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The anti-inflammatory compound lisofylline prevents Type I diabetes in non-obese diabetic mice

Z.-D. Yang[2], **M. Chen**[2], **R. Wu**[2], **M. McDuffie**[2, 3], **J.-L. Nadler**[1, 2]

[1] Division of Endocrinology and Metabolism, Department of Internal Medicine
[2] Department of Microbiology, University of Virginia, Charlottesville, Virginia, USA

Abstract

Aims/hypothesis. Pro-inflammatory cytokines are increased during the active stages of Type I (insulin-dependent) diabetes mellitus. The aim of this study was to investigate the applicability of using a new anti-inflammatory compound, Lisofylline, to prevent diabetes in non-obese diabetic (NOD) mice. Lisofylline has previously been shown to block Th1 cell differentiation and to reduce IL-1β-induced dysfunction in rat islets.
Methods. Lisofylline was added to isolated NOD islets in vitro, with or without IL-1β. Insulin secretion and DNA damage of the islets was assessed. Lisofylline was administered to female non-obese diabetic mice starting at 4, 7 and 17 weeks of age for 3 weeks. Cytokines and blood glucose concentrations were monitored. Histology and immunohistochemistry were carried out in pancreatic sections. Splenocytes isolated from donor mice were intravenously injected into immunodeficient NOD (NOD.scid) mice.
Results. In vitro, Lisofylline preserved beta-cell insulin secretion and inhibited DNA damage of islets in the presence of IL-1β. In vivo, Lisofylline suppressed IFN-γ production, reduced the onset of insulitis and diabetes, and inhibited diabetes after transfer of splenocytes from Lisofylline-treated donors to NOD.scid recipients. However, cotransfer of splenocytes from both Lisofylline-treated and diabetic NOD donors did not suppress diabetes in recipient mice.
Conclusion/interpretation. Lisofylline prevents the onset of autoimmune diabetes in NOD mice by a mechanism that does not seem to enhance the function of regulatory T cells, but could be associated with suppression of proinflammatory cytokines and reduction of cellular infiltration in islets. This study suggests that Lisofylline could have therapeutic benefits in preventing the onset of Type I diabetes. [Diabetologia (2002) 45:1307–1314]

Keywords Lisofylline, NOD mice, macrophage, IL-1β, insulitis, Type I diabetes mellitus.

Type I (insulin-dependent) diabetes mellitus results from the immune-mediated inflammatory destruction of insulin-producing beta cells in pancreatic islets of Langerhans [1]. Although specific pathogenic mechanisms in Type I diabetes are still not defined, it is clear that activated T cells are required for the initiation of diabetes. In the NOD mouse model of Type I diabetes, several studies have shown that macrophages are among the initial mononuclear cells recruited to sites of inflammation in islets, suggesting macrophages could also be important in the progression of insulitis by both their antigen-presenting function and their ability to release inflammatory mediators [2, 3, 4]. Once activated, macrophages directly secrete several inflammatory cytokines, such as interleukin 1 β (IL-1β), interleukin 12 (IL-12) and tumour necrosis

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Corresponding author: Dr. J.-L. Nadler, Division of Endocrinology and Metabolism, Department of Internal Medicine, University of Virginia, Charlottesville, VA 22908-1405, USA, E-mail: jln2n@virginia.edu
Abbreviations: Th, T helper cell; LSF, lisofylline; NOD, non-obese diabetic; NOD.scid, severe combined immune deficient NOD mice; NK, natural killer; NOS, nitric oxide synthase; STAT, signal transducer and activator of transcription

factor α (TNF-α), and indirectly trigger interferon-γ (IFN-γ) production from activated T cells [5, 6, 7]. These cytokines coordinately enhance Th1-cell-mediated inflammatory responses, which seem to be responsible for cell-mediated insulitis and beta-cell destruction [8, 9].

In our study, we used Lisofylline (LSF) to test the hypothesis that this new anti-inflammatory agent could be capable of suppressing Th1-cell differentiation and reducing the onset of diabetes in NOD mice. Previous results suggest that LSF reduces both non-antigen-specific inflammatory reactions and antigen-specific autoimmune responses. In vivo, LSF suppresses release of proinflammatory cytokines in lung injury [10] and down-regulates migration and degranulation of neutrophils in a porcine sepsis model [11]. In vitro, LSF potently inhibits both production of TNF-α, IFN-γ, IL-6 and IL-1β from human whole blood cells [12] and IL-1β-induced dysfunction in isolated rat islets [13]. LSF also selectively suppresses certain neutrophil and leukocyte functions, including adhesion and phagocytic activity [14]. LSF has been shown to prevent autoimmune experimental allergic encephalomyelitis (EAE) in mice, another model of a T-cell and/or Th1-cytokine-mediated autoimmune disorder. In the EAE model, protection by LSF was associated with suppression of IL-12-mediated Th1-cell differentiation, but not IL-12 production [15, 16]. Since Type I diabetes in the NOD model is associated with both up-regulation of pro-inflammatory cytokines and increases in cellular migration/infiltration at the site of inflammation, we hypothesised that LSF might reduce the development of diabetes in the NOD mouse model.

Materials and methods

Animals and reagents. All NOD/LtJ (NOD) and NOD.CB17-*Prkdc*^{scid}/*J* (NOD.scid) mice were obtained from the Jackson Laboratory (Bar Harbor, Me., USA). In our colony, 80% of female NOD/LtJ mice develop spontaneous diabetes by 20 weeks of age. For this study, principles of laboratory animal care (NIH publication No. 85-23, revised 1985) were followed. The experimental protocol was approved by the Institutional Animal Care and Use Committee at the University of Virginia. Lisofylline (LSF, 1-(5-*R*-hydroxyhexyl)-3,7-dimethylxanthine, or CT-1501R) was obtained from Cell Therapeutics, (Seattle, Wash., USA). Mouse recombinant IL-1β was purchased from R & D Systems (Minneapolis, Minn., USA); anti-MAC-3 antibody (M3/84) from BD/Pharmingen (San Diego, Calif., USA) and anti-insulin antibody (H-86) from Santa Cruz Biotechnology (Santa Cruz, Calif., USA); Collagenase P from Boehringer Mannhem (Indianapolis, Ind., USA); concanavalin A from Canavalia ensiformis type IV (ConA) from Sigma (St. Louis, Mo., USA).

Islet isolation, treatment and functional assays. Pancreatic islets were isolated from 5-week-old male NOD mice [17]. Each set of 200 islets was treated with one of following conditions overnight: (i) 50 µmol/l of LSF; (ii) 50 ng/ml of murine IL-1β; (iii) 30–100 µmol/l of LSF plus 50 ng/ml of IL-1β and (iv) vehicle (saline). After washing, islets were preincubated in 3 mmol/l glucose Krebs-HEPES buffer for 30 min at 37°C in a shaking water bath under an atmosphere of 95% O_2/5% CO_2, then resuspended in either 3 mmol/l or 28 mmol/l glucose Krebs-HEPES media for an additional 2-h incubation. The supernatants were assayed for insulin using a radioimmunoassay (RIA) with rat insulin standards [18]. Islets from these cultures were used for DNA fragmentation detection using an Apoptotic DNA Ladder Kit (Roche, Indianapolis, Ind., USA).

Experimental design and treatment. Female NOD mice received LSF or saline beginning at 4, 7 and 17 weeks of age. Mice in the LSF group received 25 mg per kg LSF, twice daily for 3 weeks, by i.p. injection. Mice in the control group were given normal saline at equivalent volumes, IP, for 3 weeks. Blood glucose was monitored weekly using an Accu-Chek blood glucose monitor (Roche Diagnostics, Indianapolis, Ind., USA). Mice were considered hyperglycaemic when non-fasting blood glucose was higher than 13.75 mmol/l for 3 consecutive days.

In vitro NOD splenocyte treatments. Splenocytes were purified from 7-week-old non-diabetic female NOD mice and treated with 1 µg per ml ConA ±50 µmol/l LSF for 48 h, followed by immunoprecipitation and immunoblotting.

Splenocyte adoptive transfer. Splenocytes were purified from female NOD mice that either (i) had previously received LSF (50 mg per kg per day) for 3 weeks and remained euglycaemic for an additional 4 to 6 weeks or (ii) were overtly diabetic age-matched saline-treated control mice. 3-week-old NOD.scid mice received 4×10^7 cells per mouse of freshly purified splenocytes by IV injection. For co-transfer, NOD.scid recipients received 2×10^7 splenocytes per mouse from both LSF-treated and saline-treated NOD donors (total 4×10^7cells per mouse).

Histology and immunohistochemistry. Pancreatic tissues were fixed with Bouin's Solution and embedded in paraffin. Paired adjacent sections were stained separately with either hematoxylin and eosin or aldehyde fuchsin. Cellular infiltration and beta-cell granulation were graded in a blinded fashion [19]. In situ immunohistochemical staining with anti-MAC-3 or anti-insulin antibodies was carried out [20] using frozen sections. Vectastain peroxidase ABC and DAB kits (Vector Laboratories, Burlingame, Calif., USA) were used for signal development.

Cytokine detection by RNase protection assay (RPA) and ELISA. Total RNA was extracted from NOD pancreata using a total RNA isolation kit (Promega, Madison, Wis., USA) and assessed for cytokine mRNA expression using the RiboQuant RPA System with the mCK-2 multi-cytokine probe set (BD/Pharmingen, San Diego, Calif., USA) [16]. Cytokine mRNA expression was quantitated by normalising to the L32 gene. NOD sera and splenocyte supernatants treated with or without ConA ± LSF were assayed for IFN-γ, IL-4 and IL-10 using ELISA kits (BioSource International, Camarillo, Calif., USA).

Statistics. Data are presented as mean values ± SEM. The statistical significance of difference between groups was evaluated using analysis of variance (ANOVA), followed by Student-Newman-Keuls test for multiple comparisons. A p value of less than 0.05 was considered significant.

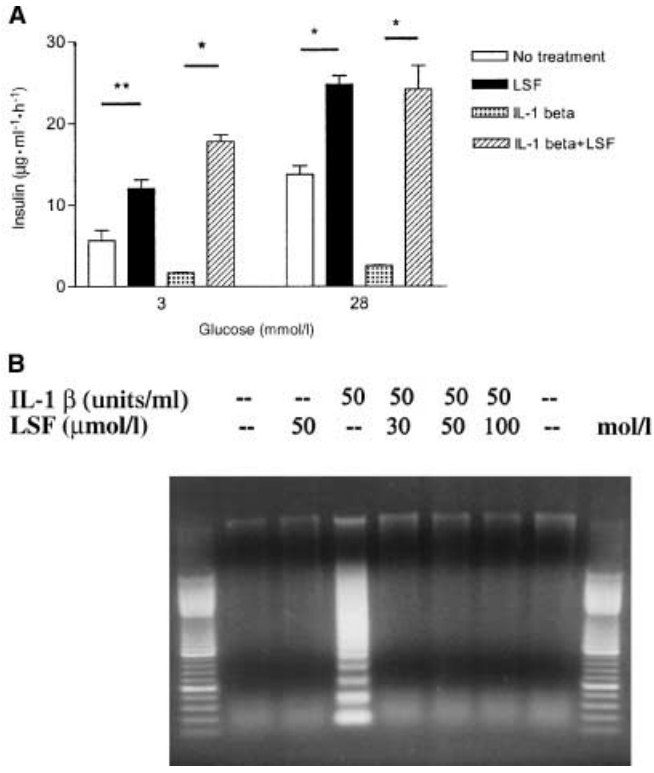

Fig. 1A,B. **A** Insulin release by isolated NOD islets. Freshly isolated islets were cultured overnight with LSF 50 μmol/l, IL-1β 50 ng/ml, IL-1β plus LSF in addition to no treatment control. Islets were then treated with either 3 mmol/l or 28 mmol/l glucose. Insulin concentration was measured in the supernatants by RIA. Each test used 200 islets in triplicate sets. The figure is representative of three repeated studies. **p*<0.001, ***p*<0.01. **B** DNA fragmentation of NOD islets. Islets were isolated from non-diabetic female NOD mice and cultured with overnight treatments as indicated. Concentration of treatments: IL-1β 50 ng/ml, LSF 30–100 μmol/l. 5 μg of each islet DNA sample was loaded on a 0.8% agarose gel. This figure is representative of three separate experiments. M: 100 bp DNA ladders

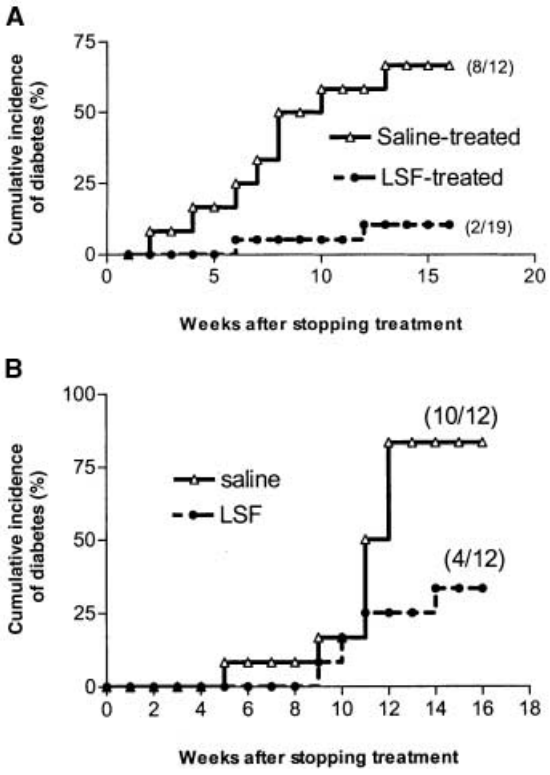

Fig. 2A, B. The cumulative incidence of diabetes in LSF-treated female NOD mice. LSF treatment (50 mg/kg per day, IP) was started at the age of 7 weeks (**A**) or 4 weeks (**B**), and continued daily for 3 weeks. Controls were age-matched female NOD mice treated with saline. The time represented a period after LSF withdrawal. *p*: LSF-treated vs saline-treated mice. $p<0.01$ in both (**A**) and (**B**)

Results

LSF prevented beta-cell damage caused by IL-1β in isolated islets. Isolated NOD islets were treated with IL-1β ± LSF. Insulin release from the IL-1β-treated islets was clearly reduced in both basal (3 mmol/l) and stimulatory (28 mmol/l) concentrations of glucose. LSF treatment enhanced insulin release by twofold to 2.5-fold in both conditions and restored insulin secretion in the presence of IL-1β. (Fig. 1A). Islet cells were morphologically intact in all groups after overnight culture. However, DNA fragmentation was seen in the islets treated with IL-1β alone. In contrast, DNA fragmentation was undetectable in the islets treated with LSF and IL-1β (Fig. 1B).

LSF prevented the onset of spontaneous diabetes in NOD mice. LSF was first given to female NOD mice IP beginning at 7 weeks of age to test its effectiveness on spontaneous disease. Treatment was continued daily for 3 weeks at 50 mg per kg per day. Treatment was then withdrawn and mice were observed for 4 months. A remarkable reduction of diabetes development was observed in LSF-treated mice, compared to saline-treated control mice (Fig. 2A). By the 15th week after the treatment was withdrawn, only 2 of 19 (10.5%) of LSF-treated mice were diabetic, compared with 8 of 12 (66.7%) saline-treated control mice. LSF also prevented or delayed the onset of diabetes in female NOD mice that started LSF therapy at 4 weeks of age (Fig. 2B). Delaying the short-term treatment with LSF until 17 weeks of age decreased the incidence of diabetes, but the results were not different from saline-treated control mice by 32 weeks of age (75% in LSF-treated vs 80% in saline-treated mice).

Splenocytes from LSF-treated NOD mice did not suppress diabetes after adoptive co-transfer. Adoptive transfer of splenocytes from NOD mice results in a rapid induction of diabetes in lymphocyte-deficient NOD.scid recipients. We used this approach to test for functional changes in immune cells induced by LSF. Female NOD mice that had received 3 weeks of LSF or saline beginning at 7 weeks of age, and kept for an

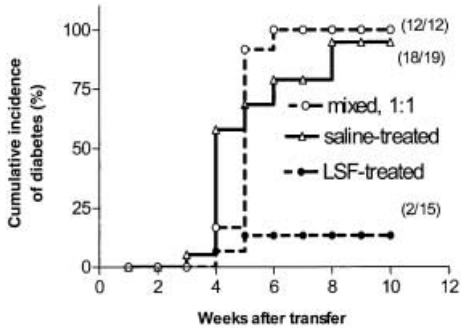

Fig. 3. The cumulative incidence of diabetes transfer in NOD.scid mice. LSF or saline was given to pre-diabetic female NOD donor mice for 3 weeks. Splenocytes were obtained from these NOD donor mice at 4 to 6 weeks after treatment withdrawal, and transferred to 3- to 4-week old NOD.scid mice at 4×10^7 splenocytes per mouse intravenously. In the co-transfer study, mixed 4×10^7 splenocytes from both LSF-treated and saline-treated donors (2×10^7 each) were injected to each recipient. $p<0.01$ (in the pairs of LSF vs saline, and LSF vs L/S mix). $p=0.1$ (saline vs L/S mix)

additional 4 to 6 weeks after stopping treatments, served as donors. All LSF-treated donor mice were normoglycaemic. Saline-treated donor mice were diabetic at the time when spleens were collected. Splenocytes were transferred i.v. into 3-week-old NOD.scid mice. By 10 weeks after splenocyte transfer, 18 of 19 (94.4%) NOD.scid recipients that had received the saline-treated splenocytes were diabetic. In contrast, only 2 of 15 (13.3%) NOD.scid recipients of the splenocytes from LSF-treated NOD donors had become diabetic (Fig. 3). However, splenocytes from LSF-treated donors were not able to suppress diabetes induction in NOD.scid mice when co-transferred with equal numbers of splenocytes from saline-treated diabetic donors (Fig. 3).

LSF suppressed macrophage infiltration into islets in vivo. Mononuclear cell infiltration into islets (insulitis) was diminished in the pancreata from LSF-treated

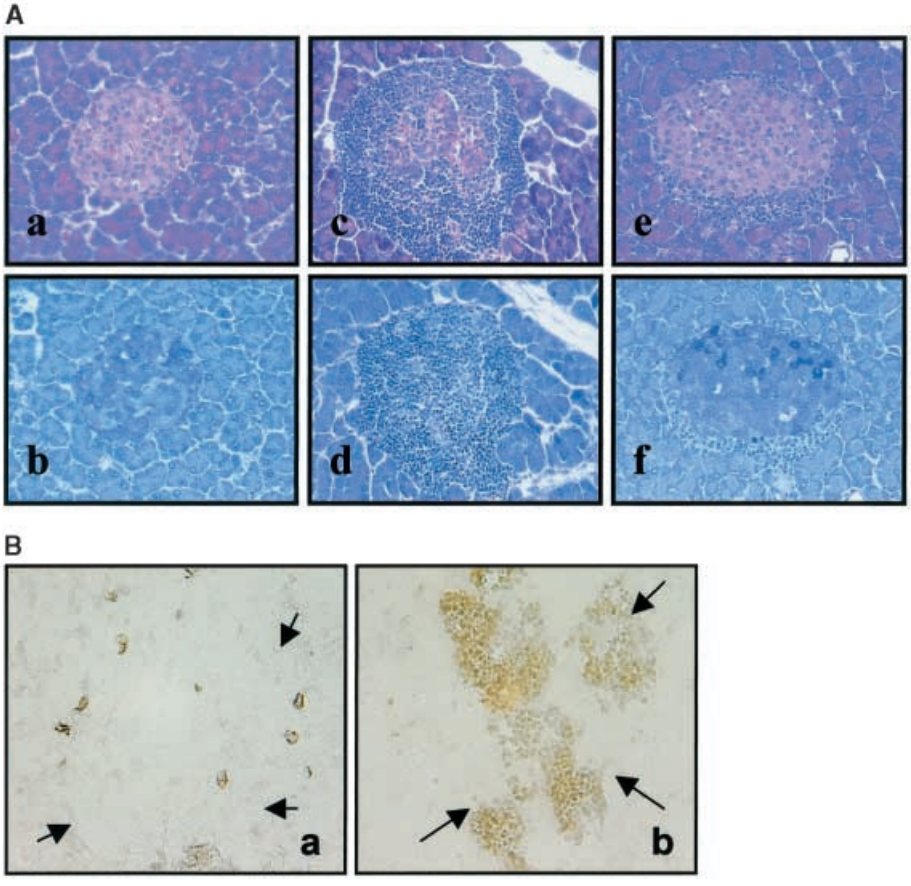

Fig. 4A, B. Pancreatic histology and immunohistochemistry. **A** Pancreatic histology from NOD mice. Panel a and b: normal histology of non-diabetic NOD at 4 weeks old; Panel c and d: representative sections from diabetic female NOD mice from saline control group, 3 weeks after saline withdrawal; Panel e and f: representative sections from euglycaemic female NOD mouse, 3 weeks after stopping LSF treatment. Panel a, c and e were stained with hematoxylin and eosin. Panel b, d and f were adjacent sections stained with aldehyde fuchsin. (×400). **B** In situ immunohistochemical staining for macrophages using anti-MAC-3 antibody in NOD pancreata. Panel a: LSF-treated NOD, 3 weeks after stopping treatment; Panel b: saline control female mouse, at 3 weeks after stopping treatment. All microphotographs (×200) were representative of pancreatic specimens from 4 to 6 mice in each group

mice when we evaluated them 3 weeks after the treatment was stopped. The morphological appearance of islets from the LSF-treated NOD mice was near normal. In contrast, abundant mononuclear cell invasion into islets was seen in saline-treated mice at the same time point. The number of islets decreased in saline-treated mice, and the remnants of residual islets showed decreases in stored insulin as determined by failure to stain with aldehyde fuchsin (Fig. 4A, Table 1) or with an anti-insulin antibody. Immunohistochemical staining using an anti-MAC-3 monoclonal antibody clearly showed that macrophages were abundant among the infiltrates in the islets of saline-treated mice. Only a few macrophages were detected around the islets in LSF-treated mice at 3 weeks after treatment withdrawal, consistent with the decrease in infiltrating mononuclear cells (Fig. 4B).

LSF suppressed IFN-γ gene expression and protein production. Expression of IFN-γ and IL-12 mRNA from pancreatic tissues was measured using a quantitative multi-probe ribonuclease protection assay. The levels of IFN-γ gene expression in pancreata from saline-treated mice were 20-fold to 30-fold higher than those in LSF-treated mouse pancreata. In contrast, IL-12 gene expression was similar in pancreata from both groups 3 weeks after stopping treatment (Fig. 5). Protein concentrations of IL-12 and IFN-γ were assessed by ELISA in sera from both groups of mice and in the supernatants of untreated NOD splenocytes stimulated in vitro. A deep reduction of IFN-γ was seen in sera from LSF-treated NOD mice and in supernatants of splenocyte cultures treated with LSF and ConA (Fig. 6). No difference in IL-4 and IL-10 concentrations was found in either sera or splenocyte supernatants from these groups (Table 2). NOD.scid

Table 1. The result of histological examination of NOD pancreatic tissue sections

	LSF-treated	Saline-treated
Total number of islets	83	27
Total number of mice examined	6	7
Average % of cells with insulin granules/islet	93.33[a]	11.14
Average inflammation score	0.58[b]	2.86

[a] $p<0.01$ in LSF-treated verse saline-treated samples
[b] $p<0.05$ in saline-treated verse LSF-treated mice
The area of tissue examined for each slide was approximately the same. For scoring cellular infiltration, a score of 0 to 3 represents no visible infiltration, polar, peripheral per peri-islet and extensive intra-islet infiltration, respectively

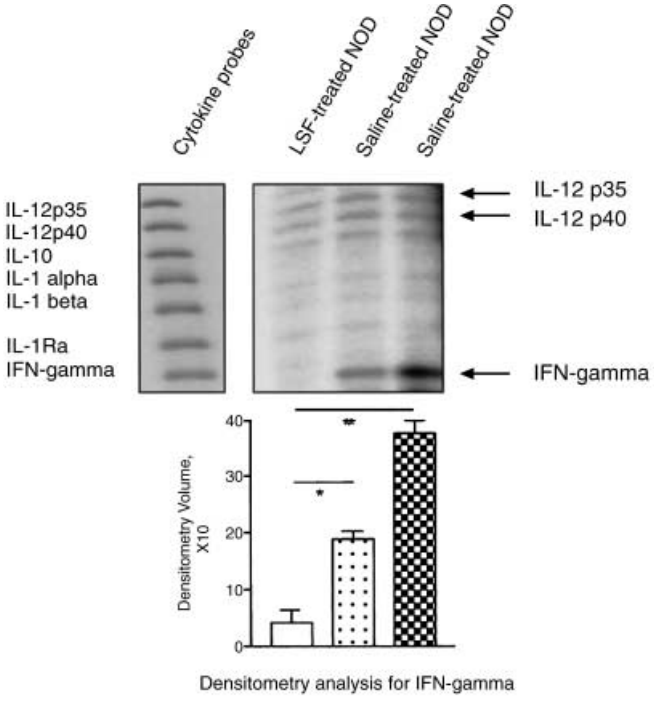

Fig. 5. Cytokine gene expression in NOD pancreata. Total RNA was extracted from NOD pancreata at 3 weeks after treatment withdrawal. Equal amounts (5 µg) of RNA were hybridized with ^{32}P-labelled cytokine probes and quantified by autoradiography with a densitometer. The data are representative from three individual experiments with four mice in each group. *p*: LSF-treated mice vs saline-treated mice, $*p<0.01$, $**p<0.005$

Table 2. The cytokine measurement by the ELISA method

	Sera of NOD mice			Supernatants of splenocyte cultures			
	UT	saline	LSF	UT	LSF	ConA	ConA + LSF
IL-4	30±8	40±12	38±25	50±20	85±25	ND	ND
IL-10	18±8	20±6	22±12	80±30	85±30	185±90	178±65
IFN-γ	60±15	75±12[b]	27±8[b]	520±20	480±50	4500±400[a]	1200±280[a]

Serum concentrations (pg/ml) of cytokines were detected from the NOD mice one week after initiation of treatment. UT indicates untreated; saline or LSF are for saline or LSF treated, respectively (n=4 for each condition). Supernatant samples were collected from splenocyte cultures of untreated female NOD mice at age 6 weeks. Freshly isolated splenocytes were cultured under different conditions overnight. 50 µmol/l of LSF and/or 1 µg/ml of ConA were used for treatment. Each condition used 10×10^6 cells and in triplicate settings. The experiment was repeated three times. 50–100 µl of each sample was used for ELISA
[a] and [b] indicate $p<0.05$ in comparison LSF-treated with saline- or ConA-treated controls. ND was not carried out

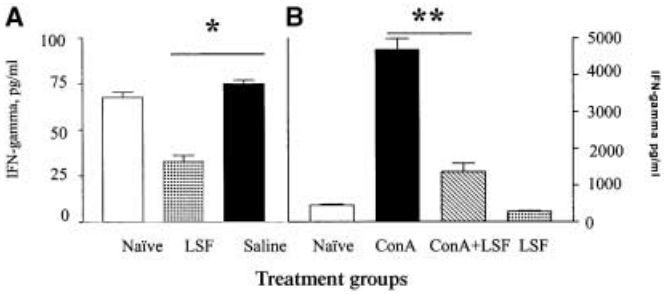

Fig. 6A, B. IFN-γ concentrations assessed by ELISA in sera obtained from female NOD mice (n=5 for each group) at 3 weeks after stopping LSF or saline (**A**), and in the supernatants of splenocytes (**B**) from 7-week-old non-diabetic female NOD mice after culture ± 50 μmol/l LSF ± 1 μg/ml ConA for 48 h in vitro (1×10^7 cells per treatment, representative of five individual experiments done in triplicate). The results from untreated age-matched female NOD mice were also shown as "Naïve" controls. *p<0.002 (LSF vs saline), **p<0.0001 (ConA + LSF vs ConA)

mice that received splenocytes from LSF-treated mice also showed a reduction in serum IFN-γ concentrations when compared with the control mice (25±6 in adoptive transferred mice vs 77±9 pg per ml in control mice, p<0.05, n=4 for each group).

Discussion

The results of this study indicate that short-term treatment with LSF induces sustained inhibition of insulitis and diabetes in NOD mice. LSF treatment reduces IFN-γ in NOD mice in vivo and IFN-γ production by activated splenocytes in vitro. LSF inhibits cellular infiltration in islets, which could further reduce local concentrations of cytokine production in islet area. Taken together, these findings suggest that suppression of both IFN-γ production and mononuclear cell infiltration to the islets contribute to substantial protection of islets from destruction in NOD mice.

In this study, splenocytes from LSF-treated donors alone reduced diabetes in NOD.scid recipients. However, co-transfer study showed no evidence that a regulatory T-cell population had been induced by LSF treatment. Therefore, it is most likely that LSF action results from inhibition of the expansion or activation of diabetogenic effector cells. Once effector cells had expanded and become activated in vivo, LSF could not fully interrupt progression of the disease process and provided less protection from beta-cell loss as shown by the short term LSF treatment to protect 17-week-old NOD mice from diabetes. These results indicate that appropriate timing of intervention with LSF will be important in preventing disease progression but long-term constant treatment might not be necessary. The current results with NOD islets confirm our earlier study using rat islets and show that LSF blocks cytokine-induced reduction of insulin secretion [13, 22]. The mechanism by which LSF can directly prevent islet damage and preserve beta-cell function is not clear. Although nitric oxide is an important mediator in IL-1β-induced beta-cell damage [23, 24], the protection of islets from IL-1β effect by LSF is not likely to be associated with inhibition of nitric oxide synthase (NOS) expression since LSF does not alter IL-1β-induced increase of inducible NOS expression in cultured rat islets [13]. However, LSF might act downstream of nitric oxide to protect islets. We have recently shown that LSF can improve mitochondrial function and ATP levels in pro-inflammatory cytokine-treated islets and mouse beta-cell lines [22]. Therefore, the protection from mitochondrial damage could explain the beneficial effects of LSF on islet function in part. Whether this direct effect on islets plays a role in disease prevention is not clear, since islet-associated infiltrates and IFN-γ production were clearly decreased after treatment in vivo.

LSF has been previously recognised as a selective inhibitor of IL-12-dependent Th1 cell differentiation and IL-12 signalling [16]. Macrophages and dendritic cells produce IL-12, which in turn induces T cells and NK cells to generate IFN-γ [25, 26]. In our study, reduction of mononuclear cells, including T cells and macrophages, in and around the islets might decrease local IFN-γ concentrations, which probably reduced stress of the islets. Substantial evidence also shows that IL-12 plays an important role in immunity and autoimmunity, including autoimmune diabetes [27, 28, 29, 30, 31, 32, 33, 34]. STAT4 signalling is an important mediator of IL-12 receptor ligation in T lymphocytes [35, 36, 37, 38, 39, 40]. Experiments are currently underway to assess the effects of LSF on STAT4 activation in our laboratory. Although LSF has no effect on production of IL-4 and IL-10, its inhibitory action on IL-12 signalling and IFN-γ production could be sufficient to regulate T cell and macrophage functions, leading to the prevention of autoimmunity.

An extensive list of therapeutic strategies has been shown to provide some protection from the development of overt diabetes in the NOD mouse model [42]. A number of immunomodulatory compounds, such as cyclosporin, FK-506 and rapamycin can suppress disease activity, but some of these agents are not applicable for clinical use in humans with diabetes because of general and islet-specific toxicities or the risks of infection and tumorigenesis [43, 44, 45].

Several less immunosuppressive anti-inflammatory agents have also been used in small studies to prevent autoimmune diabetes and disease recurrence in transplanted islets. Pentoxifylline (PTX), a structurally related compound to LSF, has been shown to have anti-inflammatory action and can reduce diabetes in NOD mice [46]. However, the mechanisms for the anti-inflammatory effects of LSF and PTX are different. LSF inhibits unsaturated phosphatidic acid generation [13], in contrast, PTX is primarily a phosphodiesterase

inhibitor. Both compounds show anti-inflammatory effects and inhibit pro-inflammatory cytokine production [47]. However, only LSF interrupts IL-12 signalling and reduces IL-12-driven cytokine production, while PTX directly inhibits IL-12 secretion through increasing intracellular cAMP [46]. In addition, PTX has no effects on IL-12-induced Th1-cell differentiation. In comparison, LSF inhibits IL-12-induced Th1 differentiation in a dose-dependent fashion [15]. Experimentally, PTX does not reduce ConA-stimulated IFN-γ secretion in mouse T cells, while LSF does [16]. Adverse effects of PTX have limited clinical use of this agent [46, 48]. In contrast, LSF has shown no significant toxicity in humans receiving bone marrow transplantation [49].

Our studies show that LSF could provide a new strategy for the prevention and treatment of Type I diabetes mellitus, even at the early stage of the disease with residual beta-cell function.

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