Selective involution of thymic medulla by cyclosporine A with a decrease of mature thymic epithelia, XCR1⁺ dendritic cells, and epithelium-free areas containing Foxp3⁺ thymic regulatory T cells

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Abstract
Immunosuppressive drugs such as cyclosporine A (CSA) can disrupt thymic structure and functions, ultimately inducing syngeneic/autologous graft-versus-host disease together with involuted medullas. To elucidate the effects of CSA on the thymus more precisely, we analyzed the effects of CSA on the thymus and T cell system using rats. In addition to confirming the phenomena already reported, we newly found that the proportion of recent thymic emigrants also greatly decreased, suggesting impaired supply. Immunohistologically, the medullary thymic epithelial cells (mTECs) presented with a relative decrease in the subset with a competent phenotype and downregulation of class II major histocompatibility complex molecules. In control rats, thymic dendritic cells (DCs) comprised two subsets, XCR1⁺SIRP1α⁻CD4⁻ and XCR1⁻SIRP1α⁺CD4⁺. The former had a tendency to selectively localize in the previously-reported epithelium-containing areas of the rat medullas, and the number was significantly reduced by CSA treatment. The epithelium-free areas, another unique domains in the rat medullas, contained significantly more Foxp3⁺ thymic Tregs. With CSA treatment, the epithelium-free areas presented strong involution, and the number and distribution of Tregs in the medulla were greatly reduced. These results suggest that CSA inhibits the production of single-positive thymocytes, including Tregs, and disturbs the microenvironment of the thymic medulla, with a decrease of the competent mTECs and disorganization of epithelium-free areas and DC subsets, leading to a generation of autoreactive T cells with selective medullary involution.

Keywords Immunosuppressive drug • Thymus • Dendritic cells • Thymic epithelial cells • Regulatory T cells • Thymic structure

Introduction
Graft-versus-host disease (GVHD) is a common complication accompanying allogeneic hematopoietic stem cell transplantation and is caused primarily by differences in the major histocompatibility complex (MHC) and/or minor antigens. However, even after transplantation of autologous stem cells, GVHD-like syndrome can occur, called engraftment syndrome or autologous GVHD (Cornell et al. 2015; Kline et al. 2008). Animal models of this syndrome, induced by a combination of hematopoietic stem cell transplantation followed by 3–4 weeks of cyclosporine A (CSA) administration and ~3 weeks of CSA-free period after withdrawal have been reported in rats and mice (Cheney and Sprent 1985; Glazier et al. 1983), and called syngeneic GVHD. In these long-term induction models, the thymus has been suggested to be involved in the development of the syndrome because thymectomized animals fail to exhibit the disease (Sorokin et al. 1986). Furthermore, short-term CSA administration can also induce autoreactive T cells within 4 days in the thymus, and 7 days in the lymph nodes (LNs), as assessed by the local popliteal LN swelling after injection of the cells into the footpads of recipient animals (Wu and Goldschneider 1999, 2001). These T cells were considered to be involved in the pathogenesis of autologous GVHD and, thus, this short-term protocol may be useful for analyzing the early effects of CSA treatment leading to the manifestation of the disease. Therefore, we deployed this model in this study.

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Concerning abnormalities in thymi and T cell generation after CSA administration, there have been many reports demonstrating involution of the thymic medulla (Schuurman et al. 1990), decreased single-positive thymocytes and peripheral T cells (Kosugi et al. 1989), downregulated expression of class II MHC molecules (MHCII) on medullary thymic epithelial cells (mTECs) (Fletcher et al. 2009), suppressed regulatory T cell (Treg) generation (Coenen et al. 2007), and the generation of autoreactive T cells (Wu and Goldschneider 1999, 2001). However, these reports mostly studied isolated cells, and in situ distribution of TECs, thymic dendritic cell (tDC) subsets, and thymic Tregs (tTregs) after CSA treatment have not yet been studied. To perform positive or negative selection, cortical TECs (cTECs) and mTECs, and medullary tDCs present MHC molecules and antigens (Klein et al. 2014; Wang et al. 2019). However, the effects of CSA on these antigen-presenting cells are still unclear.

Previously, using mAbs ED18 and ED21, we found two novel subsets of TECs, mTEC1 (ED18+ED21−) and mTEC2 (ED18+ED21+). mTEC1 is more competent with abundant expression of functional molecules, such as AIRE and MHCII (Sawanobori et al. 2014). This subset may be affected by CSA. In contrast to the medullary areas containing mTECs (medullary epithelium-containing areas, mECAs), rat thymi have unique areas lacking mTECs (medullary epithelium-free areas, mEFAs). The properties and roles of these areas in normal and CSA-affected states are not yet known.

In this study, we investigated the effects of short-term CSA treatment on thymocytes and T cell subsets in the thymus and LNs and the autoreactivity of the peripheral T cell pool. We focused on the immunohistological analysis of the thymic medulla, in regards to the in situ distribution of mECAs and mEFAs, mTEC subsets, tDC subsets, and Tregs.

Materials and methods

Animals

Eight-weeks old inbred male Lewis rats were purchased from SLC Co. (Shizuoka, Japan). The administration of CSA solution or control solvent was started at 8 weeks of age. Normal (untreated) thymi were collected at 8 or 9 weeks of age. All rats were reared under specific pathogen-free conditions. Animal handling and care protocols were approved by Dokkyo Medical University’s Regulations for Animal Experiments and with Japanese Governmental Law (No. 105).

Antibodies

The antibodies used for immunohistology and flow cytometric analysis are listed in Table 1. Some antibodies were purified from culture supernatants and conjugated in-house.

CSA administration

CSA and olive oil were purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan). CSA was dissolved into olive oil at a concentration of 30 mg/ml in a 65 °C water bath with stirring (Sorokin et al. 1986). This solution was stored at −20 °C until administration. A total of 100 μl of the CSA solution per 200 g of body weight was administered subcutaneously into rats, for a final dosage at 15 mg/kg/day (Wu and Goldschneider 1999). The control group received solvent only. Rats were administrated CSA solution or solvent for 16 consecutive days. The next day of final administration, rats were sacrificed and analyzed.

Histology and immunohistology

Freshly frozen samples were sectioned at 4 μm thickness for general histology, or 2 μm for serial sections. For hematoxylin–eosin (H&E) staining, sections of freshly frozen tissues were air-dried for 2 h, and then hydrated in PBS. After fixation in 1% glutaraldehyde/PBS and 4% paraformaldehyde-1% calcium chloride solution, sections were stained in hematoxylin solution (Sakura Finetek Japan, Tokyo, Japan) for 5–10 min. After washing in water for 10 min, sections were stained in eosin solution (Sakura Finetek Japan) for 3 min, and then dehydrated in 70, 80, 90, and 99.5% ethanol for 1 min each, followed by clearing in xylene for 5 min. Sections were mounted with Entellan new (Merck, Kenilworth, NJ).

For immunohistology, Sections of freshly frozen tissues were fixed in acetone and immunostained as described previously (Sawanobori et al. 2014). Briefly, acetone-fixed sections were hydrated in Tris-buffered saline (pH 7.4), then fixed again in 4% paraformaldehyde-1% calcium chloride solution. After blocking with Block Ace (KAC, Kyoto, Japan), sections were incubated with antibodies. Conjugations of primary antibodies and used secondary antibodies are described in the legends of each figure.

For light microscopy, sections were colored with Vector Blue substrate (Vector Laboratories) or using the New Fuchsin Substrate System (Agilent, Santa Clara, CA, USA) after incubated with alkaline phosphatase-conjugated secondary antibodies. Then type IV collagen, which reveals the tissue...
| Antigen         | Isotype  | Clone    | Conjugate                                      | Source                                                                 |
|----------------|----------|----------|------------------------------------------------|------------------------------------------------------------------------|
| Unknown        | Mouse IgM| ED18     | Biotin\(^a\), Alexa Fluor 594\(^b\)            | Produced at Neuroscience Campus Amsterdam (the Netherlands)\(^c\)      |
| Unknown        | Mouse IgM| ED19     | Biotin\(^a\)                                    | Produced at Neuroscience Campus Amsterdam (the Netherlands)\(^c\)      |
| Unknown        | Mouse IgM| ED21     | Unconjugated, biotin\(^a\), Alexa Fluor 488\(^b\)| Produced at Neuroscience Campus Amsterdam (the Netherlands)\(^c\)      |
| CD4            | Mouse IgG1| W3/25    | FITC, PerCP-Cy5.5, phycoerythrin (PE)            | BioLegend (San Diego, CA, USA)                                        |
| CD8α           | Mouse IgG1| OX8      | FITC, PE                                        | BioLegend                                                              |
| CD11b/c        | Mouse IgG2a| OX42     | PE                                              | BioLegend                                                              |
| CD25           | Mouse IgG1| OX39     | FITC, PE                                        | BioLegend                                                              |
| CD45R          | Mouse IgG2b| HIS24    | PE                                              | eBioscience (Waltham, MA, USA)                                        |
| CD80           | Mouse IgG1| 3H5      | PE                                              | BioLegend                                                              |
| CD86           | Mouse IgG1| 24F      | PE                                              | BD Bioscience (Franklin Lakes, NJ, USA)                                |
| CD90           | Mouse IgG1| OX7      | PE, PerCP-Cy5.5                                 | BioLegend                                                              |
| CD103          | Mouse IgG1| OX62     | Unconjugated, FITC                             | ECACC\(^c\)-\(^d\), BioLegend                                        |
| CD172a         | Mouse IgG2a| OX41    | PE                                              | BioLegend                                                              |
| CD205          | Mouse IgG1| HD83     | PE                                              | BioLegend                                                              |
| Foxp3          | Rat IgG2a| FJK-16s  | Biotin, eFluor660                               | eBioscience                                                           |
| Helios         | Armenian hamster IgG | 22F6 | PE                                              | BioLegend                                                              |
| MHC II         | Mouse IgG1| OX6      | Unconjugated, Alexa Fluor 647\(^b\)             | AbD Serotec, CEDARLANE (Ontario, Canada)                               |
| MHC II         | Mouse IgG1| OX3      | Alexa Fluor 647\(^b\)                           | ECACC\(^c\)-\(^d\)                                                   |
| TCRαβ          | Mouse IgG1| R73      | Alexa Fluor 647\(^b\)                           | ECACC\(^c\)-\(^d\)                                                   |
| XCR1           | Mouse IgG2b| ZET      | Unconjugated, biotin                            | BioLegend                                                              |
| Type IV collagen | Rabbit IgG | Polyclonal | Unconjugated,                                   | Jackson ImmunoResearch (West Grove, PA, USA)                           |
| Isotype control | Mouse IgG | Polyclonal | Unconjugated,                                   | Jackson ImmunoResearch (West Grove, PA, USA)                           |
| Isotype control | Mouse IgG1| MOPC-21  | Alexa Fluor 647                                 | BioLegend                                                              |
| Isotype control | Mouse IgG2b| MPC-11   | Unconjugated, PE                                | BioLegend                                                              |
| Isotype control | Mouse IgM | 11E10    | Biotin\(^a\), Alexa Fluor 488\(^b\)             | eBioscience                                                           |
| Isotype control | Rat IgG2a| RTK2758  | Biotin                                          | BioLegend                                                              |
| Anti-mouse IgG | Goat IgG  | Polyclonal | Alkaline phosphatase                           | Sigma-Aldrich (Saint Louis, MO, USA)                                  |
| Anti-mouse IgG | Horse IgG | Polyclonal | Biotin                                          | Vector Laboratories (Burlingame, CA, USA)                              |
| Anti-mouse IgM | Donkey IgG| Polyclonal | Alkaline phosphatase                           | Jackson ImmunoResearch                                                |
| Anti-rabbit IgG| Goat F(ab\(^\prime\))\(_2\) | Polyclonal | Peroxidase                                     | Jackson ImmunoResearch                                                |
| Anti-biotin    | Goat IgG  | Polyclonal | Alkaline phosphatase                           | Sigma-Aldrich                                                         |
| Streptavidin   |                        |          |                                                | Invitrogen (Waltham, MA, USA)                                         |

\(^a\)Conjugated in our facility using the Biotin Labeling Kit—NH2 (Dojindo Molecular Technologies, Kumamoto, Japan)

\(^b\)Conjugated in our facility using the Alexa Fluor® conjugate kit (Thermo Fisher)

\(^c\)Hybridomas were cultured and the produced antibodies were purified in our facility

\(^d\)The European Collection of Authenticated Cell Cultures (Salisbury, UK)
framework (Matsuno et al. 1996), was stained with anti-
type IV collagen serum (Cosmo Bio, LSL, Tokyo, Japan)
followed by peroxidase-conjugated anti-rabbit IgG anti-
body and 3,3′-diaminobenzidine (DAB) substrate (Dojindo
Molecular Technologies). Photomicrographs were captured
with a Microphot-FX microscope using a Plan Apo objec-
tive lens series (Nikon, Tokyo, Japan) and a DP26 digital
camera (Olympus, Tokyo, Japan), or a BX53 microscope
with a UPlanFL N objective lens series and a DP27 (Olym-
pus). Montage images were synthesized and area meas-
urements were processed using cellSens software (Olympus).
The original resolution of the pictures is 1224 × 960 pixels.
Exposure settings were fixed for each experiment.

For fluorescent microscopy, sections were fixed in 4%
paraformaldehyde-PBS and mounted with Fluorescent
Mounting Media (KPL, Gaithersburg, MD, USA) after immuno-
staining. Multicolor fluorescence images were cap-
tured using an Axioskop 2 Plus fluorescence microscope
equipped with a Plan-Apo objective lens series and an Axi-
oCam MRm camera (Zeiss, Oberkochen, Germany). The
original resolution of the pictures is 1388 × 1040 pixels.
Exposure settings were fixed for each experiment. Filter Sets
17, XF406, and 32 were used to capture Alexa Fluor 488,
594, and 647 respectively.

**Fluorescent image analysis**

For the analysis of immunofluorescent images, multicolor
fluorescence images were captured using a BZ-9000 fluo-
rescent microscope (Keyence, Osaka, Japan) with the CFI
Plan Fluor Objective lens series (Nikon), BZ Filter GFP-BP,
Texas Red, and Cy5 were used to capture Alexa Fluor 488,
594, and 647 respectively. The original resolution of the pic-
tures is 680 × 512 pixels. Exposure settings were fixed for
each antibody. The images were converted into BZ-X format
and analyzed by BZ-X analyzer software (Keyence) for areas
of thymic epithelial cell subsets or tDC subsets and MHCII
expression. For tDCs, we estimated either XCR1+MHCII+ surface
area/mm² because the outline of each cell was dif-
cult to determine. For this, the ECA/EFA ratio was cal-
culated as XCR1+MHCII+ area/mm² in ECA to those of
in EFA.

**Cell isolation and flow cytometry**

For flow cytometric analysis, thymi, spleens, and LNs were
injected with 0.2% collagenase D (Roche Diagnostics, Indi-
anapolis, IN, USA), 0.01% DNase I (Roche), and HBSS (37
°C, pH 7.4), and then cut into slices 1 to 2 mm-thickness and
incubated in 0.1% collagenase D/0.1% DNase I/HBSS at 37
°C for 25 min. After incubation, samples were supplemented
with EDTA to 2.5 mM, teased, and filtered using 50-μm
nylon mesh. The digested cell suspension was centrifuged
and resuspended in 15% OptiPrep (Axis-Shield, Oslo, Nor-
way)/PBS(−) in centrifuge tubes, and 12% OptiPrep/PBS(−)
and then PBS(−) overlaid on the cell suspension. The tubes
were centrifuged at 600 g for 25 min at room temperature.
Cells at the interface between the 15 and 12% OptiPrep, and
the 12% OptiPrep and PBS(−) were considered thymocytes/
lymphocytes, and DC-containing low-density cells respect-
vically. These cells were collected and subjected to flow cyto-
metric analysis. Cells were stained using the conventional
method. For analysis of thymocyte subsets, single-positive
cells were further defined as the TCRhi population, because
strong inhibition of single-positive cells by CSA made con-
tamination of double-positive or double-negative cells into
single-positive gates non-negligible. For analysis of tDCs,
low-density cells were further purified with anti-DC (OX62:
CD103) microbeads and an autoMACS (Miltenyi Biotech,
North Rhine-Westphalia, Germany). To stain Foxp3, the
Foxp3/Transcription Factor Staining Buffer Set (eBiosci-
ence) was used. Stained cells were acquired using an Attune
NxT flow cytometer (Thermo Fisher). Data were analyzed
using FlowJo V10.5.3 (FlowJo LLC, Ashland, OR).

**Mixed leukocyte reaction**

The LNs of CSA-administered or control rats and spleens
of control rats were digested and cells isolated as described
above. To prepare responder T cells, LN cells were sus-
pended in GIT medium (FUJIFILM Wako Pure Chemi-
cal) and stained with PE-conjugated anti-CD45R and anti-
CD11b/c antibodies at 4 °C for 15 min. Because CD25 is
often exploited for the depletion of Tregs (Yamazaki et al.
2006; Mikulic et al. 2017), PE-conjugated anti-CD25 anti-
body was added to obtain Treg-depleted responder T cells.
Cell suspensions were washed with 2 mM-EDTA/PBS(−)
(MACS buffer) twice, followed by incubation with anti-
PE microbeads (Miltenyi Biotech) in GIT medium at 4 °C
for 15 min. Cells were washed again and subjected to the
autoMACS (Miltenyi Biotech) depletion protocol to obtain
purified T cells. The purity of the T cells was > 96%. Stimu-
lator DCs were isolated from the spleen cells using Anti-
DC (OX62) MicroBeads (Miltenyi Biotech) and the positive
selection protocol of the AutoMACS. Isolated T cells were
stained with CytoTell™ green (AAT Bioquest, Sunnyvale,
CA, USA) reagent following the manufacturer’s protocol.
A total of 1 × 10⁵ responder cells and 9 × 10⁴ stimulator
cells were seeded into each well of 96-well flat bottomed
culture plates (Bio-Rad, Hercules, CA, USA) and incubated at 37 °C, in 5% CO₂ for 7 days. After incubation, cells were stained with Alexa Fluor 647-conjugated anti-MHCII antibody to distinguish DCs and T cells, and then counted and captured using an Attune NxT flow cytometer. To exclude dead cells, propidium iodide (PI, Dojindo Molecular Technologies) was added to samples at a final concentration of 1 μg/ml just before the capture. Proliferating cells were defined as Cytotrak dull or negative cells.

Statistical analysis

Statistical analysis was performed using the Student’s t test. For comparison between control and CSA-administered rats, data sets were considered as two-tailed distribution and heteroscedastic. For examination of cell distribution, data sets were considered as two-tailed distribution and paired. Error bars indicate standard deviations.

Results

Effects of CSA on T cell lineages of the thymus and lymph nodes

First, we confirmed decreased CD4 and CD8 single-positive thymocytes and increased double-positive thymocytes (data not shown), as well as a decrease of peripheral T cells as previously reported (Kosugi et al. 1989). As a new finding, recent thymic emigrants (RTEs), defined by the expression of CD90 (Hosseinzadeh and Goldschneider 1993), were strongly reduced, suggesting impaired egress and supply of newly developed T cells from the thymus (Fig. 1a).

For the detection of autoreactivity of peripheral T cells, total T cells were purified from peripheral LNs and cocultured with splenic DCs of the same strain (Fig. S1). T cells from CSA-treated LNs exhibited enhanced proliferation when assessed for the number of living T cells in each well

![Graphs](https://via.placeholder.com/150)

Fig. 1 Effects of CSA on T cell homeostasis and autoreactivity. Thymi and peripheral LNs from control and CSA-administered rats were digested and analyzed by flow cytometry. a, c Ratio of CD90+ RTEs in peripheral LNs (a) and ratio of CD4+CD25+Foxp3+ Tregs in peripheral LNs (c) are displayed. Each group contains three rats in (a) and five rats in (c). b, d MLR assays were performed following the scheme in Fig. S1 to examine the autoreactivity of total peripheral T cells (b) and CD25-depleted T cells (d). Each culture was prepared in triplicate. Representative data from two independent experiments are shown. *p < 0.05, **p < 0.005, ***p < 0.0005
a  

weight of thymi

Isotype control

b  

HE

c  

MHC II

d  

control

e  

CSA

ED21

g  

control

CSA

--- medullas

--- mEFAs

h  

i  

areas of medullas

j  

mEFAs/medullas
We also observed that CD25− conventional T cells in CSA-treated rats contained significantly more autoreactive cells than control rats. In auto MLR, it was detectable without Treg involvement. For Tregs, LNs exhibited a significant decrease in CD4+CD25+Foxp3+ cells (Fig. 1c). To examine the presence of autoreactivity of conventional LN T cells per se, we depleted CD25+ cells from the responders and compared the reactivity between the CSA and control groups. CD25+ T cells from CSA-administered rats still exhibited enhanced reactivity compared to control CD25+ T cells (Fig. 1d), indicating that conventional T cells in CSA rats comprise significantly more autoreactive cells than control rats. Collectively, these data indicate that our CSA protocol essentially reproduced results reported by other laboratories (Wu and Goldschneider 1999, 2001) in another experimental system, and that it may lead to autologous GVHD. A new finding was the stronger reduction in RTEs than peripheral T cells. In the control thymus, medullas were clearly identifiable with H&E staining (Fig. 2d), appearing as pale thymocyte-sparse areas and highly MHCII-expressing cells (presumably mTECs and tDCs), respectively. As we reported previously, ED21 could depict mECAs and mEFAs within medullas (Fig. 2g).

With CSA treatment, the thymic medulla was involuted and the boundaries of the cortexes and medullas became rather obscure on the H&E-stained sections (Fig. 2d). Besides, MHCII expression was not homogeneous in the involuted medullas and presented spotty patterns (Fig. 2e), making discrimination of the medulla from the cortex difficult. To depict the medulla precisely, we compared several markers. As we demonstrated in our previous paper (Sawabornobori et al. 2014), anti-keratin 5 (K5) antibody and Ulex europaeus lectin 1 (UEA-1), which are commonly used to identify thymic medulla of mice, also stained cTECs in the rat thymus (Fig. S2a, b). ED18, the antibody that we used to identify mTEC1 in combination with ED21, also stained cTECs (Fig. 2c). On the other hand, ED21 could depict rat medullas consistently even in the cases of CSA-treated thymi (Fig. S2d, Fig. 2h). Accordingly, we deployed ED21 to assess the proportions of medullas, mECAs, and mEFAs. The medullary areas of CSA-treated thymi decreased to one-third of the control, with a decrease in both mECAs and mEFAs (Fig. 2g–i). In particular, the decrease in mEFAs was more profound (Fig. 2j), while immunofluorescent staining did not reveal a skewed distribution of single-positive thymocytes in mEFAs and mECAs (data not shown). However, in the thymic cortex, the number of cTECs detected with ED19 antibody and their MHCII expression seemed unchanged (Fig. S3).

Impairment of the competent mTEC1 subset in the CSA-treated thymus

To clarify the mechanism of reduced development of T cells in CSA-treated thymi, we analyzed thymic sections with multicolor fluorescent immunostaining, and photomicrographs of immunostained sections were taken. Pixels corresponding to the surface areas of mTEC1 and mTEC2 cells were extracted using BZ-X analyzer software (Fig. 3a–c), and MHCII expression by the mTEC subsets (overlap of MHCII on the areas of mTEC subsets) was quantified (Fig. 3d). In reduced medullary areas, a proportion of the mTEC2 area was significantly increased in the CSA-treated thymi (Fig. 3e). In addition, the expression of MHCII molecules was decreased in both subsets (Fig. 3f). The relative increase in mTEC2 and suppression of MHCII expression in both subsets indicate a decrease in mTEC1 subset and suggest that the mTEC population as a whole became less mature and competent.
Fig. 3 Effects of CSA on the structure and epithelial cells of thymi. 

**a–d** Scheme of the analysis. 

a Sections of thymi were stained with Alexa Fluor 488-conjugated ED21, Alexa Fluor 594-conjugated ED18, and Alexa Fluor 647-conjugated anti-MHCII (OX3) antibodies or corresponding isotype control antibodies. Five randomly selected medullary portions in each sample were captured with BZ-9000. A 20× objective lens was used. Pictures were combined to recreate the whole medulla. Haze was removed, and mECAs were extracted from each combined picture by BZ-X analyzer software. 

b ED18 single-positive pixels and ED21 single-positive → ED18*ED21* double-positive pixels were gated as mTEC1 and mTEC2 respectively. The gates were applied to the original pictures. Ratios of the gated mTEC1 or mTEC2 areas against mTECA areas were calculated and displayed in (e). 

c MHCI expression in the gated mTEC1 or mTEC2 areas were displayed in (f). Each group contains five rats. *p < 0.05, ***p < 0.0005.
Specific tDC subset distribution in mECAs and mEFAs

Recently, all conventional DCs in the mouse were reported to be universally subdivided into either XCR1+signal regulatory protein 1 α (SIRP1α, CD172a)-negative CD8α+ DCs or XCR1−SIRP1α+CD8α− DCs, regardless of their activation status (Klein et al. 2014; Hasegawa and Matsumoto 2018). We recently reported that rat splenic DCs can also be divided into two populations with several differences from mouse: XCR1+SIRP1α−CD4−CD8α− cells and XCR1−SIRP1α+CD4+CD8α− cells (Kitazawa et al. 2019). Although both subsets were CD8α−, the XCR1+ subset was considered to be the rat counterpart of mouse CD8α+ DCs. Accordingly, we analyzed rat tDCs, defined as CD103+MHCII+ cells (Kitazawa et al. 2019) in flow cytometry, and confirmed the presence of two subsets, XCR1+SIRP1α− and XCR1−SIRP1α+ DCs (Fig. 4a). XCR1− DCs expressed relatively higher levels of CD205 and MHCII than XCR1+ DCs.

To examine the localization of tDC subsets in the thymus, we performed multicolor fluorescent immunohistology of thymic sections considering XCR1+MHCII+ cells as XCR1+ DCs (Fig. 4b–d). XCR1+MHCII+ cells seemed to accumulate in mECAs rather than mEFAs (Fig. 4b). This was confirmed with image analysis (Fig. 4d). For further confirmation, we deployed immunohistology of serial sections to detect CD103+XCR1+ cells as XCR1+ DCs (Fig. 4e–h). CD103-stained (Fig. 4e) and XCR1-stained (Fig. 4f) serial sections were carefully compared to identify CD103+XCR1+ cells and the distribution was calculated (Fig. 4h), then the accumulation of XCR1+ tDCs into mECAs was proven.

Unfortunately, we could not examine the distribution of XCR1− DCs. Although they are SIRP1α+, it is difficult to exploit it as a marker because many macrophages express it (Damoiseaux et al. 1989; Hashimoto et al. 2011). Actually, most of SIRP1α+ cells were macrophage marker (CD68, CD163, CD169) positive on immunohistology, and an attempt to identify CD103+SIRP1α+ and macrophage marker negative cells on serial sections was unreliable (data not shown).

Impairment of XCR1+ tDCs in the CSA-treated thymus

To investigate the effects of CSA on tDCs, the number of tDCs in CSA-administered rats was calculated as the number of low-density cells obtained from gravity separation multiplied by the ratio of live CD103+MHCII+ cells. Total tDCs decreased significantly in CSA-administered thymi (Fig. 5), as the XCR1+ subset exhibited selective depletion, whereas the XCR1− subset did not change.

Treg accumulation in mEFAs and effects of CSA on Tregs.

To identify Tregs by immunohistology, we utilized Foxp3 as a marker. A majority of Foxp3+ cells in the thymus were CD4+CD25+ and tTreg marker Helios+ (Thornton et al. 2010) (Fig. S4). In the thymus of control rats, Foxp3+ Tregs significantly accumulated in mEFAs compared to mECAs (Fig. 6a). In CSA rats, the total number of Foxp3+ Tregs and distribution of Foxp3+ Tregs in the thymic medullas were greatly reduced, suggesting impaired generation of tTregs (Fig. 6b).

Discussion

The present study has several novel findings. First, we found that the proportion of RTEs were greatly reduced. We also observed that CD25− conventional T cells in CSA-administered rats contained significantly more autoreactive cells than control rats. Second, with CSA treatment, the involuted thymic medulla presented a stronger decrease in the mEFA. The fluorescent image analysis revealed that mTECs had a relative decrease in the mTEC1 subset, which has a competent phenotype, as well as downregulation of MHCII molecules in both mTEC1 and mTEC2. Third, in control rats, we observed the presence of two DC subsets equivalent to mouse conventional DCs, XCR1+SIRP1α−CD4− and XCR1−SIRP1α+CD4+ DCs. XCR1+ subsets in the medulla had a predominant localization in the previously reported mECAs. On the other hand, the mEFAs contained significantly more Helios+Foxp3+ tTregs than the ECAs. Finally, with CSA treatment, the XCR1+ tDC subset exhibited a selective depletion. Immunohistologically, the total number and distribution of tTregs in the thymic medullas were greatly reduced.

The relative decrease in RTEs defined as CD90+ T cells (Hosseinzadeh and Goldschneider 1993) even within decreased peripheral CD4 and CD8 T cells (Fig. 1a) has not yet been reported because no specific markers are available in mice (Fink and Hendricks 2011). This suggests an impaired supply of newly developed T cells from the thymus or higher sensitivity of RTE to CSA than peripheral T
**Distribution of XCR1+ MHCII+ cells**

- **a**
  - Sorted thymic DCs
  - XCR1-biotin + streptavidin-PerCP-Cy5.5
  - mECA, mEFA

- **b, c**
  - ED21 MHCII
  - XCR1
  - Isotype

- **d**
  - Distribution of XCR1+/MHCII+ cells
  - ECA/EFA ratio

- **e, f, g**
  - ED21
  - Type IV collagen
  - --- medulla
  - ---- mEFA

- **h**
  - Distribution of XCR1+ DCs
  - CD103+FITC
  - Normalized to Mode

- **Legend**
  - * ED21
  - mECA, mEFA
  - mECA, mEFA
The major effects of CSA are inhibition of the calcineurin and mitogen-activated protein kinase (MAPK) pathways, both of which are involved in signaling pathways under TCR (Barbarino et al. 2013). Although some publications indicate that MAPK pathways are also involved in the functions of TECs (Colombara et al. 2005; Mainiero et al. 2003; Ramarli et al. 1998), there is no direct evidence that medullary involution and impaired differentiation of mTECs are caused by direct effects of CSA against TECs. On the other hand, signals from the T cell receptor are essential for positive selection (Klein et al. 2014). Moreover, in in vitro experiments, CSA has been shown to inhibit positive selection (Anderson et al. 1995). In turn, although TECs induce the development and selection of thymocytes, they also require molecular interactions with, for example, RANK-RANKL, CD40-CD40L, and lymphotixin-LTβR for maturation and survival (Alexandropoulos and Danzl 2012). We speculate that CSA affects positive selection first, then it causes the impaired influx of single-positive thymocytes into the medulla, inducing a decrease in medullary areas (Fig. 2i). Eventually, mTECs cannot mature due to a lack of interaction with single-positive thymocytes (Fig. 3e).

The selective accumulation of tTregs in the mEFAs in control rats suggests the presence of a specific domain for the Treg induction in the rat thymus (Fig. 6a), which has not been reported in mice. The reduction of mEFAs (Fig. 2i, j) and depletion of tTregs to one-sixth (Fig. 6b) by CSA administration suggest that the microenvironment of mEFAs loses its function for the Treg induction. However, we could not find an accumulation of particular cells that are responsible for Treg development into these areas. The actual significance of mEFAs is still left to be revealed.

Concerning XCR1+tDCs distribution, as XCR1 ligand XCL1 is secreted by mTECs in an AIRE-dependent manner (Lei et al. 2011), it is reasonable that XCR1+ tDCs are specifically distributed in mTEC-resident mECAs (Fig. 4d, h). Our finding that XCR1+ tDCs decreased in number (Fig. 5) under influence of CSA also reconciles with this XCR1-XCL1 axis concept considering mTEC suppression (Fig. 3e). On the other hand, although we could not examine the distribution of XCR1+ tDCs, decreased medullary areas and mEFAs may suggest that these tDCs may have lost their original localization and become unable to play their role.

Although we did not perform syngeneic GVHD induction, the simple CSA administration model altered the immunological condition of the animals that can contribute to the onset of the disease. The presence of more autoreactive cells among LN CD25− T cells of CSA rats than control rats indicates a significant increase in the autoreactivity in conventional T cells per se. How was autoreactivity of the T cell pool induced? The increase in the mTEC subset relatively lacking functional molecules and decrease in XCR1+ tDCs by CSA treatment suggest impaired negative selection, leading to an increase in autoreactive LN T cells. However, CSA cannot inhibit negative selection directly because it can only partly interfere with some signal pathways during negative selection (DeRyckere et al. 2003). Therefore, it is plausible to speculate that autoreactive TCR can evade negative selection under the effect of CSA via the impaired competency of mTECs. Although it has been reported that a segment Vβ8.5 is abundant among lesion-infiltrating T cells and the periphery of syngeneic GVHD rats (Fischer et al. 1995; Chen et al. 1998), we could not find any skewed Vβ usage when peripheral T cells of CSA rats had increased autoreactivity (data not shown). Autoreactive T cells may be only a small part of Vβ8.5 or other segment positive T cells, and their increase may be undetectable only with Vβ segments. In addition to the enhanced autoreactivity of
conventional T cells, the paucity of Tregs should contribute to the onset of syngeneic GVHD.

In this research, CSA induced the impairment of thymic structure, mTEC maturation, tDC localization, conventional T cell generation, Treg generation, and exclusion of autoreactive T cells. After the withdrawal of CSA, the development of autologous GVHD seems to proceed on the balance between these disturbances and recovery from them. Revealing the full view of this phenomenon will give us not only therapeutic medications for autologous GVHD but also more a profound perspective for the understanding of autoimmunity and the treatment of autoimmune diseases and GVHD.

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**Data availability** No datasets were generated or analyzed during this study. Therefore data sharing is not applicable.

**Declarations**

**Conflict of interest** All authors declare that no support, financial or otherwise, has been received from any organization that may have an interest in the submitted work, and there are no other relationships or activities that could appear to have influenced the submitted work.

**Ethical approval** This research does not include data or materials derived from human patients. Animal handling and care protocols were approved by the Dokkyo Medical University’s Regulations for Animal Experiments and with Japanese Governmental Law (No. 105).

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