siderably lower than that observed in age- and sex-matched NOD-*scid* mice or young prediabetic NOD mice [which are estimated to have ≥2000 islets per NOD mouse (*5*)]. The average diameter of the islets from Rx-NOD mice was 5.2 ± 0.1 times as large as that of age-matched NOD-*scid* mice (Fig. 1, C, D, and F; $P < 0.05$). This increase in islet size was due not to hypertrophy of individual β cells but to increased numbers of β cells and not of glucagon-expressing α cells (fig. S2). We estimated that the total β cell volume in the Rx-NOD mice was 22.5 ± 4.1% of that of age-matched NOD-*scid* mice. Similarly low numbers (12.2 ± 1.7) of significantly enlarged islets ($P < 0.05$) were observed in age-matched NOD mice that failed to develop spontaneous diabetes (Fig. 1, D to F).

To determine whether the β cells in the Rx-NOD were of host origin or derived from donor spleen cells, we examined all the Rx-NOD islets for expression of GFP. No β cells expressing GFP were detected in any of the islets examined (Fig. 1, G and H) or in the organs outside of the pancreas. Thus, our data do not support a conclusion of β cell regeneration from spleen-derived stem cells. All the hyperplastic islets from the five cured Rx-NOD mice were associated with circumferentially distributed lymphocytes (peri-insulitis) of donor origin (fig. S3) and devoid of invasive insulitis. The majority of these peri-islet lymphocytes were $CD4^+CD25^+$ cells, with a small portion expressing the transcriptional factor $FoxP3^+$ (fig. S3) (*6*). These observations suggest that regulatory T cells may function to control autoimmunity locally where β cells are situated. The islets that stained very weakly or did not stain for insulin in the one Rx-NOD mouse without hyperplasia did not have a notable peri-islet lymphocytic infiltrate.

The finding that autoimmune diabetes in NOD mice can be reversed by a complex protocol combining islet transplantation, FCA treatment, and transfer of semi-allogeneic splenocytes suggests a potential for novel β cell replacement therapies for human T1DM (*2*, *7*). We confirmed these observations and demonstrated restoration of diabetic NOD mice to normoglycemia with this therapeutic protocol. We did not observe β cell reconstitution from the infused spleen cells, and these studies were not designed to determine whether the observed host-derived β cells were the result of replication of preexisting β cells or differentiation from stem-cell precursors. Our studies indicate that normoglycemia in Rx-NOD mice can be maintained by a very low number of hyperplastic islets or, in one case, by weakly insulin-positive or insulin-negative islets surviving the autoimmunity. The consistent association between islet hyperplasia and peri-insulitis (*8*, *9*) suggests a hypothesis that autoreactivity, when restrained by regulatory T cells, may facilitate the development of islet hyperplasia. The limited β cell mass required to maintain normoglycemia in this model contrasts with the large numbers of transplanted islets required to maintain normal glycemia (*1*, *10*). Our studies confirm that autoimmune diabetes can be reversed and that sufficient endogenous β cell mass can be restored to cure diabetic NOD mice with the treatment protocol developed by Faustman and colleagues (*2*, *7*). Translating these findings into therapies useful for curing T1DM in humans remains a challenge.

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Islet Recovery and Reversal of Murine Type 1 Diabetes in the Absence of Any Infused Spleen Cell Contribution

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A cure for type 1 diabetes will probably require the provision or elicitation of new pancreatic islet β cells as well as the reestablishment of immunological tolerance. A 2003 study reported achievement of both advances in the NOD mouse model by coupling injection of Freund's complete adjuvant with infusion of allogeneic spleen cells. It was concluded that the adjuvant eliminated anti-islet autoimmunity and the donor splenocytes differentiated into insulin-producing (presumably β) cells, culminating in islet regeneration. Here, we provide data indicating that the recovered islets were all of host origin, reflecting that the diabetic NOD mice actually retain substantial β cell mass, which can be rejuvenated/regenerated to reverse disease upon adjuvant-dependent dampening of autoimmunity.

Type 1 diabetes, an autoimmune disease that targets pancreatic islet β cells, is an important and increasing health problem. It is generally believed that effective therapy for autoimmune diabetes will require two scientific advances: (i) restoration of insulin production by providing or eliciting new β cells, and (ii) repair of the breakdown in immunological tolerance that precipitated the disease in the first place.

Recently, successful achievement of both of these advances was reported, culminating in disease abrogation in a fraction of severely diabetic NOD mice (*1*), a widely used murine model of the human type 1 disorder. The protocol incorporated injection of a single dose of Freund's complete adjuvant (FCA) into severely diabetic mice, coupled with repeated infusion of allogeneic splenocytes, resulting in restoration of normoglycemia and permanent disease extinction. The conclusion of this study was that the FCA had eliminated anti-islet autoimmunity and that the donor splenocytes had differentiated into insulin-producing (presumably β) cells, ultimately leading to islet regeneration.

Although FCA has been used for a number of years in a variety of experimental protocols to modulate diabetes in NOD mice (*2*, *3*), the concept of donor splenocytes giving rise to islets in a host pancreas was a novel and exciting one, prompting the suggestion that the function of the spleen should be radically reappraised to include a role as a reservoir of multilineage stem cells (*4*). We set out to further dissect the underlying mechanisms of diabetes reversal by this treatment regime.

As a first step, we attempted to replicate the original findings, following closely the protocol published by Kodama *et al.* (*1*) and incorpo-

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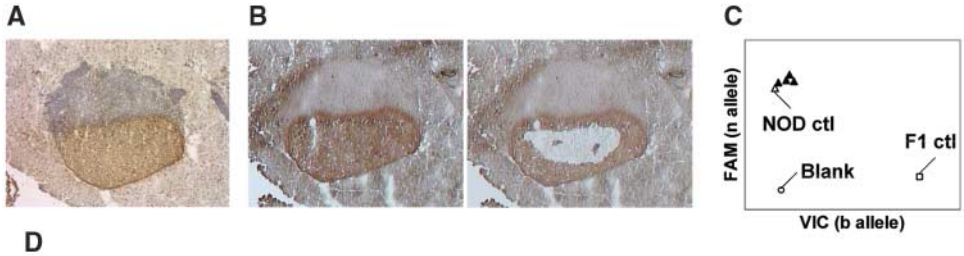
Table 1. Response profiles of treated mice. The treatment protocol is described in (*5*). BG, blood glucose.

	Mouse ID	Age at onset (weeks)	Maximum BG before transplantation	Days with BG >400 mg/dl	Number of transplanted islets	BG on first day after transplantation (mg/dl)	Number of days with BG <250 mg/dl after transplantation*	Overall course
Successful transplantation for 120 days → nondiabetic after nephrectomy	132	23	476	3	650	129	151	Nephrectomy on day 120, still nondiabetic at day 151
	138	30	499	3	800	147	186	Nephrectomy on day 120, still nondiabetic at day 186
	154	26	401	3	800	120	180	Nephrectomy on day 120, still nondiabetic at day 180
	155	35	406	7	800	74	151	Nephrectomy on day 124, still nondiabetic at day 151
	89	19	459	7	800	135	144	Nephrectomy on day 124, still nondiabetic at day 144
Successful transplantation for 120 days → diabetic after nephrectomy	78	37	403	3	650	51	122	Nephrectomy on day 120, diabetic 3 days later
	114	39	458	7	800	105	120	Nephrectomy on day 120, diabetic 1 day later
	11	29	597	7	800	83	123	Nephrectomy on day 123, diabetic 1 day later
	76	27	458	3	800	50	123	Nephrectomy on day 124, diabetic 1 day later
Recurrent diabetes before day 120	15	29	586	3	800	68	60	Spontaneously diabetic
	174	20	>600	3	800	139	46	Spontaneously diabetic
	74	24	584	3	800	101	46	Spontaneously diabetic
	78	18	547	3	800	136	25	Spontaneously diabetic
	84	24	591	14	650	134	24	Spontaneously diabetic
	128	27	505	3	800	125	22	Spontaneously diabetic
	120	44	>600	7	650	93	11	Spontaneously diabetic
	10	25	532	3	800	104	11	Spontaneously diabetic
	81	18	450	3	800	97	11	Spontaneously diabetic
	159	26	502	3	800	97	5	Spontaneously diabetic
	141	21	513	1	650	86	4	Spontaneously diabetic
	72	17	514	3	800	64	4	Spontaneously diabetic
	89	25	>600	8	650	134	2 to 7	Spontaneously diabetic
	86	26	>600	12	650	125	2 to 7	Spontaneously diabetic
	98	26	>600	1	650	90	2 to 7	Spontaneously diabetic
	136	19	541	7	650	83	2 to 3	Spontaneously diabetic
	170	16	581	7	800	172	2 to 3	Spontaneously diabetic
	36	30	543	3	800	84	2 to 3	Spontaneously diabetic
	81	40	>600	7	650	81	2	Spontaneously diabetic
	23	25	432	7	800	113	2	Spontaneously diabetic
	47	24	412	3	800	86	2	Spontaneously diabetic

*A few mice showed transient blood glucose values >250 mg/dl during the follow-up period, but these normalized in the following days, and these excursions were not considered in the evaluation of posttransplant success or failure.

rating additional details from more extensive protocols provided by the authors (*5*). Severely diabetic NOD mice (blood glucose >400 mg/dl) were transplanted under the kidney capsule with syngeneic islets in order to maintain insulin levels; engraftment was deemed a failure when hyperglycemia reappeared before 2 days. Thirty successfully transplanted mice were injected with FCA and infused with live allogeneic [C57BL/6 × Balb/c (referred to as CB6 F1)] splenocytes (Table 1). The majority (21 of 30) developed diabetes before the end of the 120-day observation period (2 to 60 days after islet transfer). We do not know why the 70% reversion rate observed here was higher than the 8% reported by Kodama *et al*. (*1*). After removal of the transplanted islets from the nine mice that had completed the 120-day observation period, four animals became diabetic shortly thereafter, indicating that the insulin that permitted their survival prenephrectomy was produced mainly by the grafted islet cells. The other five animals remained normoglycemic to termination, indicating that they carried insulin-producing cells outside the islet graft, which the immune system no longer destroyed. This 56% of long-term survivors that were insulin–self-sufficient was similar to the 67% reported by Kodama *et al*. (*1*).

Histology of the pancreas revealed islets of various sizes (fig. S1A), including a hyperplastic subset found in both the "cured" and "revertant" long-term survivors, although more frequent in the former (e.g., fig. S1B). All mice had insulitis (fig. S1, A to D) as well as some islets free of



D

Mouse ID	DNA sample ID	Dissected area	Nucleotide at SNP		
			rs4223216 (Chr 2)	rs4230624 (Chr 15)	rs4151928 (Chr 16)
#132	#132_1	normal size islet without insulitis	x	A	x
#138	#138_2	hyperplastic islet with peri-insulitis	x	x	x
	#138_3	hyperplastic islet with peri-insulitis	G	A	C
	#138_4	hyperplastic islet with peri-insulitis	G	A	C
	#138_5	exocrine tissue	G	A	C
#154	#154_1	hyperplastic islet with peri-insulitis	n.d.	n.d.	C
	#154_2	normal size islet without insulitis	G	A	C
	#154_3	normal size islet without insulitis	G	A	C
	#154_4	normal size islet without insulitis	G	A	C
	#154_5	exocrine tissue	G	A	C
control	NOD	islet	G	A	C
	F1	islet	A	C	G

Fig. 1. Laser-capture microdissection and genotyping of islets from "reverted" mice. (**A**) Anti-insulin staining of an islet from mouse 154. (**B**) An adjacent section before (left) and after (right) microdissection. (**C**) Typical genotyping by fluorogenic polymerase chain reaction for SNP rs4151928, detecting the alleles present in NOD or CB6 F1 mice as FAM or VIC fluorescence, respectively. Controls shown are DNA from microdissected islets of a nondiabetic NOD mouse and from an F_1 mouse. Solid triangles denote several samples microdissected from islets of mouse 154. (**D**) Summary of SNP genotyping of microdissected islets from mice 132, 138, and 154 (all from the first group in Table 1). Nucleotides detected for three different SNP positions (rs4223216, rs4230624, and rs4151928) in microdissected islets are shown, with the corresponding nucleotides for control NOD and F_1 DNA (x, unsuccessful amplification; n.d., not done).

infiltrate (fig. S1, A and E). Many islets exhibited an innocuous-looking infiltrate like that described in a number of contexts wherein insulitis does not progress to overt diabetes (*6*). Indeed, there was a striking similarity to the islets of recent-onset diabetic NOD mice treated with anti-CD3 monoclonal antibody (mAb) (fig. S1F). Insulitis was also observed in the syngeneic islet graft (fig. S1G).

We next evaluated the possibility that pancreatic islets were regenerated through differentiation of donor splenocytes. Because the fluorescence in situ hybridization (FISH) assays used in the previous study (*1*) can be challenging and their results can be equivocal, we established a more robust test based on single-nucleotide polymorphism (SNP) analysis of DNA derived from islet tissue isolated from histological sections by laser-capture microdissection (LCM). Islet tissue and any associated inflammation could be definitively identified by staining sections with hematoxylin and/or anti-insulin mAb (Fig. 1A) and could be cleanly excised (Fig. 1B). The SNP analysis assessed three polymorphic loci on three separate chromosomes (Fig. 1C) and clearly distinguished cells of NOD and CB6 F1 origin. In all "cured" animals tested, all tissue was derived from NOD host or graft cells; none came from CB6 F1 donor splenocytes (Fig. 1D).

This result is actually the expected one, given that the NOD host should make a strong alloresponse to the splenocytes' large major histocompatibility complex (MHC) divergence (K^b, D^b, L^d, A^d, E^d, and A^b in the CB6 F1s). We found no evidence of chimerism in the spleen or lymph nodes subsequent to the splenocyte infusions (fig. S2). Instead, strong alloreactivity was evident from the development of antibodies to the allogeneic splenocytes at titers up to 1/5000 (*7*).

The fleeting presence of the splenocytes raised the possibility that the protocol's beneficial effects might derive primarily from the FCA injection. This adjuvant is known to have immunomodulatory effects in the NOD context (*2*, *3*), but a FCA-alone control was not included in the report by Kodama *et al.* (*1*). Therefore, under otherwise the same protocol, we treated 13 severely diabetic NOD mice with FCA and a syngeneic islet transplant but not with allogeneic splenocytes. Four of these animals maintained normal glucose levels during the 120-day observation period, and three of these remained normoglycemic after removal of the transplanted islets (table S1).

A likely source of the insulin that permitted certain long-term survivors to remain nondiabetic is residual islets that expanded through either differentiation or replication once autoimmunity had been muted. Such a scenario is consistent with recent reports that substantial β cell mass can be detected in diabetic NOD mice (*8*). To test this notion, we performed a morphometric analysis of pancreatic β cell mass both in the prediabetic state (n = 3) and in the first weeks after the development of severe hyperglycemia (n = 23). Residual β cell mass was detected in essentially all of the diabetic mice (fig. S3), although it was variable in quantity and was always diminished relative to that of prediabetic comparators. There was no correlation between the blood glucose value and residual β cell mass (*9*). These results indicate that host β cells can contribute to islet regeneration promoting disease reversal in severely diabetic NOD mice.

Our experiments, like those of Kodama *et al.* (*1*), yielded a fraction of previously diabetic NOD mice that survived for a long term after syngeneic islet transplantation, a proportion of which remained normoglycemic even after the removal of the grafted islets. On the other hand, we found no evidence that the source of insulin underlying the reversal of diabetes was islet cells derived from donor splenocytes. Rather, we found that the diabetic hosts had substantial residual β cell mass and that the recovered/expanded islets were all of NOD origin rather than splenocyte CB6 F1 origin. Our conclusions match very well those of two accompanying reports (*10*, *11*). Given recent reports of active β cell replication in both nondiabetic (*12*) and diabetic (*8*) states, the most likely explanation for islet recovery in this context is that the dampening of autoimmunity permitted β cell replication to outdo β cell death. However, other explanations remain possible: differentiation of new host β cells or seeding and expansion of islet graft–derived cells (also of NOD genotype).

Should the autonomous efficacy of FCA demonstrated in our study prompt a reconsideration of the use of the analogous reagent Bacille Calmette-Guérin (BCG), in isolation, to treat diabetic humans? Although a preliminary study with 17 newly diagnosed individuals appeared to show some therapeutic efficacy (*13*), three more extensive, double-blind trials failed to demonstrate a positive effect (*14*). Likely explanations for the divergent outcomes are simply that FCA and BCG are different reagents, or that the footpad injection of FCA

used for mice and the subcutaneous injection of BCG applied to humans are radically different interventions, the former provoking a massive systemic inflammatory response (*15*) difficult to contemplate for human patients.

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Immunological Reversal of Autoimmune Diabetes Without Hematopoietic Replacement of β Cells

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Type 1 diabetes mellitus results from the autoimmune destruction of the β cells of the pancreatic islets of Langerhans and is recapitulated in the nonobese diabetic strain of mice. In an attempt to rescue islet loss, diabetic mice were made normoglycemic by islet transplantation and immunization with Freund's complete adjuvant along with multiple injections of allogeneic male splenocytes. This treatment allowed for survival of transplanted islets and recovery of endogenous β cell function in a proportion of mice, but with no evidence for allogeneic splenocyte–derived differentiation of new islet β cells. Control of the autoimmune disease at a crucial time in diabetogenesis can result in recovery of β cell function.

It is now generally accepted that two issues must be addressed for the successful treatment of type 1 diabetes mellitus (T1DM), an autoimmune disease in which T cells kill the β cells responsible for insulin production (*1*). The persistent autoreactivity to β cell antigens that characterize the disease needs to be controlled and the β cell mass, which is extensively reduced at the time of onset of clinical disease, must be restored. A treatment protocol was recently developed in nonobese diabetic (NOD) mice, an extensively used model of T1DM, that resulted in cessation of autoimmunity and reversal of diabetes through the generation of new β cells from splenic cells (*2*, *3*). Because of the importance of these conclusions in offering novel clinical strategies for disease intervention, we repeated the same experimental protocol as a prelude to further analysis of the process.

The major protocol from the paper of Kodama *et al*. involved three experimental manipulations in female diabetic NOD mice made between 7 and 20 days after the development of hyperglycemia (*2*, *3*). First, a single subcutaneous injection of Freund's complete adjuvant (FCA) was administered, which is known to stop the autoimmune process in diabetic NOD mice (*4*–*6*). Second, the mice were given a series of intravenous injections of spleen cells derived from major histocompatibility complex (MHC)–mismatched donor F1 (CByB6F1) male mice. Finally, to control the hyperglycemia, syngeneic islets were transplanted under the capsule of one kidney. The mice were followed for a period of at least 120 days after transplant, at which time nephrectomy was performed to remove the islet grafts. Most mice remained normoglycemic, which indicates that β cell function in the pancreas was restored during the time of immunological control (*2*). Moreover, the islets were reported to be derived from the injected spleen cells because they contained the male Y-chromosome marker of the injected male cells (*2*).

In our experiments, female NOD mice were maintained on insulin for 7 to 20 days before the three treatments after the first indications of hyperglycemia, to ensure that the islet β cell mass was depleted (*7*). Mice that remained persistently normoglycemic (22 out of 53) were followed for at least 120 days after treatments. (The 31 transplanted mice that developed periods of hyperglycemia before the 120-day observation period were eliminated.) A nephrectomy, which also removed the transplanted islets placed under the capsule, was performed between days 120 and 146 after transplantation. Examples of experimental mice that reverted back to diabetes, or that maintained normoglycemia after nephrectomy, are shown in Fig. 1. The large majority of the mice (82%) subsequently reverted to the diabetic state (Table 1), which suggests that endogenous β cell function had not been restored in these individuals, and histological analysis confirmed the presence of very few small islets made up entirely of glucagon-positive cells (*8*). Thus, no insulin-positive cells were detected either in the few remaining small islets or in the ducts of revertant mice (*8*). In other experiments, the same manipulations were performed with FCA injections and islet transplants (*5*) but without the inocula of allogeneic cells. In this case, 20 of 29 mice became normoglycemic, some for as long as >100 days (*8*), an indication that the

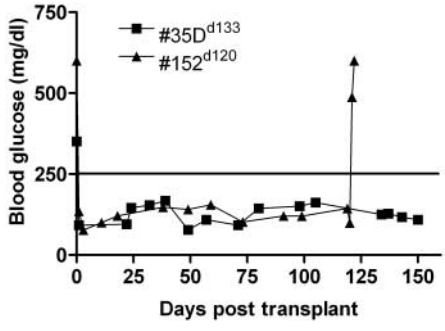

Fig. 1. Serum glucose levels of two representative mice treated according to the major protocol of Kodama *et al*. (*2*, *7*). Transplanted mice were followed for at least 120 days, after which the islet transplant under the kidney capsule was removed by nephrectomy. The day of nephrectomy for each animal is indicated in superscript. Of the two mice, one became diabetic immediately after nephrectomy, whereas the other remained normoglycemic.

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