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Regulation of Multiple Cytokine Signalling Pathways by SOCS3 is Independent of SOCS2

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Abstract

Suppressor of cytokine signalling (SOCS) 3 is an essential regulator of cytokine signalling, and in turn its expression is tightly regulated. Data from overexpression studies in cell lines suggest that SOCS2 regulates SOCS3 protein degradation, by forming a molecular bridge to an E3 ubiquitin-ligase complex. Whether this regulation is relevant in primary cells is unknown. In this study, we utilized *Socs2*$^{-/-}$ mice to examine the role of SOCS2 in modulating SOCS3 expression and degradation, and its impact on IL-2 and IL-6 signalling in primary haemopoietic cells. Both biochemical and biological analyses demonstrated unperturbed SOCS3 expression and cytokine signalling in the absence of SOCS2. Our results suggest that SOCS2 is not a physiological regulator of SOCS3 expression and action in primary haemopoietic cells.

Keywords

SOCS2; SOCS3; IL-6; IL-2; STAT; macrophages; lymphocytes

Introduction

Cytokines initiate a spectrum of biological effects through activation of a range of intracellular signalling cascades. JAK-STAT signalling downstream of cytokine receptors is tightly regulated by members of the suppressor of cytokine signalling (SOCS) protein family (Croker et al. 2008; Murray 2007). The inhibitory effects of SOCS proteins on cytokine signalling have been definitively demonstrated through analyses of mice harboring loss of functional alleles. In the absence of SOCS1 or SOCS3, mice exhibited profound pathologies including multiple haemopoietic and immunological defects as a consequence of prolonged STAT activation (Alexander et al. 1999; Cornish et al. 2003; Croker et al. 2003; Croker et al. 2004; Lang et al. 2003; Naka et al. 1998; Starr et al. 1998; Yasukawa et al. 2003).

Mice that lack SOCS3 die at midgestation due to placental defects consequent upon unrestrained LIF signalling (Robb et al. 2005; Roberts et al. 2001). Subsequent studies using conditional alleles have revealed the importance of SOCS3 in haemopoietic cells, with

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absence of SOCS3 leading to inflammatory disorders in young mice and hypersensitivity to IL-6, G-CSF and IL-1 stimulation (Croker et al. 2003; Croker et al. 2004; Wong et al. 2006).

SOCS3 contains an N-terminal region, a central SH2 domain and a C-terminal SOCS box motif (Hilton et al. 1998). Each of these modules contributes to attenuation of the duration and intensity of JAK-STAT signalling. The SH2 domain is responsible for SOCS3 binding to both the phosphotyrosine residues within cytoplasmic domain of the receptor and JAK kinase following cytokine-mediated activation (Dunn et al. 2005; Hortner et al. 2002a; Hortner et al. 2002b; Nicholson et al. 2000; Sasaki et al. 1999). As a consequence, SOCS3 is thought to inhibit signal transduction by directly inhibiting JAK activity through the kinase inhibitory region (KIR) located in the SOCS3 N-terminus (Sasaki et al. 1999; Yasukawa et al. 1999). In addition, the SOCS box motif recruits an E3 ubiquitin-ligase complex that targets SOCS3 binding partners for ubiquitin-mediated proteasomal and/or lysosomal degradation to reinforce inhibition of signalling (Boyle et al. 2008; Irandoust et al. 2007; Zhang et al. 1999).

Being a regulator itself, SOCS3 expression is also tightly controlled at multiple levels. Transcriptionally, STATs are responsible for upregulation of *Socs3* transcripts following cytokine stimulation. (Auernhammer et al. 1999; Gatto et al. 2004; He et al. 2003; Lejeune et al. 2001; Naka et al. 1997). Recently, it has been demonstrated that other factors such as Specificity protein 3 (Sp3), c-Fos, c-Jun and FOXO3a and coactivators CREB-binding protein (CBP) and p300 are also involved in the transcriptional activation of *Socs3* in a cytokine- and cell type-dependent manner (Barclay et al. 2007; Ehlting et al. 2005; Qin et al. 2007). On the other hand, proto-oncoprotein growth factor independence-1B (GFI-1B) and hepatocyte nuclear factor-1β (HNF-1β) have been identified as repressors of *Socs3* transcription (Jegalian and Wu 2002; Ma et al. 2007). At the post-transcriptional level, *Socs3* mRNA stability can be affected by TNFα-mediated activation of the MAPK Kinase 6 (MKK6)/p38^{MAPK} cascade. MAPK-activated protein kinase 2 (MK2), a downstream effector of the cascade, is an important facilitator of this process (Ehlting et al. 2007).

At the protein level, SOCS3 can be targeted for both non-proteasomal and proteasomal-mediated degradation. Modification of SOCS box, Lys6 and PEST sequences of the SOCS3 protein have all been reported to influence the stability of the protein (Babon et al. 2006; Haan et al. 2003; Sasaki et al. 2003; Zhang et al. 1999). However, the exact molecular mechanisms by which SOCS3 protein stability is influenced remain unclear. It has been proposed that SOCS2 regulates SOCS3 stability by forming a molecular bridge between an E3 ubiquitin-ligase complex and other SOCS proteins, targeting them for degradation (Piessevaux et al. 2008). Acceleration of SOCS3 degradation has been observed in cell lines overexpressing SOCS2, and was associated with deregulated IL-2 and IL-3 signalling (Piessevaux et al. 2006; Tannahill et al. 2005). Such data suggest that SOCS2 is a negative regulator of SOCS3 protein levels, and thus a positive modulator of SOCS3-inhibited cytokine signalling cascades.

A corollary of this model is that, in the absence of SOCS2, enhanced or prolonged SOCS3 expression would be expected, resulting in excessive inhibition of signalling. To examine the physiological requirement for SOCS2 in the regulation of SOCS3, we have analyzed SOCS3 protein levels and SOCS3-dependent signal transduction in cells from *Socs2*$^{-/-}$ mice.

Materials and Methods

Generation and maintenance of mice

Generation of *Socs2*$^{-/-}$ mice on a C57BL/6 background has been described previously (Metcalf et al. 2000). For conditional deletion of *Socs3*, a new floxed allele of the Socs3 locus was generated via homologous recombination in ES cells. The targeted allele incorporated three *loxP* sites allowing initial removal of the hygromycin (*hygro*) selectable marker via transient expression of Cre in targeted ES cells, followed by generation of mice bearing the floxed allele (*fl*) (Fig. S1A). *Socs3*$^{-/fl}$ *vavCre*$^{+}$ mice with a conditional deletion of SOCS3 in haemopoietic cells (hereafter referred to as *Socs3*$^{-/\Delta vav}$) were then generated by crossing *Socs3*$^{fl/fl}$ mice with *Socs3*$^{+/-}$ *vavCre*$^{+}$ mice (generated as described previously (Croker et al. 2003)). Efficient deletion of *Socs3* was verified by Southern and absence of protein expression was monitored by Western blot analysis (Fig. S1B and C). Prolonged IL-6 signalling, which is characteristic of absence of SOCS3 (Croker et al. 2003), was confirmed in *Socs3*$^{-/\Delta vav}$ bone marrow derived macrophages (BMM) cells by Western blot analysis (Fig. S1C). Mice were housed at the Walter and Eliza Hall Institute of Medical Research (WEHI) and all animal experiments were performed with the approval of the Animal Ethics Committees of the Melbourne Health Research Directorate or WEHI.

Generation of Monoclonal Antibody

SOCS2 monoclonal antibodies (mAb) were generated by immunization of mice with NusA SOCS2-SH2 fusion protein (Greenhalgh et al. 2002) three times with Freund's complete and incomplete adjuvant, before spleens were removed and fused. Antibodies were screened for SOCS2 specific binding before being cloned and grown for use.

Bone marrow-derived macrophages (BMM) culture

Bone marrow cells were cultured overnight at 37 °C in a 10% (v/v) CO_2 incubator in 10-cm tissue culture-treated plates (2×10^7/dish) in 20 mL of Dulbecco's modified Eagle's medium with 10% (v/v) fetal calf serum (DMEM/10% FCS) and 20% (v/v) L-cell-conditioned medium (LCM), a source of macrophage colony-stimulating factor. 10 mL of cells and media were then transferred to 10 cm Petri dishes and incubated for a further 4 days, with 1 mL of LCM being added to the cells on day 3. On day 5 adherent cells, comprising a pure population of macrophages, were washed in mouse-tonicity phosphate-buffered saline (MT-PBS) and resuspended in 4 mL of cell dissociation buffer (Invitrogen, Carlsbad, CA). After incubation at 37 °C for 10 min, cells were harvested and resuspended in DMEM/10% FCS.

Cytokine stimulation of BMM

BMM were plated into 6-well tissue culture treated plates (1×10^6 cells/well), and deprived of LCM by incubation in DMEM/10% FCS for 1h. Cells were then pulse stimulated with mouse IL-6 (mIL-6) (100 ng/mL, a gift generously given by Prof. Richard Simpson, Ludwig Institute for Cancer Research, Melbourne) for 0.5 h, then the media was removed and replaced with DMEM/10% FCS for up to 8 h. In some experiments, BMM were pre-incubated with mouse IL-4 (mIL-4) (10 ng/mL, a gift generously given by the Division of Immunology, WEHI) for 3.5 h.

BMM proliferation assay

Cells were plated into 96-well plates (1×10^4 cells/well) in 100 μL of DMEM/15% FCS, 10^3 U/mL of human macrophage-colony stimulating factor (hM-CSF) (Cetus, Emeryville, CA) and various concentrations of mIL-6. Cells were cultured at 37 °C, 10% CO_2 for 36 h followed by addition of 1 μCi/well of [3H]thymidine for 16 h. Cell associated radioactivity

was then harvested onto glass fibre filters using an automatic cell harvester, and measured using a scintillation counter (MicroBeta TriLux, Waltham, MA). Data were normalized to the [3H]thymidine incorporation of cells cultured in the same media without mIL-6.

Culture and stimulation of lymph node (LN) cells

A single-cell suspension was prepared from pooled cervical, brachial, axillary, inguinal, and mesenteric lymph nodes in MT-RPMI supplemented with 10% (v/v) heat inactivated (HI) FCS and 40 μM β-mercaptoethanol (RPMI/HI FCS/β-Me) and incubated at 37 °C in a 10% CO_2 incubator for 1 h. For biochemistry experiments, cells were either stimulated with 10 ng/mL of mouse IL-2 (mIL-2) (Peprotech, Rocky Hill, NJ) for up to 8 h or stimulated with anti-CD3 (5 μg/mL) and anti-CD28 (2 μg/mL) mAb and human IL-2 (hIL-2) (10 ng/mL) (Peprotech) in the presence of anti-mIL-2 neutralizing antibody (2 μg/mL) (R&D Systems, Minneapolis, MN) for 4 h. For the latter stimulating condition, the media was removed and replaced with RPMI/HI FCS/β-Me in the presence of anti-mIL-2 neutralizing antibody for up to a further 8 h incubation with or without cycloheximide (15μg/mL) (Sigma, St. Louis, MO).

T-cell purification

Lymph node T cells were enriched by negative selection using Mouse T cell Enrichment Columns (R&D Systems) according to the manufacturer's instructions. Purity was assessed by flow cytometry with anti-CD3 antibody (BD Pharmingen, San Jose, CA) and was routinely >95% (data not shown).

T-cell proliferation assay

5×10^4 purified T-cells were incubated in 100 μL of RPMI/HI FCS/β-Me, 2 μg/mL anti-mIL-2 (R&D Systems) and various concentrations of human IL-2 (hIL-2) (Peprotech) in the presence or absence of soluble anti-CD3 (5 μg/mL) and anti-CD28 (2 μg/mL) mAb. Cells were cultured at 37 °C in a 10% CO_2 incubator for 3 days followed by addition of 1 μCi/well of [3H]thymidine for 17h. Cell associated radioactivity was then harvested onto glass fiber filters using an automatic cell harvester, and measured using a scintillation counter (MicroBeta TriLux).

Western blot Analysis

LN cells or BMM were lysed in 1% (v/v) Triton X-100, 1 mM EGTA, 1 mM EDTA, 10 mM Na pyrophosphate, 1 mM Na_3VO_4, 10 mM Na β-glycerophosphate, 150 mM NaCl, 50 mM NaF, 50 mM Tris-HCl pH 7.4, containing protease inhibitors (Complete Mixture tablets, Roche Applied Science, Indianapolis, IN). Lysates were subjected to SDS-PAGE Western blotting with antibodies specific for either phospho-STAT3 Y705, total STAT3 (Cell Signaling, Danvers, MA), phospho-STAT5A/B Y694/677 (Upstate Biotechnology, Lake Placid, NY), total STAT5 (Zymed, San Francisco, CA), phospho-STAT1 Y701 (Cell Signaling), total STAT1 (Cell signaling), total SOCS3 (IBL, Gunma, Japan) and total SOCS2 (in-house mAb raised against NusA-SOCS2 SH2 fusion protein) proteins. Antibody binding was visualized with either horseradish peroxidase-conjugated anti-rabbit (Chemicon, Temecula, CA) or anti-mouse-IgG (GE Health, Piscataway, NJ) and the ECL Western blotting detection reagent (Amersham, Uppsala, Sweden). Protein expression was quantified by densitometric analyses (Bio-Rad GS-800 Calibrated Densitometer, Bio-Rad Laboratories, Hercules, CA) of Western blots.

RNA extraction and real-time PCR Analysis

DNase-treated RNA was extracted from BMM or LN cells using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA was then reverse-

transcribed into complementary DNA (cDNA) with an Oligo-dT primer (Promega, Madison, WI). cDNA was mixed with SYBR Green PCR master mix (Qiagen) and primers then amplified by PCR using ABI7900 Sequence Detector to quantify *Socs2* and *Socs3* mRNA levels. PCR conditions were 15 sec at 94°C, 30 sec at 60°C and 30 sec at 72°C with 45 cycles. The forward and reverse primers used for *Socs2*, *Socs3* and *Gapdh* were (*Socs2*) TCCAGATGTGCAAGGATAAACG and AGGTACAGGTGAACAGTCCCATT; (*Socs3*) ATTTCGCTTCGGGACTAGCTC and AGCTGTCGCGGATAAGAAAGG; (*Gapdh*) TTGTCAAGCTCATTTCCTGGT and TTACTCCTTGGAGGCCATGTA. The expression level of each target gene was normalized to *Gapdh* expression. Data represent mean ± standard deviation of the mean (SD) from 3–4 independent experiments.

Statistical Analysis

To evaluate changes in gene expression over time, data were log-transformed and one-way analysis of variance (ANOVA) was performed (GraphPad Prism Version 5.0, GraphPad Software, San Diego, CA). Variation in expression between WT and $Socs2^{-/-}$ at given timepoints were analysed by both *t*-test with equal variance assumption and paired *t*-test (Microsoft Excel, Microsoft Corporation, Redwood, WA, USA) on the log-transformed data.

Results

SOCS3 expression and IL-6-mediated responses in WT and $Socs2^{-/-}$ BMM

Previous work identified SOCS3 as a negative regulator of IL-6 signalling in macrophages (Croker et al. 2003). We utilized this system to assess whether the presence or absence of SOCS2 affected the level of SOCS3 expression and/or perturbed STAT3 activation by IL-6. In primary macrophages, expression of *Socs2* mRNA was induced from a low baseline upon stimulation with mIL-6 for 30 min (Fig. 1A). No differences in transcript levels were observed between wildtype (WT) and $Socs3^{-/-}$ cells. Even with stimulation, the level of SOCS2 protein expression remained below the level of detection by Western blotting with available antibodies (data not shown). In WT ($Socs2^{+/+}$) cells, SOCS3 protein expression was undetectable prior to pulse stimulation with mIL-6, then peaked at 1 h and was downregulated by 2 h. In the absence of SOCS2, this pattern of mIL-6-induced SOCS3 expression was unaltered (Fig. 1B). Consistent with maintenance of normal regulation of SOCS3 expression, the induction and duration of mIL-6-dependent STAT3 activation in $Socs2^{-/-}$ BMM were identical to that of the WT cells (Fig. 1B). As expected, $Socs3^{-/-}$ BMM displayed prolonged activation of STAT3 (Fig. 1B).

To examine IL-6 action in a biological context, we monitored its recognized inhibitory effect on M-CSF-induced BMM proliferation in WT macrophages (Takeda et al. 1999) and those lacking either SOCS2 or SOCS3. Consistent with the unaltered STAT3 phosphorylation observed in Fig. 1B, following mIL-6 stimulation of $Socs2^{-/-}$ BMM, $Socs2^{-/-}$ and WT cells displayed equivalent inhibition of proliferation when incubated with the cytokine. As expected, greater inhibition was seen in $Socs3^{-/\Delta vav}$ cells due to unrestrained IL-6 signalling (Fig. 1C). Thus, in BMM, SOCS3 expression and regulation of signal transduction and biological responses to mIL-6 appear independent of SOCS2. Certainly there was no evidence for aberrant SOCS3 activity in the absence of SOCS2.

Effect of SOCS2 upregulation on SOCS3 expression and IL-6 signalling in WT and $Socs2^{-/-}$ BMM

As IL-6 is a relatively weak stimulus of SOCS2, we sought to explore any regulatory role of SOCS2 at higher levels of expression. BMM were pre-incubated with mIL-4. SOCS2 expression was markedly upregulated and was readily detectable by Western blot (Fig. 2).

Despite this higher level of SOCS2 expression in WT control cells, the kinetics and magnitude of STAT3 activation and the regulation of SOCS3 expression induced by subsequent mIL-6 stimulation remained indistinguishable from those observed in similarly treated SOCS2-deficient cells (Fig. 2).

Expression of SOCS3 and response to IL-2 in lymphocytes in the absence of SOCS2

Previously, it was demonstrated that enforced expression of SOCS2 in BaF/3 cells enhanced IL-2 signalling by accelerating SOCS3 degradation (Tannahill et al. 2005). Since IL-2 is an essential proliferative cytokine for primary lymphocytes, we examined signal transduction and SOCS3 expression in *Socs2*$^{-/-}$ lymphocytes stimulated with mIL-2. In WT lymphocytes from lymph nodes, both SOCS2 and SOCS3 mRNA and protein were detectable and did not alter substantially after mIL-2 stimulation (Fig. 3A and B). No differences in the intensity of SOCS3 expression nor the duration of mIL-2 activated STAT5 were observed between WT and *Socs2*$^{-/-}$ LN cells (Fig. 3A). Identical results were also seen in purified T cells (data not shown).

To further examine whether the presence or absence of SOCS2 influences SOCS3-regulated cellular responses, we monitored T cell proliferation in response to hIL-2. Given that *Socs2*$^{-/-}$ mice display a normal distribution of T-cell subsets in various lymphoid compartments (data not shown), LN T cell proliferation in response to hIL-2 treatment was measured by [3H]thymidine incorporation. Endogenous IL-2 was neutralized with anti-mIL-2 antibody. Consistent with unaltered IL-2 signalling in *Socs2*$^{-/-}$ cells, proliferation of *Socs2*$^{-/-}$ T cells in response to hIL-2 was indistinguishable from WT (Fig. 3C).

SOCS3 expression by, and proliferation of, anti-CD3/CD28 activated Socs2$^{-/-}$ LN cells

To examine regulation of SOCS3 in an inducible system, T cells were stimulated for 4 h with anti-CD3 and anti-CD28 mAb, and the level of SOCS3 expression monitored without stimulation for a further 8 h. In both WT and *Socs2*$^{-/-}$ LN cells, SOCS3 expression was induced to maximal levels at 4.5 h and downregulated at equivalent rates in the absence of further stimuli (Fig. 4A and B). Similarly, no difference between genotypes was observed in SOCS3 expression when cycloheximide was added to prevent further protein synthesis after removal of the stimuli at 4 h (Fig. S2). Consistent with normal regulation of SOCS3 expression, anti-CD3 and anti-CD28 mAb activated *Socs2*$^{-/-}$ T-cells displayed a normal proliferative response to hIL-2 (Fig. 4C).

Discussion

Although SOCS3 is recognized as a key suppressor of cytokine signalling, our understanding of the regulation of SOCS3 protein levels is still inexact. Both transcriptional and post-translational mechanisms have been proposed for controlling the rapid induction of SOCS3 following cytokine stimulation, as well as the timely decay of protein levels to allow ongoing cellular responses to cytokines where appropriate. Within this context, SOCS2 has been proposed to interact with, and target SOCS3 for proteasomal-mediated degradation. In a cell line-based ectopic expression model, SOCS2 was shown to accelerate SOCS3 degradation, leading to prolonged IL-2 and IL-3 signalling (Piessevaux et al. 2006; Tannahill et al. 2005). If this model reflected physiology, we would predict that in the absence of SOCS2, enhanced or prolonged SOCS3 protein expression would ensue, resulting in curtailed duration and/or intensity of cytokine signalling pathways normally inhibited by SOCS3. To formally explore a physiological role for SOCS2 in the regulation of SOCS3 we analyzed primary cells from *Socs2*$^{-/-}$ mice for regulation of SOCS3 protein levels and control of signalling pathways known to be regulated by SOCS3 under

physiological conditions (IL-6) (Croker et al. 2003) as well as cytokine pathways sensitive to SOCS3 when overexpressed (IL-2) (Cohney et al. 1999).

Our data revealed that SOCS3 protein expression and STAT3 activation upon mIL-6 stimulation was indistinguishable in *Socs2*$^{-/-}$ BMM from that in WT cells (Fig. 1B and 2). Consistent with this, the absence of SOCS2 did not desensitize cells to IL-6-induced growth inhibition (Fig. 1C). We also observed similar phenomena in lymphocytes. Both SOCS3 expression and mIL-2-dependent signal transduction in SOCS2-deficient cells remained identical to the WT patterns (Fig. 3A) and the decline in cellular SOCS3 protein levels following induction in activated T-cells was also comparable between the two genotypes (Fig. 4A and B and Fig S2). In line with this, biological assays revealed that loss of SOCS2 made no difference to the proliferation of T cells in response to hIL-2 in either the quiescent or activated states (Fig. 3C and 4C). Taken together, the data presented here suggest that any interaction between SOCS2 and SOCS3 leading to targeting of SOCS3 for degradation does not play a significant role in regulation of SOCS3 expression in primary macrophages and T cells.

Thus, our data do not support there being a physiological role for SOCS2 in the regulation of SOCS3 expression and activity in the haemopoietic cells. Since the previously reported actions of SOCS2 in this regard were described following ectopic expression in cells lines, the regulatory action of SOCS2 on SOCS3 may require levels of SOCS2 not normally achieved *in vivo*. There are examples in the literature where ectopic or over-expression of SOCS proteins revealed activities that are not physiological. For example, ectopic expression of SOCS3 effectively inhibits interferon-γ (IFNγ) signalling (Karlsen et al. 2001; Song and Shuai 1998; Woldman et al. 2001), but SOCS3 appears dispensable for normal regulation of IFNγ *in vivo* (Croker et al. 2003; Lang et al. 2003) and similar observations have been made for SOCS1 and IL-6 signalling (Croker et al. 2003; Lang et al. 2003; Starr et al. 1997). It may be possible that crosstalk between SOCS2 and SOCS3 occurs in pathological states if levels of cytokines and SOCS proteins are extreme. Our data cannot exclude a role for SOCS2 in modulating SOCS3 regulation of other signalling systems, such as has been proposed for growth hormone (Piessevaux et al. 2006; Piessevaux et al. 2008), although our data do suggest that assessment of such models under physiological conditions is warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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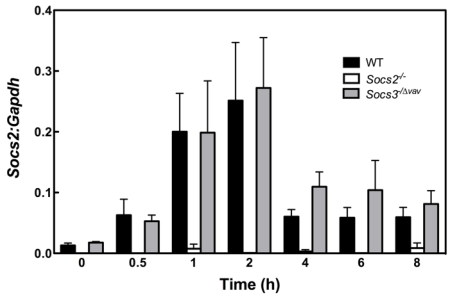

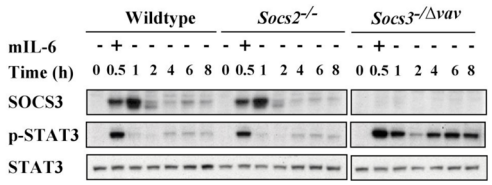

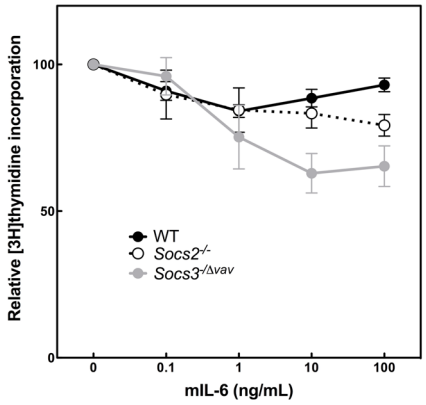

Figure 1.

Normal SOCS3 expression, STAT3 phosphorylation and SOCS3-dependent inhibition of proliferation in IL-6 stimulated *Socs2*$^{-/-}$ macrophages. (A) Expression of *Socs2* transcript relative to *Gapdh* transcript. BMM from WT, *Socs2*$^{-/-}$ and *Socs3*$^{-/\Delta vav}$ mice were stimulated with mIL-6 (100 ng/mL) for 0.5 h, then incubated in cytokine-free media for up to 8 hours. Quantification of *Socs2* mRNA was performed by Real-Time PCR after RNA extraction and reverse transcription. Data represent mean ± standard error of the mean (SEM) of replicates from 4 independent experiments. (B) Western blot analyses of cell lysates of BMM cells treated as described for A. Indicative example of 3 independent experiments. (C) BMM from WT, *Socs2*$^{-/-}$ and *Socs3*$^{-/\Delta vav}$ mice were cultured in presence of hM-CSF (10^3 U/mL) and various concentrations of mIL-6. Cell proliferation was determined by measuring [3H]thymidine incorporation after 52 h, and normalized to [3H]thymidine incorporation of cells cultured without mIL-6. Data represent mean ± SEM of 4 independent experiments.

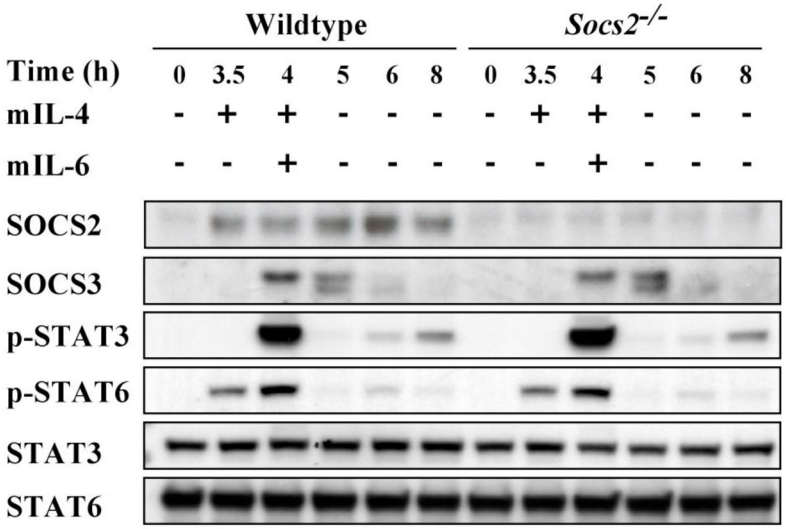

Figure 2.

SOCS3 expression and STAT3 phosphorylation are unaltered by IL-4-induced expression of SOCS2 in macrophages. BMM from WT and *Socs2*$^{-/-}$ mice were preincubated with mIL-4 (10 ng/mL) for 3.5 h to induce SOCS2 expression, then stimulated with mIL-6 (100 ng/mL) for 0.5 h, and subsequently incubated in cytokine-free media for up to 8 hours. Cell lysates were collected for Western blot analyses. Indicative example of 3 independent experiments.

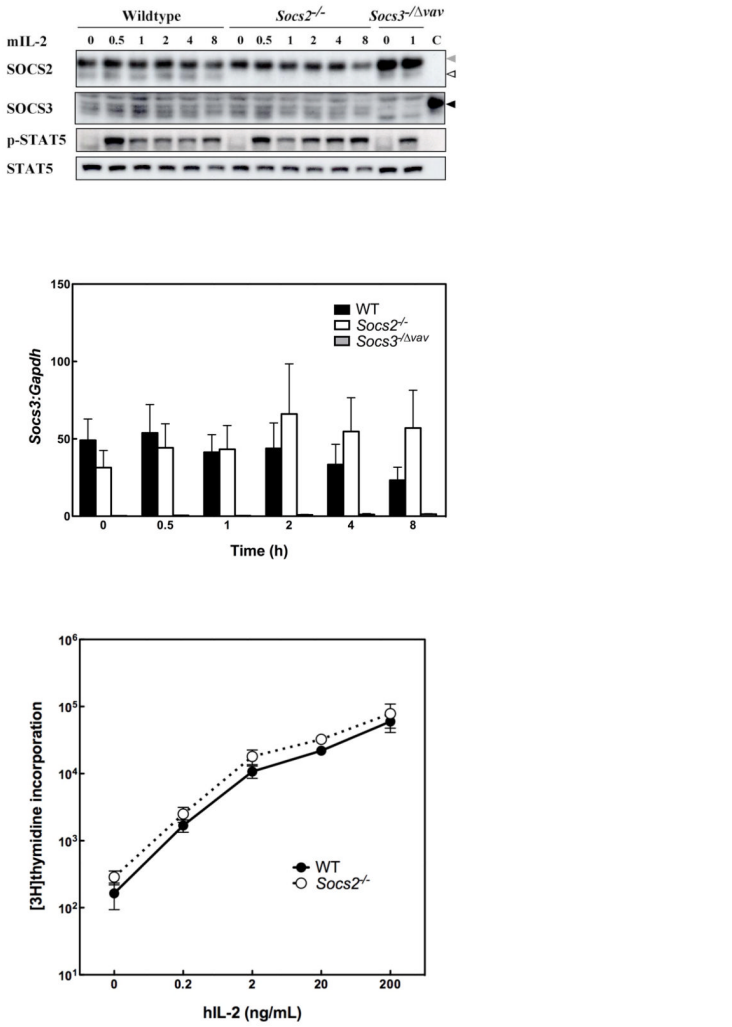

Figure 3.

SOCS3 expression, STAT5 phosphorylation and IL-2 induced proliferation are unaltered in *Socs2*$^{-/-}$ lymphocytes stimulated with IL-2. (A) WT, *Socs2*$^{-/-}$ and *Socs3*$^{-/\Delta vav}$ LN cells were stimulated with mIL-2 (10 ng/mL) for up to 8 h. Indicative Western blot of cell lysates from one of three experiments. SOCS2 and SOCS3 are indicated by white arrow and black arrows, respectively. The grey arrow indicates non-specific bands (light chains). "C" indicates SOCS3 positive control from LPS stimulated BMM. (B) Expression of *Socs3* mRNA in WT and *Socs2*$^{-/-}$ cells in response to mIL-2 (10 ng/mL) quantified by real-time PCR analysis, and expressed relative to *Gapdh* expression. Data represent mean ± SEM of 3–4 independent experiments. The variation in SOCS3 expression level between the time points for each genotype was not statistically significant as judged by one-way analysis of variance (ANOVA) on log-transformed data ($p=0.6192$ for WT and $p=0.7782$ for *Socs2*$^{-/-}$). (C) Purified LN T-cells from WT and *Socs2*$^{-/-}$ mice were cultured with various concentrations of hIL-2 for 3.5 days in the presence of anti-mIL-2. Cell proliferation was determined by measuring [3H]thymidine incorporation. Data represent mean ± SEM of 5 independent experiments.

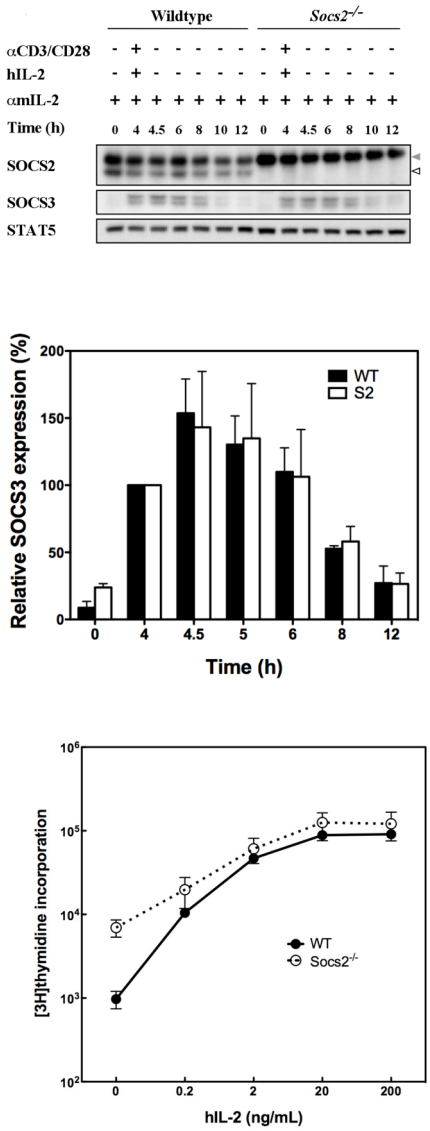

Figure 4.

Induction and decay of SOCS3 expression and proliferation in activated LN cells is independent of SOCS2 expression. (A) LN cells collected from WT and *Socs2*$^{-/-}$ mice were incubated with anti-CD3 (5μg/mL), anti-CD28 (2 μg/mL) and hIL-2 (10ng/mL) for 4 h. Thereafter, cells were washed and incubated in media for a further 8 h. Throughout the experiment cells were exposed to anti-mIL-2 (2 μg/mL). Cell lysates were collected for Western blot analyses. SOCS2 and non-specific bands (light chains) are indicated by white arrow and grey arrows, respectively. Indicative example of 3 independent experiments. (B) SOCS3 expression in above experiments was quantified by densitometry. Expression levels were normalized to the 4 h time point for each genotype and presented as relative SOCS3 expression (%). Data represent mean ± standard deviation (SD) of 3 independent experiments. (C) Purified LN T-cells from WT and *Socs2*$^{-/-}$ mice were cultured with anti-CD3 (5μg/mL), anti-CD28 (2 μg/mL) and hIL-2 or hIL-2 alone at various concentrations for 2.5 days in the presence of anti-mIL-2. Cell proliferation was determined by measuring [3H]thymidine incorporation. Data represent mean ± SD of 2 independent experiments.