Local immunomodulatory gene therapy for Sjögren’s syndrome
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CHAPTER 7

NOD Mouse Model for Sjögren’s Syndrome: Lack of Longitudinal Stability

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Abstract

OBJECTIVE: The non-obese diabetic (NOD) mouse is not only a widely used model for diabetes mellitus type I, but also for the chronic autoimmune disease Sjögren’s syndrome (SS), mainly affecting salivary and lacrimal glands. Our laboratory studies the efficacy of local recombinant serotype 2 adeno-associated viral (rAAV2) vector transfer of immunomodulatory transgenes to alter the SS-like disease in NOD mice. Data collected over a two-year period indicated a changing SS phenotype in these mice and this phenomenon was investigated. METHODS: $10^{10}$ particles rAAV2LacZ/gland were delivered to both salivary glands of NOD/LtJ mice at 8 weeks of age (before sialadenitis onset). Salivary flow rates were determined at 8 weeks and time of sacrifice. Blood glucose levels and body weights were measured weekly. After sacrifice, saliva and salivary glands were harvested. Analyses of salivary output, inflammatory infiltrates (focus score), salivary cytokine profile, body weight, and diabetes mellitus status were performed. Data from six different experimental studies over two years were analyzed and compared. RESULTS: Salivary flow rate, focus score, and salivary gland cytokines IL-2, IL-4, IL-6, IL-10, IL-12(p70), TNF-α and IFN-γ showed changes over time. There were no differences for body weight, diabetes mellitus prevalence, or blood glucose level of NOD mice. CONCLUSIONS: This retrospective report is the first to describe longitudinal variability in the NOD mouse as a model for SS. We advise other investigators to continuously monitor the SS phenotype parameters and include appropriate controls when studying this disease in NOD mice.
Introduction

The non-obese diabetic (NOD) mouse is regularly employed to study type I, insulin-dependent diabetes mellitus (IDDM).\(^1,2\) Since it also develops age and gender dependent exocrine gland infiltrates and decreased salivary glandular secretion, it is currently the most commonly used animal model to investigate the disease properties of Sjögren’s syndrome (SS).\(^3,5\)

SS is a chronic autoimmune disease mainly affecting the lacrimal and salivary glands, resulting in ocular and oral dryness (keratoconjunctivitis sicca and xerostomia).\(^6\) Apoptosis-resistant CD\(^4^+\) T cells, and to a lesser extent CD\(^8^+\) T cells, B cells and macrophages, and secreted pro-inflammatory cytokines result in inflammatory infiltrates, acinar atrophy and destruction.\(^3,7\) The etiology and exact pathogenesis are largely unknown and currently only palliative treatment is available for SS patients.

Our laboratory has focused on the use of recombinant adeno-associated virus (rAAV)-mediated gene transfer directly in the submandibular glands to alter the course of the developing sialadenitis in NOD mice. The small, single-stranded DNA, non-pathogenic AAV serotype 2 is capable of infecting numerous dividing, as well as non-dividing, mammalian cells with only a minimal host immune response.\(^8,9\) Indeed, our previous studies demonstrated that immunomodulatory transgenes, such as human interleukin-10 (hIL-10),\(^10\) vasoactive intestinal peptide (VIP),\(^11\) and NF-\(\kappa\)B inhibitor \(\alpha\) (IkB\(\alpha\); Chapter 6) upon delivery to the submandibular glands of the NOD mouse can have disease-modifying effects.

However, over time we have observed that the SS phenotype of NOD mice used in different experimental studies was altered. In this report we have analyzed and compared the changes in SS-like features in NOD/LtJ mice obtained from The Jackson Laboratory over a two-year time period. We have studied several SS parameters (salivary flow rate, inflammatory infiltrates [focus scores], and salivary gland cytokine profile) after retrograde instillation of a control vector, rAAV\(2\)LacZ, in both submandibular glands of NOD mice. In addition, we monitored the health status and incidence of IDDM in these mice. All SS parameters changed, whereas the diabetes status remained the same. The potential underlying causes involved in the observed variability of the SS model are discussed.

Materials and Methods

Construction of rAAV\(2\)LacZ

Previously, we reported construction, expression and function of rAAV\(2\)LacZ...
Variability in NOD Mouse Model for SS encoding β-galactosidase; described as rAAVRnLacZ in Chiorini et al. One virus prep was used for the first two studies and a different prep for studies III-VI (aliquoted and stored at -80°C), both prepared using well accepted standard operating procedures (e.g., see Di Pasquale, et al., Kaludov, et al. and Kata-no, et al.) by the same AAV vector core facility at the Gene Therapy and Therapeutics Branch. The titer of the second preparation used was 5.4 times higher (i.e., better) and one would expect less contaminants were administered. Thus, vector titer likely does not explain the altered SS phenotype seen in the latter studies.

Mice, gene transfer, and saliva 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; age 6-7 weeks) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and were maintained throughout the course of the study in the NIDCR animal facilities (Bethesda, MD, USA) in accordance with Federal guidelines. For studies I and II (Table 1), mice were housed in a facility that was subsequently renovated (for use in studies III-VI). Mice in study III were housed in an adjunct facility from age week 6 to 8.5 while renovations were completed. All facilities were Animal Biosafety Level 2 (ABSL-2). Mice ordered from The Jackson Laboratory were certified as pathogen-free, specifically free of mouse parvovirus and Helicobacter sp. However, our animal facility has had a confirmed contamination with both pathogens for over six years now. Otherwise, it is specific pathogen free (SPF). Mice from this company are not routinely genotyped for SS. Cages, containing up to five mice, were not overcrowded and placed on different shelf levels. Autoclaved water, autoclaved chow diet, and γ-irradiated transgenic dough diet (Bioserv, Frenchtown, NJ, USA; originally added at 12 weeks of age to facilitate alimentation because of evident hyposalivation) were supplied to the mice. The housing facilities had controlled temperature and lighting (12-hour dark-light cycles).

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 considered diabetic and were administered Ultralente insulin (Eli Lilly, Indianapolis, IN, USA) injections subcutaneously (5 U/mouse, every 24 hours) to limit diabetes-related dehydration, as described.

Six different experimental studies with group sizes ranging from 7 to 14 mice, testing several different immunomodulatory transgenes and where rAAV2LacZ
(10\textsuperscript{10} particles/gland; both salivary glands targeted) acted as a negative control, were employed over a two-year time period. Previously, we have shown that rAAV2LacZ administration or no treatment result in similar effects on the SS phenotype in NOD mice.\textsuperscript{10} Vector delivery, saliva collection and body weight measurement (at 8 weeks of age), and saliva and salivary gland collections (16 weeks) were performed, as previously described.\textsuperscript{10,11,16-18} Briefly, 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. Salivary flow rates were measured at 8 weeks (baseline, untreated, not manipulated before saliva collection) and 16 weeks of age (time of sacrifice) by the same person throughout the different experimental studies. After stimulation of secretion, using pilocarpine (0.5 mg/kg body weight; Sigma) administered subcutaneously, whole saliva was collected from the oral cavity with a microhematocrit capillary tube (Fisher Scientific, Hampton, NH, USA) for 20 minutes. This microcapillary tube was placed in a pre-weighed 0.5-mL microcentrifuge tube and saliva volume was determined. Two days later, after induction of anesthesia and an intramuscular injection of atropine (0.5 mg/kg body weight; Sigma, St. Louis, MO, USA), rAAV2LacZ was administered to both submandibular glands of NOD mice by retrograde ductal instillation (10\textsuperscript{10} genomes per gland) at 8 weeks of age. For experiment V, rAAV2LacZ was administered at 14 weeks; saliva and salivary glands were collected at 20 weeks.

\textbf{Histological assessment of inflammatory infiltrates in salivary glands}

The number of foci present (one focus is an aggregate of 50 or more lymphocytes or histiocytes per \(4 \text{ mm}^2\))\textsuperscript{19} in salivary glands were examined histologically and counted, as previously described.\textsuperscript{10,11,20} The scoring was done blindly by three examiners (BML, FM, APC) and the mean of all focus scores per animal was calculated.

\textbf{Quantification of cytokines}

Cytokine levels were determined in salivary glands after extraction of soluble protein. Immediately after sacrifice, salivary glands were snap-frozen in 2-methylbutane 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 Molecular Biochemical, Indianapolis, IN, USA) on ice. Thereafter, homogenates were centrifuged at 1,500 \(\times\) 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. Levels of IL-2, IL-4, IL-6, IL-10, IL-12(p70), interferon-\(\gamma\) (IFN-\(\gamma\)), tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), and RANTES in salivary gland protein extracts were measured commercially with SearchLight...
proteome arrays (Pierce Biotechnology, Woburn, MA, USA), which are multiplexed assays involving a sandwich ELISA procedure, as previously described.\textsuperscript{10,11}

**Statistical analysis**

Data analysis consisted of descriptive statistics, Student’s $t$ test, ANOVA, ANOVA Rank, and $\chi^2$ tests. $p$-Values less than or equal to 0.05 were considered statistically significant.

**Results**

We collected data from six different experimental studies testing different immunomodulatory transgenes packaged in rAAV2s, which were administered to salivary glands by retrograde cannulation. Here, only results of mice treated with the same negative control rAAV2LacZ are shown. Three diabetic mice died before 16 weeks of age (Table 1) and a total of nine were diabetic at that time-point. For study V, vector administration was performed at 14 weeks and mice were sacrificed, salivary flow rate was measured and salivary glands collected at 20 weeks. Therefore, data from this study were omitted from the statistical analysis.

<table>
<thead>
<tr>
<th>Study number</th>
<th>Study dates</th>
<th>Number of animals (survival rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Sept. 2003 – Nov. 2003</td>
<td>7 (100)</td>
</tr>
<tr>
<td>II</td>
<td>Nov. 2003 – Jan. 2004</td>
<td>7 (86)</td>
</tr>
<tr>
<td>III</td>
<td>June 2004 – Aug. 2004</td>
<td>8 (88)</td>
</tr>
<tr>
<td>IV</td>
<td>Sept. 2004 – Nov. 2004</td>
<td>8 (100)</td>
</tr>
<tr>
<td>V*</td>
<td>Jan. 2005 – Apr. 2005</td>
<td>7 (86)</td>
</tr>
<tr>
<td>VI</td>
<td>Apr. 2005 – June 2005</td>
<td>14 (100)</td>
</tr>
</tbody>
</table>

**Table 1. Experimental NOD mice studies using rAAV2LacZ**

Time periods of six performed studies using $10^{10}$ particles/gland of rAAV2LacZ (both salivary glands targeted). Percentage survival of total number of animals is shown in the third column [n (%)].

rAAV2LacZ administration at 14 weeks; mice sacrificed, salivary flow rate measured, and salivary glands collected at 20 weeks. In all other studies rAAV2LacZ was administered at 8 weeks; mice sacrificed, and saliva and salivary glands collected at 16 weeks.

**Salivary function in NOD mice treated with rAAV2LacZ**

Female NOD mice show a progressive decline in salivary flow rates starting between 8 and 12 weeks of age.\textsuperscript{20} We examined pilocarpine-stimulated salivary flow before virus administration at 8 weeks of age (baseline) and at time of sacri-
The salivary flow rate of rAAV2LacZ-treated NOD mice was not consistent over a two-year period (Figure 1; $p < 0.001$). The first two studies showed a characteristic low salivary flow rate at 16 weeks (Figure 1), but higher rates were seen for the later studies (III, IV and VI). In addition, the salivary flow rate change between the start and end of the studies increased from a significant deficit to little to no change in studies IV and VI (Figure 1; $p < 0.001$). Mice from study V showed a mean ± SEM salivary flow rate of $4.81 ± 0.71$ µl/gram body weight in 20 minutes at 20 weeks; there was a mean deficit in salivary flow rate change of $-0.24 ± 0.71$ compared to week 8.

Figure 1. Salivary flow rate of NOD mice treated with rAAV2LacZ
Mice were anesthetized and pilocarpine-stimulated whole saliva was collected. A. Salivary flow rate (SFR; microliters per gram body weight in 20 minutes) at 8 and 16 weeks of age. B. Change in salivary flow rate between start and end of studies. Bars represent means ± SEM. A One-Way ANOVA test was performed and $p$-value for difference between results at 16 weeks is indicated.
Variability in NOD Mouse Model for SS

**Inflammatory infiltrates in salivary gland extracts of NOD mice treated with rAAV2LacZ**

The presence of focal inflammatory infiltrates in salivary glands is an important clinical feature in SS patients and NOD mice. Therefore, we assessed the focus scores of salivary gland sections of NOD mice, as described in Materials and Methods. Mean focus scores of mice treated with rAAV2LacZ were significantly different between the six studies reported. In the first two studies (2003-2004) high focus scores were evident. Thereafter, lower focus scores, indicating reduced inflammation, were seen in studies III-VI during 2004 and 2005 (Figure 2; \( p = 0.009 \)). The focus score of study VI was significantly higher than that of study IV \( (p = 0.004) \); the focus scores of studies I and VI, and II and VI were not significantly different as determined by Student’s \( t \) tests. Study V mice had a median focus score of 1.49 at 20 weeks.

![Figure 2. Focus score of NOD mice treated with rAAV2LacZ](image)

Salivary glands were removed at time of sacrifice (16 or 20 weeks of age) for histological analysis, as described in Materials and Methods. Histopathologic scoring of three sections, each one 50 µm apart from the previous, was performed by counting the number of foci present (a focus is an aggregate of 50 or more lymphocytes or histiocytes per 4 mm²). The scoring was done blindly by three examiners. Bars represent means ± SEM. A One-Way ANOVA test was performed and the \( p \)-value for the difference between results is indicated.

**Cytokine expression in salivary gland extracts of NOD mice treated with rAAV2LacZ**

Local cytokine production contributes to the inflammatory process in SS. We examined protein expression of several pro- and anti-inflammatory cytokines and one chemokine in aqueous salivary gland extracts of rAAV2LacZ-treated mice at time of sacrifice. Significant differences were seen for IL-2, IL-4, IL-6, IL-10, IL12(p70), TNF-α, and IFN-γ (all \( p < 0.001 \); Table 2). The chemokine RANTES showed too much intra-study variability to detect a difference between studies.
Table 2. Levels of inflammatory molecules in salivary gland extracts
Mean (SEM) protein expression of inflammatory molecules (pg/mg wet weight) in salivary gland extracts after administration of rAAV2LacZ (of experimental studies III, IV and VI). A One-Way ANOVA test was performed and the \( p \)-value for the difference between the results is indicated. *Medians, as determined by an ANOVA on Ranks Test.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>III (SEM)</th>
<th>IV (SEM)</th>
<th>VI (SEM)</th>
<th>( p )-Value</th>
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<td>IL-2</td>
<td>0.11 (0.02)</td>
<td>0.65 (0.03)</td>
<td>0.34 (0.04)</td>
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</tr>
<tr>
<td>IL-4*</td>
<td>0.00</td>
<td>0.02</td>
<td>0.00</td>
<td>&lt; 0.001</td>
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<tr>
<td>IL-6</td>
<td>0.07 (0.05)</td>
<td>0.65 (0.08)</td>
<td>0.12 (0.02)</td>
<td>&lt; 0.001</td>
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<tr>
<td>IL-10</td>
<td>0.06 (0.03)</td>
<td>0.18 (0.01)</td>
<td>0.05 (0.01)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>IL-12(p70)*</td>
<td>0.10</td>
<td>0.22</td>
<td>0.04</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>TNF-( \alpha )*</td>
<td>0.05</td>
<td>0.11</td>
<td>0.00</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>IFN-( \gamma )*</td>
<td>5.65</td>
<td>0.14</td>
<td>15.45</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>RANTES*</td>
<td>3.63</td>
<td>1.51</td>
<td>3.08</td>
<td>0.26</td>
</tr>
</tbody>
</table>

**Body weights of NOD mice treated with rAAV2LacZ**
To assess the general health status of the mice body weights were measured weekly. Diabetic mice display polyuria and dehydration leading to weight loss. There was no difference in body weight between the five different experimental studies (Figure 3; \( p = 0.17 \)). Mice from study V had a mean body weight of 27.0 gram at 16 weeks.

![Figure 3. Body weight of NOD mice treated with rAAV2LacZ.](image)

**Prevalence of diabetes mellitus type I of NOD mice treated with rAAV2LacZ**
Several studies have pointed out that the incidence of diabetes detected in NOD mice can vary between different laboratories.\(^{21-23}\) We measured blood glucose levels weekly to monitor the potential for diabetes-related dehydration that could
interfere with salivary flow measures. The incidence of IDDM at 16 weeks of age is shown in Table 3; there was no significant difference between the experimental studies \( (p = 0.93) \). Additionally, the blood glucose levels of rAAV2LacZ-treated mice that were not diabetic at time of sacrifice were similar \( (p = 0.47) \). Mice in study V had a median blood glucose level of 125 mg/dL.

<table>
<thead>
<tr>
<th>Study number</th>
<th>DM+ (n)</th>
<th>DM- (n)</th>
</tr>
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<td>II</td>
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<td>6 (86)</td>
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<tr>
<td>III</td>
<td>2 (25)</td>
<td>6 (75)</td>
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<tr>
<td>IV</td>
<td>2 (25)</td>
<td>6 (75)</td>
</tr>
<tr>
<td>VI</td>
<td>4 (29)</td>
<td>10 (71)</td>
</tr>
</tbody>
</table>

Table 3. Presence (DM+) or absence (DM-) of diabetes mellitus type I at 16 weeks of age

DM+, diabetic mice; DM-, non diabetic mice. Diabetes mellitus type I was defined as blood glucose level ≥400 mg/dL and Ultralente insulin (5 U/mouse subcutaneously, every 24 hours) was administered to diabetic mice. The numbers displayed are n (%). \( \chi^2 = 0.89, p = 0.93 \).

Discussion

This is a retrospective and first report investigating the longitudinal expression of the SS phenotype in NOD/LtJ mice. We compared data from NOD mice treated with a control vector, rAAV2LacZ, in six different experimental studies over almost two years. Previously, we have shown there is no difference in SS parameters of untreated NOD mice or mice treated with an irrelevant AAV vector.\(^1\) We have also observed no differences in NOD mice between saline- and rAAV2LacZ-administered animals (see Figures 1 and 2, and the Table in the supplementary data). As shown herein, over time the NOD mice, treated with this same control vector, showed significant differences in several parameters important to SS: salivary flow rate, focus score, and salivary gland cytokines IL-2, IL-4, IL-6, IL-10, IL-12(p70), TNF-\(\alpha\) and IFN-\(\gamma\). However, body weights, prevalence of IDDM, and blood glucose levels were similar indicating that there was variation only in SS-like disease in these mice, but not IDDM. Indeed, the incidence of IDDM in the mice studied by us was low (14-29%). One caveat to this observation is the high level of intra-group variability of blood glucose levels seen, which could mask the presence of some inter-group variability. Mice in study V were older (20 weeks) at time of sacrifice, but showed a similar trend as mice in other studies, indicating the instability was not due to a delayed effect on SS phenotype expression. Interestingly, some experimental studies also included saline-treated animals and they showed a similar phenotypic variability, indicating the effect of the rAAV could be ruled out (Lodde et al., unpublished). Several reports studying intervening influences on diabetogenesis in NOD mice
exist, but there are no such studies for the SS component. Based on susceptibility factors documented in IDDM studies,\textsuperscript{21-23} it is possible to speculate on possible causes of this divergence in phenotype in NOD mice.

All colonies of NOD mice are derived from a single diabetic female detected during the breeding of a cataract-prone strain of mice, but some of the dispersed colonies have been separated for many generations and express varying levels of diabetes.\textsuperscript{21-23} A 1989 international workshop report concluded that diabetes is a complex multifactorial syndrome in which environmental factors strongly interact to modulate the penetrance of susceptibility genes;\textsuperscript{21} the NOD mouse represents one of the best models of diabetes available for demonstrating this critical interaction.\textsuperscript{24} We hypothesize, based on these discussions of diabetogenesis (see below), that the following factors are likely of most importance to account for the alterations in the SS phenotype seen by us: genetic drift, pathogenic contamination, and/or other environmental factors such as housing and diet.

Genetics are important in the development of SS in NOD mice.\textsuperscript{25-27} The question if the NOD mouse variability is due to genetic drift is difficult to answer, since genotyping was not performed by the company from which animals were purchased or us. It seems prudent that for future studies of SS using NOD mice, genotyping should be performed at regular intervals.

Baxter \textit{et al.} compared a low incidence diabetes mellitus mouse line (NOD/Wehi) with a high incidence mouse (NOD/Lt) substrain and their F1 crosses.\textsuperscript{22} The progeny was found to express a disease incidence comparable to

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\textbf{Figure 4. Blood glucose levels of non-diabetic mice treated with rAAV2LacZ}

Box plot of mice not diabetic at time of sacrifice (16 weeks of age). Medians, whiskers and outliers are shown. An ANOVA on Ranks test was performed ($p = 0.47$).
That of the low incidence line. The finding was consistent with either (a) dominant resistance gene(s) or a transmissible environmental agent in NOD/Wehi mice. Housing, diet, and water were identical for both groups and, therefore, could not be held responsible for the difference; mitochondrial transmission was eliminated. SS is also considered to exhibit a genetic predisposition, as well as an unknown environmental trigger.\textsuperscript{25}

The interaction of environmental factors, in fact, complicates genetic mapping of susceptibility loci.\textsuperscript{21} Bowman \textit{et al.} concluded that while genetic divergence may explain some of the colony differences in NOD mice, most differences seem to be environmentally driven.\textsuperscript{28} Defects in the antigen-presenting cells (APCs) of NOD mice appear to disrupt presentation of self antigens in the course of tolerance induction. Viral and/or bacterial infections, for example, are often reported to reduce the incidence of IDDM, possibly through an upregulation of the APCs by inflammatory cytokines release.\textsuperscript{28} Indeed, Ohsugi and Kurosawa saw an increased incidence of IDDM in the offspring of low incidence substrains of mice (NOD/Ju and NOD/shi) after eradication of pathogens by embryo transfer.\textsuperscript{23} These authors concluded that the diabetes incidence in NOD mice is influenced by environmental factors, in particular murine hepatitis virus. It is particularly noteworthy that the lower incidence NOD mouse substrains had the same genotype as the original NOD mouse colony.

We obtained NOD/LtJ mice from The Jackson Laboratory at 6-7 weeks of age. The company certified that housing and caretaking were unchanged during the studied time period. The status of our own facility, however, was significantly changed over this time. Mice were always housed in ABSL-2 facilities, but in three different locations over the two years studied. Additionally, the complete eradication of all pathogens in our facility was not achieved. In particular, there has been contamination of mouse parvovirus and \textit{Helicobacter} sp. for over six years. Nonetheless, it is unlikely that this latter feature is a contributing factor to the longitudinal instability since our NOD mice studied before 2004 showed the accepted SS phenotype.\textsuperscript{3,10,11,20}

Delayed onset of IDDM in NOD mice has also been shown to be associated with high stress and with being housed on the top of the rack, but not group size.\textsuperscript{29} Our mice were housed on different shelf levels in an animal room containing other immunocompetent mice. In contrast, reports on other IDDM models in the rat\textsuperscript{30} and mouse\textsuperscript{31} described an accelerating effect of stress on diabetes incidence. Taken together, the observations suggest that stressors can modulate the expression of spontaneous autoimmune diabetes by exerting pleiotropic effects on immune and/or inflammatory components at the level of the pancreas and on peripheral glucose metabolism.\textsuperscript{32} It has also been suggested that cow’s
milk casein and an unidentified substance in commercial mouse chow could be dietary diabetes triggers in NOD mice, when introduced at weaning, but the contribution of cow’s milk is controversial. Overall, the influence of diet may actually depend on the sanitation of the facility.

Although we cannot eliminate genetic drift as a cause of the altered SS phenotype in NOD mice studied by us, it seems more likely that environmental changes were most significant. There were key changes in housing and caretaking over the two years studied. Mice in the third cohort were shortly housed in an adjunct facility, while thereafter these mice and mice in studies IV-VI were housed in a new, permanent ABSL-2 facility. Interestingly, it was at this point (study III) that we began to observe SS phenotypic drift. All facilities were ABSL-2 and mice were routinely fed autoclaved water, autoclaved commercial chow, and γ-irradiated transgenic dough diet. Since the diet was γ-irradiated and already used in 2003, it is highly unlikely there is an involvement. Additionally, all virus preparations were produced by a single vector core facility in the same manner, using established and well accepted standard operating procedures (e.g., see Di Pasquale, et al., Kaludov, et al. and Katano, et al.). Thus, we think it is unlikely that the different vector preparations used herein were a source of variability in the NOD mouse model.

**Conclusion**
The SS phenotype of NOD/LtJ mice showed significant longitudinal variability in this first report describing the phenomenon. Currently, we cannot specifically discern what caused the SS divergence, but changes in environment seem possible. We advise other investigators to continuously monitor SS parameters and include appropriate controls when studying this disease in NOD mice.

**Acknowledgements**
We would like to thank Norah van Mello, Milton Papa, Coreen Johnson, and Rebecca Martinez for technical assistance during the animal experiments and Sandra Afione for her assistance in viral preparations.
Supplementary Figure 1. Effect of saline and rAAV2LacZ on salivary flow rate in NOD mice
Salivary flow rate (SFR; microliters per gram body weight in 20 minutes) at 16 weeks of age either treated with saline (n = 8) or rAAV2LacZ (LacZ, n = 7) is shown. Bars represent means ± SEM. A Student’s t test was performed; there was no difference between the groups (p = 0.96). See Figure legend 1 for additional information.

Supplementary Figure 2. Effect of saline and rAAV2LacZ on focus score in NOD mice
Focus score at 16 weeks of age either treated with saline (n = 8) or rAAV2LacZ (LacZ, n = 7) is shown. Bars represent means ± SEM. A Student’s t test was performed and indicated no significant difference between the test groups (p = 0.92). See Figure legend 2 for additional information.

Supplementary Table. Levels of immunomodulatory molecules in salivary gland extracts
Protein expression of immunomodulatory molecules (pg/mg wet weight) in salivary gland extracts after administration of saline (n = 7) or rAAV2LacZ (LacZ, n = 7). Means (SEM) are shown unless otherwise noted. A Student’s t test was performed and the difference between the groups is indicated. *Medians, as determined by a Mann-Whitney Rank Sum Test.
References