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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*$^{-/-}$ recipients, even when CD25^{+} cells were predepleted. In vitro assays suggested that CD137 deficiency had a limited effect on the suppressive function of CD4^{+}CD25^{+} 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.

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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

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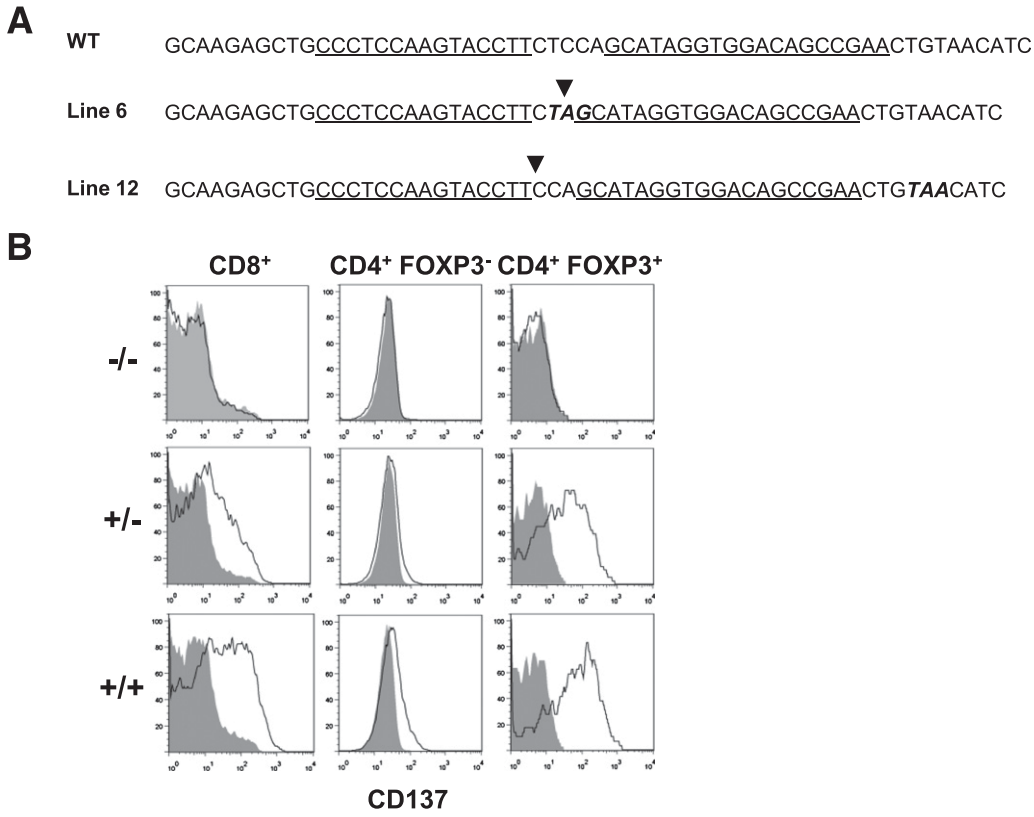

Figure 1—ZFN-mediated modification of the *Tnfrsf9* gene in NOD mice. (*A*) The wild-type (WT) sequence of the ZFN target site in exon 4 (the second coding exon) of the *Tnfrsf9* gene is shown at the top. The respectively altered sequences for line-6 and line-12 are indicated below WT. Each of the ZFN-binding 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. Splenocytes from 7- to 9-week-old male littermates of indicated genotypes were stimulated with 1.25 μg/mL anti-CD3 overnight, and cells were harvested and analyzed for CD137 protein expression by flow cytometry the following day. Representative histograms show the expression of CD137 on $CD8^+$, $CD4^+FOXP3^-$, or $CD4^+FOXP3^+$ gated cells. The solid line indicates the staining of CD137, and the shaded area depicts the isotype control. Same results were obtained in more than three independent experiments using line-6 or line-12 littermates.

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 Serreze'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 CCCTCCAAGTACCTTctccaGCATAGGTGGACAGCCGAA; ZFNs bind to each sequence shown in uppercase on

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′-AATGCCAGTCATTGTGATGC-3′) and reverse (5′-TCAAAGCTTAACTCTGCCCAA-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 BpmI 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*$^{-/-}$ 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*$^{-/-}$ mice (21) were injected intravenously with 5×10^6 purified T-cells to test their diabetogenic activity. In some experiments, CD25$^+$ 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$^+$ cells was confirmed by flow cytometry (<0.5% remaining).

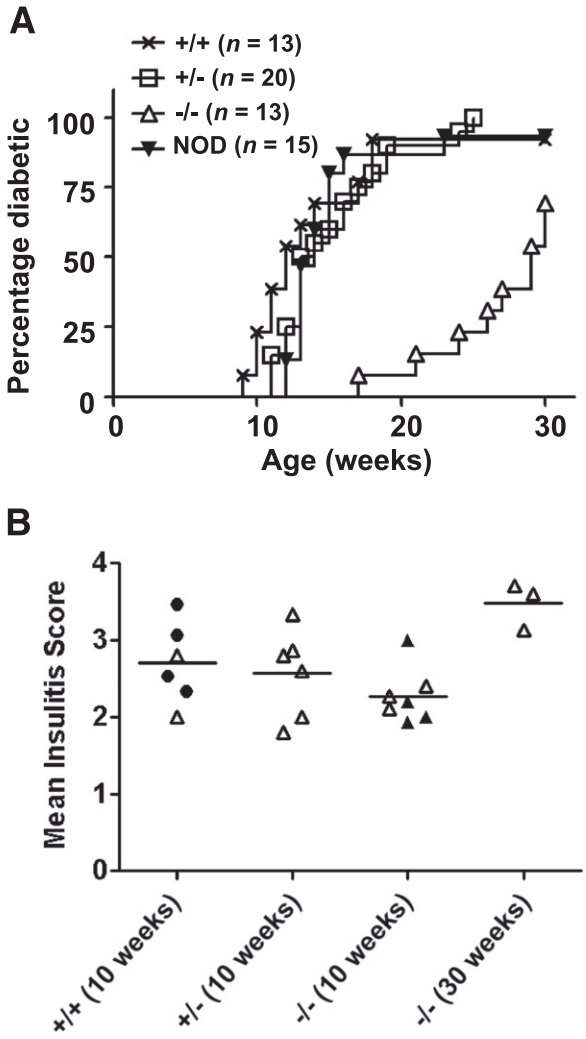

Figure 2—CD137 deficiency suppresses T1D but not insulitis development in NOD mice. (*A*) Female littermates of the indicated genotypes were monitored weekly for T1D development for 30 weeks. Diabetes onset was determined by two consecutive positive readings of glycosuria on a urine test strip (>250 mg/dL). A cohort of standard wild-type NOD females (not from line-6 or line-12 littermates) was also included for comparison. T1D progression in CD137-deficient ($^{-/-}$) mice was significantly suppressed when compared with all other groups ($P < 0.005$, Kaplan-Meier log-rank analysis). (*B*) Mean insulitis scores were determined in 10- and 30-week-old female littermates of indicated genotypes (open triangles), 10-week-old female progeny from CD137-deficient parents that had been fixed to homozygosity (closed triangles), or 10-week-old standard wild-type NOD females (closed circles). Pancreatic islets were individually scored as follows: 0, no lesions; 1, peri-insulitis but no penetration; 2, up to 25% islet destruction; 3, up to 75% islet destruction; and 4, end-stage–to–complete islet destruction. At least 15 islets were scored for each mouse, and the mean was calculated.

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.

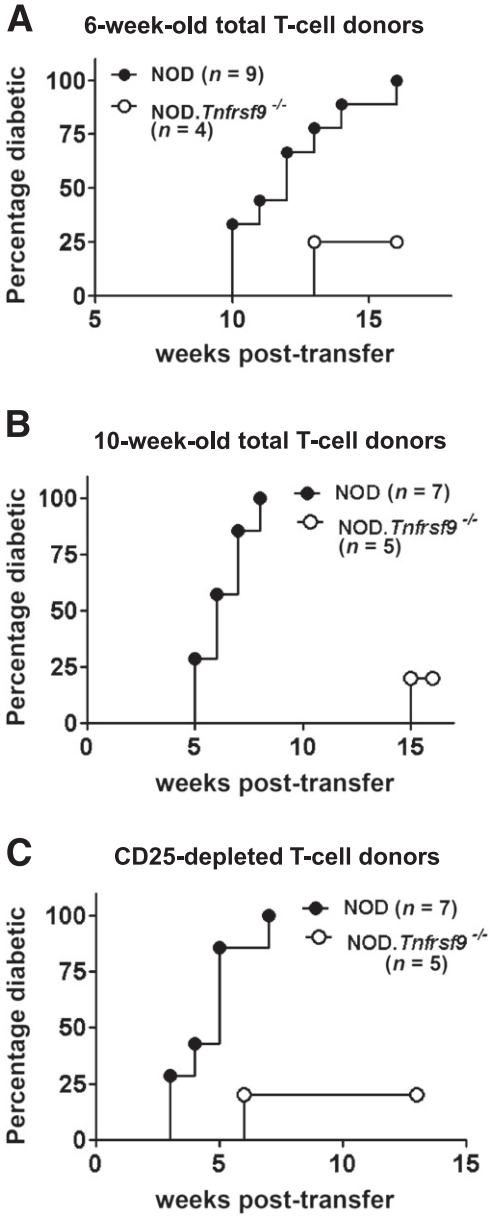

Figure 3—CD137 deficiency reduces the diabetogenic activity of T-cells. (*A*) To compare the diabetogenic activity of CD137-sufficient and -deficient T-cells, we transferred 5×10^6 purified splenic T-cells isolated from 6-week-old standard NOD or NOD.*Tnfrsf9*$^{-/-}$ (line-12) females into 6–8-week-old 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 ($P <$ 0.01, 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 5×10^6 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, 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). (*C*) To determine if the reduced diabetogenic activity of CD137-deficient T-cells was due to Tregs, we transferred CD25-depleted T-cells (5×10^6) isolated from 20-week-old NOD.*Tnfrsf9*$^{-/-}$ (line-12) females into NOD.*Rag1*$^{-/-}$ recipients. The control group received CD25-depleted T-cells isolated from 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).

mRNAs encoding the ZFNs were delivered by pronuclear injection. Of 329 transferred injected embryos, 19 pups were born, and the 13 that survived to weaning were screened for mutations. Two male founders (line-6 and line-12) were identified and sequence analyses of both showed a two base-pair deletion at the targeted site, resulting in premature stop codons (Fig. 1*A*). This translates to a gene-target mutation efficiency of 15% of screened potential founders, consistent with previous reports in other genetic backgrounds (22). Both founders were then crossed to standard wild-type NOD mice to test for germline transmission. Screening the offspring from the line-12 founder showed that close to 50% of them inherited the mutant allele (12 of 27 from three litters), indicating that the mutation was likely introduced into one allele at the one-cell stage. We needed to screen 28 pups (four litters) from the line-6 founder to obtain one heterozygous mouse that was then crossed again to a standard wild-type NOD mouse, suggesting germline mosaicism. Subsequent generations from line-6 showed a normal Mendelian inheritance of wild-type and the mutant alleles.

To confirm the absence of CD137 protein expression, we stimulated splenocytes isolated from wild-type, heterozygous, and homozygous mutant littermates from both line-6 and line-12, with anti-CD3 in culture. CD137 was highly expressed on anti-CD3-stimulated wild-type CD8 and CD4$^+$FOXP3$^+$ T-cells (regulatory T-cells [Tregs]) and, to a lesser extent, on CD4$^+$FOXP3$^-$ T-cells (Fig. 1*B*). No detectable level of CD137 expression was found on T-cells isolated from the homozygous mutant mice (Fig. 1*B*). Expression of CD137 was reduced on heterozygous T-cell subsets compared with their wild-type counterparts (Fig. 1*B*). 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.

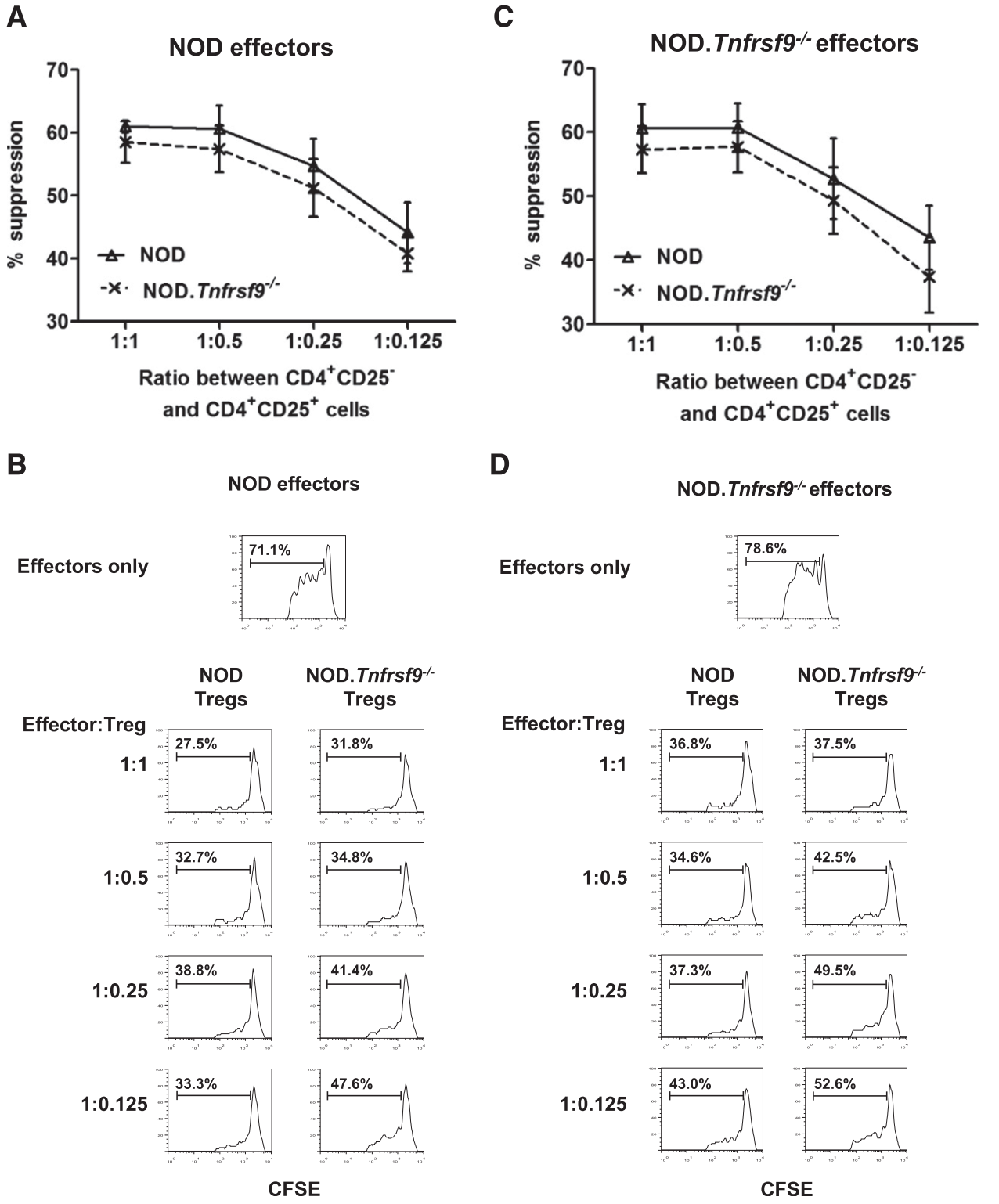

Figure 4—Functional comparison of CD137-sufficient and CD137-deficient Tregs. To test the suppressive function of Tregs in vitro, $CD4^+CD25^-$ (effectors) and $CD4^+CD25^+$ (Tregs) T-cells were isolated from the spleens of 7–9-week-old standard NOD or NOD.*Tnfrsf9*$^{-/-}$ (line-12) females. CFSE-labeled 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. 2*A*). 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. 2*A*). Histological analyses of nondiabetic NOD.*Tnfrsf9*$^{-/-}$ mice at 30 weeks of age revealed severe insulitis (Fig. 2*B*). 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. 2*B*). 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. 3*A*, 6-week-old donors; Fig. 3*B*, 10-week-old donors). Similar results were obtained when CD25^{+} cells were also predepleted in the transferred T-cells (Fig. 3*C*). 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^{+} Tregs exhibited superior function than their negative counterpart, possibly due to the production of soluble CD137 (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.

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