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

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Naturally occurring regulatory T cells (nTregs) are crucial for the regulation of immune responses and maintenance of self-tolerance (1–3). nTregs developed in the thymus are known to be capable of presenting self-peptide/MHC complexes to CD4+ thymocytes through their TCRs with self-peptide. APCs, thymic epithelial cells are known to be capable of presenting MHC complexes presenting on thymic APCs (1–3). As one of the transcription factor for nTreg differentiation (4), in their nuclei. The online version of this article contains supplemental material.

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The online version of this article contains supplemental material.

Abbreviations used in this article: cDC, conventional dendritic cell; CGH, comparative genome hybridization; DC, dendritic cell; DN, double-negative; DP, double-positive; HHR, Hirosaki hairless rat; iTreg, induced regulatory T cell; nTreg, naturally occurring regulatory T cell; pDC, plasmacytoid dendritic cell; SDR, Sprague-Dawley rat; SP, single-positive; TSLP, thymic stromal lymphopoietin.

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A wide variety of tissue-specific self Ags on their MHC class II molecules, possibly through mechanisms governed by transcription factors such as AIRE (5). nTreg precursors with high avidity for self-peptide/MHC complexes are thought to be selected for maturation to generate an nTreg repertoire.

Evidence has established that thymic dendritic cells (DCs), another type of thymic APC, also contribute to the nTreg generation process (6–8). For example, in humans, conventional DCs (cDCs) activated by thymic stromal lymphopoietin (TSLP) from epithelial cells of Hassall’s corpuscles have been reported to induce the differentiation of CD4+CD25+ T cells within the thymic medulla (6). More recently, TSLP activated another main subset of DCs, plasmacytoid DCs (pDCs), which have also been demonstrated to mediate Treg generation from CD4+CD25+ T cells in the medulla of the human thymus (9, 10). After being activated with TSLP, DCs upregulate the expression of MHC class II molecules and costimulatory molecules, such as CD80 and CD86 (11, 12), enhancing signaling events between DCs and T cells.

However, the process of nTreg development in rats is not fully understood. For example, it remains obscure whether thymic DCs contribute to the developmental process and what kind of costimulatory molecules are involved. In our laboratory, we maintain a mutant strain of rats, the Hirosaki hairless rat (HHR), which was spontaneously derived from the Sprague–Dawley rat (SDR) in 1985, with a nearly bare phenotype that is inherited in an autosomal recessive manner (13). Well-known examples of hairless animals, the nude mouse and rat, are also characterized by an abnormal immune system devoid of T cells owing to a deficiency in thymic development (14, 15). In these animals, mutations in the Foxn1 gene are responsible for both the immunodeficiency and the hairless phenotype because Foxn1 is a critical transcription factor for the differentiation and survival of both thymic and skin epithelial cells.
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 the 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 Hirosaki University Graduate School of Medicine (Hirosaki, 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, Hirosaki 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 Cellica 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 were resolved in a 2% agarose gel containing ethidium bromide. 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 pLV SIN-EBI 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 used for transduction of thymocytes in 1 ml culture medium in 24-well tissue culture plates. For Ab inhibition experiments, 5 µg mouse anti-mHC class I RT1a Ab (Serotec), and 5 µg mouse anti-mHC class II RT1b Ab (BD Pharmingen), were 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 pLV SIN-EBI 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 a 5 × 10^4 IU/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 pLV SIN-EBI vector, the vector and mock vector were transduced into 293T cells with Advartec 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

Ly49s3 RECEPTOR AND nTREG DIFFERENTIATION IN RAT THYMUS
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), graph), the population of DP thymocytes was shifted to the lower quadrant (Fig. 2D, Table I). These results suggest that the low WBC count in HHR peripheral blood was due to a decrease in lymphocytes.

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 \textit{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 \textit{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 \textit{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 \textit{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 distribution 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 \textit{Cd4} and \textit{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.

### Table I. PCR primer sequences

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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 marked 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−CD25−Foxp3+ nTregs in the thymus were calculated using the values of the proportion of CD4+CD25+ cells in the thymus and those of Foxp3+ cells in the CD4+CD25+ cell fraction obtained from flow cytometric analysis and are shown in the graph. Data are shown as mean ± SD. *p < 0.05.

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 nTreg cells in the HHR thymus. (A) Expression levels of Cd25 and Foxp3 genes in CD4-SP thymocytes 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. (B) Upper. The proportion of CD4+CD25+ cells in the thymus was determined by flow cytometric analysis. Representative results of three SDRs and HHRs are shown. Averaged values from three independent analyses for the proportion of CD4+CD25+ cells are shown in the graph. Data are shown as mean ± SD. Lower. The proportion of Foxp3+ cells in the CD4+CD25+ cell fraction was determined by flow cytometric analysis. Representative results of three SDRs and HHRs are shown. Averaged values from three independent analyses for the proportion of Foxp3+ cells in the CD4+CD25+ cell fraction are shown in the graph. Data are shown as mean ± SD. *p < 0.05. (C) Upper. Estimated values for the proportion of CD4+CD25+Foxp3− nTregs in the thymus were calculated using the values of the proportion of CD4+CD25+ cells in the thymus and those of Foxp3+ cells in the CD4+CD25+ cell fraction obtained from flow cytometric analysis and are shown in the graph. Data are shown as mean ± SD. *p < 0.05. Lower. Estimated values for the absolute number of CD4+CD25+Foxp3+ nTregs in the thymus were calculated using the values of the proportion of CD4+CD25+ cells in the thymus and those of total thymus cell number (Fig. 2A) and are shown in the graph. Data are shown as mean ± SD. *p < 0.05.
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 (Fig. 7A) 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, Cilad, 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 Rt1-Ba and Rt1-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 thymic cDCs and CD4-SP 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 genes 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

**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, Ctla4, and Pd-1 and MHC class II genes Rt1-Bα and Rt1-Bβ 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-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, Ctla4, 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 × 10^5 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 × 10^6 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, Ctlax4, and Pd-1 and MHC class II genes Rt1-Bα and Rt1-Bβ 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 × 10^5 cDCs from the HHR thymus were transduced with the lentiviral vector of FLAG-tagged Ly49s3 and then mixed with 1 × 10^6 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 non-polymorphic 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 \( u \) 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 years. It is thought that, during the period of this long and closed crossing, the MHC constitution has developed exclusively to suit the afferent pathway by, instead of, or in addition to, these molecules. It is, therefore, tempting to speculate that Ly49s3 affects not only the afferent pathway by enhancing MHC class I expression in rat thymic cDCs, but also the efferent pathway by inhibiting MHC class II expression in rat thymic cDCs.

It is interesting to know what kinds of signal transduction pathway are activated to enhance the expression of MHC class II genes by the Ly49s3 molecule in cDCs. It has been shown that Ly49-activating receptors associate with immunoreceptor tyrosine-based activating motif—containing adaptors, such as DAP-12, and these adaptors then recruit kinases, such as ZAP-70 (17). It is supposed that the signaling cascade finally phosphorylates and activates transcription factors essential for the expression of MHC class II genes in cDCs.

In our experiments, the expression of MHC class II genes was not diminished but was further enhanced upon addition of the anti-MHC class I Ab to the culture. The actual reason for this finding is obscure at present, but it is hypothesized that negative signaling pathway(s), suppressing MHC class II gene expression, Ag processing, and loading and/or recycling of MHC class II molecules, may also be activated for precise adjustment of MHC class II levels after the stimulation of Ly49s3 by MHC class I molecules. The negative pathway(s) may require stronger stimulation to be activated than the positive one(s), and may be selectively inactivated in the presence of the anti-MHC class I Ab. Further investigation is required to assess this issue.

In the literature, murine Ly49Q has been reported to be expressed on cDCs and pDCs and has been suggested to regulate their functions (21, 26). It has also been reported that human thymic pDCs regulate the nTreg developmental process (9, 10). Therefore, it remains to be clarified whether rat thymic pDCs are involved in nTreg differentiation and express the Ly49 family genes, including Ly49s3. In contrast to rodents, in which many Ly49 family genes cluster in the same chromosomal region, only one Ly49 family gene in 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 may also contribute to the nTreg 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 peripheral blood. Considering the population size of Tregs (6–10%) the impaired expression of nTreg marker genes was not observed in HHR CD4-SP thymocytes when cultured alone.

References


