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

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**Supplementary Material**

[http://www.jimmunol.org/content/suppl/2009/10/30/jimmunol.0900975.DC1](http://www.jimmunol.org/content/suppl/2009/10/30/jimmunol.0900975.DC1)

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

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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. The Journal of Immunology, 2009, 183: 6320–6329.

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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 stimulus 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-2 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) 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

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Received for publication March 27, 2009. Accepted for publication September 9, 2009.

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This work was supported by Grants from the National Natural Science Foundation of China (30530700, 30625003, 30600568, 30721065, 90710344, 30600308, 30801011, 30870126) and the CAS project (KSCX1-YW-R-43), a grant from the SIBS project (2007KIP301), grants from the Ministry of Science and Technology (2006CB503000, 2007CB512404, 2006AA02A247, 20072714), grants from the Technology Commission of Shanghai Municipality (88014199, 07DZ22916, 07XD14033, 06341034, 08451003004, 0207110206, 08DZ2291703), the EU project (FP6-2005-SSP-B, SPSB-CT-2006-044161), the National Basic Research Program of China (973 Program, No. 2007CB513001) and a grant from the B-institutes of Shanghai Universities Immunology Division.

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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 latent 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 Va-hCD2, 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 are as follows: sense primer TTCTGTGCTCGGTCCTCT on the hCD2 promoter, and anti-sense primer TGATGTGCTGCTGTTCCA on the mouse Dec2 gene. The mice were initially created on the 129 × C57BL/6 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-2 were purchased from R&D Systems. Recombinant mouse IL-1α, IL-6, and human TGF-β1 were purchased from PeproTech. Anti-mouse CD3 (145–2C11), anti-CD28 (37.51), and anti-mouse IL-4 (1B11) were obtained from BD Pharmingen. Anti-mouse IL-12 (C17.8), anti-mouse IFN-γ (XM12), FITC anti-mouse IL-2 (JE6-1A12), anti-mouse CD25 (PC61.5), and anti-mouse IL-2Rβ (TM-β1) were purchased from eBioscience. For flow cytometry analysis, allophycocyanin anti-mouse IFN-γ (YXM12), FITC anti-mouse IFN-γ, allophycocyanin anti-mouse-IL-4 (1B11), and allophycocyanin anti-mouse CD25 (PC61.5) were obtained from eBioscience. PE anti-mouse IL-4 (BV42–1D11), PE anti-mouse CD132 (TUGm2), PE anti-mouse CD62L (MEL-14), anti-mouse IL-2D4 (4A3R-4L), PE anti-mouse CD80 (16–10A1), PE anti-human CD4 (RPA-T4), and PerCP anti-mouse CD4 (RM4–5) were purchased from BD Pharmingen. For Western blot assay, Abs against c-Maf, p-Stat6, and Gata3 were obtained from Santa Cruz Biotechnology. Abs against total Stat6 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

Naive CD4+ (CD4+ CD44low CD62Lhigh) 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 μM), penicillin (50 U/ml), streptomycin (50 μg/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-12), or induced regulatory T cell (iTreg) (10 ng/ml ILT-β1). 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), MCFV-ires-GFP, MSCV-ires-human CD4 (hCD4), PCMV/Eco-Envelope, and PKF3-RSV/Gag-Pol were provided by Dr. Xingxing Zang (University of California, Berkeley, CA). The C57BL/6 mouse Dec2 and Gata3 were cloned into MCV-ires-GFP while Dec2 cDNA was also inserted into MSCV-ires-hCD4. To construct the retrovirus-mediated Dec2 RNA interference (RNAi) vector, mouse Dec2 RNAi target sequence (GCCATC TGGAGAAGACGATG) was designed and the complementary oligonucleotides were synthesized and cloned into pBS/Us6 (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/Us6 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 reincubated 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 Master Mix (ABI) and the reactions were run on an ABI PRISM 7000HT Sequence Detection System (PE 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-CAGAAATCTACCATCCCTGCA, reverse-tCCCGT TGGAAAGTCACTCA; Dec2, forward-CTGGAAAGATCTTGACCA, reverse-AAAATGCCCCAGTGTTGCAAAT; HPRT, forward-TGGC TCAATGCTAGAAGGAG, reverse-CAGAAGGCCCCAATACTGATG ATG; IL-4Rα, forward-GGAGAACGTATCATACAGTCC, reverse-CCCTCACTCAATTTTGCA; CD25, forward-CATGATACCCATGTTGCTG CG, reverse-ACCTGGTGCGGGGTACACAG, forward-GGAGG GGTGTTGCTTGG, reverse-ACCGTGGGGAGACCAGAG.

Western blot assay

T cells (106 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).
were considered statistically significant.

secreted IFN-γ, IL-4, and IL-17 after stimulation. The data are representative of two experiments. B, mRNA levels of Dec1 and Dec2 in these subsets restimulated with ConA for 4 h were examined by real-time PCR. The data are representative of two experiments. C, RNA was isolated from freshly isolated naive CD4+ cells (day 0) and from differentiating Th1 and Th2 cultures at the indicated time points over 5 days. Dec1 and Dec2 expression was determined by real-time PCR. The data are representative of two experiments. D, Lysates of 293T cells transfected with plasmids expressing HA-tagged Dec1 or Dec2 were immunoblotted with polyclonal anti-Dec2 or anti-HA Abs. E, The expression of Dec2 in T cells was detected by immunoblot assay. Lysate of 293T cells expressing exogenous Dec2 was loaded as a control. The data are representative of two experiments. F, The expression of Dec2 and Gata3 was assessed in differentiating Th1 and Th2 cells harvested at the indicated time points. G, Naïve CD4+ T cells from wild-type C57BL/6 or Stat6 deficient mice were differentiated under Th1 and Th2 conditions respectively for 3 days, and harvested for analysis of Dec2 expression by Western blotting. The data are representative of two experiments. Error bars indicate mean ± SD between duplicates.

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 FACS Calibur 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.

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, naïve CD4+ T cells were purified and differentiated into Th0, Th1, Th2, or Th17 cells, and the cytokine profile of each 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. 1G). 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 by Western blotting (Fig. 2A). Intracellular cytokine labeling analysis demonstrated that the introduction of Dec2 into Th0 or Th1 cells had no effect on their cytokine profiles. Intriguingly, overexpression of Dec2 under Th2-inducing conditions led to a marked increase in the percentage of IL-4 producers (from 21.76 to 37.59%) (Fig. 2B). The cytokine
level was also measured in the supernatants of restimulated GFP+ 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. 2A). Because GFP+ and GFP− 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 transduced 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, and this effect was abolished when IL-4 signaling was disrupted, in keeping with our observations above. It has been reported that addition of anti-IL-2 and anti-IL-2R (anti-CD25 and anti-CD122) to the culture efficiently shuts down IL-2 signaling (11, 26). Remarkably, blockade of IL-2 signaling with these neutralizing Abs normalized Th2 cytokine secretion between control and exogenous Dec2-expressing cells, even though IL-4 signaling remained intact.

Next, we adopted a complementary approach in which retrovirus-transduced cells were treated with hIL-2 in the presence of excessive anti-mIL-2 Ab. Because the neutralizing Ab could only recognize endogenous mouse IL-2, the source for initiation of the IL-2R mediated signaling came solely from hIL-2. The data showed that addition of hIL-2 to the culture was able to restore the enhancement of Th2 polarization by Dec2 in a dose dependent manner (Fig. 4H). Taken together, the data lead to the conclusion that Dec2 can up-regulate CD25, thus promoting Th2 differentiation through enhancement of the IL-2R mediated signaling pathway.

Dec2-induced CD25 expression is dependent upon Stat6 activation

Because the increase of CD25 expression by Dec2 was readily detected under Th2 skewing conditions, it appears that this effect might be dependent on IL-4 signaling. To further explore this possibility, the expression of CD25 was compared among the cells transduced with Dec2-expressing or control retrovirus under Th0, Th1, and Th2 conditions. FACS analysis showed that overexpression of Dec2 resulted in a marked increase in the level of CD25 expression in Th2 cells, with unchanged expression levels in Th0 and Th1 cells (Fig. 5A). Conversely, the enhancement of CD25 expression was completely eliminated if IL-4 signaling was defective due to Stat6 deficiency (Fig. 5B). These data indicate that Stat6 activation is necessary for CD25 induction by Dec2.

Knockdown of Dec2 attenuates CD25 expression and Th2 differentiation

Given the important role of Dec2 in the promotion of Th2 development, it was assumed that decreased Dec2 expression would reciprocally reduce Th2 cytokine production. To examine this possibility, we used RNAi and constructed a bicistronic retroviral vector containing a cassette that expresses a short hairpin RNA (siRNA) driven by a U6 promoter. Under Th2-inducing conditions, transduction of Dec2 siRNA dramatically reduced the expression of endogenous Dec2 protein (Fig. 6A) and concomitantly led to an ~40% decrease in the level of surface CD25 expression (Fig. 6B). In addition, knockdown of Dec2 expression resulted in a significant reduction of Th2 cytokine (IL-4, IL-5, and IL-13) production (Fig. 6C). These results indicate that the up-regulated Dec2 is able to impact the outcome of Th2 differentiation.

Dec2 transgenic mice are capable of mounting a stronger Th2 response

To investigate the physiological role of Dec2 in T cells, we created transgenic mice that constitutively express Dec2 under the control of a human CD2 promoter. The expression of the transgene was confirmed by Western blot assay showing that CD4+ T cells from transgenic mice expressed substantially more Dec2 protein than that of wild-type controls (Fig. 7A). As reflected in Fig. 7B, Dec2 transgenic mice exhibited normal distribution of CD4+ and CD8+ T cells in the thymus and peripheral organs. In addition, the proportion of naive CD4+ T cells was similar between wild-type and Dec2 transgenic mice (Fig. 7C). These data suggest that transgenic Dec2 expression does not alter normal T cell development, nor does it affect its activation threshold. In vitro cultured Dec2 transgenic cells displayed higher...
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 neg-
ative 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 cy-
kotines (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 peribronchial infil-
tration and accumulation of more inflammatory cells (Fig. 8C).
These data indicate that Dec2 transgenic mice can mount a stron-
ger 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

FIGURE 4. Dec2 promotes Th2 polarization through induction of CD25. A, CD25 expression was determined by flow cytometry on Th0 and Th2 cells transduced with either control or Dec2-expressing retrovirus. Numbers indicate MFI of CD25 gating on GFP+ cells in the form of Mock/Dec2. The data are representative of at least three experiments. B, CD25, CD122, and CD132 expression was determined by flow cytometry on Th2 cells transduced with either control or Dec2-expressing retrovirus. Numbers indicate MFI of indicated molecules. The data are representative of three experiments. C, Naive CD4+ T cells were activated under Th0 and Th2 conditions and infected with control and Dec2-expressing retrovirus. Forty-eight hours after infection, MFI of indicated surface molecules was determined and the data are shown as fold induction by overexpression of Dec2. D, MFI of CD25 labeling on Th0 and Th2 cells was measured at the indicated time points following retroviral infection. E, GFP+ Th2 cells were isolated after transduction with either control or Dec2-expressing retrovirus for examination of CD25 and Foxp3 expression using real-time PCR. In vitro induced Tregs served as a control. F, Isolated GFP+ T cells transduced with either control or Dec2-expressing retrovirus were either left untreated or treated with IL-2 for 30 min before harvest. Activation of Stat5 was tested by Western blot analysis. The data are representative of three experiments. G, Naive CD4+ T cells were activated under Th2 conditions for 30 h and infected with control and Dec2-expressing retrovirus followed by an additional 4-day culture under the indicated conditions of cytokines and blocking Abs. Then GFP+ cells were sorted and restimulated for cytokine production measured using ELISA. The data are representative of two experiments. H, Retrovirally transduced Th2 cells were cultured with anti-IL-2 (50 ng/ml) and incremental hIL-2 for 4 days and then GFP+ cells were sorted and restimulated for cytokine production measured with ELISA. The data are representative of two experiments. Shaded histograms (A and B) represent cells labeling with isotype-matched control Ab. Error bars indicate mean ± SD between duplicates.
virus gating on GFP was measured. Data are shown as fold induction relative to control retroviral infection with isotype-matched control Ab. The data are representative of GFP 48 h after retroviral infection. Numbers indicate MFI of CD25 gating on CD25 expressing retrovirus. CD25 expression was determined by flow cytometry

Between duplicates.

A

B

C

FIGURE 5. Induction of CD25 by overexpressed Dec2 is dependent upon Stat6 activation. A, Naive CD4+ T cells were activated under Th0, Th1, and Th2 conditions for 30 h and infected with control or Dec2-expressing retrovirus. CD25 expression was determined by flow cytometry 48 h after retroviral infection. Numbers indicate MFI of CD25 gating on GFP+ cells in the form of Mock/Dec2. Shaded histograms represent cells labeling with isotype-matched control Ab. The data are representative of two experiments. B, Wild-type and Stat6 deficient Th0 and Th2 cells were infected with control or Dec2-expressing retrovirus and CD25 expression was determined as in A. Data are shown as MFI fold induction by overexpressed Dec2. Error bars indicate mean ± SD between duplicates.

A

anti-DEC2

anti-actin

RNAi control Dec2

B

control

RNAi Dec2

C

IL-4 receptor

IL-13 receptor

IL-5 receptor

control RNAi

Dec2 RNAi

Dec2 RNAi

FIGURE 6. RNAi of Dec2 attenuates CD25 expression and Th2 differentiation. A, GFP+ Th2 cells transduced with retrovirus expressing either control or Dec2 siRNA were isolated for analysis of Dec2 expression by Western blotting. B, Th2 cells were infected as in A and CD25 expression was measured. Data are shown as fold induction relative to control retrovirus gating on GFP+ events. C, T cells in A were restimulated for analysis of secreted cytokine levels using ELISA. Error bars indicate mean ± SD between duplicates. *, p < 0.05.

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 and its function is restricted to a Th2-priming environment. In contrast, Dec2 facilitates Th2 polarization as characterized by an increase in the production of the entire set of Th2 cytokines. This phenomenon indicates that it is unlikely that Dec2 binds to all these gene loci; instead, it may strengthen central Th2-inducing signal(s). This provided a rationale to focus on the IL-4R mediated signaling pathway, the central impetus for Th2 development. However, the changes in the major events of this signaling pathway caused by overexpressed Dec2 were relatively minor, leading us to consider other possibilities.

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 Stat5a1*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 perceivable difference in the level of IL-4Rα expression at different time points after activation (supplementary Fig. 1). The online version of this article contains supplementary material.

In addition to the early induction of IL-4Rα expression, IL-2R mediated signaling is actually indispensable in the later period of
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 on 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. In addition, we detected more phosphorylated Stat5 in Dec2-transduced T cells. As the downstream mediators of IL-2 and IL-4 signalings, Stat5 and Gata3 act in a relatively independent manner during Th2 differentiation. They function synergistically in promotion

**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 Vγ 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 either by Dec2 alone or in combination with constitutive active Stat6. In contrast, it has been shown that Dec2 functions as a transcription suppressor in vitro (23, 30). Therefore, we assume that Dec2 may up-regulate the expression of CD25 indirectly and the mechanism remains to be clarified in future studies.

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

Supplementary Fig. 1. IL-4Rα expression level is similar between WT and Dec2 transgenic CD4⁺ T cells. Naïve CD4⁺ T cells from WT and Dec2 transgenic mice were activated with anti-CD3 (5 μg/ml) and anti-CD28 (2 μg/ml) under Th0 or Th2 conditions, and harvested for analysis of CD124 and CD25 expression levels by flow cytometry at the indicated time points after activation. Shaded histograms represent cells labeling with isotype-matched control antibody.