Comment on Papers by Chong *et al.*, Nishio *et al.*, and Suri *et al.* on Diabetes Reversal in NOD Mice

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Chong *et al.*, Nishio *et al.*, and Suri *et al.* (Reports, 24 March 2006, pp. 1774, 1775, and 1778) confirmed that treating nonobese diabetic (NOD) mice with an immune adjuvant and semisyngenic spleen cells can reverse the disease but found that spleen cells did not contribute to the observed recovery of pancreatic islets. We show that islet regeneration predominately originates from endogenous cells but that introduced spleen cells can also contribute to islet recovery.

Treating end-stage diabetic NOD mice with Freund's complete adjuvant combined with matched major histocompatibility complex class I and self-peptide–bearing splenocytes permanently reverses the disease (*1*, *2*). The successful use of the above treatment protocol has been confirmed by several independent studies (*3*–*7*). Although all groups documented restoration of normal blood sugars by the reappearance of large pancreatic islets, Chong *et al.* (*3*), Nishio *et al.* (*4*), and Suri *et al.* (*5*) did not find that donor spleen cells contribute to the regeneration of the pancreas. On the other hand, we reported that spleen cells, although not obligatory for the cure, hasten the time to restore blood sugar levels and contribute to the regenerative process (*1*). Here, we point to several methodological issues that might explain these results.

Lineage tracking of cells can be performed in a number of ways. We used Y-chromosomal fluorescence in situ hybridization (FISH) to follow the fate of male cells transplanted into female recipients. We prefer this method to tagging cells with green fluorescent protein (GFP), which can be difficult to detect if the GFP transgene is expressed inefficiently. It is also possible for the transgene to be completely silenced in the progeny of GFP-labeled cells (*8*). Furthermore, it is well known that detection of GFP, even if it is present, requires perfusion of the tissue (most commonly with formaldehyde-based fixatives) to prevent diffusion of the marker out of cells (*9*). Thus, the inability of Chong *et al.* (*3*) to detect GFP in spleen cells or their progeny in recipient islets using innate fluorescence of nonperfused (frozen) tissue is not surprising and is more likely to be due to technical shortcomings than to the complete lack of splenocytes.

Nishio *et al.* (*4*) used laser-capture microdissection to harvest islets from recipient mice and then looked for donor-specific single-nucleotide polymorphisms in DNA prepared from the tissue. Although this is a clever strategy, the sensitivity of the method was not examined. Others have reported that this technique cannot be used to detect low levels of chimerism (*10*).

Suri *et al.* (*5*) observed the lowest rate of restoration of islet function, i.e., normoglycemia.

Only 4 out of 22 mice were treated successfully. According to their report, the islets of their normoglycemic mice not only lacked donor spleen cells but were entirely free of lymphocytic infiltration. This is in contrast to the data in (*3*, *4*) and our own experience and suggests a possible problem with the mice or the tissue.

Despite the inability of these groups to detect donor spleen cells in treated mice, we have confirmed a direct splenocyte contribution to insulin-expressing cells of the islets using the treatment protocols described in (*1*). We used Y-chromosomal FISH to look for male (donor) cells in 6-μm sections of pancreas from treated and untreated female NOD mice. FISH was simultaneously combined with insulin staining in the same section. These NOD mice received treatment at 14 to 16 weeks of age and were then maintained for 4 months after the last treatment. All treated NOD mice consistently showed a small number of Y chromosome–positive insulin-producing islet cells (Fig. 1). No Y chromosome was detected in untreated control female NOD mice or C57BL/6 mice. Three-dimensional reconstruction was performed by image capturing at 0.5-μm distance and building Z series using a motorized stage. Figure 1 shows the restored images, which demonstrate colocalization of the Y chromosome with insulin in all views.
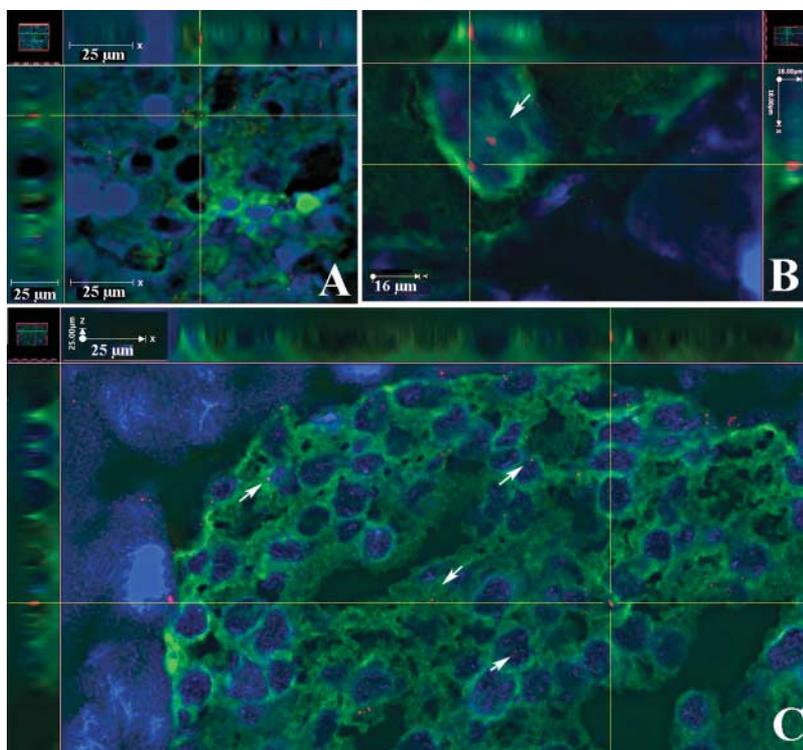
Fig. 1. Pancreatic sections from successfully treated female NOD mice show insulin-containing (green) islet cells with Y chromosomes (red) (**A**, **B**, and **C**). Each image is from a different successfully treated NOD mouse that received live spleen cells (*11*, *12*). A Y chromosome in each islet that is colocalized with insulin is shown with the grid in all three planes from one level of the Z stack. Arrows in each image point to additional colocalizations of Y chromosomes and insulin-producing cells.

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Table 1. Frequency and extent of splenic male cell engraftment (Y chromosomes) in islets of treated and untreated female NOD mice. Early diabetes was defined as normoglycemic female NOD mice at 14 to 16 weeks of age. Late diabetes was defined as hyperglycemic female NOD mice with blood sugars of 600 to 800 mg/dl of at least 2 weeks duration with insulin therapy; these mice ranged in age from 22 to 35 weeks of age. Specimens were analyzed in a blinded fashion by S. Kodama

	Treated		Untreated
	Early diabetes	Late diabetes	
Sample size	$n = 5$	$n = 14$	$n = 16$
Proportion of mice having donor male islet cells	30%	100%	3%
Percentage of male insulin-positive cells per islet	4%	33%	1%

We further examined the extent of engraftment of male donor spleen cells and the contribution of these cells to islet regeneration (Table 1). The results indicate that islet regeneration predominantly originates from endogenous cells but that introduced spleen cells can also contribute to islet recovery. Furthermore, the age of the NOD mouse at the start of the disease-reversing treatment influenced the degree of splenocyte contribution compared with endogenous regrowth of the islet. Older NOD mice with more advanced diabetes showed a higher contribution of splenocytes to regenerating islets. The extent of chimerism in transplanted islets may thus depend in part on the extent of islet destruction at the time

therapy was initiated and the duration of follow-up after therapy. Chong et al. (3), Nishio et al. (4), and Suri et al. (5) may have followed their animals for shorter time periods after therapy and used earlier time points, which might also explain their observations of exclusively endogenous islet regeneration.

Whatever the origins of the insulin-secreting cells that we observe, the treatment protocol described in (1) enables the permanent restoration of normoglycemia in treated NOD mice. As originally reported, all groups have observed the restoration of normoglycemia in the recipients; endogenous restoration of insulin from the pancreas is intact in these NOD mice. The efficacy of this intervention in permanently halting destruc-

tive autoimmunity and restoring normoglycemia merits continued efforts.

References and Notes

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11. These specimens were prepared and analyzed in a blinded fashion by investigators at NIH and McGill University (S.D.T., B.M.L., I.S., S.K., Zs.E.T., and É.M.).
12. Frozen 6-μm thin sections of female pancreata from NOD mice injected with CD45⁻ male splenocytes were immunostained for insulin and visualized with FITC-Tyramide Plus (Invitrogen). Sections were then hybridized to visualize the Y chromosome using an Alexa-fluor-594 Tyramide conjugate and analyzed using a Leica DMX6000 microscope and Volocity software. The settings for iterative restoration were 90% or 25 iterations; the sections were taken at 0.5-μm intervals.
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