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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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Abbreviations used: AD, atopic dermatitis; AHR, airway hyperresponsiveness; R_L, lung resistance; SEA, schistosome soluble egg antigen; SOCS, suppressor of cytokine signaling.

Allergic diseases such as atopic dermatitis (AD) and asthma are orchestrated and enhanced by CD4+ Th2 lymphocytes and the cytokines they produce, namely IL-4, IL-5, and IL-13. These type two cytokines drive elevated serum IgE and infiltration of Th2 cells, eosinophils, and basophils, which have been observed in patients with asthma and AD (Venge et al., 1996; Sudowe et al., 2000; Walter et al., 2001; Nakajima and Takatsu, 2007). IL-4 and IL-13 mediate their responses by activating JAK kinases and STAT6, a transcription factor responsible for activating many allergy-associated genes (Kato and Schleimer, 2007).

The suppressor of cytokine signaling (SOCS) family of proteins is a key controller of cytokine responses, which can down-regulate specific cytokine signals and consequently modify the immune response (Alexander, 2002; Croker et al., 2008; Dimitriou et al., 2008). SOCS1 and SOCS3 have been shown to influence the Th1/Th2 balance (Kubo and Inoue, 2006; Yoshimura et al., 2007; Palmer and Restifo, 2009). In particular, SOCS3 expression correlates with the severity of asthma and AD, as well as serum IgE levels in patients with allergy (Seki et al., 2003). Although SOCS2 is expressed in activated CD4+ T cells and its expression can regulate IL-2 and IL-3 signals (Tannahill et al., 2005), no role for SOCS2 in CD4+ T cell differentiation has yet been defined.

In this paper, we demonstrate that activated SOCS2$^{-/-}$ CD4+ T cells show significantly higher IL-4, IL-5, and IL-13 production and Th2 differentiation and that they had elevated SOCS3- and IL-4–induced STAT6 but blunted IL-6–induced STAT3 phosphorylation. Moreover, SOCS2$^{-/-}$ mice were highly susceptible to AD with a marked eosinophilic infiltrate and, after helminth antigen challenge, produced

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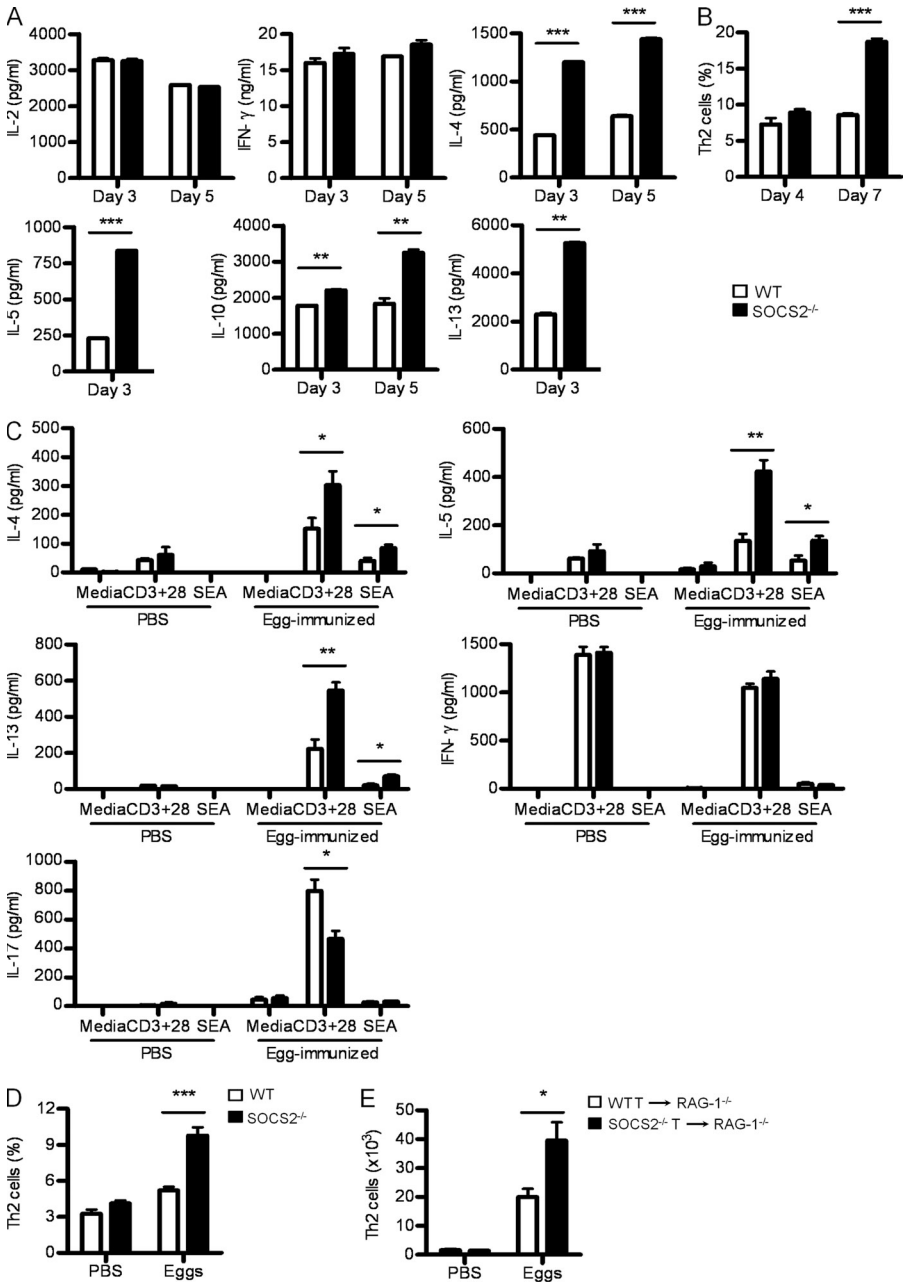

Figure 1. Increased secretion of Th2-associated cytokines in SOCS2$^{-/-}$ mice in vitro and in vivo. (A) CD4$^+$CD25$^-$ splenocytes purified from WT and SOCS2$^{-/-}$ mice were cultured for 3 or 5 d with 2 µg/ml of plate-bound anti-CD3, 1 µg/ml of plate-bound anti-CD28, and 5 U/ml IL-2 in triplicate and cytokine production was evaluated by ELISA. Data are representative of at least three independent experiments. (B) Naive CD4$^+$CD25$^-$CD44$^-$ splenocytes were cultured for 7 d under Th2 polarization conditions. Frequency of CD4$^+$IL-4$^+$ cells (Th2 cells) was evaluated at days 4 and 7 by intracellular staining. Data are representative of three independent experiments performed in triplicate. (C) SOCS2$^{-/-}$ and WT mice (n = 5 per group) were i.p. injected with 10,000 *S. mansoni* eggs or PBS. 10 d later, splenocytes were restimulated with anti-CD3 and anti-CD28 or SEA and cytokine production was measured by ELISA. (D and E) The footpads of WT and SOCS2$^{-/-}$ mice (n = 5 per group; D) or RAG-1$^{-/-}$ mice reconstituted with CD4$^+$ T cells sorted from WT (n = 4 per reconstituted group; E) or SOCS2$^{-/-}$ mice (n = 7 per reconstituted group; E) were injected with 5,000 *S. mansoni* eggs or PBS. 10 d later, the amount of Th2 cells in popliteal LNs was determinate by IL-4 intracellular staining (D) or T1/ST2 (IL-33 receptor) staining (E). Data are representative of at least three experiments and presented as the mean and SEM of each group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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$^-$CD44^{low} T cells and drove de

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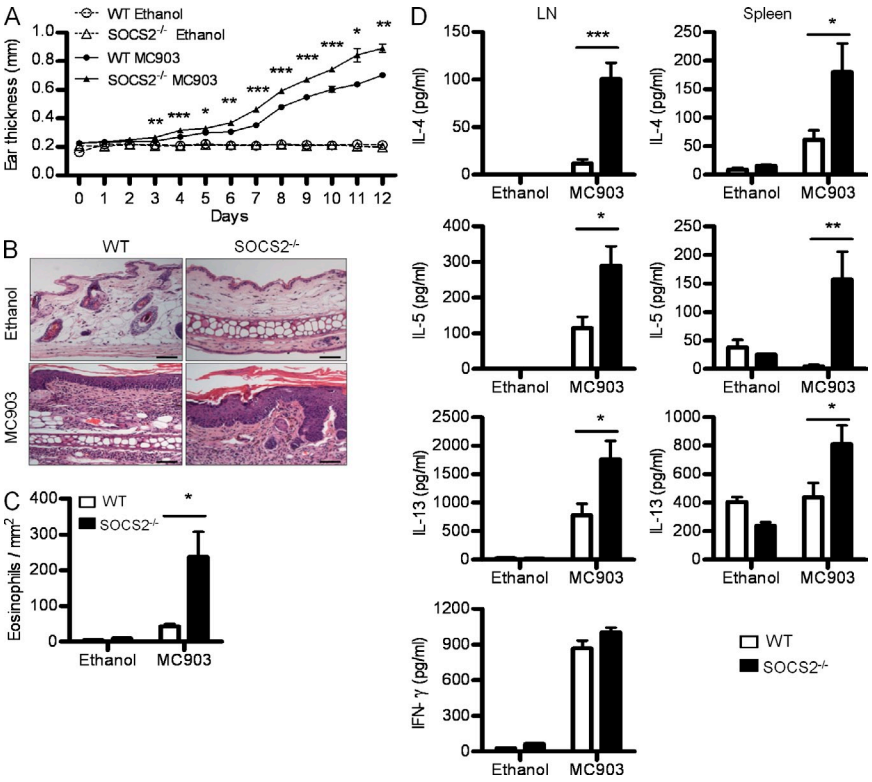

Figure 2. SOCS2$^{-/-}$ mice develop more severe AD than WT mice. (A) Calcipotriol (MC903) or ethanol was applied daily to the right ear of WT and SOCS2$^{-/-}$ mice (n = 6 per group) and ear thickness was measured daily. (B) Representative pictures of H&E-stained ear sections from WT and SOCS2$^{-/-}$ mice at the end of study (bars, 150 µm). (C) Quantitation of ear eosinophil infiltrates from 10 sections randomly selected out of one representative mouse per group. (D) Three mice per group were sacrificed at day 5, and auricular draining LN and spleen cells were isolated and restimulated in triplicate for 48 h with anti-CD3 and anti-CD28. Secreted cytokines were then measured by ELISA. Data are representative of three independent experiments and are presented as mean and SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

novo Th2 differentiation in vitro. As shown in Fig. 1 B and Fig. S1 D, in naive CD4$^+$ cells the absence of SOCS2 resulted in a significantly higher frequency of Th2 cells (CD4$^+$ IL-4$^+$ cells; P < 0.001), thus indicating that SOCS2 must control polarization toward the Th2 lineage.

Because parasitic helminth *Schistosoma mansoni* eggs induce a potent Th2 response (Fallon and Mangan, 2007), we used this model to compare Th2 responses in vivo in SOCS2$^{-/-}$ mice relative to WT animals. SOCS2$^{-/-}$ and WT mice were injected intraperitoneally with 10,000 *S. mansoni* eggs and, 10 d later, splenocytes were restimulated with anti-CD3/CD28 or with schistosome soluble egg antigen (SEA). Both approaches induced marked type 2 responses in WT mice relative to PBS-challenged mice (Fig. 1 C). Moreover, there was significantly (P < 0.05) greater production of the type 2 cytokines IL-4, IL-5, and IL-13 by splenic T cells from egg-injected SOCS2$^{-/-}$ mice relative to WT mice after both nonspecific and antigen-specific TCR stimulation (Fig. 1 C). Interestingly, splenocytes from egg-injected SOCS2$^{-/-}$ mice also produced significantly lower amounts of IL-17 compared with WT mice (Fig. 1 C). To specifically enumerate the in vivo expansion of Th2 cells, *S. mansoni* eggs were injected into the footpads of SOCS2$^{-/-}$ and WT mice. After 10 d, the frequency of Th2 (CD4$^+$ IL-4$^+$) cells in the popliteal LN was significantly higher (P < 0.001) in SOCS2$^{-/-}$ mice than in WT animals (Fig. 1 D). To confirm that SOCS2 deficiency in CD4$^+$ T cells was directly responsible for the observed Th2 bias, naive SOCS2$^{-/-}$ or WT CD4$^+$ T cells were transferred into 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 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.

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.

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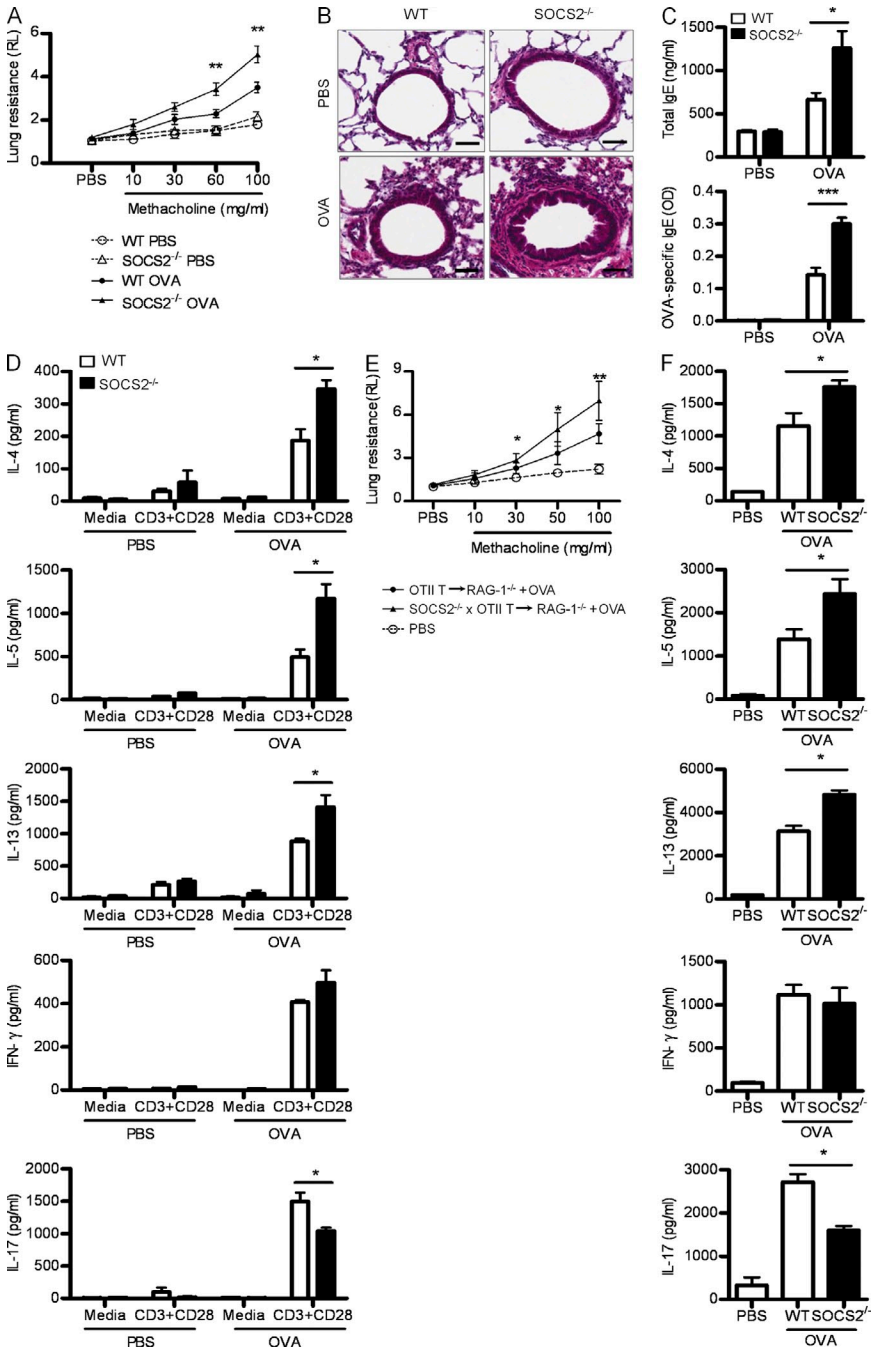

Figure 3. Increased allergic lung inflammation in SOCS2$^{-/-}$ mice. (A) Methacholine-induced R_L in PBS or OVA-sensitized SOCS2$^{-/-}$ and WT mice (n = 7 mice per group). (B) Representative image of lung sections from each group stained with periodic acid-Schiff stain (bars, 50 µm). (C) Levels of total and OVA-specific IgE in serum of mice from each group. (D) Cells from mediastinal LN were restimulated in triplicates with media or anti-CD3 and anti-CD28 and cytokine secretion was measured by ELISA. (E and F) RAG-1$^{-/-}$ mice were reconstituted with PBS as control or with sorted CD4$^+$ T cells isolated from OTII or SOCS2$^{-/-}$ OTII mice. The number of mice per group of reconstituted RAG-1$^{-/-}$ mice was between five and nine. The mice were then sensitized and challenged with OVA and methacholine-induced R_L 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. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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 (R_L), was not induced in PBS-treated SOCS2$^{-/-}$ mice, with these mice having R_L 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 R_L, 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$^{-/-}$xOT/II mice. RAG-1$^{-/-}$ mice were reconstituted either with CD4$^+$ T cells from SOCS2$^{-/-}$xOT/II or OT/II mice. Recipients were then sensitized and challenged as previously described

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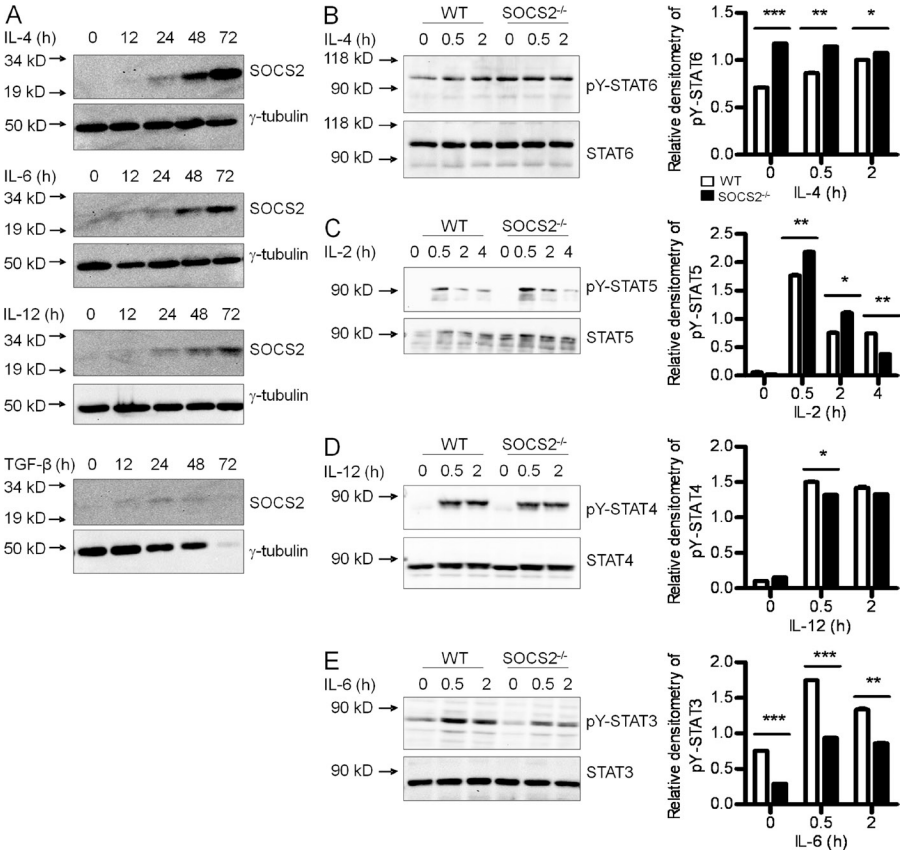

Figure 4. SOCS2 deficiency results in constitutive STAT6 activation, enhanced IL-2–mediated STAT5 activation, and reduced IL-6–mediated STAT3 activation. (A) CD4+ splenocytes from WT mice were cultured with anti-CD3 and anti-CD28 in the presence of 20 ng/ml IL-4, 200 U/ml IL-6, 20 ng/ml IL-12, or 5 ng/ml 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.

with OVA and AHR evaluated. RAG-1$^{-/-}$ animals that received the SOCS2$^{-/-}$xOT/II cells had significantly higher R_L responses than 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 T 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). 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-2–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),

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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. STAT5 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; Piessevaux 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 antigen. 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-$1^{-/-}$ 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 elevated 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 (Kinjyo 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 (Metcalf et al., 2000). C57BL/6 mice were either purchased from Harlan Laboratories or were WT littermates. RAG-$1^{-/-}$ and OT/II strain mice on a C57BL/6 background were purchased from The Jackson Laboratory. 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 10^6/ml in complete

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RPMI medium. Cells were activated 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 (Miltenyi Biotec). Naive $CD4^+CD25^-CD44^{low}$ T cells were preenriched using CD4 negative selection kit and sorted to >99% purity with a FACSAria II (BD) using anti-CD4 (BD), anti-CD25 (eBioscience), and anti-CD44 (BD) antibodies. Naive 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). Th17 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 rhIL-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′-ACAGGAGAAGGGACGCCAT-3′; reverse, 5′-GAAGCCCTACAGACGAGCTCA-3′), IL-5 (forward, 5′-AGCACAGTGGTGAAAGAGACCTT-3′; reverse, 5′-TTCAATGCATAGCTGGTGATTT-3′), IL-13 (forward, 5′-AGACCAGACTCCCCTGTGCA-3′; reverse, 5′-TGGGTCCTGTAGATGGCATTG-3′), GATA3 (forward, 5′-AGGCAAGATGAGAAAGAGTGCCTC-3′; reverse, 5′-CTCGACTTACATCCGAACCCGGTA-3′), RORγt (forward, 5′-CCGCTGAGAGGGCTTCAC-3′; reverse, 5′-TGCAGGAGTAGGCCACATTACA-3′), SOCS1 (forward, 5′-ACCTTCTTGGTGCGCGAC-3′; reverse, 5′-AAGCCATCTTCACGCTGAGC-3′), SOCS2 (forward, 5′-GGTTGCCGGAGGAACAGTC-3′; reverse, 5′-GAGCCTCTTTTAATTTCTCTTTGGC-3′), and SOCS3 (forward, 5′-CCTTCAGCTCCAAAAGCGAG-3′; reverse, 5′-GCTCTCCTGCAGCTTGCG-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 50 ng/ml PMA (Sigma-Aldrich), 500 ng/ml ionomycin (Sigma-Aldrich), and GolgiPlug (BD) or for 6 h with 2.5 μg/ml Concanavalin A (Sigma-Aldrich) and 10 μg/ml Brefeldin A (Sigma-Aldrich). Cells were fixed and permeabilized using the Cytofix/Cytoperm kit (BD), followed by intracellular anti–IL-4, anti–IFN-γ, or anti–IL-17 (BD or eBioscience) staining. For phospho-STAT analysis, the cells were fixed after stimulation, permeabilized with the Perm III buffer (BD), and stained with antibodies specific for phospho-STAT5, phospho-STAT3, and phospho-STAT6 according to the Phosflow (BD) instructions. Data were collected on a CyAn flow cytometer (Beckman Coulter) or on BD FACSCanto II (BD) and analyzed using FlowJo software (Tree Star).

Helminth egg injections. Eggs from *S. mansoni*–infected mice were obtained as described previously (Smith et al., 2009). SEA at <1 endotoxin unit/mg (Chromogenic LAL; BioWhittaker) was prepared as described previously (Smith et al., 2009). Mice were given an i.p injection of either 10,000 *S. mansoni* eggs or PBS. For the adoptive transfer experiments, $RAG\text{-}1^{-/-}$ mice were reconstituted with $CD4^+$ sorted from WT or $SOCS2^{-/-}$ mice before injection. In separate experiments, 5,000 *S. mansoni* eggs or PBS were injected into the footpad of mice to examine local cellular responses to SEA. Mice were culled and spleens or popliteal LNs removed 10 d after injection.

AD. Calcipotriol (MC903; Sigma-Aldrich) was resuspended in ethanol at 50 μM. A total of 1 nM was applied daily to the outer and inner surfaces of the right ear (20 μl/ear) as described previously (Li et al., 2006; Elentner et al., 2009). Ethanol (20 μl/ear) was applied as a vehicle control. Ear thickness was monitored daily using digital calipers (Kroeplin). Spleens and auricular draining LNs of the MC903 or vehicle-treated mice were extracted at day 5 and at the end of the study. The cells were restimulated with anti-CD3 and anti-CD28 and the supernatants harvested after 48 h. MC903 or vehicle-treated ear samples were formalin fixed, embedded in paraffin, and stained with hematoxylin and eosin. 10 fields were randomly defined throughout the tissue and counted blindly by two people using a graticule at 400×. The number of eosinophils per square millimeter of tissue was then calculated.

Experimental allergic airway inflammation. Mice were sensitized with OVA to induce allergic airway inflammation in a protocol optimized for mice on C57BL/6J strain background, as described previously (Fallon et al., 2009). Mice were administered 50 μg OVA in alum (Thermo Fisher Scientific) i.p. on days 0 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 was analyzed by an invasive method where mice were tracheostomized and ventilated using a whole-body plethysmography with a pneumotachograph linked to a transducer (EMMS) to determine changes in R_L 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 taken and fixed 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 and $SOCS2^{-/-}$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 1.5×10^7 purified $CD4^+$ cells isolated from OT/II or $SOCS2^{-/-}$xOT/II mice were injected i.v. into $RAG\text{-}1^{-/-}$ recipient mice. Control mice received PBS. $RAG\text{-}1^{-/-}$ 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 9–13. 24 h after the last OVA aerosol challenge, lung function and immunological analysis was performed as described in the previous section. Lavage was performed and cytokines were detected in BAL fluid as 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 prestimulated 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-STAT6 (Imgenex), phospho-STAT3 (Abcam), and phospho-STAT4 (Invitrogen). Blots were subsequently reprobed with total STAT6, 3, or 4 (Santa Cruz Biotechnology, Inc.). To analyze SOCS2 and SOCS3 expression, total splenocytes from WT or $SOCS2^{-/-}$ mice were prestimulated with anti-CD3, anti-CD28, and rIL-2 (R&D Systems) as shown. Cell lysates were immunoblotted 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.

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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 $RAG\text{-}1^{-/-}$ 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, SOCS3 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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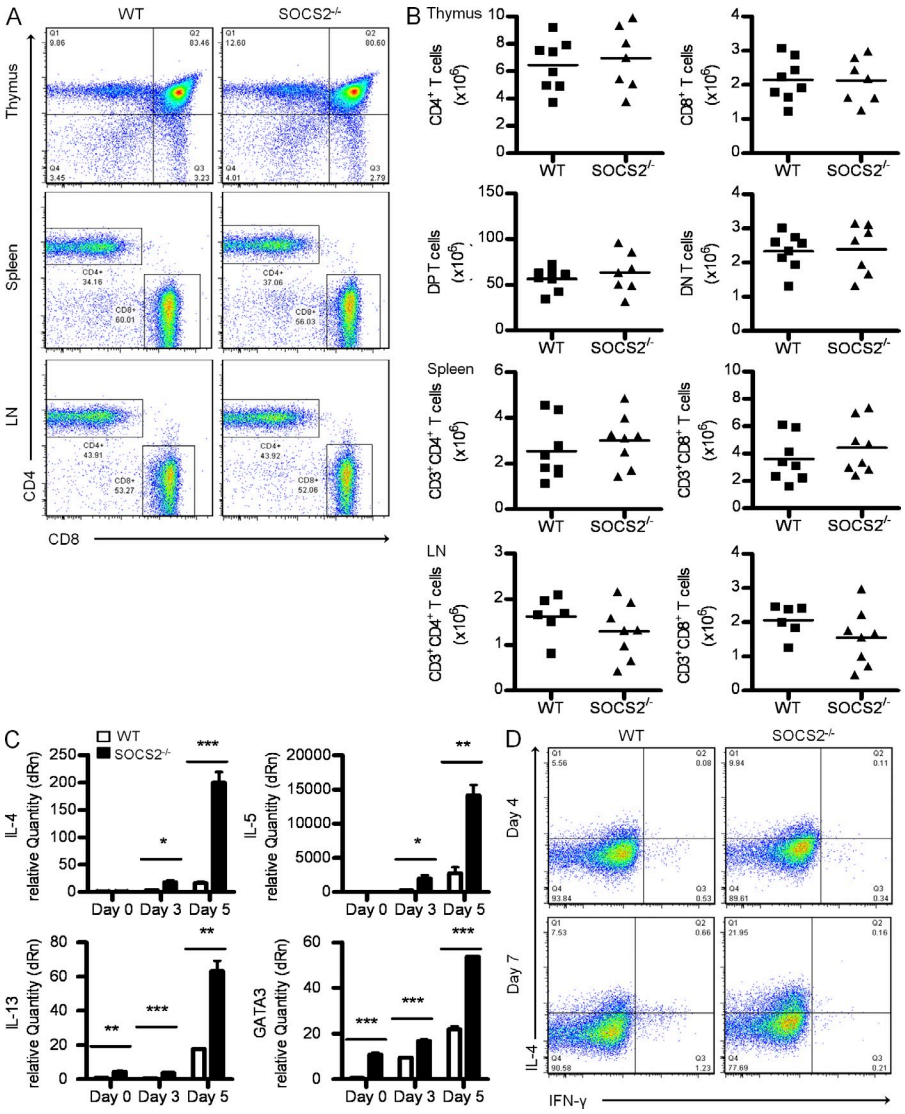

Figure S1. SOCS2 deficiency has no effect on CD4⁺ and CD8⁺ T cells development but is associated with increased Th2-associated cytokine secretion in vitro. (A and B) Lymphocytes were extracted from thymus, spleen, and axillary, brachial, and inguinal LN of WT (n = 6 per group) and SOCS2$^{-/-}$ (n = 8 per group) mice and stained for flow cytometry analysis of CD3, CD4, and CD8 markers. (A) Representative dot plots of CD4⁺ and CD8⁺ populations gated on total (thymus) or CD3⁺ (spleen and LN) lymphocytes. Numbers in quadrants indicate percentage of cells. (B) Absolute numbers of CD4⁺ and CD8⁺ populations in thymus, spleen, and LN. DP, double positive (CD4⁺CD8⁺ T cells); DN, double negative (CD4⁻CD8⁻ T cells). The horizontal bars represent the mean. (C) CD4⁺CD25⁻ splenocytes were purified from WT and SOCS2$^{-/-}$ mice, cultured as described in Fig. 1 A, and cytokine production was evaluated by real-time PCR. The results represent the mean of triplicates of fluorescent intensity relative to β-actin and unstimulated control. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (D) Representative dot plot from triplicates of naive T cells stimulated under Th2 polarizing conditions and intracellularly stained for IL-4 and IFN-γ at days 4 and 7. Data are representative of at least three independent experiments.

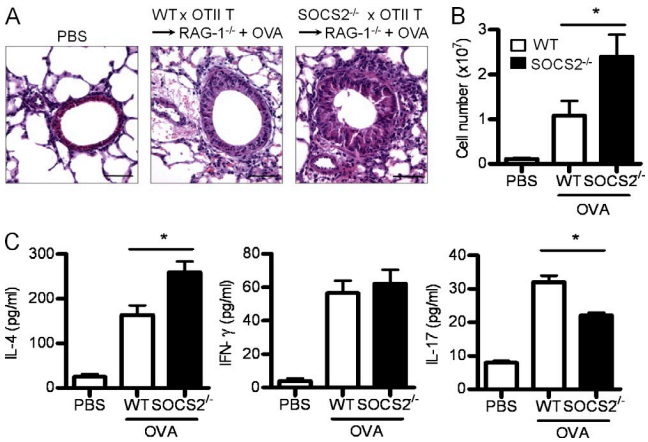

Figure S2. Increased allergic lung inflammation in RAG-1$^{-/-}$ mice reconstituted with SOCS2$^{-/-}$xOT/II CD4^{+} T cells. RAG-1$^{-/-}$ mice were reconstituted with PBS or CD4^{+} T cells isolated from OT/II or SOCS2$^{-/-}$xOT/II mice and challenged with ovalbumin as described in Materials and methods. The number of mice per group of reconstituted RAG1$^{-/-}$ mice was between five and nine. 24 h after the final OVA challenge, lung histology and BAL fluid composition were examined. (A) Representative image of lung sections from each group stained with PAS (bars, 50 µm). (B) Cell infiltration was measured by counting the cells present in the BAL fluid. (C) The cytokine concentration in the BAL fluid was measured by ELISA. Data shown are representative of three separate experiments. Error bars represent mean ± SEM. *, $P < 0.05$.

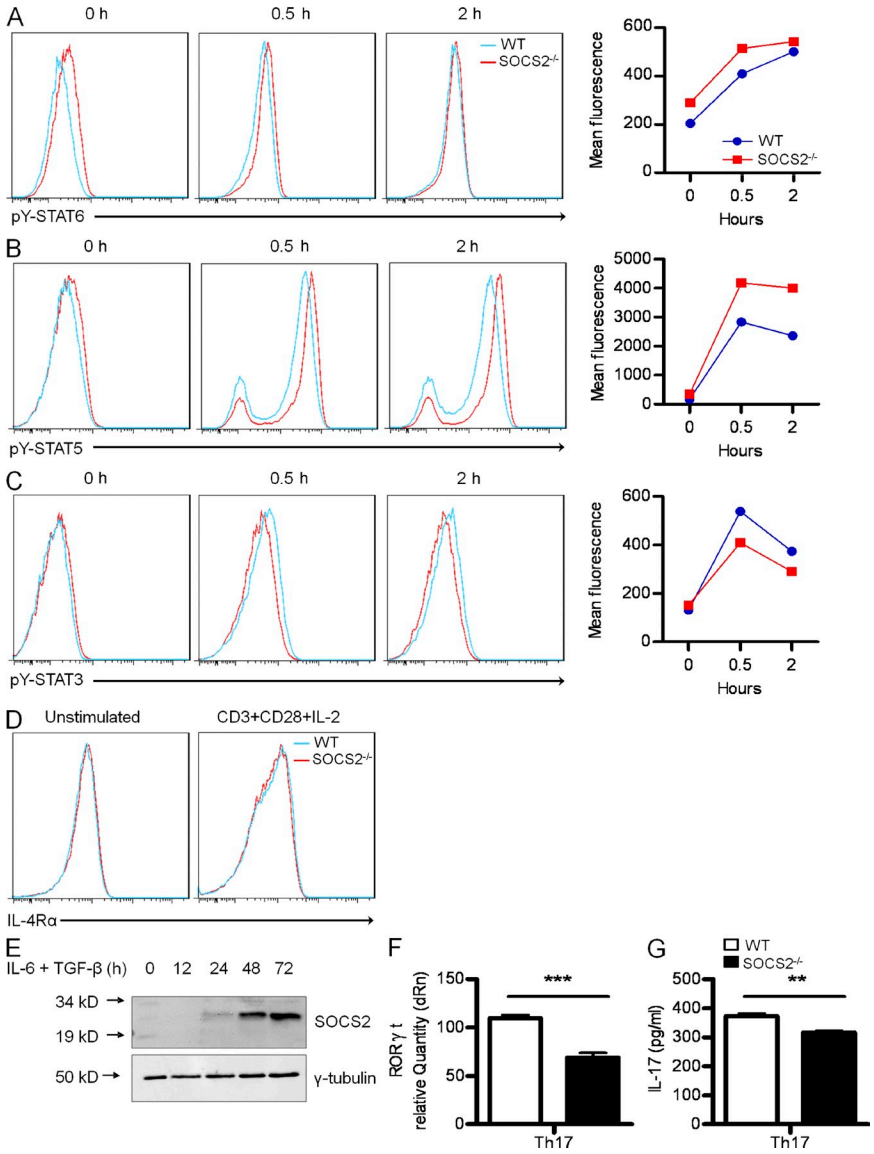

Figure S3. SOCS2 deficiency affects STAT5, STAT6, and STAT3 phosphorylation levels and IL-17 secretion in vitro. The effect of SOCS2 deficiency on phospho-STAT protein was examined by intracellular staining. (A) $CD4^+CD25^-$ T cells were sorted from WT and $SOCS2^{-/-}$ mice and cultured 48 h with 2 μg/ml of plate-bound anti-CD3 and 1 μg/ml of plate-bound anti-CD28. The cells were then restimulated with 200 ng/ml IL-4 for the indicated times, fixed, permeabilized, and stained with anti–phospho-STAT6 antibody. (B) $CD4^+CD25^-$ T cells were cultured as in A but with 3 ng/ml of exogenous TGF-β. Cells were then rested for 2 h in serum-free media before restimulation with 100 U/ml IL-2 for the indicated times. The cells were then stained as previously described with anti–phospho-STAT5 antibody. (C) $CD4^+CD25^-$ T cells were stimulated as in A, rested for 2 h in serum-free media, reactivated with 100 U/ml IL-6 for the indicated times, and intracellularly stained with anti–phospho-STAT3 antibody. On the left, the overlays of the indicated STAT phosphorylation at the different time points for both WT and $SOCS2^{-/-}$ T cells are presented. The mean fluorescence is presented on the right. (D) IL-4Rα expression was examined by flow cytometry, on freshly isolated splenocytes (left), or on splenocytes stimulated 4 h with 2 μg/ml of plate-bound anti-CD3, 1 μg/ml of plate-bound anti-CD28, and 100 U/ml IL-2. These experiments were performed three times. (E) $CD4^+$ T cells were purified from WT mice and stimulated with 2 μg/ml of plate-bound anti-CD3, 1 μg/ml of plate-bound anti-CD28, 200 U/ml IL-6, and 3 ng/ml TGF-β for the indicated times, and SOCS2 and γ-tubulin expression was examined by immunoblotting. (F and G) $CD4^+CD25^-$ T cells sorted from WT and $SOCS2^{-/-}$ mice were stimulated under Th17 polarization conditions in triplicates. (F) Cellular mRNA was then extracted and the expression of RORγt was evaluated by real-time PCR. (G) IL-17 concentration in the cell culture supernatant was measured by ELISA. These experiments were performed three times. Error bars represent mean ± SEM. **, $P < 0.01$; ***, $P < 0.001$.

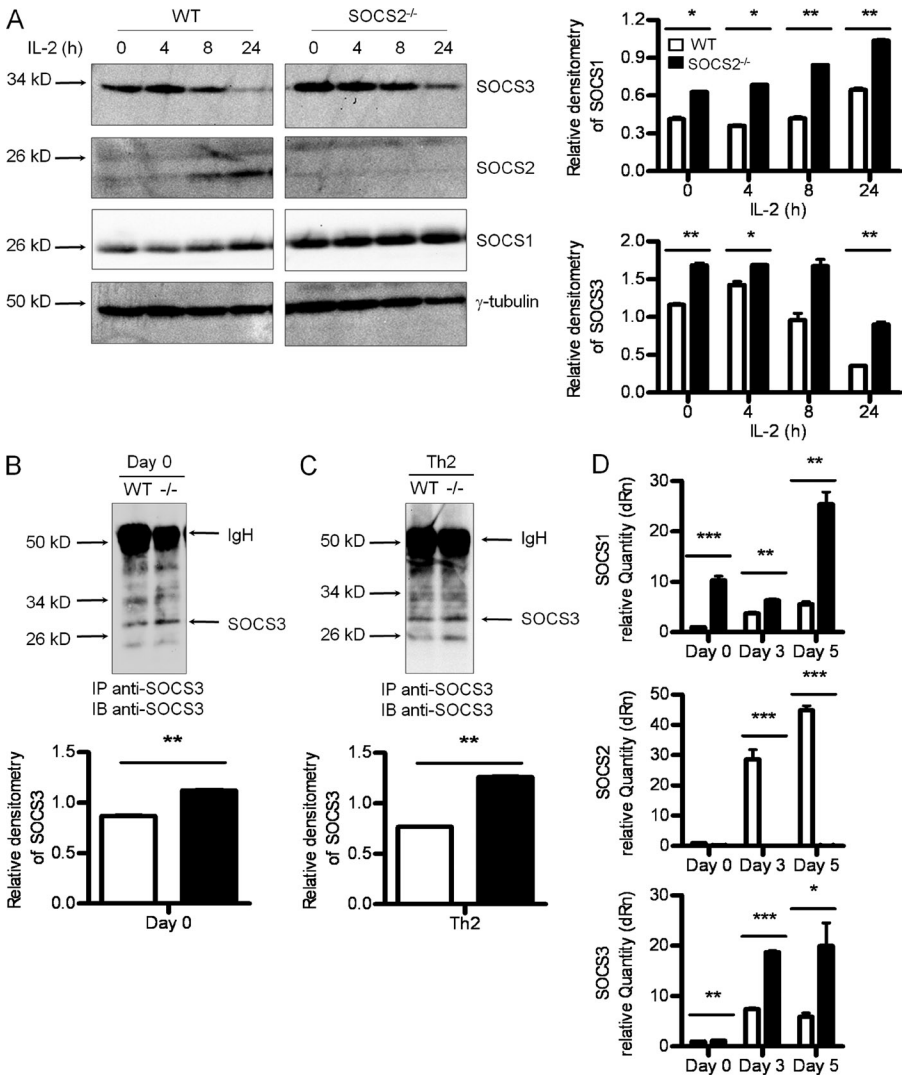

Figure S4. SOCS3 and SOCS1 expression levels are higher in the absence of SOCS2. (A) Splenocytes were isolated from WT and SOCS2$^{-/-}$ mice and stimulated with 2 µg/ml of plate-bound anti-CD3, 1 µg/ml of plate-bound anti-CD28, and 50 U/ml IL-2 for the indicated times. At each time point, cells were lysed and SOCS1, SOCS2, SOCS3, and γ-tubulin expression was examined by immunoblotting. Densitometry of SOCS1 and SOCS3 proteins relative to γ-tubulin is shown on the right. (B and C) SOCS3 expression in freshly isolated CD4$^+$ T cells (B) or after 7 d under Th2 polarization (C). Cell lysates were immunoprecipitated and blotted for SOCS3 expression. Densitometry of SOCS3 relative to background is shown on the bottom. Data are mean and SEM from triplicates. *, $P < 0.05$; **, $P < 0.01$. Data are representative of three independent experiments. (D) CD4$^+$CD25$^-$ T cells were sorted from WT and SOCS2$^{-/-}$ mice and stimulated for the indicated times with 2 µg/ml of plate-bound anti-CD3 and 1 µg/ml of plate-bound anti-CD28 in the presence of 5 U/ml IL-2 in triplicates. Cell mRNA was extracted and the expression of SOCS1, SOCS2, and SOCS3 was measured by real-time PCR. The results represent the mean fluorescent intensity relative to β-actin and unstimulated control. Error bars represent mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.