Table 3 ESSDAI and ESSPRI baseline values in real-life cohorts and clinical trials

	ESSDAI at inclusion			ESSPRI at inclusion	
	≥4	≥5	≥6	≥5	≥6
Real-life cohorts					
EULAR[3] (n=395)	256/385 (66.5%)	236/385 (61.3%)	207/385 (53.8%)	267/392 (68.1%)	214/392 (54.69%)
ASSESS[6] (n=395)	167/383 (43.6%)	140/383 (36.6%)	124/383 (32.4%)	219/356 (61.5%)	156/356 (43.8%)
Clinical trials					
TRIPPS[11] (n=103)	NA	NA	NA	98/103 (95.2%)	79/103 (76.7%)
RTX trial (the Netherlands)[14] (n=30)	28 (93.3%)	23 (76.7%)	22 (73.3%)	NA	NA
BELISS[16] (n=30)	21 (70.0%)	18 (60.0%)	18 (60.0%)	28 (93.3%)	21 (70.0%)
ASAP[15] (n=15)	14 (93.3%)	14 (93.3%)	14 (93.3%)	12/14 (85.7%)	12/14 (85.7%)
TEARS[12] (n=122)	108 (88.5%)	93 (76.2%)	89 (73.0%)	97/122 (79.5%)	76/122 (62.3%)
JOQUER[13] (n=120)	47/119 (39.5%)	38/119 (31.9%)	32/119 (26.9%)	83/117 (70.9%)	64/117 (54.7%)

ASSESS, Assessment of Systemic Signs and Evolution of SS; EULAR, European League Against Rheumatism; ESSDAI, EULAR Sjögren's syndrome disease activity index; ESSPRI, EULAR Sjögren's syndrome patient-reported index; NA, not available.

DISCUSSION

This study aimed to determine disease activity levels, PASS and MCII of the EULAR SS indexes, ESSDAI and ESSPRI. This study involved patients with primary SS from two large independent cohorts, one of which had been conducted internationally and specifically for that purpose. The results displayed reliable thresholds for defining activity levels and changes in scores that could be used for designing future clinical trials. Nevertheless, since the number of observational cohorts already used ESSDAI and ESSPRI for the evaluation of their real-life patients,[6 26] the usefulness of these thresholds in clinical practice will be further evaluated.

Our study has some limitations. Both cohorts were observational; therefore, no therapeutic intervention was systematically applied. This might have affected the evaluation of clinically relevant changes. Nevertheless, to date no treatment has formally demonstrated superiority versus placebo. In addition, since therapeutic management was left to the discretion of the treating physician, when treatment was needed, patients were treated. Furthermore, this limitation has been encompassed by the use of external anchors to assess whether the patient's condition has changed. In the EULAR cohort, physicians were instructed to include approximately half of the patients with systemic complications, not in the ASSESS cohort. This might explain why at the baseline visit in the EULAR cohort the estimates of disease activity levels were slightly higher than at the 6-month visit and that in the ASSESS cohort. Nevertheless, these discrepancies also underlined the fact that in multisystem disease with multiorgan involvement, such as pSS, no single score can perfectly 'translate' expert's evaluation of disease activity.

Table 4 Definition of MCII with ESSDAI and ESSPRI

	EULAR cohort Baseline—6 months	ASSESS cohort Baseline—1 year
ESSDAI MCII [95% CI]		
In the whole cohort	N=47	N=49
Absolute change	−4 [−5 to −2]	−1 [−3 to 0]
Relative change (% of baseline value)	−52.8% [80.0 to 9.1]	−14.9% [−57.1 to 0.0]
Among patients with ESSDAI≥5 at baseline	N=37	N=18
Absolute change	−5 [−7 to −2]	−3 [−5 to 0]
Relative change (% of baseline value)	−60.1% [83.3 to 22.2]	−27.1% [−66.7 to 0.0]
ESSPRI MCII [95% CI]		
In the whole cohort		
Improved patients according to Likert scale	N=87	NA
Absolute change	−0.67 [−1.00 to −0.33]	
Relative change (% of baseline value)	−11.1% [−18.8 to −5.0]	
Improved patients according to binary question	N=69	
Absolute change	−0.67 [−1.00 to 0.00]	
Relative change (% of baseline value)	−10.7% [−18.7 to −4.5]	
Among patients with ESSPRI≥5 at baseline		
Improved patients according to Likert scale	N=49	NA
Absolute change	−1.00 [−1.33 to −0.67]	
Relative change (% of baseline value)	−16.6% [−21.7 to −10.5]	
Improved patients according to binary question	N=50	
Absolute change	−1.00 [−1.33 to −0.67]	
Relative change (% of baseline value)	−15.6% [−23.1 to −10.0]	

ASSESS, Assessment of Systemic Signs and Evolution of SS; EULAR, European League Against Rheumatism; ESSDAI, EULAR Sjögren's syndrome disease activity index; ESSPRI, EULAR Sjögren's syndrome patient-reported index; MCII, minimal clinically important improvement; NA, not available.

Table 5 Relevance of MCII tentative cut-offs for ESSDAI and ESSPRI

MCII thresholds	Decrease of ESSDAI *Among patients with baseline ESSDAI≥5*			Decrease of ESSPRI *Among patients with baseline ESSPRI≥5*			
Clinical trials	**≥2**	**≥3**	**≥4**	**≥0.67**	**≥1**	**≥10%**	**≥15%**
TRIPPS[11]							
Placebo arm	NA	NA	NA	14/45 (31.1%)	12/45 (26.7%)	13/45 (28.9%)	11/45 (24.4%)
Infliximab arm				17/53 (32.18%)	9/53 (17.0%)	15/53 (28.3%)	12/53 (22.6%)
Rituximab trial (the Netherlands)[14]							
Placebo arm	3/7 (42.9%)	1/7 (14.3%)	1/7 (14.3%)	NA	NA	NA	NA
Rituximab arm	10/13 (76.9%)	10/13 (76.9%)	9/13 (69.2%)				
TEARS[12]							
Placebo arm	19/40 (47.5%)	13/40 (32.5%)	12/40 (30.0%)	20/43 (46.5%)	16/43 (37.2%)	20/43 (46.5%)	17/43 (39.5%)
Rituximab arm	21/47 (44.7%)	15/47 (31.9%)	14/47 (29.8%)	26/45 (57.8%)	21/45 (46.7%)	25/45 (55.6%)	21/45 (46.7%)
JOQUER[13]							
Placebo arm	12/18 (66.7%)	10/18 (55.6%)	10/18 (55.6%)	15/35 (42.9%)	13/35 (37.1%)	14/35 (40.0%)	12/35 (34.3%)
Hydroxychloroquine arm	11/14 (78.6%)	7/14 (50.0%)	4/14 (28.6%)	15/35 (42.9%)	13/35 (37.1%)	14/35 (40.0%)	10/35 (28.6%)
BELISS[16]							
Belimumab arm	15/18 (83.3%)	12/18 (66.7%)	10/18 (55.6%)	16/28 (57.1%)	12/28 (42.9%)	15/28 (53.6%)	11/28 (39.3%)
ASAP[15]							
Abatacept arm	13/14 (92.9%)	12/14 (85.7%)	12/14 (85.7%)	6/12 (50.0%)	6/12 (50.0%)	6/12 (50%)	6/12 (50%)

EULAR, European League Against Rheumatism; ESSDAI, EULAR Sjögren's syndrome disease activity index; ESSPRI, EULAR Sjögren's syndrome patient-reported index; NA, not available.

The treatment of primary SS remains a challenge. Effectively, whatever the drug used, hydroxychloroquine,[13] tumour necrosis factor-blockers[11 27] and even B-cell-targeted therapies, such as rituximab,[12] most of the RCTs did not achieve their primary endpoints. Regarding rituximab, the results remains however controversial with positive results in a small trial[14] and at 6 weeks in a larger recent study, but not confirmed at 24 weeks.[12] However, since not available, none of these trials used a systemic disease activity measure as the primary outcome, but patient-reported outcomes or dryness measures, which have been shown to be less sensitive to change.[3] In addition, some of the recent trials used ESSDAI as a secondary outcome and found it sensitive to change.[15 16 28 29]

Learning from these results, methodological insight was needed to improve the design of clinical trials in primary SS. The present study helps addressing main methodological questions for designing RCT: determining the key inclusion criteria and defining response criteria.

Better definition of inclusion criteria is a key issue. The objective is to select the patients who are most likely to benefit from the treatment in order to increase the chances of demonstrating treatment efficacy. For biologics or immunosuppressants, they might be a combination of factors, such as the inclusion of patients with active systemic complications, very early and/or biologically active disease.[16 30] An alternative, or complementary, approach is to require a minimal ESSDAI score, such as moderate activity threshold (ESSDAI≥5), to ensure inclusion of patients with active disease. We showed that most of the patients in recent trials had at least moderate activity, which demonstrates that this will not be a limit for recruitment in future RCTs.

The other key issue is to determine an optimal primary endpoint. To evaluate biologically active and immunosuppressive drugs, preferentially dedicated to patients with active systemic disease, the use of more objective endpoints, for example, ESSDAI, seems preferable. This study had determined MCII of ESSDAI (decrease of at least three points), which can be a first step for the definition of response criteria. The MCII value was of five points in the EULAR cohort and three points in the ASSESS cohort, both above the respective MDC found in each cohort. Thus, an MCII of 3 is the minimal required, but it can be increased in case of trials involving highly systemic patient populations.

In systemic lupus erythematosus (SLE), such a process of determining better definition of inclusion criteria to select active patients more likely to respond to treatment[31] and using a powerful response criteria (the SLE responder index[32]) has been applied with success to show the effectiveness of belimumab.[33 34]

Even though in clinical practice most of patients with pSS has low-disease activity, a part of patients had severe systemic complications. Nevertheless, among these systemic patients who might require and/or benefit from immunosuppressive treatment or biologic, we might keep in mind that patients included in the RCTs have usually less severe systemic manifestations than those treated in real life. Effectively, the most severe patients cannot be exposed to the risk of receiving placebo. This might explain the discrepancies between the results of RCTs and real-life experience, as for rituximab.[35] Also, this might make it more difficult to demonstrate drug efficacy since RCTs mainly included patients with mild to moderate activity. Therefore, even though they are less sensitive to change, a PRO such as ESSPRI can be considered as an outcome in an RCT whatever the drug used. Effectively, PROs and particularly ESSPRI or its individual components have been shown to significantly improve in some recent trials.[14–16] The treatment of chronic diseases finally aims to improve quality of life and patient-perceived health status. PROs are the only tools addressing this issue. Thus, considering the inclusion of patients having an ESSPRI above the threshold of unsatisfactory symptom state (ESSPRI≥5) and a defining response as an improvement of ESSPRI MCII (ESSPRI decrease of at least one point or 15%) might also be considered for future clinical trials.

In conclusion, this study, including two large cohorts of patients, has defined disease activity levels, PASS and MCII of ESSDAI and ESSPRI. Hopefully this will help to homogenise and determine the most effective way to conduct clinical trials. To evaluate treatments of systemic complications, the proposal is to include patients with at least moderate activity (ESSDAI≥5) and to define response to treatment as an improvement of

ESSDAI of at least three points. For addressing patient-reported outcomes, inclusion of patients with unsatisfactory symptom state (ESSPRI≥5) and defining response as an improvement of ESSPRI of at least one point or 15% seems reasonable. The choice of ESSDAI or ESSPRI or a combination of both will depend on the type of the drug to evaluate the study design and remains to be determined by future studies.

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The Anergy Induction of M3 Muscarinic Acetylcholine Receptor–Reactive CD4+ T Cells Suppresses Experimental Sialadenitis-like Sjögren's Syndrome

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Objective. **Autoreactive CD4+ T cells are involved in the pathogenesis of Sjögren's syndrome (SS). The aim of the present study was to clarify the dominant T cell epitopes of M3 muscarinic acetylcholine receptor (M3R) and to establish a new antigen-specific therapy for SS using an experimental mouse model.**

Methods. **Production of cytokines from M3R-reactive CD4+ T cells, after culture with various M3R peptides, was analyzed by enzyme-linked immunosorbent assay. Adoptive cell transfer was performed using splenocytes from M3R$^{-/-}$ mice that were immunized with M3R peptides or phosphate buffered saline plus H37Ra as a control. Rag1$^{-/-}$ mice were inoculated with the splenocytes and examined for the development of sialadenitis. Altered peptide ligands (APLs) of the T cell epitopes, with substitutions in amino acid residues at T cell receptor contact sites, were synthesized, and the ability of the APLs to suppress sialadenitis was evaluated. The mechanisms underlying such effects were assessed.**

Results. **CD4+ M3R-reactive T cells produced interleukin-17 (IL-17) and interferon-γ (IFNγ) in response to the N-terminal 1 (N1) and 1st extracellular loop peptides of M3R, and Rag1$^{-/-}$ mice that received N1- and/or 1st peptide–immunized splenocytes developed sialadenitis. Among the designed APLs, N1-APL7 (N→S at amino acid 15) significantly suppressed IFNγ production in vitro, and also suppressed sialadenitis in vivo. Levels of early growth response 2 in CD4+ T cells from the cervical lymph nodes of N1-APL7–treated mice were significantly higher than those of control mice, and cell proliferation was reversed by administration of exogenous IL-2. Levels of the anergy-related molecules itchy homolog E3 ubiquitin-protein ligase, Casitas B-lineage lymphoma b, gene related to anergy in lymphocytes, and Deltex-1 were significantly higher in CD4+ T cells cultured with N1-APL7.**

Conclusion. **The major T cell epitopes were from the N1 and 1st peptide regions. Moreover, N1-APL7, selected as the antagonistic APL in vitro, also suppressed sialadenitis through the induction of anergy. This is a potentially useful strategy for regulating pathogenic T cell infiltration in SS.**

Sjögren's syndrome (SS) is an autoimmune disease characterized by lymphocytic infiltration of the lacrimal and salivary glands, leading to dry eyes and dry mouth. Immunohistochemical studies have shown that most infiltrating lymphocytes around the labial salivary glands, lachrymal glands, and kidneys are CD4+ α/β T cells (1). Moreover, the restricted usage of T cell receptor (TCR) α/β has been reported, suggesting that the antigen-specific immune response is the main pathologic mechanism of SS, and that non–antigen-specific immune responses are secondary mechanisms (2). Autoantigens, dumped from the damaged salivary glands, are presented by antigen-presenting cells (APCs), and autoantigen-specific T cells are activated to induce the proliferation of polyclonal T cells.

Autoantigens recognized by T cells have been analyzed, and several candidates for autoantigens, such

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as Ro/SSA 52 kd (3–5) and α-amylase (6), have been identified. Recently, M3 muscarinic acetylcholine receptor (M3R) has been advanced to the front as one of the etiologic autoantigens of SS. M3R is important for the secretion and contraction of exocrine glands (e.g., lacrimal and salivary glands) (7). The presence of anti-M3R autoantibodies has been reported in SS patients, and M3R-reactive T cells have been detected in peripheral blood mononuclear cells (8–12). These findings emphasize the important role of the autoimmune response against M3R in the development of SS (8,13).

In experimental mouse models of SS, previous studies have demonstrated that inoculation of $Rag1^{-/-}$ mice with splenocytes from $M3R^{-/-}$ mice that had been immunized with a mixture of M3R peptides (peptides from the N-terminal region and from the 1st, 2nd, and 3rd extracellular loops) developed sialadenitis mimicking SS (M3R-induced sialadenitis [MIS]) (14,15). Moreover, MIS is characterized by the presence of anti-M3R autoantibodies and the production of interleukin-17 (IL-17) and interferon-γ (IFNγ) by M3R-reactive T cells (16,17).

Although SS is a relatively common rheumatic disease (affecting ~0.1–0.6% of the total population), its treatment is based on the use of substitute agents for the amelioration of sicca features, and there is only limited curative treatment at present (18). Several trials using biologic agents have been evaluated, but no significant clinical improvement was observed; rather, such treatment was reported to be associated with the development of serious infections (19).

Altered peptide ligands (APLs) are peptides with substitutions in amino acid residues at TCR contact sites. APLs are known to be involved in changes to TCR affinity, and can be antagonistic with partial activation (20). Antagonistic APLs can inhibit the function of specific T cell populations and are potentially useful as antigen-specific therapy for immune diseases such as SS. The aim of the present study was to delineate the major T cell epitopes of M3R molecules and to establish antigen-specific therapy for SS using an experimental mouse model.

MATERIALS AND METHODS

Mice. C57BL/6J mice ($M3R^{+/+}$) were purchased from Charles River Laboratory. $Rag1^{-/-}$ mice were purchased from The Jackson Laboratory. $M3R^{-/-}$ mice, generated from B6 mice, were kindly provided by one of the authors (MM) (14). Mice were maintained under specific pathogen–free conditions in the Laboratory Animal Resource Center at the University of Tsukuba. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals at the University of Tsukuba.

Synthesized peptides encoding M3R extracellular regions. Six types of peptides encoding murine M3R extracellular domains were synthesized chemically using a solid-phase procedure. These peptides were N-terminal 1 (MTLHSNS TTSPLFPNISSSWVHSPSEAGLP) (N1 peptide), N-terminal 2 (VHSPSEAGLPLGTVSQLDSYNISQTSGNFS) (N2 peptide), N-terminal 3 (NISQTSGNFSSNDTSSDPLGGHTI WQV) (N3 peptide), 1st extracellular loop (FTTYIIMNRWA LGNLACDLW) (1st peptide), 2nd extracellular loop (QYFVGKRTVPPGECFIQFLSEP) (2nd peptide), and 3rd extracellular loop (VLVNTFCDSCIPKTYWNLGY) (3rd peptide). The synthesized peptides were >90% pure, as confirmed by high-performance liquid chromatography (HPLC) (Scrum). The purity of all APLs (as discussed below) was also >90%, as determined by HPLC.

Immunization of mice. $M3R^{+/+}$ and $M3R^{-/-}$ mice were immunized intradermally at the base of the tail with M3R extracellular peptides (20 μg each) or, as a control, phosphate buffered saline (PBS) emulsified with Freund's incomplete adjuvant (IFA; Difco) containing 250 μg of inactivated *Mycobacterium tuberculosis* (H37Ra; Difco). Pertussis toxin (500 ng; Sigma-Aldrich) was injected intraperitoneally on the day of immunization. On day 10, each mouse received another intradermal injection of the same peptides or PBS emulsified with IFA containing H37Ra.

Analysis of cytokine profile. On day 20 after the first immunization, the spleens of mice were isolated, and red blood cells were removed using 0.16*M* NH_4Cl solution. Single-cell suspensions were prepared in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1,000 units/ml penicillin, 100 μg/ml streptomycin, and 55 μ*M* 2-mercaptoethanol. CD4+ T cells and CD11c+ cells were isolated by magnetic-activated cell sorting (Miltenyi Biotec). CD11c+ cells were treated with 50 μg/ml mitomycin C and used as APCs. The purity of each collected cell type (CD4+ T cells >95%, CD11c+ cells >88%) was confirmed by flow cytometry.

The CD4+ T cells and CD11c+ APCs were cocultured with each M3R extracellular peptide (5 μg/ml) at a ratio of 5:1 in 96-well round-bottomed plates (Nunc) in an atmosphere of 5% CO_2 at 37°C, adjusted to 1.8×10^6 cells/well. After 72 hours, the levels of IFNγ, IL-17, and IL-4 were measured by enzyme-linked immunosorbent assay (ELISA; R&D Systems). After 48 hours, the production of IL-2 was measured by ELISA (R&D Systems) in combination with an anti–major histocompatibility complex class α monoclonal antibody (BioLegend) or isotype-matched rat monoclonal antibody (BD Biosciences).

Fluorescence-activated cell sorting (FACS) staining. For FACS analyses, the cells were washed in FACS buffer (2% FBS in PBS) and then incubated with FACS antibodies. Intracellular staining was performed using Treg cell staining kits (eBioscience) according to the protocol supplied by the manufacturer. All antibodies were used at a concentration of 5–10 μg/ml and allowed to bind over 30 minutes on ice. The anti-mouse antibodies used included antibodies against CD3, CD4, CD8, CD19, IFNγ, IL-17 (all from BioLegend), and early growth response 2 (Egr-2; eBioscience).

Pre-pulse assay. CD4+ and CD11c+ cells were isolated as described above. CD11c+ cells were cultured with a suboptimal concentration of N1 peptide (1.5 μ*M*) or 1st peptide (4.5 μ*M*) for 2 hours, and then washed and adjusted to

3.0×10^4 cells/well, after which each APL (100 μM) was added. After 18 hours of incubation, CD4+ T cells (1.5×10^5 cells/well) were added. After 72 hours, the levels of IFNγ and IL-17 were measured by ELISA. The inhibition ratio, expressed as a percentage, was calculated as follows: (1 − [cytokine concentration in the presence of native peptides and APLs/cytokine concentration in the presence of native peptides only]) × 100.

Adoptive transfer and treatment with APLs. On day 20 after the first immunization, cells from the spleens of M3R$^{-/-}$ mice were isolated. The cells were resuspended in PBS and 1×10^7 cells were injected intravenously into Rag1$^{-/-}$ mice. Each mouse was also treated with an intravenous injection of 100 μg of APLs on days 7 and 10 after the cell transfer.

Measurement of salivary volume. Mice were anesthetized with an intraperitoneal injection of pentobarbital (30 mg/kg), and then injected subcutaneously with pilocarpine (1.0 mg/kg). This was followed by collection of saliva from the oral cavity over a period of 15 minutes using a 200-μl micropipette. The volume of the collected sample was measured. Changes in saliva volume were calculated as the saliva volume at day 45 relative to the volume measured at baseline, with results expressed as μl saliva per gram of body weight.

Analysis of messenger RNA (mRNA) expression levels. Total RNA was extracted from CD4+ T cells in the cervical lymph nodes of mice using the Isogen method, and complementary DNA (cDNA) was synthesized using a cDNA synthesis kit (Takara, Japan). In vitro assays also involved total RNA extraction from the cervical lymph node cells. Expression of mRNA for *Egr2* (Mm00456650_m1), itchy homolog E3 ubiquitin-protein ligase (*Itch*) (Mm00492683_m1), Casitas B-lineage lymphoma b (*Cbl-b*) (Mm01343092_m1), *Rnf128* (encoding gene related to anergy in lymphocytes [*GRAIL*]) (Mm00480990_m1), Deltex-1 (*Dtx1*) (Mm00492297_m1), lymphocyte-activation gene 3 (*Lag3*) (Mm00493071_m1), *Ctla4* (Mm00486849_m1), *Pdcd1* (Mm01285676_m1), *Il4* (Mm00445259_m1), *Il10* (Mm00439614_m1), and *Foxp3* (Mm00475156_m1) was determined by quantitative polymerase chain reaction (Applied Biosystems). *Gapdh* was used as the internal control gene. The reported gene expression data are representative of 3 independent experiments (n = 3 mice per group per experiment).

Histologic scoring. The salivary glands of mice were examined for inflammatory lesions, which were graded histologically using the method proposed by Greenspan et al (21), as follows: focus score of 1 = mild infiltration, 2 = intermediate infiltration, 3 = a single focus composed of >50 mononuclear cells per 4 mm^2 of tissue, and 4 = >2 foci. Histologic evaluation was performed in a blinded manner.

Immunohistochemical analysis. Sections of the salivary glands were thawed, dried, and fixed with formalin for 10 minutes. The sections were blocked in PBS with 1% bovine serum albumin for 30 minutes and then incubated with Alexa Fluor 488–conjugated rat anti-mouse CD3 or Alexa Fluor 647–conjugated rat anti-mouse B220 antibodies (all from Invitrogen) for 30 minutes. The stained sections were counterstained with DAPI and mounted with aqueous medium. Apoptotic cells were detected using an in situ apoptosis detection kit (Takara Biomedicals).

Immunoblotting. Cells were lysed in lysis buffer (0.5% Nonidet P40, 5 mM $MgCl_2$, 50 mM Tris HCl, and 2 mM phenylmethylsulfonyl fluoride). Equal volumes of proteins were subjected to immunoblotting using antibodies to Itch, Cbl-b (Cell Signaling Technology), GRAIL (AbCam), and actin (Sigma). The proteins were visualized using an ImageQuant LAS 4000 densitometer (GE Healthcare). The fluorescence intensity of the visualized proteins was analyzed using ImageJ, with values adjusted to the intensity of actin.

Statistical analysis. Results are expressed as the mean ± SD. Statistically significant differences between groups were examined using Dunnett's t-test or Student's t-test. P values less than 0.05 were considered statistically significant.

RESULTS

High production of IL-17 and IFNγ from M3R-reactive T cells in response to N1 and 1st peptides. To compare the immune response of M3R-reactive T cells to each M3R region, M3R$^{-/-}$ mice were immunized with a mixture of M3R extracellular peptides (hereafter designated the total peptide mix) or with PBS as a control. CD4+ T cells and CD11c+ APCs were isolated from the splenocytes of the immunized mice and then cocultured with each M3R peptide in vitro. As shown in Figure 1A, the production levels of IL-17 and IFNγ, but not those of IL-4, in M3R-reactive T cells in response to coculture with either the M3R N1 peptide or the M3R 1st peptide were significantly higher than those in response to peptides from the other M3R regions. Intriguingly, N1 peptide–reactive T cells produced larger amounts of IFNγ than did 1st peptide–reactive T cells (Figure 1A). There was no difference in the production of IL-17 between N1 peptide–reactive and 1st peptide–reactive T cells. To confirm the immune response of M3R N1– and 1st peptide–reactive T cells, M3R$^{-/-}$ mice were immunized with only the N1 peptide or only the 1st peptide. Thereafter, as described above, CD4+ T cells and CD11c+ cells were cocultured with each M3R peptide in vitro. M3R N1– and 1st peptide–reactive T cells produced higher amounts of IL-17 and IFNγ, but not IL-4, after stimulation with each corresponding peptide alone (Figures 1B and C and results not shown).

In addition, we evaluated cytokine production from the splenocytes of M3R$^{-/-}$ mice that had been immunized with the combination of N1 and 1st peptides (hereafter designated the N1 + 1st peptide mix) compared to cytokine production from the splenocytes of mice that had received a mixture without the N1 and 1st peptides (hereafter designated the empty mix). As expected, cytokine production by the splenocytes from mice that received the N1 + 1st peptide mix showed the same tendency as that described above in mice immunized with the total peptide mix, and mice that received

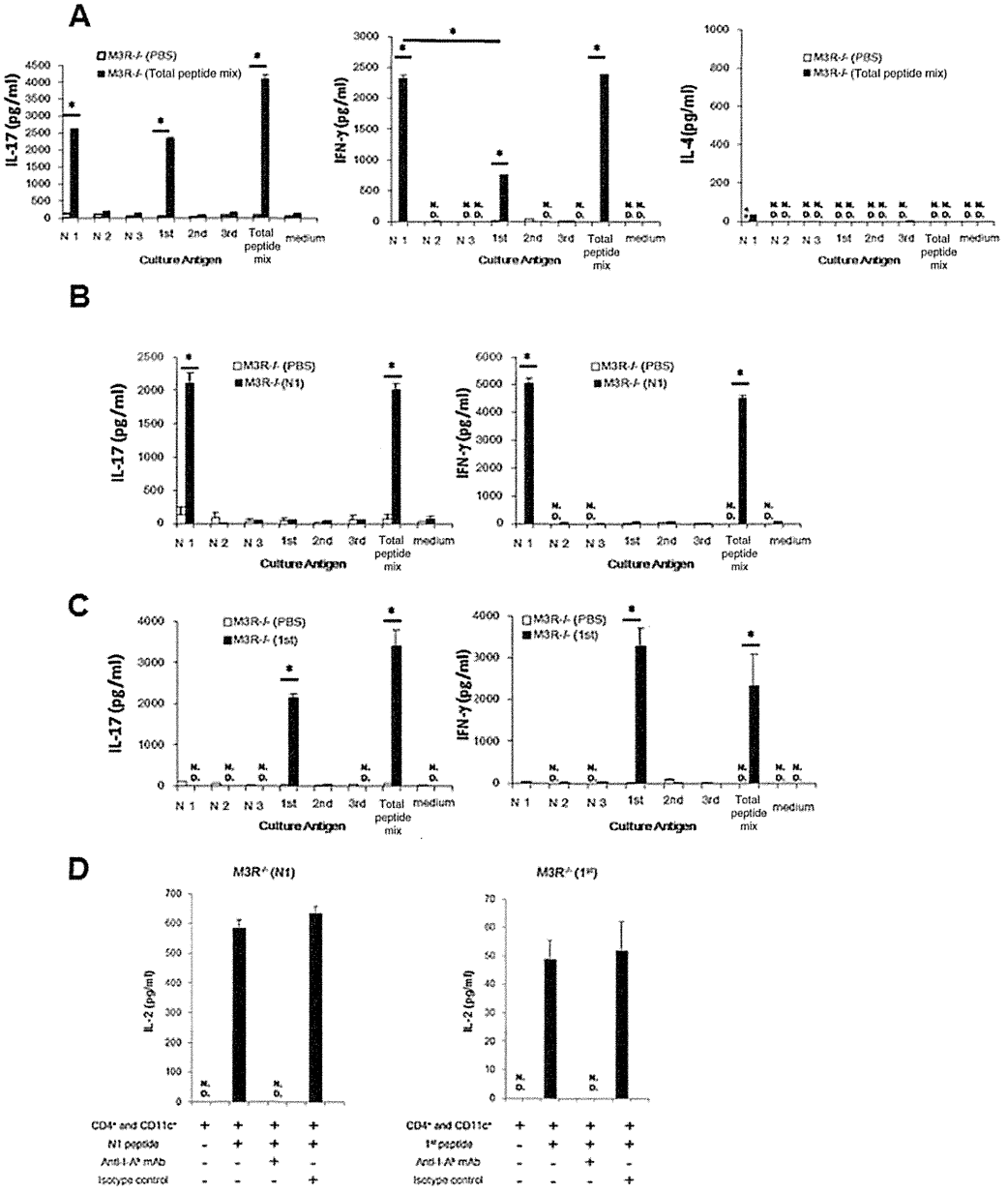


Figure 1. Cytokine profiles of M3 muscarinic acetylcholine receptor (M3R)–reactive T cells in $M3R^{-/-}$ mice. **A–C,** $M3R^{-/-}$ mice were immunized, followed by a booster dose, with a mixture of M3R extracellular peptides (total peptide mix) (**A**), the M3R N-terminal 1 (N1) peptide alone (**B**), or the M3R 1st extracellular loop (1st) peptide alone (**C**), or with phosphate buffered saline (PBS) emulsified with Freund's incomplete adjuvant containing *Mycobacterium tuberculosis*. Ten days later, the spleens were extracted, CD4+ and CD11c+ cells were isolated, and the cells were cocultured with each M3R peptide, total peptide mix, or medium alone. Concentrations of interleukin-17 (IL-17), interferon-γ (IFNγ), and IL-4 in the culture supernatants were measured by enzyme-linked immunosorbent assay. **D,** Production of IL-2 by N1- and 1st peptide–reactive T cells from $M3R^{-/-}$ mice in response to coculture with each corresponding peptide was evaluated in the presence or absence of an anti–major histocompatibility complex class II antibody. Values are the mean ± SD of triplicate cultures. Data are representative of 3 independent experiments (n = 2–4 mice per group per experiment). $* = P < 0.05$ by Dunnett's *t*-test. ND = not detected.

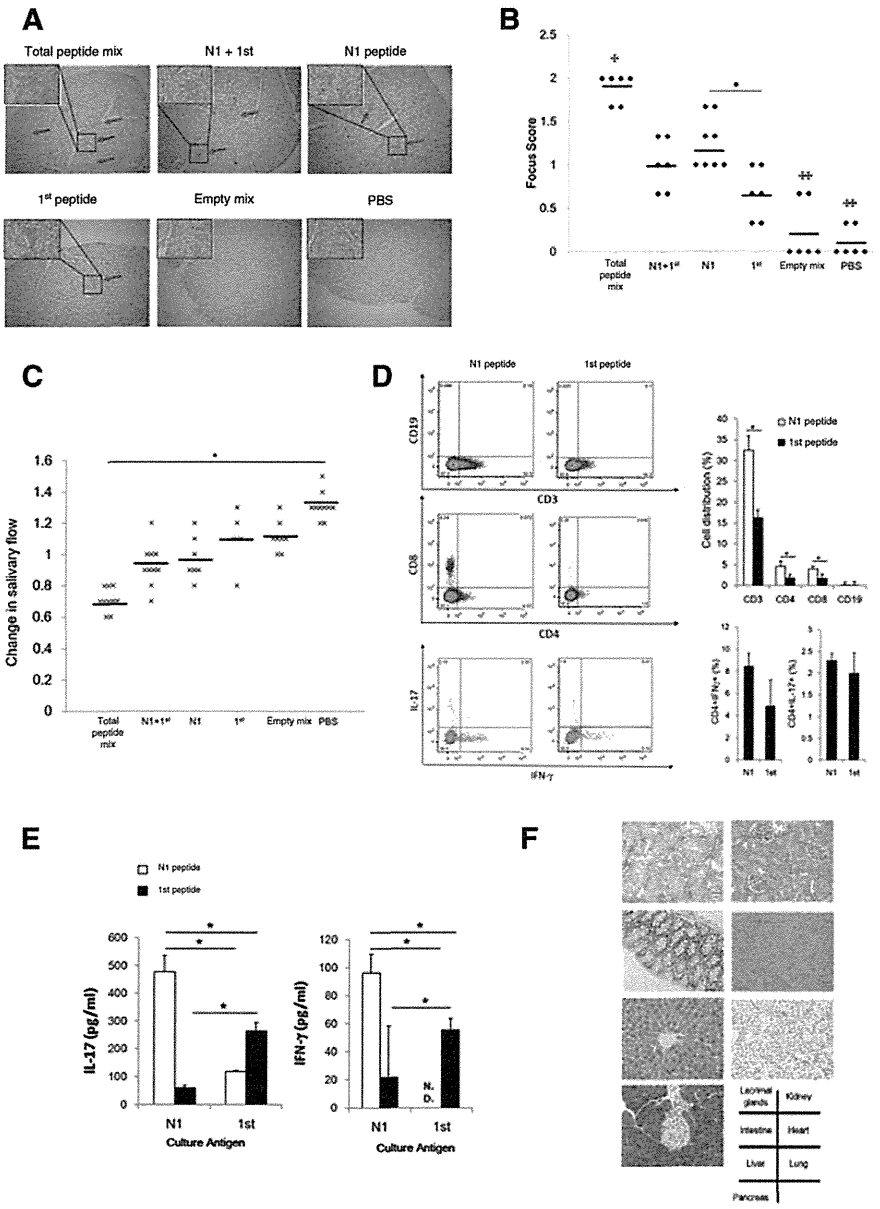


Figure 2. Phenotype of Rag1$^{-/-}$ mice after adoptive transfer of cells from immunized M3R$^{-/-}$ mice. Various organs, including the salivary glands, were isolated from Rag1$^{-/-}$ mice on day 45 after cell transfer from M3R$^{-/-}$ mice immunized with total peptide mix, N1 + 1st peptide, N1 peptide alone, 1st peptide alone, empty mix without N1 or 1st peptides, or PBS. **A,** Salivary glands of the Rag1$^{-/-}$ mice in each inoculation group were stained with Mayer's hematoxylin and eosin. **Arrows** indicate cell infiltration. **Insets** show amplified views of the boxed areas. Original magnification × 40; original magnification of **insets** × 200. **B,** Histologic focus scores were assigned to evaluate severity of inflammation in the salivary glands of Rag1$^{-/-}$ mice after adoptive transfer. Symbols indicate individual mice (n = 6–8 per group); horizontal bars show the mean. **C,** Saliva was collected from Rag1$^{-/-}$ mice after adoptive transfer, and the saliva volume was measured on day 45, calculated relative to the volume at baseline (all values adjusted for body weight). Symbols indicate individual mice (n = 8–10 per group); horizontal bars show the mean. **D,** Lymphocytes from the salivary glands of Rag1$^{-/-}$ mice in the N1 peptide alone and 1st peptide alone inoculation groups were assessed by flow cytometry for expression of cell subsets and cytokine production after stimulation with phorbol myristate acetate and ionomycin for 6 hours (left), and the percentages of each cell subset and IFNγ-producing CD4+ T cells were quantified (right). Results are the mean ± SD of pooled cells from 2–5 mice per group. **E,** Production of IL-17 and IFNγ from the splenocytes of Rag1$^{-/-}$ mice was assessed in the N1 peptide alone and 1st peptide alone inoculation groups after coculture with each corresponding peptide. Results are the mean ± SD of 2–5 mice per group per experiment. **F,** Tissue sections from the indicated organs of Rag1$^{-/-}$ mice after cell transfer were stained for histologic assessment. Original magnification × 100. * = $P < 0.05$ between the indicated groups; ✣ = $P < 0.05$ versus all other groups; ✣✣ = $P < 0.01$ versus all other groups. See Figure 1 for definitions.

the empty mix showed the same pattern of cytokine production by splenocytes as that in mice immunized with PBS (results not shown).

The cytokine production from N1- or 1st peptide–reactive T cells in response to coculture with the corresponding peptide was inhibited when these cells were cultured in the presence of anti–I-A^b antibody, suggesting that each TCR recognizes each peptide in an I-A^b–restricted manner (Figure 1D). These results suggest that the T cell epitopes in MIS might be located in both the N1 and 1st regions of M3R.

Induction of sialadenitis as validation of the T cell epitopes of M3R in both the N1 and 1st regions. We have already reported that M3R-reactive T cells are crucial in the development of sialadenitis through the production of both IFNγ and IL-17. To evaluate the T cell epitopes of MIS, the salivary glands of $Rag1^{-/-}$ mice were histologically examined on day 45 after inoculation with splenocytes from $M3R^{-/-}$ mice that had been immunized with one of the following mixtures: total peptide mix, N1 + 1st peptide mix, N1 peptide alone, 1st peptide alone, empty mix, or PBS. Sialadenitis developed in $Rag1^{-/-}$ mice after adoptive transfer of cells from mice immunized with the total peptide mix, N1 + 1st peptide mix, N1 peptide alone, or 1st peptide alone, whereas no sialadenitis was evident in $Rag1^{-/-}$ mice that had been inoculated with cells from mice immunized with the empty mix or PBS (Figure 2A). The histologic focus score of $Rag1^{-/-}$ mice inoculated with N1 peptide–expressing mouse splenocytes was significantly higher than that of $Rag1^{-/-}$ mice inoculated with 1st peptide–expressing cells (Figure 2B). Moreover, the saliva volume of $Rag1^{-/-}$ mice in the total peptide mix recipient group was significantly lower than that of $Rag1^{-/-}$ mice in the PBS control group on day 45 (Figure 2C).

When we evaluated the infiltrating cells in the salivary glands of $Rag1^{-/-}$ mice in the N1 peptide and 1st peptide recipient groups, we found that the majority of cells were T cells. Moreover, both CD4+ and CD8+ T cells were significantly more abundant in the salivary glands of mice that received N1 peptide–expressing cells compared with those that received 1st peptide–expressing cells (Figure 2D).

Since both IL-17 and IFNγ have been reported to be important for the induction of sialadenitis, we also assessed their expression levels in the mouse salivary glands. The percentages of IL-17– and IFNγ-producing CD4+ cells were higher in the salivary glands of $Rag1^{-/-}$ mice in the N1 peptide inoculation group compared with $Rag1^{-/-}$ mice in the 1st peptide inoculation group (Figure 2D).

To assess the cytokine production from M3R-reactive T cells circumstantially, CD4+ T cells and CD11c+ APCs were isolated from the splenocytes and cocultured with either the N1 or 1st peptide (Figure 2E). As expected, CD4+ T cells from $Rag1^{-/-}$ mice inoculated with N1 peptide–expressing mouse splenocytes produced significantly higher levels of IFNγ in cocultures with the N1 peptide when compared with CD4+ T cells from $Rag1^{-/-}$ mice inoculated with 1st peptide–expressing mouse splenocytes in cocultures with the 1st peptide.

Furthermore, in $Rag1^{-/-}$ mice in the total peptide mix inoculation group, cell infiltration was also detected in the lacrimal glands, but not in the other organs examined in this study (Figure 2F). Comparable results were also detected in $Rag1^{-/-}$ mice inoculated with cells expressing the N1 peptide alone or 1st peptide alone (results not shown).

In summary, these findings indicate that both the N1 and 1st peptides of M3R are T cell epitopes in MIS. These reactive T cells could play important roles in the development of experimental SS.

Designing and screening of antagonistic APLs. Since both M3R N1– and 1st peptide–reactive T cells are involved in the development of autoimmune sialadenitis, we hypothesized that suppression of these T cells might inhibit the development of sialadenitis. In order to suppress M3R-reactive T cells, we first designed APLs in the N1 and 1st regions of M3R (N1-APLs and 1st-APLs, respectively). APLs are designed to alter the affinity of the TCR, an action that has the possibility of changing the functions of T cells. Since I-A^b is thought to bind with peptides at positions p1, p4, p6, and p9 (22,23), the amino acid residues of the TCR contact sites at p2, p3, p5, and p8 were substituted with other amino acids to construct the APLs (Figures 3A and B).

To select antagonistic APLs, CD4+ T cells primed with CD11c+ cells and with M3R N1 or 1st peptides were cocultured with each N1-APL or 1st-APL in the pre-pulse assay (Figure 3C). Among the analog peptides, N1-APLs 5, 6, and 7 significantly reduced the production of IFNγ by CD4+ T cells primed with M3R N1 peptide. In contrast, 1st-APL8 significantly reduced the production of IL-17. These results suggest that both peptides act as antagonistic APLs by inhibiting the production of pathogenic cytokines by M3R-reactive T cells.

Suppression of sialadenitis in vivo by APLs. To evaluate the effects of APLs on autoimmune sialadenitis in vivo, antagonistic APLs, selected in vitro, were administered intravenously to $Rag1^{-/-}$ mice after transfer of total peptide mix–immunized mouse cells. As shown in Figures 4A and B, among the antagonistic APLs tested in this study, N1-APL7 significantly suppressed the induction of sialadenitis. On day 45 after

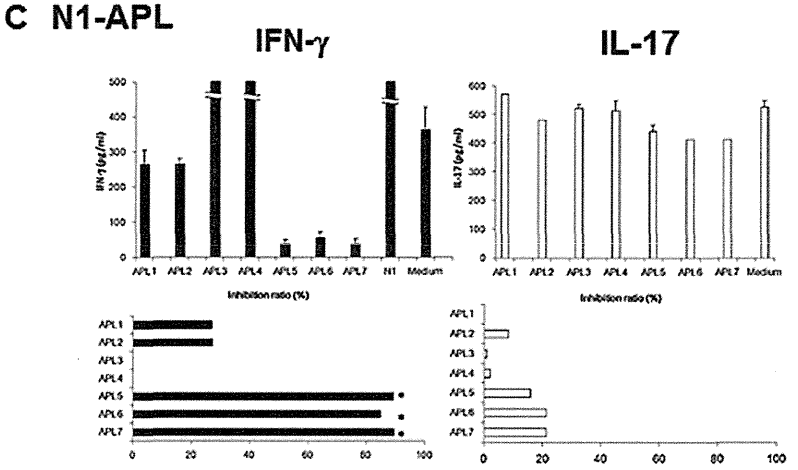
A N1-APL

M3R N1 derived altered peptide ligands										IC_{50} (nM)
M3R N1 (AA8-16)	T	T	S	P	L	F	P	N	I	101.62
N1-APL1	-	C	-	-	-	-	-	-	-	143.88
N1-APL2	-	-	C	-	-	-	-	-	-	147.23
N1-APL3	-	-	-	-	I	-	-	-	-	246.60
N1-APL4	-	-	-	-	P	-	-	-	-	290.40
N1-APL5	-	-	-	-	-	-	-	T	-	89.95
N1-APL6	-	-	-	-	-	-	-	C	-	92.47
N1-APL7	-	-	-	-	-	-	-	S	-	92.47

B 1st-APL

M3R 1st derived altered peptide ligands										IC_{50} (nM)
M3R 1st (AA133-141)	R	W	A	L	G	N	L	A	C	81.28
1st-APL1	-	F	-	-	-	-	-	-	-	72.78
1st-APL2	-	Y	-	-	-	-	-	-	-	72.78
1st-APL3	-	-	V	-	-	-	-	-	-	114.55
1st-APL4	-	-	M	-	-	-	-	-	-	139.00
1st-APL5	-	-	-	-	A	-	-	-	-	69.82
1st-APL6	-	-	-	-	V	-	-	-	-	81.28
1st-APL7	-	-	-	-	-	-	-	V	-	227.51
1st-APL8	-	-	-	-	-	-	-	M	-	227.51

C N1-APL



D 1st-APL

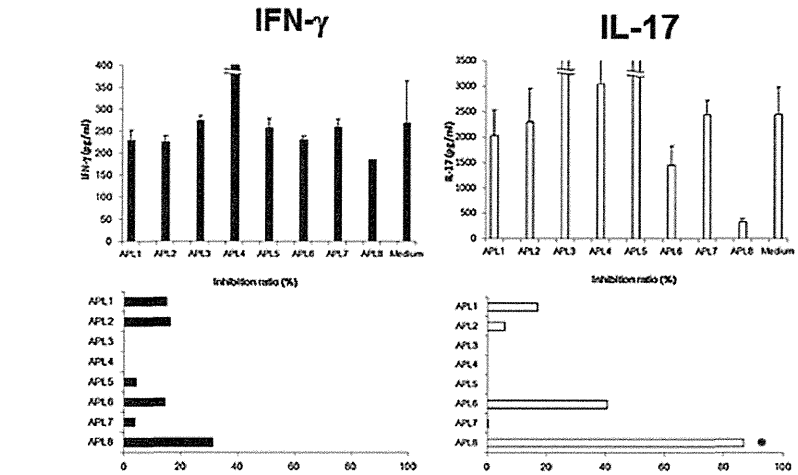


Figure 3. Designing and screening of altered peptide ligands (APLs) derived from the M3R N1 region and 1st extracellular loop. **A** and **B,** APLs of N1 peptides **(A)** and 1st peptides **(B)** were designed. The T cell receptor contact sites at p2, p3, p5, and p8 were substituted with other amino acids. **C** and **D,** CD4+ T cells were primed with CD11c+ cells and M3R N1 or 1st peptides and cocultured with each N1-APL **(C)** or 1st-APL **(D),** and antagonistic activities of the N1-APLs and 1st-APLs were evaluated by pre-pulse assay on day 20 after first immunization. Concentrations of IFNγ and IL-17 in the culture supernatants were measured by enzyme-linked immunosorbent assay (top), and inhibition ratios of each APL were determined (bottom). Cytokine concentrations are the mean ± SD of 4 mice per group per experiment. Data are representative of 3 independent experiments. * = $P < 0.05$ versus medium alone, by Dunnett's *t*-test. aa = amino acids; IC_{50} = half-maximal (50%) inhibitory concentration (see Figure 1 for other definitions).

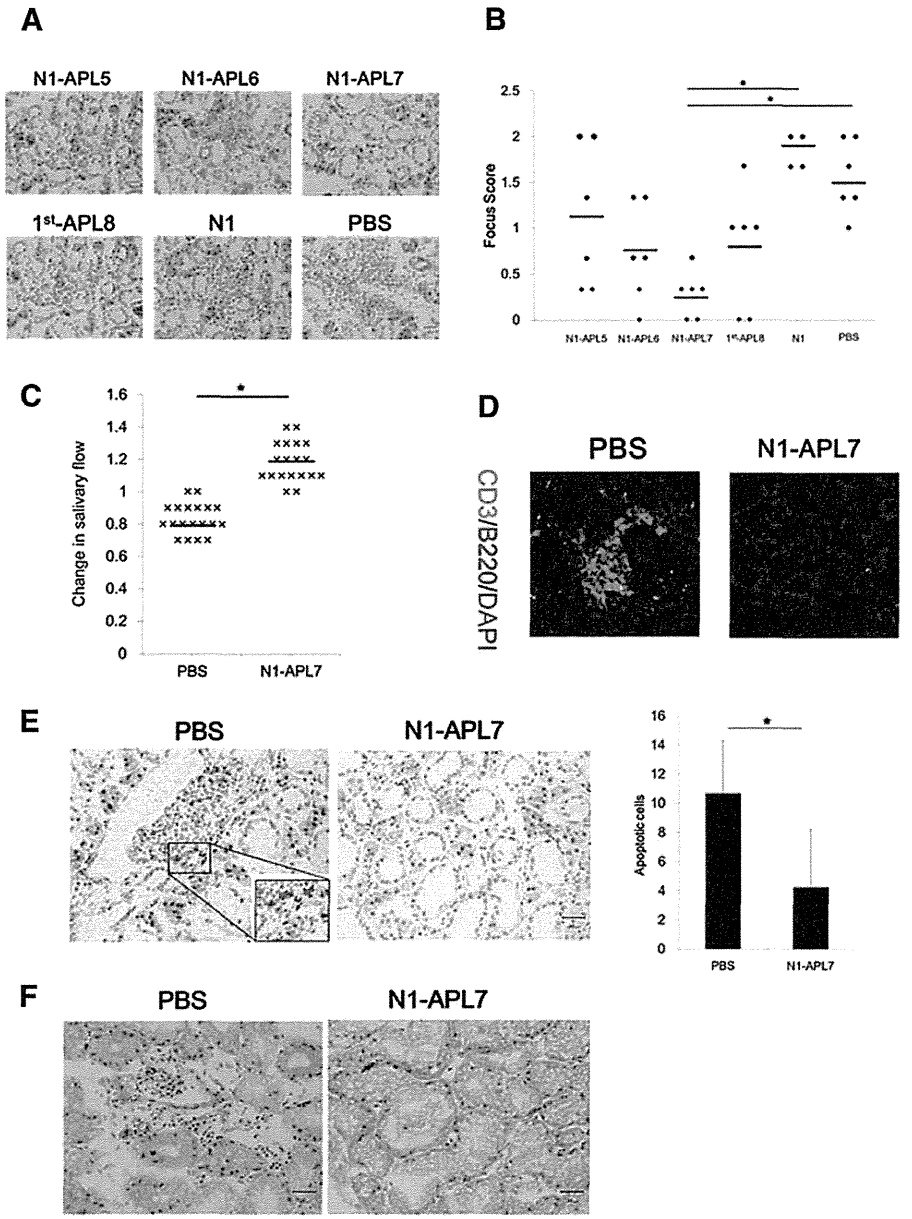


Figure 4. Inhibition of sialadenitis by antagonistic altered peptide ligands (APLs). The effect of antagonistic APLs in vitro was evaluated in vivo. $Rag1^{-/-}$ mouse recipients of splenocytes from $M3R^{-/-}$ mice immunized with the total M3R peptide mix were treated with intravenous APLs, or with N1 peptide alone or PBS, on days 7 and 10 after cell transfer. **A,** Salivary glands were isolated from the $Rag1^{-/-}$ mice on day 45 after adoptive transfer, and each section was stained with Mayer's hematoxylin and eosin (H&E). **B,** Histologic focus scores of inflammation were determined in the salivary glands of the $Rag1^{-/-}$ mice. Symbols indicate individual mice (n = 4–6 per group); bars show the mean. **C,** Saliva was collected from $Rag1^{-/-}$ mice on day 45 after adoptive transfer, and the saliva volume was measured, calculated relative to the volume at baseline (all values adjusted for body weight). Symbols indicate individual mice (n = 19 per group); bars show the mean. **D,** Inflammatory lesions in the salivary glands of $Rag1^{-/-}$ mice were assessed by staining with anti-CD3/anti-B220 antibodies. **E,** The proportion of apoptotic cells was evaluated by TUNEL staining (left), and results are expressed as the mean ± SD number of apoptotic cells in the objective area of 4 mm^2 in 3 fields per section (in 3 mice per group) (right). **Thin arrows** indicate apoptosis of the infiltrating cells; **thick arrows** in the higher-magnification view of the boxed area **(inset)** indicate apoptosis of the glandular epithelial cells. **F,** Lacrimal gland sections were stained with H&E to assess histologic features. Original magnification × 100; bars = 30 μm. * = $P < 0.05$ by Student's t-test. See Figure 1 for other definitions.

cell transfer, the saliva volume secreted by N1-APL7–treated Rag1$^{-/-}$ mice was significantly higher than that in PBS-treated control mice (Figure 4C).

Both infiltrating cells and glandular epithelial cells were apoptotic in PBS-treated Rag1$^{-/-}$ mice, whereas N1-APL7–treated mice exhibited very few apoptotic cells (Figures 4D and E). Furthermore, N1-APL7 prevented the induction of dacryoadenitis (Figure 4F). These results indicate that N1-APL7 suppresses M3R-reactive T cells, a mechanism that seems to suppress the development of sialadenitis as well as dacryoadenitis in vivo.

Induction of T cell anergy channels suppression of sialadenitis. To further analyze the mechanisms of the suppressive effects of N1-APL7, we evaluated the draining cervical lymph nodes of N1-APL7–treated or PBS-treated Rag1$^{-/-}$ mice that had received total peptide mix–immunized mouse cells. Previous reports have shown that APLs up-regulate the expression of co-molecules, induce regulatory T cells, induce apoptosis, or change the cytokine production pattern from Th1 cytokines to Th2 cytokines (24). Accordingly, we evaluated the expression of CD4+ T cell–related molecules. No major differences in the expression of mRNA for *Lag3*, *Ctla4*, *Pdcd1*, *Il4*, *Il10*, and *Foxp3* were detected between the N1-APL7–treated mice and control mice (Figure 5A).

APLs are also reported to induce T cell anergy (25), and for this reason, we also evaluated the expression of Egr-2, one of the markers of T cell anergy. Intriguingly, compared with control mice, the expression of *Egr2* mRNA was significantly higher in CD4+ T cells of N1-APL7–treated Rag1$^{-/-}$ mice that had received total peptide mix–immunized mouse splenocytes (Figure 5A). In addition, exogenous IL-2 administration reversed the cell reactivation with M3R peptides, a finding that supports the hyporesponsive state of these cells (Figure 5B). These results suggest that N1-APL7 seems to induce a state of anergy in M3R-reactive T cells, leading to suppression of cytokine production and, thus, inhibition of the induction of sialadenitis in vivo.

As indicated by the findings from ex vivo analysis, when the cells were cultured with N1-APL7, the proportion of Egr-2+CD4+ T cells was significantly higher than that in cultures with the N1 peptide (Figure 5C). Recent studies have highlighted the direct role of Egr-2 in the up-regulation of certain anergy-related molecules (26–29). To confirm these observations, we determined the expression levels of anergy-related E3 ubiquitin-protein ligases, such as Itch, Cbl-b, and GRAIL (Figures 5D and E). The mRNA expression levels of these molecules were significantly higher in N1-APL7–treated cell cultures than in control cell cultures, and their protein levels also tended to increase with N1-APL7 treatment. Furthermore, the expression of *Dtx1*, another anergy-related molecule, was up-regulated with N1-APL7 treatment (Figure 5D). These results add further confirmation of the induction of the anergy state. Thus, these findings from both ex vivo and in vitro assays suggest that N1-APL7 can induce M3R-reactive T cell anergy through the up-regulation of Egr-2 expression and anergy-related molecules.

DISCUSSION

The major finding of the present study was that the major T cell epitopes of M3R molecules in MIS, and one of the antagonistic APLs in vitro, N1-APL7, could inhibit the development of sialadenitis in vivo through the induction of T cell anergy.

Major similarities have been described between MIS and SS. The main infiltrating cells in the salivary gland are CD4+ T cells, with surrounding CD8+ T cells and B cells. Similar to the effects of M3R-reactive T cells among peripheral blood mononuclear cells of SS patients, M3R-reactive T cells are important for the induction of MIS (12,15). In the salivary glands of mice with MIS, we observed not only the infiltration of lymphocytes, but also the apoptosis of glandular epithelial cells. The infiltration of active CD4+ and/or CD8+ T cells into salivary glands results in apoptosis of mononuclear cells and glandular cells, possibly by the release of perforin and granzyme B or the interaction with Fas and Fas ligand. These processes might cause the dysfunction of the salivary glands.

Furthermore, high titers of anti-M3R antibodies have been described in MIS, similar to that described in patients with SS (8,10,11). These results support the view that M3R acts as an autoantigen to induce an autoimmune reaction in both SS and MIS. There are 2 major T cell epitopes of the M3R molecule in MIS; the N1 peptide and the 1st peptide. Intriguingly, the severity of sialadenitis was significantly different between Rag1$^{-/-}$ mice that had received N1 peptide–expressing mouse splenocytes and those that had received 1st peptide–expressing mouse splenocytes. Previous studies showed that IFNγ-producing M3R-reactive T cells play a crucial role in the development of MIS (16). As indicated, when M3R$^{-/-}$ mice were immunized with the total peptide mix and their splenocytes were cocultured with M3R N1 or 1st peptide, N1-reactive T cells could produce larger amounts of IFNγ compared with 1st peptide–reactive T cells.

Moreover, we have previously shown that IL-17 is crucial for the generation of MIS, based on experiments using M3R$^{-/-}$ × IL-17$^{-/-}$ mice (17). As there

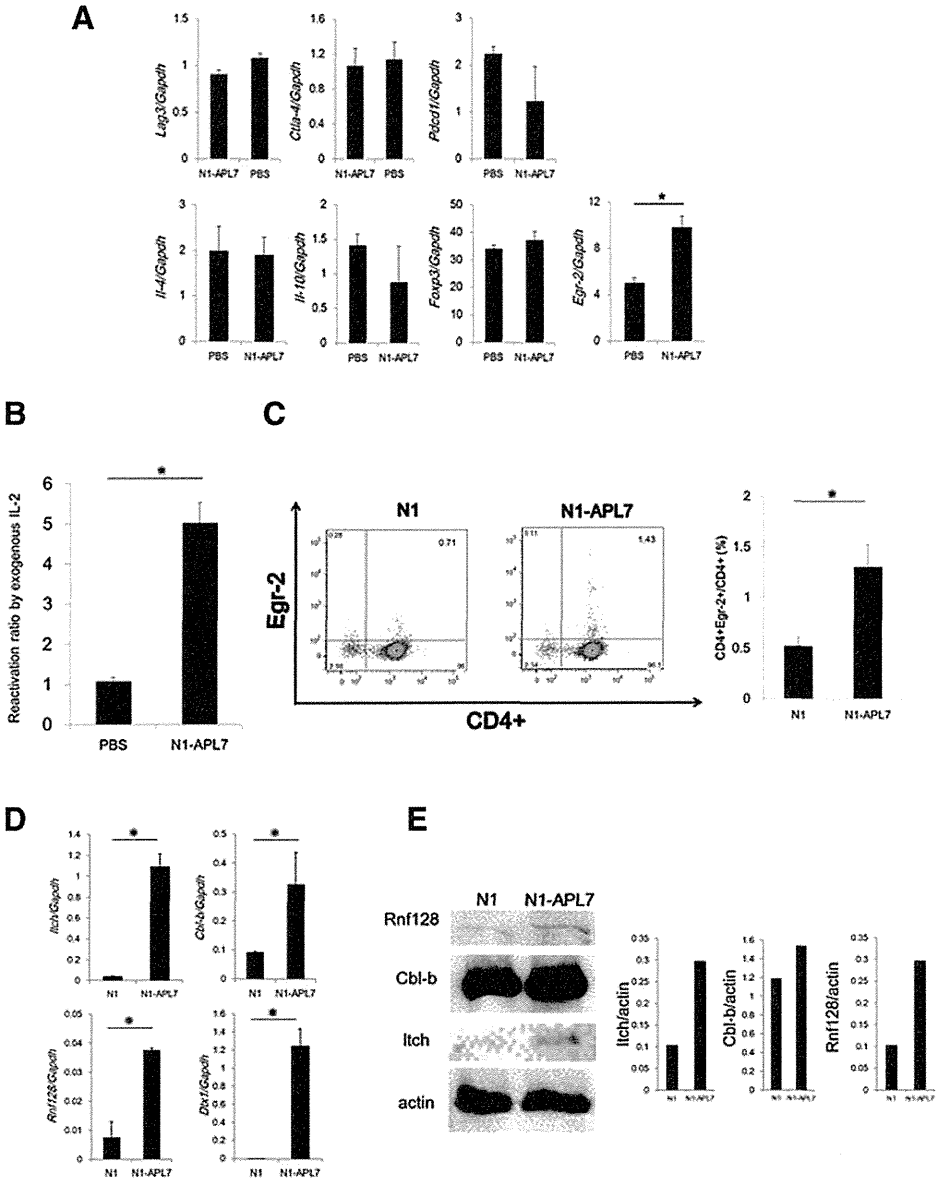


Figure 5. Induction of T cell anergy suppresses sialadenitis. Various experiments were conducted to understand the underlying mechanisms of the effects of the N1 altered peptide ligand 7 (N1-APL7) on M3R-reactive T cells. **A,** CD4+ T cells from the cervical lymph nodes of recipient $Rag1^{-/-}$ mice were isolated on day 45 after cell transfer, and mRNA expression levels of the indicated regulatory molecules were evaluated. **B,** The cervical lymph nodes were cultured with or without total M3R peptide mix and in the absence or presence of exogenous IL-2 (50 units/ml) for 72 hours, with bromodeoxyuridine added in the last 18 hours. Results are the reactivation ratio, calculated as the index of the recall proliferative response in the presence of exogenous IL-2 divided by the index in the absence of exogenous IL-2. **C,** The splenocytes of $M3R^{-/-}$ mice immunized with N1 peptide alone were isolated and cocultured for 12 hours with N1 peptide or N1-APL7, and the expression of early growth response 2 (Egr-2) on CD4+ T cells was determined by fluorescence-activated cell sorting analysis. **D** and **E,** CD4+ cells and CD11c+ cells were cocultured in the same manner as that described in **C**. After 48 hours, the mRNA expression levels of itchy homolog E3 ubiquitin-protein ligase (*Itch*), Casitas B-lineage lymphoma b (*Cbl-b*), *Rnf128* (encoding gene related to anergy in lymphocytes [GRAIL]), and Deltex-1 (*Dtx1*) were evaluated (**D**). After 96 hours, the protein expression levels of Itch, Cbl-b, and Rnf128 were evaluated by immunoblotting, with actin used as a positive control (**E**). Results are the mean ± SD of 3–6 mice per group per experiment. Data are representative of 3 independent experiments. $* = P < 0.05$ by Student's *t*-test. See Figure 1 for other definitions. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.39163/abstract.

was no significant difference in IL-17 production between M3R N1– and 1st peptide–reactive T cells, we speculated that the higher production of IFNγ by N1-reactive T cells could be responsible for the more severe form of sialadenitis observed in $Rag1^{-/-}$ mice that had received N1 peptide–expressing cells when compared with mice that had received 1st peptide–expressing cells. In analyses comparing $Rag1^{-/-}$ mice in the total peptide mix and N1 peptide inoculation groups, we found that production of IL-17, but not production of IFNγ, from M3R-reactive T cells might also affect the severity of sialadenitis (Figure 1A).

Elucidation of the major T cell epitopes of M3R molecules could enhance the establishment of an antigen-specific therapeutic strategy. Nonspecific immunosuppressive drugs have numerous side effects, and long-term treatment has questionable effects on host survival. No or little therapeutic help is currently available for patients with SS with sicca syndrome (19). We focused on a strategy utilizing APLs, peptides that have substitutions in amino acid residues only at TCR contact sites and that act as antagonists (20,30). APLs lack potential metabolic activity and can limit the range of response to the desired pathogenic peptide epitopes. Antagonistic APLs can inhibit the function of specific T cell populations and are potentially useful for antigen-specific therapy in immune diseases such as SS, in which T cells play a major pathogenic role. From the theoretical design of the APLs and the findings from the in vitro pre-pulse assay, we showed that all of the antagonistic APLs might suppress cytokine production in an I-Ab–dependent manner (31). Furthermore, N1-reactive T cells preferentially produce IFNγ rather than IL-17, and 1st peptide–reactive T cells produce IL-17 rather than IFNγ. Thus, we observed that N1-APLs 5, 6, and 7 effectively suppressed the production of IFNγ, and 1st-APL8 suppressed IL-17. These findings suggest that APLs have a cytokine-specific suppressive effect. Of all the antagonistic APLs, N1-APL7 significantly suppressed the induction of sialadenitis in vivo.

We also evaluated the mechanisms through which N1-APL7 suppressed the induction of MIS. Previous studies have described 4 possible mechanisms of action of APLs: up-regulation of co-molecules, alteration in Th2 cytokine production, induction of Treg cells, and induction of anergy. We focused first on the expression of T cell–related regulatory co-molecules. Kaushansky et al (25) showed that APLs can up-regulate the expression of CTLA-4. In our study, we analyzed the expression levels of various co–inhibitory molecules, such as *Ctla4*, *Pdcd1*, and *Lag3*, but did not detect any difference between N1-APL7– and PBS-treated mice. These results suggest that up-regulation of co-molecules is not likely the main mechanism of suppression of MIS.

Previous reports have described both Th1 cell– and Th17 cell–dependent cytokine profiles in the Th2 response (24). Several studies have demonstrated that the low affinity of the TCR induces a predominant Th2 response by reducing ERK activity, resulting in the production of IL-4 or IL-10 (32,33). We also focused on the expression of these cytokines in the cervical lymph node cells of mice, but did not find any notable differences in expression. These results rule out the possibility of any major effect of Th2 cytokine production.

We also examined the potential role of Treg cell induction. Although cytokines play a major role in the induction of Treg cells, signals based on differences in TCR affinity have also been reported to affect these T cell subsets. In fact, several groups have reported on the possible induction of Treg cells (25,34). We analyzed the expression of *Foxp3*, the master control gene in the development and regulation of CD4+ Treg cell function, but did not detect any change. These results rule out the possibility of any role of the induction of FoxP3+ Treg cells.

Finally, we speculated that the inhibitory effect of N1-APL7 on MIS is mediated through the induction of anergy, and focused on the expression of Egr-2 (35–39). Egr-2 is a member of the Cys_2His_2-type zinc finger transcription factor family, and is involved in the regulation of the state of anergy, in addition to the regulation of IL-10–producing *Lag3* Treg cells. Anergic T cells are characterized by defective DNA binding of the activation protein 1 heterodimer, as well as abnormal activation of molecules upstream of the MAPK family, although they show no defects in the activation and nuclear translocation of NF-AT (40,41). One immediate target of NF-AT–mediated transcription in T cells is the Egr family of transcription factors, and of those, a high expression level of Egr-2 has been reported in the anergic state (26). Our results confirmed the significantly high expression of Egr-2 in CD4+ T cells from the cervical lymph nodes of N1-APL7–treated mice. Thus, N1-APL7 might affect TCR affinity, which leads to the up-regulation of NF-AT signaling and induction of overexpression of Egr-2. Furthermore, ex vivo experiments showed reversible cell proliferation following exogenous IL-2 administration, confirming that N1-APL7 suppressed MIS through the induction of T cell anergy.

Recent studies have demonstrated that Egr-2 can alter the expression of anergy-related molecules, especially E3 ubiquitin ligases such as Itch, Cbl-b, and GRAIL. These molecules are known to function at different levels of the signal network and induce proteolysis of signaling molecules, which ultimately results in

T cell anergy (27–29,42). As expected, our in vitro assays confirmed the up-regulation of these molecules. Intriguingly, our experiments also showed up-regulation of *Dtx1*, which is also reported as one of the anergy-related molecules and is known to interact with Egr-2 (42–44). Although further studies are needed to evaluate the exact relationship between the expression of these molecules and the affinity of the TCR, our ex vivo and in vitro studies suggest that N1-APL7 most likely induces the anergy state of CD4+ M3R-reactive T cells, resulting in the suppression of sialadenitis.

Anergy is a long-term state of functional hyporesponsiveness. Since different experimental conditions and model systems have been used in previous studies, it has been difficult to construct a mechanistic model for T cell anergy (27). Comparison of in vitro T cell clonal anergy with in vivo T cell anergy (also known as adaptive tolerance) allows the identification of a few differences. In contrast to our results, previous findings showed that T cells could not be rescued by exogenous IL-2, and also that CTLA-4 could play a role in adaptive tolerance, but not in T cell clonal anergy (45–48). In other previously described models, however, adaptive tolerance could be induced in naive T cells, and in our model, sialadenitis was induced by splenocytes from $M3R^{-/-}$ mice immunized with M3R peptides, and N1-APL7 affected these previously activated T cells, thereby resembling some clonal T cell anergy models. In fact, other cell transfer studies, in which the cells were activated before inoculation, also showed T cell rescue by exogenous IL-2 (25). Although further studies on T cell anergy are needed, our results suggest that induction of antigen-specific T cell anergy is a potentially useful therapeutic approach.

In conclusion, the present study demonstrated that major T cell epitopes of M3R in this murine model of experimentally induced SS were from both the N1 and 1st regions of M3R, and that N1-APL7, selected as the antagonistic APL in vitro, suppressed sialadenitis through the induction of anergy. These results suggest that this approach might be useful as a therapeutic strategy for suppressing pathogenic T cell infiltration in SS.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Sumida had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Asashima, Tsuboi, Takahashi, Hirota, Iizuka, Kondo, Matsui, Matsumoto, Sumida.

Acquisition of data. Asashima, Tsuboi, Takahashi, Hirota, Iizuka, Kondo, Matsui, Matsumoto, Sumida.

Analysis and interpretation of data. Asashima, Tsuboi, Takahashi, Hirota, Iizuka, Kondo, Matsui, Matsumoto, Sumida.

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A Crucial Role of RORγt in the Development of Spontaneous Sialadenitis-like Sjögren's Syndrome

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The nuclear receptor retinoic acid–related orphan receptor (ROR)γt is required for the generation of Th17 cells, which are involved in various autoimmune diseases, including Sjögren's syndrome (SS). However, the pathological role of RORγt in SS remains to be elucidated. The present study was designed to clarify the role of RORγt in the pathogenesis of sialadenitis-like SS. Histological analysis of RORγt transgenic (Tg) mice was determined, and then Tg mice developed severe spontaneous sialadenitis-like SS. The analysis of infiltrating cells showed that most infiltrating cells were $CD4^+$ T cells. RORγt-overexpressing $CD4^+$ T cells induced sialadenitis as a result of transferred $CD4^+$ T cells from Tg mice into $Rag2^{-/-}$ mice. The examination of IL-17–deficient Tg mice indicated that IL-17 was not essential for the development of sialadenitis. The number of $CD4^+CD25^+Foxp3^+$ regulatory T (Treg) cells was significantly decreased in Tg mice, and CD25 expression and IL-2 stimulated STAT5 activation were inhibited in Treg cells. The inhibitory function of Treg cells of Tg mice was equal to that of wild-type mice in vitro. The abundant Treg cells of Tg mice could suppress the development of sialadenitis, but the reduced Treg cells of Tg mice could not inhibit the induction of sialadenitis in $Rag2^{-/-}$ mice transferred with effector cells from Tg mice. These results suggest that both RORγt-overexpressed $CD4^+$ T cells and reduced Treg cells might contribute to the development of SS-like sialadenitis. ***The Journal of Immunology*****, 2015, 194: 56–67.**

Sjögren's syndrome (SS) is a chronic autoimmune disease characterized by infiltration of lymphocytes into lacrimal and salivary glands, and clinically by dry eyes and dry mouth (1). Immunohistochemical studies have shown that most infiltrating lymphocytes in the labial salivary and lacrimal glands are $CD4^+$ αβ T cells (2). Autoantigens recognized by T cells infiltrating the labial salivary glands of patients with SS have been analyzed and several candidate autoantigens such as Ro/SSA 52 kDa (3), α-amylase (4), heat shock protein and TCR BV6 (5), and M3 muscarinic acetylcholine receptor (M3R) (6, 7) have been identified. However, the pathological role of the T cell subsets in SS remains to be elucidated.

Infiltrating Th1-type cells that mainly produce IFN-γ have been found in salivary glands of patients with SS (8, 9). These cells enhance the activity of T cells, B cells, and macrophages, resulting in the destruction and dysfunction of these glands (8, 9). In experimental models, transgenic (Tg) expression of IL-12 in the thyroid gland also leads to mononuclear infiltration of the salivary glands (10). Thus, the Th1 response is considered to play a role in the pathogenesis of SS.

However, a high level of IL-17 and Th17-related cytokines (TGF-β, IL-6, IL-23) have recently been found in the salivary glands and plasma of patients with SS and the mice model of SS (11–13). We also have reported the involvement of IL-17 production by M3R-reactive T cells in the pathogenesis of sialadenitis based on studies in a mouse model of M3R-induced sialadenitis, which is also considered as a mouse model of SS (14). Moreover Th17-associated cytokines, IL-17, IL-1β, and IL-23 are reported to form ectopic germinal centers in patients with SS (15).

The nuclear receptor retinoic acid–related orphan receptor (ROR)γt is a transcription factor induced by TGF-β and IL-6, and it directs the differentiation of inflammatory Th17 cells as well as cytokine production (16, 17). RORγt cooperates with RORα and IκBζ to enhance *Il17a* expression by binding directly to the regulatory region of the *Il17a* gene (18). Although several studies have indicated that Th17-related cytokines may be involved in development of SS, there is little information on the pathological role of RORγt in SS. To address this issue, we used RORγt Tg mice, which have been found to spontaneously develop severe sialadenitis-like SS.

Materials and Methods

Mice

RORγt Tg mice, which were generated from C57BL/6 mice, were provided by Prof. S. Takahashi (University of Tsukuba, Ibaraki, Japan). We used female mice for analysis in this study. IL-17A knockout RORγt Tg ($IL\text{-}17^{-/-}$/Tg) mice were generated by crossing RORγt Tg mice with IL-17A knockout mice provided by Prof. Y. Iwakura (Tokyo University of Science). For the isolation of regulatory T (Treg) cells, Tg and C57BL/6 mice were crossed with knock-in mice with Foxp3-IRES-GFP (RORγt-$Foxp3^{GFP}$, C57BL/6-$Foxp3^{GFP}$). C57BL/6-$Foxp3^{GFP}$ mice were provided by Prof. B. Malissen (Université de la Méditerranée, Marseille, France). All animals were maintained in specific pathogen-free conditions in the

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Abbreviations used in this article: ANA, anti-nuclear Ab; cLN, cervical lymph node; CNS, conserved noncoding sequence; LTi, lymphoid tissue inducer; M3R, M3 muscarinic acetylcholine receptor; ROR, retinoic acid–related orphan receptor; SS, Sjögren's syndrome; Teff, effector T; Tfh, T follicular helper; Tg, transgenic.

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Laboratory Animal Resource Center. All experiments were performed according to the *Guide for the Care and Use of Laboratory Animals* at Tsukuba University.

Measurement of saliva volume

Mice were first anesthetized with i.p. injection of pentobarbital (25 mg/kg), and then injected s.c. with pilocarpine (1.0 mg/kg). This was followed 15 min later by collection of saliva from the oral cavity using a 200-μl micropipette. The volume of the sample was measured and expressed relative to body weight.

Histological score

The inflammatory lesions were graded histologically (histological focus sore) using the method proposed by Greenspan et al. (19) as follows: focus score 1, a single focus composed of >50 mononuclear cells per 4 mm^2 tissue. The salivary glands were evaluated histologically by researchers blinded to the experiment, and at least one tissue section from each salivary gland was examined.

TUNEL staining

Apoptotic cells were detected by in situ apoptosis detection kit (Takara Bio, Otsu, Japan). Briefly, frozen sections were fixed with 4% paraformaldehyde for 30 min. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxidase/methanol. After washing in PBS, the sections were treated for 5 min on ice with the permeabilization buffer and then incubated with TdT enzyme reaction for 90 min at 37°C. Nuclei were counterstained with DAPI (Sigma-Aldrich, St. Louis, MO).

Isolation of lymphocytes from salivary glands

For flow cytometric analysis of infiltrating lymphocytes in salivary glands, tissue was cut into small pieces, digested by 2 mg/ml collagenase (Worthington Biochemical, Lakewood, NJ), and homogenized, and the lymphocytes were purified by centrifugation with Ficoll-Paque Plus (GE Healthcare Biosciences, Uppsala, Sweden).

Quantitative PCR analysis

Total RNA was prepared using Isogen (Nippon Gene, Toyama, Japan). First-strand cDNA was synthesized at 37°C for 15 min using the PrimeScript reverse transcriptase master mix (TaKaRa Bio) and 1 μl of this 10 μl reaction mixture was used for the PCR. A TaqMan Assay-on-Demand gene expression product was used for real-time PCR (Applied Biosystems, Foster City, CA). The expression levels of *Ifng*, *Il17a*, *Il17f*, *Ccr6*, *Ccl20*, *Il6*, *Il12p35*, *Tnf*, *Il22*, *Il21*, *Il4*, *Il23r*, *Tbx21*, *Rorc*, *Bcl6*, *Irf4*, and *Ahr* were expressed relative to the expression of *hprt1*. Analyses were performed with an ABI Prism 7500 apparatus (Applied Biosystems).

Flow cytometry

Staining for CD3, CD4, CD8, CD19, CD25, CD44, and CD62L (all from BioLegend, San Diego, CA) expression was performed for 20 min using a mixture of Abs. Intracellular staining for Foxp3, CTLA-4, GITR, and CD103 (all from BioLegend) was performed after fixation and permeabilization according to the protocol supplied by the manufacturer (eBioscience, San Diego, CA). Samples were analyzed with a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA), and data were analyzed with FlowJo software (Tree Star, Ashland, OR). For phospho-STAT5 (Cell Signaling Technologies, Danvers, MA) staining, Phosflow Lyse/Perm buffer and Perm Buffer III (BD Biosciences, San Jose, CA) were used according to the instructions provided by the manufacturer.

Immunohistochemical analysis

Sections of the salivary glands were thawed, dried, and then fixed with 1% formalin for 10 min. Immunofluorescence staining was performed as follows: the sections were incubated with Alexa Fluor 488–conjugated CD4 and Alexa Fluor 647–conjugated B220 Abs (all from Invitrogen, Carlsbad, CA) for 30 min at room temperature. The nuclear DNA was stained with DAPI.

Adoptive transfer and cell sorting

$CD4^+$ T cells were sorted from splenocytes of mice by using the $CD4^+$ MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were resuspended in PBS and inoculated intravenously in $Rag2^{-/-}$ mice. For the isolation of Treg cells, $CD4^+$ T cells were selected with $CD4^+$ MACS bead, and $CD4^+CD25^+GFP^+$ or $CD4^+CD25^-GFP^-$ cells were further purified from C57BL/6-$Foxp3^{GFP}$ or RORγt-$Foxp3^{GFP}$ mice using a MoFlo cell sorter (DakoCytomation, Glostrup, Denmark).

In vitro suppression assay and Treg cell culture

Responder cells ($CD4^+CD25^-GFP^-$) were labeled with 5 μM CFSE (Invitrogen) and then cultured with or without unlabeled Treg cells at the indicated ratio for 96 h in the presence of Dynabeads mouse T-activator CD3/CD28 (Invitrogen) in round-bottom 96-well dishes at a density of one bead per cell. The percentage inhibition rate on the proliferation of responder was calculated as [1 – (CFSE percentage of Treg cell plus responder cell coculture/responder cells alone)] × 100%. After collection of supernatant from Treg cultures, the levels of IFN-γ, IL-17, and IL-10 were measured by ELISA (R&D Systems, Minneapolis, MN).

Detection of autoantibodies by ELISA

The anti-M3R Abs were detected by ELISA as previously described (14). The peptide solution containing all types of M3R extracellular (N terminus, first, second, and third extracellular loop) peptides (each 5 μg/ml) was mixed with 0.1 M Na_2CO_3 buffer (pH 9.6) absorbed onto a Nunc Immunoplate (Nalge Nunc International, Rochester, NY) at 4°C overnight. Serum was incubated for 2 h at room temperature, and HRP-conjugated anti-mouse IgG Ab diluted 1:1000 was added for 1 h at room temperature. After washing, 100 μl tetramethylbenzidine solution (Kirkegaard & Perry Laboratories, Gatithersburg, MD) was added as a substrate and then the OD was measured at 450 nm by plate spectrophotometry (PerkinElmer Cetus, Norwalk, CT).

Anti-nuclear Abs (ANAs), autoantibodies against SSA/Ro and SSB/La, were measured by a commercially available ELISA assay according to the protocols of the manufacture (Alpha Diagnostic International, San Antonio, TX).

Results

Spontaneous development of autoimmune sialadenitis in RORγt Tg mice

Various body organs from RORγt Tg mice were analyzed histologically to determine the effects of RORγt overexpression in T cells under hCD2 promoter. Infiltrating mononuclear cells were detected in salivary glands and lacrimal glands of RORγt Tg mice (Fig. 1A). We also detected mild cell infiltration in lungs, which is a frequent complication of SS (20), but not in other organs of RORγt Tg mice and all organs of littermates control (C57BL/6) (Supplemental Fig. 1). The histological scores of inflammatory lesions in the salivary glands of RORγt Tg mice at 8, 12, 16, and 20 wk were significantly higher than those of C57BL/6 mice (Fig. 1B). We also detected the infiltrating mononuclear cells in salivary glands and lacrimal glands, as well as mild cell infiltrations in lungs from male RORγt Tg mice (Supplemental Fig. 2A, 2B), and the focus score of inflammatory lesions in salivary glands of those mice was significantly higher than that of male C57BL/6 mice (Supplemental Fig. 2C).

To verify the secretory function of the salivary glands, saliva was collected from C57BL/6 and RORγt Tg mice. The amount of saliva collected from RORγt Tg mice was significantly lower than that from C57BL/6 mice at 8 and 12 wk (Fig. 1C). However, there was no difference in saliva secretion between C57BL/6 and RORγt Tg mice at 3 wk, which is the predisease time point. To investigate tissue-specific autoantibodies, we examined sera for Abs against M3R, which are highly expressed in the exocrine glands and are candidate autoantigens in patients with SS and mice with experimentally induced autoimmune sialadenitis. The levels of anti-M3R Abs in sera of RORγt Tg mice were significantly higher than those in C57BL/6 mice (Fig. 1D). There was also a significant difference in the titer of anti-SSA Abs between C57BL/6 and RORγt Tg mice, and anti-SSB and ANAs were prone to increase in sera of RORγt Tg mice (Fig. 1D). These results indicated that overexpression of RORγt in T cells results in spontaneous autoimmune sialadenitis-like SS.

Characterization of age-dependent infiltration of inflammatory cells in salivary glands

The infiltrating cells were isolated from the inflammatory lesions of the salivary glands and then analyzed by flow cytometry. Most

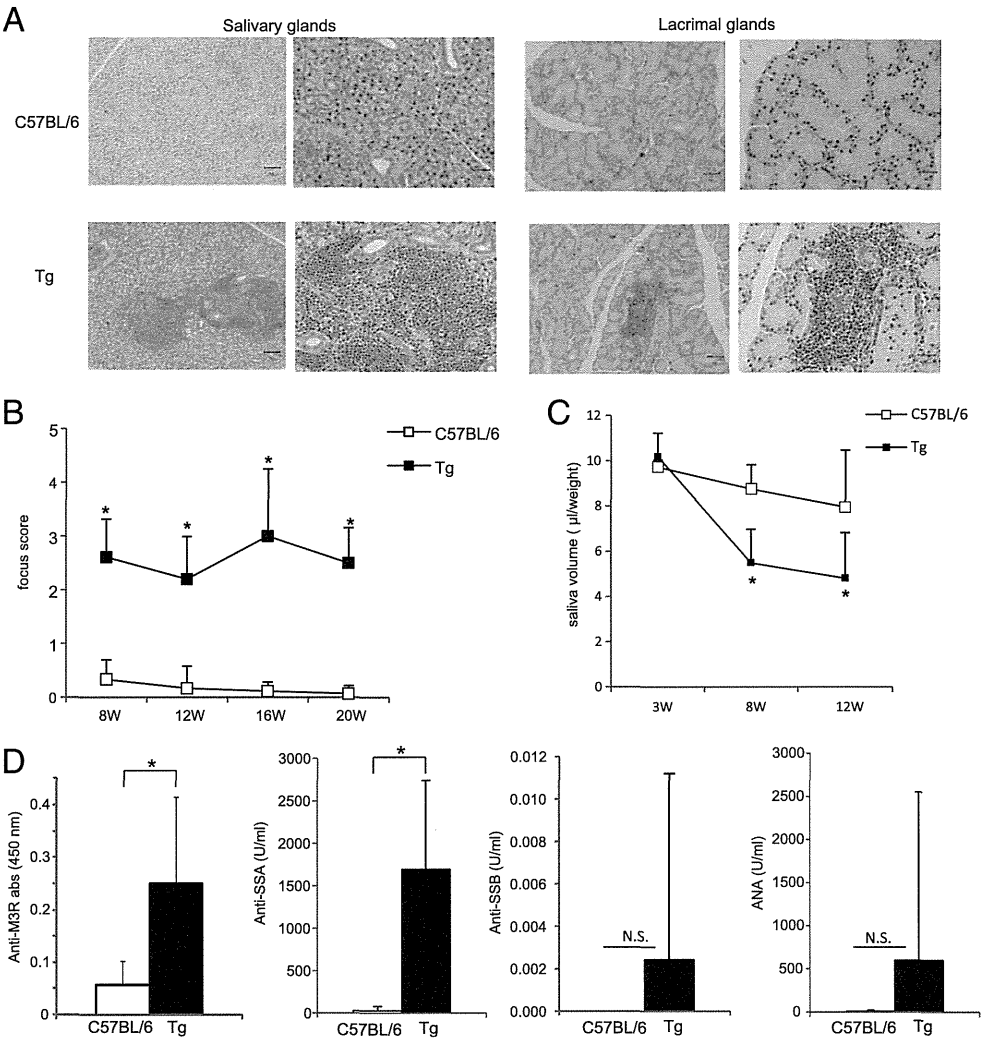

FIGURE 1. Development of spontaneous sialadenitis-like SS in RORγt Tg mice. (**A**) H&E-stained sections of the salivary and lacrimal glands from C57BL/6 and RORγt Tg mice. Scale bars, 30 μm (*left panel*), 70 μm (right panel). Data are representative of five tissue samples with similar results. (**B**) Histological score of inflammatory lesions in salivary glands of C57BL/6 and RORγt Tg mice at 8, 12, 16, and 20 wk of age. Six mice were analyzed per group. (**C**) Saliva volume from C57BL/6 and RORγt Tg mice at 3, 8, and 12 wk of age. Data are representative of two independent experiments with similar results. (**D**) Anti-M3R, anti-SSA, anti-SSB, and ANAs Abs in sera of C57BL/6 and RORγt Tg mice detected by ELISA. Data are representative of two independent experiments with similar results. $*p < 0.05$ (Mann–Whitney *U* test).

infiltrating cells were $CD3^+$ T cells although a few B cells were observed at the early phase of sialadenitis. B cells gradually increased in number with age and most infiltrating cells at later stages were B cells in RORγt Tg mice. The infiltrated $CD3^+$ cells were mainly $CD4^+$ T cells, whereas $CD8^+$ T cells formed a minor population. The CD4/CD8 T cell ratio did not change throughout the different stages of sialadenitis (Fig. 2A). Immunofluorescence staining showed similar results in salivary glands of RORγt Tg mice (Fig. 2B).

Using the TUNEL method, we were able to identify a few apoptotic cells in the inflammatory lesions of salivary glands isolated from RORγt Tg mice but not from C57BL/6 mice (Fig. 2C). This finding might account for the destruction and dysfunction of the salivary glands.

Critical role of RORγt overexpression on $CD4^+$ T cells in the development of sialadenitis

Because the main population of infiltrated cells in the early stage of sialadenitis were $CD4^+$ T cells, we then characterized these cells in RORγt Tg mice. $CD4^+$ T cells harvested from the spleen, cervical lymph nodes (cLN), and salivary glands were mainly effecter memory cells ($CD44^{hi}CD62L^{lo}$). Alternatively, only a few naive cells ($CD44^{lo}CD62L^{hi}$) and central memory cells ($CD44^{hi}CD62L^{hi}$) were found in the spleen and cLN of RORγt Tg mice relative to C57BL/6 mice (Fig. 3A, 3B).

We also analyzed the expression of *Rorc*, *Ahr*, *Irf4*, and *Ccr6*, which are related to Th17 induction, and found higher expression among splenic $CD4^+$ T cells from RORγt Tg mice compared with C57BL/6 mice. However, the expression of other T cell subset–related transcription factors *Tbx21, Gata3*, and *Bcl6* had similar levels among RORγt Tg and C57BL/6 mice (Fig. 3C). A similar result was found in $CD4^+$ T cells of cLN (Supplemental Fig. 3).

To understand the pathological role of RORγt overexpressed $CD4^+$ T cells in the development of sialadenitis, we injected purified splenic $CD4^+$ T cells into $Rag2^{-/-}$ mice. Histological analysis showed marked cell infiltration in the salivary glands of $Rag2^{-/-}$ mice inoculated with $CD4^+$ T cells of RORγt Tg mice, but not in those inoculated with $CD4^-$ cells of RORγt Tg mice or any other cell types from C57BL/6 mice (Fig. 3D). The histological score of inflammatory lesions in the salivary glands of RORγt Tg $CD4^+ \rightarrow Rag2^{-/-}$ mice was significantly higher than in RORγt Tg $CD4^- \rightarrow Rag2^{-/-}$ mice (Fig. 3E). These results emphasize the pathological role of overexpression of RORγt on $CD4^+$ T cells in the development of sialadenitis-like SS.

Various T cell subsets in inflammatory lesions of salivary glands

We checked the involvement of effector subsets of Th1, Th2, Th17, and T follicular helper (Tfh) cells in the pathogenesis of sialadenitis in RORγt Tg mice, because expression of various T cell subsets was present in the salivary glands of patients with SS. First, examination of the expression of Th17-related molecules (*Rorc, Il17a, Il23r*, and *Ccl20* [the ligand of CCR6]) showed significantly high mRNA expression levels of *Rorc, Il17a, Il23r*, and *Ccl20* in the salivary