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Lectin-like Receptor Ly49s3 on Dendritic Cells Contributes to the Differentiation of Regulatory T Cells in the Rat Thymus

Toshiyuki Yamada,* Naoki Nanashima,*† Miki Akita,* Takeshi Shimizu,* Takuya Miura,* Daisuke Yamana,* Takeyuki Sawano,* Takuya Sakurai,‡ and Shigeki Tsuchida*

Naturally occurring regulatory T cells (nTregs) are important for immune regulation and the maintenance of self-tolerance, develop in the thymus. The Hirosaki hairless rat (HHR), derived from the Sprague–Dawley rat (SDR), was shown to have decreased peripheral lymphocyte number, small thymus, and leukocyte infiltration in its dermis. In the HHR thymus, the medulla was underdeveloped and nTreg number was decreased. Array comparative genome hybridization revealed the deletion of an NK cell lectin-like receptor gene, Ly49s3, detecting MHC class I molecules on target cells, in the chromosome 4q42 region in HHRs. The gene was expressed in thymic conventional dendritic cells (cDCs) in SDRs, but not in HHRs. When CD4+ single-positive or CD4+CD8+ thymocytes were cultured with thymic cDCs, the expression of nTreg marker genes was lower when these cells were from HHRs than from SDRs, suggesting that HHR cDCs are deficient in the ability to induce and maintain nTreg differentiation. Expression of the genes was recovered when Ly49s3 was expressed on HHR thymic cDCs. Expression levels of MHC class II genes, presumably from cDCs, were parallel to those of nTreg marker genes in mixed-cell cultures. However, in the presence of an anti-MHC class I Ab, blocking interaction between Ly49s3 and MHC class I molecules, the expression of the former genes was upregulated, whereas the latter was downregulated. These results suggest that Ly49s3 contributes to nTreg regulation along with MHC class II molecules, whose effects alone are insufficient, and loss of Ly49s3 from thymic cDCs is the reason for the nTreg deficiency in HHRs. The Journal of Immunology, 2013, 191: 000–000.

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In the current study, we sought to elucidate the immunological features of the HHR. We found that the HHR thymus is small and nTreg development is inhibited in the thymus. We demonstrated that HHR deletes the DNA region containing the Ly49s3 gene, a member of the Ly49 family of genes encoding proteins originally identified as lectin-like receptors on the surface of NK cells for the detection of MHC class I molecules on target cells. As a consequence of this DNA deletion, the expression of the Ly49s3 gene is lost in HHR thymic cDCs. We cultured HHR thymic cDCs or cDCs transduced with the viral vector of the Ly49s3 gene with CD4–single-positive (SP) or CD4+CD8–CD25+ thymocytes from the HHR thymus and obtained results suggesting that Ly49s3 is involved in the regulation of nTreg differentiation and maintenance, and the absence of Ly49s3 expression from cDCs is at least one of the reasons for the failure of nTreg development in the HHR thymus.

Materials and Methods

Animals

HHRs and SDRs were maintained in our department. They were housed in an air-conditioned room and had free access to water and food in the Institute for Animal Experiments of Hiroaki University Graduate School of Medicine (Hiroaki, Japan). MHC haplotypes were a and a for HHRs and SDRs, respectively. Blood samples were obtained at 7–12 wk of age, spleen samples at 12–13 wk of age, and thymus samples at 4–6 wk of age. This study was approved by the Animal Care and Use Committee, and was carried out in accordance with the Guidelines for Animal Experimentation, Hiroaki University.

Blood cell counting, morphological analysis, and thymus cell counting

Blood was collected using an EDTA-coated syringe from a tail vein or the heart and complete blood cell counts were performed with Cellac a (Nihon Kohden). For morphological analysis of blood cells, blood smears were prepared and stained with Wright–Giemsa solution according to standard procedures. For thymus cell counting, the thymus was cut into small pieces, digested with 2 mg/ml Collagenase D (Roche Applied Science), and cells were dispersed into a single-cell suspension. RBCs were lysed by applying a Tris-buffered ammonium chloride solution. Cell numbers were counted with a Thoma hemacytometer.

Histological analysis

Tissue samples of the thymus, lung, kidney, and skin were fixed in 10% neutral buffered formaldehyde and embedded in paraffin. Tissue sections (4 μm thick) were deparaffinized, rehydrated, and stained with H&E.

Flow cytometric analysis

A single-cell suspension of thymus cells was obtained as described above. Cell surface marker proteins were stained using FITC-conjugated mouse anti-rat CD4 (Serotec), PE-conjugated mouse anti-rat CD8α (Cedarlane Laboratories), and PE-conjugated mouse anti-rat CD25 (Cedarlane Laboratories) Abs. The Foxp3 protein in nuclei was stained using the Anti-Mouse/Rat Foxp3 Staining Set APC (eBioscience). Dead cells were stained with eFluor 450 (eBioscience). Flow cytometry was performed on a BD FACSCanto II flow cytometer (BD Biosciences).

PCR

For quantitative real-time RT-PCR, total RNA was extracted from tissues and cells with the RNaseasy Mini Kit (QIAGEN), and cDNA was synthesized using an Omniscript RT Kit (QIAGEN). cDNA was quantified by real-time PCR using IQ SYBR Green Supermix (Bio-Rad) in combination with the MiniOpticon Real-Time PCR System (Bio-Rad). The expression levels of genes were calculated using the 2–ΔΔCt method as standard. An internal control, the expression level of the Gapdh gene was used. For ordinary RT-PCR, cDNA was amplified with Ex Taq (Takara) in combination with a DNA thermal cycler (PerkinElmer Cetus). For genomic PCR, genomic DNA was extracted from livers with the DNeasy Blood & Tissue Kit (QIAGEN) and amplified with Ex Taq (Takara) in combination with the DNA thermal cycler (PerkinElmer Cetus). For routine RT-PCR and genomic PCR, products were resolved in a 2% agarose gel containing ethidium bromide. Primers used are listed in Table I.

Array comparative genome hybridization analysis

Genomic DNA was extracted from livers and sent to TaKaRa Bio for genome-wide microarray comparative genome hybridization (CGH) analysis. DNA labeling and hybridization to the Agilent Rat Genome CGH 244K microarray (Agilent Technologies) were performed according to the manufacturer’s recommendations. Data were processed using Agilent Feature Extraction (Agilent Technologies). Microarray data sets are available at the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo) under accession code GSE48825.

Isolation of thymocytes and cDCs from the thymus

A single-cell suspension of thymus cells was obtained as described above. CD4+SP thymocytes were enriched by the depletion of CD4+CD8+ (double-positive (DP)) and CD8-SP thymocytes using rat CDA8 microbeads (Miltenyi Biotec), followed by positive selection using rat CD4 magnetic microbeads (Miltenyi Biotec). CD8-SP thymocytes were enriched similarly using the two kinds of bead in the opposite order. DP thymocytes were enriched by positive selection of DP and CD8-SP thymocytes using the combination of the PE-conjugated mouse anti-rat CD8α Ab and Anti-PE Multisort Kit (Miltenyi Biotec), followed by positive selection using the CD4 microbeads. CD4+CD8–CD25+ thymocytes were enriched by the depletion of CD25+ cells using the combination of the PE-conjugated mouse anti-rat CD25 Ab and anti-PE magnetic microbeads (Miltenyi Biotec), followed by the enrichment for CD4+SP thymocytes by positive selection using the CD4 microbeads. The enrichment for cDCs in the thymus was performed by positive selection using rat anti-DC (OX62) magnetic microbeads specifically bound to cDCs (Miltenyi Biotec). The morphology and purity of isolated cDCs are shown in Supplemental Fig. 1.

Cell culture

Isolated CD4+SP thymocytes and thymic cDCs were cultured in RPMI 1640 medium supplemented with 10% FBS. For the mixed-cell culture, 2.5 × 10^5 thymic cDCs were cultured with 1 × 10^6 CD4+SP or CD4+CD8+CD25+ thymocytes in 1 ml culture medium in a 24-well tissue culture plate. For Ab inhibition experiments, 5 μg mouse anti-rat MHC class I RT1A (Sero-tek), which recognizes the nonpolymorphic region of MHC class I molecule, was added to the mixed-cell culture. In the control culture, normal mouse IgG (Santa Cruz Biotechnology) was used instead of the anti-MHC class I Ab.

Lentiviral vector construction and transduction of cDCs

Ly49s3 cDNA, having the FLAG sequence at the 3′-terminus such that the FLAG is placed at the end of the extracellular domain of the Ly49s3 protein, was synthesized (GenScript) and cloned into the pLVSin-EF1α vector (Clontech). To make a mock vector, the synthesized gene was cloned into the vector in the opposite direction. The lentiviral expression vector of FLAG-tagged Ly49s3 and mock lentiviral vector were produced using the Lenti-X Ecotropic Packaging System (Clontech) and Lenti-X 293T cells (Clontech) according to the manufacturer’s recommendations. Transduction of 2.5 × 10^5 cDCs isolated from the HHR thymus was performed using the lentivirus at ∼5 × 10^5 IFU/ml. For the mixed culture of Ly49s3-expressing cDCs and CD4+SP thymocytes, cDCs were extensively washed 24 h after transduction, cultured in normal medium for an additional 2 d, and then mixed with CD4-SP thymocytes from the HHR thymus.

Western blotting

To examine the expression of the FLAG-tagged Ly49s3 protein from the recombinant pLVSin-EF1α vector, the vector and mock vector were transfected into 293T cells with Adaptatene Transfection Reagent (QIAGEN). Total cell lysates were subjected to 12% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with a mouse anti-FLAG M2 Ab (Strata- gene) and HRP-conjugated anti-mouse IgG Ab (GE Healthcare). Signal detection was performed with an ECL system (GE Healthcare).

Fluorescence microscopy

Pilot fluorescence microscopic observations were performed to confirm the expression of the FLAG-tagged Ly49s3 protein on the surface of cDCs. HHR thymic cDCs were transduced with the lentiviral expression vector of FLAG-tagged Ly49s3 and mock lentiviral vector, and, three days later, were

(14, 15). In HHRs, we demonstrated a deletion of 80 kb of genomic DNA containing five basic keratin genes as the reason for the hairless phenotype (16). However, it remains to be determined whether the HHR possesses other genetic abnormalities or exhibits deficiencies in its immune system.
stained with the anti-FLAG M2 Ab and an AlexaFluor 546 anti-mouse IgG Ab (Invitrogen).

Statistical analysis
Experiments for which a statistical analysis is indicated were performed independently at least three times. Data are presented as the mean and SD. Statistical evaluations were performed using the Student t test. Differences were considered significant with p < 0.05.

Results
Decreased number of lymphocytes in HHR peripheral blood
An analysis of peripheral blood showed that, although the RBC counts, hematocrit levels, and hemoglobin concentrations were similar between HHRs and SDRs, WBC counts were lower in HHRs (Fig. 1A). Morphological analysis of leukocytes showed a substantial decrease in the number of lymphocytes in HHRs (Fig. 1B), suggesting that the low WBC count in HHR peripheral blood was due to a decrease in lymphocytes.

Impaired differentiation of CD4-positive T cells in the HHR thymus
The above feature of peripheral blood suggested deficiencies of lymphoid tissue(s) in HHRs. We then checked their spleens and thymi and found that, although no remarkable differences were noted in weight and appearance between spleens from age- (12–13 wk) and sex-matched HHRs and SDRs (data not shown), HHR thymi were markedly smaller and had significantly lower weights and cellularities than did age- (4–6 wk) and sex-matched SDR thymi (Fig. 2A). In the histological analysis, the HHR thymus showed small lobes with underdeveloped medullae (Fig. 2B). These characteristics raised the possibility that T cell differentiation may be disturbed in the HHR thymus. To examine this issue, we carried out the following investigations with male rats 4–6 wk of age. We first performed flow cytometric analyses (Fig. 2C), which indicated that, although the proportions of CD4+CD8− [double-negative (DN)], CD4+CD8+ (DP), CD4+CD8− (CD4-SP), and CD4−CD8+ (CD8-SP) thymocytes in the HHR thymus were not significantly different from those in the SDR thymus (Fig. 2C, graph), the population of DP thymocytes was shifted to the lower CD4 levels in the HHR thymus (Fig. 2C, arrow in the diagram). Real-time RT-PCR analysis using RNAs from whole thymi confirmed lower levels of CD4 expression, but not CD8 expression, in the HHR thymus (Fig. 2D, Table I). These results suggest that the differentiation of CD4-positive thymocytes is impaired in the HHR thymus.

Developmental failure of nTregs in the HHR thymus
In contrast to CD4+ helper T cells and induced regulatory T cells (iTregs), another type of regulatory T cell, both of which mature in secondary lymphoid organs, nTregs mature in the thymus. Therefore, we focused on the differentiation status of nTregs in the HHR thymus. Real-time RT-PCR analysis showed that expression levels of the two important genes for nTregs, Cd25 and Foxp3, were decreased in CD4-SP thymocytes from the HHR thymus (Fig. 3A). Flow cytometric analysis showed that the proportion of CD4+CD25+ cells in the HHR thymus was lower than that in the SDR thymus (Fig. 3B, upper) and the proportion of Foxp3+ cells in the CD4+CD25+ cell fraction was also lower in the HHR thymus (Fig. 3B, lower). The proportion and number of CD4+CD25+Foxp3+ nTregs in the SDR and HHR thymi were calculated using the values obtained from flow cytometric analysis shown in Fig. 3B and those of total thymus cell number shown in Fig. 2A. The estimated proportion (Fig. 3C, left) and number (Fig. 3C, right) of nTregs in the HHR thymus were markedly lower than those in the SDR thymus, suggesting that nTreg development is impaired in the HHR thymus.

Leukocyte infiltration in the HHR dermis
The nTregs play fundamental roles in the maintenance of self-tolerance, and their depletion could lead to autoimmune diseases (1). To examine whether HHRs exhibit features of autoimmune diseases, we performed histological analysis of the lung, kidney, and skin. As shown in Fig. 4, leukocyte infiltration, a symptom of autoimmune diseases, was observed in the HHR dermis, suggesting that reductions in nTreg numbers may elicit inflammatory diseases in HHRs. No obvious abnormalities were observed in other tissues (data not shown).

Loss of Ly49s3 gene expression in HHR thymic cDCs
To identify the gene(s) responsible for the failure of nTreg differentiation, we next performed microarray CGH analysis using genomic DNA from SDR and HHR livers. This analysis revealed a deletion in the q42 region on HHR chromosome 4. This region encompasses four Ly49 family genes: Ly49a4, Ly49i4, Ly49s3, and Ly49i3 (Fig. 5A, left). Genomic PCR confirmed the deletion of DNA in this region in HHRs (Fig. 5A, right). To determine whether Ly49 family genes deleted in HHRs are expressed in a normal thymus, we performed RT-PCR analysis using the SDR thymus. Of the four genes, the Ly49s3 gene, but not the others, was expressed in the SDR thymus (Fig. 5B, left, and data not shown). Further
analysis using thymocytes and cDCs isolated from the SDR thymus revealed that the Ly49s3 gene was expressed in cDCs, but not in DP, CD4-SP, and CD8-SP thymocytes (Fig. 5B, middle and right). The process of identification of the gene expressed in the SDR thymus as Ly49s3 is described in Supplemental Fig. 2.

Ly49 family proteins were originally identified as lectin-like receptors on the NK cell surface for the detection of MHC class I molecules on target cells. On encountering MHC class I molecules, they transmit inhibitory signals to prevent NK cells from mediating cytotoxicity (17). At present, it is known that some members of the Ly49 family transmit activating, instead of inhibitory, signals and some are expressed on T cells and DCs and perform distinct functions (18–22). On the basis of the structural characteristics of Ly49s3—that is, the presence of a positively charged arginine residue in the transmembrane domain for association with adaptor proteins containing immunoreceptor tyrosine-based activating motifs—it has been predicted to be an activating receptor. Given that thymic DCs have been reported to play important roles in nTreg differentiation in humans, we reasoned that the developmental failure of nTregs in the HHRs thymus is due, at least in part, to the loss of Ly49s3 gene expression in cDCs. To assess this hypothesis, the following experiments were carried out.

### FIGURE 2: Characteristics of the HHR thymus and differentiation status of thymocytes in the thymus. (A) Graphs show the weights and cellularities of SDR and HHR thymi at 4–5 wk of age. Three SDRs and HHRs were used for each analysis. Data are shown as mean ± SD. *p < 0.05. (B) Histological sections of thymi from 4-wk-old SDRs and HHRs were stained with H&E. Scale bars represent 1 mm. Representative results of two SDRs and HHRs are shown. (C) The differentiation status of thymocytes was analyzed with a flow cytometer. Representative results of three SDRs and HHRs are shown. An arrow indicates a population of DP thymocytes with decreased CD4 levels. Averaged values from three independent flow cytometric analyses for the proportions of DP, CD4-SP, CD8-SP, and DN thymocytes are shown in the lower graph. Data are shown as mean ± SD. (D) Expression levels of Cd4 and Cd8 genes in the thymus were analyzed by real-time RT-PCR. Three SDRs and HHRs were used. The level of each gene is shown relative to the value for the SDR, which is set at 1. Data are shown as mean ± SD. *p < 0.05.

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Deficiency in the induction of nTreg differentiation in HHR thymic cDCs

To examine the ability of HHR thymic cDCs to govern the differentiation of nTregs, cDCs and CD4-SP thymocytes were isolated from SDR or HHR thymi and were cocultured, and the expression levels of the genes Foxp3, Cd25, Ctla4, and Pd-1, important for the differentiation and/or function of nTregs, were determined. Real-time RT-PCR analysis showed lower expression of these genes in HHR CD4-SP thymocytes cultured with HHR cDCs than in SDR CD4-SP thymocytes cultured with SDR cDCs (Fig. 6A; see “nTreg markers”). Flow cytometric analyses of Foxp3 and CD25 also showed that expression levels of these proteins were lower when the cells were from the HHR thymus (Fig. 6B). The lower expression of the nTreg marker genes in HHR CD4-SP thymocytes seemed to be attributable to HHR cDCs, rather than the intrinsic characteristics of HHR CD4-SP thymocytes, because the expression of the genes was not downregulated in HHR CD4-SP thymocytes when cultured alone, relative to that in SDR CD4-SP thymocytes (Fig. 6C; see “nTreg markers”). The combined presentation of these results (Fig. 6D) indicates that these genes were markedly induced in SDR CD4-SP thymocytes cultured with SDR cDCs, but not in HHR. These results suggest that the ability of HHR thymic cDCs to induce nTreg differentiation in CD4-SP thymocytes is impaired. However, the possibility that thymic cDCs encourage only the expansion and/or survival of mature nTregs existing in the CD4-SP thymocyte fraction cannot be ruled out.

To examine this, we next isolated CD4+CD8<sup>+</sup>CD25<sup>+</sup> thymocytes from the thymus and cultured them with thymic cDCs. As shown in Fig. 6E, expression levels of the Foxp3, Cd25, and Pd-1 genes were still lower in HHR CD4<sup>+</sup>CD8<sup>+</sup>CD25<sup>+</sup> thymocytes cultured with HHR cDCs, but the difference was not so remarkable as that seen when CD4-SP thymocytes were cultured with cDCs (Fig. 6A), and no difference was observed in the expression level of Ctla4 gene between SDR and HHR CD4<sup>+</sup>CD8<sup>+</sup>CD25<sup>+</sup> thymocytes. All this information suggests that thymic cDCs possess abilities to induce differentiation and regulate expansion and/or survival of nTregs, and both of them are impaired in HHR thymic cDCs.

Recovery of the ability to induce nTreg differentiation in HHR thymic cDCs after introduction of the Ly49s3 gene

The results so far described suggest that Ly49s3 on thymic cDCs is required for regulation of the differentiation and maintenance of...
nTregs. Therefore, we next addressed whether enforced expression of the Ly49s3 gene in HHR thymic cDCs recovers their ability to induce nTreg differentiation. To this end, HHR thymic cDCs were transduced with the lentiviral vector of the FLAG-tagged Ly49s3 gene and were cultured with HHR CD4-SP thymocytes. We used HHR CD4-SP rather than CD4+CD8+ thymocytes because the former includes the latter and the induction rate of the nTreg marker genes was expected to be higher in the former. Expression of the Ly49s3 protein from the recombinant vector was confirmed, before packaging into the virus, by Western blot analysis using the cell lysate of 293T cells transfected with the vector (Fig. 7B). The reason for the appearance of several bands is unknown. They may have been splice variants of the Ly49s3 protein, as reported in the human lectin-like protein DCAL-1 (23). Proper expression of the FLAG-tagged Ly49s3 protein on the surface of HHR thymic cDCs transduced with the viral vector of the protein, but not on cells transduced with the mock vector, was confirmed by fluorescence microscopic observations (Fig. 7C, and data not shown). Real-time RT-PCR analysis showed a higher abundance of Foxp3, Cd25, Cita4, and Pd-1 mRNAs in HHR CD4-SP thymocytes cultured with HHR thymic cDCs transduced with the expression vector of the Ly49s3 gene than that in thymocytes cultured with cDCs transduced with the mock vector (Fig. 7D; see “nTreg markers”), suggesting that Ly49s3 plays important roles in thymic cDCs for the induction of nTreg differentiation.

Given that Ly49 family proteins were originally identified as NK cell receptors for the recognition of MHC class I molecules on target cells (17), it is speculated that Ly49s3 on thymic cDCs interacts with MHC class I molecules on CD4-SP thymocytes during the induction process of nTreg differentiation. To examine this possibility, the anti-MHC class I Ab was added to the mixed-cell culture to interfere with the interaction between Ly49s3 and MHC class I molecules. By the addition of this Ab, the effects of enforced expression of Ly49s3 on nTreg differentiation were abrogated (Fig. 7E, 7F; see “nTreg markers”), suggesting that Ly49s3 interacts with MHC class I molecules on CD4-SP cells and this interaction is crucial for thymic cDCs to induce nTreg differentiation.

Involvement of MHC class II molecules in induction of nTreg differentiation by thymic cDCs

To identify the molecules involved in the regulation of nTreg differentiation and maintenance along with Ly49s3, we determined the expression levels of MHC class II Rtl1-Ba and Rtl1-BB, Cd80, Cd86, Cd40, Cd40l, and Jagged1 genes in mixed cultures of cDCs and CD4-SP thymocytes. Of these genes, the expression levels of MHC class II genes correlated well with those of nTreg marker genes; the expression of MHC class II genes was lower in mixed cultures of HHR cells than in those of SDR cells (Fig. 6A; see “MHC II”). The other genes did not correlate with nTreg marker genes (data not shown). We thought that the expression of MHC class II genes in mixed-cell cultures mainly originated from cDCs, although the possibility could not be totally ruled out that rat T cells expressed these genes after activation (24). However, the expression of these genes was hardly detected in SDR and HHR CD4-SP thymocytes cultured alone (data not shown) and, as described below, in experiments with the anti-MHC class I Ab, the expression patterns of MHC class II genes and those of nTreg were not parallel. These findings excluded such a possibility.

In the mixed culture of Ly49s3-transduced HHR cDCs and HHR CD4-SP thymocytes, the expression of MHC class II genes as well as nTreg marker genes was upregulated (Fig. 7D; see “MHC II”). However, in the presence of the anti-MHC class I Ab, the expression of MHC class II genes was further upregulated (Fig. 7E, 7F; see “MHC II”), whereas the expression of nTreg marker genes was downregulated. Thus, it is suggested that, although MHC class II molecules are upregulated after the introduction of Ly49s3 in cDCs, they cannot induce nTreg differentiation without the interaction between Ly49s3 and MHC class I molecules.

Discussion

The present study demonstrated that nTreg development is impaired in the HHR thymus, and this is presumably due to the loss of
Ly49s3 expression from thymic cDCs as a consequence of the deletion of this gene. This study also suggested that Ly49s3 expressed on cDCs interacts with MHC class I molecules on CD4-SP thymocytes and governs the differentiation and maintenance of nTregs in cooperation with MHC class II molecules.

Given that Ly49 family members are cell surface receptors involved in cell–cell interactions, it is likely that an interaction failure between cDCs and CD4-SP thymocytes leads to abrogation of the ability of cDCs to regulate the differentiation and maintenance of nTregs. Indeed, the expression of nTreg marker genes

![Image of Figure 6](https://example.com/image.png)

**FIGURE 6.** Expression of the nTreg marker and MHC class II genes in the mixed-cell culture. (A) A total of $1 \times 10^6$ CD4-SP thymocytes isolated from the SDR thymus were cultured with $2.5 \times 10^5$ cDCs from the SDR thymus, and $1 \times 10^6$ CD4-SP thymocytes from the HHR thymus were cultured with $2.5 \times 10^5$ cDCs from the HHR thymus. At 3 d later, total RNA was extracted and expression levels of nTreg marker genes Foxp3, Cd25, CIta4, and Pd-1 and MHC class II genes R11-Ba and R11-Bb were determined by real-time RT-PCR. Three independent experiments were performed for each mixed-cell culture. The level of each gene is shown relative to the value for the mixed culture of cells from the SDR thymus, which is set at 1. Data are shown as mean ± SD. *p < 0.05. (B) To confirm the expression levels of Foxp3 and Cd25, an additional mixed-cell culture experiment was performed, and the proportion of Foxp3+ or Cd25+ cells was determined by flow cytometric analysis. (C) As control experiments, $1 \times 10^6$ CD4-SP thymocytes were cultured alone for 3 d and the same real-time RT-PCR analyses as above were performed. Three independent experiments were performed for each culture. The level of each gene is shown relative to the value for SDR cells, which is set at 1. Data are shown as mean ± SD. *p < 0.05. (D) Effects of cDCs on nTreg marker gene expression. The expression levels of nTreg marker genes in mixed-cell (A) and control cultures (C) are shown in the same graph relative to the value for the SDR control culture, which is set at 1. Statistical analysis was performed between the induction ratios of gene expression. Data are shown as mean ± SD. *p < 0.05. (E) A total of $1 \times 10^6$ CD4+CD8−CD25−thymocytes isolated from SDR and HHR thymi were cultured with $2.5 \times 10^5$ cDCs isolated from SDR and HHR thymi, respectively. At 3 d later, total RNA was extracted and expression levels of nTreg marker genes Foxp3, Cd25, CIta4, and Pd-1 were determined by real-time RT-PCR. Three independent experiments were performed for each mixed-cell culture. The level of each gene is shown relative to the value for the mixed culture of cells from the SDR thymus, which is set at 1. Data are shown as mean ± SD. *p < 0.05.
FIGURE 7. Expression of the nTreg marker and MHC class II genes in the mixed-cell culture using Ly49s3-expressing HHR thymic cDCs. (A) The FLAG-tagged Ly49s3 structure is schematically represented. R: arginine residue. (B) Left, 293T cells were transfected with recombinant vectors before being packaged into the virus, and cell lysates were subjected to Western blot analysis with the anti-FLAG M2 Ab. Right, The proteins on the membrane were stained with fast green. (C) Left, cDCs from the HHR thymus were transduced with the lentiviral vector of FLAG-tagged Ly49s3, and the expression of the fusion protein on the surface of cDCs was confirmed by fluorescence microscopic observations with the anti-FLAG M2 Ab. Right, Phase contrast appearance of the identical cells shown in the left photographs. Note dendrites on the surface of the cells. The cells were suspended in buffer and all the photos were taken. Original magnification ×800. (D) A total of 2.5 × 10⁵ cDCs isolated from the HHR thymus were transduced with the lentiviral vector of FLAG-tagged Ly49s3 (Ly) or the mock vector (Mo) and then mixed with 1 × 10⁶ CD4-SP thymocytes isolated from the HHR thymus. At 3 d later, total RNA was extracted and the expression levels of nTreg marker genes Foxp3, Cd25, Ctl4a, and Pd-1 and MHC class II genes R1t-Ba and R1t-Bb were determined by real-time RT-PCR. Three independent experiments were performed for each culture. The level of each gene is shown relative to the value for the mixed culture, using cDCs transduced with the mock vector, which is set at 1. Data are shown as mean ± SD. *p < 0.05. (E) A total of 2.5 × 10⁵ cDCs from the HHR thymus were transduced with the lentiviral vector of FLAG-tagged Ly49s3 and then mixed with 1 × 10⁶ CD4-SP thymocytes from the HHR thymus. Next 5 μg of anti-rat MHC class I Ab or normal IgG was added to the culture. At 3 d later, total RNA was extracted, and the expression levels of nTreg marker genes and MHC class II genes were determined by real-time RT-PCR. Three independent experiments were performed for each culture. The level of each gene is shown relative to the value for the mixed culture in the presence of normal IgG, which is set at 1. Data are shown as mean ± SD. *p < 0.05. (F) Summary of the experiments performed using the lentiviral vector of the Ly49s3 gene and anti-MHC class I Ab. The level of each gene is shown relative to the value for mock-transduced cells, which is set at 1. Data are shown as mean ± SD. *p < 0.05.
was recovered in HHR CD4-SP thymocytes cultured with Ly49s3-introduced HHR thymic cDCs. In the presence of the anti-MHC class I Ab, however, the recovered expression of nTreg marker genes was downregulated again, suggesting that the encounter of Ly49s3 on cDCs with MHC class I molecules on CD4-SP thymocytes is crucial for cDCs to execute their functions.

Because the anti-MHC class I Ab used recognizes the non-polymorphic region of the MHC class I molecule, Ly49s3 was suggested to recognize the nonpolymorphic region, but not the polymorphic region and/or peptide Ags. As mentioned above, MHC haplotypes are u and a for HHRs and SDRs, respectively. It has been reported by Naper et al. (25) that Ly49s3 expressed on NK cells reacts with a relatively broad array of MHC haplotypes, but not with the u haplotype. It is, therefore, reasonable that, in SDR, Ly49s3 on cDCs interacts with an MHC class I molecule of the a haplotype on CD4-SP thymocytes and is involved in the regulation of nTreg differentiation. Considering the upregulation of nTreg marker genes in the mixed culture of HHR cDCs expressing Ly49s3, it likely interacted with an MHC class I molecule of the u haplotype in this setting. As shown in Fig. 7B, several bands, supposed to be splice variants, appeared in the Western blot analysis of the lysate of 293T cells transfected with the expression vector of the Ly49s3 gene. It has been reported that Ly49s3 forms a homodimer (25). If all these points are considered together, it is possible to speculate that the splice variants are translated in cDCs and form dimers between them, and some types of dimers interact with MHC class I molecules of the u haplotype. The characteristics of Ly49s3 on cDCs may be different from those on NK cells. From the time when HHR appeared from SDR in 1985, HHRs and SDRs have been maintained separately for almost 30 y. It is thought that, during the period of this long and closed crossing, basic keratin genes lost in HHRs seem not to be responsible for the hairless phenotype (16). The similarity of loci for basic keratin genes in mice and humans, hLY49L, has been identified so far (27). In humans, other lectin-like receptors may replace the function of Ly49 family proteins. In this regard, DECTIN-1 and CLEC-1, expressed in DCs, have been demonstrated to be involved in the fate determination of T cells between Tregs and Th17 cells (28–30). Lectin-like receptors seem to be more widely involved in the regulation of DC functions than was previously supposed.

We have demonstrated the deletion of 80 kb of genomic DNA containing five basic keratin genes in HHRs as the reason for their hairless phenotype (16). The similarity of loci for basic keratin genes and Ly49 genes involves the clustering of genes showing sequence homologies. Common mechanisms, such as nonallelic homologous recombination, could occur in two independent loci and lead to the hair loss and nTreg deficiency in HHRs. The five basic keratin genes lost in HHRs seem not to be responsible for the nTreg deficiency because they were not expressed in the SDR thymus and, therefore, were suggested not to correlate with nTreg development (data not shown).

The HHR thymus was small with an underdeveloped medulla and relatively normally developed cortex. DP thymocytes localized in the cortex differentiate into CD4-SP or CD8-SP thymocytes and concurrently relocate to the medulla where DCs are present (31–33). It is, therefore, natural to speculate that a correlation may exist between medullary defects and developmental deficiencies of nTregs in HHRs, although it is not known which is the cause of the other. Other possible reasons for the small thymus have also been suggested, including retardation of thymic development or acceleration of its involution. The latter seems unlikely because replacement with adipose tissue was not observed in the HHR thymus at the age used in this study and no accumulation of apoptotic cells was observed in the TUNEL assay (data not shown).

Real-time RT-PCR analyses showed that the expression level of the Cd4 gene was decreased in the HHR thymus. Flow cytometric analysis revealed that CD4 levels were decreased in a considerable number of DP thymocytes, but not in CD4-SP thymocytes. This may be another possible reason for the developmental failure of
nTregs in the HHR thymus besides the lack of Ly49s3 expression in cDCs, because a line of studies have suggested that nTreg commitment may take place at the DP stage of thymocytes in the thymic cortex (34–37). For example, Tai et al. (34) reported that simultaneous stimulation of TCR and CD28 signals in murine DP thymocytes induced Foxp3 expression, subsequently leading to Treg differentiation, and Ribot et al. (36) reported that cortical positive selection promoted the survival of Treg precursors. Therefore, it may be possible that HHR DP thymocytes with low levels of CD4 are unable to induce the differentiation and/or survival of nTregs. In addition to the deficiency in cDCs, intrinsic problems in thymocytes induced Foxp3 expression, subsequently leading to Treg failure in HHR, although the impaired expression of nTreg marker genes was not observed in HHR CD4-SP thymocytes when cultured alone.

The number of lymphocytes was markedly decreased in HHR peripheral blood. Considering the population size of Tregs (6–10%) in circulating CD4+ T cells in rodents (38), it is obvious that the decrease in nTreg number is not solely responsible for the reduction in lymphocyte numbers. Given that the significantly lower cellularity in the HHR thymus, together with the similarity in the proportion of DN, DP, CD4-SP, and CD8-SP thymocytes between SDR and HHR thymi, it is suggested that the absolute number of all of these cells is decreased in the HHR thymus. The number of not only nTregs but also conventional CD4+ T cells and/or CD8+ T cells may be decreased in HHR. Investigations are now being performed in our laboratory to assess whether HHR exhibits abnormalities in other T cell subsets.

Leukocyte infiltration, possibly owing to a disturbance in self-tolerance, was observed in the HHR dermis. The elimination of nTregs also leads to the activation of effector T cells recognizing autologous tumor Ags. On this matter, we have reported the resistance of HHRs to the development of hepatic preneoplastic lesions induced by the peroxisome proliferator clofibrate (39, 40). It seems that the depletion of nTregs results in the elicitation of effective immune responses to tumors as well as autoimmune diseases in HHRs.

In summary, during investigation of the immunological features of HHRs, we found a new functional role for the Ly49 family of lectin-like receptors expressed in thymic cDCs in the regulation of HHRs, we found a new functional role for the Ly49 family of lectin-like receptors expressed in thymic cDCs in the regulation of HHRs, we found a new functional role for the Ly49 family of lectin-like receptors expressed in thymic cDCs in the regulation of HHRs, we found a new functional role for the Ly49 family of lectin-like receptors expressed in thymic cDCs in the regulation of HHRs, and Ribot et al. (36) reported that cortical positive selection promoted the survival of Treg precursors. There-


(A) Primer 1: common 5’ primer for amplification of the 
Ly49s3 and Ly49s4 genes
Primer 1: 5’-CCT TTC CCT TCG ACT GGT TG
Ly49s3: 5’-CCT TTC CCT TCG ACT GGT TG
Ly49s4: 5’-CCT TTC CCT TCG ACT GGT TG
Ly49i3: 5’-CTG TTC AGT TCT AAT GGT AA
Ly49i4: 5’-CTG TTC AGT TCT AAT GGT AA

Primer 2: common 5’ primer for amplification of the 
Ly49i3 and Ly49i4 genes
Primer 2: 5’-CTG TTC CGT TCT GAT GGT AA
Ly49s3: 5’-CTG TTC CGT TCT GAT GGT AA
Ly49s4: 5’-CTG TTC CGT TCT GAT GGT AA
Ly49i3: 5’-CTG TTC AGT TCT GAT GGT AA
Ly49i4: 5’-CTG TTC CGT TCT GAT GGT AA

Primer 3: common 3’ primer for amplification of the 
Ly49s3, Ly49s4, Ly49i3, and Ly49i4 genes
Primer 3: 5’-AAT GGG GCA GGG ATT TGG TC
Ly49s3: 5’-AAT GGG GCA GGG ATT TGG TC
Ly49s4: 5’-AAT GGG GCA GGG ATT TGG TC
Ly49i3: 5’-AAT GGG GCA GGG ATT TGG TC
Ly49i4: 5’-AAT GGG GCA GGG ATT TGG TC

(B) RT-PCR

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Primers 1 + 3

Primers 2 + 3

Gapdh

(C) Primer 4: 5’ primer for amplification of the Ly49s3 gene
Primer 4: 5’-CAG CTC ACT GTG ATT GCT AC
Ly49s3: 5’-CAG CTC ACT GTG ATT GCT AC
Ly49s4: 5’-CAG CTC ACT GTG ATT GGT AC

Primer 5: 5’ primer for amplification of the Ly49s4 gene
Primer 5: 5’-CAG CTC ACT GTG ATT GGT AT
Ly49s3: 5’-CAG CTC ACT GTG ATT GGT AT
Ly49s4: 5’-CAG CTC ACT GTG ATT GGT AT

(D) RT-PCR

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Primers 4 + 3

Primers 5 + 3

Gapdh

Supplemental Figure 2
Supplemental Figure 1. Morphology of thymic cDCs.

Thymic cDCs were isolated from the SDR thymus using anti-DC (OX62) magnetic microbeads and cultured for three days. About 90% of cells attached to the surface of the culture dish and many of them exhibited DC morphology with dendrites.

Supplemental Figure 2. Identification of the gene(s) expressed in the normal thymus.

Since the four genes deleted in HHR, Ly49s3, Ly49s4, Ly49i3, and Ly49i4, are similar to each other, a series of RT-PCR using five primers to distinguish each gene was performed.

(A) Primer 1, a common 5’ primer for amplification of the Ly49s3 and Ly49s4 genes, and Primer 2, a common 5’ primer for amplification of the Ly49i3 and Ly49i4 genes, were set at identical regions of the four genes showing several nucleotide differences. The sequence of the 3’ terminal of Primer 1 was identical to those of the Ly49s3 and Ly49s4 genes, while that of Primer 2 was identical to those of the Ly49i3 and Ly49i4 genes. Primer 3, a common 3’ primer for amplification of all the genes, was set at identical regions of the four genes showing little nucleotide difference. The sequence of the 3’ terminal of Primer 3 was identical to the sequences of all the genes. Nucleotides of the genes not identical to those of the corresponding primers are underlined. (B) Total RNA was extracted from the thymus and thymic cDCs of SDRs and HHRs, and RT-PCR analysis was performed using Primer 1 and Primer 3 or Primer 2 and Primer 3. As shown in the figure, the former primer set amplified DNA fragments in the SDR
thymus and SDR thymic cDCs, while the latter did not, indicating that the Ly49s3 and/or Ly49s4 genes, but not the Ly49i3 and Ly49i4 genes, are expressed in the normal thymus. (C) To identify the gene(s) expressed in the normal thymus, Primer 4, a 5’ primer for amplification of the Ly49s3 gene, and Primer 5, a 5’ primer for amplification of the Ly49s4 gene, were set at identical regions of the genes showing several nucleotide differences. The sequence of Primer 4 was identical to that of the Ly49s3 gene while that of Primer 5 was identical to that of the Ly49s4 gene. The sequence of the 3’ terminal of each primer was not identical to that of the opposite genes (underline). (D) RT-PCR analysis was performed using Primers 4 and 3 or Primers 5 and 3. As shown in the figure, the former primer set amplified DNA fragments in the SDR thymus and SDR thymic cDCs, while the latter did not. Taken together, it was concluded that the gene expressed in the normal thymus is Ly49s3.