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Original article

The role of cytotoxic T cells in IgG4-related dacryoadenitis and sialadenitis, the so-called Mikulicz's disease

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Abstract

Objectives

Immunoglobulin (Ig) G4-related dacryoadenitis and sialadenitis, the so-called Mikulicz's disease (MD), is a chronic inflammatory disease. However, little is known about its pathogenesis and pathological condition. In the present study, we used immunohistological techniques to compare the roles of cytotoxic T lymphocytes (CTLs) in MD and primary Sjogren's syndrome (SS). We examined the state of CTLs (cytotoxic granule–positive rate and programmed death-1 (PD-1) expression rate) in the salivary glands.

Methods

The study samples comprised 12 submaxillary glands from untreated MD patients and 12 labial glands from SS patients. We performed immunofluorescence and multicolor immunofluorescence to stain CD8, perforin (PRF), granzyme B (GZMB), and PD-1. We measured the total number of CTLs as well as the PRF^{+}CTLs, $GZMB^{+}$CTLs, and $PD\text{-}1^{+}$CTLs.

Results

We found that the degree of infiltration of CTLs was equal in MD and SS, but the rate of CTLs with cytotoxic granules, especially PRF, in MD was less than in SS. In addition, the frequency of $PD\text{-}1^{+}$CTLs in MD was higher than in SS.

Conclusions

Cytotoxic granule–positive CTLs were in the minority in MD salivary glands, and this regulation might relate to PD-1 signals like the state of exhaustion and anergy.

Introduction

Immunoglobulin (Ig) G4-related disease (IgG4RD) is a chronic inflammatory disease characterized by elevated serum IgG4 concentrations and tumefaction or tissue infiltration by IgG4-positive plasma cells. The most well-known manifestations of IgG4RD are dacryoadenitis and sialadenitis, so-called Mikulicz's disease (MD), and autoimmune pancreatitis [1, 2, 3, 4]. However, little is known about the pathogenesis and pathological conditions in this novel disease. The pathological condition in primary Sjogren's syndrome (SS), which causes chronic dacryoadenitis and sialadenitis, is relatively well understood, and SS is often compared with MD. The mRNA levels of T helper type 1 (Th1) and Th2 cytokines, interleukin 17 (IL-17), and IL-6 were higher in labial salivary glands from SS patients as compared to glands from healthy controls. On the other hand, the cytokine profile of MD was both Th2 and regulatory T (Treg) cells predominant [5, 6]. In addition, IL-21 is overexpressed in MD and involved in germinal center formation and IgG4 class switching [7]. IL-21 is a common gamma-chain cytokine like IL-2, IL-7, and IL-15 [8]. IL-21 is related to the proliferation and activation of $CD8^+$T cells (cytotoxic T lymphocytes, CTLs) [9, 10, 11]. The cytokine profiles of MD patients have been examined; but, to the best of our knowledge, the infiltration and activity of CTLs in MD patients have not yet been studied. Many CTLs were located around the acinar epithelial cells in SS [12], so there is no contradiction between higher Th1 cytokines and activated CTLs.

CTL functions (including cytotoxic granule expression rate, cytokine-producing ability, and anti-tumor immune response etc.) are regulated by a balance between co-stimulatory and co-inhibitory signals and impaired in some chronic inflammatory diseases, leading to exhaustion in viral infections and anergy in cancer [13, 14]. This CTL impairment induces both chronicity in viral infections and the suppression of anti-tumor immune responses [13, 14, 15, 16, 17, 18]. Programmed death-1 (PD-1) is highly expressed by functionally exhausted and anergic antigen-specific CTLs, and blockade of PD-1 signaling can rescue the functionality of these CTLs [19, 20, 21, 22, 23, 24, 25, 26, 27, 28]. Therefore, CTL functional impairment is closely related to PD-1 expression. To the best of our knowledge, the relationship between CTL functions and PD-1 expression in MD has not been previously examined. In SS, PD-1 was expressed in 13 % of CTLs in saliva [29], but the relationship between CTL functions and PD-1 expression is unclear.

In the present study, we used immunohistological techniques to determine the state of CTLs (cytotoxic granule–positive rate and PD-1 expression rate) in lesions of MD and SS. We found that while the degree of infiltration of CTLs in MD was equal to that in SS, the rate of CTLs with cytotoxic granules,

particularly PRF, in MD was less than in SS. In addition, the frequency of PD-1$^+$ CTLs in MD was higher than in SS. We discuss the role of PD-1 in CTLs in MD.

Patients & Methods

Written consent to use case information was obtained from all patients prior to enrollment, in accordance with the Declaration of Helsinki. The study was approved by our institutional review board (SMU 22-57, 24-155).

Salivary gland samples

The study samples comprised 12 submaxillary glands from untreated MD patients who had been diagnosed according to the Japanese diagnostic criteria for MD [2, 30] and who were registered in the Sapporo Medical University and Related Institutes Database for Investigation and Best Treatments of IgG4-RD (SMART) database. We studied 12 labial glands from SS patients who were diagnosed according to the American–European Consensus group classification criteria [31]. Patients currently or previously treated with glucocorticoids or immunosuppressive agents were excluded.

Immunohistochemistry

For immunohistochemical analysis, 4-μm formalin-fixed and paraffin-embedded sections were prepared and stained by using a conventional avidin-biotin complex technique and the Vision BioSystems Bond Polymer Refine Detection kit DS9800 (Leica Microsystems, Wetzlar, Germany), according to the manufacturer's protocol. Sections were incubated with the primary antibody for 15 minutes at room temperature. After washing, the sections were incubated with secondary antibody. In addition, the sections were stained with Vision BioSystems diaminobenzidine (DAB) (Leica Microsystems, Wetzlar, Germany) for 10 minutes and counterstained with hematoxylin for 3 minutes. The following monoclonal mouse antibodies (Abs) were used: anti-CD8 Ab (dilution 1 : 3, clone: N1592; Dako Denmark A/S, Glostrup, Denmark), anti-PRF Ab (dilution 1 : 60, clone: LS-C87866; Lifespan Biosciences, Seattle, WA, USA), and anti-GZMB Ab (dilution 1 : 25, clone: M7235; Dako Denmark A/S). Polyclonal rabbit anti-PD-1 Ab (dilution 1 : 75, clone: AHP1706; AbD Serotec, Oxford, UK) was also used. Microscopic images of specimens were obtained in JPEG format with a resolution of 2,040×1,536 pixels by DP Controller software (Olympus, Tokyo, Japan).

Multicolor immunofluorescence

After being deparaffinized with xylene and rehydrated, the 4-μm formalin-fixed and paraffin-embedded salivary gland sections were submerged in antigen retrieval reagent (pH 9.0) (code: 415211; Nichirei,

Tokyo, Japan) and heated at 105 °C for 15 minutes to retrieve the antigens, followed by incubation with enzyme (trypsin, pepsin and protease K) for 10 minutes. Nonspecific binding of antibodies was inhibited by incubation in 10 % normal goat serum for 30 minutes. The sections were incubated with pairs of mouse primary antibody and rabbit primary antibody, such as mouse anti-PRF Ab (dilution 1 : 40) or mouse anti-GZMB Ab (dilution 1 : 25) and rabbit anti-CD8 Ab (dilution 1 : 250, clone: EP1150Y; Epitomics, Inc. Burlingame, California), and mouse anti-CD8 Ab (dilution 1 : 3) and rabbit anti-PD-1 Ab (dilution 1 : 50) for 1 hour at room temperature, followed by conjugation of the appropriate pair of secondary reagents (highly cross-adsorbed Alexa Fluor 488 (or 594) goat anti-mouse IgG (H+L) or highly cross-adsorbed Alexa Fluor 594 (or 488) goat anti-rabbit IgG (H+L)) for 30 minutes. The staining profiles were obtained with a ConfoCor3LSM510META microscope (Carl Zeiss, Oberkochen, Germany) and the images were obtained in JPEG format with a resolution of 1,728×1,728 pixels by using ZEN 2011 software (Carl Zeiss, Oberkochen, Germany).

Measurement of %CTLs

We automatically counted the number of CTLs and total cells around the alveoli and ducts, which are areas of strong lymphocyte infiltration, in CD8-stained high power field (HPF) images (about /136,000 μm^2). The automatic computer-assisted image analyses were conducted with Java-based open-source image processing software ImageJ1.42r [32]. The methods were based on Väyrynen's program [33]. In this modified program, we separated DAB and hematoxylin colors from the raw images and used the DAB images for counting CTLs and the hematoxylin images for counting total cells. Universal threshold value and cell size for each series were used, and we determined the desired threshold level and cell size by using five images from each MD and SS glands. We selected the threshold level "Default" for all studies and cell size settings "0 - 400 $pixel^2$" for CTL counting, and "0 - 200 $pixel^2$" for total cells to be the same as results by manual counts. We defined %CTLs as the percentage of CTLs among total cells and then compared the mean of %CTLs in MD with SS.

Measurement of %PRF and %GZMB

We performed multicolor immunofluorescence in MD submandibular glands with a combination of PRF and CD8 and also GZMB and CD8 with fluorescent antibody. Next, we performed immunohistochemical analysis for detection of PRF, GZMB, and CD8 by using DAB for MD submandibular glands and SS labial glands. We measured the PRF-positive cells or GZMB-positive cells (manual count) in HPF images and counted the number of CTLs in the same field of serial sections in HPF images (automatic count). We defined %PRF and %GZMB as percentage of PRF-positive cells and GZMB-positive cells among CTLs

in the same field of serial section and compared the mean of %PRF, %GZMB in MD with SS.

Measurement of %PD-1

We performed multicolor immunofluorescence with a combination of PD-1 and CD8 for MD submandibular glands and SS labial glands. We measured %PD-1 as the percentage of PD-1$^+$CTLs among whole CTLs in images (about /200,000 μm^2). We compared the mean %PD-1 in MD with SS.

Statistical analyses

Results are expressed as mean ± SE. The Mann-Whitney U test was used for statistical analysis, and *P* values less than 0.05 were considered statistically significant. Microsoft Excel 2010 (Microsoft Corporation, Redmond, Washington, USA) was used.

Results

Patient characteristics

The mean serum IgG4 levels before treatment were 886.3 ± 218.4 mg/dl in MD patients (Table 1). Two patients in the MD group and 9 patients in the SS group were antinuclear antibody-positive. No MD patients were positive for anti-SS-A antibody or anti-SS-B antibody. Nine SS patients were positive for anti-SS-A antibody, and 2 SS patients were positive for anti-SS-B antibody, and 1 SS patient was positive for anti-centromere antibody.

CTL infiltration in MD salivary glands and SS labial glands

Many lymphocytes diffusely invaded the whole field, and multiple lymphoid follicle formations were recognized in MD submandibular glands (Fig. 1A). On the other hand, lymphocytes including CTLs had heavily invaded around the ducts and acini in labial glands of SS (Fig. 1B), but the infiltrations were focal.

Mean numbers of CTLs were 141.8 ± 21.4 cells/HPF in MD and 111.3 ± 15.7 cells/HPF in SS. Average numbers of total cells were 882.0 ± 69.2 cells/HPF in MD and 707.1 ± 43.2 cells/HPF in SS (Fig. 1C). Averages %CTLs were 16.5 ± 2.7 % in MD and 16.0 ± 2.3 % in SS (Fig. 1D). The mean number of total cells was significantly higher in MD than SS ($p = 0.038$), but there was no significant difference about CTLs. Many CTLs were heavily invading MD salivary glands, which was equivalent to the invasion in SS labial glands.

Low frequency of cytotoxic granule-positive CTLs in MD

We prepared serial section images of PRF-stained sections and CD8-stained sections in almost the same field in MD glands (Fig. 2A, B) and serial section images of GZMB and CD8 (Fig. 2E, F). Similarly, in SS glands we showed PRF and CD8 serial section images (Fig. 2C, D) and GZMB and CD8 serial section

images (Fig. 2G, H).

Most PRF-positive cells (Fig. 3A, B) or GZMB-positive cells (Fig. 3C, D) could be considered to be PRF^+CTLs or $GZMB^+$CTLs by multicolor immunofluorescence. The mean rates of %PRF were 14.3 ± 3.3 % in MD and 36.3 ± 7.5 % in SS, and the frequency of PRF^+CTLs in MD was significantly lower than in SS ($p = 0.018$) (Fig. 3E). Averages of %GZMB were 14.5 ± 3.5 % in MD and 27.1 ± 6.1 % in SS (Fig. 3F). Although the mean rate of %GZMB was not significantly different, it tended to be lower in MD than in SS ($p = 0.064$). From these results, it appears that the frequency of cytotoxic granule–positive CTLs, particularly PRF-positive CTLs, was lower in MD than in SS.

High frequency of PD-1^+CTLs in MD

A PD-1-stained image and CD8-stained image from almost the same field around a lymphoid follicle in MD were combined (Fig. 4A, B). PD-1 expression was recognized in some types of cells, including CTLs. We obtained averages of %PD-1 in MD and SS from the multicolor immunofluorescence images (Fig. 4C, D, E). Averages of %PD-1 were 28.7 ± 5.2 % in MD and 7.0 ± 2.3 % in SS, and PD-1^+CTLs were significantly more frequent in MD than in SS ($p = 0.0012$) (Fig. 4F).

Discussion

IgG4RD is a comparatively rare disease; in 2009, there were approximately 8,000 patients with IgG4RD in Japan [34]. Because the disease spectrum of IgG4RD was rather recently established, many unanswered questions remain regarding its cause and pathology. To the best of our knowledge, the present study is the first to examine CTLs in MD salivary glands. Fujihara showed that CTLs in SS were connected with apoptosis of acinar epithelial cells [12]. Therefore, there is no contradiction between higher Th1 cytokines and the condition of activated CTLs in SS. Elevated expression levels of Th1 cytokines were not previously identified in MD studies [5, 6]; therefore, we were surprised to find the same extent of CTL invasion in MD as in SS. IL-21 is overexpressed in MD [7] and related to increased CTLs [8, 9, 10, 11]. Thus, IL-21 might also contribute to the induction and maintenance of CTLs in MD.

The ultimate amplitude and quality of the T-cell response, which is initiated through antigen recognition by the T-cell receptor (TCR), is thought to be regulated by a balance between positive and negative co-stimulatory signals (that is, immune checkpoints) [13, 14]. PD-1 and cytotoxic T-lymphocyte–associated antigen-4 (CTLA-4) on T cells are famous inhibitory molecules, and regulate immune responses at different levels and by different mechanisms [14, 15, 16, 35]. The major role of PD-1 is to limit the activity of lymphocytes including CTLs in peripheral tissues during an inflammatory response to infection and to limit autoimmunity [13, 14, 15, 17, 36, 37, 38]. In contrast to PD-1, even

though CTLA-4 is expressed by activated $CD8^+$effector T cells, the major physiological role of CTLA-4 seems to be through distinct effects on the two major subsets of $CD4^+$T cells: downmodulation of Th cell activity and enhancement of Treg cell immunosuppressive activity [14, 39, 40, 41]. PD-1 is expressed on T and B cells, natural killer cells, monocytes, and dendritic cells [17], and its expression in T cells is inducible upon some stimulations (e.g. IL-2, IL-7, IL-12, IL-21 and interferon α) [8, 42, 43, 44]. The ligands for PD-1, PD-1 ligand 1 (PD-L1) and PD-L2, are members of the B7 family [45, 46].

PD-1 plays an interesting role in some chronic inflammatory diseases. Some chronic viral infections such as HIV, HBV, HCV [19, 20, 21, 22, 23, 24, 25, 26, 49, 50], and cancer [18, 27, 28, 47, 48], can lead to high levels of persistent PD-1 expression on CTLs, which induces a state of exhaustion or anergy among cognate antigen-specific CTLs. This state seems to be partially reversible by PD-1 pathway blockade [19, 20, 21, 22, 23, 24, 25, 26, 27, 28]. These data indicate that PD-1 might transmit an inhibitory signal that dominates TCR signaling and decreases T-cell responses during prolonged exposure to antigens in chronic infection and that PD-1 might play a key role in the control of CTL activation in peripheral tissues.

We compared the expression of PRF, GZMB, and PD-1 in CTLs infiltrating the salivary glands. The frequency of PRF^+CTLs was significantly lower in MD than in SS, and the frequency of $GZMB^+$CTLs tended to be low in MD. The frequency of PD-1^+CTLs was greater in MD than in SS. Therefore, the functions of CTLs, especially cytotoxic granules expression, might be impaired and/or inhibited, and it is possible that the deletion of CTL functions is closely related to PD-1-expression on CTLs in MD peripheral tissues.

Cytotoxic granules are important in the cytotoxic mechanism of CTLs [51]. High levels of cytotoxic granules have been implicated in the programmed cell death that occurs in SS peripheral tissues [12, 52]. IL-2 [53, 54] and IL-21 [9] promoted the production and release of cytotoxic granules in CTLs, and Treg cells reduced the function of CTLs [55, 56]. In addition, a lower frequency of CTLs containing cytotoxic granules and a relationship with the high frequency of PD-1^+CTLs was identified in a study of exhaustion CTLs [19, 20, 21, 25, 49], and incubation with anti-PD-L1 antibody caused increased production of cytotoxicity granules [19, 20].

There is no evidence in MD about the relationship between CTL functions and PD-1 expression. In SS, PD-1 expression was identified in about 13 % of CTLs in saliva [29], but unfortunately the relationship between CTL functions and PD-1 is unclear.

It was reported that PD-1 controls the molecules (CD3ζ, Zap70, PKC-θ, and PI3K) in TCR downstream

signaling [16, 17, 35, 57, 58, 59]; but, the direct pathway to transcription inhibition and the effect of PRF and GZMB production in CTLs were unclear. However, the low expression of cytotoxicity granules and the high expression of PD-1 on CTLs seem closely related [20, 21, 25, 49]. We think that CTLs in MD salivary glands are maybe derived and activated by IL-21, and PD-1 is up-regulated following CTL activation. CTLs are functionally suppressed by PD-1 signals, like in the exhaustion state.

Whether or not PD-1 affects CTLs directly and/or indirectly through changes in the cytokine microenvironment (decreased IL-2 and increased IL-10 etc.) must be determined [20, 25, 45, 57]. If PD-1 is up-regulated on $CD4^+$T cells, PD-1^+CD4^+T cells might contribute to the pathological condition of IgG4RD through IL-10 production. Because PD-1^+CD4^+ T cells seemed characterized by IL-10 production and lesser IL-2 production reportedly [60].

Furthermore, it should be determined if the up-regulation of PD-1 in CTLs could lead to an anti-apoptotic effect in the salivary gland. Regarding PD-1^+CTLs as a therapeutic target, a more protective treatment may be enabled if the effective activation of the PD-1 pathway is possible [61, 62, 63]. But, more knowledge is needed prior to the realization of PD-1 stimulation therapy, because the functions of PD-1 on hematocyte except CTL are unknown in MD [13, 16, 50, 64, 65].

Our study has some limitations. We studied the rate of cytotoxic granule–positive CTLs among whole CTLs, including not only PD-1^+CTLs but also PD-1^-CTLs. So, the influences of several factors such as the cytokine microenvironment and Treg cells may be mixed [9, 53, 54, 55, 56, 66]. In addition, it is necessary to examine how increased PD-1 expression and how decrease cytotoxic granules production in CTLs, because we did not observe any change over time.

Fortunately, early intervention often results in clinical recovery of the glandular secretion ability of each organ in IgG4RD [67, 68], but not in SS and chronic pancreatitis. So, cytotoxicity is lower in early IgG4RD, but fibrosis progresses over time and leads to irreversible organ damage [69]. The degree of CTL activity and the strength of PD-1 expression on CTLs might be relevant for determining the appropriate treatment. In conclusion, cytotoxic granule–positive CTLs were in the minority, and this regulation might be related to PD-1 signaling in MD salivary glands.

Conflict of interest None.

Figure legends

Table 1. Serologic, biochemical, and demographic characteristics of patients

The mean serum IgG4 levels before treatment were 886.3 ± 218.4 mg/dl in MD patients (n = 12). Two patients in the MD group and 9 patients in the SS group had antinuclear antibody levels greater than ×160. None of the MD patients had anti-SS-A antibody or anti-SS-B antibody. Nine SS patients were positive for anti-SS-A antibody, and 2 SS patients were positive for anti-SS-B antibody, and 1 SS patient was positive for anti-centromere antibody.

Figure 1.

Sections of an MD submaxillary gland (A, ×100) and an SS labial gland (B, ×100) were stained for CTLs. The mean number of total cells (black bars) and CTLs (gray bars) in MD (n = 12) and SS (n = 12) are shown in bar chart (C), and the average %CTLs are shown in the bar chart (D). Many CTLs invaded the salivary glands in MD and the labial glands in SS.

Figure 2.

Almost identical fields of serial sections of an MD gland were stained for PRF (A) and CD8 (B), and GZMB (E) and CD8 (F) (×400). Similarly, serial sections of an SS gland were stained for PRF (C) and CD8 (D), and GZMB (G) and CD8 (H).

Figure 3.

Multicolor immunofluorescence images of PRF-positive cells (CD8 (green), PRF (red), nucleus (blue)) (A, B) or GZMB-positive cells (CD8 (green), GZMB (red), nucleus (blue)) (C, D), which correspond to PRF^+CTLs (white arrows) or $GZMB^+$CTLs (white arrows), are shown. Mean rates of %PRF (E) and %GZMB (F) are shown. The frequencies of cytotoxic granule–positive CTLs, particularly PRF-positive CTLs, are lower in MD patients (n = 12) than in SS patients (n = 12).

Figure 4.

The combination of a PD-1-stained section and a CD8-stained section in almost the same field around a lymphoid follicle in MD is shown (A, B, ×200). The following MD images stained by multicolor immunofluorescence are shown (×630): CD8 (green) mono-stained image (C); PD-1 (red) mono-stained image (D); and CD8 (green), PD-1 (red) merged image (E). The average %PD-1 was significantly higher in MD (n = 12) than in SS (n = 12) (F).

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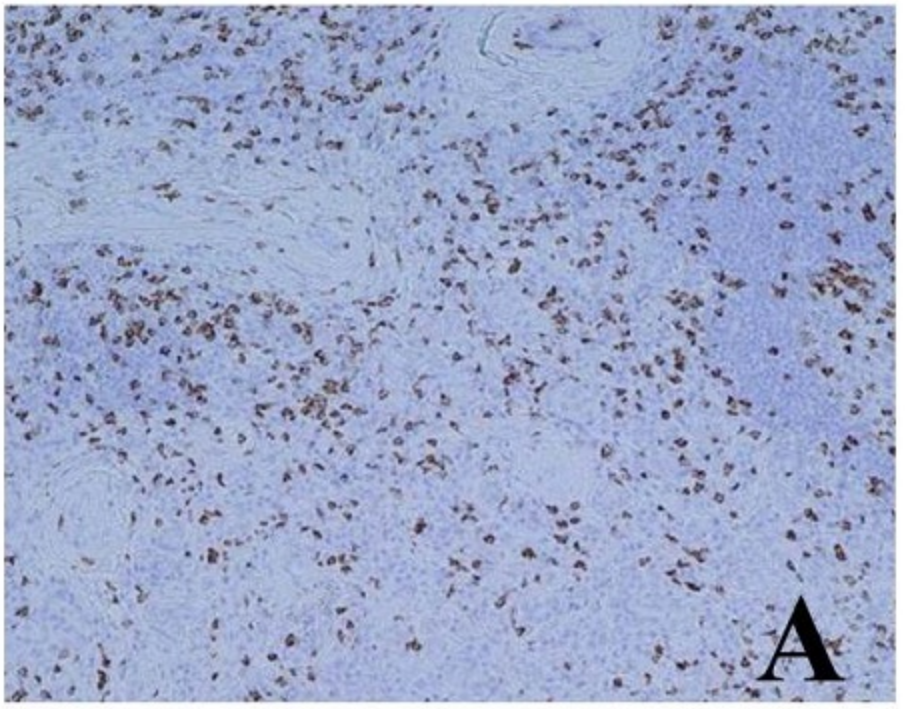
Table 1
Serologic, biochemical and demographic characteristics of patients

Patient	sex	age	IgG4 (mg/dl)	ANA	anti-SS-A ab
MD01	F	68	164	-	-
MD02	F	58	1230	-	-
MD03	F	63	160	-	-
MD04	M	62	968	-	-
MD05	M	75	1480	+	-
MD06	M	81	167	+	-
MD07	F	55	870	-	-
MD08	M	70	1420	-	-
MD09	M	64	2210	-	-
MD10	M	65	548	-	-
MD11	M	71	507	-	-
MD12	M	68	911	-	-
SS01	F	48	ND	+	-
SS02	F	16	ND	-	+
SS03	F	71	ND	+	+
SS04	F	53	13.2	+	+
SS05	F	47	ND	+	+
SS06	F	39	4.0	+	+
SS07	F	56	11.4	+	-
SS08	F	48	ND	-	+
SS09	F	47	ND	+	+
SS10	F	62	19	+	-
SS11	F	57	ND	+	+
SS12	F	72	ND	-	+

MD, IgG4-related dacryoadenitis and sialadenitis;
SS, primary Sjogren's Syndrome; ANA antinuclear antibody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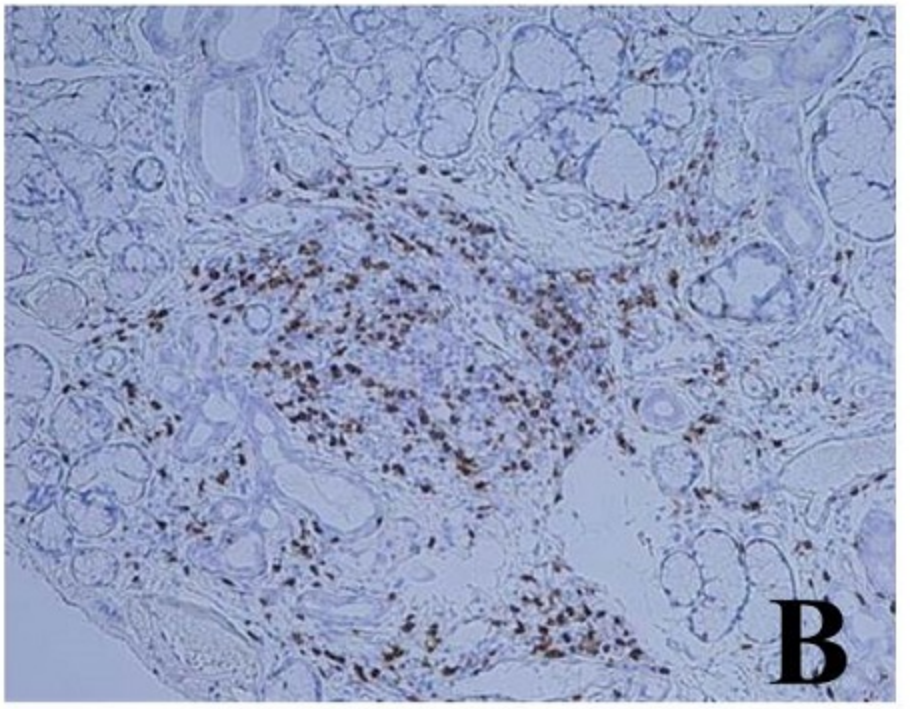
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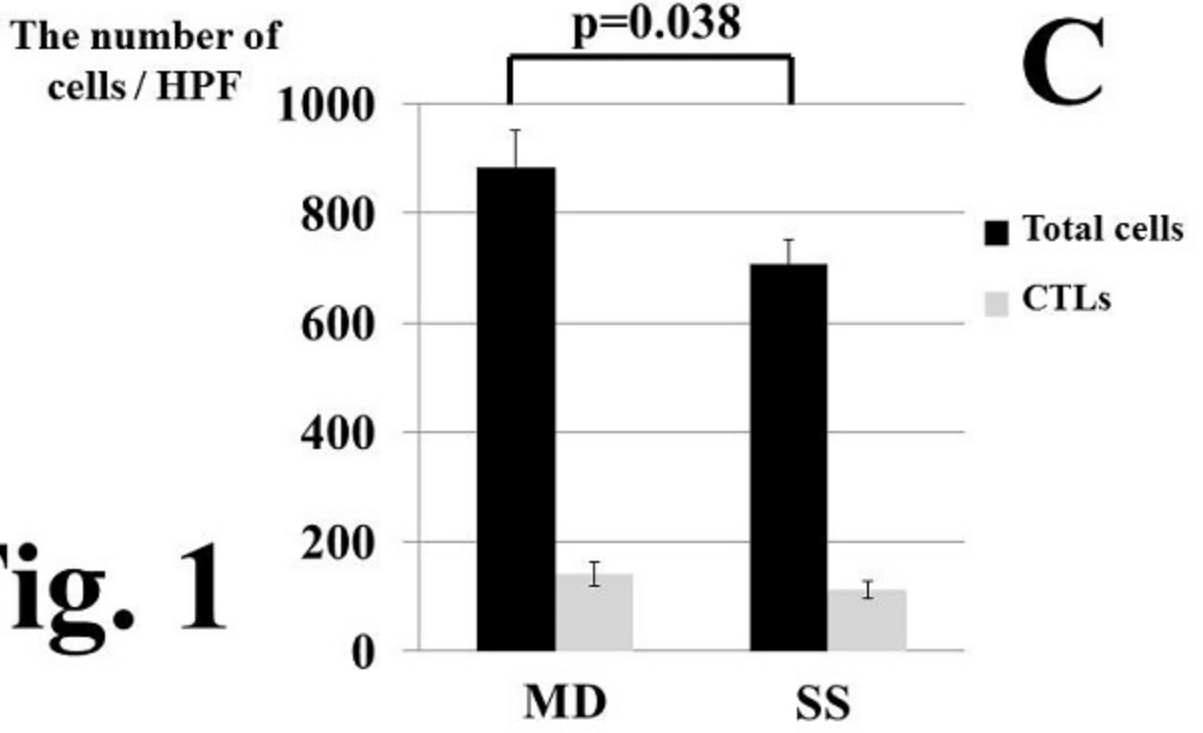
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MD
SS
Total cells
CTLs

C

D

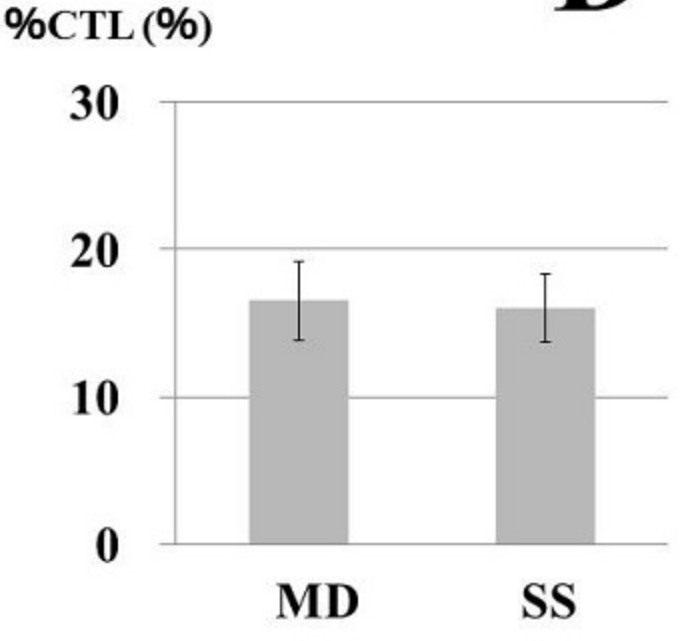

%CTL (%)
30
20
10
0
MD
SS

Fig. 1

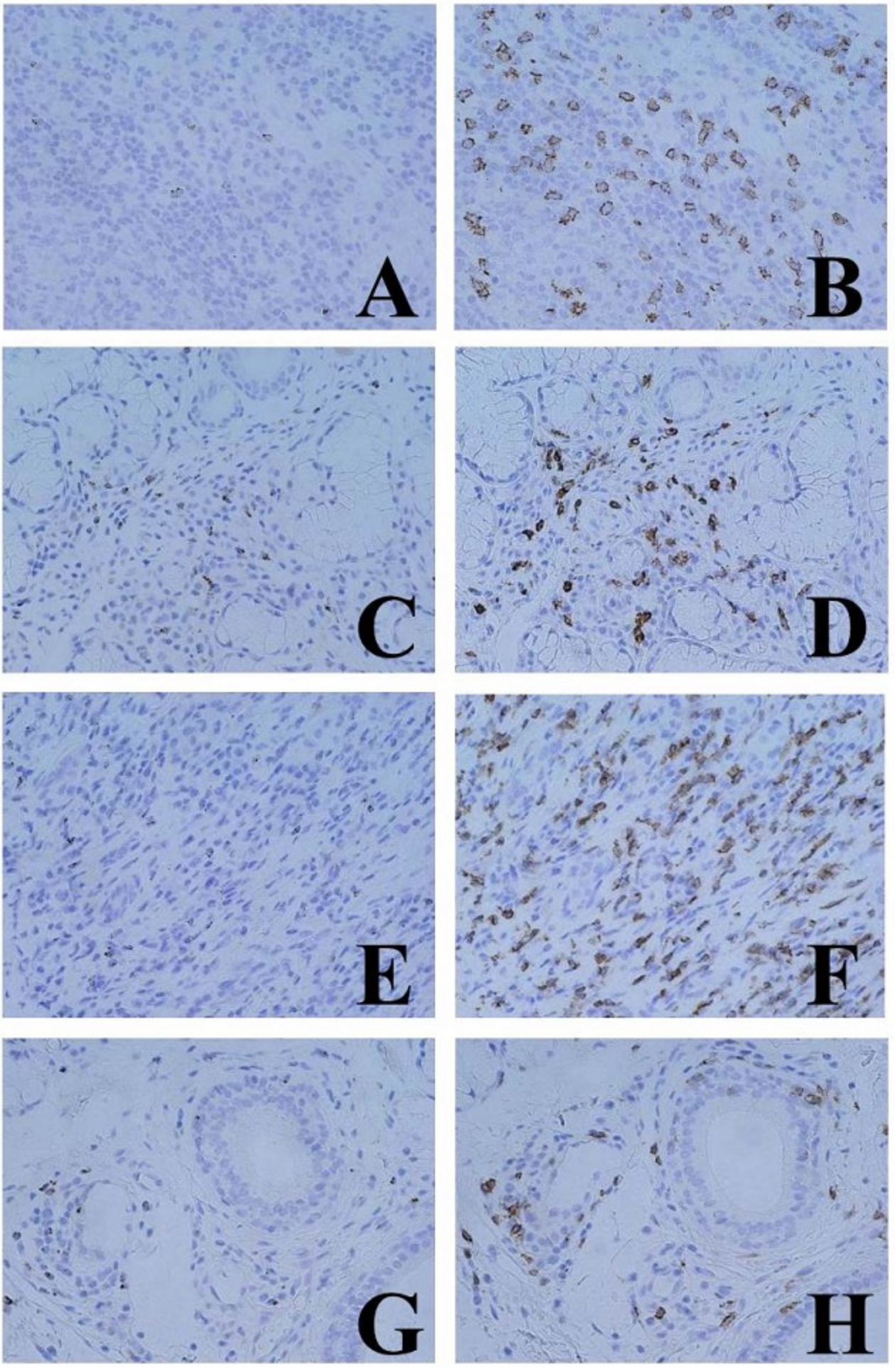
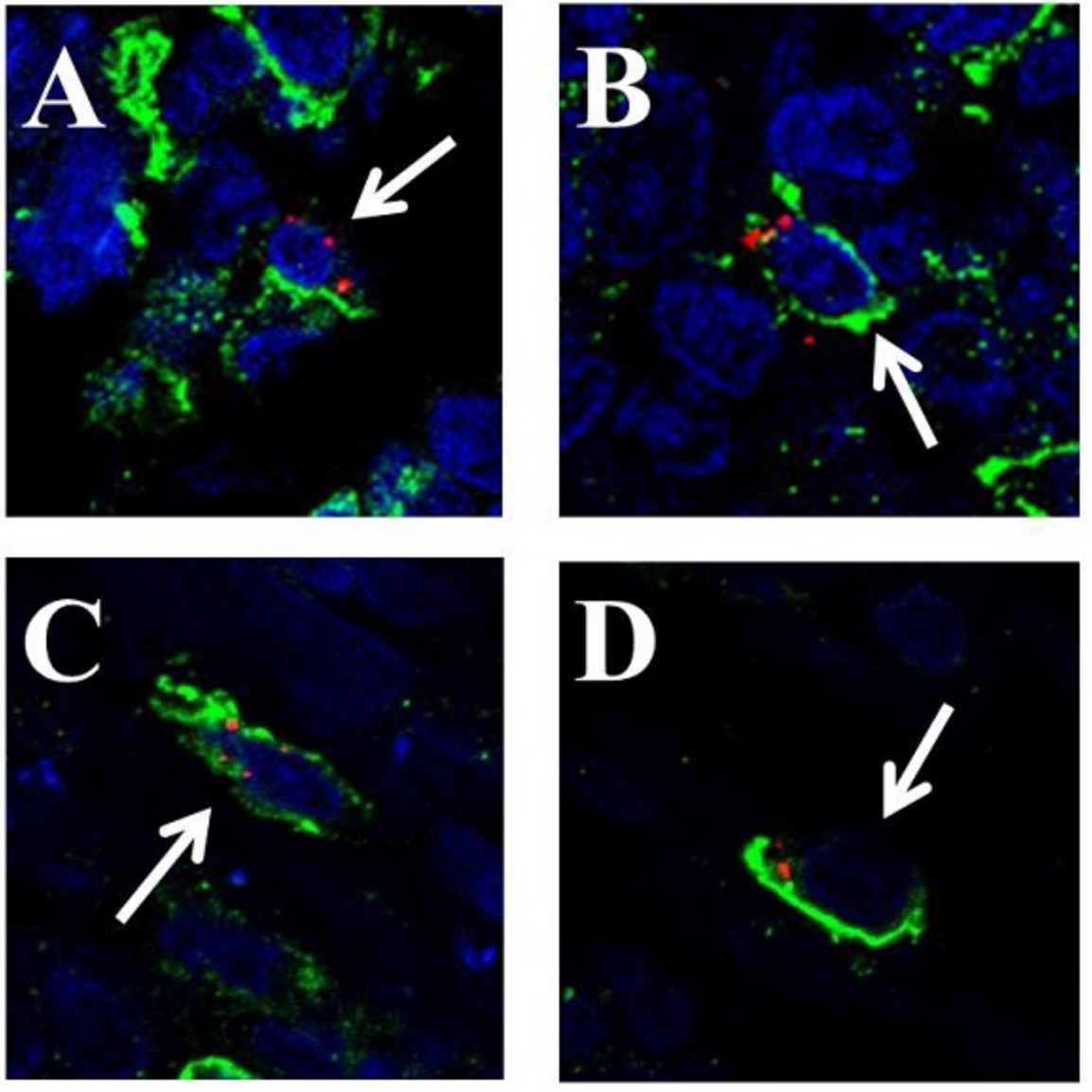


Fig. 2

Fig. 3



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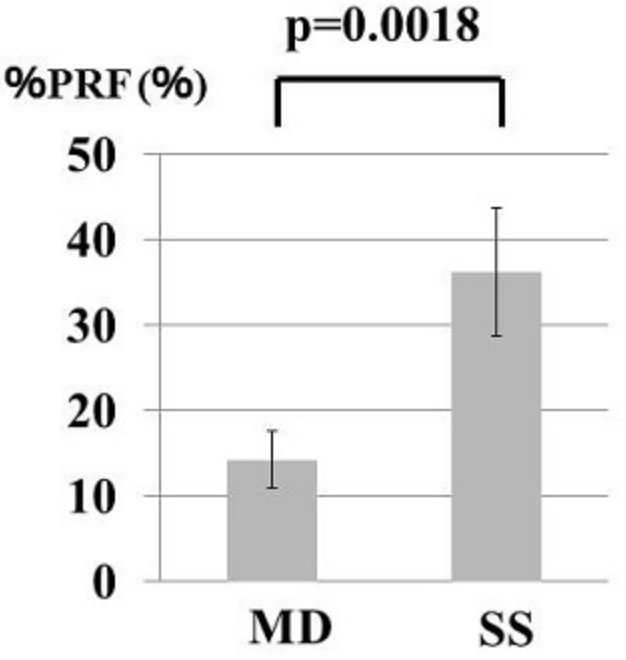

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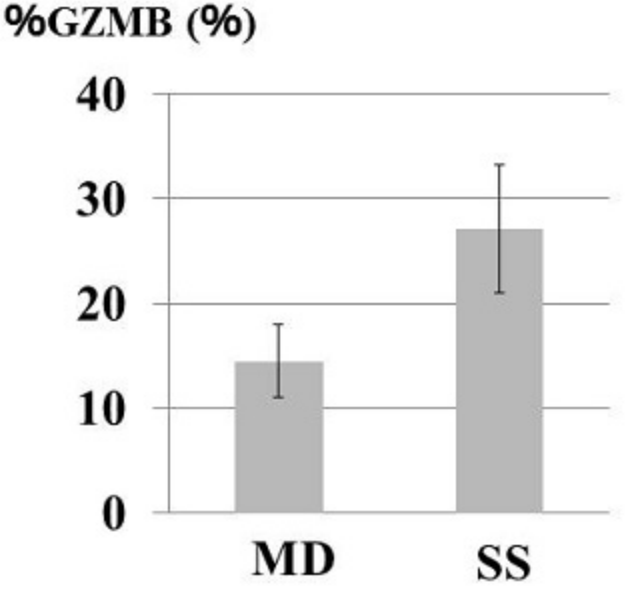

Fig. 4