CD33 responses are blocked by SOCS3 through accelerated proteasomal-mediated turnover

Running Title: SOCS3 Blocks CD33-Mediated Responses


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Abstract

CD33 is a member of the Sialic acid binding immunoglobulin-like lectin (Siglec) family of inhibitory receptors and a therapeutic target for acute myeloid leukaemia (AML). It contains a cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM), which can recruit SHP-1 and SHP-2. How CD33 expression is regulated is unclear. Suppressor of cytokine signalling 3 (SOCS3) is expressed in response to cytokines, LPS and other PAMPs, and competes with SHP-1/2 binding to ITIMs of cytokine receptors thereby inhibiting signalling. In this study, using peptide pulldown experiments we find that SOCS3 can specifically bind to the phosphorylated ITIM of CD33. Additionally, following cross-linking SOCS3 can recruit the ECS E3 ligase resulting in accelerated proteasomal degradation of both CD33 and SOCS3. Our data suggests the tyrosine motifs in CD33 are not important for internalisation while they are required for degradation. Moreover, SOCS3 inhibited the CD33-induced block on cytokine-induced proliferation. This is the first receptor shown to be degraded by SOCS3 and where SOCS3 and its target protein are degraded concomitantly. Our findings clearly suggest that during an inflammatory response, the inhibitory receptor CD33 is lost by this mechanism. Moreover, this has important clinical implications as tumours expressing SOCS3 may be refractory to α-CD33 therapy.
Introduction

Regulation of the immune response is essential to balance the inflammatory process. The immunoreceptor tyrosine-based inhibition motif (ITIM) is found in the intracytoplasmic domain of inhibitory receptors such as Siglecs (Sialic acid-binding Immunoglobulin like lectin) and acts as a regulatory molecule to inhibit activation. The Siglec family of receptors includes Sialoadhesin (Sn), CD22, CD33, Myelin-associated glycoprotein (MAG) and CD33-related Siglecs 5-11.\textsuperscript{1} This family is characterised by an amino-terminal V-set immunoglobulin (Ig) domain, a varying number of C2-set Ig domains and a transmembrane domain, followed by a short cytoplasmic tail. The CD33-related Siglecs not only contain a cytoplasmic ITIM but also an immunoreceptor tyrosine based switch-like motif (ITSM). Ligand induced clustering of inhibitory receptors results in tyrosine phosphorylation of these ITIMs and recruitment of SH2 (Src homology 2) containing phosphatases (SHP-1/2) and inositol phosphatase (SHIP).\textsuperscript{2} The ITSM has previously been shown to switch between binding to SAP (SLAM-associated protein) and EAT-2 or between SAP and SHP-2 in other receptors.\textsuperscript{3} The Siglec receptors are all expressed on cells of the haematopoietic system, except for MAG, which is found exclusively in the nervous system. Siglec family members bind to specific glycan structures containing sialic acid.\textsuperscript{4} These are a large family of 9-carbon sugars, which are all derivatives of neuraminic (Neu) or ketodeoxynonulosonic acid (KDN). More than forty forms exist in nature, attached in a variety of linkages to other sugars, thereby generating a considerable degree of molecular diversity and specificity.\textsuperscript{5}

CD33 is a 67 kDa transmembrane glycoprotein that contains one V-set and one C2-set Ig-like domain\textsuperscript{6} and is specifically expressed on the myeloid lineage. It is a biomarker and therapeutic target for acute myeloid leukemia (AML). The recruitment of SHP-1 and SHP-2 by CD33 results in inhibition of tyrosine phosphorylation and Ca\textsuperscript{2+} mobilisation.\textsuperscript{7, 8} SHP-1 and SHP-2 are recruited to Y340 of CD33, whereas Y358
functions primarily in the recruitment of SHP-2. Engagement of CD33 on chronic and acute myeloid leukemias inhibits the proliferation of these cells and activates a process leading to apoptotic cell death on AML cells.\textsuperscript{9,10}

The suppressor of cytokine signalling (SOCS) proteins, particularly SOCS1, are essential for regulating the inflammatory process. Gene targeting approaches have shown that they play a non-redundant role limiting the inflammatory response. SOCS expression is induced by cytokines, infective PAMPs (Pathogen-associated molecular patterns) and other stimuli and they regulate cytokine signal transduction via a negative feedback loop.\textsuperscript{11} They are characterised by a phosphotyrosine binding SH2 domain and the SOCS box motif\textsuperscript{12} that interacts with Elongin B/C, Cul-5 and Rbx1/2 to form an ECS-like (Elongin B/C-Cul2/Cul5-SOCS-box protein) E3 ubiquitin ligase complex to target signalling intermediate proteins for proteasomal degradation.\textsuperscript{13}

SOCS3 can interact with a number of phosphorylated receptors and appears to potently inhibit JAKs in the presence of these receptors.\textsuperscript{14} SHP-1, SHP-2 and SOCS3 bind via their SH2 domains to ITIM-like motifs on cytokine receptors such as EpoR, LeptinR, GCSFR and gp130.\textsuperscript{15,16} Interestingly, the SOCS3 SH2 domain exhibits 39\% and 41\% homology to the SH2 domains of SHP-2 and SHP-1 respectively and is thought to compete with SHP-1/2 for binding.\textsuperscript{17} Given that SOCS3 is induced by cytokines, LPS and other PAMPs, we investigated whether SOCS3 could interact with the ITIMs on CD33 to regulate inhibitory responses. Here we show that SOCS3 binds to the phosphorylated ITIM and ITSM of CD33 resulting in accelerated CD33 proteasomal degradation and that SOCS3 blocks CD33-mediated inhibition of proliferation in a cytokine-inducible cell line.

Materials and methods

Antibodies
Phosphotyrosine monoclonal antibody, PY20 was purchased from Zymed (San Francisco, CA, USA). Monoclonal anti-CD33 (α-CD33) (My9) was a kind gift from Immunogen (Dr. W.A. Blattler, MA, USA). Flag M2 monoclonal antibody, Goat-Anti-Mouse whole molecule, monoclonal anti-His and anti-Myc were purchased from Sigma Aldrich (Dorset, UK). Polyclonal rabbit anti-SHP-2 and anti-SOCS3 (M20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal anti-CD33 (Clones 1C7/1 and 3D6/1) were provided by Cancer Research UK (London, UK). Polyclonal rabbit anti-STAT5B was a generous gift from Dr. J.J. O’Shea (NIH Bethesda, MD, USA). Anti-SOCS3 (008) was purchased from Fusion Antibodies (Belfast, N. Ireland). PE-conjugated anti-CD33 and IgG1 isotype control were obtained from BD Biosciences (San Jose, CA, USA).

**Plasmids and mutagenesis**

Complementary DNA (cDNA) for CD33WT was sub-cloned into pME18S Flag vector and site-directed mutagenesis was performed using the QuikChange Site-Directed Mutagenesis Kit according to the manufacturers’ instructions (Stratagene, La Jolla, CA, USA). CD33Y340F was created using the following mutagenic primers: 5’ GAT GAG GAG CTG CAT TTT GCT TCC CTC AAC TTT C 3’ and 5’ GAA AGT TGA GGG AAG CAA AAT GCA GCT CCT CAT C 3’. CD33Y340FY358F was created using both sets of mutagenic primers. Flag-tagged SOCS3-pME18S, Flag-tagged Elongin B and Myc-tagged Elongin C expression constructs were described previously. Rbx-1 and Cullin-5 cDNA constructs were a kind gift from Dr. J.W. Conaway (Stowers Institute for Medical Research, Kansas City, MO, USA).
Peptides

Biotinylated phosphorylated and unphosphorylated peptides that spanned the tyrosines (Y340 and Y358) of CD33 were synthesized by Chiron Biotechnologies (Raleigh, NC, USA). Each peptide contains four N-terminus amino acids (SGSG) for anchoring to the biotin moiety. Peptide sequences were as follows: Phosphorylated ITIM peptide (Biotin SGSGDEELHpYASLNF-OH), Unphosphorylated ITIM peptide (Biotin SGSGDEELHYASLNF-OH), Phosphorylated ITSM peptide (Biotin SGSGDTSTEpYSEVRT-OH), Unphosphorylated ITSM peptide (Biotin SGSGDTSTEYSEVRT-OH), Control TCR zeta chain ITAM Peptide (Biotin SGSGGHDGLYQGLST-OH).

Cells and transfections

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation of whole blood from healthy consenting adults. Informed consent was provided according to the Declaration of Helsinki and approval was obtained from the Northern Ireland Blood Transfusion Service for this study. The cells were plated in RPMI-1640 growth medium supplemented with 2% fetal calf serum (FCS), 100U/ml penicillin and 100U/ml streptomycin in 175cm² flasks for 2 h to allow monocytes/macrophages to adhere. The cells were washed with RPMI-1640 to remove contaminating T and B cells and the adherent monolayer was treated with 100ng/ml LPS in RPMI containing 2% FCS prior to cross-linking with α-CD33 (IC7/1) and Goat anti-mouse (GAM) whole molecule (IgG). CD33 stable Ba/F3 (mouse pro-B cell line) and Ba/F3-SOCS3 cells were maintained in RPMI-1640 supplemented with 5% FCS, 100U/ml penicillin, 100U/ml streptomycin and 10U/ml IL-3. CD33 stable Ba/F3 and Ba/F3-SOCS3 cells were produced by retroviral infection. Plat.-E (retroviral packaging cell line) cells were transfected with 5µg of pMX-IRES-EGFP CD33 constructs using FuGENE™ 6 (Roche, East Sussex, UK)
transfection reagent according to manufacturers’ instructions. The Plat.-E medium was used to infect Ba/F3 cells during centrifugation at 37˚C for 4 h. Live cells were sorted (EPICS ALTRA) for the presence of EGFP and cultured. The Ba/F3-SOCS3 cells were maintained in 4µg/ml tetracycline and were removed from tetracycline 48 h prior to stimulation to allow SOCS3 gene expression. 293T (human epithelial) cells were maintained in DMEM (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% FCS, 100U/ml penicillin and 100U/ml streptomycin. 293T cells were transfected with 2µg of the relevant constructs using FuGENETM 6 transfection reagents. After 24 h the transfection medium was replaced with fresh medium and cells were harvested after a further 24 h.

**Immunoprecipitations and western blotting**

The cells were washed once with ice cold phosphate buffered saline (PBS) containing 1mM Na3V04 and lysed on ice in RIPA lysis buffer (50mM Tris-HCl pH7.4, 150mM NaCl, 1% NP-40, 0.25% Na-Deoxycholate, 1mM EDTA, 1mM Na3V04, 10µg/ml leupeptin, 10µg/ml aprotinin and 1mM phenylmethylsulfonyl fluoride). After 15 min, the cell lysates were clarified by centrifugation at 12,000rpm at 4˚C and the supernatants immunoprecipitated with the appropriate antibodies preassociated to protein A-sepharose beads. For peptide pull-down experiments, cell lysates were incubated with 10µg of peptide bound to Streptavidin agarose beads for 2 h, washed, boiled in Laemmli buffer and separated by SDS-PAGE. For immunoblotting, whole cell lysates or immunoprecipitates were separated by SDS-PAGE, transferred to PVDF membrane and probed with appropriate antibodies, followed by peroxidase-labelled secondary antibodies. The proteins were visualized using the ECL detection method (Amersham, Buckinghamshire, UK).
**CD33 cross-linking**

CD33 stable Ba/F3 and Ba/F3-SOCS3 cells and PBMCs were incubated with α-CD33 (IC7/1) (10µg/ml) in serum free medium for 20 min or 1 h at room temperature and the cells were then pelleted and re-suspended in serum free medium. GAM (20µg/ml) was added and cross-linking allowed to occur for the indicated times. The cells were either lysed or washed in 1 X PBS and incubated with PE-conjugated isotype control/α-CD33 (different from cross-linking Ab) for 15 min. The samples were washed in 1 X PBS and fixed in 1% paraformaldehyde for FACS analysis.

**Trypan blue exclusion assay**

CD33WT stable Ba/F3-SOCS3 cells were seeded at a density of 1×10^5 cells per ml and grown in RPMI-1640 containing 5% FCS and 5U/ml IL-3 in the presence or absence of tetracycline (4µg/ml). Samples were treated with α-CD33 (IC7/1) (10µg/ml) and GAM (10µg/ml) for 48 h. Fresh antibody was added at 24 h. Viable cell numbers were counted by trypan blue exclusion every 24 h.

**MTT assay**

CD33WT and CD33Y340F/Y358F stable Ba/F3-SOCS3 cells were seeded as above and MTT (0.5mg/ml) was added to samples at 24 h intervals and incubated at 37°C for 2 h. The cell suspension was pelleted by low-speed centrifugation, the supernatant removed and 200µl DMSO was added to the cell pellet. Cells were mixed, placed in a 96 well plate and incubated for 10 min at 37°C. The optical density of each sample was read at OD_{570} using a microplate reader.

**Results**

**SOCS3 binds the phosphorylated ITIM and ITSM peptides**
The CD33 Y340 ITIM recruits SHP-1 and SHP-2, whereas the Y358 ITSM functions primarily to recruit SHP-2. The CD33 ITIM exhibits close homology to motifs found in EpoR and gp130 (Table 1) suggesting that they may bind similar SH2 domains. SOCS3 binds ITIM-like pY motifs on these receptors via its SH2 domain. 17 To establish whether SOCS3 could bind to the CD33 ITIM, precipitation experiments were performed using phosphorylated and unphosphorylated CD33-ITIM and ITSM biotinylated peptides. Empty Vector (EV) or Flag-tagged SOCS3 pME18S were transiently transfected into 293T cells and lysates were incubated for 2 h with the CD33 peptides and immunoblotted.

While neither the unphosphorylated CD33-derived peptides nor the TCR Zeta chain derived phosphopeptide bound detectable amounts of SOCS3 (Figure 1, Panel 1, lanes 4, 6 and 7), both the phosphorylated ITIM and ITSM peptides associated with SOCS3 (lanes 3 and 5). However, significantly more SOCS3 associated with the phosphorylated CD33 ITIM peptide (lane 3) than the phosphorylated ITSM peptide (lane 5). The experiment was performed three times and consistently indicated that SOCS3 bound to the CD33 ITIM, and to a lesser extent the ITSM, in a phosphotyrosine dependent manner.

**Tyrosine motifs are required for degradation of CD33 following cross-linking**

Cross-linking CD33 is thought to result in activation via phosphorylation of the cytoplasmic ITIM and ITSM tyrosines. 8 CD33 was cross-linked in CD33WT and CD33Y340F/Y358F stable Ba/F3 cell lines in order to determine the importance of the tyrosine residues. CD33WT and CD33Y340F/Y358F stable Ba/F3 cells (1x10^7 cells per point) were incubated with α-CD33 (IC7/1) for 20 min +/- GAM for 30 min. Cells were pre-treated for 30 min with and without the proteasome inhibitors MG132 and LLNL to determine whether proteasomal degradation of CD33 occurred. The cells were lysed and immunoprecipitated with α-Flag (M2).
Cross-linking for 30 min resulted in CD33WT degradation (Figure 2A, Panel 1, lane 5), while cross-linking had little effect on CD33Y340F/Y358F (lane 9). This degradation was partially restored by pre-treatment with the proteasome inhibitors MG132 and LLNL (lane 14) suggesting that after activation, CD33 may be targeted to the proteasome for degradation in a tyrosine dependent manner. Immunoblotting with α-STAT5B confirmed equal loading in each lane (Panel 2).

To determine whether the tyrosine motifs were important for the internalisation of CD33 following activation, CD33WT and CD33Y340F/Y358F Ba/F3 cells (1x10^6 cells per point) were incubated with α-CD33 (IC7/1) for 20 min and GAM for 5 min. This was followed by FACS analysis to determine surface CD33 levels after incubation with PE conjugated-α-CD33/isotype control. Cross-linking CD33 for 5 min resulted in the immediate down-regulation of surface expression in CD33WT and CD33Y340F/Y358F Ba/F3 cells (Figure 2B). However, only CD33WT was proteasomally degraded as shown by the immunoblot analysis. This data suggests that the tyrosine motifs are not important for the internalisation of CD33, however they appear to play an important role in its subsequent degradation.

**SOCS3 accelerates degradation of CD33**

Like F-box proteins, SOCS proteins play a pivotal role in facilitating ubiquitination and proteasomal degradation of protein substrates. SOCS3 has been suggested to target IRS1 and IRS2 to the 26S proteasome for degradation. Since SOCS3 can clearly associate with CD33, we aimed to establish whether SOCS3 can target CD33 for degradation.

Since the Ba/F3 cells grown in IL-3 constitutively express SOCS3, its effect on CD33 was investigated in 293T cells transiently transfected with either EV, CD33WT or CD33Y340F pME18S Flag in the presence and absence of Flag-tagged SOCS3. The cells were treated with the protein tyrosine phosphatase inhibitor pervanadate for 15
min. Pervanadate stimulation resulted in marked CD33WT tyrosine phosphorylation (Figure 3A, Panel 2, lanes 2 and 5) and as expected CD33WT associated with SHP-2 (Panel 4, lanes 2 and 5). However, no phosphorylation of the Y340F mutant was observed (Panel 2, lanes 3 and 6), suggesting that phosphorylation of CD33 required an intact ITIM. Interestingly, in the presence of SOCS3, expression and tyrosine phosphorylation of CD33WT were significantly reduced (Panels 1 & 2, lane 5). Tyrosine phosphorylation of CD33WT correlated with protein degradation (Panel 1, lane 2). This degradation was accelerated in the presence of SOCS3 (Panel 1, lane 5) and simultaneous turnover of SOCS3 occurred (Panel 3, lane 5).

To further clarify the effect of SOCS3 on CD33 degradation, we examined the half-life of CD33 upon phosphorylation, in the presence and absence of SOCS3. 293T cells were transiently transfected with either EV, CD33WT or CD33Y340F/Y358F with or without SOCS3. The cells were pre-treated with cycloheximide and pervanadate for the periods indicated. The cells were lysed and treated as previously described.

Tyrosine phosphorylation of CD33WT was evident after 10 min pervanadate treatment, significantly enhanced after 30 min and undetectable after 60 min (Figure 3B, Panel 2, lanes 4-6). Markedly less CD33WT expression and phosphorylation was observed in the presence of SOCS3 (Panels 1 & 2, lanes 8-10). Remarkably SOCS3 expression was lost rapidly in correlation with CD33WT (Panel 3, lanes 8-10). In comparison, the expression of CD33Y340F/Y358F mutant did not fluctuate in the presence or absence of SOCS3 (Figure 3C Panel 1, lanes 3-10) and SOCS3 levels remained the same (Panel 3, lanes 8-10). This suggests mutation of the tyrosine residues within the ITIM and ITSM prevents SOCS3 binding via its SH2 domain thereby blocking the targeting of CD33 and SOCS3 for proteasomal degradation.

In order to further investigate the effect of SOCS3 on the degradation of CD33, the more physiologically relevant system of receptor cross-linking was used in stable
CD33WT tetracycline (Tet)-regulated SOCS3 Ba/F3 cells. SOCS3 was present only in the absence of Tet. CD33WT stable Ba/F3-SOCS3 cells (1x10^7 cells per point) were incubated with α-CD33 (IC7/1) for 20 min +/- GAM for the times shown. The cells were lysed and immunoprecipitated with α-Flag.

Cross-linking for 5, 10 and 15 min in the presence of over-expressed SOCS3 resulted in CD33 degradation in a time dependent manner (Figure 3D, Panel 1, lanes 14-16). This degradation was not observed in the presence of very low levels of SOCS3 (lanes 6-8). SOCS3 degradation occurred in correlation with CD33 following its cross-linking (Panel 2, lanes 14-16). This is the first time that SOCS3 has been shown to undergo degradation while in complex with another protein and would suggest that after activation, SOCS3 targets CD33 for degradation and that both proteins are degraded concomitantly.

**CD33 is degraded by SOCS3 and the ECS E3 ligase complex**

Previous studies have established that the SOCS box of SOCS3 interacts with Elongin B/C, Cullin 5 and Rbx1/2 to form an active E3 ligase, which targets substrates for degradation via the ubiquitin proteasomal pathway. To determine whether the E3 ligase complex was required for SOCS3-mediated CD33 degradation during cross-linking, 293T cells were transiently transfected with EV and CD33WT with the E3 ligase complex (Elongin B/C, Cul5 and Rbx1) in the presence and absence of SOCS3. The cells were treated with and without proteasome inhibitors (MG132 and LLNL) for 30 min prior to incubation with α-CD33 (IC7/1) for 20 min +/- GAM for 30 min. In the absence of the E3 ligase complex, degradation of CD33 or SOCS3 was not observed following cross-linking in 293T cells (Figure 4A).

Cross-linking for 30 min in the presence of the E3 ligase complex and SOCS3 resulted in significant CD33 degradation (Figure 4B, Panel 1, lane 9) in comparison to the absence of SOCS3 (lane 5). As shown previously in Figure 3D, cross-linking for
30 min in the presence of the E3 ligase complex resulted in SOCS3 degradation in correlation with CD33 (Panel 2, lane 9). This degradation was rescued by pre-treatment with the proteasome inhibitors MG132 and LLNL (Panel 1, lanes 14 and 18; Panel 2, lane 18). This result indicates that cross-linking CD33 can result in the loss of both CD33 and SOCS3 in a proteasome-dependent manner and the E3 ligase complex is necessary for this degradation to occur. Our data implies that SOCS3 binds to CD33 (see Figure 1) via its SH2 domain, while its SOCS box interacts with the ECS E3 ligase complex containing Elongin BC/Cullin-5/Rbx-1 proteins, resulting in the proteasomal degradation of CD33 and SOCS3.

Degradation of CD33 by SOCS3 in PBMCs

An experiment was performed to determine the effect of SOCS3, induced during an inflammatory response, on CD33 expression levels in an endogenous setting. LPS has previously been shown to induce SOCS3 expression. The adherent monolayer from human PBMCs was treated with LPS for 2 h to induce SOCS3 and cross-linked with \( \alpha \)-CD33 (IC7/1) for 1 h +/- GAM for 2 h. The cells were lysed and immunoprecipitated with \( \alpha \)-CD33 (My9) and \( \alpha \)-SOCS3 (008). SOCS3 was induced by LPS (Figure 5, Panel 2, lanes 3-4) and cross-linking CD33 in the presence of SOCS3 resulted in the degradation of both CD33 and SOCS3 (Panels 1 & 2, lane 4), while no effect was observed in the absence of SOCS3 (Panel 1, lane 2). The experiment was repeated three times and gave consistent results. A similar experiment was performed in THP-1 cells and degradation was observed following CD33 cross-linking (data not shown). This suggests that during the early stages of an infection, SOCS3 can target CD33 for degradation, thereby enhancing the inflammatory response.
SOCS3 inhibits the effect of CD33 engagement on cytokine-induced proliferation

Cross-linking CD33 has previously been shown to inhibit proliferation of chronic and acute myeloid leukaemias.\textsuperscript{9, 10} It has also been reported that CD33 has constitutive repressor activity on human monocytes, as siRNA depletion of CD33 resulted in increased cytokine production.\textsuperscript{24} Here we investigated whether engagement of CD33 could inhibit proliferation of the IL-3 dependent Ba/F3 cell line and the effect of SOCS3 on this. Stable CD33WT tetracycline-regulated SOCS3 Ba/F3 cells were seeded in the presence and absence of Tet. SOCS3 is an inhibitor of IL-3 signalling\textsuperscript{12} so we performed a trypan exclusion assay to compare the proliferation of cells in the absence and presence of SOCS3. The results in Figure 6A demonstrated that CD33WT stable Ba/F3-SOCS3 cells in the absence of SOCS3 proliferated faster than the same cells in the presence of SOCS3 after 48 h (p<0.005). Results were taken in triplicate and were representative of three separate experiments. These results agree with published data indicating that SOCS3 inhibits IL-3 signalling which must be taken into account for Figure 6C and 6D. Tetracycline regulation of SOCS3 expression was confirmed by immunoblotting (Figure 6B).

To examine the effect of SOCS3 on CD33-mediated responses to cytokine-induced proliferation, a trypan blue exclusion assay was set up using CD33WT stable Ba/F3-SOCS3 cells in the presence and absence of Tet. The cells were cross-linked with and without α-CD33 (IC7/1) and GAM whole molecule as described in “Materials and methods”. F(ab’)\textsubscript{2} was also used to ensure that the cross-linking effect was not due to the Fc portion of the cross-linking antibody and similar results were obtained (data not shown). Fresh antibody was added every 24 h and samples were analysed. Viable cells were counted using the trypan blue exclusion assay in triplicate and the results demonstrate that proliferation of CD33WT stable Ba/F3-SOCS3 cells in the absence of SOCS3 was significantly inhibited by cross-linking CD33 compared to cells treated with GAM after 48 h (p<0.005) (Figure 6C). Cross-linking CD33 in CD33WT stable
Ba/F3-SOCS3 cells in the presence of SOCS3 did not have a significant effect compared to cells treated with GAM. These results clearly demonstrate that engagement of CD33 in the absence of SOCS3 can inhibit cytokine-induced proliferation following IL-3 stimulation, while engagement of CD33 in the presence of SOCS3 disrupts this effect.

To confirm this observation, cytokine-induced proliferation was also examined using the MTT assay. CD33WT and CD33Y340F/Y358F stable Ba/F3-SOCS3 cells were cultured in the presence or absence of Tet and cross-linked as described. As shown before, CD33WT cells in the absence of SOCS3 showed a significant inhibition of cytokine-induced proliferation (p<0.05) while SOCS3 blocked this effect (Figure 6D). CD33Y340F/Y358F cells showed no significant effect on cytokine-induced proliferation either in the absence or presence of SOCS3 indicating a role for the tyrosine motifs of CD33 in inhibition of IL-3 induced proliferation. Signalling through CD33 inhibits cytokine responses, however, cytokine-induced SOCS3 can target CD33 for proteasomal degradation thereby blocking its inhibitory effect.

Discussion

To date SOCS3 and other SOCS have solely been implicated in the regulation of cytokine, or TLR responses and our data is the first to associate SOCS3 with the regulation of inhibitory receptors. The findings presented in this study demonstrate that SOCS3 can bind to phosphorylated CD33, and enhance its proteasomal degradation, with the concurrent loss of SOCS3. Moreover, CD33 can normally inhibit cytokine-induced proliferation but during an inflammatory response, SOCS3 can block the inhibitory effect of CD33 on cytokine signalling by enhancing receptor degradation. This implies two roles for SOCS3 involving the negative regulation of cytokine pathways and a new role in modulating inhibitory receptors during inflammation. For example, SOCS3 is constitutively present in activated T cells
during a TH2 response\textsuperscript{25} and for up to 24 h following LPS stimulation,\textsuperscript{26} therefore this suggests that SOCS3 may be pro-inflammatory in these circumstances, by overriding the inhibitory response from ITIM-bearing receptors.

SOCS3 predominantly associates with the phosphorylated ITIM of CD33. The SH2 domain of SOCS3 displays marked homology with the N-terminal SH2 domain of SHP-1 or SHP-2 and these proteins competitively bind to the same pY residues of the EpoR, gp130 and the leptin receptor.\textsuperscript{17, 27, 28} This is analogous to the findings that SAP and EAT-2 share 42\% homology with the SHP-2 SH2 domain and bind competitively to the same motif on various receptors. Binding of SAP to 2B4 in NK cells and CD150 in T cells prevented their association with SHP-2.\textsuperscript{29, 30} Since SOCS3 can bind to the ITIM of CD33, this suggests that competitive binding may occur between SOCS3 and SHP-1/2 to this motif.

For paired receptor systems such as KIRs, inhibitory receptors contain intracellular ITIMs, while their activatory counterparts associate with ITAM-containing adaptor proteins such as DAP12.\textsuperscript{31} These receptors are essential to commence, amplify and then cease immune responses.\textsuperscript{32} Siglecs recognise sialic acids, which are ubiquitously expressed endogenous molecules.\textsuperscript{4} They do not have activatory counterparts, therefore, an immune cell expressing CD33-related Siglecs normally displays continuous repressor activity via ITIMs.\textsuperscript{24, 33} However, murine Siglec H, a DAP12 coupled Siglec, has recently been shown to inhibit cytokine production in an ITIM-independent manner.\textsuperscript{34} To date, the mechanisms by which the inhibitory activity of CD33 is regulated have not been identified.

SOCS proteins have previously been implicated in facilitating ubiquitination and proteasomal degradation of signalling intermediates. SOCS1 accelerates the ubiquitination and degradation of Vav, TEL-JAK2, IRS1/2, FAK and JAK2.\textsuperscript{11} This mirrors SOCS1 null studies showing that SOCS1 strongly and specifically regulates IFN\(\gamma\) responses as IFN\(\gamma^{-/-}\) SOCS1\(^{-/-}\) are normal whereas SOCS1\(^{-/-}\) succumb to massive
systemic inflammation at 3 weeks post-partum. SOCS3 has been shown to target IRS1/2 and FAK for proteasomal degradation. The most compelling evidence comes from murine knockout studies showing that SOCS3 is essential for the regulation of ITIM-bearing receptors such as gp130 and leptin receptor.

Our findings demonstrated activation or tyrosine phosphorylation of CD33WT resulted in degradation that was accelerated by SOCS3 via the 26S proteasome. Various SOCS-box and F-box proteins have previously been shown to undergo degradation in complex with their associated targets via the 26S proteasome. For example, HIV-1 Vif (Viral infectivity factor) protein forms an SCF-like E3 ubiquitin ligase that targets APOBEC3G for proteasomal degradation. Vif is ubiquitinated by the same E3 as its target APOBEC3G which is reminiscent of F-box proteins that are auto-ubiquitinated within their own SCF (Skp1-Cul1-F-box protein) complex.

Since the E3 ligase complex enhanced the degradation of CD33 and SOCS3, this result implies the formation of an ECS (Elongin B/C-Cul2/Cul5-SOCS-box protein) complex with CD33. It would appear that the SH2 domain of SOCS3 interacts with the pITIM of CD33, while its SOCS box interacts with the ECS Elongin B/C, and Cullin-5 and Rbx1/2. Since CD33 is expressed on myeloid cells, and SOCS3 is induced by pro-inflammatory cytokines and LPS in these cells, the degradation of CD33 by SOCS3 provides a mechanism to enhance the acute inflammatory response. Decreased expression of CD33 by siRNA treatment or removal of the CD33 ligand sialic acid resulted in increased cytokine production indicating that CD33 has repressor activity against cytokine signalling. It is reasonable to suggest that SOCS3 can function to overcome this inhibitory signalling to enhance the cytokine response. Cross-linking CD33 on chronic and acute myeloid leukaemia cell lines hampers cell proliferation and CD33, Siglec 8 and Siglec 9 engagement has previously been shown to result in apoptosis of leukaemic cells, eosinophils and neutrophils, respectively. We have shown that cross-linking CD33 can result in inhibition of
cytokine-induced proliferation, however our investigations indicated that this was not due to the induction of apoptotic cell death (data not shown). SIRP1α can inhibit the proliferation of cells induced by hormones and growth factors via protein tyrosine kinase receptors. It has been proposed that this inhibition occurs via an interaction with the tyrosine phosphatases SHP-1/2 leading to a reduction in cell activation. It is therefore possible that CD33-induced inhibition of proliferation may occur via an interaction with SHP-1/2 resulting in attenuated proliferation signals.

SOCS3 has been implicated in the regulation of inflammatory and autoimmune diseases such as Inflammatory Bowel Disease (IBD) and Rheumatoid Arthritis (RA). CD33 is a marker of acute myeloid leukaemia (AML) and our findings may be important in describing a mechanism of regulation of CD33 for therapeutic benefits. It would be interesting to examine the possible role of proteasome-mediated degradation of the SOCS3/CD33 complex in the development or treatment of these diseases.

In summary, our data indicates that SOCS3 can bind to the phosphorylated ITIM of CD33 resulting in the proteasomal degradation of both CD33 and SOCS3. Furthermore, SOCS3 can block the inhibitory effect of CD33 on cytokine-induced proliferation suggesting an intricate mechanism of regulation during an inflammatory response. This may have important clinical implications in the treatment of AML with α-CD33 therapy.

Acknowledgments

We would like to thank Dr. W. Blattler from Immunogen for kindly providing the CD33 antibody (My9); Dr. J.J. O’Shea for the STAT5B antibody; Dr. J.W. Conaway for the Rbx1 and Cul5 constructs; Dr. P.R. Crocker for reviewing this manuscript and Mr. Gerry Clarke for sorting the stable cell lines created during this study.
Figure Legends

Figure 1. **CD33 and SOCS3 interaction.** 293T cells were transfected with 2μg of SOCS3 pME18S-Flag. Lysates were co-precipitated with appropriate CD33 peptide and immunoblotted with α-Flag (M2) (Panel 1) or the lysates were immunoprecipitated with α-Flag and immunoblotted with α-Flag (Panel 2). Peptide sequences were as follows:

1 & 3. Phosphorylated ITIM peptide (Biotin SGSGDEELHPYASLNF-OH),
2 & 4. Unphosphorylated ITIM peptide (Biotin SGSGDEELHYASLNF-OH),
5. Phosphorylated ITSM peptide (Biotin SGSGDTSTEpYSEVRT-OH),
6. Unphosphorylated ITSM peptide (Biotin SGSGDTSTYEYSEVRT-OH),
7. Control Peptide (Biotin SGSGHDGLYQGLST-OH),
8. Positive SOCS3 control (IP α-Flag)

Signal intensity of SOCS3 levels pulled down by pY ITIM and pY ITSM peptides were normalized to SOCS3 expression levels and are illustrated as a graph.

Figure 2. **Tyrosine motifs are important for degradation of CD33 following cross-linking.** (A) CD33WT and CD33Y340F/Y358F stable Ba/F3 cells were incubated with α-CD33 (IC7/1) for 20 min and cross-linked with GAM for 30 min. The cells were pretreated with and without MG132 (0.5μM) and LLNL (0.5μM) for 30 min. Lysates were immunoprecipitated with α-Flag and immunoblotted with α-Flag (Panel 1). WCL was immunoblotted with α-STAT5B as a loading control (Panel 2). (B) CD33WT and CD33Y340F/Y358F stable Ba/F3 cells were incubated with α-CD33 (IC7/1) for 20 min and cross-linked with Goat-Anti-Mouse for 5 min. Samples were incubated with PE-conjugated α-CD33 or isotype control for 15 min and analysed by FACS. In the lower panel, ≈70% of the Ba/F3 cells are stably infected with the expression construct for CD33Y340F/Y358F. As a result the peak in the low-left corner represents the cells negative for CD33Y340F/Y358F expression.
Figure 3. SOCS3 accelerates proteasomal degradation of CD33. (A) 293T cells were transiently transfected with 2µg of EV, CD33WT and CD33Y340F in the presence and absence of 2µg of SOCS3 and treated with pervanadate (1.4mM) for 15 min. Lysates were immunoprecipitated with α-CD33 (My9) and immunoblotted with either α-Flag (Panel 1), α-phospho-Tyr (PY20) (Panel 2) or α-SHP-2 (Panel 4). Lysates were also immunoprecipitated with α-SOCS3 (008) and immunoblotted with α-Flag (Panel 3). WCL was immunoblotted with α-STAT5B as a loading control (Panel 5). (B and C) 293T cells were transiently transfected with 2µg of CD33WT or CD33Y340F/Y358F in the presence and absence of SOCS3 and treated with cycloheximide for 30 min prior to pervanadate treatment for 10, 30 and 60 min. Lysates were immunoprecipitated with α-CD33 and immunoblotted with α-Flag (Panel 1) or α-Phospho-Tyr (Panel 2). WCL was immunoblotted with α-Flag (Panel 3). (D) Stable CD33WT Tet-regulated SOCS3 Ba/F3 cells were incubated with α-CD33 (IC7/1) for 20 min and cross-linked with GAM for 5, 10 and 15 min. Lysates were immunoprecipitated with α-Flag and immunoblotted with α-Flag (Panels 1 and 2). WCL was immunoblotted with α-STAT5B as a loading control (Panel 3).

Figure 4. E3 ligase complex is required for proteasomal degradation of CD33 and SOCS3. (A) 293T cells were transiently transfected with 2µg of EV and CD33WT in the presence and absence of SOCS3. Cells were incubated with α-CD33 (IC7/1) for 20 min and cross-linked with GAM for 30 min. Lysates were immunoprecipitated with α-Flag and immunoblotted with α-Flag (Panels 1-2). (B) 293T cells were transiently transfected with 2µg of EV and CD33WT with E3 ligase complex (Elongin B/C, Cul5 and Rbx1) in the presence and absence of SOCS3. Cells were incubated with α-CD33 (IC7/1) for 20 min and cross-linked with GAM for 30 min. The cells were pretreated for 30 min with and without MG132 and LLNL.
Lysates were immunoprecipitated with α-Flag and immunoblotted with α-Flag (Panels 1-3) and with α-Myc (9E10) (Panel 4). WCL was immunoblotted with α-His (Panel 5).

**Figure 5. Endogenous degradation of CD33 and SOCS3.** The adherant monolayer from PBMCs was treated for 2 h with LPS (100ng/ml), incubated with α-CD33 (IC7/1) for 1 h and cross-linked with GAM for 2 h. Lysates were immunoprecipitated with α-CD33 and immunoblotted with α-CD33 (IC71/3D6) (Panel 1). Lysates were immunoprecipitated with α-SOCS3 and immunoblotted with α-SOCS3 (M20) (Panel 2). WCL was immunoblotted with α-STAT5B as a loading control (Panel 2). Signal intensities of CD33 and SOCS3 expression levels were normalized to signal intensity of STAT5B and are illustrated as graphs.

**Figure 6. SOCS3 blocks the inhibitory effect of CD33 on proliferation.** (A) CD33WT stable Ba/F3-SOCS3 cells seeded at a density of 1×10^5 cells/ml were cultured in the presence or absence of tetracycline (4µg/ml). Trypan blue exclusion assay determined the viability of the cells at 24 h intervals. **p< 0.005 Students T test

(B) CD33WT stable Ba/F3-SOCS3 cells were cultured in the presence or absence of tetracycline for 48 h. They were lysed, immunoprecipitated and immunoblotted with α-Flag. (C) CD33WT stable Ba/F3-SOCS3 cells seeded at a density of 1×10^5 cells/ml were cultured in the presence or absence of tetracycline. They were incubated with α-CD33 (IC7/1) and cross-linked with GAM. Fresh antibody was added at 24 h. Trypan blue exclusion assay determined the viability of the cells at 24 h intervals. **p< 0.005. (D) CD33WT and CD33Y340F/Y358F stable Ba/F3-SOCS3 cells seeded at a density of 1×10^5 cells/ml were cultured in the presence or absence of tetracycline. They were incubated with α-CD33 (IC7/1) and cross-linked with GAM. Fresh antibody was added at 24 h. 10µl of MTT (0.5mg/ml) was added to 100µl cell culture
and incubated at 37°C for 2 h at 24 h intervals. The cells were centrifuged at 1,800rpm for 3 min and the supernatant was removed. 200µl of DMSO was added and incubated for 10 min at 37°C. Plates were read at OD\textsubscript{570} using a microplate reader. *p< 0.05.

References


29. Shlapatska LM, Mikhalap SV, Berdova AG, et al. CD150 association with either the SH2-containing inositol phosphatase or the SH2-containing protein tyrosine phosphatase is regulated by the adaptor protein SH2D1A. J Immunol. 2001;166:5480-5487.


Table 1. ITIM-like motifs that recruit SHP-1/2 and SOCS3

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CD33WT Ba/F3

CD33Y340F/Y358F

Ba/F3

Isotype control

IC7/1 & GAM

GAM
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IP α-Flag
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CD33
SOCS3

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CD33
SOCS3
Elongin B
Elongin C
Rbx1
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STAT5B

CD33 Levels

SOCS3 Levels
Figure 6

A

CD33WT Ba/F3 (+/- Tet)

B

Tet

IP α-Flag

IB α-Flag

SOCS3

1

2

C

CD33WT Ba/F3-SOCS3 (+Tet)

CD33WT Ba/F3-SOCS3 (-Tet)

D

CD33WT Ba/F3-SOCS3 (+Tet)

CD33WT Ba/F3-SOCS3 (-Tet)

CD33Y340F/Y358F Ba/F3-SOCS3 (+Tet)

CD33Y340F/Y358F Ba/F3-SOCS3 (-Tet)
CD33 responses are blocked by SOCS3 through accelerated proteasomal-mediated turnover

Selinda J Orr, Nuala M Morgan, Joanne Elliott, James F Burrows, Christopher J Scott, Daniel W McVicar and James A Johnston