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RESEARCH ARTICLE

Simultaneous stimulation of Fas-mediated apoptosis and blockade of costimulation prevent autoimmune diabetes in mice induced by multiple low-dose streptozotocin

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Type I diabetes is the result of a selective destruction of insulin-producing β cells in pancreatic islets by autoreactive T cells. Depletion of autoreactive T cells through apoptosis may be a potential strategy for the prevention of autoimmune diabetes. Simultaneous stimulation of the Fas-mediated pathway and blockade of costimulation by a CTLA4-Fas ligand (FasL) fusion protein has been reported to lead to enhanced in vitro apoptosis of peripheral lymphocytes. To test the feasibility of CTLA4-FasL-based gene therapy to prevent autoimmune diabetes, we developed a recombinant adenovirus containing the human CTLA4-FasL gene (Ad-CTLA4-FasL). A single injection of 2×10^8 plaque-forming units of AdCTLA4-FasL via the tail vein of mice greatly reduced the incidence of autoimmune diabetes (13%, $n=15$) induced by multiple low-dose streptozotocin. AdCTLA4-FasL administration abrogated pancreatic insulitis, significantly increased apoptosis of pancreatic T-lymphocytes, and altered splenocyte response to mitogenic and antigenic stimulation. These results indicate the therapeutic potential of simultaneous stimulation of the Fas-mediated pathway and blockade of costimulation by adenovirus-mediated CTLA4-FasL gene transfer in the prevention of autoimmune diabetes.
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Introduction

Insulin-dependent diabetes mellitus (IDDM) is currently believed to result from the autoimmune destruction of insulin-producing β cells in the islets.[1] Experimental evidence suggests that T cells mediate this process. In certain mouse strains, diabetes can be induced by multiple low-dose streptozotocin (MLDS) and is likely mediated through a T-cell-dependent chronic autoimmune process.[2] Depletion or suppression of T-cell responses has been reported to prevent the development of autoimmune diabetes efficiently.[3,4]

The development of autoimmune diseases may be the failure of the normal mechanism of peripheral tolerance. One of the key mechanisms for the peripheral regulation of T cells and maintenance of immune tolerance is the induction of apoptosis of activated T cells, referred to as activated-induced cell death.[5] After activation, the expression of Fas in T cell is upregulated, and T cells are susceptible to apoptosis mediated by the Fas pathway. Anti-Fas antibodies and soluble Fas ligand (sFasL) effectively induce T-cell apoptosis *in vivo* and *in vitro* and show promise in preventing autoimmune diabetes in mice.[6–8] However, the *in vivo* direct administration of anti-Fas antibodies and sFasL renders severe side effects, such as massive hepatocyte apoptosis, leading to the death of treated animals.[9,10] Therefore, an engineered Fas ligand (FasL) with less toxicity, but maintained apoptotic function is a desirable strategy for developing FasL as a therapeutic agent. FasL dendritic cell transfectants induce antigen-specific immunosuppression and have less systemic toxicity.[11] IL-2-IgG-FasL triple fusion protein, consisting of the extracellular domain of FasL in-framed fused with IL-2-IgG, suppresses a murine delayed-type hypersensitivity response *in vivo* with significant apoptosis of lymphocytes and is associated with tolerated hepatocyte apoptosis.[12]

In MLDS-induced diabetic models, an injury to islet cells resulting from streptozotocin (STZ) is the prerequisite for the initiation of the disease. However, evidence exists that macrophages and lymphocytes are required for the pathogenesis of IDDM.[13,14] Macrophages are activated with increasing cellular antigens available from β-cell apoptosis and become capable of activating T cells. It has been shown that the interaction of B7 on antigen-presenting cells (APCs), such as macrophages, and CD28 on T cells plays a pivotal role in the activation of naïve T cells. Blockade of the CD28-B7 pathway by a competitive inhibitor, CTLA4Ig, a fusion protein consisting of the CTLA-4 ectodomain in-frame linked to

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the Fc domain of IgG1, renders T cells anergic, and therefore inhibits T-cell immunopathogenesis in animal models of alloimmune and autoimmune diseases.[15–17] Furthermore, blockade of the CD28-B7 pathway with CTLA4Ig promotes T-cell susceptibility to Fas-dependent apoptosis.[18] Therefore, simultaneously disrupting CD28-B7 costimulation and delivering FasL's apoptosis-inducing activity should synergistically suppress T-cell responses. Previous studies have shown that the CTLA4-FasL fusion protein, containing the ectodomains of CTLA4 and FasL, can bind their cognate ligand B7 and Fas, and displays substantially inhibitory activity by simultaneously masking B7 cosimulators and delivering FasL signals *in trans* to T cells when anchored on the surface of APCs.[19,20]

Successful treatment of autoimmune disorders may require prolonged inhibition of peripheral T-cell activation. Gene transfer technologies have demonstrated efficient and prolonged expression of the target gene *in vivo*[21,22] and shown efficacy in preventing autoimmune disorders.[23–25] Owing to their high efficiency in transducing a wide variety of cell types, including nonreplicating cells, replication-deficient adenoviral vectors are appealing candidates for gene therapy in autoimmune disorders.

In this study, an adenovirus containing the CTLA4-FasL gene (AdCTLA4-FasL) was constructed, and its efficacy in preventing IDDM was investigated with the use of an MLDS-induced mouse model. We studied whether exogenously expressed CTLA4-FasL by a single systemic injection of AdCTLA4-FasL would promote its therapeutic potential of simultaneous stimulation of the Fas-mediated pathway and blockade of costimulation in the prevention of IDDM, and thus reduce the incidence of IDDM in MLDS-induced mice.

Results

Detection of CTLA4-FasL protein in supernatant of AdCTLA4-FasL 293 transfectants

The expression of CTLA4-FasL was confirmed by *in vitro* infection of cells, followed by Western blot analysis. The expression of the CTLA4-FasL protein by 293 cells infected with AdCTLA-FasL was demonstrated by immunodetection of the FasL ectodomain with anti-FasL monoclonal antibodies. As shown in Figure 1, the expected size of bands was detected under reduced and nonreduced conditions, the former indicating a band of ~45 kDa, and the latter indicating a band of ~45 kDa and another band of ~70–90 kDa. However, no band was detected in the supernatant of AdEGFP 293 transfectants.

Serum levels of CTLA4-FasL protein in AdCTLA4-FasL-treated mice

To confirm AdCTLA4-FasL infection and transduction *in vivo*, serum CTLA4-FasL levels were analyzed at different times by using enzyme-linked immunosorbent assay (ELISA). The serum concentration of CTLA4-FasL peaked 7 days after mice received AdCTLA4-FasL. Serum levels decreased gradually but were maintained at about 5 μg/ml on day 35 after administration (Figure 2a). However, serum levels of CTLA4-FasL in AdEGFP-treated control mice were not detected by using our method.

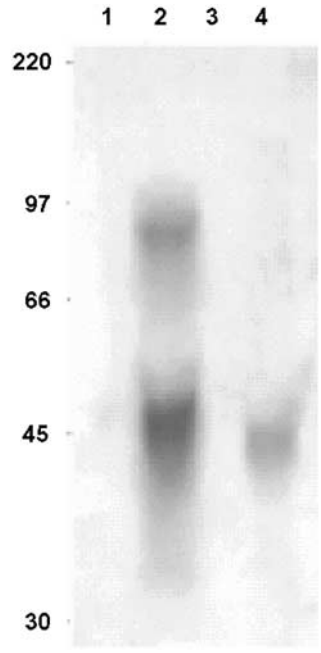

Figure 1 *CTLA4-FasL protein was detected in the supernatant of AdCTLA4-FasL 293 cell transfectants. The supernatant from AdCTLA4-FasL 293 cell transfectants (lanes 2 and 4) or AdEGFP 293 cell transfectants (lanes 1 and 3) were prepared for SDS-PAGE in the presence (reduced) or absence (nonreduced) of 2-ME (mercaptoethanol). Immunoblots were incubated sequentially with anti-FasL monoclonal antibodies and goat anti-mouse IgG-HRP, and immunoreactivity was visualized. Molecular weight markers (kDa) are indicated. In the absence of reducing agent, a ~70–90 kDa and ~45 kDa band were detected (lane 2). In contrast, under the reducing condition, the larger band disappeared, leaving only the ~45 kDa one (lane 4). As expected, no significant FasL immunoreactivity was detected in the supernatant from a negative control (ie, AdEGFP 293 cell transfectants).*

A similar expression pattern of serum CTLA4Ig was found in AdCTLA4Ig- and AdEFGP-treated mice (Figure 2b).

Greatly reduced incidence of diabetes in MLDS-induced mice administrated AdCTLA4-FasL

Low doses of STZ were given each day for 5 days to male C57BL mice. Plasma glucose levels were measured, and diabetes was defined as a blood glucose level higher than 12 mmol/l. In MLDS-induced diabetic mice, the blood glucose level was increased within 1 week after STZ injection, reaching the level of more than 15 mmol/l within 3 weeks, and becoming stable for 5 weeks (Figure 3a). Only 13% of the AdCTLA4-FasL-treated mice ($n=15$) developed diabetes, compared with mice treated with phosphate-buffered solution (PBS) (80%, $n=10$), AdEGFP (80%, $n=10$), and AdCTLA4Ig (60%, $n=15$) ($^*P<0.05$, Figure 3b). The identical incidence of diabetes in PBS- and AdEGFP-treated mice indicated that the preventive effect of AdCTLA4-FasL did not result from adenoviral vectors. Furthermore, the increased incidence of diabetes in AdCTLA4Ig-treated mice suggests that blockade of the costimulatory pathway is not sufficient in abrogating autoimmune-reactive T cells. Histologic sections of the pancreases taken from PBS- and AdEGFP-treated mice at day 7 after the last dose of STZ showed inflammatory changes involved in both $CD4^+$ and $CD8^+$ T cells, while few inflammatory

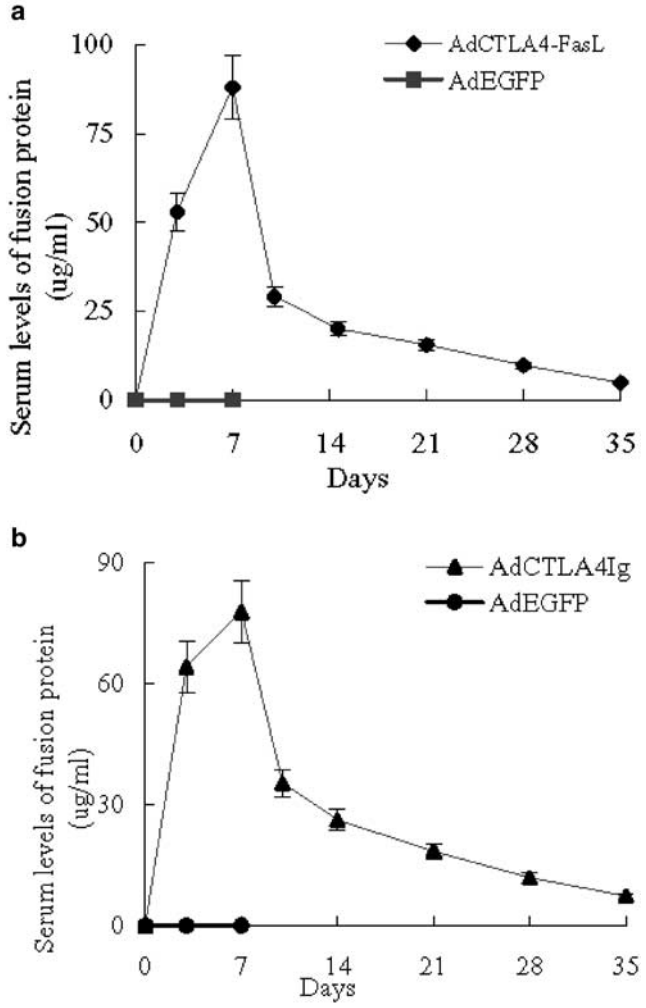

Figure 2 *Serum levels of exogenously expressed fusion protein. Serum CTLA4-FasL and CTLA4Ig were detected with the help of ELISA. The average levels of CTLA4-FasL reached a maximum (53–88 μg/ml) 7 days after injection (Figure 2a, n = 5), whereas that of CTLA4Ig reached 64–78 μg/ml (Figure 2b, n = 5). The levels were 4.8 and 7.3 μg/ml when the experiment ended. The mice treated with AdEGFP did not have any measurable CTLA4-FasL or CTLA4Ig at any time (n = 5). Data represent mean ± s.e.*

changes were indicated in pancreatic sections from AdCTLA4-FasL-treated mice (Figure 4). Furthermore, this point is underscored by the finding of a reduction in inflammatory lesions in the islets in AdCTLA4-FasL-treated mice. Only 14% of the islets ($n = 36$) exhibited infiltrates, compared with mice treated with PBS (52%, $n = 27$), AdEGFP (47%, $n = 30$), and AdCTLA4Ig (35%, $n = 31$) ($^aP < 0.01$, Table 1).

Inhibition of diabetes transfer by AdCTLA4-FasL gene transfer

Splenocytes from overtly diabetic mice include pathogenic $CD4^+$ and $CD8^+$ T cells that adoptively transfer

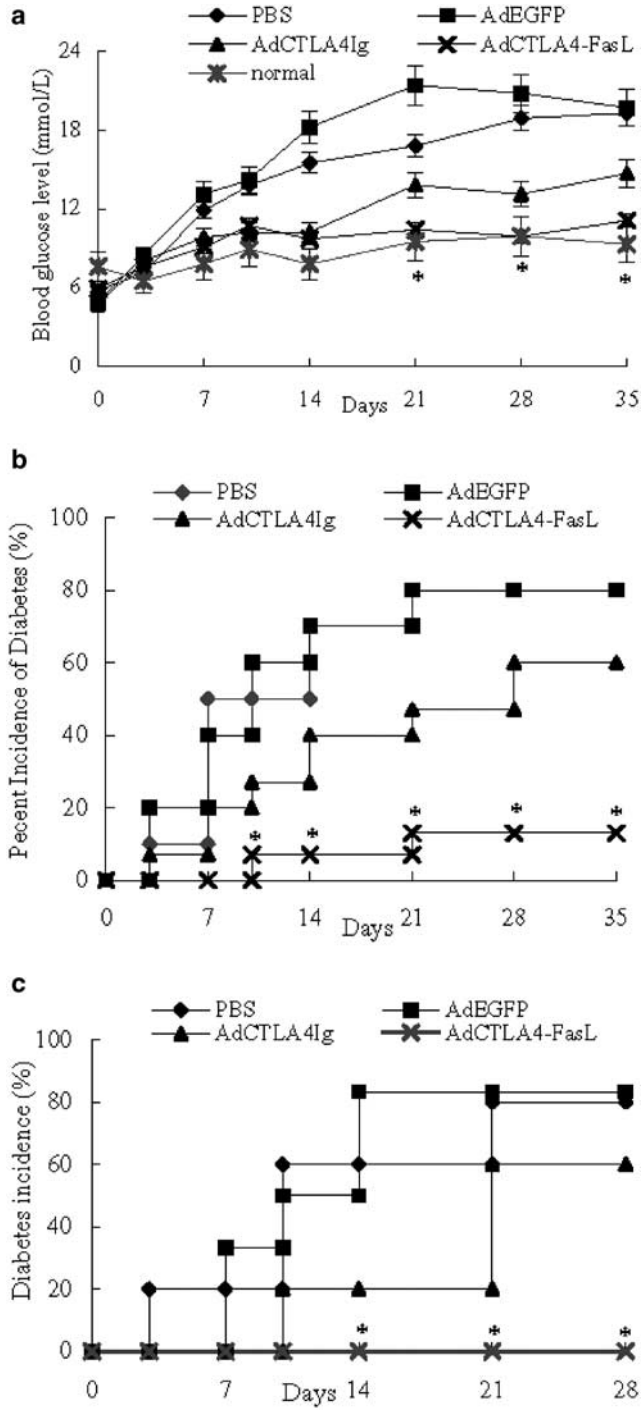

Figure 3 *Preventive effects of adenovirus-mediated CTLA4-FasL gene transfer on MLDS-induced diabetic mice. (a) Changes in the mean blood glucose levels in the treated mice. Blood samples were collected from the tail vein and blood glucose levels were measured with the help of an instant detector. The average blood glucose levels in AdCTLA4-FasL-treated mice (n = 15) were significantly lower than those of mice treated with PBS (n = 10), AdEGFP (n = 10), and AdCTLA4Ig (n = 15) (data represent mean ± s.e., *P < 0.05). (b) A significant decrease in the incidence of diabetes in mice treated with AdCTLA4-FasL (n = 15) compared with control mice treated with AdCTLA4Ig (n = 15), PBS (n = 10) and AdEGFP (n = 10) (*P < 0.05). (c) Diabetes transfer in syngeneic mice. In total, 2×10^6 splenocytes from PBS- and AdEGFP-treated diabetic mice, and the same dose of splenocytes from AdCTLA4-FasL-treated mice were injected into syngeneic mice via the tail vein. Diabetes was assessed by serial monitoring of hyperglycemia. No diabetes was found in receipts (n = 6) injected with splenocytes from AdCTLA4-FasL-treated mice, 60% of recipients (n = 5) injected with splenocytes from AdCTLA4Ig-treated mice developed diabetes at the end of the experiments (4 weeks after injection), and most recipients injected with splenocytes from PBS- (80%, n = 5) and AdEGFP-treated mice (83%, n = 6) developed diabetes. Data are results of a single experiment representative of three independent experiments with identical results (*P < 0.05).*

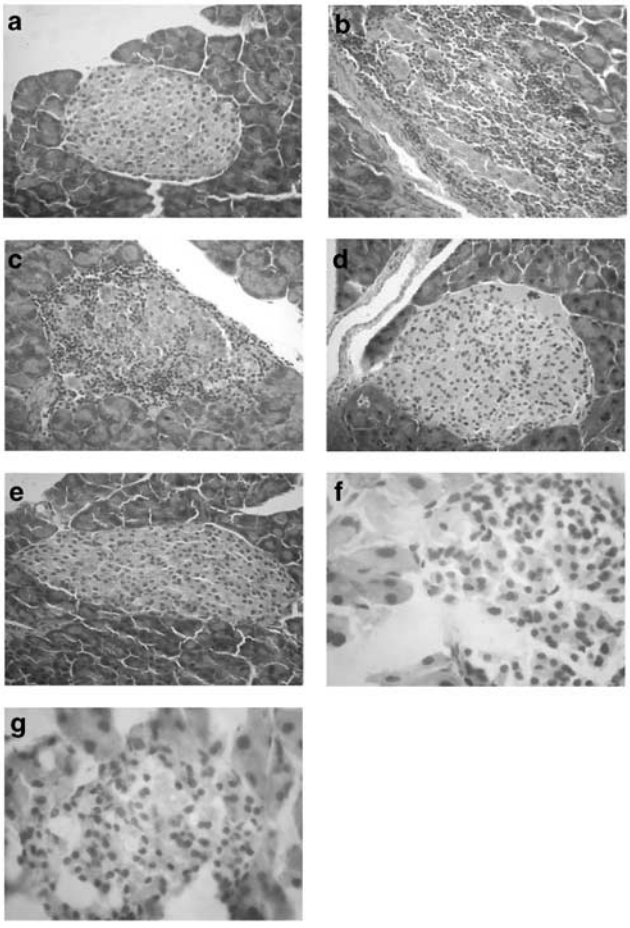

Figure 4 *Preventive effects of exogenously expressed CTLA4-FasL on MLDS-induced mice. Histological sections (5 μm) of the pancreases from mice 1 week after the last dose of STZ were prepared. Representative islets are shown from (a) normal mice, (b) control mice treated with PBS, (c) control mice treated with AdEGFP, (d) mice treated with AdCTLA4Igp, and (e) mice treated with AdCTLA4-FasL. Insulitis was found in sections (a) and (b), but little lymphocyte infiltration was shown in sections (c) and (d). (f–g) Immunohistological staining for CD4$^+$ and CD8$^+$ lymphocytes in the islets of AdEGPF-treated mice. Magnification = 200 (a–e) or 400 (f–g).*

Table 1 Insulitis profile after intravenous injection of PBS, AdEGFP, AdCTLA4Ig, or AdCTLA4-FasL in multiple low-dose streptozotocin-induced mice

Treatment	*Animals*	*Grade of insulitis, no. (%)*			*Islets, no.*
		0	1	2	
PBS	5	13 (48%)	11 (41%)	3 (11%)	27
AdEGFP	5	16 (53%)	8 (27%)	6 (20%)	30
AdCTLA4Ig	5	20 (65%)	10 (32%)	1 (3%)	31
AdCTLA4-FasL[a]	5	31 (86%)	5 (14%)	0	36

With the first dose of STZ, mice simultaneously received PBS (0.5 ml/mouse), AdEGFP (2×10^8 PFU/0.5 ml/mouse), AdCTLA4Ig (2×10^8 PFU/0.5 ml/mouse), or AdCTLA4-FasL (2×10^8 PFU/0.5 ml/mouse). In total, four experimental groups were analyzed and each group included five mice. Insulitis was graded as 0 = no intraislet cellular infiltrates, 1 = few intraislet mononuclear cells but preservation of islet architecture, and 2 = copious intraislet inflammatory cells with or without the loss of islet architecture.
[a]$P < 0.01$ for PBS, AdEGFP, and AdCTLA4Ig groups.

the disease into syngeneic mice.[26] Results from three independent experiments showed that diabetes transfer was reproducibly inhibited when splenocytes were harvested from AdCTLA4-FasL-treated mice. Nevertheless, most recipients developed diabetes when injected with splenocytes from PBS-, AdEGFP-, and AdCTLA4Ig-treated mice; the incidence of diabetes was 80% ($n = 5$), 83% ($n = 6$) and 60% ($n = 5$), respectively (Figure 3c), and the mean blood glucose levels were significantly higher than in animals passively transferred splenic lymphocytes from AdCTLA4-FasL-treated mice ($^*P < 0.05$, Table 2). These results suggest that autoreactive T cells in AdCTLA4-FasL-treated mice are inactivated and fail to render diabetic transfer.

Lymphoproliferative response

To test the ability of splenic T-lymphocytes from MLDS-induced mice to respond to mitogen and antigen *in vitro*, splenic T-lymphocytes were isolated at day 7 after the last STZ administration. The proliferation reaction under the stimulation with and without Con A, anti-CD3 antibody/phorbol 12-myristate 13-acetate (PMA) or glutamic acid decarboxylase (GAD) was measured by using of standard [^{3}H]-thymidine incorporation methods. As shown in Figure 5a, the proliferation of splenic T cells from MLDS-induced diabetic mice was significantly higher than that of normal mice ($^aP < 0.05$). However, the proliferation of splenic T cells in AdCTLA4-FasL-treated mice was significantly reduced when T cells were stimulated with GAD, compared with PBS-, AdEGFP-, and AdCTLA4Ig-treated mice ($^cP < 0.05$). The proliferation of splenic T cells from AdCTLA4Ig-treated mice was significantly reduced in comparison with PBS- and AdEGFP-treated mice ($^bP < 0.05$). Nevertheless, stimulation with Con A and anti-CD3/PMA resulted in no difference among the groups, probably because of elevated autoreactivity of splenocytes. The functional state of activated splenic T cells was determined by the addition of exogenous IL-2 to the culture. Splenic T cells from AdCTLA4Ig-treated mice were again responsive to GAD (Figure 5b), which suggests that the reduced incidence of autoimmune diabetes was primarily involved with autoreactive T-cell anergy. However, hyporesponsiveness to GAD in splenic T cells from AdCTLA4-FasL-treated mice was not reversed by exogenously added IL-2 (Figure 5b), which suggests that autoreactive T cells may be deleted through the Fas-mediated pathway.

Apoptosis of peripheral T-lymphocytes

In the *in vitro* allogeneic mixed lymphocyte culture, apoptosis of lymphocytes was observed with the addition of the CTLA4-FasL fusion protein.[20] As shown in Figure 6, pancreatic T-lymphocytes from AdCTLA4-FasL-treated mice demonstrated enhanced apoptosis (50.8%), compared with mice treated with PBS (3.7%) and AdEGFP (3.3%) and AdCTLA4Ig (15.1%), which suggests that significant apoptosis of pancreatic lymphocytes in AdCTLA4-FasL-treated mice may be attributed to the preventive effects of the CTLA4-FasL fusion protein on the development of MLDS-induced insulitis. To investigate further the effect of exogenous-expressed CTLA4-FasL protein on other peripheral T cells, the apoptosis of splenic T-lymphocytes was analyzed. The results indicated no significant difference among the

Table 2 Blood glucose levels in mice after splenic T-cell transfer (mmol/l)

Donor treatment	*Animals*	*Days after passive transfer*						
		0	*3*	*7*	*10*	*14*	*21*	*28*
PBS	15	7.5±0.7	12.0±3.3	12.4±3.6	14.5±3.5	15.2±3.8	15.7±3.7	16.3±4.5
AdEGFP	18	7.4±0.9	10.7±0.8	12.2±2.4	13.7±3.2	15.4±3.1	16.4±3.4	15.9±3.3
AdCTLA4Ig	15	7.5±0.7	9.9±0.9	10.5±1.6	11.7±2.8	12.3±3.8	14.1±4.4	14.8±4.5
AdCTLA4-FasL	18	7.4±0.5	9.5±1.1	9.4±1.0	8.8±0.9	8.7±1.0*	8.6±1.2*	8.6±0.9*

In total, 2×10^6 viable splenocytes obtained from MLDS-induced mice were infused into the tail vein of each 6- to 8-week-old syngeneic mouse. Blood glucose level was measured at different times to assess the development of diabetes. The results indicated that average blood glucose levels in mice ($n = 18$) injected splenic lymphocytes from AdCTLA4-FasL-treated mice were significantly lower than those of mice passively transferred splenic lymphocytes from PBS- ($n = 15$), AdEGFP- ($n = 18$), and AdCTLA4Ig-treated mice ($n = 15$), respectively. Data are combined results of three separated experiments. Data are mean±s.e, $^*P < 0.05$.

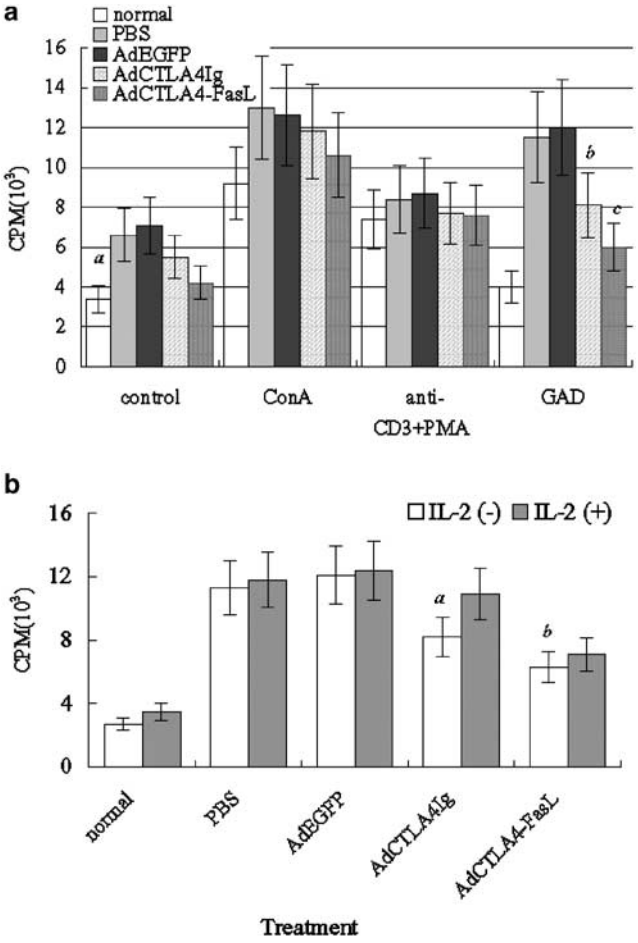

Figure 5 *Proliferation of splenic T-lymphocytes in MLDS-induced mice. Splenic T-lymphocytes were isolated on day 7 after the last dose of STZ.* (a) *Cell proliferation was measured with [³H]-thymidine incorporation plus ConA, GAD, or anti-CD3 antibody/PMA. The proliferation of splenic T-lymphocytes from PBS- and AdEGFP-treated mice was significantly elevated as compared with normal mice ($^aP < 0.05$). GAD-induced proliferation of T cells was significantly inhibited in AdCTLA4Ig-treated mice compared with PBS- and AdEGFP-treated mice ($^bP < 0.05$), whereas the proliferation of splenic T cells from AdCTLA4-FasL-treated mice was substantially lower than that of PBS-, AdEGFP- and AdCTLA4Ig-treated mice ($^cP < 0.05$). However, no significant difference occurred among the groups when splenic T cells stimulated with ConA and anti-CD3/PMA. (b) Splenic T-cell proliferation in the absence or presence of IL-2 when stimulated with GAD. In the presence of IL-2, hyporesponsiveness to GAD in splenic T cells from AdCTLA4Ig-treated mice was substantially reversed ($^aP < 0.05$ for non-IL-2), whereas the reduced response to GAD was maintained in AdCTLA4-FasL mice ($^bP > 0.05$ for non-IL-2). Data represent mean±s.e., $n = 4$ in each group.*

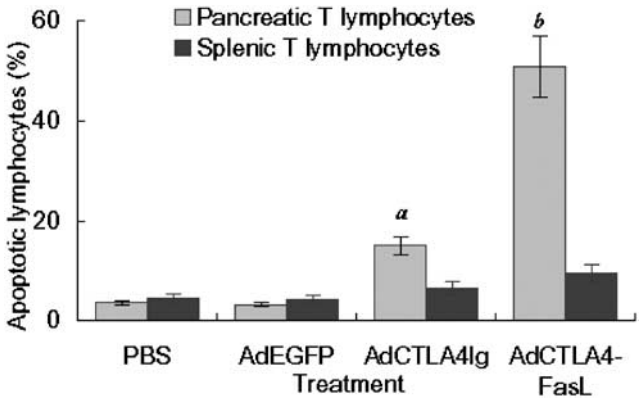

Figure 6 *Apoptosis of pancreatic and splenic T-lymphocytes in MLDS-induced mice. At day 7 after the last dose of STZ, pancreatic and splenic T-lymphocytes were isolated and analyzed on flow cytometric-PI staining. Significant apoptosis of pancreatic T-lymphocytes occurred in the AdCTLA4-FasL-treated mice ($^aP < 0.05$ for PBS-, AdEGFP-, and AdEGFP-treated mice) and AdCTLA4Ig-treated mice ($^bP < 0.05$ for PBS- and AdEGFP-treated mice), whereas less apoptosis occurred in PBS- and AdEGFP-treated mice. However, splenic T-lymphocyte apoptosis did not differ significantly among those groups. The data represent results of three independent experiments with similar results.*

groups (Figure 6), which suggests selective deletion of local antigen-activated T cells.

CTLA4-FasL gene transcription in the pancreas

Adenovirus-mediated gene transfer renders the expression of exogenous genes in multiple organs and tissues.[27] To investigate the expression of CTLA4-FasL in AdCTLA4-FasL-treated mice, the production of CTLA4-FasL mRNA was analyzed by reverse-transcriptase polymerase chain reaction (RT-PCR) with specific primers. A weak expression of CTLA4-FasL in the pancreas and a strong expression in the liver were detected in AdCTLA4-FasL-treated mice, while no expression was detected in PBS- and AdEGFP-treated mice. A similar pattern of expression of CTLA4Ig was indicated in AdCTLA4Ig-treated mice (see Figure 7).

Hepatotoxicity

It has been documented that the administration of anti-Fas antibodies or recombinant FasL causes liver failure and eventual death.[9,10] In our study, low and transiently elevated alanine aminotransferase (ALT) activities and little hepatic damage occurred in AdCTLA4-FasL-treated mice (Figure 8). Recently, it was reported that the subcutaneous and intravenous injection of up to

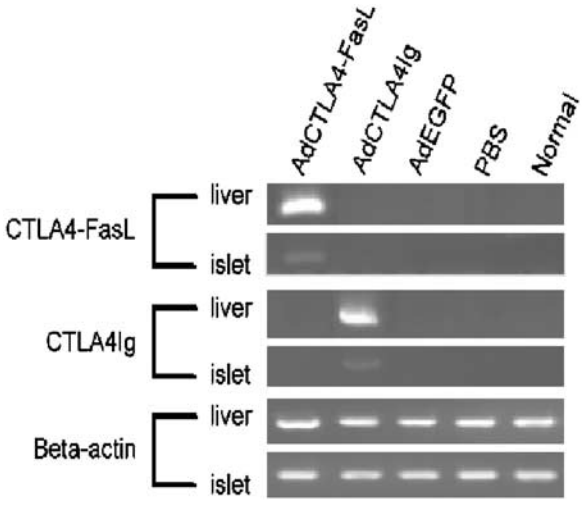

Figure 7 *RT-PCR analysis. The pancreas and liver were isolated from MLDS-induced mice 1 week after the last dose of STZ. RNA and cDNA were prepared and equivalent amounts of cDNA were subject to PCR amplification of CTLA4-FasL and CTLA4Ig, with β-actin used as a positive control. CTLA4-FasL and CTLA4Ig were highly expressed in the liver of AdCTLA4-FasL- and AdCTLA4Ig-treated mice, respectively, with weak expression in the islets of the mice. Data are results of a single experiment representative of three experiments with similar results.*

120 μg/mouse of his_6CTLA4-FasL, which displays bioactivities similar to CTLA4-FasL, was found to be well tolerated.[20] These results suggest that the hepatic toxicity of the CTLA4-FasL fusion protein is not lethal when given at therapeutic levels.

Discussion

IDDM is a T-cell-dependent autoimmune disorder. Suppression of T-cell activation by costimulation blockade or induction of T-cell apoptosis through a Fas-mediated pathway has been reported to prevent the development of IDDM efficiently.[7,8,28] However, simultaneous stimulation of the Fas-mediated pathway and blockade of the costimulatory pathway to prevent IDDM has not been reported. Previous studies have reported that the CTLA4-FasL fusion protein substantially inhibits *in vitro* allogeneic mixed lymphocyte response by inducing enhanced Fas-mediated lymphocyte apoptosis, especially when anchored on APCs while bridging APCs and T cells (Huang and Tykocinski[19] and Elhalel *et al*[20] Y Jin and S Xie, unpublished data). The function of the CTLA4-FasL fusion protein that can bridge APCs and T cells and thereby promote apoptosis of 'bridged' T cells has been well described. In a dual-chamber experiment involving CTLA4-FasL-coated Daudi cells plated below semipermeable membranes in culture wells and Jurkat target cells added to the same wells above and below the semipermeable membranes, significant Jurkat cell apoptosis is observed only in the lower compartment, where CTLA4-FasL-coated Daudi effector cells and Jurkat target cells are in direct contact on the same side of the semipermeable membrane; Jurkat cells present in isolation above the membrane demonstrate little Annexin V positivity.[19] In allogeneic mixed lymphocyte reaction, CTLA4Ig and sFasL in combination do not induce similar apoptosis of lymphocytes as CTLA4-FasL, even

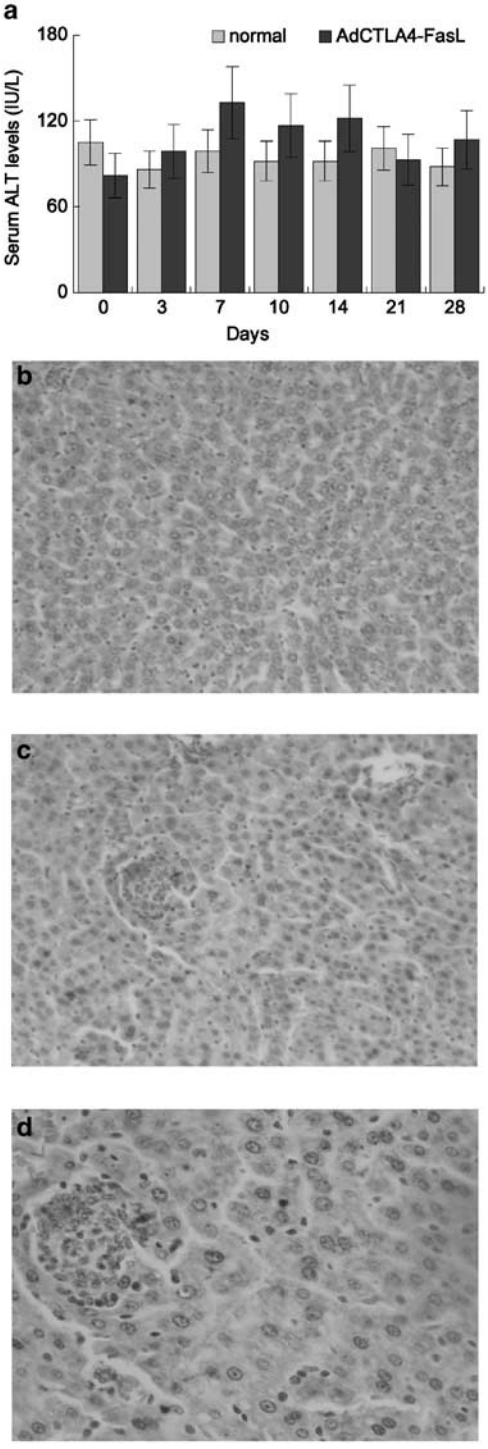

Figure 8 *ALT activities and hepatic morphologic features in AdCTLA4-FasL-treated mice. (a) ALT activities. Blood samples were taken from the tail vein of mice at intervals and ALT activities determined with the help of a standard diagnostic kit. Low and transiently elevated ALT activities occurred but were not significant in AdCTLA4-FasL-treated mice. The data represent mean ± s.e., n = 5 in each group. (b–d) Histological sections (5 μm) were prepared of livers on day 7 after the AdCTLA4-FasL administration and stained with H&E. Representative results are shown from (b) normal mice; (c and d) AdCTLA4-FasL-treated mice. Little apoptosis of hepatocytes was shown in sections (c) and (d). Magnification = 200 (b and c) or 400 (d).*

when the former two are used at a 10-fold higher molar concentration.[20] Furthermore, the proapoptotic potential of CTLA4-FasL can be substantially reduced when its anchoring of APCs has been competitively abrogated by B7.1 and B7.2 antibodies (Y Jin and S Xie, unpublished data), which suggests that membrane association is apparently a critical functional feature of CTLA4-FasL. In addition, in contrast to the *in vivo* lethality of anti-Fas and sFasL administration, no evidence for such severe side effects of CTLA4-FasL has been observed in animals treated with the CTLA4-FasL fusion protein; ALT activities and hepatic morphology indicate few significant changes in comparison with normal animals. Recently, it was reported that the subcutaneous and intravenous injection of up to 120 μg/mouse his_6CTLA4-FasL was found to be well tolerated,[20] which suggests that the hepatic toxicity of CTLA4-FasL fusion protein is not lethal. In the present study, we demonstrate that an adenovirally administrated CTLA4-FasL fusion protein efficiently prevent the development of IDDM in MLDS-induced mice, with evidence for significant apoptosis of pancreatic T-lymphocytes.

Gene transfer of target molecules to the recipients can result in an increased bioavailability as a consequence of their continuous production and circumvent the repeated administration of costly recombinant protein. Adenovirus-mediated gene therapy with advantages of high efficient transduction i*n vivo* has been widely used in the prevention of autoimmune disorders by delivering immunomodulatory molecules to the recipients.[22,23] In this study, the lasting expression of the CTLA4-FasL protein is detected in AdCTLA4-FasL-treated mice, therefore obviating the necessity of repeated administration of costly recombinant fusion protein and rendering prolonged inhibition of peripheral T-cell activation. Adenovirus-mediated gene transfer results in the expression of exogenous genes in multiple organs and tissues. The transcription of CTLA4-FasL can be detected in the pancreas and liver of AdCTLA4-FasL-treated mice by using RT-PCR. The expression of CTLA4-FasL is low in the pancreas but high in the liver, which suggests that the stimulation of local damage to islets by the Fas pathway of β cells is unlikely to occur.

The importance of Fas-mediated apoptosis has been manifested in the maintenance of T-cell tolerance and the prevention of autoimmune disease.[29,30] The stimulation of the Fas pathway by the Fas receptor agonist, anti-Fas antibodies and sFasL, has been reported to prevent diabetes efficiently but accompanied by severe side effects.[7,8] In this study, MLDS-induced mice have been treated with a Fas receptor agonist, the CLTA4-FasL protein, through adenoviral-mediated gene transfer. No lethality in AdCTLA4-FasL-treated mice has been observed. The incidence of diabetes in AdCTLA4-FasL-treated mice has been significantly reduced. Islet sections from AdCTLA4-FasL-treated mice at day 7 after the last dose of STZ show significantly reduced lymphocyte infiltration. Interestingly, significant apoptosis of pancreatic T lymphocytes has been indicated in AdCTLA4-FasL-treated mice, while no significant apoptosis of splenic T-lymphocytes is shown, which suggests that selective deletion of local antigen-activated T cells has occurred. The expression of exogenous Fas ligand in islets renders the neutrophilic destruction of islet transplants.[31] Nevertheless, the prolonged survival of islet grafts expressing human soluble FasL has been reported,[32] which suggests that the pattern of Fas ligand expressed in islets plays a pivotal role in the survival of islet grafts. In the present study, CTLA4-FasL is an alternative soluble form of human FasL and no neutrophilic destruction of pancreatic islets has been observed in AdCTLA4-FasL-treated mice. In addition, when anchoring B7-expressing cells, CTLA4-FasL executes its apoptotic function as a membrane Fas ligand, which confers the protection of islets, as described in reports that cotransplantation of islets and myoblasts expressing Fas ligand significantly improves the survival of islet grafts.[33,34]

Spontaneous as well as mitogenic- and antigenic-stimulated lymphoproliferative response in splenocytes is significantly elevated in MLDS-induced diabetic mice.[35] When stimulated with GAD, splenic T-lymphocytes from AdCTLA4-FasL-treated mice show significantly reduced proliferative activity, even with the addition of exogenous IL-2. However, hyporesponsiveness to GAD in splenic T cells from AdCTLA4Ig-treated mice is substantially reversed in the presence of exogenously added IL-2. These results suggest that autoreactive T cells may be deleted in AdCTLA4-FasL-treated mice, while autoreactive T cells are rendered anergic in AdCTLA4Ig-treated mice. Previous studies have shown that splenocytes from MLDS-induced diabetic mice are able to transfer diabetes to syngeneic mice.[26] Adoptive transfer of splenocytes from PBS-, AdEGFP-, and AdCTLA4Ig-treated diabetic mice induce diabetes in syngeneic recipients, while diabetes transfer is significantly and reproducibly inhibited with splenocytes from AdCTLA4-FasL-treated mice, which suggests that diabegenic T cells have been depleted in AdCTLA4-FasL-treated mice.

In conclusion, adenovirus-mediated CTLA4-FasL gene transfer render a lasting therapeutic level of expression of CTLA4-FasL in MLDS-induced mice and induce significant apoptosis of autoreactive T-lymphocytes such that the incidence of diabetes has been significantly reduced, and no evidence of *in vivo* lethality has been observed. The potency of the CTLA4-FasL fusion protein, targeting APCs that simultaneously disrupt B7 costimulation and deliver FasL inhibitory signals *in trans* to T cells, raises the possibility of selectively deleting antigen-activated immunoreactive T cells through apoptosis, which is a more efficient immunosuppression method and associated with fewer side effects than nonspecific standard therapies. This therapy shows promise in the treatment of autoimmune disorders, although a detailed mechanism should be further investigated. Furthermore, we are considering studies of the NOD model of autoimmune diabetes, in which more information about the preventive potentials of CTLA4-FasL is available, because the MLDS-induced diabetic model does not necessarily represent 'natural' autoimmunity in IDDM.

Materials and methods

Animals

Male C57BL mice, aged 4–6 weeks, 18–20 g in weight, were obtained from the Experimental Animal Laboratory of Peking University Health Science Center (Beijing,

China). All animals were housed at 22°C under a 12-h light/dark cycle in pathogen-free conditions and fed rodent food and water at 20:00 every day to avoid disturbance for blood glucose measurement.

All care and handling of animals was performed with the approval of the Institutional Authority for Laboratory Animal Care of the Health Science Center.

Recombinant adenovirus construction

We made human cDNA encoding amino acids 127–281 of human FasL by PCR amplification of the plasmid pBX-hFL1, containing human FasL cDNA, (a gift from Dr S Nagata, Osaka University Medical School, Osaka, Japan), with the following primers: sense, 5′-TTTAAGCTTCCA GAATCTGGGCACGGTTC-3′, antisense, 5′-TTTTCTCG AGTTAGAGCTTATATAAGCCGAA-3′, for 35 cycles of: 94°C at 45 s, 55°C at 45 s, and 72°C at 45 s. The PCR product was digested with *Hind*III (Promega, Madison, WI, USA) and *Xho*I (Promega), and the resulting 480-bp DNA fragment was subcloned into the same treated plasmid pBluescript II SK$^+$ to generate pBx-eFL. The presence of an open-reading frame for the ectodomain of human FasL was verified by sequencing. The cDNA fragment containing the oncostatin M signal sequence and the ectodomain of human CTLA-4 (amino acids 1–127) was amplified from the plasmid pSRαSD7-CTLA4Ig (a gift from Dr JF Elliott, University of Alberta, Edmonton, Canada) with the following primers: sense, 5′-TAAGAATTCCACCAATGGGTGTACTGCTCACAC AGAGGACGCTGCTCAGTCTGGTC-3′, antisense, 5′-GCTAAGCTTCCAGAATCTGGGCACGGTTC-3′, for 35 cycles of: 94°C at 45 s, 60°C at 50 s, and 72°C at 45 s. The PCR product was digested with *EcoR*I (Promega) and *Hind*III, subcloned into the plasmid pBluescript II SK$^+$ to generate pBX-eCTLA4, and sequenced, with the result indicating that amino acid 56 in the ectodomain of CTLA4 was changed from proline to threonine. To construct an expression cassette of CTLA4-FasL, pBX-eFL was digested with *Hind*III and *Xho*I, and the resulting fragment was subcloned into the respective site in pBX-eCTLA4 to produce pBX-CTLA4-FasL. An adenovirus containing CTLA4-FasL (AdCTLA4-FasL) was generated, propagated, and purified as described previously.[36] Briefly, the fragments of CTLA4-FasL cDNA were cut from pBX-CTLA4-FasL with *EcoR*I and *Xho*I and subcloned into the plasmid pShuttleCMV (a gift from Dr Tong-chuan He, The Howard Hughes Medical Institute, Chevy Chase, MD, USA) to generate the recombinant plasmid pShuttleCMV-CTLA4-FasL. Then, a recombinant adenovirus vector containing human CTLA4-FasL cDNA (pAdCTLA4-FasL) was made by cotransfection of the plasmid pShuttleCMV-CTLA4-FasL and the adenoviral genome plasmid pAdEasy1 (a gift from Dr Tong-chuan He, The Howard Hughes Medical Institute) into competent BJ5183 *Escherichia coli* (a gift of from Tong-chuan He, The Howard Hughes Medical Institute) by electroporation. The pAdCTLA4-FasL vector was subsequently transfected into 293 cells with Lipofectin reagent (Invitrogen, Carlsbad, CA, USA) to generate AdCTLA4-FasL. The culture supernatant from AdCTLA4-FasL-transfected 293 cells was analyzed on Western blotting with anti-FasL monoclonal antibodies (Clone: G247-4, PharMingen, San Diego, CA, USA) as a primary antibody to detect the CTLA4-FasL fusion protein. Then, AdCTLA4-FasL was propagated, and purified by using the CsCl precipitation method and resuspended in PBS. After being titered by using a plaque-forming assay, adenoviral preparations were added with glycerol to a final concentration of 10%, and the solution was stored at −80°C.

An adenovirus containing EGFP gene (AdEGFP) was generated by using pAdTrack and pAdEasy1 and purified, titered, and stored as described above. An adenovirus-containing CTLA4Ig gene (AdCTLA4Ig) was previously prepared in our lab.[37]

Experimental design

To induce diabetes, animals were treated with STZ (Sigma, St Louis, MO, USA) as described.[38] Briefly, C57BL mice intraperitoneally received 40 mg/kg body weight STZ dissolved in 0.01 M sodium citrate buffer solution (pH 4.2) daily for 5 consecutive days. On the first day, 2×10^8 of plaque-forming units (PFU) of AdCTLA4-FasL in 0.5 ml was injected via the tail vein, and PBS (0.5 ml), AdEGFP (2×10^8 PFU/0.5 ml), and AdCTLA4Ig (2×10^8 PFU/0.5 ml) were used as controls. Blood glucose level was measured by using an instant glucose detector (ONE TOUCH®II, Johnson and Johnson Medical, Shanghai, China) on samples taken from the tail vein on repeated occasions over the next 4 weeks. Animals with a plasma glucose level above 12 mmol/l were considered diabetic. Blood samples were collected from the tail vein and serum CTLA4-FasL or CTLA4Ig were measured by enzyme-linked immunoabsorbent assay (ELISA) at different times.

Histologic examination

The mice were killed by cervical dislocation and their pancreases removed and fixed in 10% formalin solution and embedded in paraffin. Thick sections (5-μm) were cut and stained with hematoxylin and eosin (H&E). The inflammatory lesions in the islets were graded by a pathologist unaware of the origin of the sections, a procedure described previously.[39] The lesions were graded as follows: 0 = no intraislet cellular infiltrates, 1 = few intraislet mononuclear cells but preservation of islet architecture, and 2 = copious intraislet inflammatory cells with or without loss of islet architecture.

To verify that both CD4$^+$ and CD8$^+$ T-lymphocytes are involved in the autoimmune destruction of β cells in MLDS mice, the pancreatic sections were incubated in primary polyclonal antibodies for CD4$^+$ and CD8$^+$, respectively (diluted to 1:300, kindly donated by Professor GF Ding, Department of Immunology, Peking University). Sections were rinsed and incubated for 1 h in ABC–horseradish peroxidase (HRP) complex (Sino-American Biotechnology Co., Beijing, China). HRP activity was developed in a solution of 0.025% diaminobenzidine (Sino-American Biotechnology Co.) and 0.025% H_2O_2 for 10 min.

Isolation of splenic T-lymphocytes

At day 7 after the last dose of STZ, spleens were harvested and splenic T-lymphocytes obtained as described previously.[40] Briefly, a single splenocytes suspension removing red blood cells with Tris-NH_4Cl buffer was incubated for 2 h at 37°C so that macrophages in the suspension adhered to the culture Petri dishes. The nonadherent cells were collected and incubated in Petri dishes coated with mouse anti-IgG (Sino-American

Biotechnology Co.) for 1 h. The non-antibody-conjugated cells were collected by using PRMI1640 media (Life Technologies, MD, USA) supplemented with 15% fetal bovine serum (FBS, HyClone, Logan, UT, USA), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.05 mM 2-mercaptoethanol, and 10 mM HEPE buffer. The purity of T cells was shown to be >85% determined by the percentage of $CD3^+$ cells with flow cytometry. The viability of T cells was consistently greater than 95% as determined by the Trypan blue exclusion method.

Adoptive transfer

Adoptive transfer of diabetes was conducted as described previously.[26] In brief, the spleens of MLDS-induced mice were harvested at day 7 after the last dose of STZ and homogenized. Lymphocytes from this cell suspension were separated over a density gradient (Ficoll). Cell number and viability were determined by using the Trypan blue exclusion method. A total of 2×10^6 viable splenocytes were infused into the tail vein of each 6- to 8-week-old syngeneic mouse. The blood glucose level was measured at different times to assess the disease development.

In vitro lymphoproliferative response

The isolated splenic T cells (5×10^5/well in 200 μl) were cultured in triplicate in flat-bottomed 96-well plates with [^{3}H]-thymidine (1-μCi/well) at 37°C in 5% CO_2 for 24 h and harvested on an automatic harvester; [^{3}H]-thymidine incorporation was measured in a liquid scintillation counter. To determine proliferative response induction, cells were cultured with or without ConA (1 μg/ml; Sigma), GAD (0.1 U/ml; Sigma), or anti-CD3 antibody (kindly provided by Dr DL Ma, Peking University, China) plus PMA (1 ng/ml, Sigma) for 48 h, then pulsed with [^{3}H]-thymidine for the last 18 h and counted by liquid scintillation.

For secondary analysis, selected experiments were carried out in the presence of exogenously added recombinant mouse interleukin (IL)-2 (20 U/ml; Sino-American Biotechnology Co.). T-cell reactivity in secondary experiments was assayed and assessed as detailed above.

Pancreatic T-lymphocyte isolation

At day 7 after the last dose of STZ, pancreases, including pancreatic lymph nodes, from three to four mice within the same treatment group were pooled, chopped, and digested with a mixture of collagenase (1 mg/ml, Sigma, MO, USA), hyaluronidase (0.1 mg/ml; Sigma), and DNase (300 U/ml; Sigma) in 50 ml complete media as described previously.[30] After incubating at room temperature for 60 min with constant stirring, the mixture was supplemented with 1 ml FBS and incubated for additional 60 min. A single-cell suspension was obtained by passing the mixture through a nytex screen. The cells were washed twice, resuspended in 10 ml media, and lymphocytes were purified with Ficoll (Sigma) gradient centrifugation. Then, pancreatic T-lymphocyte was isolated as described above. The purity of T cells was greater than 85%, as determined by the proportion of $CD3^+$ cells revealed by flow cytometry. The viability of T cells was consistently greater than 95%, as determined by Trypan blue exclusion method.

Quantification of apoptotic T-lymphocytes

Pancreatic and splenic T-lymphocytes were stained with propidium iodide (PI), and apoptosis was determined by flow cytometry as described.[12] Briefly, 2×10^5 cells were pelleted and washed twice with PBS. Cell pellets were resuspended in 300 μl of buffer containing 0.1% sodium citrate, 0.1% Trixon X-100, and 50 μg/ml PI, and stored overnight at 4°C in the dark. The samples were measured by use of FACScan (Becton Dickinson, San Jose, CA, USA) and were analyzed with the help of Cell Quest acquisition software (Becton Dickinson).

Analysis of CTLA4-FasL gene expression in the pancreas and liver

Use of RT-PCR analysis determined the CTLA4-FasL mRNA expression in the pancreas and liver. RNA and cDNA were prepared from isolated pancreases and liver with Trizol and Superscript (Gibco/BRL, Gaithersburg, MD, USA) according to the manufacturer's directions. PCR of β-actin was first carried out to verify the integrity of the cDNA by using the following primers: sense, 5′-GTTGGATACAGGCCAGACTTTGTTG-3′, antisense, 5′-GAGGGTAGGCTGGCCTATAGGCT-3′, for 35 cycles of: 94°C at 45 s, 68°C at 15 s, and 72°C at 45 s. The primers used to amplify CTLA4-FasL cDNA are those described above for constructing CTLA4-FasL cDNA. CTLA4-FasL and CTLA4Ig gene products were amplified with the help of the following primer pairs: CTLA4-FasL (945 bp): sense, 5′-TAAGAATTCCACCAATGGGTGTACTGCTCA CACAGAGGACGCTGCTCAGTCTGGTC-3′, antisense, 5′-TTTTCTCGAGTTAGAGCTTATATAAGCCGAA-3′; CTLA4Ig (957 bp): sense, 5′-GCTTGGTCCTTGCACTCC TGTTT-3′, antisense, 5′-GGCGTGGTCTTGTAGTTGTTC TCC-3′, for 35 cycles of 94°C at 50 s, 57°C (for CTLA4-FasL) or 67°C (for CTLA4Ig) at 50 s, and 72°C at 60 s. The amount of cDNA used in the PCR reaction was adjusted by the amplification of serially diluted cDNA with the help of β-actin primers and a comparison of the intensity of the amplified bands. The diluted cDNAs that gave the same intensity of β-actin were considered to contain the same amount of cDNA and were used to compare the cytokine gene expression. PCR products amplified were subjected to electrophoresis through 1.5% agarose-gel stained with ethidium bromide and photographed.

Hepatotoxicity

To investigate the effects of exogenously expressed CTLA4-FasL fusion protein on the liver in AdCTLA4-FasL-treated mice, serum alanine aminotransferase (ALT) activities were determined by using a standard diagnostic kit (Sino-American Biotechnology Co.). At day 7 after AdCTLA4-FasL administration, livers were harvested and hepatic sections (5 μm) stained with H&E for morphological analysis. Representative photomicrographs are shown.

Statistical analysis

Results are expressed as mean ± s.e. Statistical significance was determined by the analysis of variance (ANOVA), followed by Student–Newman–Keuls test for multiple comparisons between treatment groups, and unpaired Student's *t*-test for means between two groups. A *P*-value of less than 0.05 was considered to be statistically significant.

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