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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-2rα locus. Collectively, these data show how Dec1 mechanistically acts in Treg cells. The Journal of Immunology, 2010, 185: 7330–7339.

Dec1 (also known as Stra13 or Sharp2) is a basic helix-loop-helix (bHLH) transcription factor that has been reported to play a role in the circadian system (16, 17). Its expression is substantially elevated in response to different stimuli such as light pulses, growth factors, or hypoxia (16, 18, 19). Dec1 is also reported to be involved in TGF-β signaling in the circadian system and in cancer cells (20–22). Curiously, aged Dec1−/− mice develop autoimmune disease, even though their naïve CD4 T cells are normal or hyporesponsive to TCR stimulation in terms of proliferation and secretion of cytokines, such as IFN-γ (23). Therefore, we considered that a dysregulated immunosuppressive function might account for lymphoproliferative disease in older Dec1−/− mice.

In this paper, we report that Dec1 is highly expressed in Treg cells and that Dec1 deficiency causes a decrease in Treg cell number in aged mice and that a significant proportion of aged Dec1-deficient...
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 BshIII–BssHII 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-EGFP 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). C57BL/6 (B6, Ly5.2) 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 secondary reagents. FITC-, PE-, allophycocyanin-, allophycocyanin-Cy7, biotin-labeled, and purified mAbs were purchased from BD Pharmingen (San Diego, CA) (CD4, CD8α, CD3ε, CD44, CD62L, CD25, CD45R/B220, Mac-1, Gr-1, Ter-119, 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 in vivo staining. Biotinylated Abs were revealed with streptavidin-PE, -PerCP-Cy5.5, -allophycocyanin, or -allophycocyanin-Cy7. Clone 4.2G2 anti-CD25/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 sorting by staining with propidium iodide. Reanalysis of the sorted cells indicated a purity >99% for each cell population. Rabbit polyclonal Ab against Dec1 was prepared as follows. GST-DEC1 fusion protein was used for immunization for rabbit to produce an anti-Dec1 Ab. The resulting mouse Ab was affinity purified on a Sepharose-4B column. Anti-Dec1 Ab was then purified by immunoprecipitation using a mouse anti-Flag (M2; Sigma-Aldrich, St. Louis, MO).

Immunoprecipitation and Western blotting

Immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay of thymocyte with anticycatedylated 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 by 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 (6290 and 6178) 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 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 indicated Abs for 1 h and subsequently with protein G-Sepharose overnight at 4°C. Beads were washed with TEG buffer (20 mM Tris-Cl [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-Flag (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 bone 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 0.3% Triton X-100 for 10 min at room temperature. After washing with PBS, the cells were stained with anti-Dec1 Ab for 1 h at room temperature, washed and stained with Alexa Fluor 550-conjugated anti-rabbit secondary Ab. Subsequently, the cells were stained with anti-Runx1 Ab (Active Motif), which was directly-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'-GGGAATTAATACGGACGAGCAGACGG-3' (forward) and 5'-CACAAGTCTGGAAACCTCAGAGC-3' (reverse); mouse CD25, 5'-CCATTTGCTCAACCCCTTCTCCTG-3' (forward) and 5'-CCACAGGAGTCAACACACCTGTC-3' (reverse); mouse RUNX1, 5'-CTCTGTGAAAGGCTCCTCCTG-3' (forward) and 5'-CCACAGGAGTCAACACACCTGTC-3' (reverse); and mouse β-actin, 5'-TCTCAACACCCAGGCAATGTA-3' (forward) and 5'-TCTGAGGAGGCAATGACTC-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- naive 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
proliferation or IL-2 production on TCR stimulation in Dec1−/− CD4 or CD8 naïve T cells on the C57BL/6 genetic background (Supplemental Fig. 1). In addition, we observed less marked differentiation from naïve Dec1−/− CD4 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 naïve Dec1-ablated 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, Supplemental 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 hi 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 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. 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 tested examine the ability of Dec1−/− Treg cells to suppress autoimmune pathology on cotransfer with CD4+CD25−CD45RBhigh T (Teff) cells into lymphopenic Rag1−/− mice. To distinguish between Treg and Teff cells, we isolated the latter cells from Ly5.1+ B6 mice (Fig. 2B). As anticipated, Teff cells alone induced wasting 2–3 wk after transfer, but cotransfer with Dec1+/+ Treg cells prevented this. Interestingly, Dec1−/− Treg cells similarly inhibited the wasting disease in the early phase, but body weight began to decrease after 8 wk after transfer (Fig. 2C). In addition, the percentage of CD25+ and Foxp3+ Treg cells was decreased in mice into which Teff plus Dec1−/− Treg cells were cotransferred (Supplemental Fig. 8), and we especially noted for the suppression of Teff cell-mediated inflammation.

Dec1−/− mice had a normal capacity to inhibit the proliferation of irradiated splenocytes and anti-CD3 Ab (0.3 μg/ml). Cell proliferation was analyzed by CFSE dilution after 72 h. Fig. 2B shows the proportion of dividing cells. Two independent experiments produced similar results. B–D, A total of 1 × 10^5 purified CD4+CD25−CD45RBhigh 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).

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 (Supplemental 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/IIL-6/IL-23/IL-1β (Supplemental Fig. 11), indicating that reduced iTreg might not be simply caused by defects of TGF-β signaling in Dec1−/− cells.

Interestingly, exogenous IL-2 completely overcame the impairment in 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−neg 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).

Our investigations of Dec1 Tg thymocyte development, we...
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 CD25^{hi} 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-2rα 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 Tg DP cells by ChIP assays. The degree of H3 acetylation Dec1 acetylation status of histone H3 at these PRRs (PRRI–V) in

![Image](http://www.jimmunol.org/DownloadedFrom/49x187to278x534)

**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 ll2ra locus shows exons1 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 amplon in the ll2ra locus. Representative data from two independent experiments are shown.

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**FIGURE 6.** Impaired maintenance of Dec1^{−/−} Treg cells is restored in CD25 Tg mice A. A total of 2 × 10^{5} purified CD4^{+}CD8^{+} 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). Simi-

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 from 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. The Dec1 expression in the lysates was monitored by immunoblotting with an anti-HA Ab (bottom panel). 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. The Dec1 expression in the lysates was monitored by immunoblotting with an anti-HA Ab (bottom panel). 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 and 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-y-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-κB, 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, Soc1/3, 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-7Ra 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, Runx1flo/x flo cells 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 Cbfβ-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 Cbfβ-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 Runx1–Foxp3 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 inflammatory 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

The authors have no financial conflicts of interest.

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Figure S1
Cell proliferation and IL-2 production of CD4 or CD8 T cell upon TCR stimulation in Dec1−/− mice. Purified CD4+CD25−CD62Lhigh or CD8+CD62Lhigh T cells from Dec1+/+ or Dec1−/− mice were labeled with CFSE, and stimulated with plate-bound anti-CD3 plus anti-CD28Abs (5μg/ml). After 72h, cell were harvested, counted, and analyzed for CFSE dilution by flow cytometry. Concentration of IL-2 in the supernatants was determined by Cytometric Beads Assay (BD). Data represent the mean ±SD from three wells.
**Figure S2**

**Histological analysis of** *Dec1*<sup>−/−</sup> **mice with lymphadenopathy.** (upper) Morphology of Kidney and Liver. White bars represent 10mm. (lower) Representative hematoxylin-eosin (H&E)-stained sections of spleen, LN, Heart, Colon, Kidney, Liver and Lung from *Dec1*<sup>+/+</sup> and *Dec1*<sup>−/−</sup> mice are shown. Numbers represent the magnification.
Figure S3
T cell activation in Dec1−/− mice with lymphoproliferative disease. Flow cytometric analyses of CD62L, CD44, CD69 expression in CD4 T cells from 1.2-year-old Dec1−/− mice with lymophadenopathy or littermate Dec1+/+ mice are shown. Numbers beneath bracketed lines indicate percentage of positive cells.
No accumulation of CD4–CD8– (DN) T cells in peripheral lymphoid organs in 1.2-year-old $Dec1^{-/-}$ mice. Flow cytometric analysis of CD4 versus CD8 expression gated on Thy1.2+ T cells in spleen and lymph node are shown.
Figure S5
Proliferative status and bcl-2 expression of Treg cells in Ki67 expression in young or aged Dec1^+/− mice. Ki67 expression of CD4^+CD25^+ Treg cells from 12-week-old Dec1^+/+ or Dec1^−/− mice (upper). Numbers above bracketed line indicate percent Ki67 positive cells in CD4^+CD25^+ cells. The percentage of ki67^+ cells among CD25^+CD4^+ cells in littermate 6-10-month-old Dec1^+/+ or Dec1^−/− mice (middle). Representative flow cytometric analysis of bcl-2 expression in CD25^+CD4^+ cells from littermate 6-10-month-old Dec1^+/+ or Dec1^−/− mice.
Figure S6
Activated T cells in aged but not young Dec1−/− mice. Representative flow cytometric analyses of CD44, CD62L, and CD69 expression in CD4 and CD8 T cells from 12-week-old (young) and 10-month-old (aged) Dec1+/+ or Dec1−/− mice are shown. More than three independent littermates produced with similar results.
**Figure S7**

**Normal in vitro suppressive function of Dec1−/− Treg cells.** Representative FACS data in Figure 2A. The percentage of CFSE diluted cells (1-3) was defined as the proportion of dividing cells in Figure 2A.
Figure S8
CD4+CD25+ or Foxp3+ cells were reduced in mice transferred with Dec1−/− Treg cell. The percentage of CD25+ (upper) or Foxp3+ (lower) cells among CD4+ cells in mesenteric LN (MLN) and spleen (Spl) at 10 weeks after transfer in Figure 2b. ♦, p<0.01, t test.
Figure S9
The percentage of Ly5.1+ cells in CD25+, CD25–, or Foxp3+CD4+ cells in Figure 2b. The graph shows the percentage of Ly5.1 cells in Lymph node (L) or spleen (S) CD4 T cell populations.
Figure S10
Demethylation status of CpG motifs within the *foxp3* locus in CD4*+*CD25*+* Treg cells from *Dec1*+/+ and *Dec1*−/− mice. (upper) Schematic view of *foxp3* locus shows the CpG-rich region upstream from exon1, in which CpG Motifs are highly demethylated in Treg cells but methylated in non-Treg cells. Bisulfate-treated DNA from *Dec1*+/+ and *Dec1*−/− Treg cells was sequenced at this region. Methylated CpGs (closed circles) and unmethylated CpGs (open circles) are shown for representative examples of 16 alleles. Each circle represents an individual CpG motif.
Figure S11
The development of IL-17-producing CD4 T cells derived from Dec1−/− naïve CD4 T cell. Purified CD4+CD25−CD62Lhigh T cells were stimulated with anti-CD3ε antibody in the presence of irradiated B6 splenocytes and indicated cytokines for 5 days. After stimulation, cells were restimulated with PMA and ionomycin for 5 h and GolgiStop (last 2h) and were then subjected to intracellular cytokine staining for IL-17 and IFNγ. Numbers in quadrants represent the percentages.
**Figure S12**
Proliferative status of Treg cells in Ki67 expression in *Dec1 Tg* mice. CD4+CD25+ Treg cells from 12-week-old wild-type or *Dec1 Tg* mice. Numbers above bracketed line indicate percent Ki67 positive cells in CD4+CD25+ cells.
Figure S13
Enhanced expression of \textit{Dec1} mRNA in \textit{Dec1 Tg} thymocyte. Semi-quantitative RT-PCR analyses of \textit{Dec1} and \textit{\(\beta\)-actin} mRNA expression in the developmental fractions.
Figure S14
Thymocyte differentiation was affected in Dec1 Tg mouse. Representative flow cytometric analyses of CD4 versus CD8 expression on total thymocytes (upper) and of CD44 versus CD25 expression on lineage (CD4, 8, 3, B220, Mac1, Gr1, Ter119, Nk1.1)-negative thymocytes (lower) from 8-12-week-old Dec1 Tg and littermate control mice are shown. Numbers in quadrants represent the percentages. The results are representative of two independent Dec1 Tg mouse lines.
**Figure S15**

Dec1 and Runx1 were co-localized in nucleus. 293T cells were transfected with vectors encoding Flag-tagged Dec1 or HA-tagged Runx1. 24 h after transfection, cells were fixed with paraformaldehyde and permeabilized, and then stained with anti-Flag (Dec1: red) and anti-HA (Runx1: green) antibodies and Hoechist 33342 (DAPI: blue).