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**Altered morphology of mucosa-associated lymphoid tissues and epithelium in the nasal cavity
and lacrimal apparatus in autoimmune disease-prone MRL/MpJ-*Fas*^{lpr/lpr} mice**

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Abstract

The mucosal epithelium and the mucosa-associated lymphoid tissues (MALTs) protect the mucosa such as eye and nose, from constant exposure to foreign antigens. As autoimmune disorders can target both epithelium and MALTs, we morphologically investigated the head of MRL/MpJ-*Fas^{lpr/lpr}*, an autoimmune disease-prone mouse, to discuss the pathological crosstalk among autoimmune disorders, mucosal epithelium, and MALTs. Compared to healthy control MRL/MpJ mice, MRL/MpJ-*Fas^{lpr/lpr}* mice had more lymphoid tissues that were diffusely localized beneath the mucosa epithelium in their lacrimal tracts and nasal cavity. Particularly, lacrimal duct- and nasopharynx-associated lymphoid tissues (LDALT, NALT) were identified as the major MALTs in the mouse head. In the nasal mucosa, MRL/MpJ-*Fas^{lpr/lpr}* mice exhibited larger NALT, more frequent non-ciliated epithelial cells, and more goblet cells than in MRL/MpJ mice. NALT in MRL/MpJ-*Fas^{lpr/lpr}* mice contained more proliferating cells than in MRL/MpJ mice. Moreover, LDALT in some MRL/MpJ-*Fas^{lpr/lpr}* mice developed by being directly connected to the bone marrow surrounding the nasolacrimal duct. These data suggested systemic autoimmune disorders could alter the mucosal immunity of the head by affecting both mucosal epithelium and MALTs. These findings are crucial for understanding the pathology of the head mucosal immunity.

Keywords

Mucosal immunity, Mucosal epithelium, Mucosa-associated lymphoid tissue, Autoimmune disease, three-dimensional reconstruction

Introduction

Mucosal areas, especially those in the head, such as the ocular and nasal mucosa, are frequently exposed to exogenous antigens, including bacteria, viruses, and pollen. Crucial immune-related structures protect mucosal areas from foreign substances, such as epithelial tissue and mucosa-associated lymphoid tissue (MALT). Mucosal surfaces are covered by epithelial cells, and the arrangement of these cells differs among organs; simple, pseudostratified, or stratified. Mucosal epithelial cells and their intercellular junctional complexes function as barriers that prevent luminal substances from being transmitted to the submucosal area (Saitou et al. 2000; Fukuoka and Yoshimoto 2018). MALTs are localized beneath the mucosal epithelium in various parts of the body, including the eyes, nose, mouth, gut, and bronchi (Liebler-Tenorio and Pabst 2006; Lohrberg et al. 2018). MALTs mainly consist of lymphocytes and secrete antigen-specific antibodies to the luminal side (Cesta 2006).

Epithelial tissue and MALT coordinately perform their functions. M cells in the mucosal epithelium transfer antigens from the luminal side to the submucosal side, and goblet cells have recently been reported to be involved in this process (Kernéis et al. 1997; McDole et al. 2012; Date et al. 2017). Antigen uptake allows antigen-presenting cells in MALTs to receive antigen information and initiate an immune response (Cesta 2006). After differentiation of antigen-specific antibody-secreting plasma cells and their return to the mucosal area, the mucosal epithelium transfers the plasma cell-produced

antibodies from the submucosal side to the luminal side; thereby, maintaining mucosal immunity. Furthermore, these tissues affect each other under pathological conditions; morphological changes in the epithelium can influence the underlying MALTs. Previous studies reported that damaged mucosal epithelium in the female genital organs or the urinary tract affected the development of underlying lymphoid structures (Ichii et al. 2022; Chuluunbaatar et al. 2022). In particular, the loss of urothelium integrity, as evidenced by the altered expression of tight junction protein 1 (ZO-1) or occludin and leakage of injected dye from urothelium to the renal pelvis parenchyma, may have stimulated the subepithelial lymphoid tissues in the latter study. However, excessive lymphoid tissue development can also affect the morphology of their adjacent epithelium. Lymphoepithelial lesions, which exhibit distortion and cell infiltration in epithelial structure, are the typical pathological features of MALT lymphoma (Bacon et al. 2007).

Epithelial tissue can show pathological abnormalities due to physical damage, infections, tumors, or immune-related diseases. Previous studies have revealed that representative mouse strains for systemic autoimmune diseases, such as MRL/MpJ-*Fas*^{lpr/lpr} (Lpr) and BXSB/MpJ-*Yaa*, exhibit morphologically and functionally impaired epithelial cells; thinning or lack of intact microvilli in the cornea, increased goblet cells in the conjunctiva, and uncoordinated ciliary movement in the trachea (Hosotani et al. 2020; Hiraishi et al. 2020). Moreover, decreased epithelial cell size was also observed with the progression of autoimmune disease in tear-secreting glands, including lacrimal, Harderian,

and meibomian glands of BXSB/MpJ-*Yaa* mice (Hiraishi et al. 2020). Remarkably, enlarged or ectopic MALT have been reported beneath the intestinal and bronchial epithelium of the *Lpr* mice (Sunderrajan et al. 1986; Zahir et al. 2024) and the lacrimal glands of BXSB/MpJ-*Yaa* mice (Hiraishi et al. 2020). These data suggest that both epithelial tissue and MALT in systemic organs, including the mucosa localized to the head, can be targeted in systemic autoimmune diseases.

Several clinical cases of autoimmune diseases, such as Sjögren's syndrome (SS), manifest their main symptoms in the mucosal area of the head. Patients with SS develop inflammation of the ocular, nasal, and oral mucosae, which results from exocrine gland dysfunction (García-Carrasco et al. 2006). Although 0.04–3.1 million adults in the US are estimated to suffer from SS and certain cases of canine SS-like syndrome have been also reported, no effective treatment is available for SS as its pathology is largely unclear (Helmick et al. 2008; Nabeta et al. 2019). Furthermore, other refractory autoimmune diseases, such as systemic lupus erythematosus and IgG4-related diseases also cause inflammation or ulcers in the head tissues (Ebbo et al. 2017; Kudsi et al. 2021).

Therefore, the pathological crosstalk between autoimmune disorders, mucosal epithelium, and MALT is complicated and should be clarified to elucidate the pathology of head mucosal immunity. As lacrimal duct-associated lymphoid tissues (LDALTs) and nasopharynx-associated lymphoid tissues (NALTs) are recognized as the major MALTs in the head mucosal system of rodents (Chodosh et al. 1998; Casteleyn et al. 2011), the present study mainly focused on the pathological changes in lymphoid

and epithelial tissues in the ocular and nasal mucosa of autoimmune disease-prone Lpr mice. Our data would contribute to the comprehensive understanding of the pathology of head mucosal immunity in patients with autoimmune disorders.

Materials & Methods

Animals and sample preparation

Male MRL/MpJ (MpJ) and Lpr aged thirty to thirty-one weeks were purchased from Japan SLC Inc. (Hamamatsu, Japan). All animal experiments were approved by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine, Hokkaido University (approval no. 21-0008, 23-0062) and were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of Hokkaido University, Faculty of Veterinary Medicine, which has been approved by the Association for Assessment and Accreditation of Laboratory Animal Care International. These mice were euthanized via cervical dislocation under deep anesthesia using a mixture of 0.3 mg/kg medetomidine (Kyoritsu Seiyaku; Tokyo, Japan), 4 mg/kg midazolam (Astellas Pharma; Tokyo, Japan), and 5 mg/kg butorphanol (Meiji Seika Pharma; Tokyo, Japan). Head samples were fixed in 10% neutral buffered formalin for histologic analysis or stored in RNA later (Thermo Fisher Scientific; Waltham, MA, USA) for gene expression analysis. Histological analyses were conducted in two independent replicates, whereas RNA analyses were conducted once.

Histological analysis

Fixed head samples were dehydrated and embedded into paraffin after decalcification with 5% EDTA-2Na for two weeks at 4 °C. Twelve-micrometer-thick serial sections were prepared and stained with hematoxylin and eosin (HE) for the three-dimensional (3D) reconstruction of lymphoid structures. From the same mice, 3- μ m-thick sections were obtained as semi-serial sections with 120- μ m intervals and stained with HE or periodic acid-Schiff (PAS) to analyze the histopathology of lymphoid and epithelial tissues in the head. The stained sections were scanned with a Nano Zoomer 2.0 RS virtual slide scanner (Hamamatsu Photonics; Shizuoka, Japan) and observed under a BZ-X710 microscope (Keyence; Osaka, Japan).

3D reconstruction, volume, and area measurement

HE-stained images of serial sections from each mouse were spatially aligned using an open-source platform for biological-image analysis, Fiji (GitHub, <https://github.com/fiji>) (Schindelin et al. 2012). The aligned images were imported into Image-Pro 10 (Media Cybernetics; Rockville, MD, USA). Using this software, 3D images of tissues were constructed from manually drawn edges of them in 2D images. To measure the volume of MALT, the area (mm^2) in each HE-stained image, which was obtained by NDP.view2 software (Hamamatsu Photonics; Shizuoka, Japan), was multiplied by 0.012

(mm), and the volume (mm^3) was defined as the sum of these values. To measure the area of the MALT-covering epithelium, its length (mm) in each HE-stained image was measured and multiplied by 0.012 (mm). The area (mm^2) was calculated as the sum of these values. Thirty lymphocytes in MALTs were randomly selected and the area of each lymphocyte was measured using NDP.view2 to estimate the volume, cross-sectional area, and diameter of a single lymphocyte. The values of the volume and area of MALT and the MALT-covering epithelium were normalized to the volume and cross-sectional area of a single lymphocyte to compensate for the morphological differences between sections during specimen preparation.

Immunohistochemistry and immunofluorescence

Immunohistochemistry (IHC) and immunofluorescence (IF) were performed to evaluate the localization of B cells (B220), T cells (CD3), macrophages (IBA1), helper T cells (CD4), cytotoxic T cells (CD8), tight junction-forming proteins (ZO-1 and Occludin), M cells (GP2), interleukin-22, high endothelial venules (HEVs; PNAd), CXC-chemokine 13 (CXCL13), vimentin, proliferating cells (Ki67), and apoptotic cells (ssDNA). Information on all the antibodies used in this study is summarized in Supplemental Table 1. Three-micrometer-thick sections were deparaffinized, and antigen retrieval was conducted in optimized ways, as shown in Supplemental Table 1. Internal peroxidase activity was blocked for IHC and tyramide signal amplification (TSA) technology by soaking the sections in 0.3%

H₂O₂-containing methanol for 20 min at room temperature. All the sections were serially incubated with blocking serum (1 h at room temperature), primary antibodies (overnight at 4 °C), and secondary antibodies (30 min at room temperature). The sections were washed with PBS before incubation with the blocking serum and before and after incubation with the secondary antibody. The IHC sections were subsequently incubated with streptavidin-conjugated horseradish peroxidase (SABPO(R) kit; Nichirei, Tokyo, Japan) for 30 min at room temperature and their immunopositivity reaction was finally visualized using 3,3'-diaminobenzidine tetrahydrochloride-H₂O₂ solution with counterstain by hematoxylin. The IF sections were incubated with Hoechst 33342 (1:500; FUJIFILM Wako Pure Chemical Corporation; Osaka, Japan) at room temperature for 30 min for nuclear staining. Sections that were subjected to TSA were incubated with Cyanine 3 Amplification Reagent (1:100; Perkin Elmer; Connecticut, USA) after incubation with HRP-conjugated secondary antibody. IHC images were examined in the same manner as the HE- or PAS-stained sections, and BZ-X710 microscope (Keyence) was used to observe IF images.

Histoplanimetry

Twenty sections were examined to determine the number of lymphoid structures in the tissues of each mouse. The ratio of positive cells or reactions in PNAd⁺ HEVs, Ki67⁺ proliferating cells, and ssDNA⁺ apoptotic cells was measured using QuPath-0.5.0 (<https://qupath.github.io/>) (Bankhead et al.

2017). The density of PAS⁺ goblet cells and the height of mucosal epithelium were measured using NDP.view2 software. The heights of epithelium in the nasolacrimal duct and nasal mucosa were measured at the central point of epithelium covering the LDALT and NALT. The ratio of CD4⁺ cells and CD8⁺ cells among CD3⁺ cells and the density of GP2⁺ M cells were enumerated from the photographs captured using a BZ-X710 microscope. The positive area ratios of B220, CD3, IBA1, and CXCL13 were measured using ImageJ software (NIH, <https://imagej.nih.gov/ij/download.html>). At least three sections from each mouse were used to obtain all the data.

Estimation of total cell volume or number in or over a MALT

The total amount of PNAd⁺ HEVs, Ki67⁺ cells, or ssDNA⁺ cells in a MALT was estimated using the following calculation: density of positive HEVs or cells (%) × the volume of MALT normalized to one lymphocyte volume. For the total expression level of CXCL13 in a MALT, the positive area ratio of CXCL13 (%) × the volume of MALT. The total number of M and goblet cells in a certain area of mucosal epithelium that covers an entire MALT was estimated using the following calculation: density of M or goblet cells in MALT-covering region (number/one-lymphocyte area) × area of MALT-covering region normalized to one lymphocyte area + density of M or goblet cells in MALT non-covering region (number/one-lymphocyte area) × [70,000 or 210,000 (for nasolacrimal duct or nasal cavity, respectively) - area of MALT-covering region].

RNA analysis

The head samples for RNA analysis were divided into three parts: rostral, central, and caudal, using the border of the hard and soft palate and the lateral canthus as landmarks. The central part was then cut longitudinally, and right and left samples were processed and analyzed separately. Total RNA was purified by TRIzol reagent (Thermo) and was used as a template to synthesize complementary DNA (cDNA) with ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo; Osaka, Japan).

qPCR analysis was performed using the THUNDERBIRD SYBR qPCR Mix (Toyobo), gene-specific primers, cDNA, and a real-time thermal cycler. The primers used in this study are summarized in the Supplemental Table 2. The qPCR cycling conditions were as follows: an initial denaturation at 95 °C for 1 min, followed by 40 cycles of denaturation at 95 °C for 15 sec, annealing at 60 °C for 30 sec, and extension at 72 °C for 30 sec. Data were normalized to the values of actin, beta (*Actb*) and MpJ using the delta-delta Ct method.

Statistical analysis

The results are expressed in box plots displaying the minimum, first quartile, median, third quartile, and maximum. Given the sample sizes, which ranged from 4 to 6, a non-parametric test, the Mann–Whitney *U* test, was used to compare MpJ and Lpr mice in order to avoid potentially erroneous

assumptions of normal distribution of the data obtained in this study. The statistically significant difference was set at $P < 0.05$.

Results

Lymphoid tissues found in the mouse head

The ratio of spleen weight to body weight was significantly higher in Lpr mice than that in MpJ mice (Fig. S1), indicating the development of systemic autoimmune diseases in Lpr mice used in this study (Watanabe-Fukunaga et al. 1992).

Further, we comprehensively investigated the localization of lymphoid tissue in the mouse head using serial sections (Fig. 1). These mice lacked conjunctiva-associated lymphoid tissues and tonsils, as previously reported (Chodosh et al. 1998; Casteleyn et al. 2011). Histological examination of lacrimal apparatus revealed diffuse lymphoid tissue beneath the epithelium of the upper or lower eyelids, third eyelid, meibomian gland (MG), Harderian gland (HG), and nasolacrimal duct in both MpJ and Lpr (Fig. 1A,B). In particular, well-developed lymphoid tissues beneath the dorsal side of the nasolacrimal duct were observed in all the examined mice in this study (Fig. 1B) and we identified this lymphoid tissue as LDALT, per a previous report (Lohrberg et al. 2018).

Observation of the nasal cavity revealed that both strains of mice had lymphoid tissues beneath the respiratory mucosa and in the exocrine glands surrounding the paranasal cavity and that only Lpr mice

had lymphoid tissues in the olfactory mucosa (Fig. 1C). In particular, all the examined mice had clear lymphoid tissue (NALT) immediately beneath the ventral side of the nasal cavity (Lohrberg et al. 2018); NALT was larger in the Lpr mice than in MpJ. NALT and the paranasal sinus-surrounding exocrine gland were separated by the bone tissue in MpJ; however, NALT in Lpr mice developed in such a way that it enlarged the gap in the bone and invaded the exocrine gland (Fig. 1C).

For histoplanimetry, the average number of lymphoid tissues in upper and lower eyelids, MG, and HG in one mouse was higher for the Lpr mice than that for MpJ mice and the ratio of mice that exhibited at least one lymphoid tissue in the upper and lower eyelid, MG and HG among all the examined mice of the same strain was higher for the Lpr mice than that for MpJ mice (Table 1). Lpr mice showed a higher average number of lymphoid tissues and a higher ratio of mice with lymphoid tissues in the respiratory and olfactory mucosa than MpJ mice (Table 1). Furthermore, gene expressions of B cells, T cells, and macrophages were higher in the tissue containing the lacrimal apparatuses and the nasal mucosa of Lpr than that of MpJ (Fig. S2).

Thus, all examined mice, regardless of the strain, exhibited well-developed LDALTs and NALTs and these MALTs were directly attached to epithelium without a connective tissue layer between them. Therefore, the subsequent analyses focused on these MALTs as they were easier to observe than other lymphoid tissues in the head and appeared to be ideal for investigating the relationship between MALT and the epithelium.

Morphological changes of MALTs in the mouse head

To clarify the spatial localizations of LDALT and NALT in the mouse heads, 3D reconstruction was performed using serial sections (Fig. 2; Fig. S3,4). While LDALT is typically found on the dorsal side of the nasolacrimal duct near its connection with the lacrimal canaliculus, part of LDALT was observed between nasolacrimal duct and lacrimal canaliculus as well as the rostral portion of nasolacrimal duct in the Lpr mice (Fig. 2A; Fig. 1B). Three-dimensional reconstruction of NALT revealed that it was not only larger in cross-sections, but also in the rostral-caudal direction (Fig. 2B).

For MALT volume calculations, the values of LDALT in Lpr mice and MpJ mice were not different though LDALT in Lpr mice ranged in wider distribution. In contrast, the value of NALT was significantly higher in Lpr mice than in MpJ mice (Fig. 2C). Consistent with these results, the area of LDALT- or NALT-covering epithelium was larger in Lpr, and significance was observed in the NALT-covering epithelium (Fig. 2D).

Immune cell composition of both MALTs was also evaluated by IHC (Fig. 2E). B220-positive (+) B cells, CD3⁺ T cells, and IBA1⁺ macrophages were detected in LDALT and NALT, and no consistent tendencies were observed in the localization patterns or the expression level of these immune cells in these MALTs between the strains (Fig. S5A). Most CD3⁺ T cells in LDALT and NALT were positive for either CD4 or CD8, and a few CD3⁺CD4⁻CD8⁻ T cells, as previous studies reported in Lpr mice

(Watanabe-Fukunaga et al. 1992), were observed in LDALT and NALT in both strains (Fig. S5B, C).

Notably, there were more CD3⁺CD4⁺CD8⁻ T cells than CD3⁺CD4⁻CD8⁺ T cells in both LDALT and NALT of both strains.

Morphological changes in mucosal epithelium in the mouse head

The epithelium covering LDALT or NALT was morphologically examined from the images displayed in Figs 3 and 4. The LDALT-covering epithelium was stratified squamous epithelium and was partially thinner in all examined MpJ and Lpr mice (Fig. 3A). The NALT-covering epithelium appeared to be a pseudostratified ciliated epithelium, which is typical of the respiratory region in the nasal mucosa. Epithelial cells with shorter cilia or lacking cilia were more frequently observed in Lpr mice than in MpJ mice (Fig. 3B). In histoplanimetry, the average height of epithelium covering LDALT or NALT did not significantly differ between the strains (Fig. 3C).

The expression of tight junction proteins ZO-1 and Occludin was detected in the LDALT- and NALT-covering epithelium, without remarkable strain differences. A ZO-1⁺ reaction was observed on the apical side of epithelial cells in a linear pattern in the nasal cavity and the nasolacrimal duct, and surrounded epithelial cells in the middle layer of the nasolacrimal duct epithelium. The occludin⁺ reaction surrounded each epithelial cell along the cell membrane in both strains (Fig. 3D). Thus, the morphology of the tight junction of epithelium in these mucosal areas was maintained in Lpr mice,

similar to that in MpJ mice.

Further, we observed GP2⁺ M cells in the mucosal epithelium, which are known to contribute to antigen uptake, and measured their densities in the MALT-covering and adjacent areas. In the epithelium of the nasolacrimal duct, M cells were detected regardless of the presence of lymphoid tissue beneath it (Fig. 4A). In Figure 4B, the density of M cells was compared between the epithelium of LDALT-covering and uncovered areas in Lpr and MpJ mice. MpJ mice showed significantly lower M cell density in the LDALT-covering area compared to the uncovered area. However, no significant differences were noted between the LDALT-covering area and the uncovered area in Lpr mice or strains (Fig. 4B). M cells were also detected in the NALT-covering epithelium and adjacent areas (Fig. 4C). No remarkable differences were present in M cell density between strains or areas (Fig. 4D). Based on the data from the epithelial area that covered each MALT (Fig. 2D), the total M cell numbers in a certain epithelium area, including MALT-covering epithelium, were calculated (Fig. 4E). Those values were comparable between both strains in nasolacrimal duct and nasal cavity (Fig. 4F, G).

The epithelium in the respiratory region of the nasal mucosa also contained PAS⁺ goblet cells in both MpJ and Lpr mice without showing noticeable strain differences in their densities in histological images (Fig. 5A). Both strains showed a higher density of goblet cells in the NALT-covering epithelium than in the adjacent respiratory epithelium (Fig. 5B). As the area of the NALT-covering epithelium was larger in Lpr mice (Fig. 2D), the total goblet cell number in the area of nasal mucosa,

estimated with the 3D data, was significantly higher in Lpr mice (Fig. 5C). Positive reaction of interleukin 22, a cytokine produced from lymphocytes which can foster the differentiation of goblet cells(Zhang and Wu 2020), was detected beneath the NALT-covering epithelium, though not beneath non-covering epithelium in both strains (Fig. S. 6).

Lymphocyte recruiting factors to the MALTs of the mouse head

Further, we focused on the mechanism of MALT development in Lpr mice. HEV, a gateway for lymphocytes to migrate into lymphoid tissues, and chemotactic molecules have been observed as immune cell-recruiting factors. HEVs were detected in the LDALT and NALT in both strains, and the HEV cell densities in both MALTs were not different between the strains (Fig. S7A,B). To estimate the exact number of HEVs in the MALT, the total HEV cell number was calculated based on the data of each MALT volume (Fig. 2C). Total number of HEV cells was not significantly different between the strains in LDALT and NALT, although Lpr in NALT showed the highest number among the examined area (Fig. S7C).

For IF, the oval- or spindle-shaped cells were positive for CXCL13 in both MALTs (Fig. S7D). Several CXCL13-expressing cells were positive for B220 or vimentin. No strain differences were present in the density or total expression level of CXCL13⁺ reactions in each MALT (Fig. S7E, F).

Cell proliferation and apoptosis in the MALTs

To determine the factors involved in the development of MALTs in the Lpr mouse head, we examined the proliferative activity of cells composing LDALT or NALT. In particular, Lpr mice showed abnormal proliferation of lymphocytes due to an apoptosis-regulating factor, FAS (Nagata and Suda 1995). The LDALT and NALT in both strains contained Ki67⁺ proliferating cells (Fig. 6A). Histological images showed no significant differences in the density of proliferating cells in LDALT or NALT between the strains (Fig. 6B). However, the total number of Ki67 in NALT, estimated with the 3D data, was significantly higher in Lpr mice than that in MpJ mice (Fig. 6C).

ssDNA⁺ apoptotic cells were also detected in the LDALT and NALT in both strains (Fig. S8A). The density of apoptotic cells in the MALTs did not differ between the strains, and the total number of ssDNA⁺ cells in NALT was significantly higher in Lpr mice than that in MpJ mice (Fig. S8B, C).

Communication of MALTs and the bone marrow

Close observation of serial sections discovered that two out of six examined Lpr mice and none of MpJ mice exhibited “Bone Marrow-connected LDALT (BM-LDALT)”; LDALT directly connected to the bone marrow in the skull bone surrounding the nasolacrimal duct (Fig. 6D, Fig. S9). Bone marrow cells, such as metamyelocytes with contain less dense chromatin in indented or notched nucleus (Fig. 6D-i, iii), were not observed in LDALT in MpJ mice or in a region of BM-LDALT closer to the

epithelium (Fig. 6D-ii, v). However, cells resembling these bone marrow cells were observed in a region of BM-LDALT closer to the bone marrow (Fig. 6D-iv). The ratio of Ki67⁺ cells appeared higher in the bone-marrow-side half than the luminal-side half in BM-LDALT (Fig. 6E). The average volume of BM-LDALT was larger than that of normal LDALT (Fig. S10A). Similar to normal LDALT, BM-LDALT was mainly composed of B220⁺ B cells, CD3⁺ T cells, IBA1⁺ macrophages, and CD3⁺ T cells in BM-LDALT expressed either CD4 or CD8 (Fig. S10B, C). PNA⁺ HEVs, CXCL13⁺ cells, and ssDNA⁺ apoptotic cells were also detected in BM-LDALT (Fig. S10D).

Two out of six examined Lpr mice exhibited NALT facing an opening of the bone marrow as well. However, those NALTs were not integrated with the bone marrow (Fig. S10E). Some lymphoid tissues beneath the nasal mucosa in Lpr mice were also directly connected to the bone marrow in the nasal concha in a similar way as BM-LDALT (Fig. S10F). These structures were not observed in MpJ mice in this study.

Discussion

This study compared an autoimmune disease-prone mouse, Lpr, to its healthy control, MpJ to investigate the pathological changes of head mucosal immunity in systemic autoimmune disorders. Morphological observation focusing on the lacrimal apparatuses and nasal cavity demonstrated that the Lpr head had more lymphoid tissues and a larger volume of a major MALT in mouse heads, NALT,

compared to MpJ. B cells in secondary lymphoid tissues, such as the spleen and lymph nodes, are the primary source of autoantibodies responsible for nephritis, vasculitis, and other autoimmune phenotypes in Lpr mice (Shlomchik et al. 1994). The developed MALTs in the head of Lpr mice might also contribute to these phenotypes by producing autoantibody-secreting cells.

Although previous reports have demonstrated the existence of LDALT and/or NALT in various mammals, including mice, humans, dogs, and pigs (Knop and Knop 2001; Debertin et al. 2003; Casteleyn et al. 2011; Kuper et al. 2012; Mahdy and Author 2017), detailed and comprehensive knowledge of their morphology is limited because they are small and surrounded by bone tissues in the head. In this study, the small size of the mouse head allowed serial sectioning of the MALTs and reconstruction of their 3D images. This method revealed that LDALT was localized on a part of nasolacrimal duct close to the conjunction with the lacrimal canaliculus, and that both LDALT and NALT were enlarged in three axes (medial–lateral, dorsal–ventral, and rostral–caudal). Moreover, 3D analyses enabled the quantification of the volume of tissues and total expression levels of specific cells and molecules, while the data obtained by observation of two-dimensional histological images was limited to the density. In this way, we detected several differences in the volumes and total expression levels of cells or molecules between the two strains, emphasizing its usefulness of the 3D analyses.

Similar to MALTs, the mucosal epithelium can be affected by systemic autoimmune diseases via various pathological mechanisms. For instance, in the pathology of ulcerative colitis, autoantibody

reacting to integrin $\alpha v \beta 6$, a protein expressed on intestinal mucosal epithelial cell membrane, has been reported to cause epithelial barrier defect in the large intestine (Kuwada et al. 2021). As for the head, SS are reported to have deformed and shorter microvilli on conjunctival epithelial cells, supposedly due to desiccation caused by dysfunction of tear-secreting glands (Koufakis et al. 2006). In this study, prominent abnormalities in the mucosal epithelium of the lacrimal apparatuses and nasal cavity were not observed in Lpr mice, and the tight junctions among these cells were also intact, suggesting that the epithelium maintained its function as a physical barrier. However, importantly, Lpr partially lacked cilia on the apical side of mucosal epithelial cells in the nasal cavity. This may have been caused by desiccation of the nasal cavity because Lpr has been reported to show dysfunction of the exocrine glands in the head (Saito et al. 2013; Umazume et al. 2015). Another possible mechanism of the cilia-lacking epithelium in the nasal cavity of Lpr mice is the alterations of homeostasis-maintaining components in the nasal epithelium, as Lpr has been reported to exhibit abnormal morphology of the ciliated epithelium in the oviductal infundibulum, which are associated with changes in local hedgehog signaling-related molecules (Hosotani et al. 2023).

The population of cells with specific functions in the mucosal epithelium of the head was also examined in this study. M cells exist in the mucosal epithelium and provide luminal antigens for MALTs (Kernéis et al. 1997; Date et al. 2017). Previous studies have demonstrated the presence of M cells in the epithelium of the nasolacrimal duct and nasal cavity in mice (Mutoh et al. 2016; Date et al.

2017; Oya et al. 2021), and both MpJ and Lpr also showed M cells in these areas. The higher frequency of M cells in the ventral side of the nasolacrimal duct observed in MpJ mice might contribute to efficient antigen uptake from lacrimal fluid, whereas this difference between regions was not observed in Lpr mice. As M cell differentiation in the nasolacrimal duct epithelium is reported to be based on the receptor activator of nuclear factor-kappa B (RANK) -RANK ligand signaling (Oya et al. 2021), the expression of such receptor or ligand might be different between in LDALT-covering regions and in non-covering regions of MpJ mice. However, no strain differences were observed in terms of the total number of M cells in the nasolacrimal duct or nasal cavity, suggesting that antigen uptake through M cells was similar between MpJ and Lpr in the examined head area.

Both MpJ and Lpr possessed goblet cells in the nasal epithelium and the epithelium covering the NALT contained a higher density of these cells than adjacent parts of the epithelium in both strains. Goblet cells are differentiated from epithelial cells, and several cytokines produced from immune cells, such as transforming growth factor β or IL-22, are known to regulate the differentiation of goblet cells (Ouyang et al. 2010; Zhang and Wu 2020). Therefore, the goblet cell differentiation in the epithelium immediately above NALT might have been promoted by cytokines produced by NALT. As the NALT-covering epithelial area was larger in Lpr mice, the total number of goblet cells in the nasal mucosa tended to be higher in Lpr mice. The contribution of increased goblet cells to the nasal mucosa of Lpr mice could be interpreted in at least two ways. The widely known function of goblet cells is secreting

mucin that covers and protects mucosal epithelium. Since compensatory increase in goblet cells has been reported in mice with impaired exocrine glands (Inaba et al. 2018), the increase in the number of goblet cells in Lpr may have reinforced the physical barrier of the mucosal epithelium. Another function of goblet cells that has been under investigation is to contribute to antigen uptake by letting luminal antigens into the submucosal part, known as “Goblet cell-associated antigen passage (McDole et al. 2012; Barbosa et al. 2017).” Although the cellular biology of this passage remains unclear, acetylcholine-dependent transcytosis has been suggested to form this passage (Gustafsson et al. 2021). The larger number of goblet cells in Lpr mice may accelerate antigen uptake in the nasal cavity and be associated with excessively developed NALT in Lpr mice. Further studies are required to clarify the significance of goblet cell increase.

Further, we uncovered the mechanism underlying abnormal MALT development in Lpr. Normally, MALT develops in two ways: lymphocyte migration from circulation and local lymphocyte proliferation (Randall and Mebius 2014). Since the cell composition of LDALT and NALT in Lpr was similar to that in MpJ and other strains in previous studies, which were mainly composed of B cells, T cells, and a smaller number of macrophages, we hypothesized that the MALTs in Lpr mice should have grown larger based on these two methods. This led us to examine how they contributed more actively. The HEVs and CXCL13 levels were measured as the indices of lymphocyte migration. HEVs are the specialized blood vessels through which lymphocytes in blood circulation migrate to lymphoid

tissues (Girard and Springer 1995). CXCL13 has been reported to be a major chemoattractant for lymphocytes, particularly B cells and is essential to the trafficking of lymphocytes through HEVs (Kanemitsu et al. 2005). However, no significant differences were detected in the density, distribution, or total number of HEVs and CXCL13 between the strains in LDALT and NALT. In contrast, the total number of proliferating cells in the NALT, estimated using 3D data, was significantly higher in the Lpr mice than in the MpJ mice. Therefore, the abnormal development of MALTs in Lpr mice might be associated with the accelerated proliferation of local cells rather than an increase in the number of recruited cells. This is consistent with the genetic trait of Lpr, a mutation in apoptosis-regulating factor that causes abnormal lymphocyte proliferation and systemic autoimmunity.

LDALT in mice is normally surrounded by bones but separated from the bone marrow. However, we discovered LDALT directly connected to the bone marrow, BM-LDALT, in Lpr mice. As the connected area was abundant with B cells, T cells, and macrophages, LDALT might invade the bone marrow through an opening of the bone. The mechanism of enlarged opening of the bone is still unclear, but it might be caused by combination of multiple factors, such as physical pressure by the increased sizes of LDALT and impaired bone remodeling in Lpr mice. Systemic autoimmunity has been reported to cause imbalance between osteoclasts and osteoblasts (Namba et al. 2019), and the lack of Fas-mediated apoptosis in Lpr mice, which is the primary mechanism of apoptosis in osteoclasts (Wu et al. 2003), could increase the number of osteoblasts, resulting in impaired bone remodeling. Patients

with autoimmune diseases, such as systemic lupus erythematosus, are also known to have a higher risk of bone diseases represented by osteoporosis (Rella et al. 2022). Further study on BM-LDALT may discover a new pathological relationship between the bone and MALTs in autoimmune diseases.

Commonly, lymphocytes differentiate from stem cells in the bone marrow and T cell precursors transmit to the thymus through the vascular system. Immature self-reactive B cells and T cells are negatively selected and deleted in the bone marrow and the thymus, respectively, and only self-tolerant lymphocytes migrate from those primary lymphoid tissues to secondary lymphoid tissues, such as lymph nodes and MALTs, to be involved in the immune reaction (Von Boehmer 1988; Melchers et al. 2000). However, the presence of cells with less condensed chromatin in indented or notched nucleus, which is characteristic of bone marrow cells including metamyelocytes (Ghane et al. 2019), in BM-LDALT indicated direct cell migration from the bone marrow to LDALT. This passage might allow immature lymphocytes in the bone marrow to infiltrate into LDALT without undergoing the selection in the primary lymphoid tissues, and BM-LDALT might be a unique source of autoreactive lymphocytes and contribute to the pathology of autoimmune diseases in Lpr mice. Bone marrow-connected lymphoid tissues beneath the nasal mucosa in Lpr mice might play the same pathological role in autoimmunity as BM-LDALT. To the best of our knowledge, this study would be the first report on “Primary-secondary lymphoid tissue communication” and continued investigation is required to understand the interaction between primary and secondary lymphoid tissues in such unique structures

and their pathological significance.

In summary, this study examined the pathological changes in the tissues that protect the mucosal areas in the heads of autoimmune disease-prone mice, Lpr, using a novel digital technology. Viewing MALTs and epithelium as tissues that interact with each other, we demonstrated that systemic autoimmune disorders may affect local head mucosal immunity by changing both MALTs and mucosal epithelium, and developing MALT by local proliferation of its composing cells and direct connection with the bone marrow. These data would contribute to a comprehensive understanding of head pathology relating to autoimmune disorders.

Data availability

The materials described in the manuscript, including all relevant raw data, are available from the corresponding author upon reasonable request.

Competing interests

The authors declare no competing financial or non-financial interests to disclose.

Ethics approval

All procedures involving animals were approved by the Institutional Animal Care and Use

Committee of the Faculty of Veterinary Medicine, Hokkaido University (approval no. 21-0008, 23-0062) and were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of Hokkaido University, Faculty of Veterinary Medicine, which has been approved by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Author contributions

M.H., Y.K., and O.I. conceptualized the study and designed the experiments; M.H. performed the experiments, analyzed the data, and wrote the manuscript; Y.K. supervised the study and contributed to manuscript writing; O.I. supervised the experiments and contributed to manuscript writing; Te.N., Ta.N., and M.Z.U. provided critical feedback, assisted with experiments, data analysis, and manuscript preparation. All authors reviewed and approved the final manuscript. O.I. is the guarantor of this work, having full access to all the study data and bearing responsibility for ensuring the integrity of the data and the accuracy of the data analysis.

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Figure legends

Fig. 1. Lymphoid tissues in the head of MRL/MpJ (MpJ) and MRL/MpJ-*Fas^{lpr/lpr}* (Lpr) mice.

A Lymphoid tissues in lacrimal apparatuses. Lymphoid tissues are observed beneath the mucosal epithelium or in exocrine glands.

B Lacrimal duct-associated lymphoid tissues. Lymphoid tissues are observed beneath the dorsal epithelium of the nasolacrimal duct. NLD: Nasolacrimal duct. LC: Lacrimal canaliculus.

C Lymphoid tissues in nasal cavity (NC). Lymphoid tissues are observed beneath the mucosal epithelium.

HE-staining. The arrowheads indicate ectopic lymphoid tissues. Black and white arrows indicate lacrimal duct-associated lymphoid tissues and nasopharynx-associated lymphoid tissues, respectively. Scale bars represent 100 μ m.

Fig. 2. Mucosa-associated lymphoid tissues in lacrimal apparatus and nasal cavity of MRL/MpJ (MpJ) and MRL/MpJ-*Fas^{lpr/lpr}* (Lpr) mice.

A 3D images of the left lacrimal duct-associated lymphoid tissue (LDALT) (red) and the lacrimal duct composed of the lacrimal canaliculus and nasolacrimal duct (yellow). The gray dotted line in the left drawing indicates the area of 3D images. The blue dotted arrows indicate the flow of tear fluid, and the arrowheads indicate LDALT.

B 3D images of left nasopharynx-associated lymphoid tissue (NALT). The gray dotted line in the left drawing indicates the area of 3D images.

C Volume of LDALT and NALT normalized to the volume of a single lymphocyte.

D Area of epithelium that covers LDALT and NALT normalized to the cross-sectional area of a single lymphocyte.

E Immune cell composition of LDALT and NALT. B cells, T cells, and macrophages are denoted as B220 (green), CD3 (red), and IBA1 (white). Dotted lines surround LDALT and NALT. NLD: Nasolacrimal duct. NA: Nasal cavity. Immunofluorescence.

Box plot represents the minimum, first quartile, median, third quartile, and maximum. $n=6$, $*P < 0.05$, Mann–Whitney U test (C, D). Scale bars represent 1 mm (A, B) or 100 μm (E).

Fig. 3. Mucosal epithelial cells covering mucosa-associated lymphoid tissues of MRL/MpJ (MpJ) and MRL/MpJ-*Fas*^{*lpr/lpr*} (Lpr) mice.

A Lacrimal duct-associated lymphoid tissue (LDALT)-covering epithelium. The upper images show the average epithelium, lower images show thinner parts, and black lines represent magnified images. HE-staining.

B Nasopharynx-associated lymphoid tissue (NALT)-covering epithelium. The asterisk indicates epithelial cells with shorter or no cilia. HE-staining.

C Height of LDALT- or NALT-covering epithelium normalized to the diameter of a lymphocyte.

D, E Tight junction-composing protein of mucosal epithelial cells in the nasolacrimal duct (D) and nasal cavity (E). ZO-1⁺ reaction is observed on the apical side of epithelial cells in a linear pattern, and occludin⁺ reaction surrounded each epithelial cell along epithelial cells. White lines represent magnified images. Immunofluorescence.

Box plot represents the minimum, first quartile, median, third quartile, and maximum. n=6. Scale bars represent 100 μ m.

Fig. 4. Antigen-sampling cells in mucosa-associated lymphoid tissues (MALT) of MRL/MpJ (MpJ) and MRL/MpJ-*Fas*^{lpr/lpr} (Lpr) mice.

A GP2⁺ M cells in the nasolacrimal duct epithelium. M cells indicated by the arrowheads are observed in the epithelium of LDALT-covering and non-covering regions. White dotted lines indicate the epithelium and white lines surround the magnified images showing M cells. Immunofluorescence.

NLD: nasolacrimal duct. LDALT: Lacrimal duct-associated lymphoid tissue.

B Density of GP2⁺ M cells in the nasolacrimal duct epithelium normalized to the diameter of a lymphocyte. LDALT indicates the epithelium that covers lacrimal duct-associated lymphoid tissue (LDALT), and NLD indicates the epithelium that does not cover LDALT.

C GP2⁺ M cells in epithelium of nasal cavity. M cells indicated by the arrowheads are observed in the epithelium of LDALT-covering and non-covering regions. White dotted lines indicate epithelium and white lines surround magnified images showing M cells. Immunofluorescence. NC: nasal cavity.

D Density of GP2⁺ M cells in the nasal cavity epithelium normalized to the diameter of a lymphocyte. NALT indicates the epithelium that covers nasopharynx-associated lymphoid tissue (NALT), and Res indicates the epithelium that does not cover NALT.

E Schematic showing mucosa epithelium and GP2⁺ M cells. The area in which the total number of GP2⁺ M cells is estimated is indicated by the dotted line. This area is fixed through all the mice (70000 and 210000-lymphocyte area for NLD and NC, respectively).

F, G Total GP2⁺ M cell number in a certain epithelial area of nasolacrimal duct (F) or nasal cavity (G) that includes the LDALT- or NALT-covering area.

Box plot represents the minimum, first quartile, median, third quartile, and maximum. n=6. [#]*P*, **P* < 0.05, Mann–Whitney *U* test (#: vs. NALT, *: vs. MpJ). Scale bars represent 100 μm.

Fig. 5. Mucin-secreting cells in mucosa-associated lymphoid tissues (MALT) of MRL/MpJ (MpJ) and MRL/MpJ-*Fas*^{*lpr/lpr*} (Lpr) mice.

A PAS⁺ goblet cells in the epithelium of the nasal cavity respiratory mucosa. The arrowheads indicate goblet cells and black lines surround magnified images showing goblet cells. Goblet cells are more frequently observed in NALT-covering regions in both strains. PAS-staining.

B Density of PAS⁺ goblet cells in the epithelium of nasal cavity normalized to the diameter of a lymphocyte. NALT indicates the epithelium that covered the nasopharynx-associated lymphoid tissue (NALT), and Res indicates the epithelium that did not cover NALT.

C Total PAS⁺ goblet cell number in a certain epithelial area of the nasal cavity, equivalent to 210,000 lymphocytes, including the NALT-covering area.

Box plot represents the minimum, first quartile, median, third quartile, and maximum. n=6. [#]P, *P < 0.05, Mann–Whitney U test (#: vs. NALT, *: vs. MpJ). Scale bars represent 100 μm.

Fig. 6. Cell proliferation in mucosa-associated lymphoid tissues of MRL/MpJ (MpJ) and MRL/MpJ-*Fas^{lpr/lpr}* (Lpr) mice.

A Ki67⁺ proliferating cells in lacrimal duct-associated lymphoid tissue (LDALT) and nasopharynx-associated lymphoid tissue (NALT). Immunohistochemistry. The arrowheads indicate Ki67⁺ cells.

B Positive ratio of Ki67⁺ proliferating cells in LDALT and NALT.

C Total Ki67⁺ cell number in MALTs, estimated from the volume of each MALT and the density of Ki67 and normalized to the cross-sectional area of a single lymphocyte.

D “Bone-marrow connected LDALT (BM-LDALT)”. HE-staining. Bone marrow cells (e.g. metamyelocytes with nuclear indentation or notching), which is characterized by less condensed chromatin (indicated by white arrowheads) compared to mature lymphocytes (indicated by black arrowheads) localized in LDALT (panels ii and v), are observed in the bone marrow of both strains (panels i and iii) as well as in the BM-LDALT of Lpr mice (panel iv).

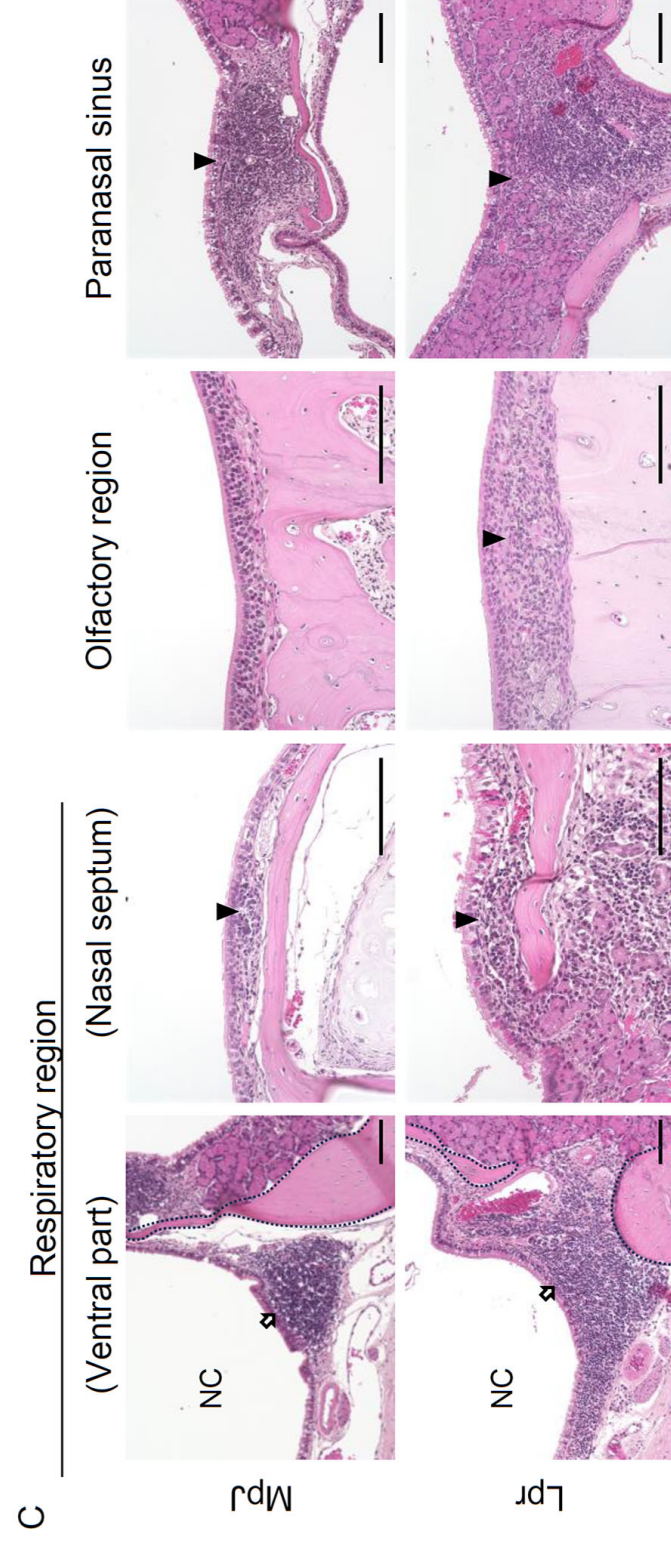
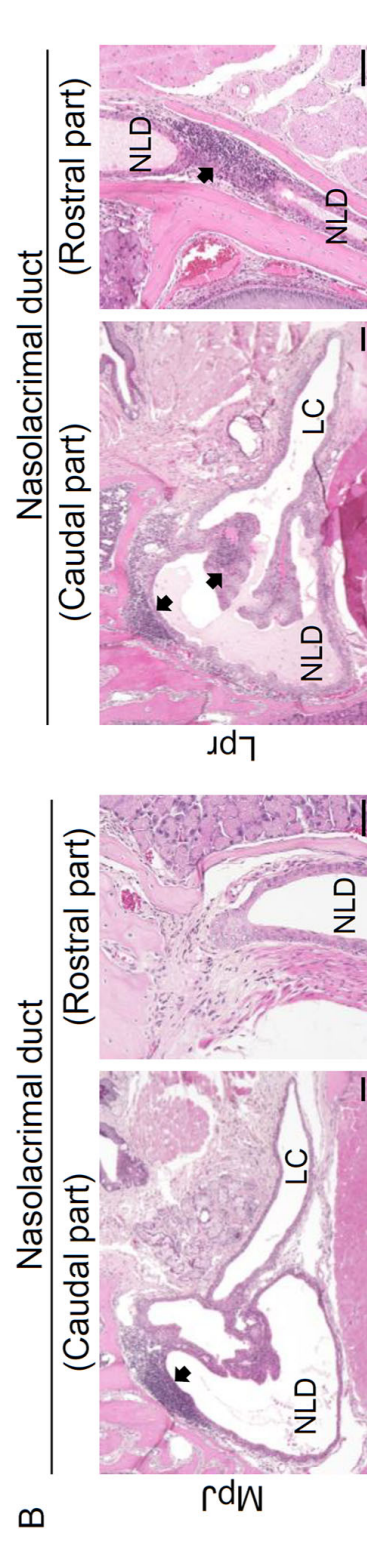
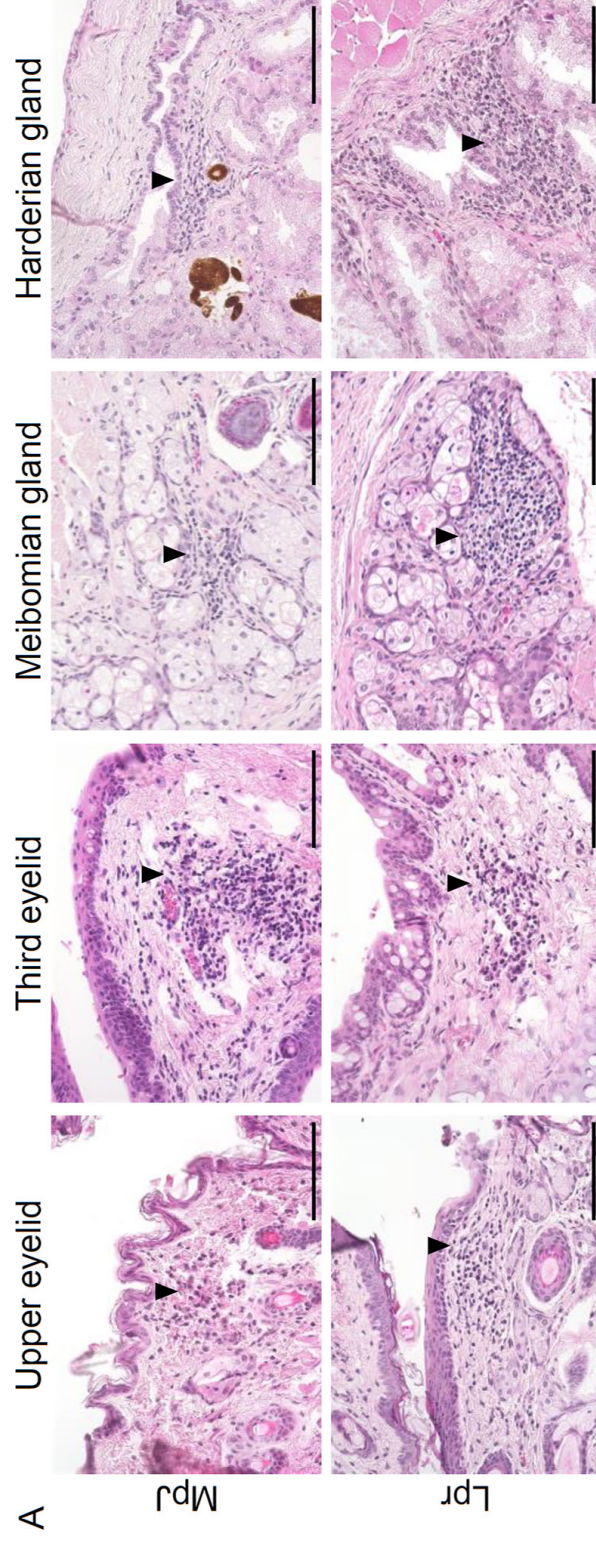
E Ki67⁺ proliferating cells in BM-LDALT. Immunohistochemistry. The black dotted line divides the bone-marrow-side half and the luminal-side half, and Ki67⁺ reaction is more frequently observed in the bone-marrow-side half.

Box plot represents the minimum, first quartile, median, third quartile, and maximum. n=6. **P* < 0.05, Mann–Whitney *U* test. Scale bars represent 50 μm (A), or 100 μm (D).

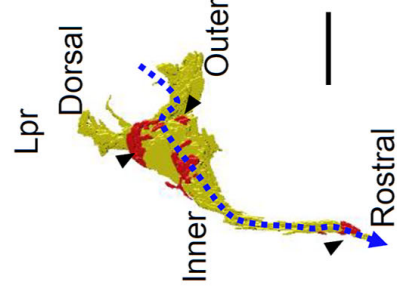
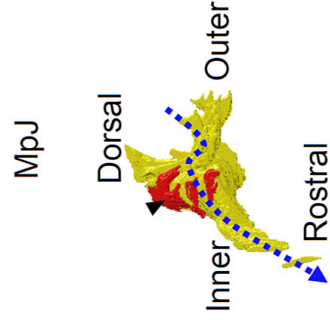
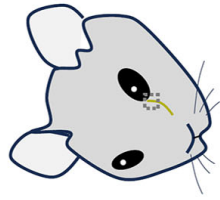
1 **Table 1. Lymphoid tissue numbers and prevalence in lacrimal apparatuses and nasal cavity of**
2 **MRL/MpJ (MpJ) and MRL/MpJ-*Fas^{lpr/lpr}* (Lpr) mice.**

	Conjunctiva		Meibomian gland	Harderian gland	
	Upper or lower eyelid	Third eyelid			
MpJ	(No./mouse)	0.25	1.50	0.33	0.67
	(%)	17 (1/6)	100 (6/6)	33 (2/6)	50 (3/6)
Lpr	(No./mouse)	0.50	1.83	1.83	1.83
	(%)	50 (3/6)	83 (5/6)	67 (4/6)	100 (6/6)
	Nasal cavity			Paranasal sinus	
	a) Respiratory region		Olfactory region		
MpJ	(No./mouse)	0.50	0.00	4.17	
	(%)	50 (3/6)	0 (0/4)	100 (6/6)	
Lpr	(No./mouse)	5.17	1.33	1.67	
	(%)	100 (6/6)	50 (3/6)	100 (6/6)	

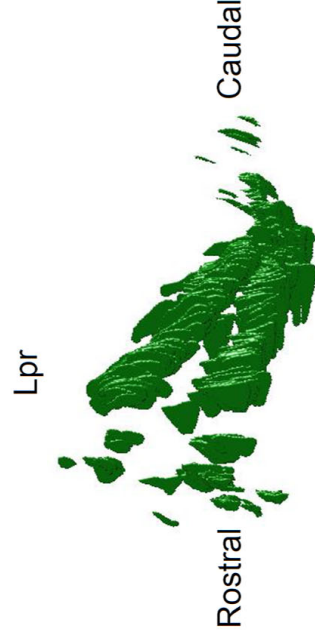
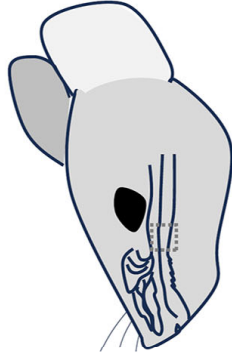
3 The numbers of lymphoid tissues in each lacrimal apparatus that were observed in one mouse on average
4 (No. /mouse) and ratios of mice that exhibited one or more lymphoid tissues in each lacrimal apparatus to
5 all the examined mice (%)
6 a) Nasopharynx-associated lymphoid tissue was excluded.



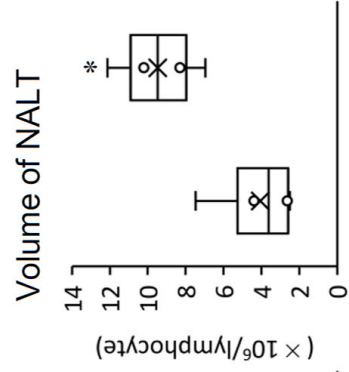
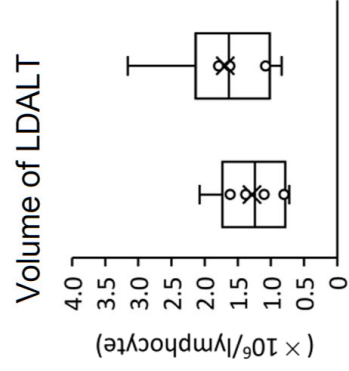
A LDALT



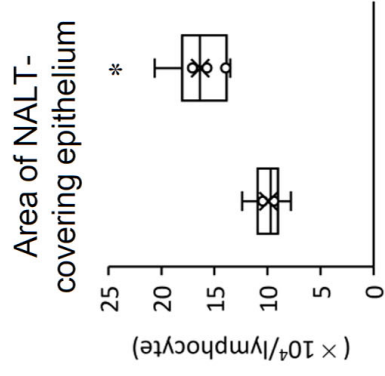
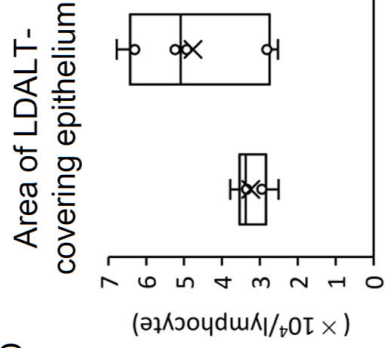
B NALT



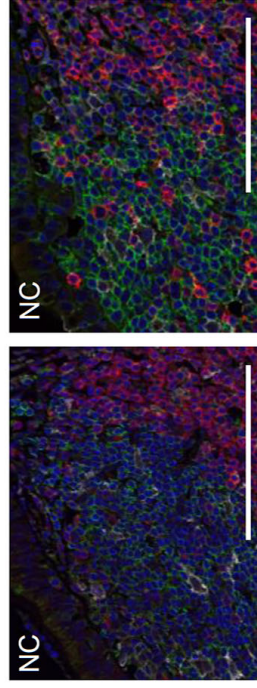
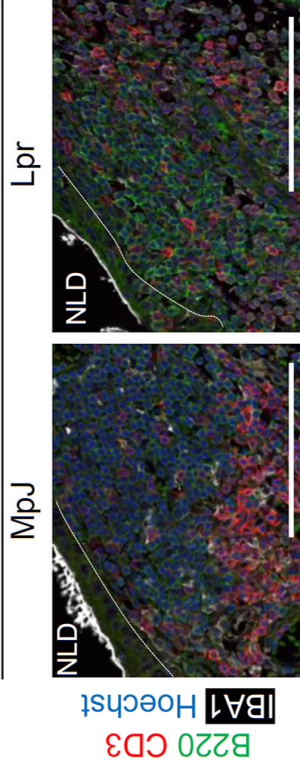
C



D



E



IBA1
B220
CD3
Hoechst

