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A critical role for suppressor of cytokine signalling 3 in promoting M1 macrophage activation and function *in vitro* and *in vivo*

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Summary

Macrophages respond to their microenvironment and develop polarized functions critical for orchestrating appropriate inflammatory responses. Classical (M1) activation eliminates pathogens while alternative (M2) activation promotes regulation and repair. M1 macrophage activation is strongly associated with suppressor of cytokine signalling 3 (SOCS3) expression *in vitro*, but the functional consequences of this are unclear and the role of SOCS3 in M1-macrophage polarization *in vivo* remains controversial. To address these questions, we defined the characteristics and function of SOCS3-expressing macrophages *in vivo* and identified potential mechanisms of SOCS3 action. Macrophages infiltrating inflamed glomeruli in a model of acute nephritis show significant up-regulation of SOCS3 that co-localizes with the M1-activation marker, inducible nitric oxide synthase. Numbers of SOCS3^{hi}-expressing, but not SOCS1^{hi}-expressing, macrophages correlate strongly with the severity of renal injury, supporting their inflammatory role *in vivo*. Adoptive transfer of SOCS3-short interfering RNA-silenced macrophages into a peritonitis model demonstrated the importance of SOCS3 in driving production of pro-inflammatory IL-6 and nitric oxide, while curtailing expression of anti-inflammatory IL-10 and SOCS1. SOCS3-induced pro-inflammatory effects were due, at least in part, to its role in controlling activation and nuclear accumulation of nuclear factor-κB and activity of phosphatidylinositol 3-kinase. We show for the first time that SOCS3 also directs the functions of human monocyte-derived macrophages, including efficient M1-induced cytokine production (IL-1β, IL-6, IL-23, IL-12), attenuated signal transducer and activator of transcription 3 activity and ability of antigen-loaded macrophages to drive T-cell responses. Hence, M1-associated SOCS3 was a positive regulator of pro-inflammatory responses in our rodent models and up-regulated SOCS3 is essential for effective M1-macrophage activation and function in human macrophages.

Keywords: glomerulonephritis; inflammation; M1 macrophage; nuclear factor-κB; suppressor of cytokine signalling 3.

Introduction

Macrophages are key cellular components of innate immunity. They detect and respond to pathogenic and tissue-derived signals to clear pathogens, remodel injured tissue and restore tissue homeostasis.[1] Their diverse but polarized functional phenotypes, driven by micro-environmental cues, allow them to adapt readily to changing conditions within tissues.[2] The extent of macrophage heterogeneity and the polarizing stimuli that exist *in vivo* are far from clear but two main functional phenotypes have been well described experimentally.[3,4] Classical or M1

Abbreviations: BMDM, bone-marrow-derived macrophages; iNOS, inducible nitric oxide synthase; NTN, nephrotoxic nephritis; PI3K, phosphatidylinositol-3-kinase; siRNA, short-interfering RNA; SOCS, suppressor of cytokine signalling

activated macrophages, induced by microbial products in the presence or absence of interferon-γ (IFN-γ) are micro-bicidal, tumoricidal and pro-inflammatory. Alternative or M2 activation by IL-4 or IL-13 gives rise to anti-inflammatory tissue-remodelling cells, while immunoregulatory or M2-like macrophages up-regulate IL-10 to dampen immune responses. As many pathologies such as chronic inflammation, fibrosis and cancer are driven by dysregulated macrophage function,[1,3,5] there is growing interest in how macrophage polarization in vivo can be manipulated to alter disease outcome. Identifying the molecular mechanisms that counteract or re-programme inappropriate activation would not only improve understanding of disease but also open up new avenues for more targeted therapies. Recent studies, including our own, have established the potential of suppressor of cytokine signalling (SOCS) proteins to be such targets.[6-9]

SOCS proteins are a family of intracellular cytokine-inducible proteins, consisting of eight members (CIS and SOCS1–SOCS7).[10] They negatively regulate Janus kinase (JAK)/signal transducer and activator of transcription (STAT) induced cytokine signalling through mechanisms including their association with key phosphorylated tyrosine residues on JAK proteins and/or cytokine receptors and degradation of signalling molecules through the ubiquitin–proteasome pathway.[10,11] SOCS1 and SOCS3 are rapidly induced in macrophages and have distinct roles in shaping macrophage activation and controlling immune and inflammatory responses.[6,7] SOCS1 inhibits macrophage responses to IFN-γ and Toll-like receptor (TLR) signalling and SOCS1-deficient mice succumb to T-cell-mediated autoimmune inflammatory disease, characterized by leucocyte infiltration and destruction of vital organs.[12,13] SOCS1, but not SOCS3, expression is strongly up-regulated in an M2 polarizing environment in vitro and in vivo, where it controls the activation phenotype.[7] By contrast, SOCS3 attenuates gp130 signalling, limiting IL-6-induced STAT1 gene expression and inhibiting IL-6-induced STAT3 anti-inflammatory effects.[14-16] In SOCS3$^{-/-}$ macrophages, IL-6 behaves more like the immunosuppressive cytokine IL-10 through prolonged STAT3 activation and suppression of lipopolysaccharide (LPS) signalling.[16] Consequently, mice lacking SOCS3 in myeloid cells are resistant to endotoxic shock with reduced production of pro-inflammatory cytokines.[16] Moreover, studies of rodent SOCS3-deficient macrophages show that SOCS3 positively regulates TLR4 signaling,[6,16-18] despite earlier over-expression studies suggesting negative regulation.[19,20]

We have previously established that macrophages infiltrating inflamed glomeruli in experimental models are rapidly polarized to express either SOCS1 or SOCS3 but rarely both, with most exclusively expressing SOCS3.[6] We have also shown that SOCS3 is necessary to drive an M1-polarized phenotype in vitro supporting a pro-inflamma-

tory role for this protein.[6] However, two recent papers, both using myeloid-specific SOCS3 knockout mice, have shown conflicting results for the role of SOCS3 in M1 macrophage polarization in vivo, proposing that it either promotes[21] or inhibits[22] pro-inflammatory responses. These phenomena and mechanisms therefore need further characterization. Moreover, the relevance of SOCS3 to the inflammatory functions of human macrophages is unknown. The present study was designed to address these questions. Using robust models of inflammation, we show that SOCS3 expression by macrophages within injured tissue co-localizes with M1 macrophage activation markers and that the proportion of these SOCS3-expressing macrophages correlates strongly with the severity of immune-mediated injury. In line with this, we show that SOCS3 controls M1-induced nuclear factor-κB (NF-κB) activity, highlighting a new mechanism by which SOCS3 could promote M1 macrophage activation. The translational relevance of elevated SOCS3 in inflammation is further illustrated by establishing its role in driving the polarization and function of human M1 macrophages.

Materials and methods

Cells and reagents

Rat bone-marrow-derived macrophages (BMDMs) were isolated by aspiration of the femur and tibia and suspended in culture medium comprising Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin with the addition of 20% L929-conditioned medium produced using a standard protocol.[23] This yields a population of > 95% macrophages after 7 days of culture. Blood was donated by consenting healthy volunteers as approved by the Ethics Review Board of the College of Life Science & Medicine, University of Aberdeen. Human macrophages and T cells were prepared from peripheral blood mononuclear cells separated by density gradient centrifugation using a Lymphoprep™ gradient medium (Axis-Shield, Dundee, UK) according to the manufacturer's instructions. CD14$^+$ monocytes and CD3$^+$ T cells were isolated by positive selection from peripheral blood mononuclear cells using antibody conjugated MicroBeads (Miltenyi Biotec, Bisley, Surrey, UK). Monocytes were matured to macrophages by culture for 7 days in Dulbecco's modified Eagle's medium supplemented with penicillin/streptomycin and 10% de-complemented sterile-filtered human AB$^+$ serum. Nephrotoxic serum was produced according to our standard protocol.[23]

Induction of peritonitis and nephrotoxic nephritis

Inbred male, Sprague Dawley rats (200–250 g) were bred under standard conditions and maintained in all cases in

accordance with United Kingdom Home Office regulations. The animals were permitted access to food and water *ad libitum*, and were maintained in a temperature- and humidity-controlled room under a 12 hr day–night cycle. Peritonitis was induced by intraperitoneal injection of 1 ml 8% [weight/volume (wt/vol)] thioglycollate in sterile saline followed 4 days later by intraperitoneal injection of 3 mg/kg LPS from *Escherichia coli* (Sigma Aldrich, Poole, UK) and adoptively transferred macrophages (6×10^6 to 10×10^6 cells per rat). Animals were killed 4 hr after adoptive transfer of cells. Nephrotoxic nephritis was induced by pre-immunization with subcutaneous injection of 1 mg of rabbit IgG (Sigma-Aldrich) in Freund's complete adjuvant and intravenous injection 1 week later with rabbit nephrotoxic serum. This results in macrophage infiltration, acute glomerular injury, and crescent formation. Rats were housed in metabolic cages for 14 hr for urine collection. Urine albumin concentration was determined using rocket electrophoresis[23] and albuminuria was calculated as the total albumin excreted over 24 hr. Sections of renal tissue fixed in methyl Carnoy's fixative were paraffin embedded, and 5-μm sections were cut before routine staining with haematoxylin & eosin.

Gene knockdown

Knockdown experiments were performed using pre-designed and validated short interfering RNA (siRNA) for SOCS3 (rat 192802, human s17191, Invitrogen, Life Technologies, Paisley, UK), SOCS1 (s16469, Invitrogen) or a non-targeting siRNA sequence control (4390844, Invitrogen) containing at least four mismatches to any mouse, human or rat gene. BMDM or human macrophages were transfected using Lipofectamine RNAi Max (Invitrogen) in serum-free medium according to the manufacturer's instructions. Three SOCS3 siRNA sequences were compared for level of knockdown and the one giving most efficient silencing was used in both cases. Mock transfection using Lipofectamine RNAi Max, but no siRNA and no transfection controls, was included. In brief, at day 6 of differentiation, macrophages were plated in antibiotic-free medium and transfected. After overnight incubation, the medium was supplemented with antibiotics and cells incubated at 37° for 48 hr before treatment. SOCS siRNA transfection efficiency was 89 ± 9% as determined by counts for the number of macrophages containing fluorescently tagged siRNA (Invitrogen). Knockdown efficacy was analysed by means of PCR and Western blotting. Cell viability was not altered by transfection reagent alone (mock transfection 98·9 ± 1·2%) or by transfection with siRNA (99·0 ± 0·7%). Moreover, the siRNA transfection procedure used did not stimulate IFN-γ responses in macrophages as assessed by analysis of expression of IFN-γ-responsive genes.

Adoptive cell transfer and conditioning in inflamed peritoneum

Control siRNA or SOCS3 siRNA transfected BMDM were labelled with the fluorescent dyes PKH2 or PKH26L (Sigma Aldrich), injected into the activated peritoneum and conditioned for 4 hr. Peritoneal exudate cells were harvested from the animals by injecting 20 ml sterile PBS through a small incision in the wall of the peritoneal cavity. The abdomen was massaged and the contents were transferred into a sterile polypropylene tube. Adoptively transferred cells that had been conditioned in the inflamed peritoneum were recovered by cell sorting based on positivity in the FL1 channel in a Becton Dickinson BD FACSVantage SE Cytometer System, with FACSDiVa High speed cell sorter (Becton Dickinson, Oxford, UK).

Measurement of cytokine and NO levels and NF-κB specific activation

Cell-sorted, control siRNA-treated or SOCS3 knockdown BMDM recovered from the inflamed peritoneum were plated at 2×10^5 cells in 200 μl per well in a 48-well plate and cultured for 12 hr *ex vivo* for determination of cytokines (IL-6 and IL-10 kits; BD Biosciences, Oxford, UK) by cytometric bead arrays according to the manufacturer's instructions. NO in the culture medium was assessed by nitrite production using a Greiss reaction.[23] In some experiments rat BMDM were pre-cultured in 24-well plates with the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 (20 μM) or with vehicle control (0·15% DMSO) for 40 min before the addition of IFN-γ (20 ng/ml) and LPS (100 ng/ml) for 10 hr to determine IL-6 or NO concentrations as above. Concentrations of IFN-γ, IL-10 and IL-17 in human cell culture supernatants were determined using ELISA-matched antibody pairs (BD Biosciences). Human IL-1β and IL-23 were analysed using ELISA kits (eBiosciences, San Diego, CA). Human IL-6, tumour necrosis factor-α (TNF-α) and IL-12p70 were measured by cytometric bead array (BD Biosciences) according to the manufacturer's instructions. NF-κB-specific activation in control siRNA- and SOCS3 siRNA-treated macrophages was measured using a TransAM ELISA-based kit (Active Motif, Carlsbad, CA) performed on nuclear extracts of BMDM according to the manufacturer's protocol. Nuclear extracts were obtained using a nuclear extraction kit (Active Motif) according to the manufacturer's instructions.

Immunohistochemistry

Total numbers of glomerular CD68+ macrophages were detected in methyl Carnoy's-fixed tissue by ED1 antibodies (AbD Serotec, Kidlington, UK) and the Vectastain

Elite ABC-Kit (Vector laboratories, Peterborough, UK) with appropriate biotinylated secondary antibodies. The peroxidase reaction product was visualized using 3,3′-diaminobenzidine (Sigma-Aldrich) as a chromogenic substrate and H_2O_2 as co-substrate. The numbers of CD68-positive glomerular macrophages that stained for SOCS1 and/or SOCS3 were determined semi-quantitatively by co-localization studies using triple immunostaining in the same methyl Carnoy's fixed kidney sections as follows. After quenching of endogenous peroxidase activity with 3% (vol/vol) H_2O_2 in methanol, tissues were blocked with 1% (wt/vol) horse serum. To analyse the numbers of SOCS1/SOCS3-positive glomerular macrophages, kidney sections were incubated with goat anti-rat SOCS1 (1/200; Abcam, Cambridge, UK) and rabbit anti-rat SOCS3 (1/500; Abcam), followed by the secondary antibodies anti-rabbit TRITC and anti-goat FITC, respectively. The sections were subsequently stained with ED1 antibody, peroxidase-conjugated secondary antibody and 3,3-diaminobenzidine (Sigma-Aldrich) as above. Negative controls without primary antibody confirmed the specificity of the reactions. Images were captured using an Olympus 1 × 70 microscope. At least five glomeruli were imaged per section for SOCS1/SOCS3-positive, CD68-positive macrophages. The basal fluorescence (or chromogenic staining) was obtained, and a threshold was automatically calculated, using sections without primary antibody (negative control). Positive (SOCS1^{hi} or SOCS3^{hi}) macrophages were counted as those with a fluorescence intensity that exceeded the thresholds set for the red and green channels; macrophages exhibiting no more than background fluorescence were considered as SOCS1^{lo} or SOCS3^{lo}, as described previously.[6] The intensity of staining was typically bimodal – those macrophages with strong fluorescence and those with staining indistinguishable from background, as previously illustrated.[6] Captured images were merged to identify SOCS1/SOCS3-positive macrophages and the proportions of SOCS1[hi/lo] SOCS3[hi/lo] macrophages per glomerulus were determined.

Alternatively, to determine the proportions of SOCS1[hi/lo] iNOS[hi/lo] and SOCS3[hi/lo] iNOS[hi/lo] macrophages, sections were quenched and blocked as above and triple stained with ED1 and rabbit anti-rat inducible nitric oxide synthase (iNOS),[24] followed by fluorescently labelled secondary antibodies and then goat anti-rat SOCS3 or SOCS1 (1 : 200; Abcam) with peroxidase-conjugated secondary antibody and 3,3-diaminobenzidine. To minimize inter-assay variation, all samples were processed in the same batch. Images were captured and staining intensity thresholds were set based on negative controls, without application of the primary antibody as above. As with SOCS1/SOCS3-positive macrophages, captured images were merged to identify proportions of SOCS1[hi/lo] iNOS[hi/lo] and SOCS3[hi/lo] iNOS[hi/lo] macrophages and expressed as percentage of positive macrophages per glomerulus.

RNA isolation and RT-PCR

Macrophages recovered from the inflamed peritoneum were pelleted and lysed and total RNA was prepared using the Trizol extraction reagent (Invitrogen) followed by RNA clean-up using an RNeasy mini kit (Qiagen, Manchester, UK) according to the manufacturer's instructions. A total of 5 μg from each sample was reverse transcribed using the first-strand cDNA synthesis kit and oligo(dT) primer as recommended by the manufacturer (Invitrogen) in a 15-μl reaction volume. Relative quantification of the genes of interest was measured as described elsewhere.[7] For each gene, the PCR was performed on 50, 100 and 200 ng of the cDNA using the following primers:

> SOCS1 forward 5′-ATGGTAGCACGTAACCAG-3′
> SOCS1 reverse 5′-CTCCAGCAGCTCGAAGAG-3′;
> SOCS3 forward 5′-ACCAGCGCCACTTCTTCACA-3′
> SOCS3 reverse 5′-GTGGAGCATCATACTGGTCC-3′;
> iNOS forward 5′-TCTTGGTGAAAGCGGTGTT-3′
> iNOS reverse 5′-TGTTGCGTTGGAAGTGTAGC-3′;
> arginase I forward 5′-TATCTGCCAAGGACATCGTG-3′
> arginase I reverse 5′-GTTCTGTTCGGTTTGCTGT-3′;
> IL-10 forward 5′-CTCCACTGCCTTGCTTTTA-3′
> IL-10 reverse 5′-CACTGCTATGTTGCCTGCTC-3′;
> CD206 (mannose receptor) forward 5′-AACAAGAATGGTGGGCAGTC-3′
> CD206 (mannose receptor) reverse 5′-AACTCCTCGTCCGTCTGTC-3′;
> GAPDH forward 5′-TGACATCAAGAAGGTGGTGAAG-3′
> GAPDH reverse 5′-TCTTACTCCTTGGAGGCCATGT-3′.

Western blotting

Protein lysates were prepared from BMDM using lysis buffer and 20 μg separated by SDS–PAGE and transferred to Immobilon P membrane (Millipore, Watford, UK) for Western blot analysis with specific primary antibodies for SOCS3, IκB and p-IκB (Abcam), AKT and p-AKT ser473 (Cell Signaling Technology, Herts, UK), p65 and p-p65 ser536 (New England Biolabs, Herts, UK) and β-actin (Sigma-Aldrich). Immunolabelled proteins were detected by using appropriate horseradish peroxidase-conjugated secondary antibodies, followed by visualization with ECL (GE Healthcare, Buckinghamshire, UK). Bands were normalized to β-actin.

T-cell proliferation assays

Human macrophages (1×10^5/well) were pre-treated with or without LPS or IFN-γ/LPS (20/500 ng/ml) for 7 hr and pulsed with 5 μg/ml of a nominal antigen (keyhole limpet haemocyanin) that elicits T-cell responses indirectly via

macrophages. Macrophages were washed several times and co-cultured with autologous CD4^{+} T cells (ratio 1 : 5). After 8 days, T-cell proliferation was measured by [³H] thymidine incorporation and cytokine secretion by ELISA.

Statistical analysis

Results are presented as mean ± SD and differences between groups of cells or animals were determined by Student's *t*-test or two-way analysis of variance followed by a multiple range test (Tukey analysis). For experiments with human macrophages a Wilcoxon rank sum test was used to compare differences between groups. Correlations between macrophage numbers and albuminuria were assessed by linear regression analysis. Values of $P < 0.05$ were considered significant.

Results

SOCS3 expression in macrophages correlates with an M1 phenotype and severity of nephritis

Most glomerular infiltrating macrophages in nephrotoxic nephritis are strongly polarized to express abundant SOCS3 without detectable SOCS1.[6] There is *in vitro* evidence that such macrophages are pro-inflammatory[6] but direct data from *in vivo* studies are lacking. Accordingly, we double-stained SOCS3-positive macrophages within inflamed glomeruli to determine whether they also expressed the well-established rodent M1 macrophage marker iNOS. Few macrophages were detected in normal glomeruli (0·3 ± 0·2 per glomerulus, mean ± SD) and these expressed little or no SOCS1, SOCS3 or iNOS (Fig. 1a). Induction of nephrotoxic nephritis resulted in a marked increase in glomerular macrophages that were heterogeneous for SOCS expression but with SOCS1^{lo}/SOCS3^{hi} macrophages predominating (Fig. 1b). Around 80% of glomerular macrophages strongly expressed iNOS but this was largely restricted to those that were SOCS3^{hi} (69·6 ± 12·4% total macrophages, 80·3 ± 14·1% SOCS3-expressing macrophages) supporting their proposed pro-inflammatory nature (Fig. 1c,d). In contrast, the majority of SOCS1^{hi}-expressing macrophages (72 ± 6·9%) did not co-express iNOS (Fig. 1e,f), which is consistent with a less inflammatory role.

Given the strong co-expression of SOCS3 and the M1 macrophage marker iNOS, we next determined if the number of SOCS3-expressing macrophages within an
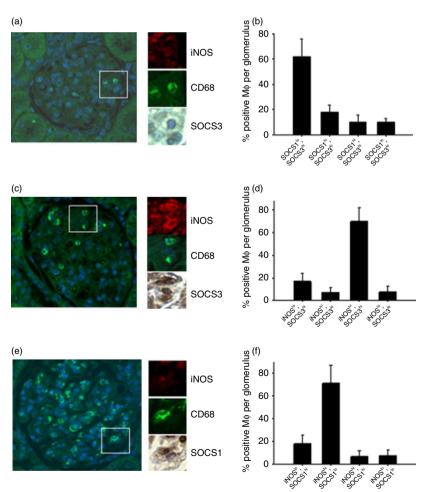

Figure 1. The majority of suppressor of cytokine signalling 3-high (SOCS3^{hi}) expressing macrophages strongly co-expressed the M1 marker inducible nitric oxide synthase (iNOS). Triple immunostaining of a representative glomerulus from a non-diseased (a) and diseased (c, e) animal after induction of nephrotoxic nephritis. The number of CD68-positive macrophages (green fluorescence) that were SOCS3-positive (brown chromogenic stain) and iNOS-positive (red fluorescence) (a, c), or SOCS1-positive (brown chromogenic stain) and iNOS-positive (red fluorescence) (e) are shown. Original magnification × 200. The mean ± SD percentage of macrophages expressing, SOCS1 and SOCS3 (b); iNOS and SOCS3 (d); or iNOS and SOCS1 (f) in glomeruli of rat kidney 3 days after induction of nephrotoxic nephritis. The mean number of total macrophages per nephritic glomerulus was 18·6 ± 8·2; $n = 8$ rats, at least five glomeruli per animal were analysed.

inflamed environment was significantly associated with the severity of inflammation. Injury in nephrotoxic nephritis is macrophage-dependent and the intensity of the macrophage infiltrate correlates with albuminuria,[25] as we show again here ($r^2 = 0.385$, $P = 0.010$; Fig. 2a). Importantly, this correlation was attributable to the numbers of infiltrating SOCS3^{hi}/SOCS1^{lo} macrophages, which correlated more strongly than total macrophages ($r^2 = 0.509$, $P = 0.001$; Fig. 2b), whereas the correlation

with SOCS3^{lo}/SOCS1^{hi} was not significant ($P = 0.120$; Fig. 2c). Collectively, the data demonstrate that despite the heterogeneity of glomerular infiltrating macrophages, SOCS3 expression identifies M1 activated cells whose numbers correlate with levels of albuminuria, which reflects the extent of damage to the glomerular filtration barrier.

SOCS3 is essential for effective polarization of pro-inflammatory macrophages *in vivo*

Using myeloid-specific knockout mice, SOCS3 has been shown to either positively or negatively control M1 macrophage polarization *in vivo*.[21,22] To address these discrepancies and further define the importance of SOCS3 in directing M1 activation *in vivo*, we used SOCS3 siRNA-silenced macrophages[6] that were activated within an inflamed rat peritoneum. The use of knockdown techniques rather than gene-deleted macrophages avoided the risk of compensatory effects in mice lacking SOCS3 and modelled the effects of more subtle modulation of levels rather than complete ablation.

Fluorescently labelled BMDM, transfected with SOCS3 or control siRNA, were injected into an inflamed rat peritoneum, recovered after 4 hr conditioning, purified by cell sorting and analysed for mRNA expression or secretion of inflammatory mediators. No significant difference between kinetics of control siRNA- and SOCS3 siRNA-transfected macrophages was observed in terms of the numbers of injected cells remaining within the rat peritoneum after transfer (control siRNA-transfected macrophages represented $9.8 \pm 4.9\%$ total peritoneal cells; SOCS3 siRNA-transfected macrophages represented $11.2 \pm 8.1\%$ total peritoneal cells after 4 hr). Macrophages conditioned in the M1-activating environment again show strong expression of SOCS3 that was attenuated in SOCS3 siRNA-transfected cells (Fig. 3a). Instead, there was a simultaneous and reciprocal up-regulation of SOCS1 (Fig. 3a) that, in the absence of SOCS3, has been shown to be associated with inhibition of pro-inflammatory responses, M2 activation and reparative macrophages.[7,11] The well-recognized M2 activation marker mannose receptor, as well as arginase I, which is induced by STAT3 and down-regulates NO production in M1 activated cells,[26] were also significantly enhanced in SOCS3 knockdown macrophages activated *in vivo* (Fig. 3a,b), similar to the changes observed with SOCS3 knockdown macrophages conditioned more simply by IFN-γ/LPS *in vitro*.[6] In contrast to *in vitro* experiments, the expression of IL-10 was significantly increased. There was no change in mRNA expression of the M1 activation marker iNOS with SOCS3 knockdown. However, secretion of nitrite as a marker of iNOS activity was significantly decreased by cells conditioned *in vivo* before culture *ex vivo* (Fig. 3c), probably due to an increase in arginase I activity. Furthermore, a
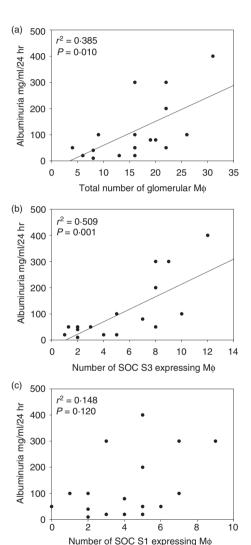

Figure 2. Numbers of glomerular macrophages expressing suppressor of cytokine signalling 3 (SOCS3) correlate strongly with severity of injury in experimental nephritis. Triple immunostaining identified SOCS1 and SOCS3 expressing CD68-positive macrophages. Macrophages exclusively expressing SOCS3 but not SOCS1 show a more significant correlation with levels of albuminuria as a measure of severity of disease ($P = 0.001$) as compared with total macrophages ($P = 0.01$), whereas, the number of macrophages exclusively expressing SOCS1 but not SOCS3 did not correlate with albuminuria ($P = 0.120$) suggesting they do not play a main role in driving disease severity.
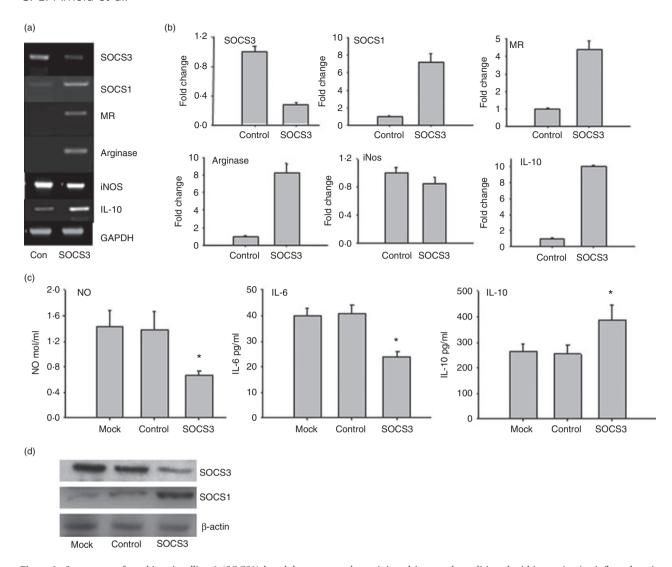
Figure 3. Suppressor of cytokine signalling 3 (SOCS3) knockdown macrophages injected into, and conditioned within, an *in vivo* inflamed environment show decreased pro-inflammatory and enhanced anti-inflammatory characteristics. (a) PCR analysis showing SOCS3 knockdown macrophages (SOCS3) have enhanced mRNA for SOCS1, mannose receptor (MR), arginase I and interleukin-10 (IL-10) compared with control short interfering RNA (siRNA) transfected cells (Con) after conditioning within an inflamed rat peritoneum *in vivo*. (b) Gene expression was quantified using RT-PCR and normalized to the housekeeping gene. The results are shown as mean ± SEM from $n = 6$ rats. (c) Production of macrophage inflammatory mediators in supernatant of mock transfected, control siRNA transfected, or SOCS3 siRNA transfected macrophages isolated from an *in vivo* conditioning environment and cultured for 12 hr before analysis for nitrite (NO), IL-10 and IL-6. Values represent mean concentration ± SD, $n = 6$ animals/group (*$P < 0.05$). # Knockdown of SOCS3 in macrophages conditioned in an inflamed environment for 4 hr show a reciprocal up-regulation of SOCS1 protein when analysed by immunoblotting with SOCS1, SOCS3 and β-actin antibodies.

decrease in IL-6 production and, consistent with mRNA analysis, significantly enhanced IL-10 secretion (Fig. 3c) and up-regulation of SOCS1 at the protein level (Fig. 3d) were observed. Therefore, consistent with our *in vitro* polarization, SOCS3 is essential for effective M1 macrophage polarization *in vivo* and in its absence, macrophages not only have an impaired ability to develop pro-inflammatory properties when they enter an inflammatory environment but instead acquire anti-inflammatory or homeostatic properties and some characteristics of M2 macrophage subtypes (enhanced mannose receptor, IL-10, SOCS1^{hi}/SOCS3^{lo}).

SOCS3 modulates NF-κB signalling in M1 activated macrophages

NF-κB/p65 is central in driving M1 macrophage activation and expression of pro-inflammatory genes[1] therefore the importance of SOCS3 in directing M1 macrophage characteristics through this pathway was determined. As expected, NF-κB/p65 nuclear translocation and DNA binding activity in nuclear extracts was up-regulated on M1 activation in control siRNA-transfected macrophages (Fig. 4a). This up-regulation was, however, significantly less in SOCS3 siRNA-transfected cells, supporting a
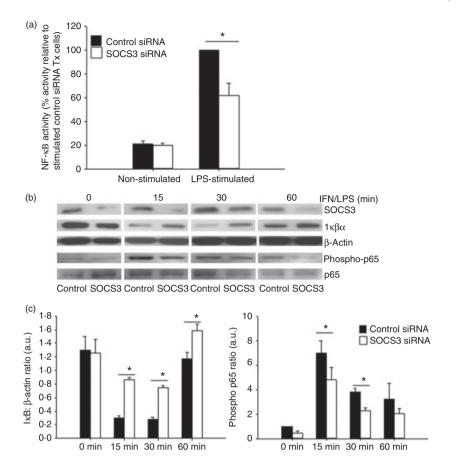
Figure 4. Nuclear factor-κB (NF-κB)/p65 activity is decreased in suppressor of cytokine signalling 3 (SOCS3) knocked down M1 activated macrophages. Bone marrow-derived macrophages (BMDM) were transfected with control short interfering RNA (siRNA) or SOCS3 siRNA. Forty-eight hours following transfection, cells were stimulated without or with lipopolysaccharide (LPS). (a) Nuclear protein extracts were prepared and assessed for NF-κB/p65 activity by a DNA binding ELISA as described in the Materials and methods. Values show mean percentage ±SD of NF-κB/p65 activity in cells transfected with SOCS3 siRNA relative to LPS-stimulated cells transfected with control siRNA; $n = 6$. (b) Immunoblot analysis of SOCS3, IκB and phospho-p65 in lysates of BMDM transfected with control siRNA or SOCS3 siRNA treated with interferon-γ (IFN-γ)/LPS for 0, 15, 30 and 60 min with β-actin as a loading control. SOCS3 knockdown decreased SOCS3 protein expression, decreased the rate of IκB degradation and increased phosphorylation of p65. Figure shows expression of proteins as determined by an immunoblot, representative of three individual experiments (b) and normalized densitometric analysis of scanned bands in arbitary units (a.u.) from three separate immunoblots (c). *$P < 0.05$.

functional role for SOCS3 in NF-κB/p65 responses. NF-κB nuclear activity is controlled upstream by the cytoplasmic inhibitory protein IκBα so we repeated time-series experiments and analysed IκBα degradation in cell lysates by Western blotting. M1 activation resulted in obvious IκBα degradation in control siRNA-treated cells, peaking at 30 min and showing evidence of re-synthesis at 60 min (Fig. 4b,c). However, knockdown of SOCS3 substantially inhibited M1 activation-induced IκBα degradation confirming that SOCS3 knockdown suppresses NF-κB activation upstream of DNA promoter binding. Furthermore, IκB kinase (IKK) typically showed a decrease in activity in the SOCS3 knockdown macrophages after M1 activation as assessed by Western blotting (see Supplementary material, Fig. S1). There was, however, no detectable interaction between SOCS3 and the IκB/p65 complex in macrophages as assessed by immunoprecipitation (data

not included). Phosphorylation of p65 (Ser536), which regulates the strength of transcriptional activity, was also decreased in M1 activated SOCS3 knockdown cells over the time course (Fig. 4b,c). Together, these results show that SOCS3 can positively regulate early NF-κB signalling in M1 activated macrophages.

Effects of SOCS3 knockdown on NF-κB activity is not mediated via enhanced SOCS1

SOCS3 knockdown reciprocally up-regulates SOCS1 (Fig. 3). SOCS1 causes ubiquitination and proteosomal degradation of IL-1 receptor-associated kinase 1, the adaptor protein TIRAP/Mal as well as nuclear p65, to abrogate NF-κB activation.[10,27–29] To determine whether up-regulated SOCS1 was responsible for the SOCS3-silencing mediated effects, SOCS1 and SOCS3 were

knocked down simultaneously and NF-κB/p65-activity was determined. Knockdown of SOCS3 at the protein level was efficient (68 ± 5%) and resulted in the expected reciprocal up-regulation of SOCS1 protein by at least twofold in M1 activated cells (Fig. 5a). Double knock-down silenced SOCS1 or SOCS3 as efficiently as in single knockdown cells but critically, did not restore NF-κB/p65 activity to control levels (Fig. 5b). The decrease in NF-κB-activity with SOCS3 knockdown was therefore not simply due to up-regulated SOCS1 expression.
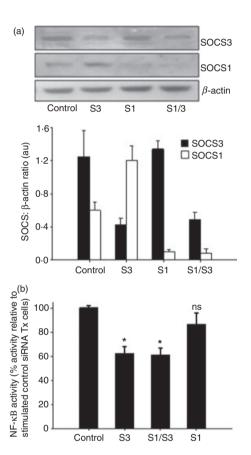

Figure 5. Knocking down suppressor of cytokine signalling 1 (SOCS1) and SOCS3 simultaneously does not restore the SOCS3-mediated decrease in nuclear factor-κB (NF-κB)/p65 activity in M1 activated macrophages. Bone marrow-derived macrophage (BMDM) were transfected with control short interfering RNA (siRNA), SOCS3 siRNA or SOCS1 siRNA (Invitrogen) in a single or combined manner. (a) Forty-eight hours following transfection, cells were incubated with interferon-γ (IFN-γ)/lipopolysaccharide (LPS) for 30 min. SOCS1, SOCS3 and β-actin protein expression was detected by Western blotting showing successful knockdown at the protein level. The intensity of SOCS1 and SOCS3 bands on the blots were determined by densitometry and normalised to β-actin; n = 3. (b) Nuclear NF-κB/p65 DNA binding activity was measured using a TransAM NF-κB kit and demonstrated simultaneous SOCS1&3 knockdown does not restore nuclear NF-κB activity. Data shows the NF-κB/p65 activity in cells transfected with SOCS1 and/or SOCS3 siRNA relative to cells transfected with control siRNA, *P < 0·02, n = 5.

SOCS3 knockdown results in an increase in PI3K (pAKT) activity on M1 activation

Effects of SOCS3 silencing on the down-regulation of TLR4 signalling have been proposed to occur indirectly through STAT3, which can inhibit NF-κB and could account for the decrease in NF-κB activity in our knockdown macrophages.[16] However, the change in NF-κB activity in our SOCS3 silenced macrophages is an early response (30 min post stimulation) and likely to occur before the LPS-induced up-regulation in STAT3 activity that is typically found at least 1 hr after stimulation.[16,22,30] Induction of PI3K-AKT signalling by TLRs is an important early feedback inhibitory mechanism that limits NF-κB activation and inflammatory cytokine production.[31,32] SOCS3 binds phosphorylated receptors and inhibits downstream PI3K-AKT activity in other systems,[33–38] therefore we next determined whether silencing SOCS3 also affected this pathway in M1 activated macrophages. On M1 activation, SOCS3 knockdown significantly increased p-AKT levels (serine 473), a surrogate marker of PI3K activation (Fig. 6a,b) supporting the view that SOCS3 also regulates this pathway in macrophages. To determine whether this provided a potential mechanism by which SOCS3 controls macrophage NF-κB signalling, effects were determined after pre-incubation with the well-known PI3K inhibitor, LY294002. As previously shown, SOCS3 knockdown resulted in a significant decrease in nuclear NF-κB/p65 activity in M1 activated macrophages; LY294002 pre-incubation abrogated this decrease (Fig. 6c). Moreover, pre-incubation with LY294002 prevented the inhibition of nitrite production (34 ± 6%, P < 0·05) by M1 activated SOCS3-silenced cells compared with cells transfected with control siRNA (Fig. 6d). Similar results were observed when IL-6 production was measured (Fig. 6e) indicating that the SOCS3-induced inhibition of PI3K is upstream of its effects on NF-κB activation. Taken together, these results suggest that, as well as modulating STAT3 to control the NF-κB activity, SOCS3 could limit PI3K/AKT activation to ensure effective pro-inflammatory M1 responses.

SOCS3 silencing alters cytokine production and activation of adaptive responses by human M1-activated macrophages

Given the disparities in macrophage activation and functions between species,[37] it was important to address the potential for SOCS3 in driving the polarization of human macrophages, and, critically, functional consequences of SOCS3 silencing in these cells. As with the rodent cells, SOCS3 protein is strongly induced in M1-activated human macrophages (see Supplementary material, Fig. S2a). Moreover, SOCS3 knockdown results in a
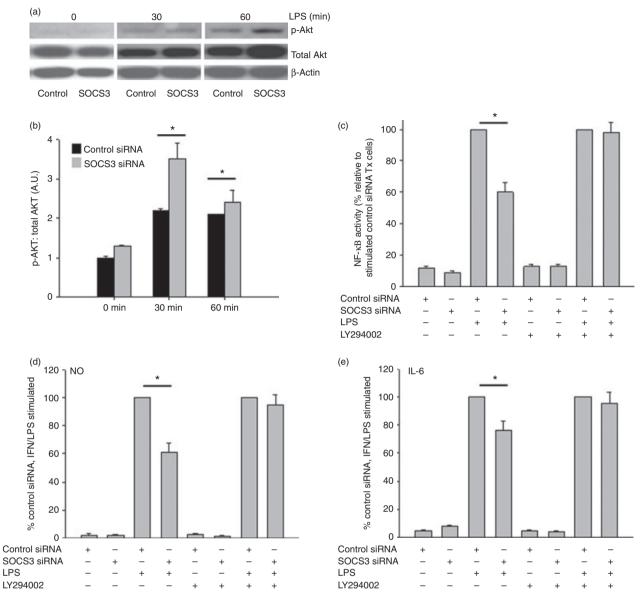
Figure 6. Suppressor of cytokine signalling 3 (SOCS3) knockdown results in an increase in phosphatidylinositol 3-kinase (PI3K) (pAKT) activity on M1 activation and inhibition of PI3K activity reverses the SOCS3 knockdown inhibitory effects on nuclear factor-κB (NF-κB). (a) Bone marrow-derived macrophages (BMDM) were transfected with control short interfering RNA (siRNA) or SOCS3 siRNA. Forty-eight hours following transfection, cells were stimulated without or with interferon-γ (IFN-γ)/lipopolysaccharide (LPS) and PI3K activity assessed by Western blotting for pAKT with loading control β-actin. Figure shows expression of proteins as determined by an immunoblot, representative of three individual experiments. (b) Normalized densitometric analysis of scanned bands in arbitary units (A. U.); $n = 3$; *$P < 0.05$. (c) Incubation of SOCS3 knockdown macrophages for 1 hr with the PI3K inhibitor LY294002 (20 μM) before M1 activation for 30 min abrogated the significant decrease in NF-κB/p65 activity in SOCS3 siRNA-transfected macrophages; DMSO (0.15%) served as a vehicle control. The inhibition of nitrite (d) and IL-6 (e) production in the culture supernatant of M1 activated, SOCS3 silenced cells compared with control siRNA-transfected cells was also prevented after pre-incubation with LY294002 for 40 min before M1 activation for an additional period of 10 hr. As LY294002 can interfere with basal NF-κB activity, values for control siRNA transfected cells with or without LY294002 were set to 100% for (c) to (e). $n = 4$; *$P < 0.05$.

reciprocal up-regulation of SOCS1 and a substantial increase in STAT3 but not STAT1 activity upon LPS stimulation (Fig. S2b–d). Next we defined the effects of SOCS3 knockdown on the inflammatory cytokine profile of LPS and LPS/IFN-γ-stimulated human macrophages by

analysing the supernatants collected from transfected cells. SOCS3 knockdown significantly decreased the secretion levels of macrophage-derived IL-1β, IL-6, IL-12p70 and IL-23 in IFN-γ/LPS-activated SOCS3 knockdown macrophages and IL-6 and IL-12p70 in LPS-activated
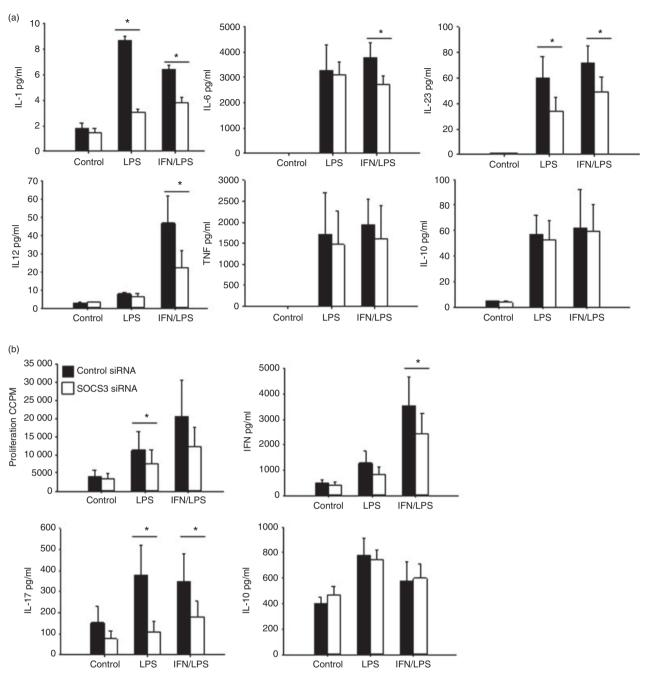
Figure 7. Suppressor of cytokine signalling 3 (SOCS3) knockdown macrophages show distinct cytokine secretion profile following differential activation. (a) Macrophages were transfected with control or SOCS3-specific short interfering RNA (siRNA). Macrophages were differentially activated with 100 ng/ml lipopolysaccharide (LPS) alone or in combination with 20 ng/ml IFN-γ and cells were loaded with nominal antigen (keyhole limpet haemocyanin). Supernatants of macrophage cultures were collected 24 hr after activation and analysed by cytometric bead array [interleukin-6 (IL-6), IL-12, tumour necrosis factor-α (TNF-α), IL-10] and ELISA (IL-1β, IL-23); $n = 6$. (b) Autologous T cells were added to macrophages treated as above and cells were co-cultured for 8 days after which T-cell proliferation ([³H]thymidine incorporation) and T-cell cytokine secretion levels (ELISA) were measured. The plots show the mean ± SEM. Statistical significance was based on Wilcoxon matched-pairs signed rank test. $n = 8$; * $P < 0.05$.

macrophages (Fig. 7a). In contrast, concentrations of M1-induced TNF-α, IL-10 and transforming growth factor β (TGF-β; data not included) were not significantly altered. This demonstrates for the first time that SOCS3 selec-

tively regulates the extent of macrophage pro-inflammatory potential in activated human cells.

M1 macrophages function as antigen-presenting cells and can drive T-cell responses. As SOCS3 knockdown

resulted in a decrease in the T helper type 17 (Th17) polarizing cytokines IL-1β, IL-6, IL-23 and the Th1 polarizing cytokine IL-12 by activated macrophages, we tested if this was functionally important and would alter their ability to induce responses to antigens by autologous T cells. Macrophages transduced with SOCS3 siRNA or control siRNA were loaded with a nominal antigen, (keyhole limpet antigen) and activated with LPS or IFN-γ/LPS to induce T-cell polarizing cytokines. This method avoids the non-specific effects that can result with simultaneous anti-CD3 activation, or synthetic T-cell polarizing conditions using anti-cytokine antibodies. As shown in Fig. 7b, activation in the presence of antigen resulted in a marked increase in T-cell proliferation and T-cell cytokine production. Knockdown of SOCS3 attenuated both the capacity to stimulate T-cell proliferation and the secretion of cytokines in the supernatant of macrophage-activated T-cell cultures. Specifically, there was a decrease in the secretion of IFN-γ and IL-17, important signature cytokines associated with pro-inflammatory Th1 and Th17, respectively, while levels of IL-10 from activated T-cell cultures (associated with Th2 and T regulatory cells) were maintained. Taken together these results show that SOCS3 is also essential in regulating the pro-inflammatory and functional properties of human macrophages and its knockdown results in an overall dampening of innate and downstream adaptive immune responses.

Discussion

We show for the first time that SOCS3 expression identifies a population of macrophages *in vivo* that express M1 markers and correlates specifically with the intensity of tissue injury. The enhanced SOCS3, as well as modulating STAT activity, restrains signalling through the PI3K/Akt pathway, which in turn augments NF-κB activity to drive efficient expression of pro-inflammatory mediators. Consequently, in the absence of SOCS3, macrophages default to a less inflammatory state on entering a pro-inflammatory milieu. These effects are not confined to rodents and we show the translational relevance and functional significance of SOCS3 in driving M1 activation in human monocyte-derived macrophages, where it fine tunes the production of inflammatory cytokines and downstream T helper cell priming, especially that of IL-17-producing effector cells. Hence, our results demonstrate that SOCS3 is not simply a feedback inhibitor of cytokine responses but selectively controls multiple pathways to regulate tightly M1 macrophage activation and tailor an appropriate inflammatory response.

An important aspect of our studies was that the majority of M1 macrophages activated in an *in vivo* pro-inflammatory conditioning environment show strong up-regulation of SOCS3 expression and without this, these macrophages have a reduced ability to develop pro-

inflammatory features. This strongly contrasts with macrophages infiltrating an *in vivo* M2-activating environment, where macrophages with enhanced SOCS1 but not SOCS3 are prominent and SOCS1 is critical in the acquisition of M2 characteristics.[7] Collectively, these studies highlight the heterogeneity of macrophages *in vivo* and the disparate effects of SOCS proteins in controlling effective subset activation. Our up-regulation of SOCS3 by macrophages activated *in vivo* (Fig. 1) and their strong association with tissue injury (Fig. 2) is remarkably similar to the phenotype and role of SOCS3-expressing macrophages induced by IFN-γ/LPS *in vitro*,[6] despite the much more complex conditioning environment. A key difference, however, is the strong induction of IL-10 gene expression and secretion by SOCS3-deficient macrophages activated within the inflamed peritoneum. Interleukin-10 is up-regulated by TLR agonists to prevent overshooting of TLR-mediated pro-inflammatory responses and *in vivo* SOCS3 appears to actively repress this feedback to maintain sufficiently the natural cytotoxicity of M1-polarized macrophages. NF-κB p50/p50 homodimers have been shown to promote LPS-induced IL-10 expression and drive macrophages to an immunosuppressive M2-like phenotype, during resolution of inflammation.[38,39] Whether SOCS3 exerts inhibitory effects on IL-10 expression *in vivo* via inhibition of this mechanism needs to be addressed further.

Initial studies using over-expression systems suggested that SOCS3, like SOCS1, was a negative regulator of TLR4 signalling and synthetically high SOCS3 expression resulted in a less pro-inflammatory phenotype.[19,20] However, more recent studies, including our own, have concluded that SOCS3 positively regulates TLR4 and inflammatory responses.[6,17,18,40] Genetically modified mice, selectively lacking SOCS3 in myeloid cells, are resistant to LPS-induced acute inflammation[16,21] and polymicrobial sepsis.[21] The same mice exhibited resistance to the tumour transplantation model because of reduced tumour-promoting cytokines such as TNF-α and IL-6 and enhanced production of anti-tumorigenic chemokine MCP2/CCL8 with anti-inflammatory effects relating to increased phosphoSTAT3.[41] An opposing study in LysM-Cre-SOCS3 [fl/fl] mice, however, found exacerbated inflammation in response to septic shock and, in spite of elevated STAT3 activity,[22] suggested a role for SOCS3 in limiting M1 activation. The reason for this discrepancy is unclear but may relate to differences in purity and dose of LPS used in each study, the difference in control group mice (wild-type control versus SOCS3 floxed transgenic control) and the genes and time-points analysed after macrophage activation. We clearly show here that SOCS3 knockdown macrophages conditioned in an LPS-activated peritoneum also show a reduced ability to become polarized to an M1 subtype *in vivo*, consistent with an essential role for SOCS3 in driving pro-inflammatory responses in macrophages.

Previously, SOCS has been shown to modulate LPS-induced IL-6-, IL-10- and IFN-γ-dependent STAT pathways,[16–18] possibly accounting for some of the changes in inflammatory properties in knockdown macrophages in our study. SOCS3 has also been shown to promote TLR4 responses in macrophages *in vitro*, by feedback inhibiting TGF-β1/Smad3 signalling that down-regulates LPS-induced cytokine production.[18] Here we propose an alternative and novel mechanism whereby SOCS3 promotes LPS-induced inflammatory responses, through enhancing early NF-κB signalling and subsequently secretion of pro-inflammatory mediators. We show that silencing of SOCS3 resulted in a decrease in M1 macrophage-induced IκB degradation, phospho-p65 levels as well as downstream nuclear p65-NF-κB activity; it has been proposed that SOCS3 exerts positive effects on the NF-κB promoter activity in a RelA-dependent manner.[42] Interestingly, our SOCS3 knockdown macrophages show a strikingly similar phenotype to those transduced with an IκB super repressor that decreases NF-κB nuclear activity, attenuates pro-inflammatory responses, enhances IL-10 and, when adoptively transferred, decreases the severity of experimental nephritis.[25]

Silencing of SOCS3 resulted in a strong up-regulation of human and rodent macrophage SOCS1 that inhibits NF-κB activity at several levels.[10,27,29] Notably, the inhibition of NF-κB signalling in SOCS3 knockdown macrophages was not, however, due to the reciprocally induced up-regulation of SOCS1. Nor is it likely to be solely due to hyper-activation of STAT3, which represses LPS-induced NF-κB activity.[43] In contrast to IL-6, LPS induces STAT3 activity with delayed kinetics[18] (> 1 hr) and in our study the decrease in LPS-induced NF-κB activity in SOCS3-deficient macrophages was observed within 30 min post activation. However, we show an early increase in PI3K-AKT activity in M1-activated SOCS3 knockdown macrophages. This is consistent with the proposed inhibitory role of SOCS3 for PI3K in other systems, where it competes with SH2-containing adaptor molecules that recruit PI3K for activation, or inhibits JAK2 activity and subsequent PI3K phosphorylation.[33–36] PI3K negatively regulates early TLR-induced pro-inflammatory cytokine production, enhances IL-10 and suppresses NF-κB cascades in response to LPS in monocytes.[31,32,44] PI3K-induced AKT activity also inhibits glycogen synthase kinase (GSK)-3β, which facilitates NF-κB activation via the IKKβ-IκBα-p65 signalling pathway.[45] A notable feature of our studies was that inhibition of PI3K reversed the repressive effects of SOCS3 knockdown on NF-κB activity, IL-6 and nitrite production in M1 activated macrophages. This supports the view that up-regulated SOCS3 restricts PI3K to augment NF-κB activity and promote an efficient M1 activation response. Although the exact mechanism by which SOCS3 controls M1-induced NF-κB activity is still unclear, we propose that there is an important contribution from this pathway, in addition to effects on STAT3 and TGF-β1/Smad3 activity.[16,18]

Our results in rodent macrophages proposed SOCS3 as a target molecule to redirect macrophage functions as a potential treatment of inflammatory disease and therefore we translated our findings to human macrophages. Studies on the mechanistic role of SOCS3 in human macrophage activation are limited, but its expression is elevated in several acute inflammatory disorders.[8,9] As with rodent cells, the production of pro-inflammatory cytokines (IL-1β, IL-6, IL-23, IL-12) was significantly decreased by SOCS3 silencing in human monocyte-derived macrophages in our studies but with specificity in their regulation and no significant change in TNF-α levels. This is in keeping with previous studies in human macrophages where SOCS3 over-expression had no effect on LPS-induced TNF-α mRNA levels[46] and demonstrating SOCS3 regulates expression of pro-inflammatory mediators in a gene-specific manner.

A key attribute of macrophages at sites of inflammation is that they can present antigen and secrete cytokines to prime and control the magnitude of T-cell responses.[47] A striking feature of our results is the reduced potential of SOCS3 silenced macrophages to drive T effector cell differentiation, especially that of Th17. This reduced ability may be partially explained by a decrease in expression of co-stimulatory molecules in SOCS3-silenced cells.[6] However, we speculate that the change in cytokine polarizing environment plays a much more significant role. All three Th17 polarizing cytokines (IL-1β, IL-6 and IL-23) show reduced production with SOCS3 knockdown, with IL-1β showing the most significant decrease. In line with this, our unpublished work and that of others show that IL-1β is key to driving Th17 polarization in human macrophages.[48] The addition of these polarizing cytokines to cultures, rather than reliance on their inherent production by macrophages, may explain the apparently paradoxical report that macrophages from LysMCre-SOCS3^{fl/fl} mice exhibited an enhanced Th1 and Th17 polarizing ability.[22] Studies in mouse SOCS3^{−/−} dendritic cells showed a similar reduced potential to drive T effector cell responses and a tolerogenic phenotype and this was due to their enhanced TGF-β production and selective expansion of Foxp3-positive regulatory T cells.[49] However, we found no evidence of enhanced TGF-β production or regulatory T-cell polarization by SOCS3 knockdown macrophages in our study. Taken together, it is clear that the regulation of intracellular signalling pathways by SOCS3 in innate cells is critical for the decision of adaptive responses such as T-cell fates. The depletion of macrophage SOCS3 in a clinical situation would therefore be predicted to dampen both pro-inflammatory innate and adaptive immune responses.

In conclusion, macrophage functions depend on their polarizing microenvironment and controlling their

responses is a key factor for the outcome of both inflammation and autoimmune disease. Our data identify an additional and novel role for SOCS3 in fine-tuning macrophage polarization, via its effects on intracellular signalling pathways including PI3K and NF-κB that direct M1 macrophage function. Therefore modulation of macrophage-specific SOCS3 expression could provide new opportunities for therapeutic manipulation of immune responses.

Acknowledgements

Authorship

CEA designed and performed experiments and analysed data. CSW and PG designed and performed experiments. RNB and AJR analysed data and contributed to manuscript preparation. HMW designed study and experiments, acquired, analysed and interpreted data, and prepared the manuscript.

Disclosures

The authors have no financial conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Phosphorylated IκB kinase (p-IKK) levels are decreased in suppressor of cytokine signalling 3 (SOCS3) knocked down M1 activated macrophages. Macrophages were transfected with control short interfering RNA (siRNA) or SOCS3 siRNA. 48 hr following transfection, cells were stimulated without or with lipopolysaccharide (LPS) for 0, 15 and 30 min and p-IKK and IKK levels were assessed by Western blotting. Densitometric analysis was performed on scanned bands and results presented as the ratio of p-IKK to β-actin in arbitrary units (a.u.). Figure is representative of five individual experiments.

Figure S2. Human monocyte derived macrophages were activated with 100 ng/ml lipopolysaccharide (LPS) for 4 hr or left untreated (non-stimulated). Total macrophage protein was isolated and expression of protein and loading control β-actin detected by Western blot and normalized by densitometric analysis of scanned bands in arbitrary units ($n = 4$). (a) Suppressor of cytokine signalling 3 (SOCS3) expression increases upon LPS stimulation in human monocyte derived macrophages. (b) SOCS3 knockdown (S3) in human monocyte derived macrophages decreases SOCS protein expression as compared with control (con) short interfering RNA (siRNA) transfected cells. (c) Knockdown of SOCS3 (S3) in human monocyte derived macrophages increases phosphorylated signal transducer and activator of transcription 3 (pSTAT3), but not pSTAT1, levels and (d) increases SOCS1 expression after LPS stimulation.