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Autoimmune sialadenitis is associated with the upregulation of chemokine/chemokine receptor pairs in T cell-specific TRAF6-deficient mice

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ABSTRACT

Sialadenitis is an inflammatory condition affecting the salivary glands including the parotid, submandibular, and sublingual glands. There are several different types of sialadenitis, each with different sites of predilection. However, the pathogenic mechanism underlying the tissue specificity of sialadenitis is largely unknown. TRAF6 is a cytoplasmic adaptor protein that is necessary for the activation of dendritic cells in response to Toll-like receptor ligands, thereby regulating innate immune responses. We previously demonstrated that T cell-specific TRAF6-deficient mice (TRAF6ΔT mice) spontaneously develop systemic inflammatory disease. Here, we show that salivary secretion is reduced in TRAF6ΔT mice due to sialadenitis that occurs in the parotid and submandibular glands, but not the sublingual glands. Consistent with pathological findings, both $CD4^+$ and $CD8^+$ T cells predominantly infiltrated the submandibular glands; however, sublingual infiltration was rare in TRAF6ΔT mice. The TH1 cytokine IFN-γ, the TH1 cell attractant chemokine CCL2, and its cognate receptor CCR2 were upregulated concomitantly in both the submandibular and sublingual glands. Interestingly, the TH17 cell attractant chemokine CCL20 and its cognate receptor CCR6 were selectively increased in the submandibular glands, but not in the sublingual glands of TRAF6ΔT mice. Thus, the expression of TRAF6 in T cells might be implicated in tissue-specific sialadenitis by regulating the chemokine-chemokine receptor system.

1. Introduction

Sialadenitis is an inflammatory condition affecting the salivary glands. Several studies have shown that sialadenitis is caused by viral or bacterial infection, autoimmune disorders such as IgG4-related disease [1], primary and secondary Sjögren's syndrome, salivary calculus, and tumors [2]. Mammalian salivary glands consist of three pairs of major glands including the parotid, submandibular, and sublingual glands. These glands secrete serous, mucous, or mixed saliva into the oral cavity via the excretory ducts [3].

Each major salivary gland, which is composed mainly of acinar and duct cells, appears similar in macroscopic appearance but produces different types of saliva [4,5]. For example, the parotid glands produce a serous, watery secretion, the submandibular glands produce a mixed serous and mucous secretion, and the sublingual glands secrete saliva that is predominantly comprised of mucous. There are several different types of sialadenitis, each with different sites of predilection. For example, mumps and Heerfordt syndrome cause parotitis [6,7], whereas chronic sclerosing sialadenitis (Kuttner's tumor) is predominantly found in the submandibular glands [8]. Moreover, Sjogren's syndrome and Mikulicz disease are highly associated with both parotitis and submandibular sialadenitis [9,10]. However, the pathogenic mechanism underlying the tissue specificity of these types of sialadenitis is not fully understood.

Chemokines, which are small chemoattractant proteins that guide cellular migration, are highly involved in the development of sialadenitis [11–13]. For example, the expression of CXCL13 is upregulated in patients with Sjogren's syndrome [14]. In Mikulicz's

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disease, CCL18 secretion by M2 macrophages is critical for the development of severe fibrosis [15].

Upon Toll-like receptor (TLR) stimulation, TNF receptor-associated factor 6 (TRAF6) transmits signals to elicit an inflammatory response in myeloid cells via activation of nuclear factor κB (NF-κB) and MAP kinase pathways [16]. In addition, we have demonstrated that TRAF6 regulates T cell activation since T cell-specific TRAF6-deficient mice (TRAF6ΔT mice) spontaneously develop systemic inflammatory disease [17]. In TRAF6ΔT mice, multiple organs including the intestine, liver, lung, and kidney are infiltrated by mononuclear cells producing IL-4, which is accompanied by elevated serum IgE levels, indicating T helper type 2 (TH2)-skewed T cell responses.

In this study, we found that saliva secretion is reduced in TRAF6ΔT mice, which is associated with sialadenitis. Interestingly, sialadenitis was found to be localized to the parotid and submandibular glands, and not the sublingual glands. Submandibular sialadenitis was characterized by the infiltration of T cells accompanied by elevated levels of the T helper type 1 (TH1) cytokine IFN-γ and TH17 cytokine IL-17, whereas the TH1 cell attractant chemokine CCL2 and its cognate receptor CCR2 were upregulated in both the submandibular and sublingual glands. Interestingly, the TH17 cell attractant chemokine CCL20 and its cognate receptor CCR6 were selectively increased in the submandibular glands of TRAF6ΔT mice. Thus, in T cells, the loss of TRAF6 is critically implicated in T cell-dependent sialadenitis with predilection for the submandibular glands via inflammation-induced activation of the chemokine-chemokine receptor system. Our findings provide novel insights into the tissue-specific onset of autoimmune sialadenitis and show that TRAF6ΔT mice could be used as an effective model system for autoimmune sialadenitis.

2. Materials and methods

2.1. Mice

TRAF6ΔT (CD4-Cre; TRAF6fl/fl) mice were previously described [17].

Mice were maintained in a conventional environment and bred in-house at the Animal Facility of the Oita University. Age-matched TRAF6ΔT and control mice were used for each experiment. Genotyping was performed by PCR. All animal studies were approved by Oita University Animal Ethics Committee.

2.2. Histological evaluation of salivary gland inflammation

TRAF6ΔT and the control mice were sacrificed at > 12 weeks of age. The parotid, submandibular, and sublingual salivary glands were then fixed in 10% neutral buffered formalin for 24 h. Tissues were embedded in paraffin and sectioned to a thickness of 4 μm. After mounting on slide glasses, sections were deparaffinized and stained with hematoxylin and eosin dye. Each section was examined by microscopy.

To evaluate the severity of sialadenitis, inflammatory foci comprising more than 50 cells were counted. The sialadenitis score was assessed using the following scale: Score 1: no foci, Score 2: 1–4 foci, Score 3: >4 foci.

2.3. Immunohistochemical assessment of submandibular glands

Submandibular glands were removed and fixed with 4% paraformaldehyde overnight. After sucrose replacement, tissues were embedded in optimal-cutting temperature compound (Sakura, Tokyo, Japan). Samples were then quickly frozen in liquid nitrogen. The samples were cut into 5-μm sections and mounted on slide glasses. After fixing using ice-cold acetone and blocking for 30 min (Blocking one, Nacalai tesque, INC, Kyoto, Japan), samples were stained with the following antibodies for 30 min. Anti-CD4 (clone RM4-5, BD Biosciences, Franklin, NJ, USA) and anti-CD8 (clone 53–6.7, BD Bioscience, Franklin, NJ, USA) were used as primary antibodies. Anti-rat Alexa 488 and anti-rat Alexa 546 (Molecular Probes, OR, USA) were used as secondary fluorescent antibodies. All samples were washed with PBS and the nucleus was stained with 4′,6-diamidino-2-phenylindole (DAPI).

2.4. Saliva collection

Mice were anesthetized with a mixture of medetomidine, midazolam, and butorphanol, and intraperitoneally injected with pilocarpine hydrochloride (Wako 161-07201, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) at 0.5 μg/g bodyweight and isoprenaline hydrochloride (IPR) (SIGMA15627-5G, St. Louis, MO, USA) at 0.2 μg/g bodyweight of. Saliva samples were collected for 20 min from the oral cavity.

2.5. Quantification of cytokines and chemokines by real-time PCR

Total RNA from submandibular and sublingual glands was isolated using TRI REAGENT (Molecular Research Center, INC, OH, USA); reverse-transcription into cDNA was carried out using Verso cDNA Synthesis Kit (Thermo Fisher Scientific, INC, CA, USA). Real time PCR was performed using the SYBR green kit (KAPA SYBR FAST qPCR kit, Kapa Biosystem, Inc, Wilmington, MA, USA) with a LightCycler 96 (Roche, Merck, Darmstadt, Germany). Each mRNA level was normalized to *β-actin* mRNA expression. Amplification conditions were: 45 cycles of 95 °C (5 s) and 60 °C (30 s). The sequences of primers are listed in Supplementary Table S1.

2.6. Flow cytometric analysis

Submandibular glands were minced into 1–2-mm pieces in RPMI, and incubated at 37 °C with 0.5 mg/mL collagenase D (Roche) for 30 min to obtain single cell suspensions. Cell suspensions were filtered through a 200-μm strainer. After Fc receptor blockade, cells were stained using the following fluorochrome-conjugated antibodies: CD45-FITC (Clone 30-F11, BD Biosciences, Franklin Lakes, NJ,USA), CD3ε-PE (Clone 145-2C11, BD Biosciences), CD4-PE-Texas Red (Clone L3T4,Ly-4, Thermo Fisher Scientific), CD8a-PerCPcy5.5 (Clone 53–6.7, Tonbo Biosciences, Inc., San Diego, CA), and B220-PEcy7 (Clone RA3-6B2, Bio Legend, San Diego, CA). Samples were fixed with 4% Paraformaldehyde and then analyzed using a FACS fortessa X-20 flow cytometer (BD Biosciences). Data were analyzed with FlowJo software (BD Biosciences).

2.7. Statistical analysis

Data sets were analyzed for statistical significance by performing two-tailed Mann Whitney U or un-paired Student's t tests using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. Lymphocytes infiltrate into the submandibular and parotid glands of TRAF6ΔT mice

In TRAF6ΔT mice, swelling of the lymph node surrounding the hypoplastic sublingual and submandibular glands was easily identified by macroscopic observation (data not shown). We then performed histological analysis of the three major salivary glands.