Silencing of *SOCS1* in macrophages suppresses tumor development by enhancing antitumor inflammation

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Inflammation has been shown to contribute to both tumor development and antitumor immunity. However, conditions determining these opposing effects are not well understood. Suppressor of cytokine signaling 1 (SOCS1) has been shown to play an important role in regulating inflammation and tumor development. It has been reported that silencing of *SOCS1* gene in dendritic cells potentiates antitumor immunity, while *SOCS1*-deficiency in whole organs except for T and B cells enhances inflammation-mediated colon tumor development. To determine which types of cells are important for the suppression of tumor development by *SOCS1*-deficiency, we employed the conditional knockout strategy. *SOCS1* gene was deleted in macrophages and neutrophils by crossing *SOCS1-flox/flox* mice with *LysM-cre* mice. Resulting conditional knockout (cKO) mice showed enhanced sensitivity to endotoxin shock. *SOCS1*-cKO mice survived much longer than wild-type mice after B16 melanoma transplantation. Colon carcinogenesis induced by 1,2-dimethylhydrazine (DMH) plus dextran sulfate sodium (DSS) was also reduced in *SOCS1*-cKO mice. *SOCS1*-deficiency in monocytic cells enhanced tumor-killing activity of macrophages and tumor-specific cytotoxic T cell activity. These results suggest that inflammation induced by *SOCS1*-deficiency in monocytes potentiates antitumor immune responses rather than tumor-promoting inflammation. (*Cancer Sci* 2009; 100: 730–736)

Chronic inflammation has long been associated with increased incidence of malignancy and similarities in regulatory mechanisms have been suggested for more than a century. As well as supporting tumor cells evading antitumor immunity, infiltration of innate immune cells such as macrophages and neutrophils into tumors promotes tumor development by various mechanisms including elevation of activities of matrix metalloproteases, increased angiogenesis and vasculature density. Conversely, the elimination of early malignant lesions by immune surveillance, which relies on the cytotoxic activity of tumor-infiltrating T cells or intra-epithelial lymphocytes, is thought to be rate-limiting for the risk of developing cancer. Tumor-associated macrophages (TAMs) and related myeloid-derived suppressor cells are key prototypic components of smoldering inflammation driving neoplastic progression. However, mononuclear phagocytes can exert antitumor activity by killing tumor cells, which may contribute to suppression of the early phases of carcinogenesis of immunogenic tumors. Therefore, shifting the macrophage balance from tumor promotion to tumor suppression represents a viable therapeutic target. However, little is know about factors which determine this balance.

The suppressor of cytokine signaling-1 (SOCS1) emerged as an important physiological regulator of cytokine responses, including interferon (IFN)γ and IFNα. SOCS1 is strongly induced by cytokines and inhibits signal transduction by suppressing janus kinase (JAK) tyrosine kinase activity.[1,2] Analysis of knockout mice indicates that SOCS1 is indispensable for the negative regulation of IFNγ.[3,4] *SOCS1*-deficiency in T cells resulted in hyper Th1 and reduced Th17 responses.[5] Furthermore, bone marrow dendritic cells (BMDCs) with reduced SOCS1 expression elicited stronger tumor-reactive immunity *in vivo*.[6,7] However, we found that *SOCS1*-KO-Tg mice, in which the *SOCS1* gene was deleted in all tissues except for T and B cells, developed IFNγ-dependent colorateral tumors spontaneously.[8] In addition, *SOCS1* gene silencing was frequently observed in various tumors including hepatocellular carcinoma (HCC).[9,10] These data suggest that *SOCS1*-deficiency enhances both antitumor immunity as well as tumor-promoting inflammation, depending on the cell type-specific expression of the *SOCS1* gene. Macrophages have been considered to be not only effector cells against tumors but also tumor-promoting micro-environmental cells. However, the role of macrophage SOCS1 in tumor development has not been clarified.

To determine the role of macrophage SOCS1 in tumor development, we generated macrophage-specific *SOCS1*-conditional knockout (cKO) mice. *SOCS1*-cKO mice survived much longer than $SOCS1^{flox/flox}$ control wild-type (WT) mice after B16 melanoma transplantation, which was dependent on IFNγ. In the DSS/DMH colon tumor model, tumor progression was also suppressed in *SOCS1*-cKO mice. *SOCS1*-deficient macrophages showed stronger tumor-killing activity. These results suggested that SOCS1 is a determining factor in the balance between antitumor immunity and tumor-promoting immunity in macrophages.

Materials and methods

Mice. $IFN\gamma^{-/-}$ and $SOCS1^{-/-}\ IFN\gamma^{-/-}$ mice have been described previously.[9] All experiments using these mice were approved by and performed according to the guidelines of the animal ethics committee of Kyushu University, Fukuoka, Japan.

Tissue-specific *SOCS1*-deficient mice were generated using the cre/loxP system by breeding $SOCS1^{lox/lox}$ mice, which carry a *SOCS1* allele flanked by loxP sites, with various cre transgenic mice. Mice expressing cre under the endogenous lysozyme M promoter (*LysM-cre*),[11] were used to delete $SOCS1^{lox}$ in the myeloid and lymphoid compartment. $SOCS1^{lox/lox}$ mice were generated on a C57BL/6 genetic background, while all other mice were 5th to 10th generation backcrosses to C57BL/6. All mice were housed in clean animal rooms with specific pathogen free (SPF) conditions.

Dendritic cell (DC) preparation and MLR. BMDCs were prepared from bone marrow suspensions from femurs and tibias of mice as described previously.[12] Bone marrow cells were cultured in 10 ng/mL mouse granulocyte/macrophage colony-stimulating factor (GM-CSF) (PeproTech, Rocky Hill, NJ, USA) or J558L-GM-CSF conditioning media and 7-day-cultured BMDCs were used

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for the experiment. Syngeneic mixed lymphocyte reactions (MLR) experiments were performed using $CD4^+$ T cells from C57BL/6 mice. $CD4^+$ T cells were purified using MACS magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) as described previously.[13] $CD4^+$ T cells were cultured with lipopolysaccharide (LPS)-stimulated BMDC in the presence of 1 μg/mL antimouse CD3e antibody (eBioscience, San Diego, CA, USA).

Macrophage preparation. Bone marrow-derived macrophages (BMDM) were prepared from bone marrow suspensions from femurs and tibias in mice as described previously.[12] Bone marrow cells were cultured in L929-M-CSF conditioning media and the 7-day-cultured bone marrow-derived macrophages were used for the experiment. Resident peritoneal macrophages were obtained by peritoneal lavage with 5 mL of cold phosphate-buffered saline (PBS). Cells were incubated in plastic dishes overnight and then washed with PBS to eliminate nonadherent cells. Adherent cells were dispersed in each well of a 24-well tissue culture plate in Dulbecco's Modified Eagles Medium (DMEM) containing 10% fetal bovine serum (FBS). Following stimulation with 10 ng/mL LPS and 100 U/mL IFNγ for 24 h, the production of NO_2^- was measured as nitrite using the Griess reagent.

Western blot analysis. Immunoblotting to detect phosphorylated signal transducers and activator of transcription (STAT)1 and I-κB was performed as previously described.[2] Anti-phospho-specific STAT1, I-κB, p65, Erk, p38, and JNK antibodies were purchased from Cell Signaling Co., Danvers, MA, USA Anti-STAT1, p65, Erk and inducible nitric oxide synthase (iNOS) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, US). Anti-phospho-specific STAT3 and Bcl-X_L antibodies were purchased from BD Transduction Laboratories, San Jose, CA, USA. Anti-COX-2 antibody was purchased from Cayman Chemical, Ann Arbor, MI, USA. Anti-actin antibody was purchased from SIGMA, St. Louis, MO, USA.

Reverse transcription – polymerase chain reaction (RT-PCR). Total RNA was isolated from cells using Trizol (Invitrogen, Carlsbad, CA, US) according to the manufacturer's instructions. The expression level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was first evaluated as an internal control using serially diluted reverse-transcribed cDNA. The expression levels of *SOCS1* were then assessed using appropriate pairs of primers. Primer sequences were as follows: *SOCS1*, sense 5′-CGCCAACGGAA-CTGCTTCTTC-3′, antisense 5′-TCAGGTAGTCACGGAGTACC-3′; GAPDH, sense 5′-ACCACAGTC CATGCCATCAC-3′, antisense 5′-TCCACCACCCTGTTGCTGTA-3′. For quantitative determination of *SOCS1* expression, quantitative real-time PCR was performed using an ABI 7000 sequence detector system (Applied Biosystems, Foster City, CA, US). *SOCS1* mRNA expression was assessed using the abovementioned primers in combination with SYBR Green (Applied Biosystems).

Cytotoxic T cell (CTL) assay. Carboxyfluorescein succinimidyl ester (CFSE)-labeled target cells were incubated in duplicate with effector cells in a 96-Well Cell Culture Cluster V-Bottom with Lid (Corning Incorporated, NY, USA) in a final volume of 200 μL. Effector to target cell (E : T) ratios of 25 : 1, 12.5 : 1 and 6.25 : 1 were used. Cultures were incubated for 4 h at 37°C in 5% CO_2, and then samples were placed on ice until analysis by flow cytometry. Spontaneous target cell death was determined in target cells cultured alone. As a positive control for cytotoxicity, labeled target cells were treated with cisplatin.

Histopathological and immunohistochemical studies. Subcutaneous tumors were isolated from each mouse. Colons were isolated and opened longitudinally to inspect for mucosal tumors. Tissue samples were fixed in 10% buffered formalin and embedded in paraffin. Sections (5-μm-thick) were cut and stained with hematoxylin and eosin (HE). The severity of colitis was determined by the histological scoring system as described previously.[14] For immunohistochemistry, paraffin-embedded sections were dehydrated and then microwaved in 10 mM citrate buffer (pH 6.0) twice for 5 min each. Then, the sections were incubated with the following antibodies: anti-iNOS (Santa Cruz Biotechnology, Inc.; 1 : 100 dilution), anti-F4/80 (CI:A3-1; Serotec; 1 : 50 dilution) and anti-CD8α (Santa Cruz Biotechnology, Inc.; 1 : 50 dilution). VECTASTAIN ABC kits (Vector Laboratories, Burlingame, CA, US) and HISTOFINE MAX-PO kits (Nichirei, Tokyo, Japan) were used for detection. All sections were counter-stained with hematoxylin.

Results

Enhanced IFNγ and LPS signaling in *SOCS1*-cKO mice. *SOCS1* knockout (–/–) mice die within 3 weeks with severe inflammation in almost all organs.[15,16] Thus, the consequence of *SOCS1*-deficiency in myeloid lineages was investigated by breeding *SOCS1*$^{lox/lox}$ mice with *SOCS1-flox/flox* and *LysM-cre* mice which has previously been shown to mediate efficient loxP recombination in myeloid cells. At first, we confirmed *SOCS1*-deficiency in macrophages from *SOCS1*-cKO mice. Peritoneal macrophages were obtained by lavage and then used for experiments *in vitro*. Peritoneal macrophages were treated with IFNγ (20 U/mL) for 3 h. *SOCS1* mRNA level was determined by RT-PCR and the tyrosine phosphorylation of STAT1 was measured by Western blotting. Macrophages from *SOCS1*-cKO mice rarely expressed *SOCS1* mRNA compared with WT mice (Fig. 1a). *SOCS1* was normally expressed in the thymus and spleen in *SOCS1*-cKO mice (data not shown).

First, to confirm the effect of *SOCS1*-deficiency in macrophages, we investigated the response to IFNγ and LPS. As expected, IFNγ-induced STAT1 activation was much stronger in macrophages from *SOCS1*-cKO mice than from WT mice (Fig. 1b). We have shown that *SOCS1*$^{+/-}$ mice as well as *SOCS1*$^{-/-}$*IFNγ*$^{-/-}$ mice were more sensitive to LPS than WT control mice. Age- and sex-matched cohorts of mice were challenged intraperitoneally with LPS and observed for 120 h. As shown in Fig. 1(c), *SOCS1*-cKO mice were more sensitive to LPS-induced lethality than were WT mice. Since *SOCS1*-cKO mice exhibited no signs of inflammation before the LPS challenge, these data confirm that SOCS1 levels modify LPS sensitivity. Next, we examined signal transduction in response to LPS; tyrosine phosphorylation of STAT1, STAT3 and serine phosphorylation of I-κB, p65, Erk, p38, and JNK were examined. In macrophages from *SOCS1*-cKO mice, STAT1 and Erk phosphorylation was stronger than in those from WT mice, although phosphorylation of other molecules showed no significant difference between *SOCS1*-deficient and WT macrophages (Fig. 1d). Although the reason for a selective enhancement of LPS-signaling by *SOCS1*-deficiency is not clear at present, these data confirm that SOCS1 negatively regulates innate immunity both *in vitro* and *in vivo*.

***SOCS1*-cKO mice were highly resistant to tumor transplantation.** We then examined the effect of *SOCS1*-deficiency in macrophages during tumor progression. We challenged WT, *SOCS1*-cKO, *IFNγ*$^{-/-}$, and *SOCS1*$^{-/-}$ *IFNγ*$^{-/-}$ mice with injection of B16 melanoma cells. We noticed a striking enhancement of survival in *SOCS1*-cKO mice (Fig. 2a). Tumor size in *SOCS1*-cKO mice was much smaller than that in WT mice (Fig. 2b). To investigate the effect of IFNγ, we compared *IFNγ*$^{-/-}$ and *SOCS1*$^{-/-}$*IFNγ*$^{-/-}$ mice. As expected, tumor development was accelerated in *IFNγ*$^{-/-}$ mice compared with *IFNγ*$^{+/+}$ mice (Fig. 2a), indicating that IFNγ plays a critical role in tumor suppression. However, *SOCS1*$^{-/-}$ *IFNγ*$^{-/-}$ mice showed sensitivity similar to *IFNγ*$^{-/-}$ mice (Fig. 2a,b), suggesting that enhanced survival of *SOCS1*-cKO mice was dependent on IFNγ. These data suggest that IFNγ is a strong candidate effector for the enhanced antitumor immunity in *SOCS1-cKO* mice.

Next, to investigate the role of macrophages and CTLs in tumor suppression, we inoculated WT and *SOCS1*-cKO mice

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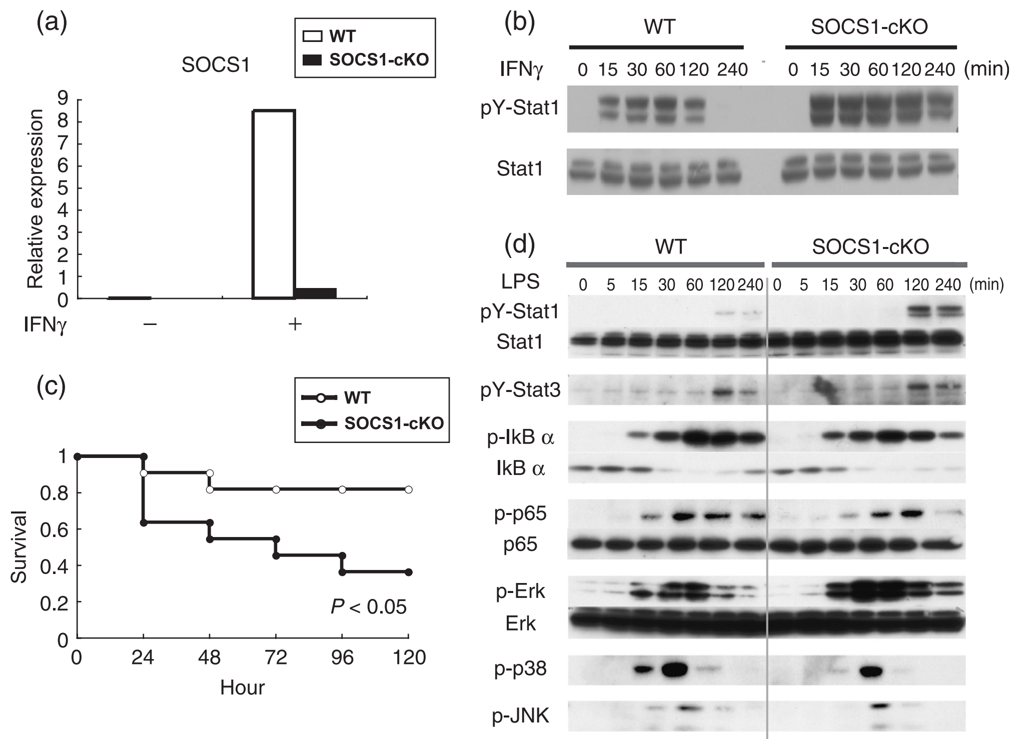

Fig. 1. Deletion of suppressor of cytokine signaling-1 (SOCS1) in monocytic cells in mice. (a) Quantitative real-time reverse transcription – polymerase chain reaction analysis of *SOCS1* expression level. Peritoneal macrophages were stimulated with 20 U/mL interferon (IFN)γ for 3 h. *GAPDH* was used as a housekeeping gene. (b) Phosphorylation of STAT1 in peritoneal macrophages in response to IFNγ. Resident peritoneal macrophages from littermate $SOCS1^{fl\ ox/fl\ ox}$ wild type (WT) and *LysM-cre* $SOCS1^{fl\ ox/fl\ ox}$ (*SOCS1*-cKO [conditional knock out]) mice were stimulated with 20 U/mL IFNγ for the indicated periods, and then cell extracts were immunoblotted with total anti-STAT1 and antiphospho-specific STAT1 antibody. (c) LPS sensitivity *in vivo*. 5- to 10-week-old *SOCS1*-cKO and WT mice were intraperitoneally injected with 1.2 mg LPS ($n = 11$). Lethality was observed for 120 h after this challenge. The results were assessed by the logrank test. (d) Phosphorylation of STAT1, STAT3, IκBα, p65, Erk, p38, and JNK in peritoneal macrophages in response to LPS. Resident peritoneal macrophages from *SOCS1*-cKO mice were stimulated with 100 ng/mL LPS for the indicated periods, and then cell extracts were immunoblotted with the indicated antibody.

with the Lewis lung carcinoma (3LL). The results of histological staining revealed the presence of F4/80-positive macrophages and $CD8^+$ CTLs around the tumor region in both WT and *SOCS1*-cKO mice (Fig. 2c). iNOS-positive cells were also observed in the tumor region (Fig. 2c). We counted the number of these infiltrated immune effector cells (Fig. 2d). The number of $CD8^+$-cells was not significantly different between *SOCS1*-cKO and WT mice. On the other hand, F4/80-positive macrophages and iNOS-positive cells around the tumor region of *SOCS1*-cKO mice were about two times more than that of WT mice (Fig. 2d). These data were consistent with previous study showing that the macrophage progenitor cells are enriched in *SOCS1*-deficient mice.[17]

***SOCS1*-cKO mice were resistant to a DSS/DMH colon tumor model.** We then investigated the role of SOCS1 in inflammation-mediated tumor development using a well-defined DSS/DMH colon cancer model.[18] The chemical carcinogen DMH was injected intraperitoneally (i.p), and then 1.5% DSS, which induces colitis, was administered in the drinking water for a week, followed by 2 weeks of regular water. This cycle was repeated three times and the mice were sacrificed (Fig. 3a). Tumor numbers in WT mice were much higher than in *SOCS1*-cKO mice (Fig. 3b). Cancer progress was also accelerated in colons of WT mice in the DSS/DMH colon cancer model (Fig. 3c). Histological staining revealed higher infiltration of F4/80- and iNOS-positive mononuclear cells in colons from both WT and *SOCS1*-cKO mice. The number of F4/80-positive cells in non-tumor regions of *SOCS1*-cKO mice was larger than that of WT mice. In addition, iNOS-positive cells were more evident in non-tumor regions of *SOCS1*-cKO mice, while these were rare in non-tumor regions in WT mice (Fig. 3d). To confirm this, we assessed activation levels of STAT1 and expression of iNOS and cylooxygenase (COX)-2 in whole colon. As shown in Fig. 3(e), STAT1, iNOS and COX-2 expression of *SOCS1*-cKO mice were higher than that of WT mice. Phosphorylation of STAT1 was also stronger in *SOCS1*-cKO mice compared to WT mice. *SOCS1*-deficiency did not influence STAT3 expression or activation in colons. These data are consistent with the results of immunohistochemical staining. The results indicate that *SOCS1*-cKO mice show higher resistance to inflammation-induced tumor development, which is associated with higher infiltration of macrophages and higher expression of inflammation mediator molecules.

Enhanced antitumor ability in *SOCS1*-deficient APCs. To investigate the mechanism of tumor resistance in *SOCS1*-cKO mice, we examined the ability of antigen-presenting cells (APCs) to induce IFNγ-producing T cells and CTLs. When naïve $CD4^+$ T cells were cocultured with *SOCS1*-deficient DCs, the levels of IFNγ were much higher in the culture supernatants of T cells cocultured with *SOCS1*-deficient DCs than those cocultured with WT-DCs (Fig. 4a). In contrast, the levels of Th2-type cytokine interleukin (IL)-4 and Th17-type cytokine IL-17 were not so different (Fig. 4a). These results indicate that *SOCS1*-deficient APCs induce higher IFNγ production from T cells compared with WT APCs.

Then, we examined CTL activities in WT and *SOCS1*-cKO mice. WT and *SOCS1*-cKO mice were challenged with B16 cells subcutaneously. Five days after B16 challenge, the spleen

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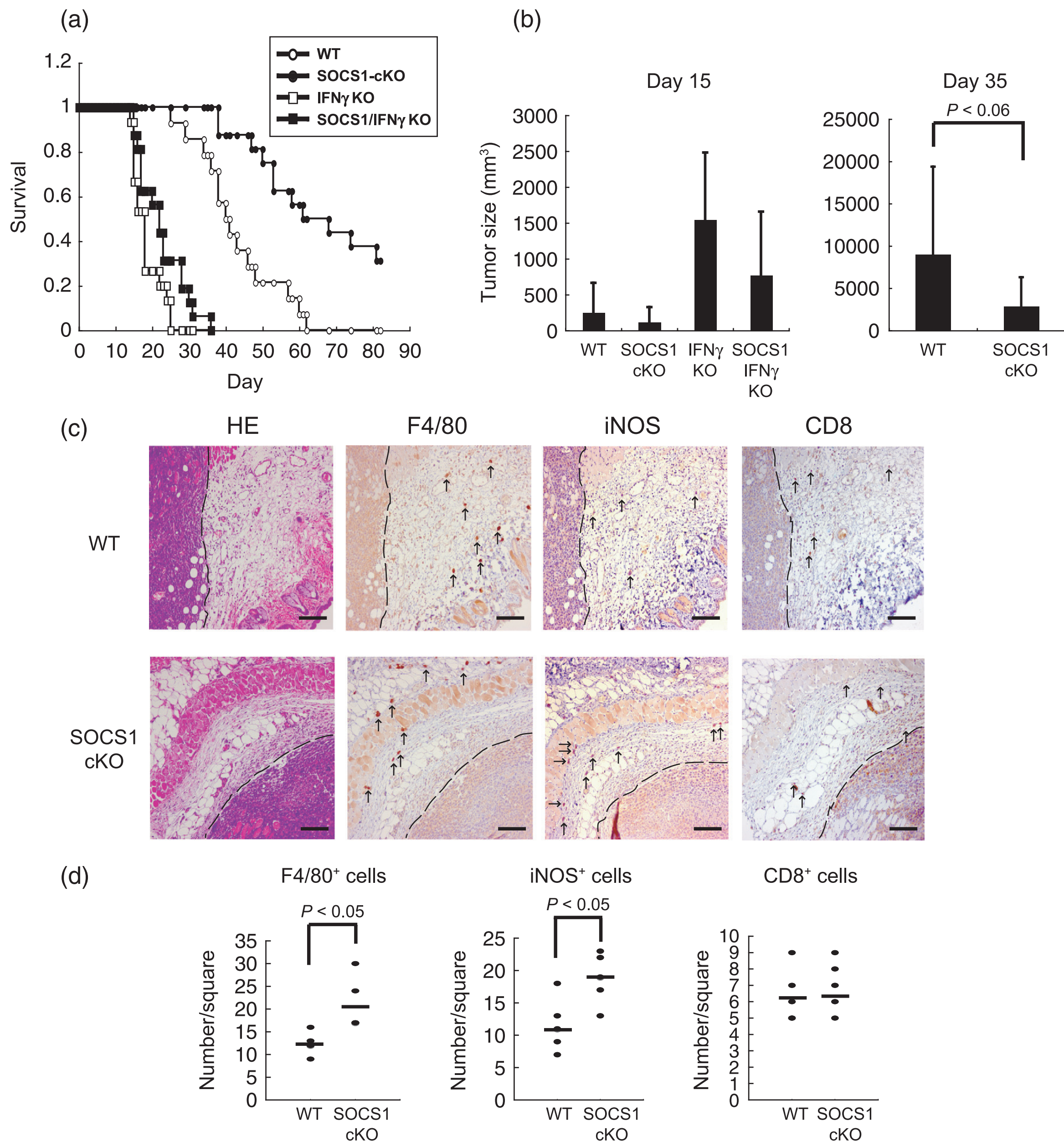

Fig. 2. Resistance of suppressor of cytokine signaling-1 (SOCS1)-conditional knock out (cKO) mice to B16 melanoma transplantation. (a) Wild type (WT), *SOCS1*-cKO, *interferon (IFN)* $\gamma^{-/-}$ and *SOCS1*$^{-/-}$ *IFN*$\gamma^{-/-}$ mice were subcutaneously challenged with 2×10^5 B16 melanoma cells (WT: $n = 14$, *SOCS1 LysM-cre*: $n = 16$, *IFN*$\gamma^{-/-}$: $n = 15$, *SOCS1*$^{-/-}$ *IFN*$\gamma^{-/-}$: $n = 16$). Lethality was observed for 80 days after this challenge. Kaplan-Meier survival curves are depicted as time after tumor challenge. The results were assessed by the logrank test. $P < 0.001$ (b) Tumor size was assessed by measuring three perpendicular diameters of the skin tumor 15 days and 35 days after tumor challenge (WT: $n = 14$, *SOCS1*-cKO: $n = 16$, *IFN*$\gamma^{-/-}$: $n = 8$, *SOCS1*$^{-/-}$*IFN*$\gamma^{-/-}$: $n = 14$). (c) 3LL tumor cells (2×10^5) were injected into WT and *SOCS1*-cKO mice subcutaneously. Seven days after tumor challenge, subcutaneous tumor samples were isolated and used for hematoxylin and eosin (HE) staining and immunostaining for F4/80, inducible nitric oxide synthase (iNOS) and CD8. Arrowheads show each positive cell. Bars, 50 μm. (d) The stained cell numbers in the setting square. Bar indicates the average of the number of positive cell.

was taken from these B16-challenged mice. Splenocytes were further cocultured with mitomycin C-treated B16 cells for 5 days, then CTL activity against B16 cells was assayed. As shown in Fig. 4(b), CTL activity against B16 cells was slightly higher in the splenocytes from *SOCS1*-cKO mice than those from WT mice. The proportion of $CD4^+$ cells and $CD8^+$ cells in the splenocytes was not significantly different (data not shown). These data indicate that killing activity of CTLs derived from *SOCS1*-cKO mice is higher than that of CTLs from WT mice, which may partly contribute to the enhanced antitumor immunity in *SOCS1*-cKO mice. However, the differences in the killing activity of CTLs between *SOCS1*-cKO mice and WT mice were small, other mechanisms seem to exist.

It has been shown that macrophages can directly kill B16 cells by a NO-dependent mechanism.[19,20] Thus, we investigated whether *SOCS1*-deficient macrophages possess higher killing activity against B16 cells. As shown in Fig. 4(c), proliferation of B16 cells was more strongly inhibited by *SOCS1*-deficient macrophages than by WT macrophages (Fig. 4c, left panel).

SOCS1-deficient macrophages produced a higher level of NO compared to WT macrophages (Fig. 4c, right panel). NO production was not detected from B16 cells alone (data not shown).

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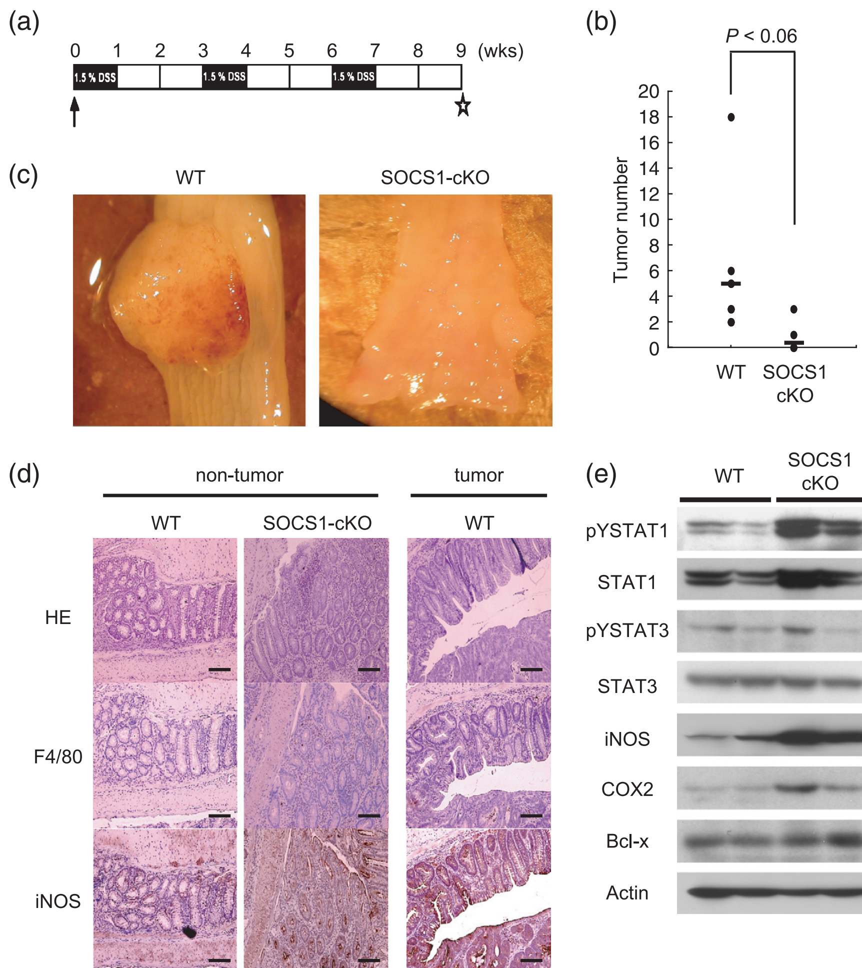

Fig. 3. Tumor formation in wild type (WT) and *suppressor of cytokine signaling-1 (SOCS1)*-conditional knock out (cKO) mice in the DSS/DMH colon cancer model. (a) Protocol of DSS/DMH. DMH (20 mg/kg body weight [b.w]) was intraperitoneally injected at indicated points. DSS (1.5%) was administrated in drinking water. Star indicates the point of sacrifice. (b) Tumor number was counted in individual mice (WT: $n = 10$, *SOCS1*-cKO: $n = 6$) (c) Representative photographs of the colon from these mice in the DSS/DMH colon cancer model. (d) Hematoxylin and eosin (HE) and immunostaining for F4/80 and inducible nitric oxide synthase (iNOS). Bars, 50 μm. (e) Western blot analysis of indicated proteins in whole colonic extracts. Actin was used as a loading control.

Next, we investigated whether macrophages induce apoptosis of B16 cells using annexin V and PI staning. When B16 cells were cocultured with activated macrophages, the apoptotic cells (annexin V and PI double positive cells) appeared in B16 cells. *SOCS1*-deficient macrophages induced apoptosis of B16 cells more efficiently than WT macrophages (Fig. 4d). These results suggest that *SOCS1*-deficiency in monocytic cells lead to higher production of IFNγ from T cells, thereby enhancing CTL-inducing activity and tumor-killing activity of macrophages. Higher number of monocytes in *SOCS1*-cKO mice may also contribute to the suppression of tumor growth.

Discussion

This study demonstrates that *SOCS1*-deficiency in monocytic cells including macrophages and neutrophils results in the suppression of tumor development. This is likely due to enhanced antitumor immunity since IFNγ plays a critical role in the suppression of tumor progression in the B16 melanoma transplantation model. Reduced expression of SOCS1 in APCs enhanced IFNγ-producing Th1 and CTL development. Moreover, tumor-killing activity of macrophages was also enhanced by *SOCS1*-deficiency. These data suggest that suppression of SOCS1 expression or function in myeloid cells is an effective strategy for enhancing antitumor immunity.

Tumor-associated macrophages (TAMs) in established metastatic malignancy are shown to be a key element of tumor-promoting inflammatory reactions. However, these cells also have the potential to express antitumor activity. In the context of established malignancy, when tumor cells have escaped by immune editing, smoldering inflammation orchestrated by TAMs and by humoral adaptive immunity promotes progression. Therapeutic strategies targeting different sides of the macrophage balance[21,22] should take into account the dual potential of these cells. However, mechanisms determining macrophage balance have not been well established.

It has been shown that *SOCS1*-deficeint DCs can potentiate antitumor immunity through an antigen-specific manner due to induction of stronger Th1 and CTLs.[6,7] However, deletion of the floxed gene in dendritic cells has been shown to be marginal in the LysCreM mice system.[11] Therefore, we think that tumor killing activities of TAMs in SOCS1-cKO mice are mostly dependent on NO, reactive oxygen species (ROS) and cytokines, but major histocompatibility complex (MHC)-independent.

It has been shown that IL-12 has a strong potential to induce IFNγ-mediated antitumor immunity. Thus, IL-12-producing TAMs have a higher potential for tumor-suppressing myeloid cells. On the other hand, Langowski *et al.* recently reported that the expression of heterodimeric cytokine IL-23, but not expression of its close relative IL-12, is increased in human colon tumors, and that IL-23 promotes inflammatory responses such as up-regulation of the matrix metalloprotease (MMP)9, and increases angiogenesis but reduces $CD8^+$ T cell infiltration.[23] Genetic deletion or antibody-mediated elimination of IL-23 leads to increased infiltration of cytotoxic T cells into the transformed tissue, rendering a protective effect against chemically induced carcinogenesis. They concluded that IL-23 is an important molecular link between tumor-promoting pro-inflammatory processes and the failure of adaptive immune surveillance to infiltrate tumors. This idea is consistent with our observation

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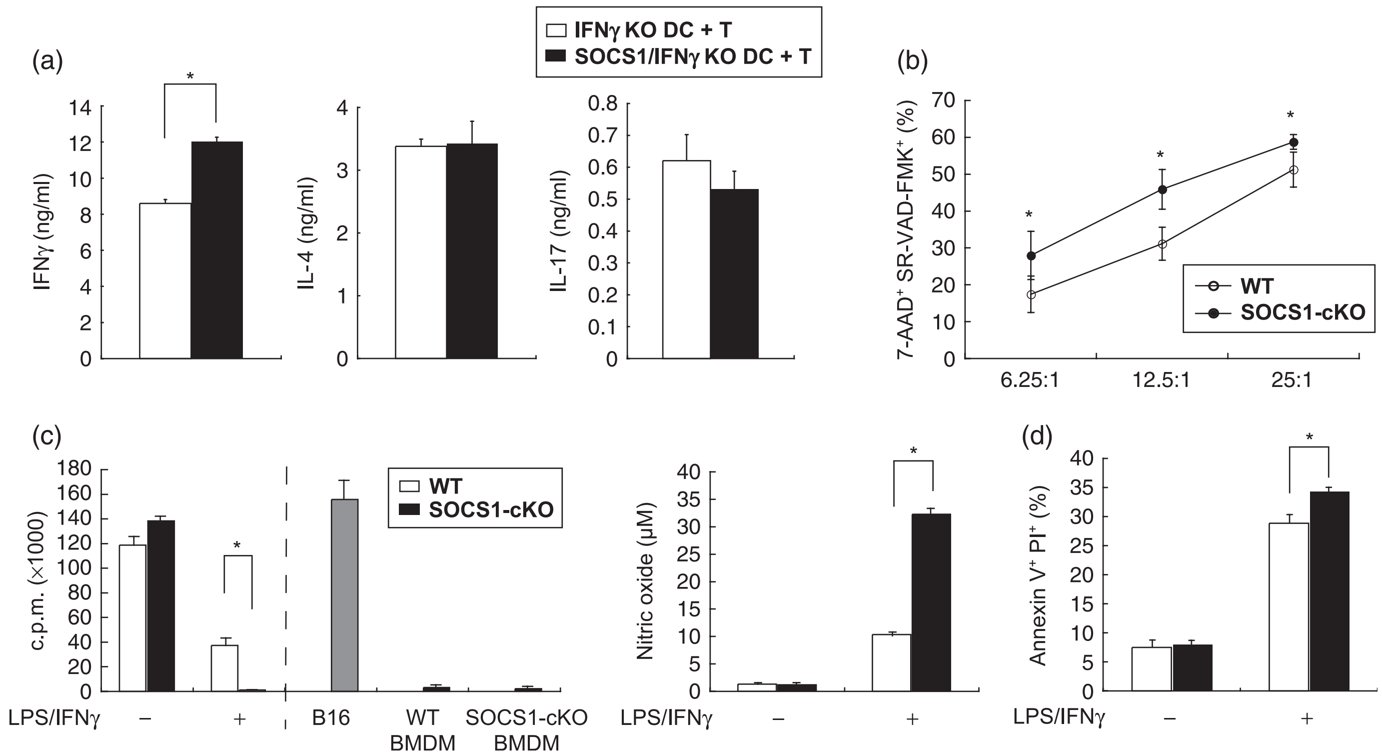

Fig. 4. Tumor killing activity of cytotoxic T cells (CTLs) and macrophages. (a) Cytokine production from wild type (WT) T cells activated with WT and mutant dendritic cells (DCs), bone marrow dendritic cells (BMDCs) from *interferon (IFN)γ$^{-/-}$* or *suppressor of cytokine signaling-1 (SOCS1)$^{-/-}$IFNγ$^{-/-}$* mice were cocultured with naïve T cells from WT mice for 72 h. Co-cultured T cells were collected and stimulated with 1 μg/mL anti-CD3 antibody platebound for 24 h, then IFNγ, interleukin (IL)-4 and IL-17 in the culture supernatant were measured by enzyme-linked immunosorbent assay. The results are expressed as mean with SD for triplicate samples. Asterisk, $P < 0.05$. (b) CTL assay. Target B16 melanoma cells were injected to WT or *SOCS1*-cKO (conditional knock out) mice. The spleen was taken from these mice 5 days after this challenge, and then CD8$^+$ cells were cocultured with mitomycine C-treated B16 cells for 5 days. CFSE-labeled B16 target cells were cocultured with effector cells at indicated ratio for 6 h. The results are expressed as mean with SD for four independent samples. Asterisk, $P < 0.05$. CFSE$^+$-gated sulforhodamine-valyl-alanyl-aspartyl-fluoromethylketone (SR-VAD-FMK)$^+$, 7 Aminoactinomycin D (AAD)$^+$ double-positive cells were killed by effector cells. (c) Tumor-killing activity of macrophages. Bone marrow-derived macrophages (BMDM) was activated with or without LPS (10 ng/mL) and IFNγ (100 U/mL). B16 cells were cocultured with BMDM from WT or *SOCS1*-cKO mice for 24 h, and then B16 cell proliferation in all conditions was measured using the ^{3}H-thymidine uptake test (left panel). NO production was measured as nitrite using the Griess reagent (right panel). (d) Peritoneal macrophages was activated with or without LPS (10 ng/mL) and IFNγ (100 U/mL), then cocultured with B16 cells for 24 h. Apoptosis of B16 was assessed by Annexin V and propidium iodide (PI) staining. Asterisk, $P < 0.001$. The results are expressed as mean with SD for four independent samples.

that *SOCS1*-deficient T cells differentiate into Th1 cells rather than Th17 cells,[5] and T cell-specific *SOCS1*-conditional KO mice have stronger antitumor potential (Hashimoto *et al.* unpublished data).

However, Kaiga *et al*. showed that IL-23 can elicit efficient antitumor immunity.[24] Furthermore, Muranski *et al*. showed that tumor-specific Th17-polarized cells play an important role in eradication of established melanoma.[25] Therefore, it is too early to conclude that IL-12 is tumor-eliminating while IL-23 is a tumor-promoting cytokine, and that the macrophage balance is determined by these two cytokines. Probably a number of environmental factors including tumor necrosis factor (TNF), IFNγ and Toll-like receptor (TLR) ligands are involved in determining the macrophage balance. Cytokine production, as well as sensitivity to cytokines and TLR ligands, is regulated by various transcription factors such as STAT, AP-1 and NF-κB, which are activated by intracellular signal transduction pathways. In this sense, regulation of intracellular signaling pathways is a way to modulate the balance. Therefore, we propose that SOCS1 is a candidate for modification of the macrophage balance. Further study is necessary to demonstrate that silencing of the *SOCS1* gene in macrophages actually reduces the tumor-promoting activity of TAMs.

We have shown that *SOCS1*-KO-Tg mice develop spontaneous colon tumors IFNγ-dependently. The *SOCS1* gene is deleted in myeloid cells as well as epithelial cells. Our current study suggests that silencing of the *SOCS1* gene in epithelial cells is important for tumor progression. Many studies have demonstrated that DNA methylation of the *SOCS1* gene is frequently found in HCC.[9,10,22] In this case, *SOCS1* gene silencing occurred in liver parenchimal cells rather than hematopoietic cells. Since *SOCS1* gene deletion in the liver cells resulted in enhanced hepatitis, we suspect that *SOCS1*-deficiency in the liver promotes regeneration of parenchimal cells during liver inflammation, thus resulting in the enhanced accumulation of transformed cells. Thus, signals in parenchimal cells and epithelial cells must be considered in addition to TAMs for tumor development and elimination.

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References

1 Sakamoto H, Yasukawa H, Masuhara M *et al*. A Janus kinase inhibitor, JAB, is an interferon-gamma-inducible gene and confers resistance to interferons. *Blood* 1998; **92**: 1668–76.

2 Yasukawa H, Misawa H, Sakamoto H *et al*. The JAK-binding protein JAB inhibits Janus tyrosine kinase activity through binding in the activation loop. *EMBO J* 1999; **18**: 1309–20.

3 Marine JC, Topham DJ, McKay C *et al*. SOCS1 deficiency causes a lymphocyte-dependent perinatal lethality. *Cell* 1999; **98**: 609–16.

4 Alexander WS, Starr R, Fenner JE *et al*. SOCS1 is a critical inhibitor of interferon gamma signaling and prevents the potentially fatal neonatal actions of this cytokine. *Cell* 1999; **98**: 597–608.

5 Tanaka K, Ichiyama K, Hashimoto M *et al*. Loss of suppressor of cytokine signaling 1 in helper T cells leads to defective Th17 differentiation by enhancing antagonistic effects of IFN-gamma on STAT3 and Smads. *J Immunol* 2008; **180**: 3746–56.

6 Hanada T, Tanaka K, Matsumura Y *et al*. Induction of hyper Th1 cell-type immune responses by dendritic cells lacking the suppressor of cytokine signaling-1 gene. *J Immunol* 2005; **174**: 4325–32.

7 Shen L, Evel-Kabler K, Strube R, Chen SY. Silencing of SOCS1 enhances antigen presentation by dendritic cells and antigen-specific anti-tumor immunity. *Nat Biotechnol* 2004; **22**: 1546–53.

8 Hanada T, Kobayashi T, Chinen T *et al*. IFN gamma-dependent, spontaneous development of colorectal carcinomas in SOCS1-deficient mice. *J Exp Med* 2006; **203**: 1391–7.

9 Yoshida T, Ogata H, Kamio M *et al*. SOCS1 is a suppressor of liver fibrosis and hepatitis-induced carcinogenesis. *J Exp Med* 2004; **199**: 1701–7.

10 Yoshikawa H, Matsubara K, Qian GS *et al*. SOCS-1, a negative regulator of the JAK/STAT pathway, is silenced by methylation in human hepatocellular carcinoma and shows growth-suppression activity. *Nat Genet* 2001; **28**: 29–35.

11 Clausen BE, Burkhardt C, Reith W, Renkawitz R, Forster I. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic Res* 1999; **8**: 265–77.

12 Lutz MB, Kukutsch N, Ogilvie AL *et al*. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods* 1999; **223**: 77–92.

13 Fukao T, Frucht DM, Yap G, Gadina M, O'Shea JJ, Koyasu S. Inducible expression of Stat4 in dendritic cells and macrophages and its critical role in innate and adaptive immune responses. *J Immunol* 2001; **166**: 4446–55.

14 Chinen T, Kobayashi T, Ogata H *et al*. Suppressor of cytokine signaling-1 regulates inflammatory bowel disease in which both IFN gamma and IL-4 are involved. *Gastroenterology* 2006; **130**: 373–88.

15 Naka T, Matsumoto T, Narazaki M *et al*. Accelerated apoptosis of lymphocytes by augmented induction of Bax in SSI-1 (STAT-induced STAT inhibitor-1) deficient mice. *Proc Natl Acad Sci USA* 1998; **95**: 15577–82.

16 Starr R, Metcalf D, Elefanty AG *et al*. Liver degeneration and lymphoid deficiencies in mice lacking suppressor of cytokine signaling-1. *Proc Natl Acad Sci USA* 1998; **95**: 14395–9.

17 Metcalf D, Alexander WS, Elefanty AG *et al*. Aberrant hematopoiesis in mice with inactivation of the gene encoding SOCS-1. *Leukemia* 1999; **13**: 926–34.

18 Wang JG, Wang DF, Lv BJ, Si JM. A novel mouse model for colitis-associated colon carcinogenesis induced by 1,2-dimethylhydrazine and dextran sulfate sodium. *World J Gastroenterol* 2004; **10**: 2958–62.

19 Cui S, Reichner JS, Mateo RB, Albina JE. Activated murine macrophages induce apoptosis in tumor cells through nitric oxide-dependent or -independent mechanisms. *Cancer Res* 1994; **54**: 2462–7.

20 Farias-Eisner R, Sherman MP, Aeberhard E, Chaudhuri G. Nitric oxide is an important mediator for tumoricidal activity in vivo. *Proc Natl Acad Sci USA* 1994; **91**: 9407–11.

21 Colombo MP, Mantovani A. Targeting myelomonocytic cells to revert inflammation-dependent cancer promotion. *Cancer Res* 2005; **65**: 9113–16.

22 Yoshimura A. Signal transduction of inflammatory cytokines and tumor development. *Cancer Sci* 2006; **97**: 439–47.

23 Langowski JL, Zhang X, Wu L *et al*. IL-23 promotes tumour incidence and growth. *Nature* 2006; **442**: 461–5.

24 Kaiga T, Sato M, Kaneda H, Iwakura Y, Takayama T, Tahara H. Systemic administration of IL-23 induces potent antitumor immunity primarily mediated through Th1–type response in association with the endogenously expressed IL-12. *J Immunol* 2007; **178**: 7571–80.

25 Muranski P, Boni A, Antony PA *et al*. Tumor-specific Th17-polarized cells eradicate large established melanoma. *Blood* 2008; **112**: 362–73.

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