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A combination treatment of low-dose dexamethasone and aspirin-triggered resolvin D1 reduces Sjögren syndrome–like features in a mouse model

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Abstract

Background.—Sjögren syndrome (SS) is an autoimmune disease characterized by lymphocytic infiltration and diminished secretory function of the salivary glands. Dexamethasone (DEX) resolves dry mouth and lymphocytic infiltration; however, this treatment is difficult to maintain because of multiple adverse effects (eg, osteoporosis and skin thinning); likewise, aspirin-triggered resolvin D1 (AT-RvD1) increases saliva secretion but cannot eliminate lymphocytic infiltration. Previous studies showed that a combination of low-dose DEX with AT-RvD1 before disease onset prevents SS-like features in a mouse model; however, this is not clinically practical because there are no reliable indicators of SS before disease onset. Therefore, the authors applied the combined treatment at disease onset to show its efficacy and comparative lack of adverse effects, so that it may reasonably be maintained over a patient's lifetime.

Methods.—NOD/ShiLtJ mice were treated with ethanol (vehicle control), high-dose DEX alone, AT-RvD1 alone, or a combination of low-dose DEX with AT-RvD1 at disease onset for 8 weeks. Then saliva flow rates were measured, and submandibular glands were harvested for histologic analyses.

Results.—A combined treatment of low-dose DEX with AT-RvD1 significantly decreased mast cell degranulation and lymphocytic infiltration, increased saliva secretion, and restored apical aquaporin-5 expression in submandibular glands of NOD/ShiLtJ mice.

Conclusions.—Low-dose DEX combined with AT-RvD1 reduces the severity of SS-like manifestation and prevents the development of advanced and potentially irreversible damage, all

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in a form that can reasonably be administered indefinitely without the need to cease treatment because of secondary effects.

Graphical Abstract

A combination treatment of low-dose dexamethasone and AT-RvD1 reduces SS-like features in a mouse model

	Reduces Lymphocyte Infiltration	Reduces Mast Cell Degranulation	Increases Saliva Secretion	Increases Apical Aquaporin-5 Localization
Ethanol (Vehicle Control)	No	No	No	No
High-Dose DEX	Yes	Yes	Yes	No
AT-RvD1	No	Yes	Yes	Yes
Low-Dose DEX + AT-RvD1	Yes	Yes	Yes	Yes

Keywords

Salivary glands; inflammation; resolvins; steroids; lipid mediators

Introduction

Sjögren syndrome (SS) is a systemic autoimmune disease characterized by loss of saliva and tear secretion mediated by salivary and lacrimal glands, respectively.[1] Specifically, the glandular inflammation induced by lymphocytic infiltration and mast cell degranulation results in a loss of epithelial acinar cells that mediate fluid secretion.[1] Primary and secondary SS occur in the absence or presence of other rheumatic disorders such as lupus erythematosus, rheumatoid arthritis, or scleroderma.[2] Genetic predisposition and environmental factors influence the development of SS; however, the specific causes and effective therapies are not known.[3–7] Given the heterogeneity of the clinical manifestations in SS, early diagnosis is difficult, and patients are often only identified once saliva secretion has been significantly diminished.[8]

Dexamethasone (DEX) is a common treatment for SS because it reduces salivary gland inflammation, restores saliva secretion, and greatly diminishes lymphocytic infiltration[9,10] that will inevitably lead to a reversal of treatment gains if left to proliferate; however, DEX treatment has significant issues. Specifically, treatments must be continued throughout the life span to maintain the desired effects, but significant adverse effects accrue that negatively affect a patient's quality of life.[11–14] To make DEX treatment for salivary gland inflammation more viable for lifelong use, we explore the use of a diminished dosage of DEX with aspirin-triggered resolvin D1 (AT-RvD1), which is reported to be effective in managing salivary gland inflammation, as detailed below. In so doing, we aim to preserve treatment gains achieved with higher doses of DEX but at a dosage threshold that will not discourage patients from continuing treatment indefinitely.

Resolution of inflammation is an actively regulated process mediated in part by a family of specialized proresolving lipid mediators (SPM), which include resolvins, maresins, lipoxins, and protectins as well as their AT forms, which are comparable in their properties to naturally occurring SPM[15–22] but have a longer half-life.[23] SPM and their AT forms can be an alternative for treating inflammatory diseases by limiting uncontrolled inflammation in response to injury or environmental challenges[24–30] while promoting its termination and leading to tissue repair and functionality.[31–34] SPM have been detected in human tears,[35]

plasma,[35–39] milk,[40] and saliva[41] as well as in animal models of infection and chronic inflammation.[36,42–46] Studies of SPM and AT forms within the salivary glands have been largely confined to AT-RvD1, one of many in the resolvin family, that has shown particular promise in treating some key features of hyposalivation.[9,10] Previous studies showed that mouse and human salivary glands express SPM[41,47] with their biosynthetic machinery[24] and receptors.[48] In salivary glands, the SPM family members, most notably RvD1 and its aspirin-triggered epimer AT-RvD1, have been shown to activate formyl peptide receptor 2 (ALX/FPR2) and promote prosurvival signals both in vitro and in vivo.[49–53]

Despite the promise shown by SPM in general and AT-RvD1 in particular for treating hyposalivation, a critical obstacle must be overcome for the potential use of this treatment in SS. Specifically, AT-RvD1 does not completely eradicate lymphocytic infiltration, which leads to the secretion of SS-associated proinflammatory cytokines that are known to disrupt epithelial integrity and invariably lead to loss of function.[54,55] Subsequent to this observation, a previous study showed that coadministration of low-dose DEX with AT-RvD1 enhances saliva secretion and prevents lymphocytic infiltration in an SS-like mouse model when administered before disease onset.[10] However, this treatment to date has been limited by a lack of screening techniques for SS. The disease has no reliable early genetic markers and is typically identified with a loss of saliva secretion not attributable to other causes.[8,56] Therefore, we sought to extend prior findings to the early disease onset phase (ie, after lymphocytic infiltration and reduced saliva secretion) by determining whether combined treatment with low-dose DEX with AT-RvD1 reduces SS-like responses in the NOD/ShiLtJ mouse model of SS. Should our study prove successful, a later investigation will seek to extend these findings to cases of late-stage disease manifestation with the aim of reversing the most severe damage caused by SS.

Methods

Animals

Forty female 12-week-old NOD/ShiLtJ mice were randomly divided into 4 groups: ethanol (vehicle control) treated, high-dose DEX treated, AT-RvD1 treated, and a combination of low-dose DEX with AT-RvD1 treated. Specifically, animals were treated twice a week for 8 weeks via tail vein injection with ethanol (3.5% [vol/vol], vehicle control), high-dose DEX (8.25 mg/kg) (Sigma Aldrich), AT-RvD1 alone (0.1 mg/kg) (Cayman Chemical) or a low-dose of DEX (4.125 mg/kg) with AT-RvD1 (0.1 mg/kg). The doses of DEX and AT-RvD1 used in this study were chosen on the basis of a pilot study indicating that these compounds produce a significant downregulation of systemic inflammatory genes.[9] In this study, only female NOD/ShiLtJ mice were used in light of the predominance of females affected by SS, with a 9:1 ratio compared with males.[57,58] For submandibular gland (SMG) harvesting, mice were euthanized using carbon dioxide at 20 weeks of age, followed by abdominal exsanguination. SMGs were then removed and processed, as detailed in Figure 1. Animals were housed in cages in a room with a controlled environment (12-hour day/night cycles) and provided with a standard pellet diet and water. Moreover, mice were ear-tagged to minimize potential confounding variables, and group allocation at the different stages of the study was controlled by all the people who obtained experimental data. Finally, this

study was performed using protocols approved by the Institutional Animal Care and Use Committee and the Animal Research: Reporting In Vivo Experiments guidelines.[59]

Tissue processing

SMGs were fixed in 10% (vol/vol) formalin for 24 hours at room temperature and then transferred to 70% (vol/vol) ethanol. Next, SMGs were dehydrated through a series of graded ethanol washes (30%, 50%, 70%, twice at 95%, and 3 times at 100%), embedded in paraffin, and cut into 5-μm sections. Paraffin-embedded slides were then deparaffinized by washing 3 times for 5 minutes in 100% (vol/vol) xylene. Slides were washed for 5 minutes in xylene:ethanol (1:1), twice for 5 minutes in 100% (vol/vol) ethanol, followed by 5 minute washes in 95%, 80%, 70%, and 50% ethanol, then twice in distilled water.

Hematoxylin-eosin staining

Deparaffinized and rehydrated tissue sections were stained with hematoxylin for 10 minutes, washed twice for 5 minutes each with tap water, then destained with 0.3% hydrogen chloride for 3 seconds and rinsed twice for 1 minute each with tap water. Next, sections were washed with 95% (vol/vol) ethanol for 1 minute, stained with eosin for 10 minutes, and washed 3 times with 95% (vol/vol) ethanol for 1 minute. Subsequently, samples were rinsed 3 times with 100% (vol/vol) ethanol, cleared in xylene, and mounted with a xylene-based mounting medium (Poly-sciences). Finally, to evaluate histopathologic features, samples were examined using a Leica DMI6000B inverted microscope (Leica Microsystems), and lymphocytic foci size was divided by the total SMG area using ImageJ (National Institutes of Health).

Toluidine blue staining

Deparaffinized and rehydrated tissue sections were stained with toluidine blue working solution (VitroView) for 3 minutes. Next, specimens were washed 3 times with distilled water, and tissue sections were dehydrated by washing 3 times for 3 minutes each in 95% and 100% (vol/vol) alcohol. Then, specimens were washed twice for 3 minutes in xylene and mounted with a xylene-based mounting medium. Finally, to assess mast cell degranulation, samples were examined using a Leica DMI6000B inverted microscope.

Confocal microscopy analyses

Deparaffinized tissue sections were incubated with sodium citrate buffer (10 mM sodium citrate, 0.05% [vol/vol] polyethylene glycol sorbitan monolaurate [Tween 20, Sigma-Aldrich], pH 6.0) at 95 °C for 30 minutes for antigen retrieval. Next, samples were rinsed twice with distilled water and permeabilized with 0.1% (vol/vol) *t*-octylphenoxypolyethoxyethanol (Triton X-100, Sigma-Aldrich) in phosphate-buffered saline (PBS) at room temperature for 45 minutes. Sections were then blocked with 5% (vol/vol) goat serum in PBS at room temperature for 1 hour and incubated with rabbit–anti-mouse aquaporin-5 (1:100 [ab78486; Abcam]) and rabbit–anti-mouse chymase (1:100 [ab233103; Abcam]) antibodies at 4 °C overnight. Then, specimens were washed 3 times with PBS and incubated with Alexa Fluor 488-conjugated anti-rabbit immunoglobulin G (1:500 [A-11008; ThermoFisher]) at room temperature for 1 hour. Finally, sections were washed 3 times with

PBS, nuclei were counterstained with 4′,6-diamidino-2-phenylindole at room temperature for 15 minutes (1:1,000 dilution), and images were analyzed using a confocal Stellaris 5 microscope (Leica Microsystems). The positive area of chymase was calculated by measuring positive pixels using ImageJ in each tissue section.

Measurement of saliva flow rate

Mice were anesthetized with 100 mg/kg of ketamine and 5 mg/kg of xylazine followed by intraperitoneal injection of 50 mg/kg of pilocarpine-hydrogen chloride (Sigma) and 0.5 mg/kg of isoproterenol (Sigma) in PBS to stimulate saliva secretion. Then, the whole saliva was collected for 5 minutes using a 200-μL pipette, and the saliva flow rate was calculated by dividing the total amount of stimulated saliva (μL) by the product of the mouse body weight (g) and the collection time (5 min).

Statistical analyses

Data are presented as mean (SD) from 3 or more determinations. Prism software (GraphPad) was used for statistical analyses by *t* test or 1-way analysis of variance. $P \leq 0.05$ represents significant differences between experimental groups.

Results

A results summary describing treatment effects by groups is provided in the graphical abstract.

Treatment with low-dose DEX with AT-RvD1 reduces lymphocytic infiltration in SMG of SS-like mice

To determine the degree of lymphocytic infiltration in SMG from NOD/ShiLtJ SS-like mice, tissues were stained with hematoxylin-eosin, and histopathologic analysis was performed as described in the Methods section. As shown in Figure 2A, SMG from ethanol (vehicle control) and AT-RvD1–treated mice showed extensive lymphocytic infiltration. Moreover, lymphocytic foci covered more than 40% of the glandular tissue, indicating that systemic AT-RvD1 treatment alone has no appreciable impact on reducing immune cell infiltration or expansion. In contrast, SMG from mice treated with high-dose DEX alone or low-dose DEX with AT-RvD1 showed a significant reduction in lymphocytic foci size per glandular area (Figure 2B).

A combination treatment with low-dose DEX with AT-RvD1 reduces mast cell degranulation in SMG of SS-like mice

Given that mast cells are involved with SS initiation and progression by releasing inflammatory mediators (eg, prostaglandin and leukotrienes),[60–63] the effect of combination low-dose DEX with AT-RvD1 treatment on mast cell degranulation was determined. As shown in Figure 3A, SMG from ethanol (vehicle control) showed extensive mast cell degranulation, as evidenced by the presence of toluidine blue-stained granules (ie, purple) outside the mast cells. In contrast, treatment with high-dose DEX alone or low-dose DEX combined with AT-RvD1 significantly diminished mast cell degranulation. To confirm and quantify these results, confocal analysis using a selective antibody for mast cells (ie, rabbit–

anti-mouse chymase antibody) was performed as described in the Methods section. Results show that mice treated with high-dose DEX alone, AT-RvD1 alone, or low-dose DEX with AT-RvD1 all displayed a significant reduction of mast cell degranulation compared with ethanol (vehicle control) (Figure 3B, C).

A combination treatment with low-dose DEX and AT-RvD1 restores saliva secretion in SS-like mice

To determine the effects of DEX with AT-RvD1 treatment on saliva flow rates in the NOD/ShiLtJ SS-like mouse, mice were treated as described in the Methods section. Results show that mice treated with high-dose DEX alone, AT-RvD1 alone, or low-dose DEX with AT-RvD1 all showed a significant increase in saliva flow rates compared with vehicle controls (Figure 4).

A combination treatment with low-dose DEX with AT-RvD1 induces apical localization of the SMG water channel aquaporin-5

Given the increased saliva secretion in all treatment groups, specific effects on aquaporin-5 apical localization were investigated. Specifically, aquaporin-5 is the major water channel involved in polarized fluid secretion[64–66] and is known to abnormally translocate from the apical to the basolateral membrane (ie, loss of polarity) in salivary glands of SS patients.[9] For our study, confocal analysis using a selective antibody for aquaporin-5 (ie, rabbit–anti-mouse aquaporin-5 antibody) was performed as described in the Methods section. Our data show that treatment with AT-RvD1 alone or low-dose DEX with AT-RvD1 results in apical aquaporin-5 localization in SMG compared with mice treated with high-dose DEX or ethanol (vehicle control) (Figure 5). Although high-dose DEX alone did increase saliva flow rate (Figure 4), this treatment failed to induce aquaporin-5 apical localization (Figure 5), thereby suggesting damage to this secretory protein that may compromise saliva secretion in the long term. These results indicate that treatment with both AT-RvD1 alone and low-dose DEX with AT-RvD1 ensures an apical expression of aquaporin-5, the main conduit of saliva secretion in SMG.

Discussion

A previous study showed that the potent anti-inflammatory drug DEX when administered alone and at high strength, significantly reduces lymphocytic infiltration in SMG of NOD/ShiLtJ mice when administered at the predisease stage,[10] and our study extends these findings to disease onset. However, DEX has major limitations, including multiple secondary effects such as hyperglycemia, obesity, hypertension, osteoporosis, cataract formation, and striatal and skin thinning.[10] Moreover, treatment with high-dose DEX cannot maintain apical expression of aquaporin-5 in salivary glands, consistent with a previous study.[67] Similarly, although a clinical study showed that the use of corticosteroids relieves oral symptoms in SS patients (eg, dry mouth, increased water drinking frequency, sticky sensations, and lip dryness),[68] such beneficial effects are not sustained when the drug is no longer taken,[69] and long-term use of DEX at high doses has been shown to in fact lead to loss of saliva secretion in both humans and mice.[14,70]

Considerable benefits were likewise noted with AT-RvD1. Specifically, a previous study showed that activation of ALX/FPR2 with RvD1 blocks proinflammatory signals caused by tumor necrosis factor-α while enhancing salivary gland epithelial integrity in the rat parotid Par-C10 cell line.[50] Moreover, previous studies confirmed that ALX/FPR2 is expressed in primary salivary gland epithelial cells.[37,49] Activating AT-RvD1 increases a diverse set of intracellular prosurvival signaling pathways, such as calcium ion, Erk1/2, and Akt, which block TNF-α–mediated caspase-3 activation.[49] A subsequent pilot study showed that AT-RvD1 treatment at 0.1 mg/kg significantly reduces SS-associated proinflammatory genes in SMG from NOD/ShiLtJ compared with vehicle-treated mice.[9] Next, a preclinical study showed that AT-RvD1 treatment administered at disease onset reduced the number of T helper 17 cells in SMG and successfully restored salivary gland function in NOD/ShiLtJ mice.[71] Together, these reports indicate that treatment with AT-RvD1 alone achieves proresolving effects by reestablishing normal tissue architecture and functionality in salivary gland epithelium while reducing proinflammatory signals in the NOD/ShiLtJ SS-like mouse model.[9,10,49–53,71] However, treatment with AT-RvD1 alone does not reduce lymphocytic infiltration in SMG. T lymphocytes can develop into ectopic lymphoid structures, whereas B lymphocytes become hyperactive and form autoantibodies, lymphoepithelial lesions, and SS-related mucosa-associated lymphoid tissue lymphoma, all of which alter salivary epithelial integrity.[72–78] Thus, AT-RvD1 has proven insufficient as a stand-alone treatment given its inability to reduce lymphocytic infiltration.

In light of the clear benefits of both of these candidates for the treatment of SS-like features (ie, DEX with AT-RvD1) as well as their significant deficits (adverse effects for long-term high strength DEX use and inability to impact lymphocytic infiltration for AT-RvD1), a combination of the 2 was used in this study, with results indicating a significant treatment effect that could reasonably be continued throughout the life span of the SS patient. Specifically, our findings indicate that low-dose DEX combined with AT-RvD1 is highly effective for blocking lymphocytic infiltration and mast cell degranulation (benefits previously seen with high-dose DEX[9,10]) while also increasing apical aquaporin-5 expression and saliva secretion in SMG of SS-like NOD/ShiLtJ mice (benefits previously seen with AT-RvD1 alone[9,10]), all without the significant adverse effects consistently seen with the higher dosage of DEX. Given that the proposed treatment combining low-dose DEX with AT-RvD1 is intended to be administered indefinitely to maintain treatment gains, we would highlight the overriding importance of reducing secondary effects associated with high-dose DEX.

The limitations of this study include a lack of experiments to prove that lymphocytic infiltration will lead to glandular dysfunction; however, previous studies indicate that proinflammatory cytokines released by infiltrating lymphocytes lead to secretory dysfunction by damaging salivary gland tight junctions,[50,79–84] a finding that we will seek to expand on in our future investigation. Moreover, lymphocyte receptors may prove unresponsive to AT-RvD1, thereby allowing for the continuation of inflammatory cytokine secretion and leading to tight junction disruption.[50,79–84] Such an effect could result in relapse among the AT-RvD1-treated cohort, and future studies are warranted to determine if this is the case. Finally, adverse effects of DEX are well-known, and lower doses will predictably reduce adverse effects[85–88]; however, we have yet to determine the lowest DEX

concentration at which point treatment effects would be lost. As such, future studies will conduct dose-response experiments to determine the appropriate minimum dosage of DEX with AT-RvD1. Having thus determined the lowest permissible DEX dosage, later studies may be extended to establish the sustainability of treatment gains.

Conclusions

This study showed that a combination of low-dose DEX with AT-RvD1 reduced the severity of SS-like features and prevented the development of advanced and potentially irreversible damage, all in a form that can be administered indefinitely without the need to cease treatment because of secondary effects. Finally, it is worth noting that the cohort in our study was composed entirely of female mice in light of the heavy predominance of females affected by SS, with a ratio of 9:1 compared with males.[57] That said, once the management of the primary treatment group has been established, further studies would seek to apply these techniques to the remaining cases appearing among males while also extending these findings to the potential reversal of late-stage SS damage.

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Why Is This Important?

Treatments for Sjögren syndrome are ineffective in that they either address only part of the problem, do so at a considerable cost in terms of secondary effects when used indefinitely, or both. This study aimed to combine 2 promising treatments for Sjögren syndrome (ie, dexamethasone and aspirin-triggered resolvin D1) to retain their benefits when given in isolation while reducing their respective deficits.

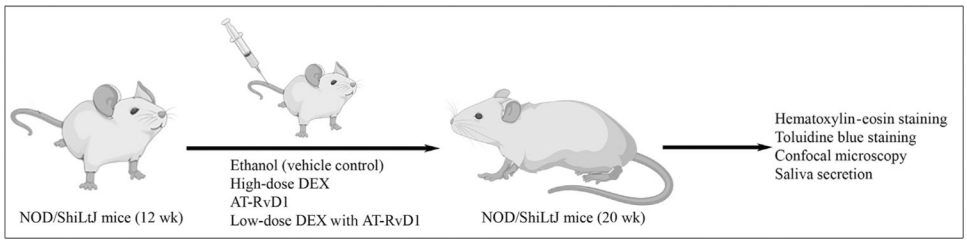

Figure 1.

Diagram summarizing the treatments used in the study. Sjögren syndrome–like mice were randomly divided into 4 groups and treated twice a week for 8 weeks via tail vein injection: ethanol (vehicle control), high-dose dexamethasone (DEX), aspirin-triggered resolvin D1 (AT-RvD1), and low-dose DEX with AT-RvD1. After the indicated times, specimens were collected as described in the Methods section. The study was performed using protocols approved by the Institutional Animal Care and Use Committee and the Animal Research Reporting In Vivo Experiments guidelines,[59] and figures were generated using biorender.com.

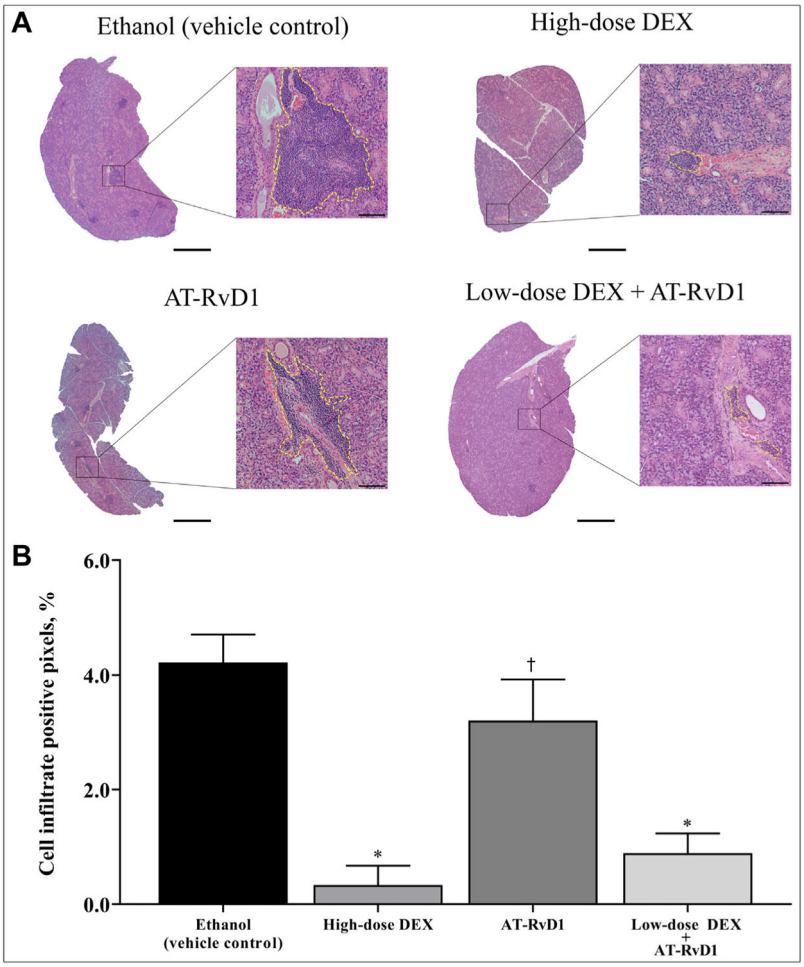

Figure 2.

Combined treatment with low-dose dexamethasone (DEX) with aspirin-triggered resolvin D1 (AT-RvD1) reduces submandibular gland lymphocytic infiltration in submandibular glands of Sjögren syndrome–like mice. Mice were treated as described in the Methods section, and submandibular glands were harvested, sectioned, and stained with hematoxylin-eosin. **A**. Lymphocytic foci are shown within yellow dotted lines in which scale bars in low and high magnification images are 500 μm and 50 μm, respectively. **B**. Lymphocytic foci were quantified, and data were expressed as mean (SD), in which * indicates $P = .001$ and † indicates not significant.

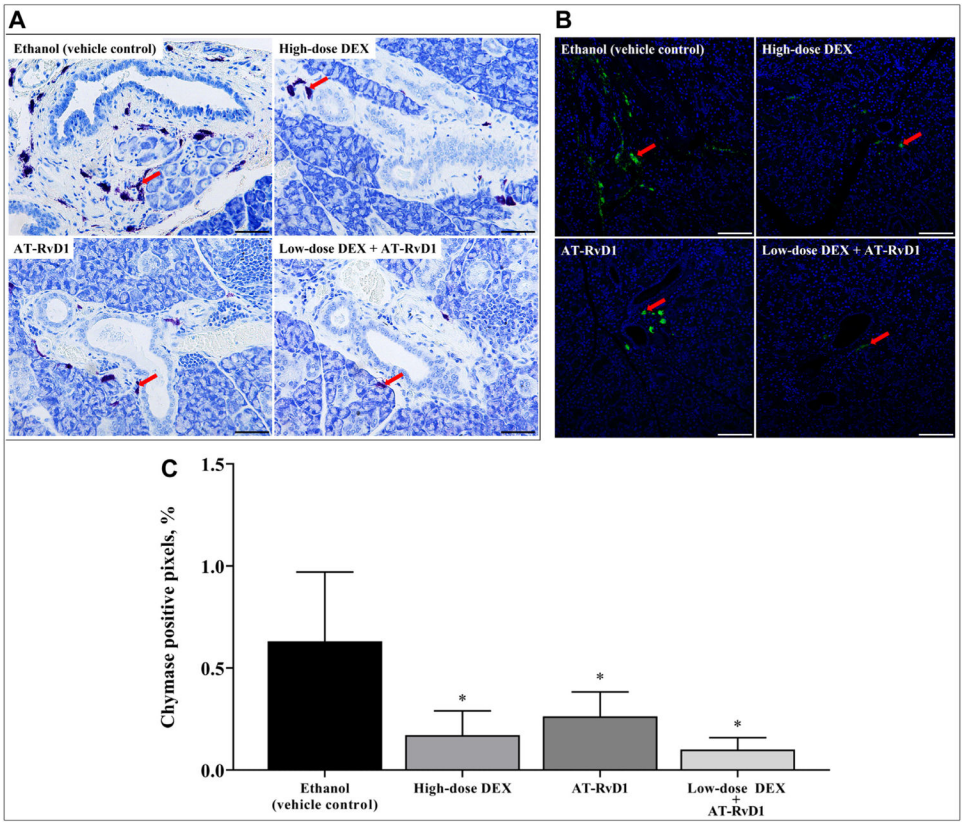

Figure 3.

Combined treatment with low-dose dexamethasone (DEX) with aspirin-triggered resolvin D1 (AT-RvD1) reduces mast cell degranulation in submandibular glands of Sjögren syndrome–like mice. Submandibular glands were harvested, formalin-fixed, paraffin-embedded, and sectioned. **A**. Mast cell degranulation in submandibular glands was observed using toluidine blue staining (red arrows), in which scale bars represent 50 μm. **B**. Chymase was detected with rabbit–anti-mouse chymase antibody (green; red arrows), and nuclei were stained for nucleic acids with 4′,6-diamidino-2-phenylindole (blue) with images analyzed using confocal microscopy. Representative fluorescence images are shown from 4 samples, in which scale bars represent 100 μm. **C**. Mast cell degranulation was quantified and expressed as mean (SD), in which * indicates $P = .05$.

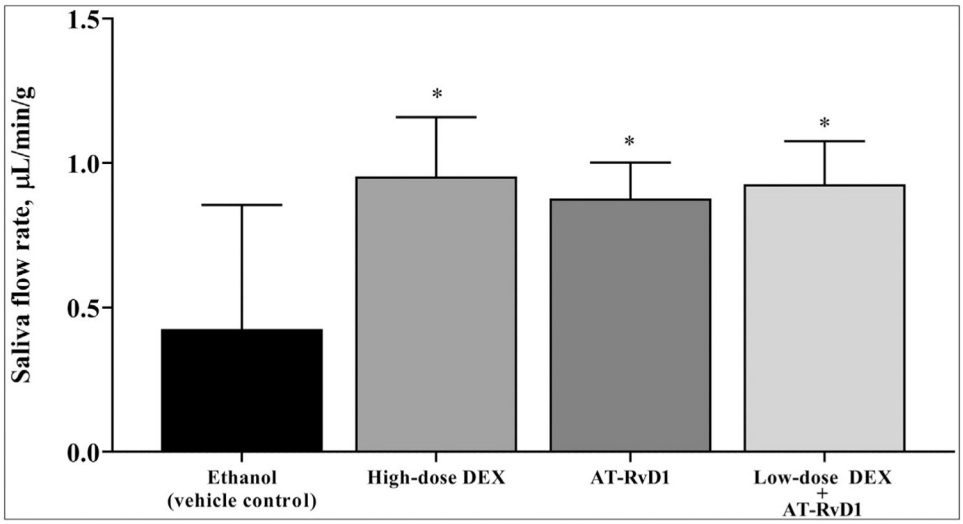

Figure 4.

Combined treatment with low-dose dexamethasone (DEX) with aspirin-triggered resolvin D1 (AT-RvD1) increases saliva secretion in Sjögren syndrome–like mice. Mice were treated as described in Methods. Then, saliva was collected after intraperitoneal injection with pilocarpine-hydrogen chloride (50 mg/kg) and isoproterenol (0.5 mg/kg). Results are representative of 5 mice per condition, and data are expressed as mean (SD) in which * indicates $P = .01$.

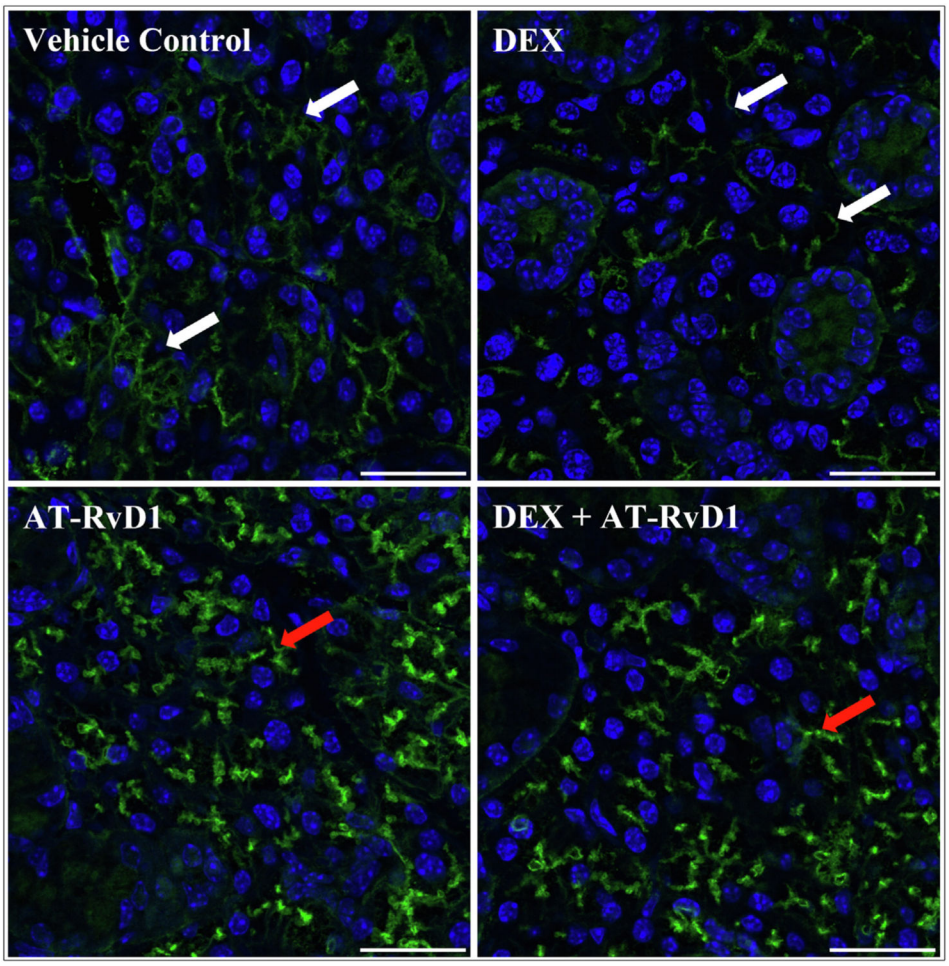

Figure 5.

Combined treatment with low-dose dexamethasone (DEX) with aspirin-triggered resolvin D1 (AT-RvD1) enhances the expression and apical distribution of aquaporin-5 in submandibular glands of Sjögren syndrome–like mice. Submandibular glands were harvested, formalin-fixed, paraffin-embedded, and sectioned. Aquaporin-5 staining was detected with rabbit–anti-mouse aquaporin-5 (green), nuclei were stained with 4′,6-diamidino-2-phenylindole (blue), and images were analyzed using confocal microscopy. Representative fluorescence images from 4 samples, in which scale bars represent 25 μm. White arrows indicate basolateral staining, and red arrows indicate apical staining for aquaporin-5.