

The signaling suppressor CIS controls proallergic T cell development and allergic airway inflammation

Xuexian O Yang^{1,2}, Huiyuan Zhang^{1,9}, Byung-Seok Kim^{1,9}, Xiaoyin Niu^{1,3}, Juan Peng^{1,4}, Yuhong Chen⁵, Romica Kerketta², Young-Hee Lee¹, Seon Hee Chang¹, David B Corry^{6,7}, Demin Wang⁵, Stephanie S Watowich¹ & Chen Dong^{1,8}

Transcription factors of the STAT family are critical in the cytokine-mediated functional differentiation of CD4⁺ helper T cells. Signaling inhibitors of the SOCS family negatively regulate the activation of STAT proteins; however, their roles in the differentiation and function of helper T cells are not well understood. Here we found that the SOCS protein CIS, which was substantially induced by interleukin 4 (IL-4), negatively regulated the activation of STAT3, STAT5 and STAT6 in T cells. CIS-deficient mice spontaneously developed airway inflammation, and CIS deficiency in T cells led to greater susceptibility to experimental allergic asthma. CIS-deficient T cells showed enhanced differentiation into the T_H2 and T_H9 subsets of helper T cells. STAT5 and STAT6 regulated IL-9 expression by directly binding to the *IL9* promoter. Our data thus demonstrate a critical role for CIS in controlling the proallergic generation of helper T cells.

CD4⁺ helper T cells are the central organizers of adaptive immunity and various immunological diseases. After recognizing antigens, naive precursors of CD4⁺ T cells undergo clonal expansion and functional differentiation into cytokine-secreting effector cells. The effector differentiation of cells into the T_H1, T_H2 and T_H17 and T_H9 subsets of helper T cells is determined by the cytokine environment^{1,2}. T_H1 differentiation is promoted by interleukin 12 (IL-12), which signals through the intracellular signaling molecule STAT4. The interferon- γ (IFN- γ)-STAT1 pathway in turn sustains T_H1 development, which leads to induction of expression of the transcription factor T-bet. During T_H2 polarization, IL-4 serves an essential role by activating STAT6, and phosphorylated STAT6 in turn induces expression of the transcription factor GATA-3 (ref. 3). In addition to IL-4, IL-2 regulates T_H2 differentiation by STAT5-mediated induction of the α -chain of the receptor for IL-4 (IL-4R α), the main STAT6-activating component of the IL-4 and IL-13 receptors^{4–6}. T_H17 differentiation is mediated by a combination of signaling via IL-6 and transforming growth factor- β (TGF- β)⁷. IL-6, as well as IL-21 and IL-23, supports T_H17 development via STAT3 (ref. 7), whereas IL-2-STAT5 signaling negatively regulates T_H17 differentiation⁸. IL-9-expressing T_H9 cells also require TGF- β , in addition to IL-4, for their generation^{9–11}; the mechanism for this remains unclear.

Thus, cytokine-mediated activation of STAT (signal transducer and activator of transcription) proteins has a pivotal role in the differentiation of helper T cells. Moreover, overactivation of STAT

proteins may lead to excessive immune responses and tissue damage. Thus, signaling from STAT proteins is controlled by several negative regulatory mechanisms^{12–14}. Among those, members of the SOCS (suppressor of cytokine signaling) family, induced by cytokine stimulation, inhibit STAT signaling^{12–14}. For example, genetic deletion of SOCS1 in mice results in greater responsiveness to IFN- γ and causes a fatal inflammatory disease¹⁵. The eight members of the SOCS family (CIS ('cytokine-induced SH-2 protein'; also called CIS1, SOCS or CISH)¹⁶ and SOCS1–SOCS7) share a common structure, with a central Src-homology 2 (SH2) domain, a carboxyl-terminal SOCS box and a divergent amino-terminal domain. The SOCS box interacts with ubiquitination enzymes, such as elongin B, elongin C, cullin-5 and RING box 2, and an E2 ubiquitin transferase. The central SH2 domain determines the interaction with target proteins, such as kinases of the Jak family, which brings them close to the ubiquitinating scaffold associated with the SOCS box. SOCS-mediated ubiquitination of the target proteins leads to rapid degradation via the proteasome and inhibition of cytokine signaling.

As negative regulators of STAT proteins, some members of the SOCS family are involved in the differentiation of helper T cells. SOCS1 negatively regulates T_H1 differentiation through inhibition of the IFN- γ -STAT1 and IL-12-STAT4 pathways^{17–20}. SOCS3 is 'preferentially' expressed in T_H2 cells²¹, and forced expression of SOCS3 in T cells promotes a T_H2 phenotype by blocking STAT4 signaling^{22–24}. However, conditional removal of SOCS3 in T cells seems

¹Department of Immunology and Center for Inflammation and Cancer, MD Anderson Cancer Center, Houston, Texas, USA. ²Department of Molecular Genetics and Microbiology, University of New Mexico School of Medicine, Albuquerque, New Mexico, USA. ³Shanghai Jiao Tong University School of Medicine and Shanghai Institute of Immunology, Shanghai, China. ⁴College of Basic Medicine, Wuhan University, Wuhan, China. ⁵Blood Research Institute, Blood Center of Wisconsin, Milwaukee, Wisconsin, USA. ⁶Department of Medicine, Baylor College of Medicine, Houston, Texas, USA. ⁷Department of Pathology and Immunology, Baylor College of Medicine, Houston, Texas, USA. ⁸Tsinghua University School of Medicine, Beijing, China. ⁹These authors contributed equally to this work. Correspondence should be addressed to C.D. (cdong@mdanderson.org) or X.O.Y. (xyang@salud.unm.edu).

Received 15 February; accepted 30 April; published online 2 June 2013; doi:10.1038/ni.2633

to suppress both T_H1 responses and T_H2 responses, an unexpectedly broad effect that may indirectly result from greater secretion of the immunosuppressive cytokines TGF- β and IL-10 (ref. 25). In addition, SOCS3 blocks STAT3 signaling and inhibits T_H17 polarization^{26,27}. SOCS5 is 'preferentially' expressed in T_H1 cells. Although expression of SOCS5 negatively regulates IL-4-dependent activation of STAT6 and T_H2 differentiation during T_H1 differentiation²⁸, the absence of SOCS5 from CD4⁺ T cells does not seem to alter normal T_H1 or T_H2 differentiation²⁹. Regulatory T cells (T_{reg} cells) have high expression of SOCS2, and it may be involved in their differentiation and/or function^{12–14}. Except for SOCS1, SOCS3 and SOCS5, the function of the SOCS family in the effector differentiation of helper T cells has not been established.

CIS was first shown to inhibit signaling from the receptors for erythropoietin and growth hormone^{16,30,31}. In CD4⁺ T cells, CIS inhibits STAT5 signaling^{32,33}, which suggests that it may be involved in the differentiation of helper T cells. In this study, we found that CIS, strongly induced by IL-4 in T cells, inhibited the phosphorylation of STAT3, STAT5 and STAT6. The absence of CIS in T cells led to more T_H2 and T_H9 differentiation and exacerbated airway allergic disease.

RESULTS

CIS expression in CD4⁺ T cells

T cell differentiation is initiated by signals from the T cell antigen receptor, costimulatory molecules and cytokine receptors. To characterize the role of CIS in T cell differentiation, we first assessed CIS expression during early activation of T cells. We sorted CD4⁺CD25[–]CD44^{lo}CD62L^{hi} naive T cells from C57BL/6 mice by flow cytometry and activated the cells for various times with plate-bound antibody to CD3 (anti-CD3) and anti-CD28, then analyzed the expression of mRNA encoding CIS (*Cish*; called 'Cis' here), IL-2 (*Il2*) and IL-2R α (*Il2ra*; called 'Cd25' here) by quantitative RT-PCR. We detected upregulation of *Cis* mRNA expression at 0.5 h, which reached a peak at 2 h, followed by downregulation at 8 h and a second peak at 48 h (Fig. 1a). *Il2* and *Cd25* shared a similar pattern of expression; both had low expression in the first 8 h, and their expression peaked at 48 h (Fig. 1a). These results suggested that in the early phase, *Cis* expression was independent of endogenous IL-2 production and expression of the high-affinity IL-2 receptor CD25 (IL-2R α).

Next we assessed expression of *Cis* mRNA in response to a variety of cytokines that are important in T cell differentiation. After 3 h of activation with plate-bound anti-CD3 and anti-CD28, the addition of IL-6 and IL-10 weakly upregulated *Cis* mRNA expression, whereas IL-4 substantially upregulated *Cis* mRNA expression (Fig. 1b). Consistent with that, blockade of IL-4 or IL-6 with neutralizing antibodies attenuated *Cis* mRNA expression (Fig. 1b), and deficiency in the gene encoding IL-4R α (*Il4ra*) abrogated IL-4-induced *Cis* mRNA expression (Supplementary Fig. 1a). However, in STAT6-deficient cells, IL-4-dependent *Cis* mRNA expression was only slightly lower (Supplementary Fig. 1b), which suggested that other factors in addition to STAT6 were required for IL-4-induced *Cis* mRNA expression. Consistent with that, STAT6 bound to the *Cis* promoter (Supplementary Fig. 1c), suggestive of direct regulation. Unexpectedly, at this early phase (3 h) of T cell differentiation, the addition of IL-2, a cytokine known to activate STAT5 (refs. 16,33), did not induce *Cis* expression much (Fig. 1b). Furthermore, we explored the expression of *Cis* mRNA in various helper T cell subsets and found high expression in T_H1 cells, T_H2 cells, inducible T_{reg} cells (iT_{reg} cells) and natural T_{reg} cells (nT_{reg} cells) and moderate expression in T_H9 cells (Fig. 1c). *Cis* mRNA expression

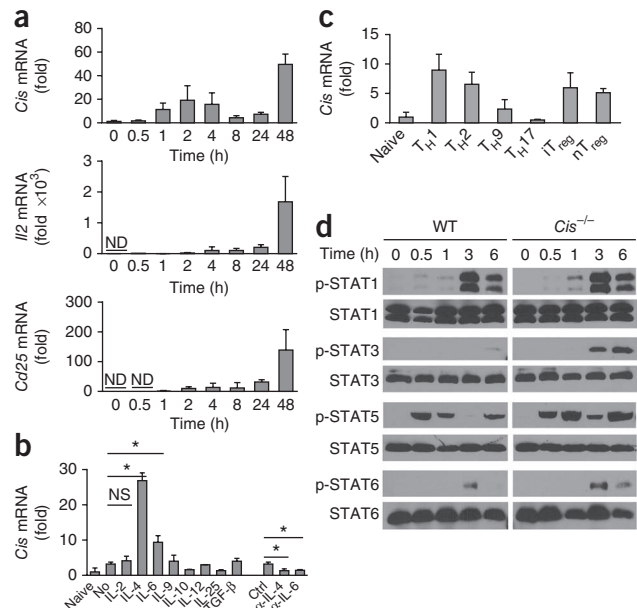


Figure 1 Expression and signaling function of CIS. (a) Quantitative real-time RT-PCR analysis of *Cis*, *Il2* and *Cd25* mRNA in naive CD4⁺ T cells activated for various times (horizontal axes) by plate-bound anti-CD3 and anti-CD28, normalized to expression of the reference gene *Actb* and presented relative to the lowest detectable expression, set as 1. ND, not detected. (b) Quantitative real-time RT-PCR analysis of *Cis* mRNA in naive CD4⁺ T cells left inactivated (Naive) or activated for 3 h by plate-bound anti-CD3 and anti-CD28 in the presence of no additional reagents (No) or various cytokines or antibodies (horizontal axis; α -, anti-), presented as in a. NS, not significant; * $P \leq 0.005$ (Student's *t*-test). (c) Quantitative real-time RT-PCR analysis of *Cis* mRNA in various helper T cell subsets, iT_{reg} cells and nT_{reg} cells, presented as in a. (d) Immunoblot analysis of total and phosphorylated (p-) STAT proteins in wild-type (WT) and *Cis*^{–/–} CD4⁺ T cells activated for various times (above lanes) with plate-bound anti-CD3 and anti-CD28 in the presence of recombinant human IL-2. Data are from two (a,c) or three (b) independent experiments (mean and s.d.) or are from one experiment representative of two independent experiments (d).

in T_H17 cells was similar to that in naive cells (Fig. 1c). Together these data suggested possible involvement of CIS in the regulation of cytokine signaling during helper T cell differentiation.

CIS antagonizes STAT activation

To further understand the role of CIS in the differentiation and function of T cells, we generated mice with conditional deletion of *Cis*. We introduced two loxP sites into the *Cis* locus that flanked a region spanning exon 2 to the coding region of exon 3 (Supplementary Fig. 2a). We generated a *Cis*-deficient allele by breeding mice with that loxP-flanked *Cis* allele (*Cis*^{+/fl}) with mice that express Cre recombinase from the cytomegalovirus promoter (Supplementary Fig. 2b). We backcrossed *Cis*^{+/–} or *Cis*^{+/fl} mice to C57BL/6 mice for seven to eight generations and then interbred the resulting mice to generate *Cis*^{–/–} or *Cis*^{fl/fl} mice. In *Cis*^{–/–} mice, *Cis* mRNA was not detectable in bone marrow or T_{reg} cells by RT-PCR (Supplementary Fig. 2c), and CIS protein was completely absent from CD4⁺ T cells by immunoblot analysis (Supplementary Fig. 2d), which indicated that a null mutation was generated and that CIS was not required for normal mouse development. The lymphoid populations in the thymus, spleen and lymph nodes of *Cis*^{–/–} mice seemed grossly normal, although they had slightly more cells

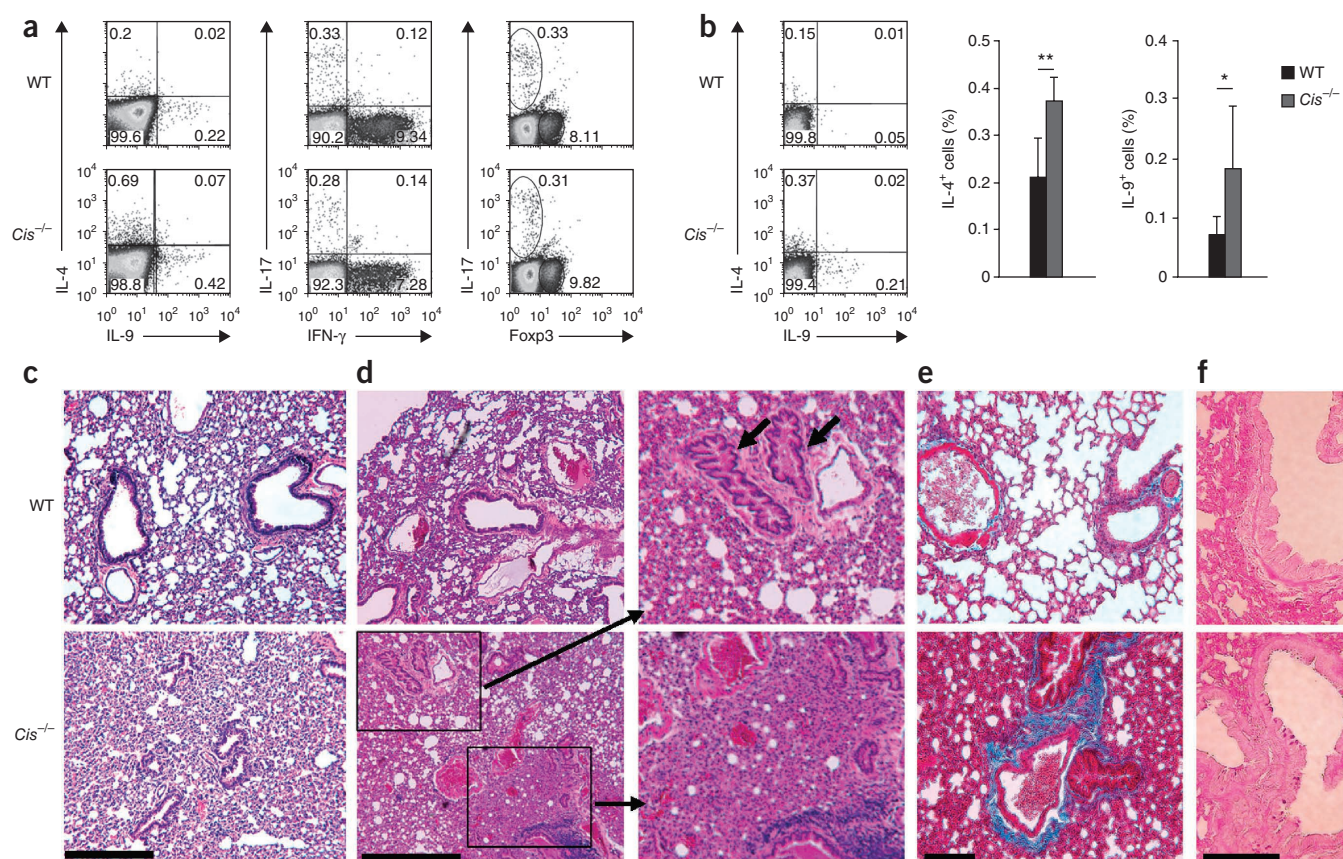


Figure 2 CIS deficiency leads to spontaneous pulmonary disease. **(a,b)** Intracellular staining of cytokines and Foxp3 in splenocytes **(a)** and lung-associated lymph node cells **(b)** from 6-month-old wild-type and *Cis*^{-/-} mice ($n = 4$ –5 per group), presented on a CD4⁺ gate. Numbers in quadrants indicate percent cells in each throughout or adjacent to outlined areas. * $P \leq 0.05$ and ** $P \leq 0.005$ (Student's *t*-test). **(c,d)** Hematoxylin-and-eosin staining of lung sections from 10-month old **(c)** or 18-month old **(d)** wild-type mice ($n = 3$ –4 per group) and *Cis*^{-/-} mice ($n = 4$ per group). Right **(d)**, enlargement ($\sim 2.1\times$) of outlined areas at left; arrows (top right) indicate smooth-muscle hyperplasia in a collapsed airway; bottom right, lung parenchymal consolidation and mononuclear cell inflammatory infiltrate. **(e,f)** Staining of lung sections from wild-type mice ($n = 3$ –4 per group) and *Cis*^{-/-} mice ($n = 4$ per group) with Masson's trichrome **(e)** or Periodic acid–Schiff **(f)** showing enhanced collagen deposition (blue filaments, **e**) and metaplastic goblet cells (red epithelial cells, **f**) in *Cis*^{-/-} mice. Scale bars, 1 mm **(c,d)** (left) or 200 μ m **(e,f)**. Data are representative of two independent experiments (mean and s.d. in **b**).

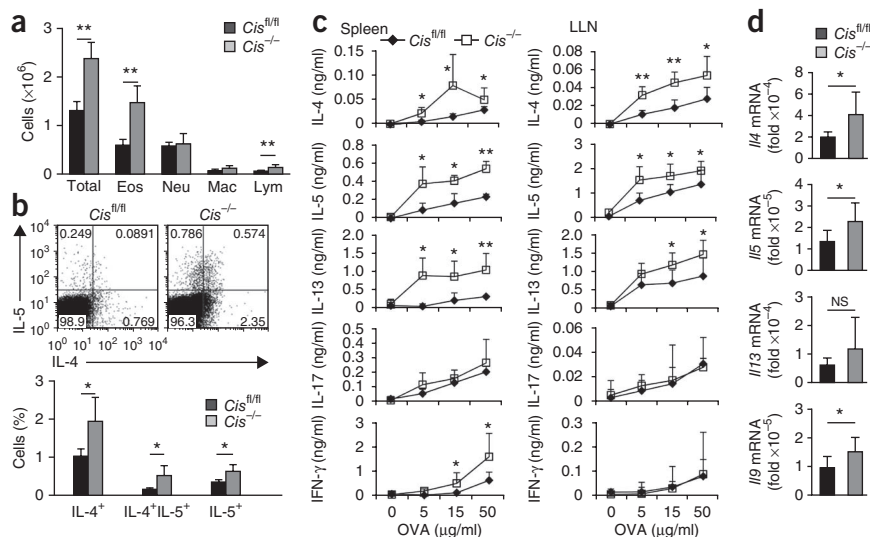
than did their wild-type counterparts (**Supplementary Fig. 3** and data not shown). Also, the development of CD4⁺CD25⁺Foxp3⁺ nT_{reg} cells in the thymus and spleen from *Cis*^{-/-} mice seemed normal (**Supplementary Fig. 3b,c**).

When activated with plate-bound anti-CD3 and anti-CD28 in the presence or absence of exogenous IL-2, CIS-deficient CD4⁺ T cells had enhanced proliferation, but endogenous IL-2 secretion was not altered (**Supplementary Fig. 4**). Moreover, *Cis*^{-/-} nT_{reg} cells had suppressive activity similar to or marginally less than that of their wild-type counterparts (**Supplementary Fig. 5a**). In addition, *Cis* deficiency did not alter the expression of T_{reg} cell-specific genes, such as *Foxp3*, *Tgfb1*, *Il10*, *Il35* and *Ctla4*, as assessed in nT_{reg} cells expressing the transcription factor Foxp3 linked to green fluorescent protein (GFP), sorted by flow cytometry (**Supplementary Fig. 5b**).

As CIS is reported to inhibit STAT activation, we assessed the phosphorylation of STAT proteins in T cells. We activated wild-type and *Cis*^{-/-} CD4⁺ T cells for various times with plate-bound anti-CD3 and anti-CD28 in the presence of recombinant human IL-2, then assessed phosphorylated and total STAT proteins by immunoblot. *Cis*^{-/-} CD4⁺ T cells had more phosphorylated STAT3, STAT5 and STAT6 than did

wild-type CD4⁺ T cells at various times, whereas the abundance of phosphorylated STAT1 remained the same in cells of each genotype (**Fig. 1d**). Of note, wild-type cells had less phosphorylated STAT5 at 3 h (**Fig. 1d**), which inversely correlated with CIS expression at 2–4 h (**Fig. 1a** and **Supplementary Fig. 2d**). To further understand the original signals that activate STAT3, STAT5 and STAT6, we first assessed the effect of IL-2. In the absence of exogenous IL-2 (endogenous IL-2 was expressed only 8 h after activation; **Fig. 1a**), phosphorylated STAT3 and STAT6 retained their enhanced activation in CIS-deficient cells, whereas the increase in phosphorylated STAT5 was greatly delayed and diminished, and we found no difference between wild-type and CIS-deficient cells (**Supplementary Fig. 6a**), which suggested that phosphorylated STAT5 was triggered by IL-2. Moreover, after the addition of IL-4-neutralizing antibody, phosphorylated STAT6 signals were no longer detectable (**Supplementary Fig. 6b**), which suggested that the activation of STAT6 required IL-4 in both wild-type cells and CIS-deficient cells. However, neutralization of IL-6 and/or IL-21 or IL-4 did not affect the activation of STAT3 (**Supplementary Fig. 6c,d**). In summary, these results indicated involvement of CIS in control of the IL-2–STAT5, IL-4–STAT6 and STAT3 pathways in CD4⁺ T cells.

Figure 3 CIS negatively regulates allergic asthma responses. (a) Quantification of cells in bronchoalveolar lavage fluid and lung infiltrates from *Cis*^{-/-} and *Cis*^{fl/fl} mice (*n* = 4–5 per group) in which allergic asthma was induced. Eos, eosinophil; Neu, neutrophil; Mac, macrophage; Lym, lymphocyte. (b) Expression of the T_H2 cytokines IL-5 and IL-4 in lung-draining lymph nodes from the mice in a, assessed by flow cytometry on a CD4⁺ gate. (c) ELISA of cytokines in splenocytes (Spleen) or cells from the lung-draining lymph nodes (LLN) of the mice in a, stimulated for 3 d with various concentrations of ovalbumin (OVA). (d) Quantitative RT-PCR analysis of mRNA encoding T_H2 and T_H9 cytokines in lung tissues from the mice in a, presented relative to expression of the internal reference *Actb*. **P* ≤ 0.05 and ***P* ≤ 0.005 (Student's *t*-test). Data represent three independent experiments (mean and s.d.).



CIS deficiency causes spontaneous pulmonary disease

The aberrant activation of STAT proteins in *Cis*^{-/-} CD4⁺ T cells suggested that CIS may be involved in regulating the differentiation and function of T cells. To confirm this, we first analyzed helper T cells from unmanipulated 6-month-old wild-type and *Cis*^{-/-} mice by intracellular staining. Splenocyte and lung-associated lymph node cell populations from *Cis*^{-/-} mice had significantly more IL-4- and IL-9-expressing T cells but not more IFN- γ - or IL-17-secreting cells than did those from wild-type mice (Fig. 2a,b and Supplementary Fig. 7a). The frequency of Foxp3⁺ cell was also similar for the two strains (Fig. 2a and Supplementary Fig. 7a). These data suggested that CIS may negatively regulate the proallergic differentiation of T cells *in vivo*.

We prepared lung, heart, liver, kidney and intestinal tissue sections from 10-month-old wild-type and *Cis*^{-/-} mice and stained the sections with hematoxylin and eosin. We observed no difference between the genotypes for any tissues tested except that in contrast to wild-type lungs, *Cis*^{-/-} lungs had parenchymal consolidation, narrowed airways and alveoli, hyperplasia of airway smooth muscle cells and goblet cell

metaplasia (incidence, 80%; Fig. 2c). Those histological features were more pronounced in all lungs from 18-month-old *Cis*^{-/-} mice and included more severe airway obstruction due to mucus impaction and scattered infiltration of mononuclear cells (Fig. 2d). By staining with Masson's trichrome and Periodic acid-Schiff, we found more collagen deposition in the peribronchovascular spaces (Fig. 2e) and more mucin staining (goblet-cell metaplasia) in the airway epithelium (Fig. 2f) in *Cis*^{-/-} lungs than in wild-type lungs. Moreover, we observed significantly more infiltration of eosinophils in *Cis*^{-/-} lungs than in wild-type lungs (*P* ≤ 0.05; Supplementary Fig. 7b). Together these results indicated that CIS deficiency resulted in a spontaneous lung disorder that resembled allergic asthma; it was associated with a greater abundance of IL-4- and IL-9-secreting CD4⁺ T cells, which suggested that T_H2 and T_H9 cells may be involved in the disease process in these mice.

T cell–intrinsic role of CIS

Because *Cis*^{-/-} mice had spontaneous allergic responses, we induced allergic asthma in CIS-sufficient (*Cis*^{fl/fl}) mice and their CIS-sufficient counterparts (*Cis*^{fl/fl} mice (Fig. 3) or wild-type littermates (Supplementary Fig. 8), as a control) by a standard protocol³⁴. *Cis*^{-/-} mice had significantly more total cells, eosinophils

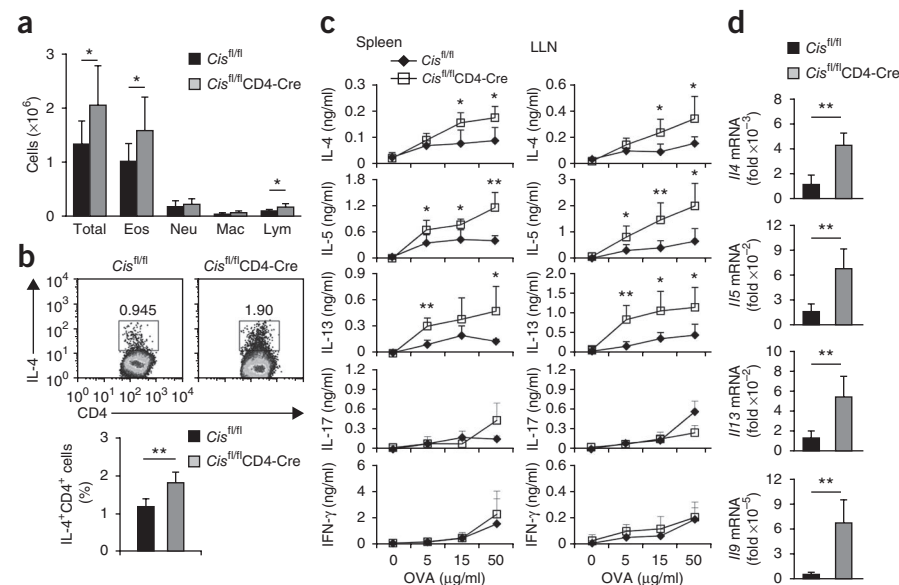
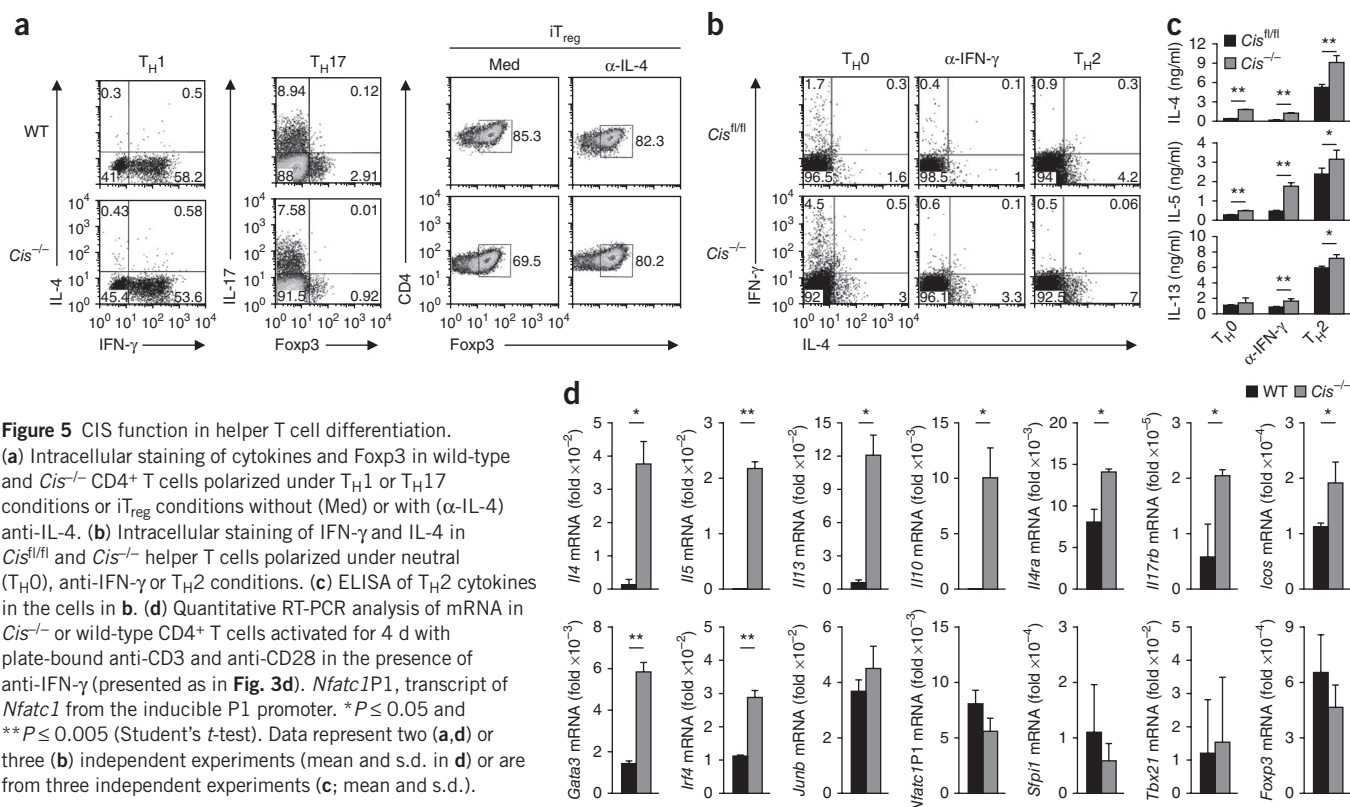


Figure 4 CD4⁺ T cell-specific *Cis* deletion leads to enhanced allergic asthma disease. (a) Quantification of cells in bronchoalveolar lavage fluid from *Cis*^{fl/fl} and *Cis*^{fl/fl}CD4-Cre mice (*n* = 4–5 per group) in which allergic asthma was induced. (b) Expression of T_H2 cytokines in lung-draining lymph node cells from the mice in a, assessed by flow cytometry on a CD4⁺ gate. (c) ELISA of cytokines in splenocytes and cells from the lung-draining lymph nodes from the mice in a, stimulated for 3 d with various concentrations of ovalbumin. (d) Quantitative RT-PCR analysis of mRNA encoding T_H2 and T_H9 cytokines in lung tissues from the mice in a (presented as in Fig. 3d). **P* ≤ 0.05 and ***P* ≤ 0.005 (Student's *t*-test). Data represent two independent experiments (mean and s.d.).



and lymphocytes in lung infiltrates than did CIS-sufficient mice (Fig. 3a and Supplementary Fig. 8a). There were more IL-4⁺ or IL-5⁺ CD4⁺ cells in lung infiltrates from *Cis*^{-/-} mice than in those from CIS-sufficient mice (Fig. 3b and Supplementary Fig. 8b). Moreover, after *ex vivo* restimulation with various doses of ovalbumin, lung-associated lymph node cells and splenocytes from *Cis*^{-/-} mice produced significantly more IL-4, IL-5 and IL-13 than did similarly restimulated cells from CIS-sufficient mice (Fig. 3c and Supplementary Fig. 8c). Consistent with that, total lung tissue from *Cis*^{-/-} mice had higher expression of *Il4*, *Il5*, *Il13* and *Il9* mRNA than did that from CIS-sufficient mice (Fig. 3d and Supplementary Fig. 8d). These results suggest that the enhanced T_H2 and T_H9 responses in CIS-deficient mice were independent of the polymorphisms in the genome of the 129 mouse strain that surrounds the *Cis* locus because the *Cis*^{fl/fl} mice had same *Cis* allele (that originated from the 129 strain) that the *Cis*^{-/-} mice had.

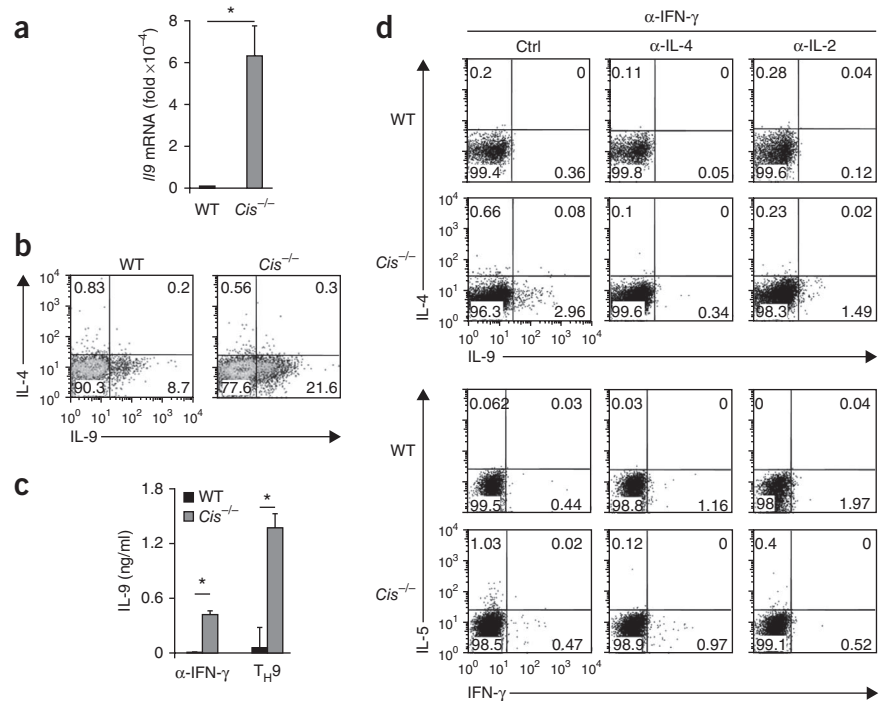
Next we assessed the T cell-intrinsic role of CIS in allergic lung responses. We induced asthma in *Cis*^{fl/fl}CD4-Cre mice (the offspring of *Cis*^{fl/fl} mice and mice that express Cre from the *Cd4* promoter), *Cis*^{fl/fl}Foxp3-Cre mice (the offspring of *Cis*^{fl/fl} mice and mice that express Cre from the *Foxp3* promoter) and *Cis*^{fl/fl} mice. The T cell-specific deletion of *Cis* in *Cis*^{fl/fl}CD4-Cre mice resulted in enhanced asthmatic responses, whereas the T_{reg} cell-specific deletion of *Cis* in *Cis*^{fl/fl}Foxp3-Cre mice did not (Fig. 4 and Supplementary Fig. 9). *Cis*^{fl/fl}CD4-Cre mice had more total cells and eosinophils accumulation in the bronchoalveolar lavage fluid than did *Cis*^{fl/fl} mice, whereas *Cis*^{fl/fl}Foxp3-Cre mice and *Cis*^{fl/fl} mice had similar numbers of all populations analyzed (Fig. 4a). Lung infiltrates from *Cis*^{fl/fl}CD4-Cre mice contained more CD4⁺IL-4⁺ cells than did those of *Cis*^{fl/fl} mice (Fig. 4b). In addition, in response to restimulation *ex vivo* with ovalbumin, lung-associated lymph node cells and splenocytes from *Cis*^{fl/fl}CD4-Cre mice secreted significantly higher concentrations of IL-4, IL-5 and IL-13 than did

those from *Cis*^{fl/fl} mice (Fig. 4c). Total lung tissue from *Cis*^{fl/fl}CD4-Cre mice also had more *Il4*, *Il5*, *Il13* and *Il9* mRNA than did that from *Cis*^{fl/fl} mice (Fig. 4d). Notably, we observed greater enhancement of T_H2 responses in *Cis*^{fl/fl}CD4-Cre mice (Fig. 4c,d) than in *Cis*^{-/-} mice (Fig. 3c,d). That might have partially resulted from the higher expression of IL-10 observed in T_H2 cells, which would further sustain the T_H2 polarization, as reported in an allergic skin model³⁵. To obtain further evidence of the T cell-intrinsic role of CIS in allergic T cell development, we reconstituted mice with a congenital deficiency in mature B cells and T cells (deficient in recombination-activating gene 1 (*Rag1*^{-/-})) with T cell-depleted bone marrow from wild-type (CD45.1⁺) mice and *Cis*^{-/-} (CD45.2⁺) mice (1:1 mixture) and induced asthma in the resulting mice. We found that *Cis*^{-/-} cell populations had a higher frequency of IL-4⁺ and IL-13⁺ cells in lung infiltrates and draining lymph nodes than did their wild-type counterparts (Supplementary Fig. 10a–c). In addition, flow cytometry-sorted *Cis*^{-/-} (CD45.2⁺) CD4⁺ T cells had higher expression of *Il4*, *Il5* and *Il9* mRNA than did their wild-type (CD45.1⁺) counterparts (Supplementary Fig. 10d). Therefore, CIS inhibited the induction of allergic lung disease by inhibiting the production of T_H2 and T_H9 cytokines in a T cell-intrinsic but T_{reg} cell-extrinsic manner.

CIS inhibits T_H2 differentiation

Our *in vivo* data collectively supported the proposal of a role for CIS in the negative regulation of proallergic T_H2 and T_H9 responses. We next assessed the role of CIS in helper T cell differentiation *in vitro*. We sorted naive CD4⁺ T cells from *Cis*^{-/-} mice and their wild-type littermates by flow cytometry and skewed the cells toward T_H1, T_H2, T_H17 or iT_{reg} differentiation, then assessed the lineage-specific markers IFN-γ, IL-17, IL-4 and Foxp3, respectively, by intracellular staining. Under T_H1 conditions, the frequency of IFN-γ⁺ cells among *Cis*^{-/-} cells was similar to that among wild-type cells (Fig. 5a),

Figure 6 Enhanced T_H9 differentiation in the absence of CIS. (a) Quantitative RT-PCR analysis of *Il9* mRNA in wild-type and *Cis*^{-/-} cells treated with anti-IFN- γ (presented as in Fig. 3d). (b) Intracellular staining of IL-4 and IL-9 in wild-type and *Cis*^{-/-} T_H9 cells. (c) ELISA of IL-9 in wild-type and *Cis*^{-/-} cells cultured under anti-IFN- γ or T_H9 conditions. (d) Intracellular staining of IL-4 and IL-9 (top) or IL-5 and IFN- γ (bottom) in naive *Cis*^{-/-} or wild-type CD4⁺ T cells activated in the presence of anti-IFN- γ plus a control antibody (Ctrl) or anti-IL-4 or anti-IL-2. * $P \leq 0.05$ (Student's *t*-test). Data are from two independent experiments (a,c; mean and s.d.) or represent three independent experiments (b,d).



consistent with the normal activation of STAT1 in *Cis*^{-/-} cells (Fig. 1d). Under T_H17 conditions, the frequency of IL-17⁺ cells was also similar for both strains, but there were fewer Foxp3⁺ cells among *Cis*^{-/-} cells than among their wild-type counterparts (Fig. 5a); this may have been due to enhanced STAT3 signaling. Under iT_{reg} conditions, *Cis*^{-/-} cell populations had a lower frequency of Foxp3⁺ cells than did their wild-type counterparts (Fig. 5a). That difference was diminished when IL-4 was blocked (Fig. 5a), which suggested that the enhanced T_H2 program mediated by CIS antagonized the iT_{reg} program.

As expected, given the *in vivo* data, by intracellular staining or enhanced immunosorbent assay (ELISA), CIS deficiency led to enhanced T_H2 differentiation and expression of T_H2 cytokines in naive T cells activated under neutral, anti-IFN- γ or T_H2 conditions (Fig. 5b,c and Supplementary Fig. 11a), which correlated with the enhanced activation of STAT5 and STAT6 observed in *Cis*^{-/-} cells (Fig. 1d). Furthermore, by quantitative RT-PCR, we found that when treated with anti-IFN- γ , *Cis*^{-/-} cells had higher expression of not only mRNA encoding T_H2 cytokines (*Il4*, *Il5*, *Il13* and *Il10*) but also mRNA encoding several T_H2 -associated cell-surface receptors (*Il4ra*, *Il17rb* and *Icos*) and transcription factors (*Gata3* and *Irf4*; Fig. 5d). These results suggested that CIS inhibited the T_H2 differentiation program but not the T_H1 or T_H17 differentiation program and may have also regulated the iT_{reg} program indirectly by restricting the T_H2 pathway.

CIS deficiency enhances T_H9 differentiation

In addition to IL-4, IL-5 and IL-13, IL-9 is also associated with allergic asthma^{36–38}. TGF- β , together with IL-4, greatly enhances IL-9 expression, which results in T_H9 polarization^{9–11}. Because we found higher expression of *Il9* mRNA mice with germline or T cell-specific *Cis* deletion than in their CIS-sufficient counterparts (Figs. 3d and 4d), we sought to determine whether CIS also inhibits T_H9 differentiation. We first assessed *Il9* mRNA expression in wild-type and *Cis*^{-/-} T cells differentiated from flow cytometry-sorted naive cells and activated for 4 d in the presence of anti-IFN- γ . We found that *Il9* mRNA expression was much higher in the absence of CIS (Fig. 6a). Next we polarized naive CD4⁺ T cells to differentiate into T_H9 cells and found that the resultant CIS-deficient (*Cis*^{-/-}) cell populations had many more IL-9 expressing cells than did their CIS-sufficient (wild-type (Fig. 6b) or *Cis*^{fl/fl} (Supplementary Fig. 11b)) counterparts. IL-9 production was also much greater in *Cis*^{-/-} cells than in wild-type cells in the presence of anti-IFN- γ and T_H9 conditions (Fig. 6c). Finally, to determine the

cytokine-STAT signals that drive T_H9 differentiation, we activated naive CD4⁺ cells from wild-type and *Cis*^{-/-} mice in the presence of anti-IFN- γ . We found more IL-4⁺IL-9⁺ cells and IL-5⁺ cells among *Cis*^{-/-} cells than among wild-type cells in this condition (Fig. 6d). The addition of neutralizing antibody to IL-4 or IL-2 diminished the enhanced T_H9 and T_H2 responses of *Cis*^{-/-} T cells (Fig. 6d), which suggested signaling via both IL-4 and IL-2 was important in T_H9 differentiation as well as in T_H2 differentiation.

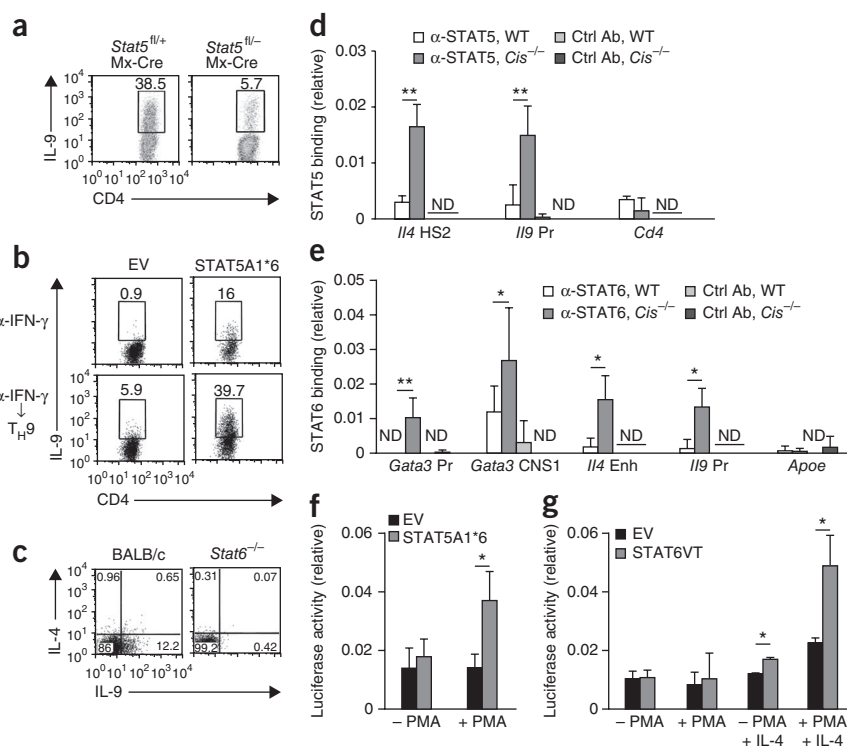
STAT5 and STAT6 regulate T_H9 differentiation

Signaling via IL-4 and IL-2 is known to activate STAT6 and STAT5, respectively. Our results indicated that CIS deficiency led to enhanced phosphorylation of STAT5 and STAT6 that was dependent on signaling via IL-4 and IL-2, respectively, as well as enhanced T_H9 and T_H2 responses *in vitro* and *in vivo*. Although both STAT5 and STAT6 are well known to regulate T_H2 differentiation, their roles in T_H9 development are unclear. We next examined the effect of STAT5 and STAT6 on T_H9 differentiation. To assess the role of STAT5, we studied the offspring of mice with one loxP-flanked *Stat5a* allele (*Stat5a*^{fl/+} or *Stat5a*^{fl/-}) crossed with Mx-Cre Ai3 mice (which express yellow fluorescent protein (YFP) after the induction of Cre expressed from the *Mx1* promoter through the use of IFN- α). We obtained naive YFP⁺CD4⁺ T cells from those offspring and differentiated the cells toward the T_H9 lineage. We found that STAT5-deficient (*Stat5a*^{fl/-}Mx-Cre) cell populations included considerably fewer IL-9⁺ cells than did their STAT5-sufficient (*Stat5a*^{fl/+}Mx-Cre) counterparts (Fig. 7a). Conversely, forced expression of a constitutively active form of the STAT5A isoform of STAT5 (STAT5A1*6)³⁹ greatly enhanced T_H9 differentiation in the presence of anti-IFN- γ or under T_H9 -differentiation conditions after 1 d of activation in the presence of anti-IFN- γ and infection with virus encoding STAT5A1*6 or control virus (Fig. 7b).

To assess the role of STAT6, we differentiated naive CD4⁺ T cells from *Stat6*^{-/-} and BALB/c mice into T_H9 cells and found considerably fewer IL-9-expressing cells among *Stat6*^{-/-} cells than among wild-type cells, consistent with a published report⁴⁰. *Stat6*^{-/-} cell populations also had a lower frequency of IL-4⁺ cells than did their wild-type

Figure 7 CIS controls T_H9 differentiation by modulating the activity of STAT5 and STAT6.

(a) Intracellular staining of IL-9 in T_H9 cells polarized from naive YFP+CD4⁺ T cells from *Stat5^{fl/+}*Mx-Cre or *Stat5^{fl/-}*Mx-Cre mice (which have a reporter gene encoding YFP). (b) Intracellular staining of IL-9 in wild-type CD4⁺ T cells polarized with anti-IFN- γ alone or with anti-IFN- γ followed by switching to T_H9 conditions (left margin) with (STAT5A1*6) or without (empty vector (EV)) forced expression of constitutively active STAT5A, assessed in a GFP⁺ gate. (c) Intracellular staining of IL-9 and IL-4 in wild-type (BALB/c) and *Stat6^{-/-}* T_H9 cells. (d,e) ChIP assay of the binding of STAT5 (d) or STAT6 (e) to various gene regions (horizontal axes) in wild-type or *Cis^{-/-}* naive CD4⁺ T cells activated for 1 h in the presence of IL-2 (d) or incubated for 3 h in the absence of IL-2 (e), precipitated with anti-STAT5, anti-STAT6 or control antibody (Ctrl Ab), followed by quantitative PCR analysis of DNA enrichment; results are presented relative to input. *Cd4* and *Apoe* serve as negative controls. Pr, promoter; Enh, enhancer. ND, not done. (f,g) Luciferase activity in EL4 cells transduced to express a luciferase reporter driven by the *Il9* promoter plus empty vector (EV) or vector encoding constitutively active STAT5A (STAT5A1*6; f) or STAT6 (STAT6VT; g) and treated with various combinations of PMA and/or IL-4 (horizontal axis); results are presented relative to those of renilla luciferase (used to normalize transfection efficiency), set as 1. * $P \leq 0.05$ and ** $P \leq 0.005$ (Student's *t*-test). Data represent two independent experiments (a–c) or are from two (d,e,g) or three (f) independent experiments (mean and s.d.).



counterparts, as expected, although both *Stat6^{-/-}* and wild-type cell populations included very few IL-4⁺ cells (Fig. 7c). In summary, our observations demonstrated that CIS negatively regulated the IL-4–STAT6 and IL-2–STAT5 pathways that were both important in T_H9 differentiation.

Our results thus far showed that CIS inhibited T_H2 and T_H9 differentiation by antagonizing the activation of STAT5 and STAT6. STAT5 has been shown to bind to the *Il4* locus-control region⁴¹, whereas STAT6 binds to the *Il4* and *Gata3* loci⁴². By comparison of mouse and human genomic DNA sequences with the ECR browser (of evolutionary conserved regions)⁴³ and rVISTA program⁴⁴, we found potentially overlapping STAT5- and STAT6-binding sites (TGCCAG GAAATACCATTCCTCAGA and AGGAAATA, respectively) in the *Il9* promoter. We sought to determine whether CIS controls the T_H2 and T_H9 programs by targeting the DNA-binding activity of STAT5 and STAT6. To address this question, we did chromatin immunoprecipitation (ChIP) with anti-STAT5, anti-STAT6 or a control antibody. We activated naive CD4⁺ T cells from wild-type and *Cis^{-/-}* mice with plate-bound anti-CD3 and anti-CD28 in the presence (for STAT5 analysis) or absence (for STAT6 analysis) of IL-2. Through the use of anti-STAT5, we observed that the absence of CIS resulted in significantly more binding of STAT5 at the DNase I-hypersensitivity site HS2 of the *Il4* locus and at the *Il9* promoter (Fig. 7d). Furthermore, as shown by the use of anti-STAT6, CIS deficiency led to significantly enhanced binding of STAT6 to the promoter and conserved noncoding sequence CNS1 of *Gata3*, the *Il4* locus enhancer and the *Il9* promoter (Fig. 7e). Furthermore, in a luciferase reporter assay, STAT5 and STAT6 transactivated the transcription of a luciferase reporter driven by the *Il9* promoter in the presence of the phorbol ester PMA (in the presence of IL-4, for STAT6 analysis; Fig. 7f,g). These results therefore suggested that CIS attenuated T_H2 and T_H9

differentiation through inhibition of the phosphorylation of STAT5 and STAT6 and, in turn, their binding to genes encoding T_H2 and T_H9 signature cytokines.

DISCUSSION

Effector T cell differentiation is directed by the environmental cytokine milieu. STAT proteins are broadly engaged in cytokine signaling transduction and critically regulate the differentiation of helper T cells into the T_H1, T_H2 and T_H17 subsets. However, the involvement of STAT proteins in T_H9 cells is unclear. Because T_H9 differentiation requires IL-4, STAT6 is probably involved. STAT activity is negatively regulated by members of the SOCS family. SOCS1, SOCS3 and SOCS5 have been shown to regulate the differentiation of helper T cells through inhibition of STAT activation^{12–14}. In this paper, we have reported an essential role for the SOCS family member CIS in the control of the proallergic differentiation of T_H2 and T_H9 cells and allergic disease.

SOCS expression is induced by cytokine signals, mainly via activated STAT proteins. We tested a panel of cytokines that are important in the differentiation of helper T cells in the presence of signaling via the T cell antigen receptor and the coreceptor CD28. We found at the early phase of activation of helper T cell progenitors, IL-6 and IL-10 weakly induced CIS expression, whereas IL-4 strongly induced CIS expression. Consistent with that, in response to IL-4, IL-4R α -deficient CD4⁺ T cells failed to upregulate *Cis* mRNA expression. However, STAT6-deficient CD4⁺ T cells had lower expression of *Cis* mRNA, approximately 50% that of wild-type cells. These data indicated in addition to STAT6, unknown factors mediated IL-4-induced CIS expression. *Cis* has been shown to be induced by STAT5 in liver cells³¹, erythroid progenitor cells³⁰ and human T lymphocytes³³. Our results suggest that CIS may be regulated by distinct cytokines in a cell type-specific

manner. As CIS negatively regulates allergic responses, IL-4-induced CIS expression may serve as a negative feedback mechanism.

As IL-4 was the main inducer of CIS expression in the early phase of the differentiation of helper T cells, CIS may be involved in the IL-4-directed differentiation of helper T cells, such as T_H2 and T_H9 cells. Indeed, CIS deficiency led to spontaneously augmented T_H2 and T_H9 differentiation but not to the generation of T_H1 or T_H17 cells. As for iT_{reg} cells, when differentiated with TGF- β and IL-2, CIS-deficient cell populations included fewer cells that were Foxp3⁺, whereas after the addition of anti-IL-4, we did not observe this difference. Thus, in T_{reg} cell differentiation, CIS seemed to act indirectly by enhancing the T_H2 response. Although T_{reg} cells had high expression of CIS, genetic deletion of CIS in T_{reg} cells did not lead to enhanced allergic diseases, which indicated a dispensable role for CIS in Foxp3⁺ nT_{reg} or iT_{reg} cells. It is also possible that other SOCS proteins, such as SOCS2, have redundant function in these cells.

Members of the SOCS family attenuate cytokine signaling by inhibiting the phosphorylation of STAT proteins. CIS has been shown to associate with the IL-2 receptor β -chain and to inhibit IL-2-dependent signaling by disruption of the Jak-STAT5 pathway^{32,33}. In this study, we found that CIS deficiency enhanced the phosphorylation of STAT3, STAT5 and STAT6 but not that of STAT1. The STAT5 signals were initiated by IL-2 because in the absence of exogenous IL-2, phosphorylation of STAT5 was delayed and there were no differences between wild-type and CIS-deficient cells in the phosphorylation of STAT5. With or without exogenous IL-2, the phosphorylation of STAT3 and STAT6 was not altered, which suggested that other cytokines were responsible for the activation of STAT3 and STAT6. When IL-4 was blocked with a neutralizing antibody, phosphorylated STAT6 signals disappeared, which indicated that a trace amount of IL-4 in the medium or from autocrine secretion was sufficient to induce the activation of STAT6. However, blockade of IL-6 and/or IL-21 or IL-4 did not change the activation of STAT3 in wild-type or CIS-deficient cells. At present, the cytokine that activates STAT3 is unclear. Together, the enhanced signaling via STAT5 and STAT6 may have accounted for the enhanced T_H2 response of CIS-deficient cells. Consistent with the lack of an effect of CIS deficiency on T_H1 differentiation, the activation of STAT1 was intact in CIS-deficient cells. CIS deficiency did not alter T_H17 differentiation; this may have been due to enhanced signaling via STAT5, a negative regulator of T_H17 differentiation⁸, counterbalanced by enhanced activation of STAT3.

T_H9 polarization is driven by IL-4 and TGF- β , which can activate the transcription factors PU.1 and IRF4 to establish the T_H9 program^{45,46}. In our study, CIS deficiency led to higher expression of *Il9* mRNA and *Irf4* mRNA but not of mRNA encoding PU.1 (*Sfp1*), which suggested a more important role for IRF4 in T_H9 differentiation when CIS is absent. However, the STAT proteins that mediate T_H9 differentiation are not well understood. We found that in the presence of anti-IFN- γ , blockade of IL-4 or IL-2 resulted in a low frequency of CIS-deficient IL-9⁺ cells, whereas we observed no wild-type IL-9⁺ cells, which suggested involvement of signaling via IL-2-STAT5 and/or IL-4-STAT6 in T_H9 differentiation. To confirm that, we assessed the effect of STAT5 and STAT6 on T_H9 polarization. Conditional removal of STAT5A resulted in less T_H9 polarization, whereas forced expression of a constitutively activated form of STAT5A (STAT5A1*6) resulted in more T_H9 polarization. In addition to STAT5 deficiency, STAT6 deficiency profoundly impaired T_H9 skewing, consistent with a published report on STAT6-dependent regulation of T_H9 differentiation⁴⁰. Moreover, we also demonstrated that in addition to binding to T_H2 signature genes (*Gata3* and *Il4*), STAT5 and STAT6 bound to the *Il9* promoter

and transactivated its transcription. These data indicated essential roles for STAT5 and STAT6 in the generation of T_H9 cells.

In vivo, CIS-deficient mice were more susceptible to experimental allergic asthma, associated with enhanced T_H2 and T_H9 responses, in which more influx of eosinophils may manifest the acute allergic responses. In the asthma model, we also observed moderately more airway remodeling in CIS-deficient mice (data not shown). With aging, CIS-deficient mice spontaneously developed allergic pulmonary disease with alveolar epithelial cell and airway muscle cell hyperplasia and more eosinophils in lungs. This may have been due to chronically higher expression of the T_H2 cytokine IL-13 (refs. 47–49) and the T_H9 cytokine IL-9 (refs. 36–38,49). As both T_H2 differentiation and T_H9 differentiation require IL-4, the higher IL-4 expression in CIS-deficient mice may have further augmented the T_H2 and T_H9 responses. Moreover, IL-9 also promotes IL-13 expression in airway epithelial cells⁵⁰, which further amplifies the effect of IL-13. Notably, we did not observe the spontaneous airway remodeling in 1-year-old *Cis*^{fl/fl}CD4-Cre mice (data not shown), which suggested that the enhanced T_H2 and T_H9 responses were not the only pathogenic factors and that defective CIS signaling in other cells (cells of the innate immune system and/or airway structural cells, for example) may have had a role in this. Notably, innate sources of IL-9 and IL-13 have been shown to be important regulators of lung inflammation, especially in acute allergic responses^{51,52}. The role of CIS in cell types other than T_H2 and T_H9 cells needs further investigation.

In summary, we found that CIS, induced by IL-4 (and also IL-2 at the late phase), attenuated signaling via IL-2-STAT5 and IL-4-STAT6 during T_H2 and T_H9 differentiation by diminishing the binding of STAT5 and STAT6 to T_H2 and T_H9 signature genes. Therefore, our data suggest negative feedback by the CIS-STAT5 and CIS-STAT6 pathways in T_H2 and T_H9 differentiation. Our results may help to elucidate the pathogenesis of human allergic diseases.

METHODS

Methods and any associated references are available in the [online version of the paper](#).

Note: Supplementary information is available in the online version of the paper.

ACKNOWLEDGMENTS

We thank S. Rivera and J. Parker-Thornburg of the MD Anderson Genetic Engineered Mouse Facility for help in generating gene-targeted mice; A.Y. Rudensky (Memorial Sloan-Kettering Cancer Center) for Foxp3-GFP and Foxp3-Cre mice; C.B. Wilson (University of Washington) for CD4-Cre mice; M. Kaplan (Indiana University School of Medicine) for the STAT6VT-expressing construct; and members of the Dong laboratory for help and discussion. Supported by the US National Institutes of Health (C.D., S.S.W., D.W. and D.B.C.), the Leukemia and Lymphoma Society (C.D. and D.W.), MD Anderson Cancer Center (C.D.), the Biology of Inflammation Center at Baylor College of Medicine (D.B.C.), the American Heart Association, the American Cancer Society (X.O.Y.), the Shanghai Board of Health Foundation, Shanghai Jiao Tong University (X.N.) and the Ministry of Education of China (J.P.).

AUTHOR CONTRIBUTIONS

C.D. and X.O.Y. conceived of the project, designed the experiments and wrote the manuscript; X.O.Y., H.Z. and B.-S.K. did most of the experiments; and X.N., J.P., Y.C., R.K., Y.-H.L., S.H.C., D.B.C., D.W. and S.S.W. participated in specific experiments.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Reprints and permissions information is available online at <http://www.nature.com/reprints/index.html>.

1. Dong, C. Diversification of T-helper-cell lineages: finding the family root of IL-17-producing cells. *Nat. Rev. Immunol.* **6**, 329–333 (2006).

2. Glimcher, L.H. & Murphy, K.M. Lineage commitment in the immune system: the T helper lymphocyte grows up. *Genes Dev.* **14**, 1693–1711 (2000).
3. Dong, C. & Flavell, R.A. Control of T helper cell differentiation—in search of master genes. *Sci. STKE* **2000**, PE1 (2000).
4. Cote-Sierra, J. *et al.* Interleukin 2 plays a central role in Th2 differentiation. *Proc. Natl. Acad. Sci. USA* **101**, 3880–3885 (2004).
5. Yamane, H., Zhu, J. & Paul, W.E. Independent roles for IL-2 and GATA-3 in stimulating naive CD4⁺ T cells to generate a Th2-inducing cytokine environment. *J. Exp. Med.* **202**, 793–804 (2005).
6. Liao, W. *et al.* Priming for T helper type 2 differentiation by interleukin 2-mediated induction of interleukin 4 receptor α -chain expression. *Nat. Immunol.* **9**, 1288–1296 (2008).
7. Dong, C. Differentiation and function of pro-inflammatory Th17 cells. *Microbes Infect.* **11**, 584–588 (2009).
8. Laurence, A. *et al.* Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. *Immunity* **26**, 371–381 (2007).
9. Schmitt, E. *et al.* IL-9 production of naive CD4⁺ T cells depends on IL-2, is synergistically enhanced by a combination of TGF- β and IL-4, and is inhibited by IFN- β . *J. Immunol.* **153**, 3989–3996 (1994).
10. Dardalhon, V. *et al.* IL-4 inhibits TGF- β -induced Foxp3⁺ T cells and, together with TGF- β , generates IL-9⁺IL-10⁺Foxp3⁺ effector T cells. *Nat. Immunol.* **9**, 1347–1355 (2008).
11. Veldhoen, M. *et al.* Transforming growth factor- β 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat. Immunol.* **9**, 1341–1346 (2008).
12. O'Shea, J.J., Gadina, M. & Schreiber, R.D. Cytokine signaling in 2002: new surprises in the Jak/Stat pathway. *Cell* **109** (suppl.), S121–S131 (2002).
13. Yoshimura, A., Naka, T. & Kubo, M. SOCS proteins, cytokine signalling and immune regulation. *Nat. Rev. Immunol.* **7**, 454–465 (2007).
14. Palmer, D.C. & Restifo, N.P. Suppressors of cytokine signaling (SOCS) in T cell differentiation, maturation, and function. *Trends Immunol.* **30**, 592–602 (2009).
15. Zhang, J.G. *et al.* The SOCS box of suppressor of cytokine signaling-1 is important for inhibition of cytokine action *in vivo*. *Proc. Natl. Acad. Sci. USA* **98**, 13261–13265 (2001).
16. Yoshimura, A. *et al.* A novel cytokine-inducible gene CIS encodes an SH2-containing protein that binds to tyrosine-phosphorylated interleukin 3 and erythropoietin receptors. *EMBO J.* **14**, 2816–2826 (1995).
17. Sakamoto, H. *et al.* A Janus kinase inhibitor, JAB, is an interferon- γ -inducible gene and confers resistance to interferons. *Blood* **92**, 1668–1676 (1998).
18. Alexander, W.S. *et al.* SOCS1 is a critical inhibitor of interferon γ signaling and prevents the potentially fatal neonatal actions of this cytokine. *Cell* **98**, 597–608 (1999).
19. Eyles, J.L., Metcalf, D., Grusby, M.J., Hilton, D.J. & Starr, R. Negative regulation of interleukin-12 signaling by suppressor of cytokine signaling-1. *J. Biol. Chem.* **277**, 43735–43740 (2002).
20. Diehl, S. *et al.* Inhibition of Th1 differentiation by IL-6 is mediated by SOCS1. *Immunity* **13**, 805–815 (2000).
21. Egwuagu, C.E. *et al.* Suppressors of cytokine signaling proteins are differentially expressed in Th1 and Th2 cells: implications for Th cell lineage commitment and maintenance. *J. Immunol.* **168**, 3181–3187 (2002).
22. Kubo, M. & Inoue, H. Suppressor of cytokine signaling 3 (SOCS3) in Th2 cells evokes Th2 cytokines, IgE, and eosinophilia. *Curr. Allergy Asthma Rep.* **6**, 32–39 (2006).
23. Seki, Y. *et al.* SOCS-3 regulates onset and maintenance of Th2-mediated allergic responses. *Nat. Med.* **9**, 1047–1054 (2003).
24. Kubo, M., Ozaki, A., Tanaka, S., Okamoto, M. & Fukushima, A. Role of suppressor of cytokine signaling in ocular allergy. *Curr. Opin. Allergy Clin. Immunol.* **6**, 361–366 (2006).
25. Kinjo, I. *et al.* Loss of SOCS3 in T helper cells resulted in reduced immune responses and hyperproduction of interleukin 10 and transforming growth factor- β 1. *J. Exp. Med.* **203**, 1021–1031 (2006).
26. Chen, Z. *et al.* Selective regulatory function of Socs3 in the formation of IL-17-secreting T cells. *Proc. Natl. Acad. Sci. USA* **103**, 8137–8142 (2006).
27. Ogura, H. *et al.* Interleukin-17 promotes autoimmunity by triggering a positive-feedback loop via interleukin-6 induction. *Immunity* **29**, 628–636 (2008).
28. Seki, Y. *et al.* Expression of the suppressor of cytokine signaling-5 (SOCS5) negatively regulates IL-4-dependent STAT6 activation and Th2 differentiation. *Proc. Natl. Acad. Sci. USA* **99**, 13003–13008 (2002).
29. Brender, C. *et al.* SOCS5 is expressed in primary B and T lymphoid cells but is dispensable for lymphocyte production and function. *Mol. Cell Biol.* **24**, 6094–6103 (2004).
30. Iwatsuki, K. *et al.* STAT5 Activation correlates with erythropoietin receptor-mediated erythroid differentiation of an erythroleukemia cell line. *J. Biol. Chem.* **272**, 8149–8152 (1997).
31. Ram, P.A. & Waxman, D.J. SOCS/CIS protein inhibition of growth hormone-stimulated STAT5 signaling by multiple mechanisms. *J. Biol. Chem.* **274**, 35553–35561 (1999).
32. Matsumoto, A. *et al.* CIS, a cytokine inducible SH2 protein, is a target of the JAK-STAT5 pathway and modulates STAT5 activation. *Blood* **89**, 3148–3154 (1997).
33. Aman, M.J. *et al.* CIS associates with the interleukin-2 receptor β chain and inhibits interleukin-2-dependent signaling. *J. Biol. Chem.* **274**, 30266–30272 (1999).
34. Yang, X.O. *et al.* Regulation of inflammatory responses by IL-17F. *J. Exp. Med.* **205**, 1063–1075 (2008).
35. Laouini, D. *et al.* IL-10 is critical for Th2 responses in a murine model of allergic dermatitis. *J. Clin. Invest.* **112**, 1058–1066 (2003).
36. Temann, U.A., Geba, G.P., Rankin, J.A. & Flavell, R.A. Expression of interleukin 9 in the lungs of transgenic mice causes airway inflammation, mast cell hyperplasia, and bronchial hyperresponsiveness. *J. Exp. Med.* **188**, 1307–1320 (1998).
37. McLane, M.P. *et al.* Interleukin-9 promotes allergen-induced eosinophilic inflammation and airway hyperresponsiveness in transgenic mice. *Am. J. Respir. Cell Mol. Biol.* **19**, 713–720 (1998).
38. Ying, S., Meng, Q., Kay, A.B. & Robinson, D.S. Elevated expression of interleukin-9 mRNA in the bronchial mucosa of atopic asthmatics and allergen-induced cutaneous late-phase reaction: relationships to eosinophils, mast cells and T lymphocytes. *Clin. Exp. Allergy* **32**, 866–871 (2002).
39. Onishi, M. *et al.* Identification and characterization of a constitutively active STAT5 mutant that promotes cell proliferation. *Mol. Cell Biol.* **18**, 3871–3879 (1998).
40. Goswami, R. *et al.* STAT6-dependent regulation of Th9 development. *J. Immunol.* **188**, 968–975 (2012).
41. Zhu, J., Cote-Sierra, J., Guo, L. & Paul, W.E. Stat5 activation plays a critical role in Th2 differentiation. *Immunity* **19**, 739–748 (2003).
42. Scheinman, E.J. & Avni, O. Transcriptional regulation of GATA3 in T helper cells by the integrated activities of transcription factors downstream of the interleukin-4 receptor and T cell receptor. *J. Biol. Chem.* **284**, 3037–3048 (2009).
43. Ovcharenko, I., Nobrega, M.A., Loots, G.G. & Stubbs, L. ECR Browser: a tool for visualizing and accessing data from comparisons of multiple vertebrate genomes. *Nucleic Acids Res.* **32**, W280–W286 (2004).
44. Loots, G.G. & Ovcharenko, I. rVISTA 2.0: evolutionary analysis of transcription factor binding sites. *Nucleic Acids Res.* **32**, W217–W221 (2004).
45. Chang, H.C. *et al.* The transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation. *Nat. Immunol.* **11**, 527–534 (2010).
46. Staudt, V. *et al.* Interferon-regulatory factor 4 is essential for the developmental program of T helper 9 cells. *Immunity* **33**, 192–202 (2010).
47. Kasaian, M.T. & Miller, D.K. IL-13 as a therapeutic target for respiratory disease. *Biochem. Pharmacol.* **76**, 147–155 (2008).
48. Wynn, T.A. IL-13 effector functions. *Annu. Rev. Immunol.* **21**, 425–456 (2003).
49. Doherty, T. & Broide, D. Cytokines and growth factors in airway remodeling in asthma. *Curr. Opin. Immunol.* **19**, 676–680 (2007).
50. Temann, U.A., Laouar, Y., Eynon, E.E., Homer, R. & Flavell, R.A. IL9 leads to airway inflammation by inducing IL13 expression in airway epithelial cells. *Int. Immunol.* **19**, 1–10 (2007).
51. Wilhelm, C. *et al.* An IL-9 fate reporter demonstrates the induction of an innate IL-9 response in lung inflammation. *Nat. Immunol.* **12**, 1071–1077 (2011).
52. Spits, H. & Di Santo, J.P. The expanding family of innate lymphoid cells: regulators and effectors of immunity and tissue remodeling. *Nat. Immunol.* **12**, 21–27 (2011).

ONLINE METHODS

Mice. For the generation of CIS-deficient mice and mice with *loxP*-flanked *Cis* alleles, a targeting vector was constructed by the introduction of two *loxP* sites into the *Cis* locus with a neomycin-resistance cassette (positive selection marker) and sequence encoding diphtheria toxin A (negative selection marker; **Supplementary Fig. 2**). Targeted TC-1 embryonic stem cell clones (derived from 129S6/SvEvTac mice) were selected and injected into C57BL/6 (B6) blastocysts to generate chimeras. Chimeras with a high percentage of cells of the 129 strain were bred with B6 mice for germline transmission. Targeted mice were crossed with FLPe mice (which have a knock-in allele with widespread expression of the FLPe variant of the *Saccharomyces cerevisiae* FLP1 recombinase; Jackson Laboratory) to generate mice bearing a *loxP*-flanked *Cis* allele (*Cis*^{fl/fl}) for conditional deletion. The *Cis*^{fl/fl} mice were then bred with mice that express Cre recombinase from the cytomegalovirus promoter (Jackson Laboratory), and the excision of exon 2 and the coding region of exon 3 resulted in the generation of *Cis*^{+/-} mice. *Cis*^{fl/fl} and *Cis*^{+/-} mice were backcrossed with B6 mice for seven to eight generations and were then interbred to generate *Cis*^{fl/fl} and *Cis*^{+/-} mice. *Cis*^{-/-} mice on the B6 background were studied with age- and sex-matched *Cis*^{fl/fl} mice on the same background or wild-type littermates as controls. In some experiments, *Cis*^{fl/fl} mice were bred with CD4-Cre mice⁵³ or Foxp3-Cre mice⁵⁴ to generate mice with T cell- or T_{reg} cell-specific deletion of *Cis*, respectively. The genotyping primers were as follows: F, 5'-TGAGTAACCTGCCCTTTGGT; R1, 5'-TGGGGTTCTCAGGGAGACCTC; and R2, 5'-CCTAGAGGCGTTGACCTCAG. The primers F and R1 amplify a 260-base pair band (wild-type) or a 452-base pair band (*loxP*-flanked), whereas the primers F and R2 produce a 408-base pair band (*Cis* deletion). *Stat5a*^{fl/+}Mx-Cre Ai3 and *Stat5a*^{fl/-}Mx-Cre Ai3 mice have been described⁵⁵⁻⁵⁸. *Stat6*^{-/-} mice (on the BALB/c background), BALB/c mice, *Il4ra*^{-/-} mice (on the B6 background) and *Rag1*^{-/-} mice (on the B6 background) were from the Jackson Laboratory. Animals were housed in a specific pathogen-free facility, and animal experiments were done with protocols approved by Institutional Animal Care and Use Committees of the MD Anderson Cancer Center and the University of New Mexico Health Sciences Center.

Immunoblot analysis. Total CD4⁺ T cells sorted by magnetic-activated cell sorting were activated for various times by plate-bound anti-CD3 (2C11; BioXCell) and anti-CD28 (37.51; BioXCell) in the presence or absence of IL-2 or anti-IL-4 (11B11; BioXCell) or anti-IL-6 (MP520F3; R&D Systems) and/or anti-IL-21 (AF594; R&D Systems). Whole-cell lysates were subjected to immunoblot analysis. The antibodies were as follows: anti-STAT1 (9172; Cell Signaling Technology), anti-phosphorylated STAT1 (9171; Cell Signaling Technology), anti-STAT3 (sc-482; Santa Cruz Biotechnology, Santa Cruz, CA), anti-phosphorylated STAT3 (91345; Cell Signaling Technology), anti-STAT5 (sc-835; Santa Cruz), anti-phosphorylated STAT5 (93515; Cell Signaling Technology), anti-STAT6 (sc-981; Santa Cruz), anti-phosphorylated STAT6 (sc-981-G; Santa Cruz) and anti-CIS (N19; sc-1529; Santa Cruz).

T cell differentiation. Naive CD4⁺CD25⁻CD62L^{hi}CD44^{lo} T cells were sorted by flow cytometry and activated with 1 μg/ml plate-bound anti-CD3 and 1 μg/ml soluble anti-CD28 in the presence of polarizing cytokines and/or blocking antibodies. For T_H1 polarization, 5 μg/ml anti-IL-4 (11B11; BioXCell) and 5 ng/ml IL-12 (Peprotech) were used. For T_H2 polarization, 5 μg/ml anti-IFN-γ (XMG1.2; BioXCell) and 5 ng/ml IL-4 (Peprotech) were used. For T_H9 polarization, 2 ng/ml TGF-β (Peprotech) and 20 ng/ml IL-4 were used. For T_H17 polarization, 20 ng/ml IL-6 (Peprotech) and 2.5 ng/ml TGF-β were used. For iT_{reg} differentiation, 5 ng/ml TGF-β and 50 unit/ml recombinant human IL-2 with or without anti-IL-4 were used.

Asthma induction. Allergic asthma was induced and analyzed as described³⁴.

Retroviral transduction. Flow cytometry-sorted naive CD4⁺CD25⁻CD62L^{hi}CD44^{lo} T cells from B6 mice were activated with anti-CD3 and anti-CD28 in the presence of anti-IFN-γ. Then, 24 h later, cells were transduced with pMXires-GFP vector or retrovirus encoding STAT5A1*6 (ref. 39) and were maintained in medium containing anti-IFN-γ with or without switching to the T_H9-polarization condition after infection on day 2.

Quantitative real-time RT-PCR. Gene expression was examined with a Bio-Rad iCycler Optical System with an iQ SYBR green real-time PCR kit (Bio-Rad Laboratories). Data were normalized to an *Actb* reference gene. The primers for CIS were as follows: forward, 5'-GGACATGGTCCTTTGCGTACAG, and reverse, 5'-GGAGAACGTCTTGCTATGCAC. The following genes were amplified with primers as described: *Cd25* (ref. 59); *Il9*, *Il4*, *Il5* and *Il13* (ref. 60); *Il10* (ref. 61); *Gata3*, *Irf4*, *Il17rb* and *Il2* (ref. 62); *Junb* (ref. 63); *Nfatc1p1* (ref. 64); and *Actb* and *Tbx21* (ref. 65).

ChIP. For ChIP analysis of STAT proteins, cytoplasmic STAT proteins were removed by cell fractionation by the following procedure: 3 × 10⁶ to 5 × 10⁶ CD4⁺ T cells were suspended in 500 μl of cold buffer containing 10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl and 0.5% NP-40, plus proteinase inhibitors, 0.5 mM PMSF, 2 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM Na₃VO₄. After incubation for 15–30 min on ice, the cells were spun for 5 min at 10,000 r.p.m. at 4 °C. The pellet (nuclear fraction) was then subjected to ChIP assay as described⁶⁶. The antibodies used were polyclonal anti-STAT5 (sc-835; Santa Cruz), polyclonal anti-STAT6 (sc-981; Santa Cruz) or control immunoglobulin G (sc-2763; Santa Cruz). The resulting DNA was analyzed by real-time PCR. The primers for STAT5-binding sites were as follows: *Il9* promoter, forward, 5'-AC TGATACCCAGTGGCCAC, and reverse, 5'-ACACAGACCTGGGCTTTCA; *Cis* promoter, forward, 5'-CACGTCAGTTCAGGGTCCCT, and reverse, 5'-CGTCTAGTGCTTTGGACCGA; and *Il4* HS2 primers as described⁴¹. The primers for STAT6-binding sites were as follows: *Il9* and *Cis* promoter primers, same as the primers for the STAT5-binding sites; *Il4* enhancer and *Gata3* promoter and CNS1 primers as described⁴². Negative control primers were as follows: *Cd4*, forward, 5'-TTGTGGCTGTTGCTTTTGGAG, and reverse, 5'-CCAGGCTGCTACAAGAGACC; *Apoe*, forward, 5'-GCC TAGCCGAGGGAGAGCCG, and reverse, 5'-TGTGACTTGGGAGCTCT GCAGC.

Luciferase reporter assay. Vector encoding STAT5A1*6 (ref. 39) or STAT6V1*67 or empty vector was transfected into EL4 T cells with a luciferase construct containing the *Il9* promoter (cloning primers: forward, 5'-GATCCTCAAGGCCAATGCT, and reverse, 5'-ACATGTTGAC GGGAGTCTGG) with or without PMA (phorbol 12-myristate 13-acetate; Sigma) and IL-4. Firefly and renilla luciferase activity were measured by dual-luciferase reporter system (Promega) and renilla luciferase was used to normalize transfection efficiency and luciferase activity.

Generation of mixed-bone marrow chimeras. Irradiated *Rag1*^{-/-} mice were reconstituted with T cell-depleted bone marrow from wild-type (CD45.1⁺) mice and *Cis*^{-/-} (CD45.2⁺) mice on the B6 background (1:1 mixture) as described⁶⁶. For induction of asthma, the chimeric mice were used 6 weeks later.

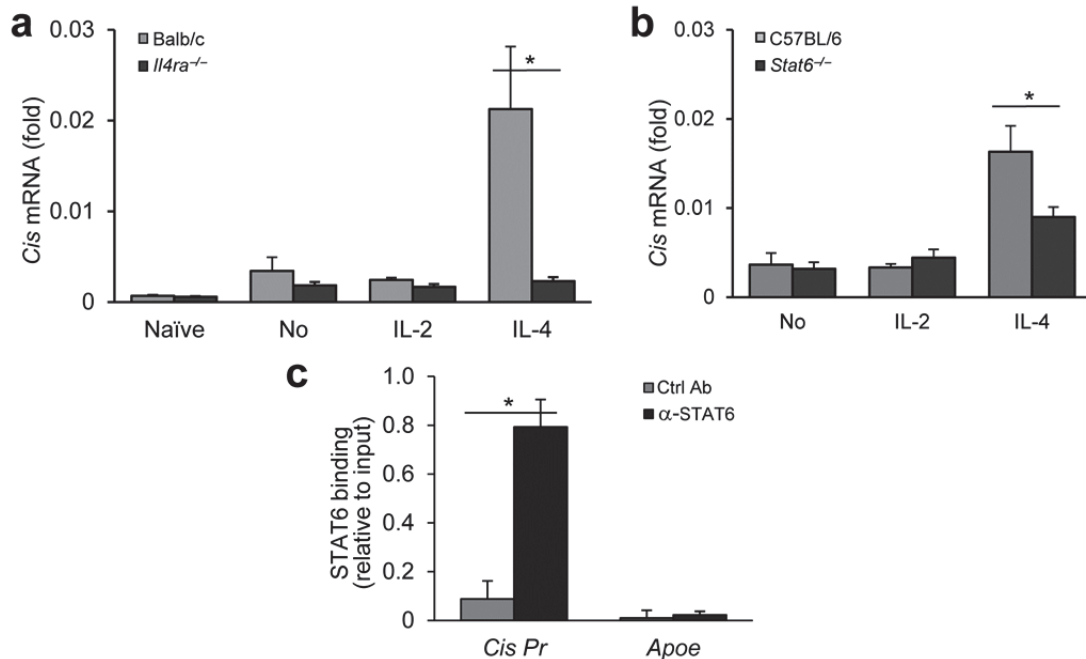
Statistical analysis. The statistical significance of differences between groups was calculated with the unpaired Student's *t* test. *P* values of 0.05 or less were considered significant.

53. Lee, P.P. *et al.* A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. *Immunity* **15**, 763–774 (2001).
54. Rubtsov, Y.P. *et al.* Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity* **28**, 546–558 (2008).
55. Dai, X. *et al.* Stat5 is essential for early B cell development but not for B cell maturation and function. *J. Immunol.* **179**, 1068–1079 (2007).
56. Fu, G. *et al.* Phospholipase Cγ1 is essential for T cell development, activation, and tolerance. *J. Exp. Med.* **207**, 309–318 (2010).
57. Kuhn, R., Schwenk, F., Aguet, M. & Rajewsky, K. Inducible gene targeting in mice. *Science* **269**, 1427–1429 (1995).
58. Madisen, L. *et al.* A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nat. Neurosci.* **13**, 133–140 (2010).
59. Hwang, E.S., White, I.A. & Ho, I.C. An IL-4-independent and CD25-mediated function of c-maf in promoting the production of Th2 cytokines. *Proc. Natl. Acad. Sci. USA* **99**, 13026–13030 (2002).
60. Angkasekwinai, P., Chang, S.H., Thapa, M., Watarai, H. & Dong, C. Regulation of IL-9 expression by IL-25 signaling. *Nat. Immunol.* **11**, 250–256 (2010).
61. Lang, R., Rutschman, R.L., Greaves, D.R. & Murray, P.J. Autocrine deactivation of macrophages in transgenic mice constitutively overexpressing IL-10 under control of the human CD68 promoter. *J. Immunol.* **168**, 3402–3411 (2002).

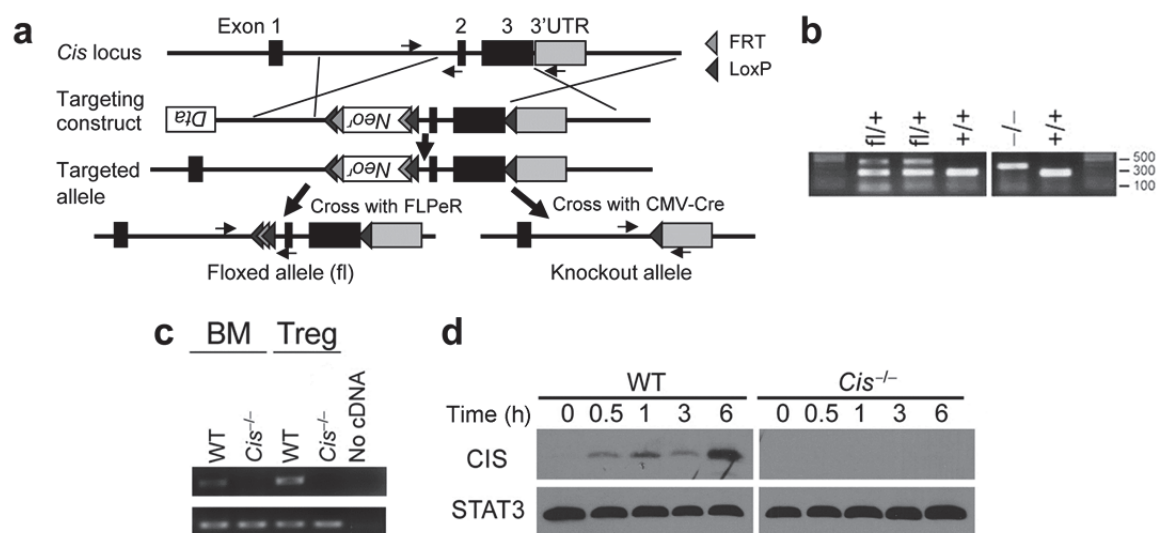
62. Yang, X.O. *et al.* Requirement for the basic helix-loop-helix transcription factor Dec2 in initial T_H2 lineage commitment. *Nat. Immunol.* **10**, 1260–1266 (2009).
63. Voice, J. *et al.* c-Maf and JunB mediation of Th2 differentiation induced by the type 2 G protein-coupled receptor (VPAC2) for vasoactive intestinal peptide. *J. Immunol.* **172**, 7289–7296 (2004).
64. Evans, K.E. & Fox, S.W. Interleukin-10 inhibits osteoclastogenesis by reducing NFATc1 expression and preventing its translocation to the nucleus. *BMC Cell Biol.* **8**, 4 (2007).
65. Yang, X.O. *et al.* STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *J. Biol. Chem.* **282**, 9358–9363 (2007).
66. Yang, X.O. *et al.* T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR α and ROR γ . *Immunity* **28**, 29–39 (2008).
67. Bruns, H.A., Schindler, U. & Kaplan, M.H. Expression of a constitutively active Stat6 *in vivo* alters lymphocyte homeostasis with distinct effects in T and B cells. *J. Immunol.* **170**, 3478–3487 (2003).

The signaling suppressor CIS controls proallergic T cell development and allergic airway inflammation

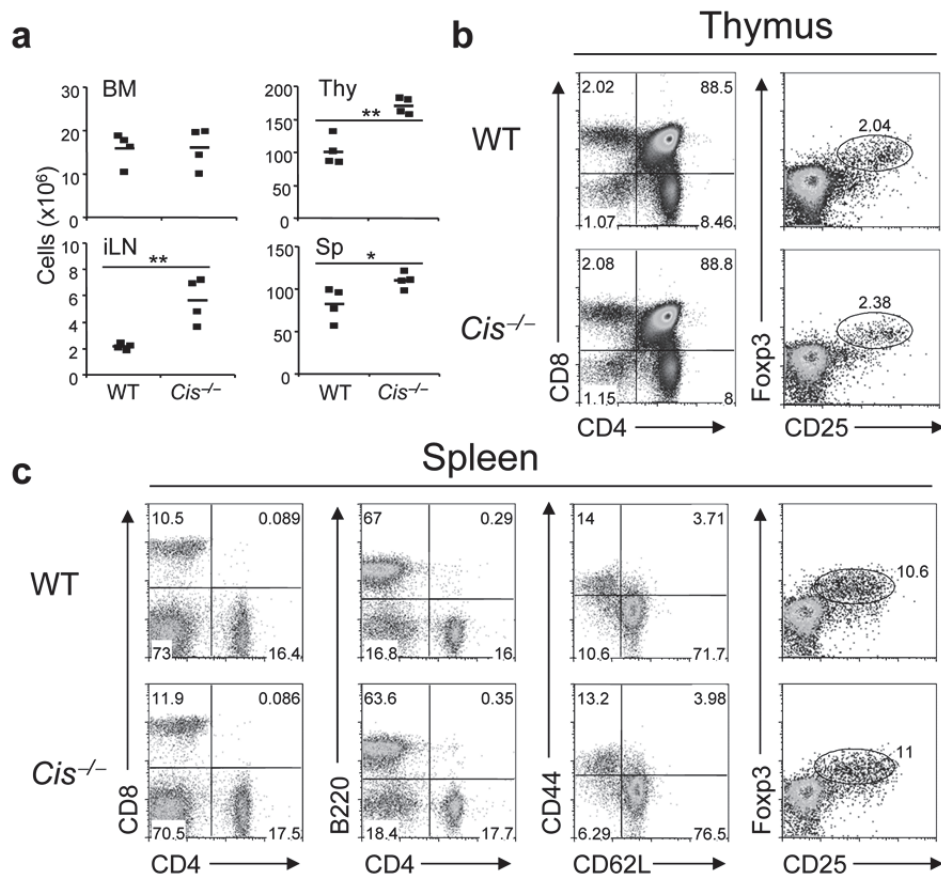
Xuexian O Yang, Huiyuan Zhang, Byung-Seok Kim, Xiaoyin Niu, Juan Peng, Yuhong Chen, Romica Kerketta, Young-Hee Lee, Seon Hee Chang, David B. Corry, Demin Wang, Stephanie S Watowich, and Chen Dong



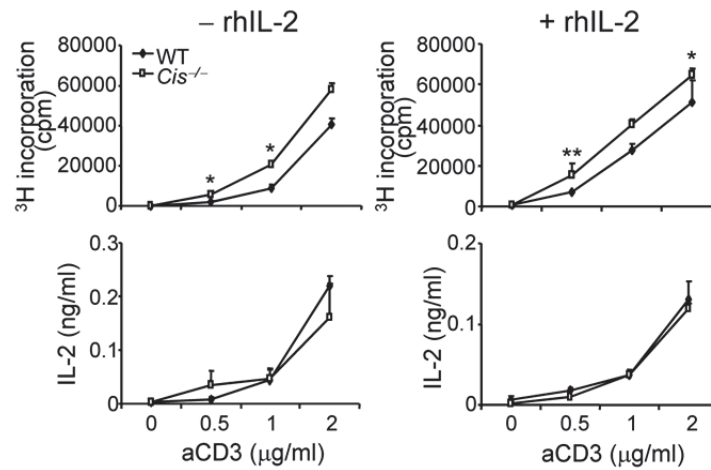
Supplementary Fig. 1. IL-4-induced *Cis* mRNA expression is dependent on IL-4R α and partially on STAT6. (**a**, **b**) Quantitative RT-PCR of *Cis* mRNA expression in naïve CD4⁺ T cells from Balb/c and *Il4ra*^{-/-} (**a**) or C57BL/6 and *Stat6*^{-/-} (**b**) mice activated with plate-bound anti-CD3 and anti-CD28 for 3 h. *Actb* was used as a loading control. Values are mean and s.d. *, student *t* test, $p \leq 0.05$. Data shown are a combination of 2 experiments. (**c**) Chromatin immunoprecipitation assay (ChIP) of STAT6 binding to the *Cis* promoter. Values (mean and s.d.) were measured by quantitative PCR with the *Apoe* locus as a negative control. Pr, promoter. *, student *t* test, $p \leq 0.05$. Data shown are a combination of 2 independent experiments.



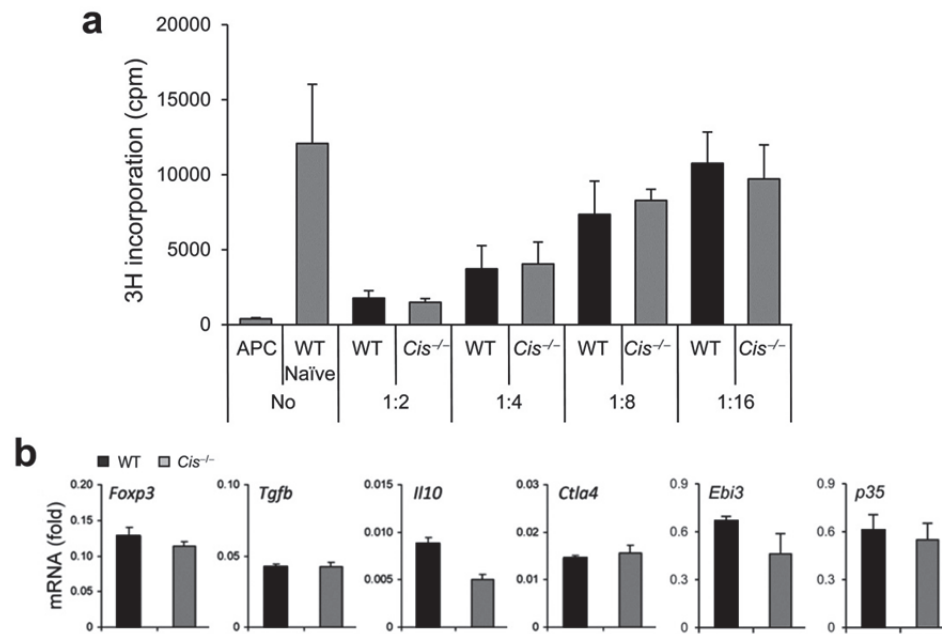
Supplementary Fig. 2. Generation of *Cis*^{-/-} mice. **(a)** Targeting strategy. **(b)** Genotyping of floxed (*fl*)/+, +/+, and -/- mice. **(c)** *Cis* mRNA expression in bone marrow (BM) and CD4⁺CD25⁺ regulatory T (T_{reg}) cells from *Cis*^{-/-} and WT mice was assessed by RT-PCR. **(d)** CIS protein expression. MACS sorted total CD4⁺ T cells were activated with plate bound anti-CD3 and anti-CD28 for various times and CIS and STAT3 protein expression was assessed by immunoblot.



Supplementary Fig. 3. Lymphocyte compartments in WT and *Cis*^{-/-} mice. **(a)** Cellularity of bone marrow (BM), Thymus (Thy), inguinal lymph nodes (iLN) and spleen (Sp) from WT and *Cis*^{-/-} mice. Student *t* test, *, $p \leq 0.05$; **, $p \leq 0.005$. **(b-c)** CD4⁺ and CD8⁺ T cell profiles in thymus **(b)** and spleen **(c)**. Data shown are a representative of 4 individual sex matched mice in each group at 8 weeks old. Numbers in dot plots indicate percentage. Data shown represent 2 independent experiments.



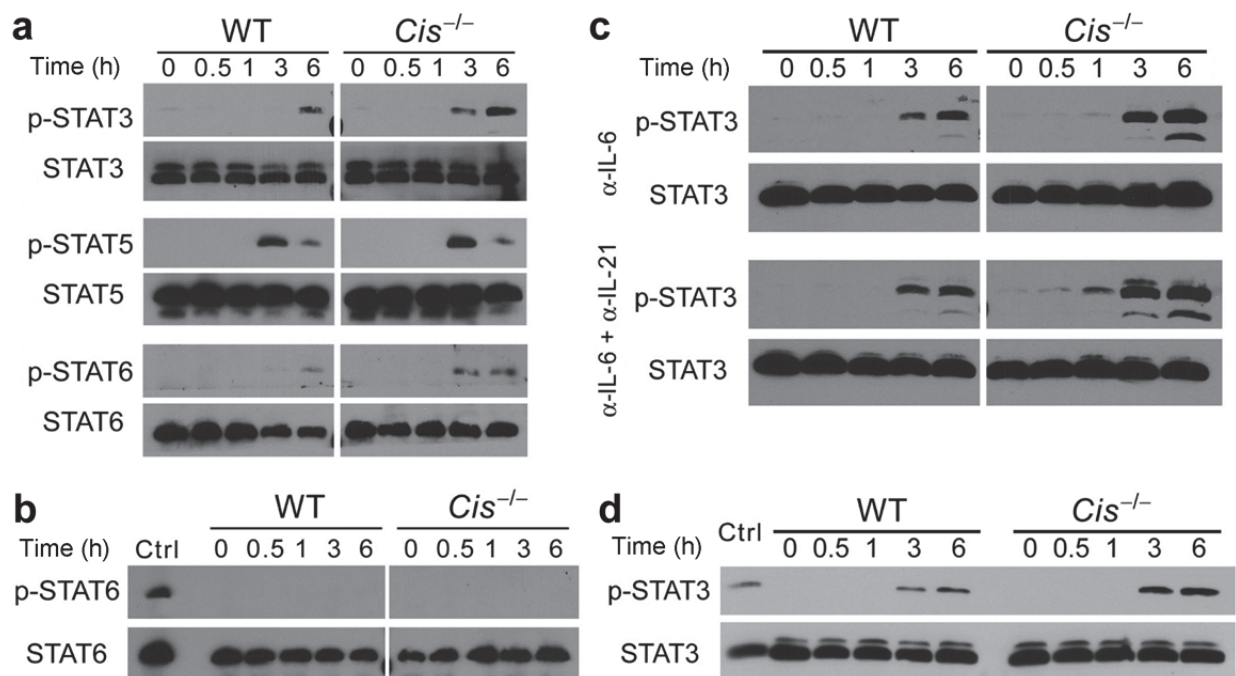
Supplementary Fig. 4. Cis-deficiency enhances CD4^+ T cell proliferation. Naïve CD4^+ T cells from WT and $Cis^{-/-}$ mice ($n = 3-4$ in each group) were activated with indicated concentrations of plate-bound anti-CD3 and fixed concentration of anti-CD28 ($1 \mu\text{g/ml}$) in the presence or absence of rhIL-2. Upper, ELISA of IL-2 production on day 2. Lower, ^3H -thymidine incorporation for the last 8 h on day 3. Student t test, *, $p \leq 0.05$; **, $p \leq 0.005$. Data shown represent at least 3 independent experiments.



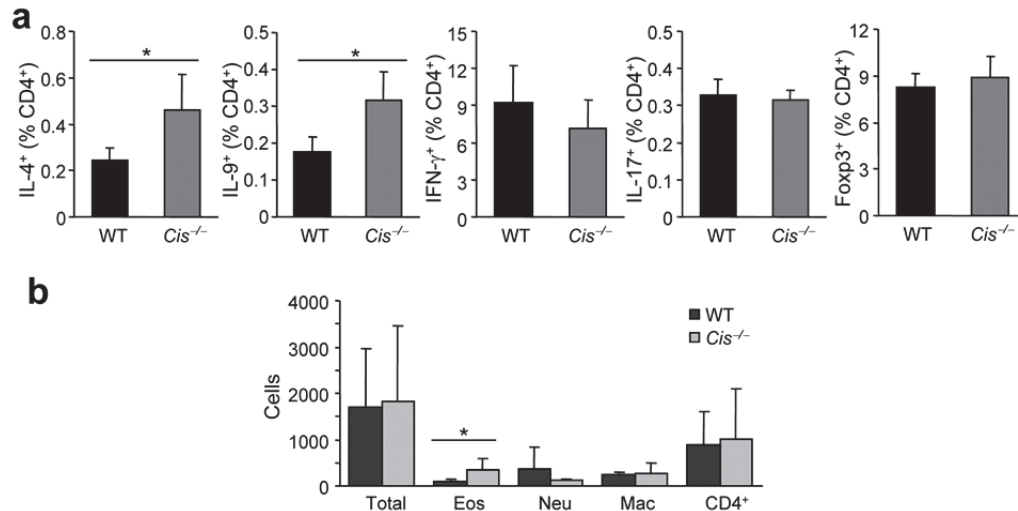
Supplementary Fig. 5. *Cis*-deficient natural regulatory T cells (nT_{reg}) remain repression

function. (a) T_{reg} cell repression assay. FACS sorted GFP⁺ regulatory T cells from WT × and *Cis*^{-/-} × Fcpx3-GFP reporter mice were co-cultured with WT naïve CD4⁺ T cells and irradiated splenic APCs for 3 d and proliferation was measured by ³H-thymidine incorporation. Ratios are T_{reg} to T_{eff} (effector). Data shown are a combination of 2 independent experiments. (b)

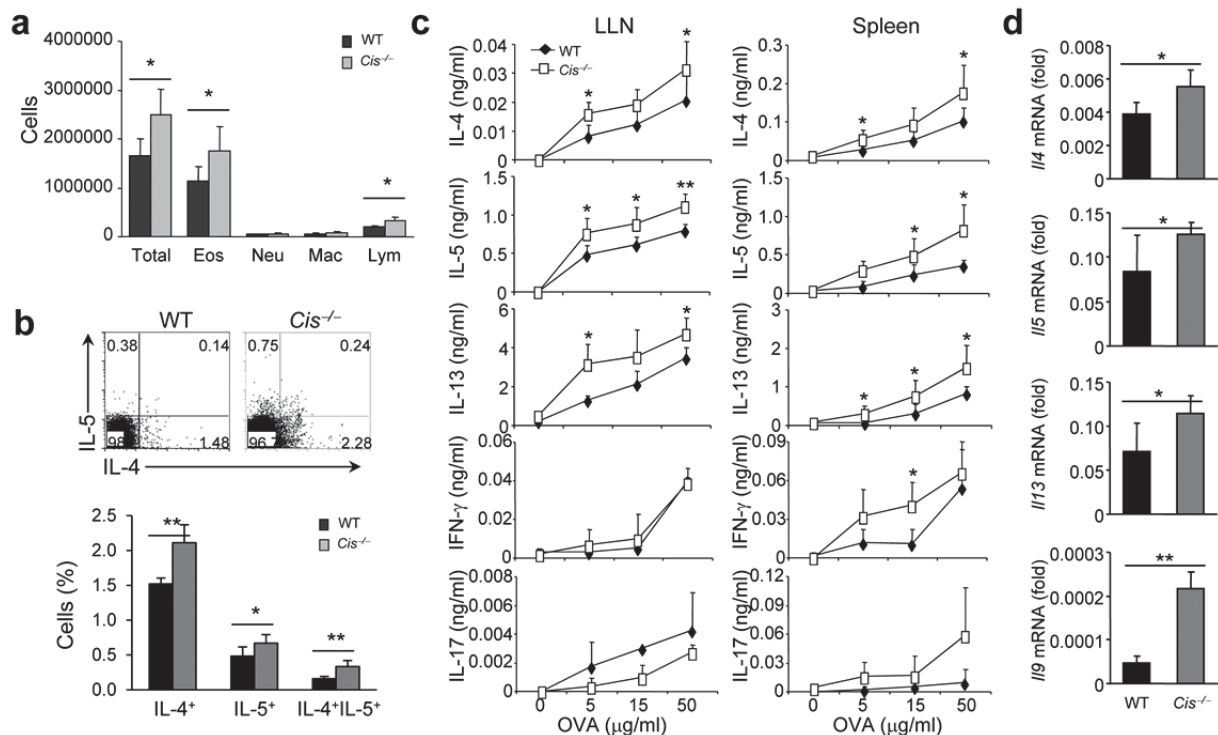
Quantitative real-time RT-PCR of gene expression in Fcpx3-GFP⁺ Treg cells FACS-sorted from *Cis*^{-/-} Fcpx3-GFP mice or their *Cis*^{+/+} Fcpx3-GFP littermates. Data are expressed as folds of the internal reference *Actb*. *Ebi3* and *p35* encode IL-35.



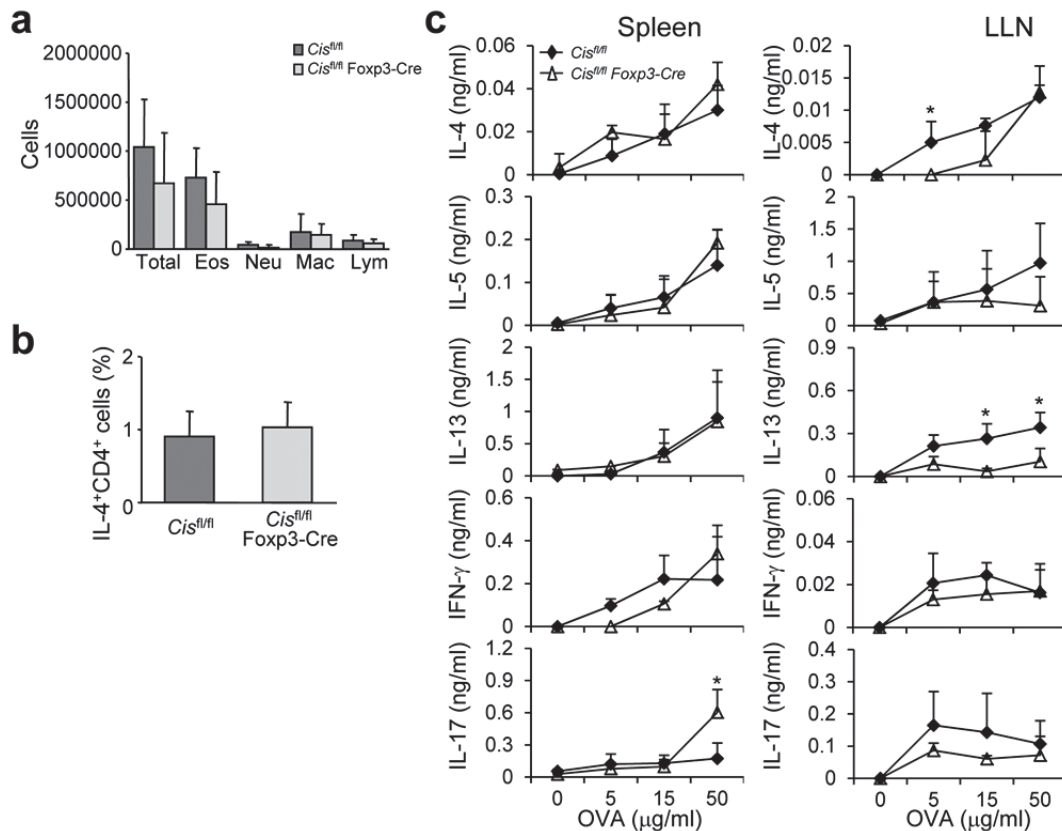
Supplementary Fig. 6. Regulation of STAT phosphorylation by CIS. Immune blot of total or phospho-STATs in WT and *Cis*^{-/-} CD4⁺ T cells activated by plate-bound anti-CD3 and anti-CD28 in the absence of recombinant human (rh) IL-2 (**a**), or in the presence of neutralizing antibody anti-IL-4 (**b**), or anti-IL-6 or anti-IL-6 + anti-IL-21 (**c**) for various times. (**d**) Blockade of IL-4 did not affect STAT3 activation. Data shown represent 2 independent experiments.



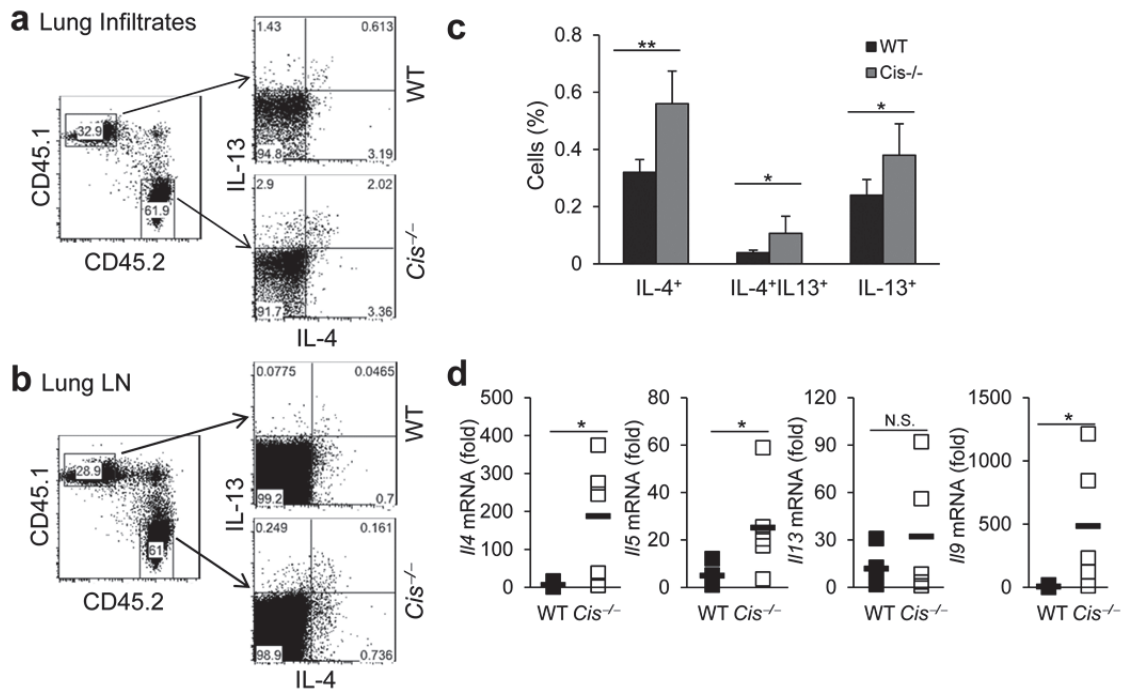
Supplementary Fig. 7. *Cis*-deficiency resulted in increased T_H2 and T_H9 cell responses in spleen and enhanced eosinophil influx in lung. **(a)** Intracellular staining of cytokine and Foxp3 positive cells in splenocytes from 6-month old *Cis*^{-/-} and WT mice. Data shown are on a CD4⁺ gate. **(b)** Cellular profile of lung infiltrates. Eos, eosinophil; Neu, neutrophil; Mac, macrophage. Values (mean and s.d.) were calculated from 4-5 individual animals in each group. Student *t* test, *, $p \leq 0.05$; **, $p \leq 0.005$. Data shown represent 2 independent experiments.



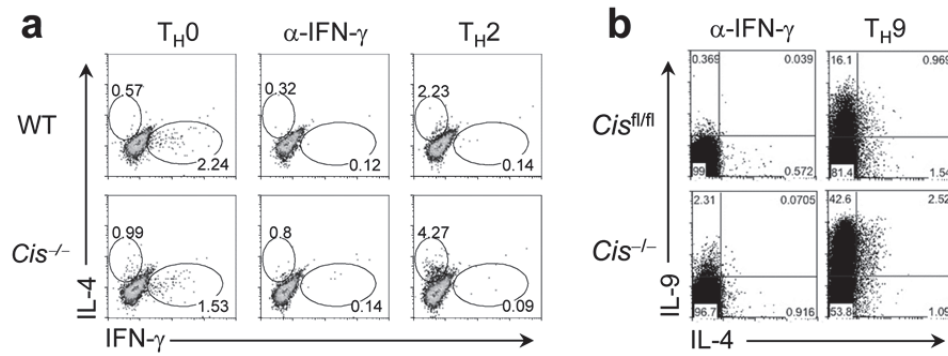
Supplementary Fig. 8. CIS negatively regulates allergic asthma disease. Allergic asthma was induced in *Cis*^{-/-} and WT mice ($n = 4-5$ in each group). **(a)** Profiles of lung infiltrates from the asthmatic mice. Eos, eosinophil; Neu, neutrophil; Mac, macrophage; Lym, lymphocyte. **(b)** FACS analysis of T_H2 cytokine expression in lung infiltrates on a CD4⁺ gate. Numbers in the quadrants indicate percentage of positive cells. **(c)** ELISA analysis of T_H2 cytokine production in lung-associated lymph node cells or splenocytes stimulated with various concentrations of OVA protein for 3 d. **(d)** Quantitative RT-PCR analysis of T_H2 cytokine production in lung tissues. Data are expressed as folds of the internal reference *Actb*. Values are means and s.d. Student *t* test, *, $p \leq 0.05$; **, $p \leq 0.005$. Data shown represent 3 independent experiments.



Supplementary Fig. 9. CIS regulates allergic responses independent of its expression in regulatory T cells. Allergic asthma was induced in *Cis^{fl/fl} Foxp3-Cre* and *Cis^{fl/fl}* mice ($n = 4-5$ in each group). **(a)** Profiles of lung infiltrates from the asthmatic mice. Eos, eosinophil; Neu, neutrophil; Mac, macrophage; Lym, lymphocyte. **(b)** FACS analysis of T_H2 cytokine expression in lung infiltrates on a CD4⁺ gate. Numbers in the quadrants indicate percentage of positive cells. **(c)** ELISA analysis of T_H2 cytokine production in lung-associated lymph node cells or splenocytes stimulated with various concentrations of OVA protein for 3 d. Values are means and s.d. Student *t* test, *, $p \leq 0.05$; **, $p \leq 0.005$. Data shown represent 2 independent experiments.



Supplementary Fig. 10. T cell intrinsic defect in CIS leads to enhanced T_H2 and T_H9 responses. Asthma was induced in *Rag1*^{-/-} mice reconstituted with 1:1 mix of T cell-depleted bone marrow cells from WT (CD45.1⁺) and *Cis*^{-/-} (CD45.2⁺) mice. **(a, b)** Intracellular staining of IL-4 and IL-13 expression in WT and *Cis*^{-/-} CD4⁺ T cells in lung infiltrates **(a)** and draining lymph nodes **(b)** on a CD4⁺ gate. Numbers in the quadrants indicate percentage of positive cells. **(c)** Statistical analysis of IL-4⁺ and IL-13⁺ CD4⁺ cells in draining lymph nodes from WT and *Cis*^{-/-} mice. **(d)** Quantitative RT-PCR of mRNA expression of indicated cytokine genes in FACS-sorted WT and *Cis*^{-/-} CD4⁺ T cells. **(c, d)** Values are means and s.d. n = 5 in each group. Student *t* test, *, *p* ≤ 0.05; **, *p* ≤ 0.005. Data shown represent 2 independent experiments.



Supplementary Fig. 11. CIS negatively regulates T_H2 and T_H9 cell differentiation. **(a)** Intracellular staining of IL-4 and IFN- γ expression in *Cis*^{-/-} or WT T_H cells polarized under neutral (T_H0), anti-IFN- γ or T_H2 conditions. **(b)** Intracellular staining of IL-9 and IL-4 expression in *Cis*^{-/-} or WT T_H cells polarized in the presence of anti-IFN- γ or under the T_H9 condition. Numbers in the quadrants indicate percentage of positive cells.