Gene Targeting in NOD Mouse Embryos Using Zinc-Finger Nucleases

Studies in NOD mice have provided important insight into the genetics and pathogenesis of type 1 diabetes (T1D). Our goal was to further explore novel methods of genetic manipulation in this mouse model. We tested the feasibility of using zinc-finger nucleases (ZFNs) to knock out a gene directly in a pure NOD background, bypassing the need of embryonic stem cells. We report here the successful application of ZFN pairs to specifically and efficiently knock out Tnfrsf9 (encoding CD137/4-1BB) directly in the NOD mouse by embryo microinjection. Histology and T1D incidence studies indicated that CD137 was dispensable for the development of insulitis but played a role to promote progression to overt diabetes in NOD mice. We also demonstrated that CD137-deficient T-cells were less diabetogenic than their wild-type counterpart when adoptively transferred into NOD.Rag1<sup>−/−</sup> recipients, even when CD25<sup>+</sup> cells were predpleted. In vitro assays suggested that CD137 deficiency had a limited effect on the suppressive function of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T-cells (Tregs). Therefore, CD137 deficiency predominately affected effector T-cells rather than Tregs. Our study demonstrates the ability to generate gene-targeted knockouts in a pure NOD background by using ZFNs without potential confounding factors introduced by contaminating genetic materials obtained from other strains.

Diabetes 2014;63:68–74 | DOI: 10.2337/db13-0192

The NOD mouse has been used as an animal model for type 1 diabetes (T1D) since its development three decades ago (1). In both NOD mice and humans, T1D is regulated by a large number of genetic loci (named Idd in the mouse), with certain major histocompatibility complex haplotypes being the primary risk (2). Genetic studies in NOD mice have provided important insights into how defective IL-2 and CTLA4 pathways contribute to T1D development (3–5). One major limitation of genetic studies in NOD mice has been the lack of germline competent embryonic stem cells (ESCs) that allow efficient gene targeting. Two recent studies reported the establishment of germline competent NOD ESC lines amenable to genetic modifications (6,7), one of which has been successfully used to generate NOD mice deficient in the nonconventional class II molecule DM (8). The availability of these ESC lines has not led to routine generation of gene-targeted mutations in NOD mice, presumably due to the delicate culture conditions required to maintain these cells. The standard approach...
has been to introduce a modified allele generated in a different genetic background into the NOD mouse through generations of backcrossing. This process is time-consuming, and it inevitably cotransfers unwanted genetic contamination that may contribute to the observed phenotypes. Another approach is to use RNA interference to knock down the expression of the targeted gene in NOD mice (9, 10). However, the efficiency of RNA interference knockdown may vary among different cell types and animals. Furthermore, the knockdown approach can only be used to reduce the expression level but cannot be used to generate a null mutation.

In the past three years, the zinc-finger nuclease (ZFN) technology has emerged as a powerful genetic tool to specifically target genes in a variety of cells and organisms with high efficiency (11, 12). Therefore, we decided to test if such genetic modification approaches could be applied to NOD mice. The goal of the current study was to assess the potential of ZFN-mediated mutagenesis directly in a pure NOD background. To test this, we targeted Tnfrsf9 (encoding CD137/4–1BB), a tumor necrosis factor receptor family member expressed by multiple cell types, including activated T-cells (13). We chose Tnfrsf9 to prove the feasibility of ZFN-mediated gene targeting because its protein expression can be easily detected, it has been implicated as a candidate gene of Idd9.3 (14–16), and its role in T1D has not been directly tested.

**RESEARCH DESIGN AND METHODS**

**Generation of CD137-Deficient NOD Mice**

NOD/LtDVS (hereafter NOD) mice were originally imported from Serrezée’s colony at The Jackson Laboratory and subsequently maintained at the Medical College of Wisconsin by brother–sister mating. Constructs of the ZFN pairs specifically targeting exon 4 (the second coding exon) of the mouse Tnfrsf9 gene were designed, assembled, and validated by Sigma-Aldrich (target sequence CCCTCCAAGTACCTTctccaGATAGGTGAGCCGAA; ZFNs bind to each sequence shown in uppercase on

---

**Figure 1**—ZFN-mediated modification of the Tnfrsf9 gene in NOD mice. (A) The wild-type (WT) sequence of the ZFN target site in exon 4 (the second coding exon) of the Tnfrsf9 gene is shown at the top. The respectively altered sequences for line-6 and line-12 are indicated below WT. Each of the ZFN-binding sequences on the opposite strands is underlined. The arrowheads indicate the respective locations of the two base-pair deletions in line-6 and line-12. The bold and italic letters depict the premature stop codons introduced as a result of the deletion at the ZFN target site. (B) Lack of CD137 protein expression confirms its deficiency in the homozygous Tnfrsf9 mutant.
opposite strands). mRNAs encoding ZFN pairs were prepared in injection buffer (1 mM Tris-Cl, 0.1 mM EDTA, pH 7.4) at a concentration of 5–10 ng/µL and injected into the pronucleus of fertilized NOD one-cell embryos at the Medical College of Wisconsin Transgenic Core. Injected embryos were transferred to pseudopregnant CD-1 females. At weaning, DNA was extracted from tail tissues and screened for ZFN-induced mutation by the Surveyor nuclease assay as previously described (11,17). Extracted tail DNA was PCR-amplified with forward (5’-AATGC-CAGTCATTGTGATGC-3’) and reverse (5’-TCAAAGCCT-TAACTCTGCCCCA-3’) primers. The PCR products of the two identified male mutants (line-6 and line-12) were cloned into the TOPO TA-cloning vector (Invitrogen) and subjected to standard sequencing. The line-12 founder was backcrossed to an NOD female, and heterozygous progeny were intercrossed to generate littermates of all three genotypes for the indicated studies. In parallel, we also further backcrossed line-12 mice to NOD for two additional generations followed by intercrossing to fix the mutation to homozygosity. Similarly, the line-6 founder was backcrossed to NOD for two generations followed by intercrossing to generate littermates for indicated studies. The mutations in line-6 and line-12 eliminate a Bpm1 restriction site (CTCCAG), which was subsequently used for genotyping the PCR products.

**T1D Incidence Study and Analysis of Insulitis**

Assessment of diabetes and insulitis was done as previously described (18).

**Flow Cytometry Analysis**

Splenocytes cultured overnight with 1.25 µg/mL anti-CD3 (clone 145–2C11) were harvested and stained with anti-CD8 (clone 53–6.7) and anti-CD137 (clone 17B5) or with anti-CD4 (clone RM4–5) and anti-CD137, followed by anti-Foxp3 (clone FJK-16s) using an intracellular staining kit from eBioscience. In separate tubes, golden Syrian hamster IgG isotype control antibodies were used instead of anti-CD137. All antibodies were purchased from eBioscience or BD Bioscience. Antibody staining and flow cytometry analysis procedures have been described previously (19). In vitro T-cell suppression assay was performed essentially as previously described but with 1 µg/mL anti-CD3 and NOD. Rag1<sup>−/−</sup> splenocytes as antigen-presenting cells (20).

**Adoptive T-Cell Transfer**

Splenic total T-cells were isolated by negative selection using the Pan T-cell Isolation Kit II (Miltenic Biotec). Isolated total T-cells were confirmed by flow cytometry to be >95% pure and consisted of CD4 and CD8 T-cells (approximately a 2:1 ratio). NOD.Rag1<sup>−/−</sup> mice (21) were injected intravenously with 5 × 10<sup>6</sup> purified T-cells to test their diabetogenic activity. In some experiments, CD25<sup>+</sup> cells were also depleted in the T-cell preparation by a biotin-conjugated anti-CD25 antibody (clone 7D4) and antibiotin microbeads (Miltenic Biotec). Depletion of CD25<sup>+</sup> cells was confirmed by flow cytometry (<0.5% remaining).

**RESULTS**

**Generation of Tnfrsf9 Knockout NOD Mice**

A pair of fingers designed to specifically target the Tnfrsf9 gene was used to test if engineered ZFNs can be used to directly modify genomic sequence of NOD embryos.
Chen and Associates

showed various levels of insulitis in three nondiabetic NOD. 0.01, Kaplan-Meier log-rank analysis). Histological analysis

Tnfrsf9

approximately a 2:1 ratio). T1D development was monitored weekly
to be

significantly lower than those infused with wild-type T-cells (P <

0.01, Kaplan-Meier log-rank analysis). (B) The diabetogenic activity of CD137-deficient and -deficient T-cells was compared by transferring 5 × 10⁶ purified splenic T-cells isolated from 10-week-old standard NOD or NOD. Tnfrsf9 -/- (line-12) females into 10-week-old standard NOD females. T1D development was monitored weekly for 13 weeks. Total splenic T-cells were isolated as in A and B, and CD25 + cells were also depleted by

flow cytometry (mean insulitis scores of 1.3, 2.68, and 2.88, respectively). (B) The diabetogenic activity of CD137-sufficient and -deficient T-cells was compared by transferring 5 × 10⁶ purified splenic T-cells isolated from 10-week-old standard NOD or NOD. Tnfrsf9 -/- (line-12) females into 6-week-old NOD.Rag1 -/- recipients. Splenic total T-cells were isolated by negative selection using the Pan T-cell Isolation Kit II that depletes CD11b, CD11c, CD4, CD8, and CD4+FOXP3+ T-cells (regulatory T-cells [Tregs]) and, to a lesser extent, on CD4+FOXP3+ T-cells (Fig. 1B). No detectable level of CD137 expression was found on T-cells isolated from the homozygous mutant mice (Fig. 1B). Expression of CD137 was reduced on heterozygous T-cell subsets compared with their wild-type counterparts (Fig. 1B). Both line-6 and line-12 homozygous mutants lacked CD137 expression, verifying complete knockout of the gene. Since we did not find phenotypic differences between line-6 and line-12 mice, we report combined results of both lines or as indicated hereafter.

Figure 3—CD137 deficiency reduces the diabetogenic activity of T-cells. (A) To compare the diabetogenic activity of CD137-sufficient and -deficient T-cells, we transferred 5 × 10⁶ purified splenic T-cells isolated from 6-week-old standard NOD or NOD. Tnfrsf9 -/- (line-12) females into 6-8-week-old NOD.Rag1 -/- recipients. Splenic total T-cells were isolated by negative selection using the Pan T-cell Isolation Kit II that depletes CD11b, CD11c, CD19, B220, DX5, and CD105 - cells, confirmed by flow cytometry to be >95% pure, and consisted of CD4 and CD8 T-cells (approximately a 2:1 ratio). T1D development was monitored weekly for 16 weeks. T1D incidence of CD137-deficient T-cell recipients is significantly lower than those infused with wild-type T-cells (P < 0.005, Kaplan-Meier log-rank analysis). Histological analysis showed various levels of insulitis in three nondiabetic NOD.Rag1 -/- recipients infused with CD137-deficient T-cells at 16 weeks post-transfer (mean insulitis scores of 1.3, 2.68, and 2.88, respectively). (A) To compare the diabetogenic activity of CD137-sufficient and -deficient T-cells, we transferred 5 × 10⁶ purified splenic T-cells isolated from 6-week-old standard NOD or NOD. Tnfrsf9 -/- (line-12) females into 6-week-old NOD.Rag1 -/- recipients. Splenic total T-cells were isolated by negative selection using the Pan T-cell Isolation Kit II that depletes CD11b, CD11c, CD19, B220, DX5, and CD105 - cells, confirmed by flow cytometry to be >95% pure, and consisted of CD4 and CD8 T-cells (approximately a 2:1 ratio). T1D development was monitored weekly for 16 weeks. T1D incidence of CD137-deficient T-cell recipients is significantly lower than those infused with wild-type T-cells (P < 0.005, Kaplan-Meier log-rank analysis). Histological analysis showed various levels of insulitis in three nondiabetic NOD.Rag1 -/- recipients infused with CD137-deficient T-cells at 16 weeks post-transfer (mean insulitis scores of 1.3, 2.68, and 2.88, respectively). (B) The diabetogenic activity of CD137-sufficient and -deficient T-cells was compared by transferring 5 × 10⁶ purified splenic T-cells isolated from 10-week-old standard NOD or NOD. Tnfrsf9 -/- (line-12) females into 10-week-old standard NOD females. T1D development was monitored weekly for 13 weeks. Total splenic T-cells were isolated as in A and B, and CD25 + cells were also depleted by

a biotin-conjugated anti-CD25 antibody and antibiotin microbeads. Depletion of CD25 + cells was confirmed by flow cytometry (<0.5% remaining). T1D incidence of CD137-deficient T-cell recipients is significantly lower than those infused with wild-type T-cells (P < 0.01, Kaplan-Meier log-rank analysis).
Figure 4—Functional comparison of CD137-sufficient and CD137-deficient Tregs. To test the suppressive function of Tregs in vitro, CD4+CD25− (effectors) and CD4+CD25+ (Tregs) T-cells were isolated from the spleens of 7–9-week-old standard NOD or NOD.Tnfrsf9−/− (line-12) females. CFSE-labeled effector cells (5 × 10^4) isolated from (A and B) standard NOD or (C and D) NOD.Tnfrsf9−/− mice were cocultured in triplicate with graded numbers (5 × 10^4 to 0) of NOD or NOD.Tnfrsf9−/− Tregs in the presence of 2 × 10^5 NOD.Rag1−/− splenocytes and 1 μg/mL anti-CD3 in round-bottomed 96-well tissue culture plates in a final volume of 200 μL. Proliferation of effector cells pooled from triplicate wells was determined after 4 days of culture by CFSE dilution. (A and C) Summarized results of Treg-mediated suppression. The percentage of suppression is defined by the percentage reduction in the proportion of divided effector T-cells relative to that of the control without Tregs. The results are presented as the mean ± SEM from four independent experiments. (B and D) CFSE profiles of labeled effectors after 4 days of culture from one representative experiment.
**CD137 Deficiency Significantly Delays Spontaneous T1D in NOD Mice**

Female littermates of all three genotypes were followed for T1D development. Both the wild-type and heterozygous mice developed high levels of T1D similar to our standard NOD females (Fig. 2A). This indicates that one wild-type *Tnfrsf9* allele is sufficient to drive T1D development in NOD mice. In contrast, CD137-deficient NOD mice developed much later onset of T1D (Fig. 2A). Histological analyses of non-diabetic NOD. *Tnfrsf9*−/− mice at 30 weeks of age revealed severe insulitis (Fig. 2B). When analyzed at 10 weeks (preonset), levels of insulitis were slightly lower in NOD. *Tnfrsf9*−/− than in wild-type females, although it did not reach statistical significance (Fig. 2B), CD137 deficiency may suppress T1D by altering normal development of lymphoid and myeloid cells. However, no significant difference in the proportions and the numbers of CD4 and CD8 T-cells, B-cells, NK cells, and myeloid cells was found in the spleens of 7-week-old sex-matched wild-type and homozygous knockout littermates (cells were stained with antibodies against CD3/CD4/CD8, CD3/CD19/DX5, or CD11b/CD11c; data not shown).

**CD137 Deficiency Reduces the T-Cell Diabetogenic Activity**

To test if CD137 is important for the diabetogenic activity of T-cells, we transferred purified total splenic T-cells from NOD or NOD. *Tnfrsf9*−/− mice into NOD. *Rag1*−/− recipients. CD137-deficient T-cells had significantly lower ability than their wild-type counterpart to induce T1D in the NOD. *Rag1*−/− mice (Fig. 3A, 6-week-old donors; Fig. 3B, 10-week-old donors). Similar results were obtained when CD25+ cells were also depleted in the transferred T-cells (Fig. 3C). To test the function of Tregs, we performed in vitro T-cell suppression assays. Carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled CD4+CD25+ cells (effectors) were cultured with graded numbers of CD4+CD25− (Tregs) for 4 days. In all culture conditions, CD137-deficient Tregs showed a trend of less suppressive than the wild-type control but they were not statistically different (Fig. 4).

**DISCUSSION**

Development of the ZFN technology provides a means to specifically target a gene in rodents bypassing the need of ESCs. In this report, we demonstrated the feasibility of ZFNs to introduce gene-targeted mutations into NOD mice. The availability of such technology has several important implications for future genetic studies using this mouse model. These include elimination of time-consuming backcrosses to generate NOD knockout mice to study the diabetogenic function of a gene and, most importantly, avoidance of genetic contaminants that potentially interfere with the interpretation of a phenotype. As ESCs are not required, the ZFN approach will also provide a means to target candidate genes within an *Idd* region of a NOD congenic strain, allowing identification of diabetes susceptibility/resistance alleles. The application of the ZFN technology is not limited to generation of knockouts. Allele-specific modification could also be achieved when a DNA template is coinjected with the ZFN-coding mRNAs (12,23), a possibility we are currently testing in the NOD mouse.

CD137 has been implicated as the *Idd9.3* gene (14–16). NOD mice have a hypofunctional allele compared with the C57BL/10 (B10) variant (15). The functional difference was associated with an increased frequency of CD137-expressing Tregs in the *Idd9.3* congenics compared with standard NOD mice (16). CD137- expressing Tregs exhibited superior function than their negative counterpart, possibly due to the production of soluble CD137 (14–16). Our in vitro suppression assay did not show a significant difference between CD137-sufficient and CD137-deficient Tregs. As only a small proportion (~10–20%) of NOD Tregs expressed CD137 (16), it is possible that the difference between wild-type and CD137-deficient Tregs became less detectable in our assay. It remains to be determined if a different approach can reveal a functional role of CD137 in Tregs.

Although we showed that diabetes development was suppressed in CD137-deficient NOD mice, this does not exclude *Tnfrsf9* as the *Idd9.3* gene. As shown in Fig. 3, CD137-deficient T-cells are less diabetogenic than their wild-type counterpart, albeit potential effects of a small number of contaminating cells expanding in the lymphopenic recipients cannot be completely ruled out. Injection of anti-CD137 agonist antibodies also accelerated T1D in NOD-scid recipients infused with T-cells isolated from diabetic NOD mice (24). These results indicate that CD137 also plays a role in pathogenic T-effectors in the progression of T1D in NOD mice. Collectively, our results suggest that complete deficiency of CD137 predominantly affects the pathogenic function of β-cell autoreactive T-cells in NOD mice, resulting in delayed progression to T1D. Further studies are needed to conclusively dissect the role of CD137 in pathogenic T-effectors and Tregs.

In summary, we demonstrated an ability to use ZFNs to generate gene-targeted mutations directly in the NOD background, bypassing the need of ESCs. This provides a critical foundation for future applications of this technology to conduct genetic studies in NOD mice and will greatly facilitate the dissection of T1D pathogenesis.

**Acknowledgments.** The authors thank the personnel at the Medical College of Wisconsin Transgenic Core for their excellent technical support. The authors also thank D. Serreze of The Jackson Laboratory for providing the NOD/LtDVS breeder pairs.

**Funding.** This work was supported by the National Institutes of Health (grant DK077443 to Y.-G.C.), a basic science award (1-10-BS-26) from the American Diabetes Association (to Y.-G.C.), and the Children’s Hospital of Wisconsin Foundation.
Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. Y.-G.C. designed and performed the experiments, analyzed data, and wrote the manuscript. M.H.F., S.K., and A.E.C. performed experiments and edited the manuscript. M.J.H. contributed to discussion and edited the manuscript. A.M.G. contributed to design and edited the manuscript. Y.-G.C. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

References

1. Makino S, Kunimoto K, Muraoka Y, Mizushima Y, Katagiri K, Tochino Y. Breeding of a non-obese, diabetic strain of mice. Jikken Dobutsu 1980;29:1–13

2. Driver JP, Serreze DV, Chen YG. Mouse models for the study of autoimmune type 1 diabetes: a NOD to similarities and differences to human disease. Semin Immunopathol 2011;33:67–87

3. Vijayakrishnan L, Slavik JM, Illés Z, et al. An autoimmune disease-associated CTLA-4 splice variant lacking the B7 binding domain signals negatively in T cells. Immunity 2004;20:563–575

4. Yamanouchi J, Rainbow D, Serra P, et al. Interleukin-2 gene variation impairs regulatory T cell function and causes autoimmunity. Nat Genet 2007;39:329–337

5. Gerold KD, Zheng P, Rainbow DB, Zernecke A, Wicker LS, Kissler S. The soluble CTLA-4 splice variant protects from type 1 diabetes and potentiates regulatory T-cell function. Diabetes 2011;60:1955–1963

6. Hanna J, Markoulaki S, Mitalipova M, et al. Metastable pluripotent states in NOD-mouse-derived ESCs. Cell Stem Cell 2009;4:513–524

7. Nichols J, Jones K, Phillips JM, et al. Validated germline-competent embryonic stem cell lines from nonobese diabetic mice. Nat Med 2009;15:814–818

8. Morgan MA, Muller PS, Mould A, et al. The nonconventional MHC class II molecule DM governs diabetes susceptibility in NOD mice. PLoS ONE 2013;8:e56738

9. Kissler S, Stern P, Takahashi K, Hunter K, Peterson LB, Wicker LS. In vivo RNA interference demonstrates a role for Nramp1 in modifying susceptibility to type 1 diabetes. Nat Genet 2006;38:479–483

10. Chen Z, Stockton J, Mathis D, Benoist C. Modeling CTLA4-linked autoimmune with RNA interference in mice. Proc Natl Acad Sci USA 2006;103:16400–16405

11. Geurts AM, Cost GJ, Rémy S, et al. Generation of gene-specific mutated rats using zinc-finger nucleases. Methods Mol Biol 2010;597:211–225

12. Urnov FD, Rebar EJ, Holmes MC, Zhang HS, Gregory PD. Genome editing with engineered zinc finger nucleases. Nat Rev Genet 2010;11:636–646

13. Wang C, Lin GH, McPherson AJ, Watts TH. Immune regulation by 4-1BB and 4-1BBL: complexities and challenges. Immunol Rev 2009;229:192–215

14. Lyons PA, Hancock WW, Denny P, et al. The NOD Idd9 genetic interval influences the pathogenicity of insulitis and contains molecular variants of Cd30, Tnfr2, and Cd137. Immunity 2000;13:107–115

15. Cannons JL, Chamberlain G, Howson J, et al. Genetic and functional association of the immune signaling molecule 4-1BB (CD137/TNFRSF9) with type 1 diabetes. J Autoimmun 2005;25:13–20

16. Kachapati K, Adams DE, Wu Y, et al. The B10 Idd9.3 locus mediates accumulation of functionally superior CD137(+) regulatory T cells in the nonobese diabetic type 1 diabetes model. J Immunol 2012;189:5001–5015

17. Geurts AM, Cost GJ, Freyvert Y, et al. Knockout rats via embryo microinjection of zinc-finger nucleases. Science 2009;325:433

18. Serreze DV, Osborne MA, Chen YG, et al. Partial versus full allogeneic hematopoietic chimerization is a preferential means to inhibit type 1 diabetes as the latter induces generalized immunosuppression. J Immunol 2006;177:6675–6684

19. Chen YG, Scheuplein F, Driver JP, et al. Testing the role of P2X7 receptors in the development of type 1 diabetes in nonobese diabetic mice. J Immunol 2011;186:4278–4284

20. Chen YG, Scheuplein F, Osborne MA, Tsaih SW, Chapman HD, Serreze DV. Idd9/11 genetic locus regulates diabetogenic activity of Cd4 T-cells in nonobese diabetic (NOD) mice. Diabetes 2008;57:3273–3280

21. Shultz LD, Lang PA, Christianson SW, et al. NOD/LtSz-Rag1null mice: an immunodeficient and radioresistant model for engraftment of human hematolymphoid cells, HIV infection, and adoptive transfer of NOD mouse diabeticogenic T cells. J Immunol 2000;164:2496–2507

22. Carbery ID, Ji D, Harrington A, et al. Targeted genome modification in mice using zinc-finger nucleases. Genetics 2010;186:451–459

23. Cui X, Ji D, Fisher DA, Wu Y, Briner DM, Weinstein EJ. Targeted integration in rat and mouse embryos with zinc-finger nucleases. Nat Biotechnol 2011;29:64–67

24. Irie J, Wu Y, Kachapati K, Mittler RS, Rigway WM. Modulating protective and pathogenic CD4+ subsets via CD137 in type 1 diabetes. Diabetes 2007;56:186–196