Protection from Autoimmune Diabetes and T-Cell Lymphoproliferation Induced by FasL Mutation Are Differentially Regulated and Can Be Uncoupled Pharmacologically

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Spontaneous mutation of Fas (lpr) or FasL (gld) completely protects nonobese diabetic mice from autoimmune diabetes but also causes massive double-negative T-cell lymphoproliferation. In this study, we used bone marrow chimeras and adoptive transfer analysis to investigate further the role of FasL in the pathogenesis of autoimmune diabetes and to determine whether gld-induced tolerance and double-negative T-cell lymphoproliferation can be uncoupled from each other. We show that FasL expressed on hematopoietic and nonhematopoietic compartments plays nonredundant roles in the pathogenesis of autoimmune diabetes. Mutation of FasL in either compartment interferes with the autoimmune process and prevents onset of diabetes, but FasL expressed in the hematopoietic compartment is the dominant regulator of T-cell homeostasis. Furthermore, pathogenesis of diabetes is dependent on normal FasL expression in both compartments, whereas only minimal FasL function is required to maintain T-cell homeostasis. Consequently, partial disruption of FasL protects from autoimmune diabetes without causing T-cell lymphoproliferation. This is demonstrated genetically in nonobese diabetic-gld/+ mice and pharmacologically by using FasL-neutralizing antibody. These results have important implications for understanding the role of the Fas pathway in pathogenesis of autoimmune diseases and for designing novel FasL-

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Autoimmune diabetes results from a systemic breakdown in central and peripheral mechanisms of tolerance, leading to expansion of autoreactive T cells. Recognition of autoantigens by autoreactive T cells leads to their priming and initiation of the autoimmune process. Thus, it is conceivable that many immunotherapy strategies are focused on targeting molecules critical for initiation of T-cell activation.¹ Nevertheless, clues for an alternative approach that avoids T-cell activation pathways is suggested by spontaneous loss-of-function mutation in Fas (lpr) or its ligand (gld) that completely prevents autoimmune diabetes.^{2–10} Female nonobese diabetic (NOD) mice bearing homozygous gld/gld mutation are completely protected from autoimmune diabetes that otherwise affects more than 80% of wild-type (wt) NOD females.¹¹ It was initially thought that the protection is due to abrogation of Fas-mediated death of β cells.² Subsequent studies, however, showed no or only limited role for the Fas/FasL system in the death of β cells.^{4,5,7,9,12} Defects in the Fas pathway also protect against experimental autoimmune encephalomyelitis in animal models of multiple sclerosis,^{13,14} suggesting that blockade of the Fas pathway has a general protective effect against organ-specific autoimmune diseases.

The Fas system is a major apoptosis pathway that is important for maintenance of peripheral T-cell homeostasis¹⁵ but not for T-cell activation, and there are no reports of serious immune suppression or incidence of tumors in mice bearing gld or lpr mutations. However, the Fas pathway has not previously been considered a viable therapeutic target

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because homozygosity for either *gld* or *lpr* mutation leads to T-cell lymphoproliferation. Although benign, the lymphoproliferation is massive and is dominated by a population of double negative (DN) $\alpha\beta$ T cells that lack CD4 and CD8 coreceptors and express the B220 isoform of CD45 that is normally expressed by B cells.¹⁶ Such DN T cells are rare in the peripheral immune system but progressively accumulate in mutant mice, reaching up to 80% of peripheral T cells depending on the mouse strain.¹⁶ Understanding whether DN T-cell lymphoproliferation and the protective effect of inactivating the Fas pathway are separable is important for understanding the pathogenesis of autoimmune diabetes and for harnessing the Fas pathway for therapy of autoimmune disease.

In this study, we show that FasL expressed on hematopoietic and nonhematopoietic compartments plays nonredundant roles in the pathogenesis of autoimmune diabetes. Mutation of FasL in either compartment interferes with the autoimmune process and prevents onset of diabetes. Moreover, FasL expressed in the hematopoietic compartment is the dominant regulator of T-cell homeostasis. In addition, we demonstrate genetically, in bone marrow chimeras and haploinsufficient NOD-*gld*/+ mice, and pharmacologically, using FasL-neutralizing antibody, that the protective effect of FasL inactivation can be achieved without causing DN T-cell lymphoproliferation. These findings provide the basis for developing new therapeutic strategies that avoid interfering with pathways that play primary roles in initiating normal immune responses.

Materials and Methods

Mice

NOD, NOD-*gld*/+, and NOD-*gld*/*gld* mice were bred and maintained at the Animal Care Facility of the Johns Hopkins School of Medicine. NOD-*gld*/*gld* mice were generated by crossing FasL-deficient C3H/HeJ-*gld*/*gld* mice (The Jackson Laboratory, Bar Harbor, ME) with NOD/LtJ mice, and the *gld* gene was backcrossed to NOD for six generations and then intercrossed, as described in detail by Su et al.¹⁷ NOD-*gld*/*gld* and NOD-*gld*/+ mice and their NOD-*wt* intercross littermates were typed for polymorphic microsatellites linked to the insulin-dependent diabetes mellitus susceptibility (*Ida*) genes. Microsatellite markers were selected from the published data¹⁷ and from the database released by the Whitehead Institute/Massachusetts Institute of Technology Center for Genome Research (Cambridge, MA). NOD-severe combined immunodeficient (*scid*)-*gld*/*gld* mice were generated by crossing NOD-*gld*/*gld* mice with NOD-*scid* mice. F₁ generations were intercrossed, and the offspring were screened for the *gld* mutation and *scid* mutations. All experiments were performed by following protocols that are approved by the Animal Care and Use Committee of Johns Hopkins University.

Genotyping of NOD Mice for *gld* Expression

The *gld* genotype was determined by polymerase chain reaction (PCR) on tail DNA by using a pair of primers

(5'-CAGCAGCCCCAAAGCTTTATG-3' and 5'-CTCAACTCTCTGATCAATTTTGAGGA-3') as previously described.¹⁷ The 320-bp PCR products were then digested with *Stu*I (New England BioLabs, Beverly, MA) at 37°C overnight and resolved on a 1.2% Nusieve agarose gel (FMC BioProducts, Rockland, ME). The digestion yielded 280- and 40-bp fragments for the wild-type allele, whereas *Stu*I does not digest the 320-bp PCR product for the mutated allele. Homozygous (*gld*/*gld*) mice express only the 320-bp band, wt mice express the 280 band, and heterozygous *gld*/+ mice express both bands.

Staining and Flow Cytometry

Directly conjugated fluorescein isothiocyanate, phosphatidylethanolamine, peridinin chlorophyll protein (PerCP), and allophycocyanin antibodies purchased from BD Pharmingen (San Jose, CA) or eBioscience (San Diego, CA) were used in our experiments, unless otherwise indicated. In some instances, CD4 and CD8 T cells were analyzed as one subset labeled "CD4⁺ or CD8⁺ T cells" by using a combination of PerCP-conjugated anti-CD4 and anti-CD8 monoclonal antibodies. Fas was detected with fluorescein isothiocyanate-labeled Jo2, whereas FasL was detected with phosphatidylethanolamine-conjugated MFL-3. Intracellular staining for Foxp3 was performed according to the manufacturer's protocol (eBioscience) with phosphatidylethanolamine-conjugated Foxp3 FJK-16s.

Analysis of Diabetes and Insulinitis

Induction of diabetes was monitored by measuring blood glucose levels once a week. Mice with two consecutive readings of ≥ 250 mg/dl blood glucose were considered diabetic. For analysis of insulinitis, pancreata were fixed in formalin, sectioned, stained with hematoxylin and eosin (H&E), and analyzed for insulinitis. Slides were read blindly, and the frequency of islets with insulinitis (penetrating infiltration in the islets), peri-insulinitis (inflammation around the islets), and no insulinitis (no inflammation in or around the pancreatic islets) was determined relative to the total number of pancreatic islets counted in each mouse.

Bone Marrow Chimeras

Chimeras were prepared as previously described.¹⁸ In brief, bone marrow (BM) cells derived from the femurs and tibia of donors (1×10^7) were injected intravenously into lethally irradiated recipients. Thy1.1⁺ and Thy1.2⁺ allelic markers were used to assess chimerism and reconstitution. Mice were given antibiotics for 4 weeks and were periodically assessed for reconstitution by fluorescence-activated cell sorting.

Adoptive Transfers

Single-cell suspensions were prepared from spleens of donors and 1 to 2×10^7 cells transferred i.v. into the designated NOD-*scid* hosts. Depending on the specific

experiment, NOD-gld/gld, NOD-gld/+, or NOD-wt mice were used as donors, and NOD-scid-wt or NOD-scid-gld/gld mice were used as recipients. Mice were monitored for induction of diabetes as described above.

Antibody Treatment

Monoclonal antibodies specific for cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (clone UC10-4F10-11) and interleukin (IL)-2 (clone S4B6-1) were purified from hybridoma culture supernatants (American Type Culture Collection, Manassas, VA). Anti-glucocorticoid-induced tumor necrosis factor receptor (anti-GITR) (clone 108619) was purchased from R&D Systems (Minneapolis, MN). All injections were given intraperitoneally. Each experiment was performed independently with its own set of control mice (age- and sex-matched NOD-gld/+ mice treated in parallel with phosphate-buffered saline). For anti-CTLA-4 treatment, mice received three injections of 100 μ g each with 1-week intervals between injections. Anti-IL-2 treatment consisted of a single injection of 1 mg. Anti-GITR treatment consisted of two weekly injections of 50 μ g for 4 consecutive weeks. We used 12-week-old NOD-wt for CTLA-4 and 8-week-old NOD-wt mice for IL-2 and anti-GITR experiments. Mice were monitored for development of diabetes as indicated above.

Measurement of Antinuclear Antibodies

Antibodies generated against nuclear constituents in the serum were measured using a semiquantitative enzyme-linked immunosorbent assay kit from Alpha Diagnostic International (San Antonio, TX) according to the manufacturer's instructions. Measured antinuclear antibodies (ANAs) included autoantibodies that bind to double-stranded-DNA, single-stranded-DNA, Smith antigens, histones, and ribonucleoproteins and SS-A, SS-B, Jo-1, and Scl-70. Serum samples (1:100) were added in triplicate to the enzyme-linked immunosorbent assay plate coated with extracted nuclear antigen. Standard positive and negative control samples provided by the manufacturer were run in parallel. After washing, goat anti-mouse IgG-horseradish peroxidase was added. After another washing, tetramethyl benzidine was added, leading to color development that was directly proportional to the amount of ANAs in the sample. The reaction was terminated by adding stopping solution. Absorbance was measured on an enzyme-linked immunosorbent assay reader at 450 nm. The concentration of ANAs in each sample was calculated as the ANA index (AI), defined as the ratio of absorbance of the test sample and net absorbance of the negative (endpoint-cutoff) controls.

In Vivo Blockade of FasL

Neutralizing anti-FasL monoclonal IgG (MFL4) was previously described.¹⁹ Four-week-old NOD-wt mice were injected intraperitoneally with 500 μ g of anti-FasL MFL4 antibody ($n = 10$) or control hamster IgG ($n = 9$) for 2

consecutive weeks, followed by 300- μ g injections until the age of 20 weeks. Age-matched control mice were treated similarly with control hamster IgG. Mice were monitored weekly for onset of diabetes and periodically for induction of DN T cells as described in Results.

Annexin V Analysis

To determine the percentage of apoptotic T cells, spleen cells and lymph node cells were freshly isolated from three mice in each group, surface-stained with allophycocyanin-TCR, and PerCP-CD4/PerCP-CD8 monoclonal antibodies, and analyzed for Annexin V binding by using an apoptosis detection kit from BD Pharmingen according to the manufacturer's instruction. For detecting apoptotic DN T cells, we gated TCR⁺CD4⁺CD8⁻ cells, and the percentage of Annexin V⁺ cells was determined. Apoptosis of CD4⁺ and CD8⁺ T cells was determined by measuring the percentage of Annexin V⁺ and TCR⁺ cells that were CD4⁺ or CD8⁺. Results represent mean $\pm$ SEM from two different experiments.

Results

Segregation of the Protective Effect of gld Mutation from DN T-Cell Lymphoproliferation

FasL is induced after TCR activation to mediate deletion of excess effector cells and maintains T-cell homeostasis; hence hematopoietic tissues are major sites of FasL expression. Nonhematopoietic tissues, including immune-privileged sites and gut epithelia, can also express FasL and contribute to peripheral T-cell deletion.²⁰ We therefore compared the impact of gld mutation in the hematopoietic and nonhematopoietic compartments on induction of DN T-cell lymphoproliferation and resistance to diabetes. Our objective was to determine whether inactivating FasL in either compartment differentially affected lymphoproliferation and incidence of diabetes. This idea was tested in BM chimeras that expressed gld/gld mutation in the hematopoietic or nonhematopoietic compartment as depicted in Figure 1A. Donors and recipients of BM were littermates produced by intercrossing NOD-gld/+ mice.¹⁷ In the first set of mice, lethally irradiated NOD-gld/gld mice were reconstituted with BM from NOD-wt littermates. In a second set of mice, we did the reciprocal experiment by reconstituting lethally irradiated wt mice with BM from NOD-gld/gld mice. In a third set, lethally irradiated wt mice were reconstituted with wt BM and used as positive controls. Successful reconstitution was determined by examining chimeras for the ratio of donor and recipient T cells in the blood, which were distinguished by differential expression of Thy1.1⁺ and Th1.2⁺ congenic markers (Figure 1B). Chimeras also established a normal ratio of CD4 and CD8 T cells (Figure 1D; data not shown). Development of diabetes in different groups was assessed weekly by measuring blood glucose levels (Figure 1C). All wt into wt-positive control chimeras developed diabetes within 25 weeks. By contrast, none of wt into gld/gld chimeras and only 1 of 11

Figure 1. Expression of homozygous gld mutation on either hematopoietic or nonhematopoietic tissues is sufficient to protect NOD mice from spontaneous diabetes. Lethally irradiated 8-week-old NOD-wt or NOD-gld/gld mice recipients and 6-week-old wt or gld/gld Thy1.1⁺ NOD donors were used to generate BM chimeras as described in Materials and Methods. **A:** Schematic illustration of the adoptive transfer protocol. **B:** Assessment of BM chimeras. Fluorescence-activated cell sorting analysis of representative blood samples from Thy1.2⁺ NOD-gld/gld mice before irradiation and 2 and 16 weeks after reconstitution with BM from Thy1.1⁺ NOD-wt mice. All mice used were reconstituted to more than 90% (Thy1.1/Thy1.1 + Thy1.2) by donor T cells. **C:** Diabetes incidence in four chimeras during a 27-week monitoring period after reconstitution. Four or five recipients were used in each group except in gld/gld into wt chimeras where 11 recipients were used. **D:** Frequency of DN T cells in various chimeras. Mice were analyzed at 35 weeks of age (27 weeks after reconstitution). Spleen and lymph node cells from different chimeras were stained for TCR, CD4, CD8, and B220. TCR⁺ cells were gated, and the frequency of DN T cells in various chimeras was determined. Numbers in quadrants represent average $\pm$ SEM. Only gld/gld BM into wt chimeras developed significantly higher frequency of DN T cells in the lymph nodes ($P < 0.003$) and spleens ($P < 0.008$) than wt BM into gld/gld chimeras. There was no statistically significant difference in the frequency of DN T cells in the lymph nodes and spleens of wt into gld/gld chimeras relative to wt into wt chimeras as determined by unpaired Student's *t*-test, which was performed using SigmaPlot v.8.0. *P* values of < 0.05 were considered significant.

gld/gld into wt chimeras developed diabetes. Thus, the gld/gld mutation on either hematopoietic or nonhematopoietic tissues is protective against autoimmune diabetes (Figure 1C). On the other hand, the site of gld expression

significantly influenced the level of DN T-cell lymphoproliferation. Chimeras expressing homozygous gld mutation on the hematopoietic tissues (gld/gld into wt) developed significant DN T-cell lymphoproliferation. In contrast, chimeras expressing gld/gld mutation on the nonhematopoietic tissues did not develop DN T-cell lymphoproliferation, indicating that DN T-cell lymphoproliferation is mainly due to the gld mutation expressed on the hematopoietic tissues (Figure 1D). Thus, inactivating FasL in the hematopoietic compartment induces protection from diabetes but also causes DN T-cell lymphoproliferation, whereas the gld mutation expressed on nonhematopoietic tissues induces protection without causing DN T-cell lymphoproliferation.

Next, we determined whether gld-induced tolerance is acquired during T-cell development in the thymus or whether wt T cells that developed in FasL-sufficient thymi could be controlled by gld mutation expressed in other tissues. We tested the ability of splenocytes (depleted from DN T cells) from diabetic NOD-wt mice to cause diabetes in NOD-scld-gld/gld hosts or NOD-scld-wt controls (Table 1). We also examined the ability of splenocytes from age- and sex-matched nondiabetic NOD-gld/gld mice (depleted from DN T cells) to cause diabetes in NOD-scld wt hosts. The results of the adoptive transfer confirmed the conclusion obtained from the analysis of BM chimeras. None of the NOD-scld-gld/gld hosts reconstituted with wt splenocytes from diabetic mice developed diabetes, whereas the same wt donor splenocytes caused diabetes in NOD-scld-wt controls. Moreover, splenocytes from NOD-gld/gld mice failed to cause diabetes in NOD-scld-wt mice. Only very low numbers of DN T cells are seen in any of the hosts, consistent with the reported data that DN T cells develop in the thymus.²¹ The results of adoptive transfer extend the BM chimeric mice findings and show that T-cell tolerance in gld/gld hosts is not acquired during T-cell differentiation in the thymus and that diabetogenicity of wt T cells that developed in wt thymic environment can be controlled by mutation of FasL expressed in other tissues.

FasL Haploinsufficiency Confers Indefinite Protection from Diabetes without Inducing Lymphoproliferation Because of Differences in FasL Threshold Requirement

FasL functions as a homotrimer made of randomly associated single molecules^{22,23} and hence all but a few (12%) FasL homotrimers in NOD-gld/+ mice are expected to be inactive because of incorporation of at least a single gld molecule in each homotrimer.^{22,23} Thus, gld/+ mice are FasL haploinsufficient but with some preserved FasL activity; analysis of their susceptibility to diabetes and DN T-cell lymphoproliferation has therapeutic implications because partial blockade of FasL can potentially be recapitulated by using FasL-neutralizing antibody. We therefore generated NOD-gld/+ mice and determined whether FasL haploinsuf-

Table 1. NOD-scid-gld/gld Mice Are Resistant to Transfer of Diabetes by Splenocytes from Diabetic NOD-wt Mice

Donor	Recipient	Diabetic	Mean frequency of DN T cells	
			Lymph node	Spleen
NOD-gld/gld	Scid-wt	0 of 5	7.2	8.1
NOD-wt	Scid-gld/gld	1 of 6	4.1	5.0
NOD-wt	Scid-wt	5 of 5	1.7	2.2

NOD-scid-wt mice that received splenocytes from NOD-wt mice were used as controls. Mice in each group were monitored for induction of diabetes as described in Materials and Methods. The experiment was terminated after all control mice developed diabetes. Spleen and lymph node cells were stained for TCR, CD4, CD8, and B220 surface markers. After gating on TCR⁺ cells, the frequency of DN T cells in each organ was determined.

iciency could prevent T-cell autoimmunity without induction of DN T-cell lymphoproliferation. All NOD-gld/+ heterozygote mice had normal-sized lymphoid organs and developed no lymphadenopathy or splenomegaly that otherwise affected all age-matched NOD-gld/gld mice (Figure 2A). None of the NOD-gld/+ mice developed T-cell lymphoproliferation at any time during their lifespan (up to 2 years) indicating that they had maintained sufficient FasL activity to preserve T-cell homeostasis. This was confirmed by flow cytometry because DN T cells in the periphery of NOD-gld/+ mice expressed normal level of Fas similar to wt and gld/gld mice. Importantly, the percentage of gld/+ DN T cells that expressed significant FasL on their surface was comparable with that of FasL-expressing wt DN T cells. Moreover, even though not all surface FasL molecules on gld/+ DN T cells were expected to be functional, gld/+ DN T cells underwent spontaneous apoptosis that was similar to that of DN T cells in wt mice; as a result, less than 10% of T cells in the lymph nodes and spleens of NOD-gld/+ mice were DN T cells compared with approximately 80% in age-matched NOD-gld/gld mice and 5% in wt mice (Figure 2, B and C). We confirmed that apoptosis of DN T cells was Fas-mediated by showing that it was inhibitable by FasL-neutralizing antibody *in vivo* (data not shown).

NOD-gld+ mice were completely protected from developing diabetes (Figure 2D). We have not detected a single incidence of diabetes in NOD-gld/+ mice of various ages in our colony, whereas NOD-wt littermates developed diabetes with a predicted incidence rate. Insulinitis was also curtailed; the majority of NOD-gld/+ mice remained free of insulinitis, and fewer animals developed peri- or mild insulinitis that did not progress to full destruction of islets and diabetes (Figure 2E). NOD-gld/+ mice did not develop significant levels of antinuclear antibodies, whereas modest and infrequent ANAs were observed in NOD-gld/gld mice (Figure 2F). We concluded that complete inactivation of FasL was not essential for prevention of spontaneous diabetes in NOD mice and that partial blockade could be used to induce protection from autoimmune diabetes in wt mice without impairing T-cell homeostasis. Thus, the NOD-gld/+ mouse provides a model to study the role of FasL in T-cell tolerance without the complication caused by the massive DN T-cell accumulation.

Disease-Free NOD-gld/+ Mice Harbor Pathogenic Cells Capable of Causing Diabetes in Adoptive Hosts and in Situ

The resistance of NOD-gld/+ mice to diabetes could be due to the deletion or active control of diabetogenic T cells. To distinguish between these possibilities, we assessed the diabetogenicity of NOD-gld/+ splenocytes in NOD-scid hosts (Figure 3). As positive controls, we used scid hosts reconstituted with splenocytes from age- and sex-matched diabetic NOD-wt donors. All NOD-scid hosts that were reconstituted with splenocytes from NOD-gld/+ mice or NOD-wt mice developed diabetes by day 45 after transfer (Figure 3). Splenocytes from NOD-gld/+ mice, however, caused diabetes at slightly lower rate than did splenocytes from wt mice. Thus, NOD-gld/+ mice harbor a diabetogenic population within their T-cell repertoires that was able to cause diabetes in susceptible hosts. However, because homeostatic expansion in lymphopenic hosts might lead to extensive remodeling of the T-cell repertoire and favored expansion of diabetogenic cells, we determined whether self-tolerance in adult NOD-gld/+ mice could be reversed *in situ* using agents that augment T-cell pathogenicity or abrogate immune suppression. Blockade of CTLA-4 or IL-2 signaling as well as GITR stimulation (Materials and Methods) led to development of diabetes in adult NOD-gld/+ mice (Figure 4). CTLA-4 is a negative regulator of T-cell activation, and hence blockade of CTLA-4 can directly augment T-cell autoreactivity by releasing negative signaling²⁴ or indirectly by abrogation of suppression with or without deletion of Foxp3⁺CD4 regulatory T cells.^{25,26} Reverse of tolerance by anti-CTLA-4 treatment was associated with slight or no significant change in the frequency of Foxp3⁺CD4 regulatory T cells in NOD-gld/+ mice (Figure 4A). Treatment of NOD-gld/+ mice with anti-IL-2 antibody led to temporary loss of regulatory T cells (data not shown) and reversed resistance to diabetes in a significant number of treated mice (Figure 4B). Protection was also abrogated by anti-GITR treatment, which can both co-stimulate effector T cells²⁷ and inhibit suppressive activity of regulatory T cells (Figure 4C). These data show that NOD-gld/+ mice harbor diabetogenic T cells that can be turned on after transfer into susceptible hosts or by blockade of pathways that regulate T-cell tolerance.

Figure 2. Heterozygous gld mutation confers indefinite protection from diabetes without causing lymphoproliferation. **A:** Comparison of sizes of spleens and lymph nodes of 16-week-old NOD-gld/gld, NOD-gld/+, and NOD-wt mice. **B:** Expression of Fas (top) and FasL (bottom) by freshly isolated T cells from gld/gld, gld/+, and wt (+/+) mice. Isolated cells were stained with allophycocyanin-TCR β , PerCP-CD4, PerCP-CD8 α , fluorescein isothiocyanate-Fas, and phosphatidylethanolamine-FasL antibodies and analyzed by fluorescence-activated cell sorting. Gated TCR $^{+}$ cells were analyzed for Fas and FasL expression. Inclusion of CD4 and CD8 T cells in one subset by using PerCP-conjugated anti-CD4 and anti-CD8 antibodies allowed us to compare Fas and FasL expression on CD4 $^{+}$ or CD8 $^{+}$ T cells versus CD4 $^{-}$ /CD8 $^{-}$ DN T $^{+}$ cells, simultaneously. Only 9% of DN T cells from gld/gld mice expressed surface FasL compared with 42% of DN T cells in gld/+ mice and 48% in wt mice. **C:** Expression of a single functional FasL allele in NOD-gld/+ mice is sufficient to prevent peripheral DN T-cell accumulation (left) via apoptosis (middle) but with minimal effect on CD4 and CD8 T cells (right). Apoptosis was measured using Annexin V binding as described in Materials and Methods. Three mice were used per group. Results are mean $\pm$ SEM from two different experiments. **D:** Cumulative diabetes incidence in NOD-gld/gld ($n = 14$), NOD-gld/+ ($n = 35$), and NOD-wt ($n = 29$) mice that were up to 65 weeks of age. **E:** NOD-gld/+ mice develop mild or no insulinitis. Pancreata from 12-week-old NOD-wt, NOD-gld/+, or NOD-gld/gld mice were formalin-fixed, sectioned, H&E-stained, and compared with age-matched NOD-wt mice for insulinitis. Three mice per group were examined. The **inset** indicates incidence of either peri-insulinitis or insulinitis in the three mice (designated 1, 2, and 3) analyzed in each group. **F:** Heterozygote gld mutation does not cause production of ANAs. ANAs in the sera from NOD-gld/gld, NOD-gld/+, and NOD-wt mice between the ages of 15 to 30 weeks were measured using a ANA detection kit (Alpha Diagnostic International) as described in Materials and Methods ($n = 6$ per group). The concentration of ANAs in each sample is calculated as AI, which is defined as the ratio of absorbance of the test sample and net absorbance of the negative (endpoint-cutoff) control. AI >22 is considered positive.

Figure 3. Disease-free NOD-gld/+ mice harbor diabetogenic lymphocytes that transfer diabetes to NOD-scid mice. Diabetogenicity of 2×10^7 splenocytes from diabetes-free NOD-gld/+ mice was tested in adoptive NOD-scid hosts after intravenous transfer and compared with that of equal number of splenocytes from age-matched diabetic NOD-wt mice ($n = 7$ per group). Blood glucose levels were monitored weekly to determine diabetes induction. Mice with two consecutive blood glucose levels of ≥ 250 mg/dl were considered diabetic. Results are from one of two similar experiments.

FasL-Neutralizing Antibody Prevents Diabetes in NOD-wt Mice

Our analysis of NOD-gld/+ mice indicated that manifesting the pathogenic role of FasL required a high level of FasL expression, whereas low FasL expression was sufficient for prevention of DN T-cell lymphoproliferation. Thus, we sought to determine whether pharmacological partial blockade of FasL abrogated its pathogenic role but not its role in prevention of DN T-cell accumulation. To test this idea, we treated prediabetic NOD female mice with MFL4 FasL-neutralizing antibody²⁸ as described in Materials and Methods and monitored them for development of diabetes. Control mice that were treated in parallel with hamster IgG developed diabetes with the normal incidence rate. In contrast, none of the mice in the treated group developed diabetes (Figure 5, A and B). Analysis of pancreata from treated mice showed that blockade of FasL also prevented insulinitis in the majority of the mice in the group, although a few mice developed perinsulinitis, whereas severe insulinitis was observed in the control group (Figure 5C). Furthermore, anti-FasL treatment was associated with only a small and transient increase in the frequency of DN T cells, which did not exceed 6% of T cells in any of the treated mice (Figure 5D). As in NOD-gld/+ mice, protection from diabetes was not associated with systemic increase in the level of antinuclear antibodies (Figure 5E). Thus, it seems that there is a wide window for maneuvering to block most FasL activity to inhibit its pathogenic effect without interfering with T-cell homeostasis. These studies show the feasibility and suggest that FasL-based intervention may prove beneficial in the future to protect high-risk individuals from type 1 diabetes.

Discussion

This study shows that protection from autoimmune diabetes induced by gld mutation of FasL is genetically dissociable from gld-induced DN T-cell lymphoproliferation. Inactivation of FasL in either the hematopoietic or nonhematopoietic compartment is equally protective

Figure 4. Tolerance in NOD-gld/+ mice is reversed by blockade of the CTLA-4, IL-2 signaling, or anti-GITR treatment. NOD-gld/+ female mice were injected intraperitoneally with three doses of 100 μ g of anti-CTLA-4 (**A**), a single dose of 1 mg of anti-IL-2 (**B**), or two doses (50 μ g) per week for 4 consecutive weeks of anti-GITR (**C**). Age- and sex-matched NOD-gld/+ control mice in each group received parallel injections of phosphate-buffered saline. Mice were monitored for onset of diabetes by measuring blood glucose each week. We used 12-week-old NOD-gld/+ mice for CTLA-4 experiments and 8-week-old NOD-gld/+ mice for IL-2 and anti-GITR experiments. There were four mice in each group.

from diabetes, whereas inactivation of FasL in the hematopoietic tissues is largely responsible for DN T-cell lymphoproliferation. Hence, protection from diabetes without DN T-cell lymphoproliferation is seen when wt FasL is expressed on the hematopoietic tissues and the gld mutation is restricted to the nonhematopoietic tissues in BM chimeras (wt>chimeric gld) and adoptive hosts (wt>NOD-scid-gld/gld). Furthermore, analysis of NOD-gld/+ mice demonstrated that maintenance of some FasL activity is sufficient to control DN T-cell homeostasis

Figure 5. Treatment with FasL-neutralizing antibody prevents diabetes development in NOD-wt mice. Four-week-old NOD-wt mice were injected intraperitoneally with 500 μ g of anti-FasL MFL4 antibody ($n = 10$) or control hamster IgG ($n = 9$) for 2 consecutive weeks, followed by weekly 300- μ g injections until age 20 weeks. **A** and **B**: Diabetes incidence and blood glucose levels in mice treated with FasL-neutralizing antibody or hamster IgG control antibody. **C**: Anti-FasL treatment of NOD-wt mice curtailed development of insulinitis. Pancreata from three nondiabetic mice from each group were collected 9 weeks after termination of treatment and analyzed for insulinitis as described in Materials and Methods using H&E staining. Top shows representative islets with no insulinitis (left) or peri-insulinitis (right) from mice that received anti-FasL treatment. Bottom shows an islet with insulinitis from hamster IgG-treated control mice. The **inset** shows percentage of insulinitis, peri-insulinitis, and no insulinitis in the pancreatic islets of anti-FasL-treated (120 islets) and control mice (42 islets). **D**: Blockade of FasL leads to mild and transient increase in the frequency of DN T cells in peripheral blood. Blood samples were collected and stained with TCR-, CD4-, and CD8-specific antibodies and analyzed by fluorescence-activated cell sorting. After gating on TCR⁺ cells, the frequency of DN T cells was determined. **E**: AI in mice in the anti-FasL ($n = 10$) or hamster IgG ($n = 9$) groups or in untreated NOD-wt ($n = 3$). ANAs were measured as described in Figure 2F. AI value >27.5 is considered positive. Results are presented as mean $\pm$ SEM.

and that complete inhibition of FasL is not essential for restoring tolerance in NOD mice. These genetic findings are potentially translatable into therapeutic strategies, because administration of FasL-neutralizing antibody protects NOD-wt mice from diabetes without causing DN T-cell lymphoproliferation.

The T cell is a primary site of FasL induction,²³ and hence mutation of FasL on hematopoietic cells eliminates

a major source of FasL. In addition, FasL expressed on hematopoietic cells, including T cells, has a more direct role than does FasL expressed on the nonhematopoietic tissues in mediating activation-induced cell death in T-cell-autonomous or fratricide Fas/FasL interactions between neighboring T cells.^{29,30} Thus, the ability of FasL expressed on hematopoietic cells to prevent DN T-cell lymphoproliferation is consistent with the role of the Fas

pathway in regulating T-cell homeostasis. On the other hand, the crucial role of FasL expressed on the nonhematopoietic tissues in regulating T-cell tolerance is unexpected and provides new insight into the pathogenetic process of autoimmune diabetes. This novel role is demonstrated by the potent protective effect of the *gld* mutation expressed in the nonhematopoietic compartment from spontaneous (Figure 1) and adoptively transferred (Table 1) autoimmune diabetes. Interestingly, expression of the *gld* mutation on hematopoietic tissues is equally protective from autoimmune diabetes. These data indicate that FasL expressed in either compartment plays a specific pathogenic role in the autoimmune process that cannot be substituted for by FasL expressed in the other compartment. FasL is not only a ligand for Fas, but also transduces inflammatory signals.³¹ Hence, failure of FasL expressed on the hematopoietic tissues to substitute for the pathogenic role of FasL expressed on the nonhematopoietic tissues can be due to specificity of inflammatory signals derived from engagement of FasL expressed in either compartment. Alternatively, limited accessibility of FasL expressed in one tissue to Fas-expressing cells in the other tissue could also explain why development of autoimmune diabetes depends on expression of FasL in both compartments. We therefore propose a two-signal model for FasL in the pathogenesis of autoimmune diabetes. FasL expressed in the hematopoietic compartment provides one signal, whereas FasL expressed in the nonhematopoietic compartment provides the second signal. Consistent with this view, parenchymal tissue cells, including vascular smooth muscle,³² intestinal epithelia,³³ astrocytes,³⁴ and synoviocytes,³⁵ secrete proinflammatory cytokines and chemotactic factors in response to Fas engagement and thus can represent an important source of Fas-induced proinflammatory mediators. Intrinsic signals generated by engagement of FasL on the nonhematopoietic cells could induce secretion of soluble mediators that target and modulate responses of hematopoietic cells. On the other hand, engagement of Fas expressed on the hematopoietic tissues, including T cells,³⁶ dendritic cells,^{37–39} and neutrophils,^{40,41} by FasL modulates proliferation, maturation, and production of inflammatory cytokines. Interaction of Fas on β cells by FasL on T cells is an example of potential *trans*-interaction between Fas expressed in one compartment with FasL expressed in the other compartment. Thus, it is possible that some of the diabetogenic signals are dependent on interactions of Fas expressed on hematopoietic cells with FasL expressed on the nonhematopoietic tissues and vice versa and that this critical pathogenic role is inhibited by the *gld* mutation in either compartment. Similar protective effect is achieved by partial but global mutation of FasL in NOD-*gld*/+ mice. Likewise, the long-term protective effect of FasL-neutralizing antibody is probably mediated by partial neutralization of FasL in both the hematopoietic and nonhematopoietic compartments.

Mature wt T cells that developed in a FasL-sufficient environment and isolated from diabetic NOD mice failed to cause diabetes in NOD-*scid*-*gld*/*gld* mice, although they readily caused diabetes in NOD-*scid* mice with an

intact Fas pathway (Table 1). The failure of wt T cells to cause diabetes in mice expressing the *gld*/*gld* mutation on the nonhematopoietic tissues is not due to impaired Fas-mediated killing of β cells, which were Fas-sufficient in both host types, whereas T cells are FasL sufficient. Therefore, the protection induced by heterozygous global *gld* mutation or compartmentalized *gld* mutation in chimeras and adoptive host or by FasL blockade in NOD-wt mice using FasL-neutralizing antibody is not due to inhibition of Fas-mediated apoptosis of β cells. These results corroborate previous studies that show no or a limited role for the Fas/FasL system in the death of β cells.^{4,5,7–9,12} Last but not least, *gld*-mediated protection from diabetes is not dominated by a specific pathway because blockade of CTLA-4 or IL-2 signaling pathway reversed tolerance in NOD-*gld*/+ mice that otherwise have life-long protection from diabetes. Likewise, treatment of NOD-*gld*/+ mice by anti-GITR antibody reversed tolerance and caused diabetes in NOD mice. Future studies should define specific effects of the *gld* mutation on these pathways and on the homeostasis of natural and induced regulatory T cells.

In summary, these data provide the basis for future mechanistic and therapeutic studies for understanding further how targeting FasL on specific compartments regulates T-cell tolerance mechanisms. The NOD-*gld*/+ mouse provides a model for analyzing the long-term consequences of partial FasL blockade on T-cell tolerance and potential side effects in the absence of DN T-cell lymphoproliferation. Moreover, our findings that genetic or therapeutic inhibition of FasL protects against diabetes without causing lymphoproliferation indicate the potential for developing a new class of FasL-based therapeutics that do not interfere with vital activation pathways.

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References

- Steinman L: Blocking adhesion molecules as therapy for multiple sclerosis: natalizumab. *Nat Rev Drug Discov* 2005, 4:510–518
- Chervonsky AV, Wang Y, Wong FS, Visintin I, Flavell RA, Janeway Jr CA, Matis LA: The role of Fas in autoimmune diabetes. *Cell* 1997, 89:17–24
- Kreuwel HT, Sherman LA: The role of Fas-FasL in CD8+ T-cell-mediated insulin-dependent diabetes mellitus (IDDM). *J Clin Immunol* 2001, 21:15–18
- Su X, Hu Q, Kristan JM, Costa C, Shen Y, Gero D, Matis LA, Wang Y: Significant role for Fas in the pathogenesis of autoimmune diabetes. *J Immunol* 2000, 164:2523–2532
- Savinov AY, Tcherepanov A, Green EA, Flavell RA, Chervonsky AV: Contribution of Fas to diabetes development. *Proc Natl Acad Sci USA* 2003, 100:628–632
- Itoh N, Imagawa A, Hanafusa T, Waguri M, Yamamoto K, Iwahashi H, Moriwaki M, Nakajima H, Miyagawa J, Namba M, Makino S, Nagata S, Kono N, Matsuzawa Y: Requirement of Fas for the development of autoimmune diabetes in nonobese diabetic mice. *J Exp Med* 1997, 186:613–618
- Kim S, Kim KA, Hwang DY, Lee TH, Kayagaki N, Yagita H, Lee MS:

- Inhibition of autoimmune diabetes by Fas ligand: the paradox is solved. *J Immunol* 2000, 164:2931–2936
8. Thomas HE, Darwiche R, Corbett JA, Kay TW: Evidence that beta cell death in the nonobese diabetic mouse is Fas independent. *J Immunol* 1999, 163:1562–1569
9. Apostolou I, Hao Z, Rajewsky K, von Boehmer H: Effective destruction of Fas-deficient insulin-producing beta cells in type 1 diabetes. *J Exp Med* 2003, 198:1103–1106
10. Kim YH, Kim S, Kim KA, Yagita H, Kayagaki N, Kim KW, Lee MS: Apoptosis of pancreatic beta-cells detected in accelerated diabetes of NOD mice: no role of Fas-Fas ligand interaction in autoimmune diabetes. *Eur J Immunol* 1999, 29:455–465
11. Anderson MS, Bluestone JA: The NOD mouse: a model of immune dysregulation. *Annu Rev Immunol* 2005, 23:447–485
12. Allison J, Strasser A: Mechanisms of beta cell death in diabetes: a minor role for CD95. *Proc Natl Acad Sci USA* 1998, 95:13818–13822
13. Sabelko KA, Kelly KA, Nahm MH, Cross AH, Russell JH: Fas and Fas ligand enhance the pathogenesis of experimental allergic encephalomyelitis, but are not essential for immune privilege in the central nervous system. *J Immunol* 1997, 159:3096–3099
14. Waldner H, Sobel RA, Howard E, Kuchroo VK: Fas- and FasL-deficient mice are resistant to induction of autoimmune encephalomyelitis. *J Immunol* 1997, 159:3100–3103
15. Nagata S, Golstein P: The Fas death factor. *Science* 1995, 267:1449–1456
16. Cohen P, Eisenberg R: Lpr and gld: single gene models of systemic autoimmunity and lymphoproliferative disease. *Annu Rev Immunol* 1991, 9:243–269
17. Wicker LS, Todd JA, Peterson LB: Genetic control of autoimmune diabetes in the NOD mouse. *Annu Rev Immunol* 1995, 13:179–200
18. Serreze DV, Leiter EH: Defective activation of T suppressor cell function in nonobese diabetic mice: potential relation to cytokine deficiencies. *J Immunol* 1988, 140:3801–3807
19. Hattori K, Hirano T, Ushiyama C, Miyajima H, Yamakawa N, Ebata T, Wada Y, Ikeda S, Yoshino K, Tateno M, Oshimi K, Kayagaki N, Yagita H, Okumura K: A metalloproteinase inhibitor prevents lethal acute graft-versus-host disease in mice. *Blood* 1997, 90:542–548
20. Pinkoski MJ, Droin NM, Lin T, Genestier L, Ferguson TA, Green DR: Nonlymphoid Fas ligand in peptide-induced peripheral lymphocyte deletion. *Proc Natl Acad Sci USA* 2002, 99:16174–16179
21. Pestano GA, Zhou Y, Trimble LA, Daley J, Weber GF, Cantor H: Inactivation of misselected CD8 T cells by CD8 gene methylation and cell death. *Science* 1999, 284:1187–1191
22. Orlicki JR, Vaishnav AK, Elkon KB: Structure and function of Fas/Fas ligand. *Int Rev Immunol* 1999, 18:293–308
23. Siegel RM, Chan FK, Chun HJ, Lenardo MJ: The multifaceted role of Fas signaling in immune cell homeostasis and autoimmunity. *Nat Immunol* 2000, 1:469–474
24. Korman AJ, Peggs KS, Allison JP: Checkpoint blockade in cancer immunotherapy. *Adv Immunol* 2006, 90:297–339
25. Read S, Greenwald R, Izcue A, Robinson N, Mandelbrot D, Francisco L, Sharpe AH, Powrie F: Blockade of CTLA-4 on CD4+CD25+ regulatory T cells abrogates their function in vivo. *J Immunol* 2006, 177:4376–4383
26. Maker AV, Attia P, Rosenberg SA: Analysis of the cellular mechanism of antitumor responses and autoimmunity in patients treated with CTLA-4 blockade. *J Immunol* 2005, 175:7746–7754
27. Shevach E: Regulatory T cells in autoimmunity. *Annu Rev Immunol* 2000, 18:423–449
28. Kayagaki N, Yamaguchi N, Nagao F, Matsuo S, Maeda H, Okumura K, Yagita H: Polymorphism of murine Fas ligand that affects the biological activity. *Proc Natl Acad Sci USA* 1997, 94:3914–3919
29. Brunner T, Mog R, LaFace D, Yoo N, Mahboubi A, Echeverri F, Martin S, Force W, Lynch D, Ware C, Green D: Cell autonomous Fas (CD95)/Fas ligand interaction mediates activation induced apoptosis in T cell hybridomas. *Nature* 1995, 373:441–444
30. Dhein J, Walczack H, Baumler C, Debatin K-M, Krammer P: Autocrine T cell suicide mediated by APO-1 (Fas/CD95). *Nature* 1995, 373:438–441
31. Desbarats J, Duke RC, Newell MK: Newly discovered role for Fas ligand in the cell-cycle arrest of CD4+ T cells. *Nat Med* 1998, 4:1377–1382
32. Schaub FJ, Han DK, Liles WC, Adams LD, Coats SA, Ramchandran RK, Seifert RA, Schwartz SM, Bowen-Pope DF: Fas/FADD-mediated activation of a specific program of inflammatory gene expression in vascular smooth muscle cells. *Nat Med* 2000, 6:790–796
33. Abreu-Martin MT, Vidrich A, Lynch DH, Targan SR: Divergent induction of apoptosis and IL-8 secretion in HT-29 cells in response to TNF-alpha and ligation of Fas antigen. *J Immunol* 1995, 155:4147–4154
34. Saas P, Boucraut J, Quiquerez AL, Schnuriger V, Perrin G, Desplat-Jego S, Bernard D, Walker PR, Dietrich PY: CD95 (Fas/Apo-1) as a receptor governing astrocyte apoptotic or inflammatory responses: a key role in brain inflammation? *J Immunol* 1999, 162:2326–2333
35. Sekine C, Yagita H, Kobata T, Hasunuma T, Nishioka K, Okumura K: Fas-mediated stimulation induces IL-8 secretion by rheumatoid arthritis synoviocytes independently of CPP32-mediated apoptosis. *Biochem Biophys Res Commun* 1996, 228:14–20
36. Vence L, Benoist C, Mathis D: Fas deficiency prevents type 1 diabetes by inducing hyporesponsiveness in islet beta-cell-reactive T-cells. *Diabetes* 2004, 53:2797–2803
37. Ashany D, Savir A, Bhardwaj N, Elkon KB: Dendritic cells are resistant to apoptosis through the Fas (CD95/APO-1) pathway. *J Immunol* 1999, 163:5303–5311
38. Guo Z, Zhang M, Tang H, Cao X: Fas signal links innate and adaptive immunity by promoting dendritic-cell secretion of CC and CXC chemokines. *Blood* 2005, 106:2033–2041
39. Rescigno M, Piguat V, Valzasina B, Lens S, Zubler R, French L, Kindler V, Tschopp J, Ricciardi-Castagnoli P: Fas engagement induces the maturation of dendritic cells (DCs), the release of interleukin (IL)-1beta, and the production of interferon gamma in the absence of IL-12 during DC-T cell cognate interaction: a new role for Fas ligand in inflammatory responses. *J Exp Med* 2000, 192:1661–1668
40. Seino K, Kayagaki N, Okumura K, Yagita H: Antitumor effect of locally produced CD95 ligand. *Nat Med* 1997, 3:165–170
41. Hohlbaum AM, Moe S, Marshak-Rothstein A: Opposing effects of transmembrane and soluble Fas ligand expression on inflammation and tumor cell survival. *J Exp Med* 2000, 191:1209–1220