Suppressor of cytokine signaling 1 regulates an endogenous inhibitor of a mast cell protease*

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Running Title: SOCS1 regulates mast cell protease activity

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Summary

Suppressor of cytokine signaling 1 (SOCS1) is a negative regulator of c-Kit and IL-3 receptor signaling. We examined the role of SOCS1 in regulating IL-3-induced cell growth of primary bone marrow-derived mast cells (BMMC) from SOCS1-/- mice. Instead of showing increased proliferation, SOCS1 deficient BMMC responded poorly to IL-3 and stem cell factor. SOCS1-/- BMMC showed increased apoptosis and defective cell cycle entry. We show that the growth retardation of SOCS1-/- BMMC was due to a cell intrinsic defect. Protein tyrosine phosphorylation following IL-3 stimulation was markedly diminished in SOCS1-/- BMMC. Intriguingly, JAK2 and STAT5 proteins were selectively diminished in SOCS1-/- BMMC, which also showed lower molecular mass products of p85 and Vav suggesting proteolytic degradation. Incubation of the SOCS1-/+ BMMC lysate with STAT5, p85 and Vav immunoprecipitated from SOCS1+/+ cells directly demonstrated the dysregulated proteolytic activity in SOCS1-/- BMMC. The proteolytic activity in SOCS1-/- BMMC was selectively inhibited by phenylmethysulfonylfluoride and soybean trypsin inhibitor, suggesting that the protease regulated by SOCS1 is a tryptase. The dysregulated tryptase in SOCS1-/- BMMC is unlikely to be mMCP6 or mMCP7 as the enzyme activity was not inhibited by polybrene, but was inhibited by normal mouse plasma. SOCS1+/+ BMMC lysate inhibited the proteolytic activity present in SOCS1-/- BMMC lysate, indicating that SOCS1-/- BMMC are lacking an endogenous protease inhibitor. These results show that SOCS1 is required for the expression and/or stability of an endogenous protease inhibitor which protects mast cells from their own proteolytic enzymes.
Introduction

The generation, survival, proliferation and functions of most hematopoietic cells critically depend on cytokines (1,2). A majority of the growth-promoting cytokines transduce signals by activating intracellular Janus family of protein tyrosine kinases (JAKs) non-covalently associated with the cytokine receptor subunits (see (3,4) for reviews). The catalytic activity of JAK kinases is low in the absence of cytokine stimulation. Upon ligand binding, receptor oligomerization allows trans-autophosphorylation of the JAKs in their activation loop tyrosine, which stimulates their kinase activity. This leads to phosphorylation of the receptor chains, and recruitment of adaptor proteins and second messengers to the receptor complex. The primary mediators of cytokine receptor signaling downstream of JAKs are signal transducers and activators of transcription (STATs) (5), which become tyrosine phosphorylated, dimerize, translocate into the nucleus and induce transcription of specific sets of genes to mediate the cellular responses of cytokine stimulation.

The cytokine stimulated JAK-STAT pathway is highly regulated so that the activating signals of appropriate magnitude are delivered for only the required duration (reviewed in (6,7)). These signals are promptly attenuated to prevent excessive signaling and uncontrolled cellular stimulation. This is achieved by several mechanisms regulating the JAK kinase activity and the functioning of the STAT molecules. Protein tyrosine phosphatases recruited to the cytokine receptor complex dephosphorylate the JAK kinases at their activation loop tyrosine thereby attenuating their enzymatic activity. PIAS family proteins bind to activated STAT molecules preventing their DNA-binding activity (8). However, the major regulatory pathway attenuating cytokine receptor signal transduction is mediated by the SOCS family of negative feedback regulators (9). The SOCS family contains at least 8 known members. Genes encoding CIS1,
SOCS1, SOCS2 and SOCS3 are rapidly induced following cytokine stimulation, and many cytokines induce the expression of several SOCS family members.

All SOCS proteins have a conserved C-terminal motif called the SOCS box, which is also present in many other proteins of diverse functions (10). A central SH2 domain is also a feature of the SOCS family members, whereas the N-terminal end is quite variable in sequence and length. The regulatory function of SOCS molecules is mediated by the binding of the SH2 domain to the activation loop tyrosine of the JAK kinases (11-16). A stretch of amino acids N-terminal to the SH2 domain of SOCS1 is required for its inhibitory function. After the binding of the SH2 domain to phosphotyrosine, the pre-SH2 region is believed to occlude the substrate binding pocket of the kinase. In this manner, SOCS1 functions as a competitive inhibitor of JAK kinase substrates. This physical interaction subsequently leads to the destruction of the JAK kinase via ubiquitin dependent protein degradation (17-20). This latter function of SOCS1 requires the SOCS box, which interacts with components of an E3-ubiquitin protein ligase that ubiquitinates the JAKs and targets them for proteasomal degradation (10,21). Several other proteins also interact with SOCS1 via its SH2 domain and the N-terminal proline-rich sequences, and some of these proteins are also ubiquitinated and targeted for proteasomal degradation (16,22).

We have shown that SOCS1 is rapidly induced in mast cells following stimulation with stem cell factor (SCF) and IL-3 (16). Mast cells play diverse roles in inflammation and anti-parasitic immunity, and are the central effector cells in allergic reactions (23,24). They differentiate from hematopoietic stem cells into committed progenitor cells which circulate in the blood before migrating to mucosal and connective tissues of peripheral organs where they mature into phenotypically distinct mucosal or connective tissue mast cells (25). Development of
mast cells and their survival in the peripheral tissues are critically dependent on SCF. In addition to SCF, IL-3 also stimulates mast cell differentiation and proliferation. Mast cells are unique among hematopoietic cells in that they can be cultured from bone marrow progenitors using either SCF or IL-3 alone (26). Therefore, we used bone marrow-derived mast cells (BMMC) from \textit{SOCS1}/⁻/ mice to address whether SOCS1 is a critical regulator of IL-3 signaling. To our surprise, we observed that SOCS1 deficient BMMC showed a poor proliferative response to IL-3 and SCF, because SOCS1 deficiency dysregulated mast cell proteolytic enzymes leading to selective degradation of several signaling molecules.

\textbf{Materials and Methods}

\textit{Mast cell cultures from \textit{SOCS1}/⁻/ mice.} \textit{SOCS1}/⁻/ mice were generous gifts from Dr. J. Ihle. The \textit{SOCS1}/⁻/ were bred in our animal care facility from SOCS1 heterozygous parents because \textit{SOCS1}/⁻/ mice die within 2-3 wk after birth. Mast cell cultures were established from the bone marrow of femurs obtained from \textit{SOCS1}/⁻/ mice as soon as they were phenotypically detectable, usually around 8-14 days. All animal work was performed following institutional guidelines. BMMC were cultured in OPTI-MEM (Invitrogen/Life Technologies, Burlington, ON) containing 10% fetal bovine serum (FBS), 50 \( \mu \)M \( \text{`}-\text{mercaptoethanol} \) and IL-3. Culture supernatants (CS) from X630/mIL-3 cell line (27) and BHK/mKL (a gift from S. Tsai) were used at 1:100 dilution as sources of IL-3 and SCF, respectively. Non-adherent cells were subcultured every 3-4 days at 0.5 \( \times \)10⁶ per ml concentration. After 4 weeks, the cultures were highly enriched for mast cells, representing more than 95% of all cells based on Toluidine blue staining (data not shown). The cultures were maintained in culture for up to 6 months, and the cells were used for experiments from 4 weeks onwards.
**Antibodies and reagents.** Antibodies against mouse IgE, CD16/CD32 (Fc-RIII/II) CD45 (Ly5.2), CD117 (c-Kit) and IL-3Rα conjugated to FITC, PE or biotin were purchased from BD Pharmingen Biosciences (Palo Alto, CA). Streptavidin-spectral red (ST-SPRD) was from Southern Biotechnology Associates, Inc. (Birmingham, AL). Mouse monoclonal antibody against phosphotyrosine (4G10) and rabbit polyclonal antibodies against p85, JAK2 and phospho-JAK2 were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Rabbit polyclonal antibody against phospho-STAT5 (pSTAT5) was from Cell Signaling Technology (Beverly, MA). Antibodies against STAT5, Vav and p85 were from SantaCruz Biotechnology (Santa Cruz, CA). Antibodies to Bax and p27Kip were from BD Transduction Labs. 5-(6)carboxyfluorescein diacetate succinimidyl ester (CFSE) was from Molecular Probes (Eugene, OR). Mouse monoclonal IgE against TNP (clone C38-2) and anti-actin antibody (clone AC-15) were from Sigma. Anti-HSP70 mAb was a kind gift from R. Miller (Ontario Cancer Institute, Toronto). Complete protease inhibitor cocktail, AEBSF, leupeptin and aprotinin were from Roche Molecular Biology. Protease inhibitors PMSF, soybean trypsin inhibitor (STI), benzamidine, TPCK, TLCK, 3,4-dichloroisocoumarin (DIC), (2S,3S)-trans-epoxysuccinyl-L-leucyl-amido-3-methylbutane ethyl ester (E-64), ethylenediamine tetra acetic acid (EDTA) and STI-conjugated agarose beads were from Sigma. Proteasome inhibitors MG132, Proteasome inhibitor I (ZAL) and Lactacystin were from Calbiochem. N-acetyl-Leu-Leu-Norleu-al (LLnL; calpain inhibitor-I), N-acetyl-Leu-Leu-Met-al (LLM; calpain inhibitor-II), and Ala-Ala-Phe chloromethylketone (AAF-CMK) were from Sigma. Epoxomycin was from Affinity (Exeter, UK). Neutralizing anti-IFNα antibody was from BD Pharmingen. Mouse fibrinogen was purchased from Sigma.
Flow cytometry. Single cell suspensions in PBS containing 5% FBS and 0.05% sodium azide were incubated with indicated fluorochrome or biotin conjugated antibodies for 15 min. After washing twice in PBS/FBS, biotin conjugated primary antibodies were followed by ST-SPRD. Washed cells were analysed in a FACS Calibur® using CellQuest software (Becton Dickinson, Mountainview, CA). To detect FcRI, mast cells were incubated first with IgE monoclonal antibody against TNP followed by anti-mouse IgE. For detecting cells undergoing apoptotic death, cells in steady state growth were washed and resuspended in annexin-V binding buffer followed by Annexin-V staining and flow cytometric analysis.

Cell proliferation assays and cell cycle analysis. 1×10^5 BMMC were stimulated with indicated concentrations of IL-3 or SCF for 48 h in 96 well U bottom plates. One μCi of methyl-[^3]H-thymidine (NEN, Boston, MA) was added per well during the last 8 h. Neutralizing antibodies were added at the initiation of the culture. The cells were harvested onto glass fiber filter mats and the incorporated radioactivity was measured in a TopCount® liquid scintillation counter (Canberra/Packard, Missisauga, ON).

The CFSE dye dilution assay for cell proliferation was used to address whether SOCS1 deficient BMMC can cross-inhibit wild type BMMC. Wild type BMMC were labeled with CFSE by incubating them at 2×10^7 cells/ml in PBS containing 5 μM CFSE for 10 min at room temperature. The reaction was quenched with an equal volume of FBS. The cells were washed twice and stimulated with IL-3 (at 1:100 dilution of the CS), in the presence of unlabeled SOCS1 deficient or wild type cells. At indicated time points, the cells were analyzed by flow cytometry to evaluate the dilution of CFSE fluorescence intensity which is indicative of cell division.
Cell cycle analysis was done on BMMC in steady state growth or on cells starved overnight in OPTI-MEM containing 0.5% FBS and 1 mg/ml BSA. Following stimulation with IL-3 for indicated periods of time, 1×10^6 cells were pelleted and chilled on ice. The cells were resuspended directly in low salt Vindelov’s solution containing propidium iodide (100 mM NaCl, 3.4 mM Tris pH 7.6, 0.1% v/v NP-40, 700 U/ml RNase, 7.5 µM propidium iodide) with continuous mixing. The cells were analyzed by flow cytometry and the proportions of cells in G1, S, M and G2 phases were estimated using ModFit™ (Verity Software House).

*RT-PCR.* For RT-PCR analysis of cytokine gene expression in mast cells, total RNA was isolated from 1×10^6 BMMC in steady state growth using 1 ml of Trizol (Gibco-BRL) following the manufacturer’s instructions. Equivalent amount of RNA was primed with oligo(dT), and first strand cDNA was synthesized using Thermoscript™ Reverse Transcriptase (Life Technolgies, Inc.) according to the manufacturer’s protocol. PCR was performed on equivalent amounts of cDNA using Platinum Taq DNA polymerase (Life Technologies, Inc.) in a GeneAmp PCR System 9700 thermocycler (PE Applied Biosystems). The sense and antisense primers used for PCR detection of specific mRNA are as follows: IFNα, AACGCTACACACTGCATCT and TGCTCATTTGTAATGCTTGG; TNF-α, GAAAGCATGATCCGCGACGTGGA and TACGACGTGGGCTACAGGCTTG; TGFβ, CGGGGCGACCTGGGCACCATCCATGAC and CTGCTCCACCTTGGGCTTGCGACCCAC; lymphotoxin (LT), TCAGAAGCACTTGACCCAT and AAGTCCCGGATACACAGACT; G3PDH, TGATGGGTGTGAACCACGAG and TCAGTGTAGCCCAAGATGCC; mMCP-2, ATTTCCATTCCTGATTTCCCTCTTGAC and CAGGATGAGAACGGCTGGGAT; mMCP-4, GTAATTTCTCCTCGCTCCTCTGAC and TGACAGGATGGACACATGCTTT; mMCP-5,
GGCAGAACAAACGTGAATGAGCC and AAGAACCTTCTGGAAGCTCAGGG; mMCP-6, GCACATCAAAAGCCACAGC and TAGACAGGGGAGACAGAGGAC; mMCP-7, GCACTACTCCTCACTGTG and CGCATTATTGAGGCATAGCAGA; mouse \( \beta\)-actin, GGCATTGTTACCAACTGGGAC and ACCAGAGGCATACAGGGACAG.

**Western blot.** Cytokine-stimulated and unstimulated control BMMC were pelleted and lysed either directly in SDS-PAGE sample buffer (50 mM Tris pH 6.8, 1% w/v SDS, 1 mM EDTA, 1 mM dithiothreitol) by boiling, or in TNN lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.5% v/v NP-40) by incubating on ice for 30 min. Lysates containing equivalent amounts of total proteins, quantitated by BCA assay (Pierce, Rockford, IL), were electrophoresed in SDS-PAGE gels, transferred to PVDF membranes, and probed with phospho-STAT5 antibody following the manufacturer’s instructions. The blots were developed by enhanced chemiluminescence reagent from Amersham Pharmacia Biotech (Piscataway, NJ). The blots were stripped by incubating in stripping solution (2% SDS, 62.5 mM Tris pH 6.8, 100 mM \( \beta\)-mercaptoethanol) for 30 min at 55°C, blocked and reprobed for STAT5. A similar protocol was followed for other phosphospecific antibodies.

**Fibrinolysis assay.** BMMC were lysed in TNN lysis buffer containing the CÔmplete\O protease inhibitor cocktail at 10 × 10^6 cells per ml. Mouse fibrinogen was dissolved in 25 mM sodium phosphate buffer pH 7.4 containing 1 mM EDTA at 1 mg/ml concentration. Fifteen \( \mu\)g of fibrinogen was incubated with BMMC lysates containing 20 \( \mu\)g of total proteins at 37°C for 1 h in a total volume of 30 \( \mu\)l. To inhibit the fibrinolytic activity of the proteolytic enzymes, PMSF or normal mouse plasma was added to a final concentration of 1 mM and 1%, respectively, to the
BMMC lysate and incubated for 10 min at room temperature before adding to fibrinogen. At the end of the reaction, the protein content of the reaction mixture was denatured by boiling in sample buffer. The proteins were separated in SDS-PAGE gels and visualized by Coomassie brilliant blue staining.

**Results**

*SOCS1 deficient mast cells exhibit retarded growth due to decreased proliferative response to IL-3 and stem cell factor.* We have previously shown that SOCS1 is rapidly induced in primary BMMCs following stimulation with IL-3 or SCF, and forced expression of SOCS1 inhibits SCF-mediated cell proliferation without interfering with cell survival signals delivered via the c-Kit receptor (16). In addition to SCF, IL-3 also stimulates mast cell differentiation and proliferation. To address whether SOCS1 is a critical regulator of IL-3 signaling, we established BMMCs from *SOCS1*<sup>−/−</sup> mice using IL-3.

Flow cytometric analysis of the mast cells for surface markers showed comparable levels of CD45 and Fc-RIII/II (CD16/32) on SOCS1 deficient and control cells (Fig. 1A). Even though the expression of Fc-RI was slightly lower in *SOCS1*<sup>−/−</sup> cells (Fig. 1A), these cells did not significantly differ from SOCS1 sufficient cells in IgE-mediated degranulation (data not shown). Surprisingly, SOCS1 deficient BMMCs exhibited a very slow growth rate compared to that of wild type BMMCs in response to IL-3 or SCF (Fig. 1B and data not shown), despite expressing comparable levels of the receptors for IL-3 and c-Kit (Fig. 1A). The decreased proliferative response of SOCS1 deficient BMMCs remained unchanged over a wide concentration range of IL-3 or SCF (Fig. 1C). These results show that SOCS1 is essential for normal growth response of mast cells to cytokines.
SOCS1 deficient mast cells show defective cell cycle entry and increased apoptosis. To examine whether the decreased proliferative response of SOCS1 deficient BMMC to IL-3 was due to increased cell death, we stained the SOCS1−/− BMMC with Annexin V. IL-3 stimulated SOCS1 deficient mast cell cultures contained at least three times more apoptotic cells than the wild type cultures (Fig. 2A). To investigate whether the increased cell death in SOCS1 deficient mast cell cultures arises from a cell cycle defect, we carried out a cell cycle analysis of these cells at different time points after IL-3 stimulation and in steady state growth. At all time points after IL-3 stimulation, the proportion of cells in the S phase was 5-10 fold less in SOCS1−/− cultures compared to wild type cultures (Fig. 2B). In steady state cultures, SOCS1 deficient BMMC did not accumulate at the G2-M phase (Fig. 2B). These results suggested that the decreased proliferative response of SOCS1 deficient BMMC and their increased apoptosis could result either from active growth suppression or from an intrinsic inability of these cells to pass through the cell cycle restriction point.

Decreased proliferation of SOCS1 deficient mast cells results from a cell autonomous defect. SOCS1−/− mice die from unbridled IFN-α signaling (28,29) and SOCS1 deficient fibroblasts are hypersensitive to growth inhibition by IFN-α (30). Since IFN-α is cytostatic for several cell types, and IL-3 dependent mast cell lines are reported to express IFN-α mRNA and other growth inhibitory cytokines such as TGF-β (see (31) for review), we examined the cytokine profile of SOCS1 deficient BMMC by RT-PCR. Whereas the mitogen-stimulated lymph node cells showed the induction of the IFN-α gene, we could not detect IFN-α message in wild type BMMC or in SOCS1 deficient BMMC (Fig. 3A). Moreover, neutralizing IFN-α antibody, which blocked IFN-α
induced STAT1-phosphorylation in fibroblasts (data not shown), failed to restore the proliferation of SOCS1 deficient BMMC to IL-3 or SCF (Fig. 3B). Further, addition of exogenous IFN-α did not decrease the growth response of wild mast cells to IL-3 or SCF (data not shown). These results strongly suggest that endogenous IFN-α is unlikely to be responsible for the retarded growth of SOCS1 deficient BMMC. Expression of TNFα, lymphotoxin (TNFβ) and TGFβ mRNA showed no appreciable difference between SOCS1 deficient and SOCS1 sufficient BMMC (Fig. 3A), however, we do not rule out the possibility that SOCS1 deficient BMMC may be more susceptible to the growth inhibitory effects of these cytokines.

To investigate whether SOCS1 deficient BMMC may be secreting an unknown growth inhibitory factor, we cultured wild type BMMC in conditioned media from SOCS1 deficient BMMC. Proliferation of wild type BMMC was unaffected by conditioned media from SOCS1 deficient BMMC generated with or without IL-3 stimulation (Fig. 3C). To further explore whether SOCS1 deficient BMMC may be expressing on their surface a hitherto unidentified growth inhibitory molecule, we labeled wild type BMMC with CFSE, a green fluorescent dye that labels cellular proteins and gets equally partitioned into daughter cells upon cell division. Co-culture with unlabelled SOCS1 deficient BMMC did not alter the cell division profile of the CFSE labeled wild type BMMC (Fig. 3D). These results showed that SOCS1 deficient BMMC are not expressing any growth inhibitory molecule that may act on the adjacent cells, and strongly suggested that the retarded growth of SOCS1 deficient BMMC is due to a cell intrinsic defect.

_Cytokine-stimulated protein tyrosine phosphorylation is defective in SOCS1 deficient mast cells._ IL-3 signals through a receptor complex composed of the IL-3Rα chain and the βc
chain. Upon IL-3 binding, the Jc-associated JAK2 is activated and phosphorylates the receptor chains and several signaling proteins including STAT5 (32). To examine whether these membrane proximal signaling pathways are intact in SOCS1 deficient BMMC, we first evaluated the profile of Tyr-phosphorylated proteins in mast cells stimulated with IL-3. Equivalent number of cells were lysed directly in denaturing lysis buffer and blotted with 4G10 anti-P-Tyr antibody. Strikingly, SOCS1 deficient BMMC showed a less pronounced protein tyrosine phosphorylation following cytokine stimulation compared to wild type cells (Fig. 4, left panels). Stimulation with SCF also elicited weaker tyrosine phosphorylation in SOCS1 deficient BMMC than in wild type cells (data not shown). Probing the blots with phospho-specific antibodies showed negligible quantities of phospho-JAK2 and phospho-STAT5 in SOCS1 deficient BMMC (Fig. 4, right panels). Strikingly, reprobing the blots revealed that the total amounts of JAK2 and STAT5 were markedly lower in SOCS1 deficient BMMC, whereas the levels of other control proteins such as Bax, p27kip or actin were comparable between SOCS1 deficient and SOCS1 sufficient BMMC (Fig. 4, right panels). Since the cells were lysed by boiling in SDS containing lysis buffer immediately after stimulation, these results suggested that SOCS1 deficiency impaired cytokine receptor signaling as a result of decreased steady state levels of critical signal transduction molecules.

**SOCS1 deficiency causes a selective loss of several signaling proteins in mast cells.** To further investigate the selective loss of certain signaling proteins in SOCS1 deficient BMMC, we examined other signaling molecules known to be phosphorylated by IL-3R signaling. When the proteins were immunoprecipitated after lysing cells in non-ionic detergents, the loss of JAK2 and STAT5 in SOCS1 deficient cells was even more dramatic (Fig. 5A). In addition to JAK2 and
STAT5, the levels of the p85 regulatory subunit of PI3K and Vav were markedly diminished in SOCS1–/– BMMC, while the protein levels of the loading control Hsp70 was comparable between the SOCS1–/– and SOCS1+/+ BMMC (Fig. 5A). Examination of the insoluble pellet showed that the vanishing proteins did not redistribute into the nuclear/cytoskeletal pellet (Fig. 5B). Fibroblasts and macrophages derived from SOCS1–/– mice did not show a decrease in the levels of STAT5 or p85, indicating that the defect is mast cell-specific (Fig. 5C and data not shown). The observation that the protein loss was accentuated by non-ionic detergent lysis on ice for 30 min (Fig. 5) compared to SDS lysis (Fig. 4) suggested that the protein loss might be mediated by degradation rather than by decreased expression. This notion was further supported by the observation that p85 and Vav immunoprecipitated from SOCS1 deficient BMMC, but not wild type BMMC, showed low molecular mass protein bands (Fig. 5A, arrowheads) which could represent degradation products.

Absence of SOCS1 in mast cells dysregulates mast cell protease activity. Mast cells are rich sources of proteases stored in secretory granules, which mediate the diverse pathophysiological functions of mast cells (33). In normal mast cells, the proteolytic activity of these enzymes is tightly regulated to prevent cellular protein degradation and tissue injury (33). To directly address whether SOCS1 deficiency leads to the degradation of cellular proteins by dysregulating the mast cell proteases, or by somehow altering the susceptibility of cellular proteins to mast cell proteases, we immunoprecipitated STAT5, p85, Vav and Lyn from wild type BMMC. The immunoprecipitated proteins bound to the protein G-speharose beads were washed thoroughly, and exposed to serially diluted whole cell lysates of SOCS1 deficient BMMC. The beads were subsequently washed to remove the SOCS1–/– mast cell lysate and
examined for the decrease in the amount of proteins retained on the beads. Following incubation, the amount of immunoprecipitated STAT5 was decreased by the SOCS1 deficient mast cell lysate in a dose-dependent manner (Fig. 6). Exposure to SOCS1 deficient mast cell lysate caused only a marginal reduction in the signal intensity of immunoprecipitated p85 or Vav, but interestingly, generated the same lower molecular mass products as seen in SOCS1 deficient mast cell lysates (Fig. 6 and 5A). This effect was specific to a subset of cellular proteins, as SOCS1 deficient mast cell lysate did not degrade Lyn immunoprecipitated from wild type cells (see Fig. 7B.2). These results strongly supported the notion that SOCS1 deficiency dysregulated the mast cell proteolytic enzymes.

Mast cell protease dysregulated by the absence of SOCS1 is a tryptase-like serine protease. Non-ionic detergent-lysates of both SOCS1 deficient and SOCS1 sufficient BMMC (Fig. 5) contained Complete protease inhibitor cocktail with broad specificity for serine, cysteine and metallo- proteases, which was apparently ineffective in inhibiting the dysregulated protease(s) of SOCS1 deficient BMMC. In an attempt to find an inhibitor of the dysregulated proteases of SOCS1 deficient BMMC and to characterize the protease activities, we lysed the SOCS1 deficient BMMC in buffers containing a wide variety of protease and proteasome inhibitors. Clearly, PMSF and soybean trypsin inhibitor (STI), and to a lesser extent AEBSF, blocked the degradation of STAT5 in SOCS1 deficient BMMC (Fig. 7A and 7B.1). PMSF did not affect STAT5 in wild type cells (Fig. 7B.1). All other inhibitors were ineffective in preventing STAT5 degradation in knockout cell lysates. PMSF and AEBSF also completely inhibited the SOCS1 deficient mast cell lysate from degrading STAT5 and p85 immunoprecipitated from wild type BMMC (Fig. 7B.2). In addition, the dysregulated protease
activity present in SOCS1 deficient mast cell lysate was completely depleted by STI bound to agarose beads (Fig. 7C). These results demonstrated that SOCS1 deficiency dysregulated a tryptase-like serine protease in mast cells.

Whereas PMSF and AEBSF totally inhibited the dysregulated proteases of SOCS1 deficient BMMC, Complete protease inhibitor cocktail and leupeptin only partially inhibited the proteases generating the lower molecular mass degradation product of p85, and aprotinin was completely ineffective against the proteolytic enzymes (Fig. 7B.2). These results suggested that either the dysregulated mast cell protease shows differential susceptibility to various inhibitors, or SOCS1 deficient BMMC contained more than one species of dysregulated proteolytic enzymes.

Dysregulated protease in SOCS1 mast cell is not mMCP-7. During the course of our studies, we observed that the phenotype of SOCS1 deficient mast cells showed considerable variability (Fig. 8A). The expression of many mast cell proteases is controlled by transcriptional, post-transcriptional, translational and post-translational regulatory mechanisms (33). GATA family transcription factors and the mi-transcription factor (MITF) regulate tissue and strain-specific expression of mast cell proteases. We have been breeding the SOCS1-/- mice into C57BL6 background, and C57BL6 mice do not express mMCP-7 due to a point mutation which creates an alternate splice site resulting in 98-bp deletion within the transcript and a premature stop codon (34). Therefore, to examine whether the variable phenotype of SOCS1-/- BMMC could be due to variable expression of a protease such as mMCP-7, we examined the mRNA levels of several mast cell proteases, including mMCP-7, by RT-PCR. Our results show that the growth phenotype of SOCS1 deficient BMMC did not correlate with the transcription of any of the mMCP genes we have tested (Fig. 8B). Since the protease inhibitor experiments have
indicated that the dysregulated protease is a tryptase-like serine protease, we further examined whether the dysregulated protease in SOCS1 deficient cells is mMCP-7 using a specific assay described by Stevens and colleagues. Recombinant mMCP-7 specifically cleaves the $\alpha$-isomer of fibrinogen and this activity is not blocked by the natural protease inhibitors present in mouse plasma (35). When incubated with fibrinogen, SOCS1 deficient mast cell lysate specifically cleaved the $\alpha$-isomer (Fig. 9, upper panel), however this cleavage generated a smaller sized product which was also generated by trypsin, but not by mMCP-7 (35). In addition, unlike mMCP-7, cleavage of $\alpha$-fibrinogen by the dysregulated protease of SOCS1 deficient BMMC was completely inhibited by pre-incubation with normal mouse plasma (Fig. 9, lower panel). These results indicate that expression of the mast cell protease regulated by SOCS1 is genetically variable, however it is not mMCP-7.

*Dysregulated proteolytic activity in SOCS1 deficient mast cells is due to the lack of an endogenous protease inhibitor.* The enzymatic activity of proteases inside mast cells is regulated mainly by compartmentalization within the secretory granules by molecular chaperones (33). Dysregulation of proteases in SOCS1 deficient BMMC did not result from the disruption of this compartmentalization because lysis of wild type BMMC did not cause protein degradation. To investigate whether dysregulation of mast cell proteases in the absence of SOCS1 was due to destabilization of a putative chaperone or inhibitor, we exposed STAT5, p85 and Vav immunoprecipitated from wild type BMMC to SOCS1 deficient mast cell lysate serially diluted with wild type cell lysate. While undiluted SOCS1 deficient mast cell lysate degraded STAT5, p85 and Vav, a 20% supplementation with wild type lysate was enough to inhibit this activity (Fig. 10A). Dilution of the SOCS1 deficient mast cell lysate with buffer alone did not inhibit the
proteolytic activity (see Fig. 6). The inhibitory activity was present in $SOCS1^{+/+}$ and $SOCS1^{+/-}$ BMMC, but absent from all $SOCS1^{-/-}$ BMMC which showed loss of STAT5 (Fig. 10B). The endogenous inhibitor present in wild type BMMC was inactivated by heat treatment at 70°C for 10 min (Fig. 10C), indicating that the inhibitor is a heat-labile factor.

**Discussion**

Stimulation of the IL-3 receptor activates the JAK-STAT pathway primarily involving JAK2 and STAT5, although other JAK kinases and STAT molecules have also been implicated (32). According to the current model of SOCS1 as a negative feedback regulator of JAK kinases, SOCS1 deficiency was expected to cause uncontrolled growth of mast cells if SOCS1 played a non-redundant role in regulating IL-3R signaling and was not expected to cause growth retardation in response to IL-3 or SCF. Investigating this unexpected mast cell phenotype led to the finding that mast cell proteolytic activity is dysregulated in the absence of SOCS1, and SOCS1 is a critical regulator of an endogenous inhibitor of a tryptase type mast cell serine protease.

Upon activation via cross-linking of the FcRI by antigens, mast cells liberate a number of bioactive molecules including several proteases (23). Proteases constitute as much as 30% of the total protein mass in a mature mast cell (36). While the expression of these proteases is regulated at transcriptional, post-transcriptional, translational and post-translational levels, their activity appears to be regulated primarily by compartmentalization (33). Newly synthesized inactive zymogens of the proteases, by virtue of their association with the glycosaminoglycan
side chains of serglycin proteoglycans, are targeted to the secretory granules where they become activated and remain tethered to the serglycin proteoglycans. However, the activity of these enzymes is prevented from causing cellular autolysis by the low pH within the confines of the secretory granules. A decrease in the steady state protein levels of STAT5, p85 and Vav, but not Lyn or several other proteins in SOCS1−/− BMMC, and a direct demonstration of such substrate-specific proteolytic activity in SOCS1−/− cells suggest that SOCS1 deficiency may compromise the intracellular regulation of mast cell proteases leading to the degradation of susceptible target proteins which are vital for cell proliferation.

Most of the mast cell granule proteases are active at neutral pH, and fall into 3 families, namely chymases, tryptases and carboxy peptidase-A (33). The identity of the protease(s) dysregulated in SOCS1−/− BMMC is not known, however they are susceptible to inhibition by only certain protease inhibitors. The in vitro activity of the dysregulated protease(s) in SOCS1−/− BMMC was unaffected by several protease inhibitors capable of blocking diverse classes of proteolytic enzymes. Among the serine protease inhibitors, PMSF, soy bean trypsin inhibitor and AEBSF but not Aprotinin, 3,4-dichloroisocoumarin, TPCK or TLCK, suggesting that the dysregulated protease(s) in SOCS1−/− BMMC is a tryptase type serine protease. Murine mast cells contain at least four tryptases, namely mMCP-6, mMCP-7, transmembrane tryptase (mTMT) and mT4 (37-40). Failure to inhibit the proteolytic activity of SOCS1−/− mast cells by heparin antagonist polybrene (Fig. 7) suggests that the dysregulated enzyme is unlikely to be mMCP-6 (41). Purification and biochemical characterization of the mast cell protease(s), which are deregulated because of SOCS1 deficiency, is hampered by the very slow growth of SOCS1−/− BMMC. Our attempts to immortalize SOCS1−/− BMMC by Ablesson murine leukemia virus (42) was unsuccessful probably because v-Abl-mediated cellular transformation proceeds via
constitutive activation of the JAK-STAT pathway (43). Scanning the primary amino acid sequence of murine p85 has revealed a few potential cleavage sites with Ser-Ser-Arg/Lys-residues at P3, P2 and P1 positions specific for mMCP-7 (35), raising the possibility that mMCP-7 could be one of the mast cell proteases dysregulated in the absence of SOCS1. Direct examination of this possibility using the -fibrinogen degradation assay (35), revealed that the dysregulated protease of SOCS1 deficient BMMC resembles mMCP-7 in specifically cleaving -fibrinogen. However, the protease regulated by SOCS1 is unlikely to be mMCP-7 because (i) it generates a proteolytic fragment that was not observed following mMCP-7 digestion, and (ii) unlike mMCP-7, the dysregulated protease of SOCS1 deficient BMMC was completely inhibited by mouse plasma. Whether the mast cell protease regulated by SOCS1 is mTMT, mT4 or an unknown tryptase remains to be explored.

After degranulation, most of the mast cell proteases are still in their complex form around the degranulated cells and thus have restricted substrate availability, whereas mMCP-7 is destabilized from its complex form and diffuses freely into the bloodstream (36). A majority of the exocytosed enzymes are inactivated following endocytic clearance by the adjacent cells (44) as well as by protease inhibitors which constitute >10% of the plasma proteins (33). Susceptibility of the mast cell proteases to these natural protease inhibitors seems to vary greatly, as mMCP-6 and mMCP-7 are ~75% identical in their amino acid sequence, yet only mMCP-7 is resistant to inhibition by plasma protease inhibitors (45). The mechanism by which SOCS1 deficiency activates the mast cell proteases appears to be indirect via regulation of an endogenous mast cell protease inhibitor (Fig. 11). The genetic variability in the growth phenotype and degradation of signaling proteins in SOCS1 deficient mast cells (Fig. 8) suggests that, in addition to the proteases, the endogenous protease inhibitor(s) of mast cells may also be
differentially expressed in different mouse strains, resulting in differential activity. The putative tryptase inhibitor, which is present in SOCS1 sufficient BMMC but lacking in SOCS1+/− BMMC, is likely to regulate the proteolytic enzymes directly rather than by regulating their compartmentalization, because breakdown of this compartmentalization by detergent solubilization did not degrade the target proteins in SOCS1+/− BMMC. Therefore, by regulating the expression and/or the stability of the putative inhibitor, SOCS1 complements the control of mast cell proteases by compartmentalization. Such a positive regulatory role for SOCS1 could be similar to its requirement for inducible MHC-class-II expression in fibroblasts (30).

In addition to being the primary mediators of atopic responses, mast cells play crucial roles in innate and acquired immunity (24,46,47). The precise roles for many of the secretory granule proteases in mast cell functions are unclear, however some mast cell proteases have been demonstrated to mediate critical immune defense mechanisms (33). Mice deficient in mMCP-1 are defective in the expulsion of the nematode Trichinella spiralis (48). The distinct biochemical and functional characteristics of mMCP-6 and mMCP-7 suggest a co-ordinate action of these proteases in inflammatory lesions (33). While the localized action of mMCP-6 stimulates IL-8 synthesis from bystander cells, the fibrinolytic activity of mMCP-7 keeps the blood/endothelial barrier open for the influx of neutrophils migrating towards the IL-8 gradient (35,49). The mast cell tryptases mediate diverse biological activities which contribute to airway hyper-reactivity in asthma (50). Naturally, the activities of mast cell proteolytic enzymes must be regulated both within the cell as well in the extracellular fluid to prevent indiscriminate damage to tissues. Since mast cells secrete as well as respond to many cytokines that can induce SOCS1 expression, it is conceivable that SOCS1 may function as a house-keeping gene in mast cells to prevent them from autolysis by their own proteases. Identification of the mast cell proteases
regulated by SOCS1 and understanding the underlying mechanisms will unravel the cytokine regulation of mast cell proteolytic machinery.

References

1. Watowich, S. S., Wu, H., Socolovsky, M., Klingmuller, U., Constantinescu, S. N., and Lodish, H. F. (1996) Annu Rev Cell Dev Biol 12, 91-128
2. Ward, A. C., Touw, I., and Yoshimura, A. (2000) Blood 95, 19-29.
3. Leonard, W. J., and Lin, J. X. (2000) J Allergy Clin Immunol 105, 877-888.
4. O'Shea, J. J., Gadina, M., and Schreiber, R. D. (2002) Cell 109 Suppl, S121-131.
5. Ihle, J. N. (2001) Curr Opin Cell Biol 13, 211-217.
6. Starr, R., and Hilton, D. J. (1999) Bioessays 21, 47-52.
7. Yasukawa, H., Sasaki, A., and Yoshimura, A. (2000) Annu Rev Immunol 18, 143-164
8. Shuai, K. (2000) Oncogene 19, 2638-2644.
9. Alexander, W. S. (2002) Nat Rev Immunol 2, 1-7
10. Kile, B. T., Schulman, B. A., Alexander, W. S., Nicola, N. A., Martin, H. M., and Hilton, D. J. (2002) Trends Biochem Sci 27, 235-241.
11. Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Matsumoto, A., Tanimura, S., Ohtsubo, M., Misawa, H., Miyazaki, T., Leonor, N., Taniguchi, T., Fujita, T., Kanakura, Y., Komiya, S., and Yoshimura, A. (1997) Nature 387, 921-924.
12. Naka, T., Narazaki, M., Hirata, M., Matsumoto, T., Minamoto, S., Aono, A., Nishimoto, N., Kajita, T., Taga, T., Yoshizaki, K., Akira, S., and Kishimoto, T. (1997) *Nature* **387**, 924-929.

13. Narazaki, M., Fujimoto, M., Matsumoto, T., Morita, Y., Saito, H., Kajita, T., Yoshizaki, K., Naka, T., and Kishimoto, T. (1998) *Proc Natl Acad Sci U S A* **95**, 13130-13134.

14. Nicholson, S. E., Willson, T. A., Farley, A., Starr, R., Zhang, J. G., Baca, M., Alexander, W. S., Metcalf, D., Hilton, D. J., and Nicola, N. A. (1999) *Embo J* **18**, 375-385.

15. Yasukawa, H., Misawa, H., Sakamoto, H., Masuhara, M., Sasaki, A., Wakioka, T., Ohtsuka, S., Imaizumi, T., Matsuda, T., Ihle, J. N., and Yoshimura, A. (1999) *Embo J* **18**, 1309-1320.

16. De Sepulveda, P., Okkenhaug, K., Rose, J. L., Hawley, R. G., Dubreuil, P., and Rottapel, R. (1999) *Embo J* **18**, 904-915.

17. Frantsve, J., Schwaller, J., Sternberg, D. W., Kutok, J., and Gilliland, D. G. (2001) *Mol Cell Biol* **21**, 3547-3557.

18. Kamizono, S., Hanada, T., Yasukawa, H., Minoguchi, S., Kato, R., Minoguchi, M., Hattori, K., Hatakayama, S., Yada, M., Morita, S., Kitamura, T., Kato, H., Nakayama, K., and Yoshimura, A. (2001) *J Biol Chem* **276**, 12530-12538.

19. Ungureanu, D., Saharinen, P., Juntila, I., Hilton, D. J., and Silvennoinen, O. (2002) *Mol Cell Biol* **22**, 3316-3326.

20. Rottapel, R., Ilangumaran, S., Neale, C., La Rose, J., Ho, J. M., Nguyen, M. H., Barber, D., Dubreuil, P., and de Sepulveda, P. (2002) *Oncogene* **21**, 4351-4362.

21. Tyers, M., and Rottapel, R. (1999) *Proc Natl Acad Sci U S A* **96**, 12230-12232.
22. De Sepulveda, P., Ilangumaran, S., and Rottapel, R. (2000) *J Biol Chem* **275**, 14005-14008.

23. Stevens, R. L., and Austen, K. F. (1989) *Immunol Today* **10**, 381-386.

24. Gurish, M. F., and Austen, K. F. (2001) *J Exp Med* **194**, F1-5.

25. Galli, S. J., and Hammel, I. (1994) *Curr Opin Hematol* **1**, 33-39.

26. Metcalfe, D. D., Baram, D., and Mekori, Y. A. (1997) *Physiol Rev* **77**, 1033-1079.

27. Karasuyama, H., and Melchers, F. (1988) *Eur J Immunol* **18**, 97-104.

28. Alexander, W. S., Starr, R., Fenner, J. E., Scott, C. L., Handman, E., Sprigg, N. S., Corbin, J. E., Cornish, A. L., Darwiche, R., Owczarek, C. M., Kay, T. W., Nicola, N. A., Hertzog, P. J., Metcalf, D., and Hilton, D. J. (1999) *Cell* **98**, 597-608.

29. Marine, J. C., Topham, D. J., McKay, C., Wang, D., Parganas, E., Stravopodis, D., Yoshimura, A., and Ihle, J. N. (1999) *Cell* **98**, 609-616.

30. Ilangumaran, S., Finan, D., La Rose, J., Raine, J., Silverstein, A., De Sepulveda, P., and Rottapel, R. (2002) *J Immunol* **169**, 5010-5020.

31. Gordon, J. R., Burd, P. R., and Galli, S. J. (1990) *Immunol Today* **11**, 458-464.

32. Reddy, E. P., Korapati, A., Chaturvedi, P., and Rane, S. (2000) *Oncogene* **19**, 2532-2547.

33. Huang, C., Sali, A., and Stevens, R. L. (1998) *J Clin Immunol* **18**, 169-183.

34. Hunt, J. E., Stevens, R. L., Austen, K. F., Zhang, J., Xia, Z., and Ghildyal, N. (1996) *J Biol Chem* **271**, 2851-2855

35. Huang, C., Wong, G. W., Ghildyal, N., Gurish, M. F., Sali, A., Matsumoto, R., Qiu, W. T., and Stevens, R. L. (1997) *J Biol Chem* **272**, 31885-31893.

36. Matsumoto, R., Sali, A., Ghildyal, N., Karplus, M., and Stevens, R. L. (1995) *J Biol Chem* **270**, 19524-19531.
37. Reynolds, D. S., Gurley, D. S., Austen, K. F., and Serafin, W. E. (1991) *J Biol Chem* **266**, 3847-3853.
38. McNeil, H. P., Reynolds, D. S., Schiller, V., Ghildyal, N., Gurley, D. S., Austen, K. F., and Stevens, R. L. (1992) *Proc Natl Acad Sci U S A* **89**, 11174-11178.
39. Wong, G. W., Tang, Y., Feyfant, E., Sali, A., Li, L., Li, Y., Huang, C., Friend, D. S., Krilis, S. A., and Stevens, R. L. (1999) *J Biol Chem* **274**, 30784-30793.
40. Wong, G. W., Li, L., Madhusudhan, M. S., Krilis, S. A., Gurish, M. F., Rothenberg, M. E., Sali, A., and Stevens, R. L. (2001) *J Biol Chem* **276**, 20648-20658.
41. Hallgren, J., Karlson, U., Poorafshar, M., Hellman, L., and Pejler, G. (2000) *Biochemistry* **39**, 13068-13077.
42. Pierce, J. H., Di Fiore, P. P., Aaronson, S. A., Potter, M., Pumphrey, J., Scott, A., and Ihle, J. N. (1985) *Cell* **41**, 685-693.
43. Danial, N. N., Losman, J. A., Lu, T., Yip, N., Krishnan, K., Krolewski, J., Goff, S. P., Wang, J. Y., and Rothman, P. B. (1998) *Mol Cell Biol* **18**, 6795-6804.
44. Atkins, F. M., Friedman, M. M., and Metcalfe, D. D. (1985) *Lab Invest* **52**, 278-286.
45. Ghildyal, N., Friend, D. S., Stevens, R. L., Austen, K. F., Huang, C., Penrose, J. F., Sali, A., and Gurish, M. F. (1996) *J Exp Med* **184**, 1061-1073.
46. Wedemeyer, J., Tsai, M., and Galli, S. J. (2000) *Curr Opin Immunol* **12**, 624-631.
47. Malaviya, R., Navara, C., and Uckun, F. M. (2001) *Immunity* **15**, 313-321.
48. Knight, P. A., Wright, S. H., Lawrence, C. E., Paterson, Y. Y., and Miller, H. R. (2000) *J Exp Med* **192**, 1849-1856.
49. Cairns, J. A., and Walls, A. F. (1996) *J Immunol* **156**, 275-283.
50. Sommerhoff, C. P. (2001) *Am J Respir Crit Care Med* **164**, S52-58.
Footnotes

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1Abbreviations used in this paper: BMMC, bone marrow-derived mast cells; SCF, stem cell factor; SOCS1, suppressor of cytokine signaling 1.

Figure legends

**FIG. 1.** **SOCS1 deficiency compromises the cytokine-dependent growth of mast cells.** (A) Phenotypic characterization of *SOCS1*^-/-^ BMMC. Bone marrow-derived mast cell lines established from *SOCS1*^-/-^, *SOCS1*^+/-^ and *SOCS1*^+/-^ mice were stained for the indicated cell surface markers as described in Materials and Methods, and analyzed by a flow cytometer. Solid lines represent the expression level whereas the broken lines show the binding of isotype-specific control antibodies. In the FcRI panel, the broken line represents the binding of anti-IgE antibody in the absence of anti-TNP IgE. (B) *SOCS1*^-/-^ BMMC show retarded growth in IL-3-stimulated cultures. 2.5 × 10^5 *SOCS1*^+/-^, *SOCS1*^+/-^ or *SOCS1*^-/-^ BMMC were stimulated with 1:100 dilution of the IL-3 containing CS in 1 ml volume in 48-well culture dishes. At indicated time points, the cells were harvested and counted. Growth kinetics of one *SOCS1*^+/-^ cell line and three lines each of *SOCS1*^+/-^ and *SOCS1*^-/-^ cells are shown. (C) *SOCS1*^-/-^ mast cells respond poorly to IL-3 and SCF. 2.5 × 10^3 cells of *SOCS1*^+/-^, *SOCS1*^+/-^ and *SOCS1*^-/-^ mast cell lines were seeded in 96 well microtiter plates and stimulated with indicated concentrations of IL-3 or SCF.
in a total volume of 200 µl. After 36 h the cultures were pulsed with 1 µCi of [3H]-thymidine for 12 h and the incorporated radioactivity was quantitated. Representative results from one of several experiments including three lines each of SOCSI⁺/⁻ and SOCSI⁻/⁻ cells, and one SOCSI⁺/+ cell line are shown.

**FIG. 2. SOCSI⁻/⁻ mast cells undergo increased apoptosis and show defective cell cycle entry.** (A) Increased frequency of Annexin-V positive cells in SOCSI⁻/⁻ mast cell cultures. SOCSI⁺/⁻, SOCSI⁺/+ and SOCSI⁻/⁻ mast cell lines were starved of IL-3 overnight and then stimulated with 1:100 dilution of the IL-3 containing CS at a final concentration of 2.5 ×10⁵ cells/ml. At 24 h and 72h after stimulation, the cells were harvested and stained for apoptosis with Annexin V. Representative data from several mast cell lines of each SOCSI genotype are shown. (B) Cell cycle analysis of SOCSI⁻/⁻ mast cell cultures. SOCSI⁺/⁻, SOCSI⁺/+ and SOCSI⁻/⁻ mast cell lines in steady state growth, or 12, 36 or 60 h after IL-3 stimulation were labeled with propidium idodide for cell cycle analysis. The percentage of cells in the S phase, indicated within each histogram, was calculated using the ModFit software. Representative data from several independent experiments are shown.

**FIG. 3. Retarded growth of SOCSI⁻/⁻ mast cells is due to a cell autonomous defect.** (A) SOCSI⁻/⁻ BMMC do not express IFNα. SOCSI⁺/⁻, SOCSI⁺/+ and SOCSI⁻/⁻ mast cell lines in steady state growth in IL-3 containing medium, and total lymph node (LN) cells stimulated with PMA+ionomycin were evaluated for the expression of indicated cytokine genes by RT-PCR. (B) Neutralizing anti-IFNα antibody did not restore the proliferative potential of SOCSI⁻/⁻ BMMC. 2.5 ×10³ SOCSI⁺/⁻, SOCSI⁺/+ or SOCSI⁻/⁻ BMMC were seeded in 96 well microtiter plates and
stimulated with IL-3 or SCF, or left unstimulated, in the presence or absence of neutralizing anti-
IFN-α antibody in a total volume of 200 µl. After 36 h the cultures were pulsed with 1 µCi of
[3H]-thymidine for 12 h and then harvested onto glass fiber filters to measure the incorporated
radioactivity. (C) SOCS1−/− BMMC do not secrete a growth inhibitor. 2.5 × 10^3 wild type BMMC
were cultured without or with IL-3 in conditioned medium collected from 24 h cultures of
SOCS1+/−, SOCS1+/+ or SOCS1−/− BMMC diluted 1:1 with fresh culture medium. After 36 h, the
cultures were pulsed with 1 µCi of [3H]-thymidine for 12 h and the incorporated radioactivity
was quantitated. (D) Defective growth response of SOCS1−/− BMMC is cell intrinsic. SOCS1+/+
BMMC were labeled with CFSE as described in Materials and Methods, mixed with unlabelled
knock out (KO) or WT BMMC at 1:1 ratio and cultured in the presence of IL-3. At indicated
time points, the cells were analyzed for dilution of CFSE. The unlabelled cells which give a
negative peak are identified with an U. Data from one of the two experiments performed is
shown.

**FIG. 4.** Defective IL-3 receptor signaling in SOCS1−/− mast cells. After overnight starvation of
IL-3, equivalent numbers of SOCS1+/− and SOCS1−/− BMMC were stimulated with IL-3 for 10
min before lysis in SDS-PAGE sample buffer. Proteins separated by SDS-PAGE were
transferred to PVDF membranes for probing with indicated antibodies followed by ECL
detection of specific proteins.

**FIG. 5.** SOCS1 deficiency causes a selective loss of certain signaling proteins in mast cells.
(A) Non-ionic detergent lysis accentuates the loss of some signaling proteins in SOCS1−/−
BMMC. Equivalent numbers of SOCS1+/−, SOCS1+/+ and SOCS1−/− BMMC, after IL-3
deprivation overnight, were stimulated with IL-3 for 10 min. The cells were lysed in TNN lysis buffer on ice for 30 min at 10 ×10^6 cells/ml and cleared by centrifugation at 10,000 g for 10 min. JAK2, STAT5, p85 and Vav were sequentially immunoprecipitated from 1 ml of the lysate and detected by western blot. The smaller molecular mass bands of p85 and Vav detected only in SOCS1 deficient mast cell lysates are indicated by arrow heads. Hsp70 in total lysates was used as control. (B) The loss of signaling proteins from SOCSI−/− mice was not due to detergent insolubility. The detergent-insoluble pellet from (A) was solubilized by boiling in SDS-PAGE sample buffer, diluted 1:10 with TNN buffer before immunoprecipitation and western blot detection. (C) The loss of specific signaling proteins due to SOCS1 deficiency is mast cell-specific. Lysates from several SOCSI+/+ and SOCSI−/− fibroblasts and macrophages were probed for STAT5, p85 or actin.

**Fig. 6. SOCS1 deficiency deregulates mast cell proteolytic activity.** SOCSI−/− BMMC were lysed at 10 ×10^6 cells/ml concentration in TNN lysis buffer containing Complete protease inhibitor cocktail. STAT5, p85 and Vav proteins were immunoprecipitated from wild type BMMC. The immunoprecipitated beads were incubated overnight at 4°C with SOCSI−/− mast cell lysate diluted with the lysis buffer at indicated ratios. The beads were washed and proteins were eluted by boiling in SDS sample buffer, separated by SDS-PAGE, blotted onto PVDF membranes and probed for the respective antibodies. The degradation products of p85 and Vav from wild type SOCS1 cells are indicated by arrow heads.

**Fig. 7. Dysregulated protease of SOCSI−/− mast cells is a tryptase-like serine protease.** (A) Protease inhibitor profile of the dysregulated proteases of SOCSI−/− BMMC. SOCSI−/− BMMC
were lysed in TNN buffer containing the indicated protease and proteasome inhibitors, and the lysates were probed for STAT5b. SOCS1+/+ BMMC lysed in TNN buffer containing the CÔmplete® protease inhibitor cocktail was used as a control. (B) Differential susceptibility of the dysregulated proteases of SOCS1−/− BMMC to serine protease inhibitors. (a) SOCS1−/− BMMC lysed in TNN buffer containing indicated protease and protease inhibitors were probed for STAT5b and Lyn. (b) STAT5, p85 and Lyn proteins immunoprecipitated from SOCS1+/+ BMMC were incubated with SOCS1−/− mast cell lysates made with different protease inhibitors as described in Fig. 6. After overnight incubation, proteins that remained bound to the beads were eluted and probed for the respective proteins. (C) Depletion of the dysregulated proteases of SOCS1−/− BMMC by STI-agarose. SOCS1−/− mast cell lysate prepared without any protease inhibitor was precleared overnight with soy bean trypsin inhibitor bound to agarose beads, and then applied to STAT5b immunoprecipitated from SOCS1+/+ BMMC. After overnight incubation at 4°C, STAT5b remained bound to the beads was evaluated by western blot. For comparison, SOCS1−/− mast cell lysates supplemented with STI or PMSF were used to show direct inhibition of the proteases.

**FIG. 8. Expression of mMCP-7 does not correlate with the phenotype of SOCS1−/− mast cells.** (A) Variability in the phenotype of SOCS1+/+ BMMC. Different batches of BMMC derived from SOCS1−/− mice in F4-F6 generation into C57BL6 background were tested for the degradation of STAT5 by western blot. The growth phenotype of the cells correlated with STAT5 levels (not shown). (B) Expression of several mast cell proteases in SOCS1−/− BMMC does not correlate with the growth phenotype. Expression of various mMCPs was examined by RT-PCR analysis of
RNA extracted from several mast cell lines as described in materials and methods. Expression of actin and G3PDH served as cDNA loading controls. Growth phenotype: N, normal; R, retarded.

**Fig. 9.** The mast cell protease regulated by SOCS1 specifically cleaves fibrinogen, and is inhibited by protease inhibitors present in plasma. Fifteen µg of fibrinogen was incubated for 1 h at 37°C with SOCS1−/− or SOCS1+/+ BMMC lysate containing 20 µg of total proteins, or 1 µg of trypsin. At the end of the reaction, the proteins were separated in SDS-PAGE gels and stained with coomassie brilliant blue. In the lower panel, PMSF or normal mouse plasma was added to the BMMC lysates and trypsin 10 min before incubation with fibrinogen.

**Fig. 10.** Lack of an endogenous protease inhibitor dysregulates the protease activity in SOCS1−/− mast cells. (A) Dyregulated proteolytic activity of SOCS1−/− BMMC is inhibited by SOCS1+/+ mast cell lysate. SOCS1−/− mast cell lysate prepared without any protease inhibitor was used either alone, or after dilution with SOCS1+/+ mast cell lysates containing Complete protease inhibitor cocktail at indicated ratios, on STAT5b, p85 or Vav immunoprecipitated from SOCS1+/+ BMMC as described in Fig. 6. After overnight incubation at 4°C, proteins that remained bound to the beads were evaluated by western blot using corresponding antibodies. (B) SOCS1+/+ BMMC contain an endogenous protease inhibitor. Representative SOCS1+/− and SOCS1−/− mast cell lysates were tested for the presence of the inhibitor of the proteases as described in Fig. 8A. (C) Endogenous inhibitor of the mast cell proteases is heat-labile. SOCS1+/+ mast cell lysates containing Complete protease inhibitor cocktail was heat inactivated at 70°C for 10 min. STAT5 immunoprecipitated from SOCS1+/+ BMMC was incubated with SOCS1−/− mast cell lysate diluted 1:1 with untreated or heat-inactivated SOCS1+/+
mast cell lysate. After overnight incubation at 4°C, STAT5b remaining on the beads was evaluated by western blot.

**FIG. 11. Schematic representation of the role of SOCS1 in regulating mast cell proteases.**

Following IL-3 or c-Kit stimulation of mast cells, SOCS1 is rapidly induced as a negative feedback regulator of JAK kinases. In the absence of SOCS1, the mast cell proteases stored in secretory granules seem to degrade some of the important signaling proteins downstream of the cytokine receptors. While compartmentalization prevents such degradation of cellular proteins to a large extent, SOCS1 seems to be required for absolute control of the proteases by regulating the expression and/or stability of a putative, endogenous inhibitor. The protease(s) regulated by SOCS1 appears to be a tryptase-like serine protease, however the identity of both the dysregulated protease and the putative endogenous inhibitor are not yet known.
Fig. 1, Ilangumaran et al.,
Fig. 2, Ilangumaran et al.
Fig. 3, Ilangumaran et al.,
### Fig. 4, Ilangumaran et al.,

| SOCS1  | +/- | -/- |
|-------|-----|-----|
| IL-3  |    - |   + |
|       |    - |   + |
| P-Tyr |    - |   + |

| SOCS1  | +/- | -/- |
|-------|-----|-----|
| IL-3  |    - |   + |
|       |    - |   + |

- P-JAK2
- JAK2
- P-STAT5
- STAT5
- Bax
- p27kip
- Actin
Fig. 5, Ilangumaran et al.,

| SOCS1 | MC Fibroblasts |
|-------|----------------|
| ++    |                 |
| +/-   |                 |
| -/    |                 |

IL-3

SOCS1

IL-3

SOCS1

++

+/-

-/

JAK2

STAT5

p85

Vav

Hsp70

Soluble Insoluble

Actin

p85

STAT5

JAK2

IL-3

SOCS1

SOCS1

+
SOCS1<sup>−/−</sup> Lysate:

| Buffer | 5:0 | 4:1 | 3:2 | 2:3 | 1:4 | 0:5 |
|--------|-----|-----|-----|-----|-----|-----|

- STAT5
- p85
- Vav

Fig. 6, Ilangumaran et al.,
Fig. 7, Ilangumaran et al.,
Fig. 8, Ilangumaran et al.
Fig. 9, Ilangumaran et al.
Fig. 10, Ilangumaran et al.
Secretory granule proteases
Inhibitor X
IL-3R
c-KIT
Mast cell
SOCS1
Inhibitor X
Secretory granule proteases
JAK2
STAT5
p85
Vav1
Proliferation

Fig. 11, Ilangumaran et al.
A

|   | 1 | 2 | 3 |
|---|---|---|---|
| phospho-STAT1 | None | IFN | (IFN + anti-IFN) |
| STAT1 | None | IFN |  |

B

| None | +IFN |
|------|------|
| x'' | x' |
| SOCS1 | STAT5 |
| p85 | Actin |

C

| IFN -/- | +/+ | +/+ | -/- | -/- | +/+ |
|---------|-----|-----|-----|-----|-----|
| SOCS1 | | | | | |
| STAT5 | | | | | |
| Lyn | | | | | |
| IFN | | | | | |

Supplement Fig. 1, Ilangumaran et al.,
LEGEND FOR SUPPLEMENTARY FIG. 1

(A) Neutralization of IFNα by anti-IFNα antibody. Semi-confluent monolayer of wild type mouse embryo fibroblasts in 35 mm dishes were starved of serum overnight and then stimulated with medium alone, 500 U/ml of IFNα or 500 U/ml of IFNα pre-incubated with 10 µg/ml of neutralizing anti-IFNα antibody. After 15 min stimulation, the cells were lysed in denaturing lysis buffer and examined for STAT1 phosphorylation by western blot. The blots were stripped and reprobed for STAT1.

(B) IFNα does not induce degradation of signaling proteins in wild type mast cells, or augment the protein loss in SOCS1−/− mast cells. Wild type and SOCS1−/− mast cells were stimulated with 1000 U/ml of IFNα for 16 h, lysed and probed for STAT5, p85 and actin by western blot. The arrow on p85 blot corresponds to the degradation product seen in SOCS1−/− mast cells.

(C) IFNα is not responsible for the degradation of signaling proteins in SOCS1−/− mast cells. BMMC mast cells from SOCS1−/−IFNα−/−, SOCS1+/−IFNα−/− and SOCS1+/− IFNα−/− mice (in F4-F6 generation into C57BL6 background) were examined for STAT5 expression with or without IFNα treatment (1000 U/ml for 16 h). Lyn served as loading control, and was probed in the same blot.
Suppressor of cytokine signaling 1 regulates an endogenous inhibitor of a mast cell protease

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