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Suppressors of Cytokine Signaling Promote Fas-Induced Apoptosis through Downregulation of NF-κB and Mitochondrial Bfl-1 in Leukemic T Cells

Jiyoung Oh,*,1,2 Seol-Hee Kim,*,1,2 Sinae Ahn,* and Choong-Eun Lee*

Suppressors of cytokine signaling (SOCS) are known as negative regulators of cytokine- and growth factor–induced signal transduction. Recently they have emerged as multifunctional proteins with regulatory roles in inflammation, autoimmunity, and cancer. We have recently reported that SOCS1 has antiapoptotic functions against the TNF-α- and the hydrogen peroxide–induced T cell apoptosis through the induction of thioredoxin, which protects protein tyrosine phosphatases and attenuates Jaks. In this study, we report that SOCS, on the contrary, promote death receptor Fas-mediated T cell apoptosis. The proapoptotic effect of SOCS1 was manifested with increases in Fas-induced caspase-8 activation, truncated Bid production, and mitochondrial dysfunctions. Both caspase-8 inhibitor c-Flip and mitochondrial antiapoptotic factor Bfl-1 were significantly reduced by SOCS1. These proapoptotic responses were not associated with changes in Jak or p38/Jnk activities but were accompanied with down-regulation of NF-κB and NF-κB–dependent reporter gene expression. Indeed, p65 degradation via ubiquitination was accelerated in SOCS1 overexpressing cells, whereas it was attenuated in SOCS1 knockdown cells. With high NF-κB levels, the SOCS1-ablated cells displayed resistance against Fas-induced apoptosis, which was abrogated upon siBfl-1 transfection. The results indicate that the suppression of NF-κB–dependent induction of prosurvival factors, such as Bfl-1 and c-Flip, may serve as a mechanism for SOCS action to promote Fas-mediated T cell apoptosis. SOCS3 exhibited a similar proapoptotic function. Because both SOCS1 and SOCS3 are induced upon TCR stimulation, SOCS would play a role in activation-induced cell death by sensitizing activated T cells toward Fas-mediated apoptosis to maintain T cell homeostasis.

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The regulation of T cell survival and apoptosis is crucial for lymphocyte homeostasis (1). In particular, activation-induced cell death (AICD) is necessary for clearing activated T cells generated by Ag stimulation from the host through apoptosis mechanisms (2). Deficiency in AICD involving dysregulation of CD95 (Fas), TNFR1, and TRAILR may cause autoimmune disorders and cancer (3, 4). The Fas-mediated apoptosis is ensued following engagement of the receptor by Fas ligand (FasL) expressed on activated T cells (5). The receptor ligation followed by binding with Fas-associated death domain protein leads to the recruitment of caspase-8 to form the death-inducing signaling complex (DISC), resulting in the cleavage and activation of caspase-8 and downstream caspases (6). The caspase-8–induced cleavage of Bid, cytochrome c (Cyt c), release p50, p100/p52, and RelB. Under resting conditions, NF-κB heterodimers (p65/p50) are trapped in the cytoplasm by the inhibitory protein IκB. T cell activation induces the phosphorylation and degradation of IκB, leading to the translocation of p65/p50 or p52/RelB into the nucleus, where they trigger the transcription of various cell survival genes (17). In T cells, a number of NF-κB target genes have been identified, including antiapoptotic factors Bfl-1, Mcl-1, Bcl-2, and Bcl-xL, which act against proapoptotic Bax, Bad, Bid, and Bim (18–20).

As a prosurvival Bcl-2 family member, Bfl-1 expression is generally confined to immune cells or tissues and it is highly induced by proapoptotic cytokines such as TNF-α and IL-1 (21, 22). A number of studies support the role of NF-κB in Bfl-1 induction. The Bfl-1 transcripts were found transiently elevated in Jurkat T cells activated with PMA plus ionomycin in an NF-κB–dependent manner (23). Lymphocytic cells from c-Rel−/− mice exhibit an absolute defect in the ability to express Bfl-1 in response to cell activation, indicating that Bfl-1 gene is strongly controlled by the NF-κB pathway (24). Furthermore, it has been shown that NF-κB...
regulates Bfl-1 gene expression via kB binding sites in the 5′ regulatory region of the Bfl-1 promoter (25).

Suppressors of cytokine signaling (SOCS) are known as negative feedback regulators of cytokine as well as growth factor signaling in diverse cell types. SOCS are also recognized for multiple roles in development, differentiation, survival, and apoptosis in T cells (26). The SOCS family consists of eight distinct members (SOCS1–7 and CIS), all of which contain a central SH2 domain, a variable N-terminal region, and a conserved C-terminal SOCS box. SOCS proteins inhibit Jak activities by binding to the catalytic domain and/or binding directly to cytokine receptors at phosphotyrosine residues to suppress cytokine signal transduction mediated by STATs (27). The SOCS box binds with E3 ubiquitin ligase complex such as elongin B and C, Cullin, and Rbx proteins. This mediates the degradation of target proteins bound via the SH2 domain of SOCS and regulates the downstream signal transduction.

In this regard, SOCS1 has been shown to interact with Tel-Jak-2, IRS-2, and Vav, leading to the target protein degradation via ubiquitination machinery (28–30). Additionally, SOCS1 has been proposed to control the transactivator function of NF-kB for suppression of NF-kB–responsive genes likely through the SOCS box-mediated p65 degradation (31, 32).

Both proapoptotic and antiapoptotic functions of SOCS have been observed depending on the cell types and stimulations that cells receive. For example, SOCS3 exhibited the growth-inhibitory effects and promoted radiation-induced apoptosis in lung cancer cells (33). It is also shown to inhibit cell cycle progression, suppressing the androgen-mediated proliferation of prostate cancer cells (34). In contrast, SOCS3 suppressed the cytokine-induced apoptosis of pancreatic β cells (35). Similarly, SOCS1 has been reported for antiapoptotic effects. The overexpression of SOCS1 affected the apoptotic proteins and MAPK members to induce survival response against TNF-α–induced cell death (36, 37). More recently, we have reported that SOCS1 prevents the reactive oxygen species (ROS)–mediated immune cell apoptosis through the induction of thioredoxin, which protects SHP-1/CD45, and through the attenuation of Jak5 acting upstream of p38 MAPK (38). In contrast to these findings, the proapoptotic role of SOCS1 regulating T cell numbers in lymphoid organs has been suggested in studies with SOCS1 deletion mice through mechanisms unknown (39).

In this study, we report a novel function of SOCS to promote Fas-mediated T cell apoptosis by downregulation of NF-kB activities through binding and protesomeal degradation of p65. This results in the decrease in the expression of its potential prosurvival target genes, c-Flip and Bfl-1, acting at the level of death receptor signaling complex and mitochondria during Fas signaling, respectively. The SOCS-mediated downregulation of these survival factors would provide a molecular basis of SOCS action in immune cell homeostasis by facilitating the removal of activated T cells by Fas-induced apoptosis.

Materials and Methods

Antibodies

Anti-cleaved caspase-3, anti–procaspase-8, anti–procaspase-2, anti–procaspase-9, anti–procaspase-3, anti–cleaved poly(ADP-ribose) polymerase, anti-p38, anti–phospho-p38, anti–phospho-Jnk1/2, anti–Jnk1/2, anti–truncated Bid (t-Bid), anti–hemagglutinin (HA), and anti–Myc Abs were from Cell Signaling Technologies (Beverley, MA). Anti-cleaved caspase-8, anti–cleaved caspase-9, anti–Flip, anti–Cyt c, anti–Bcl-2, anti–Bcl-xL, and anti–FasL Ab were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p65, anti–p53/p105, anti–Fas, and anti–FasL Abs were from Upstate Biochemicals (Charlottesville, VA). Anti–Cox IV Ab was from Sigma-Aldrich (St. Louis, MO). Anti–Jnk1/2 and anti–Jnk1, anti–truncated Bcl-2, and anti–Bcl-xL Abs were from BD Pharmingen (San Diego, CA). Rabbit Abs against SOCS1, as well as SOCS3, were from AnaSpec (San Jose, CA). The Ab for Bfl-1 was a gift from Dr. C. W. Kim (25).

Cell lines and constructs

The human T cell line Jurkat and B cell line Ramos was obtained from the American Type Culture Collection. Jurkat and Ramos cells were cultured in complete RPMI media supplemented with 10% FBS (Invitrogen, Carlsbad, CA), 10 mm HEPES, 2 mM L-glutamine, 50 μM 2-ME, 10,000 U/ml penicillin, and 10,000 μg/ml streptomycin (Life Technologies, Grand Island, NY) at 37˚C under 5% CO2. The establishment of SOCS1 overexpressing or knockdown stable Jurkat cell lines in our laboratory was described (38). The HA-SOCS1/Jurkat and Myc-SOCS3/Jurkat cells were cultured and maintained with 200 μg/ml G418 (Invitrogen). The short hairpin RNA SOCS1 (shSOCS1)/Jurkat cells were maintained with 100 ng/ml puromycin (Sigma-Aldrich). SOCS1-transduced Ramos B cells and FCTT116 cells were also prepared by transfection with the HA-SOCS1 construct.

Primary cell cultures

PBMCs were isolated from peripheral blood of healthy donors and then purified using Opti-Prep. Mouse splenocytes were isolated from BALB/c mice as described (38). The cells were cultured in RPMI 1640 medium and treated with mouse anti-Fas Ab or anti–CD3 plus anti–CD28 Abs, after which they were harvested and analyzed for apoptosis and/or SOCS expression levels.

Gene transfection

Cells were transiently transfected using electroporation. Jurkat T cells (5 × 10^6) resuspended in 500 μl Opti-MEM buffer (Life Technologies) were mixed with validated stealth negative control RNA, siBfl-1 (5′-AAUC-GUUUCCUAUCAUGUC-3′), or siBcl-xL (5′-CAGGGACAGCAUACAAGAG-3′). The mixture was then transferred into a 0.4 cm cuvette and subjected to five pulses of 270 V for 5 ms by the Gene Pulse Xcell electroporation system (Bio-Rad, Herbercules, CA).

RNA isolation and RT-PCR

Total RNAs were isolated from TRizol reagent (Invitrogen, Camarillo, CA) according to the manufacturer’s instructions. cDNA was synthesized from total RNA (1 μg) using oligo(dT). The PCR primers for Bfl-1 were gifts from Dr. C. W. Kim (25). PCR amplification was then performed using the specific primers described below for 30 cycles using a thermal cycler (GeneAmp PCR System 2400; Applied Biosystems, Warrington, U.K.). Primers used were: Bfl-1 5′ primer, AGTCTCAAGATTTCTCTCCAC; 3′ primer, TGGAAGTGCTTTTCTGTCACAG; Bcl-xL 5′ primer, GTTGAAAGGACGGTACC; 3′ primer, GCGTGGGCTCGAAATG; Bcl-xL 5′ primer, CACCGAAGATACAGCTG; 3′ primer, CTCCTGATCAAGGCTCTA.

Real-time RT-PCR

Total RNA was reverse-transcribed, after which real-time PCR amplification with Power SYBR Green (Applied Biosystems) was performed using a Mastercycler realplex thermocycler (Eppendorf, Hamburg, Germany). The results were normalized against GAPDH by comparing the fold change in the expression of Bfl-1, Bcl-2, and Bcl-xL mRNA to the expression of GAPDH. Primers used were: Bfl-1 5′ primer, TACGGACGAAATGTCGCCCG; 3′ primer, TGAATGTCAGCCTTCCCCCA; Bcl-xL 5′ primer, TCTGTCGCCCTGCACTT; 3′ primer, CAGGGAGCCTGAAGGT; Bcl-2 5′ primer, GGATGCTTTTGTGGAACGT; 3′ primer, AGGCTGCACGTGTTCCAT.

 Luciferase assays

p65-luc or control promoter constructs were transfected in SOCS1 overexpressing or knockdown cells. pCMV β-galactosidase was used to normalize the transfection efficiencies. After 24 h, cells were treated with Fas Ab for 30 min and lysed. The supernatant was used in the luciferase assay using a luminometer.

Flow cytometric analysis

Apoposisis measured by flow cytometry using an apoptosis detection kit (BD Pharmingen) according to the manufacturer’s protocols. Both early apoptotic (annexin V+, propidium iodide ) and late apoptotic (annexin V+, propidium iodide ) cells were included in cell death determinations. For the detection of mitochondrial membrane potential, cells were stained with MitoTracker Red CMXRos (Invitrogen) and then analyzed by flow cytometry.

Western blot and immunoprecipitation

Whole cell, cytosolic, or nuclear lysates were isolated as previously described (40). The lysates (40 μg each) were separated on SDS-PAGE and transferred to polyvinylidene difluoride membranes (Amersham Biosciences). The mem-
branes were then incubated with the specific Abs. The immunoblots were revealed by incubation with HRP-conjugated anti-mouse or anti-rabbit secondary Abs (Cell Signaling Technologies) and subjected for detection with Immobilon Western (Millipore, Billerica, MA). For immunoprecipitation, cell extracts were prepared in immunoprecipitation buffer (10 mM HEPES [pH 7.6], 15 mM KCl, 2 mM MgCl2, 0.1% Nonidet P-40, 1 mM PMSF) and complete protease inhibitor (Roche). The extracts (500 μg proteins) were then incubated with mouse monoclonal anti-p65, anti-p50/p105, anti-HA, or anti-SOCS1 Abs for 8 h at 4˚C. Protein A/G-agarose beads were then added (Santa Cruz Biotechnology), after which the bound proteins were released, resolved on SDS-PAGE, transferred to polyvinylidene difluoride membranes, and analyzed by immunoblots.

Densitometry analysis

The densitometric analysis of immunoblots was performed with MCID analysis software version 7.0 (Imaging Research, St. Catherines, ON, Canada).

Statistical analysis

All experiments were performed at least in three independent sets. The values are presented as means ± SE. Statistical significance was determined by a Student t test. A value of p < 0.05 was considered statistically significant.

Results

Fas-mediated apoptosis is promoted by SOCS1 overexpression and suppressed by SOCS1 ablation in T cells

In our recent report, SOCS1 was shown to suppress apoptotic death of immune cells in response to oxidative stress. Upon the ROS-generating stimuli such as hydrogen peroxide, etoposide, and TNF-α, T cell apoptosis was significantly attenuated by SOCS1 overexpression (38). Alternatively, we have noted that SOCS1 promoted apoptosis induced by the Fas receptor cross-linking by anti-Fas Ab treatment of Jurkat T cells. The cleavage of caspase-8 and -3 as well as PARP was increased in cells overexpressing SOCS1 upon treatment with 10–50 ng/ml anti-Fas Ab (Fig. 1A). Whereas SOCS1 overexpression per se exerted no apoptosis-inducing effect (6.7 versus 5.5%; Fig. 1B), a prominent proapoptotic effect of SOCS1 was seen in cells treated with anti-Fas Ab, exhibiting a significant increase in apoptotic populations as measured by annexin V staining (25.8 versus 41.8% at 10 ng/ml anti-Fas Ab in Fig. 1B; 47.2 versus 61.4% at 20 ng/ml anti-Fas Ab, data not shown).

FIGURE 1. SOCS1 promotes Fas-induced apoptosis in Jurkat T cells. (A) Jurkat T cells with stable overexpression of SOCS1 (Jurkat/HA-SOCS1) or mock vector HA (Jurkat/HA) were treated with 50 μM H2O2 or anti-Fas Abs at the indicated concentrations for 8 h. Whole cell lysates were assessed by Western blot analysis for cleaved caspase-8, cleaved caspase-3, cleaved PARP, and HA. (B) Jurkat/HA-SOCS1 or Jurkat/HA cells were either untreated or treated with 10 ng/ml anti-Fas Abs for 8 h, and cells were then assessed for apoptosis by flow cytometric analysis. The bar graph is shown as means ± SE as obtained from three independent experiments. (C) Stably established Jurkat/shRNA2 (sh) or Jurkat/shSOCS1 cells were treated with 10 ng/ml anti-Fas Abs at indicated concentrations for 8 h, after which the whole cell lysates were analyzed by Western blotting for cleaved PARP, cleaved caspases, SOCS1, and β-actin. (D) shSOCS1 or mock cells were treated with or without 10 ng/ml anti-Fas Abs for 8 h, and apoptotic cells were determined by FACS using annexin V staining. The bar graph is shown as means ± SE (n = 3 independent experiments). (E) Jurkat/HA or Jurkat/HA-SOCS1 cells and (F) Jurkat/sh or Jurkat/shSOCS1 cells were treated with or without anti-Fas Abs (10 ng/ml) for the indicated times. Whole cell lysates were assessed by Western blotting for FLIPL, FLIPS, procaspases, cleaved caspases, and β-actin.
To confirm the role of SOCS1 in the regulation of apoptosis, a stable Jurkat T cell line lacking SOCS1 expression established by introducing shRNA of SOCS1 (38) was employed. The SOCS1 knockdown cells exhibited an opposite response upon Fas ligation such that the Fas-mediated apoptosis was significantly suppressed. The cleavage of PARP and caspases was substantially reduced (Fig. 1C), which is accompanied by an ∼50% decrease in annexin V–stained populations in SOCS1-ablated cells as compared with control cells (31.4 versus 17.1%; Fig. 1D). These data suggest that SOCS1 has a proapoptotic function in T cells in response to Fas stimulation.

When the kinetics of apoptosis induction was analyzed, caspase-8 activation was accelerated in SOCS1 overexpressing cells as compared with the mock transfectants (2 versus 4 h). In HA-SOCS1 cells, a significant decrease in c-Flip ligand levels from 2 h coincides with caspase-8 activation, which was followed by the activation of caspase-2 and -3 (Fig. 1E). The overall c-Flip levels were maintained lower in SOCS1-transduced cells and higher in SOCS1 knockdown cells than in respective mock transfectant cells (Fig. 1E, 1F). The result indicates that the promotion of caspase-8 activation, shown by a rapid disappearance of the proform and a strong induction of the cleaved form, induced by SOCS1 correlates with the reduced level of c-Flip, a negative regulator of apoptosis through the inhibition of DISC function and caspase-8 processing (14).

SOCS1 accentuates Fas-mediated mitochondrial dysfunction

Caspase-8, as the initiator caspase in the Fas-mediated apoptosis signaling, also leads to mitochondrial dysfunction by the cleavage of Bid, a well-defined substrate of activated caspase-8 predominantly located in the cytoplasm (41). Upon the cleavage, t-Bid translocates to the mitochondria where it participates, as a proapoptotic factor, in permeability transition pore complex formation (7, 41). Previous work indicated that Fas-induced Cyt c release is associated with decreased levels of mitochondrial antiapoptotic proteins such as Bcl-2, Bcl-xL, Bfl-1, and/or increased translocation of t-Bid (41, 42). This was accompanied with mitochondrial membrane depolarization, disruption of mitochondrial membrane potential, and permeability change. To elucidate the mechanism by which SOCS1 promotes the Fas-mediated apoptotic response, the effect of SOCS1 on Fas-induced changes in mitochondrial function was examined. Thus both Jurkat/HA-SOCS1 cells and Jurkat/mock cells were treated with Mitotracker, a mitochondrial specific dye, after which cells were analyzed for mitochondrial membrane potential and examined for Cyt c release upon Fas stimulation. The result shows that when compared with mock cells, mitochondrial membrane potential was further reduced with a corresponding decrease in Cyt c levels in the mitochondria following Fas ligation in cells expressing SOCS1 (Fig. 2A, 2B).

To determine whether this effect is related to the expression of mitochondrial apoptosis regulatory proteins, the levels of Bcl-2 family members including Bcl-2, Bcl-xL, and Bfl-1 as well as t-Bid were examined by real time-PCR and immunoblots. It was noted that Fas ligation led to the early induction of Bfl-1 both at mRNA and protein levels peaking at 20 min by 2-fold, which was prominently suppressed in HA-SOCS1 cells (Fig. 2C, 2D). The expression levels of Bcl-xL and Bcl-2 were not significantly

FIGURE 2. SOCS1 accentuates Fas-mediated mitochondrial dysfunction. (A) Stable Jurkat/HA-SOCS1 or Jurkat/HA cells were treated with or without anti-Fas Abs (10 ng/ml) for 8 h and and stained with 250 nM MitoTracker Red CMXRos. The stained cells were then analyzed by FACS. (B) Stable cells were treated as in (A), and mitochondrial fraction was prepared and analyzed for Cyt c release. (C and D) Stable cells were incubated with anti-Fas Abs for the indicated times. Whole cell lysates and total RNAs were then prepared and analyzed for Bfl-1, Bcl-xL, Bcl-2, and t-Bid by Western blot (C) and quantitative RT-PCR using specific primers for Bfl-1, Bcl-xL, or Bcl-2 (D), respectively. The line and scatter plot graph data for densitometric analysis of blots obtained from three independent experiments are shown.
changed by SOCS1 under Fas signaling. Alternatively, the Fas ligation–induced increase in t-Bid levels was notably upregulated upon SOCS1 overexpression (Fig. 2C). The effect of SOCS1 on these apoptosis regulatory factors was examined using SOCS1-ablated cells as well. As expected, upregulation of Bfl-1 and down-regulation of t-Bid levels were observed upon SOCS1 knockdown without appreciable changes in other Bcl-2 members (Fig. 3A, 3B). The results indicate a possibility that SOCS1, although promoting t-Bid levels, abrogates Bfl-1 expression thereby accentuating the mitochondrial dysfunction induced by Fas signal.

Bfl-1, an NF-κB–dependent gene, is a target of apoptosis-promoting function of SOCS1 in T cells under Fas signaling

The above data suggest that SOCS1-induced apoptosis is mediated by promoting caspase-8 activity and mitochondrial membrane perturbation through downregulation of distinct antiapoptotic factors c-Flip and Bfl-1, respectively. Because the expression of c-Flip and Bfl-1 is known to be both regulated via transcriptional activation by NF-κB (13, 22, 24), a key prosurvival factor, the role of NF-κB on the Fas-mediated induction of c-Flip and Bfl-1 expression in SOCS1-depleted cells was examined using an inhibitor of NF-κB. Indeed, treatment with the IκB kinase inhibitor thalidomide (43) significantly attenuated the Fas-mediated induction of c-Flip and Bfl-1 in SOCS1-ablated cells, suggesting that NF-κB activity is critical for the upregulation of these antiapoptotic genes in T cells to exhibit resistance to Fas-mediated apoptotic response (Fig. 3C).

Next, to further investigate the biological role of Bfl-1 in mediating SOCS1 function through mitochondrial control of apoptosis, the effect of Bfl-1 ablation in SOCS1 regulation of apoptosis by Fas signal was examined. Thus, the SOCS1 knockout cells, which exhibited an antiapoptotic response to Fas signal, were transiently transfected with siRNAs of Bfl-1 or Bcl-xL and treated with anti-Fas Ab. The expression levels of Bfl-1 and Bcl-xl mRNA were confirmed using RT-PCR to ensure the specificity of depletion (Fig. 4A). The subsequent analysis of cell death revealed that whereas shSOCS1 cells display a reduced sensitivity to Fas-mediated cell death (31.4–17.1% in Fig. 1D), the depletion of Bfl-1 in these cells resulted in a full recovery in sensitivity to apoptosis by Fas signal (27.5–45.8% in Fig. 4B). Alternatively, depletion of Bcl-xL induced only a modest change in the apoptotic population in SOCS1 knockout cells (27.5–34.3%). Taken together, these data indicate that SOCS1 promotes Fas-mediated apoptosis of T cells mainly through the regulation of Bfl-1 expression induced by NF-κB activation.

SOCS1 downregulates NF-κB stability and activity under Fas signaling

Having observed that NF-κB–dependent antiapoptotic factors are downregulated in SOCS1-transduced cells, which accounts for, at least in part, the proapoptotic effect of SOCS1 (Figs 1–4), we examined whether SOCS1 regulates NF-κB during Fas signaling. In Jurkat T cells, it is observed that Fas signal induced a significant decrease of total p65 level after 1 h in mock cells (Fig. 5A). All

FIGURE 3. Regulation of antiapoptotic factors in SOCS1 knockdown cells. (A) Jurkat/sh or Jurkat/shSOCS1 stable cells were incubated with or without anti-Fas Abs for the indicated times. Whole cell lysates were then analyzed by Western blot for Bfl-1, Bcl-xl, Bcl-2, and t-Bid. (B) Stable cells were incubated with or without anti-Fas Abs for the indicated times. Bfl-1, Bcl-xl, or Bcl-2 expression was analyzed by quantitative RT-PCR using specific primers. (C) shSOCS1 stable cells were treated with anti-Fas Abs for indicated times in the absence or presence of an IκB kinase inhibitor thalidomide pretreatment (200 μM, 1 h). Whole cell lysates were then prepared and analyzed by Western blot analysis for antiapoptotic proteins. Scatter plot graph data for densitometric analysis of blots obtained from two independent experiments are shown.
though this is in agreement with the earlier report that NF-κB undergoes proteolysis, leading to eventual cell death upon Fas signaling (44), we have further noted that the Fas-induced reduction in p65 level was accelerated in SOCS1-transduced cells as compared with mock cells (Fig. 5A). On the contrary, the reduction was blocked and p65 was maintained over the basal level in SOCS1-ablated cells (Fig. 5A versus 5B). The basal level of p65 per se is downregulated in SOCS1 overexpressing cells and up-

FIGURE 4. Role of Bfl-1 in the apoptosis control by SOCS1 upon Fas stimulation. (A) siBfl-1 or siBcl-xL was transfected to Jurkat/shSOCS1 stable cells, and then the mRNA levels of Bfl-1 and Bcl-xL were analyzed by RT-PCR. (B) SOCS1 knockdown cells from (A) were treated with or without 10 ng/ml anti-Fas Abs for 8 h. Apoptotic cells were determined by FACS using annexin V Ab. The bar graph is shown as means ± SE as obtained from three independent experiments.

FIGURE 5. Downregulation of p65 stability and NF-κB activity by SOCS1. (A and B) Stably established Jurkat/HA-SOCS1 (A) or Jurkat/shSOCS1 cells (B) along with their mock counterpart cells were treated with or without 10 ng/ml anti-Fas Abs for the indicated times. The whole cell lysates were analyzed by Western blotting for p65, cleaved PARP, cleaved caspase-3, SOCS1, and β-actin. (C and D) Jurkat/HA and Jurkat/HA-SOCS1 cells were treated with or without 10 ng/ml anti-Fas Abs for the indicated times, after which cytoplasmic and nuclear extracts were prepared from harvested cells. The status of p65 was analyzed by Western blot (C). The nuclear/cytoplasmic ratio of p65 was shown in the line and scatter plot (D). (E and F) The cells were transiently transfected with luciferase reporter construct containing the p65 binding site or pGL3 control vector. The pCMV β-galactoside constructs were used to monitor transfection efficiency. Cells were treated with anti-Fas Abs, and the reporter activity was determined after 30 min. Data are shown as mean ± SE (n = 3 independent experiments).
regulated in SOCS1-depleted cells. The results indicated that SOCS1 acts as a potential regulator of both constitutive and Fas signal-induced p65 levels.

The subcellular fractionation analysis revealed a prominent activation of p65 shown as nuclear translocation by 20 min Fas stimulation in control Jurkat transfectants, which was almost abrogated in HASOCS1 cells (Fig. 5C, 5D). Next, the changes in transactivating function of NF-κB were examined by reporter gene assays. As shown in Fig. 5E, the κB-responsive promoter activation and the subsequent induction of luciferase expression induced by Fas signaling in 30 min were significantly decreased in SOCS1-transduced Jurkat T cells as compared with mock cells. Conversely, SOCS1 knockdown T cells exhibited increased κB-dependent promoter activities (Fig. 5F). These results demonstrate that SOCS1 downregulates the expression and the consequent activation of NF-κB induced within 20–30 min in response to Fas signal in Jurkat T cells.

We then investigated the mechanism of SOCS1 action on NF-κB. Because it has been suggested that NF-κB is a target of SOCS box–mediated proteosomal degradation, we tested the possibility that SOCS1 reduces NF-κB stability in T cells under Fas signaling through protein degradation process. Indeed, the Fas-induced p65 protein level was significantly reduced in SOCS1-transduced T cells and was substantially restored upon treatment with proteasome inhibitor MG132 in a time-dependent manner, indicating that NF-κB stability is regulated by SOCS1 through proteosome-mediated pathways during Fas signaling (Fig. 6A). To further define whether SOCS1 affects NF-κB degradation, SOCS1 overexpressing Jurkat T cells were examined for p65 ubiquitination. As expected, the Fas-induced polyubiquitinated p65 was sustained at a higher level in Jurkat/HA-SOCS1 cells than in control Jurkat cells (Fig. 6B). In SOCS1 knockdown cells, in contrast, Fas-mediated p65 ubiquitination was reduced (data not shown). The data suggest that SOCS1 promotes p65 ubiquitination and the subsequent degradation to reduce p65 levels upon Fas stimulation.

Next, to investigate whether this ubiquitination of p65 is mediated by binding with SOCS1, the molecular interaction between the two proteins in response to Fas signal was examined. The coimmunoprecipitation experiments have demonstrated that the interaction between SOCS1 and p65 or p50 is constitutively present and further promoted upon Fas ligation by 15–30 min in SOCS1 overexpressing Jurkat cells (Fig. 6C). A similar interaction pattern was observed in PBMCs, which were also responsive to Fas-mediated apoptosis. In these primary cells, the increased association between SOCS1 and p65 was noted upon Fas stimulation with MG132 treatment (Supplemental Fig. 1). Additionally, SOCS1 strongly interacted with p65 in the nucleus (Fig. 6D), which is correlated with preferred localization of SOCS1 to the nucleus in SOCS1-transduced cells (45, 46). Taken together, the results indicate that SOCS1 interacts with p65 and promotes its ubiquitination for proteosomal degradation to reduce NF-κB activities upon Fas signaling.
SOCS3 also promotes Fas-mediated apoptosis with downregulation of Bfl-1 and NF-κB

To determine whether the apoptosis-promoting function of SOCS1 in Fas-induced signal transduction is a property shared with other SOCS members, we have examined the effect of SOCS3. Being most abundantly expressed isoforms in T cells, SOCS1 and SOCS3 possess common structural features and often, but not always, display similar functional characteristics. For example, both SOCS1 and SOCS3 inhibit Jak/STAT pathways by binding with phosphotyrosine-Jaks via the SH2 domain and the subsequent degradation of Jaks through proteosomal activation mediated via the SOCS-box domain in many cell types. However, SOCS1 but not SOCS3 has been reported to suppress TNF-α or ROS-induced apoptosis in T cells (36, 38). By employing Jurkat T cells stably overexpressing SOCS3 (Fig. 7A), we have observed that similar to SOCS1, SOCS3 profoundly enhanced the Fas signal–induced apoptosis as seen in increase of annexin V* populations (26.8–46.4%; Fig. 7B). The change in apoptotic response was accompanied with a significant downregulation of Fas-induced Bfl-1 expression levels as assessed by real-time PCR (7.2- versus 3.7-fold).

The kinetics of Bfl-1 induction corresponds with the kinetics of NF-κB induction upon Fas signaling, with both of them peaking at 60 min after Fas stimulation (Fig. 7C, 7D). As in the case for SOCS1-transduced cells, a significant decrease in total as well as nuclear NF-κB levels was obtained in SOCS3 overexpressing cells (Fig. 7D). The data strongly suggest that both SOCS1 and SOCS3 act in a similar mechanism to promote Fas-mediated apoptosis by suppression of NF-κB–dependent Bfl-1 expression, an important antiapoptotic factor in T cells.

Induction of SOCS and FasL during T cell activation

The above data strongly indicate that SOCS1 and SOCS3 act as a potential regulator of Fas-mediated apoptosis. Because Fas-induced T cell death occurs after T cell activation upon TCR stimulation through Fas L induction (as AICD), we have examined the induction of SOCS and FasL and the kinetic correlation of these apoptosis-promoting molecules. When Jurkat T cells were stimulated by anti-CD3 and anti-CD28, the induction of SOCS1 and SOCS3 was observed from 30 min at both mRNA and protein levels (Fig. 8A). In these T cells SOCS1 induction gradually increased and preceded FasL induction which appears at later times, 16–24 h after TCR stimulation (Fig. 8B). In mouse splenocytes, the induction of SOCS and FasL was also noted upon anti-CD3 and anti-CD28 stimulation, albeit with a faster kinetics of induction for FasL as compared with Jurkat T cells (Fig. 8C). The relatively early induction of SOCS proteins may play a role in the attenuation of Jak-mediated TCR signaling and in the sensitization of T cells to AICD.

Discussion

We have previously reported that SOCS1 exhibits antiapoptotic functions against oxidative stress-induced immune cell death. The exogenously applied hydrogen peroxide and cytokine TNF-α signal both induced an increase in intracellular ROS levels, causing the inhibition of protein tyrosine phosphatases, and the activation of Jaks, which led to the apoptotic response of T cells. Under such conditions, the antiapoptotic effect of SOCS1 was manifest by reducing ROS levels and protection of protein tyrosine phosphatases by thioredoxin (38). Although the ROS-mediated NF-κB activation was noted in this system, the antiapoptotic...
function of SOCS1 did not involve NF-κB, as NF-κB activities remained unchanged upon SOCS1 overexpression (38). Alternatively, in the present study we have noted the proapoptotic effect of SOCS1 during Fas signaling in T cells, which involves downregulation of Fas-induced NF-κB activation.

The activation of NF-κB noted within 20–60 min upon Fas receptor cross-linking seems to partly counteract the apoptosis signal ensued by Fas. In fact, it has been proposed that during the Fas signal–induced apoptosis, a low level of ROS is generated and the ROS-mediated NF-κB activation can serve as a survival signal in Jurkat T cells (25). Unlike the case for hydrogen peroxide or TNF-α stimulation, which induces ROS-mediated Jak activation and the upregulation of thioredoxin (38), Fas ligation did not lead to a significant induction of Jak activities or thioredoxin (Supplemental Fig. 2A, 2B). Whereas the aforementioned Jak activation–dependent TNF-α signaling for apoptosis was counterregulated by SOCS1, the increased Fas-mediated apoptotic response by SOCS1 was not associated with noticeable changes in Jaks, p38, or Jnk activation involved in apoptosis signaling (Supplemental Fig. 2B, 2C).

These data suggest that SOCS1-mediated promotion of Fas signal leading to apoptosis is likely to occur by attenuating survival pathways such as those mediated by NF-κB. In support of this notion, the critical role of NF-κB in the resistance against apoptotic signal in various cell types has been demonstrated. For example, blocking NF-κB activation is found to convert the survival agent into an apoptotic effector in Jurkat leukemic T cells (23), and Fas resistance of leukemic eosinophils is shown to be due to the activation of NF-κB induced by Fas receptor ligation (47). In this regard, we have noted a transient induction of NF-κB activation upon Fas ligation in Jurkat T cells, which is then downregulated as apoptosis proceeds (Fig. 5C, 5D). Whereas Fas ligation did not upregulate SOCS levels (Supplemental Fig. 1A), forced expression of SOCS1 or SOCS3 promoted the overall decrease of both total and nuclear p65 levels (Figs. 5A, 5C, 7D).

Through a series of experiments we demonstrate that decreased NF-κB levels caused by SOCS-mediated NF-κB ubiquitination and the subsequent degradation are responsible for the reduced nuclear localization and the subsequent downregulation of transcriptional activities of NF-κB. Two NF-κB–dependent antiapoptotic factors, c-Flip and Bfl-1, suppressing caspase-8 activation and mitochondrial permeability transition pore complex forma-

**FIGURE 8.** Induction of SOCS and FasL upon TCR stimulation in primary immune cells and T cell lines. Jurkat T cells (A, B) and mouse splenocytes (C) were stimulated with anti-CD3 (1 μg/ml) and anti-CD28 (0.5 μg/ml) for indicated durations. Cell lysates were prepared and analyzed for SOCS1, SOCS3, or FasL expression levels by RT-PCR (A) and Western blotting (B, C) as indicated. Scatter plot graph data for densitometric analysis of blots obtained from two independent experiments are shown.

**FIGURE 9.** Proposed model for SOCS action to promote Fas-mediated apoptosis in T cells. Fas signal–induced apoptosis in T cells is promoted by SOCS, which, through degradation of p65, downregulates NF-κB–dependent expression of c-Flip and Bfl-1. The suppression of caspase-8 inhibitor c-Flip leads to efficient cleavage of Bid. Increased propapoptotic t-Bid production together with the decrease in antiapoptotic Bfl-1 will promote mitochondrial dysfunction through MTPC formation. The subsequent release of Cyt c and caspase-9/3 activation lead to enhanced apoptotic response.
tion, respectively, appear to play a key role in the regulation of Fas-mediated apoptosis in T cells (Fig. 9).

Both c-FlipL and c-FlipS have been demonstrated to have a prosurvival function. They are known to participate in the inhibition of DISC formation, blocking caspase-8–mediated downstream signaling (13). Members of c-Flip have been also reported to induce NF-κB and Erk activation, thus acting in a positive feedback loop to promote cell survival (14). Additionally, the induction of lymphocyte-specific antiapoptotic factor Bfl-1 in an NF-κB–dependent manner during Fas signaling may constitute an important survival response in T cells. Thus, in control cells with basal NF-κB levels, the transient induction of Bfl-1 and the low production of t-Bid by attenuated caspase-8 activities controlled by c-Flip during the early phase of Fas ligation would keep the cleavage of caspase-3 weak up to 4 h (Figs. 1E, 2C, 5A). Alternatively, in SOCS1-transduced cells with NF-κB levels downregulated, Bfl-1 induction was abrogated and t-Bid production was enhanced by active caspase-8 by 2 h. The balance of Bfl-1 and t-Bid is considered important in the mitochondrial control of apoptosis as Bfl-1 sequesters t-Bid from its interaction with Bak or Bax (42). In SOCS1-ablated cells with the high levels of NF-κB and c-Flip, Bfl-1 induction is maintained and t-Bid production is kept low. This would protect the cells from both caspase-8– and mitochondria-mediated apoptosis initiated by Fas ligation (Figs. 1C, 1F, 3A, 5B).

We have then demonstrated, by employing coimmunoprecipitation, that the Fas signal–induced and SOCS1-mediated NF-κB downregulation occurs through stability control via direct molecular interaction between SOCS1 and p65 in JurkT T cells and PBMCs, likely involving SOCS box–mediated p65 degradation. NF-κB has been proposed as a target of SOCS action since its identification as an interacting partner of SOCS1 followed by demonstration of p65 degradation control by SOCS1 (31). Our data further show that SOCS1 coimmunoprecipitates p65 as well as p50, suggesting the molecular interaction of SOCS1 with the NF-κB complex of p65/p50. Of note, while this manuscript was in preparation, SOCS1 action to limit the duration NF-κB signaling through SOCS box–mediated p65 degradation in the nucleus was reported using κB-dependent reporter gene assays in SOCS1-transfected HEK 293 cells (32). Whereas these preceding studies suggest the mechanism of SOCS1 action in suppressing nuclear NF-κB activity and the target gene expression at the molecular level, our work presents a complete system where such inhibitory action of SOCS on the NF-κB activation via expression of specific gene products translates into the regulation of cell survival response against apoptotic stimuli in a defined cell type. Strebovskov et al. (32) reported a lack of SOCS3 regulation of p65 in their experimental system. We have, however, shown in human leukemic JurkT T cells that SOCS3 also acted as a potent proapoptotic factor during the Fas-induced cell death with suppressive effects on NF-κB activation and Bfl-1 expression. Whereas the SOCS-mediated NF-κB/p65 degradation was observed within 1 h Fas stimulation, SOCS levels also tend to be decreased as the apoptosis proceeds, at the time of caspase activation (Fig. 1A, 5A, Supplemental Fig. 1A). In fact, the reduction in SOCS levels during Fas-mediated apoptosis is found to be a caspase-dependent response, as the downregulation was blocked by pretreatment of cells with a pan-caspase inhibitor z-VAD (data not shown).

In the immune scenarios, activated and clonally expanded T cells generated by Ag stimulation are destined to undergo apoptosis upon Fas receptor ligation. Because Bfl-1 has been identified as a key antiapoptotic factor in T cells induced by Fas signaling via NF-κB–dependent pathways (25), the downregulation of NF-κB activity by proapoptotic SOCS will aid in the effective T cell removal through targeting Bfl-1. In relationship to this, we have noted that during the early stage of TCR stimulation, SOCS1 and SOCS3 are induced both in splenocytes and JurkT T cells (Fig. 8). These SOCS proteins would act in a negative feedback loop of T cell activation and sensitize activated T cells to the FasL/Fas-mediated apoptosis by downregulating NF-κB. Although there have been reports indicating NF-κB as a target of Fas signal to counteract TCR-mediated T cell activation and proliferation (44, 48), to our knowledge this study is the first to demonstrate that SOCS proteins act as a modulator of Fas-induced apoptosis in T cells by regulation of NF-κB and Bfl-1. The observation that the proapoptotic effect of SOCS on Fas signal was prominent in T cells but not in other cell types including B cells and epithelial cells (data not shown) also suggests that SOCS1 and SOCS3 act as regulators of T cell survival critical in immune homeostasis. The use of T cell–specific knockout animal models for SOCS1 and SOCS3 would be useful to obtain in vivo evidence of SOCS action in Fas-mediated apoptosis signaling in T cells (49, 50). As much as defective Fas signaling by functional polymorphism of Fas/FasL and Fas-associated death domain protein has been implicated in genetic susceptibility to T cell malignancies and autoimmune lymphoproliferative syndrome (51), further elucidation on the mechanism of SOCS action in the promotion of AICD would provide a basis for novel approaches to control such lymphoproliferative disorders.

Disclosures
The authors have no financial conflicts of interest.

References