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

Xuexian O Yang[1,2], **Huiyuan Zhang**[1,8], **Byung-Seok Kim**[1,8], **Xiaoyin Niu**[1,3], **Juan Peng**[1,4], **Yuhong Chen**[5], **Romica Kerketta**[2], **Young-Hee Lee**[1], **Seon Hee Chang**[1], **David B Corry**[6], **Demin Wang**[5], **Stephanie S Watowich**[1], and **Chen Dong**[1,7]

[1]Department of Immunology and Center for Inflammation and Cancer, Maryland Anderson Cancer Center, Houston, Texas, USA

[2]Department of Molecular Genetics and Microbiology, University of New Mexico School of Medicine, Albuquerque, New Mexico, USA

[3]Shanghai Jiao Tong University School of Medicine and Shanghai Institute of Immunology, Shanghai, China

[4]College of Basic Medicine, Wuhan University, Wuhan, China

[5]Blood Research Institute, Blood Center of Wisconsin, Milwaukee, Wisconsin, USA

[6]Department of Medicine and Department of Pathology and Immunology, Baylor College of Medicine, Houston, Texas, USA

[7]Tsinghua University School of Medicine, Beijing, China

Abstract

Transcription factors of the STAT (signal transducer and activator of transcription) family are critical in the cytokine-mediated functional differentiation of $CD4^+$ helper T cells. Members of the SOCS (suppressor of cytokine signaling) 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 direct binding to its gene. Our data thus demonstrate a critical role for CIS in controlling proallergic generation of helper T cells.

Correspondence should be addressed to C.D. (cdong@mdanderson.org) or X.O.Y (xyang@salud.unm.edu).
[8]These authors contributed equally to this work.

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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.

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$CD4^+$ 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 helper T cells into the T_H1, T_H2 and T_H17 and T_H9 subsets 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 the 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 αchain of the receptor for IL-4 (IL-4Rα), the main STAT6-activating component of the IL-4 and IL-13 receptors[4–6]. T_H17 differentiation is mediated by the combination of signaling via IL-6 and transforming growth factor-β (TGF-β)[7]. IL-6, as well as IL-23 and IL-21, supports T_H17 development via STAT3 (ref. 7), whereas IL-2–STAT5 signaling negatively regulates T_H17 differentiation[8]. IL-9-expressing T_H9 cells also require TGF-β, in addition to IL-4, for their generation[9–11]; the mechanisms of this remain unclear.

Thus, cytokine-mediated activation of STAT proteins has a pivotal role in the differentiation of helper T cells. Moreover, overactivation of STATs 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, proteins 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 disease[15]. 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 cells[21], 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 to suppress both T_H1 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 STAT6 activation and T_H2 differentiation during T_H1 differentiation[28], the absence of SOCS5 from

$CD4^+$ T cells does not seem to alter normal T_H1 or T_H2 differentiation[29]. 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 receptor for erythropoietin and the receptor for 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 C67BL/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-2Rα (*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 that peaked at 48 h (Fig. 1a). These results suggested that at the early phase, *Cis* expression was independent of endogenous IL-2 production and expression of the high-affinity IL-2 receptor CD25 (IL-2Rα).

Next we assessed expression of *Cis* mRNA in response to a variety of cytokines that are important in T cell differentiation. After 3 h 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-4Rα (*Il4ra*) abrogated IL-4-induced *Cis* mRNA expression (Supplementary Fig. 1a). However, in STAT6-deficient cells, IL-4-dependent expression of *Cis* mRNA 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 Treg cells (nT_{reg} cells) and moderate expression in T_H9 cells (Fig. 1c). In T_H17 cells, *Cis* mRNA expression was similar to that in naive cells (Fig. 1c). Together these data suggested possible involvement of CIS in the regulation of cytokine signals 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 knockout of *Cis*. We introduced two *lox*P 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 *lox*P-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 with C57BL/6 mice for seven to eight generations and then interbred the offspring 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 than their wild-type counterparts (Supplementary Fig. 3 and data not shown). Also, the development of $CD4^+CD25^+Foxp3^+$ natural T_{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, naturally occurring $Cis^{-/-}$ T_{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 natural T_{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 showed an increase in phosphorylated STAT3, STAT5 and STAT6 when compared with WT cells at various times, whereas the abundance of phosphorylated STAT1 remained the same (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. Next, 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 and *Cis*-deficient cells. However, neutralization of IL-6 and/or IL-21 did not affect the activation of STAT3

(Supplementary Fig. 6c). 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.

CIS deficiency causes spontaneous pulmonary disease

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. Splenocytes 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 in the two strains (Fig. 2a and Supplementary Fig. 7a). These data suggested that CIS may negatively regulate proallergic differentiation of T cells *in vivo*.

We prepared lung, heart, liver, kidney and intestine tissue sections from 10-month-old wild-type and $Cis^{-/-}$ mice and stained the sections with hematoxylin and eosin. We observed no difference between genotypes for any tissues tested except that in contrast to wild-type lungs, $Cis^{-/-}$ lungs exhibited lung 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-months-old $Cis^{-/-}$ mice and included more severe airway obstruction due to mucus impaction and scattered mononuclear cell infiltration (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 eosinophil infiltrates $Cis^{-/-}$ lungs than in wild-type lungs (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 $Cis^{-/-}$and $Cis^{fl/fl}$ (or wild-type littermate as indicated) control mice by a standard protocol[34]. $Cis^{-/-}$mice had significantly more total cells, eosinophils and lymphocytes in lung infiltrates than did CIS-sufficient mice (Fig. 3a and Supplementary Fig. 8a). There were more $IL\text{-}4^+$ or $IL\text{-}5^+$ $CD4^+$ 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 expressed more *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 *ex vivo* restimulation 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 than in Cis$^{-/-}$ mice (Fig. 3c,d). This might have partially resulted from the higher expression of IL-10 observed in T_H2 cells, which would further sustain the T_H2 polarization of cells, as reported in an allergic skin model[35]. 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*, *Il13* 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 together 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 them 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), 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 the 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). This 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 predicted from the *in vivo* data, by intracellular staining or enhanced immunosorbent assay (ELISA), CIS deficiency led to enhanced T_H2 differentiation and T_H2 cytokine expression 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 expressed not only more mRNA encoding T_H2 cytokines (*Il4*, *Il5*, *Il13* and *Il10*) but also more 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* deficiency 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 in the absence of CIS, *Il9* mRNA expression was much higher (Fig. 6a). Next we polarized naive CD4$^+$ T cells from wild-type (Fig. 6b) (or *Cis*$^{fl/fl}$, Supplementary Fig. 11b) mice and *Cis*$^{-/-}$ mice to differentiate into T_H9 cells and found that *Cis*$^{-/-}$cell populations had many more IL-9 expressing cells. 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). Third, 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 in these conditions (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 is important in T_H9 differentiation in addition to 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 *lox*P-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 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 report[40]. *Stat6*$^{-/-}$ cell populations also had a lower frequency of IL-4^+ cells than did their wild-type counterparts, as predicted, 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 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 region[41], whereas STAT6 binds to the *Il4* and *Gata3* loci[42]. By comparison of mouse and human genomic DNA sequences with the ECR browser (of evolutionary conserved regions)[43] and rVISTA program[44], we found potentially overlapping STAT5- and STAT6-binding sites (TGCCAGGAAATACCATTCCTCAGA 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 *Il4* locus HS2 (DNase I hypersensitive site 2) and *Il9* promoter (Fig. 7d). Furthermore, as shown by the use of anti-STAT6, CIS deficiency led to significantly enhanced binding of STAT6 to the *Gata3* promoter and conserved noncoding sequence CNS1, the *Il4* locus enhancer and the *Il9* promoter (Fig. 7e). Furthermore, in a luciferase reporter assay, STAT5 and STAT6 trans-activated 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) (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 the T_H9 subset of helper T 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 diseases.

SOCS expression is induced by cytokine signals, activated STAT mainly through 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 progenitor activation of helper T cells, 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-4Rα-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 mediate IL-4-induced CIS expression. *Cis* has been shown to be induced by STAT5 in liver cells[31], erythroid progenitor cells[30] and human T lymphocytes[33]. 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 is the major inducer of CIS at 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 of T_H17 cells. As for inducible T_{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 an dispensable role for CIS in $Foxp3^+$ T_{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, as 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 STAT6 activation. However, blockade of IL-6 or/and IL-21 did not change the activation of STAT3 in wild-type or CIS-deficient cells. At this time, 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, and this may have been due to the enhanced signaling via STAT5, a negative regulator of T_H17 differentiation[8], 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 (*Sfpi1*), 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 had not been identified before, to our knowledge. We found that in the presence of IFN-γ, blockade of IL-4 or IL-2 resulted in a much lower frequency of CIS-deficient IL-9^+ cells, whereas we observed no wild-type IL-9^+ cells, which suggested involvement of IL-2–STAT5 and/or IL-4–STAT6 signaling 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 constitutive activation 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 differentiation[40]. Moreover, we also demonstrated that in addition to binding to genes of the T_H2 signature (*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 enhanced eosinophil influx 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 increased eosinophils in lungs. This may have been due to chronically enhanced 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 further augment T_H2 and T_H9 responses. Moreover, IL-9 also promotes IL-13 expression in airway epithelial cells[50], which further amplifies the effect of IL-13. Notably, we did not observe the spontaneous airway remodeling in older *Cis*$^{fl/fl}$CD4-Cre mice (1 year of age; data not shown), which suggested that enhanced T_H2 and T_H9 responses are 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 a role. 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 needs further investigation.

In summary, we found that CIS, induced by IL-4 (and also IL-2 at the late phase), attenuated IL-2–STAT5 and IL-4–STAT6 signaling 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 CIS-STAT5-STAT6 pathway in T_H2 and T_H9 differentiation. Our results may help elucidate the pathogenesis of human allergic diseases.

ONLINE METHODS

Mice

For the generation of CIS-deficient mice and mice with *lox*P-flanked *Cis* alleles, a targeting vector was constructed by the introduction of two *lox*P sites into the *Cis* locus with a neomycin--resistance cassette (*neo*^{r}; positive selection marker) and sequence encoding diphtheria toxin A (negative selection marker; Supplementary Fig. 2). Targeted 129S6/SvEvTac derived TC-1 embryonic stem cell clones were selected and injected into C57BL/6 (B6) blastocysts to generate chimeras. Chimeras wih high percentage of 129 cells were bred with B6 mice for germline transmission. Targeted mice were crossed with FLPeR 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 *lox*P-flanked conditional *Cis* allele (*Cis*$^{fl/+}$). The *Cis*$^{fl/+}$ 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*$^{fl/+}$ 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*$^{fl/+}$ mice were bred with CD4-Cre mice[53] or Foxp3-Cre mice[54] to generated 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 (*lox*P-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 described[55,56,57,58]. *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 Committee 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) or/and 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 or/and 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 described[34].

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 pMX-ires-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′-GGAGAACGTCTTGGCTATGCAC. *Cd25* (ref. 59), *Il9*, *Il4*, *Il5*, and *Il13* (ref. 60), *Il10* (ref. 61), *Gata3*, *Irf4*, *Il17rb*, *Il2* (ref. 62), *Junb* (ref. 63), *Nfatc1p1* (ref. 64), *Actb*, *Tbx21* (ref. 65) were amplified as described before.

ChIP

For ChIP analysis of STAT proteins, cytoplasmic STAT proteins were removed by cell fractionation by the following procedure: 3×10^6 to 5×10^6 $CD4^+$ T cells were suspended in 500 μl of cold buffer containing 10 mM HEPES, pH7.9, 1.5 mM $MgCl_2$, 10 mM KCl and 0.5% NP-40, plus proteinase inhibitors, 0.5 mM PMSF, 2 μg/ml aprotenin, 10 μg/ml leupeptin and 1 mM Na_3VO_4. 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 described[66]. 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′-ACTGATACCCAGTGCCCAC, and reverse, 5′-ACACAGACCTGGGCTTTCA; *Cis* promoter, forward, 5′-

CACGTCAGTTCAGGGTCCCT, and reverse, 5′-CGTCTAGTGCTTTGGACCGA; and *Il4* HS2 primers as described[41]. 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 described[42]. Negative control primers were as follows: *Cd4*, forward, 5′-TTGTGGCTGTTGCTTTTGAG, and reverse, 5′-CCAGGCTGCTACAAGAGACC; *Apoe*, forward, 5′-GCCTAGCCGAGGGAGAGCCG, and reverse, 5′-TGTGACTTGGGAGCTCTGCAGC.

Luciferase reporter assay

Vector encoding STAT5A1*6 (ref. 39) or STAT6VT[67] or empty vector was transfected into EL4 T cells with luciferase constructs containing the *Il9* promoter (cloning primers: forward, 5′-GATCCTCAAGGCCAATGCT, and reverse, 5′-ACATGTTGACGGGAGTCTGG) 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 nd *Cis*$^{-/-}$ (CD45.2^{+}) mice on the B6 background (1:1 mixture) as described[66]. For induction of asthma, the chimeric mice were used 6 weeks later.

Statistical analysis

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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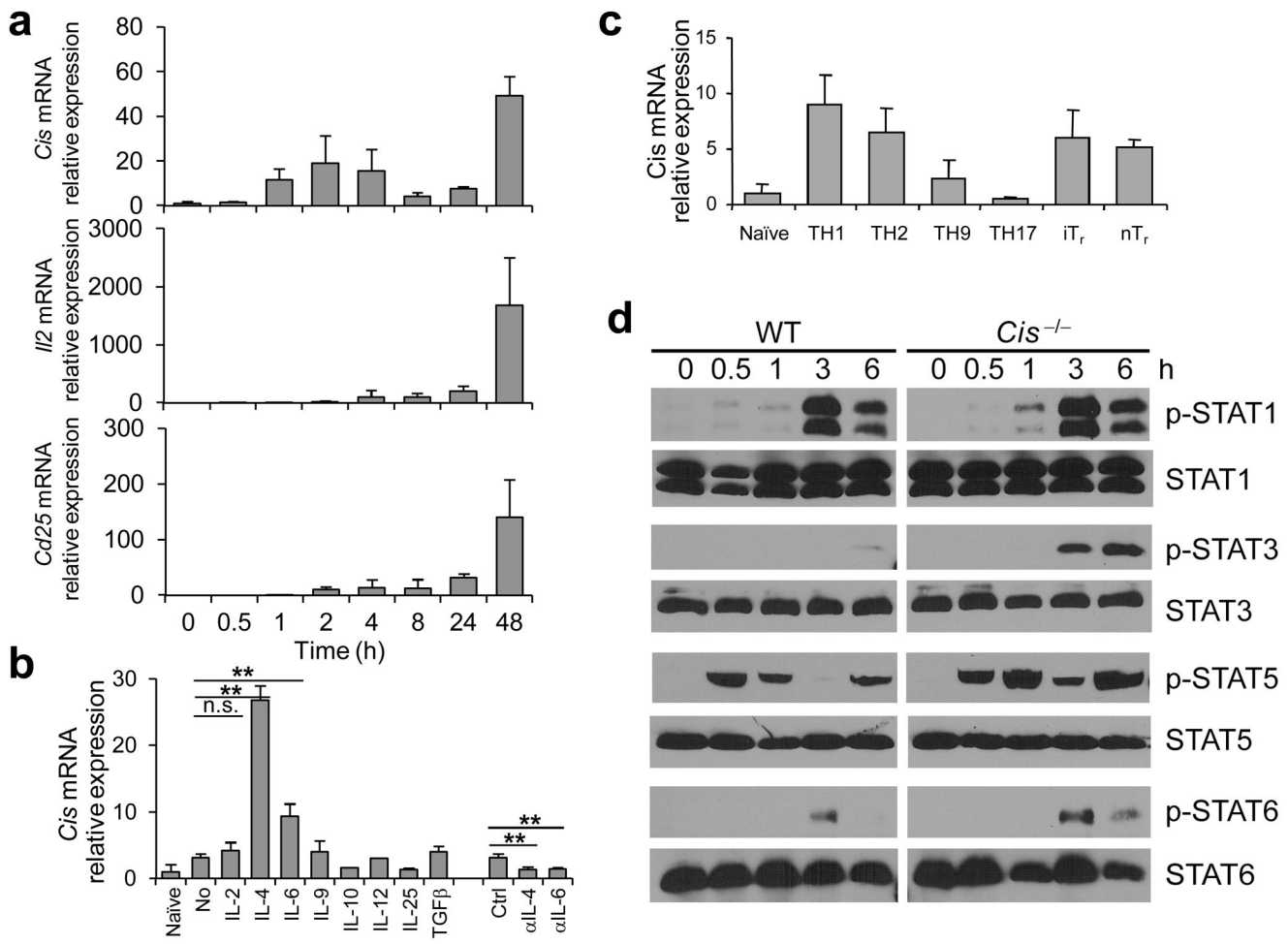

Figure 1.

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. (**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-), normalized as in **a** and presented relative to the lowest detectable level, set as 1. 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 and iT_{reg} cells and nT_{reg} cells, presented as in **b**. (**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**).

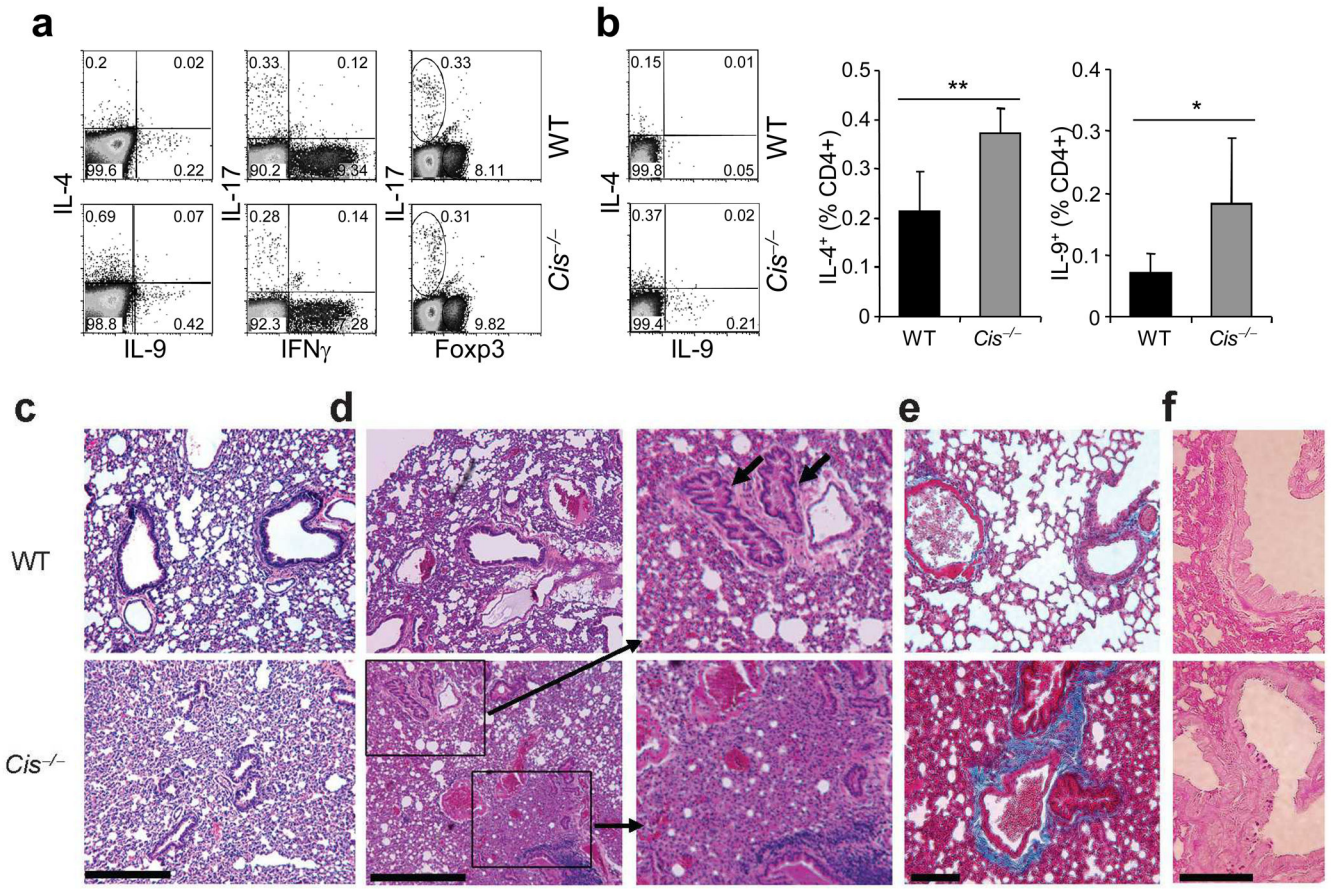

Figure 2.

CIS deficiency leads to spontaneous pulmonary diseases. (**a**,**b**) Intracellular staining of cytokine- and Foxp3-expressing splenocytes (**a**) and lung-associated lymph node cells (**b**) from 6-month-old wild-type and *Cis*$^{-/-}$ mice, presented on a CD4^{+} gate. Numbers in quadrants indicate percent cells in each throughout. N = 4–5 per group. *$P \leq 0.05$ and **$P \leq 0.005$ (Student's *t*-test). (**c**,**d**) Hematoxylin-and-eosin staining of lung sections prepared from 10-month old (**c**) or 18-month old (**d**) wild-type and *Cis*$^{-/-}$ mice. Right (**d**), enlargement of outlined area at left; arrows indicate airway smooth-muscle hyperplasia in collapsed airway (top right); bottom right, lung parenchymal consolidation and mononuclear cell inflammatory infiltrate. (**e**,**f**) Staining of wild-type and *Cis*$^{-/-}$ lung sections 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 μm (**e**,**f**). (**c**–**f**) WT, n = 3–4; *Cis*$^{-/-}$, n = 4. Data are representative of two independent experiments (mean and s.d. in **a**–**c**).

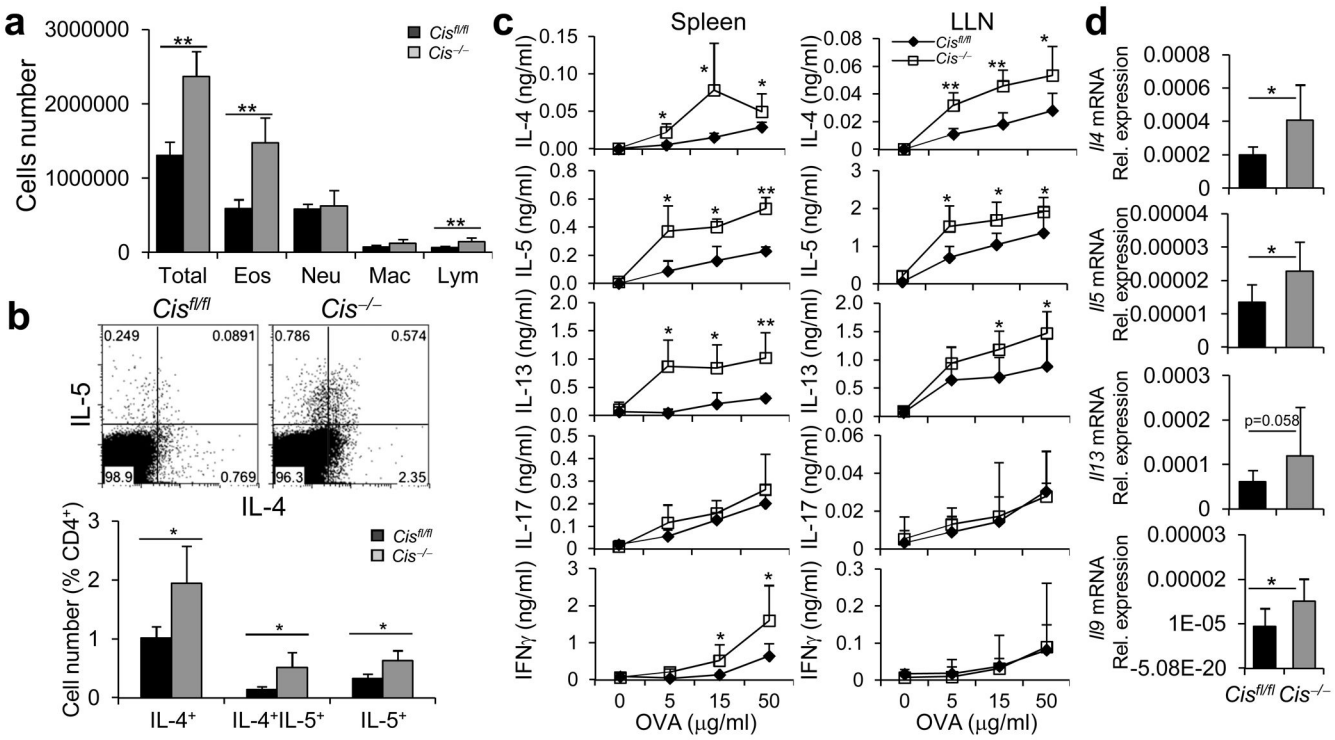

Figure 3.

CIS negatively regulates allergic asthma responses. (**a**) Quantification of cells in bronchoalveolar lavage fluid and lung infiltrates from *Cis*$^{-/-}$ and *Cis*$^{fl/fl}$ mice in which allergic asthma was induced (n = 4–5 per group). 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 T_H2 cytokines in splenocytes (Spleen) or cells from the lung-draining lymph nodes (LLN) of mice as 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 mice in **a**, presented relative to the internal reference *Actb*. *$P \leq 0.05$ and **$P \leq 0.005$ (Student's t-test). Data represent three independent experiments (mean and s.d.).

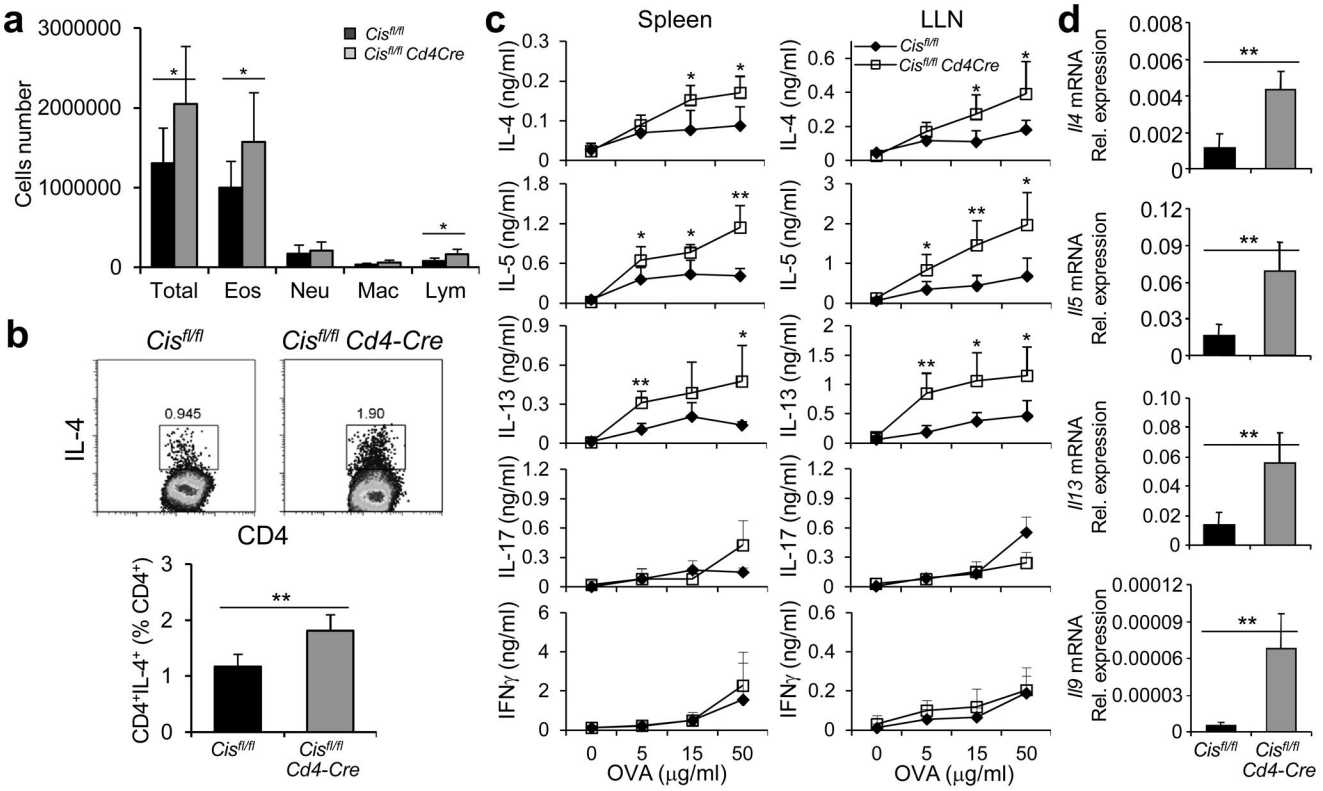

Figure 4.

CD4$^+$ T cell–specific *Cis* deficiency 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 mice in **a**, assessed by flow cytometry on a CD4$^+$ gate. Numbers adjacent to outlined areas (top) indicate percent IL-5$^+$CD4$^+$ cells. (**c**) ELISA of T_H2 cytokines in cells from lung-draining lymph nodes or splenocytes from 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 mice in **a** (presented as in Fig. 3d). *$P \leq 0.05$ and **$P \leq 0.005$ (Student's t-test). Data represent two independent experiments (mean and s.d.).

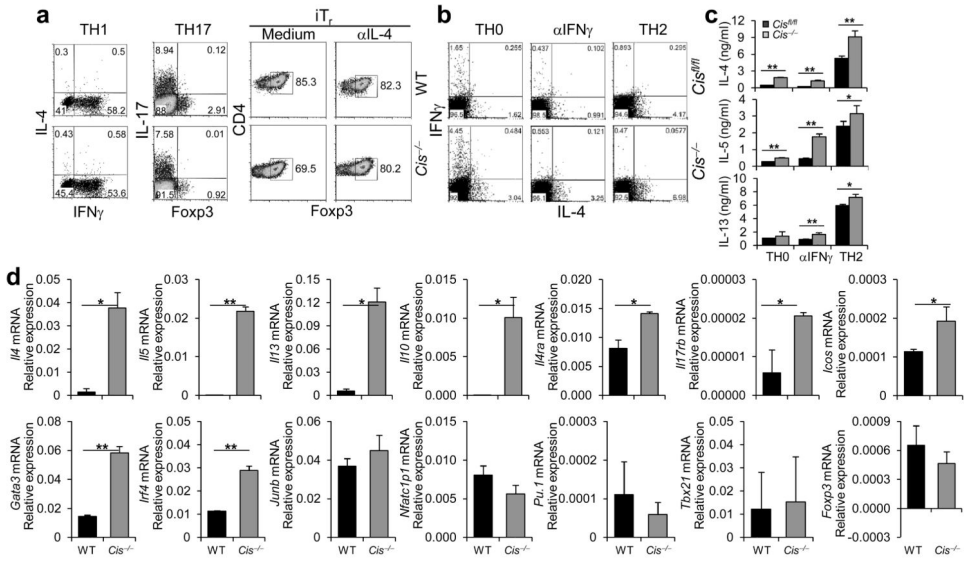

Figure 5.

CIS function in helper T cell differentiation. (**a**) Intracellular staining of wild-type and *Cis*$^{-/-}$ CD4^{+} T cells polarized under T_H1 or T_H17 conditions or iT_{reg} conditions without (Med) or with (α-IL-4) anti-IL-4. Numbers adjacent to outlined areas (right) indicate percent CD4^{+}Foxp3^{+} cells. (**b**) Intracellular staining of *Cis*$^{fl/fl}$ and *Cis*$^{-/-}$ helper T cells polarized under neutral (T_H0), anti-IFN-γ or T_H2 conditions. (**c**) ELISA of T_H2 cytokines in the cells in **b**. (**d**) Quantitative RT-PCR analysis of mRNA in CD4^{+} T cells from *Cis*$^{-/-}$ or wild-type mice activated for 4 d with plate-bound anti-CD3 and anti-CD28 in the presence of anti-IFN-γ (presented as in Fig. 3d); *Nfatc1p1*, transcript of the gene encoding the transcription factor NFATc, from the inducible P1 promoter. *$P \leq 0.05$ and **$P \leq 0.005$ (Student's *t*-test). Data represent two (**a**,**d**) or three (**b**) independent experiments (mean and s.d. in **d**) or are from three independent experiments (**c**; mean and s.d.).

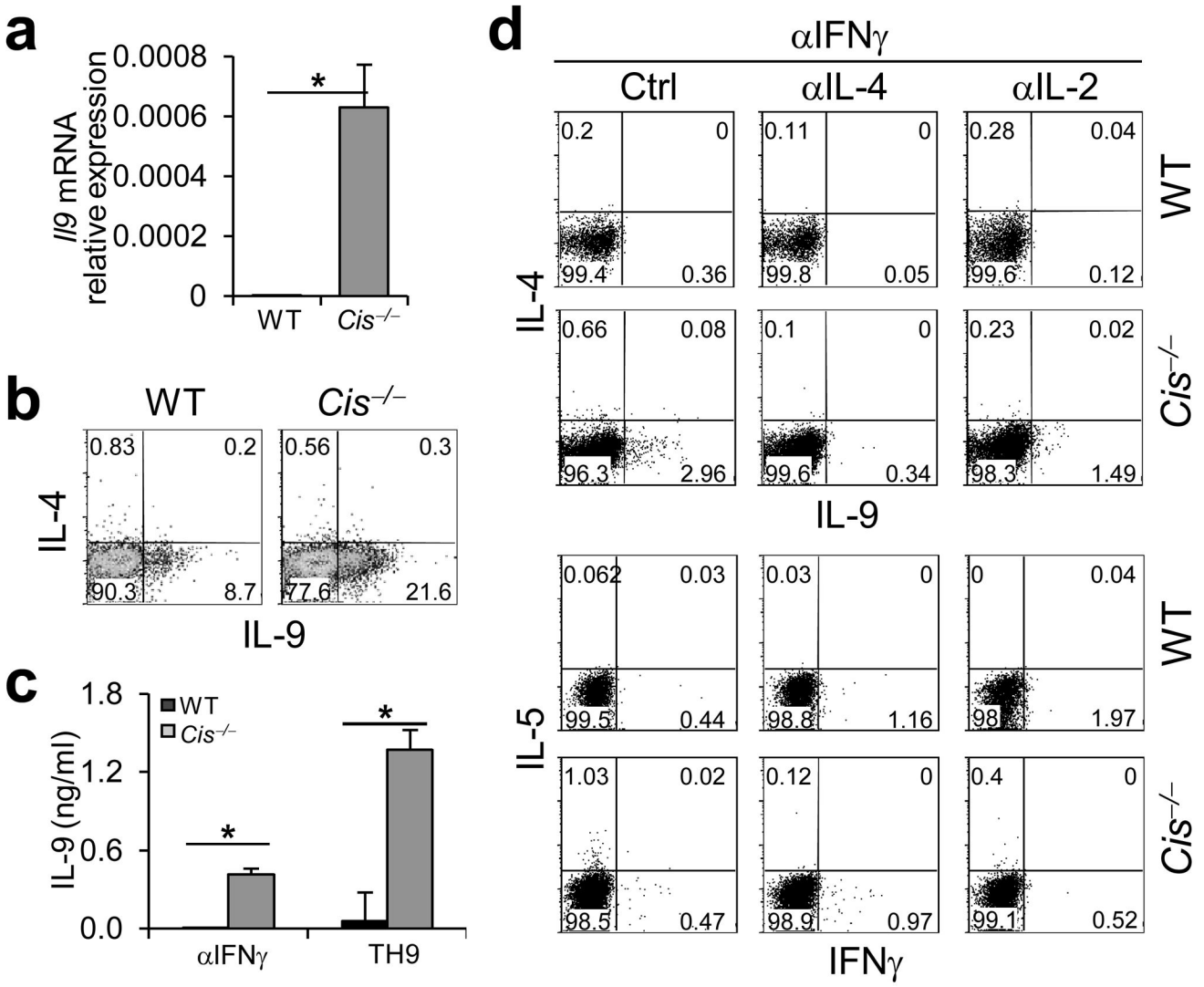

Figure 6.

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 under anti-IFN-γ or T_H9 conditions *, student *t* test, $P \leq 0.05$. (**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 with a control antibody (Ctrl) or anti-IL-4 or anti-IL-2. Data are from two independent experiments (**a**,**c**; mean and s.d.) or represent three independent experiments (**b**,**d**).

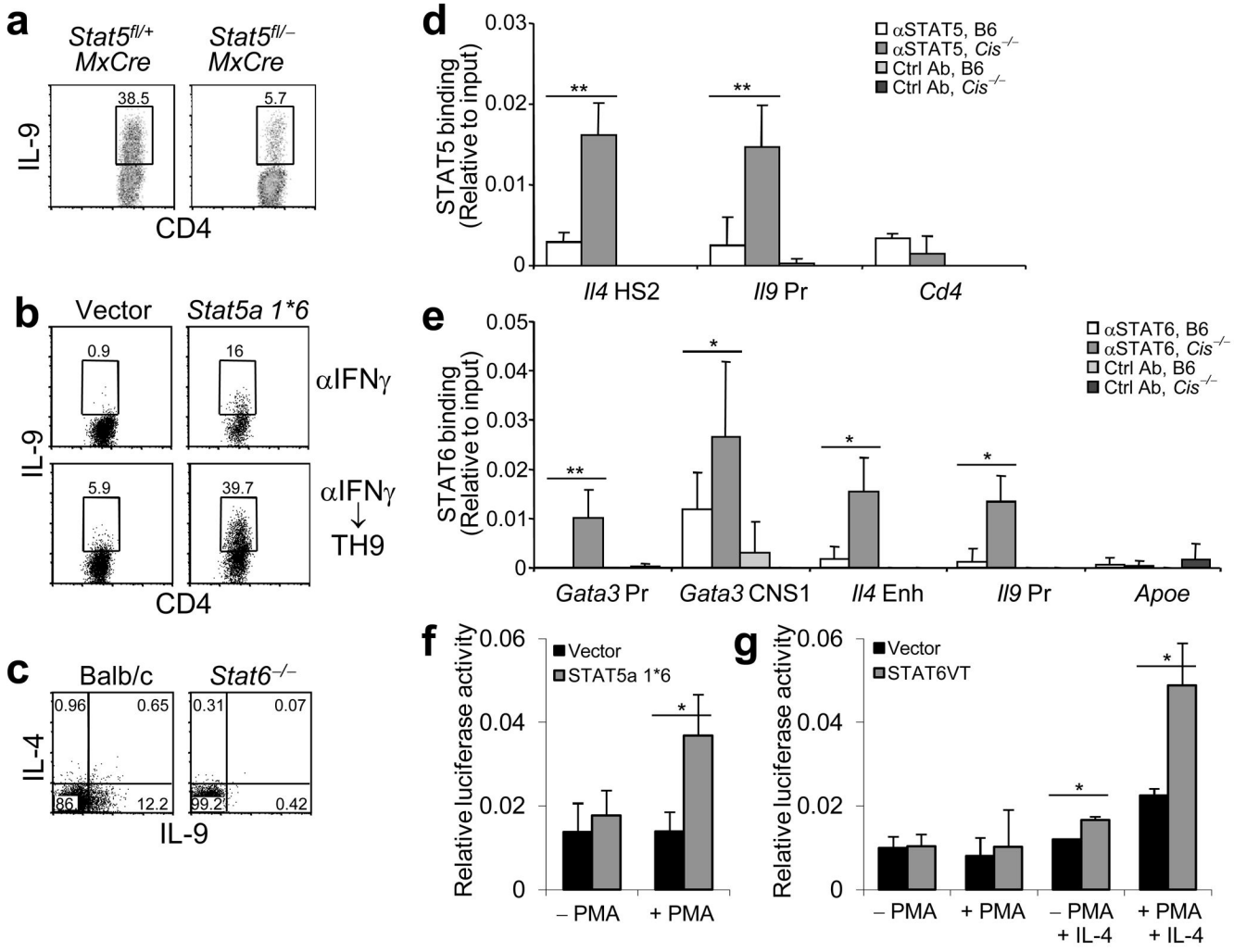

Figure 7.

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 *Stat5a*$^{fl/-}$Mx-Cre or *Stat5a*$^{fl/-}$Mx-Cre mice. Numbers above outlined areas indicate percent IL-9^+CD4^+ cells. (**b**) Intracellular staining of IL-9 in wild-type $CD4^+$ T cells polarized with anti-IFN-γ alone or anti-IFN- γ plus T_H9 conditions (left margin) with (right) or without (left) forced expression of constitutively active STAT5, assessed in a GFP^+ gate. (**c**) Intracellular staining of IL-9 and IL-4 in 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, relative to input; *Cd4* and *Apoe* serve as negative controls. Pr, promoter; Enh, enhancer, HS, DNase I hypersensitive site; CNS, conserved non-coding sequence. (**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 STAT5 (STAT5A1*6; **f**) or STAT6

(STAT6VT; **g**) and treated with various combinations of PMA and/or IL- 4 (horizontal axis); renilla luciferase activity was used to normalize transfection efficiency and set as 1. **P* ≤ 0.05 and ***P* ≤ 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.).