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Requirement for the basic helix-loop-helix transcription factor Dec2 in initial T_H2 lineage commitment

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Abstract

How naïve $CD4^+$ T cells commit to the T helper type 2 (T_H2) lineage is poorly understood. Here we show that the basic helix-loop-helix transcription factor Dec2 is selectively expressed in T_H2 cells. $CD4^+$ T cells from Dec2-deficient mice exhibits defective T_H2 differentiation *in vitro* and *in vivo* in an asthma model and in response to challenge with a parasite antigen. Dec2 promotes interleukin 4 (IL-4), IL-5 and IL-13 expression during early T_H2 differentiation, and directly binds to and activates transcription of the *Junb* and *Gata3* genes. As GATA3 induces Dec2 expression, these findings also indicate a feed-forward regulatory circuit during T_H2 differentiation.

INTRODUCTION

$CD4^+$ T helper (T_H) cells are the central regulators of adaptive immunity and allergic diseases. Upon activation by antigen-presenting cells (APCs), naïve $CD4^+$ precursors undergo clonal expansion and functional differentiation into cytokine-secreting effector cells. T_H1 cells make interferon (IFN)-γ and promote antigen presentation and cellular immunity 1, 2. T_H2 cells produce interleukin (IL)-4, -5 and -13, which together regulate anti-parasite and allergic responses 1, 2. T_H-17 cells, distinct from T_H1 and T_H2 cells, secret IL-17, IL-17F, IL-22 and IL-21 and mediate tissue inflammation 3, 4.

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Author Contributions

C.D., X.O.Y. and B.S. designed the research, analyzed and interpreted the results. X.O.Y., P.A., J.Z., J.P., Z.L., R.N., X.L., Y.C. and S.H.C. performed the experiments, and X.O.Y., B.S. and C.D. prepared the manuscript.

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T_H effector differentiation is determined by the cytokine environment, which ultimately directs the expression of lineage-specific transcription factors 1, 2. During T_H2 cell polarization, IL-4 is essential, as it activates STAT6, which induces the expression of the transcription factor GATA3 5. Naive $CD4^+$ T cells start to express IL-4 mRNA 24 hours after stimulation of the T cell receptor (TCR) and costimulatory receptors; IL-4 mRNA expression increases even further at 48 hours post-stimulation6. The costimulatory receptor ICOS and the cytokine IL-25 are also important for early IL-4 production, as they promote expression of the transcription factors NFATc1 and JunB (http://www.signaling-gateway.org/molecule/query?afcsid=A001301)7–9. In addition to IL-4, IL-2 is involved in the initiation of T_H2 differentiation 10, 11. When stimulated with low concentrations of antigenic peptide, T_H cells produced IL-4, and this IL-4 production is regulated by IL-2–dependent STAT5 phosphorylation and IL-4–independent early GATA-3 expression 11. IL-2 also facilitates early T_H2 differentiation by inducing expression of the IL-4 receptor α-chain in a manner dependent on STAT5 but independent of IL-4 12.

However, the precise mechanism controlling initial T_H2 lineage commitment is not well understood. Here we show that Dec2 (also called Sharp1, Bhlhb3; Bhlhb2l or Bhlhe41) 13, 14, a basic helix-loop-helix transcription factor previously implicated in regulating circadian rhythm 15 and differentiation of a range of cell types 16, 17, is selectively expressed in the T_H2 subset among all tested T helper cell subsets. We found that Dec2 promotes expression of GATA3 and JunB, the latter of which induces expression of IL-4 5, 18, 19 and IL-2 20–22 to promote T_H2 differentiation.

Results

Regulation of Dec2 expression in T_H2 cells

In a gene expression microarray (B.S. and Z.L., data not shown, and Z. Li, K. Mao, J. Zou, Y. Wang, Z. Tao, G. Lin, L.Tian, Y. Ji, X. Wu, X. Zhu, S. Sun, C. Xiang, and W. Chen, personal communication), expression of Dec2 mRNA was greatly elevated in T_H2 cells compared to T_H1 cells (13.5 fold). We thus further assessed the expression of Dec2 mRNA in T_H1, T_H2, T_H-17, and inducible regulatory T (iT_{reg}) cell subsets as well as naïve $CD4^+$ and $CD8^+$ T cells. $CD4^+$ T cells purified from OT-II TCR transgenic mice were cultured in T_H1, T_H2, T_H-17 or iT_{reg} cell-polarizing conditions *in vitro*. As measured via quantitative real-time RT-PCR, *Dec2* mRNA was highly expressed in T_H2 cells but not in the other types of T cells (Fig. 1a). *Dec2* mRNA was also expressed in non-T lineage immune cells, including plasmacytoid dendritic cells (DCs) and eosinophils (Supplementary Fig. 1).

To understand the function of Dec2 in T cells, we tested the kinetics of Dec2 induction during T_H2 differentiation. $CD4^+CD25^-CD62L^{hi}CD44^{lo}$ naïve T cells from C57BL/6 (B6) mice purified by fluorescence-activated cell sorting (FACS) were activated with plate-bound anti-CD3 and anti-CD28 under T_H1 or T_H2-polarizing conditions for 1 to 4 days and gene expression was assessed daily. Similar to *Gata3* mRNA, *Dec2* mRNA expression increased with time in developing T_H2 but not in T_H1 cells; in contrast, T_H1 but not T_H2 cells expressed abundant *Tbx21* mRNA (Fig. 1b). To test whether Dec2 is expressed in T_H2 cells generated under physiological conditions, we induced experimental asthma in *Il4-Gfp* reporter (4-get) mice 23 and purified IL-4-GFP^+ and GFP^- cells by FACS from the

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$CD4^+CD44^{hi}$ population among lung-associated lymph node cells. As expected, Dec2 was highly expressed in IL-4-GFP$^+$ T_H2 cells but not in GFP$^–$ non-T_H2 cells (Fig. 1c). Thus, Dec2 is selectively expressed in T_H2 cells generated *in vitro* and *in vivo*.

We next asked how Dec2 expression is regulated during T_H differentiation. Costimulatory signals especially those emanating from ICOS are important for T_H2 differentiation [9 11]. We thus sorted naïve CD4$^+$ T cells from B6 mice and activated them with plate-bound anti-CD3 in the presence or absence of anti-CD28 or/and anti-ICOS for 1 or 2 days. On day 1, stimulation with anti-CD3 plus anti-CD28, but not with anti-CD3 alone, induced minimal upregulation of Dec2 mRNA expression (Fig. 1d). Addition of anti-ICOS further enhanced *Dec2* expression. On day 2, *Dec2* mRNA was much more abundant in cells receiving ICOS costimulation (Fig. 1d). These results indicate an important role of costimulation, particularly via ICOS, in regulation of *Dec2* mRNA expression.

IL-25 facilitates the initiation of T_H2 differentiation 7. Therefore, we also tested the effect of IL-25 on *Dec2* mRNA expression. Naïve CD4$^+$ T cells from B6 mice were activated with plate-bound anti-CD3 and anti-CD28 in the presence or absence of IL-25 for 1 to 3 days. *Dec2* mRNA expression was strongly boosted in response to IL-25 stimulation (Fig. 1e).

As *Dec2* mRNA is selectively expressed in T_H2 cells, we assessed whether Dec2 expression is regulated by the transcription factor GATA3, which promotes T_H2 differentiation. Naïve CD4$^+$ T cells from *Il4*$^{+/+}$ or *Il4*$^{–/–}$ mice were activated with plate-bound anti-CD3 and anti-CD28 for 36 hours and infected with bicistronic retroviruses encoding GATA3-IRES-GFP or GFP alone. Two days later, GFP$^+$ cells were FACS sorted and *Dec2* and *Il4* mRNA expression was measured by quantitative RT-PCR. In both *Il4*$^{+/+}$ and *Il4*$^{–/–}$ cells, over-expression of GATA3 increased *Dec2* mRNA expression (Fig. 1f). In addition, we tested whether GATA3 is required for Dec2 expression. Established *Gata3*$^{fl/fl}$ or *Gata3*$^{+/+}$ T_H2 cells were infected with a bicistronic retrovirus encoding the Cre recombinase and GFP (hCre-IRES-GFP-RV)24. GFP$^+$ cells were FACS-sorted after transduction and cultured under T_H2-polarizing conditions for 1 or 2 weeks. *Dec2* and *Il13* mRNA expression was then measured. Unlike *Il13* mRNA, the expression of which was quickly reduced after deletion of *Gata3*, *Dec2* mRNA expression remained stable for 1 week after deletion of *Gata3* but diminished after 2 weeks (Fig. 1g). Next, we tested the impact of acute GATA3 ablation on *Dec2* mRNA expression during early T_H2 differentiation. Naïve CD4+ T cells from *Gata3*$^{fl/fl}$ mice were activated under T_H2-polarizing conditions and were infected with Cre-expressing or control retroviruses. Deletion of *Gata3* at this early stage of T_H2 differentiation did not substantially influence *Dec2* mRNA expression, but it did markedly reduce *Il13* mRNA expression (Fig. 1h). Taken together, these findings indicate that *Dec2* mRNA expression is induced by costimulatory signals and by IL-25. GATA3 is sufficient to induce *Dec2* mRNA expression but is not necessary for induction or maintenance of *Dec2* mRNA expression.

Dec2 in T_H2 differentiation *in vitro*

To assess the function of Dec2 in T cell differentiation, we generated Dec2-deficient mice. Two LoxP sites were introduced into the Dec2 locus, flanking the promoter region and exons 1 to 3. Germline Dec2-deficient mice were produced by breeding this floxed mouse

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with a CMV-Cre mouse (Supplementary Fig. 2a–b). *Dec2*$^{+/-}$ mice were backcrossed with B6 for 6–8 generations and were then interbred to generate *Dec2*$^{-/-}$ mice. In all experiments, *Dec2*$^{-/-}$ mice on a B6 background were used unless indicated. In *Dec2*$^{-/-}$ mouse, *Dec2* messenger RNA in bone marrow was not detectable by RT-PCR (Supplementary Fig. 2c) and Dec2 protein expression in splenocytes was completely absent when tested by immunoblot (Supplementary Fig. 2d). The development of lymphoid populations in thymus and spleen appeared grossly normal in *Dec2*-deficient mice (Supplementary Fig. 3).

To analyze the function of Dec2 in T cell function, we first examined CD4$^+$ T cell activation in response to anti-CD3 and anti-CD28 stimulation. Naïve CD4$^+$ T cells were FACS sorted from Dec2-deficient and control mice and activated with various concentrations of plate-bound anti-CD3 in the presence of a fixed amount anti-CD28. IL-2 production was measured by ELISA on day 2 and ^{3}H-thymidine incorporation on day 3. *Dec2*-deficient CD4$^+$ T cells exhibited reduced proliferation and IL-2 production compared with wild-type cells (Fig. 2a).

Next, we assessed the function of Dec2 in T_H differentiation. Naïve CD4$^+$ T cells from wild-type and Dec2-deficient mice were differentiated under neutral (T_H0), T_H1, T_H2, T_H-17 and iT_{reg} conditions for 4 days. As revealed by intracellular staining, no IL-4$^+$ cells were detected under T_H0 or T_H1 conditions. Under the T_H0 condition, *Dec2* deficiency resulted in reduced numbers of IFN-γ-producing cells (Fig. 2b). Reduced percentages of IFN-γ-producing cells were also observed under the T_H1 condition (Fig. 2b). T_H-17-polarizing conditions (TGF-β plus IL-6) induced greater percentages of IL-17-producing cells in Dec2-deficient than wild-type populations; addition of anti-IFN-γ and anti-IL-4 diminished this difference (Fig. 2b), suggesting that the enhanced T_H-17 differentiation in Dec2-deficient T cells may be due to their impaired production of IFN-γ and/or IL-4. Under the iT_{reg} condition, Dec2-deficient T cells developed into Foxp3$^+$ cells as efficiently as wild-type cells (Fig. 2b). During T_H2 differentiation in the presence of increasing doses of exogenous IL-4, *Dec2* deficiency led to greatly reduced percentages of IL-4-producing cells (Fig. 2c). As measured by ELISA, Dec2-deficient T cells in these experiments secreted reduced amounts of the T_H2 cytokines IL-4, IL-5 and IL-13 (Fig. 2d). High doses of exogenous IL-4 partially restored IL-4 and IL-5 but not IL-13 expression in *Dec2*-deficient cells. In summary, our analysis of Dec2-deficient T cells indicates that Dec2 is needed for maximal IL-2 production and T_H2 differentiation *in vitro*.

Dec2 promotes T_H2 responses *in vivo*

To analyze the function of Dec2 *in vivo*, we immunized *Dec2*-deficient and B6 mice subcutaneously with chicken ovalbumin (OVA) protein in Complete Freund's Adjuvant (CFA). Five days later, splenocytes were isolated and stimulated *ex vivo* with OVA protein. OVA immunization in CFA elicited a strong inflammatory response, and Dec2-deficient cells expressed comparable amounts of IFN-γ and IL-17 as wild-type cells, and neither Dec2-deficient nor wild-type cells expressed detectable amounts of IL-4 (Fig. 3a). These results indicate that Dec2 is dispensable for T cell activation and T_H1 and T_H-17 differentiation *in vivo*.

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To determine the role of Dec2 in T_H2 responses *in vivo*, we immunized mice intraperitoneally (i.p.) with OVA and the adjuvant alum. Upon restimulation with OVA, T cells from *Dec2*-deficient animals exhibited significantly reduced IL-4, IL-5 and IL-13 expression and comparable IL-17 and IFN-γ expression compared with those from wild-type mice (Fig. 3b). Next we adoptively transferred $CD4^+$ T cells from *Dec2*-deficient or wild-type mice together with wild-type B cells into $Rag1^{-/-}$ mice. Recipients of Dec2-deficient T cells produced less IL-4, IL-5 and IL-13 after immunization with OVA and alum, suggesting that the defective T_H2 cytokine expression in *Dec2*-deficient mice was due to a $CD4^+$ T cell-intrinsic defect (Fig. 3c).

T_H2 cells play an important role in immunity against parasite infection. To characterize the role of Dec2 in type 2 immunity, we injected *Dec2*-deficient and wild-type mice i.p. with *Schistosoma mansoni* eggs, which elicit strong T_H2 responses 25. In response to restimulation with soluble egg antigen (SEA), splenocytes from *Dec2*-deficient animals exhibited defective T_H2 cytokine expression compared with those from wild-type mice (Fig. 3d). In addition, IFN-γ but not IL-17 expression was reduced in *Dec2*-deficient cells.

As T_H2 cells are pathogenic in asthma, we induced experimental allergic asthma in *Dec2*-deficient and wild-type mice by using a standard protocol 26. Compared with wild-type mice, *Dec2*-deficient mice contained significantly fewer total cells, eosinophils, neutrophils and lymphocytes in bronchoalveolar lavage (BAL) fluid (**Fig. a**). Moreover, upon *ex vivo* restimulation with different doses of OVA protein, lung-associated lymph node cells from *Dec2*-deficient mice produced lower amounts of IL-4, IL-5 and IL-13 than did cells from wild-type mice (Fig. 4b). To rule out the possibility that *Dec2* deficiency affects eosinophil development, we injected recombinant murine IL-5 i.p. into Dec2-deficient and wild-type mice and examined blood eosinophils with Wright-Giemsa staining. No significant difference was found in the numbers of blood eosinophils in wild-type and Dec2-deficient mice (Supplementary Fig. 4). Thus, the reduced eosinophila in asthmatic Dec2-deficient mice was not likely due to a defect in eosinophils or IL-5 signaling, but rather was caused by less T cell IL-5 expression in response to OVA + alum challenge. In summary, our data indicate that Dec2 is required for T_H2 responses *in vivo*.

Dec2 in early T_H2 lineage commitment

We next addressed how Dec2 regulates T_H2 differentiation by asking whether Dec2 is required for T_H2-specific gene expression during early T_H2 development. Naïve $CD4^+$ T cells from Dec2-deficient or wild-type mice were activated under the T_H2-polarizing condition or with only anti-IFN-γ for 2 or 4 days. As measured by quantitative RT-PCR, *Il4* mRNA expression was reduced in Dec2-deficient cells under both conditions on days 2 and 4 (Fig. 5a). These results indicate a critical role for Dec2 in early IL-4 expression in activated T cells. To further understand the function of Dec2, we differentiated naïve $CD4^+$ T cells from Dec2-deficient or wild-type mice under T_H0 or T_H2 conditions for 2 days and analyzed their gene expression profiles. Dec2-deficient cells failed to up-regulate *Gata3*, *Junb*, *Il4ra* and *Il17rb* mRNA, but expressed wild-type amounts of *Irf4*, *Nfatc1* and *Tbx21* mRNA (Fig. 5b). Notably, IL-2 expression was reduced in *Dec2*-deficient cells under the

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T_H0 but not T_H2 conditions (Fig. 5b). These results indicate that Dec2 plays an important role in early T_H2 differentiation and is required for GATA3 and JunB expression.

To understand if Dec2 is sufficient to initiate T_H2 differentiation, we over-expressed Dec2 by retroviral transduction. Naïve $CD4^+$ T cells from wild-type mice were activated with plate-bound anti-CD3 and anti-CD28 in the presence or absence of a blocking antibody specific for IFN-γ. Thirty-six hours later, the activated cells were infected with bicistronic retroviruses encoding GFP alone or with Dec2. Two days after infection, GFP^+ cells were FACS-sorted and their gene expression profiles were assessed using real-time RT-PCR. In both the presence and the absence of anti-IFN-γ, Dec2 over-expression greatly enhanced *Il2*, *Il4*, *Il5*, *Il13*, *Gata3*, *Junb*, *Il4ra* and *Il17rb* mRNA expression, while *Ifng* and *Tbx21* expression was not changed (Fig. 6a). Thus, Dec2 is sufficient to up-regulate T_H2-specific gene expression, even under neutral conditions.

We then asked whether Dec2 can up-regulate T_H2-specific gene expression under non-favorable conditions. First, we over-expressed Dec2 in cells cultured in T_H1 or T_H-17-polarizing conditions or in the presence of a blocking antibody specific for IL-4. In the presence of anti-IL-4, low amounts of *Il5* and *Il13* mRNA expression were detected after over-expression of Dec2 (data not shown), which correlated with a very modest increase in *Gata3* and *Junb* mRNA expression; no change in *Tbx21* or *Ifng* expression was detected (Fig. 6b and data not shown). Under T_H1-polarizing conditions, over-expression of Dec2 moderately elevated only JunB expression (Fig. 6b). Under T_H-17-polarizing conditions, both *Gata3* and *Junb* mRNA were significantly increased in cells infected with Dec2-encoding compared to control retrovirus, but no T_H2 cytokine expression was detected (Fig. 6c and data not shown). In summary, Dec2 overexpression drives T_H2 differentiation under neutral conditions but is not sufficient to promote T_H2 differentiation in the T_H1 or T_H-17-polarizing conditions.

Dec2 binds to and activates *Junb* and *Gata3* genes

Dec2 can promote T_H2 differentiation, which is IL-4 dependent. *Junb* and *Gata3* expression was elevated by Dec2 overexpression. We thus reasoned that Dec2 may promote T_H2 differentiation by directly regulating the expression of these factors. Using the VISTA program 27, 28, we searched for conserved non-coding sequences (CNSs) throughout the *Gata3*, *Junb* and *Il5-Il13-Il4* gene loci by comparing mouse versus human sequences (data not shown). To predict Dec2 binding motifs in the promoters and CNS, we utilized the PROMO program 29, 30; we found a cluster of potential Dec2-binding sites in a *Junb* CNS approximately 6–7 kb upstream of the transcriptional start site (Supplementary Fig. 5) as well as some scattered putative Dec2 binding motifs in the *Junb* and *Gata3* promoters and in the T_H2 locus control region (data not shown). To determine if Dec2 directly binds these sites *in vivo*, we utilized a transgenic mouse that expresses a Flag-tagged Dec2 protein under a *CD2* mini-locus 31 (Supplementary Fig. 6). Naïve $CD4^+$ T cells from these mice were cultured in T_H2-polarizing conditions *in vitro*. The cells were then crosslinked with paraformaldehyde, sonicated and immunoprecipitated with an antibody specific for the Flag tag, a polyclonal antibody specific for Dec2 with rat IgG or rabbit IgG control, respectively. The crosslinks were then reversed and the immunoprecipitated nucleic acid subjected to

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real-time PCR using primers specific for the predicted Dec2-binding regions. In *Il17* exon 2, a negative control, there was no detectable binding of Dec2 (Fig. 7a). The *Junb* CNS and *Gata3* promoter region were strongly bound by Dec2, but no Dec2 binding was detected in the *Junb* promoter region or in the T_H2 locus control region (Fig. 7a). To substantiate the above results, we differentiated naïve $CD4^+$ T cells from OT-II mice into T_H2 cells *in vitro* using OT-II peptide and splenic APCs and then repeated the chromatin immunoprecipitation (ChIP) experiments using anti-Dec2 and control antibody. Consistent with the ChIP experiments from the Dec2 transgenic mice, we detected Dec2 binding to the *Junb* CNS and *Gata3* promoter in these OT-II T_H2 cells (Fig. 7b). This binding appears specific as no Dec2 binding to *Junb* CNS and *Gata3* promoter was detected in Dec2-deficient T cells (Fig. 7c). To examine whether Dec2 regulates chromatin modifications in the *Junb* and *Gata3* loci, naïve T cells from Dec2-deficient and wild-type mice were activated under T_H2-polarizing conditions for 2 days and the abundance of acetylated histone H3 in the *Junb* CNS and *Gata3* promoter were assessed by ChIP. Dec2-deficiency resulted in partially reduced histone H3 acetylation in the *Gata3* promoter and the *Junb* CNS regions (Fig. 7d), suggesting a role for Dec2 in regulation of chromatin modifications in the *Junb* and *Gata3* loci.

As Dec2 was originally identified as a transcriptional repressor, we tested the activity of Dec2 on *Gata3* promoter and *Junb* CNS using a dual luciferase reporter system. Dec2 overexpression significantly increased *Gata3* promoter activity (Fig. 7e). Moreover, Dec2 enhanced the activity of the *Junb* promoter but only in the presence of the *Junb* CNS (Fig. 7e). Therefore, Dec2 may directly bind to the *Junb* CNS and the *Gata3* promoter to activate their transcription.

Our above results suggest *Gata3* and *Junb* are direct and biologically meaningful downstream targets of Dec2. To confirm this hypothesis, we overexpressed GATA3 and JunB in *Dec2*-deficient T cells. Naïve T cells from Dec2-deficient and wild-type mice were activated in the presence of a blocking antibody specific for IFN-γ and were infected with GATA3- or JunB-encoding retroviruses or a control retrovirus. Two days after infection, GFP^+ cells were FACS-sorted and expression of *Il4*, *Il5* and *Il13* mRNA was analyzed by real-time RT-PCR. Overexpression of JunB fully restored T_H2 cytokine mRNA expression in *Dec2*-deficient cells (Fig. 7f). However, overexpression of GATA3 only partially restored enhanced T_H2 cytokine mRNA expression in *Dec2*-deficient cells (Fig. 7g). Taken together, Dec2 directly binds to *Gata3* and *Junb* loci and activates their transcription; reciprocally, GATA3 induces Dec2 expression. Our results thus suggest a feed-forward regulatory circuit during T_H2 differentiation (Supplementary Fig. 7).

Discussion

Effector T cell differentiation is regulated by environmental cytokine milieu which establishes T cell-intrinsic lineage-specific transcriptional programs. T_H2 differentiation is driven by autocrine IL-4 production. However, the initiation and maintenance of IL-4 expression is not well studied. In the current study, we showed that the transcription factor Dec2 is selectively expressed by T_H2 cells, that Dec2 promotes expression of JunB and GATA3, and that Dec2 facilitates autocrine IL-4 and IL-2 expression and T_H2

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differentiation. Our data support a crucial role for Dec2 in T_H2 cell differentiation *in vitro* and *in vivo*.

In response to anti-CD3 and anti-CD28 stimulation, *Dec2*-deficient naïve $CD4^+$ T cells also exhibited impaired proliferation compared with wild-type cells; this defect is consistent with reduced IL-2 production by Dec2-deficient T cells. Notably, under neutral conditions, *Dec2*-deficient T cell populations also contained greatly reduced numbers of IFN-γ^+ cells. Reduced IL-2 and IFN-γ expression also likely accounted for the enhanced IL-17 expression by Dec2-deficient T cells cultured with TGF-β and IL-6. However, Dec2-deficient mice did not exhibit any significant defect in T_H-17 differentiation *in vivo*, and Dec2-deficient mice showed reduced T_H1 responses in the *S. mansoni* egg challenge but not in the OVA + alum or OVA + CFA challenge models. In contrast, both *in vitro* and *in vivo*, *Dec2*-deficiency led to defective production of T_H2 cytokines. Dec2-deficient mice showed impaired T_H2 cytokine expression in response to *S. mansoni* egg challenge. In an allergic asthma model, *Dec2*-deficient mice had greatly reduced numbers of cells including eosinophils in BAL; this reduction was associated with a defect in T_H2 cytokine production but not in IL-5-mediated eosinophilia.

Our analysis of Dec2 expression provides an interesting insight into the mechanisms underpinning T_H2 differentiation. Keeping with the importance of costimulatory signals, especially those emanating from ICOS, in T_H2 cell polarization, Dec2 expression was boosted by simultaneous stimulation of ICOS and the TCR. In addition, IL-25, a cytokine established as able to promote T_H2 differentiation 7, also enhanced *Dec2* expression.

During T_H2 cell differentiation, Dec2 shared a similar expression pattern and induction kinetics to GATA3, indicating that they are co-regulated. In fact, overexpression GATA3 greatly enhanced *Dec2* mRNA expression, independently of IL-4 signaling. However, after deleting *Gata3* in established T_H2 cells, *Dec2* expression remained stable for 1 week, although it diminished thereafter. In addition, deletion of *Gata3* during the early T_H2 differentiation did not significantly alter Dec2 expression. Thus, GATA3 may be dispensable for the induction and maintenance of Dec2 expression. When T cells were activated under the neutral conditions or in the presence of anti-IFN-γ, overexpression of *Dec2* markedly elevated expression of GATA3 as well as IL-4, JunB, IL-4Rα and IL-17RB. However, Dec2 could not upregulate GATA3 expression when T cells were activated under T_H1-polarizing conditions. Thus, Dec2 may require other factors, such as STAT6, to activate GATA3 expression. Nonetheless, our results altogether suggest a reciprocal regulation model in which Dec2 and GATA3 participate in regulating T_H2 cell development.

High doses of exogenous IL-4 partially restored T_H2 cytokine expression in *Dec2*-deficient cells *in vitro*, suggesting that Dec2 may be involved in the regulation of early autocrine IL-4 production. This hypothesis was confirmed using *Dec2*-deficient T cells. NFATc1 and JunB are important in regulating early IL-4 production 32. As NFATc1 expression was not altered in *Dec2*-deficient cells, we focused on GATA3 and JunB, whose expression was greatly reduced by *Dec2* deficiency. Under neutral and T_H-17 but not T_H1-polarizing conditions, forced expression of Dec2 greatly enhanced *Junb* and *Gata3* mRNA expression. As shown

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in a ChIP assay, a CNS upstream the *Junb* gene and the *Gata3* promoter were directly bound by Dec2. *Dec2*-deficiency led to reduced histone H3 acetylation at the *JunB* CNS and the *Gata3* promoter, implicating a role of Dec2 in regulation of chromatin modification at these two loci. Although Dec2 is considered as a transcriptional repressor, overexpression of Dec2 greatly enhanced *Gata3* promoter and *Junb* CNS activity. Our data thus indicated that GATA3 and JunB are direct targets of Dec2. In fact, overexpression of JunB fully restored T_H2 cytokine expression in Dec2-deficient cells. Overexpression of GATA3 partially restored T_H2 cytokine expression. Our results thus indicate that Dec2 activates JunB as well as GATA3 expression, that GATA3 further regulates Dec2 expression to reinforce an autoregulatory loop and that in turn JunB and GATA3 together with NFATc1 regulate early IL-4 and IL-2 production, which aid in establishing the genetic program of T_H2 differentiation.

Methods

Mice

A targeting vector was generated by introduction of 2 LoxP sites into the *Dec2* locus with *Neo*^{r} as positive and Diphtheria toxin A as negative selection markers (Supplementary Fig. 1). Targeted ES cell clones were selected and injected into C57BL/6 (B6) blastocysts to generate chimeras. High percentage chimeras were bred with B6 mice for germline transmission. Targeted mice were crossed with CMV-Cre mice (Jackson Laboratory), and the excision of the promoter and exons 1–3 resulted in the generation of *Dec2*$^{+/-}$ mice. *Dec2*$^{+/-}$ mice were backcrossed with B6 for 6–8 generations and were then interbred to generate *Dec2*$^{-/-}$ mice. *Dec2*$^{-/-}$ mice on the B6 background were used with age and sex matched B6 wild-type controls except those in Fig. 2b were on a mixed B6.129 background. The genotyping primers were F, 5′-tgctgcaaaacaagccctgtcg, R1, 5′-cccaaatgcacgcgcactggagc and R2, 5′-gctgctcagttaaggctgttagc. The primers F and R1 amplify a 233-bp wild-type band and/or a 439-bp LoxP band, while the primers F and R2 give a 331-bp KO band. For the generation of Flag-Dec2 transgenic mice, Flag-Dec2 cDNA was inserted into phCD2 containing a human CD2 mini-locus 31 and a *Xho*I and *Xba*I fragment was isolated and microinjected into B6 mice at the Genetic Engineering Mouse Facility at M. D. Anderson Cancer Center. Transgenic founders were maintained by breeding with B6 mice. *Gata3*$^{fl/fl}$ mice were previous described 24. 4-get and *Il4*$^{-/-}$ mice were purchased from the Jackson laboratory. The animal experiments were performed at the age of 6–10 weeks using protocols approved by Institutional Animal Care and Use Committee.

Dec2-specific antibody

A Dec2-specific rabbit polyclonal antibody was raised against bacterially produced recombinant mouse Dec2 protein fragments encompassing amino acids 245–410. The specific antibody was purified using Affinity Chromatography.

T cell differentiation

Naïve $CD4^+CD25^-CD62L^{hi}CD44^{lo}$ T cells were FACS sorted as described 33, 34 and were activated with plate-bound 1 µg/ml anti-CD3 and 2 µg/ml anti-CD28 and 50 unit/ml rhIL-2 in the presence of polarizing cytokines or/and blocking antibodies. For T_H1 polarization we

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used 10 μg/ml anti-IL-4 (11B11) and 5 ng/ml IL-12 (Peprotech). For T_H2 polarization, we used10 μg/ml anti-IFN-γ (XMG1.2) and 5 ng/ml (or indicated doses of) IL-4 (Peprotech). For T_H-17 polarization we used 20 ng/ml IL-6 (Peprotech) and 2.5 ng/ml TGF-β (Peprotech) with or without anti-IFN-γ and anti-IL-4. For iT_{reg} differentiation we used 5 ng/ml TGF-β.

OVA immunization

Mice (3–5 per group) were immunized with OVA (0.5 mg/ml) emulsified in CFA (0.5 mg/ml) at the tail base (100 μl each mouse) or with OVA (0.5 mg/ml) mixed in aluminium hydroxide (alum) intraperitoneally. Five to seven days later, splenocytes from the immunized mice were cultured *in vitro* in the presence of OVA protein for 2–3 days and cytokine expression was analyzed by ELISA. For adoptive transfers, $CD4^+$ T cells from wild-type or *Dec2*$^{-/-}$ mice were enriched by MACS using anti-CD4 beads (Miltenyi Biotec.) and mixed with B cells from wild-type mice at 1:1 ratio before injection i.v. into *Rag1*$^{-/-}$ mice (10×10^6 $CD4^+$ T cells per mouse). The recipient mice were immunized with OVA in alum i.p. on the next day. Five days after immunization, splenocytes were restimulated with OVA protein for 3 days. Cytokine expression was determined by ELISA.

Schistosoma mansoni egg challenge

Mice were i.p. injected with *S. mansoni* eggs (gift of Y. Belkaid, NIAID, NIH) that were inactivated by repeated freeze-thaw cycles (3000 eggs per mouse). Eight days later, splenocytes from the challenged mice were restimulated with SEA for 3 days and cytokine expression was analyzed by ELISA. SEA was prepared as described 35 with modification. In brief, 5000 eggs were homogenized in 1 ml ice-cold PBS and centrifuged at 10000×*g* for 20 min. The supernatant was collected and sterilized by Spin-X columns (Corning). SEA protein concentration was determined by a Bradford method (Bio-Rad).

Asthma induction

Allergic asthma was induced and analyzed as described 26.

Retroviral transduction

pGFP-RV containing IRES-regulated GFP, and pGFP-RV-GATA3 were gifts from K. Murphy (Washington University, St. Louis, MO) 36. The genes encoding JunB (GenBank Acc. # NM_008416.1) and Flag-tagged Dec2 (GenBank Acc. # NM_024469) were ampilified (the latter using a primer attached to a Flag-tag sequence) and cloned into pGFP-RV. Naïve $CD4^+CD25^-CD62L^{hi}CD44^{lo}$ T cells from B6 mice were FACS sorted and transduced as described 37.

Quantitative real-time PCR

Gene expression was examined with a Bio-Rad iCycler Optical System using an iQ™ SYBR green real-time PCR kit (Bio-Rad Laboratories, Inc.). The data were normalized to an *Actb* or *Cd4* reference gene. The primers were, *Dec2*, forward, 5′-aacatggacgaaggaatccctc, and reverse, 5′-taaggctgttagcgctttcaag; *Irf4*, forward, 5′-tcctctggatggctccagatgg, and reverse, 5′-caccaaagcacagagtcacctg; *Gata3*, forward, 5′-agggacatcctgcgcgaactgt, and reverse, 5′-catcttccggtttcgggtctgg; *Il17rb*, forward, 5′-ccatccctccagatgacaac, and reverse, 5′-

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tgctccttccttgcctccaagtta; *Il2,* forward, 5′-*cactcctcacagtgacctcaag,* and reverse, 5′-*gggcaagtaaaatttgaaggtg. Cd4* primters were purchased from ABI (Cat# Mm00442754_m1). *Junb* 38, *Nfatc1p1* 39, *Actb, Rorc(γt)*, *Tbx21*, *Ifng*, *Il4*, *Il5*, and *Il13* 7, 37 were amplified as described before.

Chromatin immunoprecipitation

ChIP assays were carried out as described 37, 40. The antibodies used were a polyclonal antibody to Dec2, anti-Flag (M2, Sigma), anti-acetyl-Histone H3 (Milipore) or rat IgG. The resulting DNA was analyzed by real-time PCR. The primers were, *Junb* CNS, forward, 5′-tttggcagagcctatcgtggca, and reverse, 5′-gtaggtgttttctgctgggcac; *Junb* promoter, forward, 5′-tggcgctcaacctggcggatcc, and reverse, 5′-gatcaagcgctccagttccgtg; *Gata3* promoter, forward, 5′-ctggctgtcggaggtgtgctgc, and reverse, 5′-tgggtgctgaccgttgaggacc; *Il5-Il13-Il4* LCR, forward, 5′-agctcgcttaggagcactgcca, and reverse, 5′-cagtgtgctttactctgagacg, and a negative control, *Il17* exon 2, forward, 5′-tcaaccgttccacgtcaccctggac, and reverse, 5′-tcagcattcaacttgagctctcatgc.

Luciferase reporter assay

Dec2-encoding or empty vector was transfected into 293T cells with luciferase constructs containing the *Junb* promoter (−876 to +590 relative to the translation start site, GenBank Acc. # NM_008416.1) with or without CNS element (504 bp, the cloning primers were 5′-ttggcagagcctatcgtggca and 5′-agggactagcccaacaggttcc), or the *Gata3* promoter (−830 to +398 relative to translation start site, GenBank Acc. # NM_008091). Firefly and Renilla luciferase activity were measured by using a dual-luciferase reporter system (Promega) and Renilla luciferase was used to normalize transfection efficiency and luciferase activity.

Statistical analysis

Results were expressed as mean ± standard deviation (s.d.). Differences between groups were calculated for statistical significance using the unpaired Student's t test. *P* 0.05 was considered as significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank S. Rivera and J. Parker-Thornburg at MD Anderson Genetic Engineered Mouse Facility for their help in generation of knockout and transgenic animals, K. Murphy (Washington University, St. Louis, MO) for GFP-RV and GFP-RV-GATA3 vectors, Y. Belkaid (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) for *S. mansoni* eggs and suggestion, and the Dong lab members for their help and discussion. The work is supported by research grants from NIH, and MD Anderson Center for Targeted Therapy (to CD and RN), and American Heart Association (to XOY) and by the Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health (to JZ). RN receives a Scientist Development Award from American Heart Association, JP receives a fellowship from Ministry of Education of China, and CD is a Trust Fellow of the MD Anderson Cancer Center, and a Leukemia and Lymphoma Society Scholar.

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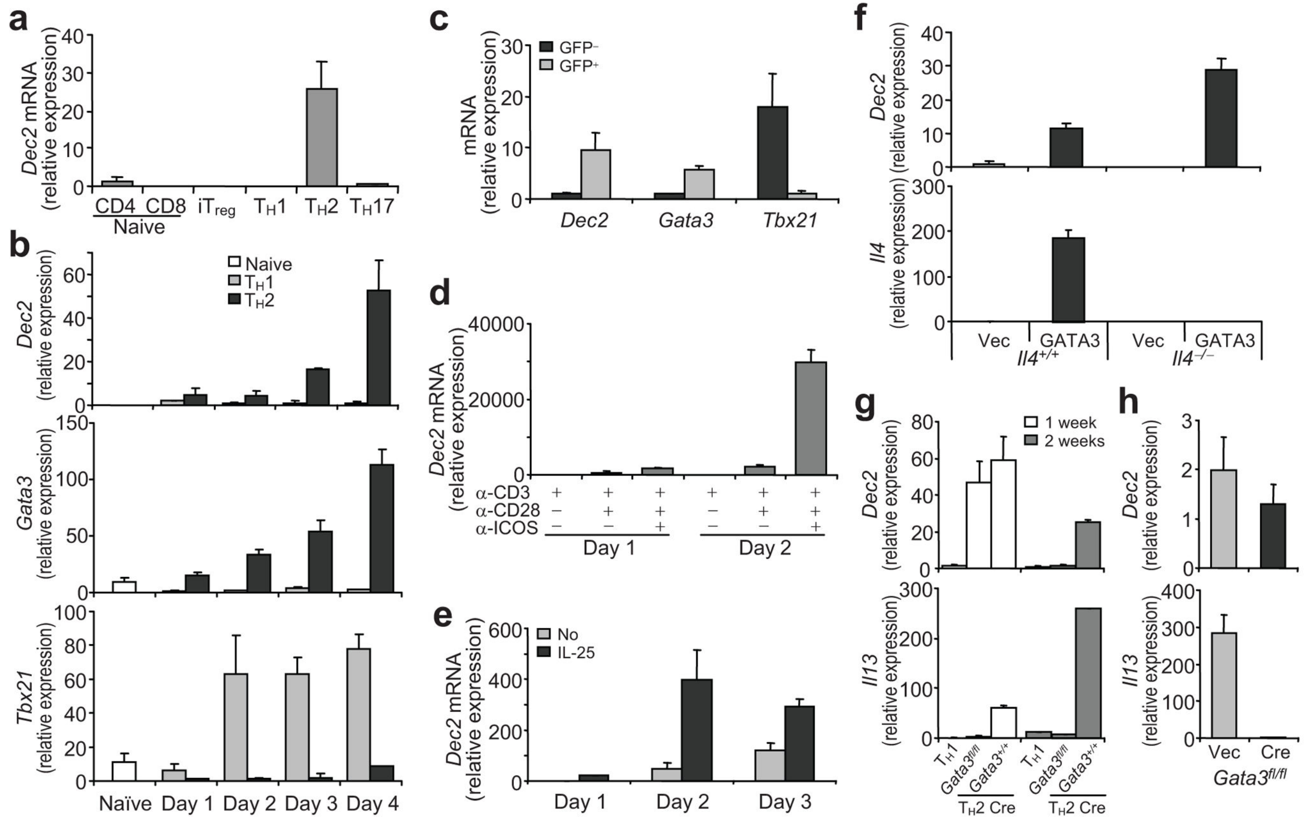

Fig. 1. Expression and regulation of Dec2

(**a**) *Dec2* mRNA expression in T_H1, T_H2, T_H-17 or iT_{reg} cells was measured by quantitative RT-PCR. *Dec2* expression in naïve $CD4^+$ T cells was set as 1. (**b**) Kinetic expression of *Dec2*, *Gata3* and *Tbx21* mRNA in T_H1 or T_H2 cells. (**c**) Lung-associated lymph node cells from 4-get mice with asthma were restimulated with OVA for 48 hr.and IL-4-GFP^+ and $GFP^–$ cells were sorted on a $CD4^+CD44^{hi}$ gate and mRNA expression was examined by real-time RT-PCR. (**d**, **e**) Naïve $CD4^+$ T cells were activated with the indicated plate-bound antibodies (**d**) or with plate-bound anti-CD3 and anti-CD28 in the presence or absence of IL-25 (**e**) and *Dec2* mRNA expression was analyzed daily. (**f**) *Dec2* and *Il4* mRNA expression in naïve $CD4^+$ T cells from $Il4^{–/–}$ or $Il4^{+/+}$ mice infected with retrovirus expressing GATA3-IRES-GFP (GATA3) or GFP only (Vec).(**g**) *Dec2* and *Il13* mRNA expression in naïve $CD4^+$ T cells from $Gata3^{fl/fl}$ or $Gata3^{+/+}$ mice polarized under T_H2 conditions, infected with the bicistronic retrovirus hCre-IRES-GFP and further cultured under T_H2 conditions for 1 or 2 weeks. (**h**) *Dec2* and *Il13* mRNA expression in naïve $CD4^+$ T cells from $Gata3^{fl/fl}$ mice activated under T_H2 conditions and infected with a Cre-encoding virus or a control virus. (**f–h**) GFP^+ fractions were sorted and analyzed. Data were normalized to *Actb* (**a–f, h**) or *Cd4* (**g**) and show mean and s.d. (**b–h**) The sample with lowest detectable expression was set as 1. All experiments were repeated 2–3 times with consistent results.

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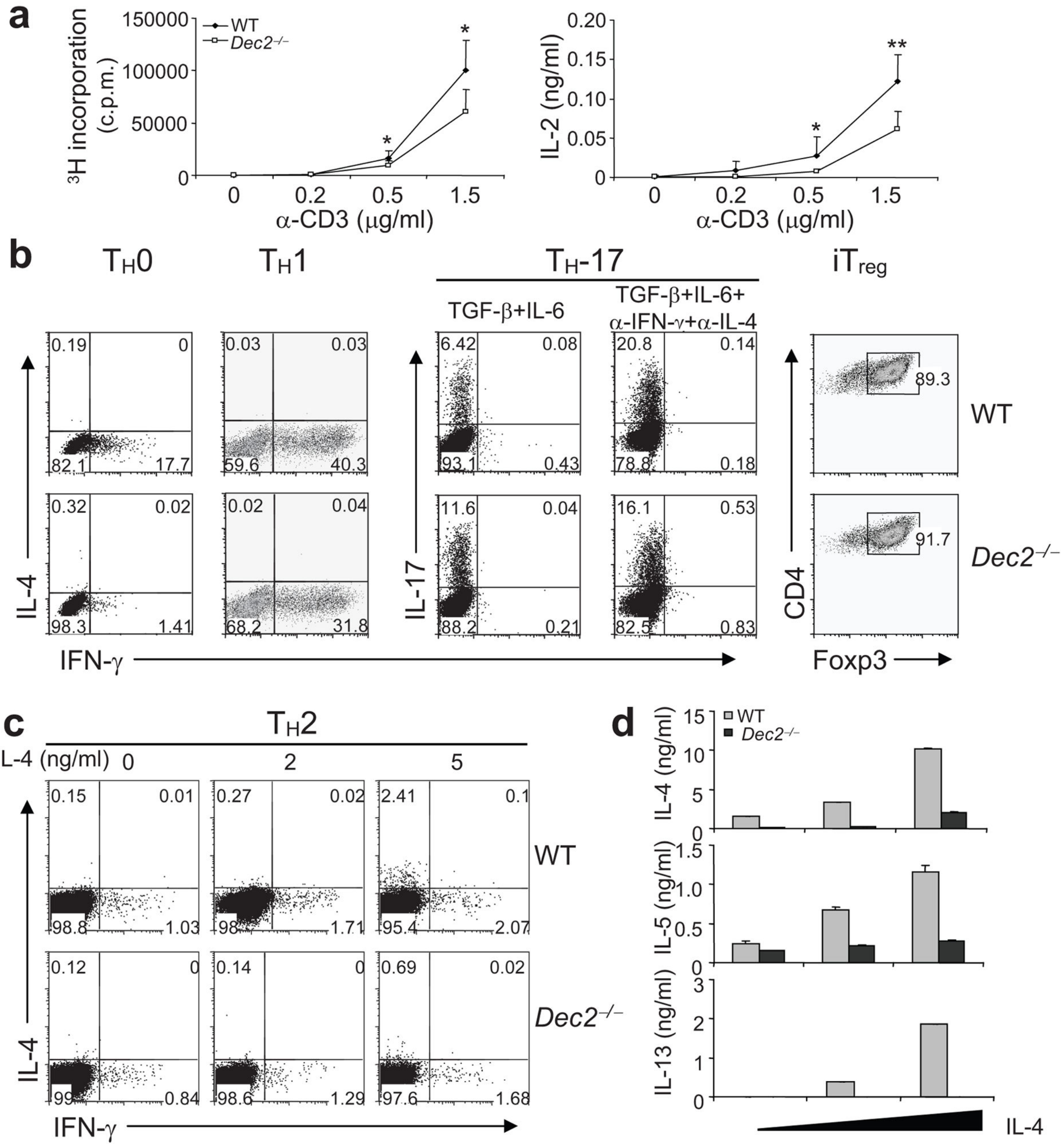

Fig. 2. Dec2 deficiency results in impaired T_H2 differentiation *in vitro*

(**a**) Naïve $CD4^+$ T cells from the indicated mice ($n = 3$ per group, analyzed individually) were activated with indicated concentrations of anti-CD3 in the presence of anti-CD28. On day 3, proliferation was measured by ^{3}H-thymidine incorporation (left). IL-2 protein expression was measured by ELISA on day 2 (right). (**b**) Naïve T cells from indicated mice were polarized under neutral (T_H0), T_H1, T_H-17 and iT_{reg} conditions for 4 days and cytokine production or Foxp3 expression was assessed by intracellular staining. (**c**). Naïve T cells from the indicated mice were polarized into T_H2 cells with various concentrations of IL-4 for 4 days and were assessed by intracellular staining. (**d**) IL-4, IL-5 and IL-13 expression in

the T_H2 cells from (**c**) was measured by ELISA. All data are representative of 2 or 3 independent experiments with consistent results. (**a**, **d**) Values are means and s.d. Student *t* test, * $P < 0.05$; ** $P < 0.005$.

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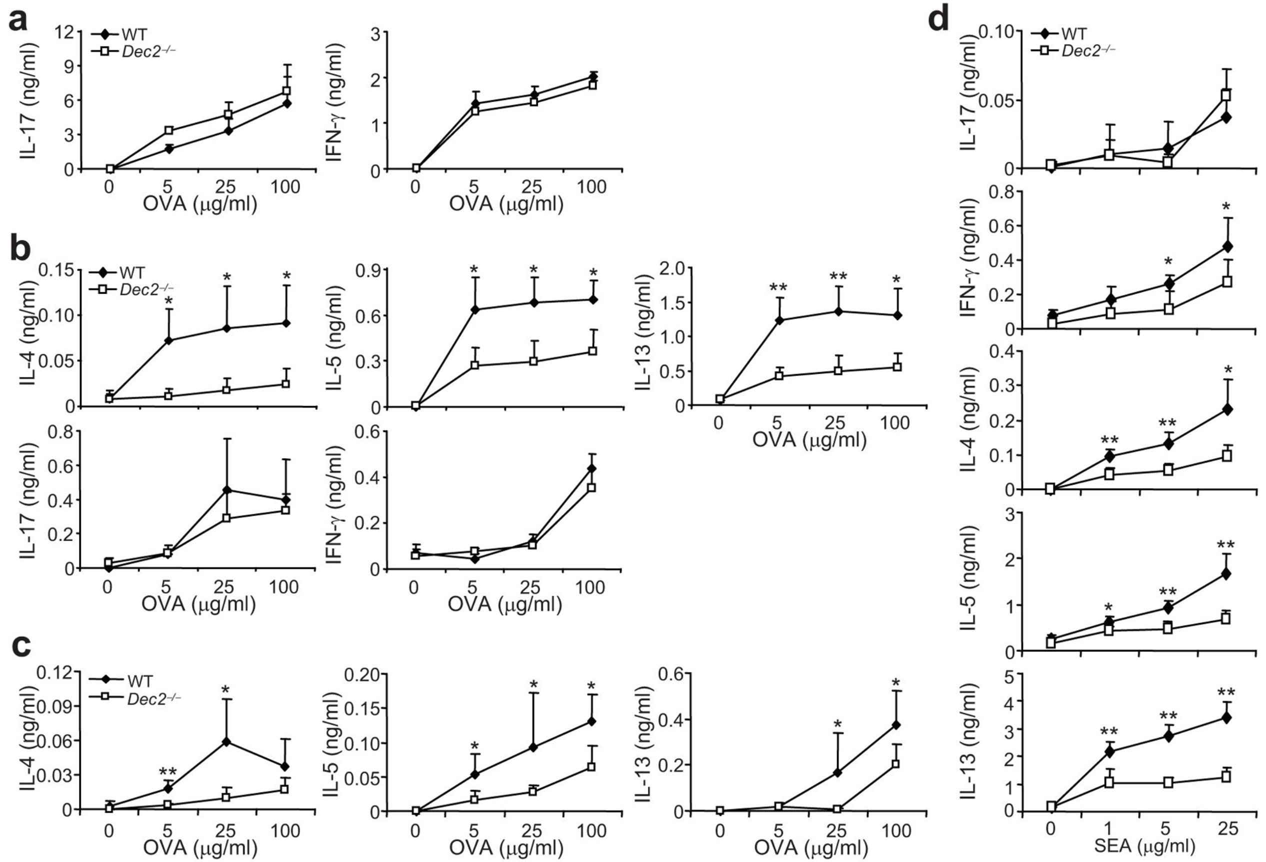

Fig. 3. Defective T_H2 responses *in vivo* in the absence of Dec2

(**a**) The indicated mice ($n = 4$ in each group, analyzed individually) were subcutaneously immunized with OVA in CFA. Five days later, splenocytes were collected and restimulated with OVA protein for 2 days and IFN-γ and IL-17 were measured by ELISA. (**b**) The indicated mice ($n = 4$ in each group, analyzed individually) were i.p. immunized with OVA in alum. Sox days later, splenocytes were collected and restimulated with OVA protein for 3 days. IL-4, IL-5, IL-13, IFN-γ and IL-17 were measured by ELISA. (**c**) $CD4^+$ T cells enriched from indicated naïve mice were mixed with B cells from B6 mice and transferred into *Rag1*$^{-/-}$ mice. Recipients were i.p. immunized with OVA in alum the next day. Five days later, the splenocytes were harvested and restimulated with OVA protein for 3 days. Cytokine expression was determined by ELISA. (**d**) Dec2-deficient ($n = 5$) and wild-type B6 ($n = 4$) mice were i.p. injected with inactivated Schistosoma mansoni eggs. Eight days later, splenocytes were collected and restimulated with soluble SEA for 3 days. IL-4, IL-5, IL-13, IFN-γ and IL-17 were measured by ELISA. Values are means and s.d. *, Student *t* test, $P < 0.05$.

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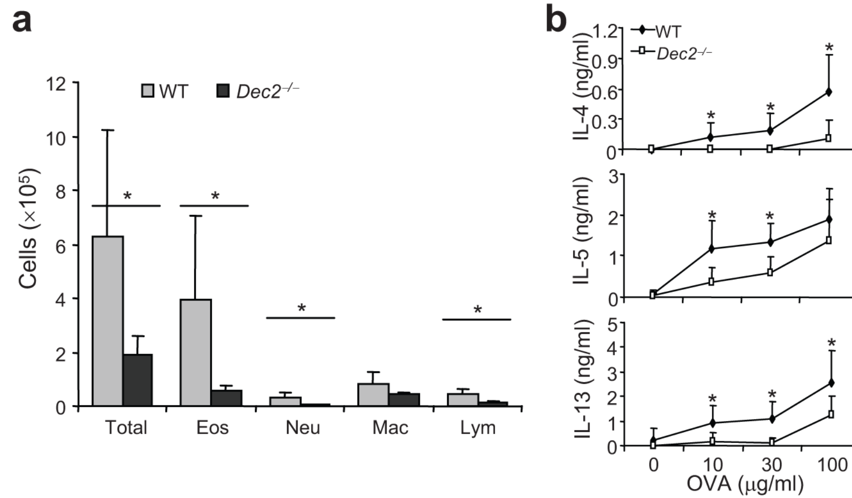

Fig. 4. Dec2 is required in allergic asthma disease

Allergic asthma was induced in *Dec2*$^{-/-}$ and wild-type (WT) mice (*n* = 5 in each group, analyzed individually) by i.p. injection of OVA in alum followed by intranasal challenges with OVA protein. (**a**) Cells in BAL fluid. Eos, eosinophil; Neu, neutrophil; Mac, macrophage; Lym, lymphocyte. (**b**) T_H2 cytokine expression in lung-associated lymph node cells, which were harvested and stimulated with OVA protein for 3 days. Cytokine production were measured by ELISA. Values are means and s.d. *, Student *t* test, $P < 0.05$. Data shown represent two independent experiments with consistent results.

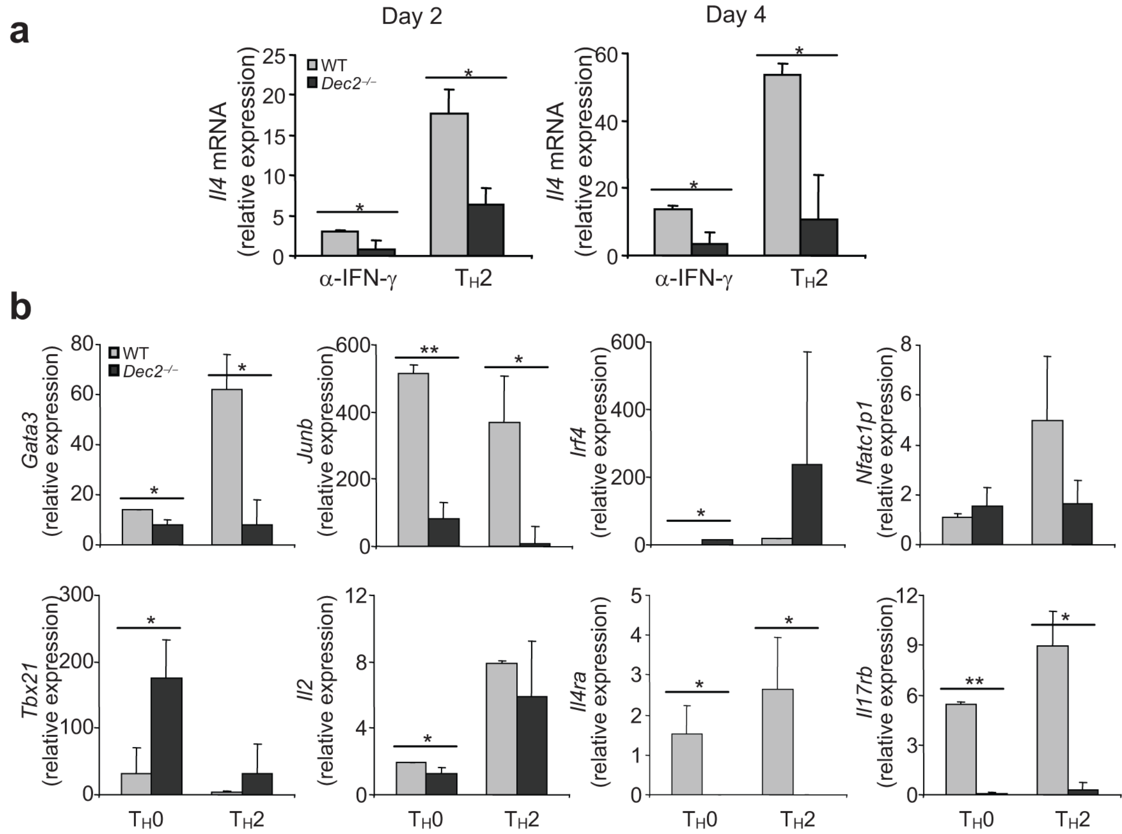

Fig. 5. Dec2 is required for early T_H2 differentiation

(**a**) Naïve CD4+ T cells from *Dec2*−/− and wild-type (WT) mice were differentiated with anti-IFN-γ or under T_H2-polarizing conditions for 2 or 4 days and *Il4* mRNA expression was assessed by real-time RT-PCR. (**b**) Naïve CD4+ T cells from *Dec2*−/− and WT mice were differentiated under T_H0 and T_H2 conditions for 2 days and mRNA expression was assessed by real-time RT-PCR. Values are means of 2–3 independent experiments, and s.d. are indicated. The results were normalized to a reference *Actb* and presented in arbitrary units. *, Student *t* test, $P < 0.05$.

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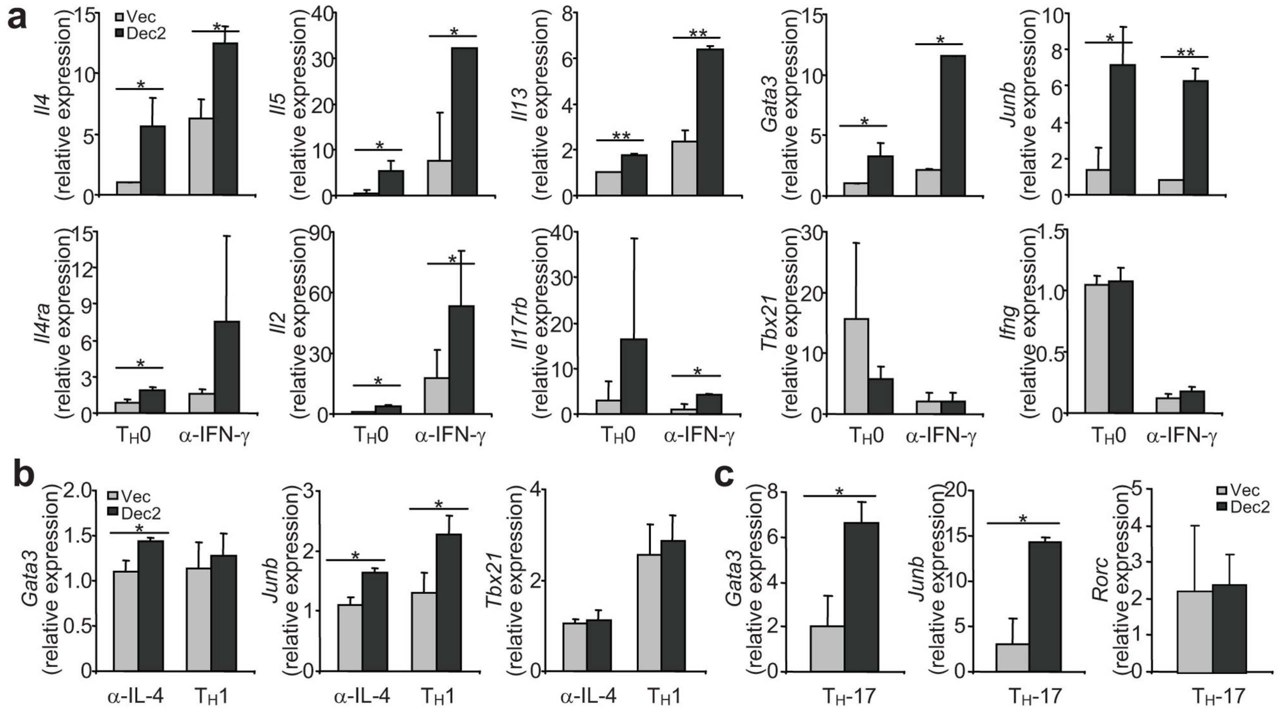

Fig. 6. Dec2 activates T_H2 gene expression

(**a–c**) Naïve $CD4^+$ T cells from B6 mice were activated under (**a**) neutral conditions or with anti-IFN-γ, (**b**) with anti-IL-4 or under T_H1 conditions, or (**c**) T_H-17 conditions. Thirty-six hours later, cells were infected with bicistronic retroviruses encoding Dec2 and GFP or GFP alone. Two days after infection, GFP^+ cells were sorted and mRNA expression was analyzed by real-time RT-PCR. Results were normalized to *Actb* and presented in arbitrary units. Values are means of 2–3 independent experiments and s.d. are indicated. Student *t* test, *, $P < 0.05$; **, $P < 0.005$.

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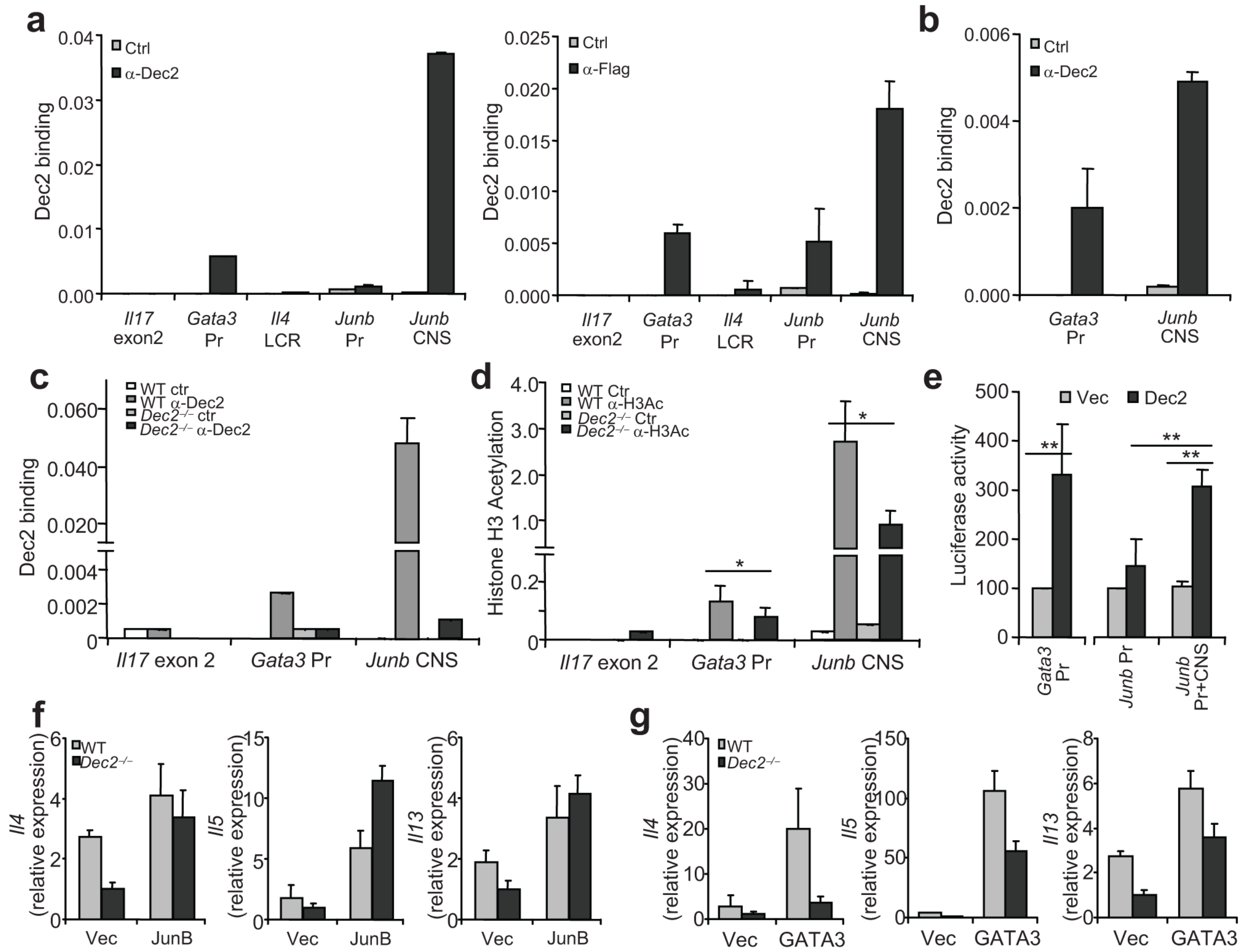

Fig. 7. Dec2 and regulates *Junb* and *Gata3* transcription

Naïve CD4+ T cells from CD2-flag-Dec2 transgenic mice (**a–c**), from OT-II mice (**b**) or from *Dec2*−/− and wild-type (WT) mice (**c**) were differentiated into T_H2 cells and Dec2 binding to the indicated loci was assessed by ChIP. Pr, promoter. (**d**) Naïve CD4+ T cells from *Dec2*−/− and WT mice were cultured under T_H2-polarizing conditions for 2 days and Histone H3 acetylation at the indicated loci was assessed by ChIP. Data represent 2 independent experiments with consistent results. *, Student *t* test, $P < 0.05$. Values were relative to the input abundance. (**e**) Dec2-expressing vector (Dec) or empty vector (Vec) was transfected into 293T cells with luciferase constructs containing the *Junb* promoter with or without the CNS element, or the *Gata3* promoter. Renilla luciferase was used to normalize transfection efficiency and luciferase activity. Data shown are a combination of 2 (*Gata3* Pr) or 3 (*Junb* Pr and Pr + CNS) independent tests. **, Student *t* test, $P < 0.005$. (**f–g**) Naïve CD4+ T cells from *Dec2*−/− and WT mice were activated in the presence of neutralizing antibody against IFN-γ and were infected with vectors encoding JunB (**f**) or GATA3 (**g**) and GFP, or GFP alone (Vec). Four days later, GFP+ cells were sorted and T_H2 cytokine mRNA

expression was assessed by real-time RT-PCR. Data shown represent 2 (**f**) or 3 (**g**) independent experiments with consistent results. Values are means and s.d.

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