SUPPLEMENTARY INFORMATION

Materials and Methods

Cell lines

The cell lines used were human embryonic 293 HEK cells and 293 T cells, which express the simian virus 40 (SV40) large T antigen in a stable manner [59] (ATCC, Manassas, VA, USA). 293 HEK cells were grown in Improved Minimum Essential Medium, Eagle's (IMEM) and 293 T cells in Dulbecco's Modified Eagle's Medium (DMEM). All media were supplemented with 10% 55°C 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).

Construction of viral vector encoding functional hVIP

Two days after transfection, cells were harvested. Clarified cell lysates were treated with benzonase (100 U/mL lysate, incubated for 45 min at 37°C) and 0.5% sodium deoxycholate, adjusted to a refractive index of 1.372 by addition of CsCl and centrifuged at 38,000 rpm for 65 hr at 20°C. Equilibrium density gradients were fractionated and fractions with a refractive index of 1.366-1.3765 were collected and stored at 4°C. The particle titer was determined by real-time Quantitative (Q)-PCR using an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA), the SYBR Green PCR Master Mix (Applied Biosystems) and a specific 5' (0.3 μM; GATGCGGTTTTGGCAGTACATC) and 3' (0.3 μM; TGGGGTGGAGACTTGGAAATC) primer pair for the CMV promoter. The Q-PCR was performed with the following conditions: holding at 95°C for 10 min, denaturing at 95°C for 15 s, and extension and annealing at 60°C for 1 min for 40 cycles. A standard curve employing pAAV2CMVhVIP was included and duplicate samples were assayed; the detection limit of the assay was 10 vector copies.

Mice

Over the past two years, we have observed variability in many different cohorts of NOD mice purchased from The Jackson Laboratory with regard to prevalence and severity of sialadenitis and type I diabetes mellitus. More specifically, the salivary flow rate reductions and focus scores in these cohorts were not to the same extent as we have seen previously. Therefore, we compared untreated NOD mice from two different sources, i.e. Taconic (Germantown, NY, USA) and The Jackson Laboratory, as SS models. In our study, Taconic NOD mice showed no decrease in salivary flow rate, lower focus scores and decreased prevalence of type I diabetes mellitus at 20 weeks of age, compared to mice from The Jackson Laboratory. We concluded the Taconic NOD mice did not comprise a reliable SS model in which to study the efficacy of gene transfer procedures, and all studies reported here were conducted with NOD mice purchased from The Jackson Laboratory.

Gene transfer, and salivary and serum collection

Mild anesthesia was induced with a ketamine (100 mg/mL, 1 mL/kg body weight; Fort Dodge Animal Health, Fort Dodge, IA, USA) and xylazine (20 mg/mL, 0.7 mL/kg body weight; Phoenix Scientific, St. Joseph, MO, USA) solution given intramuscularly. After an intramuscular injection of atropine (0.5 mg/kg body weight; Sigma, St. Louis, MO, USA) rAAV2hVIP or rAAV2LacZ (n = 15 each) were administered to both SMGs of NOD mice by retrograde ductal instillation (10^{10} genomes per gland) at 8 weeks of age. Salivary flow rates were measured at 8 weeks (baseline, untreated, not manipulated before saliva collection) and 16 weeks of age (time of sacrifice). After induction of anesthesia and stimulation of secretion, using pilocarpine (0.5 mg/kg body weight; Sigma) administered subcutaneously [18,25], whole saliva was collected from the oral cavity with a microhematocrit capillary tube (Fisher Scientific, Hampton, NH, USA). This microcapillary tube was placed in an ice-cold pre-weighed 0.5-mL microcentrifuge tube, containing complete protease inhibitor cocktail (Roche Molecular Biochemical, Indianapolis, IN, USA) and 10 mM Ethylenediaminetetraacetic Acid (EDTA), and saliva volume was determined gravimetrically as previously described, with comparable results [18,20,25]. After sacrifice, blood was collected in ice-cold 1.1-mL Z-gel tubes (Sarstedt, Newton, NC, USA), containing complete protease inhibitor cocktail (Roche) and 3.4 mM EDTA, and kept on ice. Blood samples were centrifuged at 10,000 rpm for 5 minutes and serum was collected. All samples were stored at -80°C until analysis.

Quantification of cytokines

Cytokine levels were determined in SMGs (see text), after extraction of soluble protein, and serum. Immediately after sacrifice, SMGs were snap-frozen in 2-methyl butane on dry ice and stored at -80°C until further analysis. Wet weight was measured and the glands were homogenized in ice-cold buffer (phosphate-buffered saline [PBS] and complete protease inhibitor cocktail; Roche) on ice. Thereafter, homogenates were centrifuged at 1,500 x *g* for 15 minutes at 4°C and amount of total protein in the supernatants was determined with a Bio-Rad (Hercules, CA, USA) protein assay according to the manufacturer's instructions.

Results

Experimental study design

A time line of the studies is shown in supplementary Figure 1. Two diabetic mice treated with rAAV2LacZ died during the study and were not included. Another mouse in the LacZ group and one in the hVIP group developed diabetes during the study; both were treated with insulin daily. Blood glucose levels and body weights at 16 weeks of age were not different between the two groups (data not shown).

Supplementary Table 1. VIP levels in serum and SMG extracts.

	LacZ (SEM)	hVIP (SEM)	*p*-value
Serum‡	0.13 (0.01)	0.16 (0.01)	0.030
SMG*	0.36 (0.05)	0.49 (0.03)	0.039

VIP expression (ng/mL) in vivo after administration of rAAV2LacZ (LacZ) or rAAV2hVIP (hVIP). Mean (SEM) is shown. ‡LacZ, n = 13; hVIP, n = 15. *LacZ, n = 7; hVIP, n = 8

Supplementary Table 3. Levels of inflammatory molecules in serum

Cytokine	LacZ	hVIP	*p*-value
IL-2	23.04 (0.70)	19.80 (1.87)	0.150
IL-4†	0.00	0.00	0.189
IL-6	80.40 (33.49)	34.85 (5.74)	0.175
IL-10	7.14 (3.10)	6.11 (0.74)	0.736
IL-12(p70)	11.84 (0.73)	9.93 (0.84)	0.113
TNF-α†	0.00	0.00	1.000
IFN-γ	620.56 (84.23)	682.90 (71.23)	0.579
RANTES†	12.20	6.80	0.040

Protein expression of immunomodulatory molecules (pg/mL) in serum after administration of rAAV2LacZ (LacZ, n = 7) or rAAV2hVIP (hVIP, n = 8). Means (SEM) are shown unless otherwise noted. †Mann-Whitney Rank Sum Test with median values.

Discussion

Local pro-inflammatory cytokines, such as TNF-α, were decreased after rAA2VhVIP delivery, although randomized, blind clinical trials using systemically administered TNF-α protein antagonists [60,61] have not proven beneficial for SS. This could be due to the fact these studies featured recombinant proteins, with a limited half-life, administered i.m. distally. Local immunomodulatory gene transfer could perhaps change the outcomes.

SMG delivery is relatively non-invasive with easy access through the excretory duct orifices. SMGs are also non-critical for life organs, and they are well-encapsulated, therefore limiting undesirable viral spread. Furthermore, high systemic VIP concentrations, e.g. as seen with a VIPoma, are disadvantageous [12]. The results of the present study indeed suggest predominantly local vector effects. Although not shown herein, blood glucose levels showed no differences between the two vector treatment groups, suggesting no influence by transgenic hVIP after SMG delivery on developing diabetes in NOD mice. Importantly, none of the serum cytokines studied by us were different between both treatment groups, with the only significant change observed in serum levels of the chemokine RANTES. The significance of this latter effect is unclear at present. Additionally, we saw no differences in anti-VIP neutralizing antibody levels when comparing the sera from rAAV2LacZ- and rAAV2hVIP-treated mice.

Supplementary References

59. **DuBridge RB**, Tang P, Hsia HC, Leong PM, Miller JH, Calos MP. Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. *Mol Cell Biol* 1987;**7**:379-87.
60. **Sankar V**, Brennan MT, Kok MR, Leakan RA, Smith JA, Manny J*, et al.* Etanercept in Sjogren's syndrome: a twelve-week randomized, double-blind, placebo-controlled pilot clinical trial. *Arthritis Rheum* 2004;**50**:2240-5.
61. **Mariette X**, Ravaud P, Steinfeld S, Baron G, Goetz J, Hachulla E*, et al.* Inefficacy of infliximab in primary Sjogren's syndrome: results of the randomized, controlled Trial of Remicade in Primary Sjogren's Syndrome (TRIPSS). *Arthritis Rheum* 2004;**50**:1270-6.

Supplementary Figure Legends

Supplementary Figure 1

Study design

Two days before viral vector delivery pilocarpine-stimulated salivary flow rates were measured in 8-week old female NOD mice. On day 0, 10^{10} particles of rAAV2hVIP or rAAV2LacZ (n = 7 first study, n = 8 second study, each) were instilled in murine submandibular glands (SMGs). From age week 10-16 blood glucose levels and body weights were measured. At 16 weeks of age salivary flow rates were determined, all mice were sacrificed, and saliva, serum and SMGs were collected and stored at -80°C. Thereafter, analysis of salivary output, inflammatory infiltrates (focus scores), VIP protein expression, cytokine profile, and serum anti-VIP antibodies was performed. For a detailed description refer to text.

Supplementary Figure 2

Evaluation of serum anti-VIP antibodies in NOD mice

Pooled sera of 16-week old mice treated with rAAV2LacZ or rAAV2hVIP were incubated with standard concentrations of recombinant VIP and assessed by an ELISA, as described in Materials and Methods. The standard VIP protein was also tested in the absence of sera. The optical density (OD) of these reaction mixtures is shown. Note that there are no differences between VIP standard protein incubated with sera from both vector treatment groups. The figure represents the results from samples obtained with the second study and is representative of both studies.