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RORγt antagonist improves Sjögren's syndrome-like sialadenitis through downregulation of CD25

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Abstract

Objective: We reported previously that T cells specific RORγt-transgenic-mice under human CD2 promoter (RORγt-Tg mice) developed severe spontaneous Sjögren's syndrome (SS)-like sialadenitis, induced by RORγt-overexpressing $CD4^{+}$T cells and reduced regulatory T cells. The purpose of this study was to clarify the effectiveness and mechanisms of action of A213, a RORγt antagonist, in RORγt-Tg mice with SS-like sialadenitis.

Methods: 6-week-old RORγt-Tg mice were administered orally of A213 or PBS every three days for two weeks. We analyzed saliva volume, histopathology of salivary glands, populations of T cells in splenocytes and cervical lymph nodes (cLNs), and the protein expression levels of CD69 on $CD4^{+}CD25^{+}Foxp3^{-}$ and $CD4^{+}CD25^{+}Foxp3^{+}$ cells in cLNs. We also investigated *in vitro* the potential immunomechanisms of action of A213.

Results: A213 significantly increased saliva volume, reduced mononuclear cell infiltration in salivary glands and reduced the focus score of sialadenitis. Analysis of the immunomechanisms using cLNs showed A213 significantly reduced the proportion of $CD4^{+}CD25^{+}/CD4^{+}$ T cells and the protein expression levels of CD69 on $CD4^{+}CD25^{+}Foxp3^{-}$ cells. *In vitro* experiments showed that A213 suppressed CD25 expression on $CD4^{+}$ T cells and reduced IL-2 production from $CD4^{+}$ T cells derived from RORγt-Tg mice.

Conclusion: A213 improves SS-like sialadenitis through inhibition of $CD4^{+}CD25^{+}$ cells in cLNs.

Key words:

RORγt antagonist, CD25, Sjögren's syndrome

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Introduction

Sjögren's syndrome (SS) is a chronic autoimmune disease characterized histopathologically by infiltration of lymphocytes into salivary and lacrimal glands, and in which $CD4^+$ T cells and activated B cells seem to play important pathogenic roles (Fox and Stern, 2002). High levels of IL-17 and Th17-related cytokines (TGF-β, IL-6, IL-23) have been recently described in the salivary glands and plasma of patients with SS and mouse model of SS (Sakai et al., 2008, Nguyen et al., 2008, Katsifis et al., 2009). The nuclear receptor retinoic acid-related orphan receptor t (RORγt) plays an indispensable role in the differentiation of Th17 cells (Yang et al., 2008, Ivanov et al., 2006, Ivanov et al., 2007, Manel et al., 2008). We showed previously that T cells specific RORγt-transgenic mice under human CD2 promoter (RORγt-Tg mice) developed severe spontaneous SS-like sialadenitis and that RORγt-overexpressing $CD4^+$ T cells and reduction of Treg cells contributed to the development of SS-like sialadenitis in these mice (Iizuka et al., 2015). RORγt-overexpressing $CD4^+$ T cells include various T cell subsets, such as Th1, Th2, Th17 and T follicular helper (Tfh) cells, and are known to produce IFNγ, IL-4, IL-17 and IL-21, which play significant roles in the immunopathogenesis in RORγt-Tg mice (Iizuka et al., 2015). Surprisingly, IL-17 was not essential for the development of sialadenitis in this model (Iizuka et al., 2015).

Several synthetic ligands, which bind to and inhibit RORγt (RORγt antagonists), have been developed in recent years (Takaishi et al., 2017, Solt et al., 2015). RORγt antagonism seems to be a promising therapeutic strategy against Th17-mediated autoimmune disorders, such as SS, psoriasis, type 1 diabetes and collagen-induced arthritis, acting by the suppression of differentiation and function of Th17 cells (Takaishi et al., 2017, Solt et al., 2015, Chang et al., 2014). More recently, Bassolas-Molina H et al. have reported that RORγt antagonist inhibited the Th17 related genes and proteins, while upregulating Treg and preserving Th1 and Th2 signatures in peripheral blood mononuclear cells (PBMCs) from Crohn's disease patients (Bassolas-Molina et al., 2018). Furthermore, one of the RORγt antagonist is known to be well tolerated and safe in healthy volunteers and that has shown a signal of efficacy in a phase Ⅱ study in patients with psoriasis (NCT02555709) (McGeehan et al., 2016).Thus, RORγt antagonist has potential for clinical application.

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On the other hand, we reported previously that Rag1$^{-/-}$ mice inoculated with splenocytes from M3 muscarinic acetylcholine receptor (M3R) knockout mice immunized with M3R peptides mixture developed SS-like sialadenitis (M3R induced sialadenitis; MIS) (Iizuka et al., 2010) In MIS, both IFNγ and IL-17 produced by M3R-specific T cells had crucial roles in the development of sialadenitis (Iizuka et al., 2013, Iizuka et al., 2015). We also found that oral administration of A213, a RORγt antagonist, improved sialadenitis in MIS through the suppression of IFNγ and IL-17 production from M3R-specific T cells, suggesting that RORγt antagonism can be potentially useful in the treatment of SS (Tahara et al., 2017).

In the present study, we examined the efficacy of A213 and explored its mechanisms of action in RORγt-Tg mice, which is known to develop spontaneous SS-like sialadenitis, to establish the therapeutic potential of RORγt antagonism in autoimmune diseases, including SS.

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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 in this study. All animals were maintained in specific pathogen-free conditions in the Laboratory Animal Resource Center. All experiments were performed adequate measures which were taken to minimize pain for mice according to the Guidelines laid down by the National Institute of Health (NIH) in the USA regarding the care and use of animal for experimental procedures and the Guide for the Care and Use of Laboratory Animals at University of Tsukuba.

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Treatment protocol with A213

RORγt antagonist (A213) was kindly provided by Daiichi-Sankyo Company. Details of its chemical structure have been described previously (Tahara et al., 2017). The compound was dissolved in phosphate buffered saline (PBS) at 30 mg/ml, and 6-week-old female RORγt-Tg mice received orally 300 mg/kg of A213 (10 μl/g body weight) or PBS (10 μl/g body weight of mice) every three days for 2 weeks (total 5 doses) until 8-week-old, when untreated RORγt-Tg mice developed spontaneous sialadenitis (Iizuka et al., 2015).

Measurement of salivary volume

Mice were first anesthetized with intraperitoneal injection of pentobarbital (1.0 mg/kg), and then injected subcutaneously with pilocarpine (25 mg/kg). We collected saliva from the oral cavity over a period of 15 minutes using a 200 μl micropipette. The volume of the sample was measured and expressed relative to the body weight. Changes in saliva volume were calculated relative to the volume measured at baseline, using the following formula; [day-14 saliva volume (ml) / body weight (g)] / [day-0 saliva volume (ml) / body weight (g)]. According to the Categories of Biomedical Experiments Based on Increasing Ethical Concerns for Non-human Species by the Scientists Center for Animal Welfare (SCAW), collecting saliva is equivalent to the category B, which has no uncomfortable for animals. However, it took for times to collect saliva, we used the pentobarbital for the sedation. This procedure was performed adequate measure which had no pain for mice according to the Guidelines laid down by the National Institute of Health (NIH) in the USA

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regarding the care and use of animal for experimental procedures and the Guide for the Care and Use of Laboratory Animals based on the SCAW categories at University of Tsukuba.

Histopathological analysis

The salivary glands were surgically excised, fixed in 10% formalin, paraffin embedded, and 5 μm-thick sections were prepared. For analysis, 4-5 μm tissue sections were stained with hematoxylin and eosin (H&E) by standard technique. The inflammatory lesions were graded histologically using the focus score (number of focuses per 4 mm^2 of each section; one focus was defined as >50 mononuclear cells found around the salivary gland ducts). Histological evaluation was performed in a blinded manner.

Detection of autoantibody by ELISA

Autoantibody against Ro (SS-A) was measured by commercially available enzyme-linked immunosorbent assay (ELISA) according to the protocols of the manufacture (Alpha Diagnostic International, San Antonio, TX).

***Ex vivo* experiments**

After treatment of RORγt-Tg mice with A213 or PBS for two weeks, the spleen and cLN were harvested and stained with anti-CD4, CD44, CD62L, CD25 and CD69 antibodies (all from BioLegend, San Diego, CA) for 20 min using a mixture of antibodies. Intracellular staining with anti-Foxp3, RORγt, and Tbet antibodies (all from BioLegend) was performed after fixation and permeabilization according to the protocol supplied by the manufacturer (eBioscience, San Diego, CA). All samples were analyzed with BD FACSVerse (BD Biosciences), and the data were analyzed with FlowJo software (TreeStar, Ashland, OR).

For quantitative PCR analysis, $CD4^+$ T cells were isolated from the cervical lymph nodes (cLNs) by magnetic activated cell sorting (MACS)-positive selection using anti-CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and preserved in RNA later™ Solution (Invitrogen by Thermo Fisher Scientific, Carlsbad, CA). 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 Inc, Otsu, Japan) and 1 μl of this 20 μl reaction mixture was used for the PCR. For qRT-PCR, we

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used a TaqMan Assay-on-Demand gene expression product (Applied Biosystems, Foster City, CA). The expression levels of *Il2* (Mm00434256_m1), *Il17a* (Mm00439618_m1) and *Ifng* (Mm01168134_m1) were normalized relative to the expression of *Gapdh* (Mm99999915_g1). All analyses were performed with an ABI Prism 7500 apparatus (Applied Biosystems).

Effects of A213 on expression levels of cell surface molecules and cytokines *in vitro*

$CD4^+$ T cells were isolated from the splenocytes of 6-week-old RORγt-Tg mice by MACS-positive selection using anti-CD4 microbeads (Miltenyi Biotec). The cells (2.0×10^5 cells/well) were cultured in RPMI-1640 medium (Sigma-Aldrich, St Louis, MO) containing 10% fetal bovine serum (FBS), 100 units/ml of penicillin and 100 mg/ml of streptomycin, with or without various concentrations of A213 (0, 0.01, 0.1, 1 µM) under stimulation with CD3/CD28 in 96-well flat-bottomed plates (Nunc, Rochester, NY). The A213 concentrations used in the in vitro were nearly equaled that of in vivo, based on the data from Daiichi Sankyo (unpublished data) that the peak concentration of A213 was 0.6µM and the trough concentration was 0.05µM in vivo. After 72 hours culture, the cells were stained with anti-CD4, CD25 and CD69 antibodies (all from BioLegend, San Diego, CA) for 20 min using a mixture of antibodies. For intracellular cytokine staining, phorbol myristate acetate (50 ng/ml), ionomycin (0.5 mg/ml) and GolgiStop (eBioscience) were added during the last 4 hours of each culture. Cells were fixed and permeabilized with fixation/permeabilization solution (eBioscience). Then, intracellular staining was performed according to the protocol supplied by the manufacturer, using anti-IL-17 and IFNγ antibodies (all from BioLegend). Each sample was analyzed with a BD FACSVerse flow cytometer (Biosciences), and data were analyzed with FlowJo software (Tree Star, Ashland, OR). Furthermore, after 72 hours of culture under the conditions described above, IL-2 concentrations in the culture supernatant were measured using the Duoset enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN), according to the instructions supplied by the manufacturer.

Statistical analysis

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Data were expressed at mean ± standard deviation (SD). Differences between groups were examined for statistical significance using the Student's t-test or Kruskal-Wallis test. *P* values less than 0.05 were considered significant.

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Results

Oral administration of A213 recovered salivary secretion and severity of sialadenitis in RORγt-Tg mice

The treatment protocol is shown in Figure 1a. Compared with PBS, oral administration of A213 significantly suppressed the decrease in salivary volume in RORγt-Tg mice (changes in saliva volume at day 14; 1.3±0.3 in PBS-treated group, 3.1±1.4 in A213-treated group, $P<0.05$) (Figure 1b). Moreover, A213 dramatically inhibited mononuclear cell infiltration into the salivary glands compared with PBS (Figure 1c). The severity of sialadenitis in RORγt-Tg mice at day 14 assessed by focus score was significantly lower in A213- than in PBS-treated group (2.5±0.6 in PBS-treated group, 0.3±0.3 in A213-treated group, $P<0.05$) (Figure 1d). These results demonstrated that oral administration of A213 significantly suppressed the decrease in salivary volume and severity of SS-like sialadenitis in RORγt-Tg mice. The titer of anti-Ro (SS-A) IgG antibody in serum was tended to be lower in A213-treated group than PBS-treated group ($P=0.09$) (Figure 1e).

Effects of A213 on $CD4^+$ T cell population in RORγt-Tg mice

We examined the effects of treatment with A213 on RORγt and Tbet expression in $CD4^+$ T cells. The mean fluorescence intensities (MFI) of RORγt and Tbet in splenic $CD4^+$ T cells were comparable between A213- and PBS-treated groups (Figure 2a, b).

The main population of infiltrated cells in the early stage of sialadenitis in RORγt-Tg mice are $CD4^+$ T cells, mainly effector memory cells ($CD44^{hi}CD62L^{lo}$), with a few naïve cells ($CD44^{lo}CD62L^{hi}$) and central memory cells. These cells were also found in the spleen and cLNs (Iizuka et al., 2015). Therefore, we compared the population of these cells in spleen and cLNs between A213- and PBS-treated groups. The population of these cells in spleen and cLNs were comparable in A213- and PBS-treated groups (Figure 2c-f).

We also checked the expression of CD25, which could be expressed on activated T cells, on $CD4^+$ T cells in spleen and cLNs. The proportion of $CD4^+CD25^+$ cells among total $CD4^+$ T cells in spleen was comparable between A213- and PBS-treated groups (Figure 2g, h). Surprisingly, the proportion of $CD4^+CD25^+$ cells in total $CD4^+$ T cells in cLNs was significantly lower in A213-(17.8±6.0%) than in PBS-treated group (45.5±10.5%)

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(P=0.007) (Figure 2i, j). These results suggested that A213 suppressed $CD4^+CD25^+$ cells in the draining lymph nodes of salivary glands much more than spleen cells.

Effects of A213 on $CD4^+$ T cells in cervical lymph node

We focused on $CD4^+$ T cells in cLNs since A213 specifically decreased the population of $CD4^+CD25^+$ T cells in cLNs alone. We reported previously the presence of low number of $CD4^+CD25^+Foxp3^+$ regulatory T (Treg) cells in RORγt-Tg mice (Iizuka et al., 2015). In the next step, we examined whether A213 changes the number of $CD4^+CD25^+Foxp3^+$cells. Contrary to our expectation, the proportion of $CD4^+CD25^+Foxp3^+$ among $CD4^+$ T cells was significantly lower in the A213-(1.5±0.1%) compared with PBS-(5.8±1.9%) treated group (P=0.01) (Figure 3a, b). Next, we focused on the population of $CD4^+CD25^+Foxp3^-$ cells. The proportion of $CD4^+CD25^+Foxp3^-$ in $CD4^+$ T cells was also significantly lower in the A213-(9.6±9.5%) than PBS-(35.2±7.4%) treated group (P=0.02) (Figure 3a, c). However, the proportion of $Foxp3^+$ and $Foxp3^-$ cells gated on $CD4^+CD25^+$ cells were comparable between A213- and PBS-treated groups (Figure 3d, e). On the other hand, the MFI of Foxp3 in $CD4^+CD25^+$ T cells was significantly lower in the A213-(424±7.37) treated group compared with PBS-(457±15.5) treated group (Figure 3f, g). These results indicate that A213 suppresses $CD4^+CD25^+$ T cells regardless of the expression of Foxp3.

We also checked CD69, which is thought to be an activation marker in T cells, on $CD4^+CD25^+Foxp3^+$ and $CD4^+CD25^+Foxp3^-$ cells. The MFI of CD69 on $CD4^+CD25^+Foxp3^+$ cells tended to be lower in A213- (127±35.4) than PBS-treated groups (247±108) (P=0.08) (Figure 3h, i). The MFI of CD69 on $CD4^+CD25^+Foxp3^-$ cells was significantly lower in A213-(106±33.1) than PBS-treated group (175±33.7) (P=0.04) (Figure 3j, k). These results suggest that A213 can suppress T cell activation in the draining lymph nodes.

We also analyzed the expression of *Il2*, *Il17a* and *Ifnγ*, which were related to Th17 and Th1 cells and highly expressed in RORγt-Tg mice (Iizuka et al., 2015). The expression of *Il2*, *Il17a* and *Ifnγ* in $CD4^+$ T cells of cLNs was comparable between the A213- and PBS-treated groups (Figure 3l).

Effects of A213 on activation marker in $CD4^+$T cells derived from RORγt-Tg mice *in vitro*

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To further confirm the down-regulatory effect of A213 on CD25 expression in $CD4^+$ T cells derived from RORγt-Tg mice, we examined the effects of A213 *in vitro*. The protein expression level of CD25 in $CD4^+$ T cells decreased significantly in an A213-dose dependent manner (0.01-1 μM) *in vitro* (Figure 4a, b). These results suggest the effect of A213 on $CD4^+$ T cells and suppression of CD25 expression. However, A213 had no significant effect on the CD69 protein expression level *in vitro* (Figure 4c, d).

Effect of A213 on cytokine production by $CD4^+$ T cells derived from RORγt-Tg mice *in vitro*

To examine the effects of A213 on IL-2 production, $CD4^+$ T cells derived from RORγt-Tg mice were cultured with various concentrations of A213 under stimulation with anti-CD3 and anti-CD28 antibodies. A213 at high concentration (1 μM) significantly decreased IL-2 levels in the culture supernatants (Figure 5a), suggesting that A213 seems to alter IL-2 production from $CD4^+$ T cells.

We reported previously that infiltrating $CD4^+$ T cells found in the salivary glands of RORγt-Tg mice produce IL-17 and IFNγ (Iizuka et al., 2015). In the next step, we checked the inhibitory effects of A213 on the production of these cytokines by $CD4^+CD25^+$ cells using flow cytometry. The percent $IFN\gamma^-IL\text{-}17^+$ in $CD4^+CD25^+$ cells was significantly lower in the presence of moderate to high concentrations of A213 (0.1-1 μM) (Figure 5b, c), compared with the control. On the other hand, both $IFN\gamma^+IL\text{-}17^-$ and $IFN\gamma^+IL\text{-}17^+$ cells in $CD4^+CD25^+$ cells were significantly decreased in the presence of high concentration of A213 (1 μM) (Figure 5b, d, e). These results indicate that A213 suppresses both IL-17 and IFNγ production by $CD4^+CD25^+$ T cells.

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Discussion

In this study, we showed that A213 improved saliva production and sialadenitis in RORγt-Tg mice. Immunotherapeutically, A213 modulated immune cell numbers and functions; it significantly reduced both $CD4^+CD25^+$ $Foxp3^+$ and $CD4^+CD25^+Foxp3^-$ cells and down-regulated CD69 expression on $CD4^+CD25^+Foxp3^-$ cells in cLNs. In contrast, A213 did not suppress RORγt and T-bet proteins, although it significantly downregulated Foxp3 expression. The *in vitro* studies demonstrated that A213 suppressed CD25 expression on $CD4^+$ T cells and reduced IL-2 production from $CD4^+$ T cells. These findings support the notion that A213 suppresses $CD4^+CD25^+$ T cells through inhibition of IL-2-mediated CD25 expression and down-regulation of CD69 on $CD4^+CD25^+Foxp3^-$ cells, resulting in improvement of sialadenitis.

What are the mechanisms of A213 suppressive action on SS-like sialadenitis in RORγt-Tg mice? We propose the following five possible mechanisms. First, A213 can suppress the activated $CD4^+CD25^+$ T cells, resulting in improvement of sialadenitis in RORγt-Tg mice. Our study showed that A213 decreased $CD4^+CD25^+$ cells in cLNs *in vivo*. Moreover, A213 suppressed CD25 expression on $CD4^+$ T cells derived from RORγt-Tg mice *in vitro*. CD25, the α-chain of the IL-2 receptor, is not a specific marker expressed exclusively on Treg cells. It is also expressed on activated effectors, such as T cells, both in humans and mice (Theze et al., 1996). The expression of CD25 is defined by the presence of IL-2 (Fontenot et al., 2005). Interestingly, we showed that A213 suppressed IL-2 production by $CD4^+$ T cells *in vitro*. These results suggest that A213 suppresses IL-2 production by $CD4^+$ T cells, which in turn result in decrease in CD25 expression. However, our data had differences of IL-2 expression between in vivo and in vitro. We speculate the reasons as follows; the concentration of A213 reached the trough concentration when we sacrified (unpublished data from Daiichi Sankyo), while the concentration of A213 was kept continuously during the cell culture in vitro. Thus, IL-2 expression in vivo would be recovered. Moreover, A213 significantly decreased the MFI of CD69 in $CD4^+CD25^+Foxp3^-$ cells *in vivo*. CD69 is an early leukocyte activation molecule expressed at sites of chronic inflammation (Sancho et al., 2005). Considered together, these findings suggest that A213 targets activated $CD4^+$ T cells that express both CD25 and CD69 at sites of chronic inflammation. Furthermore, the titer of anti-Ro antibody in serum of A213-treated mice

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was tended to be lower than PBS-treated mice. We speculated that it would be caused by the reduction of activated $CD4^+$ T cells expressing CD25 and CD69, which could help activation of autoreactive B cells.

Second, we could speculate that A213 directly binds to RORγt in Th17 cells and reduces IL-17 production, resulting in the suppression of sialadenitis. In RORγt-Tg mice, RORγt-overexpressing $CD4^+$ T cells seem to contribute to sialadenitis (Iizuka et al., 2015). In the present study, RORγt protein expression in $CD4^+$ T cells and *Il17a* mRNA expression in cLNs $CD4^+$ T cells were comparable between A213- and PBS-treated groups. Thus, A213 did not alter RORγt protein expression and did not influence IL-17 production, suggesting the suppressive effect of A213 on sialadenitis is unlikely to be due to the down-regulation of RORγt-IL-17 pathway. One cannot rule out, however, the possibility that the amount of RORγt antagonist *in vivo* was insufficient to cancel the effect of RORγt overexpression on $CD4^+$ T cells in RORγt-Tg mice.

Third, A213 could ameliorate sialadenitis by altering the balance between $Foxp3^+$ and $Foxp3^-$ $CD4^+CD25^+$ T cells. In this mouse model of SS, reduced number of Treg cells contributes to the development of sialadenitis (Iizuka et al., 2015). Our studies showed that A213 significantly decreased both $CD4^+CD25^+Foxp3^-$ and $CD4^+CD25^+Foxp3^+$ cells in cLNs. Moreover, A213 also significantly reduced the MFI of Foxp3 in $CD4^+CD25^+$ cells. In contrast, the proportions of $Foxp3^+$ and $Foxp3^-$ cells in $CD4^+CD25^+$ cells were comparable in A213- and PBS-treated groups. Taken together, the suppression of sialadenitis observed in A213-treated mice was not due to the recovery of Foxp3 expression on $CD4^+CD25^+$ T cells and the imbalance of $Foxp3^+$ Tregs and $Foxp3^-$ T cells. Further studies are needed to elucidate the true role of $CD4^+CD25^+Foxp3^-$ cells in the immunopathogenesis of sialadenitis.

Forth, A213 acts by regulating IFNγ–producing cells, resulting in refinement of sialadenitis. As we previously reported, the expression of IFNγ in salivary gland of RORγt-Tg mice was higher than that of C57BL/6 mice. Furthermore, the source of IFNγ were infiltrating $CD4^+$ T cells in salivary gland (Iizuka et al., 2015). In RORγt-Tg mice, all $CD4^+$T cells over-expressed RORγt, indicating the existence of $ROR\gamma t^+Tbet^+$ $CD4^+$ T cells. These cells are thought to be convertible Th17 cells to Th1 cells (i.e., exTh17) (Tahara et al., 2017). Our experiments showed that A213 decreased not only $IFN\gamma^+IL\text{-}17^-$ producing

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but also IFNγ^{+}IL-17^{+} double producing CD4^{+}CD25^{+} T cells *in vitro*. However, A213 neither altered *Ifng* mRNA expression nor Tbet protein expression *in vivo*. These results suggest that A213 does not influence IFNγ production by both IFNγ^{+}- and IFNγ^{+}IL-17^{+}-producing CD4^{+}CD25^{+} T cells in RORγt-Tg mice. Thus, it is unlikely that A213 reduced sialadenitis through IFNγ–producing RORγt^{+}T cells.

Fifthly, A213 has possibility to affect the thymocytes in RORγt-Tg mice. Guo et al. have reported that RORγt antagonist controlled the thymocyte development and altered thymic-emigrant recognition of self and foreign antigens, resulted in delayed development of experimental autoimmune encephalomyelitis (EAE) (Guo et al., 2016). In RORγt-Tg mice, it would be possible like that A213 could affect thymocytes and reduce the autoreactive T cells. Further studies are needed to examine the thymocytes in A213-treated RORγt-Tg mice.

We demonstrated in our previous study that A213 improves sialadenitis in MIS mice by reducing both M3R-specific Th17 cells and exTh17 cells, which express RORγt and can convert into Th1 cells (Tahara et al., 2017). In the present study, A213 downregulated CD25 expression via inhibition of IL-2 production and CD69 expression on CD4^{+} T cells, resulting in improvement of sialadenitis in RORγt-Tg mice. However, A213 had no effects on IL-17 and IFNγ production in RORγt-Tg mice. Thus, we propose two separate mechanisms to explain the effects of A213 on the two different experimental sialadenitis mouse models. One is the suppression of antigen-specific Th17 and exTh17 cells in MIS mice, while the other is alleviation of activated CD25^{+}CD69^{+} T cells in RORγt-Tg mice. Moreover, Juan Liu et al. have recently reported that the differences of chemical structure in RORγt antagonist had discrepant effects on T cell subsets (Liu et al., 2019). Importantly, our results proposed that A213 could inhibit CD4^{+}CD25^{+}CD69^{+} T cells which could be activated T cells, as well as antigen specific Th1 and Th17 cells (Tahara et al., 2017).

Although, present study showed that A213 suppressed IL-2 production from CD4^{+} T cells, resulting in downregulation of CD25, the molecular mechanisms remain unclear. Thus, identification of target genes by RORγt using RNA sequencing and Chromatin Immunoprecipitation (ChIP) assay of A213 treated CD4^{+} T cells would be promising methods.

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Moreover, exploring the effects of A213 on other SS mouse models such as female New Zealand Black X New Zealand White F1 mice repeatedly treated with toll-like receptor 3 agonist poly (I:C), in which activation of innate immunity accelerated sialadenitis (Nandula et al., 2011), might confirm the therapeutic usefulness of A213 as well as reveal the novel therapeutic mechanisms of A213.

Recently, some biologics such as rituximab, abatacept, belimumab, and anti-CD40 monoclonal antibody have been reported to improve salivary gland disease of SS in a systematic review (Gueiros et al., 2019). In comparison with these biologic agents, RORγt antagonist could be a novel alternative treatment strategy by oral administration for SS, if it's effectiveness and safety are confirmed in the future studies.

In conclusion, our results suggest that A213 suppresses SS-like sialadenitis through the inhibition of IL-2-mediated CD25 expression and CD69 expression on $CD4^+$ T cells. The results highlight the potential usefulness of RORγt antagonism as a new therapeutic strategy for SS. To our knowledge, this is the first report to demonstrate that RORγt antagonism results in the suppression of $CD4^+CD25^+$ T cells.

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Figure Legends

Figure 1. Oral administration of A213 results in recovery of salivary volume and reduced severity of sialadenitis in RORγt-Tg mice.

(a) In vivo study protocol. Six-week-old female RORγt-Tg mice received orally 300 mg/kg of A213 (10 μl/g body weight) or phosphate buffered saline (PBS, 10 μl/g body weight of mice) every three days for two weeks (total 5 doses) until 8 weeks of age when untreated RORγt-Tg mice developed spontaneous sialadenitis. A213 was dissolved in PBS. Arrows indicate the day of administration of A213 or PBS.

(b) Saliva was collected from RORγt-Tg mice. Saliva volume was measured on days 0 (baseline) and 14 after treatment with A213 or PBS. Changes in saliva volume were calculated relative to the volume measured at baseline, using the formula; [day-45 saliva volume (ml)/weight (g)]/ [day-0 saliva volume (ml)/weight (g)]. Data are mean ± SD of five mice per group (representative data of three independent experiments). *$P<0.05$, between A213 and PBS-treated mice at day 14 (by Student's t-test).

(c) (d) Comparison of hematoxylin and eosin (H&E)-stained salivary gland sections of RORγt-Tg mice treated with A213 or PBS on day 14. Representative images obtained from 5 mice per each group. Arrows point to the inflammatory lesions in the salivary glands of PBS-treated mice (c). Bottom panels are the enlarged images of the yellow boxes in the top panels (c). Original magnifications are x40 (top) and x200 (bottom). Data are representative of three independent experiments. Histological focus scores of inflammatory lesions in salivary glands of RORγt-Tg mice treated with A213 and PBS on day 14. The focus score was assessed in a blinded manner.

(e) Anti-Ro (SS-A) IgG antibody in serum of A123- and PBS-treated RORγt-Tg mice detected by enzyme-linked immunosorbent assay (ELISA).

Data are mean±SD of 5 mice per group and representative of three independent experiments. *$P<0.05$, between A213- and PBS-treated mice (by Student's T test).

Figure 2. Effects of A213 on $CD4^+$ T cell population.

(a) Flow cytometric analysis of RORγt expression (left) and mean fluorescence intensity (MFI) of RORγt (right) gated on $CD4^+$ T cells in spleen of A213- and PBS-treated mice.

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(b) Flow cytometric analysis of Tbet expression (left) and the MFI of Tbet (right) gated on $CD4^+$ T cells in spleen of A213- and PBS-treated mice.
(c) Representative flow cytometric data of naïve ($CD44^{lo}CD62L^{hi}$), central memory ($CD44^{hi}CD62L^{hi}$), and effector memory ($CD44^{hi}CD62L^{lo}$) T cells among total $CD4^+$ cells in the spleen of A213- and PBS-treated mice.
(d) Proportion of naïve, central memory and effector memory T cells in spleen of A213- and PBS-treated mice.
(e) Representative flow cytometric data of naïve, central memory, and effector T cells among total $CD4^+$ cells in cLNs of A213- and PBS-treated mice.
(f) Proportion of naïve, central memory and effector memory T cells in cLNs of A213- and PBS-treated mice.
(g) Representative flow cytometric data of CD4 and CD25 expression gated on lymphocytes in spleen of A213- and PBS-treated mice.
(h) Proportion of $CD4^+CD25^+/CD4^+$ T cells in spleen of A213- and PBS-treated mice.
(i) Representative flow cytometric data of CD4 and CD25 expression gated on lymphocytes in cLNs of A213- and PBS-treated mice.
(j) Proportion of $CD4^+CD25^+/CD4^+$ cells in cLNs of A213- and PBS-treated mice.
Data are mean±SD of 5 mice per each group and representative of three independent experiments. Bars indicate mean values. N.S.= not significant. $^{**}P<0.01$, between A213- and PBS-treated mice (by Student's t-test). N; naïve, CM; central memory, EM; effector memory T cells

Figure 3. Effects of A213 on $CD4^+CD25^+$ cells gated on $CD4^+$ T cells in cervical lymph nodes.

(a) Flow cytometric analysis of CD25 and Foxp3 expression gated on $CD4^+$ T cells in cervical lymph node cells (cLNs) of A213- and PBS-treated mice. Five mice per group were analyzed and representative data are shown.
(b) Proportion of $CD4^+CD25^+Foxp3^+$ cells gated on $CD4^+$ T cells in cLNs of A213- and PBS-treated mice.
(c) Proportion of $CD4^+CD25^+Foxp3^-$ cells gated on $CD4^+$ cells in cLNs of A213- and PBS-treated mice.

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(d) Proportion of $Foxp3^+$ cells gated on $CD4^+CD25^+$ T cells in cLNs of A213- and PBS-treated mice.

(e) Proportion of $Foxp3^-$ cells gated on $CD4^+CD25^+$ T cells in cLNs of A213- and PBS-treated mice

(f) Flow cytometric analysis of Foxp3 in cLNs of A213- and PBS-treated mice. These cells were gated on $CD4^+CD25^+$ cells.

(g) Mean fluorescence intensity (MFI) of Foxp3 gated on $CD4^+CD25^+$ T cells in cLNs of A213- and PBS-treated mice.

(h) Flow cytometric analysis of CD69 in cLNs of A213- and PBS-treated mice. These cells were gated on $CD4^+CD25^+Foxp3^+$ cells.

(i) MFI of CD69 in cLNs of A213- and PBS-treated mice. These cells were gated on $CD4^+CD25^+Foxp3^+$ cells.

(j) Flow cytometric analysis of CD69 in cLNs of A213- and PBS-treated mice. These cells were gated on $CD4^+CD25^+Foxp3^-$ cells.

(k) MFI of CD69 in cLNs of A213- and PBS-treated mice. These cells were gated on $CD4^+CD25^+Foxp3^-$ cells.

(l) Quantitative PCR analysis of mRNA expression levels of *Il-2*, *Il-17a* and *Ifng* in $CD4^+$ cells of cLNs of A213- and PBS-treated mice. Experiments were performed in duplicate. Gene expression levels were calculated from the standard curve and expressed relative to *Gapdh* gene expression.

Data are mean±SD of five mice per group and representative of three independent experiments. Bars indicate mean values (b, c, d, e, g, i, k, l). N.S.= not significant. **P*<0.05, between A213- and PBS-treated mice (by Student's t-test).

Figure 4. Effects of A213 on CD25 and CD69 expression in $CD4^+$ cells derived from RORγt-Tg mice *in vitro*.

(a) *In vitro* effects of A213 on CD25 protein expression level in $CD4^+$ cells derived from splenocytes of 6-week-old RORγt-Tg mice. The effect was examined using different A213 concentrations (0, 0.01,0.1, 1 μM) under stimulation with anti-CD3 (plate-bound) and anti-CD28 (soluble) antibodies for 72 hours and analyzed by flow cytometry (blue line: 0 μM, light blue line: 0.01 μM, orange line: 0.1 μM, red line: 1 μM of A213, gray line: isotype control).

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(b) Mean fluorescence intensity (MFI) of CD25 in $CD4^+$ cells of RORγt-Tg mice. MFI was determined in the presence of different concentrations of A213 (0, 0.01, 0.1, 1 μM) under stimulation with anti-CD3 and anti-CD28 antibodies for 72 hours and analyzed by flow cytometry.

(c) *In vitro* effects of A213 on CD69 protein expression level in $CD4^+$ cells derived from splenocytes of 6-week-old RORγt-Tg mice. The effect was examined using various concentrations of A213 (0, 0.01,0.1, 1 μM) under stimulation with anti-CD3 and anti-CD28 antibodies for 72 hours and analyzed by flow cytometry (blue line: 0 μM, light blue line: 0.01 μM, orange line: 0.1 μM, red line: 1 μM of A213, gray line: isotype control).

(d) MFI of CD69 in $CD4^+$ cells derived from RORγt-Tg mice. MFI was examined in the presence of various concentrations of A213 (0, 0.01, 0.1, 1 μM) under stimulation with anti-CD3 and anti-CD28 antibodies for 72 hours and analyzed by flow cytometry.

Data are mean ± SD of four wells at each A213 concentration. Representative data of three independent experiments. Bars represent average values (b,d). N.S.=not significant. $P<0.05$; between and among the different concentrations of A213 (by Kruskal-Wallis test).

Figure 5. *In vitro* effects of A213 on cytokine production.

(a) $CD4^+$ T cells from 6-week-old RORγt-Tg mice were cultured in the presence of various concentrations of A213 (0, 0.01, 0.1, 1 μM) under stimulation with anti-CD3 and anti-CD28 antibodies for 72 hours, with or without PMA and ionomycin during the last four hours. Interleukin (IL)-2 level in the culture supernatants was analyzed by enzyme-linked immunosorbent assay (ELISA).

(b) $CD4^+$ T cells from 6-week-old RORγt-Tg mice were cultured in the presence of various concentrations of A213 (0, 0.01, 0.1, 1 μM) under stimulation with anti-CD3 and anti-CD28 antibodies for 72 hours, and then stimulated with or without PMA and ionomycin during the last four hours. The expression level of $IFN\gamma^+$ $IL\text{-}17^-$, $IFN\gamma^-IL\text{-}17^+$ and $IL\text{-}17^+IFN\gamma^+$ cells gated on $CD4^+CD25^+$ cells was analyzed by flow cytometry. Representative flow cytometry data are shown.

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(c) Proportion of $IFN\gamma^{-}IL\text{-}17^{+}$ cells among $CD4^{+}CD25^{+}$ cells treated with various concentrations of A213 (0, 0.01, 0.1, 1 μM) and stimulated with or without PMA and ionomycin.

(d) Proportion of $IFN\gamma^{+}IL\text{-}17^{-}$ cells among $CD4^{+}CD25^{+}$ cells treated with various concentrations of A213 (0, 0.01, 0.1, 1 μM) and stimulated with or without PMA and ionomycin.

(e) Proportion of $IFN\gamma^{+}IL\text{-}17^{+}$ cells among $CD4^{+}CD25^{+}$ cells treated with various concentrations of A213 (0, 0.01, 0.1, 1 μM) and stimulated with or without PMA and ionomycin.

Data are mean±SD of four wells under each A213 concentration. Representative data of two independent experiments. (a,c,d,e) **P<0.001 (by Kruskal-Wallis test).

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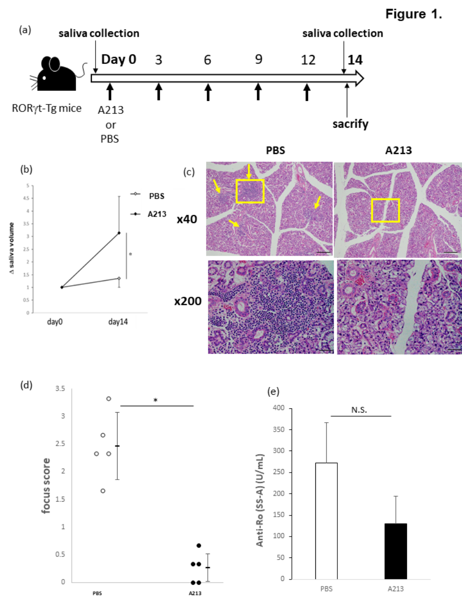

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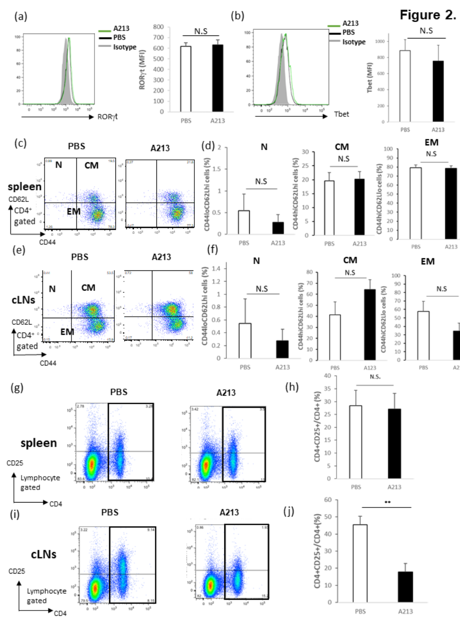

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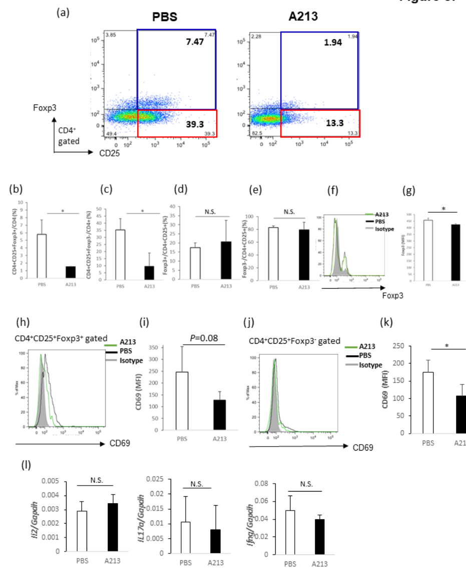

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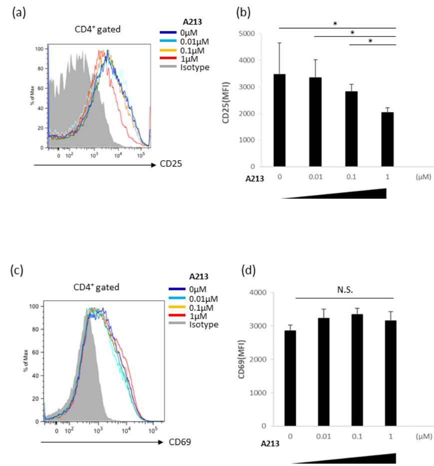
Figure 4.



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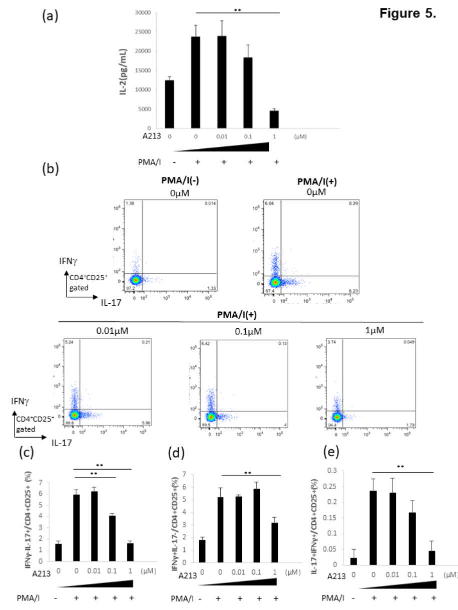

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