Local immunomodulatory gene therapy for Sjögren’s syndrome
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Local Delivery of IκBα Super Repressor cDNA Leads to Disease-Modifying Effects in the NOD Mouse Model for Sjögren’s Syndrome

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Abstract

OBJECTIVE: Sjögren’s syndrome (SS) is an autoimmune exocrinopathy of unknown etiology, mainly affecting lacrimal and salivary glands. This results in ocular and oral dryness for which currently only palliative treatment is available. NF-κB, a group of inducible dimeric transcription factors, is expressed in all cell types. Following stimulation and degradation of its inhibitory protein IκB, cytoplasmic NF-κB is translocated to the nucleus, where it plays an important regulatory role in the cellular response to inflammatory processes. An IκBα mutant that renders the inhibitor a super repressor (IκBα(sr)) is resistant to immediate degradation upon stimulation, preventing NF-κB activation. Local gene transfer of IκBα(sr) is potentially useful in management of sialadenitis in SS. METHODS: A recombinant serotype 2 adeno-associated virus encoding the human IκBα(sr) transgene (rAAV2IκBα(sr)) was constructed and its efficacy tested in the female non-obese diabetic (NOD) mouse model for SS following retrograde instillation in salivary glands. $10^{10}$ particles/gland of rAAV2IκBα(sr) or saline (control) were administered at 8 weeks of age (before sialadenitis onset). Salivary flow rates were determined before vector delivery and at time of sacrifice (16 weeks). After sacrifice, saliva and salivary glands were harvested. Analyses of salivary output, inflammatory infiltrates (focus score), and salivary gland cytokine profile were performed. RESULTS: rAAV2IκBα(sr) led to significantly improved salivary flow rate and reduction of salivary gland cytokines IL-2, IL-10, IL-12(p70), TNF-α, and RANTES, compared to control. There was no difference in focus score. CONCLUSIONS: Local rAAV2IκBα(sr) delivery can have disease-modifying and immunosuppressive effects in salivary glands of the NOD mouse model. This novel prophylactic strategy may be useful for both understanding and managing the salivary component of SS.
Introduction

Sjögren’s syndrome (SS) is a chronic autoimmune disease with local and systemic features. The lacrimal and salivary glands are mainly characterized by a progressive mononuclear cell infiltration, resulting in ocular and oral dryness (keratoconjunctivitis sicca and xerostomia). Apoptosis-resistant CD4+ T cells, and to a lesser extent CD8+ T cells, B cells and macrophages, and secreted pro-inflammatory cytokines result in inflammatory infiltrates, acinar atrophy and destruction. The etiology and exact pathogenesis are largely unknown and currently only palliative treatment is available for SS patients.

Nuclear factor κB (NF-κB), a group of inducible dimeric transcription factors, is expressed in virtually all cell types. Upon stimulation, inactive NF-κB—normally sequestered in the cytoplasm—is translocated to the nucleus after degradation of its inhibitory protein IkB. In the nucleus, it plays a pivotal regulatory role in the expression of multiple genes important in inflammatory and immune responses. Chronic inflammatory sites, like salivary and lacrimal glands of SS patients, are characterized by local expression of pro-inflammatory cytokines (e.g., interleukin-1 [IL-1] and tumor necrosis factor-α [TNF-α]). This pro-inflammatory response is regulated by the classical NF-κB pathway, which leads to translocation of the NF-κB dimer p65-p50. p65-p50 in turn activates transcription of pro-inflammatory cytokines, adhesion molecules, chemokines, growth factors, anti-apoptotic genes, and other proteins (e.g., cyclooxygenase-2 [COX-2], inducible nitric oxide synthase [iNOS], matrix metalloproteinase 9 [MMP-9]) involved in inflammation. The NF-κB inhibitor α, IkBα super repressor (IkBα(sr)), generated by serine-to-alanine mutations of residues 32 and 36, is resistant to immediate degradation preventing NF-κB’s movement into the nucleus and subsequent transcription.

To prevent undesirable and uncontrollable systemic NF-κB inhibition, local modulation, such as may be provided by tissue-directed gene transfer, is preferable to more generalized regulation. Indeed, salivary glands provide an excellent, highly localized target site following retrograde ductal infusion of vectors with minimal vector dissemination. The small, single-stranded DNA, non-pathogenic adeno-associated virus (AAV) has shown considerable promise as a viral vector for gene therapy. Recombinant serotype 2—the most widely used serotype—AAV (rAAV2s) vectors are capable of infecting numerous dividing, as well as non-dividing, mammalian cells and elicit only a minimal immune response. Our previous studies demonstrated that immunomodulatory transgenes, such as human IL-10 and vasoactive intestinal peptide (VIP), upon delivery to the submandibular glands of the non-obese diabetic (NOD) mouse can have disease-modifying effects. While the NOD mouse is regularly em-
employed to study type I, insulin-dependent diabetes mellitus, it also develops age and gender dependent exocrine gland infiltrates and decreased glandular secretion and is currently the most commonly used animal model to study SS.\textsuperscript{2,14,15}

In the present study, we have constructed the vector rAAV2IκBα(sr) and tested its ability to alter the progressive SS-like dysfunction in female NOD mice after local salivary gland delivery before disease-onset. Inhibition of the NF-κB pathway resulted not only in the reduction of several pro-inflammatory cytokines in salivary glands, but also in significantly improved salivary flow rate.

**Materials and Methods**

*Reagents and cell lines*

pNF-κB-luc (Clontech, Palo Alto, CA, USA), a firefly luciferase reporter gene coupled to a four-tandem repeat of the NF-κB consensus sequence (fused to a herpes simplex virus thymidine kinase promoter region), and ph-RL-TK (Promega, Madison, WI, USA), containing the Renilla luciferase gene, were used to transfect cells. Recombinant mouse TNF-α protein was purchased from Sigma (St. Louis, MO, USA). For immunoblotting, mouse T7-Tag Monoclonal Antibody (Novagen, San Diego, CA, USA) and anti-mouse immunoglobulin G (IgG) were used. Measurement of IκB degradation was performed with primary rabbit anti-mouse IκBα antibody (Cell Signaling Technology, Danvers, MA, USA) and primary anti-mouse vinculin antibody (Bio-Rad, Hercules, CA, USA) and secondary goat anti-rabbit HRP antibody (Bio-Rad). NF-κB translocation was determined using primary rabbit anti-NF-κB p65 (C-20) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The cell lines used were human embryonic kidney (HEK) 293 T cells expressing the simian virus 40 (SV40) large T antigen in a stable manner\textsuperscript{16} (ATCC, Manassas, VA, USA), Naut cells, a HEK 293 cell-line specifically selected for high production of rAAV vectors (Microbix Biosystems Inc., Toronto, Canada), and a murine myoblast cell line (C2C12 cells). C2C12 cells were grown in high-glucose Dulbecco’s Modified Eagle’s Medium (DMEM), and 293 T cells and Naut cells in low-glucose DMEM. All media were supplemented with 10% 55ºC heat-inactivated fetal bovine serum (Life Technologies, Rockville, MD, USA), 2 mM glutamine, penicillin (100 U/mL), and streptomycin (100 mg/mL; Biofluids, Rockville, MD, USA), unless otherwise noted.

*Construction of rAAV2IκBα(sr)*

The EcoRI/HindIII CMV promotor–Gp10-tagged IκBα(sr) fragment from expression vector pEVRF\textsuperscript{5} was subcloned by blunt/sticky ligation into the recombinant AAV2 DNA plasmid pDT1.1 (containing the ampicillin resistance gene; described as pAAV-MCS2.7 in Braddon et al.)\textsuperscript{17} after digestion with enzymes KpnI (blunt)
and HindIII. This resulted in pAAV2CMVκBα(sr) (6846 bp; 2650 bp between inverted terminal repeats, ITR’s; Figure 1). The correct construct was verified by restriction digests and sequencing, and the presence of the ITR’s was confirmed by a Smal digest (not shown). rAAV2κBα(sr) was generated and the particle titer determined according to procedures previously described.\textsuperscript{8,9,13}

\textbf{Figure 1. DNA plasmid map of pAAV2CMVκBα(sr)}

Gp10-tagged κBα(sr) from pEVRF was subcloned into the recombinant AAV2 DNA plasmid pDT1.1 (containing the ampicillin resistance gene), resulting in pAAV2CMVκBα(sr) (6846 bp; 2650 bp between inverted terminal repeats, ITRs). For further details refer to Materials and Methods.

\textit{mRNA analysis of transgene expression by RT-PCR}

293 T cells on 6-well plates were transduced with rAAV2κBα(sr) in the presence of wild-type adenovirus. After 24 hours of incubation, cells were harvested. mRNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and treated with Deoxyribonuclease I (Invitrogen), and cDNA was synthesized with the ThermoScript RT-PCR System (Invitrogen), all according to manufacturers’ instructions. PCR, using Platinum Blue PCR SuperMix (Invitrogen) and a thermocycler (MJ Research, Waltham, MA, USA), was performed as follows: denaturing at 94°C for 30 s, extension—at different temperatures depending on primers—for 30 s, and annealing at 72°C for 1 minute for 35 cycles. The primers used were GAPDH primers (Stratagene, La Jolla, CA, USA; 1 μM each; extension at 60°C) and κBα(sr) specific primers (sense: 5’-GGATCTATGGCTAGCATGAC-3’ and antisense: 3’-AGTAGCGCGCTCTTTCTTCAG-5’; 1.5 μM each; extension at 52°C).
Evaluation of immunomodulatory activity of rAAV2IkBα(sr)

Immunomodulatory effects elicited by rAAV2IkBα(sr) were determined in vitro by assessing inhibition of NF-κB activation. C2C12 cells (2x10^5 cells/well) in 24-well plates were transduced with 2 µl of CsCl gradient fractions containing rAAV2IkBα(sr). The next day, all cells were transfected with the plasmids pNF-κB-luc (0.2 µg; Biosciences Clontech, Palo Alto, CA, USA) and ph-RL-TK (0.05 µg; Promega) using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA), according to the manufacturer’s instructions. Plasmids were mixed with transfection reagent and serum free medium without antibiotics and added to the cell cultures. After five hours of incubation, additional serum containing medium was added. The following day all cells, except for the unstimulated medium control well, were stimulated with 20 ng/mL mouse TNF-α for five hours. The Dual Reporter Luciferase assay system (Promega) was used, according to the manufacturer’s recommendations, to measure luciferase activity in the cell lysates with a luminometer. The ratio of firefly to Renilla luciferase activity in the unstimulated medium control cells was calculated to correct for transfection efficiency. Fold induction of firefly luciferase expression in stimulated control cells was set at 100%. The percentage fold induction in cells transduced with rAAV2IkBα(sr) was compared to this value.

Transgene expression analysis by immunoblotting

Infectious vectors were demonstrated by transducing 2x10^6 293 T cells on 6-well plates with 2 µl from each CsCl gradient fraction in the presence of 2.4x10^8 particles of wild-type adenovirus. After 24 hours of incubation, cells were harvested and lysed with ELB buffer (50 mM Tris-HCl [pH 7.5], 300 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol). Protein extracts were resolved with SDS-PAGE 10% gel (Invitrogen, Carlsbad, CA, USA) and transferred to Immobilon-P membrane (Millipore Billerica, MA, USA), as previously described. Briefly, the membrane was blotted with antibodies (dilution 1:10,000), after which reactive bands were detected with ECL reagents (Amersham Biosciences, Piscataway, NJ, USA).

Mice, gene transfer, and saliva, serum and salivary gland collection

Animal studies were approved by the National Institute of Dental and Craniofacial Research (NIDCR) Animal Care and Use Committee and the National Institutes of Health (NIH) Biosafety Committee. All procedures were conducted in accordance with IASP (International Association for the Study of Pain) standards. Female NOD/LtJ mice (stock 001976) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and were maintained throughout the course of the study in the NIDCR animal facility (Bethesda, MD, USA) in accordance with Federal guidelines. Starting at age 10 weeks, body weights were measured weekly, as well as blood glucose levels (obtained by tail cut), using a OneTouch
monitor (LifeScan, Milpitas, CA, USA). All mice with blood glucose levels ≥400 mg/dL were given Ultralente insulin (Eli Lilly, Indianapolis, IN, USA) injections subcutaneously (5 U/mouse, every 24 hours) to limit diabetes-related dehydration, as described. The experiments described in this article represent two separate studies conducted to investigate the effects of rAAV2IκBα(sr) administration on salivary gland function in NOD mice. In the first study we tested two vector doses (10^9 and 10^10 particles/gland, 7 animals/group); for the second study, we instilled only 10^10 particles in each gland (8 animals/group), a dose that was effective in the first study. The experiments described herein report the 10^10 particles/gland data from the two separate studies, over the indicated 8- to 16-week time course. Both studies gave comparable results. Vector delivery by retrograde ductal instillation, saliva collection and body weight measurement (at 8 weeks of age), and saliva and salivary gland collection (16 weeks) were performed as described in previous reports from our laboratory.

Soluble protein was extracted from parts of the salivary glands, as previously described. Briefly, immediately after sacrifice, salivary glands were snap-frozen, wet weight was measured and the glands were homogenized in ice-cold buffer (phosphate-buffered saline [PBS] and complete protease inhibitor cocktail; Roche, Molecular Biochemical, Indianapolis, IN, USA) on ice. Thereafter, homogenates were centrifuged at 1,500 x g for 15 minutes at 4ºC and amount of total protein in the supernatants was determined with a Bio-Rad protein assay according to the manufacturer’s instructions. NF-κB p65 levels were determined in aqueous salivary gland protein extracts with an ELISA (Active Motif, Carlsbad, CA, USA), according to manufacturer’s instructions.

Histological assessment salivary glands
Salivary glands were examined histologically and the number of foci present (one focus is an aggregate of 50 or more lymphocytes or histiocytes per 4 mm²) was counted, as previously described. The scoring was done blindly by three examiners (BML, FM, APC) and the mean of all focus scores per animal was calculated.

Quantification of cytokines
Levels of IL-2, IL-4, IL-6, IL-10, IL-12(p70), interferon-γ (IFN-γ), TNF-α, and RANTES in salivary gland protein extracts were measured commercially for animals in experiment two with SearchLight proteome arrays (Pierce Biotechnology, Woburn, MA, USA), which are multiplexed assays involving a sandwich ELISA procedure, as previously described.

IkBα degradation
Based on the first two experiments, intracellular effects of IkBα(sr) gene transfer were assessed in an additional group of NOD mice: IkBα degradation was
NF-κB translocation
Inhibition of NF-κB translocation by IκBα(sr) in salivary glands of 16-week-old NOD mice was determined with immunofluorescence. Frozen salivary gland sections (8 µm thick) were mounted on slides, fixed and rehydrated. Sections were blocked with 10% horse serum in PBS for 30 minutes and incubated with primary anti-NF-κB antibody (dilution 1:50) at 4ºC overnight. Next day, sections were washed in 10% horse serum in PBS and incubated in secondary anti-rabbit antibody for 1 hour. Then, slides were washed in 10% normal human serum in PBS, incubated with DAPI (Molecular Probes, Carlsbad, CA, USA) for 15 s and mounted using Prolong Gold anti-fade medium (Molecular Probes). Tissue sections were analyzed using a Nikon Microphot FXA microscope (Melville, NY, USA) and NF-κB translocation was determined semi-quantitatively by counting NF-κB positive-stained nuclei compared to total number of DAPI-positive nuclei.

Statistical analysis
Data analysis consisted of descriptive statistics, reported as means ± SEM or medians, unpaired Student’s t tests and Mann-Whitney Rank Sum Tests. p-Values less than or equal to 0.05 were considered statistically significant.

Results
In vitro expression and function of rAAV2IκBα(sr)
The highest particle titers of rAAV2IκBα(sr) were typically found in fractions with a refractive index of ~1.372, equivalent to a buoyant density of 1.4 g/mL, and corresponded to 2.7x10^{12} particles/mL. Expression of IκBα(sr) was detected by immunoblotting (Figure 2A) after transduction of 293 T cells with rAAV2IκBα(sr) and by RT-PCR (Figure 2B). Next, we examined if transgenic IκBα(sr) possessed immunomodulatory activity. C2C12 cells were transduced with measured in extracts from salivary glands obtained at 16 weeks of age. Protein extracts were resolved with NuPAGE Novex Bis-Tris 10% gel (Invitrogen) and transferred to a Transblot nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% non-fat dry milk in TTBS for 1 hour, incubated with primary anti-mouse IκBα antibody (dilution 1:1,000) at 4ºC overnight and incubated with secondary anti-mouse vinculin antibody (dilution 1:25,000; internal loading control) at 4ºC overnight. Next day, the membrane was washed with TTBS, incubated with goat anti-rabbit HRP antibody (dilution 1:3,000) for 1 hour and washed again, after which reactive bands were detected with ECL reagents (Amersham Biosciences). Ratios of IκBα and vinculin densities in treated versus untreated mice samples were calculated using Quantity One software and GS 800 Calibrated Densitometer (Bio-Rad).
Figure 2. Detection of *in vitro* transgene expression and function
A. Immunoblotting (1) Gp10-tagged IkBa(sr) expressed by rAAV2IkBa(sr) (multiplicity of infection [MOI] 4.1x10^3) after transduction of 293 T cells in the presence of wild-type adenovirus (MOI 1.2x10^3; 45 kDa band). (2) Negative control represents identical amount of 293 T cells infected with wild-type adenovirus alone. (3) T7-Tag positive Control Extract (containing a 31.1 kDa target fusion protein, prepared from an E.coli λDE3 lysogen carrying a pSCREEN™-1b(+) recombinant; Novagen). This is representative of two experiments.

B. RT-PCR (I) GAPDH primers (arrow; 600 bp PCR product) and (II) IkBSR primers (arrow; 149 bp PCR product) were used to demonstrate presence of mRNA. (1) Transduction of 293 T cells with rAAV2IkBa(sr) (MOI 2.7x10^3), in the presence of wild-type adenovirus (MOI 1.2x10^2). (2) Negative control represents identical amount of 293 T cells infected with wild-type adenovirus alone. (3) Positive control: cDNA from HEK 293 cells (I) or 100 ng pAAV2CMVIκBa(sr) (II). (4) Non-template control. This is representative of two experiments.

C. Effect of IkBa(sr) transgene product on intracellular NF-κB activation C2C12 cells in 24-wells plates were transduced with rAAV2IkBa(sr) (MOI 5.4x10^4). After 24 hours cells were transfected with pNF-κB-luc and ph-RL-TK. The following day, cells were stimulated with 20 ng/mL mouse TNF-α for five hours. Luciferase activity in the cell lysates was determined with a luciferase assay. The ratio of firefly to Renilla luciferase activity was calculated to correct for transfection efficiency. Fold induction of luciferase expression in stimulated control cells was set at 100%. NF-κB activation by IkBa(sr) transgene product is shown. Experiments were analyzed in duplicate and are representative of two separate experiments. Bars represent means ± SEM. A Student’s *t* test was performed (*p* = 0.03).
rAAV2IκBα(sr) (multiplicity of infection [MOI] \(5.4 \times 10^4\)), then transiently transplanted with pNF-κB-luc and ph-RL-TK, and stimulated with TNF-α. Stimulation of cells with TNF-α results in activated, endogenous NF-κB that binds to the κ enhancer element of the NF-κB sequence located upstream of the firefly luciferase gene in the vector, resulting in high induction levels of the luciferase reporter. Transduction of cells with rAAV2IκBα(sr) resulted in a ~32% reduction in NF-κB activation compared to control \((p = 0.03; \text{Figure 2C})\).

In vivo experiments
Since NF-κB plays a central role in the inflammatory process we decided to investigate the effects of its inhibition on salivary gland damage and dysfunction in a mouse model of SS. As noted above, an initial study was conducted using this construct with comparable results. For that study, we tested two rAAV2IκBα(sr) vector doses \((10^9 \text{ and } 10^{10} \text{ particles})\) per submandibular gland (both glands targeted), with the latter dose yielding the best results. For the second study, we instilled \(10^{10} \text{ particles}\) in each gland. One diabetic mouse treated with rAAV2IκBα(sr) died during the first study at 15 weeks of age and was not included. At the time of sacrifice two other mice in the rAAV2IκBSR-treated and five mice in the saline-treated were diabetic; all were treated with insulin daily. Blood glucose levels, prevalence of diabetes mellitus, and body weights at 16 weeks of age were not different between the two groups (data not shown).

Effect of rAAV2IκBα(sr) on salivary function in NOD mice
female NOD mice show a progressive decline in salivary flow rates starting between 8 and 12 weeks of age.\(^{20}\) The effect of rAAV2IκBα(sr) on pilocarpine-stimulated salivary flow rates was next determined. We examined salivary flow before virus administration at 8 weeks of age (baseline) and at the time of sacrifice (16 weeks of age). At baseline, average salivary flow rate (microliters/body weight [grams] in 20 minutes; mean ± SEM) was \(2.70 \pm 0.55\). At 16 weeks of age, eight weeks after vector delivery, the salivary flow rates for the saline and rAAV2IκBα(sr) group were, respectively, \(2.43 \pm 0.34\) and \(4.33 \pm 0.40\) \((p = 0.001; \text{Figure 3})\).

Effect of rAAV2IκBα(sr) on inflammatory infiltrates in salivary gland extracts of NOD mice
To determine if rAAV2IκBα(sr) affected the development of focal inflammatory infiltrates in salivary glands, an important clinical feature in NOD mice and SS patients, we assessed salivary gland sections of 16-week-old treated mice, as previously described.\(^{8,13,20}\) For this, foci were counted and the mean focus score was calculated, as described in Materials and Methods. There was no statistical difference in focus scores (mean ± SEM) between the groups treated with saline and rAAV2IκBα(sr) \((1.78 \pm 0.21 \text{ versus } 1.48 \pm 0.21, p = 0.32; \text{data not shown})\).
Figure 3. Effect of rAAV2IκBα(sr) on salivary flow rate in NOD mice
After anesthesia, pilocarpine-stimulated whole saliva was collected. Salivary flow rate (microliters per gram body weight in 20 minutes) of mice at 8 weeks (n = 18; untreated mice, randomly selected from both groups) and at 16 weeks of age, either treated with saline (n = 15) or rAAV2IκBα(sr) (n = 14), is shown. Bars represent means ± SEM. Student’s t tests were performed and the p-values for the differences are indicated.

Effect of rAAV2IκBα(sr) on cytokine expression in salivary gland extracts of NOD mice
Suppression of NF-κB leads to downregulation of inflammatory responses by inhibiting the expression of pro-inflammatory cytokines. To determine if rAAV2IκBα(sr) could alter the local immune milieu, we examined protein expression of several pro- and anti-inflammatory cytokines and one chemokine in salivary glands of treated mice at 16 weeks of age. Aqueous salivary gland extracts from NOD mice treated with rAAV2IκBα(sr) showed significantly lower levels of IL-2 (p = 0.001), IL-10 (p = 0.02), IL-12(p70) (p = 0.001), TNF-α (p = 0.009), and RANTES (p = 0.003) than those from mice treated with saline (Table). IL-4, IL-6, and IFN-γ levels were not significantly different between the groups.

Assessment of rAAV2IκBα(sr) expression in salivary glands of NOD mice
Levels of IκB degradation and NF-κB translocation were established in an additional NOD mice study (n = 8) administered saline, rAAV2LacZ or rAAV2IκBα(sr) at 8 weeks. While no differences were seen between the groups (data not shown), it was evident that the overall degree of both IκB degradation and NF-κB translocation was low, even in the saline and rAAV2LacZ (both control) groups. In addition, salivary flow rates at 16 weeks were much higher in all three groups (data not shown) than observed in the two initial rAAV2IκBα(sr) studies here or previously reported.
Table. Levels of inflammatory molecules in salivary gland extracts

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<th>Cytokine</th>
<th>Saline (SEM)</th>
<th>IkBα(sr) (SEM)</th>
<th>p-Value</th>
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<td>0.02 (0.01)</td>
<td>0.001</td>
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<td>IL-4*</td>
<td>0.00</td>
<td>0.00</td>
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<tr>
<td>IL-6*</td>
<td>0.00</td>
<td>0.00</td>
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<td>IL-10*</td>
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<td>0.00</td>
<td>0.021</td>
</tr>
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<td>IL-12(p70)</td>
<td>0.14 (0.02)</td>
<td>0.05 (0.01)</td>
<td>0.001</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.10 (0.03)</td>
<td>0.02 (0.01)</td>
<td>0.009</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>6.67 (0.75)</td>
<td>8.17 (0.97)</td>
<td>0.254</td>
</tr>
<tr>
<td>RANTES</td>
<td>3.96 (0.46)</td>
<td>2.06 (0.29)</td>
<td>0.003</td>
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</tbody>
</table>

Discussion

This is the first report investigating the efficacy of transgenic IkBα(sr) in an animal model of SS. We characterized the rAAV vector, rAAV2IkBα(sr), in vitro and showed clearly it mediated the expression of IkBα(sr) mRNA and protein and resulted in a significant inhibition of NF-κB activity. Next, the immunomodulatory and clinical effects of rAAV2IkBα(sr) were tested in the NOD mouse model for SS, before disease onset, i.e., a prevention model. Instillation of rAAV2IkBα(sr) in salivary glands of NOD mice resulted in higher salivary flow rates, a reduction of cytokines IL-2, IL-10, IL-12(p70), TNF-α, and RANTES in salivary gland extracts, but no difference in focus scores, compared to results with saline controls. Previously, we have shown there is no difference in salivary flow rates in mice treated with saline or an irrelevant AAV vector. Additional experiments in a successive group of NOD mice did not yield the expected results: administration of saline, rAAV2LacZ, as well as rAAV2IkBα(sr) at 8 weeks of age let to high salivary flow rates and low levels of IkB degradation and NF-κB translocation at 16 weeks without significant differences. This raised questions about the extent and severity of inflammation present in these mice. In the past, 16-week-old control NOD mice had demonstrated apparent inflammatory activity as shown by low salivary flow rates, high focus scores, and high salivary gland cytokine levels. In a new study the stability of the NOD mouse’s SS phenotype was assessed (Chapter 7), however, for the current study this re-evaluation of the NOD mouse model for SS meant these two last assays could not be repeated.
Salivary flow rates were increased after rAAV2IκBα(sr) administration, but lymphocytic infiltrates (focus scores) were unaffected. We have seen similar results in our earlier study with rAAV2hVIP\textsuperscript{13} and this dissociation is not entirely unexpected. In SS patients a well-recognized incongruity exists between the reduction in salivary flow and the extent of focal lymphoid infiltration measured.\textsuperscript{23,24} Thus, in addition to destruction and atrophy of gland tissue, cytokines and/or autoantibodies (e.g., anti-muscarinic receptor antibodies) could contribute to the hypofunction of salivary epithelia observed.\textsuperscript{3,25,26}

As stated earlier, the exact pathogenesis of SS is still unidentified. Nevertheless, SS has been viewed as a local and systemic imbalance in cytokine expression: both pro- and anti-inflammatory cytokines are increased depending on disease stage and severity.\textsuperscript{3,27} In this study, saline-treated mice showed elevated levels of pro- and anti-inflammatory cytokines. Since NF-κB is involved in the transcription of many different cytokines, chemokines, intracellular and secreted proteins,\textsuperscript{28} we made a selection of cytokines to measure based on our earlier studies\textsuperscript{8,13} and current understanding of SS pathophysiology.\textsuperscript{3,27,29,30} Earlier studies have shown that mRNA levels of both pro- and anti-inflammatory cytokines (IL-1β, IL-2, IL-6, IL-10, TNF-α, IFN-γ) were increased in submandibular glands of NOD mice around 16 weeks of age.\textsuperscript{20,31,32} Here, rAAV2IκBα(sr) treatment resulted in a decrease of several pro-inflammatory cytokines (IL-2, IL-12(p70), TNF-α), an anti-inflammatory cytokine (IL-10) and a chemokine (RANTES). It is noteworthy that IL-4 levels were low or absent in previous reports, as in our study. There are no data available on cytokine protein levels in submandibular glands of NOD mice, other than our own studies, nor are data available for murine salivary gland cytokines after administration of an IκBα mutant.

Salivary glands consist of acinar cells, forming the primary saliva, and ductal cells, which primarily reabsorb NaCl but are relatively impermeable to water.\textsuperscript{10} rAAV2 vectors are thought to transduce only ductal cells, not acinar cells.\textsuperscript{9,18} In SS, lymphocytes, acinar and ductal epithelial cells secrete pro-inflammatory cytokines, together leading to inflammatory infiltrates, acinar atrophy, and destruction.\textsuperscript{3,33} We hypothesize, based on our results, that intracellular IκBα(sr) inhibits NF-κB translocation and subsequently production of pro-inflammatory cytokines in ductal cells. This decreased production affects the surrounding acinar cells and lymphocytes resulting in local immunomodulation. The validity of this hypothesis requires further experimental testing.

Conclusion
This is the first study investigating the efficacy of NF-κB inhibition in an experimental model for SS. Local administration of rAAV2IκBα(sr) to murine salivary glands led to local disease modifying and immunosuppressive effects. The data
suggest that IkBo(sr) gene transfer may be useful in managing sialadenitis in SS.

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References


