Dec2 Promotes Th2 Cell Differentiation by Enhancing IL-2R Signaling

Zhiduo Liu, Zhenhu Li, Kairui Mao, Jia Zou, Yuan Wang, Zhiyuan Tao, Guomei Lin, Lin Tian, Yongyong Ji, Xiaodong Wu, Xueliang Zhu, Shuhui Sun, Weiguang Chen, Charlie Xiang and Bing Sun

J Immunol 2009; 183:6320-6329; Prepublished online 30 October 2009; doi: 10.4049/jimmunol.0900975
http://www.jimmunol.org/content/183/10/6320

Supplementary Material
http://www.jimmunol.org/content/suppl/2009/10/30/jimmunol.0900975.DC1

References
This article cites 30 articles, 12 of which you can access for free at:
http://www.jimmunol.org/content/183/10/6320.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Dec2 Promotes Th2 Cell Differentiation by Enhancing IL-2R Signaling

Zhido Lu,* Zhenhu Li,† Kairui Mao,* Jia Zou,* Yuan Wang,* Zhiyun Tao,* Guomei Lin,* Lin Tian,* Yongyong Ji,* Xiaodong Wu,* Xueliang Zhu,* Shuhui Sun,‡ Weiguang Chen,‡ Charlie Xiang,§ and Bing Sun**

Th cell differentiation is precisely regulated by thousands of genes at different stages. In the present study, we demonstrate that Dec2, a transcription factor belonging to the bHLH (basic helix-loop-helix) superfamily, is progressively induced during the course of Th2 differentiation, especially at the late stage. The up-regulated Dec2 can strongly promote Th2 development under Th2-inducing conditions, as evidenced by retrovirus-mediated gene transfer or transgenic manipulation. In addition, an enhancement of Th2 responses is also detectable in Dec2 transgenic mice in vivo. Conversely, RNA interference-mediated suppression of endogenous Dec2 could attenuate Th2 differentiation. Finally, we show that the enhanced Th2 development is at least in part due to substantial up-regulation of CD25 expression elicited by Dec2, thereby resulting in hyperresponsiveness to IL-2 stimulation.


C

d4+ helper T cells are centrally positioned to orchestrate an adaptive immune response. We now know that when encountering Ag, naive CD4+ T cells can undergo a differentiation program leading to at least three distinct effector subsets. These have been named Th1, Th2, and Th17 cells, based upon their cytokine expression profile, as well as lineage-specific transcription factors. Th1 cells produce predominantly IFN-γ and IL-2 and mediate defense against infection with intracellular microbes, whereas Th2 cells secrete mainly IL-4, IL-5, and IL-13 and play an essential role in parasite clearance and allergic diseases. Both these subtypes can provide cytokine help to promote B cell Ab production. Recently, Th17 cells have been identified as a new CD4+ helper T cell lineage characterized by the ability to produce IL-17. They are responsible for protection against challenges from extracellular bacteria and fungi and contribute to the pathogenesis of autoimmune diseases, such as experimental autoimmune encephalomyelitis and collagen-induced arthritis (1–3).

The selective differentiation of naive helper T cells into Th2 cells is tightly regulated by the coordination of signal transduction and a transcription factor network. Although it is still a matter of debate as to whether IL-4 is responsible for initiating a Th2 immune response in vivo, in vitro studies strongly indicate that it is critical in driving T cells toward a Th2 fate via a positive feedback loop (4, 5). IL-4 triggers a rapid tyrosine phosphorylation of IL-4R. In turn, the activated IL-4R recruits and phosphorylates Stat6, allowing it to subsequently translocate into the nucleus and regulate downstream events (6). Stat6, in driving Th2 differentiation, relies mainly on the up-regulation of Gata3, whose role as a master regulator of Th2 differentiation both in vitro and in vivo, has been definitively established (7–9). Gata3 is inhibitory of the Th1 response and is indispensable for most, if not all, of the expression of Th2 cytokines.

Once thought of as a growth and survival factor for effector T cells, IL-2 has been clearly demonstrated to be a vital stimulant for Th2 cell development (5, 10, 11). IL-2R consists of three subunits, α, β, and γ. Although β and γ subunits are responsible for mediating signaling transduction, the α subunit plays a major role in the higher affinity to IL-2 in conjunction with the other two subunits (12). Following engagement of IL-2R, activated Stat5 enters the nucleus and binds to HSII and HSIII at the IL-4 locus, thereby maintaining these sites in an accessible state (13). Without IL-2 signaling, T cells fail to acquire the competence to produce Th2 cytokines even under optimal Th2 conditions (11). Collectively, the integration of IL-2 and IL-4 signaling contributes to full Th2 differentiation.

The basic helix-loop-helix (bHLH)4 proteins are transcriptional regulators that control a wide variety of biological processes, including cell proliferation and differentiation (14–16). The common structures shared among the members of this superfamily are the basic domain, which is required for DNA binding, and the helix-loop-helix domain, which is involved in dimerization. On the basis

* Laboratory of Molecular Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences, Shanghai, China; † Institute of Pasteur of Shanghai, Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences, Shanghai, China; ‡Fudan University School of Medicine, Shanghai, China; and § State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, the First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, China. E-mail addresses: bsun@sibs.ac.cn or cxiang@zju.edu.cn

† Address correspondence and reprint requests to Dr. Bing Sun, Laboratory of Molecular Immunology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yue Yang Road, Shanghai, China or Charlie Xiang, State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, the First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, China. E-mail addresses: bsun@sibs.ac.cn or cxiang@zju.edu.cn

4 Abbreviations used in this paper: bHLH, basic helix-loop-helix; hCD4, human CD4; Treg, regulatory T cell; sRNA, short-interfering RNA; MFI, mean fluorescence intensity; RNAi, RNA interference; iTreg, induced regulatory T cell; MSCV, mouse stem cell virus.
of biochemical and functional criteria, the bHLH proteins can be categorized into distinct families, one of which consists of the bHLH-O proteins and is characteristic of an Orange domain located at the C-terminal of the bHLH domain. Dec1 and Dec2 constitute a subfamily of bHLH-O proteins, because they share the highest sequence homology with each other relative to the remaining family members. In vitro studies show that Dec1 and Dec2 use multiple mechanisms, including DNA-binding and protein-protein interactions, to achieve E-box-dependent transcriptional repression (17). Dec1 is implicated in promotion of neurogenic differentiation (18) and inhibition of adipogenesis (19), while Dec2 has been shown to modulate myogenesis (20). It has been reported that the expression of Dec2 exhibits a rhythmic pattern in the suprachiasmatic nucleus and forebrain structures and that Dec1 and Dec2 are involved in the regulation of circadian rhythms in cooperation with other clock genes (21). Furthermore, they are induced in response to hypoxia (22); Dec2 appears to negatively regulate vascular endothelial growth factor expression induced by hypoxia through physical interference with HIF-1α (23). Dec1-deficient mice exhibit defects in T cell activation and ineffective elimination of activated T and B cells (24). However, the function of Dec2 in the immune system, especially in T cells, remains unclear.

In this study, we show that Dec2 is preferentially expressed in Th2 cells, and its induction is particularly pronounced during the late phase of Th2 differentiation. When over-expressed in T cells by retroviral transduction or transgenic manipulation, Dec2 can facilitate Th2 differentiation in vitro and can assist in promoting a stronger Th2 response in vivo. In contrast, knockdown of Dec2 protein impairs Th2 cytokine production. Furthermore, we demonstrate that Dec2 functions as an important modulator of IL-2Rα expression and plays a synergistic role in regulating IL-2 signaling with IL-4 to drive Th2 cell differentiation.

Materials and Methods

Mice

C57BL/6, BALB/c, and Stat6−/− mice were obtained from The Jackson Laboratory and held under specific pathogen-free conditions at the Animal Care Facility of the Chinese Academy of Sciences. Only 6- to 8-wk-old female mice were used. Animal care and use were in compliance with institutional guidelines.

To generate T cell-specific Dec2 transgenic mice, cDNA encoding mouse Dec2 was cloned into the plasmid pU-hcCD2, which was a gift from Dr. Hua Gu (Department of Microbiology, Columbia University, New York, NY). Transgene-positive mice were identified by PCR using primers derived from the transgene construct and Dec2 cDNA. The sequences of primers for real-time PCR analysis are as follows: sense primer TTTCTGTGCTCGGTCCTCT on the hCD2 promoter, and anti-sense primer TGGTATCGTCTCGCTTCA on the mouse Dec2 gene. The mice were initially created on the 129 background, then extensively backcrossed to C57BL/6.

Cytokines and Abs

For in vitro T cell culture, recombinant mouse IL-4, IL-12, IL-23, and human IL-6, and human TGF-β1 were purchased from R&D Systems and Abs against p-Stat5 (Tyr694) and total Stat5 from Cell Signaling Technology. The Dec2 rabbit polyclonal Ab was raised against recombinant bacterially produced mouse Dec2 protein fragments encompassing aa 245–410.

Cell purification and differentiation in vitro

Naïve CD4+ (CD44+ CD62L+ CD25−) T cells and retrovirus transduced GFP+ cells were purified routinely to >95% purity using a FACS Aria (BD Biosciences). For in vitro differentiation, T cells were maintained in RPMI 1640 supplemented with 10% FCS (Life Technologies), glutamine (2 mM), β-ME (50 uM), penicillin (50 U/ml), streptomycin (50 mg/ml), sodium pyruvate (1 mM), and HEPES (100 mM) and stimulated with 5 μg/ml plate-bound anti-CD3 and 2 μg/ml soluble anti-CD28 under conditions formulated to obtain cell types: Th0 (10 μg/ml anti-IFN-γ, 10 μg/ml anti-IL-12/23, and 10 μg/ml anti-IL-4), Th1 (10 ng/ml IL-12 and 10 μg/ml anti-IL-4), Th2 (10 ng/ml IL-4, 10 μg/ml anti-IFN-γ, and 10 μg/ml anti-IL-12/23), Th17 (1 ng/ml TGF-β1, 20 ng/ml IL-6, 10 ng/ml IL-1α, 10 ng/ml IL-23, 10 μg/ml anti-IFN-γ, and 10 μg/ml anti-IL-4), or induced regulatory T cell (iTreg) (10 ng/ml anti-IL-23). IL-2 (50 U/ml) was added to the cultures of Th0, Th1, Th2, and iTreg cells. To block IL-2 signaling, anti-IL-2 (S4B6, 20 μg/ml), anti-IL-2Rα (PC61, 20 μg/ml), and anti-IL-2Rβ (TM-β1, 20 μg/ml) were added to the cultures.

Retrovirus preparation and infection

Vectors for retrovirus packaging including mouse stem cell virus (MSCV), MSCV-IREs-GFP, MSCV-IREs-human CD4 (cCD4), PCMV/Eco-Envelope, and pKFT3-RSV/Gag-Pol were provided by Dr. Xingxing Zang (University of California, Berkeley, CA). The Dec2 and Gata3 were cloned into MSCV-IREs-GFP while Dec2 cDNA was also inserted into MSCV-IREs-hcCD4. To construct the retrovirus-mediated Dec2 RNA interferer (RNAi) vector, mouse Dec2 RNAi target sequence (GCCATCTGGAAGAACGATGA) was designed and the complementary oligonucleotides were synthesized and cloned into pBS/U6 (a gift from Dr. Gang Pei, Shanghai Institutes for Biological Sciences, Shanghai, China). Subsequently, DNA fragments representing the EGFP coding sequence and the cassette expressing Dec2 short interfering RNA (siRNA) under the U6 promoter were digested from pEGFPc1 (Clontech) and pBS/U6 respectively, and sequentially cloned into MCS of MSCV to generate MSCV-EGFP-Dec2 siRNA.

Retroviral vectors were transduced into 293T cells using Lipofectamine (Invitrogen). After 24 h, the medium was replaced with RPMI 1640 containing 10% FCS. Forty-eight hours post transfection, the retroviral supernatants were harvested, supplemented with 6 μg/ml polybrene, and used to infect CD4+ T cells that had been preactivated with anti-CD3 and anti-CD28 under specific conditions for 30 h. Plates were centrifuged at 1800 rpm for 90 min at room temperature. After 12 h of incubation at 32°C, viruses were removed and cells were recultured in fresh differentiation medium.

mRNA analysis

Total RNA was extracted from cultured cells with TRIzol (Invitrogen) according to the manufacturer’s instructions. Oligo(dT) primers and Superscript III reverse transcriptase (Invitrogen) were used for reverse transcription of purified mRNA. All gene transcripts were measured by quantitative PCR with SYBR Green dye (Applied Biosystems). Each sample was amplified in triplicate. Data were analyzed with SDS software. The sequences of primers for real-time PCR analysis are as follows: Dec1, forward-CAGAAAATCATTGCCCTGCA, reverse-TCCCAGCAATCAATGTA; Dec2, forward-GCTGAAAGATTTACTGCC, reverse-AGCGTGGGAAGGTGCAGAG.

Western blot assay

T cells (105 per sample) were harvested and lysed with 100 μl lysis buffer (50 mM HEPES (pH 7.0), 1% Nonidet P-40, 5 mM EDTA, 450 mM NaCl, 10 mM Na pyrophosphate, and 50 mM NaF) freshly supplemented with inhibitors (1 mM Na orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin, leupeptin, pepstatin) at room temperature for 20 min. Aliquots of the whole cell extract were prepared and subjected to 10% SDS-PAGE and then electroblotted onto nitrocellulose membranes. Membranes were then probed with Abs as indicated, followed by HRP-labeled secondary Abs and visualized with Super Signal West Pico Chemiluminescent Substrate (Pierce).
Flow cytometry and ELISA

To detect the expression of surface molecules, T cells were first incubated with an anti-Fc receptor Ab (24G2) to reduce nonspecific binding of mAbs and then labeled with appropriate fluorescein-conjugated mAbs.

For analysis of intracellular IFN-γ and IL-4 production, T cells were stimulated with PMA (50 ng/ml) and ionomycin (1 mM) for 6 h with the addition of Brefeldin A (10 μg/ml; Sigma-Aldrich) for the last 4 h of stimulation. Cells were harvested, washed, fixed, permeabilized (CALTAG FIX AND PERM), and stained with fluorescein-labeled anti-IFN-γ and anti-IL-4 mAbs, according to the manufacturer’s instructions. Appropriate fluorescein-conjugated, isotype-matched, irrelevant mAbs were used as negative controls. Cells were analyzed on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences).

For analysis of cytokine production in the supernatant, IL-2, 4, 5, 10, 13, and IFN-γ ELISA Duoset kits were purchased from R&D Systems and used according to the manufacturer’s protocols.

OVA-induced airway inflammation

Wild-type C57BL/6 and Dec2 transgenic mice were immunized twice by i.p. injection of 10 μg OVA (Sigma-Aldrich) in 200 μl PBS, and mixed with 1 mg alum on days 1 and 14. From day 21, mice were challenged 30 min/day with aerosolized OVA (1% w/v) for 5 consecutive days. Mice were sacrificed 24 h after the last aerosol challenge and mediastinal lymph node cells were isolated for restimulation with OVA (100 μg/ml) for 4 days. Supernatants were harvested for analysis of cytokine production using ELISA. Determination of serum OVA-specific IgE production and histological analysis were conducted as previously described (25).

Statistics

Results represent the mean ± SD where applicable. Student’s t test was used to compare two independent groups. For all tests, values of p < 0.05 were considered statistically significant.

Results

Dec2 is highly expressed in Th2 cells and its expression is regulated by Stat6

A comparative microarray analysis performed on T cells that had undergone Th1 vs Th2 polarization (data not shown) revealed a higher expression of Dec2 in Th2 cells. To confirm this, naive CD4+ T cells were purified and differentiated into Th0, Th1, Th2, or Th17 cells, and the cytokine profile of each Th subset was determined by ELISA (Fig. 1A). Subsequently, Dec2 mRNA expression was examined using real-time PCR. As shown in Fig. 1B, Th2 cells restimulated with ConA for 4 h exhibited a substantially higher expression of Dec2 mRNA as compared with the other subsets. In contrast, the expression level of Dec1, a homologue of Dec2, was equivalent among these four subsets. Next, we examined the kinetics of Dec2 expression during the course of helper T cell differentiation. Dec2 mRNA was induced slowly in the first 2 days in both Th1 and Th2 cells, followed by a dramatic increase in Th2 cells from days 3 to 5, whereas Dec1 mRNA was induced similarly upon stimulation in both subsets (Fig. 1C).

To study Dec2 protein expression, we raised a polyclonal Ab against a C-terminal fragment of mouse Dec2 protein to obviate the strong similarity between Dec1 and Dec2 among the bHLH domains. Western blot assays indicated that the Ab specifically recognized the mouse Dec2 protein exogenously expressed in 293T cells (Fig. 1D). In agreement with the mRNA expression pattern, endogenous Dec2 protein was selectively detected in
restimulated Th2 cells (Fig. 1E) and was markedly up-regulated at the late stage of Th2 differentiation, corresponding to the exclusive expression of Gata3 in Th2 cells throughout the whole differentiation process (Fig. 1F). The strong induction of Dec2 in Th2 cells requires the activation of Stat6 because the expression of Dec2 protein was markedly diminished when Stat6-deficient T cells were cultured in the presence of IL-4 (Fig. 1F). It should be noted that the expression of Dec2 is not entirely subset-exclusive. We could still discern a trace amount of Dec2 expression in Th1 cells, albeit much weaker than that in Th2 cells (Fig. 1G). Taken together, our results demonstrate that Dec2 is strongly induced in CD4+ T cells undergoing Th2 differentiation, and its up-regulation is dependent upon Stat6 activation.

Dec2 promotes Th2 differentiation in the presence of IL-4 signaling

To investigate a possible role for Dec2 in CD4+ T cell differentiation, we generated a Dec2-expressing bicistronic retroviral vector and infected CD4+ T cells 30 h after activation. These cells were then cultured in an environment favoring either Th0, Th1, or Th2 differentiation. Overexpression of Dec2 mediated by retroviral transduction was confirmed in GFP+ cells sorted and split into two groups with one half being reprimed and differentiating under Th0 conditions and the other half under Th2 conditions for the next 6 days. Cytokine production was measured using ELISA from supernatants taken 24 h after restimulation at the end of the second round of culture. The data are representative of two experiments. E, Wild-type or Stat6-deficient naive CD4+ T cells were infected with control or Dec2-expressing retrovirus, cultured under Th2 conditions and purified as described above. Cells were restimulated and cytokine production was assessed using ELISA. The data are representative of two experiments. Error bars indicate mean ± SD between duplicates.
level was also measured in the supernatants of restimulated GFP<sup>+</sup> cells using ELISA. Given that Dec2 failed to take effect without IL-4 stimulation, cells cultured under Th0 conditions were always incorporated into our following experiments to serve as a negative control. The data show that under Th2 conditions, the cells transduced with Dec2-expressing retrovirus acquired a stronger capacity for Th2 polarization as characterized by an increase in IL-4, IL-5, IL-13, and IL-10 production as compared with the cells transduced with control retrovirus. However, the amount of Th1-related cytokines (IL-2 and IFN-γ) was not affected. In contrast, the production of all these cytokines remained at a similar level between Th0 cells transduced with control or Dec2-expressing retrovirus (Fig. 2C). These data suggest that Dec2 may play an important role in facilitating Th2 differentiation.

To clearly elucidate whether the function of Dec2 relies on IL-4 signaling, we adopted a two-round T cell culture protocol, in which T cells were transduced with retrovirus and cultured under Th0 conditions for the first round, and then reprimed in a Th0 or Th2-inducing environment for the second round. The supernatants from restimulated cells at the end of each round of culture were harvested for analysis using ELISA. As expected, in the first round without IL-4 signaling, Dec2 could not increase Th2 cytokine secretion (data not shown). However, in the second round when these cells received IL-4 triggering, exogenous Dec2-expressing T cells gained an ability to produce more Th2 cytokines (IL-4 and IL-5) than control cells, whereas the cytokine production remained comparable between the two populations if they remained in culture under Th0 conditions (Fig. 2D).

Furthermore, overexpression of Dec2 in Stat6-deficient cells failed to increase Th2 cytokine production (IL-4 and IL-5) even with the administration of IL-4 (Fig. 2E). Together, our observations indicate that Dec2 requires IL-4 signaling to promote Th2 differentiation.

Major events in IL-4 signaling involved in the regulation of Th2 differentiation are not affected by Dec2

We next tried to identify possible mechanisms underlying the enhanced Th2 differentiation mediated by Dec2. In view of the apparent connection between IL-4 signaling and the function of Dec2, we hypothesized that as a product of IL-4 signaling, the up-regulated Dec2 might in turn act as a positive regulator of this signaling pathway. To address this possibility, we initially analyzed the data of the intracellular cytokine labeling assay and found that under Th2 conditions, the difference in the proportion of IL-4-producing cells existed only in GFP positive cells transduced with Dec2-expressing or control retrovirus (Fig. 3A). Because GFP<sup>+</sup> and GFP<sup>−</sup> cells shared the same cytokine milieu, this implied that Dec2 functions in a cell-intrinsic way, ruling out the probability that Dec2 influences soluble factors in the culture to form a positive loop for Th2 differentiation.

Subsequently, the mRNA level of the IL-4 receptor α subunit (CD124) and its protein expression on the surface of the T cells were examined. Real-time PCR and FACS analyses demonstrated that overexpression of Dec2 did not have a discernible effect on the expression of CD124, irrespective of the culture environments (Fig. 3, B and C).
Next, GFP+ cells were sorted and then treated with IL-4 to explore the activation level of Stat6. As shown in Fig. 3D, the intensity and kinetics of Stat6 activation were similar between control and Dec2-expressing cells. Consistently, under Th2 conditions, the induced expression levels of Gata3 and c-Maf were comparable between control and Dec2-expressing cells (Fig. 3E). Despite the inability to enhance Gata3 expression level, we wondered whether Dec2 is able to promote Th2 differentiation with Gata3 synergistically. To address this question, two bicistronic retroviral vectors containing GFP or hCD4 markers were used (Fig. 3F) and Stat6-deficient CD4+ T cells were transduced with these two retroviruses yielding all combinations of Dec2 and Gata3. Both marker positive cells were sorted and restimulated for cytokine production analysis using ELISA. The overexpression of Dec2 and Gata3 was confirmed by Western blot assay (Fig. 3G). As expected, introduction of Gata3 endowed cells with the competence to produce large amounts of Th2 cytokines IL-4 and IL-5, while greatly reducing the production of IFN-γ. Yet, we did not find clear enhancement of Th2 cytokine production in cells expressing both Dec2 and Gata3 as compared with that of cells expressing Gata3-only (Fig. 3H). Taken together, our results indicate that Dec2 appears unable to affect the tested major checkpoints related to the regulation of Th2 differentiation.

Dec2 promotes Th2 polarization through induction of CD25

The indispensable role of IL-2 in Th2 polarization has been documented by numerous reports in recent years (5, 10, 11). In addition to IL-4, IL-2 was also added to our culture system, raising a possibility that Dec2 may function by influencing this signaling pathway. To address this, FACS analysis was conducted to examine the expression level of IL-2R on the surface of T cells transfected with Dec2. Interestingly, although most cells in both groups displayed CD25 expression, an ectopic Dec2 expression led to an ~2-fold increase in MFI (mean fluorescence intensity) of CD25 labeling under Th2 conditions (Fig. 4A). In contrast, the levels of the other two subunits (CD122 and CD132) of IL-2R remained unchanged using the same settings (Fig. 4, B and C). Furthermore, the kinetics of CD25 expression at different time points after retroviral transduction showed a consistent pattern (Fig. 4D).

Because CD25 is also highly expressed in regulatory T cells (Tregs), to test whether the function of Dec2 is related to Tregs, we used real-time PCR to analyze the expression of CD25 and Foxp3 in sorted GFP+ cells that expressed exogenous Dec2 or GFP only. In vitro differentiated iTregs served as a positive control. In line with published reports, iTregs expressed high levels of CD25 and Foxp3. Overexpression of Dec2 resulted in a marked increase in the level of CD25 transcripts, but it had no effect on Foxp3 expression (Fig. 4E), a result indicating that Dec2 is not able to confer upon T cells a Treg-related phenotype.

To determine whether higher expression of CD25 yields stronger IL-2R signaling, GFP+ cells of two groups cultured under Th2 conditions were sorted and rested for 6 h and then treated with or without IL-2 stimulation. As shown in Fig. 4F, more phosphorylated Stat5 was detected in cells transduced with Dec2-expressing retrovirus in response to IL-2 stimulation. These data clearly demonstrate that the distinct expression level of CD25 indeed results in a difference in the strength of IL-2R downstream signaling events, leading us to propose further that the strengthened IL-2 signaling might be responsible for the enhanced Th2 development by Dec2 protein. To address this possibility, we first used neutralizing Abs to block IL-2 and IL-4 signaling separately or simultaneously in retrovirus-transduced Th cells. As shown in Fig. 4G, whether both IL-2 and IL-4 signaling were present, the overexpressed Dec2 caused a marked increase in the level of IL-4 and IL-5 production.
surface CD25 expression (Fig. 7D) and more Th2 cytokine secretion (Fig. 7E) under Th2 conditions, in agreement with the data obtained under retrovirus-mediated overexpression.

To further examine the function of Dec2 in vivo, we used the OVA-induced asthma model. Wild-type and Dec2 transgenic mice were immunized twice with OVA plus alum and challenged with aerosolized OVA. PBS plus alum immunization served as a negative control. Twenty-four hours after the last challenge, draining lymph node cells were isolated from the mice and cultured with OVA. ELISA showed that Dec2-Tg cells produced more Th2 cytokines (IL-4, IL-5, and IL-13) than did wild-type cells, while IL-2 production remained unchanged (Fig. 8A). Furthermore, Dec2 transgenic mice exhibited a higher level of OVA-specific IgE in serum (Fig. 8B) and developed a more severe lung inflammatory response characterized by perivascular and peribronchiolar infiltration and accumulation of more inflammatory cells (Fig. 8C). These data indicate that Dec2 transgenic mice can mount a stronger Th2 response in vivo.

Discussion

Once helper T cell differentiation is initiated, T cells will develop a number of mechanisms to reinforce this program until epigenetic
expression patterns of thousands of genes at different stages (28). In this study, we identify a transcription factor, Dec2, which promotes Th2 differentiation through enhanced IL-2R-mediated signaling.

We initially found that Dec2 is highly expressed in Th2 cells while Dec1 does not exhibit such a preference. More intriguingly, when we looked into their expression throughout the whole differentiation process, we noticed that Dec2 is progressively induced during the course of Th2 differentiation, and reaches a peak at the later stage of Th2 differentiation. These features of the expression pattern of Dec2 imply that it may play a role in Th2 differentiation.

Forced expression of Dec2 in vitro and in vivo markedly enhances Th2 development under Th2-inducing conditions. Conversely, RNAi of endogenous Dec2 is able to attenuate Th2 differentiation. These data not only reveal an important role for Dec2 but also provide clues to aspects of its function. On the one hand, unlike Gata3, Dec2 alone is not sufficient to achieve Th2 differentiation. These features of the expression pattern of Dec2 imply that it may play a role in Th2 differentiation.

Interestingly, we found that CD25 expression on the surface of T cells under Th2 conditions is substantially increased by ectopic Dec2 expression. Consistent with this observation, the intensity of downstream signaling events is also substantially enhanced. Furthermore, we provide evidence that by blocking or rescuing this signaling pathway it is possible to display the association of increased Th2 development with enhanced IL-2R-mediated signaling, both elicited by Dec2. Although we cannot completely exclude alternative mechanisms for promotion of Th2 differentiation by Dec2, our data strongly suggest that the action of Dec2 can be ascribed, at least in part, to the increased intensity of IL-2R signaling. First, Th2 differentiation requires strong Stat5 activation. We showed that the capacity of T cells to secrete Th2 cytokines is proportional to the amount of administered exogenous IL-2 (Fig. 4H), Moreover, Paul et al. (13) reported that Stat5a*6, a constitutively active form of Stat5a, when introduced into T cells, can give rise to a large proportion of IL-4-producing cells even under Th1-inducing conditions, an effect presumably resulting from the hyperactivation of Stat5a due to the mutation. Secondly, IL-2 signaling has the ability to influence the entirety of Th2 cytokines, which is in accord with the effect caused by overexpressed Dec2.

IL-2 has a central role in Th2 differentiation, with distinct effects at different phases. Upon TCR engagement, IL-2-mediated Stat5 activation was shown to play an essential role in inducing IL-4Rα expression level (29), thereby rendering IL-4 signal to be more efficiently delivered to the cell. However, we did not notice a substantial increase of IL-4Rα expression by retrovirus-mediated Dec2 overexpression. Because the retrovirus transduction may miss the early events, we also compared IL-4Rα expression between wild-type and Dec2 transgenic T cells. Likewise, these two cell populations did not exhibit perceptible difference in the level of IL-4Rα expression at different time points after activation (supplementary Fig. 1).5

In addition to the early induction of IL-4Rα expression, IL-2R-mediated signaling is actually indispensable in the later period of

5 The online version of this article contains supplementary material.
Th2 differentiation. Ho et al. (26) reported that the surface level of CD25 was substantially higher in developing Th2 cells than in developing Th1 cells from the fourth day after T cell priming, implying a possible role of IL-2 signaling in this particular period. More directly, Paul et al. (11) found that while Th2 development was completely arrested without IL-2 signaling, restoring this signaling as late as 46 h after T cell activation did not impair Th2 cell differentiation. The dramatic induction of Dec2 expression at the late stage of Th2 differentiation exactly matches the time window for requirement of IL-2 signaling to reinforce Th2 differentiation.

FIGURE 7. Characterization of T cell specific Dec2 transgenic mice. A, CD4+ T cells from wild-type and Dec2 transgenic mice were activated in vitro for 2 days and harvested for determination of Dec2 expression by Western blotting. B, Distribution of CD4+ and CD8+ T cells was assessed by flow cytometry in the indicated tissues from wild type C57BL/6 and Dec2 transgenic mice. Numbers in the dot plots are the percentages of each cell population. C, The percentage of naive CD4+ T cells was determined in spleens and lymph nodes isolated from wild-type C57BL/6 and Dec2 transgenic mice. Data are shown as two-color dot plots of events gated on CD4+ cells. D, Naive CD4+ T cells from WT and Dec2-Tg mice were cultured under Th0 or Th2 conditions and CD25 expression was assessed by FACS. Shaded histograms represent cells labeling with isotype-matched control Ab. E, Cytokine production of cells as in D was determined using ELISA after restimulation. Error bars indicate mean ± SD between duplicates.

FIGURE 8. Dec2 transgenic mice mount a stronger Th2 response. Wild-type C57BL/6 and Dec2 transgenic mice were immunized with OVA and alum and challenged with aerosolized OVA as described in Materials and Methods, mice immunized with PBS served as a negative control. A, Supernatants of draining lymph node cells restimulated with OVA for 4 days were harvested for analysis of cytokine production using ELISA. B, OVA-specific IgE levels in serum were determined using ELISA. C, Lung tissue sections were stained with H&E (original magnification, ×40). Error bars indicate mean ± SD between duplicates. *, p < 0.05.
of Th2 differentiation, because T cells transduced with both GATA-3 and STAT5A1*6 yield more IL-4 producers relative to cells transduced with each alone (13). In interpreting this phenomenon, Paul et al. reported that in Th2 cells, GATA3 and Stat5 can bind to different sites in the Il4 locus, with GATA3 to CNS-1 and Vα sites and Stat5 to HSII and HSIII sites (11, 13). Hence, in combination with GATA3, activated Stat5 through IL-2 signaling may maintain the accessibility of the Il4 gene and maximize its transcription. Taken together, our data indicate that it is likely that Dec2, induced by IL-4 signaling, helps to maintain a high level of IL-2R, thereby meeting the needs of Th2 cells at a late stage for strong Stat5 activation to achieve full fate commitment.

The up-regulation of CD25 expression by Dec2 is dependent upon Stat6 activation, but the specific mechanism is not clear. In studies not shown here, we did not find a marked effect on CD25 promoter activity by either Dec2 alone or in combination with Gata3, activated Stat5 through IL-2 signaling may maintain the accessibility of the Il4 gene and maximize its transcription. Taken together, our data indicate that it is likely that Dec2, induced by IL-4 signaling, helps to maintain a high level of IL-2R, thereby meeting the needs of Th2 cells at a late stage for strong Stat5 activation to achieve full fate commitment.

In conclusion, our study demonstrates that Dec2 is induced at a late stage of Th2 differentiation and that it can in turn enhance this process by increasing IL-2 signaling. These findings may be helpful in attaining a fuller understanding of the regulation of Th2 differentiation.

Acknowledgments
We thank Prof. Yongjun Liu, Dangsheng Li, Hua Gu, Rachel R. Caspi, Michael J. Lenardo, Weiguo Zhang, Binfeng Lu, and Yangxin Fu for helpful comments on this study and Dr. Sheri Skinner for reviewing the manuscript.

Disclosures
The authors have no financial conflict of interest.

References