SOCS2 regulates T helper type 2 differentiation and the generation of type 2 allergic responses

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The incidence of allergy and asthma in developed countries is on the increase and this trend looks likely to continue. CD4+ T helper 2 (Th2) cells are major drivers of these diseases and their commitment is controlled by cytokines such as interleukin 4, which are in turn regulated by the suppressor of cytokine signaling (SOCS) proteins. We report that SOCS2−/− CD4+ T cells show markedly enhanced Th2 differentiation. SOCS2−/− mice, as well as RAG−1−/− mice transferred with SOCS2−/− CD4+ T cells, exhibit elevated type 2 responses after helminth antigen challenge. Moreover, in in vivo models of atopic dermatitis and allergen-induced airway inflammation, SOCS2−/− mice show significantly elevated IgE, eosinophilia, type 2 responses, and inflammatory pathology relative to wild-type mice. Finally, after T cell activation, markedly enhanced STAT6 and STAT5 phosphorylation is observed in SOCS2−/− T cells, whereas STAT3 phosphorylation is blunted. Thus, we provide the first evidence that SOCS2 plays an important role in regulating Th2 cell expansion and development of the type 2 allergic responses.

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significantly more type 2 cytokines relative to WT animals. In addition, OVA-sensitized SOCS2−/− mice had significantly increased airway resistance, specific IgE, and airway inflammation after OVA challenge. Our data suggest a novel role for SOCS2 in controlling CD4+ lineage commitment and type 2 allergic responses.

RESULTS

SOCS2 deficiency is associated with enhanced IL-4, IL-5, and IL-13 secretion and Th2 differentiation both in vitro and in vivo

SOCS2−/− mice present with gigantism but with no reported obvious immune defect (Metcalf et al., 2000). Phenotyping of thymus, spleen, and LN of SOCS2−/− and WT mice showed similar frequency and numbers of CD4+, CD8+, double-positive, and double-negative T cells (Fig. S1, A and B), suggesting that T cell development was normal. However, as other SOCS family members have important roles in determining the cytokine responsiveness of different Th subsets (Palmer and Restifo, 2009), we examined the cytokine profile of CD4+ T cells from SOCS2−/− mice after TCR stimulation in vitro. IL-2 and IFN-γ secretion were similar in CD4+ T cells from SOCS2−/− and WT mice but, surprisingly, IL-4, IL-5, IL-10, and IL-13 levels were markedly higher in the absence of SOCS2 (Fig. 1 A). Moreover mRNA levels of these cytokines were also enhanced when examined by real-time PCR (Fig. S1 C). Importantly the level of GATA-3 mRNA was significantly higher in SOCS2−/− CD4+ T cells (Fig. S1 C), suggesting spontaneous enhancement of Th2 polarization. To explore this more closely, we sorted naive CD4+CD25−CD44low T cells and drove de
SOCS2 deficiency in naive CD4+ T cells was directly responsible for the observed Th2 bias, as indicated by the significantly higher (P < 0.001) in SOCS2−/− mice (Fig. 1 D). To confirm that SOCS2 deficiency in vivo significantly lower amounts of IL-17 compared with WT mice after both nonspecific and antigen-specific TCR stimulation (Fig. 1 C). Interestingly, splenic T cells from egg-injected SOCS2−/− mice produced greater production of the type 2 cytokines IL-4, IL-5, and IL-13 by splenic T cells from egg-injected SOCS2−/− mice (Fig. 1 C). Moreover, there was significantly (P < 0.05) higher frequencies of Th2 cells in the popliteal LN was significantly exaggerated in the SOCS2−/− mice (P < 0.05; Fig. 2 D). All together, the data demonstrates MC903-treated SOCS2−/− mice produced enhanced IL-4, IL-5, and IL-13, but not IFN-γ in restimulated auricular LN and spleen cells showed enhanced IL-4, IL-5, and IL-13 secretion in SOCS2−/− mice compared with those reconstituted with WT cells (Fig. 1 E), showing a requirement for SOCS2 expression in CD4+ T cells in controlling in vivo differentiation of Th2 cells. These observations indicate that SOCS2 deficiency unexpectedly favors Th2 polarization in vitro and in vivo and, therefore, that SOCS2 is a key regulator of Th2 differentiation.

RAG-1−/− recipients and challenged with S. mansoni eggs. 10 d after injection, the number of Th2 (CD4+ T1/ST2+) cells in popliteal LN was significantly (P < 0.05) higher in mice reconstituted with SOCS2-deficient T cells when compared with those reconstituted with WT T cells (Fig. 1 E), confirming an inhibitory effect of SOCS2 on the allergic response in vivo.

**SOCS2−/− mice are more susceptible to AD**

Atopic disorders are driven by an elevated Th2 response; therefore, we used an in vivo model of AD, which was previously described by Li et al., (2006), to assess whether SOCS2−/− mice are more susceptible to atopy. Mice were treated daily with an application of the vitamin D3 analogue calcipotriol (MC903). Topical application of MC903 triggered an AD-like response—increase in skin thickness—in all mice, but SOCS2−/− mice exhibited earlier onset and more severe disease (Fig. 2 A). Histological examination showed red scaly lesioned skin, accompanied by epidermal hyperplasia with dermal lymphocyte infiltration that was more abundant in SOCS2−/− mice (Fig. 2 B). Furthermore, the dermal inflammation was associated with a marked eosinophilic infiltrate that was significantly exaggerated in the SOCS2−/− mice (P < 0.05; Fig. 2 C). Finally, analysis of cytokine secretion in restimulated auricular LN and spleen cells showed enhanced IL-4, IL-5, and IL-13, but not IFN-γ, levels from MC903-treated SOCS2−/− compared with treated WT animals (P < 0.05; Fig. 2 D). All together, the data demonstrates that SOCS2−/− mice were more susceptible to AD in this model, confirming an inhibitory effect of SOCS2 on the allergic response in vivo.
SOCS2−/− mice have increased susceptibility to allergic airway inflammation

Given the marked predisposition of SOCS2−/− mice to AD, we investigated whether SOCS2 deficiency resulted in an increased response to OVA-induced allergic airway inflammation. SOCS2−/− and WT mice were OVA challenged systemically and by aerosol to evoke airway inflammation and airway hyper-responsiveness (AHR). In the absence of allergen challenge, AHR, determined by lung resistance (Rl), was not induced in PBS-treated SOCS2−/− mice, with these mice having Rl values comparable to those of PBS-treated WT mice in response to methacholine challenge (Fig. 3 A). In comparison, OVA-sensitized SOCS2−/− mice had a striking dose-dependent increase in Rl, relative to OVA-sensitized WT animals, that was statistically significant (P < 0.01) at higher methacholine doses (Fig. 3 A).

Histological analysis of lungs of OVA-sensitized mice showed more marked inflammation in SOCS2−/− mice relative to WT, with peribronchial infiltrating eosinophils and goblet cell hyperplasia (Fig. 3 B). Consistent with a greater allergen-specific type 2 response, OVA-sensitized SOCS2−/− mice had significantly enhanced levels of both total IgE (P < 0.05) and OVA-specific IgE (P < 0.001) compared with OVA-treated WT animals (Fig. 3 C). Furthermore, cells from the lung-draining mediastinal LN from OVA-sensitized SOCS2−/− mice had significantly (P < 0.05) greater production of type 2, but not type 1, cytokines relative to allergen-sensitized WT animals (Fig. 3 D). Again, we observed a significant reduction (P < 0.05) in IL-17 production in response to antigen challenge in T cells from SOCS2−/− mice (Fig. 3 D). To formally determine that the enhanced allergen-induced lung inflammation in SOCS2-deficient mice (Fig. 3, A–D) was attributed to specific alterations in CD4+ cell function, we generated SOCS2−/− x OT/II mice. RAG-1−/− mice were reconstituted with WT or SOCS2−/− T cells and challenged with OVA and methacholine-induced Rl was measured (E). The cytokine concentrations in the lungs were measured after homogenization (F). Data are from three independent experiments and are presented as the mean and SEM per group. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Moreover, IL-4 was the most potent inducer of SOCS2, suggesting a possible role for SOCS2 in limiting Th2 differentiation. Interestingly, TGF-β induced no detectable SOCS2 expression and IL-12 induced limited amounts in CD4+ T cells (Fig. 4 A). SOCSs are known to inhibit STAT activation, and STATs, in turn, play a key role in the differentiation and commitment of helper T cell subsets. STAT6 is associated with Th2 development, whereas STAT4 and STAT3 reciprocally control Th1 and Th17 development, respectively (O’Shea et al., 2011). To examine the mechanism of preferential Th2 differentiation in SOCS2−/− mice, we investigated the profile of STAT activation in T cells. SOCS2−/− CD4+ T cells displayed constitutive and significantly enhanced STAT6 phosphorylation compared with WT CD4+ T cells (Fig. 4 B and Fig. S3 A), suggesting that the SOCS2-deficient CD4+ T cells are hyper-responsive to IL-4 in vivo. Moreover, IL-4–induced STAT5 activation is thought to promote early Th2 cell differentiation (Zhu et al., 2003; Cote-Sierra et al., 2004; Paul and Zhu, 2010) and we found that IL-2–induced STAT5 phosphorylation is enhanced in SOCS2−/− CD4+ T cells. (Fig. 4 C and Fig. S3 B). Importantly, IL-2–mediated STAT5 activation was shown to induce and maintain IL-4Rα expression (Liao et al., 2008). However, we found that IL-4Rα expression was unchanged (Fig. S3 D). Finally, although IL-12–mediated STAT4 activation was unaffected by the absence of SOCS2 (Fig. 4 D), with OVA and AHR evaluated. RAG-1−/− mice reconstituted with OT/II CD4+ T cells (Fig. 3 E). Moreover, this was associated with enhanced peribronchial cell infiltration and goblet cell hyperplasia in recipients of CD4+ from SOCS2−/− xOT/II mice relative to cells from OT/II mice (Fig. S2 A) and increased bronchoalveolar cell infiltration (Fig. S2 B). Recipients of CD4+ from SOCS2−/− xOT/II mice had significantly increased secretion of Th2-associated cytokines, lower IL-17, and unaffected IFN-γ both in the lungs (Fig. 3 F) and in BAL fluid (Fig. S2 C). Collectively, the data demonstrate that SOCS2−/− mice are more susceptible to OVA-induced airway inflammation and this can be a direct result of increased Th2 phenotype in SOCS2-deficient CD4+ T cells.

**Enhanced STAT5 and STAT6 phosphorylation and SOCS1 and SOCS3 expression after T cell activation in the absence of SOCS2**

In the different allergic models used in this study, SOCS2−/− mice consistently had enhanced IL-4, IL-5, and IL-13 and reduced IL-17 expression. It was important, therefore, to determine which cytokines involved in Th helper cell polarization actually induced SOCS2 expression. Consequently, we next investigated which cytokine signals induced SOCS2 in purified WT CD4+ T cells. Both IL-4 and IL-6 strongly induced SOCS2 with expression being clearly up-regulated after 48 h (Fig. 4 A).

**Figure 4. SOCS2 deficiency results in constitutive STAT6 activation, enhanced IL-2–mediated STAT5 activation, and reduced IL-6–mediated STAT3 activation.** (A) CD4+ splenocytes from WT mice were cultured with anti-CD3 and anti-CD28 in the presence of 20 ng/ml IL-4, 200 U/ml IL-6, 20 ng/ml IL-12, or 5 ng/ml TGF-β for the indicated times. Cells were then lysed and immunoblotted for the expression of SOCS2 and γ-tubulin. (B) CD4+ splenocytes were cultured with anti-CD3 and anti-CD28 for 48 h. 200 ng/ml IL-4 was then added to the culture for times indicated. Cell were lysed and immunoblotted for phospho-STAT6. (C) After 48 h and preactivation with anti-CD3, anti-CD28, 5 ng/ml TGF-β, and 5 U/ml IL-2, CD4+ splenocytes were incubated for 2 h in serum-free media, restimulated with 100 U/ml IL-2 for the indicated times, and immunoblotted for phospho-STAT5. (D and E) CD4+ splenocytes were preactivated for 48 h with anti-CD3 and anti-CD28, rested 2 h in serum-free media, and restimulated with 50 ng/ml IL-12 (D) or 50 U/ml IL-6 (E) for the indicated times, lysed, and immunoblotted for phospho-STAT4 (D) or phospho-STAT3 (E). Total STAT6, STAT5, STAT4, or STAT3 expression was also assessed. Right panels show densitometry of phospho-STAT relative to total STAT. Data are mean and SEM from triplicates. *, P < 0.05; **, P < 0.01; ***, P < 0.001. The data are representative of three independent experiments.
we observed consistent blunting of IL-6–induced STAT3 phosphorylation (Fig. 4 E and Fig. S3 C). Interestingly, SOCS2 expression was induced after IL-6 and TGF-β stimulation (Fig. S3 E) and, when stimulated under Th17 polarization conditions, SOCS2-deficient T cells express less RORγt (Fig. S3 F) and secrete less IL-17 (Fig. S3 G). These results are consistent with the decreased IL-17 levels observed in vivo (Fig. 1 C and Fig. 3 D), including in the CD4+ cell reconstitution experiments (Fig. 3 F and Fig. S2 C), and they suggest that SOCS2 is required for normal Th17 differentiation. STAT3 is known to inhibit Th17 differentiation (Laurence et al., 2007; Yang et al., 2011) and, therefore, our data suggest that the reduced STAT3 activation and increased IL-2–mediated STAT5 phosphorylation in the SOCS2−/− T cells primes Th2 development, probably at the expense of Th17.

Interestingly, SOCS3 is a strong inhibitor of STAT3 signaling (Elliott and Johnston, 2004) and constitutive expression of SOCS3 in T cells was shown to favor Th2 polarization (Seki et al., 2003). Moreover, several studies suggest that SOCS2 can control the expression of both SOCS1 and SOCS3 (Tannahill et al., 2005; Fessevaux et al., 2006). We therefore hypothesized that SOCS1 and SOCS3 levels may be altered in SOCS2-deficient T cells. When splenocytes from SOCS2−/− mice were stimulated with anti-CD3/CD28 and IL-2 for periods of up to 24 h, we consistently observed significantly elevated SOCS1 and SOCS3 expression (Fig. S4 A). Interestingly, in WT cells, SOCS2 expression after T cell activation correlated with loss of detectable SOCS3 protein (Fig. S4 A). To assess whether SOCS3 levels were elevated in Th2 cells from SOCS2−/− mice, we purified and cultured CD4+ T cells for 7 d under Th2 conditions. Basal levels of SOCS3 were higher in SOCS2-deficient T cells relative to controls (Fig. S4 B), with the difference between WT and SOCS2−/− cells being amplified under polarizing conditions (Fig. S4 C). Finally, we also found that SOCS1 and SOCS3 mRNA levels are elevated in SOCS2-deficient CD4+ T cells stimulated for 3 and 5 d with anti-CD3/CD28 antibodies (Fig. S4 D). Because SOCS1 and SOCS3 are important regulators of Th differentiation (Palmer and Restifo, 2009), the elevated SOCS3 expression in SOCS2−/− CD4+ T cells would be consistent with the observation that SOCS3 blocks Th17 development by inhibiting IL-6 and IL-23 responses (Chen et al., 2006) while favoring Th2 differentiation (Seki et al., 2003). It is therefore plausible that the increased levels of SOCS1 and SOCS3 may also affect polarization toward other subsets, indirectly favoring Th2 differentiation.

**DISCUSSION**

Collectively, our findings show that SOCS2−/− mice are predisposed to AD and allergic lung inflammation and developed enhanced type 2 immune responses to helminth antigens. Because the SOCS2 mice are ubiquitous knockouts, other immune cells also lacking SOCS2 could contribute to this phenotype. Of note, we made the observation that the SOCS2-deficient B cells do not spontaneously class-switch toward IgE (unpublished data), which could have explained the predisposition of the SOCS2−/− mice for atopy. Of course, this should be further investigated, together with the effect of SOCS2 deficiency on APC, eosinophils, and mast cells in particular. However, as RAG−/− mice reconstituted with SOCS2-deficient CD4+ T cells developed enhanced pathology compared with those reconstituted with WT (Fig. 1 E and Fig. 3, E and F), these findings suggest that SOCS2 exerts an intrinsic inhibition on CD4+ T cells, which prevents polarization toward Th2 both in vitro and in vivo. Interestingly, and consistent with the normal IFN-γ production we observe, SOCS2−/− mice mount a normal Th1 response to L. major infection (Bullen et al., 2003).

SOCS2 has primarily been studied in relation to the regulation of growth hormone and cytokine signaling and has not previously been thought to influence T cell lineage commitment. CD4+ T cell lineage maturation is governed by the expression of master regulator transcription factors that drive differentiation, but recent evidence suggests that their expression is not as stable as initially thought; indeed, there are now many examples of plasticity in Th cell subsets (Murphy and Stockinger, 2010). T cell plasticity and lineage fate are also governed by the cytokines they encounter after antigen recognition. Therefore it is plausible that the SOCS proteins might play a significant role in Th cell polarization. Indeed, constitutive expression of SOCS3 during CD4+ T cell differentiation facilitates Th2 expansion (Seki et al., 2003; Ozaki et al., 2005), whereas selective deletion of SOCS3 in T cells facilitated enhanced STAT3 activation and increased IL-17 production (Chen et al., 2006). Moreover, recent studies have shown that SOCS3 may play a role in controlling regulatory T cell responses because SOCS3-deficient T cells demonstrate a Th3-like phenotype with increased TGF-β and IL-10 and increased STAT3 activation (Kinjo et al., 2006; Taleb et al., 2009).

In conclusion, therefore, we propose that SOCS2 inhibits Th2 development, and that the enhanced STAT5 and STAT6 phosphorylation in the absence of SOCS2 favors Th2 polarization. Finally, we have defined new functions for SOCS2 in the regulation of allergic inflammation and, potentially, CD4+ T cell differentiation, providing new insights on how the influence of SOCS proteins on T cell plasticity may be exploited for the development of therapies for atopic disorders.

**MATERIALS AND METHODS**

**Mice.** All experiments used 12–16-wk-old sex- and age-matched C57BL/6 and SOCS2−/− mice. SOCS2−/− mice were a gift from D.J. Hilton (Royal Melbourne Hospital, Victoria, Australia) and were generated on a C57BL/6 background as previously described (Metcalfe et al., 2000). C57BL/6 mice were either purchased from Harlan Laboratories or were WT littermates. OT/II mice were subsequently crossed to SOCS2−/− mice in house to generate SOCS2−/− xOT/II mice. Mice were housed under specific pathogen-free conditions. All animal experiments were performed in compliance with either the UK Home Office or the Irish Department of Health and Children regulations and approved either by the Queen’s University Ethical Review Committee or by the Trinity College Bioresources Ethical Review Board.

**T cell culture, purification, and differentiation.** All in vitro assays were performed by using between two and three mice per group. After extraction, splenocytes and lymphocytes were depleted of erythrocytes by lysis with ammonium chloride solution. Cells were cultured at 10⁶/ml in complete...
RIPI medium. Cells were co-cultured with 2 µg/ml (eBioscience) or 0.5 µg/ml (BD) of plate-bound anti-CD3 (145-2C11) and 1 µg/ml (eBioscience) or 4 µg/ml (BD) of plate-bound anti-CD28 (37–51). CD4+ T cells were isolated using positive or negative selection kits (Milteny Biotech). Naïve CD4+CD25−CD44− T cells were pre-enriched using CD4 negative selection kit and sorted to >99% purity with a FACS Aria II (BD) using anti-CD4 (BD), anti-CD25 (eBioscience), and anti-CD44 (BD) antibodies. Naïve cells were polarized toward Th2 with 5 µg/ml of plate-bound anti-CD3 and 5 µg/ml of plate-bound anti-CD28 in the presence of 20 ng/ml of recombinant IL-4 (R&D Systems) and 20 µg/ml of blocking IFN-γ (R&D Systems) as previously described (Watford et al., 2010). Th7 differentiation was driven by stimulating CD4+CD25− T cells with 2 µg/ml of plate-bound anti-CD3 and 1 µg/ml of plate-bound anti-CD28 antibodies in the presence of 1 ng/ml of recombinant human (rh) TGF-β (R&D Systems), 200 U/ml rIL-6 (R&D Systems), and 10 µg/ml of blocking IL-4 and IFN-γ antibodies (R&D Systems) in complete IMDM medium (Invitrogen).

Immunological analysis. For real-time PCR, mRNA was extracted with the RNeasy kit (Qiagen) and converted as first-strand cDNA with the MMLV reverse transcription (Invitrogen). Each PCR was performed on an MX3000P qPCR machine (Agilent Technologies) from 50 ng cDNA using SYBR green technology (Qiagen), and genes were amplified with the following primers (Invitrogen): IL-4 (forward, 5′-ACAGGAGAAGGAGCGCAT-3′; reverse, 5′-GAAGCCCTACAGACGCTA-3′), IL-8 (forward, 5′-AGACAGTGGTAAAGAGACCTT-3′; reverse, 5′-TTCTAATTGCA-TAGCTGGTGTATT-3′), IL-13 (forward, 5′-AGACCAGACTCCCTGTC-GCA-3′; reverse, 5′-TGGTTCTCTGTGATGTCATTTG-3′), GATA3 (forward, 5′-AGGCCAGATGAGAAGAGTGCCT-3′; reverse, 5′-CTC-GACTTACATCGCAACCCGCTA-3′), RORγt (forward, 5′-CCTGGTGA-GAGGGCTTCAC-3′; reverse, 5′-TCAGAGGAGTGGCACCATTACA-3′), SOCS1 (forward, 5′-ACACTCTTGTGAGCCGAC-3′; reverse, 5′-AAGGC- CATCTTCACGCTGACG-3′), SOCS2 (forward, 5′-GGTTGCGGAGG-AACAGTC-3′; reverse, 5′-GAGGCTCTTTAAATTCTCTTGGC-3′), and SOCS3 (forward, 5′-CCTTCAGCTCACAAGGACG-3′; reverse, 5′-GCTCTCCTGCAGCTTCG-3′). Cytokine secretion in cell culture supernatants was analyzed by ELISA (BD or R&D Systems). Total and antigen-specific serum IgE were measured by ELISA, as described previously (Mangan et al., 2007).

Flow cytometry. Surface marker expression was assessed by flow cytometry using anti-CD3 (BD), anti-CD4 (BD), or anti-CD8 (eBioscience). For cytokine staining, cells were restimulated in complete medium at 37°C for 4 h with anti-CD3, anti-CD28, and rIL-2 (R&D Systems) as shown. Cell lysates were immunoprecipitated with antibodies: phospho-STAT6 (Imgenex), phospho-STAT3 (Abcam), and phospho-STAT4 (Invitrogen). Blots were subsequently reprobed with total STAT6, 3, or 2 (Santa Cruz Biotechnology, Inc.). To analyze SOCS2 and SOCS3 expression, total spleenocytes from WT or SOCS2−/− mice were pre-enriched with anti-CD3, anti-CD28, and rIL-2 (R&D Systems) as shown. Cell lysates were immunoprecipitated with antibodies against SOCS3 (IBL International), SOCS2 (Cell Signaling Technology), and γ-tubulin (Sigma-Aldrich). For SOCS3 analysis in CD4+ cells, equal amounts of total protein were immunoprecipitated with anti-SOCS3 (IBL International) that had been precoupled to protein A Sepharose (Amersham Biosciences). Supernatants were analyzed by SDS-PAGE and Western blotting with antibodies against SOCS3 (IBL International), SOCS2 (Cell Signaling Technology), and γ-tubulin (Sigma-Aldrich). Lungs from allergen-sensitized and challenged mice were fixed and taken in 10% formaldehyde-saline for histology (Mangan et al., 2007). Sections of lungs were stained with hematoxylin and eosin for assessment of lung inflammation and Periodic acid-Schiff stained for the enumeration of goblet cells and mucus production.

OT/II CD4+ cell transfer model of allergic airway inflammation. Spleen and LNs were harvested from OT/II or SOCS2−/−xOT/II mice. CD4+ T cells were enriched from single cell suspension by negative selection using the EasySep mouse CD4+ T cell enrichment kit (STEMCELL Technologies). Enriched CD4+ T cells were subsequently sorted to >99% purity with a MoFlo (Beckman Coulter) using anti-CD4 (BD) antibodies. A total of 1.5 × 106 purified CD4+ cells isolated from OT/II or SOCS2−/−xOT/II mice were injected i.v. into Rag-1−/− recipient mice. Control mice received PBS. Rag-1−/− mice were administered 50 µg OVA adsorbed onto alum (Thermo Fisher Scientific) i.p. on days 1 and 8 and were subjected to airway exposure with 1% OVA aerosol in PBS for 20 min on days 27–31. 24 h after the last OVA aerosol challenge, lung function was analyzed by an invasive method wherein total STAXchemostome was used and ventilated using a whole-body plethysmography with a pneumotachograph linked to a transducer (EMMS) to determine changes in BR in response to increasing doses of inhaled methacholine (10, 30, 60, and 100 mg/ml; Sigma-Aldrich). Lungs from allergen-sensitized and challenged mice were harvested on days 9 and 14 and subjected to airway exposure with 1% OVA in PBS for 20 min on days 27–31. 24 h after the last OVA aerosol challenge, lung function and immunological analysis was performed as described in the previous section. Lungs were homogenized and cytokines determined (expressed as picogram of cytokine per milligram of lung protein) as previously described (Mangan et al., 2007).

Immunoblotting and immunoprecipitation. CD4+ cells were restimulated with anti-CD3 and anti-CD28 for 48 h before activation with rIL-4 (R&D Systems), rIL-12 (PeproTech), or rIL-6 (PeproTech) as indicated. Cells lysates were analyzed by Western blotting as previously described (Tannahill et al., 2005). Phosphorylated STAT proteins were detected using the following antibodies: phospho-pho-STAT6 (Imgenex), phospho-STAT3 (Abcam), and phospho-STAT5 (Invitrogen). Blots were subsequently reprobed with total STAT6, 3, or 4 (Santa Cruz Biotechnology, Inc.). To analyze SOCS2 and SOCS3 expression, total spleenocytes from WT or SOCS2−/− mice were pre-enriched with anti-CD3, anti-CD28, and rIL-2 (R&D Systems) as shown. Cell lysates were immunoprecipitated with antibodies against SOCS3 (IBL International), SOCS2 (Cell Signaling Technology), and γ-tubulin (Sigma-Aldrich). For SOCS3 analysis in CD4+ cells, equal amounts of total protein were immunoprecipitated with anti-SOCS3 (IBL International) that had been precoupled to protein A Sepharose beads. SOCS3 protein was subsequently analyzed by Western blotting as previously described (Tannahill et al., 2005). Densitometry was performed using ImageJ software (National Institutes of Health).

Statistical analysis. Data were analyzed with Prism4 (GraphPad Software). Statistical differences were determined by two-tailed unpaired or paired Student’s t test as appropriate.

Liquid chromatography/mass spectrometry (LC/MS).
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Online supplemental material. Fig. S1 shows CD4+ and CD8+ numbers in thymus, spleen, and LN of WT and SOCS2−/− mice, increased IL-4, IL-5, IL-13, and GATA-3 mRNA expression in SOCS2-deficient CD4+ T cells by real-time PCR, and dot plot showing enhanced Th2 differentiation in the absence of SOCS2. Fig. S2 shows lung histology, cell infiltration in BAL, and IL-4, IFN-γ, and IL-17 concentration in BAL of RAG-1−/− mice reconstituted with PBS, WT, or SOCS2−/− CD4+ T cells after OVA challenge. Fig. S3 shows increased STAT6 and STAT5 phosphorylation and reduced STAT3 phosphorylation in SOCS2-deficient CD4+ T cells by flow cytometry, unaffected IL-4Rα levels, SOCS2 induction in CD4+ T cells after IL-6 and TGF-β stimulation, and reduced RORγt and IL-17 levels in SOCS2 deficient CD4+ T cells polarized toward Th17 lineage. Fig. S4 shows that in the absence of SOCS2, SOCS2 and SOCS1 levels are elevated in splenocytes, SOCS3 expression is higher in freshly isolated and Th2-polarized CD4+ T cells, and SOCS1 and SOCS3 mRNA expression is higher in CD4+ cells stimulated with anti-CD3 and anti-CD28. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20101167/DC1.

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