Use of localized gene transfer to develop novel treatment strategies for the salivary component of Sjögren's syndrome

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Disease modifying effects of local soluble tumor necrosis factor α receptor-1 gene transfer in a murine model of Sjögren’s syndrome

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ABSTRACT

Background
Sjögren’s syndrome (SS) is a chronic autoimmune disease characterized by focal mononuclear infiltrates (mainly CD4+ cells) in the salivary glands (SGs) and decreased saliva production. Current therapies for SS are unsatisfactory. Disappointing results of systemic treatment with TNF-α blocking drugs in SS patients were recently reported. This could be due to the lack of sufficient levels of the TNF-α blocking drugs at the active sites of disease such as the salivary glands.

We hypothesized that transfer of the gene encoding soluble TNF receptor -1 to SGs, mediated by a recombinant adeno-associated viral (rAAV) vector, would decrease lymphocytic infiltration and increase salivary flow in non-obese diabetic (NOD) mice. NOD mice, a model of SS, exhibit focal lymphocytic infiltrates and a characteristic age and gender (female) related decline of salivary flow.

Material and methods
Vectors were delivered locally via cannulated ducts with retrograde infusion to the SGs. Mice received rAAVTNR as active treatment (T). Control groups received either saline (S), rAAV encoding β-galactosidase (rAAVLacZ) (negative control; C-), or rAAVhIL10 (positive control; C+). Mice received vector at age 8 wk, before onset of sialadenitis, and were sacrificed at week 16. Blood glucose levels were monitored weekly and mice with values >400 mg/dl were treated with insulin by injection (q24 h) to limit diabetes related dehydration. Saliva collection and focus scores were performed.

Results
Mice receiving rAAVTNR or rAAVhIL10 had a salivary flow rate increase at week 16 compared to week 8 of 46±16 (μl/20min; mean ± SEM) (T) and 37±14 (C+), respectively. Mice receiving either saline or rAAVLacZ had a decline in salivary flow of -20±14 (S) and -12±10 (μl/20min) (C-), respectively. There was a significant difference between C- versus T (p=0.028) and C- versus C+ (p=0.045). There was no statistically significant difference between T and C+ or C- and S. Mice receiving vectors had focus scores at 16 wk of 2.02±0.16 (mean ± SEM) (S), 2.14±0.18 (C-), 1.17±0.24 (T) and 1.22±0.22 (C+) with a significant difference between C- and T (p<0.001). Similarly, for mice receiving C+ versus C- there was a significant difference (p=0.005). We did not observe any significant differences between the active treatment groups C+ and T nor between the negative control groups C- and S.

Conclusion
Local rAAV mediated TNF receptor gene transfer modifies sialadenitis in NOD mice resulting in decreased tissue inflammation and increased saliva production.
INTRODUCTION

Sjögren's syndrome (SS) is characterized by focal and diffuse lymphocyte infiltration into the lacrimal and salivary glands (SG). These mononuclear cell infiltrates mainly consist of CD4 positive T lymphocytes (1). The altered secretory function of the salivary and lacrimal glands is the most prominent feature of the disease. Non-obese diabetic (NOD) mice not only exhibit a prominent lymphocytic cell infiltration of the pancreatic islets, but are also characterized by mononuclear cell infiltrates in salivary and lacrimal glands that is age dependent. Moreover, the secretory function of the SG of female NOD mice diminishes over time, comparable to SS (2).

Proinflammatory cytokines such as TNF-α may play a pivotal role in the development of autoimmune diseases (3-5). In addition, the immune response seems to be biased toward a Th1-like profile in NOD mice (6). Immunohistochemical staining of the exocrine gland lymphocytic infiltrates is most intense for TNF-α, IL-2, and IFN-γ (2, 7-9).

In the NOD mice, cells expressing and secreting TNF-α are mostly found around infiltrating CD4 positive T-cells (10). NOD mice transgenic for soluble TNF receptor -1 (sTNRp55) are protected against mononuclear cell infiltration (10). Thus, TNF-α could play an important role in SS, and sTNRp55 may prove a valuable treatment in this disease.

The current array of treatments, using secretagogues to alleviate symptoms, are unsatisfactory in their efficacy. In rheumatoid arthritis biologicals blocking the effects of TNF-α are highly effective (11). However, recent clinical trials using anti-TNF antibodies (infliximab) or sTNFR (etanercept) in SS patients did not show significant improvement (12-15). These disappointing results might be due to the fact that these proteins are delivered systemically by multiple injections, which could result in insufficient levels of the therapeutic protein locally in the salivary gland.

The SGs are by physiological nature a tissue designed for protein production (16). Adeno-associated virus (AAV) serotype 2 has proven to be especially useful for in vivo gene transfer to the SGs (17). Recently, we have shown that rAAV vectors elicit relatively modest host immune responses compared to adenoviral vectors. They result in long term expression in vivo and vector dissemination beyond the encapsulated SGs is limited (18, 19).

Because of the positive features offered by rAAV-mediated gene transfer, we hypothesized that an rAAV encoding sTNFR could be useful to modulate inflammatory reactions locally in salivary glands of patients with Sjögren's syndrome. Therefore, we investigated the ability of in vivo immunomodulatory gene transfer with rAAVstNFR p55 to alter the immunopathological and functional changes of autoimmune sialadenitis in the NOD mouse model of SS.
MATERIALS AND METHODS

Cell lines
293 T and COS cells were grown in DMEM. All media were supplemented with 10% heat-inactivated (55°C; 30 min) fetal bovine serum (Life Technologies, Rockville, MD), 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) (Biofluids, Rockville, MD) as previously described (20).

Construction of plasmids and AAV vectors
For construction of pAAV2sTNFRp55 plasmid, a PAC plasmid containing the chimeric construct encoding the human 55kDa TNF receptor extracellular domain linked to a mouse IgG heavy chain, was used (kindly provided by Dr J. Kolls) (21). The cassette containing a CMV promoter and the stFRp55 chimeric construct was excised from the PAC vector and cloned into pAAV. The preparation of rAAVhIL10, rAAVLacZ (encoding β-galactosidase (22)) and rAAVsTNFRp55 was done as previously reported (20). The titer of DNA physical particles in rAAV stocks was determined by real-time PCR as described (20). Infectivity was demonstrated by infecting COS cells with 2 μl of each CsCl fraction in the presence of 2.4 x 10⁶ particles of wild type adenovirus. As appropriate, supernatants from infected cells were analyzed by an ELISA for hIL10 or sTNFRI (p55) (see below) expression, or cells were stained for β-galactosidase activity with X-gal (22).

Animals
Female NOD/LtJ mice (stock # 001976) used in this study were obtained from The Jackson Laboratory (Bar Harbor, ME), and were maintained in the animal facilities of the National Institute of Dental and Craniofacial Research (NIDCR). Animal protocols were approved by NIDCR Animal Care and Use Committee and the National Institutes of Health (NIH) Biosafety Committee. Blood glucose levels were measured. Mice with blood glucose levels > 400mg/dl were treated by subcutaneous injection with long acting Ultralente insulin (4U/24h) to limit diabetes related dehydration, as described (20).

rAAV vector administration, saliva and serum collection
Vectors were delivered into submandibular glands by retrograde ductal administration as previously described (23,24). Briefly, 8 weeks old female NOD mice were anesthetized with ketamine (60 mg/mL, 1 μL/g body weight; Phoenix Scientific, St. Joseph, MO) and xylazine (8 mg/ml; Phoenix Scientific) intramuscularly (IM) and then injected with atropine (0.5 mg/kg body weight, IM; Sigma, St. Louis, MO). Ten minutes later 50 μL of an rAAV vector (10¹⁵ viral particles in isotonic saline) was administered to both submandibular glands by retrograde ductal instillation using a thin cannula. Eight weeks after injection the mice were anesthetized to collect saliva by subcutaneous injection with pilocarpine (0.5mg/ kg body weight). Whole saliva was gravimetrically collected with a capillary tube as previously described (23,24). Blood was collected from the animals by retro-orbital plexus bleeding, from which serum was separated by centrifugation.
Submandibular glands were carefully dissected from the animals, and were treated appropriately as described below.

**Histologic assessment of submandibular glands**

Submandibular glands were removed for histologic analyses from NOD mice at the time of sacrifice, 16 wk of age, and formalin fixed according to standard procedures. Sections were cut at 5 μm thickness, and subsequently stained with hematoxylin and eosin. Histopathologic scoring was performed as previously described (20). Briefly, the sections were evaluated using a focus score (25). The total number of foci was counted in at least three different areas of three sections (total of 9 fields) from each gland sample using a graticule at ×40 magnification. The results were calculated and expressed as foci per 4 mm². Two different examiners assessed the focus scores blindly. The results of scores assigned were determined by taking the mean of the two observers for each score.

**Detection of transgene products and cytokines**

Secretion of hIL10 and sTNFRp55 in cell culture media, and in mouse sera and saliva, were determined by ELISA. Commercial ELISA kits for hIL10 and sTNFRp55 (R&D Systems, Minneapolis, MN, and Biosource International, Camarillo, CA) were used. The lower limit of detection was 5 pg/ml for hIL10 with in vitro experiments, 5 pg/ml for sTNFRp55 with in vivo experiments, and 0.2 pg/ml using a high sensitivity assay for hIL10 with in vivo experiments. Assays were performed according to the manufacturer’s instructions. The levels of several cytokines were also determined after extraction of soluble protein from murine salivary glands. After measuring the wet weight, salivary glands were homogenized in buffer (PBS and Complete proteinase cocktail, Roche, Indianapolis, IN). Thereafter, crude extracts were centrifuged at ~325x g and total protein determined in the supernatant using the Bio-Rad protein assay (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. Mouse (m) IL2, mIL4, mIL6, mIL10, mIL12p70, mINFγ and mTNF-α were measured commercially by Pierce Biotechnology (Woburn, MA) using SearchLight Proteome Arrays, which are multiplexed assays involving a sandwich ELISA procedure. Detection limits for this assay are: mIL2 and mIL4: 0.8 pg/ml, mIL6: 5.5 pg/ml, mIL10: 0.8 pg/ml, IL12: 0.8 pg/ml, INFγ: 7.8 pg/ml, TNFα: 3.1 pg/ml. n = 5 for all groups.

**Statistical analysis**

Descriptive statistics were calculated and reported as mean ± SEM. Student’s t-test for unpaired variables was used to compare differences between groups. P values ≤ 0.05 were considered significant.
RESULTS

rAAV vector characteristics
Peak fractions were pooled following measurement of hIL10 and sTNFRp55 secretion, from, or LacZ expression in, COS cells co-infected with wild type adenovirus. All experiments reported herein were performed with a single preparation of rAAV2 of each transgene. Both the rAAVhIL10 and the rAAVsTNFRp55 transgene products, hIL10 and sTNFRp55, were functional in vitro and in vivo (20,23,26,27).

Successful transgene expression after retrograde ductal delivery of rAAV
Vectors were delivered locally to the SGs via cannulated ducts with retrograde infusion. NOD mice received rAAVsTNFRp55 as active treatment under investigation. Control groups received either saline, rAAV encoding β-galactosidase (rAAVLacZ) serving as negative control, or rAAVhIL10 serving as positive control (n = 7 for all groups). Systemic transgene expression was determined measuring serum and saliva protein expression of either hIL10 or sTNFRp55. At 16 weeks the average serum level in rAAVsTNFRp55 treated mice was (mean ± SEM) 3.75 ± 1.55 pg/ml. The salivary sTNFRp55 in these mice was 2.78 ± 1.17 pg/ml. The average serum level in rAAVhIL10 treated mice was 0.9 ± 0.78 pg/ml of detectable hIL10, while salivary hIL10 levels were 0.64 ± 0.34 pg/ml. No sTNFRp55 or hIL10 could be measured in saline or rAAVLacZ treated animals.

Increased salivary flow after sTNFRp55 vector delivery
Pilocarpine-stimulated salivary flow rates were measured at 8 and 16 wk. After the second measurement we calculated the percent change in salivary flow for each group. In the group administered rAAVsTNFRp55 the average salivary flow rate change was

![Bar graph showing percent change in salivary flow rate with error bars](image)

**Fig. 1. Effect of rAAV2 vector administration on salivary flow**
Whole saliva was collected over a 20 min time course, after stimulation of secretion using 0.5 mg pilocarpine/kg body weight administered subcutaneously. Saliva was obtained from the oral cavity by micropipette, placed into 0.5-ml microcentrifuge tubes, and volume determined gravimetrically, as described in Materials and Methods. The data are the mean ± SEM of 5 determinations and expressed as the percent change in salivary flow rate between baseline (8 weeks) and endpoint (16 weeks).
Fig. 2. Effect of rAAV-TNFp55 administration on the lymphocytic infiltration of submandibular glands of NOD mice. Mice received treatment as described. Glands were removed for histopathologic assessment at the time of sacrifice (16 weeks). Histopathologic assessment was performed and presented as a focus score. (see Materials and Methods). Arrows indicate foci in this gland. Section are stained with hematoxylin and eosin original and displayed at a 40X magnification. (A) Foci were more frequently seen in the glands of NOD mice administered either saline or rAAVLacZ. (B) Section from a submandibular gland of a NOD mouse treated with rAAV-TNFp55. A single focus of lymphocytes is present in this section (arrow).
46±16 μl/20min, an increase of ~40%. In the group that was administered rAAVhIL10, the average salivary flow rate was 37±14, an increase of ~32%. The mice receiving either rAAVLacZ or saline by retrograde ductal delivery to the SG had a decline in salivary flow change of -12±10 (~8%) and -20±14 (~13%) (Fig. 1). Salivary flow rates of animals receiving rAAVsTNFRp55 were significantly increased in comparison to those measured both in the rAAVLacZ-treated mice and saline injected animals. Mice receiving rAAVhIL10 had significantly increased flow rates compared to rAAVLacZ-treated mice (p=0.045). Importantly, we did not observe any significant differences in salivary flow rate between rAAVLacZ-treated or saline treated animals. Similar to our previous studies we could not detect any relationship between blood glucose levels and salivary flow rate in this study (20) (data not shown).

**Reduced salivary gland cell infiltrates after rAAVsTNFRp55 delivery**

As reported by Hunger et al, the presence of TNF-α mRNA in the SGs of female NOD mice appears essential to the presence of inflammatory cells within the glands (10). Sections from the submandibular glands of rAAVsTNFRp55, rAAVhIL10, rAAVLacZ and saline-treated NOD mice sacrificed at 16 wk were examined histologically for inflammatory infiltrates. Typical signs of autoimmune sialadenitis, similar to that seen in human SS are frequently observed in female NOD mice ≥12 wk old and at ~16 wk of age, inflammatory cells are widely present in the SGs of female NOD mice (2,9). The clinically developed focus score was used to determine the severity of salivary gland inflammation (25) (Fig. 2). The number of foci present in salivary glands of mice treated with either rAAVsTNFRp55 or rAAVhIL10 was clearly reduced compared with submandibular glands from animals administered rAAVLacZ or saline (Fig. 3).

### Fig. 3. Effect of rAAVsTNFRp55 administration on focus scores.

Histopathologic scoring was performed by using the focus score (y-axis: focus, an aggregate of 50 or more lymphocytes and histiocytes, per 4 mm²; see description in Materials and Methods). Two different examiners assigned focus scores after blindly reviewing at least nine different fields from each gland. Each bar represents the mean score of 7 mice. Significant differences are indicated and were determined by the Student t-test.

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Salivary gland cytokine expression of cell infiltrates after rAAVsTNFRp55 delivery

To better understand the possible pathophysiological changes in the SGs after rAAVsTNFRp55 delivery, we determined local cytokine expression in aqueous extracts of SGs. We could not detect mIL2, mIL4 and mIL6 present. No significant difference was observed for the levels of mIL10, mIL12, and mINF-γ. However, mTNF-α in the rAVTNFRp55 treated animals was significantly lower (p<0.05) compared to saline treated mice (Fig. 4).

![Graphs showing cytokine expression](image_url)

**Fig. 4. Effect of rAAVsTNFRp55 administration on cytokine expression in submandibular glands of NOD mice.** NOD mice received treatments as described in Materials and Methods. Submandibular glands were removed at age 16 weeks. Protein was obtained as described in Materials and Methods and individual cytokines were measured commercially in SearchLight proteome arrays (Endogen), multiplexed assays involving a sandwich ELISA procedure. Data are shown as picograms per milligram wet weight of the submandibular gland and represent mean values ±SEM, n=5 for all groups. Mouse (m)IL2, mIL4 and mIL6 were below detection level (data not shown). No significant difference was observed for mIL10, mIL12 and mINFγ levels. However mTNF-α in the TNFRp55 treated animals showed a lower value compared to saline treated animals. Detection limits are stated in the Material and Method section.

**DISCUSSION**

In the present study, we examined the effect of rAAV serotype 2 mediated transfer of sTNFRp55 to the SGs on salivary flow and sialadenitis in the NOD mouse model of SS. To investigate this, we created a novel rAAV2 construct containing the chimeric construct sTNFRp55. This construct containing the human extracellular domain of the TNFRp55 was extensively tested, both in vitro and in mice (21,26,27).

Previously, we have shown that the SGs are capable of extended expression of biologically and therapeutically active proteins with minimal dissemination of vector beyond the gland (18,20,23). We hypothesized that local administration of a rAAV vector encoding sTNFRp55 to the SGs might be useful in the treatment of SS-like disease in the NOD mouse model.
The presence of TNF-α in the SGs of both SS patients and animal models for SS, and TNF receptors both in mononuclear cells, endothelial cells and SG ductal cells, is well established (10, 28-30). TNF-α can induce the release of pro-inflammatory cytokines, upregulate MHC molecules, cause direct cytotoxicity and enhance the expression of chemokines and adhesion molecules like ICAM-1 and VCAM-1 in numerous tissues (10). VCAM-1 staining within focal aggregates of lymphoid cells has been demonstrated in the SGs of SS patients (31). In the NOD mice, the cells expressing TNF-α, mainly located in the mononuclear gland infiltrates, are not observed in non-affected glands (10). The diminished presence of mononuclear cell infiltrates observed in NOD mice transgenic for a soluble TNF receptor p55 fused to the Fc part of human IgG3 suggest an instrumental role of TNF-α in the development of submandibular gland infiltration (10).

The NOD mice not only exhibit mononuclear cell infiltrates, but also, as shown by our and other groups, a reduction in salivary flow rates in NOD mice starting between 8 and 12 weeks of age (9). In the present study, there was significant difference in salivary flow changes at 16 weeks between animals treated with rAAVsTNFRp55 (or rAAVhIL10) and animals receiving either rAAVLacZ or saline. These results are comparable to our earlier findings, in which we have shown that long-term (20 weeks) salivary function is significantly higher in NOD mice after rAAVhIL10 delivery directly to the submandibular glands (20). Importantly, there was no difference in salivary flow between rAAVLacZ and saline treated mice, suggesting murine SG function is not affected by rAAV vectors and β-galactosidase protein.

In addition to the increased salivary flow in rAAVsTNFRp55 treated animals, histologic assessment of SGs using the focus score revealed a significant decrease in lymphocyte infiltrates in these and rAAVhIL10 treated animals at 16 weeks, compared to rAAVLacZ or saline treated animals (Fig. 2). Moreover, there was no evidence of an additional inflammatory effect after administration of rAAVLacZ, since there was no difference in lymphocyte infiltrate between groups treated with rAAVLacZ versus saline. These findings suggest that rAAV2sTNFRp55 can act as a disease modifying treatment when administered locally in the SGs (Fig. 3).

In order to get a better insight into the possible pathophysiological processes altered by treatment with rAAV2sTNFRp55, we evaluated SG expression of cytokines. Since TNF-α can induce the production of pro-inflammatory cytokines and adhesion molecules, we hypothesized blocking TNF-α could lead to lower concentrations of these cytokines. Most of the cytokines measured, both pro and anti-inflammatory, were either expressed below detection level or showed no significant difference between the test groups. Thus, the observed beneficial effects are not due to simple local alterations of cytokine profiles. Mouse TNF-α cytokine expression however, was significantly lower in SG extracts of the rAAVTNFRp55 treated animals (Fig. 4). This could not only be the
result of successful soluble TNF receptor binding, but it could also be due to decreased salivary gland TNF-α synthesis (32).

Finally, a possible alternative explanation of the successful improvement of salivary function and lymphocytic infiltration seen here after local rAAVsTNFRp55 delivery could be the soluble receptor itself. In human studies, the soluble TNF receptor p75 (Etanercept) was used, and it had no beneficial clinical effects (12,14).

In summary, these results shown herein suggest a potentially beneficial role for local TNFRp55 gene transfer to SGs., although further investigation is needed to clarify the pathophysiological mechanisms involved.

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REFERENCES


Disease modifying effects of TNF-α receptor in SS


