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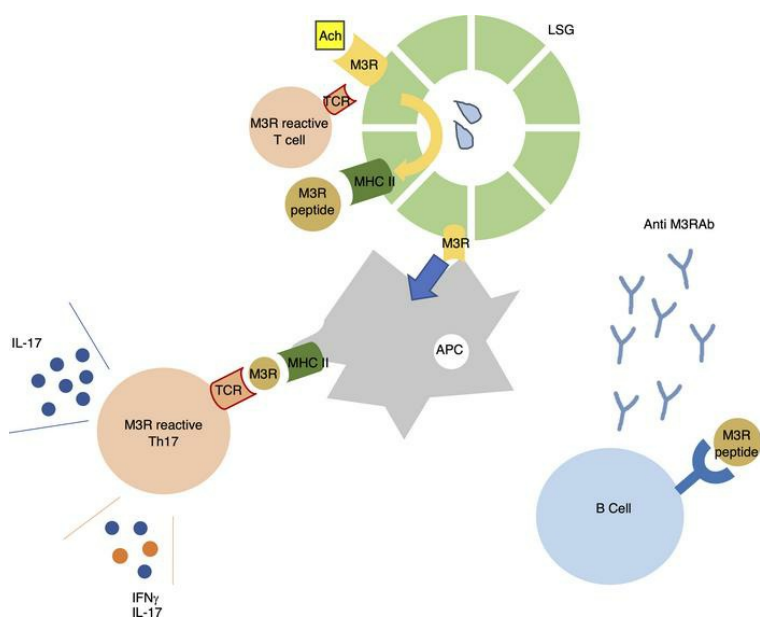
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Research Article

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### Graphical abstract



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# M3 muscarinic acetylcholine receptor–reactive Th17 cells in primary Sjögren’s syndrome

Saori Abe,<sup>1</sup> Hiroto Tsuboi,<sup>1</sup> Hanae Kudo,<sup>1</sup> Hiromitsu Asashima,<sup>1</sup> Yuko Ono,<sup>1,2</sup> Fumika Honda,<sup>1</sup> Hiroyuki Takahashi,<sup>1</sup> Mizuki Yagishita,<sup>1</sup> Shinya Hagiwara,<sup>1</sup> Yuya Kondo,<sup>1</sup> Isao Matsumoto,<sup>1</sup> and Takayuki Sumida<sup>1</sup>

<sup>1</sup>Department of Internal Medicine, Faculty of Medicine, University of Tsukuba, Ibaraki, Japan. <sup>2</sup>Faculty of Dental Science, Kyushu University, Fukuoka, Japan.

M3 muscarinic acetylcholine receptor (M3R) is one of the autoantigens associated with Sjögren’s syndrome (SS) and is localized in exocrine glands where disease-specific inflammation occurs. The inflammatory lesion is characterized by infiltration of CD4<sup>+</sup> T cells, including clonally expanded Th17 cells. We undertook this study to identify circulating M3R-specific Th17 cells and to determine functional properties of those cells. Using the enzyme-linked immunospot assay (ELISpot) method, we identified M3R-reactive Th17 cells in the peripheral blood of patients with primary SS (pSS). Among 10 examined pSS patients, 10 healthy subjects (HS), and 5 IgG4-related disease (IgG4-RD) patients, M3R-reactive IL-17 secreting cells were significantly increased in 5 pSS patients specifically. The most common T cell epitope, which was analyzed and confirmed by coculture of isolated CD4<sup>+</sup> T cells with antigen presenting cells plus M3R peptides in vitro, was peptide 83-95 of M3R. Peptide recognition was partly in an HLA-DR-restricted manner, confirmed by blocking assay. M3R-reactive Th17 cells positivity correlated with higher titers of anti-M3R antibodies, whose systemic disease activity score tended to be higher. Our studies highlight the role of tissue-specific autoantigen–derived circulating Th17 cells in pSS, for which further work might lead to antigen-specific targeted therapy.

## Introduction

Sjögren’s syndrome (SS) is an autoimmune disease characterized by lymphocytic infiltration into the lacrimal and salivary glands (1), mainly consisting of T lymphocytes and B lymphocytes, and the T/B cell ratio negatively correlates with lesion severity (2). Among the infiltrating T lymphocytes, different subsets of CD4<sup>+</sup> helper T cells, Th1, follicular T helper cells (T<sub>fh</sub>), and recently Th17 cells are reported to play pathological roles in primary SS (pSS) (3). IL-17, a cytokine secreted by Th17 cells, was detected in the salivary glands of patients with SS, predominantly in infiltrating CD4<sup>+</sup> T cells (4). In addition, Th17 cells, as defined by their chemokine receptor expression profile (CD4<sup>+</sup>CD45RA<sup>+</sup>FoxP3<sup>−</sup>CXCR5<sup>+</sup>CXCR3<sup>−</sup>CCR4<sup>+</sup>CCR6<sup>+</sup>) are increased in the peripheral blood of pSS with moderate disease activity compared with healthy controls (5). These observations suggest that circulating Th17 cells play a pathological role in pSS and correlate with disease severity, though their antigen specificity, and how they relate to pSS clinical features, remain unknown.

Studies on TCR on infiltrating T cells have shown clonal expansion of certain T cells, suggesting that pSS is an autoimmune disease, with autoantigen-specific T cells contributing to the progression of the disease (6). Furthermore, recent genome-wide association studies have reported that the major histocompatibility complex (MHC), including HLA-DRB1 locus, is a disease-associated gene in the Han Chinese population (7, 8), suggesting that interaction between a specific autoantigen presented by HLA-DRB1 and a specific TCR seems essential to the autoimmune pathology of pSS. Moreover, single cell analysis of ex vivo infiltrating T cells demonstrated that activated Th17 cells in pSS showed restricted complementarity-determining region 3 $\alpha$ -specific (CDR3 $\alpha$ -specific) motif (9), suggesting the possibility of antigen-driven selection of specific Th17 cells. Several candidate autoantigens that can be recognized by autoreactive T cells in SS have been reported, including M3 muscarinic acetylcholine receptor (M3R) (10). M3R is expressed in exocrine glands and plays an important role in exocrine gland secretion (11).

**Conflict of interest:** The authors have declared that no conflict of interest exists.

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**Table 1. Background profiles of healthy subjects (HS) and patients with pSS and IgG4RD**

	HS (n = 10)	pSS (n = 10)	IgG4-RD (n = 5)	P value (HS vs. pSS)	P value (IgG4-RD vs. pSS)
Age (years)	44.7 ± 11.4	59.8 ± 20.9	60.8 ± 10.0	0.16	0.87
Sex (male/female)	3/7	2/8 <sup>A</sup>	4/1 <sup>A</sup>	0.70	0.04
HLA-DRB1 genotype					
01:01	0	2	2	0.24	0.40
04:03	2	1	0	0.50	0.66
04:05	4	3	1	0.50	0.59
04:06	0	0	1	1.00	0.33
04:10	0	1	0	0.50	0.66
08:02	0	3	1	0.11	0.59
08:03	1	3	0	0.30	0.28
09:01	4	1	1	0.17	0.56
12:02	0	0	1	1.00	0.33
13:02	0	2	1	0.24	0.71
14:06	2	0	0	0.24	1.00
15:01	2	3	2	0.50	0.55
15:02	4	1	0	0.17	0.66
16:02	1	0	0	0.50	1.00

All participants were checked for HLA-DRB1 type to predict MHC binding peptides using Immune Epitope Database (IEDB). The healthy subjects were matched for age-, sex-, and HLA-DRB1 to pSS patients. The significantly higher proportion of males in the IgG4-RD group is due to the sex distribution of the disease. Age: <sup>A</sup> $P < 0.05$  by Kruskal Wallis test. Sex, HLA typing: <sup>A</sup> $P < 0.05$  by Fisher's exact test.

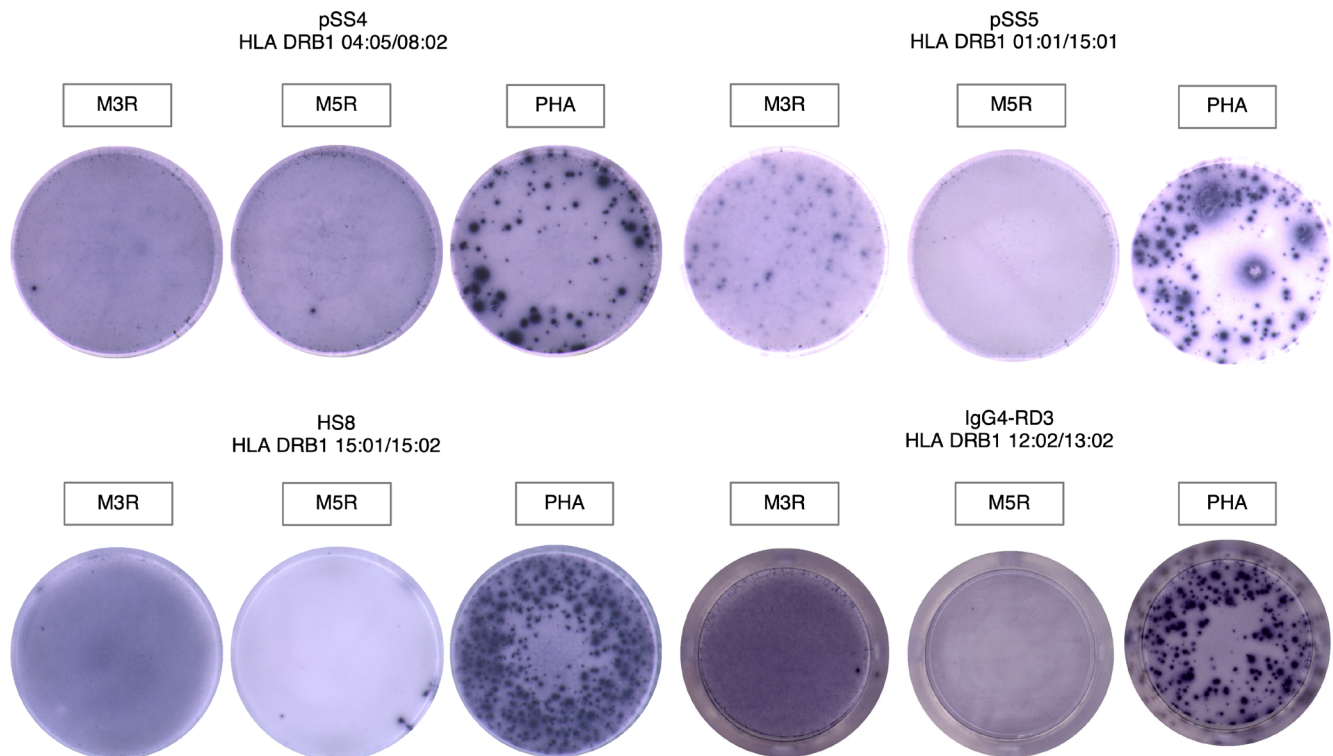
In this regard, we previously reported the presence of anti-M3R antibodies against each of the 4 extracellular domains of the M3R (12) and also identified the presence of M3R-reactive CD4<sup>+</sup>IFN- $\gamma$ -producing helper T (Th1) cells in the peripheral blood of SS patients (13). These findings suggest that M3R-reactive B cells and Th1 cells could play pathogenic roles in pSS. Importantly, studies in the SS mouse model, which is induced by immune response to M3R (14), have confirmed the pathological roles of M3R-reactive Th1 and Th17 cells in the development of autoimmune sialadenitis (15, 16).

The above background emphasizes the potentially significant role of autoantigen reactive Th17 cells in pSS and that M3R is a candidate autoantigen, although the detection of M3R-reactive Th17 cells has never been confirmed. Thus, the purpose of this study was to identify circulating M3R-reactive Th17 cells and to examine the relationship between these cells and clinical features of pSS.

## Results

**Subject characteristics.** We recruited 10 patients with pSS who met Japanese (17) and internationally accepted criteria (18) for SS, 10 healthy subjects, and 5 patients with IgG4-related disease (IgG4-RD) who were matched by age and HLA-DRB1 genotype to the pSS group. However, we could not match the sex distribution between the pSS and IgG4-RD groups, which was mainly due to the difference in the sex-related predilection of each disease; pSS is known to affect mainly females, whereas IgG4-RD more often affects males (Table 1). Supplemental Table 1 (supplemental material available online with this article; <https://doi.org/10.1172/jci.insight.135982DS1>) shows the results of HLA-DRB1 typing and top 10 highly ranked predicted epitope peptides among the full length of M3R peptide of the pSS patients and the 2 control groups.

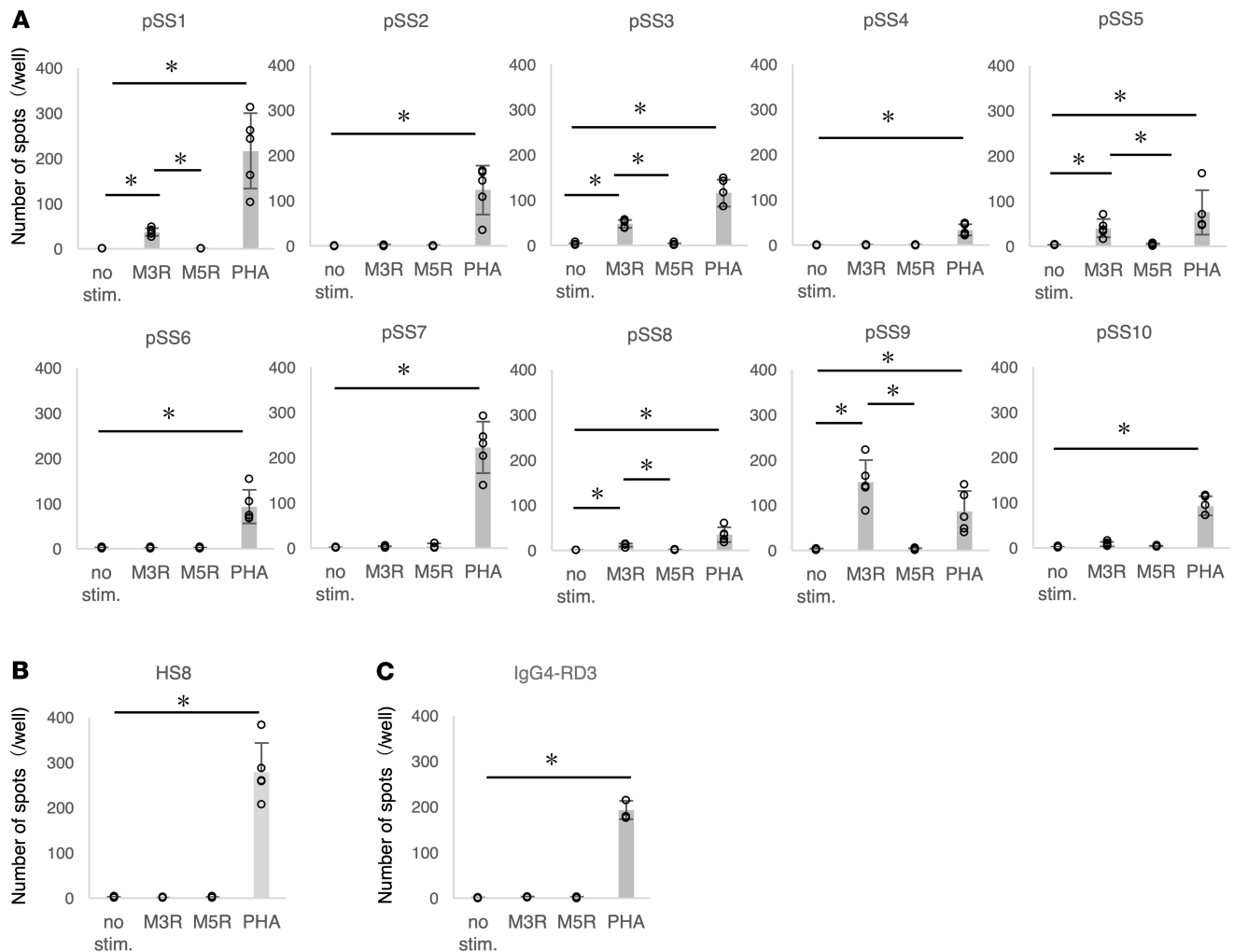
**Detection of M3R-reactive IL-17-secreting cells by ELISpot assay.** To detect M3R-reactive circulating IL-17 secreting cells in the peripheral blood, IL-17 enzyme-linked immunospot assay (ELISpot) assay was performed after culture of PBMCs with a mixture of selected M3R peptides according to subject HLA-DRB1 typing (Supplemental Table 1). Among the 10 pSS patients, 5 patients showed significantly increased spots after incubation of PBMCs with the selected M3R peptide mixture (50% total), compared with the number of spots after nonstimulation (Figure 1 and Figure 2A). Meanwhile, neither the healthy control subjects nor the IgG4-RD patients showed significantly increased spots after culture of PBMCs with M3R peptides (Figure 1 and Figure 2, B and C). Thus, we could identify HLA-DR-related M3R-specific IL-17-secreting cells in peripheral blood of pSS, and the proportion of M3R-reactive IL-17 secreting cells was significantly higher in pSS compared with the healthy subjects ( $P = 0.03$ ) (Figure 1 and Figure 2).



**Figure 1. Representative photomicrographs of ELISpot assay.** Peripheral blood mononuclear cells were incubated with a mixture of selected M3R peptides according to the HLA-DRB1 type of participants, M5R peptides (control peptides), or 1% PHA (positive control) for 40 hours. Five replicate wells were examined for each condition. Representative photomicrographs of M3R-reactive IL-17-producing cells in a negative pSS patient (pSS4), positive pSS patient (pSS5), HS (HS8), and IgG4-RD patient (IgG4-RD3) is shown. In patient pSS5, note the higher number of spots after stimulation with the indicated mixture of the selected M3R peptides, whereas no substantial increase in the spots was noted after M5R peptides stimulation. In patients pSS4, HS8, and IgG4-RD3, no spots were noted after stimulation with either M3R or M5R peptides.

*Induction of M3R-reactive IL-17 secretion by M3R AA83-95 peptide.* Based on the above findings, we next sought to identify the main peptide responsible for triggering IL-17 secretion. For this purpose, a mixture of the selected top 10 ranked M3R peptides used for stimulation in the previous exam were separately used for stimulation of samples positive for M3R-specific IL-17-secreting cells. By stimulating candidate M3R peptides separately, to our surprise, we found that 1 peptide, M3R AA83-95, triggered IL-17 secretion in all 5 pSS patients, and a significantly larger number of spots was noted after incubation of PBMCs with M3R peptides, compared with nonstimulation (Figure 3). One pSS patient (pSS8) with HLA-DRB1 09:01/15:01, also reacted to another peptide, M3R AA76-87. This indicates that recognition of M3R AA76-95 could be the trigger for IL-17 secretion because these 2 peptides have 7 overlapping amino acids. Despite the lack of statistical significance, another patient (pSS3), who also possessed HLA-DRB1 15:01 in common with pSS8, tended to show a larger number of spots after M3R AA76-87 peptide stimulation (Figure 3). Overall, these findings suggest that M3R-specific IL-17 secretion is induced mainly by M3R AA83-95 and that these peptides were probably presented by several HLA alleles, including HLA-DRB1 15:01 in pSS patients.

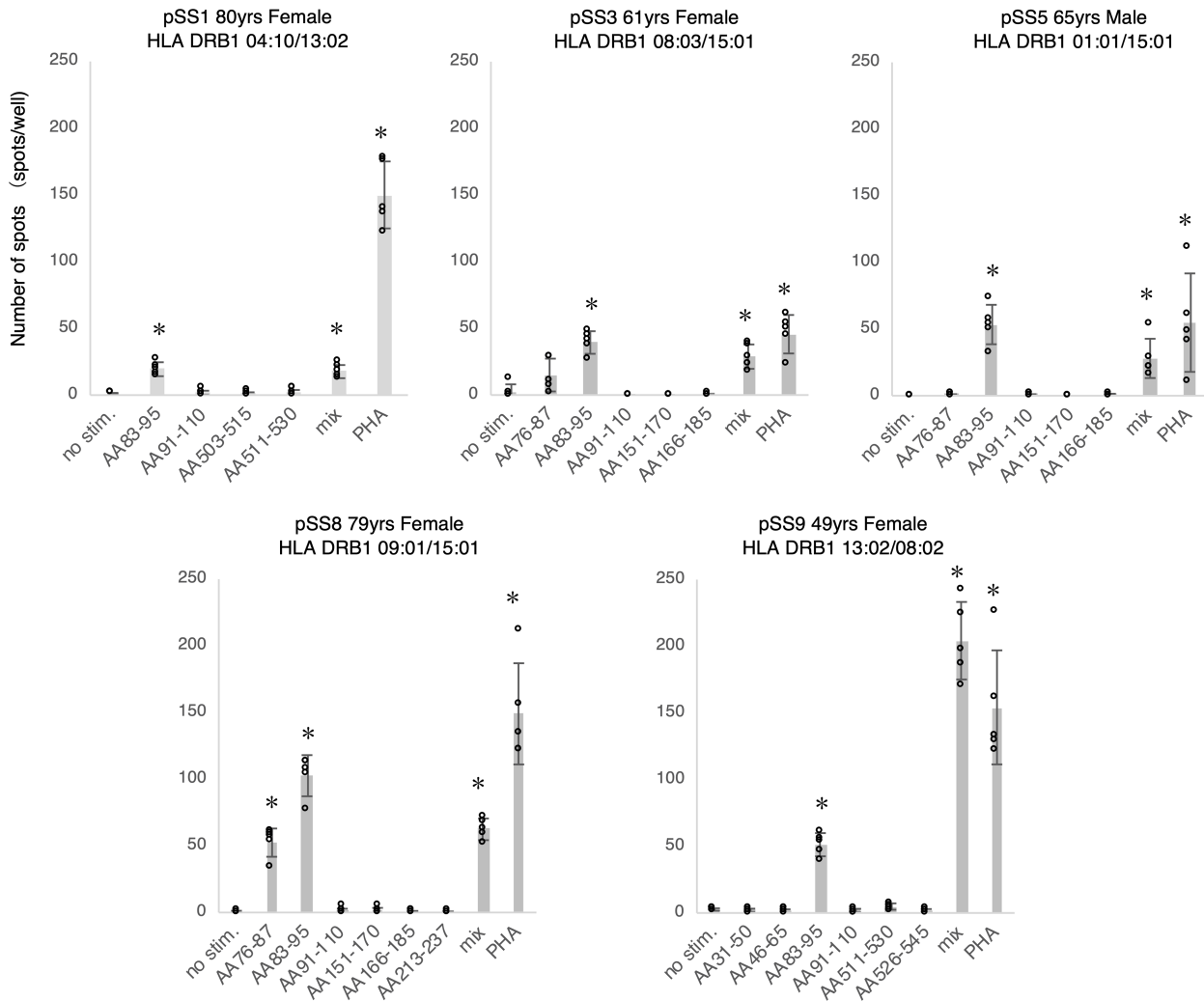
*M3R-reactive IL-17-secreting cells were CD4<sup>+</sup> Th17 cells.* IL-17 has been shown to be secreted by several different types of cells in peripheral blood, such as CD8<sup>+</sup> cells, innate lymphocytes including  $\gamma\delta$ T cells, NK cells, group 3 innate lymphoid cells, and Th17 cells (19). Therefore, to determine that the spotted IL-17-secreting cells were Th17 cells, isolated CD4<sup>+</sup> T cells were cocultured with the monocyte-derived autologous DCs and stimulated with main identified M3R peptides AA83-95 as described above. Three pSS patients positive for peripheral M3R-reactive IL-17-secreting cells and able to provide enough samples were examined. As expected, all 3 patients had significantly larger numbers of spots after M3R stimulation compared with no stimulation, confirming that the detected spots were M3R-reactive Th17 cells (Figure 4, A and B). To assess HLA restriction of M3R-specific Th17 cells, HLA-DR blocking was performed in the above-mentioned 3 patients, which showed a significant decrease in the number of spots after incubation with HLA-DR antibody, compared with isotype control (Figure 4C and Supplemental Figure 1). These results confirm that



**Figure 2. Statistical analysis of spot counts between culture conditions.** Ten pSS patients examined, and representative data of HS and IgG4-RD patients is shown. **(A)** Analysis of ELISpot of IL-17-producing cells in pSS ( $n = 10$ ). Results are expressed as mean spot counts of 5 replicate ELISpot microwells, each containing  $2.0 \times 10^5$  to  $5.0 \times 10^5$  cells of PBMCs/well. Spot counts were compared between each condition (Kruskal-Wallis test,  $P < 0.05$ ). The numbers of spots increased significantly in pSS1, pSS3, pSS5, pSS8, and pSS9 after stimulation with M3R peptides, compared with no stimulation. **(B and C)** Representative ELISpot analysis of IL-17-producing cells in HS ( $n = 10$ ) **(B)** and IgG4-RD ( $n = 5$ ) **(C)** patients. The numbers of spots did not change in any of the subjects of the 2 groups after stimulation with the M3R peptides.  $*P < 0.05$ , by Kruskal-Wallis test.

the detected peripheral M3R-reactive IL-17-secreting cells were M3R-specific Th17 cells and suggest that the recognition of M3R peptides by these Th17 cells is restricted, in part, by HLA-DR.

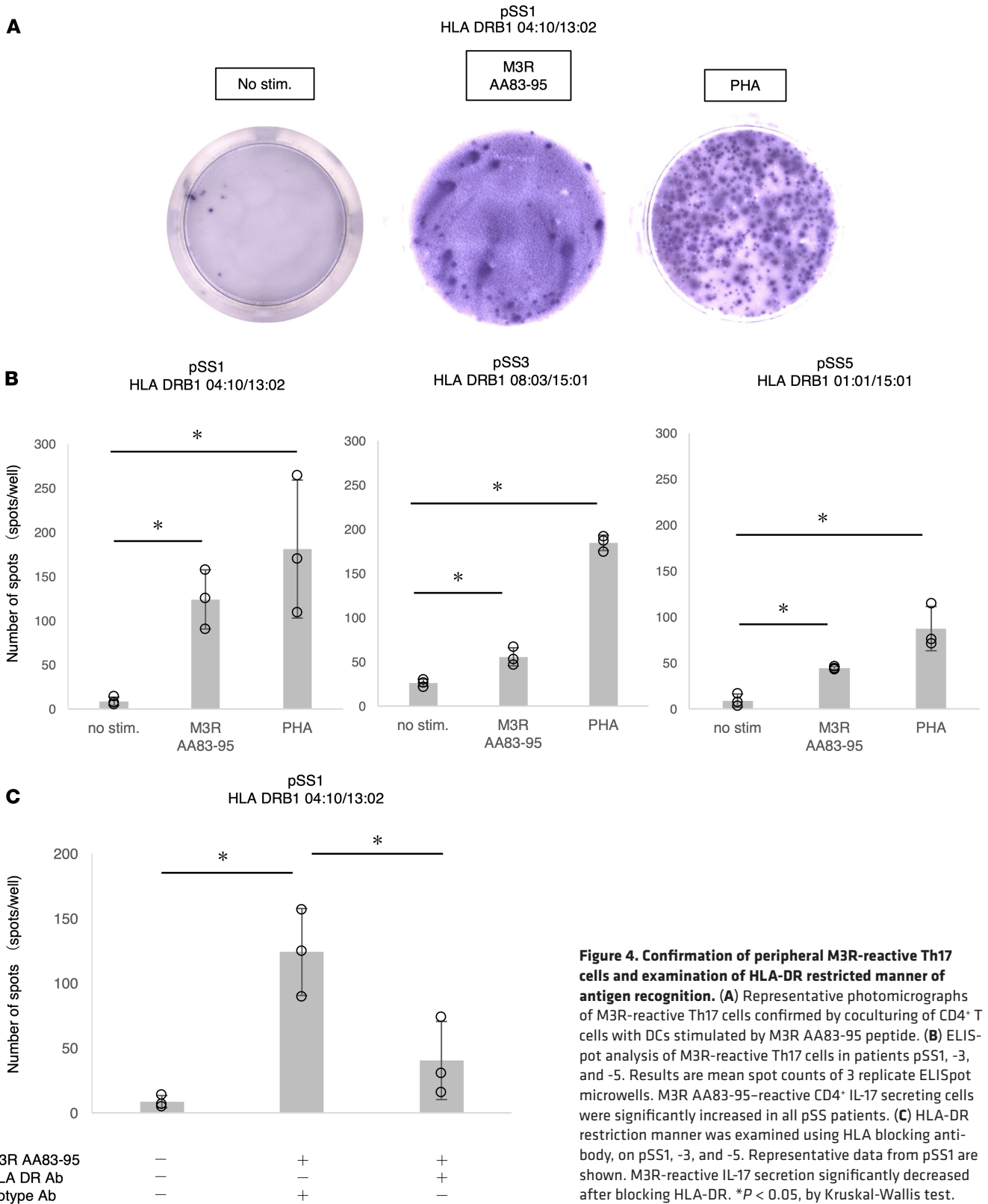
*Cytokine production profile of M3R AA83-95-reactive Th17 cells and identification of T cell repertoire overlap in salivary glands.* Since our previous report of circulating M3R-reactive IFN- $\gamma$ -secreting Th1 cells in pSS (13), questions have remained as to whether M3R-reactive Th17 cells coproduce IFN- $\gamma$ , and whether IFN- $\gamma$ -secreting cells and IL-17-secreting cells recognize the same epitope. To answer these questions, IL-17 producing main peptide M3R-reactive IL-17 and IFN- $\gamma$  cytokine secretion from CD4<sup>+</sup> Th cells were assessed in 3 of the 5 ELISpot-positive pSS patients (pSS1, pSS3, pSS5). Cytokine secretion assay kits (Miltenyi Biotec) were used after coculturing PBMCs with main M3R peptides for 8 days, as previously described (20). In accordance with ELISpot results, stimulation with M3R AA83-95, as expected, resulted in increase of IL-17 secretion compared with no stimulation, whereas increase of IL-17 secretion was not observed with M3R control peptide stimulation (Figure 5, A and B). In addition, not only IL-17 single-producing cells (CD4<sup>+</sup>IL-17<sup>+</sup>IFN- $\gamma$ <sup>-</sup> cells), but also both IL-17 and IFN- $\gamma$  double-producing cells (CD4<sup>+</sup>IL-17<sup>+</sup>IFN- $\gamma$ <sup>+</sup>), were significantly increased after M3R AA83-95 stimulation, which was also not observed with M3R control peptide stimulation (Figure 5B). Meanwhile, M3R AA83-95 stimulation increased IFN- $\gamma$  single producing Th1 cells compared with no stimulation, although the increase was also



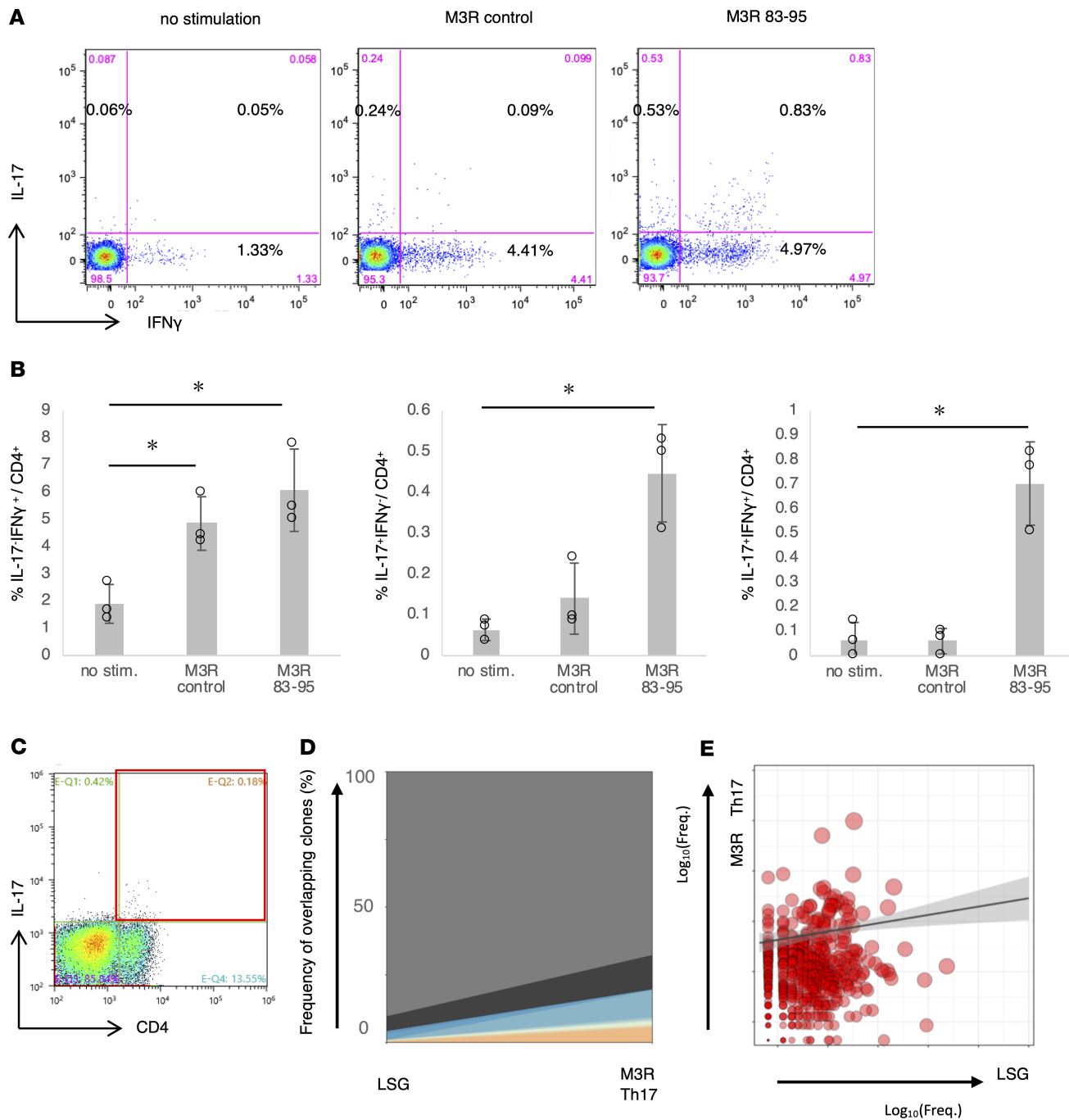
**Figure 3. Identification of the main M3R peptide responsible for IL-17 secretion.** In this study,  $2.5 \times 10^5$  to  $5 \times 10^5$  cells/well of PBMCs from pSS patients positive for peripheral M3R-reactive IL-17 secreting cells were incubated separately with each of a mixture of M3R peptides (12-20 amino acids components each) predicted to bind to each HLA-DRB1. We compared the number of counted spots for each condition. All 5 such patients (pSS1, -3, -5, -8, and -9) reacted to M3R AA83-95. Patient pSS8 also reacted to M3R AA76-87. \* $P < 0.05$ , Kruskal-Wallis test. AA, amino acid; mix, mixture of selected top 10 M3R peptides highly ranked as HLA binding epitope for each case; PHA, Phytohemagglutinin stimulation (positive control).

observed when stimulating with M3R control peptide (Figure 5B). The results show an increase in M3R AA83-95-reactive Th17 cells response, including IFN- $\gamma$  coproducer cells. However, IFN- $\gamma$ -producing Th1 cells were not specifically induced by this peptide.

Knowing that M3R is expressed in exocrine glands, we next sought to confirm the presence or absence of these M3R AA83-95-reactive Th17 cells in the labial salivary glands (LSG) of patients with pSS. For this purpose, we used the TCR sequencing method (21) to investigate whether the detected peripheral M3R-reactive Th17 cells share the same TCR repertoire with LSG-infiltrating T cells. Analysis of the TCR repertoire that overlapped with the LSG-infiltrating T cells and peripheral blood-derived, M3R-reactive IL-17-producing Th17 cells (Figure 5C) was performed in patient pSS9. T cell clones were defined by the combination of the V(D)J recombination and CDR3 nucleotide sequences. Overlapping T cell clones were defined as those clones presenting both the LSG-infiltrated and M3R-reactive Th17 T cell repertoire. We found that about 10% of the T cell repertoire identified in LSG were overlapped with the M3R-reactive Th17 repertoire (Figure 5D). Moreover, we found that the most dominant T cell clone in the M3R-reactive Th17 cells was not the dominant clone in LSG infiltrating T cells and vice versa (Figure 5E). The top 10 of the overlapped TCR- $\beta$  clonotypes in each sample are shown in Table 2. These data indicate the existence of M3R-reactive T cells in LSG, even if they were not the dominant T cell clone in LSG of the pSS patient we studied.



*Correlation between higher titers of anti-M3R antibody and M3R-reactive Th17 cells in pSS.* In the next step, we examined anti-M3R antibodies against 4 extracellular domains of M3R peptides (1st, 2nd, 3rd extracellular loop and N terminal). In addition to our previous study showing the importance of autoreactive Th17 cells on B cell infiltration in the salivary glands (16), several studies have also reported that Th17 cells can promote



**Figure 5. Cytokine production and TCR repertoire analysis of M3R Th17 cells.** (A) Representative FACS plot of 3 pSS ELISpot-positive patients (pSS1, -3, -5) shows that IL-17<sup>+</sup>CD4<sup>+</sup> cells were increased together with IL-17<sup>+</sup>IFN- $\gamma$ <sup>+</sup> double-positive CD4<sup>+</sup> cells after culturing PBMCs with M3R AA83-95 peptide for 8 days. (B) Statistical analysis of each subset in above 3 pSS. \**P* < 0.05, by Kruskal-Wallis test. (C) Sorting strategy of M3R AA83-95-induced IL-17<sup>+</sup>CD4<sup>+</sup> T cells from pSS9. Dead cells are previously gated out. (D) TCR repertoire overlap analysis between LSG infiltrating T cells and peripheral M3R-reactive Th17 cells in 1 pSS ELISpot-positive patient (pSS9). Frequency of overlapping clones between samples are shown for the top 20 overlapping clones (colored), as well as the clones those frequency were below the top 20 (black) and nonoverlapping (gray) clones. Overlapping peripheral M3R Th17 cells TCR repertoire were seen in about 10% of LSG TCR repertoire. (E) Overlapping clones between each sample were plotted with log<sub>10</sub> frequencies in each sample. Each plot represents overlapping clonotype. Major clones in peripheral M3R Th17 cells were not as dominant in LSG, and vice versa.

B cell activation (22, 23). Thus, we investigated the correlation of anti-M3R antibodies and M3R-reactive Th17 cell positivity to assess interaction of tissue-specific autoantigen-derived B cell response and the same autoantigen-specific Th17 cells. The proportions of anti-M3R antibody positivity were similar among patients with pSS and the 2 control groups (Figure 6A). Although, when comparing between M3R-reactive Th17



**Table 2. TCR $\beta$  overlap analysis between M3R reactive Th17 cells and LSG T cells.**

Rank	CDR3	V	D	J	M3R Th17 frequency ( $\times 10^{-5\%}$ )	LSG frequency ( $\times 10^{-5\%}$ )
1	TGTGCCAGCAGCATGACAGGGCTAAAGAACACTGAAGCTTTCTTT	TRB V9	TRB D1	TRB J1-1	9700	33.1
2	TGTGCCATCGATAGGACAGGGGCCACTCTCAGCCCCAGCATTTT	TRB V10-3	TRB D1	TRB J1-5	5000	7.6
3	TGTGCCAGCAAGATCGGACCGACGAACACTGAAGCTTTCTTT	TRB V7-2	ND	TRB J1-1	800	1.2
4	TGTGCCAGCAGCTTCACAGGGAACTGAAGCTTTCTTT	TRB V7-9	TRB D1	TRB J1-1	800	31.8
5	TGTGCCAGCAGATTAGTTCCTCTCGGACAGGGCAATCAGCCCCAGCATTTT	TRB V7-8	TRB D1	TRB J1-5	700	0.6
6	TGTGCCAGCTCACCGTATCCGGGACAGGGAACTGAAGCTTTCTTT	TRB V18	TRB D1	TRB J1-1	400	200.0
7	TGTGCCAGCAGCCAAGATCTTCGGGACAACCATTCTATGGCTACACCTTC	TRB V3-2	TRB D1	TRB J1-2	400	8.2
8	TGTGCCAGCAGCCCAAGTAATTCACCCCTCCACTTT	TRB V9	ND	TRB J1-6	400	7.0
9	TGTGCCAGCAGCCTGGGGACTAGCGTTCGCTACGAGCAGTACTTC	TRB V13	TRB D2	TRB J2-7	300	8.2
10	TGTGCCAGCAGCTTGACAGGGAATATGGCTACACCTTC	TRB V7-3	TRB D1	TRB J1-2	300	13.3

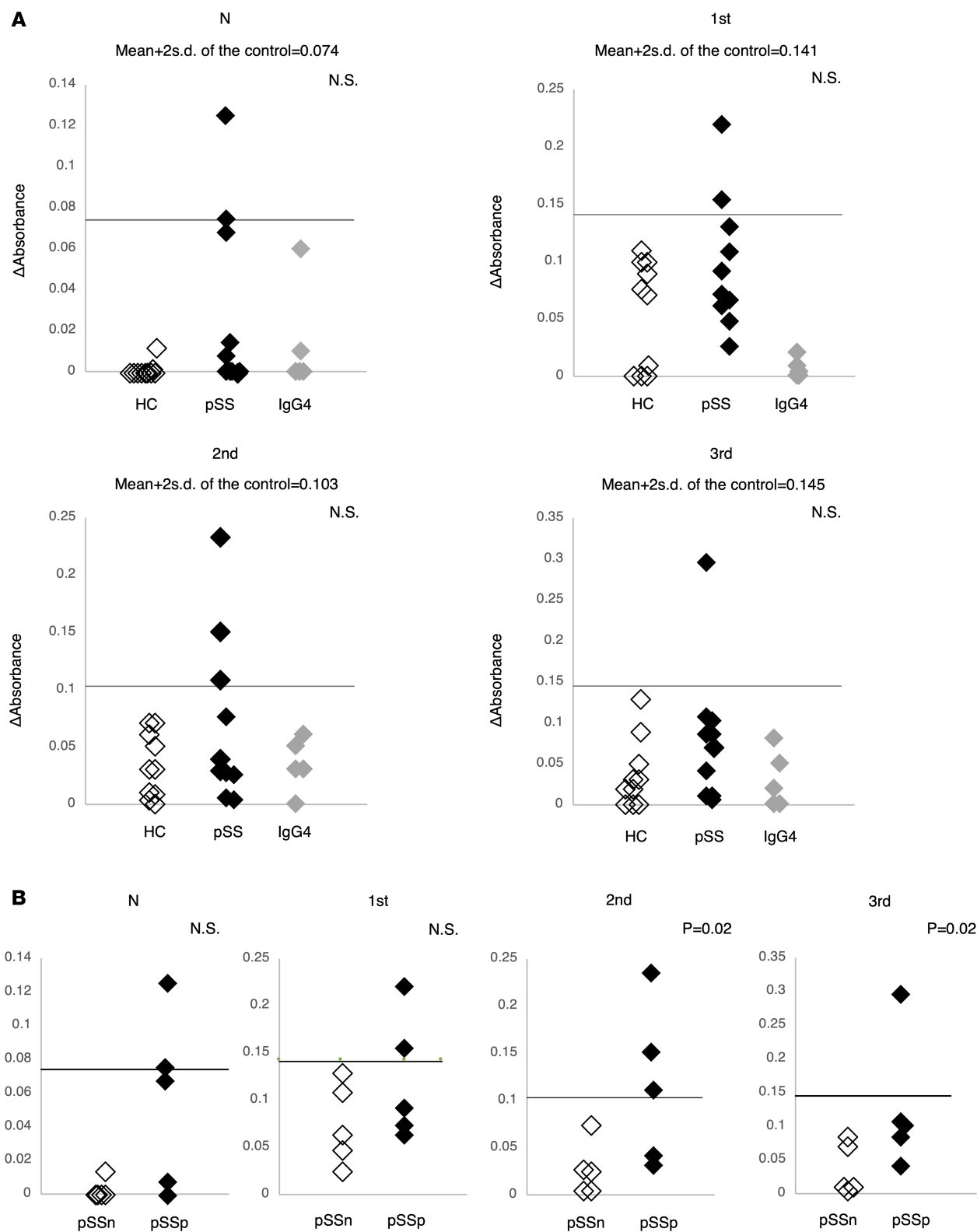
Top 10 TCR clonotype frequency of TCR- $\beta$  overlap analysis between peripheral M3R-reactive Th17 cells and LSG T cells is shown. Overlapping clones are ranked for their frequency in peripheral M3R-reactive Th17 cells. Percentage of clonotype frequency is shown for each sample. V, D, J gene name according to the international ImMunoGeneTics database (IMGT). ND, not determined.

cell-positive patients with negative patients, significantly higher titers of anti-M3R antibodies against 2nd and 3rd extracellular loops were found in pSS patients positive for M3R-reactive Th17 cells than those negative for these cells (Figure 6B). These results demonstrate the clinical relevance of M3R antibody to M3R-reactive Th17 cells, suggesting that tissue-derived autoantigen-reactive B cell activity is partly supported by interaction with the same autoantigen reactive Th17 cell among a subgroup of pSS patients.

M3R-reactive peripheral Th17 cell-positive patients tend to have higher disease activity score. To complement the above clinically relevant finding, we investigated the clinical relevance of M3R-specific peripheral Th17 cells in pSS patients. For this purpose, we compared the clinical features of M3R-reactive Th17 cell-positive and negative pSS patients (Table 3). Intriguingly, the disease activity scored according to the EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) tended to be higher among patients positive for peripheral M3R-reactive Th17 cells compared with the negative patients, though adjusted *P* values were not significant due to the small sample size. HLA-DR typing was also not significantly different between the 2 groups, although it is noteworthy that 3 of 5 patients positive for peripheral M3R-reactive Th17 cells had HLA-DRB1 15:01 genotype. Moreover, the HLA DQ 01:02 genotype was seen significantly more frequently among the M3R-reactive Th17 cell-positive group than the negative group (Table 4). There were no significant differences between the 2 groups with respect to in age, sex, LSG grade, Schirmer's test, Sialography stage, Saxon test, each domain of the ESSDAI score, and immunological findings other than anti-M3R antibody. Thus, positivity of M3R-reactive Th17 cells correlates with higher titers of anti-M3R antibodies, whose disease activity score tended to be higher than negative patients.

## Discussion

Despite several reports about the pathogenicity of Th17 cells in pSS, there is no information on antigen-specific Th17 cells in pSS. Here, we reported the identification of M3R-reactive Th17 cells in the peripheral blood and the infiltration of those identical T cell clones into the LSG of pSS patients. Potent IFN- $\gamma$  secretion from M3R-reactive Th17 cells was also clarified. Furthermore, our data provide evidence that M3R-reactive Th17 cells correlate with pSS disease activity score and antibody titer against M3R. This is the first report to our knowledge to confirm the presence of peripheral M3R-specific Th17 cells in a subgroup of pSS patients.



**Figure 6. Anti-M3R antibodies against 4 extracellular domains of M3R.** Anti-M3R antibodies against extracellular domains of M3R were examined as described previously by ELISA (12). The cutoff level between negative and positive values of each anti-M3R antibody represented the mean of the normal controls + 2 SD values, represented by the horizontal line in each figure. **(A)** The frequency and titers of anti-M3R antibody were not significantly different among HS, IgG4-RD, and pSS. Only pSS were positive for anti-M3R antibodies. \* $P < 0.05$ , by Kruskal-Wallis test. **(B)** Antibody titers against second and third extracellular domains were significantly higher in the 5 pSS patients positive for M3R-reactive Th17 cells ( $P = 0.02$ , each). \* $P < 0.05$ , by the Mann-Whitney  $U$  test. N, N terminal; 1st, first extracellular loop; 2nd, second extracellular loop; 3rd, third extracellular loop; pSSn, M3R-reactive Th17-negative patients; pSSp, M3R-reactive Th17-positive patients.

**Table 3. Clinical parameter comparison of pSS patients positive and negative for M3R-reactive Th17 cells**

	Negative pSS (n = 5)	Positive pSS (n = 5)	P value	Adjusted P value
Age (years)	48.2 ± 24.0	71.6 ± 8.4	0.14	0.58
ESSDAI	2.8 ± 1.7	8.0 ± 4.3	0.01	0.17
Constitutional	0	1.2 ± 2.6	0.31	0.59
Lymphadenopathy and lymphoma	0	2.4 ± 3.5	0.13	0.59
Glandular	0	0	ND	ND
Articular	0.4 ± 0.8	0.4 ± 0.8	1.00	1.00
Cutaneous	0	0	ND	ND
Pulmonary	1.0 ± 2.2	1.0 ± 2.2	1.00	1.00
Renal	0	0	ND	ND
Muscular	0	0	ND	ND
Peripheral nervous system	0	0	ND	ND
Central nervous system	0	0	ND	ND
Hematological	0	0.4 ± 0.8	0.31	0.59
Biological	1.4 ± 0.8	1.6 ± 0.5	0.81	0.92
RF (U/mL)	17.0 ± 9.9	39.8 ± 39.8	0.24	0.59
ANA (times)	336 ± 531.1	720 ± 1057.9	0.58	0.82
IgG (mg/dL)	1819 ± 356	2685 ± 1045	0.17	0.59
IgM (mg/dL)	114 ± 58	102 ± 34	0.60	0.82
IgA (mg/dL)	286 ± 26	355 ± 150	0.60	0.82
LSG (grade)				
1	0	0	0.63	0.82
2	1	1		
3	2	1		
4	2	3		
Schirmer's test (mm/5 minutes.)	8.6 ± 8.3	4.8 ± 8.6	0.29	0.59
Sialography (stage)				
0	2	0	0.24	0.59
1	2	2		
2	0	2		
3	1	0		
4	0	1		
Saxon test (g/2 minutes.)	2.3 ± 2.7 <sup>A</sup>	2.4 ± 1.9	0.79	0.92

Disease activity score (ESSDAI) tended to be higher among pSS patients who were positive for M3R-reactive Th17 cells than negative patients. Sialography stages are evaluated according to the classification of Rubin and Holt. *P* values for age, ESSDAI, each domain of ESSDAI, RF, ANA, IgG, IgM, and IgA, by Mann-Whitney U test. *P* value for LSG biopsy grade and Sialography stage by Cochran-Armitage test. Adjusted *P* values calculated using Benjamini-Hochberg procedure. <sup>A</sup>Missing data were treated by multiple imputation (100 imputations), because a datum from 1 negative pSS patient was lacking.

Our results confirmed the presence of peripheral M3R-reactive Th17 cells in 50% of pSS patients in this study, a similar frequency to that reported for M3R-reactive Th1 cells in SS (13). In the study, patients with M3R AA213-237-reactive IFN- $\gamma$ -producing cells most frequently had HLA-DRB1 09:01 genotype, while our results showed that patients with HLA-DRB1 15:01 genotype comprised more than half of the group with M3R AA83-95-reactive IL-17-producing Th17 cells. HLA-DRB1 15:01 allele is considered to be associated with immune-associated disease, such as antiglomerular basement membrane disease (24) and multiple sclerosis (25). The anchor motif for DRB1 15:01 has been studied previously; with the primary binding anchor at position i, also known as P1, requires nonaromatic, hydrophobic anchor (leucine, valine, or isoleucine) and an additional hydrophobic side chain represented by methionine, isoleucine, valine, or phenylalanine at position i+6 (also known as P7) (26). The T cell epitope of M3R-reactive Th17 cells identified in the present study contains a number of hydrophobic amino acids, such as valine, isoleucine, and leucine. Furthermore, starting from M3R AA81 to AA95 (VTIIGNILVIVSFKV), AA81 (V) was thought to bind to P1, and AA87 (I) to P7. Thus, although our data suggest that M3R AA83-95 is the main T cell epitope, information on the anchor residue provides explanation as to why pSS8, which

**Table 4. HLA genotype comparison of pSS patients positive and negative for M3R-reactive Th17 cells**

	Negative pSS (n = 5)	Positive pSS (n = 5)	P value
HLA-DRB1 genotype			
01:01	1	1	1.00
04:03	1	0	1.00
04:05	3	0	0.21
04:10	0	1	1.00
08:02	2	1	1.00
08:03	2	1	1.00
09:01	0	1	1.00
13:02	0	2	0.47
15:01	0	3	0.21
15:02	1	0	1.00
HLA DQA1 genotype			
01:01	1	1	1.00
01:02	0	5	0.01
01:03	3	1	0.29
03:01	3	3	0.68
03:02	2	0	0.23
04:01	1	0	1.00
HLA DQB1 genotype			
03:02	3	1	0.29
03:03	0	1	1.00
04:01	3	0	0.21
04:02	0	1	1.00
05:01	1	1	1.00
06:01	3	1	0.29
06:02	0	3	0.21
06:04	0	1	1.00
06:09	0	1	1.00

HLA-DRB1, -DQA1, and -DQB1 genotype was compared between pSS patients positive and negative for M3R-reactive Th17 cells. HLA DQA1 01:02 was associated with M3R-reactive Th17 cells. HLA-DRB1 15:01 and HLA-DQB1 06:02 was found in more than half of M3R-reactive Th17 cell-positive pSS patients. *P* values for HLA genotyping by Fisher's exact test.

carries 1 HLA-DRB1 15:01 allele, reacted to both M3R AA76-85 and AA83-95, since the predicted residue existed where those peptides overlap.

Besides the unique feature of HLA-DRB1 genotyping, recent studies indicate that no single HLA class II genotyping is likely to be required as a necessary cofactor in SS pathogenesis (27) and that HLA DQ genotypes are also associated with SS genetics (28). We found that HLA DQA1 01:02 was related to M3R-reactive Th17 cell-positive pSS patients; moreover, the DRB1 15:01, DQA1 01:02, DQB1 06:02 haplotype was seen in 3 of the 5 patients with M3R-reactive Th17 cell positive pSS patients. In patients with pSS, HLA-DR2 (including DRB1 15:01), -DQA1 01:02, -DQB1 06:02 haplotype has previously been reported to be associating with less diversified La/Ro RNP response, which reacted to Ro antigen alone (29), and the DQA1 01:02-DQB1 06:02 haplotype was associated with reactions to specific peptides of the AChR  $\alpha$ -chain among patients with myasthenia gravis (30). Together, these reports indicate a possible feature of HLA-restricted antigen reaction of HLA-DRB1 15:01, -DQA1 01:02, -DQB1 06:02 haplotype that might also be associated with M3R peptide reactivity, as observed in the present study. There is more to be assessed about influence of HLA typing on autoantigen activity. For example, 1 patient (pSS9) who did not have the most frequently identified HLA haplotype showed much higher counts by mixed peptide stimulation than by single peptide stimulation, assuming the possibility of reacting to a peptide where overlapping or specific combinations of several peptides; those possibilities were not assessed here due to the limitation of sample amount. Although more studies are needed that involve a larger sample size, our data provide insights into the epitopes of M3R-reactive Th17 cells with specific HLA class II typing.

Additionally, we also demonstrated that the presence of M3R-specific Th17 cells in pSS tend to correlate with higher disease activity score assessed by ESSDAI score. Interestingly, the presence of

M3R Th17 cells did not correlate with any of the glandular manifestations, including LSG biopsy grade, sialography, and Schirmer score. Thus, there seems to be more extensive extraglandular involvement in M3R-specific Th17 cell-positive pSS patients, which resulted in a higher ESSDAI score. This is peculiar since M3R is expressed in exocrine glands, in which autoimmunity against M3R was shown to be associated with glandular manifestation of pSS in an SS mouse model (14). In this study, identical T cell clones of M3R-reactive Th17 cells were identified in the LSG of 1 pSS patient, suggesting that M3R-recognizing Th17 cells localize in the salivary gland and might also contribute to autoimmunity against M3R in pSS patients. Based on the latter reported finding, we expected to find a strong correlation between the presence of M3R-reactive Th17 cells and glandular manifestations. However, in agreement with the findings of several previous studies (5, 31), we found that circulating Th17 cells tend to correlate with a higher ESSDAI score in at least a subgroup of pSS patients. These findings suggest that a specific group of antigen-reactive Th17 cells might act differently depending on their distribution and location. For example, glandular Th17 cells may play a role in glandular inflammation seen at the onset of the disease (4, 32), whereas circulating antigen-specific Th17 cells, including M3R-reactive Th17 cells, seem to play a role in systemic organ involvement and progression of the disease. We clarified that dominance of overlapping TCR clones between peripheral M3R-reactive Th17 cells and LSG infiltrating T cells were different in both sites; therefore, distribution and location might also influence cell expansion and function. However, because our study did not include any functional assay for M3R-specific Th17 cells, more study is needed to determine exactly how antigen-specific circulating Th17 cells play a pathogenic role in systemic inflammation and how they expand. Nevertheless, our findings suggest the potential effectiveness of a pSS therapy designed around these cells.

Our results also demonstrated the presence of high titers of anti-M3R antibody against 2nd and 3rd extracellular loops of the M3R in pSS patients positive for M3R-reactive Th17 cells. Although positivity for anti-M3R antibody did not show a significant difference, probably due to the small number of patients, it was notable that none of the pSS patients negative for M3R-reactive Th17 cells were positive for anti-M3R antibody, while 60% (3 of 5 patients) of the pSS patients positive for M3R-reactive Th17 cells were also positive for the antibody. Th17 cells were reported previously to act as an effective B cell helper in promoting B cell activation, as well as the formation of germinal centers (22) — effects that were associated with higher antibody production rate in vivo in the SS mouse model induced by adoptive transfer of Th17 cells (32). Similar effects of Th17 cells on B cells were also reported in the M3R-induced mouse model (16). To a certain extent, these findings provide indirect explanation for our results, suggesting that autoantigen reactive Th17 cells might act as effective autoantigen reactive B cell helpers, which result in higher antibody response against M3R in M3R-reactive Th17 cell-positive pSS patients. In addition, among all M3R extracellular portions, the anti 2nd extracellular loop of the M3R antibody was the most frequently positive. Since the second extracellular loop of the M3R is critical for receptor activation by agonists (33), and a functional M3R antibody against the second extracellular loop has been reported (12), the presence of activated M3R-reactive Th17 cells — with the associated higher production of M3R antibody against the second extracellular loop of the M3R — might explain the correlation between the presence of M3R-reactive Th17 cells and higher disease activity score, at least indirectly.

Finally, we clarified potent IFN- $\gamma$  secretion of M3R-reactive Th17 cells. IFN- $\gamma$  has been reported to be a proinflammatory cytokine involved in SS pathogenesis (34), and we previously identified peripheral M3R-reactive IFN- $\gamma$ -secreting cells among SS patients (13). It is known that, due to the plasticity of Th17 cells, they produce not only IL-17, but also produce IL-17 and IFN- $\gamma$ , which can also be referred to as Th1-like Th17 cells, or Th17.1 cells (35). Here, in vitro assessment of antigen-specific T cell response was assessed, clarifying that the main M3R peptide 83-95 increased IL-17-producing cells together with IL-17 and IFN- $\gamma$  double producing cells, but not IFN- $\gamma$  single producing cells, when compared with control M3R peptide. From the results, it seems that M3R-reactive Th17 cells react to a specific M3R peptide, while IFN- $\gamma$  single producing Th1 cells react to multiple peptides. Since IFN- $\gamma$  single producing Th1 cells are reported to have lineage stability, IL-17 and IFN- $\gamma$  double producing cells, which we identified after M3R stimulation, are likely to be Th17.1 cells (35). Recently, Th17.1 cells are reported to participate in the pathogenesis of autoimmune diseases, such as inflammatory bowel disease, multiple sclerosis, and rheumatoid arthritis (36–38). One of the reported features of these cells is their involvement in B cell antibody secretion (39). In SS, it is reported that Th17.1 cells increase in the salivary glands (40), which was concordant with salivary gland characteristics seen in the SS mouse model (41). Taken together, these findings suggest that coproducing IL-17 and IFN- $\gamma$

Th17.1 cells, including the M3R-reactive Th17.1 cells identified in this study, contribute to pSS disease progression (3); however, more functional studies are needed for a better understanding of their roles.

The limitations of this study include the following points. First, the study only included Japanese participants. Frequency of HLA class II genes and disease-associated genes differs between ethnic groups (27). For example, the frequency of the DRB1 03:01 allele, the associated DRB1 allele only among White pSS patients (28), is 18.6%–22.0% in White patients (Allele Frequency Net Database; <http://www.allelefrequencies.net>), but only 0.1% in Japanese patients (HLA Foundation Laboratory; <http://www.hla.or.jp>). Thus, differences in HLA-DRB1 allele distribution across populations certainly seems to affect disease susceptibility. Meanwhile, we still have no data on how allele distribution would affect antigen reactivity in pSS. However, the frequency of HLA-DRB1 allele 15:01, which was found more frequently in pSS with M3R autoreactivity in this study, is 15.9%–24.4% in White patients compared with 7.73% in Japanese patients. Therefore, because this allele frequency is not so low in a non-Japanese population as well, it leads to the idea of a possible common M3R autoreactivity among different ethnic groups. Further studies with larger and multiethnic samples are needed to address this question.

Second, the study did not include functional assessment of M3R-reactive Th17 cells. It is still unclear whether M3R-reactive Th17 cells play direct or indirect pathogenic role. Furthermore, there is no information on the involvement of these cells in SS disease onset and progression. How they activate B cells to produce anti-M3R antibody remains unclear, as well. We are currently in preparation of further studies to establish an M3R Th17 cell line for future functional studies.

In conclusion, we have reported the identification of circulating M3R-reactive Th17 cells, including Th17.1 cells, in treatment-naïve pSS patients, and the identical TCR repertoire was also detected in LSG. The presence of these cells tended to correlate with a higher disease activity score and significantly higher titers of functional anti-M3R antibodies against the 2nd and 3rd extracellular loops of the M3R. These results suggest that screening for M3R-specific Th17 cells and targeting them therapeutically is a potentially effective treatment strategy for pSS.

## Methods

**Study subjects.** We obtained blood samples from 10 pSS patients who fulfilled the 1999 Japanese Ministry of Health pSS diagnostic criteria (17), as well as the 2016 American College of Rheumatology/European League against Rheumatism (ACR/EULAR) classification criteria for SS (18). All patients were followed up at the University of Tsukuba Hospital. We also obtained peripheral blood samples from 10 age- and sex-matched healthy subjects. For disease control, we recruited 5 patients with IgG4-RD patients, who all fulfilled the diagnostic criteria proposed in 2011 by the All Japan IgG4 team (42) and were also followed up at the University of Tsukuba Hospital. All SS patients, healthy subjects, and IgG4-RD patients were naïve to any immunosuppressive therapy, including steroids. The clinical data and results of laboratory tests, imaging studies, including sialography (staged by the classification of Rubin and Holt), LSG biopsy, and EULAR primary ESSDAI score (43) were obtained from the medical records. LSG biopsies were taken at the time of diagnostic work up. Each study participant was typed for HLA-DRB1 at the HLA Foundation Laboratory (Kyoto, Japan). For the pSS patients, HLA-DQA1 and -DQB1 were examined. **Synthesis of M3R peptides and identification of candidate T cell epitopes based on HLA-DRB1 typing.** Peptides encoding M3R were synthesized chemically using a solid-phase procedure. In total, 45 types of M3R peptides, each encoding 12–20 mers, with overlapping 4–5 mers, were synthesized, altogether covering the full length of the M3R peptide, which comprises 590 amino acids (Supplemental Table 2). Because HLA-DRB1 is reported to be one of the disease-associated genes of pSS (7, 8), we sought to determine HLA-DR-related antigen specificity. T cell epitope candidates from each participants were selected according to their HLA-DRB1 typing by Immune Epitope Database (<http://www.iedb.org/>), among the full sequence of M3R peptides. We chose M3R peptides for each case that covered all top 10 selected 15-mer peptides highly ranked by IC<sub>50</sub> values, assuming that the chosen peptides were likely to bind to each specific MHC molecules.

**IL-17 ELISpot.** After obtaining whole blood samples collected using heparin tubes, peripheral blood mononuclear cells (PBMCs) were isolated by density gradient using Ficol-Paque Plus (GE Healthcare). PBMCs were cryopreserved at a density of  $5 \times 10^6$  cells/mL in cellbanker at  $-80^\circ\text{C}$  and moved to a liquid nitrogen tank after freezing for 1 week, to evaluate several samples simultaneously. During examination, PBMCs were thawed, rested for at least 1 hour to allow removal of cell debris as recommended in the protocol (catalog 3520-2A, MABTECH), washed, and resuspended in RPMI Medium 1640 containing 10% FBS

and 1% penicillin. Multiscreened Sterile ELISpot plates (catalog MSIPS4510, MilliporeSigma) treated with coating antibody and incubated overnight at 4°C–8°C were used for culture. A total of  $2.5 \times 10^5$  to  $5 \times 10^5$  cells/well were cultured in the presence of 10 µg/mL of individually selected top 10–ranked M3R peptides, all mixed, for 40 hours at 37°C in an incubator with 5% CO<sub>2</sub> under humidified atmosphere. Stimulation with 1% PHA and medium with cells were assessed as positive and negative controls, respectively. In addition, cultures containing M5 muscarinic acetylcholine receptors (M5R) peptide were assessed as the control peptide (M5R extracellular loop 3: AA464-478, STFCDKCVPTLWH). Five replicate wells were examined for each condition. Based on the instructions provided with products, all visualized spots were assessed, and ELISpot plates were analyzed and counted using ImmunoSpot S6 ENTRY analyzer (CTL Analyzers).

*Main M3R peptide reactivity.* A mixture of the selected top 10–ranked M3R peptides that were highly predicted to bind to each HLA-DRB1, and thus used for stimulation, were separately used for stimulation of samples positive for M3R-specific IL-17–secreting cells. This procedure allowed the identification of the part of M3R peptide that was mainly responsible for IL-17 secretion. Each of the selected M3R peptides comprised of 12–20 amino acids was added separately to the culture of  $2.5 \times 10^5$  to  $5 \times 10^5$  cells/well of PBMCs. As described previously, stimulation with 1% PHA and medium with cells was assessed for positive and negative controls, respectively. Furthermore, 5 replicate wells were examined for each condition.

*Cocultures of CD4<sup>+</sup> T cells and monocyte-derived DCs.* To confirm that the detected M3R-reactive IL-17–secreting cells were M3R-specific Th17 cells, we conducted IL-17 ELISpot after coculture of isolated CD4<sup>+</sup> T cells with DCs, followed by stimulation with IL-17 secretion triggering main M3R peptides identified in the previous procedure. DCs used for antigen presenting cells (APCs) were derived from peripheral autologous CD14<sup>+</sup> monocytes. First, CD14<sup>+</sup> monocytes were isolated from PBMCs by positive selection using CD14 cell isolation kit (catalog 130-050-201, Miltenyi Biotec). Next, the isolated monocytes were cultured with GM-CSF (100 ng/mL) and IL-4 (10 ng/mL). After 5 days of culture, the cells were treated with LPS (3 µg/mL), IL-4 (20 ng/mL), and TNF-α (10 ng/mL) for 3 days in RPMI-1640 medium containing 10% FBS (30). Finally, CD14<sup>+</sup> monocytes matured to DCs after a total of 8 days of culture (Supplemental Figure 2A). Differentiation of DCs was confirmed by flow cytometry and staining for HLA-DR-PE (catalog 307600, clone L243, BioLegend), CD80-PECy7 (catalog 305218, clone 2D10, BioLegend), and CD11c-APC (catalog 301614, clone 3.9, BioLegend) (Supplemental Figure 2B). The differentiated DCs were used for coculture with CD4<sup>+</sup> T cells, which were also isolated from PBMCs by positive selection using CD4-coated microbeads (catalog 130-045-101, Miltenyi Biotec) at a ratio of 5:1 and stimulated with dominant IL-17 secretion–responsible M3R peptides. The isolated CD4<sup>+</sup> T cells were rested for 6 hours before coculture at 37°C in an incubator with 5% CO<sub>2</sub> and humidified atmosphere. After 40 hour of incubation, IL-17 ELISpot assay was performed as described above. Due to the limitation of available cell numbers, 3 replicate wells were examined for each condition.

*HLA-DR blocking assay.* HLA-DR restriction of M3R-reactive Th17 cells was checked by blocking HLA-DR, using anti–HLA-DR antibody (catalog 307648, clone L243, BioLegend), in 3 pSS patients positive for M3R-reactive Th17 cells (pSS1, pSS3, pSS5) who could provide enough samples for evaluation. In this assay, 10 µg/mL of anti–HLA-DR antibody or isotype clone (catalog 400264, clone MOPC-173, BioLegend) was added to isolated DCs, 30 minutes before adding peptides. After incubation for 30 minutes, anti–HLA-DR antibody–pulsed DCs and CD4<sup>+</sup> T cells with dominant M3R peptides responsible for IL-17 secretion, were added to the ELISpot plates. Three replicate wells were examined under each condition.

*Cytokine production of M3R-reactive Th17 cells.* PBMCs were cultured with either 10 µg/mL of IL-17 secreting main M3R peptide (M3R AA83-95) or with 10 µg/mL of control M3R peptide, which were also highly predicted to bind to each HLA-DRB1 type for each case (Supplemental Table 1), or with no peptide in RPMI 1640 medium containing 10% human serum with rhIL-2 (20 IU/mL), and rhIL-7 (10 ng/mL), as described previously (44), with modification of rhIL-2 concentration in reference to the method described by Lande and colleagues (45). At day 8, 12 hours before examination, PBMCs were restimulated with M3R peptides (10 µg/mL); then, IFN-γ (PE), and IL-17 (APC) secretion were assessed using cytokine secretion assay kits (catalog 130-054-201, 130-094-536, Miltenyi Biotec), following product guidelines. Stimulated PBMCs were also stained with CD4 Alexa Fluor 488 (catalog 317420, clone OKT4, BioLegend) and 7-AAD Viability Staining Solution (catalog 420404, BioLegend). Analysis was performed using FACSVerse cytometer (Becton Dickinson) and Flowjo software (Tree Star). The gating strategy is shown in Supplemental Figure 3.

*Sorting of M3R-reactive Th17 cells.* After coculturing of the PBMCs with M3R peptide for 8 days, M3R 83-95–reactive Th17 cells were stained with anti–CD4 Alexa Fluor 488 (clone OKT4), and IL-17 APC using cytokine secretion assay kits (catalog no.130-094-536, Miltenyi Biotec, CA), as previously described.

This method enables us to isolate M3R-reactive IL-17-secreting CD4<sup>+</sup> T cells alive using FACS. Detection and cell sorting were performed using the SH800S cell sorter (SONY).

*TCR sequence of M3R-reactive Th17 cells and LSG.* TCR sequencing library construction and sequencing were performed using the sorted M3R-reactive Th17 cells and total RNA samples derived from the LSG. The LSG biopsies were preserved in RNAlater Stabilization Solution (catalog no. AM7020, Invitrogen) at -30°C, and total RNA was extracted using TRIZol reagent (catalog no. 15596018, Thermo Fisher Scientific). TCR cDNA amplification and sequencing was performed as previously described (21) with minor modifications. In brief, total mRNA was converted into cDNA, and second strand synthesis was performed using a universal primer. TCR- $\beta$  cDNAs were specifically amplified using nested PCR, and sequencing libraries were constructed using NEBNext UltraII FS library Prep Kit (New England Biolabs). Final TCR libraries were sequenced using an Ion 540 Chip Kit, an Ion 540 Kit-Chef, and an Ion Genestudio S5 next generation sequencer (Thermo Fisher Scientific). Sequencing data was quality filtered using PRINSEQ-0.20.4 and processed using MiXCR-3.0.5. Filtered reads were aligned to reference human TCR V(D)J sequences and assembled into TCR clonotypes using “analyze amplicon” command with the following parameters: -s hsa, --starting-material rna, --5-end no-v-primers, --3-end c-primers, --adapters no-adapters, --receptor-type trb, --region-of-interest CDR3, --only-productive, --align “-OvParameters.geneFeatureToAlign=VTranscript”, --align “-OvjAlignmentOrder=JThenV”, --assemble “-ObadQualityThreshold = 10”, --assemble “-OseparateByV=true”, --assemble “-OseparateByJ=true”. The list of final clones were converted into VDJtools format and analyzed with VDJtools-1.2.1. (46) and Microsoft R Open-3.5.2 (<https://mran.microsoft.com/open>). Identical TCR repertoire was defined as TCR reads with the same TCR variable (V) segment, joining (J) segment, and CDR3 nucleotide sequences.

*Anti-M3R antibody.* Anti-M3R antibodies against extracellular domains of M3R were screened by ELISA using synthetic peptide antigen as described previously (12). M3R peptide-specific absorbance values at 405 nm ( $\Delta$ absorbance) were calculated for each serum sample by subtracting absorbance value of the negative peptide from the value of M3R peptides. Dose-dependent curve at 1:50 serum dilution was within the linear part. Triplicate wells were measured under each condition and standardized between experiments by using the absorbance value of the positive control. The results were confirmed by more than 2 independent experiments. Antibody titers represented the average of experiments that could be conducted and properly evaluated.

*Statistics.* Statistical differences between 2 groups were evaluated by the 2-tailed Student's *t* test. Differences between 3 or more groups were evaluated by the Kruskal-Wallis test. Comparison of categorical values was examined by Fisher's exact test. The Mann-Whitney *U* test was used to evaluate differences between 2 independent groups with continuous dependent variable. The Cochran-Armitage test was used for analysis of categorical data. *P* values less than 0.05 denoted the presence of statistically significant differences. To adjust the FDR, the Benjamini-Hochberg procedure was used for calculating adjusted *P* values. Missing data were treated by multiple imputation (100 imputations).

*Study approval.* The study was approved by the local ethics committees of University of Tsukuba Hospital and was carried out in accordance with the Declaration of Helsinki. Patients recruited in the study were enrolled with written informed consent received before inclusion in the study.

## Author contributions

SA, HK, YO, FH, MY, SH, and YK recruited patients and collected clinical data. SA, HA, and H. Takahashi performed all experiments and analyzed the data. H. Tsuboi, IM, and TS participated in the design of the study and interpretation of the data. SA wrote the initial draft; H. Tsuboi, IM, and TS edited it. All authors accept equal responsibility for the accuracy of the contents of this paper.

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Address correspondence to: Takayuki Sumida, Department of Internal Medicine, Faculty of Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba-city, Ibaraki 305-8575, Japan. Phone: 81.29.853.7388; Email: [tsumida@md.tsukuba.ac.jp](mailto:tsumida@md.tsukuba.ac.jp).



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