

## INFLAMMATORY BOWEL DISEASE

# Suppressor of cytokine signalling 1 in lymphocytes regulates the development of intestinal inflammation in mice

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**Background and aims:** Imbalance between pro- and anti-inflammatory cytokines produced by intestinal T cells induces inflammatory bowel diseases (IBD). However, the importance of regulation of cytokine signalling in IBD has not been fully clarified. We have demonstrated that suppressor of cytokine signalling 1 (SOCS1) is expressed in inflamed tissues in an experimental colitis model. In the present study, we investigated the role of SOCS1 in colitis models to clarify the mechanism of IBD development.

**Methods:** Intestinal T cells in transgenic mice expressing high levels of SOCS1 in lymphocytes (SOCS1Tg mice) were characterised by flow cytometric analysis and cytokine production from intestinal T cells was determined by ELISA. 2,4,6-Trinitrobenzene sulphonic acid (TNBS) induced colitis was induced in SOCS1Tg mice and severity was compared with control littermates by measurement of survival rates. Intracellular signalling was assessed by western blotting analysis.

**Results:** SOCS1Tg mice developed colitis spontaneously with age. Young SOCS1Tg mice less than 15 weeks of age, before the onset of colitis, were susceptible to TNBS induced colitis. Intestinal T cells of SOCS1Tg mice showed increased interferon  $\gamma$  and tumour necrosis factor  $\alpha$  production and decreased transforming growth factor  $\beta$  production. Expression of cytotoxic T lymphocyte associated antigen 4 (CTLA-4), a negative regulator of T cell activation, in SOCS1Tg mice was severely impaired at the protein level although mRNA levels of CTLA-4 in SOCS1Tg mice were comparable with those in control mice.

**Conclusions:** Our data suggest that SOCS1 plays an important role in the regulation of colitis by controlling intestinal T cell activation mediated through CTLA-4 expression.

Inflammatory bowel diseases (IBD) are immune mediated disorders induced by a complex chronic inflammatory process. Dysregulation of T cell functions or cytokine balance has been suggested as a cause of colitis in the murine model and IBD patients.<sup>1-3</sup> In IBD models, colitis is ameliorated by transforming growth factor  $\beta$  (TGF- $\beta$ ), an immunosuppressive cytokine produced by regulatory T cells.<sup>4,5</sup> However, it is unknown how activation and regulation of intestinal T cells lead to the development of colitis.

Cytokine signalling is regulated in part by suppressor of cytokine signalling (SOCS) proteins.<sup>6-9</sup> Cytokines bind to specific receptors and activate Janus kinase (JAK)/signal transducers and activators of the transcription (STAT) signalling pathway, which is rapidly and strictly downregulated by SOCS proteins. SOCS proteins are characterised by the presence of an SH2 domain and a conserved motif termed the SOCS box.<sup>10</sup> SOCS1 is induced by a variety of cytokines, such as interferons (IFNs), and inhibits signalling by suppression of JAK kinase activity.<sup>6</sup> In addition, data accrued over the past six years indicate that SOCS1 acts not only as a kinase inhibitor but also as an E3 ubiquitin ligase that binds and regulates the turnover of certain substrates.<sup>11-14</sup> Although SOCS1 is now well known as a negative regulator of JAK signalling, the importance of SOCS1 in controlling cellular events through a JAK independent pathway remains to be uncovered.

We have demonstrated that SOCS1 is expressed in inflamed tissues in an experimental colitis model.<sup>15</sup> Expression was analysed mostly in epithelial cells (EC) and the role of SOCS1 in T cells of IBD has not been clarified. Although Fujimoto *et al* have reported that SOCS1 transgenic

mice (Tg) expressing high levels of SOCS1 in T lymphocytes show severe lymphopenia due to a block in T cell development in the thymus and expression of activated/memory markers by remaining peripheral T cells, they provided no information on intestinal inflammation in mice.<sup>16</sup> Therefore, it is still to be elucidated whether SOCS1 dysregulation in T cells is an important factor in the pathology of IBD.

The intestine is constitutively exposed to a very large number of resident bacteria. These may be a source of both antigens and proinflammatory stimulation. In the regulation of T cell activation after an encounter with antigen presenting cells, two well characterised B7 (CD80/CD86) receptors, CD28 and cytotoxic T lymphocyte associated antigen 4 (CTLA-4), play a critical role in the control of T cell activation. CD28 is present on naïve T cells and has been shown to be an important costimulatory factor for the development of optimal primary responses.<sup>17,18</sup> In contrast, CTLA-4 is induced only after T cell activation, and its ligation to B7 results in inhibition of T cell activation. CTLA-4 deficient mice developed a fatal lymphoproliferative disease and multiple organ immune pathology,<sup>19,20</sup> and treatment with

**Abbreviations:** IBD, inflammatory bowel disease; SOCS, suppressor of cytokine signalling; JAK, Janus kinase; STAT, signal transducers and activators of transcription; IEL, intestinal intraepithelial lymphocytes; LPL, lamina propria lymphocytes; EC, epithelial cells; TNBS, 2,4,6-trinitrobenzene sulphonic acid; IFN- $\gamma$ , interferon  $\gamma$ ; CTLA-4, cytotoxic T lymphocyte associated antigen 4; TCR, T cell receptor; TGF- $\beta$ , transforming growth factor  $\beta$ ; RT-PCR, reverse transcription-polymerase chain reaction; FACS, fluorescence activated cell sorting; mAb, monoclonal antibody; IL, interleukin; TNF- $\alpha$ , tumour necrosis factor  $\alpha$ ; PLC $\gamma$ 1, phospholipase-C $\gamma$ 1

anti-CTLA-4 antibody led to deterioration of colitis in CD45RB<sup>high</sup>CD4<sup>+</sup> transferred SCID mice.<sup>21</sup> These reports show that CTLA-4 is essential for maintenance of homeostasis of the immune system. Furthermore, cross linking of CTLA-4 in the presence of T cell receptor (TCR) ligation has been shown to result in TGF- $\beta$  production by naïve CD4<sup>+</sup> T cells or CD4<sup>+</sup> T cell clones,<sup>22</sup> indicating an additional mechanism for regulatory T cells.

In the present study, we showed that transgenic mice expressing high levels of SOCS1 in lymphocytes (SOCS1Tg) develop spontaneous colitis with age. Intestinal T cells obtained from SOCS1Tg mice were spontaneously activated and produced increased levels of proinflammatory cytokines due to downregulation of CTLA-4.

## MATERIALS AND METHODS

### Mice and induction of colitis

C57BL/6 mice were purchased at the age of eight weeks from Japan SLC (Hamamatsu, Japan). SOCS1Tg mice were generated using an Lck promoter and E $\mu$  enhancer<sup>23</sup> and used between eight and 12 weeks of age. Colitis was induced according to published methods.<sup>24</sup> Briefly, on days 0 and 7, mice were administered a 40  $\mu$ g/body weight 2,4,6-trinitrobenzene sulphonic acid (TNBS) enema, and three days after the last administration they were sacrificed to assess tissues and cells. All mice were approved by the institutional review board for animal experiments of the University of Miyazaki and Kyushu University.

### Experimental procedures

Mucosal samples of the intestine were obtained from fresh resection specimens of patients with ulcerative colitis (UC) and Crohn's disease (CD) with a chronic active course that was poorly responsive to corticosteroid or other medical treatments. Microscopically unaffected areas of intestinal specimens were used as normal controls. Analysis of surgical samples was approved by the ethics committee and institutional review board of the University of Miyazaki.

### Reverse transcription-polymerase chain reaction (RT-PCR)

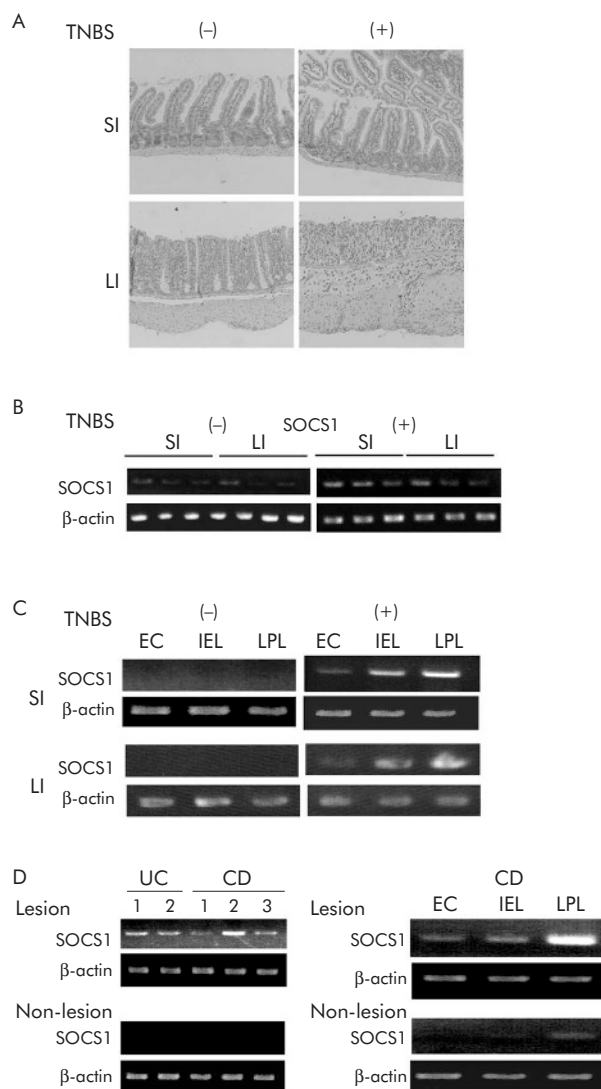
Total RNA was extracted from intestinal tissues and isolated cells using the TRIzol reagent (Invitrogen, Calsbad, California, USA) and reverse transcribed with 20 pmol of a random primer to synthesised cDNA. cDNA was amplified by PCR with gene specific primers as follows: SOCS1 sense, 5'-CTC GAG TAG GAT GGT AGC ACG CAA-3', SOCS1 antisense, 5'-CAT CTT CAC GCT GAG CGC GAA GAA-3'; SOCS1-transgene sense, 5'-GAT CCC ATC GAA TTC GAG TAG-3', SOCS1-transgene antisense, 5'-GTG ATG CGC CGG TAA TCG GA-3';  $\beta$ -actin sense, 5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3',  $\beta$ -actin antisense, 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3'.

### Detection of anaerobes

Detection of anaerobes was performed as described previously.<sup>24</sup>

### Preparation of IEL, EC, and LPL, and cell culture for cytokine ELISA

Mucosal lymphocytes were isolated and prepared according to published methods.<sup>25, 26</sup> These intestinal intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) were used for fluorescence activated cell sorting (FACS) analysis. For the cell culture, CD3<sup>+</sup> T cells were positively separated using microbeads followed by a MACS separation column (Myltenyi Biotec, Bergisch Gladbach, Germany). CD3<sup>+</sup> T cells ( $2.5 \times 10^5$ /ml) were cultured for 48 hours with anti-CD3 monoclonal antibody (mAb) in RPMI 1640



**Figure 1** Expression of suppressor of cytokine signalling 1 (SOCS1) in intestinal mucosa and cells of 2,4,6-trinitrobenzene sulphonic acid (TNBS) treated mice. (A) Haematoxylin-eosin staining of transverse sections of the small intestine (SI) and large intestine (LI) with (+) or without (-) TNBS treatment (magnification 100 $\times$ ). (B) Total RNA was prepared from the SI and LI in three C57BL/6 mice with or without TNBS administration. (C) Epithelial cells (EC), intestinal intraepithelial lymphocytes (IEL), and lamina propria lymphocytes (LPL) in the SI and LI were prepared from TNBS treated mice. (D) Tissues were prepared from ulcerative colitis (UC) and Crohn's disease (CD) patients (left). EC, IEL, and LPL, in the small intestine were prepared from CD patients (right).

supplemented with 10% fetal calf serum. Supernatants were collected to estimate the cytokine contents by an ELISA using mouse IFN- $\gamma$ , interleukin (IL)-4, and tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ) (*e*-bioscience, San Diego, California, USA), and TGF- $\beta$  (Biosource International Inc., Camarillo, California, USA) according to the manufacturer's instructions.

### FACS analysis

Molecules of the cell surface were stained as described previously.<sup>25</sup> mAbs used in this study were as follows: FITC conjugated CD4 (RM4-5) and TCR $\gamma\delta$  (GL3); PE conjugated CD8 $\alpha$  (53-6.7), CD44 (IM7), TCR $\beta$ (H57-597), and CD152 (CTLA-4; 9H10); allophycocyanin conjugated CD3 $\epsilon$  (145-2C11) mAb; biotin conjugated CD62L (MEL-14), CD28 (37.51), and isotypic control hamster IgG (G192-1), followed

by streptavidin-FITC or streptavidin-PE. These mAbs were purchased from BD PharMingen (San Jose, California, USA). Intracellular staining of CD152 and SOCS1 was performed using Cytotfix/cytopern Plus kit (BD PharMingen) according to the manufacturer's instructions. After permeabilisation, cells were stained with PE conjugated anti-CD152 or anti-SOCS1 (Immuno-Biological Laboratories Co., Ltd. Gunma, Japan) followed by antirabbit IgG conjugated with Alexa Fluor 488 (Molecular Probes, Inc., Eugene, Oregon, USA). Flow cytometry analysis was performed on a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey, USA).

### Western blotting analysis

Lysates from intestinal tissues and IEL were obtained according to a published method.<sup>27</sup> Anti-T-bet polyclonal antibody was a kind gift from Dr Glimcher (Harvard Medical School). Mouse antiphosphorylated ERK1/2 and rabbit anti-ERK2 were purchased from New England BioLabs, Inc.; rabbit antiphosphorylated Smad2/3 and anti-PLC $\gamma$ 1, and goat anti-Smad2/3 antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, California, USA); and mouse anti- $\beta$ -actin antibody from Sigma (St Louis, Missouri, USA). Anti-Flag (M2) antibody was from Sigma.

### Binding of SOCS1 mutants to GST-CTLA4 in vitro

GST-CTLA4 constructs were kindly provided by Dr Takashi Saito (Chiba University, Chiba, Japan). The GST-CTLA4 cytoplasmic domain was expressed in BL21 (Stratagene) to prepare unphosphorylated fusion proteins or in TKB-1 (Stratagene) to prepare tyrosine phosphorylated fusion proteins and purified on glutathione sepharose (Amersham Pharmacia Biotech) in accordance with the manufacturer's instructions. They were then stained with Coomassie brilliant blue and immunoblotted with anti-phospho tyrosine antibody. Flag tagged SOCS1 and SOCS3 vectors were transiently

expressed in 293 cells, and the in vitro binding assay was done as previously described.<sup>27, 28</sup>

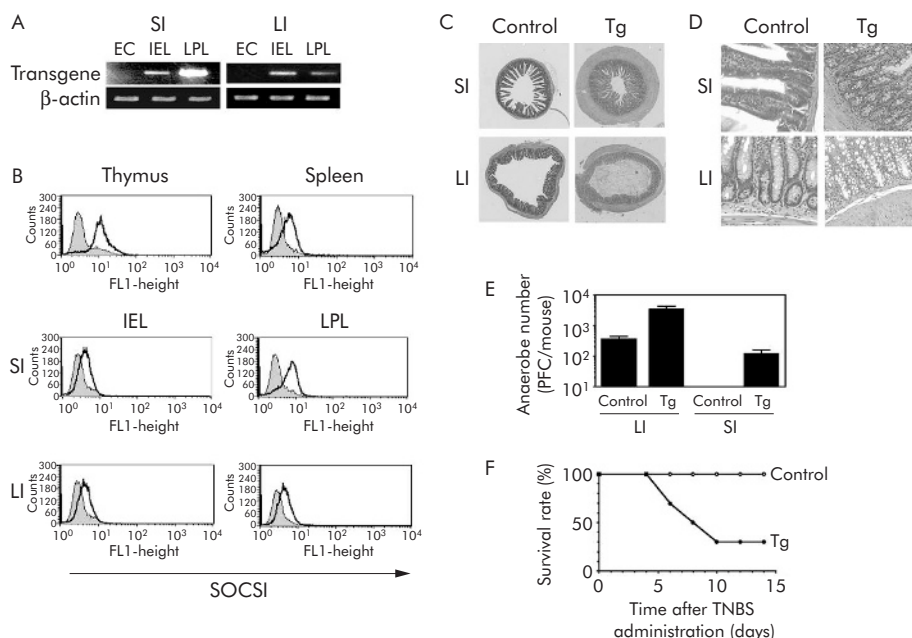
### Statistical analysis

The Student's *t* test was used to determine significant differences. A *p* value of less than 0.05 was taken as significant.

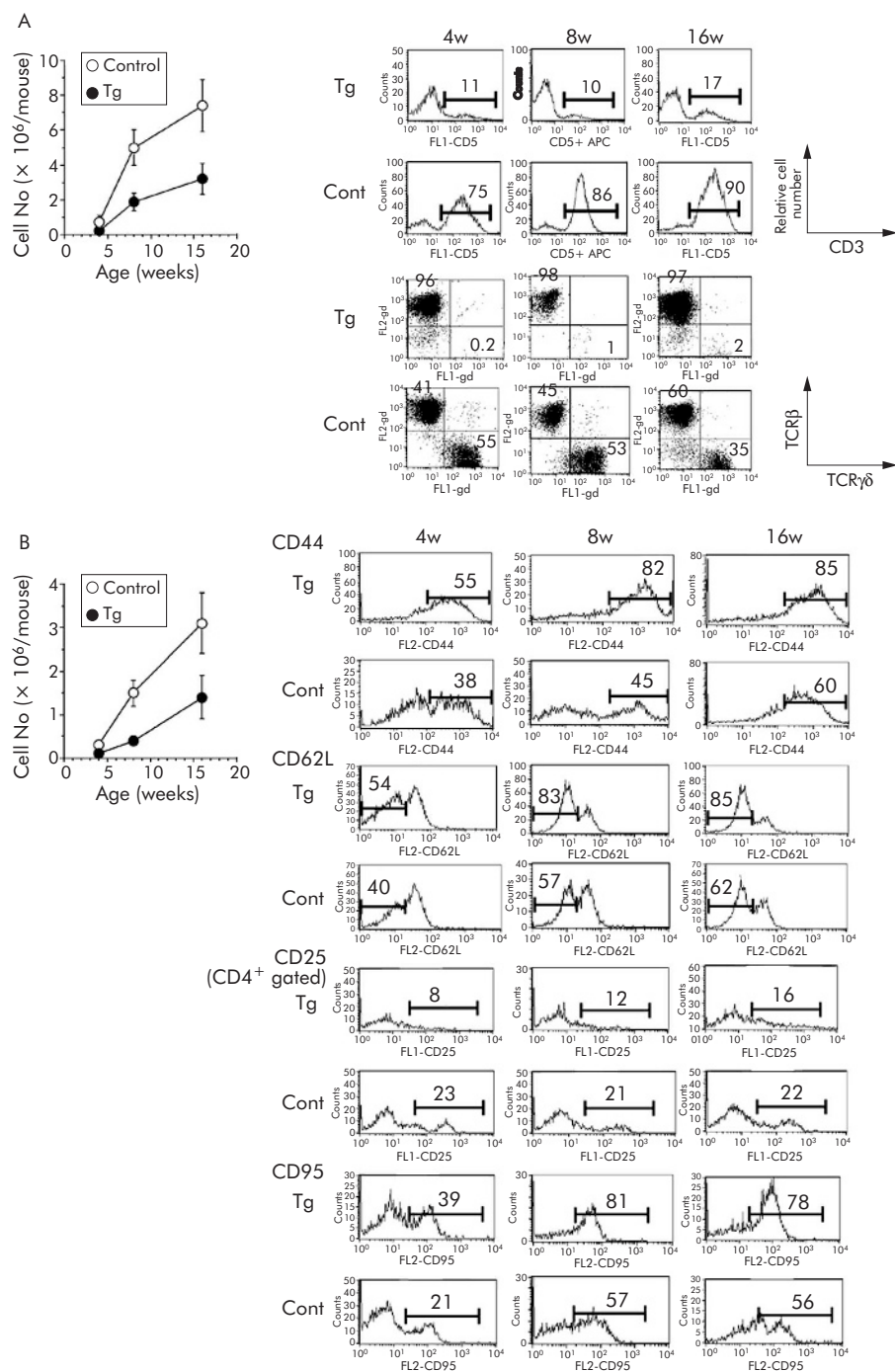
## RESULTS

### Induction of the SOCS1 gene in colitis

To determine expression of SOCS1 in the development of colitis, we examined gene expression of SOCS1 in TNBS treated C57BL/6 mice. Although TNBS induces inflammation in the large intestine but not in small intestine (fig 1A), both the small and large intestine in mice treated with TNBS showed induction of SOCS1 which was undetectable in mice not treated with TNBS (fig 1B). To identify which cells expressed a higher levels of SOCS1, we isolated EC, IEL, and LPL from the small and large intestine in TNBS treated mice. Figure 1C showed that SOCS1 was strongly induced in LPL and expressed at intermediate levels in IEL in both the small and large intestine following TNBS administration whereas low level induction was found in EC. To determine expression of SOCS1 in clinical disease, we examined gene expression of SOCS1 in both UC and CD patients. As shown in fig 1D (left), mucosal lesions in both UC and CD patients showed induction of SOCS1 whereas non-lesion control specimens showed undetectable levels of SOCS1. To identify which cells expressed higher levels of SOCS1, we isolated EC, IEL, and LPL from the small intestine of CD patients. Fig 1D (right) showed that SOCS1 was strongly induced in LPL and expressed at intermediate levels in EC and IEL in CD lesions. These results indicate that SOCS1 is strongly induced in the murine colitis model and in IBD patients. The observations suggest the possibility that high levels of SOCS1 expression in lymphocytes may contribute to the development of colitis.



**Figure 2** Spontaneous colitis in transgenic (Tg) mice expressing high levels of suppressor of cytokine signalling 1 (SOCS1Tg). (A) Expression of SOCS1 transgene in epithelial cells (EC), intestinal intraepithelial lymphocytes (IEL), and lamina propria lymphocytes (LPL) in the small (SI) and large intestines (LI). (B) Expressions of SOCS1 in thymus, spleen, IEL, and LPL were examined using fluorescence activated cell sorting. Shaded histogram shows expression of control littermates and the open histogram shows that of SOCS1Tg mice. (C, D) Spontaneously developing colitis in a SOCS1Tg mouse (five months old) with rectal prolapse. Haematoxylin-eosin staining of transverse sections of the SI and LI (magnification in (C) 100 $\times$ ; magnification in (D) 200 $\times$ ). (E) Number of anaerobes on day 10 after 2,4,6-trinitrobenzene sulphonic acid (TNBS) administration. (F) Susceptibility of SOCS1Tg mice to TNBS. SOCS1Tg mice and control littermates (*n* = 10) were administered 40  $\mu$ g TNBS/g body weight. Survival rates were obtained by daily observation after the first TNBS administration.

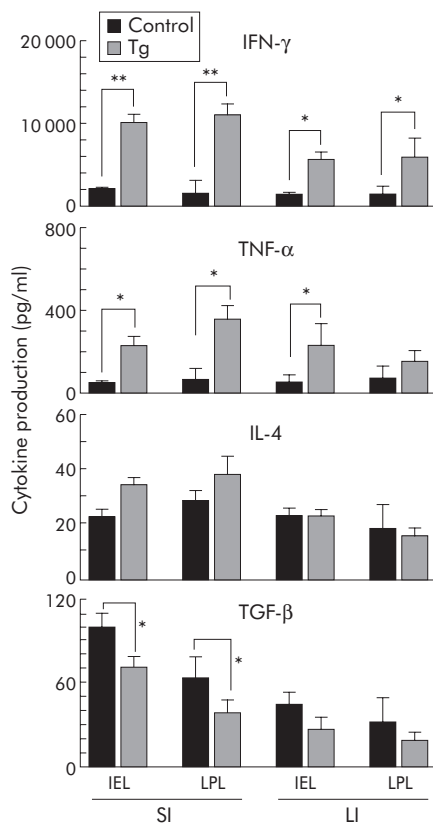


**Figure 3** Phenotype of intestinal lymphocytes in transgenic (Tg) mice expressing high levels of suppressor of cytokine signalling 1 (SOCS1Tg). (A) Total intestinal intraepithelial lymphocyte (IEL) number was counted (left). IEL in the small intestine (SI) were stained with anti-CD3, T cell receptor (TCR) $\beta$ , and TCR $\gamma\delta$  monoclonal antibodies (mAbs) and analysed using FACS (right). (B) Total lamina propria lymphocyte (LPL) number was counted (left). LP T cells of the SI were stained with anti-CD3 mAb, anti-CD44, anti-CD62L, anti-CD4, anti-CD25, and anti-CD95 mAbs. The analysis gate was set on CD3<sup>+</sup> cells (right) and gated on CD4<sup>+</sup> for CD25<sup>+</sup> cells. The numbers indicate percentages of stained cells within each area of A and B.

### Development of spontaneous colitis in SOCS1Tg mice

As demonstrated above, TNBS treated mice showed induction of the SOCS1 gene in mucosal lymphocytes. We next examined the effect of high expression of SOCS1 in lymphocytes in the regulation of colitis using a SOCS1Tg mouse line which expresses high levels of SOCS1 in lymphocytes. SOCS1Tg mice showed high levels of transgene expression in intestinal lymphocytes but not in EC (fig 2A). LPL expressed higher levels of the transgene than IEL in the small intestine whereas a similar level of transgene expression in IEL and LPL was observed in the large intestine (fig 2B). Young SOCS1Tg mice, less than 15 weeks of age, showed no symptom of colitis. However, we found that approximately 40% of SOCS1Tg mice over 15 weeks of age

developed rectal prolapse spontaneously when they were kept under conventional conditions while no colitis was observed under specific pathogen free conditions (data not shown). Aged SOCS1Tg mice showed dilatation and thickening of the colon, extending continuously up to the ileum, elongation of crypts, ulcers reaching the muscle layer, diffuse cell infiltration, and a high number of goblet cells (fig 2C, 2D). As SOCS1Tg mice spontaneously developed colitis with soft stool and diarrhoea and it has been reported that anaerobe numbers are increased in IBD patients,<sup>29</sup> we examined changes in the number of anaerobes. The number of anaerobes was greater in SOCS1Tg mice than in control littermates (fig 2E). We further examined susceptibility of young SOCS1Tg mice to TNBS induced colitis, which is a



**Figure 4** Cytokine profiles in intestinal intraepithelial lymphocyte (IEL) and lamina propria (LP) T cells in the small (SI) and large (LI) intestine. Freshly isolated IEL and LP T cells were stimulated with plate bound anti-CD3 monoclonal for 48 hours, and supernatants were collected to determine concentrations of cytokines using ELISA for interferon  $\gamma$  (IFN- $\gamma$ ), tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ), interleukin 4 (IL-4), and transforming growth factor  $\beta$  (TGF- $\beta$ ). \* $p < 0.05$ , \*\* $p < 0.01$ .

Th1-type colitis. Seventy per cent of SOCS1Tg mice died within 14 days after TNBS administration although all control mice survived (fig 2F). Another SOCS1Tg mouse line with a low level of SOCS1 did not develop spontaneous colitis (data not shown), suggesting a strong relationship between high expression of SOCS1 and spontaneous development or susceptibility to Th1-type colitis induction.

### Aberrant phenotypes and cytokine production of intestinal T cells in SOCS1Tg mice

To investigate the mechanism of colitis development in SOCS1Tg mice, we analysed cell surface markers of intestinal T cells, IEL, and LP T cells, in Tg mice before they developed colitis. As shown in fig 3A, SOCS1Tg mice exhibited a reduction in total IEL number and CD3<sup>+</sup> IEL in the small intestine; in particular, very few  $\gamma\delta$ IEL were detected. A similar change was also observed in the IEL of the large intestine (data not shown). We next analysed the expression of activation markers on LP T cell. As shown in fig 3B, total LPL number was decreased in SOCS1Tg mice. LP T cells of SOCS1Tg mice showed upregulation of activation/memory T cell markers, CD44 and CD95, and downregulation of the naïve T cell marker, CD62L, in the small intestine, indicating that LP T cells in SOCS1Tg mice are activated spontaneously in vivo and that these T cells exhibit the characteristics of activated/memory T cells. The population of regulatory T cells, CD4<sup>+</sup>CD25<sup>+</sup>, was less in SOCS1Tg than in controls. A similar increase in these markers was observed in LP T cells of the large intestine (data not shown). These results strongly

suggest that mucosal T cells in SOCS1Tg mice are activated spontaneously in vivo.

A cytokine imbalance between Th1 and Th2 is well known to be involved in induction or aggravation of colitis. Therefore, we examined cytokine production of mucosal T cells of untreated SOCS1Tg mice in vitro. IEL in both the small and large intestine from untreated SOCS1Tg mice produced higher levels of the Th1-type proinflammatory cytokines, IFN- $\gamma$  and TNF- $\alpha$ , than those from control littermates (fig 4). Higher levels of IFN- $\gamma$  production were also observed in LP T cells of SOCS1Tg mice. In contrast, no significant difference was observed in the production of IL-4, a Th2-type cytokine, between control and SOCS1Tg mice. We also found that TGF- $\beta$ , an immunoregulatory cytokine, was produced at lower levels in SOCS1Tg mice than in control mice. These results suggest that SOCS1Tg mice tend to produce higher levels of the Th1-type and proinflammatory cytokines, IFN- $\gamma$  and TNF- $\alpha$ , but lower levels of the immunosuppressive cytokine, TGF- $\beta$ .

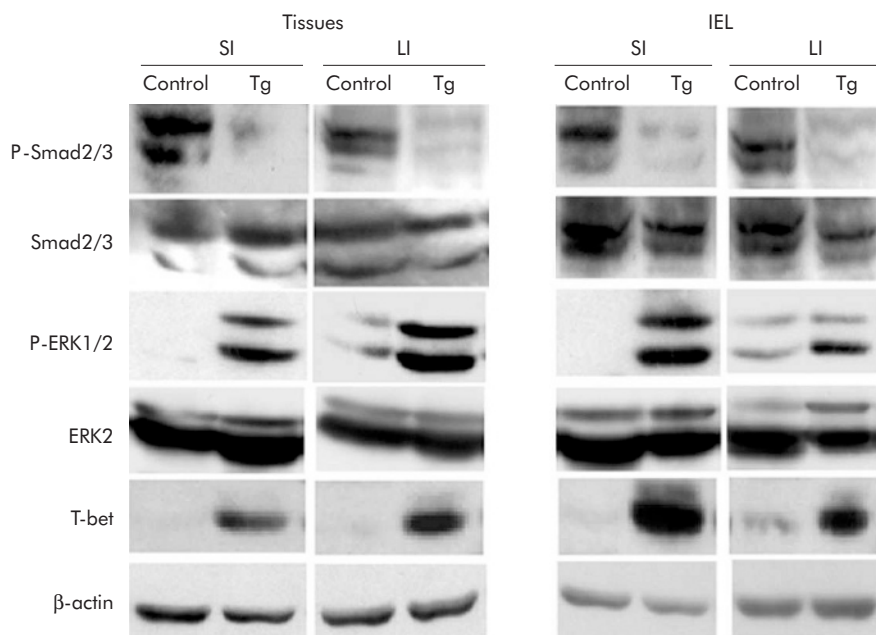
### Enhancement of ERK activation and T-bet in SOCS1Tg mice

As the data above strongly suggest that reduction of TGF- $\beta$  production is linked to higher susceptibility to colitis induction in SOCS1Tg, we analysed the TGF- $\beta$  signalling pathway in the intestine of SOCS1Tg mice. SOCS1Tg mice showed decreased phosphorylation of Smad2/3, the transducers of TGF- $\beta$  signalling, in both the small and large intestine (fig 5). TGF- $\beta$  has been shown to suppress activation of ERK1/2<sup>30</sup> and expression of T-bet, an essential factor for Th1 differentiation.<sup>31</sup> In both the small and large intestine of SOCS1Tg mice, ERK1/2 was spontaneously activated, and expression of T-bet was upregulated (fig 5). Consistent with the reduced ability of TGF- $\beta$  production in IEL and LP T cells of SOCS1Tg (fig 4), these data confirm that, in vivo, TGF- $\beta$  signalling is indeed reduced and Th1 signalling is increased in the intestines of SOCS1Tg.

### Downregulation of CTLA-4 in SOCS1Tg mice

The B7/CD28/CTLA-4 pathway plays a key role in regulating T cell activation and tolerance. CD28 is constitutively expressed on the surface of T cells whereas CTLA-4 expression is rapidly upregulated following T cell activation.<sup>19, 20</sup> As aberrant activation of T cells in SOCS1Tg mice partly resemble that of CD28 upregulated or CTLA4 suppressed mice, we analysed expression levels of CD28 and CTLA-4 in LP T cells. The data in fig 6A show that higher levels of CD28 on LP T cells were expressed in SOCS1Tg mice than in control mice. In contrast, lower levels of CTLA-4 were expressed in SOCS1Tg mice than in control mice. These results raise the possibility that SOCS1 inhibits CTLA-4 gene expression. However, as shown in fig 6B, mRNA levels of CTLA-4 in SOCS1Tg mice were almost the same as those in control mice before and after anti-CD3 antibody stimulation. These results suggest the possibility that SOCS1 affects transportation of CTLA-4 to the cell surface on T cell activation or, most likely, translation of CTLA-4 or protein stability of CTLA-4.

To investigate SOCS1 mediated regulation of CTLA-4, we tested whether SOCS1 interacts with CTLA-4 by an in vitro binding assay. As CTLA-4 is phosphorylated on T cell activation, we used GST fusion proteins carrying the phosphorylated CTLA-4 cytoplasmic domains (fig 6C). GST fusion protein was expressed in TKB-1 bacteria carrying an active tyrosine kinase, and fusion proteins were thereby tyrosine phosphorylated. SOCS1 strongly bound to phosphorylated GST-CTLA-4 (panel e in fig 6). However, SOCS1 bound to neither GST nor unphosphorylated GST-CTLA-4. The interaction between SOCS1 and GST-CTLA-4 was



**Figure 5** Biochemical analysis of transforming growth factor  $\beta$  signalling pathway in the small (SI) and large (LI) intestine in untreated transgenic (Tg) mice expressing high levels of suppressor of cytokine signalling 1 (SOCS1Tg). Lysates from tissues and intestinal intraepithelial lymphocytes (IEL) in littermate controls and SOCS1Tg mice were prepared and subsequently analysed by western blot for phosphorylated ERK1/2 and Smad2/3, and total ERK1/2, Smad2/3, and T-bet.

dependent on an intact SH2 domain of SOCS1 because point mutation at the SH2 domain (R105E) abrogated the interaction (panel b in fig 6). We measured binding of phospholipase-C $\gamma$ 1 (PLC $\gamma$ 1) as a control (panel d, fig 6) and found that the binding affinity of SOCS1 to CTLA-4 was comparable with that of PLC $\gamma$ 1. As it has been reported that SOCS3 was upregulated in a murine colitis model and in IBD patients,<sup>15</sup> we compared the binding ability of SOCS1 and SOCS3. Unlike SOCS1, SOCS3 did not bind phosphorylated GST-CTLA-4 (panel c in fig 6). This result suggests the physical coupling of SOCS1 with CTLA-4.

## DISCUSSION

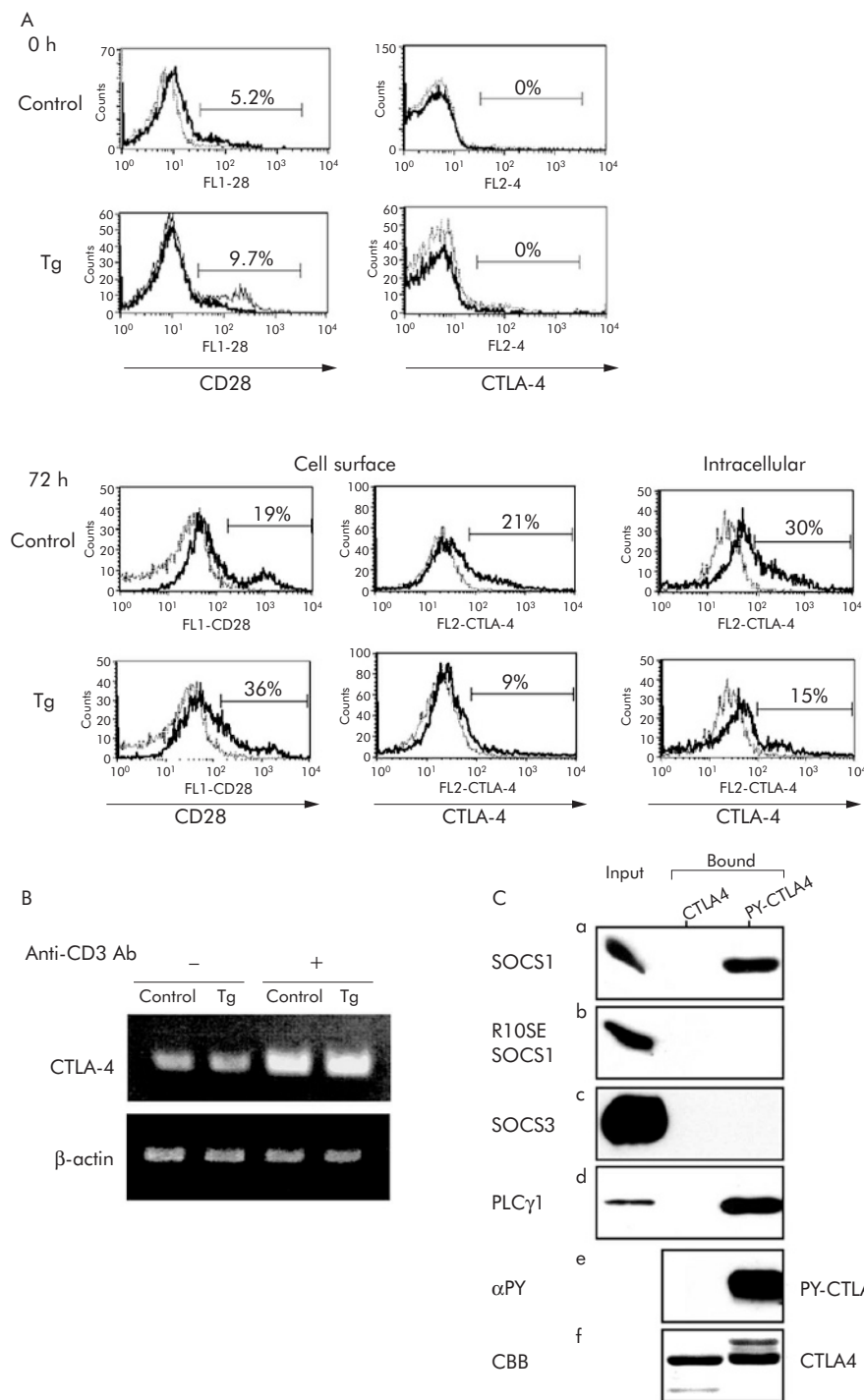
Several cytokines and their signalling molecules have been reported to be important as inducers or suppressors of colitis. SOCS1 has been shown to be expressed in the murine colitis model and IBD patients.<sup>15</sup> However, how SOCS1 is involved in clinical disease and how sustained expression of SOCS1 affects the pathogenesis has not been investigated. In this study, mice overexpressing SOCS1 in lymphocytes spontaneously develop colitis and ileitis. These results strongly support the hypothesis that high and sustained expression of SOCS1 in intestinal T lymphocytes causes colitis. Therefore, SOCS1Tg mice is a new model for understanding the pathogenesis of IBD.

We found that CTLA-4 is an important candidate molecule involved in colitis induction in SOCS1Tg mice. CTLA-4 expression was down-modulated in the intestinal T cells of SOCS1Tg mice. As CTLA-4 crosslinking leads to TGF- $\beta$  production in CD4<sup>+</sup> T cell,<sup>22</sup> we estimate that the decrease in CTLA-4 expression in SOCS1Tg mice is the reason for the decrease in TGF- $\beta$  production by intestinal T cells in colitis. CTLA-4 was shown to be constitutively expressed in a regulatory T cell subset, CD25<sup>+</sup>CD4<sup>+</sup>.<sup>32, 33</sup> Regulatory T cells, which produce TGF- $\beta$ , have been shown to exhibit a protective role against Th1-type colitis by suppressing activated T cells.<sup>4, 34</sup> TGF- $\beta$  has been reported to suppress the phosphorylation of ERK1/2 and expression of T-bet.<sup>30, 31</sup> These observations are consistent with our data showing that IEL of SOCS1Tg mice decrease TGF- $\beta$  production (fig 4) and increase phosphorylation of ERK1/2 and expression of T-bet (fig 5). Therefore, in SOCS1Tg mice, the cause of the

development of colitis is presumed to be degradation of CTLA-4 by SOCS1, which results in a decrease in TGF- $\beta$  production.

The decrease in TGF- $\beta$  production was observed on both IEL and LPL (fig 4). Suppression of TGF- $\beta$  production in IEL may be partly caused by lack of  $\gamma\delta$  T cells. Fujimoto *et al* reported that SOCS1Tg mice show few  $\gamma\delta$  T cells in the thymus and a characteristic of activated/memory T cell in peripheral T cells such as the spleen and lymph node T cells.<sup>16</sup> In spite of the fact that SOCS1Tg mice show severe lymphopenia, no symptoms were observed in young mice, as previously reported.<sup>16</sup> However, we demonstrated that these mice develop colitis with age (fig 2). Intestinal T cells, especially IEL, are known to develop intrathymically or extrathymically and exhibit unique characteristics compared with T cells in other tissues. We further showed that there were few  $\gamma\delta$  T cells in the intestine (fig 3). Recently, we discovered that  $\gamma\delta$  IEL is an important protective T cell population against colitis, as colitis induced in  $\gamma\delta$  T cell deficient (C $\delta$ <sup>-/-</sup>) mice was suppressed by adoptive transfer of  $\gamma\delta$  IEL which produce TGF- $\beta$ .<sup>24</sup> Thus lack of  $\gamma\delta$  T cells may easily skew the immune response towards activation of T cells and the onset of inflammation. However, the severity of colitis induced by TNBS was greater in SOCS1Tg mice than in C $\delta$ <sup>-/-</sup> mice (this report and Inagaki-Ohara and colleagues<sup>24</sup>). Differences in severity between SOCS1Tg and C $\delta$ <sup>-/-</sup> mice appear to be explained by the phenotypes of T cells. C $\delta$ <sup>-/-</sup> mice still have intestinal T cells with TCR $\alpha\beta$  and they may play a protective role against colitis.<sup>35</sup> In contrast, SOCS1Tg mice showed a marked decrease in both  $\gamma\delta$  T cells and  $\alpha\beta$  T cells, and the remaining  $\alpha\beta$  IEL were activated (fig 3). Less regulatory IEL of both TCR  $\gamma\delta$  and TCR $\alpha\beta$  lineages in SOCS1Tg mice may exacerbate colitis.

Our results suggest a new function for SOCS1 in immunoregulation through CTLA-4, as discussed above. Expression of CTLA-4 protein in intestinal T cells of SOCS1Tg mice was markedly lower than that of control mice, while CD28 expression was increased. Decreased CTLA-4 expression was not due to a decrease in expression levels of CTLA-4 mRNA. Therefore, downregulation of CTLA-4 by SOCS1 seems to be enhanced degradation of CTLA-4. Several reports have shown that SOCS1 acts as a ubiquitin ligase that



**Figure 6** Low expression of cytotoxic T lymphocyte associated antigen 4 (CTLA-4) of lamina propria (LP) T cells in transgenic (Tg) mice expressing high levels of suppressor of cytokine signalling 1 (SOCS1Tg). (A) LP T cells in the small intestine were stimulated with anti-CD3 antibody for 72 hours. CD28 and CTLA-4 expressed on the cell surface and intracellular CTLA-4 were examined. Cells were stained with anti-CD28 antibody or anti-CTLA-4 antibody (bold line), and with isotype control antibody (broken line). (B) mRNA levels of CTLA-4 in LP T cells stimulated with anti-CD3 antibody for 48 hours were analysed by reverse transcription-polymerase chain reaction. (C) Extracts of 293 cells expressing Flag tagged WT SOCS1 or SOCS3 or indicated mutant SOCS1 were incubated with 5  $\mu$ g of immobilised GST fusion proteins at 4°C for one hour (a-c). Proteins complexed with GST fusion proteins were resolved by 13% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoblotted with anti-Flag (a-c) or anti-PLC $\gamma$ 1 (d) antibody.

regulates the half life of several molecules, including Vav,<sup>11</sup> the oncogene Tel-JAK2,<sup>12, 13</sup> and JAK kinases, as well as IRS-1/2.<sup>14</sup> We assume that SOCS1 may induce ubiquitin mediated degradation on CTLA-4 but not on CD28. On the other hand, several reports have implied that binding of SOCS1 to target molecules is not sufficient for its degradation although binding primes the degradation of target protein. SOCS1 cannot degrade c-Kit, Tec kinase, or FGF receptor even if it strongly binds to them.<sup>6, 36, 37</sup> We speculate that SOCS1 can act as E3 ligase only when the potential ubiquitination lysine of a target molecule is located at the correct position for the SOCS1-E3 complex. Future studies on the mechanisms of SOCS1 mediated protein degradation will provide

information about the regulatory pathways of CD28 and CTLA-4 controlled by SOCS1.

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