REVIEW

Regulation of Cytokine Signaling by the SOCS and Spred Family Proteins

Akihiko Yoshimura

Department of Microbiology and Immunology, School of Medicine, Keio University, Tokyo, Japan; Japan Science and Technology Agency (JST), CREST, Tokyo, Japan

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Abstract

Various cytokines are involved in the regulation of the immune system and of hematopoiesis. Most cytokines utilize the so-called JAK-STAT pathway, but others activate the Ras-ERK pathway, which is more important than the STAT pathway for the proliferation of hematopoietic cells. Dysregulation of cytokine signaling can cause a variety of diseases, including allergy, inflammation, and cancer. We have identified two important regulator families involved in cytokine signaling: the SOCS proteins and the Spred proteins. Suppressors of cytokine signaling (SOCS) proteins bind to JAK and to certain receptors, thereby suppressing further signaling events. Spred family proteins interact with Ras and Raf, thereby suppressing ERK activation. Studies have shown that SOCS and Spred proteins are key physiological regulators of immunity, hematopoiesis, and angiogenesis. Evidence is also emerging for the involvement of these proteins in human diseases. (Keio J Med 58 (2) : 73–83, June 2009)

***Keywords*: cytokine, signal transduction, negative regulation**

Introduction

Cytokines play several essential roles in the development, differentiation, and function of myeloid and lymphoid cells. Some of them, including interleukins, interferons (IFNs), and hematopoietic growth factors, activate the Janus kinase/Signal Transducers and Activators of Transcription (JAK/STAT) pathway. In this pathway, cytokine binding induces receptor oligomerization, which initiates signaling from cytokine receptors. This signaling brings associated JAK kinases (JAK1, JAK2, JAK3, and Tyk2) into close apposition and allows their cross-phosphorylation and activation (**Fig. 1**).[1,2] The activated JAKs phosphorylate the receptor cytoplasmic domains, which creates docking sites for SH2-containing signaling proteins. Among the substrates of tyrosine phosphorylation are members of the Signal Transducers and Activators of Transcription family of proteins (STATs).[1,2] Although this pathway was initially found to be activated by IFNs, it is now known that a large number of cytokines, growth factors, and hormonal factors also activate JAK and/or STAT proteins; for example, pro-inflammatory cytokine IL-6 binds to the IL-6 receptor α chain and to gp130, both of which mainly activate JAK1 and STAT3. IFNγ utilizes JAK1 and JAK2, although it mainly activates STAT1. Interestingly, anti-inflammatory cytokine IL-10 also activates STAT3. STAT4 and STAT6 are essential for Th1 and Th2 development since these are activated by IL-12 and IL-4, respectively.

In addition, the Ras-ERK pathway is activated through adaptor proteins such as SHP2 and Gab-1 (**Fig. 1**). This pathway is essential for the proliferation of hematopoietic cells through hematopoietins including IL-3, IL-5, erythropoietin (EPO), and granulocyte colony-stimulating factor (G-CSF).[3,4] For example, STAT3 activated by G-CSF suppresses granulopoiesis induced by G-CSF, while the Ras-ERK pathway is shown to play an essential role in promoting proliferation and anti-apoptosis of granulocyte progenitors.[5]

Although our understanding of the intracellular signaling molecules that mediate the functional outcome of cytokine-receptor activation has increased profoundly, the

Reprint requests to: Akihiko Yoshimura, M.D., Department of Microbiology and Immunology, School of Medicine, Keio University, 35 Shinano-machi, Shinjuku-ku, Tokyo 160-8582, Japan, Tel: +81-3-5363-3483, Fax: +81-3-5360-1508, E-mail: yoshimura@a6.keio.jp

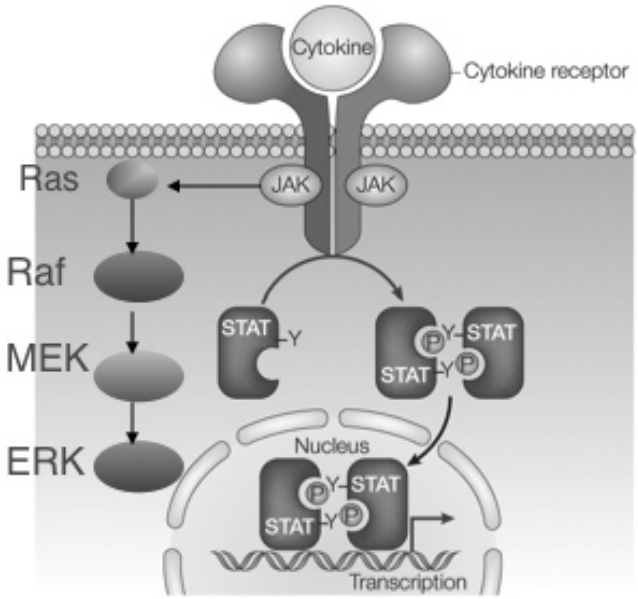

Fig. 1 Signal transduction of the cytokine receptors
Most cytokines activate receptor-associated JAK tyrosine kinases, thereby activating STAT transcription factors and the Ras-ERK pathway.

most recent research has placed increasing emphasis on the mechanisms by which cytokine signals are terminated. A number of mechanisms have been proposed, including tyrosine phosphatases and transcription suppressors. In addition to these, we have previously identified two more potential mechanisms: the large CIS/SOCS and Spred/Sprouty families of proteins.[6,7] At the time of their discovery, the SOCS proteins were recognized as an important mechanism for the negative regulation of the cytokine-JAK-STAT pathway, but recent studies using gene-disrupted (KO) mice have revealed that they play additional, unexpected, and profound roles in many immunological processes. The Spred/Sprouty family proteins, meanwhile, are more specific to the Ras-ERK pathway, although it has been shown that they are very important regulators for development, angiogenesis and neural networks. Furthermore, their relationship to human diseases has been recently discovered.

CIS/SOCS Family

Suppressor of cytokine signaling (SOCS) proteins and cytokine inducible SH2-containing (CIS; also known as CISH) protein molecules comprise a family of intracellular proteins, several of which have been shown to regulate the responses of immune cells to cytokines.[8–10] There are eight CIS/SOCS family proteins; CIS, SOCS1, SOCS2, SOCS3, SOCS4, SOCS5, SOCS6, and SOCS7, each of which has a central SH2 domain, an amino-terminal domain of variable length and sequence, and a carboxy-terminal 40-amino-acid module known as the SOCS box (**Fig. 2A**). The SOCS box is also found in other miscellaneous proteins. The SOCS box interacts with elongin B and elongin C, cullin 5, and the RING-finger-domain-only protein RBX2 (which recruits E2 ubiquitin–transferase).[11] CIS/SOCS family proteins, as well as other SOCS-box-containing molecules, probably function as E3 ubiquitin ligases and mediate the degradation of proteins that are associated with these family members through their N-terminal regions (**Fig. 2A**). The best characterized SOCS-family members are CIS, SOCS1, SOCS2 and SOCS3.

In addition to their ability to suppress signaling by ubiquitin-mediated degradation of the signaling complex, both SOCS1 and SOCS3 can inhibit JAK tyrosine kinase activity directly through their kinase inhibitory region (KIR), which has been proposed to function as a pseudosubstrate and which is important for the suppression of cytokine signals (**Fig. 2**).[12] The KIR peptides have been shown to inhibit JAK2-mediated phosphorylation of STAT1.

The central SH2 domain determines the target of each SOCS and CIS protein. The SH2 domains of CIS, SOCS2, and SOCS3 bind to phosphorylated tyrosine residues on activated cytokine receptors.[13,14] SOCS3 binds to gp130-related cytokine receptors, including the phosphorylated tyrosine 757 (Tyr757) residue of gp130 and the Tyr800 residue of IL-12 receptor β2 (**Fig. 2B**).[15] The SH2 domain of SOCS1 directly binds to the activation loop of JAKs.[12] The SH2 domain of SOCS3, in contrast, does not have a high affinity to the activation loop of JAKs; yet the KIR of SOCS3 has a higher affinity to the kinase domain of JAK2 than that of SOCS1 has.[16] Therefore, SOCS3 might also use the same strategy of first binding with high affinity to the receptor before inhibiting JAKs through KIR (**Fig. 2B**).

Physiological Functions of SOCS

SOCS1 is an essential negative regulator of IFNγ

Although *SOCS1* knockout (KO) mice are normal at birth, they exhibit stunted growth and die within 3 weeks of birth, with a syndrome characterized by severe lymphopenia, activation of peripheral T cells, fatty degeneration and necrosis of the liver, and macrophage infiltration of major organs.[17] The neonatal defects exhibited by *SOCS1*$^{-/-}$ mice appear to occur primarily as a result of unbridled IFNγ signaling, since *SOCS1*$^{-/-}$ mice that also lack the IFNγ gene or the IFNγ receptor gene do not die neonatally.[10,17] Constitutive activation of STAT1 as well as constitutive expression of IFNγ-inducible genes was observed in *SOCS1* KO mice. These data strongly suggest that the excess IFNγ is derived from the abnormally

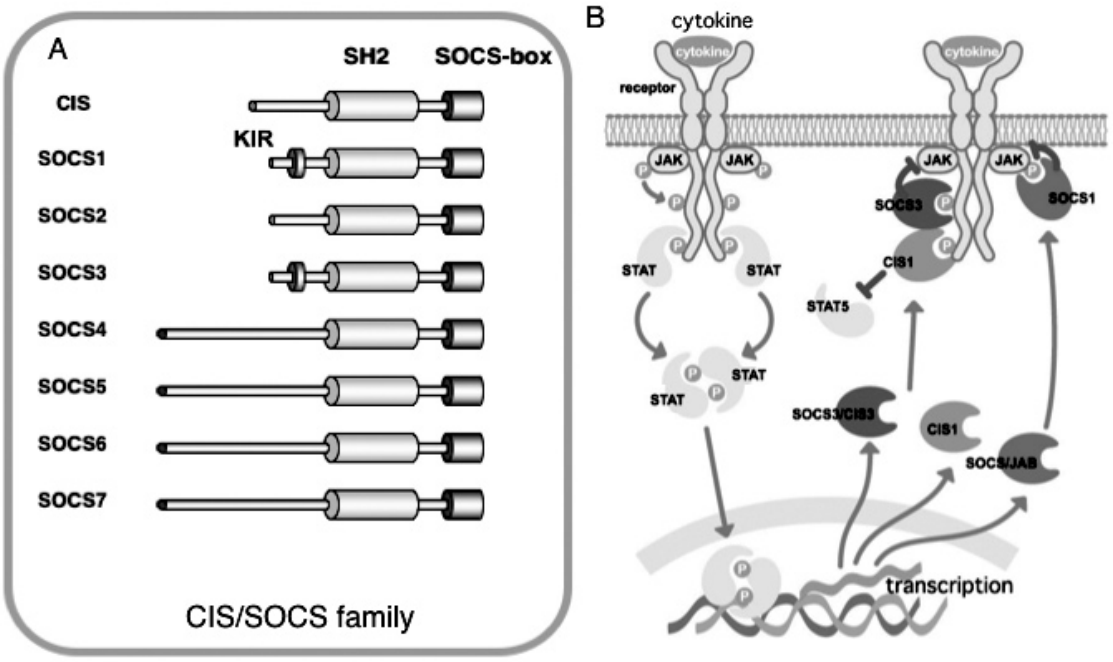

Fig. 2 The structure and function of SOCS proteins
(A) Schematic structure of the CIS/SOCS family proteins. The SOCS box is conserved in all CIS/SOCS family proteins. The function of the SOCS box is the recruitment of the ubiquitin-transferase system; thus the CIS/SOCS family proteins, as well as other SOCS-box-containing molecules, function as E3 ubiquitin ligases and mediate the degradation of proteins with which they are associated through their amino (N)-terminal regions. SOCS1 (also called JAB) and SOCS3 (also called CIS3) inhibit the tyrosine kinase activity of JAK directly, as they contain a kinase inhibitory region (KIR) immediately upstream of the central SH2 domain, which is proposed to function as a pseudosubstrate. (B) Mechanism of suppression by CIS (also called CIS1), SOCS1 (also called JAB), and SOCS3 (also called CIS3). All of these are induced by cytokine stimulation. CIS1 binds to the STAT5 activating receptors, thereby suppressing further activation of STAT5. SOCS1 binds to JAKs, and SOCS3 binds to the receptor through the SH2 domain, but both inhibit JAK activity through KIR. These complexes may be degraded by ubiquitination and proteasomal degradation recruited through the SOCS-box.

activated T cells in *SOCS1*$^{-/-}$ mice.

Using liver-specific *SOCS1*-conditional knockout mice, we demonstrated that *SOCS1* deletion in hepatocytes enhanced concanavalin A (ConA)-induced hepatitis, which has been shown to be dependent on NKT cells and IFNγ.[18] The pro-apoptotic signals, including STAT1 and JNK activation, were enhanced in SOCS1-deficient mice compared to those in wild-type (WT) mice. In contrast, SOCS1 overexpression in the liver by adenoviral gene transfer prevented ConA-induced liver injury by suppressing STAT1 activation. These findings indicate that SOCS1 plays an important negative role in fulminant hepatitis and that forced expression of SOCS1 is therapeutic in preventing hepatitis.[18]

We have recently demonstrated that SOCS1 is essential for helper T cell differentiation. Most *SOCS1*$^{-/-}$CD4 naïve T cells differentiated into Th1, even under skewing conditions, while Th17 differentiation was strongly suppressed. This was also dependent on IFNγ, since Th17 was normally developed in *SOCS1*$^{-/-}$*IFNγ*$^{-/-}$ T cells. As a result, T cell specific *SOCS1*-deficient mice were very sensitive to dextran sulfate sodium (DSS)-induced colitis (Th1 type disease)[19] but resistant to experimental autoimmune encephalomyelitis (EAE), a typical Th17 type disease.[20] Taken together, these data indicate that SOCS1 negatively regulates IFNγ signaling in various types of cells, thus fulfilling an important function for the suppression of Th1-type inflammatory diseases.

Physiological functions of SOCS3 defined by gene targeting at various tissues

SOCS3 knockout mice die during the embryonic stage of development due to placental function defects. In other words, deletion of SOCS3 causes embryonic lethality; these embryos can be saved, however, by a tetraploid rescue approach. These observations demonstrate SOCS3's essential role in placental development and non-essential role in embryo development. Rescued *SOCS3*-deficient mouse embryos exhibit prenatal lethality with cardiac hypertrophy, which suggests that SOCS3 is essential for regulating either LIF receptors or gp130 signaling.[21]

Conditional-KO mice studies have demonstrated that

SOCS3 is an important negative regulator of IL-6[15] and G-CSF.[22] Mice in which the *SOCS3* gene was deleted in all hematopoietic cells developed neutrophilia and a spectrum of inflammatory pathologies. When stimulated with G-CSF *in vitro*, *SOCS3*-deficient cells of the neutrophilic granulocyte lineage exhibited prolonged *STAT3* activation and enhanced cellular responses to G-CSF;[22] *SOCS3*-deficient mice injected with G-CSF *in vivo* displayed enhanced neutrophilia, progenitor cell mobilization, and splenomegaly, but unexpectedly also developed inflammatory neutrophil infiltration into multiple tissues and consequent hind-leg paresis. Interestingly, conditional *STAT3*-deletion in neutrophils also resulted in hyper-response to G-CSF,[23] suggesting that a major role of STAT3 in neutrophils is the induction of *SOCS3*. It seems likely that the ERK pathway induced by G-CSF play a major role in the proliferation and survival of neutrophils.

The essential roles of SOCS3 in endocrine systems have also been clarified in recent years. Administration of leptin to neural cell-specific SOCS3 conditional KO mice greatly reduces their food intake and causes enhanced body weight loss compared to WT mice, indicating that SOCS3 in the brain negatively regulates leptin signaling.[24] Moreover, the *SOCS3*-deficient mice were resistant to high fat diet-induced weight gain and hyperleptinemia, and their insulin-sensitivity was retained.[24,25] These data indicate that SOCS3 is a key regulator of diet-induced leptin as well as of insulin resistance. In addition, *SOCS3* deficient adipocytes generated from *SOCS3* KO fibroblasts are significantly protected from TNF-α -induced insulin resistance, mainly due to reduced proteasomal degradation of IRS proteins by TNF-α, suggesting that SOCS3 is an important mediator of insulin resistance *in vivo*.[26] Consistent with these ideas is the observation that the loss of SOCS3 in the liver apparently improved insulin sensitivity.[27] Unexpectedly, however, liver-specific *SOCS3* cKO mice exhibited obesity and systemic insulin resistance with age, due to constitutive activation of STAT3 which mimics chronic inflammation. Collectively, these results indicate that SOCS3 can be a potential therapeutic target for human metabolic disorders such as obesity and diabetes, although long-term treatment may cause inconvenient side effects.

SOCS and Immunity

The role of SOCS1 in innate immunity and inflammatory diseases

Toll-like receptor (TLR) signals that initiate innate immune responses to pathogens must be tightly regulated to prevent excessive inflammatory damage in the host. TLR ligands, such as LPS and CpG DNA, are potent inducers of *SOCS1* and *SOCS3*; therefore the role of SOCS1 and SOCS3 in TLR responses has been extensively investigated.

SOCS1-deficient mice are hypersensitive to LPS, which leads to increases in tumour necrosis factor (TNF) and IL-12 production.[28] The hyperactivation of macrophages in *SOCS1*$^{-/-}$ mice might be due in part to a stronger responsiveness of *SOCS1*$^{-/-}$ cells to IFNγ compared with wild-type cells. However, since *IFNγ*$^{-/-}$*SOCS1*$^{-/-}$ mice are still sensitive to LPS-induced shock, IFNγ-independent mechanisms probably exist. A direct effect of *SOCS1* on the TLR–NF-κB (nuclear factor-κB) pathway has been proposed.[29] Moreover, Kimura *et al*. indicate that LPS can activate JAK2 and STAT5, which are involved in IL-6 induction, and that SOCS1 selectively inhibits this process.[30]

SOCS1 also negatively regulates LPS- and IL-4-induced dendritic cell (DC) maturation. *SOCS1*-deficient DCs secrete larger amounts of IFNγ, IL-6, IL-12, and TNF in response to LPS and CpG compared with wild-type cells.[31,32] *SOCS1*-deficient DCs express higher levels of MHC class II and co-stimulatory molecules.[32] Therefore, SOCS1 must be deeply involved in the development, maturation, and activation of DCs.

These *SOCS1*-deficient DCs seem to be responsible for the development of systemic autoimmunity in aged *SOCS1*-deficient mice. Immunization with normal DCs can activate autoreactive T cells but rarely causes autoimmune pathology, indicating that self-tolerance is still maintained in the vaccinated hosts. However, an adoptive transfer of *SOCS1*$^{-/-}$ DCs to wild-type recipients resulted in the induction of autoantibodies through enhanced expression of B-cell-activating factor (BAFF) by the donor DCs.[31] This breaking of self-tolerance might be due to hyper-production of IL-12 by *SOCS1*-deficient DCs.[32] These data indicate that SOCS1 is an essential negative regulator for T-cell activation by DCs and for the maintenance of immunological tolerance.

Depletion of the SOCS1 protein in DCs, therefore, may enhance anti-tumour immunity. Indeed, we have shown that adoptive transfer of antigen-loaded *SOCS1*$^{-/-}$ BMDCs can prevent B16 melanoma growth in mice.[32] Similarly, Chen and colleagues reported that silencing *SOCS1* using small interfering RNA (siRNA) technology in antigen-presenting DCs strongly enhanced antigen-specific anti-tumour immunity.[33] A transfer of DCs treated with ovalbumin (OVA)-pulsed *SOCS1*-siRNA enhanced the proliferation and function of OVA-specific cytotoxic T cells (CTLs) compared with control DCs.

TLR signaling pathways are essential for macrophage-dependent inflammatory bowel diseases, and *SOCS1* has an important role in the development of colitis. *SOCS1*$^{-/-}$ *Tcra*$^{-/-}$ mice develop very severe colitis and die within two months of birth.[35] This very severe colitis is dependent on both IFNγ and IL-4. In addition, NF-κB and MAP kinases were strongly activated in the colons of

these mice; interestingly, however, this severe colitis was completely abolished when the mice were derived in germ-free conditions. This is consistent with previous observations that eventually all colitis models in mice occur in non-germ-free conditions. These findings suggest that TLR signaling is prerequisite for pro-inflammatory cytokine production and that these two signals must be mutually activated to promote colitis. Furthermore, SOCS1 has an important role in the negative regulation of both the JAK/STAT signaling cascade and the TLR pathway during the development of inflammatory bowel diseases.

SOCS3 and regulation of TLR signaling

SOCS3 has now been shown to be a key regulator for the divergent activity of IL-6 and IL-10 following TLR stimulation.[15] IL-6 is a pro-inflammatory cytokine that assumes a progressive role in many inflammatory diseases, while IL-10 is an immunoregulatory cytokine that has potent anti-inflammatory activity, including the suppression of gene activation through TLR signaling pathways. While it was known that STAT3 is essential for the biological actions of both IL-6 and IL-10, it was unclear how these two cytokines could have such precisely opposing functions. SOCS3 protein is strongly induced by both IL-6 and IL-10 in the presence of LPS, but IL-6 signaling is selectively inhibited due to the binding of SOCS3 to the IL-6R subunit gp130 (Tyr759), but not to the IL-10 receptor (IL-10R). Therefore, STAT3 activation is transient in response to IL-6, but is sustained for a long period in response to IL-10. Furthermore, a gp130 mutant lacking the SOCS3 binding site (Tyr759Phe), as well as any heterologous cytokine receptors that are mutated to induce sustained STAT3 activation by deleting the SOCS3 binding sites, can elicit an anti-inflammatory effect indistinguishable from that induced by IL-10. Therefore, the anti-inflammatory response is a generic cytokine signaling pathway dependent on STAT3 but not unique to the IL-10. This idea is consistent with recent studies showing that constitutively activated STAT3 (STAT3c) is sufficient for the suppression of LPS-induced TNF and IL-6 production in macrophages. We proposed that this sustained activation of STAT3 is essential for the anti-inflammatory effect, while transient activation of STAT3 promotes inflammation.

SOCS3 and DC-mediated T-cell differentiation

Recently, we examined the antigen-presenting cell (APC) function of *SOCS3*-deficient DCs. Like *SOCS3*$^{-/-}$ macrophages, *SOCS3*$^{-/-}$ DCs showed constitutive activation of STAT3 and expressed low levels of MHC class II molecules, co-stimulatory molecules, and IL-12.[36] Surprisingly, adoptive transfer of *SOCS3*$^{-/-}$ DCs suppressed EAE. *SOCS3*$^{-/-}$ DCs are poor activators of effector CD4^{+} T cells, but they selectively expand forkhead box P3 (FOXP3)$^{+}$ regulatory T cells (Tregs), which can suppress EAE. We have shown that IL-10 and TGFβ1 are target genes of STAT3.[37,38] FOXP3^{+} T-cell expansion can be blocked by TGFβ-specific antibody, and indeed *SOCS3*$^{-/-}$ DCs produced higher levels of TGFβ than wild-type DCs did. Thus, high STAT3 activation in DCs without *SOCS3* results in increased production of IL-10 and TGFβ, thereby inducing FOXP3^{+} Tregs. By contrast, reduced STAT3 expression due to the overexpression of SOCS3 in *SOCS3*-transduced DCs results in reduced production of IL-12, IFNγ, and IL-23, thereby inducing Th2 cells.[39] These results suggest that the expression of SOCS3 by DCs might have a crucial role in the balance between effector Th2 cells and Tregs.

SOCS3 and inflammatory diseases

A growing body of evidence suggests that, in pathological situations, SOCS3 could suppress inflammatory reactions in which IL-6-related cytokines play important progressive roles. This is because SOCS3 is a relatively specific inhibitor of gp130, as described above. STAT3 activation and high SOCS3 expression levels have been found in epithelial and lamina propria cells in the colon of intestinal bowel disease (IBD) model mice, as well as in human ulcerative colitis and CD patients[40] and in synovial fibroblasts of RA patients.[41] STAT3 activation preceded SOCS3 expression, which is consistent with the idea that SOCS3 is part of the STAT3 negative-feedback loop.[40] We have shown that overexpression of SOCS3 by adenovirus gene transfer could prevent the development of experimental arthritis.[41] Therefore, the IL-6/STAT3 pathway promotes the progression of the chronic status of diseases by contributing to cytokine and growth factor production, tissue hyperplasia, synovial fibroblast proliferation, fibrosis, and osteoclast activation. Modulation of the gp130/JAK/STAT pathway therefore represents a reasonable strategy for new anti-inflammatory drug development. In addition, STAT3 is now known to play an essential role in the development of Th17 cells, which are extremely inflammatory and pathogenic. SOCS3 has been shown to negatively regulate Th17 development by suppressing STAT3 activated by IL-6 or IL-23.[20,42] Thus, again, enforced expression of SOCS3 in T cells may also ameliorate Th17-mediated inflammatory diseases such as RA.

In contrast, enhanced action of SOCS3 may promote allergic responses, since a recent analysis has indicated that transgenic SOCS3 expression in T cells inhibits Th1 development and promotes Th2 development.[43] Indeed, the same report also proposes that the degree to which SOCS3 expression in T cells is increased correlates with the severity of human allergic diseases such as asthma

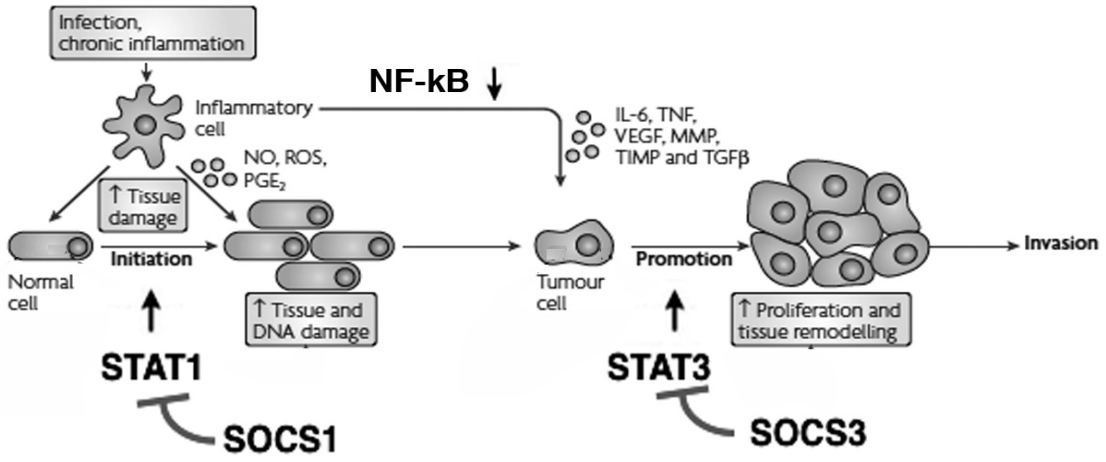

Fig. 3 Role of SOCS1 and SOCS3 in inflammation-associated tumourigenesis
Two-step carcinogenesis model associated with inflammation. STAT1/SOCS1 are involved in the early stage of inflammation which leads to tissue damages, resulting in increased cellular turnover. NO and ROS from inflammatory cells may induce DNA damage, which increases the possibility of the emergence of cells possessing a high risk of malignant transformation. STAT3/SOCS3 are involved in the late stage, in which tumour promotion occurs by cellular and extracellular signals activated by cytokines from inflammatory cells or stromal cells. This step raises immortalized cells that are resistant to growth-inhibitory signals, apoptosis, and anti-tumour immunity.

and atopic dermatitis. Modulation of SOCS3 levels in T cells could help to regulate Th1/Th2 balance for the treatment of autoimmune inflammatory diseases.

SOCS and Inflammation-associated Tumourigenesis

It has been estimated that more than 20% of all malignancies are initiated or exacerbated by inflammation; for example, most human hepatocellular carcinomas (HCCs) are a consequence of hepatitis C virus (HCV) infection. The expression of *SOCS1* is often silenced in these tumours by hypermethylation of CpG islands of the *SOCS1* promoter.[44] *SOCS1* is one of the most frequently methylated genes (65%) in HCCs, and the deletion of SOCS1 in tumour cells might enhance IL-6-mediated cell proliferation. Therefore, *SOCS1* is considered to be an anti-oncogene because it is a suppressor of signals induced by a growth factor (IL-6). *SOCS1*$^{+/-}$ mice are consistently shown to be hypersensitive to dimethylnitrosamine-induced hepatocarcinogenesis.[44]

The full story, however, may not be so simple. We found that silencing of *SOCS1* was frequently observed even in pre-malignant HCV-infected patients.[44] Liver injury is associated with hyperactivation of STAT1 and reduced activation of STAT3.[18,45] Therefore, reduced expression of SOCS1 might enhance tissue injury and inflammation by hyperactivation of STAT1, promoting the turnover of epithelial cells and enhancing their susceptibility to oncogenesis. The importance of SOCS1 for inhibition of inflammation-associated tumour development is supported by the recent finding that *SOCS1*$^{-/-}$ transgenic mice, in which *SOCS1* expression is deleted in all types of cells except T and B cells, developed chronic colitis and colon tumours.[46] This study strongly suggests that chronic activation of the IFNγ–STAT1 pathway that occurs in the absence of SOCS1 causes colitis-induced colon tumours. Therefore, SOCS1 is a unique anti-oncogene that prevents carcinogenesis by suppressing chronic inflammation (**Fig. 3**).

SOCS3 might also be involved in the development and progression of malignancies. Unlike SOCS1, SOCS3 expression levels were high in infected non-tumour areas of patients infected with HCV.[45] Reduced expression of SOCS3 has been observed in various human cancers and is associated with constitutive STAT3 activation.[47] Indeed, the levels of SOCS3 were inversely correlated with STAT3 activation in regions of human livers with and without HCC.[45] Numerous studies have shown that hyperactivation of STAT3 can contribute to tumourigenesis by inducing multiple tumour-promoting genes.[47]

Thus, we propose a two-hit model of inflammation-associated tumourigenesis (**Fig. 3**). In this model, initiation occurs in the form of an activating or disabling mutation in one of the molecules that regulate the cellular circuits and capacitors controlling cell division, survival, and senescence. Persistent inflammation leads to tissue damage, resulting in increased cellular turnover. Nitric oxide (NO) and reactive oxygen species (ROS) from inflammatory cells may induce DNA damage, which in-

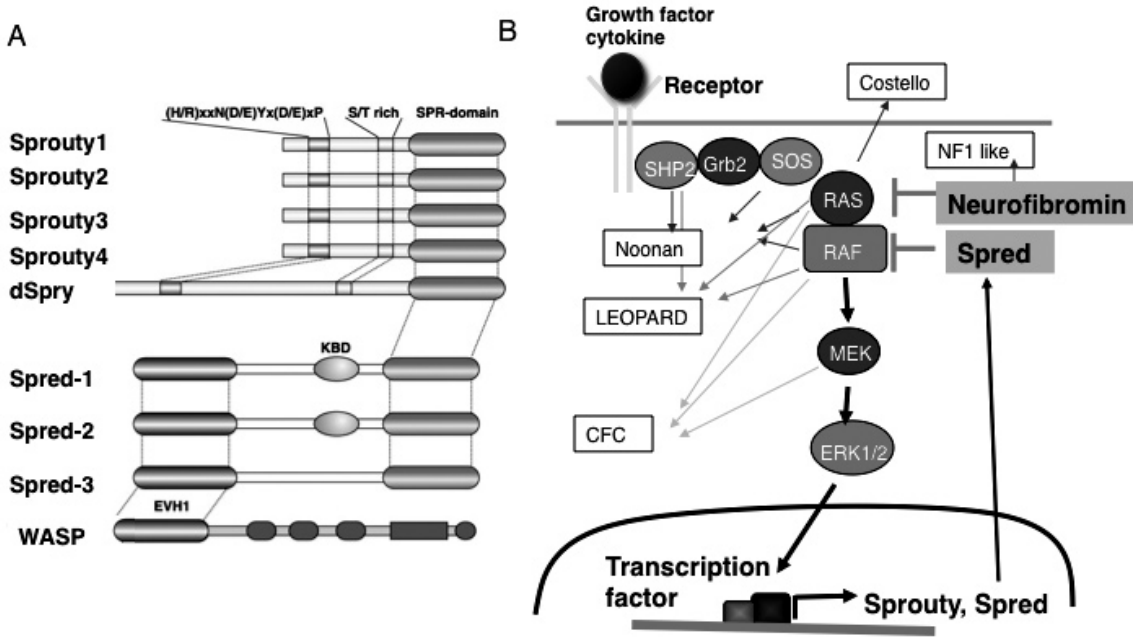

Fig. 4 Structure of the Spred/Sprouty family proteins and relationship to NCFC syndrome
(A) Conserved N-terminal tyrosine motifs of Sproutys and other domains are illustrated. S/T rich: serine/threonin rich region, SPR: Sprouty-related region, KBD: c-kit-binding domain, EVH1: Ena/VASP homology 1, WASP: Wiskott-Aldrich syndrome protein. (B) The growth factor-induced Ras-ERK pathway is illustrated and the component proteins involved in each syndrome are shown.

creases the possibility of the emergence of cells possessing a high risk of malignant transformation. STAT1 plays a positive role in non-tumour inflammatory regions in this early stage, and *SOCS1* silencing in pre-tumour cells results in strong and persistent STAT1 activation, which induces apoptosis and tissue damage, leading to DNA damage and cell regeneration which may promote the emergence of malignant cells. Then, promotion occurs by cellular and extracellular signals activated by cytokines from inflammatory cells or stromal cells, leading to immortalized cells that are resistant to growth-inhibitory signals, apoptosis, and anti-tumour immunity. Constitutive STAT3 activation in tumour cells contributes to an expansion of tumour cells by promoting cell proliferation, survival, angiogenesis, and tissue remodeling. SOCS3 silencing is one of the mechanisms for constitutive STAT3 activation. However, the mechanism of the reduction of SOCS3 expression in tumours has not been established.

Spred and Sprouty

Finding of the Spred/Sprouty family

Sprouty was originally identified in *Drosophila* as a negative regulator of FGF (fibroblast growth factor) signaling during tracheal development. Now it is regarded as a general inhibitor of the growth factor-induced RTK (receptor tyrosine kinase)-dependent Ras/Raf/ERK signaling pathways involved in development and organogenesis. In mammals, four Sprouty orthologues have been identified (Sprouty1, -2, -3, and -4).[48] In addition, we have found three Sprouty-related genes, which we call Spreds (Sprouty-related Ena/VASP homology 1 domain-containing proteins) (**Fig. 4A**).[7,49] Spreds bind to Ras and Raf, thereby suppressing activation of Raf.[7] Gene targeting and overexpression studies have demonstrated that mammalian Sproutys also inhibit growth factor-induced cellular responses by inhibiting the RTK-dependent ERK signaling pathway (**Fig. 4B**).[50–52]

Spred and hematopoiesis

Spred-1 has been implicated in hematopoiesis ever since it was observed that bone marrow-derived mast cells and eosinophils from *Spred-1*$^{-/-}$ mice were more sensitive to IL-3 and IL-5, respectively, than those from WT mice.[53,54] In *Spred-2*$^{-/-}$ mice, embryonic hematopoiesis in the aorta-gonad-mesonephros (AGM) region was enhanced compared with that observed in WT mice.[55]

Spred-1$^{-/-}$ adult animals appeared to be healthy and showed no apparent abnormalities in most organs. Studies in a murine allergic asthma model of *Spred-1*-deficient mice demonstrated that Spred-1 negatively regu-

lates allergen-induced airway eosinophilia, hyperresponsiveness, and mucus production, without affecting helper T cell differentiation. Biochemical assays demonstrated that Spred-1 suppresses IL-5-dependent cell proliferation and ERK activation. This indicates that Spred-1 negatively controls eosinophil numbers and functions by modulating IL-5 signaling in allergic asthma.[54]

Spred-1 deficient mice developed myeloproliferative diseases with age. RT-PCR analysis showed high levels of Spred-1 expression in hematopoietic stem cells and bone-marrow-derived mast cells (BMMCs), erythroid cells, and B-cells, but low levels in megakaryocytes and macrophages. Spred-1 is expressed in a particular subset of mature hematopoietic cells and is inducible by IL-3. In IL-3 dependent Ba/F3 cells expressing c-kit, forced expression of Spred-1 resulted in a reduced proliferation rate and ERK activation in response to not only SCF but also IL-3.[56] In *Spred-1*-deficient bone-marrow-derived mast cells, proliferation and ERK/MAP kinase activation was increased in response to IL-3 or SCF.[56] Therefore, Spred-1 inhibits not only growth-factor-induced ERK activation but also cytokine-induced ERK activation.

Spred and lymphangiogenesis

Although the individual physiological roles of Spred-1 and Spred-2 have been investigated using gene-disrupted mice, the overlapping functions of Spred-1 and Spred-2 have not been clarified. We demonstrate that the deletion of both *Spred-1* and *Spred-2* resulted in embryonic lethality at E12.5-15.5 with marked subcutaneous hemorrhage, edema, and dilated lymphatic vessels filled with erythrocytes.[57] The phenotype of these mice resembled that of *Syk*$^{-/-}$ and *SLP-76*$^{-/-}$ mice, with defects in the separation of lymphatic vessels from blood vessels. The numbers of LYVE-1-positive lymphatic vessels and lymphatic endothelial cells were markedly increased in *Spred-1/2* -deficient embryos compared with wild-type embryos, while the number of blood vessels was not different. *Ex vivo* colony assay revealed that *Spred-1/2* suppressed lymphatic endothelial cell proliferation and/or differentiation. In cultured cells, the overexpression of *Spred-1* or *Spred-2* strongly suppressed vascular endothelial growth factor-C (VEGF-C)/VEGF receptor (VEGFR)-3-mediated ERK activation, while *Spred-1/2*-deficient cells were extremely sensitive to VEGFR-3 signaling. Thus, Spreds play an important role in lymphatic vessel development by negatively regulating VEGF-C/VEGFR-3 signaling. Interestingly, microRNA miR-126 has been shown to enhance the proangiogenic actions of VEGF and FGF, and to promote blood vessel formation by repressing the expression of Spred-1.[58] Thus, Spreds may also be involved in the regulation of angiogenesis.

Spred-1 and human diseases

Neurofibromatosis type 1 (NF1), or von Recklinghausen disease, is an autosomal dominant condition characterized by multiple café-au-lait spots, axillary freckling, Lisch nodules in the iris, and tumours of the nervous system. Other frequently observed features are short stature, macrocephaly, and learning and behavioral problems. Most NF1 is caused by inactivating mutations in the *NF1* tumour suppressor gene encoding neurofibromin, a positive regulator of RAS inactivation. NF1 was the first human disorder shown to originate from germline mutations in a gene encoding a component of the RAS-ERK pathway. Subsequently, mutations in genes encoding other components of this pathway were implicated in disorders showing some phenotypic overlap with NF1, for example, *PTPN11*, *KRAS*, and *SOS1* were implicated in Noonan syndrome, *PTPN11* in LEOPARD syndrome, *HRAS* in Costello syndrome, and *KRAS*, *BRAF*, *MEK1*, and *MEK2* in cardio-facio-cutaneous (CFC) syndrome (**Fig. 4B**). These disorders, now known as the 'neuro-cardio-facial-cutaneous' (NCFC) syndromes, present with a variable degree of cognitive impairment, facial dysmorphism, congenital heart defects, and skin abnormalities.[59]

We reported germline loss-of-function mutations in *SPRED1* resulting in a newly identified autosomal dominant human disorder (**Fig. 5A**).[60] The clinical features of the reported disorder resemble those of neurofibromatosis type 1; they consist of multiple café-au-lait spots, axillary freckling, and macrocephaly. All mutations are found to be the loss-of -function type (**Fig. 5B**). To our knowledge, this is the first report of mutations in this family of genes causing human disease, and the existence and nature of this disorder strongly support our notion that Spred is a negative regulator of the RAS-ERK pathway.

Furthermore, Spreds are now considered to be potential tumour suppressors, as is NF-1. It has been reported that *Spred-1* and *Spred-2* expression was reduced in human HCC.[61] In addition, overexpression of Spred-1 can efficiently suppress tumourigenesis in nude mice.[62] Further study is underway to identify mutations or loss of expression of Spreds/Sproutys in cancer and to establish Spreds as a therapeutic target.

Concluding Remarks

In the past decade, following the discovery of the SOCS and Spred protein families, we have extended our understanding of the structure and function of these proteins. SOCS proteins not only act as simple negative-feedback regulators, but also play a part in the fine tuning of the immune response and in the cross-talk of the complicated cytokine signal networks. Spreds/Sproutys

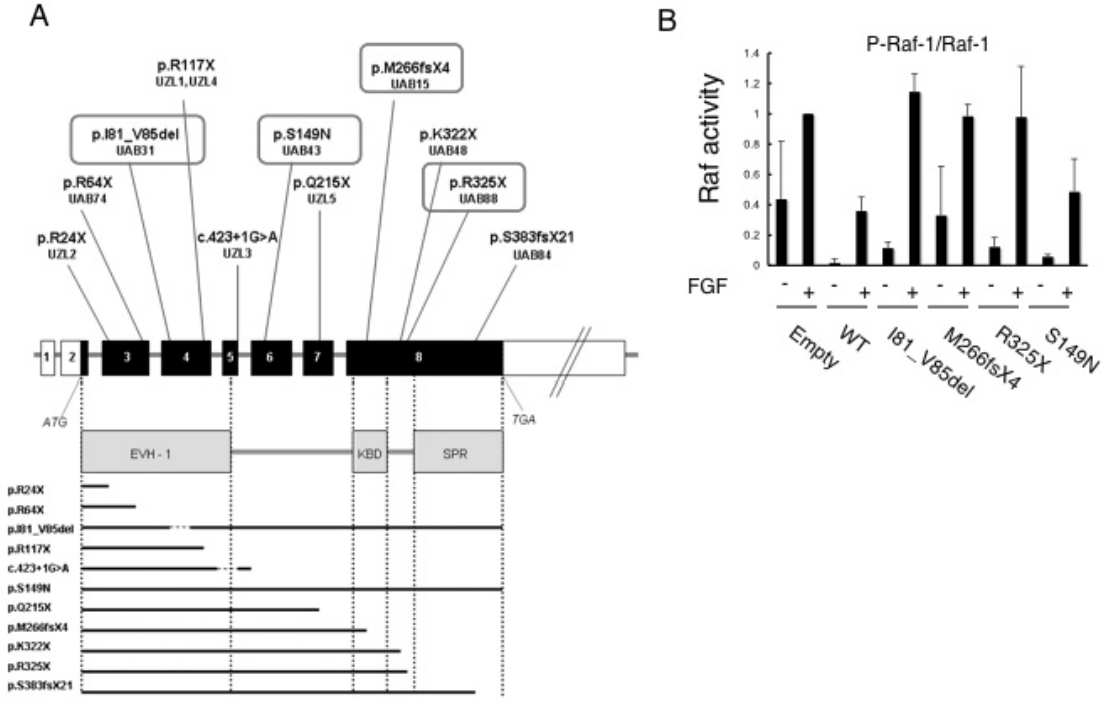

Fig. 5 Mutations in SPRED1 gene found in NF1 patients (A) and suppression of the FGF-induced Raf1 kinase activity by mutant Spred1 proteins (B). 293 cells transfected with various mutant SPRED1 genes were stimulated with FGF, then Raf1 was immunoprecipitated. *In vitro* Raf1 kinase assay was performed using MEK1 as substrate (B).

are also very important for development, hematopoiesis, angiogenesis, and neurogenesis. Since various cytokines and stimuli are constantly in the microenvironment of immune cells, hematopoietic cells, neural cells, and endothelial cells, signal regulation by those proteins and other regulators must be important to maintain the proper homeostasis. Therefore, further investigation into the function of SOCS and Spred proteins might provide us with an unexpected role for these proteins in the regulation of signaling pathways in life.

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