

# Reversal of Sjögren's-like syndrome in non-obese diabetic mice

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**Background:** Non-obese diabetic (NOD) mice exhibit autoimmune diabetes and Sjögren's-like syndrome.

**Objective:** To test whether a treatment that reverses end-stage diabetes in the NOD mouse would affect their Sjögren's-like syndrome.

**Methods:** NOD mice have a proteasome defect. Improperly selected naive T cells escape, but can be killed by reintroducing major histocompatibility complex class I self-peptides on matched normal splenocytes. The proteasome defect also impairs nuclear factor  $\kappa$ B, a transcription factor in pathogenic memory T cells, increasing their susceptibility to tumour necrosis factor-induced apoptosis stimulated through complete Freund's adjuvant (CFA). The impact of this two-limb therapy (injections of matched normal splenocytes and CFA) on the autoimmune salivary gland disease of the NOD mice was studied.

**Results:** All NOD mice receiving the above treatment had a complete recovery of salivary flow and were protected from diabetes. Restoration of salivary flow could be the result of a combination of rescue and regeneration of the gland, as confirmed by immunohistochemical analysis. All untreated NOD mice showed a continuous decline in salivary flow, followed by hyperglycaemia and death.

**Conclusion:** This study establishes that a brief intervention in NOD mice with Sjögren's-like syndrome can reverse salivary gland dysfunction.

Sjögren's syndrome is an autoimmune disease that affects 1–4 million Americans. These patients are unable to produce saliva, which can profoundly affect their quality of life, interfering with eating, speaking and sleeping. Treatments to restore saliva production would greatly increase the quality of life for these patients. Possible therapeutic approaches can be tested in non-obese diabetic (NOD) mice that present a Sjögren's-like syndrome.<sup>1</sup> NOD mice display infiltrates of lymphocytes and a gradual loss of salivary function. The reduced saliva output mimics in part the condition seen in patients with Sjögren's syndrome.

Faustman and coworkers reported that an intervention with complete Freund's adjuvant (CFA) combined with matched major histocompatibility complex (MHC) class I and self-peptide-bearing splenocytes can permanently reverse established end-stage type 1 diabetes in the NOD mouse.<sup>2,3</sup> This two-limb therapy worked by selective apoptosis of two subpopulations of disease-causing T cells.<sup>4,5</sup> One limb of this therapy allowed MHC class I and self-peptide complex on matched normal cells (splenocytes) to re-select naive pathogenic T cells. The second limb induced endogenous tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) to kill disease-causing activated T cells.<sup>5</sup> Once autoimmunity was removed, the return of normoglycaemia in the

previously hyperglycaemic animals was driven by the regeneration of pancreatic  $\beta$  cells.

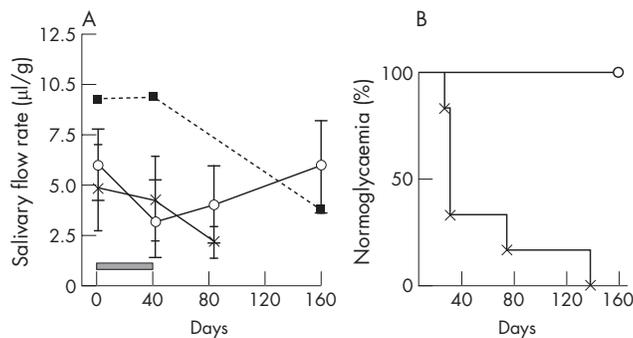
T cell selection occurs by both negative and positive selection through antigen presentation, and in the periphery with the additional aid of cytokines. Antigen presentation through MHC class I and self-peptides allows efficient negative selection (in the bone marrow and thymus) of autoreactive CD8 T cells in their naive state. Efficient self-peptide display within the MHC class I groove is driven by the preparation of peptides in the cytoplasm by the latent membrane protein 2 (LMP2)-expressing proteasome. The LMP2 protein is an obligatory subunit for the preparation of peptides for T cell self-tolerance and is encoded in the highest-risk region of the genome associated with autoimmunity—that is, the MHC class II region. After T cells reach the periphery and the T cell receptor is triggered, the LMP2 protein plays an additional role in T cell selection. In highly activated T cells, the proteasome activates nuclear factor  $\kappa$ B (NF $\kappa$ B), a transcription factor with multiple roles, including apoptosis resistance. NF $\kappa$ B activation protects activated T cells from death after exposure to cytokines such as TNF.<sup>5</sup>

NOD mice have a defect in the production of the LMP2 protein and therefore have autoreactive T cells in the periphery that are in both the naive and activated states.<sup>6,7</sup> The ability to remove established autoimmunity in this NOD model is by the re-establishment of targeted apoptosis of disease-causing T cells. Autoreactive naive T cells can be selectively induced to die, even in advanced autoimmune disease, by the reintroduction of the missing MHC class I and self-peptide complexes. Autoreactive activated T cells can be induced to die by the stimulation of NF $\kappa$ B with low-dose TNF.

These fundamental defects in self-tolerance could have a central role in Sjögren's syndrome in humans. Antigen-processing genes have previously been implicated in Sjögren's syndrome, as well as an observed altered expression of peptide-filled MHC class I structures.<sup>8,9</sup> Krause *et al*<sup>10</sup> reported that patients with Sjögren's syndrome have interrupted MHC class I presentation and that their immune cells similarly have LMP2 deficiencies. Therefore, humans with Sjögren's syndrome and the NOD mice could have a similar genetic problem resulting in improper T cell selection.

The objective of this study was to assess whether a two-limb therapy, which reversed end-stage diabetes in NOD mice by selective killing of pathogenic naive and activated T cells, could halt or reverse xerostomia (dry mouth) in established Sjögren's-like syndrome (based on the commonality of similar genetic defects in both the mouse and human).

**Abbreviations:** CFA, complete Freund's adjuvant; FISH, fluorescence in situ hybridisation; LMP, latent membrane protein; MHC, major histocompatibility complex; NF $\kappa$ B, nuclear factor  $\kappa$ B; NOD, non-obese diabetic; SFR, salivary flow rate; TNF, tumour necrosis factor



**Figure 1** Effects of treatment on the restoration of salivary flow rates (SFRs) and maintenance of normoglycaemia in autoimmune-prone non-obese diabetic (NOD) mice. (A) Stimulated SFR of treated NOD (open circles), untreated NOD (cross hatch) and control C57BL/6 mice (solid squares) during the 160-day time course. The bar indicates the 40-day treatment period. (B) Kaplan–Meier plot for normoglycaemia for treated NOD (open circle) compared with untreated NOD mice (cross hatch).

## METHODS

### Animals

Female NOD (Taconic Farms, New York, USA), female C57BL/6 and male CByF1B6F1/J mice (Jackson Laboratory, Maine, USA) were used as described previously.<sup>2</sup> CFA (Difco, Michigan, USA) was injected into each hind footpad simultaneously with the first splenocyte injection (see supplementary material at <http://ard.bmj.com/supplemental> and <http://www.sciencemag.org/cgi/data/302/5648/1223/DC1/1>). Salivary flow rates (SFRs), focus score and blood glucose were measured as described previously.<sup>11</sup>

### Histological examination

Fluorescence in situ hybridisation (FISH) combined with immunohistochemical staining was performed as outlined previously.<sup>2, 12–13</sup> The primary antibodies used were directed against salivary and pancreatic cell markers and colocalised with a repeat sequence on the mouse Y chromosome<sup>14</sup> (see supplementary materials).

### Statistical analysis

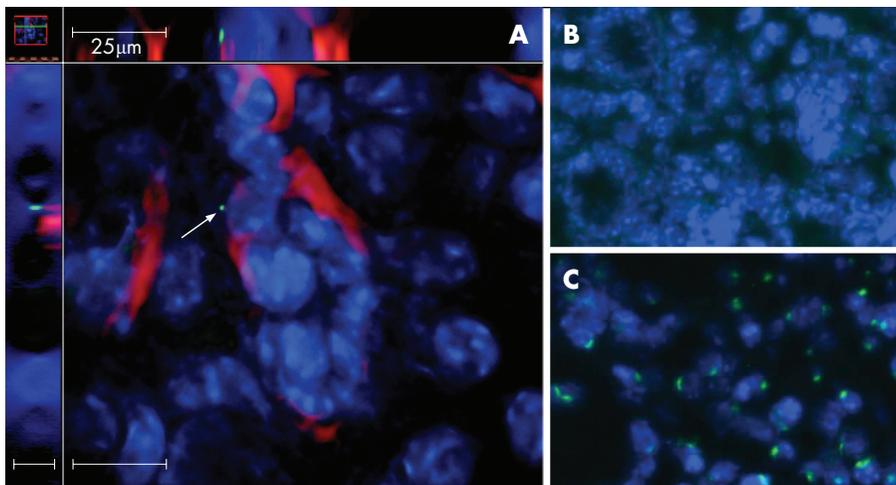
The percentage of normoglycaemia was indicated by the Kaplan–Meier method. SFR and focus scores were analysed with the Mann–Whitney U test.

## RESULTS

SFRs of 13 female NOD mice were monitored. Mice with advanced Sjögren's-like syndrome (>50% SFR loss) were randomised into two groups: untreated (n = 5) versus treated NOD (n = 8). All NOD mice were normoglycaemic at the start of treatment, at 14 weeks of age. Treated NOD mice received one injection of CFA for TNF induction and male donor live MHC class I and peptide-matched splenocytes biweekly for 40 days.<sup>2</sup> The use of male splenocytes allows for post-transplantation tracking of these Y chromosome cells (using FISH) in salivary and pancreatic tissues of female NOD mice. As a control group, C57BL/6 mice were similarly studied (n = 14).

Female NOD mice exhibit a decrease in SFR at 12–14 weeks of age. SFRs were compared between untreated NOD, treated NOD and age-matched C57BL/6 mice at 10 weeks (prior to Sjögren's-like syndrome), 14 weeks (before CFA/splenocyte treatment), 21 weeks (treatment completed) and 35 weeks of age (killed). All eight treated NOD mice exhibited a decrease in SFR during the 40 days of active treatment (fig 1A). During the next 120 days, however, there was a gradual restoration of the SFR. By 160 days, SFR of treated NOD mice was comparable to that of age-matched C57BL/6 mice (p = 0.6434). Treated NOD mice were also protected from diabetes (p < 0.001; fig 1B). The SFR of untreated NOD mice continued to deteriorate over time, and all of them died of severe hyperglycaemia within 140 days from the start of the treatment. SFR directly reflects function of the glands and its decrease is the major clinical finding in patients with Sjögren's syndrome.

To investigate the mechanism of the return of SFR in treated NOD mice, we examined the salivary tissues histologically for chimerism and inflammatory signs. Restoration of SFR could be the result of rescue of a damaged gland or a combination of rescue and regeneration. Regeneration could derive from endogenous salivary cells (after being liberated from T cell suppression) and/or from donor splenic precursor cells. We combined FISH with immunohistochemical staining in the same section to allow for the simultaneous detection of both the Y chromosome and specific markers for salivary or pancreatic cells. A small number of Y-positive salivary epithelial cells (fig 2) and a few pancreatic  $\beta$  cells were detected in the treated NOD mice (data not shown), but not in untreated NOD or C57BL/6 mice. We observed the reappearance of pancreatic islets free of invasive lymphoid infiltrates in treated NOD mice (data not shown). However, in salivary glands, the lymphocytic infiltrates (focus score) did not differ significantly between splenocyte-treated and untreated animals (p = 0.23).



**Figure 2** Double immunostaining of a section of a salivary gland of a successfully treated female non-obese diabetic (NOD) mouse (A) shown in three planes restored from a Z stack of sections taken at 0.5  $\mu$ m intervals. The Y chromosome signal is green and cytokeratin 13 (a marker of salivary epithelial cells) is red. Nuclei are stained in blue with 4,6-diamidino-2-phenylindole (DAPI). We observed Y chromosome-positive nuclei in cytokeratin-positive cells of several treated female NOD mice. The y and z dimensions are shown on the two sides of the x plane image and demonstrate the presence of the Y chromosome (green) in the same plane with the cytokeratin 13 (red) and the nucleus (DAPI—blue). Fluorescence in situ hybridisation analysis of salivary tissue sections from untreated female (B) and male (C) mice are used here as controls for the Y chromosomal probe.

## DISCUSSION

We used 14-week-old normoglycaemic NOD mice with established Sjögren's-like syndrome. The successful treatment criteria were (1) preventing progression to diabetes; (2) islet regeneration or rescue; (3) restoration of SFR; and (4) salivary tissue regeneration. Our results showed 100% protection from progression to diabetes when this treatment was administered in young (14-week-old) NOD mice. This percentage is higher than the 85% success rate reported by Faustman *et al*<sup>2</sup> for end-stage diabetic NOD mice at 22 to 40 weeks of age, suggesting the importance of early intervention. Lineage tracking methods<sup>15</sup> confirmed that although both islet rescue from the recipient and islet regeneration from donor-injected splenocytes occurred, in young animals, the islet rescue (or host-derived regeneration) dominated. We demonstrate that salivary gland function, as measured by SFR, was completely restored. A small number of donor splenocytes colonised the salivary gland and differentiated into salivary epithelial cells.

Unlike the pancreas, the salivary glands of treated mice did not show a change in focus score (lymphoid infiltrates within the glands). In the pancreas of these mice, the "benign" circumferential insulinitis increased and this patterning in the NOD model is known to never progress to disease. In the pancreas, the aggressive (active) invasive insulinitis never appeared after treatment. However, in the salivary gland, this patterning of salivary gland infiltrates does not distinguish between "benign" and active disease. This explanation may also be valid in Sjögren's syndrome in humans, where SFR did not correlate with the focus score of minor salivary gland biopsy specimens.<sup>16</sup>

This study demonstrates that a two-limb intervention can stably reverse two forms of established autoimmune disease—that is, diabetes and xerostomia in Sjögren's-like syndrome. With the recent finding that humans with Sjögren's syndrome have identical proteasome defects in their LMP2 subunit as the NOD mice,<sup>10</sup> patients with Sjögren's syndrome might indeed benefit from this treatment.

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See supplementary material at <http://ard.bmj.com/supplemental>

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