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The Role of the Basic Helix-Loop-Helix Transcription Factor Dec1 in the Regulatory T Cells

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Naturally occurring regulatory T (Treg) cells play a central role in the maintenance of immune homeostasis and in restraining the development of spontaneous inflammatory responses. However, the underlying mechanisms of Treg homeostasis remain incompletely understood. Of particular note, the IL-2Rα (CD25) is crucial for the homeostasis of Treg cells and the prevention of lymphoproliferative autoimmune disease. In this paper, we report that the basic helix-loop-helix transcription factor Dec1 is involved in the homeostasis of Treg cells and plays a role in their survival or expansion after adoptive transfer to lymphopenic recipients. Hence, it is crucial for the suppression of effector T cell-mediated inflammatory responses. Enforced expression of Dec1 upregulates CD25 expression during thymocyte development and increases the number of Treg cells in the periphery. Dec1 binds the transcription factor Runx1 and colocalizes with Runx1 in Treg cells. Specifically, we demonstrate that in Treg cells the Dec1/Runx1 complex binds to regulatory elements present in the Il-2ra locus. Collectively, these data show how Dec1 mechanistically acts in Treg cells. The Journal of Immunology, 2010, 185: 000–000.

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mice exhibit the development of lymphoproliferative autoimmune disease. In addition, we show that Dec1 is required for the long-term maintenance of Treg cells after adoptive transfer to suppress effector T cell-mediated inflammation. Dec1 appears to positively regulate CD25 expression in association with Runx1, and these data link Dec1 and Runx1 into a common pathway to regulate Treg cells.

Materials and Methods

Mice

Targeting of the Dec1 locus was performed by standard techniques. The BshHII–BshHII genomic fragment of Dec1 as previously described (23), which contains the promoter and coding region including the ATG in exon1, was replaced with a Neo cassette. The resulting chimeric mice were backcrossed to C57BL/6 more than six to eight times. Dec1 transgenic (Tg) mice were generated using the Lck proximal promoter transgene cassette vector as described previously (24). We generated two independent Dec1 Tg mouse lines. CD25 Tg mouse was generated using the cassette vector in which CD25 DNA is driven by the CD4 enhancer promoter that was provided by Dr. I. Taniuchi (The Institute of Physical and Chemical Research [Japan] Research Center for Allergy and Immunology, Yokohama, Japan). C57BL6 (B6, ly5.2) mice were obtained from CREA Japan (Tokyo, Japan), and B6.SJL PtprcPep3bBoyJ (B6, Ly5.1) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were kept in accordance with the laboratory animal science guidelines of Hiroshima University (Hiroshima, Japan).

Flow cytometry and Abs

Single-cell suspensions from thymus, spleen, and mesenteric lymph node were stained with mAbs and second reagents. FITC-, PE-, allophycocyanin-, allophycocyanin-Cy7, biotin-labeled, and purified mAbs were purchased from BD Pharmingen (San Diego, CA) (CD4, CD8α, CD3e, CD44, CD62L, CD25, CD45R/B220, Mac-1, Gr-1, Ter119, NK1.1, CD45.2, and CD45.1) or from eBioscience (San Diego, CA) (CD4, CD3e, CD127, CD25, and Foxp3). Mouse Treg cell staining kit (eBioscience) was used for Foxp3 staining. Biotinylated Abs were revealed with streptavidin-FITC, -PE, -PerCP-Cy5.5, -allophycocyanin, or -allophycocyanin-Cy7. Clone 2.4G2 anti-CD12/CD16 was used to block FcRs. FACS analysis was performed on a FACScalibur (BD Biosciences, San Jose, CA) or FACS-VantageSE (BD Biosciences), and data were analyzed with FlowJo (Tree Star, Ashland, OR) software. For cell sorting, all cells were stained with biotinylated Abs, bound to streptavidin magnetic beads, and depleted by MACS separation column (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were subsequently sorted with a FACSVantageSE (BD Biosciences). Dead cells were removed from analysis and sorted by staining with propidium iodide. Reanalysis of the sorted cells indicated a purity >95%.

Preparations of CD4+CD25+ cell populations

Splenocytes were harvested from 8- to 12-wk-old Dec1+/−, Dec1 WT, or Dec1 Tg mice, and the red cell fraction was eliminated by lysis. Splenocytes were stained with biotin-labeled anti-CD8, -TCR-γ, -CD19, -CD11b, -Gr-1, -NK1.1, allophycocyanin-Cd4, and PE-CD25 Abs and bound to streptavidin-magnetic beads. Then, cells were depleted by MACS separation column (Miltenyi Biotec) and were sorted CD4+CD25+ cells on a FACSVantageSE. These cells were used for in vitro and in vivo expression analyses.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay of thymocyte with anti-acetylated H3 (Upstate Biotechnology, Lake Placid, NY), anti-Runx1 (Active Motif, Carlsbad, CA), purified anti-Dec1 and control (Santa Cruz Biotechnology, Santa Cruz, CA) Abs were performed as described previously (24). In brief, isolated total thymocyte or sorted double-positive (DP) cells were fixed with 1% formaldehyde at room temperature and sonicated. Soluble chromatin was immunoprecipitated with Abs overnight. PCR was performed with SYBR Premix Ex Taq for real-time PCR (TaKaRa, Shiga, Japan). The sequences of the PCR primers are available on request. For ChIP assay using Treg cells, sorted CD4+CD25+ Treg cells were fixed and sonicated. Then, we used Low Cell# ChiP kit (Diagenode, Denville, NJ).

Immunoprecipitation and Western blotting

Expression vectors for Flag-tagged Runx1 and hemagglutinin (HA)-tagged Dec1, which contain their entire coding region sequences, respectively, were prepared using pcDNA3.1 vector. The Runx1 deletion constructs (6402 and 6362) were provided by Dr. S. Sakaguchi (25). Deletion constructs of Flag-tagged Runx1 (26290 and B178) and HA-tagged Dec1 were made by PCR. Flag-tagged Runx1 and HA-tagged Dec1 constructs were cotransfected into COS7 cells using FugeneHD (Roche, Basel, Switzerland), according to the manufacturer’s instructions. The cells were harvested in ice-cold PBS, and the cell pellet was resuspended in ice-cold 0.5% Triton X-100 buffer, followed by centrifugation at 4˚C for 30 min at maximal velocity in a microcentrifuge. For immunoprecipitation (IP), 400 μg of the supernatant protein aliquots was incubated with the Abs for 1 h and subsequently with protein G-Sepharose overnight at 4˚C. Beads were washed with TEG buffer (20 mM Tris-HCl [pH 7.4], 1 mM EDTA, and 10% glycerol) and boiled in sample buffer. Immunoprecipitated proteins were analyzed by SDS-PAGE, followed by immunoblotting using rat anti-HA (3F10; Roche) or mouse anti-Flag (M2; Sigma-Aldrich, St. Louis, MO).

Immunocytochemistry

Non-Treg (CD4+CD25−) or Treg (CD4+CD25+) cells were prepared from mouse lymph nodes using Abs and MACS columns as described in the section of the preparation of CD4+CD25+ cell populations. The cells were fixed with 4% paraformaldehyde and permeabilized with 0.03% Triton X-100 for 10 min at room temperature. After washing with PBS, the cells were stained by anti-Dec1 Ab for 1 h at room temperature, washed and stained by Alexa Fluor 550-conjugated anti-rabbit secondary Ab. Subsequently, the cells were stained with anti-Runx1 Ab (Active Motif), which was direct-labeled with Alexa Fluor 488 using Zenon rabbit IgG labeling kit according to the manufacturer’s instructions (Molecular Probes, Eugene, OR). After washing with PBS, Hoechst 33342 was used for DNA staining. Controls were performed by staining cells only with secondary Ab.

RT-PCR (quantitative real-time RT-PCR)

Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) and was reverse transcribed to cDNA using SuperScriptIII (Invitrogen). The cDNA was used for quantitative real-time RT-PCR or semiquantitative RT-PCR with SYBR Premix Ex Taq (TaKaRa) or Ex Taq polymerase (TaKaRa), respectively. The PCR primers sequences are as follows: mouse Dec1, 5′-GGAGGAAAAACGGAGGCAGAAGC-3′ (forward) and 5′-CACAGTTGCTGGAACACCTGGAGCA-3′ (reverse); mouse CD25, 5′-CCACATTCAAGCCTCCCTCTCA-3′ (forward) and 5′-GTTTTCCACACCTTCATCTGC-3′ (reverse); mouse Runx1, 5′-GCTGCTGAAAGCAGGACTCTCG-3′ (forward) and 5′-CCAGGACGAGTAACACGCTC-3′ (reverse); and mouse β-actin, 5′-TTCACACCCCAAGCAGGATGA-3′ (forward) and 5′-CTTCAGGAGGACAAATGACTC-3′ (reverse).

Statistical analysis

The p values were calculated with the two-tailed Student t test for two group comparison as applicable with Microsoft Excel software. The statistical significance level was 0.05.

Results

Dec1 gene deficiency results in reduced Treg cell numbers and in lymphoproliferative disease in aged mice

Dec1 mRNA expression is induced in >40-fold in CD4+CD62L− naıve T cells on TCR signaling (Fig. 1A). It is highly expressed in immature CD4−8− double-negative [DN]) and mature CD4+ or CD8+ (single-positive [SP]) cells but markedly reduced in CD4+8+ (DP) cells during thymocyte development (Fig. 1B). Furthermore, the level of Dec1 expression is higher in the Treg cell population (CD4+CD25+) than in non-Treg cells (CD4+CD25−) (Fig. 1C). It has been reported that >50% of Dec1 gene-deficient (Dec1−/−) mice exhibited lymphoid organ hyperplasia after 6–8 mo of age (23). To understand the biological role of Dec1, we generated Dec1−/− mice and analyzed T cell, especially Treg cell, status therein. Thymocyte development was not affected in Dec1−/− mice (data not shown). No major difference was observed in cell
Flow cytometric analyses in CD4+ T cells

However, at the lymphoproliferative phenotype within a 10-mo period. Lymphoproliferative disease (4 females of 20 mice at a marked reduction in the percentage of CD25+ or Foxp3+ pulp and white pulp in the spleen, or loss of germinal center organs and dissolution of the normal architecture, such as red mass. Massive infiltration of polyclonal lymphocytes into systemic CD62L (Supplemental Fig. 3). Histological analysis showed defects in the elimination of activated T and B cells (26). The expression in CD4+CD25+ cells between 6- and 10-mo-old Dec1−/− mice. We also observed no significant differences in Ki67 or bcl-2 accumulation of DN T cells is characteristic of those mutant mice (27). However, we observed no such accumulation of DN T cells in any of the aged Dec1−/− mice (Supplemental Fig. 4). These observations encouraged us to further investigate the role of Dec1 in the development of Treg cells. The percentage of Foxp3+ cells within the CD4+ population was decreased in aged (10 mo) Dec1−/− mice without lymphoid organ hyperplasia compared with their aged littermate controls, although there was no significant difference in the number of Foxp3+ cells between young adult (12 wk) Dec1+/+ and Dec1−/− mice (Fig. 1F). We did not observe obvious attenuation of Foxp3 expression (Fig. 1F), and a normal percentage of Foxp3+ cells among CD4SP thymocytes in Dec1−/− mice was present (data not shown). The proliferative status of CD4+CD25+ cells was analyzed by Ki67 expression in Dec1−/− mice, which was also found to be comparable to littermate controls (Supplemental Fig. 5). We also observed no significant differences in Ki67 or bcl-2 expression in CD4+CD25+ cells between 6- and 10-mo-old Dec1+/+ and Dec1−/− mice (Supplemental Fig. 5). In addition, increased activated T cells (CD69+ or CD44high) and less naive T cells (CD62Lhigh) were observed in several aged Dec1−/− mice without lymphoproliferative disease, whereas the expression of these markers in young adult Dec1−/− mice was normal (Supplemental Fig. 6). On the basis of these observations, we propose that a deficiency in Dec1 caused a reduction in the proliferation or IL-2 production on TCR stimulation in Dec1−/− CD4 or CD8 naive T cells on the C57BL/6 genetic background (Supplemental Fig. 1). In addition, we observed less marked differentiation from naive Dec1−/− CD4+ or CD8+ T cells to IFN-γ-secreting T cells following TCR stimulation in vitro (data not shown). We failed to find any obvious hyper-responsiveness to TCR stimulation in naive Dec1−/− T cells.

Unlike in the previous report (23), on the C57BL/6 genetic background, we did not identify any Dec1−/− mouse exhibiting the lymphoproliferative phenotype within a 10-mo period. However, at >1.2 y of age, some of these mice manifested lymphoproliferative disease (4 females of 20 mice at >1 y old showed splenomegaly and lymphadenopathy; Fig. 1D, Supplemen
tal Fig. 2), with CD4 T cells highly expressing the activation markers CD44 and CD69 together with lower expression of CD62L (Supplemental Fig. 3). Histological analysis showed massive infiltration of polyclonal lymphocytes into systemic organs and dissolution of the normal architecture, such as red pulp and white pulp in the spleen, or loss of germinal center formation in lymph nodes (Supplemental Fig. 2). We found a marked reduction in the percentage of CD25+ or Foxp3+ Treg cells among the CD4 T cells in mice with lymphoproliferative disease (Fig. 1E). It is well known that mutations in Fas and Fas ligand also result in autoimmune disease because of defects in the elimination of activated T and B cells (26). The

FIGURE 1. Dec1 gene deficiency results in reduced Treg cell numbers in aged mice, which subsequently manifest lymphoproliferative autoimmune disease. A and C, Quantitative real-time RT-PCR analysis of Dec1 expression in CD4+CD25− CD62Lhigh T cells after stimulation with anti-CD3 and CD28 Abs (A) or in thymic CD4+CD25− and CD4+ CD25high cells (C). Results are normalized to Hprt expression. B, Semiquantitative RT-PCR analysis of Dec1 and β-actin during thymocyte development. D, Sple
nose
demy and lymphadenopathy in 1.2-y-old Dec1−/− mice. Scale bar represents 10 mm. E, Flow cytometric analyses in lymph node CD4+ T cells from 1.2-y-old Dec1−/− mouse with lymphoproliferathy or littermate mouse. Numbers above bracketed lines indicate the percentage of positive cells. More than three independent pairs yielded similar results. F, Flow cytometric analyses in CD4+ T cells from 12-wk-old or 10-mo-old Dec1+/+ and Dec1−/− mice. Numbers above the bracketed line indicate percent Foxp3+ cells. Foxp3 expression levels in Foxp3+ cells were quantified by and are displayed as mean fluorescence intensity (MFI), with the value in Dec1+/+ cells defined as 1. Data were averaged from at least three mice. ♦p = 0.00325; t test.
numbers of Treg cells and subsequent dysregulation of T cell activation in aged mice while their status of proliferation and survival was not affected. Furthermore, we suggest that these defects eventually increase the susceptibility to systemic lymphoproliferative disease.

Dec1 plays a crucial role in maintaining Treg cells, which are required for the suppression of effector T cell-mediated autoimmunity

Next, we investigated the in vitro suppressive function of Treg cells from Dec1−/− mice. CD4+CD25+ Treg cells derived from Dec1−/− mice had a normal capacity to inhibit the proliferation of naive T cells in a dose-dependent manner (Fig. 2A, Supplemental Fig. 7). Furthermore, to assay the function of Treg cells in vivo, we used adoptively transferred Treg cells after adoptive transfer, which is required for the suppression of Teff cell-mediated inflammation. A, CD4+CD25 CD62Lhigh T cells were labeled with CFSE and mixed with CD4+CD25high T cells from 12-wk-old Dec1−/− and Dec1+/+ mice at the indicated ratios, and assayed for proliferation with irradiated splenocytes and anti-CD3e Ab (0.3 μg/ml). Cell proliferation was analyzed by CFSE dilution after 72 h. Values indicate the proportion of dividing cells. Two independent experiments produced similar results. B–D, A total of 1 × 105 purified CD4+CD25− CD45RBhigh T cells from Ly5.1 B6 mice (Teff) were transferred i.v. into Rag1−/− mice at the indicated ratios, and assayed for proliferation with irradiated spleens and anti-CD3e Ab (0.3 μg/ml). The percentages of CD25+ and Foxp3+ Treg cells among the CD4+ cells was significantly decreased in mice receiving Dec1−/− Treg cells (Fig. 2D). No significant difference in either Ly5.1+CD25−, Ly5.1+CD25+, or Ly5.1+ Foxp3+ cells was observed between the two groups (Teff cells + Dec1−/− Treg cells and Teff cells + Dec1+/+ Treg cells), indicating a lack of any difference in the development of Treg cells or non-Treg cells from Ly5.1 effector T cell (Supplemental Fig. 9). These results suggest that Dec1−/− Treg cells do mediate normal regulatory activity to suppress Teff cells but that they were not maintained long term. This resulted in Teff-mediated colitis at later period.

However, these results could also be explained by the possibility that adoptively transferred Dec1−/− Treg cells were rejected because of minor histocompatibility Ag differences (because of six to eight generations on the C57BL/6 background). To exclude this, we used Dec1+/+ Treg cells from littermate control mice. In addition, we also observed a decrease in the percentage of Treg cells 10 wk after transfer of CD4 T cells from Dec1−/− mice, including mixtures of Dec1-deficient Treg and non-Treg cells (data not shown). Therefore, we conclude that the reduction in numbers of transferred Treg cells was not caused by minor histocompatibility Ag differences.

To verify that Dec1 is required for the homeostasis/maintenance of Treg cells, we adoptively transferred only Treg cells into Rag1−/− mice. Four weeks thereafter, the percentage of CD25+ cells or Foxp3+ cells among the CD4+ cells was significantly decreased in mice receiving Dec1−/− Treg cells (Fig. 2E, right). Taking the above findings together, we suggest that the Dec1 gene plays a crucial role in the maintenance of Treg cells in the periphery, which is required for the suppression of effector T cell-mediated autoimmunity.

To assess impairment of Treg cell homeostasis, we focused on the maintenance of Foxp3 expression. Recent reports suggested...
that stable Foxp3 expression is regulated by DNA demethylation at the evolutionarily conserved region within the Foxp3 locus (28). However, the status of this locus in Dec1+/− Treg cells was found to be highly demethylated, similar to control Treg cells (Supple-
mental Fig. 10).

Dec1 is involved in induced Treg cell development by supporting their survival or proliferation

Next, we examined the involvement of the Dec1 gene in the induction of Foxp3 expression in CD4+CD25− non-Treg cells (induced Treg; iTreg) by TCR stimulation in the presence of TGF-β in vitro. There was a significant reduction in the development of iTreg cells from Dec1−/− non-Treg cells (Fig. 3A). TGF-β signaling is thought to be required not only for iTreg but also for the differentiation of IL-17-producing T (Th17) cells (29, 30). However, CD4+CD25−CD62Lhigh T cells from Dec1−/− mice yielded substantial numbers of Th17 cells in the presence of TGF-β/IL-6/IL-23/IL-β (Supplemental Fig. 11), indicating that reduced iTreg might not be simply caused by defects of TGF-β signaling in Dec1−/− T cells.

Interestingly, exogenous IL-2 completely overcame the impairment of iTreg cell development from Dec1−/− non-Treg cells, suggesting that Dec1 is involved in the survival or expansion of iTregs but not in their commitment and that excess exogenous IL-2 overcomes these defects even in the absence of the Dec1 gene (Fig. 3B).

Enforced expression of Dec1 increases the number of Treg cells

To investigate whether enhanced expression of Dec1 reciprocally influenced Treg cells, we generated Tg mice expressing Dec1 in a T cell lineage-specific manner, with the transgene under the control of the lck-proximal promoter. In contrast to Dec1−/− mice, we observed an increased percentage of Foxp3+ cell among CD4 T cells in Dec1 Tg mice compared with wild-type (WT) mice (Fig. 4A). The proliferative status of Treg cells in Dec1 Tg mice analyzed by Ki67 expression was comparable to WT mice (Supplemental Fig. 12). CD4+CD25+ Treg cells from Dec1 Tg mice mediated inhibitory activity on the proliferation of CD4+CD25−CD62Lhigh naive T cells comparable to WT Treg cells (Fig. 4B). In addition, CD4+CD25− non-Treg cells in Dec1 Tg mice expressed more Foxp3 than WT non-Treg cells following activation via TCR signaling plus TGF-β (Fig. 4C). These results indicate that, in contrast to Dec1 deficiency, enforced expression of the Dec1 gene increases the number of Treg cells in vivo, as well as iTreg cell induction in vitro.

Dec1 upregulates CD25 expression

We next investigated target genes of Dec1 in T cells by detailed analysis of Dec1 Tg mice. We verified the high expression of Dec1 in Dec1 Tg thymocytes (Supplemental Fig. 13). In our analysis of thymocyte development, we noticed an accumulation of DN cells and a decrease in CD4 or CD8SP cells. Among lineage+reg cells, a marked increase in DN3 cells and reduction of DN4 cells in Dec1 Tg mice was observed (Supplemental Fig. 14). These results suggest impairments in β selection and positive selection during thymocyte development. We confirmed defects in β selection and positive selection of Dec1 Tg thymocyte using fetal thymic organ cultures and reaggregation thymic organ cultures (data not shown). During our investigations of Dec1 Tg thymocyte development, we

**FIGURE 3.** Dec1 is involved in the survival or expansion of iTreg cells. A. Purified populations of CD4+CD25−CD62Lhigh cells from 8- to 12-wk-old Dec1+/+ or Dec1−/− mice were cultured on the plate-bound anti-CD3 plus anti-CD28 Abs (5 μg/ml) in the presence of TGF-β1 (2 ng/ml). After 72 h, cells were stained with anti-Foxp3 Ab and analyzed by FACS. Numbers above bracketed line indicate percentage of Foxp3+ cells (left). Data represent the mean ± SD from three wells. ♦ p = 0.01721; t test. Three independent experiments produced similar results. B. Exogenous IL-2 (1 μg/ml) can restore the development of iTreg cells from Dec1−/− naive CD4 T cells (left). The increased rate of the cell numbers of either Foxp3+ or Foxp3− cells by the addition of IL-2 (right). Foxp3− (Dec1+/+, 1.015 ± 0.089; Dec1−/−, 1.413 ± 0.091), Foxp3+ (Dec1+/+, 18.588 ± 1.554; Dec1−/−, 87.401 ± 1.081). ♦ p = 0.00014; t test.

**FIGURE 4.** Enforced expression of the Dec1 gene increases Treg and iTreg cell numbers. A. Representative flow cytometric analyses of Foxp3 expression in CD4+ cells from 12-wk-old WT or Dec1 Tg mice. Numbers above bracketed lines indicate the percentage Foxp3+ cells within the CD4+ population. Foxp3 expression was quantified by mean fluorescence intensity, and the value in WT cells was defined as 1. Data represent the mean ± SD from at least three mice. ♦ p = 0.02010; t test. B. Sorted CD4+CD25−CD62Lhigh Teff cells were labeled with CFSE and mixed with Treg cells from 12-wk-old WT or Dec1 Tg mice and assayed for proliferation in the presence of irradiated B6 splenocytes and anti-CD3e Ab as in Fig. 2A. Three days after stimulation, Teff cell proliferation was analyzed by CFSE dilution. Values indicate the proportion of dividing cells. Two independent experiments produced similar results. C. Sorted CD4+CD25−CD62Lhigh cells from 12-wk-old WT or Dec1 Tg mice were cultured with anti-CD3 plus anti-CD28 Abs in the presence of TGF-β1 as in Fig. 3A. After 72 h, cells were stained with anti-Foxp3 Ab. Numbers above bracketed lines indicate the percentage of Foxp3+ cells (left). Data represent the mean ± SD from three wells. ♦ p = 0.03010; t test. Two independent experiments produced similar results.
found abnormal expression of CD25 in the DP cells of these mice, despite little expression in WT DP cells (Fig. 5A, 5B). Although there were marked increases of CD25high cells within the CD4SP subpopulation, we found normal Foxp3 expression in Dec1 Tg CD4SP cells (Fig. 5C). These results indicate that Dec1 could induce CD25 expression but not Foxp3 directly during thymocyte development. CD25/IL-2Rα is strictly regulated during T cell development and is necessary for the maintenance of Treg cells in the periphery. Previous studies have collectively identified positive regulatory regions (PRRs) in the Il-2ra locus, to which various transcriptional factors can bind (31) (Fig. 5D, upper panel). Therefore, we investigated the acetylation status of histone H3 at these PRRs (PRRI–V) in Dec1 Tg DP cells by ChIP assays. The degree of H3 acetylation was higher at PRRI, II, III, and V and was similar at PRRIV in the Il-2ra locus in Dec1 Tg DP cells, compared with control DP cells (Fig. 5D, lower panel). These results suggest that one of the target genes of Dec1 is Cd25/Il-2ra.

We next investigated whether Dec1 was required for the induction of CD25 expression in naive T cells on TCR signaling. There were no significant differences in CD25 expression by either CD4 or CD8 T cells from Dec1+/− or Dec1−/− mice (data not shown), indicating that Dec1 is dispensable for the induction of CD25 in naive T cells.

**Enforced CD25 expression restores the long-term maintenance of Dec1−/− Treg cells**

We hypothesized that Dec1 is involved in the expression of CD25, in particular in its maintenance, and that impaired maintenance of Treg cells in Dec1−/− mice is caused by defects in sustaining CD25 expression. To test this hypothesis, we generated a mouse Tg for CD25 under the control of the CD4 promoter and crossed this with the Dec1−/− mice to determine whether enhanced expression of CD25 could overcome the defects in Dec1−/− Treg cells. We found more CD4+CD25+ cells, but an almost normal percentage of Foxp3+ cells among the CD4+ cells in either CD25-Tg; Dec1−/− or CD25-Tg; Dec1+/− mice (Fig. 6A, upper panel). As in Fig. 2D, we isolated CD25+ cells from CD25-Tg; Dec1−/− or −/− mice (Fig. 6A, lower panel), transferred them into Rag1−/− mice and then analyzed them 4 wk after transfer. The percentage of Foxp3+ cells among the CD4+ cells in mice receiving CD25+ cells from CD25-Tg; Dec1−/− animals was comparable to that in control CD25-Tg; Dec1−/− mice (Fig. 6B). On the basis of these observations, we suggest that CD25 is involved in the impaired maintenance of Dec1−/− Treg cells after adoptive transfer.

**Dec1 binds to the Il-2ra locus together with Runx1 in Treg cells**

We next sought to determine whether endogenous Dec1 actually bound to the Il2ra gene locus in Treg cells. Purified CD4+CD25+ Treg cells from Dec1+/− or Dec1−/− mice were tested in ChIP

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**FIGURE 5.** Dec1 Tg thymocytes upregulate CD25 expression. A, Representative flow cytometric analyses in CD4+CD8− (DP) and CD4+CD8+ (CD4SP) cells from WT or Dec1 Tg mice. Numbers above bracketed lines indicate the percentage of CD25+ cells. B, Quantitative real-time RT-PCR analysis of CD25 expression in WT or Dec1 Tg DP thymocytes. Results are normalized to Hprt expression, referred to in the WT as 1. Data represent the mean ± SD. C, Flow cytometric analyses in CD4SP cells from 12-wk-old WT or Dec1 Tg mice. Numbers in quadrants represent the percentages. D, Schematic view of the Il2ra locus shows exon1 and identified PRRs (PRRI–V) showing the position of each individual primer for the ChIP assay (upper panel). Purified DP cells from WT or Dec1 Tg mice were used for ChIP assays with Ab against acetylated histone H3 (AcH3) or control IgG Ab (con). Real-time PCRs were carried out with primers for each amplicon in the Il2ra locus. Representative data from two independent experiments are shown.

**FIGURE 6.** Impaired maintenance of Dec1−/− Treg cells is restored in CD25 Tg mice. A, A total of 2 × 10⁶ purified CD4+CD25+ T cells from 12-wk-old CD25-Tg; Dec1−/− or CD25-Tg; Dec1+/− mice were transferred into Rag1−/− mice (n = 4), as in Fig. 2E. Flow cytometric analyses in LN CD4+ T cells (upper panels) and isolated CD4+CD25+ cells (lower panels) are shown. B, The percentage of Foxp3+ cells among CD4+ cells was analyzed by FACS 4 wk after transfer. Error bars represent SD.
assays using Ab against the Dec1 protein. We detected Dec1 binding at PRRV, PRRIV, and intron1 (5) in Dec1+/+ Treg cells but not Dec1−− cells (Fig. 7A, upper panel). However, we could not find an E-box sequence, reported as a Dec1 binding site (32) in these regions. Thus, we hypothesized that Dec1 is recruited to the Il-2ra locus together with other transcription factors that bind to these sites. It has recently been reported that Runx1 bound to Il-2ra loci and collaborated with Foxp3 (25). We did find the Runx1-DNA binding consensus sequence in PRRV and PRRIV; especially, PRRIV had been previously reported as a Runx1 binding site. In addition, we found a Runx1-binding sequence close to the amplicon of intron1 (5). Runx proteins bind to the regulatory elements of target genes together with other transcription factors and can function as activators or repressors (33). Accordingly, we explored the binding capacity of Dec1 for Runx1. In IP experiments using lysates of COS7 cells cotransfected with genes encoding HA-tagged Dec1 and Flag-tagged Runx1 proteins, we found that Dec1 bound to Runx1 in vitro (Fig. 7B). Furthermore, IP experiments using deletion mutants showed that Runx1 lacking the C-region (aa 363–402) failed to bind to Dec1 (Fig. 7C).

**FIGURE 7.** Dec1 physically interacts with Runx1 and binds to PRRs in Il-2ra locus together with Runx1 in Treg cells. A. Purified CD4+CD25+ Treg cells from Dec1+/+ or Dec1−− mice were used for ChIP assays with Abs against Dec1 (upper panel) and Runx1 (lower panel) proteins. Real-time PCRs were carried out with primers for amplicons as seen in Fig. 5D. Two independent experiments yielded similar results. B. HA-tagged Dec1 and Flag-tagged Runx1 were cotransfected into COS7 cells, and the lysates were immunoprecipitated with anti-Flag or anti-HA Ab. The blots of the proteins before (input) or after IP with anti-Flag or anti-HA Ab were probed with anti-HA (upper panel) or anti-Flag (lower panel) Ab. C. Schematic diagram of the deletion constructs of Runx1. The Runt (Runt), activation domain (AD), and inhibition domain (ID) are shown. HA-tagged Dec1 was cotransfected with the Flag-tagged deletion constructs and immunoprecipitated with anti-Flag Ab. D. Schematic diagram of the deletion constructs of Dec1. The bHLH and ORANGE domains (ORANGE) are shown. Flag-tagged Runx1 was cotransfected with the HA-tagged deletion constructs and immunoprecipitated with anti-Flag Ab. E. The intracellular localization of endogenous Runx1 and Dec1 in non-Treg (CD4+CD25−) or Treg (CD4+CD25+) cells was analyzed by immunocytochemistry. Images of Runx1 (green) and Dec1 (red) were merged to show regions of colocalization (yellow). Nuclei were revealed with DAPI staining (blue). Original magnification ×400. Scale bar, 5 μm.
lar experiments using deletion mutants of Dec1 indicated that the Runx1-interacting domain of Dec1 was located in the region between 125 and 188 aa, including the ORANGE domain found in Hairy/E(SPL) family of bHLH proteins (34) (Fig. 7D). We observed the colocalization of these factors in the nucleus by immunofluorescent staining in vitro (Supplemental Fig. 15). Furthermore, endogenous Dec1 and Runx1 were partly colocalized in the nonheterochromatin area in the nucleus of CD4+CD25+ Treg cells, but not in non-Treg cells, in which little expression of Dec1 was found (Fig. 7E). These results caused us to investigate how Runx1 bound to the II-2ra locus in Dec1−/− Treg cells. Curiously, Runx1 was found to bind to the same sites as Dec1 in the II-2ra locus in Dec1+/+ Treg cells, and accordingly, its binding was drastically reduced in Dec1−/− Treg cells (Fig. 7A, lower panel). These results suggest that Dec1 binds to the II-2ra locus upon interacting with Runx1. We propose that Dec1 binding is required for Runx1 DNA binding to regulatory elements present in the II-2ra locus in Treg cells and that this interaction is specific for Treg cells.

Discussion

Because it was observed that the Dec1 gene is downregulated in DP cells and induced again in CD4 or CD8SP cells (Fig. 1B), we analyzed its role in positive and negative selection of DP thymocytes by intercrossing HY-TCR Tg or AND-TCR Tg mice. Interestingly, Dec1 Tg mice exhibited markedly impaired positive and negative selection of DP cells, even though no obvious defects in thymocyte selection were observed in HY-TCR Tg:Dec1+/+ mice (our unpublished data). Thus, the dysregulation of peripheral T cells in aged Dec1−/− mice is not caused by impairments in thymocyte selection.

The target cells of Treg suppressive mechanisms are of two types, responder T cells and APCs, which are affected through multiple molecules such as via cytokines (IL-10 and TGF-β). IL-2 consumption because of high-level IL-2R expression, CTLA-4 expression, and so on (9, 35–37). Treg cells do not rely only on one mechanism for their function but on the use of many alternative mechanisms to control inflammation. Although Dec1−/− Treg cells express normal levels of CD25 and CTLA-4 and mediate normal suppressive function at early time points in vivo, Treg cells failed to suppress at later time points. This was associated with a reduced number of Treg cells remaining after 8 wk in adoptive transfer models. These observations suggest that Dec1 is involved in the long-term maintenance of Treg cells after their adoptive transfer. Dec1 deficiency causes a reduction in the numbers of Treg cells in aged but not young mice, accompanied by increased activation of effector T cells. At younger age, many non-Treg as well as Treg cells are produced in the thymus and exported to the periphery. However, the supply of Treg cells is gradually reduced in mice as they age. Therefore, we propose that Treg cell retention is more important for maintaining immune homeostasis in aged than young mice because of the reduced output of new Treg cells from aged thymus and, accordingly, that the Dec1 gene is mainly required for the suppression of spontaneous inflammation by maintaining Treg cells. However, we note that the reduction of Treg cells in aged Dec1−/− mice was not severe and the proliferation status and bcl-2 expression in aged Dec1−/− Treg cells were comparable to that with control Treg cells (Supplemental Fig. 5). It has been reported that the number of Treg cells are immediately and severely decreased after thymectomy, and the remaining Treg cells exhibits increased proliferation (38). Therefore, it remains unclear whether Dec1 is required for the maintenance of Treg cells in periphery, their production in thymus, and/or their emigration from thymus in aged mice. We also observed an increasing CD25+Foxp3−CD4 SP cells in Dec1 Tg thymus (Fig. 5C). It is conceivable that this compartment contains Treg precursors. This raises another possibility that Dec1 may be involved in the production of thymic Treg precursor cells and that the reduced production of precursor cells may give rise to a decrease in peripheral Treg cells in aged Dec1−/− mice. In addition, many aged Dec1−/− mice showed less Treg cells and more activated effector T cells. However, few mice suffered from lymphoproliferative disease in >1-yr-old mice, indicating that additional factors, such as infection, are necessary for the incidence of autoimmune disease. We note that this is similar to human autoimmune diseases, some of which are induced or exacerbated by infection.

IL-2 signaling has a central role in the homeostasis of Treg cells in the mouse model (3). CD25 also plays a crucial role in several human autoimmune diseases in which lymphadenopathy and hepatosplenomegaly is observed, because these are associated with CD25 mutations (39). IL-2 signaling in Treg cells is required for their maintenance in vivo, although CD25-deficient Treg cells retain normal suppressive function in vitro (11). Although Dec1−/− naïve T cell expressed comparable levels of CD25 following TCR stimulation (data not shown), we observed that Dec1−/− Foxp3+ iTreg cells contain fewer cells expressing moderate level of CD25. This suggests that Foxp3+ cells expressing moderate levels of CD25 are most affected by Dec1 deficiency (data not shown). Many transcription factors are known to regulate CD25 expression, such as AP-1, NF-xB, and STAT5 (31). It is possible that other factors such as Foxp3 may have compensated for the loss of the Dec1 gene in Treg cells, because CD25 expression was not attenuated in Dec1−/− Treg cells at a young age.

IL-2R signaling also upregulates Foxp3 expression (40). STAT5 actually binds to Foxp3-CNS2, and a constitutively active STAT5 can expand Treg cells (41, 42). We also found that the percentages of Ly5.1 cells within the CD25+ population after transfer of Dec1−/− Treg cells were comparable to controls (Supplemental Fig. 8). This indirectly suggests that at least the majority of Ly5.2+ Dec1−/− Treg cells did not convert into effector T cells. It is possible that some elements representing less stable Treg cells, such as iTreg cells, could convert into effector T cells and that Dec1 also prevents this conversion via maintaining CD25 expression. This idea is based on the report that CD25 expression levels appear to be important for resistance to conversion in adoptive transfer models (43). Dec1 is highly expressed in Treg cells, and its expression is not altered in Foxp3-deficient Treg cells. Thus, CD25 expression could be compensated by factors other than Foxp3 in those cells because Foxp3-deficient cells still express CD25 (15). Therefore, we suggest that Dec1 is involved in CD25 expression in Treg cells and is required for Treg survival or expansion. In addition, it is possible that Dec1 regulates other target genes than CD25, independently of Foxp3.

Dec1 is reported to bind E47 (44); we also saw binding of Dec1 and E47 and their colocalization in vitro (our unpublished data). The E2a gene, which encodes E12 and E47, has a crucial role not only in B cell development, but also in T cell development (45). E47 regulates cellular expansion, developmental progression, and cell death through control of the expression of genes such as Cdk6, CD25, Gadd45a/b, Socs1/3, IL-7R, and so on (46). Thus we believe that there is a functional overlap between E47 and Dec1 in T cell development, because, in addition to upregulation of CD25, IL-7Rα was remarkably downregulated in Dec1 Tg thymocyte (our unpublished data). Further study is required to understand Dec1 and E2A interactions and their implications for T cell function.

A previous report showed that Runx1 interacts with Foxp3 and represses IL-2 expression or upregulates CD25 when introduced
into primary CD4+ cells (25). The same report documented that Runx1 binds to the regulatory region of the CD25 gene. A Runx1-overproducing T hybridoma cell line showed increased expression of CD25 induced by anti-CD3 Ab (47). In contrast, Runx1–Foxp3/mouse Cd4-cre mice did not manifest autoimmune disease, and Treg cells express almost normal levels of CD25 and a slightly lower level of Foxp3 (48). In addition, recent reports of Treg cell-specific Cbβ-deficient mice indicate that the Runx1–Cbfβ complex has a crucial role in the suppressive function of Treg cells and in maintaining Foxp3 expression. However, CD25 expression is not changed in Treg cell-specific Cbβ-deficient mice (49, 50). These results indicate that the Runx1 gene is dispensable for the expression of CD25. Moreover, CD25 expression is almost normal in Dec1−/− Treg cells despite a marked reduction of Runx1 binding at the Il-2ra gene locus. Dec1 could not bind to Foxp3 even in the presence of Runx1 in vitro (data not shown), suggesting that Dec1–Runx1 complexes might be distinct from Foxp3–Runx1 complexes. Therefore, Dec1−/− Treg cells do still mediate suppressive function. Taking these data together, we conclude that Dec1 and Runx1 coordinately bind to the Il-2ra gene, and this may be required only for its long-term expression. Although the contribution of Runx1 to the long-term maintenance of Treg cells is still unclear, we believe that Runx1 is also important for long-term maintenance of CD25 expression in addition to the suppressive function supported by Runx1–Foxp3 complexes. We observed the binding of Dec1 for Runx1 in COS7 cells cotransfected with Dec1 and Runx1 and the partial colocalization of them in Treg cells (Fig. 7). However, it was hard to assess the binding of Dec1 for Runx1 in Treg cells because of a very small amount of protein from those cells.

It has also been recently reported that Dec2, another member of the Dec subfamily, plays a role in feed-forward regulatory circuits during Th2 differentiation (51). This finding emphasizes the significance of transcriptional regulation by bHLH Dec subfamily members in immunological responses.

In summary, the data provide insight into how bHLH transcription factors modulate Treg development and how they act to regulate the inflammatory response particularly in mice beyond middle age.

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Disclosures

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