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Suppressor of cytokine signalling (SOCS) 1 and 3 enhance cell adhesion and inhibit migration towards the chemokine eotaxin/CCL11

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ABSTRACT

Suppressors of cytokine signalling (SOCS) proteins regulate signal transduction, but their role in responses to chemokines remains poorly understood. We report that cells expressing SOCS1 and 3 exhibit enhanced adhesion and reduced migration towards the chemokine CCL11. Focal adhesion kinase (FAK) and the GTPase RhoA, control cell adhesion and migration and we show the presence of SOCS1 or 3 regulates expression and tyrosine phosphorylation of FAK, while also enhancing activation of RhoA. Our novel findings suggest that SOCS1 and 3 may control chemotaxis and adhesion by significantly enhancing both FAK and RhoA activity, thus localizing immune cells to the site of allergic inflammation.

1. Introduction

Extravasation of leukocytes from the blood vessels into tissue is fundamental for the development of an effective allergic inflammatory response. Chemokines are a family of secreted proteins that regulate migration of leukocytes and mediate their biological effect through G-protein-coupled receptors on the surface of their target cells. Chemokines are also responsible for abnormal, excessive leukocyte recruitment during allergic inflammatory disease, such as asthma and contact dermatitis, which has increased attempts to therapeutically manipulate their effects [1].

CCL11 is a chemokine originally identified as an eosinophil chemoattractant, but now known to be chemotactic for many cells that express its receptor, CCR3, including macrophages and neutrophils [2,3]. CCL11 signals via activation of signalling proteins, including mitogen-activated protein kinases, extracellular signal-regulated kinases and protein kinase C [4] and we have shown that CCL11 uses suppressors of cytokine signalling (SOCS) induction to block granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin (IL)-4 signalling and thus regulate dendritic cell differentiation [5]. However, the specific regulation of CCL11 intracellular signalling or migration has not been investigated.

SOCS are a family of 8 proteins (cytokine-inducible SH2-containing protein and SOCS1-7) that act in a negative feedback loop, inhibiting responses to cytokines and a number of microbial products, including Interferon (IFN)-γ, IL-6 and lipopolysaccharide [6]. SOCS were first discovered as inhibitors of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway [7,8]; however they are increasingly thought to control other pathways, such as the focal adhesion kinase (FAK) signalling pathway [9–11]. FAK is a tyrosine kinase, crucial in signalling from integrins and receptor tyrosine kinases during migration and adhesion. FAK is recruited to sites of integrin clustering, which induces tyrosine-397 phosphorylation and creates a binding site for Src kinases leading to Rho GTPase activation. Rho GTPases are plasma-membrane bound G-proteins that control the formation and disassembly of the actin

Abbreviations: SOCS, suppressors of cytokine signalling; FAK, focal adhesion kinase; GM-CSF, granulocyte macrophage-colony stimulating factor; IL, interleukin; IFN, interferon; JAK, Janus kinase; STAT, signal transducer and activator of transcription; GEF, guanine nucleotide exchange factors; GAP, GTPase-activating proteins; MEF, murine embryonic fibroblast; HEK, human embryonic kidney; DMEM, Dulbecco's Modified Eagle Media; FCS, fetal calf serum; BMDM, bone marrow-derived macrophages; WT, wild-type; RT, room temperature

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cytoskeleton and thus direct cell mobility. GTPases cycle between an inactive (GDP-bound) and active (GTP-bound) state and their activity is positively regulated by guanine nucleotide exchange factors (GEFs) and negatively regulated by GTPase-activating proteins. FAK enhances RhoA activity via associations with GEFs and thus controls actin and microtubule polarization [12]. While SOCS1 and 3 have been shown to associate with FAK and inhibit its kinase activity, their effects on Rho GTPases are not known.

This study demonstrates that SOCS1 and 3 strongly enhance cell adhesion and inhibit migration towards CCL11. The presence of these SOCS enhanced both FAK expression and phosphorylation, while also increasing activity of RhoA, which may be responsible for the rise in adhesion and inhibition of chemotaxis. Our observations directly implicate SOCS1 and 3 in the regulation of FAK and RhoA-mediated cell mobility, which is crucial in understanding inflammatory-mediated migration.

2. Materials and methods

2.1. Cell culture

Murine embryonic fibroblasts (MEFs) and human embryonic kidney (HEK) 293T cells were grown in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% fetal calf serum, 1% L-glutamine and 1% pen/strep. Cells were cultured at 37 °C in 5% CO_2 and 95% humidity. SOCS-$1^{-/-}$ MEFs were retro-virally infected with SOCS1 pMX-IRES-EGFP and $SOCS3^{-/-}$ MEFs with SOCS3 pMX-IRES-EGFP as described previously [13]. Cells were treated with 100 ng/ml human CCL11 (Peprotech, Rocky Hill, NJ). Bone marrow-derived macrophages (BMDMs) were prepared from C57/BL6 wild-type (WT) and LysM+/cre SOCS3 fl/fl mice and cultured for 1 week in DMEM, supplemented as above. Proliferation was driven by GM-CSF derived from L929 cell supernatant.

2.2. Migration assay

Cell migration towards CCL11 was assessed using Boyden chambers with transwell membranes over 12 h (6.0-mm diameter, 8-um pore size [Receptor Technologies]) as previously described [14]. 293T migration was measured as cell count from confocal images. MEF and BMDM migration was measured by fixing the cells for 10 min in methanol and staining with crystal violet solution (0.05% crystal violet and 25% methanol) for 20 min. Membranes were destained for 20 min with destaining solution (1:1, ethanol:0.2 M sodium citrate) and optical density was measured at A_{570}.

2.3. Immunoblot analysis

Protein expression was assessed from cells lysed in Brij lysis buffer (0.05 M Tris–HCl pH7.4, 0.15 M NaCl, 1×10^{-3} M EDTA pH 8, 1% Brij 97) supplemented with aprotinin (5 μg/ml), leupeptin (5 μg/ml), phenylmethylsulfonyl fluoride (1 mM) and Na_3VO_4 (1 mM). Extracts were pelleted at 12 000×*g*, 4 °C for 10 min. Lysates were analysed by poly-acrylamide gel electrophoresis. SOCS1 (Invitrogen), SOCS3 (Santa Cruz Biotechnologies, CA), pY397FAK and FAK (Biosource), β1 integrin (Cell signal), and γ-tubulin (Sigma) were detected using the appropriate antibodies.

2.4. Confocal imaging

Cells were seeded and transfected on LabTek II, CC2 treated chamber slides. Cells were fixed using 4% paraformaldehyde in 1XPBS for 20 min. The cells were then permeabilised in 0.5% Triton X-100 in 1XPBS for 5 min, washed in 1XPBS and blocked in blocking solution (1% BSA, 10% Donkey Serum in 1XPBS) for 1 h at room temperature (RT). Cells were stained with pY397FAK or pY925FAK (Cell Signal) and Alexafluor 488 anti-rabbit secondary antibody (Molecular Probes), Talin (Sigma) and Alexafluor 568 anti-mouse secondary antibody (Molecular Probes) and also Phalloidin-FITC (Sigma) for F-actin. The slides were sealed with a cover slip and anti-fade mountant (Invitrogen) and analysed using a confocal microscope and LAS AF software (Leica).

2.5. Adhesion assay

96-well plates were coated with fibrinogen (20 g/ml) for 1 h at RT before 100 μL of cells (1×10^5/ml) were added for 0–60 min. Adhered cells were stained with crystal violet solution (as above) for 20 min, before being washed with 1XPBS and destained for 20 min in destaining solution (as above) and the optical density was measured at A_{570}.

2.6. GTPase activation assays

RhoA activation was measured by GTP pull down assay as described previously [15]. Cells were washed with 1XPBS and lysed in lysis buffer (50 mM Tris pH 7.2, 500 mM NaCl, 1% (*v*/*v*) Triton X-100, 5 mM $MgCl_2$, 1 mM DTT) containing protease inhibitors (as above). Lysates were incubated at 4 °C for 1 h with GST–Rhotekin fusion protein coupled to glutathione-sepharose beads (Amersham Biosciences). After incubation, beads were centrifuged at 8 000×*g* and washed in lysis buffer. Beads were boiled in Laemmli buffer. Active GTPases were detected by immunoblotting with anti-RhoA antibody (Santa Cruz) and lysate was analysed to quantify total Rho.

2.7. Statistical analysis

Migration and adhesion data were represented as bar graphs, created from the raw data in microsoft excel. Groups were then compared using Student's *t*-test for statistical significance ($^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.01$ and $^{***}P \leqslant 0.001$).

3. Results

3.1. SOCS1 and 3 expression inhibit chemotaxis towards CCL11

SOCS1 and 3 have been linked to a block in fibronectin-mediated mobility [10,11], but their function in chemokine-induced migration remains unexplored. Therefore, we analysed the effect of SOCS1, 2 and 3 on chemotaxis towards CCL11. CCR3 and SOCS1, 2 or 3 were transfected into HEK293T cells, before migration towards CCL11 was measured using a Boyden chamber. CCR3 levels were confirmed by flow cytometry and SOCS expression by immunoblotting (data not shown). SOCS2 had no effect on CCL11-induced migration, but both SOCS1 and 3 significantly reduced cell mobility (Fig. 1A). Having identified SOCS1 and 3 as potent inhibitors of migration, we verified their specific involvement by analysing CCL11-induced migration in WT, SOCS1 and 3 null and expressing MEFs. We found that WT, SOCS1 and 3 null cells migrated towards CCL11 significantly more than SOCS expressing cells (Fig. 1B, C and D). Interestingly, SOCS3 null cells migrated more than SOCS1 nulls. SOCS expression was confirmed by western blotting (Fig. 1E). Migration of WT and SOCS3 null BMDMs towards CCL11 was also investigated. BMDMs lacking SOCS3 showed significantly enhanced migration towards CCL11, compared to cells from WT mice (Fig. 1F). These findings support the hypothesis that both SOCS1 and 3 control chemotactic responses to CCL11 and therefore regulate cell movement during allergic inflammation.

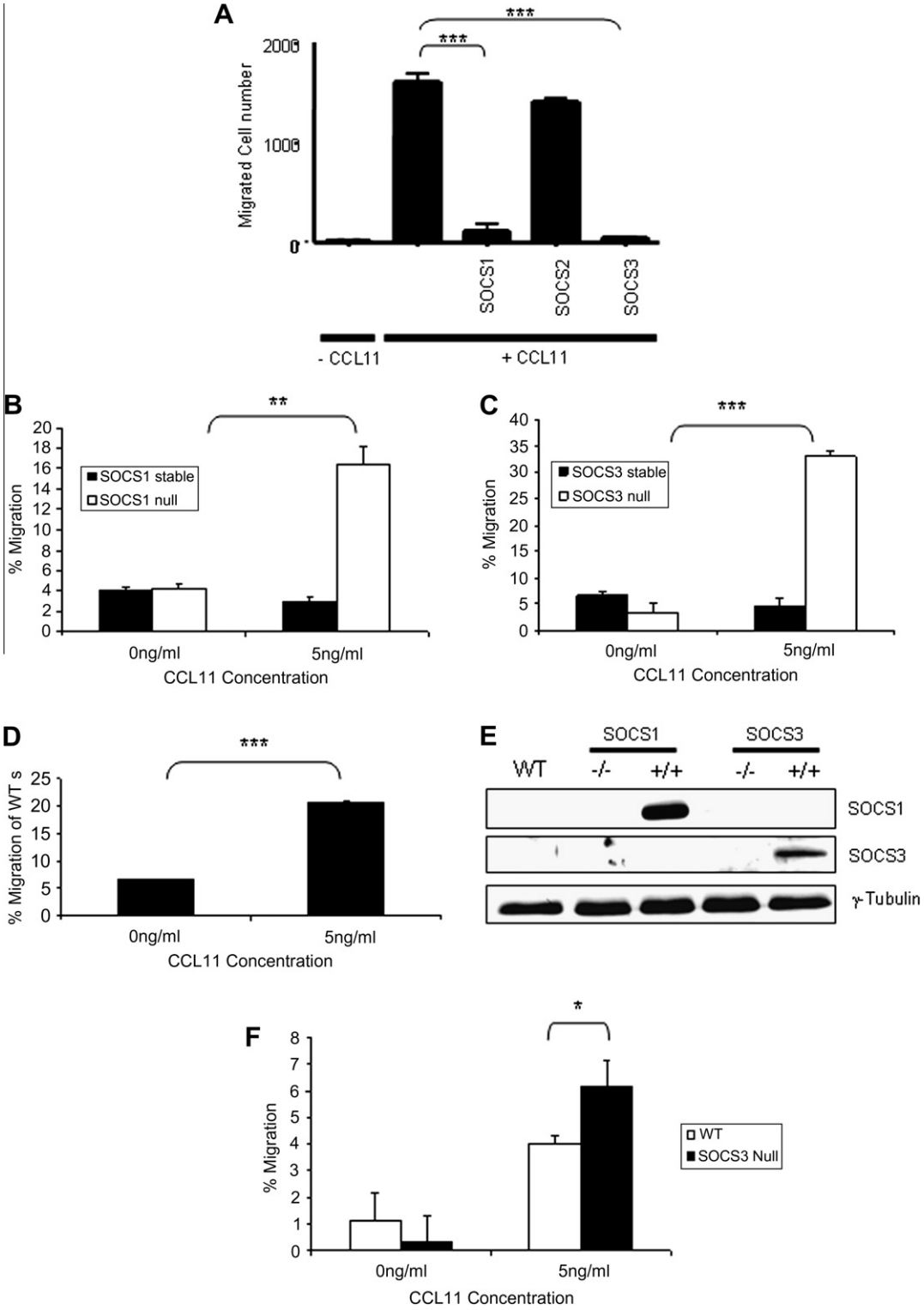

Fig. 1. SOCS1 and 3 regulate migration towards CCL11. HEK293T cells were transfected with 5 μg of CCR3 and SOCS1-3 constructs, before chemotaxis using 100 ng/ml CCL11 (A). Chemotaxis was also analysed using SOCS1 and 3 null and stable and WT MEFs towards CCL11 (5 ng/ml) (B–D). Data are mean ± S.E.M. ($n = 3$). SOCS1 and 3 expression was confirmed by immunoblotting (E) ($n = 3$). Migration of WT and SOCS3 null BMDMs towards CCL11 (5 ng/ml) was measured (F).

3.2. SOCS1 and 3 enhance adhesion

Having discovered that SOCS inhibited CCL11-induced migration of epithelial cells, fibroblasts and macrophages, we investigated the effect of SOCS1 and 3 on cell adhesion. We analysed adhesion to fibrinogen of WT, SOCS null and SOCS expressing MEFs, at 0, 5, 15, 30 and 60 min. We found WT MEFs had maximal adherence at 20 min. SOCS3 expressing cells adhered significantly more than cells lacking SOCS3, with maximal adherence being observed between 30 and 60 min. SOCS1 expressing cells also adhered sig-

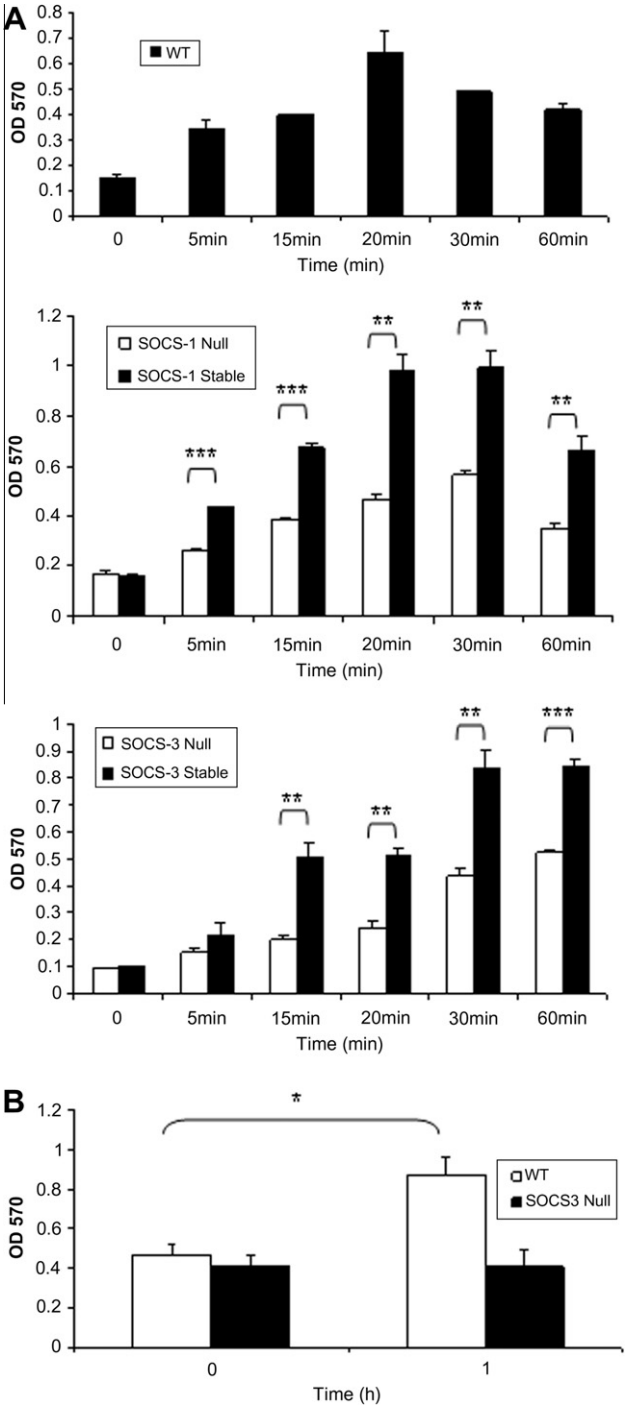

Fig. 2. SOCS1 and 3 enhance cell adhesion. WT, SOCS1 and 3 null and stable MEFs were incubated for 0–60 min on fibrinogen coated 96-well plates. Cells were stained with crystal violet and the optical density of the detaining solution was correlated to cell adhesion (A). Adherence of SOCS3 null BMDMs was also measured for 0 and 60 min (B). Data are means ± S.E.M. (n = 3).

nificantly more than their null counterparts and adherence peaked between 20 and 30 min (Fig. 2A). We also found that WT BMDMs adhered significantly more to fibrinogen than cells without SOCS3, reaffirming SOCS3's positive regulatory role in adherence (Fig. 2B). Together our results indicate that both SOCS1 and 3 reduce CCL11-mediated migration, but enhance adhesion, unveiling a novel mechanism of chemotactic regulation.

3.3. SOCS1 and 3 enhance FAK expression and tyrosine phosphorylation

Since we found SOCS1 and 3 to inhibit CCL11-mediated migration and enhance adherence, we were interested to explore which intracellular proteins were affected. Since FAK activation promotes cell attachment and migration [16], we analysed its expression and phosphorylation, in response to CCL11, in SOCS1 and 3 null and expressing cells. We found that SOCS1 and 3 null cells expressed low endogenous FAK levels and lacked tyrosine-397 phosphorylation (Fig. 3A and B). However, protein and tyrosine phosphorylation were upregulated by CCL11 stimulation (Fig. 3A and B). In contrast, constitutive FAK expression and phosphorylation were enhanced in both SOCS1 and 3 expressing MEFs (Fig. 3A and B). WT MEFs expressed low levels of FAK and low endogenous FAK phosphorylation was enhanced upon CCL11 treatment (Fig. 3C). To further investigate FAK phosphorylation, we analysed pFAK by confocal microscopy in WT, stable and null SOCS1 and 3 MEFs. Confirming the results obtained by immunoblotting, pY397FAK (Fig. 3D) and pY925FAK (Supplementary Fig. 1) had high constitutive expression in SOCS expressing cells, compared to low levels in WT or SOCS null equivalent cells. pFAK also localized at protruding cell surfaces with the adhesion marker protein, talin and the cytoskeletal protein, F-actin, in SOCS1 and 3 expressing cells, indicating enhanced FAK activation in focal adhesion sites (Fig. 3D and Supplementary Fig. 1). As with FAK, basal levels of β1 integrin were lower in WT, SOCS1 and 3 null cells compared to SOCS expressing cells. β1 integrin expression showed a similar pattern of induction to pFAK and was upregulated in SOCS1 null cells by CCL11, but remained low in SOCS3 null cells (Fig. 3E). These results indicate that SOCS1 and 3 enhance endogenous FAK expression and activation at sites of cell adhesion, providing a molecular mechanism for increased adhesion and thus reduced migration.

3.4. SOCS1 and 3 enhance RhoA GTPase activation

GTP-bound RhoA is responsible for actin remodelling and thus essential for adhesion and migration [17]. Therefore, having observed that both SOCS1 and 3 enhanced pFAK expression, we analysed the activation of RhoA by CCL11 in both SOCS1 and 3 null and stable MEFs. We discovered that GTP-bound RhoA is basally high in SOCS1 and 3 expressing cells and CCL11 induced further activation in SOCS3 stable cells. However, basal levels of RhoA were attenuated in the absence of either SOCS (Fig. 3F). These results suggest that SOCS1 and 3 expression leads to RhoA activation, which may increase cell adhesion. Taken together our results suggest that SOCS1 and 3 stabilise FAK protein expression and enhance its tyrosine phosphorylation, while also increasing RhoA activation, which may increase cell adhesion and reduce migration.

4. Discussion

This study reveals a novel role for SOCS1 and 3 in the regulation of FAK and RhoA-mediated cell movement. We have shown that SOCS1 and 3 expression blocks migration towards CCL11, possibly through increased cell adhesion. The observed effects on cell mobility may be regulated by increased levels of activated FAK and RhoA.

Our interest in SOCS-controlled migration was prompted by massive infiltration of T lymphocytes, macrophages and eosinophils into major organs, such as the liver, heart and pancreas observed in SOCS1−/− mice [7,18,19] and granulomatous lesions and chronic infiltrates of lymphocytes, macrophages, eosinophils and neutrophils into the lungs, skin, gut and abdominal organs in SOCS1−/−IFN-γ−/− mice [20]. This phenotype was thought to be

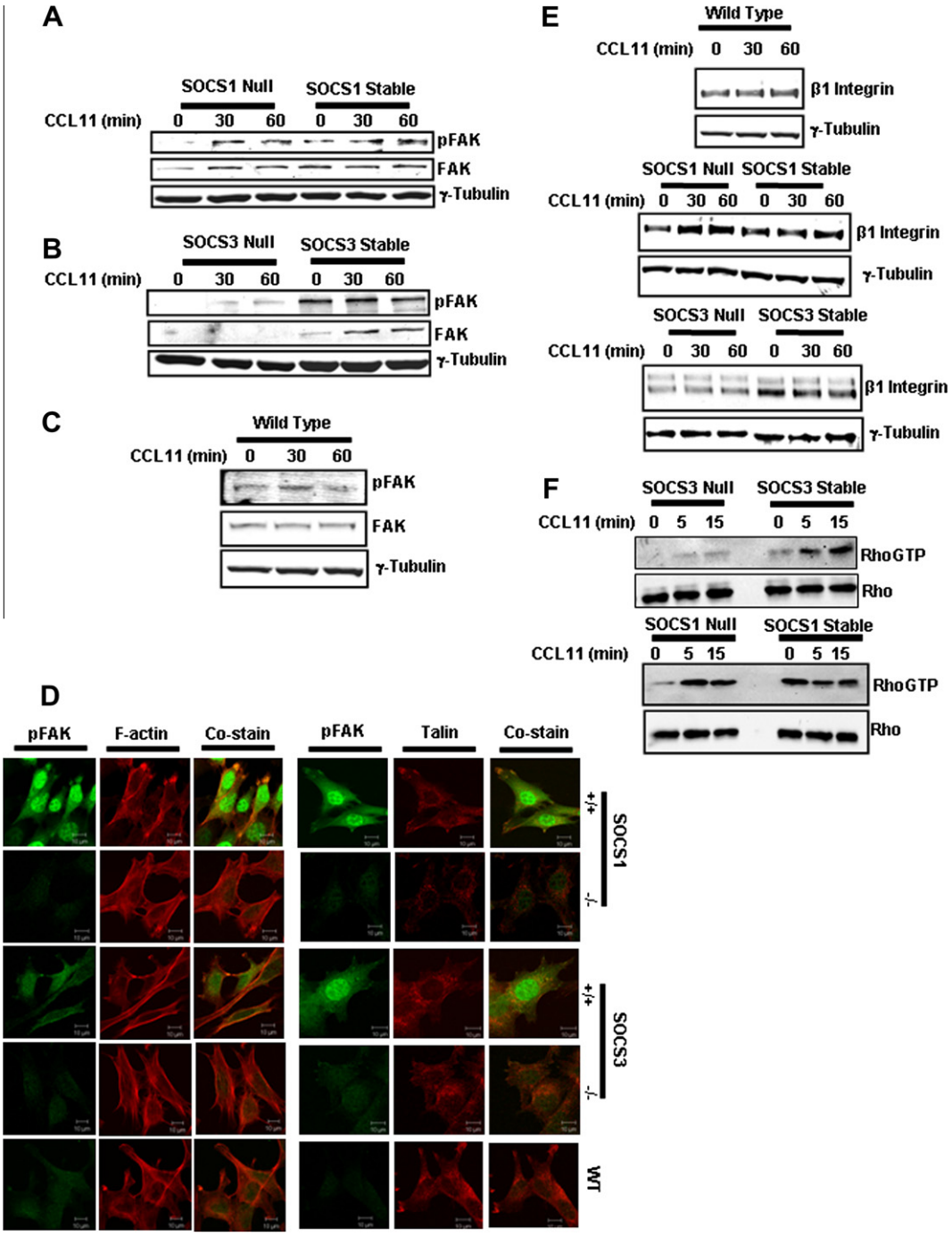

Fig. 3. SOCS1 and 3 regulate FAK activation and expression, β1 integrin expression and RhoA GTPase activation. SOCS1 (A) and 3 (B) null, stable and WT (C) MEFS were stimulated with CCL11 (100 ng/ml) for 0–60 min before lysates were analysed for pFAK, FAK and γ-tubulin by immunoblotting. pFAK (green), talin (red) and F-actin (red) were analysed in WT, SOCS1 and 3 null and stable cells by confocal microscopy (D). β1 Integrin levels were analysed by Western blotting lysates from WT, SOCS1 and 3 null and stable MEFs stimulated for 0, 30 and 60 min with CCL11 (100 ng/ml) (E). SOCS1 and 3 stable and null MEFs were treated with CCL11 (100 ng/ml) for 0, 5 and 15 min and the activation of RhoA assessed by pull down assay using GST-Rhotekin. Proteins extracted by pull down and whole cell lysates were immunoblotted with anti-RhoA antibody (F).

IFN-γ-mediated, however, our study reveals that SOCS proteins control adhesion and migration towards chemoattractants, such as CCL11, and that this may be a fundamental aspect of inflammatory control.

Previous studies have shown SOCS1 and 3 to interact with FAK, promote its degradation and thus reduce migration to fibronectin [10,11,21]. In contrast, we discovered enhanced expression and tyrosine phosphorylation of FAK in the presence of SOCS1 or 3. We went on to find that these cells, with enhanced pFAK expression, had increased cell adhesion which may explain the reduction in chemokine-induced migration. While a common finding is SOCS-mediated reduction in migration, the discrepancy in FAK expression and activation may be cell type or species specific, since previous studies used human hepatoma and monkey kidney cell lines and our investigations were carried out with human kidney cells, murine fibroblasts and macrophages.

The exact role of FAK in cell migration is debated. FAK−/− fibroblasts show reduced motility [22–24], but siRNA knockdown in

HeLa enhances migration on collagen [25] and over-expression of FAK in Chinese hamster ovary cells also enhances cell migration [26]. Therefore, it seems that FAK activity may vary between cells and migratory stimulus. Transient FAK activation is required for IL-8-mediated migration, however, desensitizing levels of IL-8 cause constitutive FAK phosphorylation [27], similar to our findings in SOCS1 and 3 expressing MEFs. We previously demonstrated that IL-8 induces both SOCS1 and 3 expression [28], which explains desensitization to IL-8. Constant FAK phosphorylation renders leukocytes unable to cycle between attachment and detachment, essential for cell movement. Therefore, consistent FAK activation, such as that observed with excess IL-8 or in SOCS1 or 3 expressing MEFs may promote excessive adhesion, resulting in reduced migration.

This study demonstrates for the first time that SOCS1 and 3 enhance FAK and RhoA activation, leading to increased cell adhesion and reduced migration towards CCL11. Our novel findings are important in the context of both normal cell migration and allergic inflammation and unveil new therapeutic targets for controlling excessive or inappropriate inflammatory responses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.10.007.

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