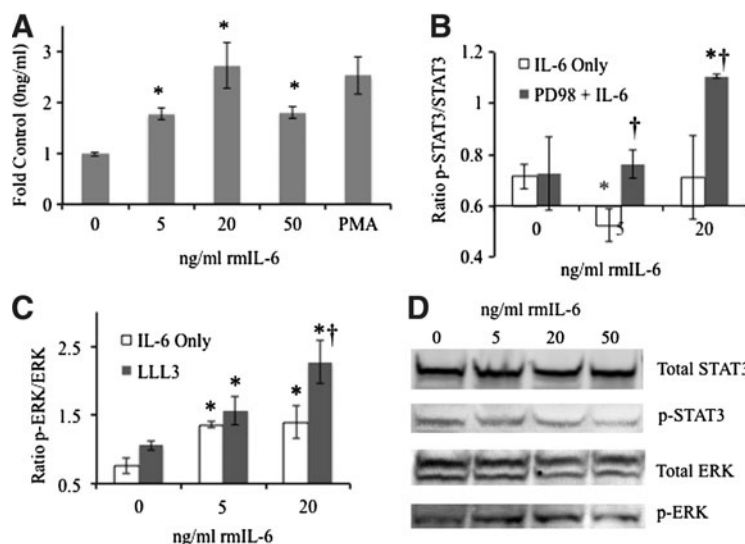


FIG. 1. Dermal fibroblast IL-6R signaling preference and alteration by chemical inhibitors. Dermal fibroblasts were grown to 80% confluency in 96-well (IL-6KO, A, B, C) or 100 mm culture plates (WT, B). Where indicated, growth media was replaced and cultures were treated with various concentration of PD98059 (ERK inhibitor, B), or LLL-3 (STAT3 Inhibitor, C) 30 min before rmIL-6 treatment as described. Total and Phosphorylated ERK (A, C) or STAT3 (B) was determined via in-cell ELISA according to manufacturer's instructions (RayBiotech, Norcross, GA). Data (A–C) are expressed as mean \pm SE, ($n=6$, *Significantly different from 0 ng/mL, †Significantly different from IL-6 alone treatment, $P \leq 0.05$). WT fibroblasts (D) grown in 100 mm culture plates were treated with rmIL-6 as described and analyzed via Western blot utilizing anti-STAT3 and anti-p-STAT3-Y705, or total ERK1/2 and p-ERK1/2 antibodies and fluorescently labeled secondary antibodies. Images were obtained utilizing a Molecular Imager ProPlus (BioRad). Representative image shown of three replicate experiments. IL, interleukin; WT, wild type; SE, standard error.

with PD98059 abrogated the inhibition of STAT3 activation at 5 and 20 ng/mL rmIL-6 (Fig. 1b). Conversely, when fibroblasts were treated with the STAT3 inhibitor LLL3, IL-6 induced ERK phosphorylation was significantly augmented compared with IL-6 treatment alone (Fig. 1c).

While these data expand on previous results, it was necessary to determine whether ERK signaling preference also occurs in WT fibroblasts. Thus, fibroblasts from C57BL/6 mice were cultured as described and exposed to rmIL-6 for 10 min. Western blot indicated that indeed STAT3 phosphorylation was not induced by IL-6 treatment, while phosphorylation of ERK was apparent, primarily in the ERK2 band (Fig. 1d) similar to Fig. 1a and previous findings (Gallucci and others 2006).

IL-6 induces phosphorylation of serine727 on STAT3

Historically it was thought that only single tyrosine residues on the STAT protein were phosphorylated as a result of

cytokine stimulation. However, serine phosphorylation of STATs can occur through MAPK and has been demonstrated to result in positive regulation of its activity (Abe and others 2001; Schuringa and others 2000). To determine if STAT3 serine phosphorylation was modulated as a result of IL-6 treatment, IL-6KO fibroblasts were treated with 0 and 20 ng/mL of rmIL-6 for 30 min, and STAT3 phospho-serine 727 (p-S727) was visualized by immunohistology. Staining for STAT3 was apparent in both cytoplasm and nucleus of untreated cells (green), while p-S727 (red) was very low (Fig. 2a). Upon treatment with 20 ng/mL of rmIL-6, p-S727 of STAT3 is readily detected, but rmIL-6 did not appear to increase nuclear translocation of STAT3 (Fig. 2b). Pretreatment with PD98059 had little effect on either STAT3 or p-S727 staining in untreated cells (Fig. 2c), while IL-6 treatment appeared to increase STAT3 nuclear staining (Fig. 2d). Pretreatment with the STAT3 inhibitor LLL3 appeared to decrease nuclear staining of STAT3 while augmenting phosphorylation of S727 (Fig. 2e). IL-6 treatment further

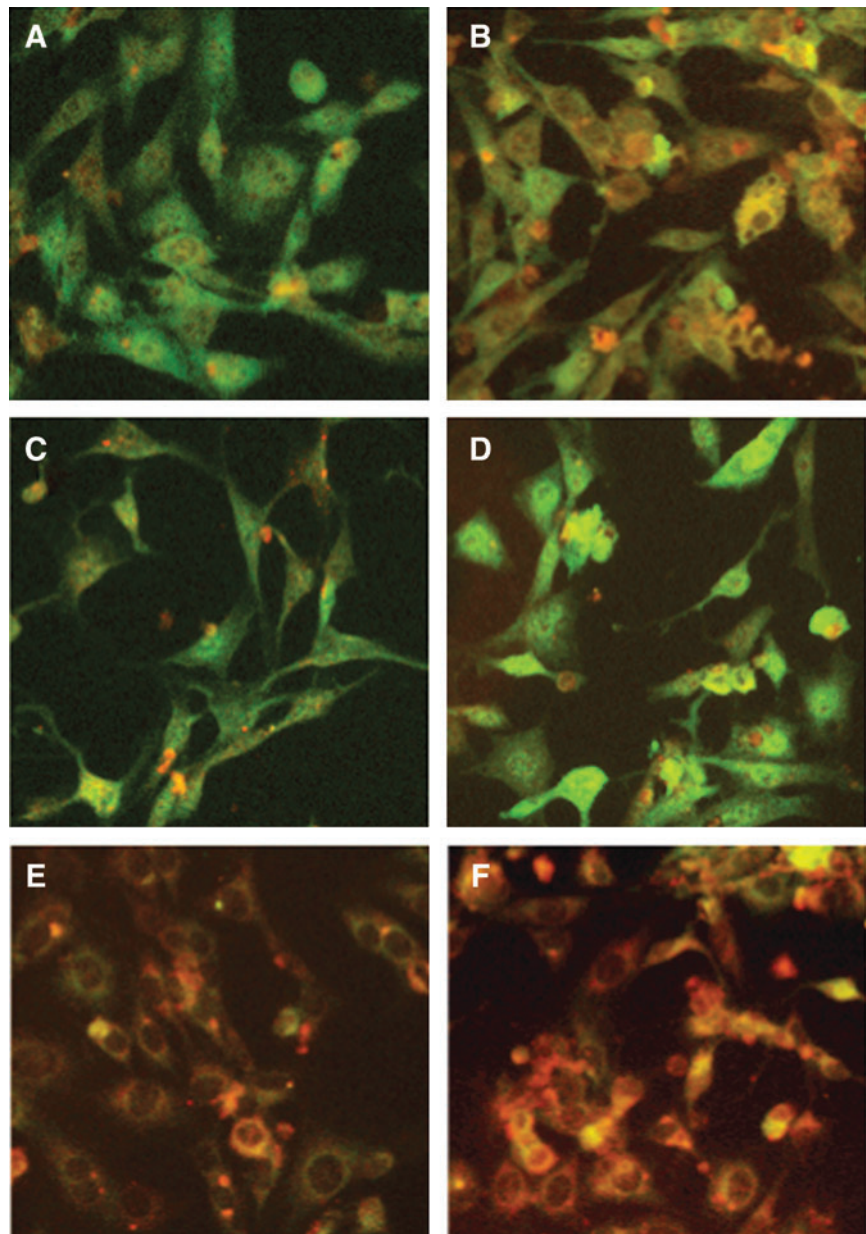


FIG. 2. IL-6 induces STAT3 serine phosphorylation in IL-6KO dermal fibroblasts. IL-6KO fibroblasts were grown to confluency on multi-chamber slides and pretreated with saline control (A, B), PD98059 (C, D), or LLL-3 (E, F). After preincubation, the cells were treated with either 0 ng/mL (A, C, E) or 20 ng/mL (B, D, F) rmIL-6. After paraformaldehyde fixation, slides were incubated with anti-phospho-S727 STAT3 (red), or anti-STAT3 (green) antibody and detected using Alexafluor 546 or 488 conjugated secondary antibodies respectively. Stained slides were visualized using a Leica DM400B fluorescence microscope and 20 \times objective, photographs are representative of three replicate experiments.

increased the intensity of p-S727 staining, while not affecting STAT3 nuclear staining (Fig. 2f).

IL-6 induces SOCS3 Phosphorylation and association with p120 RasGAP

Aside from its negative regulatory functions on STAT3 signaling, SOCS3 can be phosphorylated on key tyrosine residues and interact with the Ras inhibitor p120 RasGAP (Cacalano and others 2001). This ultimately results in disinhibition and potentiation of the MAPK signaling cascade. To investigate whether IL-6 treatment led to phosphorylation of SOCS3 and physical association with p120 RasGAP, fibroblasts were treated with rmIL-6 for various time periods and immunoprecipitation/Western blots (ip) were performed. Analysis of proteins (100 μ g total protein each sample) immunoprecipitated via a SOCS3 specific antibody showed that treatment with 20 ng/mL of IL-6 induced SOCS3 phosphorylation after 15 and 30 min of treatment, as indicated by a lone doublet band revealed by phospho-tyrosine staining (Fig. 3a). It was also found that p120 RasGAP coimmunoprecipitated with phosphorylated SOCS3 after 15 min treatment as well with 20 ng/mL of IL-6 (Fig. 3b). IL-6 treatment (15 min) did not affect SOCS3 protein (Fig. 3b) or mRNA expression (Fig. 3c).

Knockdown of SOCS3 expression restores STAT3 phosphorylation in fibroblasts

Given that SOCS3 may be a key regulator of IL-6R signal transduction in IL-6KO fibroblasts, it was of interest to determine whether the inhibition of SOCS3 expression would potentiate JAK/STAT activity in IL-6KO fibroblasts. To determine this, IL-6KO fibroblasts were electroporated (Lonza, Walkersville, MD) and transfected with SOCS3 specific siRNA oligonucleotide constructs (Qiagen, Valencia, CA). Twenty-four hours post transfection, fibroblasts were treated with 20 ng/mL rmIL-6 for 30 min. Co-transfection with green fluorescent protein allowed for optimization of >30% transfection with >50% viability in this cell type (not shown). SOCS3 mRNA expression significantly decreased in transfected cells compared with those treated with scrambled siRNA (Fig. 4a). Examination of STAT3 phosphorylation after knockdown of SOCS3 shows that treatment with 20 ng/mL of IL-6 significantly induced STAT3 phosphorylation compared with scrambled control transfected cells (Fig. 4b, c).

Modulation of IL-6R signaling regulates IL-6 induced gene expression

Matrix-metalloproteinases are important mediators of wound healing and remodeling in skin. The expression of MMP-2 in dermal fibroblasts can be modulated by the IL-6R through the MAPK pathway (Lockett and Gallucci 2007). To examine whether manipulation of either signaling pathways can have an effect on the expression of this gene, IL-6KO fibroblasts were pretreated with the inhibitors LLL3 (STAT3 inhibitor) and PD98059 (MAPK/ERK inhibitor), and IL-6 induced mRNA expression of MMP-2 was assessed. Treatment with LLL3 did not significantly affect the IL-6 mediated suppression of MMP-2 (Fig. 5, gray bars) mRNA expression. However, treatment with PD98059 and rmIL-6 significantly

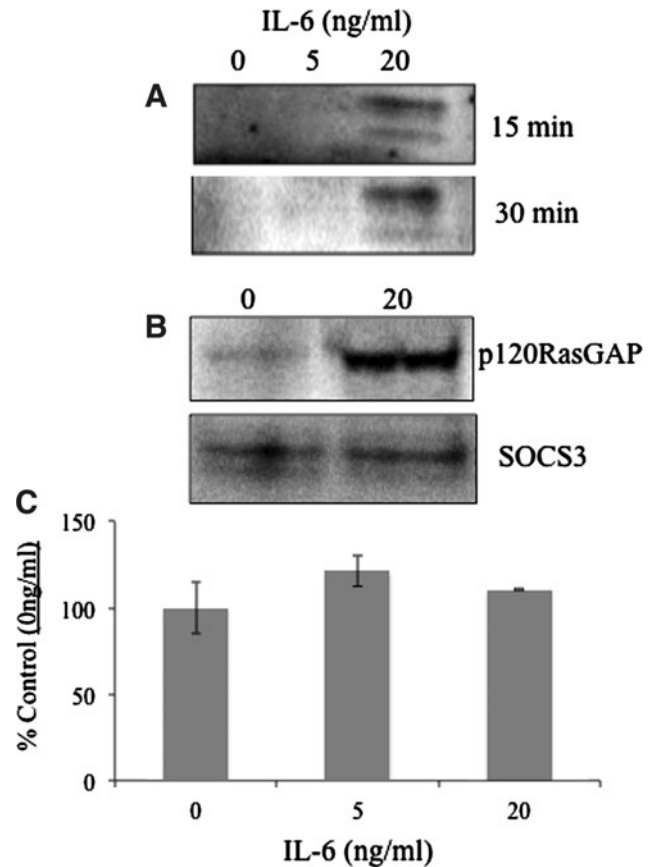


FIG. 3. IL-6 induces SOCS3 phosphorylation resulting in an association with p120 RasGAP. IL-6KO dermal fibroblasts were cultured as described and treated with the indicated concentrations of rmIL-6 for up to 30 min. Cells were lysed and 100 μ g of total protein per sample was immunoprecipitated with anti-SOCS3 antibody. SDS-PAGE gels were transferred to nitrocellulose membranes and blotted with anti-phospho-tyrosine (A), anti-p120 RasGap (B, upper panel), or anti-SOCS3 (B, lower panel). Expression of SOCS3 mRNA was evaluated after IL-6 treatment in fibroblasts (C) as determined by QPCR ($n=4$). SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; QPCR, quantitative polymerase chain reaction.

augmented MMP-2 mRNA over basal levels (Fig. 5, dark bars).

Discussion

IL-6 can activate three functionally distinct signaling pathways through its receptor, yet these pathways can influence each other to varying degrees (Kamimura and others 2003). Utilizing the IL-6KO fibroblast model it was shown that these cells signal primarily through the MAPK/ERK pathway, with ERK phosphorylation occurring in a time (Gallucci and others 2006) and dose dependent manner (Fig. 1a). However, while this laboratory has previously shown that IL-6R primarily signals through the ERK pathway in these cells, it was not determined whether WT cells responded in a similar manner. Herein, it was found that normal mouse dermal fibroblasts as well appear to only signal through the ERK pathway with no induction of STAT3 phosphorylation up to 50 ng/mL (Fig. 1d). However, these

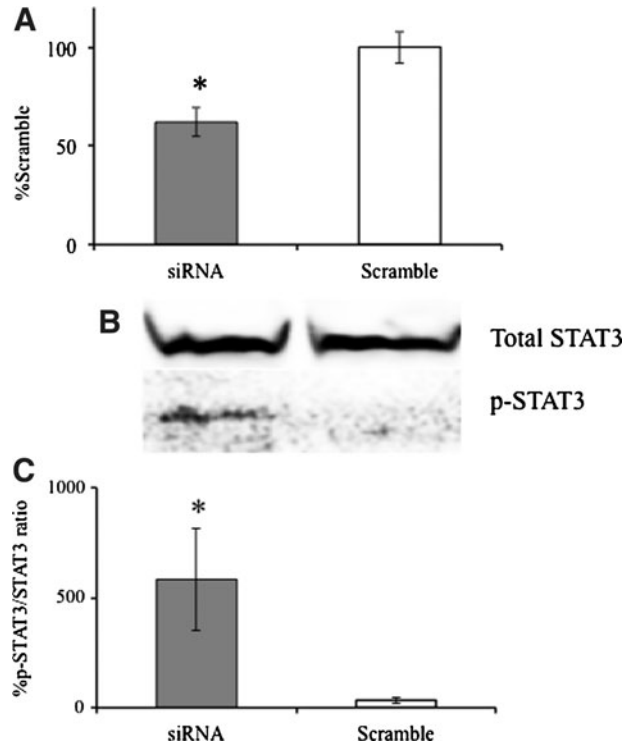


FIG. 4. Knockdown of SOCS3 expression restores STAT3 phosphorylation in IL-6KO fibroblasts. IL-6KO dermal fibroblasts were grown as described and electroporated with SOCS specific siRNA and a scrambled control. Twenty-four hours post transfection, growth media was replaced with serum free media and treated up to 20 ng/mL rmIL-6 for 30 min. Total mRNA and protein were extracted from fibroblasts as described. Expression of *SOCS3* (**A**) was determined via Real-time PCR utilizing an ABI 7000 SDS system and accompanying software. Phospho-STAT3 and total STAT3 was determined by Western blot (representative **B**) and differences determined by digital image analysis utilizing Image J (**C**). Data are expressed as a percent of untreated non-transfected control cells (**A**, **C**), mean \pm SE, ($n=4$), *Significantly different from 0 ng/mL, $P \leq 0.05$). SOCS, suppressor of cytokine signaling.

cells require repeated stringent washing with serum free media before rmIL-6 treatment to obtain consistent basal p-ERK activation (not shown), presumably to remove endogenous IL-6. Indeed, the dose response seems to have shifted toward a lower peak concentration of IL-6 compared with IL-6KO fibroblasts, which may reflect residual IL-6 in the culture media or captured on receptor. Thus, studying IL-6R function in fibroblasts is ideal in the IL-6KO model as it offers the advantages of expressing active IL-6R, but does not allow production of IL-6 protein, thus making analysis of cell function less prone to artifacts from receptor feedback inhibition, and allowing consistent and precise determination of dose response relationships of rmIL-6 treatment. It should be taken into account that the preference for ERK signaling and lack of STAT3 activation in fibroblasts reported here occurred at ≤ 50 ng/mL IL-6, which is thought to be within the physiological range. Indeed, most research on IL-6R function is carried out utilizing approximately 10–20 ng/mL concentrations (for review (Heinrich and others 2003). However, it is possible that quite different signaling might occur at su-

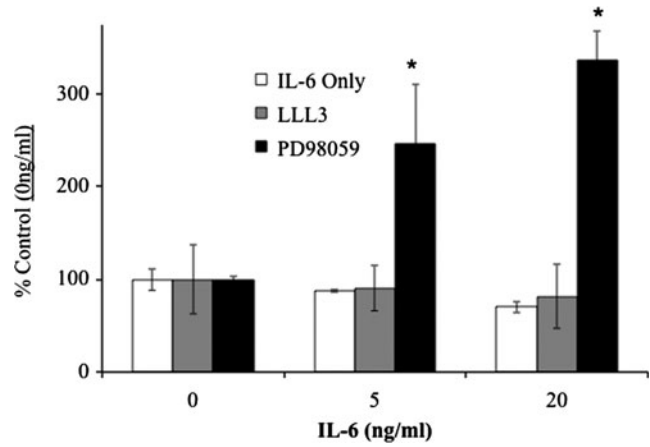


FIG. 5. Signal transduction inhibitors modulate IL-6R induced gene expression. IL-6KO dermal fibroblasts were isolated as described and grown to 80% confluency. Growth media was replaced and cultures were treated with LLL-3 (20 μ M, STAT3 Inhibitor), PD98059 (30 μ M, ERK inhibitor), or vehicle (0.1% DMSO in culture media) 30 min before rmIL-6 treatment, as described in Methods. Expression of *MMP-2* mRNA was determined via Real-time PCR utilizing an ABI 7000 SDS system, and accompanying software. Expression differences were determined relative to 28s rRNA utilizing the ddCt method. Data are expressed as mean \pm SE, ($n=4$), *Significantly different from IL-6 only treatment ($P < 0.05$). DMSO, dimethyl sulfoxide.

praphysiological or even pharmacological doses of perhaps greater than 100 ng/mL, and further study will be necessary to determine whether this is the case.

Since IL-6R signaling can activate either JAK/STAT or MAPK/ERK pathways, it was of interest to determine whether the activation of one pathway may result in an interaction that could modulate the activity of the other as explained by the "Signal Orchestration Model" (Kamimura and others 2003). As predicted by the model, chemical inhibition of either IL-6R pathway seemed to augment the IL-6 induction of the other (Fig. 1 and Fig. 2c–f), which fits well with the fact that phorbol ester augmentation of Erk activation has been shown to inhibit IL-6R induced STAT3 activation (Sengupta and others 1998). This indicates that IL-6R induced JAK/STAT3 signaling can occur, and that this pathway is inhibited by the ERK pathway in dermal fibroblasts. Interestingly, ERK can mediate the phosphorylation of STAT3 at S727 which is thought to augment the activation of this transcription factor (Chung and others 1997; Sengupta and others 1998; Decker and Kovarik 2000; O'Rourke and Shepherd 2002). Indeed, IL-6 treatment of fibroblasts increases phosphorylation of STAT3-S727, but this did not result in STAT3 nuclear translocation (Fig. 2). This lack of increased STAT3 translocation may be related to the fact that dual phosphorylation of Y705 and S727 appears to be necessary for STAT3 activation (Schuringa and others 2000; Abe and others 2001). Further, ERK activation can inhibit Y705 phosphorylation and STAT3 transcriptional activity in various cell types (Sengupta and others 1998; Venkatasubbarao and others 2005). Thus, these data seem to indicate that in IL-6 treated fibroblasts, serine phosphorylation of STAT3 does not mediate the translocation/activation of the transcription factor, and in fact may play a role in Erk mediated inhibition of STAT3 signaling in these cells.

There are numerous mechanisms known to be involved in inhibition or termination of IL-6R signaling including receptor internalization, and the actions of protein tyrosine phosphatases, protein inhibitors of activated STAT (PIAS) family, proteasomal degradation of STAT factors and the suppressor of cytokine signaling or SOCS family (Greenhalgh and Hilton 2001; Fischer and others 2004). Each mechanism results in attenuation of the IL-6 signal at different points throughout the cascade, adding redundancy and multiple levels of control to the system. The SOCS family of proteins acts in a classical negative feedback manner in that they are induced by cytokine stimulation yet negatively modulate the same cascade that initiated their production. SOCS proteins can inhibit signal transduction via several proposed mechanisms such as directly binding to the activation loop of JAK and inhibiting its activity, targeting signaling component for proteasomal degradation, and acting as a ubiquitin ligase (Kamura and others 1998; Sasaki and others 1999; Yasukawa and others 1999; Zhang and others 1999; Auernhammer and Melmed 2001; Kile and others 2002; Johnston 2004).

Aside from acting as a negative feedback inhibitor, SOCS3 may play additional roles in cytokine signaling. Cacalano and others (2001) found that in response to IL-2, erythropoietin, EGF, and PDGF, SOCS3 can be phosphorylated at Y204 and Y221 that are located within the SOCS box region of the protein and affect the stability of SOCS3 (Haan and others 2003). Further, it was shown that phosphorylated SOCS3 subsequently binds and inactivates the Ras/GTPase-activating protein p120. Activated GTP-bound Ras acts by regulating the cellular response through distinct Ras effector proteins such as Raf. Activated Raf then phosphorylates MEK-activating the MAPK/ERK cascade (Pamonsinlapatham and others 2009). The interaction of phosphorylated SOCS3 with p120 results in the inhibition of Ras, which leads to a sustained Ras/GTP and MAPK signal (Cacalano and others 2001). Herein, it was indeed found that SOCS3 is tyrosine phosphorylated in fibroblasts (Fig. 3a), and coimmunoprecipitates with p120 RasGap (Fig. 3b). Interestingly, this interaction appears to increase after rmIL-6 treatment (Fig. 3b), possibly directly through the action of JAK as has been shown via insulin receptor in 3T3 fibroblasts (Peraldi and others 2001). Furthermore, after siRNA knockdown, SOCS3 mRNA expression was greatly decreased as compared to the scrambled control (Fig. 4a), and similar to results shown by Lang and others (2003) utilizing SOCS3^{-/-} cells, STAT3 phosphorylation increased as a result of IL-6 treatment (Fig. 4b), confirming the results obtained utilizing chemical inhibitors shown in Fig. 1. Interestingly, SOCS3 itself does not appear to be induced by IL-6 treatment in these cells (Fig. 3b, c), indicating that basal expression is enough to mediate the apparent STAT3 modulation. Overall, these results indicate that the JAK/STAT pathway can indeed be activated in fibroblasts by IL-6 stimulation if the influence of SOCS3 is decreased, indicating its essential role in modulating ERK signaling preference of these cells in response to IL-6.

While it seems apparent that by perturbing the balance of signaling pathway cross talk by utilizing chemical inhibitors could artificially induce one cascade over another, it was not determined whether this might induce functional consequences in these cells. The matrix metalloproteinase family of endopeptidases is very important and tightly regulated within the wound repair process (Madlener and others 1998;

Daniels and others 2003). MMP-2 was examined as an indicator of IL-6 function in fibroblasts, as IL-6 treatment is known to decrease MMP-2 expression hepatic stellate cells (Bansal and others 2005) and dermal fibroblasts (Luckett and Gallucci 2007). Indeed, IL-6 treatment with or without STAT3 inhibition resulted a dose dependent decrease in MMP-2 expression when treated with increasing concentrations of rmIL-6, whereas the opposite trend was seen upon cytokine treatment and inhibition of the MAPK/ERK cascade (Fig. 5). These data suggest that the IL-6R system may be manipulated in dermal fibroblasts through the inhibition of either cascade, resulting in an altered outcome as suggested by the "Signal Orchestration Model" (Kamimura and others 2003). These results hint at possible therapeutic applications where an undesirable IL-6R signaling outcome could be manipulated utilizing an appropriate inhibitor, for instance to modulate insulin intolerance in the liver caused by IL-6 overexpression (DiCosmo and others 1994; Kado and others 1999),

When viewed together, the result herein seem to indicate that IL-6R mediated ERK signaling preference in dermal fibroblasts is influenced by several means. In summary, it appears that SOCS3 is tyrosine phosphorylated in response to IL-6 treatment, possibly by JAK (Peraldi and others 2001). This inactivates p120 RasGAP allowing Ras to activate the MAPK/ERK pathway unhindered. Increased ERK activity phosphorylates STAT3, (at least at S727), inhibiting its translocation. This is supported by the fact that if ERK is inhibited by PD98059, STAT3 p-S727 is decreased, and increased translocation is observed (Fig. 2d). Knockdown of SOCS3 (Fig. 4) would allow p120 to inhibit Ras mediated MAPK activation, eliminating STAT3 serine phosphorylation, and directly augmenting JAK/STAT activity as previously shown (Lang and others 2003).

However, despite the fact that induction of STAT3 activation by IL-6 in fibroblasts is not apparent, this transcription factor appears to be activated in untreated fibroblast cultures and is apparent in this (Figs. 1, 2) and a previous study (Gallucci and others 2004b). Thus, this STAT3 activation may be one source for basal levels of SOCS3 expression in these cells that directs the cells toward the ERK pathway. Thus, a question remains as to what causes basal SOCS3 and STAT3 activation in these cells? However, it is well known that IL-6 is not the only cytokine that can activate gp130, and it could be that one of the multiple ligands is inducing the apparent basal phosphorylation of STAT3 and production of SOCS3. Certainly, further study will be necessary to determine the source function of this activation in fibroblasts.

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Author Disclosure Statement

No competing financial interests exist.

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