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The transcription factor Sox4 is a downstream target of signaling by the cytokine TGF-β and suppresses T_H2 differentiation

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Sox4 is a transcription factor that regulates various developmental processes. Here we show that Sox4 was induced by TGF-β and negatively regulated the transcription factor GATA-3, the master regulator of function of T helper type 2 (T_H2) cells, by two distinct mechanisms. First, Sox4 bound directly to GATA-3, preventing its binding to GATA-3 consensus DNA sequences. Second, Sox4 bound to the promoter region of the gene encoding interleukin 5 (IL-5), a T_H2 cytokine, and prevented binding of GATA-3 to this promoter. T_H2 cell–driven airway inflammation was modulated by alterations in Sox4 expression. Thus, Sox4 acted as a downstream target of TGF-β to inhibit GATA-3 function, T_H2 differentiation and T_H2 cell–mediated inflammation.

Elucidating the mechanisms by which naive $CD4^+$ T cells differentiate into effector helper T cells is crucial for understanding T cell–dependent immune responses. Functionally distinct helper T cell subsets have been reported, including the T_H1, T_H2, T_H9, T_H17, T_H22 and iT_{reg} subsets[1–8], and several transcription factors that regulate differentiation into these subsets have been identified, including T-bet[1–8], GATA-3 (refs. 9,10) and RORγt and RORα[11,12] for differentiation into T_H1 cells, T_H2 cells and T_H17 cells, respectively. T lymphocytes have abundant expression of GATA-3, and its expression is required for the development of T cells in the thymus. In peripheral $CD4^+$ T cells, interleukin 4 (IL-4)-mediated activation of the transcription factor STAT6 induces the expression of mRNA encoding GATA-3, which drives T_H2 differentiation[13,14]. GATA-3 binds to various regulatory regions in loci encoding T_H2 cytokines and induces chromatin remodeling[15–17]. In addition, GATA-3 binds to the *Il5* promoter and acts as a transcription factor for this gene[18,19].

TGF-β is a pleiotropic cytokine that contributes to the maintenance of immune homeostasis through inhibition of the proliferation, differentiation, activation and effector function of cells of the immune system[20]. Mice with T cell–specific disruption of TGF-β signaling develop inflammation as a result of constitutive activation of T cells[21,22]. Depending on the cytokine environment, TGF-β induces the differentiation of peripheral $CD4^+$ T cells into anti-inflammatory regulatory T cells (T_{reg} cells) and also into proinflammatory T_H17 and T_H9 cells[1–8]. In contrast, TGF-β inhibits the differentiation of naive $CD4^+$ T cells into effector T_H1 and T_H2 cells[23,24]. The molecular mechanisms of TGF-β-mediated inhibition of T_H1 and T_H2 differentiation remain unclear.

TGF-β mediates its biological functions by binding to type 1 and type 2 receptors for TGF-β, both of which are serine-threonine kinases[20,25]. Binding to these receptors induces the phosphorylation of proteins of the Smad family of signal transducers and the localization of Smad proteins to the nucleus. Eight Smad proteins have been identified in vertebrates; these are grouped into the following three categories: five receptor-associated Smad proteins (Smad1, Smad2, Smad3, Smad5 and Smad8), one common Smad protein (Smad4) and two inhibitory Smad proteins (Smad6 and Smad7). After TGF-β receptor–induced phosphorylation, Smad2 and Smad3 associate with Smad4, translocate to the nucleus and induce the transcription of target genes by binding to Smad-binding motifs.

Transcription factors of the Sox family ('Sry-related high-mobility-group (HMG) box') have key roles in the regulation of transcription during developmental processes, including early embryogenesis, sex determination, neural development, chondrogenesis, cardiac development and hematopoiesis[26,27]. The HMG box is critical for the function of Sox4 through its role in binding to DNA, bending DNA and protein interactions. Sox proteins can pair with various transcription factors[28,29]. Lymphocytes have high expression of Sox4, and Sox4 regulates T cell differentiation in the thymus and the population expansion of pro-B cells[30,31]. However, its expression profile in peripheral T cells and roles in immune responses have yet to be fully elucidated.

Here we investigated the role of Sox4 in peripheral $CD4^+$ T cells and found that Sox4 was a downstream target of the TGF-β signaling pathway that negatively regulated T_H2 differentiation and T_H2 cell–dependent allergic airway inflammation. Sox4 inhibited the function

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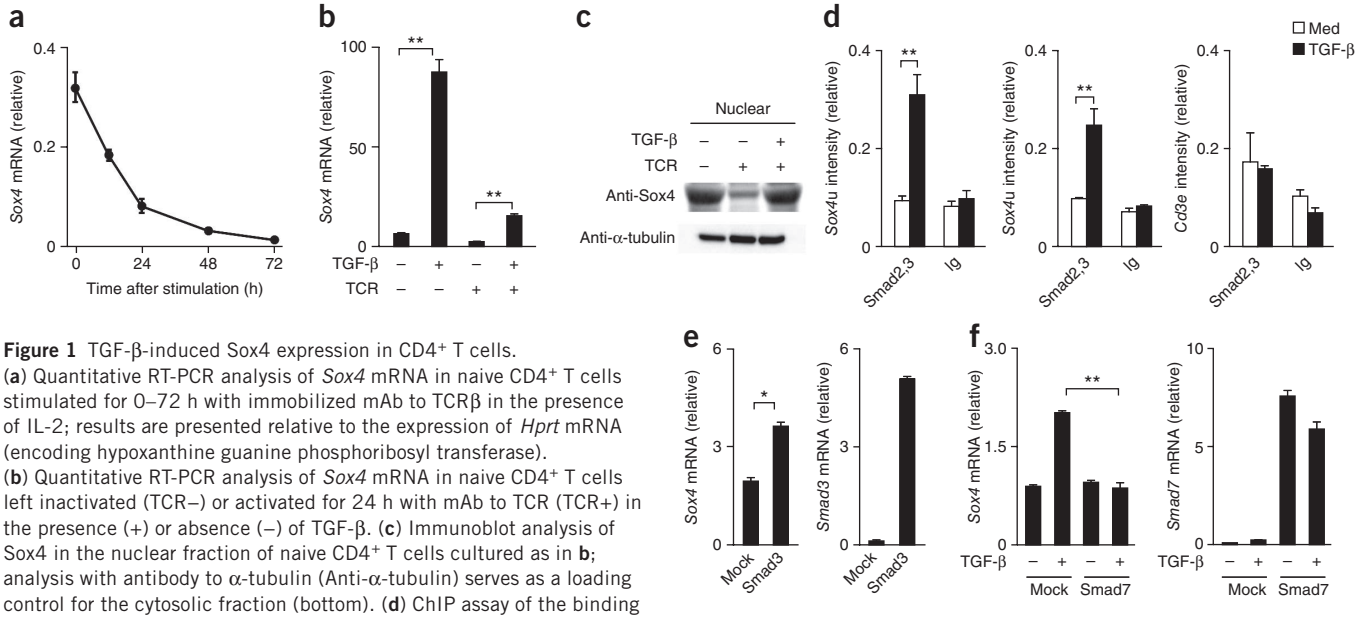

Figure 1 TGF-β-induced Sox4 expression in CD4$^+$ T cells. (**a**) Quantitative RT-PCR analysis of *Sox4* mRNA in naive CD4$^+$ T cells stimulated for 0–72 h with immobilized mAb to TCRβ in the presence of IL-2; results are presented relative to the expression of *Hprt* mRNA (encoding hypoxanthine guanine phosphoribosyl transferase). (**b**) Quantitative RT-PCR analysis of *Sox4* mRNA in naive CD4$^+$ T cells left inactivated (TCR–) or activated for 24 h with mAb to TCR (TCR+) in the presence (+) or absence (–) of TGF-β. (**c**) Immunoblot analysis of Sox4 in the nuclear fraction of naive CD4$^+$ T cells cultured as in **b**; analysis with antibody to α-tubulin (Anti-α-tubulin) serves as a loading control for the cytosolic fraction (bottom). (**d**) ChIP assay of the binding of Smad2-Smad3 (Smad2,3) or immunoglobulin (Ig; control) to a 5′ region upstream of the *Sox4* locus (*Sox4*u) or the promoter of the gene encoding the invariant signaling protein CD3ε (*Cd3e*) in naive CD4$^+$ T cells cultured for 12 h in the presence of medium (Med) or TGF-β; results are presented relative to those of input DNA. (**e**) Quantitative RT-PCR analysis of *Sox4* and *Smad3* mRNA in naive CD4$^+$ T cells mock transduced (Mock) or transduced to express Smad3, then stimulated with mAb to TCR, followed by stimulation for 24 h with TGF-β (presented as in **a**). (**f**) Quantitative RT-PCR analysis of *Sox4* and *Smad7* mRNA in naive CD4$^+$ T cells mock transduced or transduced to express Smad7, then stimulated with mAb to TCR, followed by no stimulation (–) or stimulation with TGF-β (+; presented as in **a**). $^*P < 0.05$ and $^{**}P < 0.01$ (Student's *t*-test). Data are representative of four (**a**; mean and s.d. of three samples), three (**b**,**d**) or two (**c**,**e**,**f**) independent experiments with similar results (error bars (**b**,**d**–**f**, s.d.).

of GATA-3 via two distinct mechanisms. Therefore, Sox4 is a unique negative regulator of GATA-3 function and a critical regulator of T_H2 differentiation and T_H2 cell–dependent immune responses.

RESULTS

Regulation of Sox4 expression by TGF-β

We detected substantial *Sox4* mRNA in naive CD4$^+$ T cells, but its expression decreased rapidly after stimulation mediated by the T cell antigen receptor (TCR; **Fig. 1a**). The treatment of naive CD4$^+$ T cells with TGF-β for 24 h induced significant upregulation of the expression of *Sox4* mRNA, which we also observed in CD4$^+$ T cells stimulated with monoclonal antibody (mAb) to the TCR (**Fig. 1b**). We confirmed TGF-β-mediated induction of Sox4 at the protein level by immunoblot analysis (**Fig. 1c**). Sox4 belongs to the group C subfamily of Sox proteins, which includes the following three members in mice and humans: Sox4, Sox11 and Sox12. Although we detected low expression of *Sox11* and moderate expression of *Sox12* in activated CD4$^+$ T cells, we noted no obvious effect of treatment with TGF-β (**Supplementary Fig. 1a**). Among the other helper T cell subsets, the expression of *Sox4* mRNA was highest in iT_{reg} cells and lowest in T_H2 cells (**Supplementary Fig. 1b**). We confirmed high expression of Sox4 protein in iT_{reg} cells by immunoblot analysis (**Supplementary Fig. 1c**).

To determine whether TGF-β-mediated activation of the Smad signaling pathway was involved in the induction of *Sox4* expression, we did chromatin-immunoprecipitation (ChIP) analysis with an antibody to Smad2 and Smad3. We detected significant binding of Smad2-Smad3 to the *Sox4* promoter (–480 base pairs from the transcription start site) and an upstream region of the *Sox4* locus (–1,000 base pairs from the transcription start site; **Fig. 1d**). Retrovirus vector–mediated expression of Smad3 in developing T_H2 cells significantly enhanced the TGF-β-induced expression of *Sox4* mRNA (**Fig. 1e**). Furthermore, the retroviral expression of an inhibitory Smad protein, Smad7, resulted in the inhibition of TGF-β-induced *Sox4* expression in activated CD4$^+$ T cells (**Fig. 1f**). These results indicated that TGF-β induced Sox4 expression through activation of the Smad signaling pathway.

Inhibition of T_H2 differentiation by TGF-β-induced Sox4

TGF-β inhibits the proliferation and activation of CD4$^+$ T cells and the differentiation of CD4$^+$ T cells into helper T cells[32]. Although both T_H1 differentiation and T_H2 differentiation were inhibited by TGF-β, T_H2 differentiation was 'preferentially' inhibited (data not shown). In particular, when we added TGF-β to cells cultured under T_H1- or T_H2-polarizing conditions 48 h after the initial stimulation of the TCR, T_H2 differentiation was selectively inhibited, whereas T_H1 differentiation was left intact (**Fig. 2a**). We confirmed lower expression of *Il4*, *Il5* and *Il13* and normal induction of *Ifng* (which encodes interferon-γ (IFN-γ)) by quantitative RT-PCR (**Fig. 2b**) as well as lower expression of the corresponding proteins by enzyme-linked immunosorbent assay (ELISA; **Fig. 2c**). Although TGF-β had potent inhibitory effects on T_H2 differentiation, inhibition of the expression of *Gata3* mRNA and GATA-3 protein was moderate under these conditions (**Supplementary Fig. 2a**,**b**). To assess the involvement of Sox4 in the TGF-β-mediated inhibition of T_H2 differentiation, we did a series of knockdown experiments. Inhibition of the expression of *Il4*, *Il5* and *Il13* by TGF-β was restored significantly by the introduction of short hairpin RNA (shRNA) specific for Sox4 (**Fig. 2d**). The inhibition of *Il5* expression by TGF-β was also reproducibly diminished by knockdown of Sox4 expression mediated by small interfering RNA (siRNA; **Fig. 2e**). Knockdown of Sox4 in T_H1 cells did not result in the induction of IL-4-producing T_H2 cells (data not shown). By ChIP assay, we assessed the effect of treatment with TGF-β on the binding of GATA-3 to loci encoding

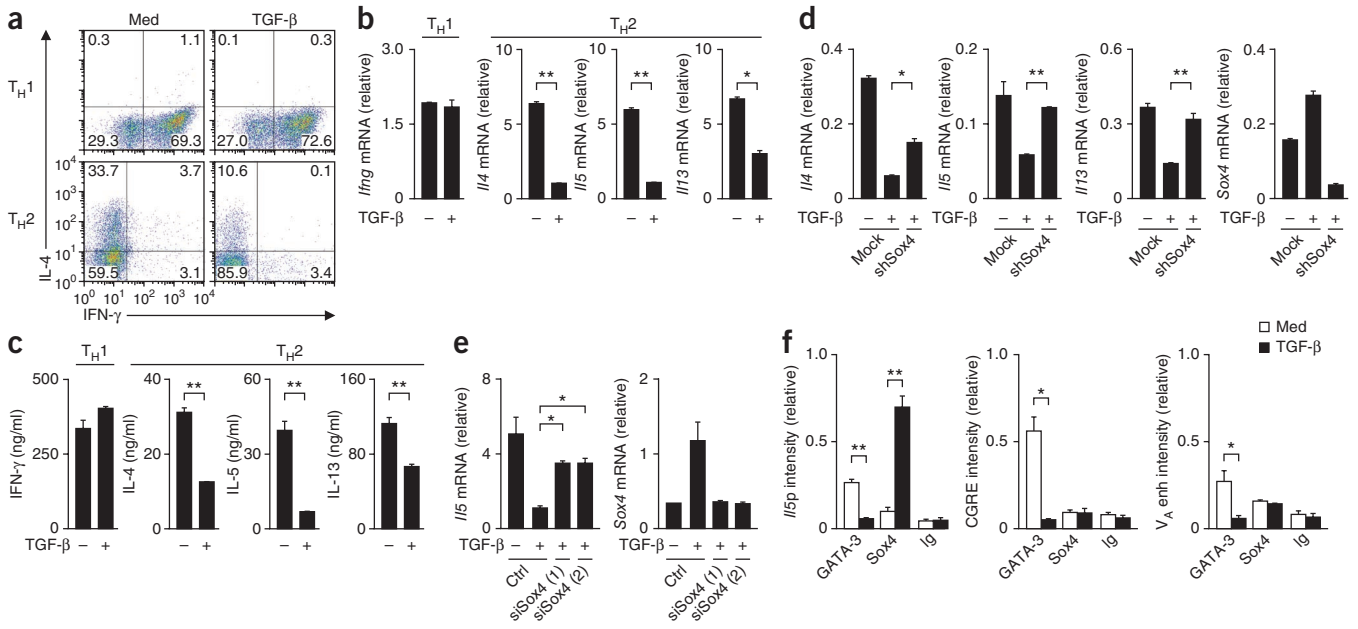

Figure 2 Involvement of Sox4 in the TGF-β-mediated inhibition of T_H2 differentiation. (**a**) Intracellular staining of IL-4 and IFN-γ in cells cultured under T_H1- or T_H2-polarizing conditions with medium alone or TGF-β. Numbers in quadrants indicate percent cells in each throughout. (**b**) Quantitative RT-PCR analysis of cytokine-encoding mRNA in T_H1 and T_H2 cells left untreated (–) or treated with TGF-β (+), then restimulated for 24 h with immobilized anti-TCR (presented as in **Fig. 1a**). (**c**) ELISA of cytokines in supernatants of the cells in **b**. (**d**) Quantitative RT-PCR analysis of cytokine-encoding mRNA in cells treated with control shRNA (Mock) or Sox4-specific shRNA (shSox4) and left untreated or treated with TGF-β (presented as in **Fig. 1a**). (**e**) Quantitative RT-PCR analysis of *Il5* and *Sox4* mRNA in cells treated with control siRNA (Ctrl) or Sox4-specific siRNA (siSox4 (1) and siSox4 (2)) and left untreated or treated with TGF-β (presented as in **Fig. 1a**). (**f**) ChIP assay of the binding of GATA-3, Sox4 and immunoglobulin to the *Il5* promoter (*Il5*p), the CGRE and the V_A site in the *Il4* enhancer (V_A enh) in developing T_H2 cells cultured with medium alone or TGF-β (presented as in **Fig. 1d**). $^*P < 0.05$ and $^{**}P < 0.01$ (Student's *t*-test). Data are representative of three (**a**,**b**,**d**,**e**) or two (**c**,**f**) independent experiments with similar results (error bars (**b**–**f**), s.d.).

T_H2 cytokines. Binding of GATA-3 to the *Il5* promoter, the conserved GATA-3-response element (CGRE) of genes encoding T_H2 cytokines, and the DNase-hypersensitive site V_A in the *Il4* enhancer was abrogated by treatment with TGF-β (**Fig. 2f**). We observed TGF-β-dependent binding of Sox4 to the *Il5* promoter but not to the CGRE or the V_A site (**Fig. 2f**). These results indicated that Sox4 mediated the TGF-β-mediated inhibition of T_H2 differentiation and expression of T_H2 cytokines.

Suppression of T_H2 differentiation by enforced expression of Sox4

To further investigate the role of Sox4 in differentiation into various helper T cell subsets, we ectopically expressed Sox4 in T_H1 cells and T_H2 cells via a retroviral vector. Ectopic expression of Sox4 resulted in loss of IL-4-producing cells under T_H2-differentiating conditions, whereas the generation of IFN-γ-producing cells under either T_H1- or T_H2-polarizing conditions was augmented (**Fig. 3a**). The differentiation of cells infected with empty vector (mock-infected cells) was not altered (**Fig. 3a**). Sox4-transduced T_H2 cells had lower expression of *Il4*, *Il5* and *Il13* mRNA (**Supplementary Fig. 3a**) and IL-4, IL-5 and IL-13 protein (**Fig. 3b**) but slightly higher expression of *Ifng* mRNA than did mock-infected T_H2 cells (**Supplementary Fig. 3a**). The introduction of Sox4 also inhibited the T_H2 differentiation of T-bet-deficient $CD4^+$ T cells (**Supplementary Fig. 3b**), which indicated that the inhibition of T_H2 differentiation by Sox4 was not dependent on T-bet expression. There was less histone H3 acetylated at Lys9 (H3K9ac) and H3K14ac, which are associated with transcriptionally active chromatin, at the *Il4*, *Il5* and *Il13* promoters, but more at the *Ifng* promoter, in Sox4-transduced T_H2 cells than in mock-infected T_H2 cells (**Supplementary Fig. 3c**). Sox4-transduced T_H2 cells had slightly higher, not lower, expression of *Gata3* mRNA and *Nfat1* and *Nfat2* mRNA (which encode transcription factors of the NFAT family) than did mock-infected T_H2 cells, whereas their expression of *Junb* mRNA (which encodes the transcription factor JunB) and *Maf* mRNA (which encodes the transcription factor c-Maf) was not affected (**Fig. 3c**). These results indicated that Sox4 negatively regulated T_H2 differentiation and the expression of mRNA encoding T_H2 cytokines without inhibiting *Gata3* expression.

To further investigate the role of Sox4 in peripheral T cell function, we generated mice with transgenic expression of Sox4 under the control of the T cell–specific *Lck* distal promoter (Sox4-transgenic mice; **Supplementary Fig. 4a**). *Sox4* mRNA expression was approximately tenfold higher in both $CD4^+$ splenic T cells and $CD8^+$ splenic T cells from Sox4-transgenic mice than in those from wild-type mice (**Supplementary Fig. 4b**). Although Sox4-transgenic mice had slightly fewer $CD4^+$ T cells in the spleen and thymus than did wild-type mice, cell-surface expression of TCRs and receptors for cytokines, as well as antigen-induced proliferative responses, were within the normal range in these mice (**Supplementary Fig. 4c–e**). There was less generation of IL-4-producing Sox4-transgenic $CD4^+$ T cells than of IL-4-producing wild-type $CD4^+$ T cells under T_H2 conditions, whereas the generation of IFN-γ-producing Sox4-transgenic $CD4^+$ T cells was modestly enhanced (**Fig. 3d**). The generation of IFN-γ-producing Sox4-transgenic $CD4^+$ T cells cells under T_H1 conditions was enhanced (**Fig. 3d**), whereas the generation of IL-17-producing T_H17 cells, transcription factor Foxp3–expressing iT_{reg} cells and T_H9 cells was not affected by the transgene encoding Sox4 (**Supplementary Fig. 4f**). We confirmed by ELISA the lower production of IL-4, IL-5 and IL-13 by Sox4-transgenic $CD4^+$ T cells

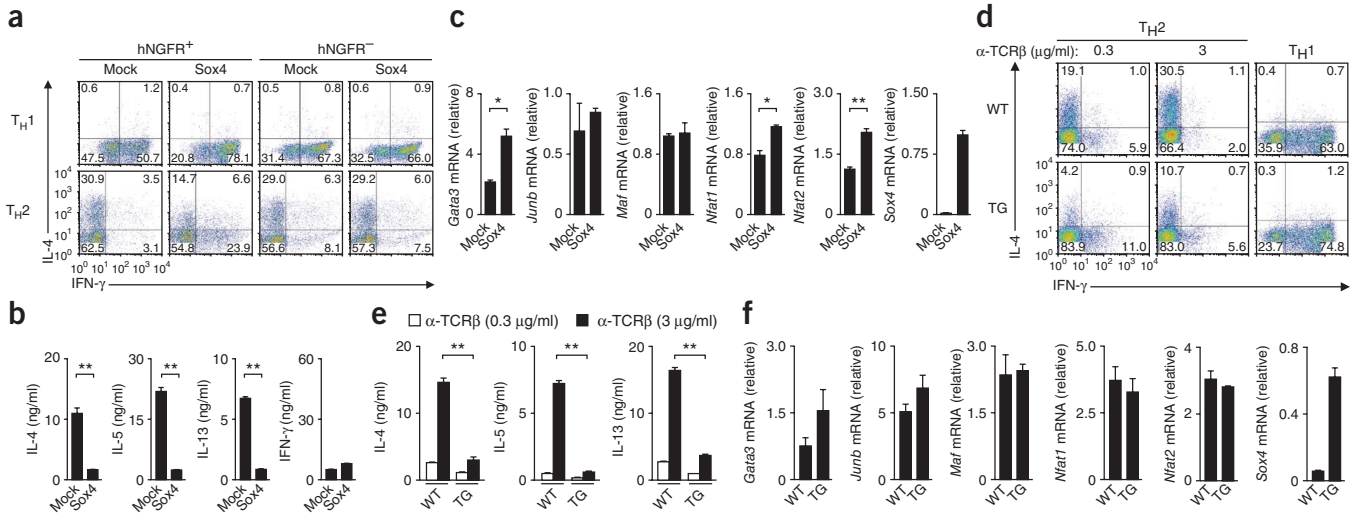

Figure 3 Enforced expression of Sox4 inhibits T_H2 differentiation. (**a**) Staining of IL-4 and IFN-γ in CD4+ T cells left uninfected (hNGFR−) or infected (hNGFR+), and transduced with empty vector (mock transduced; Mock) or with a Sox4-expressing retroviral vector (Sox4) and then cultured for 3 d under T_H1- or T_H2-polarizing conditions. (**b**) ELISA of cytokines in T_H2 cells mock transduced (Mock) or transduced with a Sox4-expressing retroviral vector (Sox4) and restimulated with immobilized mAb to TCRβ. (**c**) Quantitative RT-PCR analysis of mRNA encoding various transcription factors (vertical axes) in T_H2 cells transduced as in **b** (presented as in **Fig. 1a**). (**d**) Staining of IL-4 and IFN-γ in wild-type (WT) and Sox4-transgenic (TG) naive CD4+ T cells cultured under T_H2-polarizing conditions with various concentrations (above plots) of anti-TCRβ (α-TCRβ) or under T_H1-polarizing conditions (far right). (**e**) ELISA of cytokines in supernatants of wild-type and Sox4-transgenic T_H2 cells generated *in vitro* and stimulated for 16 h with immobilized mAb to TCRβ at a concentration of 0.3 or 3 μg/ml (key). (**f**) Quantitative RT-PCR analysis of mRNA encoding various transcription factors (vertical axes) in wild-type and Sox4-transgenic CD4+ T cells (presented as in **Fig. 1a**). $*P < 0.05$ and $**P < 0.01$ (Student's *t*-test). Data are representative of three (**a**,**b**,**d**–**f**) or four (**c**) independent experiments with similar results (error bars (**b**,**c**,**e**,**f**), s.d.).

than by wild-type cells (**Fig. 3e**). There was less H3K9ac and H3K14ac at the *Il4*, *Il5* and *Il13* promoters in Sox4-transgenic T_H2 cells than in wild-type cells (**Supplementary Fig. 4g**). Although the expression of *Gata3* mRNA was higher in Sox4-transgenic cells than in wild-type cells (not statistically significant; **Fig. 3f**), wild-type and Sox4-transgenic T_H2 cells had a similar amount of GATA-3 protein (**Supplementary Fig. 4h**). There was no significant difference between wild-type and Sox4-transgenic cells in their expression of other T_H2 cell–associated transcription factors (**Fig. 3f**). These results indicated that T_H2 differentiation was impaired without affecting the expression of T_H2-associated transcription factors, including GATA-3.

Sox4 inhibits GATA-3 function by distinct mechanisms

To identify the molecular mechanisms by which Sox4 inhibits T_H2 differentiation, we assessed the possible physical association of Sox4 with GATA-3 in three different experimental systems. Sox4 immunoprecipitated with GATA-3 in mixtures of lysates of 293T cells (human embryonic kidney T cells) transfected to express Myc-tagged Sox4 or Flag-tagged GATA-3 (**Fig. 4a**). We also observed the association of Sox4 with GATA-3 in TG40 cells, a mouse T cell line with substantial endogenous expression of both Sox4 and GATA-3 protein (**Fig. 4b**). In addition, glutathione *S*-transferase–tagged recombinant GATA-3 directly interacted with Myc-tagged recombinant Sox4, as detected in a precipitation assay with glutathione 4B Sepharose beads (**Fig. 4c**). Next we generated several Myc-tagged Sox4 mutants to determine which domains of Sox4 were important for its association with GATA-3 (**Supplementary Fig. 5a**). The association between GATA-3 and a Sox4 mutant lacking the amino-terminal region was much weaker than that of GATA-3 and wild-type Sox4; also, a mutant in which both the amino-terminal region and HMG box were deleted and a mutant in which the carboxy-terminal region, including the transactivation domain, was deleted each completely failed to associate with GATA-3 in 293T cells (**Supplementary Fig. 5a**). This indicated that both the HMG box and carboxy-terminal portion of Sox4 were important for the association with GATA-3. The arginine at position 61 (Arg61), proline at position 62 (Pro62), phenylalanine at position 66 (Phe66) and methionine at position 67 (Met67) are perfectly conserved among proteins of the Sox family. We generated several Sox4 mutants (**Supplementary Fig. 5b**) and examined their association with GATA-3 in 293T cells. A Sox4 mutant with deletion of amino acids 60–71 showed less association with GATA-3 than did wild-type Sox4, but the association with GATA-3 was unaffected by the deletion of amino acids 203–214 (**Fig. 4d**). Substitution of Arg61 and Pro62 or of Phe66 and Met67 with alanine abolished the association of Sox4 with GATA-3 (**Fig. 4d**), but substitution of the methionine at position 78 (Met78) and glutamic acid at position 79 (Glu79) with alanine had no effect on the binding of Sox4 to GATA-3. These results indicated that Arg61, Pro62, Phe66 and Met67 in the amino-terminal region of the HMG box in Sox4 were important for the association of Sox4 with GATA-3.

To further examine the effect of the Sox4 mutants described above on T_H2 differentiation, we expressed those mutants in primary developing T_H2 cells through the use of retrovirus vectors. The Sox4 mutants with substitution of Arg61 and Pro62 or of Phe66 and Met67 with alanine did not inhibit the differentiation of IL-4 and IL-5 producing T_H2 cells, but the Sox4 mutant with substitution of Met78 and Glu79 with alanine did have this effect (**Fig. 4e**). Thus, the association of Sox4 with GATA-3 was required for the Sox4-mediated inhibition of T_H2 differentiation. To identify the domains of GATA-3 critical for its association with Sox4, we generated various GATA-3 mutants (**Supplementary Fig. 5c**). A GATA-3 mutant with deletion of the amino-terminal finger and another with deletion of the carboxy-terminal finger showed much weaker association with Sox4 than did wild-type GATA-3 (**Supplementary Fig. 5c**), whereas

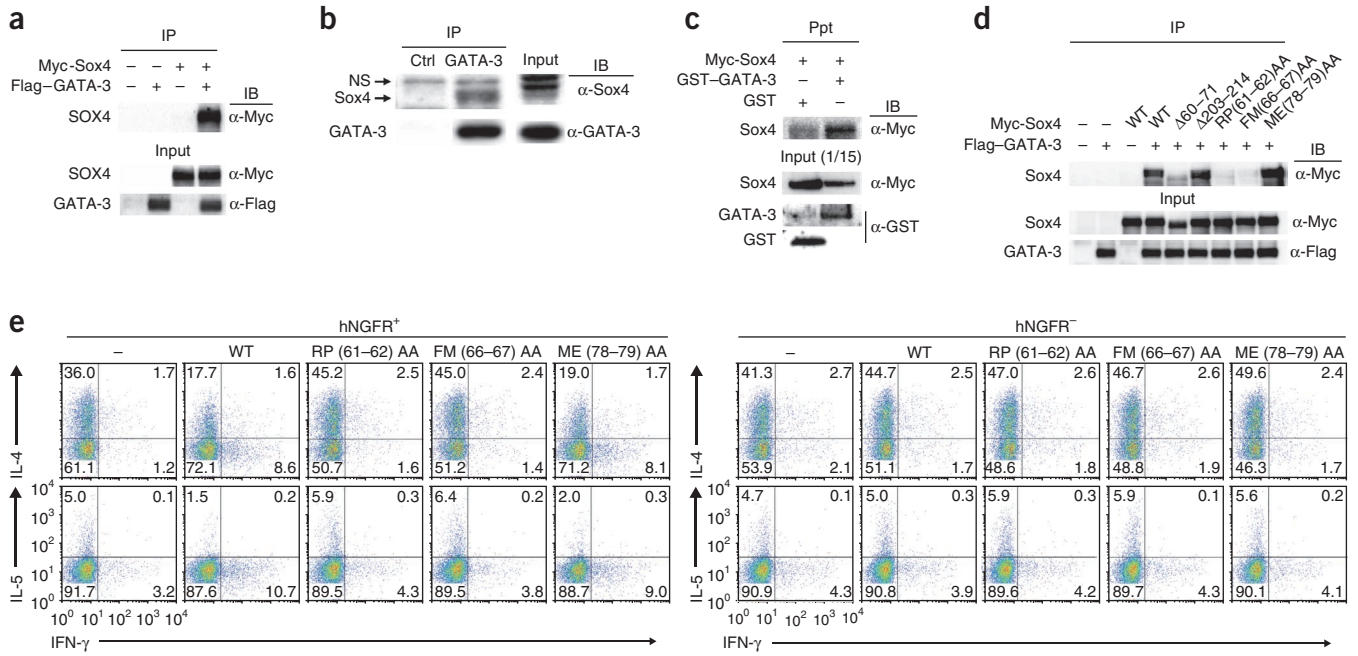

Figure 4 Physical association of Sox4 with GATA-3. (**a**) Immunoprecipitation (IP) and immunoblot analysis (IB) of the association of Sox4 and GATA-3 in 293T cells left untransfected (–) or transfected (+) to express Myc-tagged Sox4 (Myc-Sox4) and/or Flag-tagged GATA-3 (Flag–GATA-3); below (Input), parallel analysis of total cell lysates (without immunoprecipitation). (**b**) Immunoprecipitation and immunoblot analysis of the association of endogenous GATA-3 with endogenous Sox4 in TG40 cells (Input (middle right), as in **a**). NS, nonspecific band. (**c**) Precipitation assay (Ppt) of the association of Sox4 and GATA-3 in cells transfected to express Myc-tagged recombinant Sox4 (Myc-Sox4), glutathione *S*-transferase–tagged recombinant GATA-3 (GST–GATA-3) and/or glutathione *S*-transferase alone (GST), probed with anti-Myc; below (Input), immunoblot analysis of an aliquot of input samples (1/15 volume) with anti-Myc or anti-GST. (**d**) Immunoprecipitation and immunoblot analysis of the association of Sox4 and GATA-3 in 293T cells mock transfected (–) or transfected to express Flag-tagged GATA-3 and Myc-tagged wild-type Sox4 (WT) or mutant Sox4 lacking amino acids 60–71 (Δ60–71) or amino acids 203–214 (Δ203–214) or with substitution of alanine residues for arginine and proline (RP(61–62)AA), phenylalanine and methionine (FM(66–67)AA) or methionine and glutamic acid (ME(78–79)AA); Input (below), as in **a**. (**e**) Staining of IL-4, IL-5 and IFN-γ in T_H2 cells left untransduced (hNGFR$^-$) or transduced (hNGFR$^+$) and mock transfected or transfected to express wild-type or mutant Sox4 (as in **d**), assessed 3 d after retroviral infection. Data are representative of at least three independent experiments with similar results.

the association of GATA-3 with Sox4 in 293T cells was eliminated by deletion of the zinc-finger domain and carboxy-terminal region of GATA-3 (**Supplementary Fig. 5c**). These results indicated that the zinc-finger domains of GATA-3 were important for its association with Sox4.

Next we assessed the effect of Sox4 on the DNA-binding activity of GATA-3 by a precipitation assay. The binding of GATA-3 to an oligonucleotide containing a consensus GATA-binding site was decreased in a dose-dependent manner in the presence of Sox4 (**Fig. 5a**), which indicated that Sox4 interfered with the binding of GATA-3 to the GATA-binding consensus motif. To assess whether Sox4 inhibited the binding of GATA-3 to DNA in primary T_H2 cells, we did a ChIP assay with Sox4-transgenic T_H2 cells. We detected less binding of GATA-3 to its target regions in Sox4-transgenic T_H2 cells than in wild-type primary T_H2 cells (**Fig. 5b**). In addition, we examined the effect of retroviral expression of Sox4 and GATA-3 in developing T_H1 cells on T_H2 differentiation and the expression of T_H2 cytokines. Transduction of GATA-3 alone induced the expression of T_H2 cytokines[33], whereas the coexpression of GATA-3 and Sox4 resulted in lower expression of T_H2 cytokines (**Fig. 5c**). Ectopic expression of Sox4 lead to more inhibition of *Il5* transcription than of *Il4* transcription or *Il13* transcription (**Fig. 5c**).

Analysis of the *Il5* promoter sequence showed a conserved Sox-binding site that overlapped a GATA-binding site (**Fig. 5d**). To assess the DNA sequence required for the binding of Sox4 to the *Il5* promoter region, we generated oligonucleotides of the *Il5* promoter containing single-nucleotide mutation of GATA-binding site 1a only or of GATA-binding site 1a and the Sox-binding site. To exclude possible confounding effects due to the interaction of Sox4 with GATA-3, we used the Sox4 mutant lacking the carboxy-terminal region that failed to associate with GATA-3 (**Supplementary Fig. 5a**). That Sox4 mutant inhibited the binding of GATA-3 to the *Il5* promoter with mutation of GATA-binding site 1a only but did not inhibit the binding of GATA-3 to the *Il5* promoter with mutation of both GATA-binding site 1a and the Sox-binding site (**Fig. 5e**). Both wild-type Sox4 and the mutant Sox4 efficiently suppressed *Il5* promoter activity in M12 mouse B cells (**Fig. 5f**). These results indicated that Sox4 was able to inhibit *Il5* expression independently of its association with GATA-3.

Sox4 controls T_H2 cell–dependent airway inflammation

Next we investigated the role of Sox4 in T_H2 differentiation in an *in vivo* model of airway inflammation. We immunized wild-type and Sox4-transgenic mice with ovalbumin (OVA) and aluminum hydroxide (as an adjuvant) and then challenged the mice with OVA by inhalation. We observed less infiltration of inflammatory cells, including eosinophils, in the bronchioalveolar lavage (BAL) fluid of OVA-immunized Sox4-transgenic mice than in that of OVA-immunized wild-type mice (**Fig. 6a**). The expression of *Il4*, *Il5* and *Il13* mRNA in cells of the BAL fluid was also very low in Sox4-transgenic mice (**Fig. 6b**). Sox4-transgenic mice had fewer mononuclear cells infiltrating the peribronchiolar regions of the lung than did wild-type mice (**Fig. 6c**). Sox4-transgenic bronchioles had less mucus hyperproduction

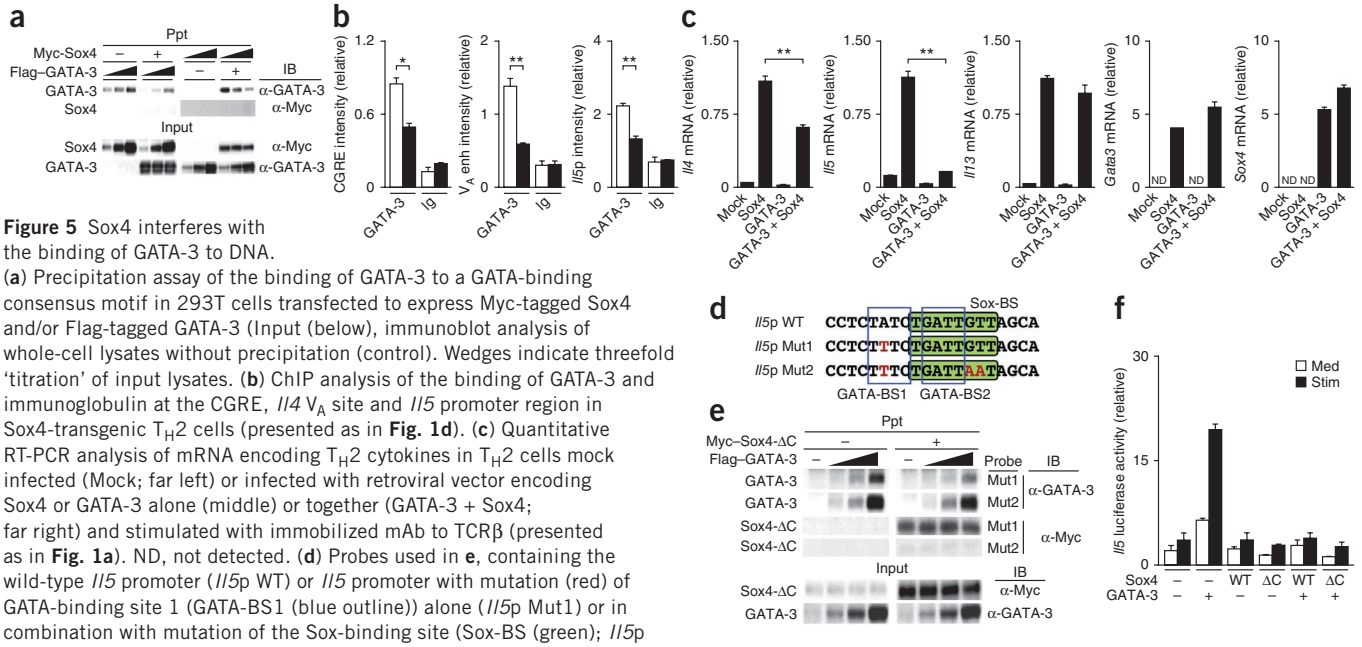

Figure 5 Sox4 interferes with the binding of GATA-3 to DNA. (**a**) Precipitation assay of the binding of GATA-3 to a GATA-binding consensus motif in 293T cells transfected to express Myc-tagged Sox4 and/or Flag-tagged GATA-3 (Input (below), immunoblot analysis of whole-cell lysates without precipitation (control). Wedges indicate threefold 'titration' of input lysates. (**b**) ChIP analysis of the binding of GATA-3 and immunoglobulin at the CGRE, *Il4* V_A site and *Il5* promoter region in Sox4-transgenic T_H2 cells (presented as in **Fig. 1d**). (**c**) Quantitative RT-PCR analysis of mRNA encoding T_H2 cytokines in T_H2 cells mock infected (Mock; far left) or infected with retroviral vector encoding Sox4 or GATA-3 alone (middle) or together (GATA-3 + Sox4; far right) and stimulated with immobilized mAb to TCRβ (presented as in **Fig. 1a**). ND, not detected. (**d**) Probes used in **e**, containing the wild-type *Il5* promoter (*Il5*p WT) or *Il5* promoter with mutation (red) of GATA-binding site 1 (GATA-BS1 (blue outline)) alone (*Il5*p Mut1) or in combination with mutation of the Sox-binding site (Sox-BS (green); *Il5*p Mut2), which overlaps GATA-binding site 2 (GATA-BS2 (blue outline)). (**e**) Precipitation assay of mixtures of lysates of 293T cells mock transfected (–) or transfected (+) to express a Myc-tagged Sox4 mutant lacking the carboxy-terminal region (Myc–Sox4-ΔC) or various concentrations (wedges) of Flag-tagged GATA-3, assessed with the mutated probes in **d** (Input (below), as in **a**). (**f**) Luciferase activity in M12 B cells left untransfected (–) or transfected (+) to express wild-type Sox4 or the Sox4 mutant in **e**, plus GATA-3, as well as a firefly luciferase reporter for the *Il5* promoter, then left unstimulated (Med) or stimulated (Stim) with the phorbol ester PMA (30 ng/ml) and dibuteryl cAMP (100 μM); results are presented relative to renilla luciferase activity. $^*P < 0.05$ and $^{**}P < 0.01$ (Student's *t*-test). Data are representative of at least three independent experiments with similar results (mean and s.d. of three samples in **c**; mean and s.d. in **b** and **f**).

and goblet-cell metaplasia than did wild-type bronchioles, as assessed by staining with periodic acid–Schiff reagent (**Fig. 6d**), and Sox4-transgenic mice had lower expression of *Clca3* mRNA (encoding the calcium-activated chloride channel CLCA3) and *Muc2* mRNA (encoding the mucin protein Mucin2) in the lungs than did wild-type mice (**Fig. 6e**). No obvious methacholine-induced airway hyper-responsiveness was induced in the Sox4-transgenic mice (**Fig. 6f**).

We also knocked down Sox4 with shRNA in DO11.10 T_H2 cells (which have transgenic expression of an OVA-specific TCRαβ) and evaluated the ability of these cells to cause airway inflammation. After restimulation of the TCR, we found higher expression of *Il4* and *Il5* mRNA in DO11.10 T_H2 cells transfected with Sox4-specific shRNA than in those transfected with control shRNA (**Supplementary Fig. 6a**). We intravenously injected DO11.10 T_H2 cells transfected with control or Sox4-specific shRNA into wild-type BALB/c mice, followed by intranasal administration of OVA[34]. The infiltration of the BAL fluid with inflammatory cells, including eosinophils, as well as airway hyper-responsiveness, were much greater in the group given cells transfected with Sox4-specific shRNA than in the group given cells transfected with control shRNA (**Supplementary Fig. 6b,c**).

To generate mice with T cell–specific deficiency in Sox4, we crossed mice with *lox*P-flanked *Sox4* alleles ($Sox4^{fl/fl}$ mice)[35] to mice with transgenic expression of Cre recombinase driven by the promoter of the gene encoding CD4 (which results in T cell–specific deletion

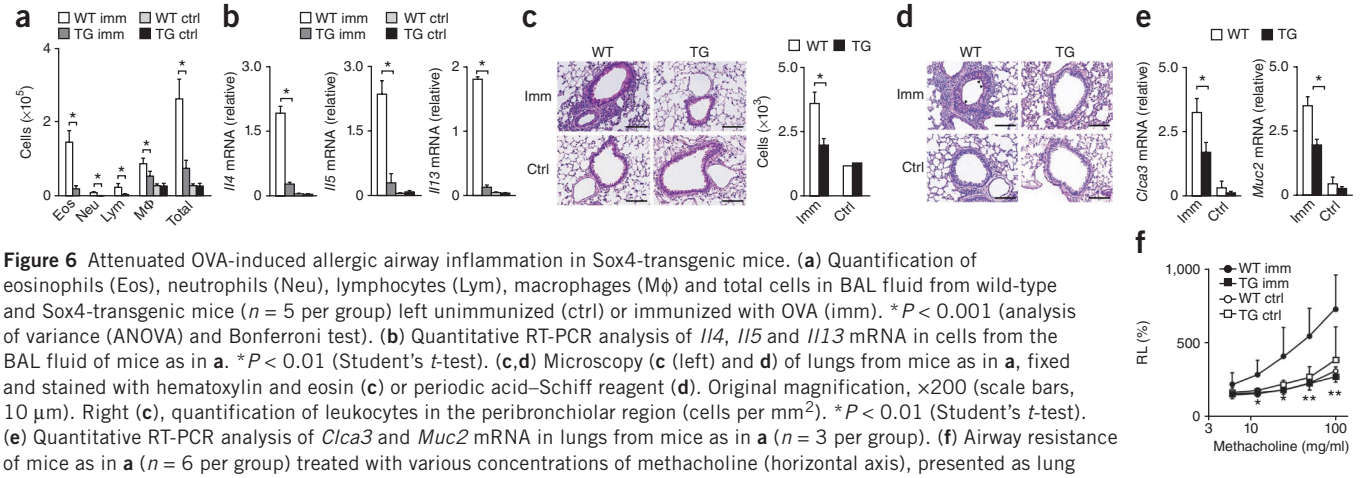

Figure 6 Attenuated OVA-induced allergic airway inflammation in Sox4-transgenic mice. (**a**) Quantification of eosinophils (Eos), neutrophils (Neu), lymphocytes (Lym), macrophages (Mϕ) and total cells in BAL fluid from wild-type and Sox4-transgenic mice ($n = 5$ per group) left unimmunized (ctrl) or immunized with OVA (imm). $^*P < 0.001$ (analysis of variance (ANOVA) and Bonferroni test). (**b**) Quantitative RT-PCR analysis of *Il4*, *Il5* and *Il13* mRNA in cells from the BAL fluid of mice as in **a**. $^*P < 0.01$ (Student's *t*-test). (**c**,**d**) Microscopy (**c** (left) and **d**) of lungs from mice as in **a**, fixed and stained with hematoxylin and eosin (**c**) or periodic acid–Schiff reagent (**d**). Original magnification, ×200 (scale bars, 10 μm). Right (**c**), quantification of leukocytes in the peribronchiolar region (cells per mm^2). $^*P < 0.01$ (Student's *t*-test). (**e**) Quantitative RT-PCR analysis of *Clca3* and *Muc2* mRNA in lungs from mice as in **a** ($n = 3$ per group). (**f**) Airway resistance of mice as in **a** ($n = 6$ per group) treated with various concentrations of methacholine (horizontal axis), presented as lung resistance (RL) relative to the lung resistance without methacholine treatment. $^*P < 0.01$ and $^{**}P < 0.001$ (ANOVA and Bonferroni test). Data are representative of three independent experiments with similar results (mean and s.d. in **a**–**c**,**e**,**f**).

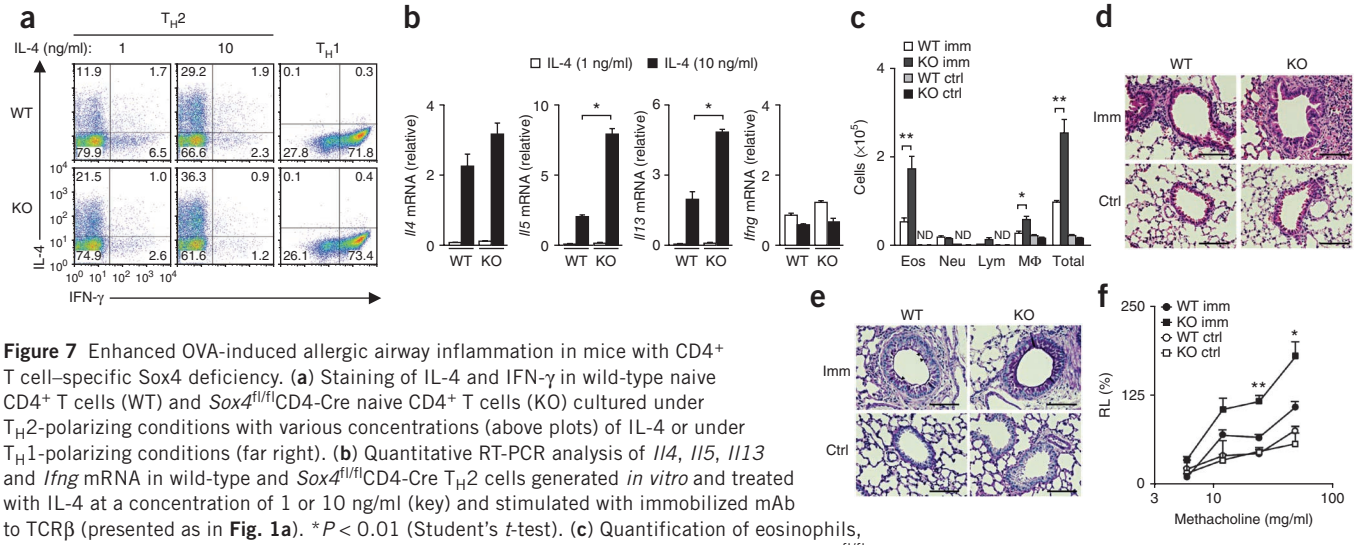

Figure 7 Enhanced OVA-induced allergic airway inflammation in mice with CD4$^+$ T cell–specific Sox4 deficiency. (**a**) Staining of IL-4 and IFN-γ in wild-type naive CD4$^+$ T cells (WT) and *Sox4*$^{fl/fl}$CD4-Cre naive CD4$^+$ T cells (KO) cultured under T_H2-polarizing conditions with various concentrations (above plots) of IL-4 or under T_H1-polarizing conditions (far right). (**b**) Quantitative RT-PCR analysis of *Il4*, *Il5*, *Il13* and *Ifng* mRNA in wild-type and *Sox4*$^{fl/fl}$CD4-Cre T_H2 cells generated *in vitro* and treated with IL-4 at a concentration of 1 or 10 ng/ml (key) and stimulated with immobilized mAb to TCRβ (presented as in **Fig. 1a**). $^*P < 0.01$ (Student's *t*-test). (**c**) Quantification of eosinophils, neutrophils, lymphocytes, macrophages and total cells of the BAL fluid from wild-type or *Sox4*$^{fl/fl}$CD4-Cre mice ($n = 5$ per group) left unimmunized or immunized with OVA. $^*P < 0.05$ and $^{**}P < 0.01$ (ANOVA and Bonferroni test). (**d**) Microscopy of lungs from the mice in **c**, fixed and stained with hematoxylin and eosin. Quantification of mononuclear cells (per mm^2): wild-type, 552 ± 37.6 (unimmunized) or 2,654 ± 308.6 (immunized); *Sox4*$^{fl/fl}$CD4-Cre, 474.7 ± 80.5 (unimmunized) or 5,694 ± 574.7 (immunized). $P < 0.01$, wild-type immunized versus *Sox4*$^{fl/fl}$CD4-Cre immunized (Student's *t*-test). (**e**) Microscopy of lungs from the mice in **c**, fixed and stained with periodic acid–Schiff reagent. Original magnification (**d**,**e**), ×200 (scale bars, 10 μm). (**f**) Airway resistance of lungs from the mice in **c** ($n = 6$ per group; presented as in **Fig. 6f**). $^*P < 0.01$ and $^{**}P < 0.001$ (ANOVA and Bonferroni test). Data are representative of three (**a**,**b**) or two (**c**–**f**) independent experiments with similar results (mean and s.d. in **b**,**c**,**f**).

of *lox*P-flanked genes) to produce *Sox4*$^{fl/fl}$CD4-Cre offspring. The number of CD4$^+$ T cells, cell-surface expression of TCR and cytokine receptors and the proliferative responses of naive CD4$^+$ T cells induced by stimulation with mAb to TCR plus mAb to CD28 were within the normal range in *Sox4*$^{fl/fl}$CD4-Cre mice (**Supplementary Fig. 7a–c**). *Sox4*$^{fl/fl}$CD4-Cre naive CD4$^+$ T cells cultured under T_H2 conditions generated more IL-4-producing cells than did wild-type cells, whereas the generation of IFN-γ-producing cells under T_H1 conditions was similar in cells of each genotype (**Fig. 7a**). The generation of IL-17-producing T_H17 cells, Foxp3-expressing iT_{reg} cells and IL-9-producing T_H9 cells was not affected by the genotype of the cells (**Supplementary Fig. 7d**). We detected significantly higher expression of *Il5* and *Il13* mRNA in *Sox4*$^{fl/fl}$CD4-Cre T_H2 cells than in wild-type T_H2 cells (**Fig. 7b**). The expression of GATA-3 protein was similar in T_H2 cells of each genotype (**Supplementary Fig. 7e**).

In the airway inflammation model, we observed significantly more infiltration of the BAL fluid by inflammatory cells, including eosinophils, in OVA-immunized *Sox4*$^{fl/fl}$CD4-Cre mice than in OVA-immunized wild-type mice (**Fig. 7c**). The expression of *Il5* and *Il13* mRNA in cells of the BAL fluid was slightly but notably higher *Sox4*$^{fl/fl}$CD4-Cre mice than in wild-type mice (**Supplementary Fig. 7f**); *Sox4*$^{fl/fl}$CD4-Cre mice had more mononuclear cells infiltrating the peribronchiolar regions of the lungs than did wild-type mice (**Fig. 7d**), and *Sox4*$^{fl/fl}$CD4-Cre mice had more mucus hyper-production and goblet-cell metaplasia in the bronchioles than did wild-type mice (**Fig. 7e**). There was also significantly more airway hyper-responsiveness in *Sox4*$^{fl/fl}$CD4-Cre mice than in wild-type mice (**Fig. 7f**). Together these results indicated that Sox4 regulated T_H2 cell–mediated allergic airway inflammation and airway hyper-responsiveness.

DISCUSSION

Here we have demonstrated that Sox4 was induced by TGF-β and downregulated T_H2 differentiation through its role in the negative regulation of GATA-3 function via two distinct mechanisms. First, Sox4 associated with GATA-3 and inhibited the binding of GATA-3 to its DNA-binding sequence, which resulted in impaired GATA-3-induced T_H2 differentiation. Second, Sox4 directly bound to the *Il5* promoter and interfered with the binding of GATA-3 to DNA, which led to the repression of *Il5* transcription. Therefore, *Il5* expression was negatively regulated by Sox4 through the inhibition of both T_H2 differentiation and transcription.

During the initiation of T_H2 differentiation, stimulation of the TCR seemed to downregulate Sox4 expression and facilitated the GATA-3 function of inducing T_H2 differentiation and *Il5* transcription. In the presence of TGF-β, however, Sox4 was induced and suppressed GATA-3 function, which resulted in the inhibition of T_H2 differentiation and *Il5* transcription. The expression of Sox4 seemed to depend on the balance between the strength of TCR stimulation and the amount of TGF-β.

Overexpression of Sox4 in developing T_H1 cells induced more IFN-γ-producing cells. However, we observed no apparent change in T_H1 differentiation in cultures of Sox4-deficient T_H1 cells. Therefore, although it is likely that Sox proteins, including Sox4, control T_H1 differentiation under some conditions, we obtained no solid evidence indicating that Sox4 directly controlled T_H1 differentiation.

TGF-β-dependent induction of *SOX4* mRNA has been demonstrated in a human pituitary tumor cell line[36]. TGF-β induces Sox4 expression in glioma-initiation cells, and Sox4 is suggested to maintain the stem-cell qualities of the glioma-initiation cells via the induction of Sox2 expression[37]. Although the expression of Sox2 was undetectable in naive CD4$^+$ T cells, *Sox4* mRNA expression was high in these cells. Therefore, Sox4 may have an important role in maintaining the quiescent state and/or the multilineage differentiation potential of naive CD4$^+$ T cells.

Several transcription factors, including ROG ('repressor of GATA'), FOG ('friend of GATA') and LEF1, have been reported to associate with GATA-3 and modulate GATA-3 function[38–40]. ROG binds to the

carboxy-terminal finger of GATA-3 and FOG binds to the amino-terminal finger of GATA-3, whereas both zinc fingers of GATA-3 are required for its association with LEF1. Similar to that[40], both zinc fingers of GATA-3 seemed to be involved in its association with Sox4. Both Sox4 and LEF1 belong to the HMG box–containing family of transcription factors, and the HMG box of these molecules is required for the association with GATA-3 (ref. 40). This is consistent with the proposal that heterodimerization occurs via the HMG box of Sox molecules and the DNA-binding domain of partner transcription factors[27,41,42]. Thus, although detailed structural analysis of the interaction between the Sox HMG box and the GATA-3 zinc fingers is required, Sox proteins may be able to associate with GATA proteins and thus may be important in the regulation of transcription factors of the GATA family.

We found that a Sox-binding motif overlapped one of the GATA-binding motifs in the *Il5* promoter. Sox4 itself did not activate the *Il5* promoter in M12 mouse B cells, but Sox4 interfered with the binding of GATA-3 to DNA via directly binding to the Sox-binding motif. A similar example of overlapping binding sites for Sox and GATA in the promoter of the gene encoding fibroblast growth factor 3 (*Fgf3*) has been reported[43]. In this case, both Sox7 and GATA-4 independently bind to and activate the *Fgf3* promoter, which indicates that Sox7 and GATA-4 are competitive activators of *Fgf3* transcription. In contrast, Sox4 seemed to compete with GATA-3 to inhibit *Il5* transcription.

TCR-induced proliferative responses of naive $CD4^+$ cells are required for T_H1 and T_H2 differentiation. In addition, it takes 2–3 d to induce GATA-3 expression similar to that in differentiated T_H2 cells[44]. Therefore, we added TGF-β to the T_H2-differentiation cultures 48 h after the initial TCR stimulation to address the role of TGF-β in T_H2-specific processes more selectively. In our experimental system, the inhibition of GATA-3 expression by TGF-β was marginal, whereas we observed selective inhibition of T_H2 differentiation. TGF-β is known to control several processes during the development of T_H2 cells, such as the proliferation of naive $CD4^+$ T cells[20]. TGF-β is also known to strongly inhibit the development of T_H2 cells even in the presence of exogenous IL-4 (refs. 23,24). However, the effect of TGF-β on T_H1 development is less clear. TGF-β is known to inhibit IL-12-dependent T_H1 differentiation[45], whereas the IFN-γ-induced development and/or enhancement of T_H1 cells is not perturbed but is instead enhanced in the presence of TGF-β[46].

$CD4^+$ T cells cultured under T_H2 conditions in the presence of TGF-β 'preferentially' differentiate into T_H9 cells[47,48]. We found that the efficiency of the generation of T_H9 cells by either Sox4-transgenic or *Sox4*$^{fl/fl}$CD4-Cre T cells was not altered and the induction of Foxp3 was not impaired in Sox4-deficient $CD4^+$ T cells. Thus, Sox4 may have only a limited role in T_H9 and iT_{reg} differentiation. In summary, Sox4 is a downstream target of TGF-β and, through its role as a negative regulator of GATA-3, functions a critical regulator of T_H2 differentiation and T_H2 cell–dependent immune responses.

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.

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AUTHOR CONTRIBUTIONS

M.K. and M.Y. designed and did experiments, analyzed data and wrote the manuscript; K.S., S.T., A.O., R.S., S.M., D.T., H.H. and C.I. designed and did experiments and edited the manuscript; V.L. established Sox4 deficient mice; and T.N. conceptualized the research, directed the study and wrote and edited the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. Mice with transgenic expression of Sox4 under the control of the distal promoter of the mouse *Lck* gene were generated on a C57BL/6 background in the Department of Immunology of Chiba University. The distal *Lck* promoter was used to minimize the effect of Sox4 overexpression on T cell development in the thymus, as this promoter is reported to be active from the late $CD4^+CD8^+$ double-positive stage to the $CD4^+$ or $CD8^+$ single-positive stage of thymocyte development. Sox4-deficient mice[35] established by V.L. were backcrossed to C57BL/6 mice eight times. Mice with transgenic expression of Cre under the control of the *Cd4* promoter, on a C57BL/6 background, were from Jackson Laboratory. DO11.10 mice (with transgenic expression of an OVA-specific TCR-αβ) were provided by D. Loh. C57BL/6 and BALB/c mice were from Clea. All mice were maintained under specific pathogen–free conditions and were used at 6–10 weeks of age. All experiments with mice received approval from the Chiba University Administrative Panel for Animal Care. All animal care was in accordance with the guidelines of Chiba University.

$CD4^+$ T cell–differentiation cultures. $CD4^+$ T cells with a naive phenotype ($CD44^{lo}CD62L^{hi}$) were purified with a FACSAria cell sorter (Becton Dickinson), which yielded a purity of >98%, and were used as naive $CD4^+$ T cells. Naive $CD4^+$ T cells (1.5×10^6) were stimulated for 2 d with immobilized mAb to TCRβ (10 μg/ml or 1 μg/ml; H57-597; BioLegend) plus soluble mAb to CD28 (1 μg/ml; 37.5; BioLegend) in the presence of IL-2 (2.5 ng/ml; Peprotech), IL-12 (3 ng/ml; PeproTech) and mAb to IL-4 (5 μg/ml; 11B11; BioLegend), for T_H1 conditions, or in the presence of IL-2 (2.5 ng/ml), IL-4 (10 ng/ml; PeproTech) and mAb to IFN-γ (5 μg/ml; R4-6A2; BioLegend), for T_H2 conditions. Cells were cultured for an additional 3 d without stimulation of the TCR in the presence of the original cytokines. Where needed, TGF-β (10 ng/ml; PeproTech) was added to the second culture. Cultured cells were restimulated for 6 h with mAb to TCRβ (10 μg/ml), and intracellular staining was done as described[34].

Expression plasmids and retrovirus-mediated gene transfer. Flag-tagged GATA-3 mutants (pFlag-CMX2-GATA-3) and Myc-tagged Sox4 mutants (pCMV Myc-Sox4) were generated by PCR-based mutation. Expression plasmids were transfected into 293T cells with Fugene reagentaccording to the manufacturer's protocol (Roche). The method for the generation of retrovirus supernatant and infection of developing T_H2 cells has been described[49]. Infected cells were detected by staining with mAb to human nerve growth factor receptor (C40-1457; BD Bioscience).

Knockdown analysis. A Sox4-containing microRNA-adapted retroviral vector (MSCV/LTRmiR30-PIG; vector pLMP; Open Biosystems) was used for Sox4 shRNA. Naive $CD4^+$ T cells were cultured for 2 d under T_H2 conditions and then infected with retrovirus vector containing control shRNA (pLMP-hNGFR) or Sox4 shRNA (pLMP-shSox4-hNGFR) and were cultured in the presence of TGF-β (10 ng/ml). Then, 3 d after infection, infected cells (positive for human nerve growth factor receptor (hNGFR)) were purified and subjected to further analysis. The siRNA was introduced into primary $CD4^+$ T cells by electroporation with a Mouse T cell Nucleofector Kit and Nucleofector I (Amaxa). Naive $CD4^+$ T cells were transfected with 675 pmol control (random) siRNA or siRNA specific for Sox4 (Applied Biosystems) and were cultured under the appropriate conditions. At 4 d after transfection, cultured cells were analyzed by quantitative RT-PCR.

Quantitative RT-PCR. Quantitative RT-PCR was done as described[34]. Primers and TaqMan probes for the detection of mouse *Gob5, Mac5a-Mac5c, Sox4*, *Gata3*, *Nfat1*, *Nfat2*, *Maf*, *Junb*, *Il4*, *Il5*, *Il13*, *Ifng* and *Hprt* were from Applied Biosystems.

ChIP assay. ChIP assays were done as described[49]. Antibody to H3K9ac-H3K14ac (06-595; Upstate Biotechnology), antibody to H3 trimethylated at Lys4 (ab8580; Abcam), mAb to GATA-3 (HG3-31; Santa Cruz Biotechnology), anti-Smad2-Smad3 (ab28379; Abcam), mAb to Smad2-Smad3 (D7G7; Cell Signaling) and anti-Sox4 antiserum (AB5803; Millipore) were used. For the detection of specific genome regions, the Roche Universal Probe Library System was used. The specific primers and TaqMan probes for the detection of the *Sox4* and *Cd3e* were as follows: Sox4 U forward, 5′-CGGGAGACAATGGGTAAG AA-3′, and reverse, 5′-CCAAAGGATAGATGGGTTCG-3′; universal probe 12 (4-685-113; Roche); Sox4 P forward, 5′-TGCACCAAAGGCTGATTC TT-3′, and reverse: 5′-TTCTGCTTAAAAGCCGAGTGA-3′; universal probe 26 (4-687-574; Roche); CD3ε P forward, 5′-ACACTTCCTGTGTGG GGTTC-3′, and reverse, 5′-CTGAAGAAGGCACCAGACG-3′; and universal probe 16 (4-686-896; Roche). Other specific primers and TaqMan probes used have been described[34].

Luciferase reporter assay. The luciferase assay for *Il5* promoter activity was done in an M12 B cell line as described[18] with a firefly luciferase reporter (pGL3; Promega) and a renilla luciferase plasmid (pRL; Promega). Luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega).

Precipitation assay. Lysates of transfected 293T cells were incubated with biotinylated oligonucleotides and bound proteins were eluted and separated by SDS-PAGE, then analyzed by immunoblot with specific antibodies. Oligonucleotide probes for the precipitation assay were as follows: GATA-3 consensus, 5′-CACTTGATAACAGAAAGTGATAACT CT-3′; *Il5*p Mut1, 5′-CCCTCTATCTGATTAATAGCA-3′; and *Il5*p Mut2. 5′-CCTCTTTCTGATTGTTAGCA-3′.

Immunoprecipitation and immunoblot analysis. Immunoprecipitation and immunoblot were done as described[40]. The TG40 T cell line[50] was used for coimmunoprecipitation. A mAb to GATA-3 (HG3-31; Santa Cruz Biotechnology), anti-Sox4 antiserum (C-20; Santa Cruz Biotechnology), mAb to Flag (M2; Sigma-Aldrich) and mAb to Myc (PL14; MBL, Japan) were used for immunoblot analysis. After immunoprecipitation with mAb to Flag, the immunoprecipitates were eluted with 3X FLAG peptide (F4799; Sigma-Aldrich) and were then separated by electrophoresis.

Glutathione *S*-transferase precipitation assay. Glutathione *S*-transferase–tagged recombinant GATA-3 (1.5 μg; Abnova) and Myc-tagged recombinant Sox4 (1.5 μg; Origene) were mixed with glutathione Sepharose 4B (GE Healthcare) in binding buffer (20 mM Tris-HCl (pH 7.4), 250 mM NaCl, 1% Triton X-100, 0.25% sodium deoxycholate) and incubated for 1 h at 4 °C. Coprecipitated Sox4 was separated by electrophoresis and visualized by immunoblot analysis with mAb to Myc. Glutathione *S*-transferase protein (ab70456; Abcam) served as a control.

OVA-induced allergic airway inflammation and hyper-responsiveness. Mice were immunized intraperitoneally with 250 μg OVA in 2 mg aluminum hydroxide gel (alum) on days 0 and 7 and then challenged with aerosolized OVA in saline (10 mg/ml) on days 14, 16, 21 and 23. At 2 d after the final inhalation of OVA, cells from BAL fluid and lung samples for histological examination were prepared as described[49]. Airway hyper-responsiveness was assessed by measurement of the change in lung resistance and dynamic compliance in response to increasing doses of inhaled methacholine as described[49]. For transfer experiments, naive DO11.10 $CD4^+$ T cells were stimulated for 2 d under neutral conditions and then infected with empty retroviral vector or retroviral vector containing Sox4-specific shRNA. Then, 3 d after infection, the infected cells were purified and stimulated for another 5 d with mAb to TCRβ plus mAb to CD28 under T_H2 conditions. The infected T_H2 cells (1×10^6) were transferred intravenously into BALB/c mice and the recipient mice were challenged by inhalation of OVA twice on days 1 and 3. The infiltration of inflammatory cells into BAL fluid and airway hyper-responsiveness were assessed on day 4.

Statistical analysis. Student's *t*-test was used. ANOVA and the Bonferroni test was used for multiple comparisons of different groups.

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50. Sussman, J.J., Saito, T., Shevach, E.M., Germain, R.N. & Ashwell, J.D. Thy-1- and Ly-6-mediated lymphokine production and growth inhibition of a T cell hybridoma require co-expression of the T cell antigen receptor complex. *J. Immunol.* **140**, 2520–2526 (1988).