In Autoimmune Diabetes the Transition from Benign to Pernicious Insulitis Requires an Islet Cell Response to Tumor Necrosis Factor α

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Summary

The islet-infiltrating and disease-causing leukocytes that are a hallmark of insulin-dependent diabetes mellitus produce and respond to a set of cytokine molecules. Of these, interleukin 1β, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ are perhaps the most important. However, as pleiotropic molecules, they can impact the path leading to β cell apoptosis and diabetes at multiple points. To understand how these cytokines influence both the formative and effector phases of insulitis, it is critical to determine their effects on the assorted cell types comprising the lesion: the effector T cells, antigen-presenting cells, vascular endothelium, and target islet tissue. Here, we report using nonobese diabetic chimeric mice harboring islets deficient in specific cytokine receptors or cytokine-induced effector molecules to assess how these compartmentalized loss-of-function mutations alter the events leading to diabetes. We found that islets deficient in Fas, IFN-γ receptor, or inducible nitric oxide synthase had normal diabetes development; however, the specific lack of TNF-α receptor 1 (p55) afforded islets a profound protection from disease by altering the ability of islet-reactive, CD4+ T cells to establish insulitis and subsequently destroy islet β cells. These results argue that islet cells play a TNF-α-dependent role in their own demise.

Key words: autoimmunity • diabetes • tumor necrosis factor α receptor • T cells • insulitis

Abbreviations used in this paper: CFSE, 5,6-carboxy-succinimidyl-fluorescein-ester; IDDM, insulin-dependent diabetes mellitus; iNOS, inducible NO synthase; NO, nitric oxide; NOD, nonobese diabetic.
lute deficiency of the IFN-γ gene in NOD mice had little effect on the overall development of diabetes (23).

To further complicate matters, many proinflammatory cytokines not only promote inflammation but may also facilitate the localized destruction of target tissue. For example, IFN-γ, TNF-α, and IL-1β have all been implicated in the cytolytic activation of a number of cell types, including pancreatic β cells (24, 25). TNF-α and IFN-γ have been shown to directly induce apoptosis (24–26), whereas IL-1β may act indirectly through the induction of reactive nitrogen intermediates (NO) on the islet cell surface (27–29). Here again, the pluripotent nature of cytokines has made it difficult to dissect and ascribe precisely what role they play in the actual destruction of β cells in vivo.

Similarly, understanding β cell death has been hampered by the inability to study individual apoptotic pathways in isolation. This is perhaps best exemplified by recent studies on Fas (CD95) as a potential inducer of cell death (30, 31). Although Fas-deficient NOD mice (NOD.scid) do not develop diabetes, the global loss of Fas expression in these mice affects not only the pancreatic β cell but the critical T and B lymphocyte populations as well. Therefore, it is difficult to assign the protection from diabetes seen in these mice to the inability to kill Fas-deficient β cells or to the gross abnormalities found in adaptive immunity.

How then does one root out the multiple effects cytokines have on a complex autoimmune disease such as IDDM? Here, we report the use of chimeric NOD mice carrying islets deficient in one of several defined cytokine receptors or cytokine-induced effector molecules as a means of specifically and physiologically isolating the target islet tissue from the effects of locally produced cytokines. In this way, we can examine the effect islet-specific deficiencies in Fas, IFN-γR, inducible NO synthase (iNOS), and TNFR on the development of IDDM in NOD mice when confronted by a population of diabeticogenic CD4+ T cells. Contrary to previous reports, we found that Fas, IFN-γR, and iNOS deficiencies did not alter the development of diabetes. However, the specific deficiency of the TNF-α receptor 1 (p55) rendered these islets profoundly impermeable to islet-reactive T cells. In fact, in the absence of an islet response to locally produced TNF-α, infiltrating T cells failed to proliferate, establish insulitis, and subsequently destroy β cells. Not only do these results argue that TNF-α is a key player in the development of diabetes, they argue that this molecule must act in part upon the target tissue. Therefore, the islet must play an active, and TNF-α-dependent, role in its own demise.

Materials and Methods

Mice. BDC2.5 TCR transgenic mice have been described previously (32). B6.lpr mice were obtained from Dr. John Ruscetti (Washington University, St. Louis, MO), who originally obtained congenic breeding pairs from The Jackson Laboratory. These mice were backcrossed >12 generations to C57Bl/6 (B6) and were maintained by brother/sister mating. IFN-γR−deficient mice (33) were obtained on a 129 background from Dr. M. Aguett (Institut de Recherche sur le Cancer, Epalinges, Switzerland). TNFR p55−/− (34) and p75−/− (35) mice were obtained as doubly deficient mice on a mixed 129 x B6 background from Dr. R.D. Schreiber (Washington University; with permission from Drs. W. Lesslauer, R. O'he, Basel, Switzerland; and M. Moore, Genentech, South San Francisco, CA). A control TNF-α wild-type line was derived from negative littermates and used as controls in all TNF-α receptor experiments. Mice deficient in IFN-γR− were obtained from The Jackson Laboratory on a pure 129/SvEv background by permission of Dr. J. Mudd (Merck Research, Raraway, NJ). All mice were bred and housed under specific pathogen-free conditions. All donor islets were derived from the original knockout or mutant lines as indicated above, unless specifically noted in the text. Mice deficient in the p55 receptor were backcrossed onto NOD.scid for seven generations and intercrossed to generate p55−/− NOD.scid mice.

Flow Cytometry. Flow cytometry was performed on a Becton Dickinson FACScalibur®. We purchased PE-conjugated anti-CD4 (Catlag Labs), anti-CD2 (Pharmingen) and goat anti-mouse IgM (Jackson ImmunoResearch Labs.). The mAb to the β chain of the transgenic TCR, anti-Vβ4 (37), was conjugated with FITC. List mode data was collected on 10⁵ cells and reanalyzed on a PC using WinMDI (version 2.7) software written by J. Trotter (http://facs.scripps.edu).

Diabetes. Diabetes was assessed by measurement of venous blood using a Bayer Glucometer Elite one-step blood glucose meter. Animals were considered diabetic after two consecutive readings >250 mg/dl (13.57 mM). NOD.scid mice were weighed and streptozotocin was injected intravenously at a dose of 180 mg/kg, after which most mice became diabetic (>400 mg/dl) within 48–72 h. Diabetic mice were transplanted with islets within 48–72 h of the onset of diabetes.

Islet Isolation and Culture. Mouse islets were isolated by collagenase technique (38) and purified on Ficoll gradient. Individual clean islets were selected and cultured overnight at 24°C in 5% CO₂ in DMEM supplemented with 5% FCS (HyClone), 10 mM Hepes, 5 x 10⁻⁵ M 2-ME, penicillin (100 U/ml), streptomycin sulfate (100 μg/ml), and glutamine (2 mM).

Islet Transplantation. 250 islets from mutant or control mice (all H-2k on either a 129 or B6 background) were transplanted under the renal capsule of streptozotocin-induced diabetic NOD.scid mice as previously described (39). In brief, under anesthesia (87 mg/kg of sodium pentobarbital), islets were transplanted under the renal capsule by exposing the left kidney through a flank incision, pushing the kidney through the incision, and holding it in place with small clamp attached to fatty tissue; with the aid of a dissecting microscope the capsule was cut with a needle and islets were then delivered through the incision by a Hamilton syringe fitted with a polyethylene catheter. After the catheter was withdrawn and the capsule was sealed by a small, pen-size eyecauter, the kidney was returned to the abdomen and the incision was closed. Normoglycemia was reestablished within 24 h of successful transplantation. Mice were then rested for 10–14 d to allow for vascularization of the graft by host vascular endothelium before the introduction of diabetic T cells. In the mixed islet grafts, the number of islets was 300 (200 p55−/− and 100 wild-type). Transplants were performed in a similar manner on bilateral kidneys with a total of 300 islets being transplanted per mouse. In all experiments, mice not receiving diabeticogenic T cells remained normoglycemic (80–110 mg/dl) for >180 d. Confirmation of the
Nephrectomy. Mice were anesthetized with 87 mg/kg of sodium pentobarbital and the engrafted kidney was exposed by a flank incision as above. The engrafted kidney was raised and freed from fatty tissue as before. The renal artery and vein along with the ureter were clamped off with a mosquito hemostat and were sutured distal to the kidney with 4-0 silk. The kidney was cut free with a scalpel. The clamp was released slowly and the suture was inspected for leaks and the incision closed.

Immunohistochemistry. Kidney graft sections were stained with antibodies against Vβ4 (KT4), macrophages (ER MP-23; reference 40), dendritic cells (NLDC-145; Harlan sera labs), mad-CAM (MECA-367; reference 41), and peripheral node addressin (MECA-79; reference 41) as previously described (42).

Results and Discussion

The ability to target loss-of-function mutations to specific organ systems remains a major challenge. With the exception of certain mutations targeted to the immune system using the Rag mutant complementation system (44) or the inducible knockouts (45), it has been difficult to study the effects of broadly expressed or broadly acting mutations on specific organ systems or disease models. This is particularly true of the ubiquitously expressed cytokine receptors and their pluripotent ligands, the cytokine molecules themselves.

Several experimental models have long suggested that cytokines influence the development of autoimmune diabetes (for review see reference 46). Although these models have been informative, it has remained difficult to attribute particular stage dependency to any given cytokine, especially under physiological conditions. Moreover, it has been difficult to determine what cellular conduit channels the action of each cytokine; is it through the effector lymphocyte, the APC, the vascular epithelium, or the islet tissue itself? Therefore, we undertook to develop a novel approach that compartmentalizes the action of cytokines and their receptors to specific cellular targets in an effort to establish the dependency of particular phases of diabetes development to the local effect of these mediators.

We did this by creating chimeric NOD mice. Unlike prior models, these mice harbored wild-type NOD APCs that express the disease-associated MHC class II, I-Aβ7, and wild-type NOD vascular endothelium. Moreover, they carried a defined population of diabetogenic CD4+ T cells (BDC2.5 TCR transgenic T cells) that respond to pancreatic β cell antigen, presented uniquely by NOD APCs, and are capable of mediating the autoimmune destruction of pancreatic β cells (32, 47–49). What distinguishes our current approach was the prior replacement of the endogenous β cells with those derived from one of several mouse strains deficient in key cytokine receptor or proapoptotic effector molecules. In so doing, we created chimeric NOD mice containing altered β cell target tissue. This allowed us to assess the potential impact of each genetic alteration on the islet cell’s ability to serve as a target for autoimmune-mediated destruction by altering the host effector lymphocyte, APC, or vascular endothelium.

As shown schematically in Fig. 1, NOD.scid mice were treated with streptozotocin, a β cell toxin, to destroy endogenous β cells, producing a chemically-induced diabetes (>400 mg/dl). N ormoglycemia (<100 mg/dl) was rapidly returned with the transplantation of ~250 islets under the left kidney capsule. (It should be noted that the donor islets can be from any strain of mouse as NOD.scid do not reject allogeneic tissue. We routinely used islets of H-2d donors.) The rescued mice were then rested for 7–10 d, allowing for host-derived vascularization of the graft. At this point, splenic T cells from diabetic BDC2.5/NOD.scid mice were transferred into these chimeric NOD.scid mice, and the mice were followed onset for diabetes.

These experiments are predicated on the following observations. First, as mentioned above, the recipient mice are scid, hence they do not reject allogeneic islet grafts, as confirmed by control experiments where each series of donor islets are engrafted under the kidney capsule and the mice are left unmanipulated for >180 d. N one of these mice develop diabetes during this period. Moreover, the transplanted islets are functional, and are responsible for the maintenance of normoglycemia, as removal of the engrafted kidney results in hyperglycemia (data not shown). Second, the BDC2.5 T cells do not recognize the β cells directly but rather require the transfer of islet antigen to NOD (H-2b) APCs, which are supplied by the NOD.scid recipient mice. Third, although the recognition of antigen...
is MHC restricted, all strains of mice express the relevant antigen in their pancreatic β cells (48). And finally, once activated by antigen, BDC2.5/NOD.scid T cells can mediate the destruction of islet β cells in NOD.scid mice (6).

Destruction of Pancreatic β Cells in the Absence of Fas. Pancreatic β cell death during the course of T cell–mediated diabetes is by apoptosis (6–8). One potential mediator of β cell apoptosis is Fas (CD95). The engagement of Fas, a pro-apoptotic member of the TNFR family, on the surface of target cells by Fas ligand–expressing T lymphocytes leads to the apoptotic destruction of the Fas-expressing target cell (for review see reference 50). Treatment with IFN-γ induces the expression of Fas on the surface of a variety of cell types including β cells (24). Moreover, islet-infiltrating T cells can induce Fas expression on β cells through localized production of IFN-γ (31). Additionally, Fas-deficient, NOD.lpr/lpr mice do not develop diabetes (30, 31); however, these mice had substantially altered T and B cell immunity (30). These results notwithstanding, it has not been formally demonstrated that Fas expression on β cells is required for their autoimmune-mediated destruction.

To test whether Fas expression on β cells is obligatory, we used our chimeric NOD mice model to create mice specifically lacking Fas expression on their islet cells. This was done by either eliminating the islet's ability to respond to IFN-γ by replacing the existing islet mass with islets lacking the IFN-γR or by using islets from B6.lpr/lpr mice that lack functional Fas expression as islet donors. After transplant, T cells from diabetic BDC2.5/NOD.scid mice were transferred into these mice and diabetes onset was followed. We found that BDC2.5 T cells destroyed B6.lpr/lpr islets as efficiently as control B6 islets, as shown in Fig. 2 a, indicating that Fas does not play an obligatory role as the critical inducer of β cell destruction at least with respect to disease transferred by diabetogenic CD4+ T cells. Similarly, when NOD.scid mice were transplanted with islets deficient in IFN-γR, the chimeric mice developed diabetes at the same rate as control islet grafts (129/SvJ; Fig. 2 b). These results clearly demonstrate that islet cell Fas expression, either induced or constitutive, is not required for islet destruction by diabetogenic CD4+ T cells. Moreover, this would suggest that much of the protection seen in Fas-deficient NOD mice results from altered lymphoid development in the absence of Fas expression on T and B lymphocytes. Parenthetically, these results help to clarify the role IFN-γ may play in diabetes development. Wang et al. in describing the introduction of systemic IFN-γ receptor deficiency onto the NOD background, found that both NOD and BDC2.5/NOD mice lacking IFN-γR had severely retarded insulitis development (21). Our results would suggest that this is probably due to an effect IFN-γ has on the T cells, APCs, or host endothelium but not on the islet mass itself.

Islet Cell Production of Reactive Nitric Oxide Intermediates Is Not Required for β Cell Destruction. Previous studies have indicated that IL-1 stimulates the production of nitric oxide (NO) either by priming for Fas-mediated apoptosis or by inducing the inducible form of the NOS synthase gene (iNOS or NOS2; references 51, 52). iNOS-deficient NOD production can lead to β cell death in vitro (27–29). NO can be produced by the islets themselves or by the infiltrating macrophage/dendritic population. We found that the in vivo neutralization of IL-1β with a cocktail of antibodies and soluble receptor did not prevent NOD mice from becoming diabetic (data not shown), leading us to question its role in β cell death. However, to examine the effect that the targeted iNOS deficiency in islets had on β cell destruction, we tested the ability of iNOS-deficient islets to resist T cell–mediated destruction. As shown in Fig. 2 b, iNOS-deficient islets were destroyed with similar kinetics and magnitude as wild-type islets, indicating that iNOS gene expression is not critical for islet cell apoptosis. Although this result does not rule out a role for NO production by infiltrating macrophage/dendritic cells, it clearly demonstrates that islets themselves do not need to produce intracellular NO to undergo T cell–mediated elimination.

TNF-αR Deficiency Affects Islets Protection from Diabetogenic CD4+ T Cell–Mediated Destruction. TNF-α, which is secreted principally by activated macrophages and CD4+ Th1 cells (12, 13), can both retard and exacerbate the disease.

Figure 2. TNF-αR deficiency affects islets protection from diabetogenic CD4+ T cell–mediated destruction. TNF-αR (p55)–deficient islets are protected from destruction by diabetogenic CD4+ T cells, whereas Fas-deficient, IFN-γR–deficient, iNOS-deficient, and TNF-αR–deficient islets are destroyed. (a) 250 B6.lpr/lpr (●, n = 12) or control B6 islets (○, n = 10) transplanted under the kidney capsule are destroyed. (□, diabetes in control NOD.scid mice transferred with the same population of T cells (n = 9). (b) 250 IFN-γR–deficient (●, n = 7) iNOS-deficient (■, n = 6) or control 129 islets (▲, n = 8) transplanted under the kidney capsule are destroyed. (c) Transplanted p55−/− islets (▲, n = 9) and p55−/−p75−/− doubly deficient islets (▲, n = 10) are protected from destruction by transferred T cells from diabetic BDC2.5/NOD.scid mice, whereas p75−/−/p55−/− islets (▲, n = 11) are destroyed. Diabetes is measured by sampling venous blood using a standard one-step glucometer. Mice are considered diabetic after two successive readings ≥250 mg/dl. In all cases, engrafted mice that did not receive diabetogenic T cells remained normoglycemic throughout the experimental period (>180 d); moreover, the engrafted islets were responsible for normoglycemia, as the hemi-nephrectomy of engrafted kidneys resulted mice to hyperglycemia.
velopment of IDDM in NOD mice largely dependent on the time of its administration (14–16). Thus, when TNF-α is given to NOD mice from birth to 3 wk of age, diabetes is accelerated, and conversely the administration of neutralizing antibody to TNF-α during this period markedly reduces both insulitis and diabetes (16). Yet, when administered to adult NOD mice with established insulitis, TNF-α attenuates the disease process, and its antibody neutralization exacerbates diabetes (16, 53). Moreover, the transgenic expression of TNF-α in the islets of adult NOD mice leads to insulitis without disease (17–19) and produces a state of T cell tolerance to islet cell antigens (19, 20). Thus, although these experiments reveal the potent ability of TNF-α to alter the development of autoimmune diabetes, the physiological role played by TNF-α has yet to be fully elucidated.

To investigate the role that localized production of TNF-α can have on the development of diabetes, we transplanted streptozotocin-treated NOD.scid mice with islets rendered doubly deficient for both TNF-αRs (TNF-αR1, p55; TNF-αR2, p75). As before, the transfer of diabeticogenic T cells led to the rapid destruction of wild-type islet grafts (7 out of 8); however, doubly deficient islets (p55−/−p75−/−) remained functional (Fig. 2 c). Mice engrafted with p55−/−p75−/− islets remained normoglycemic for up to 52 d after the transfer of T cells. To confirm that the introduced islets were responsible for the maintenance of blood glucose, normoglycemic p55−/−p75−/− islet recipients were heminephrectomized at day 28 to remove the engrafted kidney. As shown in Fig. 2 c, these mice became hyperglycemic within 24 h of nephrectomy, proving that the transplanted p55−/−p75−/− islets were indeed responsible for the maintenance normoglycemia. Interestingly, the mice carrying p55−/−p75−/− islets contained BDC2.5 T cells as measured by flow cytometric analysis of spleen and lymph node. In addition, these T cells were phenotypically normal in that they could still transfer disease to unmanipulated NOD.scid mice (data not shown).

To assess which receptor conferred the protection, we produced chimeric mice carrying islets defective in either the p55 receptor or the p75 receptor. Fig. 2 c shows that p55−/− islets were protected from T cell–mediated destruction, whereas the p75−/− islets remained normoglycemic. All the p75−/− transplanted mice (11 out of 11) became diabetic by day 12, whereas the p55−/− transplanted mice (9 out of 9) remained normoglycemic until end of the assay (≥28 d). We therefore concluded that the engagement of the p55 receptor by locally produced TNF-α was critical in the subsequent destruction of β cells.

p55−/− islets and p75−/− islets are equally antigenic to BDC2.5 T cells. One explanation for the lack of islet destruction of the p55−/− grafts is that the p55−/− islets are non- or poorly antigenic. To test this, BDC2.5 T cells were cocultured with NOD APCs in the presence of dispersed p55−/− and p75−/− islet cells for 72 h under standard conditions, and T cell proliferation was measured. As shown in Fig. 3, BDC2.5 T cells proliferated equally well to both receptor-deficient islet cells, indicating that islet cell antigenicity does not depend on TNF-αR expression. We performed a similar assay with intact islets in vitro in the presence and absence of exogenous recombinant TNF-α and were unable to detect a difference in the induced proliferation of BDC2.5 T cells (data not shown). We therefore concluded that at least in vitro, there is no difference in the antigenicity of p55−/− and p75−/− islet cells. This is somewhat discordant with recent results by Green et al., who reported that the localized production of TNF-α in β cells of transgenic NOD mice enhanced autoantigen presentation to BDC2.5 T cells in vitro (54).

The CD4+ T cell infiltration of p55−/− islet grafts does not progress to destructive insulitis. An alternative explanation for the p55-deficient islets’ resistance to T cell–mediated destruction resides with a fundamental modification in the cellular constituency of the infiltrate. To evaluate this possibility, we performed an immunohistochemical analysis of both p55−/− and wild-type (or p75−/−) islet grafts after T cell transfer. Engrafted kidneys were sampled at day 5, 7, and 9 after T cell transfer as well as at the time of diabetes or in the case of normoglycemic mice at day 28. As seen in Fig. 4, there was no infiltration in either p55-deficient or wild-type islet grafts at day 5. At day 7, however, the wild-type graft showed distinct signs of peri-islet accumulation of leukocytes, with some grafts showing evidence of intra-islet infiltration and destruction. The same was not true for the p55-deficient islet grafts, which showed only modest peri-islet infiltration and no intra-islet infiltration. As seen in Fig. 4, the most dramatic difference was revealed at day 9 when the wild-type islet grafts were completely infiltrated. These islets showed discrete foci of apoptotic β cells as revealed by TUNEL analysis (data not shown). In contrast, the p55-deficient islet grafts were only mildly infiltrated at day 9 (Fig. 4) and showed no signs of apoptosis (data not shown). Moreover, by day 13, the wild-type islet grafts were destroyed and the mice were overtly diabetic. Surprisingly, the mild infiltration of the p55−/− grafts failed to progress, and in fact resolved, so that by day 28 they were nearly indistinguishable from those seen on or before day 5. We therefore concluded that the p55−/− islet grafts could not sustain the infiltrating CD4+ T cells and that the propagation of destructive insulitis requires a TNF-α-dependent response on part of the islets.

We then asked if the composition of the infiltrate was modified between the p55−/− and wild-type lesions. We compared the cellular components of the transient infiltration of p55-deficient islet grafts at day 9 with those of the...
wild-type grafts. In general, the composition of the infiltrate was similar to that seen in the pancreata of NOD.scid recipients of T cells from diabetic BDC2.5/NOD mice (42). Moreover, we saw no difference between the p55−/− and the wild-type grafts in the activation state of the high endothelial venule (HEV) as revealed by staining for madCAM (MECA 367) and peripheral node addressin (MECA 79, data not shown). We were also able to identify the presence of roughly equal numbers of BDC2.5 T cells in both infiltrates as revealed by Vβ4 (KT4) and anti-CD4 staining. Both lesions contained similar subsets of macrophage (F4/80, MOMA 1, MOMA 2) and dendritic cells (NLDC-145) as well. Despite these similarities, there was one striking difference between the p55−/− and wild-type grafts: the complete absence of β cell apoptosis in the p55−/− grafts (data not shown). We therefore concluded that apart from the lack of continued progression of the lesions and the lack of β cell apoptosis, there was little difference in the nature of the infiltrates and the vascular endothelium between wild-type and p55−/− deficient islet grafts.

NOD.scid mice lacking the p55 receptor, but carrying wild-type islets, develop diabetes upon BDC2.5 T cell transfer. To verify that the resistance to diabetes resided with the p55−/− deficient islets rather than with the endothelium or APC populations, reciprocal transplants were performed in which wild-type islets (from 129 mice) were transplanted under the kidney capsule of streptozotocin-treated p55−/− deficient NOD.scid mice (N7 generation). Under these conditions, both the host vasculature and APC population lacked p55 receptor expression, whereas the engrafted islet mass retained full p55 functionality. When purified diabetogenic CD4+ T cells were transferred into these mice, diabetes developed with similar kinetics in both these mice and control recipients (Fig. 5 a). This indicated that functional expression of the p55 receptor on the islet mass alone was sufficient to drive β cell destruction regardless of the p55 receptor expression status of the host APCs and the vascular endothelium.

An islet graft containing both p55−/− deficient and p55−/− sufficient islet mass is destroyed upon diabetogenic BDC2.5 T cell transfer. Although the p55−/− deficient islets were no less antigenic than the p55−/− sufficient islets and were equally capable of attracting similar subsets of infiltrating leukocytes, they were clearly incapable of providing a microenvironment that supported the maturation of the immune response to a point where β cell death could occur. This could be for one of two reasons. First, the propagation of insulitis may require TNF-α–mediated β cell death. In this case, the TNF-α produced locally by the infiltrating T cells and macrophages would fail to kill the p55−/− deficient β cells and insulitis would subside due to a lack of β cell damage. This would be consistent with our failure to observe β cell apoptosis in p55−/− deficient islets during the early phase of infiltration, yet it also seemed unlikely as ectopic expression of super-physiologic levels of TNF-α by the islets of transgenic mice did not lead to β cell death or diabetes (17–19, 55). Alternatively, the evolution of insulitis from a benign accumulation of leukocytes to a destructive infiltrate may require a TNF-α–dependent change in the islet mass—either...
the release of an islet cell–produced chemoattractant or activation factor or an alteration in the secretion or production of antigen (something we are unable to mimic in vitro, but which has been observed by others, see reference 54). Either way, the net result would be the full activation of the infiltrating BDC2.5 T cell population such that it can now act to target β cells for destruction in a TNF-α–independent fashion.

To distinguish between these two possibilities, we designed and produced chimeric NOD.scid mice that carried mixed grafts containing both varying amounts of p55-deficient and p55-sufficient islets. If TNF-α acted strictly as a cytolytic agent, only the p55-sufficient islets would be destroyed upon transfer of diabeticogenic T cells, while the p55-deficient islets would be spared and normoglycemia would be maintained, provided that adequate amounts of p55-deficient islets were included in the mixed graft. On the other hand, if TNF-α acted to alter the local environment in favor of T cell activation, the presence of even a modicum of TNF-α–responsive islets would result in T cell activation and the destruction of both the p55-deficient and -sufficient islets and diabetes.

We first ascertained the minimum number of islets required in our grafts to maintain a persistent state of normoglycemia in our Streptozotocin-treated NOD.scid mice. We found that as few as 100 islets could maintain blood glucose levels at ≤100 mg/dl (data not shown). Therefore, for our initial experiments we chose to mix ≥200 p55-deficient islets with ~100 wild-type islets per graft. In this way, the “protected” p55-deficient islets were in sufficient excess to assure normoglycemia if all of the wild-type islets were destroyed. As before, mixed islet recipients received diabeticogenic T cells 7–10 d after islet transplantation. As depicted in Fig. 5 b, both the mixed islet recipients and the control mice engrafted with 300 wild-type islets developed diabetes with comparable kinetics (between days 16 and 18 after transfer). In subsequent experiments, the numbers of wild-type islets were reduced until as few as 10 wild-type islets were mixed with ~300 p55-deficient islets, yet the results (islet graft destruction and diabetes) were the same (data not shown). Therefore, we concluded from these experiments that the stimulation of islet cells through their p55 receptor altered the local environmental conditions favoring the development of a productive BDC2.5 T cell infiltrate.

The Physical Separation of p55-deficient and p55-sufficient Islet Grafts on Opposing Kidneys Still Allows for the In Vivo Activation of Diabeticogenic CD4+ T Cells, the Destruction of β Cells and the Development of Diabetes. Having determined that a small number of islet cells can, in response to locally produced TNF-α, support the transition from benign to destructive insulitis, it was now critical to determine if this was a purely localized effect. To address this, we performed kidney grafts on streptozotocin-treated NOD.scid mice in which wild-type islets (100 islets) were engrafted under the right kidney capsule and p55-deficient islets (250 islets) were engrafted under the left kidney capsule of the same animal. By physically separating the grafts we sought to minimize the effects between the wild-type and p55-deficient graft. If, upon transfer of diabeticogenic T cells, the p55-deficient graft survived in these mice, despite the destruction of the wild-type islet grafts, then the original destruction of the p55-deficient islets in the mixed islet grafts described above (Fig. 5 b) resulted merely by virtue of their intimate proximity to the wild-type islets. On the other hand, if the distant p55-deficient grafts were likewise destroyed, it is more likely that the transferred T cells were altered by an encounter with wild-type islet cells.

We found that the twin-kidney engrafted NOD.scid recipient mice did, in fact, become diabetic 12–16 d after receiving BDC2.5 T cells, at a rate coincidental with the development of diabetes in mice harboring dual wild-type grafts (Fig. 6 a). As before, those mice engrafted with only p55-deficient islets did not develop disease. Additionally, histological analysis of the p55-deficient bilateral graft showed signs of β cell apoptosis within 1 d of the onset of destructive infiltration of the p55-sufficient graft (~day 5–7 after transfer, Fig. 6 b). The ability of wild-type grafts to influence the outcome of the contralateral p55-deficient grafts indicated that TNF-α responsiveness on the part of the wild-type islets led to the activation of the transferred BDC2.5 T cells such that they were now capable of homing to and destroying the p55-deficient graft on the opposite kidney.

These results led us to assess the in vivo proliferative status of BDC2.5 T cells after transfer. We reasoned that the lack of progression in the p55-deficient islet engrafted mice may be due to the inability of the p55−/− islets to fully activate the transferred BDC2.5 T cells. In the mixed and twin-kidney graft experiments, the wild-type islets would

![Figure 6](https://jem.rupress.org/content/fig/input/059/07/01059/F6.png)
provide an environment capable of furnishing this activation and therefore of leading to the subsequent destruction of the p55−/− islets in a TNF-α-independent fashion. If this were true, T cell proliferation in vivo might differ between mice harboring only p55−/− islets and those carrying only wild-type islets. This was tested by monitoring the degree of in vivo proliferation of the BDC2.5 T cells in the efferent lymph of mice harboring one or the other islet grafts under the left kidney using the decay of the integral membrane dye, CSFE, as an indicator of cell division (43, 56, 57). As depicted in Fig. 7, draining lymph nodes from mice engrafted with p55−/− islets were devoid of reactivated BDC2.5 T cells, whereas the renal lymph nodes from mice engrafted with wild-type islets contained T cells that clearly had undergone several rounds of replication. Therefore, we concluded that the most likely explanation for the infiltration and subsequent destruction of p55−/− islets in both the mixed and twin-kidney grafts was due in part to the activation of the BDC2.5 T cells in response to wild-type islets either proximal or distal to the p55−/− islets. This process required a TNF-α response on the part of the target islet cells but the subsequent destruction of the islet tissue was TNF-α-independent. The nature of the TNF-α response on the part of islets remains unknown, but could be an increase in antigen delivery either in direct response to TNF-α or as a result of islet cell death. In either case, this leads to the subsequent activation of our infiltrating islet-reactive BDC2.5 T cells, which then act to mediate the destruction of islet β cells in a process that does not require TNF-α.

Using the same BDC2.5 TCR transgenic model, André et al. have proposed two checkpoints in the progression of diabetes; the first the formation of a benign infiltrate and the second the transition to destructive insulitis (58). We would propose that the transition through checkpoint two is dependent on the active response of islets to TNF-α. Moreover, our results are consistent with the hypothesis that early and local production of TNF-α in the islet acts to enhance the islet’s antigenicity and the subsequent activation of disease-causing lymphocytes (16, 17, 53).

In conclusion, these data, taken together, demonstrate that Fas, IFN-γ, and iNOS do not play an obligatory role in the apoptotic destruction of pancreatic β cells induced by a diabetogenic CD4+ T cell population, but that TNF-α plays a critical role in the transformation of islet-reactive CD4+ T cells from a benign state of β cell indifference to an activated state of β cell reactivity. Moreover, these results suggest, for the first time, that the islet cells themselves play an active and TNF-dependent role in facilitating their own death by providing an environment capable of perpetuating T cell-mediated insulitis.

We would like to thank Ms. Olga Strots and Mr. Larry McClendon for their excellent technical assistance and animal care. We would also like to thank Drs. Rober t O. Schreiber, John H. R ussell, Osami K anagawa, John M udgett, Werner Leslauer, and Mark M oore for gifts of reagents, mice, and critical technical advice, and Dr. Stacey Smith for use of her cryostat. We would also like to thank Dr. Paul E. Lacy for his advice on transplantation experiments and critical review of the manuscript.

We particularly wish to thank Dr. Charles Kilo and his Kilo Diabetes and Vascular Research Foundation for their generous financial support. In addition, this work was supported by grants to J.D. Katz from the Juvenile Diabetes Foundation International (JDFI; No. 197030), the Washington University Diabetes Research and Training Center, the National Institutes of Health (R01 AI44416), and a joint NIH/JDFI program project grant (P01 AI39676/995012; Dr. E.R. Unanue, program director). J.D. Katz is a recipient of an American Diabetes Association Career Development Award.

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Received for publication 19 November 1998 and in revised form 19 January 1999.

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Figure 7. T cells from the efferent lymph of p55−/−islet engrafted mice have not undergone cell division, whereas those from wild-type islet engrafted mice have. Streptozotocin-treated NOD.scid mice were engrafted with either p55-deficient (p55−/−) or p55-sufficient islets (WT) under the left kidney capsule. Several weeks later, 10⁷ CSFE-labeled BDC2.5 T cells were injected intravenously in each group of mice. On days 4 and 5 after transfer, peri-renal lymph nodes were collected and BDC2.5 T cell proliferation was assessed as measured by diminution of CSFE label on a flow cytometer. Lymph nodes from two to four animals were pooled per group.
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