Dectin-1 Stimulation Induces Suppressor of Cytokine Signaling 1, Thereby Modulating TLR Signaling and T Cell Responses

Mariel E. Eberle and Alexander H. Dalpke

*J Immunol* 2012; 188:5644-5654; Prepublished online 30 April 2012; doi: 10.4049/jimmunol.1103068

http://www.jimmunol.org/content/188/11/5644

**Supplementary Material**

[http://www.jimmunol.org/content/suppl/2012/04/30/jimmunol.1103068.DC1](http://www.jimmunol.org/content/suppl/2012/04/30/jimmunol.1103068.DC1)

**References**

This article cites 53 articles, 24 of which you can access for free at: [http://www.jimmunol.org/content/188/11/5644.full#ref-list-1](http://www.jimmunol.org/content/188/11/5644.full#ref-list-1)

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: [http://jimmunol.org/subscription](http://jimmunol.org/subscription)

**Permissions**

Submit copyright permission requests at: [http://www.aai.org/About/Publications/JI/copyright.html](http://www.aai.org/About/Publications/JI/copyright.html)

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: [http://jimmunol.org/alerts](http://jimmunol.org/alerts)
Dectin-1 Stimulation Induces Suppressor of Cytokine Signaling 1, Thereby Modulating TLR Signaling and T Cell Responses

Mariel E. Eberle and Alexander H. Dalpke

Suppressor of cytokine signaling (SOCS) proteins have been identified as negative feedback inhibitors for type I and II cytokine receptors. Eight family members, namely, SOCS1 to SOCS7 and cytokine-inducible SH2 domain-containing protein (CIS), are known (1). Induction of SOCS proteins is achieved through JAK/STAT signaling, which, in turn, is terminated by SOCS proteins. It has been shown that SOCS1, SOCS3, and CIS can also be induced in innate immune cells, including macrophages and dendritic cells (DCs). In these cells, stimulation of TLRs, among others, induces the expression of SOCS proteins (2–5). SOCS1 serves as a cross-talk inhibitor, because it regulates the sensitivity of macrophages to IFN-γ (4, 6), as well as JAK/STAT independent pathways, namely, TNF-α (7) and TLR signaling pathways (8, 9), indicating a role in innate immunity.

TLRs belong to a diverse set of proteins recognizing microbial-derived molecules of pathogens, collectively referred to as pattern recognition receptors (PRRs), which interact with conserved pathogen-associated molecular patterns (PAMPs) (10, 11). Intact pathogens, however, are composed of a variety of different PAMPs. Hence a combination of TLR and non-TLR PRRs will be triggered during infections. One class of PRRs located on the cell surface, namely, C-type lectins (i.e., Dectin-1), has the capacity to signal in a TLR-independent manner (12, 13). Dectin-1 is mainly found on cells of the myeloid lineage, namely, macrophages and DCs. Because the ability of Dectin-1 to act as autonomous receptor has been appreciated only recently, the molecular regulation of its signaling pathway is still partly unknown. On engagement of Dectin-1, tyrosine residues within a receptor ITAM motif are phosphorylated by Src family tyrosine kinases that, in turn, recruit and activate Syk kinase, thereby initiating downstream signaling (14–16). It has been shown that stimulation of Dectin-1 in Syk-deficient DCs is completely abrogated (14). In murine DCs, Syk is needed for Zymosan internalization. Pathways downstream of Syk are just beginning to be explored, and a key role for ERK has been proposed (17). Less is known about termination of Dectin-1 signaling.

Because SOCS proteins have been shown to be induced not only through the JAK/STAT pathway but can also be induced in innate immune cells through PAMPs, we addressed the question whether SOCS might contribute to fine-tuning of Dectin-1 signaling. In this study, we show that SOCS1 is induced as a consequence of stimulating murine bone marrow-derived DCs (BMDCs) and bone marrow-derived macrophages (BMMs) with depleted Zymosan (dZ) that triggers exclusively Dectin-1. SOCS1 was induced by a novel pathway using the tyrosine kinases Src and Syk, followed by activation of the downstream proline-rich tyrosine kinase 2 (Pyk2). Pyk2 activated ERK that, in turn, mediated induction of SOCS1. Moreover, we show that SOCS1 induced via Dectin-1 influences TLR signaling because it is responsible for the downregulation of IL-12p40 in BMMs costimulated via TLR9 and Dectin-1. T cell priming was influenced by SOCS1, as Zymosan-stimulated BMMs from IFN-γ−/−/SOCS1−/− mice were less ef-
Cultures were stimulated on day 5 with PMA (50 ng/ml; Sigma) and ionomycin (750 ng/ml; Calbiochem) for 4 h. Supernatant was analyzed for IL-17 secretion by ELISA.

Quantitative RT-PCR
Total RNA from 3 × 10^5 BMMs or 5 × 10^5 BMDCs was extracted with peqGOLD total RNA kit (peqlab, Erlangen, Germany) and reverse transcribed using the high-capacity cDNA transcription kit (Applied Biosystems, Life Technologies, Darmstadt, Germany). cDNA was diluted 1:4 and real-time PCR was performed in duplicate wells on a 7900HAT platform (Applied Biosystems, Life Technologies). Specificity of RT-PCR was controlled with no-template as well as no-reverse transcriptase samples and analysis of melting curves. Results are normalized to the housekeeping gene β-actin. Primer sequences are available on request.

Cytokine detection and detection of NF-κB DNA binding
After stimulation for 16–24 h, cell-free supernatant was removed and analyzed for cytokine secretion using sandwich ELISAs: IL-12p40, TNF-α, IL-10, and IL-6 (BD, Becton Dickinson, Heidelberg, Germany), TGF-β and IL-17 (R&D Systems, Abingdon, U.K.), and IL-23 (eBioscience, Frankfurt, Germany). ELISAs were used. For the measurement of transcription factor activity, nuclear extracts were prepared 6 h after stimulation of SOCS1−/−/IFN-γ−/− or IFN-γ−/− BMs (9 × 10^5) with a Nuclear extract kit (Active Motif, La Hulpe, Belgium). Lysates were analyzed using the TransAM NF-κB Transcription Factor Assay Kit (Active Motif). Analyses were performed according to manufacturer’s protocol in duplicates.

Western blotting
After stimulation, cells (BMMs or BMDCs) were lysed in 100 μl lysis buffer (6-well) and treated as described elsewhere (18). Immunoblots were performed using anti-SOCS1 Ab (1:500, clone 4H1; Millipore, Schwalbach, Germany). Abs directed against IκBα, pIκBα, pp38, pERK (p44/p42), pJNKII, Pyk2, and pPyk2 (all 1:1000) were purchased from Cell Signaling Technology (Frankfurt, Germany). Anti–β-actin (1:200; Cell Signaling Technology) was used as loading control. As secondary Abs, HRP-linked anti-mouse or anti-rabbit (1:2000; Cell Signaling Technology) was used. Signals were detected using the ECL system Immobilon Chemosiluminex EHR Substrate (Millipore). Blots were imaged digitally, and contrast adjustments were applied to all parts of a figure. Framed lanes indicate that only parts of the blot are shown for better visualization. Quantitation of bands from individual immunoblots was performed with the QuantOne software from Bio-Rad (München, Germany).

EMSA
Nuclear extracts were prepared as previously described (19). A total of 1.2 × 10^7 cells was stimulated as indicated and lysed after 6 h. Nuclear fractions were collected and EMSA was performed using the LightShift chemiluminescent EMSA Kit (Pierce, Thermo Scientific, Karlsruhe, Germany) with modifications. Instead of the provided binding buffer, a 5× binding buffer composed of 100 mM HEPES (pH 8.0), 250 mM KCl, 2.5 mM DTT, 250 μM EDTA, 5 mM MgCl₂, and 25% (v/v) glycerol was used. In addition, protein–DNA complexes were directly detected within the gel: after incubation of nuclear extracts with Cy3-labeled SOCS1 probe (15 min, RT), the DNA–protein complexes were loaded on a native gel without the usage of bromophenol blue containing loading buffer. After ~3 h at 100 V, 4°C, the gel was analyzed. Fluorescence was measured using the Typhoon Trio detector (GE Healthcare, Braunschweig, Germany). Cy3 was excised at 550 nm, and emission was detected at 570 nm. Sequence of the Cy3-labeled SOCS1 probe was 5′-Cy3-AAG ACT GGC GCA GGG GCG GGC-3′.

Statistical analysis
All experiments were performed at least two times, and the number of experiments is stated in the figure legends. Means ± SD are shown. Significant differences were evaluated by the unpaired Student t test with two-tailed distribution within GraphPad prism 4.03 (GraphPad Software, San Diego, CA); *p < 0.05, **p < 0.01, ***p < 0.001 were considered significant.

Results

**dZ induces SOCS1 in a TLR-independent manner**
To analyze whether Dectin-1 triggers SOCS1, we made use of dZ that has been reported to lack contaminating TLR2 activity.

Materials and Methods
Reagents
Cycloheximide, Syk inhibitor IV Bay 61-3606, NF-κB inhibitor Bay 11-7082, PP1, AG17, BAPTA, and KN93 were obtained from Merck, Calbiochem (Darmstadt, Germany). dZ was from InvivoGen (Toulouse, France), Phosphorothioate-modified CpG oligonucleotide 1668 (TCC ATG ACG TTC CTG ATG CT; TIB Molbiol, Berlin, Germany) was commercially purchased; LPS from Salmonella minnesota (smooth form) was provided by U. Seydel (Borstel, Germany). Recombinant murine IL-10, IFN-γ, IL-4, IL-6, IL-12, and human TGF-β were from PeproTech (Hamburg, Germany). Functional-purified monoclonal anti-mouse IL-10 Abs, anti-CD3-e, and anti-CD28 were obtained from eBioscience (Frankfurt, Germany). Neutralizing Abs against IL-4 were purchased from eBioscience and anti-IFN-γ Abs from PeproTech.

Mice
C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). IFN-α/β receptor-deficient mice (IFNAR−/−) were kindly provided by Rainer Zawatzky (Heidelberg, Germany), and MyD88/TRIF double-deficient mice by U. Kalinke (Düsseldorf, Germany). Dectin-1−/− mice were received from G. Brown (Aberdeen, U.K.). IFN-γ−/− and SOCS1−/−/IFN-γ−/− were obtained from Martin Rottenberg (Stockholm, Sweden).

Cell culture and stimulation
BMMs and BMDCs were prepared from wild-type (WT) or knockout mice on C57BL/6 background. In brief, bone marrow was collected from femurs and tibiae. Cells were plated in 150-mm tissue-culture plates in different media containing LPS (160 μg/ml) and essential FCS, 50 mM 2-ME, antibiotics penicillin G [100 IU/ml] and streptomycin sulfate [100 μg/ml]; BMMs: DMEM supplemented with 10% FCS, antibiotics penicillin G and streptomycin sulfate). BMDCs were prepared as described previously (18) using GM-CSF. For BMMs, nonadherent cells were collected after 24 h, washed, and ~2.5 × 10^7 cells were seeded into 150-mm tissue-culture plates in medium. Culture supernatant (30%) of an M-CSF–transfected cell line was used as a source of M-CSF. At day 3, fresh M-CSF containing supernatant was added, and at day 7, adherent BMMs were harvested.

For stimulation assays, cells were plated in 96-well (2 × 10^5 cells/well), 24-well (3 × 10^5 BMMs or 5 × 10^5 BMDCs), 6-well (3–6 × 10^5) plates or transwells (as indicated in the figure legends) and stimulated either with 100 or 500 μg/ml LPS, CpG-DNA, and inhibitors were used as indicated. Transwell Polycarbonate Membrane Permeable Supports were from Costar (Corning, Amsterdam, The Netherlands).

Gene knockdown
Knockdown experiments were performed using short interfering RNA (siRNA). siRNA targeting Pyk2 and control siRNA were purchased from Qiagen (Hilden, Germany) and Invitrogen (Carlsbad, CA). BMMs were transfected using Lipofectamine RNAiMax (Invitrogen). In brief, siRNA was incubated with Lipofectamine RNAiMAX in 24- or 6-well plates. After 20 min, cells were plated (3 × 10^5 for 24-well plates or 2 × 10^5 for 6-well plates) directly to the reaction mixture and incubated at 37°C. After 20 h, cells were stimulated, respectively. Knockdown efficiency was determined by Western blotting and quantitative RT-PCR.

In vitro T cell stimulation assay
CD4+ T cells were purified from spleens of WT C57BL/6 mice. Cell purification was performed using the CD4+ T cell isolation kit II according to manufacturer’s protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). Magnetically labeled cells were isolated via the autoMACS Separator (Miltenyi Biotec). A total of 1 × 10^7 sorted T cells were cocultured together with 1 × 10^5 BMMs or BMDCs from SOCS1−/−/IFN-γ−/− or IFN-γ−/− control mice and 2 μg/ml plate-bound anti-CD3e, 2 μg/ml soluble anti-CD28 in the presence or absence of 100 μg/ml DZ, 1 μM CpG, or a combination thereof. Th17 controls consisted of CD4+ T cells activated in the presence of 10 ng/ml TGF-β, 20 ng/ml IL-6, and neutralizing Abs against IFN-γ (2 μg/ml) and IL-4 (2 μg/ml) cocultured with BMMs or BMDCs. Th1 control cells were induced by IL-12 (2 ng/ml) and Th2 control cells by IL-4 (10 ng/ml).

DZ induces SOCS1 in a TLR-independent manner
To analyze whether Dectin-1 triggers SOCS1, we made use of dZ that has been reported to lack contaminating TLR2 activity.
Stimulation of BMMs and BMDCs resulted in a robust, concentration-dependent induction of SOCS1 at mRNA (Fig. 1A, Supplemental Fig. 1A, 1B) and protein level (Fig. 1B). SOCS1 mRNA induction by Zymosan was up to 40-fold and comparable with well-known SOCS1 inducers like LPS or IFN-γ, thus identifying Dectin-1 as an additional, so far unknown, trigger of SOCS1 transcription. SOCS1 transcripts were increased in a dose- and time-dependent manner with a peak induction at 4.5 h (Supplemental Fig. 1A, 1B) but lasting up to 16 h. Moreover, priming of BMMs with IFN-γ previous to stimulation with dZ led to a further increase in SOCS1 transcription (Fig. 1A).

To confirm that dZ lacks any possible TLR-binding moiety, we additionally analyzed BMDCs and BMMs from MyD88/Trif knockout mice. We observed no reduction in levels of SOCS1 when stimulating these cells with dZ as compared with cells of WT mice (Fig. 1C). In contrast, LPS-mediated SOCS1 expression was completely abolished in MyD88/Trif knockout mice (Fig. 1C) as expected from published data (8). Consistent with those findings, downstream signaling of Dectin-1 was not affected in MyD88/Trif knockout mice as demonstrated for phosphorylation of ERK (Fig. 1D, Supplemental Fig. 1C). On the contrary, LPS-induced ERK phosphorylation was entirely abrogated in the respective knockout mice. These results show that SOCS1 is induced in BMMs and BMDCs when activated via Dectin-1 in a TLR-independent manner.

SOCS1 is a direct target gene of Dectin-1

To further ensure that SOCS1 induction on stimulation with dZ was exclusively dependent on Dectin-1, we stimulated Dectin-1−/− BMMs. SOCS1 expression in Dectin-1−/− macrophages was entirely abolished with Zymosan as trigger as compared with WT mice (Fig. 2A).

To analyze the mode of SOCS1 induction by Dectin-1, we thought to exclude intermediate, secreted factors that could trigger classical JAK/STAT signaling. We therefore used a transwell system in which BMMs or BMDCs were plated in both wells, but stimulation with Zymosan, which could not pass the transwell membrane because of size, was done only in the lower compartment. Upon stimulation with dZ, induction of mRNA of SOCS1 could only be observed in the lower well showing direct induction of SOCS1 by Zymosan. In contrast, LPS that is able to enter the upper well by diffusion was capable of inducing SOCS1 expression in BMMs or DCs in both chambers of the transwell (Fig. 2B) serving as positive control. To strengthen these findings, we additionally stimulated type I IFNR-deficient mice as type I IFNs were suggested to act as secreted intermediate factors in a TLR setting (20, 21). SOCS1 induction showed no differences in IFNAR1−/− cells compared with WT when triggered with either LPS or dZ (Supplemental Fig. 2A, 2B). Because it has been shown that TLR4 triggering induces SOCS in a direct and type I IFN-independent manner, we expected no difference in LPS-induced SOCS1 in IFNAR1−/− cells (8). Furthermore, pretreatment with cycloheximide as inhibitor of protein biosynthesis did not affect SOCS1 induction in BMDCs and BMMs when triggered via Dectin-1 (Fig. 2C). We even observed a slight upregulation of SOCS1 mRNA expression. Absence of TNF-α and IL-12p40 protein in supernatants of cycloheximide-treated cells confirmed successful protein inhibition (Supplemental Fig. 2C, 2D).

It has been published that myeloid cells produce high amounts of IL-10 on infection with fungi. We therefore additionally wanted to exclude IL-10 as a possible mediator of SOCS1 transcription in Dectin-1−/−stimulated cells. Ab blocking of IL-10, however, did not affect Zymosan-induced SOCS1, thus further confirming direct induction of SOCS1 by Dectin-1 triggering (Fig. 2D). IL-10 blockade was confirmed by measuring SOCS3, which is strongly induced by IL-10 (Fig. 2E). These data therefore exclude any paracrine mediators that could play a role in SOCS1 induction and identify SOCS1 as a direct target gene of Dectin-1.

**FIGURE 1.** SOCS1 is induced in myeloid cells on engagement of Dectin-1. (A, C) BMDCs (5 × 10^5) or BMMs (3 × 10^5) from WT (A) or MyD88/TRIF−/− (C) mice were stimulated with LPS (100 ng/ml) or dZ (100 μg/ml) for 4.5 h. For priming experiments, cells were preincubated with IFN-γ (50 ng/ml) for 16 h. Cells were lysed and gene expression of SOCS1 was measured by quantitative RT-PCR. Depicted are expression levels normalized to the expression of the housekeeping gene β-actin or fold induction over mock. (B) BMDCs or BMMs (18 × 10^5) were stimulated with increasing concentrations of LPS (10 or 100 ng/ml) or dZ (100 or 500 μg/ml) for 6 h, and lysates were analyzed by immunoblot. Molecular mass is depicted on the left side of the blots and refers to SOCS1 immunoblot. (D) BMMS (3 × 10^6) from WT or MyD88/Trif−/− mice were incubated with LPS (100 ng/ml) or dZ (500 μg/ml) for 30 min. Lysates were analyzed by immunoblot. Blots were probed with Abs specific for SOCS1 (B), ERK1/2 phosphorylated on Thr202/Tyr204 (D), or β-actin as control. Results in (A) and (C) show representative data of three independent experiments (mean + SD) performed in duplicate wells, and data in (B) and (D) are representative for two independent experiments. *p < 0.05, **p < 0.01. mock, Unstimulated control; rE, relative expression.
Dectin-1–triggered SOCS1 is induced independently of NF-κB in BMMs

As shown by Goodridge et al. in 2009 (22), BMMs, in contrast with DCs, do not activate NF-κB on Dectin-1 stimulation. Our observation of SOCS1 induction by Zymosan therefore hints toward a hitherto unknown JAK/STAT and NF-κB–independent mode of transcriptional activation. We therefore further analyzed downstream pathways of Dectin-1 in both cells, BMDCs and BMMs. On stimulation with dZ, we clearly detected phosphorylation of the MAPKs ERK, JNKII, and p38, as well as Syk, the latter mediating proximal signaling by Dectin-1 (Fig. 3A) in BMMs. Unlike DCs, BMMs showed no phosphorylation of IκBα (Fig. 3B), thus confirming the published data on BMMs. Usage of an NF-κB inhibitor corroborated these findings as SOCS1 induction by dZ was not impaired in BMMs. In contrast, inhibition of NF-κB by BMDCs resulted in a downregulation of SOCS1 mRNA. LPS served as a control for the NF-κB inhibitor to be functional (Fig. 3C). Because NFAT is described as a potential transcription factor activated on stimulation of Dectin-1, we examined whether NFAT contributes to SOCS1 induction. However, usage of the NFAT inhibitor CsA did not affect SOCS1 induction in BMMs (data not shown). Furthermore, we could not detect any binding activity of early growth response factor (egr) transcription factor, a direct target of NFAT, to the SOCS1 promoter using EMSA (Fig. 3D, 3E). The findings indicate that, in BMMs, SOCS1 induction on Dectin-1 triggering is independent of NF-κB, thereby identifying an additional mode of SOCS induction.

Pyk2 and ERK MAPK are important for SOCS1 induction

It is known that the MAPK ERK participates in Dectin-1 downstream signaling (17), and we easily observed Dectin-1–mediated ERK activation (Fig. 3A). Pyk2 has been reported to act downstream of Dectin-1, Src, and Syk, and upstream of ERK MAPK, leading to IL-10 production (23).

To determine whether Dectin-1 activates the Pyk2–ERK pathway in murine BMMs, we tested for Pyk2 activation by phosphorylation. Stimulation of BMMs with dZ resulted in tyrosine phosphorylation of Pyk2 (Fig. 4A). Preincubation of murine BMMs with inhibitors directed against Src (PP1) and Syk (Syki) inhibited Pyk2 activation, thus placing Pyk2 downstream to these signaling molecules (Fig. 4A). Because Pyk2 can be activated in a calcium-dependent manner (23–25), we used inhibitors to block calcium pathways. Surprisingly, Pyk2 could still be activated in the presence of the calcium chelator BAPTA or the calmodulin–independent kinase inhibitor KN93 (Fig. 4B), whereas AG17, which prevents phosphorylation of Pyk2, strongly blocked Pyk2 activation (Fig. 4B). Importantly, dZ-induced ERK phosphorylation was largely suppressed in cells pretreated with AG17, but not with BAPTA or KN93 (Fig. 4C). In contrast, the calcium inhibitors decreased the activation of p38 and JNKII MAPK (Fig. 4C). Phosphorylation of p38 was even increased when Pyk2 was inhibited.

To study the impact of the Pyk2–ERK pathway on SOCS1 induction, we pretreated BMMs with Pyk2 and calcium inhibitors. As shown in Fig. 4D, SOCS1 induction was completely abrogated in case of pretreatment with AG17, but not when calcium signaling was blocked. Treatment with Src and Syk inhibitors abolished SOCS1 expression (Fig. 4E) and reduced ERK phosphorylation (Fig. 4F). Inhibition of Syk had no effect on p38 and JNKII activation (Fig. 4F).

To confirm the role of Pyk2 in SOCS1 induction by different means, we additionally targeted Pyk2 by siRNA. Specific knockdown of Pyk2 was observed for mRNA and protein expression (Fig. 5A, 5B). Staining the siRNA-treated BMMs with dZ led to an inhibition of ERK phosphorylation and SOCS1 induction (mRNA, protein; Fig. 5B–D). These findings could further be verified using an inhibitor directed against ERK activation (UO126). Preincubation with UO126 abolished SOCS1 expres-
FIGURE 3. NF-κB is not induced in BMMs via Dectin-1. (A and B) Immunoblots of lysates of BMMs (3 × 10^6) or BMDCs (3 × 10^6). (A) Cells were stimulated with LPS (100 ng/ml) or dZ (500 μg/ml) for 30 min. (B) Cells were stimulated for the indicated time periods with LPS (100 ng/ml) or dZ (500 μg/ml). Molecular mass is depicted on the left side of the blots and refers to pIκBα immunoblots. (C) BMMs (3 × 10^5) or BMDCs (5 × 10^5) were preincubated for 1 h with 0, 10, or 50 μM of the NF-κB inhibitor Bay 11-7082 and stimulated with LPS (100 ng/ml) or dZ (100 μg/ml). Cells were lysed after 4.5 h and analyzed for gene expression (SOCS1) by quantitative RT-PCR. (D) BMMs (1.2 × 10^5) from WT mice were stimulated with LPS (100 ng/ml), dZ (500 μg/ml), or left unstimulated (mock). After 6 h, nuclear extracts were prepared and added to SOCS1 probe containing an egr1 binding site. Samples were run in 6% acrylamide native gels, and Cy3 fluorescence was detected. Asterisk indicates unspecific binding. (E) Sequence of the probe including the binding site (gray letters). Numbers indicate relative position from the A in the ATG initiation site in the human SOCS1 gene. Consensus sequence of egr1 is depicted below the probe. Results in (C) and (D) were performed in duplicate wells.

SOCS1 does not serve as feedback inhibitor in Dectin-1 signaling

SOCS proteins have been identified as negative feedback inhibitors for cytokine signaling via the JAK/STAT pathway (26, 27). We next assessed whether SOCS1, being induced by Dectin-1 triggering, also acts as feedback inhibitor in this pathway. BMMs from SOCS1^−/− mice on IFN-γ knockout background to circumvent perinatal lethality (SOCS1^−/−/IFN-γ^−/−) were stimulated with dZ for different time periods and analyzed for phosphorylation of Syk, Pyk2, and ERK. None of the involved kinases showed a difference in activation profile in cells from SOCS1^−/−/IFN-γ^−/− mice as compared with IFN-γ^−/− control cells (Supplemental Fig. 3). egr2 is one of the first factors directly induced on triggering of Dectin-1 (28). Therefore, we tested regulation of this transcription factor in SOCS1^−/−/IFN-γ^−/− macrophages. We were not able to detect any difference in egr2 induction in SOCS1^−/−/IFN-γ^−/− compared with control IFN-γ^−/− mice (Supplemental Fig. 3C). Moreover, no difference in IL-10 secretion was observed (Supplemental Fig. 3D). Thus, SOCS1 does not act as negative feedback inhibitor in Dectin-1 signaling.

Zymosan-induced SOCS1 modulates TLR cross talk

Because SOCS1 is induced on Dectin-1 stimulation but does not interfere with this pathway, we speculated that it might act as cross-talk inhibitor for different signals. Indeed, Dectin-1 is known to modulate TLR2 signaling (13, 29), the latter being also able to induce SOCS proteins (2, 8). Moreover, mycobacteria can activate Dectin-1 (30) and mycobacterial DNA is a powerful stimulus for TLR9, the latter having the propensity to stimulate high amounts of IL-12p40 (18). Therefore, we used dZ to stimulate solely Dectin-1 and added CpG-DNA as well as other pure TLR ligands, that is, LPS.

IL-12p40 secretion in BMMs and BMDCs was markedly reduced on costimulation of Dectin-1 and TLRs. Cytokine secretion was decreased up to 60% as compared with CpG-stimulated cells (data not shown). To analyze whether SOCS1 could have an influence on this cross talk between TLR9 and Dectin-1 signaling, we examined BMMs from SOCS1^−/−/IFN-γ^−/− mice. As in WT cells, Zymosan reduced IL-12p40 secretion by CpG-
DNA in IFN-γ−/− control cells (Fig. 6A). However, costimulation by Zymosan and CpG-DNA in SOCS1−/−/IFN-γ−/− cells showed no inhibition of CpG-induced IL-12p40 secretion when compared with IFN-γ−/− BMM control cells (Fig. 6A). Thus, the inhibitory effect of Zymosan on CpG-induced IL-12p40 was SOCS1 dependent. In contrast, dZ increased CpG-induced TNF-α, and this synergistic effect was not affected by SOCS1 (Fig. 6B). Regarding IL-10, the increase in cytokine expression was 2-fold in SOCS1−/−/IFN-γ−/− as compared with control cells (Fig. 6C).

Because it was shown that SOCS1 contributes to ubiquitination and subsequent degradation of NF-κB (31, 32), thereby modulating a subset of NF-κB–induced genes, we wanted to analyze whether activation of NF-κB transcription factors after costimulation with CpG-DNA and dZ was altered. We first tested whether the co-stimulation causes an increase in SOCS1 expression. Indeed, SOCS1 induction in WT cells could clearly be amplified by concomitant application of CpG-DNA and dZ in comparison with cells that were stimulated with the respective individual ligands (Fig. 6D). dZ alone had a greater potential to induce SOCS1 as compared with CpG-DNA. We speculated that the higher expression level of SOCS1 could explain the downregulation of IL-12p40 after ligation of TLR9 and Dectin-1. Therefore, we tested IκBα degradation and phosphorylation by immunoblot and NF-κB transcription factor activation by assessing nuclear translocation and subsequent DNA binding. A prolonged and increased phosphorylation of IκBα was induced by dZ and CpG-DNA in SOCS1−/−/IFN-γ−/− mice as compared with control cells, in which IκBα phosphorylation was shorter (Fig. 6E). Importantly, a clear increase in phosphorylation could be detected on costimulation with CpG and dZ after 90 and 240 min. The results suggest regulation of IκBα activation kinetics by SOCS1. Similarly, degradation of IκBα was slightly increased in knockout BMMs when stimulated via dZ alone or together with CpG (Fig. 6E). Using an ELISA-based system that measures binding of NF-κB subunits to their respective DNA elements, we further analyzed transcription factor

**FIGURE 4.** SOCS1 induction depends on ERK and Pyk2 kinases. (A–F) Immunoblot of lysates from BMMs pretreated for 1 h with 10 μM Syki, 10 μM PP1 (A, E, F), or 10 μM BAPTA, AG17, and KN93 (B–D). Blots were probed with Abs against Pyk2 phosphorylated on Tyr402 (A, B), pp38, pERK1/2, pJNKII (C, F), and SOCS1 (D, E). (A) Cells (3 × 10^6) were stimulated for the indicated time periods with dZ (500 μg/ml). (B, C, and F) Cells (3 × 10^6) were stimulated with dZ (500 μg/ml) for 30 min. (D and E) Cells (18 × 10^6) were stimulated for 6 h with dZ (500 μg/ml). 0, no inhibitor was used; Syki, Syk inhibitor IV Bay 61-3606; PP1, Src inhibitor; BAPTA, calcium chelator; AG17, calmodulin-dependent kinase inhibitor; AG17, blocks phosphorylation of Pyk2. Results show representative data of four independent experiments. Intensities (% of mock control) of the various bands are depicted above each blot. In (A) and (C), molecular mass is depicted on the left side of each blot and refers to pPyk2 (A), pERK, pp38, and pJNKII (C). Bar graphs of the densitometry are shown in Supplementary Fig. 4.
We observed an increase in NF-κB (p50 and p52) activation on costimulation of cells from SOCS1−/−/IFN-γ−/− mice compared with IFN-γ−/− control BMMs (Fig. 6F). Furthermore, p52 and p50 were activated via dZ alone in the double-knockout cells (Fig. 6G); concomitant stimulation of these cells with dZ and CpG even increased the activation.

FIGURE 5. Pyk2 mediates ERK activation and SOCS1 induction. (A–D) BMMs were transfected with 50 nM control or Pyk2-specific siRNA for 20 h. Stimulation was performed for 4.5 h (B), 6 h (D), or 30 min (C). (A) Cells (2 × 10⁶) were lysed and blot was performed with Ab against Pyk2 to validate knockdown. (B) Cells (5 × 10⁵) were lysed and analyzed for gene expression and knockdown by quantitative RT-PCR. Transcription was compared with control siRNA. (C and D) Cells (2 × 10⁶ [C] or 18 × 10⁶ [D]) were stimulated with dZ (500 μg/ml) and immunoblot was performed with Abs against pERK (C) or SOCS1 (D). Results in (B) show representative data of two independent experiments, and results in (A), (C), and (D) are representative for three independent experiments (mean ± SD). Results in (B) were performed in duplicate wells. Intensities of the various bands (shown as % of mock control) are depicted above each blot. Molecular mass is depicted on the left side of the β-actin blot in (C). Bar graphs of the densitometry are shown in Supplementary Fig. 4. *p < 0.05, **p < 0.01. siCon, Control siRNA.

FIGURE 6. Dectin-1–induced SOCS1 modulates TLR signaling. (A–G) BMMs from SOCS1−/−/IFN-γ−/− or IFN-γ−/− mice stimulated with dZ, CpG, or a combination thereof were used. (A–C) Cells (2 × 10⁵) were stimulated with dZ (100 μg/ml), CpG (1 μM) for 16 (A, B) or 24 h (C). In case of dZ+CpG, cells were prestimulated with CpG (4 h) after incubation with dZ (12 h [A, B]; 20 h [C]). Supernatants were analyzed for secretion of IL-12p40, TNF-α, or IL-10 by ELISA. (D) Cells (18 × 10⁶) were incubated with dZ (500 μg/ml), CpG (1 μM), or dZ+CpG for 6 h. Lysates were analyzed by immunoblot with Abs specific for SOCS1. (E) Cells (3 × 10⁶) were stimulated with dZ (500 μg/ml), CpG (1 μM), or dZ+CpG for the indicated time periods, cells were lysed, and immunoblot with Abs against IκBα or pIκBα was performed. (F and G) Cells (9 × 10⁶) were incubated with dZ (100 μg/ml), CpG (1 μM), or dZ+CpG for 6 h, and nuclear extracts were analyzed for DNA binding activity of NF-κB subunits p50 (F) and p52 (G). Results in (A)–(C), (F), and (G) show representative data of three independent experiments; data in (D) and (E) are representative for two independent experiments. Results in (A)–(C), (F), and (G) are mean ± SD and were performed in duplicate wells. *p < 0.05, **p < 0.01, ***p < 0.001.

Dectin-1 STIMULATION DIRECTLY INDUCES SOCS1
The data indicate that SOCS1 induced by dZ leads to decreased and shortened NF-κB activation in CpG-DNA–triggered cells, thereby modulating the latter activity (specifically IL-12p40).

**Cross talk between TLR and Dectin-1 influences T cell priming in an SOCS1-dependent manner**

Th17 cells play an important role in the protection against fungal infections (33, 34). It has been claimed that BMDCs triggered via Dectin-1 are able to induce IL-17–producing T cells (35, 36). Stimulation of BMMs with dZ led to an inhibition of CpG-induced IL-12p40 that was reversed in SOCS1 knockout cells (Fig. 6A). This observations and the fact that IL-12 is one of the main cytokines that is responsible for Th1 polarization led us to examine the secretion of three of the main cytokines, namely, IL-6, IL-23, and TGF-β, which are involved in induction of Th17 cells. Cells of IFN-γ−/− mice showed an elevated production of these cytokines when costimulated by dZ and CpG (Fig. 7A–C). In case of TGF-β, dZ alone led to an upregulation. In contrast, in SOCS1−/−/IFN-γ−/− BMMs, the secretion of IL-6, IL-23, and TGF-β was neither increased via dZ nor when TLR9 and Dectin-1 were triggered in parallel (Fig. 7A–C). In a coculture approach, we tested the ability of BMMs and BMDCs to induce IL-17–producing T cells. BMMs or BMDCs from SOCS1−/−/IFN-γ−/− mice and the respective controls were cultured together with T cells isolated from spleens from WT mice. Concerning positive controls (i.e., Th17), cytokines to induce IL-17–producing T cells were not derived from the macrophages or DCs but were added extra. Thus, secretion of IL-17 should not differ between cultures with SOCS1−/−/IFN-γ−/− or IFN-γ−/− cells. The same holds true for the respective Th1 and Th2 negative controls. A clear increase in IL-17 production could be demonstrated when IFN-γ−/− BMMs or BMDCs were stimulated with CpG plus dZ compared with cells triggered via TLR9 or Dectin-1, respectively. In case of BMDCs, dZ alone led to a significant increase in IL-17 production. In contrast, we could not detect any difference in the SOCS1−/−/IFN-γ−/− cells/WT T cell cultures with respect to Zymosan/CpG costimulation (Fig. 7D), thus arguing for an important role of Dectin-1–induced SOCS1 to shape activation of myeloid cells and subsequent T cell stimulation.

**Discussion**

SOCS proteins, although having been identified initially as feedback inhibitors for JAK/STAT signaling, are meanwhile known to be induced by a variety of additional signals (2, 3, 5, 37). In this study, we expand this plethora of signaling pathways toward a calcium-independent pathway activated by ligation of Dectin-1 that, in the end, results in induction of SOCS1. The presented experiments clearly show a TLR-independent and, importantly, direct induction of SOCS1 as delineate as follows. Engagement of Dectin-1 by dZ leads to induction of SOCS1 in a direct manner, independently of any intermediate or paracrine-secreted factors. Furthermore, SOCS1 induction was not mediated by MyD88/Trif, ruling out TLR stimulation by contaminants within dZ. Indeed, TLRs have been reported to induce SOCS1-3 and CIS (5, 8) in macrophages and DCs. Our results now show that also PRRs belonging to the C-type lectin pathway (Dectin-1) increase transcription of SOCS regulators. Also, RIG-I, a cytosolic PRR, triggers SOCS transcription (38, 39), thus identifying SOCS proteins as regulators induced by all important classes of pathogen receptors in innate immune cells.

Concerning the mode of action, SOCS1 induced via dZ did not influence Dectin-1 signaling itself, thus excluding an inhibitory
Dectin-1 STIMULATION DIRECTLY INDUCES SOCS1

role in this pathway. Another C-type lectin DC-SIGN was recently shown to induce and be regulated by SOCS1 (40). However, in our study, we observed that SOCS1 induced through Dectin-1 cross-regulated TLR signaling as it affected cytokine secretion (IL-12, IL-10) and T cell priming (Th17) by concomitant TLR9 stimulation. Thus, SOCS1 is important to regulate the activation pattern of macrophages and DCs if whole pathogens that use different receptors act on a cell.

Because NF-κB was not activated on stimulation of Dectin-1 in macrophages, we can exclude that SOCS1 induction occurs through NF-κB signaling as previously reported for other stimuli (8). Instead, we first thought that the NFAT family could be responsible for the induction of SOCS1. It is described that NFAT, activated via Dectin-1, is responsible for the induction of egr, another family of transcription factors that, in turn, can bind to the promoter of IL-10, which is upregulated by C-type lectin receptor (CLR) engagement (28). Moreover, egr 1 transcription factor has been found to bind to the SOCS1 promoter (41). However, we were not able to ascertain any contribution of NFAT to the expression of SOCS1. Because activation and transllocation of NFAT into the nucleus is strictly calcium dependent, this strongly supports our findings of a calcium-independent SOCS1 induction.

Furthermore, we show for the first time, to our knowledge, a role for Pyk2 in induction of SOCS1, and this again occurred independent of calcium signaling (Figs. 4, 5). So far, only little is known about the Pyk2 tyrosine kinase in immune cells. Thus, different activation patterns of Pyk2, calcium dependent (25) and independent (as concluded from our experiments) are possible. Pyk2 plays an important role in cell morphology, motility, and adhesion in a variety of cells including macrophages (24, 42). Its role in inflammation is only beginning to be explored. A calcium-dependent pathway has been suggested to result in the activation of NFAT and subsequent production of inflammatory cytokines, that is, IL-10 (23, 25). In this study, we confirm that Pyk2 is activated on Dectin-1 signaling, yet show that Pyk2-mediated induction of SOCS1 occurs through ERK in a manner that is independent of calcium signaling pathways.

Thus, our findings suggest the following SOCS1 induction pathway: Engagement of Dectin-1 via dZ leads to dimerization of Dectin-1 monomers and subsequent cross-phosphorylation of intracellular ITAM-like motifs. Src kinases are recruited and activated, and thereupon phosphorylate spleen tyrosine kinases (Syk). This is followed by phosphorylation and activation of Pyk2. MAPK ERK is stimulated and upregulates SOCS1. As shown in Fig. 4, the missing activation of ERK on Pyk2 inhibition by AG17 locates ERK downstream of Pyk2. Consequently, SOCS1 induction was lost as also observed for direct ERK inhibition. Confirming these observations, MAPKs have been shown to trigger SOCS transcription (2, 8, 43), although ERK specifically had not been analyzed in detail so far. The exact mechanism by which Pyk2 regulates ERK and subsequent SOCS1 expression is still unclear, but previous work has shown that in other cell types, Syk and Pyk2 can activate ERK via either the Ras–Raf pathway or the MAPKKK Tpl2 (44, 45). As shown in Fig. 4E, the Src inhibitor PP1 also abolished phosphorylation of p38. The downstream signaling of Dectin-1 additionally comprises the MAPK kinase kinase Raf 1 (30) that is activated via the Src kinase as well. The application of an inhibitor directed against Raf-1 largely suppressed phosphorylation of p38 and JNKII, but not of ERK, thus ruling out a contribution of Raf-1 in ERK activation (data not shown).

Concerning the role of SOCS1 in Dectin-1 signaling, we were not able to find evidence for an inhibitory role of SOCS1 as feedback inhibitor (Supplemental Fig. 3). Such a mode of action was suggested for SOCS1 regulation on ligation of the CLR DC-SIGN (40). To avoid lethality of SOCS1−/− mice, we used SOCS1−/−/IFN-γ−/− BMMs to analyze a possible inhibitory potential of SOCS1 for Zymosan/Dectin-1 signaling. During an infection, fungi probably will stimulate TLRs (TLR2, TLR6) in combination with C-type lectins (29, 46, 47). Therefore, we speculated that SOCS1 might be important for cross-regulation of different receptors. In this study, we show that dZ modulated cells that were triggered by CpG-DNA. This manipulation was dependent on SOCS1. In cells from WT mice, we observed an inhibition of IL-12 secretion in a costimulatory setting with TLR9. Similar results were obtained in IFN-γ−/− BMMs. However, in SOCS1−/−/IFN-γ−/− cells, this inhibition was abrogated; secretion of IL-12 was even enhanced compared with CpG-DNA alone (Fig. 6A). In cells of WT mice, SOCS1 induction could clearly be enhanced when cells were cotreated with dZ and CpG-DNA (Fig. 6D). From these results, we conclude that the inhibition of CpG-DNA–induced IL-12 by Zymosan was due to regulation of SOCS1. In contrast, secretion of other cytokines like TNF-α, IL-8, or IL-6 was not modified in SOCS1−/−/IFN-γ−/− cells. Similarly, ligation of Dectin-1 and TLR9 resulted in an upregulation of IL-10 (Fig. 6C). Knockout of SOCS1 did further increase secretion of this cytokine as compared with control cells. Thus, SOCS1 shapes the overall reaction pattern in situations of parallel triggering of TLRs and CLRs.

The opposing regulation of TNF and IL-12, IL-10 might be caused by the discriminative characteristics of the binding of NF-κB to its promoter. Delayed-type genes, that is, IL-12 and IL-10, which need prolonged binding of NF-κB to the promoter, could be inhibited by SOCS1, whereas others might be unaffected (18). It is shown that in macrophages, transcription of IL-12 and IL-10 can be promoted by p50/p50 homodimers on stimulation (48, 49). In addition, Dectin-1 is the only known CLR to induce the non-canonical NF-κB pathway, which is primarily mediated by processed p52 (30, 50). Thus, we analyzed SOCS1−/−/IFN-γ−/− and IFN-γ−/− BMMs for activation of NF-κB subunits. Indeed, activation of p50 and p52 was significantly upregulated in SOCS1−/−/IFN-γ−/− cells costimulated with dZ and CpG-DNA (Fig. 6F, 6G). Therefore, SOCS1 supposedly blocks activation of NF-κB p50 and p52 subunits when triggered via TLR9 and Dectin-1. Because it has been published that SOCS1 induces degradation of bound p65 (32), we suggest that also other subunits (p50, p52) could be possible targets for SOCS1-induced ubiquitination and degradation. NF-κB dimers are maintained cytoplasmatically by their interaction with inhibitory IκB proteins, such as IκBα (51). In TLR9/Dectin-1 costimulated SOCS1−/−/IFN-γ−/− cells, an increase in IκBα phosphorylation was observed.

dZ alone led to a subtle phosphorylation of IκBα (Fig. 6E) when macrophages on IFN-γ knockout background were used as opposed to WT macrophages (Fig. 3B). This might reflect subtle changes in the differentiation status of the cells, and indeed, missing NF-κB activation in macrophages by Zymosan has been discussed controversially (22, 52). However, dZ alone still was not able to induce IL-12p40 secretion, and thus a functional NF-κB response in SOCS1−/−/IFN-γ−/− cells.

PPR-activated DCs will prime T cell responses. Recently, the subset of Th17 cells has been implicated in immunity against fungi and some bacteria (33, 34). As we show that the overall reaction pattern in TLR9/Dectin-1 costimulated cells is regulated by SOCS1, we speculated that SOCS1 might also affect subsequent T cell priming capacity. We show that BMMs triggered with dZ or costimulated via Dectin-1 and TLR9 induced IL-17–producing Th cells only in the presence of SOCS1 (Fig. 7D). In line with this result, control IFN-γ−/− BMMs (APCs) will not produce IFN-γ.
themselves and T cells that were WT) produced TGF-β, IL-6, and IL-23, whereas in SOCS1−/−/IFN-γ−/− cells, the expression of these cytokines was abrogated (Fig. 7). Thus, we conclude that SOCS1 plays an essential role in shifting T cell responses toward IL-17–producing Th cells. Thus, SOCS1 seems to be involved in the protection of the host organism against invading fungal pathogens (53). In conjunction with our observations, it could be shown in vivo that Dectin-1 signaling in *Aspergillus fumigatus*-infected mice enhanced Th17 responses (34). In addition, an increased Th17 response was detected on infection of mice with Candida albicans (54).

Taken together, our findings demonstrate that SOCS1 is induced in BMMs to the induction of IL-17–producing Th cells represents an of adaptive immunity. The contribution of SOCS1 expressed in vivo that Dectin-1 signaling in cytokines was abrogated (Fig. 7). Thus, we conclude that SOCS1 but cross-regulated TLR signaling (TLR9) by inhibiting IL-12 taken together, our findings demonstrate that SOCS1 is induced signaling: more than just antiviral. The Journal of Immunology 5653

Acknowledgments

We appreciate the excellent technical help of Konrad A. Bode (University of Heidelberg, Heidelberg, Germany), Ann-Katrin Reuschl (University of Stockholm, Stockholm, Sweden), and Barbara Roieder (University of Mainz, Mainz, Germany).

Disclosures

The authors have no financial conflicts of interest.

References


Suppl. Fig. 1

Time dependent expression of SOCS1 and Kinetic of ERK activation in BMMs of MyD88/Trif−/− mice

(A) and (B), 5x10^5 BMDCs (A) or 3x10^5 BMMs (B) from WT mice were stimulated with increasing concentrations of dZ (10 μg/ml, 100 μg/ml, 500 μg/ml) or 100 ng/ml LPS as control. Cells were lysed after the indicated time-points and gene expression of SOCS1 was examined by means of quantitative RT-PCR. Displayed are expression levels normalized to β-actin. n=3 (mean + SD). Experiments were performed in duplicate wells. (C), (3x10^6) from WT or Myd88/TRIF−/− mice were incubated with LPS (100 ng/ml) or dZ (500 μg/ml) for the indicated time-points. Lysates were analyzed by immunoblot. Blots were probed with antibodies specific for ERK 1/2 phosphorylated on Thr202/Tyr204 or β-actin as control. Data are representative for two independent experiments.

dZ, depleted Zymosan; rE, relative Expression.
Suppl. Fig. 2

Expression of SOCS1 in IFNAR−/− mice and in BMMs treated with MAPK inhibitors. Cytokine secretion of cycloheximid treated cells

(A), (B), BMDCs (5x10⁵) or BMMs (3x10⁵) from WT or IFNAR−/− mice were stimulated as indicated with dZ (100 μg/ml) or LPS (100 ng/ml). After 4.5 h, cells were lysed and gene expression of SOCS1 (A) or IRF7 (B) as knockout control was analyzed by quantitative RT-PCR. Relative expression levels normalized to β-actin are shown and results were performed in duplicate wells. n=2; mean ± SD; (C) and (D), BMDCs (2x10⁵) or BMMs (2x10⁵) were pretreated for 1h with 10 μM or 50 μM Cycloheximid. Stimulation was performed with 100 ng/ml LPS for 16h. IL12p40 and TNFα secretion in cell supernatants was determined by means of ELISA. none; without inhibitor; mock, unstimulated control. Experiments were carried out in duplicates. n=2, mean ± SD; (E), Cells (18x10⁶ BMMs) were treated with 10 μM of JNKIIi, SB203580 or UO126 prior to stimulation with 500 μg/ml dZ. Cells were stimulated for another 6h and proteins were measured in the lysates by immunoblot. Blots were probed with Abs specific for SOCS1 or β-actin. Results show representative data for 2 independent experiments. JNKIIi, inhibitor of JNKII; SB203580, p38 inhibitor; UO126, ERK inhibitor.
Suppl. Figure 3

SOCS1 is not a feedback inhibitor of Dectin-1 signalling

(A, B) BMMs (3x10^6) from SOCS1^-/-/IFN^-/^- or IFN^-/+ mice were stimulated for the indicated time periods with dZ (500 μg/ml) or left unstimulated (mock). Cells were lysed and immunoblot was performed with Abs specific for pPyk2, Syk phosphorylated on Tyr519/Tyr520 and pERK1/2. (C, D) BMMS from SOCS1^-/-/IFN^-/^- or IFN^-/+ control mice were stimulated with dZ (100 μg/ml) or CpG-DNA (1 μM). (C) 3x10^5 cells were lysed after 4.5h and analyzed for egr2 expression by quantitative RT-PCR. (D) Supernatants of 2x10^5 cells were analyzed for the secretion of IL10 after 24h by ELISA. Results in (A), (B) and (C) show representative data of two independent experiments and results in (D) are representative for three independent experiments (mean + SD). Results in (C, D) were performed in duplicate wells.
Suppl. Fig. 4

Quantitative analysis of western blots from main Fig.4 and Fig. 5 as bar graphs

(A)-(H), data from Figure 4 and 5 were quantified by densitometry and are depicted as percentage of unstimulated (mock) control. (A) and (B), quantitative analysis of pPyk2 immunoblot from cells pre-treated with inhibitors directed against Syk (Syki) and Src (PP1) kinases, (C), analysis of pERK, pp38 and pJNKII immunoblots from cells pre-treated with BAPTA, Ag17 or KN93, (D), quantitative analysis of SOCS1 immunoblot from cells pre-treated as in (C), (E), SOCS1 immunoblot from cells pre-treated with Syki or PP1, (F) analysis of pERK, pp38 and pJNKII western blot from cells that were pre-treated as in (E), (G) and (H), quantification of immunoblots from cells treated with siRNA against Pyk2 (siPyk2) or control siRNA (siCon). n= 4 (mean + SD). Knockdown efficiency is depicted in (G). (H), quantification of immunoblots probed with antibodies against pERK or SOCS1. n = 3 (mean + SD).