Original Article

Modulating Protective and Pathogenic CD4$^+$ Subsets via CD137 in Type 1 Diabetes

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CD137 (TNFRSF9) is an activation-inducible T-cell costimulatory molecule and a member of the tumor necrosis factor (TNF) receptor superfamily. *Cd137* is also a candidate gene (in the *Idd*9.3 interval) for autoimmune diabetes in NOD mice. Here, we demonstrate that anti-CD137 treatment protects NOD mice from diabetes. Anti-CD137–treated mice are not protected from insulitis and still harbor pathogenic T-cells, as demonstrated by transfer studies. Transfer of CD4$^+$, but not CD8$^+$, cells from anti-CD137–treated pre-diabetic NOD mice into NOD-*scid* mice delayed diabetes onset. Anti-CD137 treatment significantly increased the number of CD4$^+$CD25$^+$ cells, which demonstrated intracellular Foxp3 expression and in vitro suppressive activity. The CD4$^+$CD25$^+$ cell subset from anti-CD137–treated mice transferred complete protection from diabetes, whereas the CD4$^+$CD25$^-$ cell subset offered no significant protection. Anti-CD137 treatment of NOD-*scid* recipients of diabetic spleen cells, however, hastened the onset of disease, showing that the effect of anti-CD137 treatment depends on the balance of pathogenic and protective cells. These results support a critical role for CD137 acting in the early phase of autoimmune diabetes to enhance regulatory cell production. Disease-associated CD137 alleles are likely ineffectual at stimulating a regulatory T-cell population sufficient to prevent disease. *Diabetes* 56:186–196, 2007

The NOD mouse is a well-established immunogenetic model of spontaneous type 1 diabetes (1). NOD mice spontaneously develop insulitis (infiltration of the pancreatic islet cells) starting at 3 weeks of age and develop diabetes around 20 weeks. The NOD mouse model exemplifies the modern approach to characterizing a genetically complex autoimmune disease. The first genome scan of NOD mice demonstrated that multiple recessive loci, termed "insulin-dependent diabetes" (*Idd*) loci, were linked to diabetes resistance or susceptibility (2). At present, at least 20 genes are thought to play a role in this complex, multigenic process (1). The strongest genetic contribution is from the major histocompatibility complex (MHC) class II molecule, I-A^{g7}; however, even two copies of I-A^{g7} are insufficient to mediate diabetes when placed on a nonautoimmune non-MHC background (3). Therefore, although substitution of other MHC class II molecules can prevent diabetes (indicating that I-A^{g7} is necessary for disease), it is not both necessary and sufficient; many non-MHC *Idd* loci contribute critically to the disease process.

To establish the identity and function of non-MHC *Idd* loci, *Idd* congenic mice are bred to contain B6/B10 resistance (*Idd*) intervals on a predominant NOD (disease-related) genetic background. These congenic mice show decreased susceptibility to diabetes, confirming that the gene or genes whose B6/B10 alleles were resistant to disease were "captured" in the congenic intervals (1). The congenic genetic intervals are initially quite large, but a "congenic mapping" approach has led to the identification of several "candidate" genes in the intervals (4–9). One such candidate gene, in the *Idd9.3* locus, is *Cd137* (6). *Idd9.3* has been mapped to a 1.2-mb interval with 15 genes, including *Cd137* (10). Cannons et al. (10) recently demonstrated that the NOD allele of CD137, compared with the B10 allele, had decreased T-cell signaling; NOD T-cells stimulated with a CD137L produced less interleukin (IL)-2 than NOD.*Idd9.3* congenic T-cells.

CD137 is an inducible member of the TNF receptor superfamily expressed on activated T-cells (11). It is upregulated on T-cells 48 h after activation in vitro and functions as a T-cell costimulatory molecule (12,13). The CD137 ligand is expressed on activated antigen-presenting cells (APCs), especially at the sites of inflammation in vivo (14–17). CD137 stimulation augments T-cell proliferation and can function as a T-cell costimulatory molecule in the absence of CD28 (13,18). Anti-CD137 antibodies have elucidated the function of CD137 in vivo, causing expansion of both CD4 and CD8 T-cell subsets (12,19). T-cells from CD137$^{-/-}$ or CD137L$^{-/-}$ mice have decreased memory responses to antigen recall; CD137L$^{-/-}$ mice adoptively transferred with antigen-specific CD8 TCR TG cells showed decreased late-stage T-cell survival (20). These results combined with the expression kinetics of CD137 suggest a costimulatory role acting later in T-cell activation and critically in the activation of memory T-cells. Anti-CD137 antibodies have demonstrated activity in several murine autoimmune models. Anti-CD137 blocked development of collagen arthritis, accompanied by suppression of anti-collagen antibodies and decreased CD4$^+$ recall responses to type-2 collagen (17,21). In a murine lupus model, anti-CD137 reversed established disease and

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APC, antigen-presenting cell; EAE, experimental autoimmune encephalomyelitis; ELISA, enzyme-linked immunosorbent assay; IBD, inflammatory bowel disease; IFN-γ, interferon-γ; IL, interleukin; MHC, major histocompatibility complex; SLE, systemic lupus erythematosus; TNF, tumor necrosis factor; Treg, T regulatory.

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dramatically reduced autoantibody production (22). Anti-CD137 also prevented experimental autoimmune encephalomyelitis (EAE); notably, however, mice induced to get EAE by adoptive transfer of pathogenic T-cell clones were not protected by anti-CD137 (23). Overall, these results demonstrate a powerful effect of CD137 on T-cells and on the progression of autoimmune disease states.

Recently, $CD4^+CD25^+$ T-cells have emerged as essential regulatory cells controlling autoimmunity and antitumor immunity in human and animal models (24). In the setting of type 1 diabetes in NOD mice, quantitative and qualitative deficiencies of $CD4^+CD25^+$ T regulatory (Treg) cells have been reported (25,26), and transfer of $CD4^+CD25^+$ cells suppressed the onset of diabetes (27). Although the precise mechanisms employed by Treg cells remain unclear, costimulatory signaling is clearly important (25). Two studies reported that CD137 was upregulated in $CD4^+CD25^+$ Tregs (27,28), although conflicting results on the role of CD137 in Tregs were reported using $CD137^{-/-}$ mice (29,30).

In this study, we demonstrate that treatment of NOD mice with anti-CD137 antibody prevented autoimmune diabetes but did not eliminate pancreatic insulitis. Pathogenic cells, in fact, persisted in protected NOD mice, as shown by transfer studies. $CD4^+$ cells isolated from anti-CD137–treated mice ameliorated adoptive transfer of diabetes into NOD-*scid* mice, whereas control-treated $CD4^+$ cells did not. Using transfer of anti-CD137–treated or control pre-diabetic splenocytes into NOD-*scid*, we show that anti-CD137 significantly increased the number of $CD4^+CD25^+$ cells in treated mice, and transfer of these $CD4^+CD25^+$ cells completely prevented diabetes in NOD-*scid* recipients, whereas the $CD4^+CD25^-$ cell subset offered no significant protection. Treatment of NOD-*scid* mice with anti-CD137 after transfer of diabetic $CD4^+$ and $CD8^+$ cells not only did not prevent disease, but also hastened its onset, demonstrating that CD137 expression on autoreactive memory cells can mediate destructive effects. These results implicate a role for the *Idd9.3* candidate gene product, CD137, in type 1 diabetes pathogenesis and suggest that the balance of CD137 stimulation between regulatory and pathogenic T-cells could represent a therapeutic target.

RESEARCH DESIGN AND METHODS

NOD and NOD-*scid* colonies were maintained under specific pathogen-free conditions in our animal facilities. Mice were handled in accordance with the institutional animal care guidelines of the University of Pittsburgh School of Medicine. Urinary glucose analysis was performed using Tes-tape (Shionogi, Osaka, Japan) once a week. Plasma glucose levels were determined when glycosuria was detected, and mice with a blood glucose level above 300 mg/dl were considered diabetic. Antibodies against mouse CD3, CD4, CD8, CD25, CD28, CD49b, and T-cell β-receptor were purchased from BD Bioscience. Anti-Foxp3 antibody was purchased from eBioscience. Anti-mouse CD137 antibody (3H3) was produced by R.S. Mittler as previously described (22).

Histology. The pancreas of NOD mice was removed at the indicated times, fixed in 10% formaldehyde, and embedded in paraffin. Thin sections at six levels, 150 μm apart, were cut for staining with hematoxylin-eosin to evaluate islet-infiltrating cells by light microscopy. At least 35 islets from each recipient mouse were observed and scored using the following criteria: grade 0, islets free of insulitis; grade 1, peri-insulitis; grade 2, intra-insulitis with mononuclear cell infiltration of <50% of the area of each islet; and grade 3, intra-insulitis with mononuclear cell infiltration of >50% of the area of each islet.

Preparation, sorting, and stimulation of splenocytes and adoptive transfer. The spleen from each mouse was removed aseptically and minced. After lysing erythrocytes, the cells were washed three times with PBS. To sort $CD4^+$, $CD4^+CD25^+$, $CD4^+DX5^-$, or $CD8^+$ cells, splenocytes were prepared by magnetic separation using a magnetic sorting system (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The purities of sorted cells were >90%. In other experiments, purified CD4 populations were obtained by sorting using flow cytometry. To stimulate sorted splenocytes, cells were suspended in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Gibco-BRL) and penicillin/streptomycin (Gibco-BRL). The $CD4^+$ cells (1×10^6) resuspended in 2 ml culture medium were transferred to each well of a 24-well anti-CD3 antibody precoated plate with or without soluble anti-CD28 antibody (1 μg/ml). The cells were cultured for 72 h at 37°C in a humidified 5% CO_2 incubator, and the supernatant was collected at the end of culture.

For suppression experiments, $CD4^+CD25^+$ cells, 5×10^4 of effector or suppressor cells, or both were seeded to 96-well plate with soluble anti-CD3 antibodies and irradiated splenocytes as APCs.

Flow cytometry. Cells were incubated with Fc blocker (Pharmingen) and stained with labeled antibodies for 25 min at 4°C. Samples were analyzed on an FACS Caliber (Becton Dickinson, Miami, FL). Intracellular cytokine staining of Foxp3 was performed according to the manufacturer's instructions.

Cytokine measurement by enzyme-linked immunosorbent assay. Cytokines (interferon-γ [IFN-γ], IL-4, and IL-10) were measured by enzyme-linked immunosorbent assay (ELISA) as previously described (31). Briefly, flat-bottom 96-well plates were coated with anti–IFN-γ, anti–IL-4, or anti–IL-10 antibodies (Pharmingen), and supernatants were added. After incubation, biotinylated anti-cytokine antibodies and then europium-avidin solution (Perkin Elmer, Turku, Finland) were added. After adding enhancement solution (Perkin Elmer), plates were measured with an ELISA reader (Perkin Elmer).

Statistical analysis. Results are presented as means ± SE. Differences between the two groups were analyzed using Mann-Whitney *U* test for nonparametric unpaired observations. Log-rank tests were used to compare the incidence of diabetes between two groups using JMP IN software (SAS Institute) and Graph-pad (Graph-pad Software).

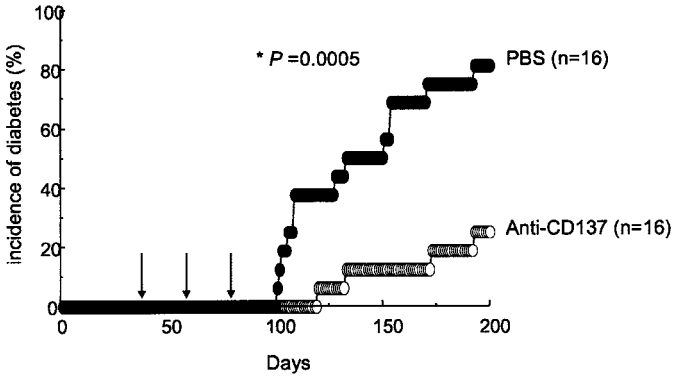

FIG. 1. Anti-CD137 antibody prevents type 1 diabetes in NOD mice. Female NOD mice aged 42–44 days were treated three times with 200 μg anti-CD137 antibody (○; $n = 16$) or 200 μg PBS (●; $n = 16$) (see arrows). The onset of diabetes was detected by checking for glucosuria every week and confirmed when the blood glucose levels were >300 mg/dl. *$P = 0.0005$ vs. control by log-rank test.

RESULTS

Anti-CD137 prevents diabetes but does not prevent insulitis nor eliminate pathogenic cells. To test the effect of anti-CD137 on the course of type 1 diabetes in NOD mice, we treated 6-week-old female NOD mice with a total of three 200-μg doses of anti-CD137 antibody (clone 3H3) (22) administered over a 9-week interval every 3rd week. Control mice received PBS alone; mice were followed for glycosuria. The administration of anti-CD137 antibody significantly suppressed type 1 diabetes (Fig. 1; $P = 0.0005$).

It has previously been published that when placed on the NOD background, the Idd9.3 region (containing the B10 allele of *Cd137*) decreased diabetes incidence but did not result in decreased insulitis (6). We tested whether anti-CD137 treatment ameliorated insulitis; as shown in Fig. 2*A*, anti-CD137 did not suppress pancreatic islet infiltration ("insulitis") in mice protected from diabetes. To further evaluate the progression of insulitis in treated

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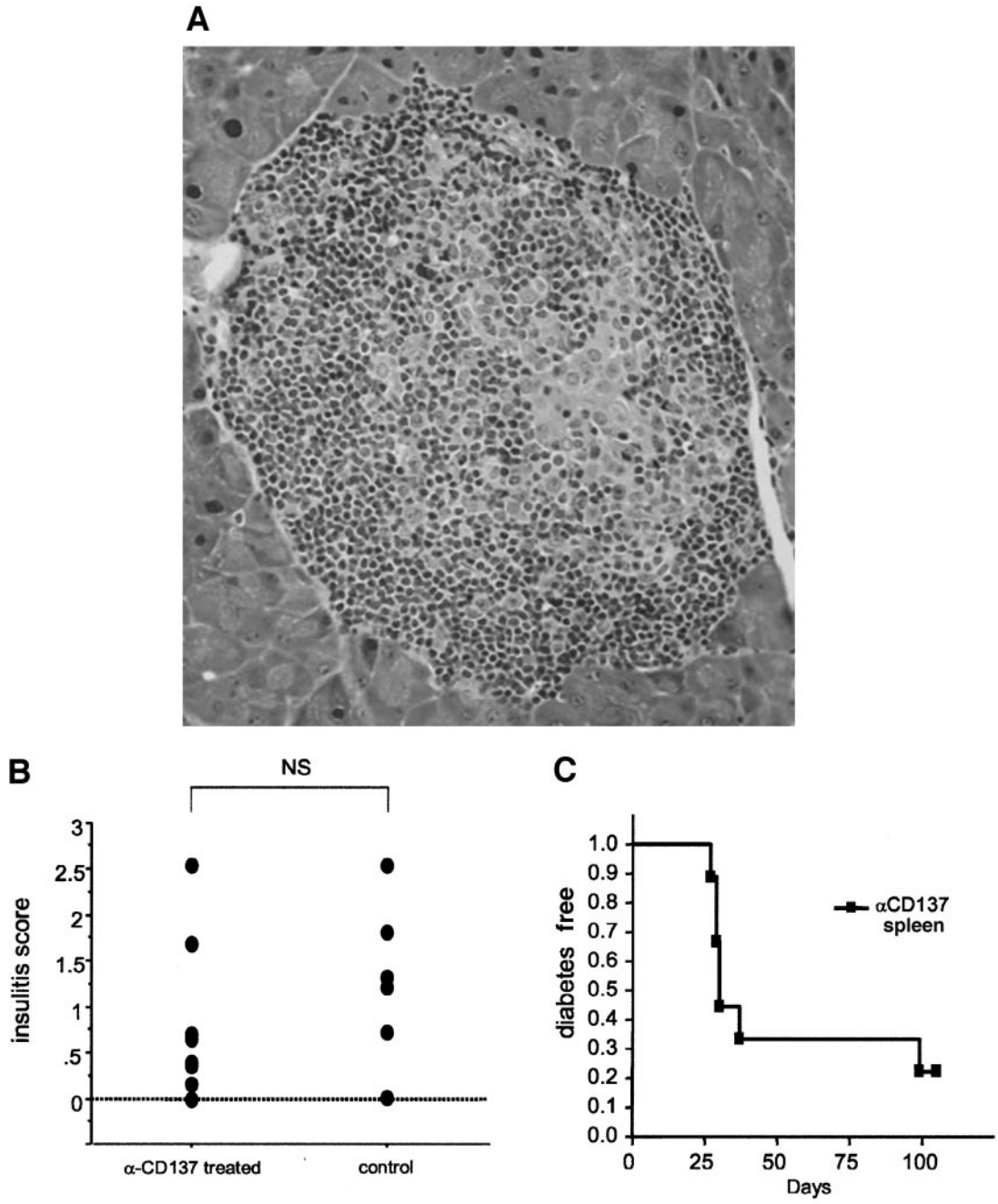

FIG. 2. Anti-CD137 does not prevent insulitis nor delete pathogenic T-cells. ***A*****: The pancreases of anti-CD137–treated, diabetes-free female NOD mice aged 207–254 days were studied by hematoxylin-eosin staining. One representative of eight mice is shown.** ***B*****: Quantification of insulitis in anti-CD137 antibody–treated female NOD mice at 10 weeks of age. Female NOD mice aged 46–55 days were treated twice with 200 μg anti-CD137 antibody (*** $n = 8$ **) or 200 μg PBS (** $n = 6$ **) and scored for insulitis 1 week after the second injection.** ***C*****: Splenocytes from anti-CD137–treated nondiabetic mice can transfer diabetes into NOD-*** ***scid*****. Spleens were harvested from three anti-CD137–treated NOD mice aged over 200 days and transferred into a total of nine NOD-*** ***scid*** **mice.**

mice, NOD mice were treated at 6 and 9 weeks of age with 200 μg anti-CD137 antibody or with PBS alone, and at 10 weeks of age the pancreas was scored for insulitis. As shown in Fig. 2*B*, the insulitis score between treated and control NOD mice was no different. Therefore we conclude that anti-CD137 treatment prevents type 1 diabetes but not by preventing insulitis.

The presence of insulitis in the treated mice raised the question of whether anti-CD137 directly or indirectly affected pathogenic T-cells, for example, by inducing deletion or regulation of autoreactive T-cells. We addressed this possibility by transferring 20×10^6 splenocytes from anti-CD137–treated nondiabetic NOD mice, aged over 200 days, into NOD-*scid* recipients. Although the donor mice were protected from diabetes, seven of nine NOD-*scid* recipients developed type 1 diabetes, indicating that the donors retained pathogenic T-cells (Fig. 2*C*). This result demonstrated that pathogenic T-cells were not destroyed or inactivated in anti-CD137–treated NOD mice.

$CD4^+$ cells from mice recently treated with anti-CD137 antibody can ameliorate adoptive transfer of diabetes. The above experiments raised the question: why did splenocytes from aged, but nondiabetic, treated mice transfer disease? We reasoned that, because some anti-

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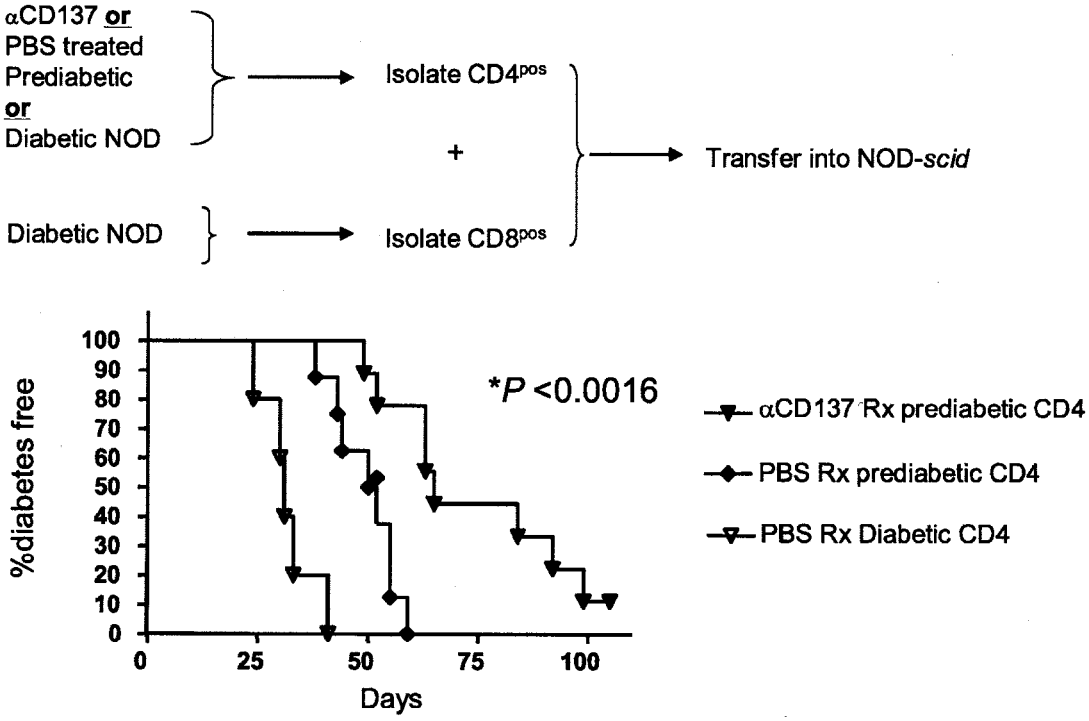

FIG. 3. $CD4^+$ splenocytes from anti-CD137–treated mice ameliorate transfer of diabetes into NOD-*scid* mice. Female NOD-*scid* mice received (0.5–2) × 10^6 diabetic CD8 cells plus 2 × 10^6 $CD4^+$ splenocytes from pre-diabetic female NOD mice, age 74 days, treated by either CD137 or PBS as above (see transfer scheme). The sum of three independent experiments is shown (anti-CD137, ▲, n = 9; PBS, ♦, n = 8), P = 0.0016 by log-rank test. A group receiving $CD4^+$ and $CD8^+$ cells from diabetic NOD donors is shown for comparison of diabetes onset between diabetic and pre-diabetic donors.

CD137–treated mice eventually develop diabetes (Fig. 1), the regulatory effect likely decreased over time. As a result, when splenocytes from aged treated mice were transferred into NOD-*scid*, homeostatic expansion of autoreactive cells in the NOD-*scid* lymphopenic environment (32) might overwhelm any remaining regulatory effect. The hypothesis resulting from this scenario is that regulatory cells from recently treated mice should be able to ameliorate disease in the NOD-*scid* transfer model. To test this hypothesis we used a pre-diabetic NOD transfer system, as previously described (33), to investigate the protective cell populations in the pre-diabetic mice. $CD4^+$ cells from anti-CD137–treated 10-week-old pre-diabetic NOD mice, but not from PBS-treated age-matched controls, significantly delayed disease transfer into NOD-*scid* recipients (Fig. 3; P = 0.0016). (Note the donor population used in this model is pre-diabetic; as shown in Fig. 3, diabetic splenocytes transfer disease more rapidly.) Therefore freshly recruited $CD4^+$ regulatory cells can counteract the disease process even in the NOD-*scid* transfer model. Notably, however, in the NOD-*scid* transfer model, the protective effect of anti-CD137 antibody treatment was less than in "normal" NOD mice (Fig. 1), likely as a result of rapid homeostatic effector expansion in the NOD-*scid* environment. It has previously been reported that anti-CD137 treatment can induce $CD8^+$ regulatory cells as a protective mechanism (17). To test the hypothesis that regulatory $CD8^+$ cells could have suppressor function in treated NOD mice, $CD8^+$ cells from either anti-CD137–treated or PBS-treated control NOD mice were transferred to NOD-*scid* recipients with diabetic $CD4^+$ cells. $CD8^+$ cells from treated NOD mice, however, did not have protective ability, indicating that $CD8^+$ regulatory cells are unlikely to participate in anti-CD137–induced diabetes protection in NOD mice (data not shown). These results are in agreement with data of Foell et al. (22), which showed that anti-CD137–mediated reversal of systemic lupus erythematosus (SLE) was independent of $CD8^+$ T-cells.

Anti-CD137 treatment alters the cytokine phenotype of $CD4^+$ cells and increases the proportion of IL-10–producing $DX5^+$ T-cells; $Dx5^+$ cells do not prevent diabetes. The co-transfer experiment shown above encouraged us to evaluate the functional properties of $CD4^+$ cells induced by anti-CD137 antibody treatment. As shown in Fig. 4, sorted $CD4^+$ splenocytes from anti-CD137–treated mice produced significantly more IL-4 and IL-10 and less IFN-γ than $CD4^+$ cells from untreated mice.

To determine the source of IL-4/IL-10–producing cells, we examined $Dx5^+$ NKT cells, because it has been recently shown that $CD137^{-/-}$ mice have decreased numbers of $CD3^+/Dx5^+$ NK T-cells, and it is well established that NKT cells produce substantial quantities of IL-4 and IL-10 (34,35). We hypothesized that one mechanism of anti-CD137–mediated protection occurred through its effects on NK T-cell numbers and function. To test this hypothesis, we isolated $Dx5^+$ cells from anti-CD137–treated and untreated NOD mice (at 10 weeks of age as above) and examined the numbers of positive cells. The number of $Dx5^+$ T-cells increased in anti-CD137–treated NOD spleen and peripheral lymph node (P = 0.0045 and 0.0012, respectively; Fig. 5*A*).

Next, we asked to what extent the change in cytokine production in the $CD4^+$ population was due to $Dx5^+$ cells. Stimulated $CD4^+$ cells from anti-CD137–treated spleens produced more IL-4 and IL-10 than $CD4^+$ cells from PBS-treated mice (Fig. 5*B*, solid bars), and removing $DX5^+$ cells from the $CD4^+$ population eliminated the enhanced

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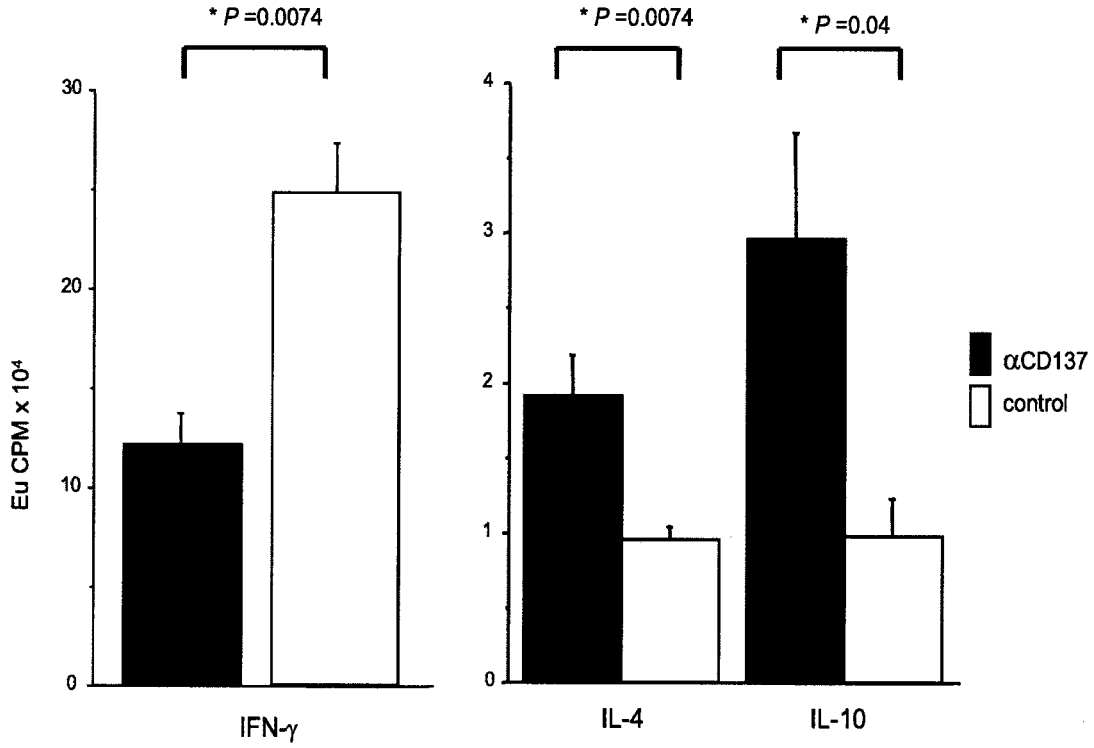

FIG. 4. Anti-CD137 treatment increases IL-4 and IL-10 production and decreases IFN-γ production. Female NOD mice aged 49–55 days were treated with 200 μg anti-CD137 antibody (n = 7) or 200 μg PBS (n = 5) every 3 weeks and studied 1 week after the second CD137 treatment. $CD4^+$ splenocytes were purified by magnetic sorting and stimulated with plate-bound anti-CD3 antibody for 3 days. The supernatants were collected and IFN-γ (i), IL-4 (ii), and IL-10 (iii) concentrations were measured by ELISA. The europium counts for each sample are shown. i, P = 0.0074; ii, P = 0.0074; and iii, P = 0.04 by Mann-Whitney U test.

level of cytokine production; the $CD4^+DX5^-$ cells from treated mice had the same production of IL-4 and IL-10 as untreated $CD4^+$ cells (Fig. 5*B*, hatched bars).

Finally, to test whether $Dx5^+$ NKT cells had a protective effect in vivo, we used two approaches. First, $CD4^+Dx5^+$ cells were purified using sorting by flow cytometry. NOD-*scid* recipients then received either $CD4^+DX5^-$ populations alone or $CD4^+DX5^-$ cells plus $CD4^+Dx5^+$ cells from anti-CD137–treated pre-diabetic mice, along with diabetic $CD8^+$ cells. As shown in Fig. 5*C*, there was no significant benefit in diabetes prevention obtained by adding $CD4^+Dx5^+$ to $CD4^+Dx5^-$ cells, compared with $CD4^+Dx5^-$ cells alone. In the second approach, NOD-*scid* mice received either whole $CD4^+$ (including $Dx5^+$) cells from untreated pre-diabetic NOD mice or $CD4^+DX5^-$ cells, along with diabetic $CD8^+$ cells. Again, as shown in Fig. 5*D*, there was no significant difference in diabetes onset between the two groups, indicating no treatment effect of depleting $Dx5^+$ cells in the transfer system. Both $CD4^+Dx5^+$ and $CD4^+Dx5^-$ transfers from anti-CD137–treated mice delayed diabetes compared with $CD4^+$ or $CD4^+Dx5^-$ populations from untreated mice (Fig. 5*C* compared with *D*, P = 0.0023), but neither $Dx5^+$ nor $Dx5^-$ fractions from untreated mice delayed diabetes onset (Fig. 5*D*; compare with PBS-treated CD4 cells in Fig. 3). This result showed that there was no protective effect of the $CD4^+Dx5^+$ population in the NOD-*scid* transfer system (although it remains possible that a protective effect of Dx5 cells exists and is not shown by our experiments) but confirmed that there must be at least one protective $CD4^+$ cell population in the anti-CD137–treated $CD4^+Dx5^-$ cell population.

Anti-CD137 treatment increases the $CD4^+CD25^+$ Treg cell population; anti-CD137–treated $CD4^+CD25^+$ cells prevent adoptive transfer of diabetes; anti-CD137 binds specifically to $CD4^+CD25^+$ cells in vivo. The transfer of type 1 diabetes from splenocytes of anti-CD137–treated nondiabetic mice and the protective capability of anti-CD137–treated $CD4^+$ subsets indicated that anti-CD137 treatment did not destroy/inactivate pathogenic $CD4^+$ T-cells but acted on regulatory cells. The presence of a protective effect in the treated $CD4^+Dx5^-$ subset led us to investigate other regulatory CD4 subsets. It has been well established that $CD4^+CD25^+$ regulatory cells can modulate the development of type 1 diabetes in the presence of pathogenic T-cells. Quantitative and qualitative defects of $CD4^+CD25^+$ Treg cells have been reported in NOD mice, and $CD4^+CD25^+$ Tregs can prevent type 1 diabetes in NOD mice (25,26). Therefore, we investigated the quantitative and qualitative phenotype of $CD4^+CD25^+$ Treg cells in anti-CD137–treated NOD mice.

As shown in Fig. 6*A*, we found that naïve $CD4^+CD25^+$ cells in NOD mice upregulate CD137 compared with $CD25^-$ cells, supporting two previous reports, which indicated that $CD4^+CD25^+$ cells upregulated CD137 compared with $CD4^+CD25^-$ cells (27,28). The upregulation of CD137 on $CD25^+$ cells suggested a mechanism whereby CD137 antibody treatment could preferentially affect $CD4^+CD25^+$ cells. As shown in Fig. 6*B*, anti-CD137 antibody treatment increased the number of $CD4^+CD25^+$ T-cells in NOD mice.

To confirm that increased $CD4^+CD25^+$ T-cells were regulatory cells and not polyclonally activated T-cells upregulating CD25, intracellular Foxp3 expression was analyzed. $CD25^+$ cells in anti-CD137–treated mice were

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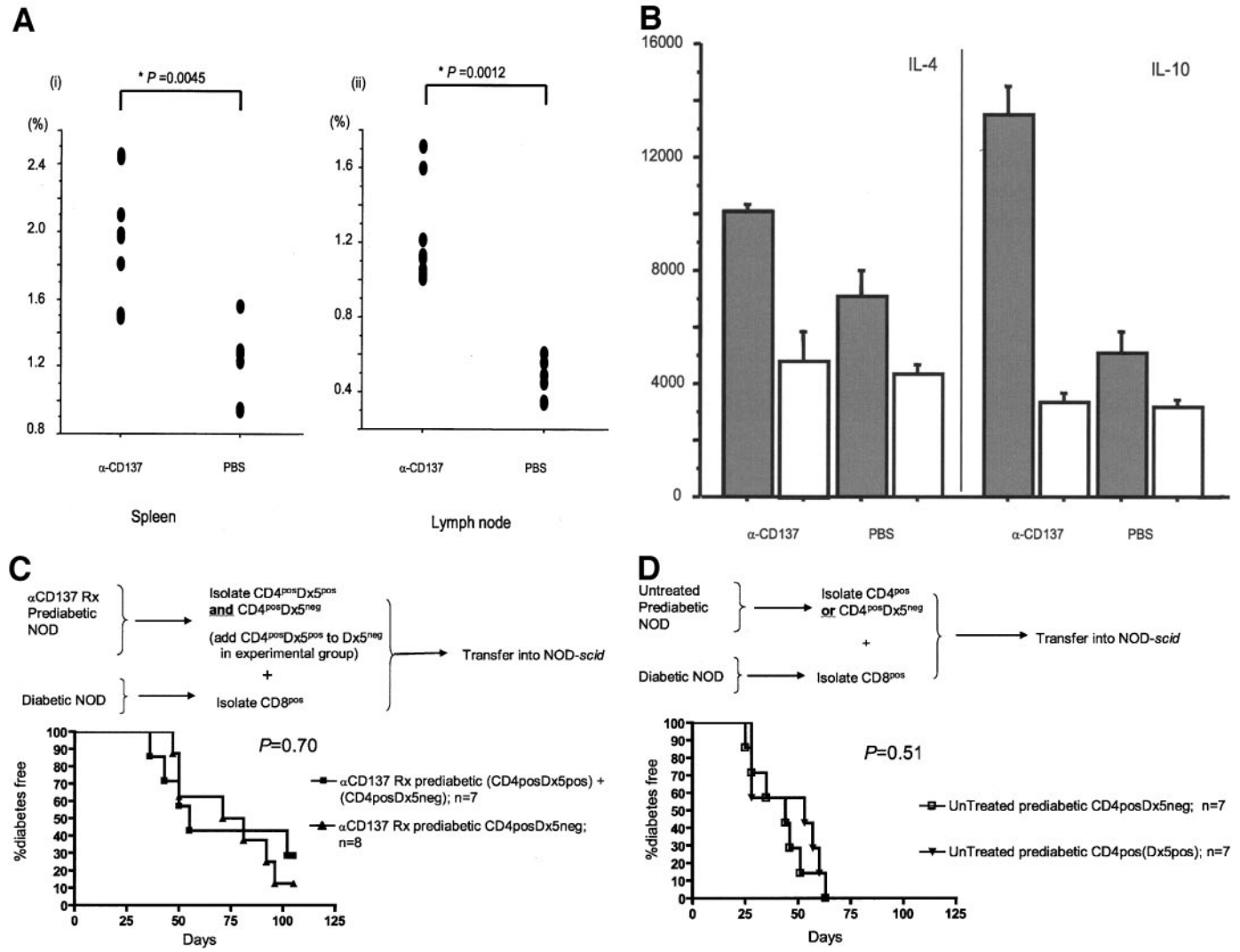

FIG. 5. Anti-CD137 treatment increases the population of cytokine-producing TCR$^+$Dx5$^+$ cells, but anti-CD137–treated CD4$^+$Dx5$^+$ cells do not ameliorate diabetes onset. *A*: Anti-CD137 treatment significantly increases the numbers of peripheral TCR$^+$DX5$^+$ cells. Female NOD mice aged 44–55 days were treated twice with 200 μg anti-CD137 antibody or PBS at a 3-week interval and studied 1 week after the second injection. Spleen and peripheral lymph nodes were analyzed by fluorescence-activated cell sorting. $P = 0.0045$ and 0.0012 for spleen and lymph node, respectively, by Mann-Whitney *U* test. Anti-CD137 ($n = 8$). *B*: Increased IL-4 and IL-10 in anti-CD137–treated mice is due to the CD4$^+$Dx5$^+$ (■) cell subset. Female NOD mice aged 44 days were treated as above and studied at 1 week after the second CD137 treatment. CD4$^+$ and CD4$^+$DX5$^-$ (□) splenocytes were purified by magnetic sorting and stimulated with plate-bound anti-CD3 antibody and soluble anti-CD28 antibody for 3 days. Supernatants were analyzed by ELISA for IFN-γ, IL-4, and IL-10 concentrations. One representative of four experiments is shown. *C*: CD4$^+$Dx5$^+$ cells from anti-CD137–treated NOD mice do not ameliorate disease onset compared with CD4$^+$Dx5$^-$ cells. CD4$^+$Dx5$^+$ and CD4$^+$Dx5$^-$ cell fractions were sorted by flow cytometry from anti-CD137–treated NOD mice, and recipient NOD-*scid* mice received either CD4$^+$Dx5$^-$ cells alone (solid triangles, $n = 8$) or CD4$^+$Dx5$^+$ cells combined with CD4$^+$Dx5$^-$ cells (solid squares, $n = 7$), along with diabetic CD8$^+$ cells. Two separate experiments were performed; the Dx5$^+$ cells offered no significant additional protective effect. *D*: Depletion of Dx5$^+$ cells from naïve pre-diabetic NOD CD4$^+$ cells does not enhance diabetes. Whole CD4$^+$ (2×10^6; inverted triangles) or DX5-depleted CD4$^+$ splenocytes (open squares) were purified by magnetic beads from 63- to 65-day-old female untreated NOD mice, and transferred into female NOD-*scid* mice with CD8$^+$ splenocytes (2×10^6) from diabetic female NOD mice ($n = 7$ each group, two separate experiments).

Foxp3$^+$ (Fig. 6*C*), indicating that the administration of anti-CD137 antibody induced Foxp3$^+$CD4$^+$CD25$^+$ Treg cells associated with diabetes protection. To prove the regulatory function of CD4$^+$CD25$^+$ Treg cells in vitro, we performed suppression studies, which showed that CD4$^+$CD25$^+$ cells from anti-CD137–treated NOD mice had suppressive activity on CD25$^-$ cells from both naïve and anti-CD137–treated mice (Fig. 6*D*). These findings indicated that CD137 antibody–induced CD4$^+$CD25$^+$ cells have the Treg cell phenotype.

To directly prove that treatment-induced CD4$^+$CD25$^+$ cells have protective ability, we transferred CD4$^+$CD25$^+$ or CD4$^+$CD25$^-$ cells from anti-CD137–treated mice, along with diabetic CD8$^+$ cells, to NOD-*scid* recipients. As shown in Fig. 6*E*, all NOD-*scid* recipients transferred with CD4$^+$CD25$^+$ cells were completely protected from diabetes compared with those that received CD4$^+$CD25$^-$ cells ($P < 0.0001$). Moreover, mice receiving anti-CD137–treated CD4$^+$ cells from which CD25$^+$ cells were depleted (CD4$^+$CD25$^-$) showed no significant difference in disease onset compared with CD4$^+$ T-cells transferred from NOD mice treated with PBS alone (Fig. 6*E*, $P = 0.91$), which strongly supports the hypothesis that anti-CD137 therapy acts on CD4$^+$CD25$^+$ cells. Interestingly, we found that some of the mice receiving CD25$^-$ cells developed inflammatory bowel disease (IBD) (manifested by wasting and rectal prolapse) or neuropathy, consistent with previous reports (data not shown; 36,37). Of mice receiving the

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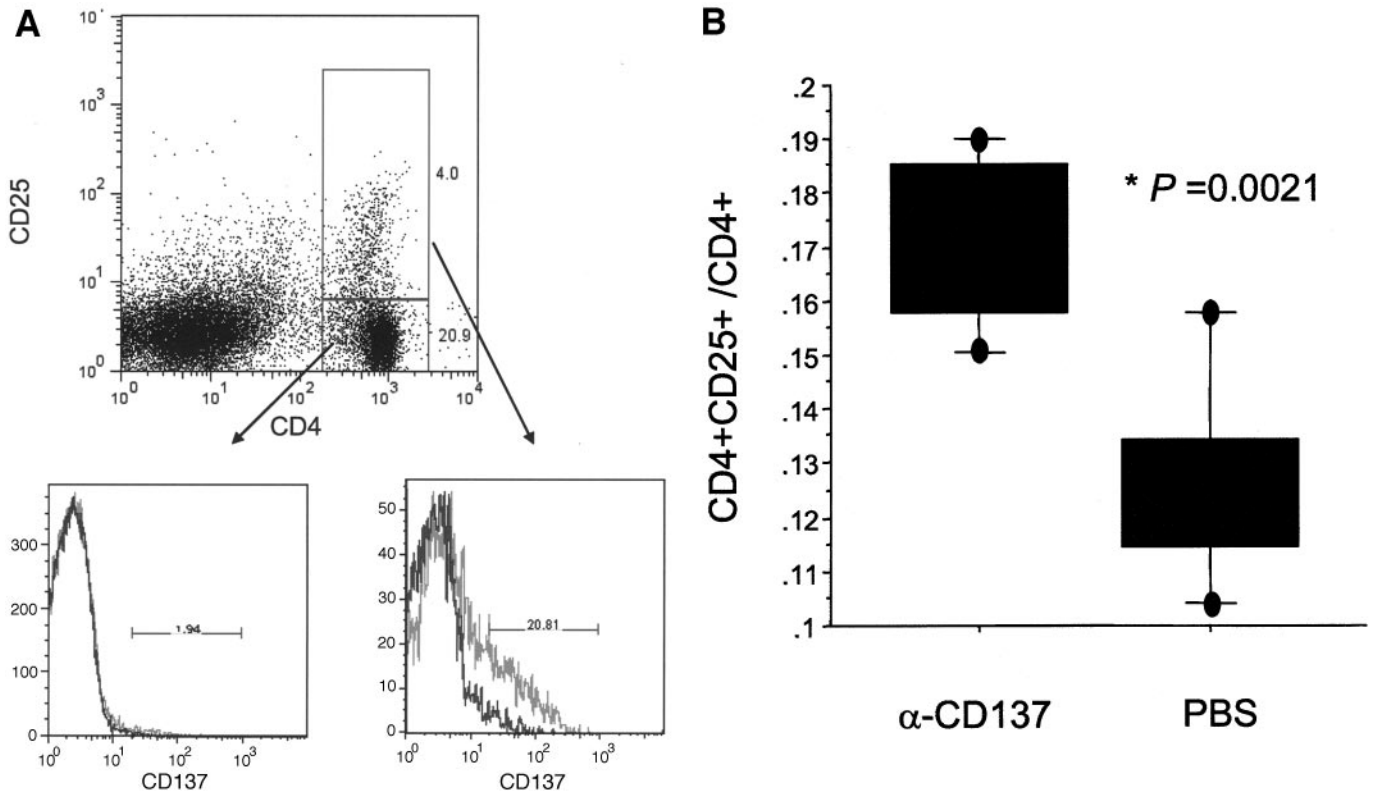

FIG. 6. CD4$^+$CD25$^+$ cells express CD137; treatment with anti-CD137 boosts CD25$^+$ cell numbers; and anti-CD137–treated CD25$^+$ cells prevent diabetes. *A*: Naïve NOD CD4$^+$CD25$^+$ cells express increased CD137 compared with CD25$^-$ cells. Splenocytes from 50- to 75-day-old NOD mice were analyzed for CD137 expression on CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ cells. One representative of 11 NOD mice is shown. *B*: Anti-CD137 treatment increases the numbers of CD4$^+$CD25$^+$ cells. Female NOD mice 5–8 weeks of age were treated with 200 μg anti-CD137 antibody (3H3) twice at 3-week intervals, and splenocytes were studied 1 week after the second treatment. The ratio of CD4$^+$CD25$^+$ cells to CD4$^+$ cells is shown. *C*: Anti-CD137–treated CD25$^+$ cells express Foxp3. Female NOD mice aged 43 days were treated with 200 μg anti-CD137 antibody (3H3) or PBS twice with 3-week intervals and studied at 1 week after the second treatment. Surface expression of CD4 and CD25 and intracellular Foxp3 expression were analyzed. Foxp3 (red) and isotype control (green) are shown. *D*: Anti-CD137–treated CD25$^+$ cells show suppressive function in vitro. Female NOD mice aged 43 days were treated with 200 μg anti-CD137 antibody (3H3) or PBS twice with 3-week intervals and studied at 1 week after second treatment. CD25$^+$ (5×10^4) or CD25$^-$ (5×10^4) cells (from either treated or naïve NOD mice) or both were cultured with irradiated splenocytes and anti-CD3 antibody for 4 days. [^{3}H]thymidine was added during the final 18 h of culture. *E*: CD25$^+$ cells from anti-CD137–treated mice protect against diabetes. Female NOD mice aged 38–49 days were treated with 200 μg anti-CD137 antibody (3H3) twice at 3-week intervals; at 10 weeks, CD25$^+$ and CD25$^-$ populations were sorted, and CD25$^+$ ($4–6 \times 10^5$) or CD25$^-$ ($4–6 \times 10^5$) splenocytes were transferred to female NOD-*scid* mice with CD8$^+$ splenocytes from female diabetic NOD mice. Results from two separate experiments are shown; the PBS-treated pre-diabetic CD4$^+$ group is the same as shown in Fig. 3. *F*: Anti-CD137 antibody specifically binds CD4$^+$CD25$^+$ cells in vivo. Female NOD mice were treated with 200 mg anti-CD137 antibody (3H3) or PBS twice at 3-week intervals and studied at 1 week after the second treatment. Splenocytes were stained with CD4, CD25 (or CD8/CD44 or CD4/Dx5), and anti-IgG2a-bio antibodies followed by strep-avidin phycoeythrin or step-avidin APC. Only CD4$^+$CD25$^+$ cells showed binding by anti-CD137. One representative of five experiments.

CD25$^-$ cells, five of nine developed diabetes, whereas four of nine developed either IBD or neuropathy. The finding that diabetes and neuropathy/IBD were mutually exclusive is consistent with the report of Setoguchi et al. (36), who had similar results.

To test whether anti-CD137 had a preferential cell target in vivo, we harvested splenocytes from NOD mice treated twice with 3H3, 1 week after the final treatment. We stained for CD4 and CD25 and used the second-step (anti-IgG2a-bio) antibody specific for 3H3, followed by strep-avidin phycoeythrin. As shown in Fig. 6*F*, 3H3 was bound only to CD4$^+$CD25$^+$ cells, not CD4$^+$CD25$^-$ cells in vivo, consistent with a mechanism of action directed to this Treg subset. There was not significant in vivo binding of 3H3 to CD8 or Dx5$^+$ cell subsets.

Anti-CD137 treatment of diabetic splenocytes can hasten disease onset in a transfer system. Finally, we tested directly whether anti-CD137 antibody could act on pathogenic CD4$^+$ cells by using a different cell transfer system, in which CD4$^+$ and CD8$^+$ cells were sorted from diabetic NOD splenocytes and transferred into NOD-*scid* recipients. At the time of transfer, recipient NOD-*scid* mice were treated with a single dose of 200 μg anti-CD137 or PBS. Treatment of NOD-*scid* recipients with anti-CD137 at the time of adoptive transfer did not prevent diabetes compared with PBS-treated control NOD-*scid* recipients and slightly but significantly increased the onset of diabetes (Fig. 7). This result confirms that the protective effect of anti-CD137 did not act by inactivating pathogenic CD4$^+$ and CD8$^+$ T-cells and, in fact, shows that activating CD137 on autoreactive T-cells could enhance disease onset. This result agrees with the data of Sun et al. (23), who showed that although anti-CD137 therapy can prevent EAE, it could not prevent EAE after adoptive transfer of a pathogenic T-cell population. The lack of effect of anti-CD137 antibody at time of transfer into NOD-*scid* is likely due to an imbalance of regulatory cells compared with pathogenic cells in diabetic NOD spleen populations.

DISCUSSION

We have demonstrated that treatment specifically directed to the *Idd9.3* candidate gene product, CD137, can prevent diabetes in NOD mice and showed that anti-CD137 therapy

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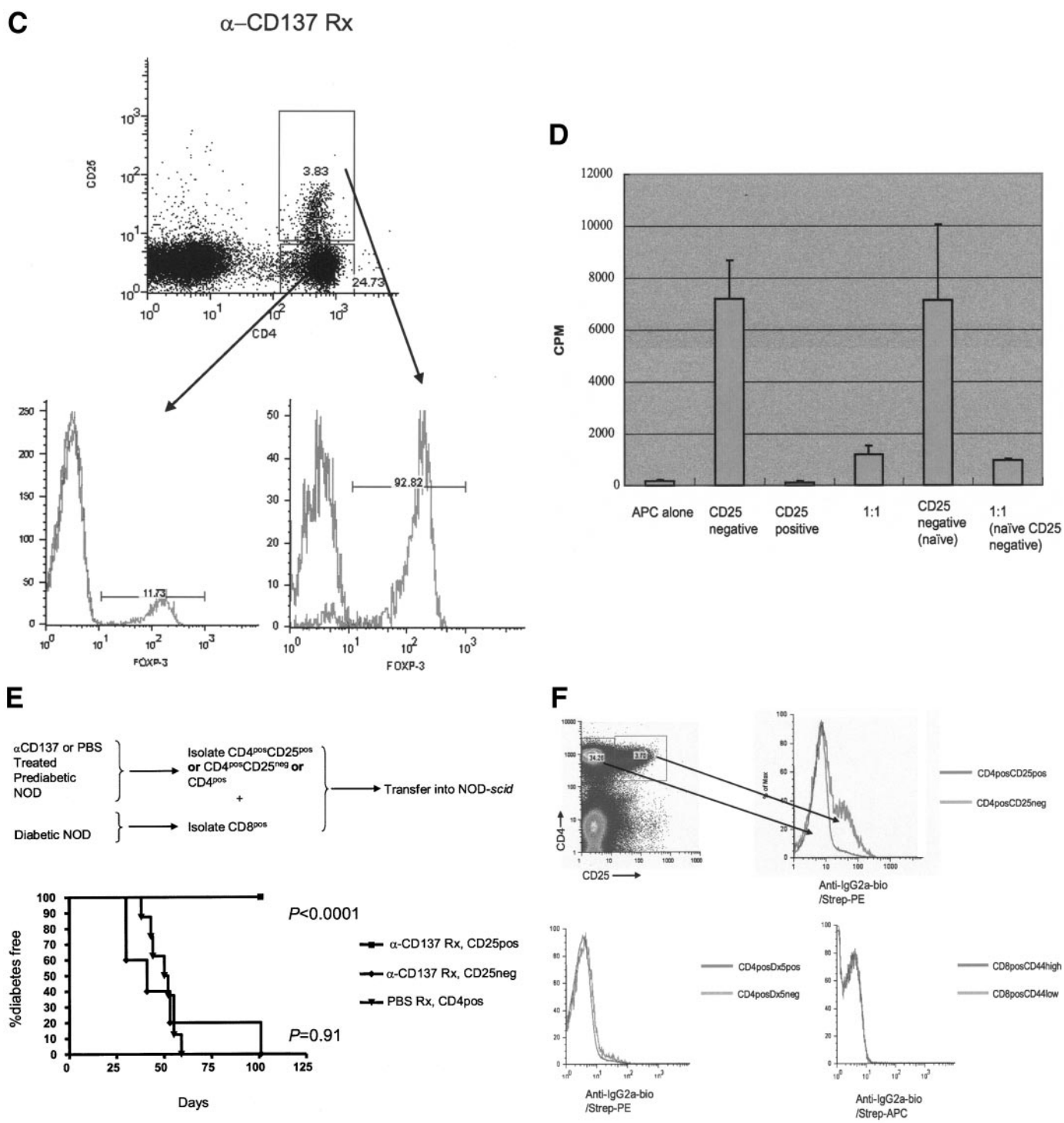

FIG. 6 Continued

increased the number of $CD4^+Dx5^+$ and $CD4^+CD25^+$ Treg cells. By using transfer models into NOD-*scid* mice, we showed that the effect of anti-CD137 treatment is not directly on the $CD4^+$ pathogenic autoreactive T-cell population (because these cells persist in treated mice and can transfer disease) but is on Treg cells that control the pathogenicity of the autoreactive cells.

Anti-CD137 therapy has previously been demonstrated to prevent murine SLE, EAE, and collagen arthritis (17,21–23). Ours is the first report, to our knowledge, to show that it prevents autoimmune diabetes. The only previous report of the effect of CD137 on the diabetes disease process is that overexpression of a *Cd137* transgene increased the incidence of diabetes (38). Although our results indicate a protective effect of CD137 stimulation in type 1 diabetes of NOD mice, our results combined with the results of Sytwu et al. (38) suggest that there can be too much of a good thing. CD137 is a member of the TNF receptor superfamily; in this regard, it is useful to recall that TNF-α had dual effects in type 1 diabetes: anti–TNF-α treatment at 9–10 weeks prevented diabetes, whereas TNF-α given to neonates increased diabetes severity (39–41). Our results show that anti-CD137 is most effective at preventing diabetes when given early in the disease process. Once a

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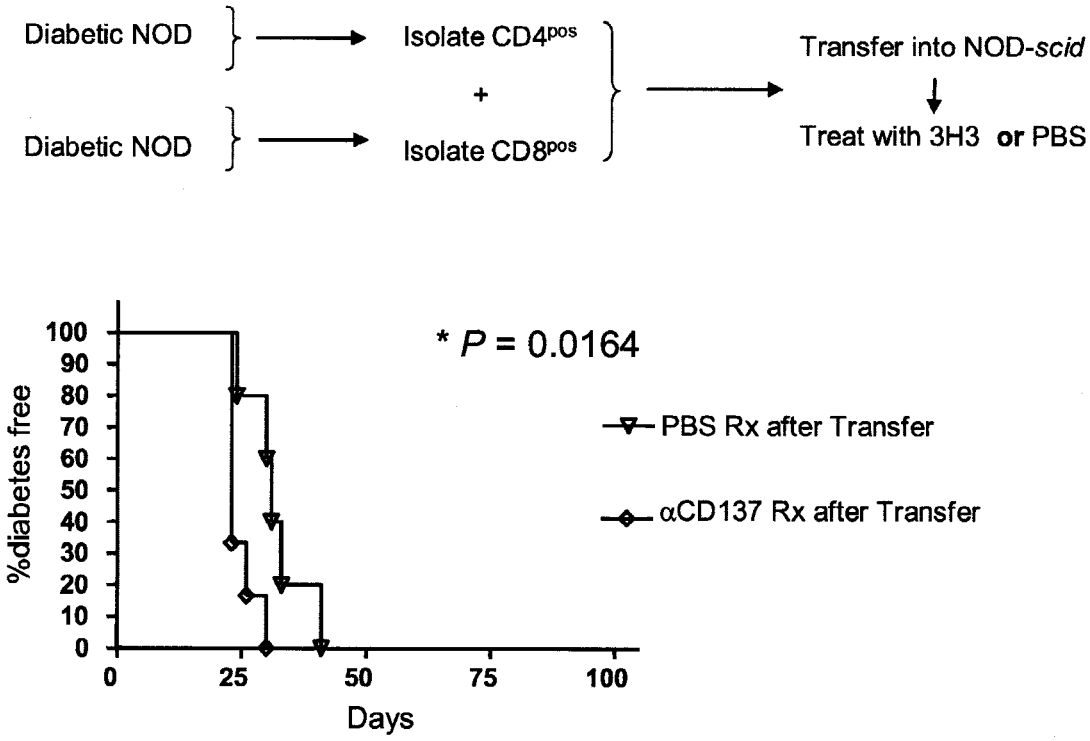

FIG. 7. Anti-CD137 treatment after transfer of diabetic splenocytes hastens onset of diabetes. Diabetic splenocytes were purified into $CD4^+$ and $CD8^+$ subsets and transferred into NOD-*scid* recipients. One day after transfer, the NOD-*scid* recipients were treated with either PBS ($n = 5$) or anti-CD137 ($n = 6$); from two separate experiments. Anti-CD137 treatment after transfer of diabetic splenocytes hastens disease onset ($P = 0.016$). Note: the PBS-treated group here is the same as shown in Fig. 3.

cadre of autoreactive T-cells is fully developed, anti-CD137 cannot prevent disease and may even worsen it (as shown by the earlier onset of diabetes when anti-CD137 is given at the time of transfer of NOD $CD4^+$ diabetic cells into NOD-*scid*; Fig. 7). Moreover, splenocytes taken from aged anti-CD137 mice can transfer diabetes, suggesting a finite regulatory capacity of the anti-CD137 induced Treg cells. Because autoimmune, activated pathogenic $CD4^+$ T-cells upregulate CD137, it is possible also that once the autoimmune repertoire has expanded, anti-CD137 activates the autoreactive cells, opposing the protective effect of $CD4^+CD25^+$ Treg cells. In this regard, it is noteworthy that we find that anti-CD137 treatment of lymphocytic chonomeningitis virus–infected mice leads to sustained high-level production of TNF-α (Mittler lab, unpublished data).

Nevertheless, our results are important for several reasons. *Cd137* is a strong candidate gene for *Idd9.3*; i.e., it is highly likely that CD137 has a significant role in the pathogenesis of spontaneous type 1 diabetes in NOD mice. Lyons et al. (6) showed that although the Idd9.3 region containing the B10 allele of *Cd137* decreased diabetes incidence on an NOD background, it did not prevent insulitis, consistent with our result. Moreover, these authors showed that the insulitic lesions in the *Idd9* congenic upregulated IL-4, consistent with our demonstration that anti-CD137–treated $CD4^+$ cells upregulate IL-4 production (6). Cannons et al. (10) have recently followed up this work by demonstrating that T-cells from NOD mice congenic for the B10 allele of *Cd137* proliferate more vigorously to anti-CD3 plus anti-CD137 stimulation than NOD cells, i.e., that the NOD *Cd137* allele may have relatively defective signaling capability. The authors speculate that decreased signaling through NOD CD137 could result in defective regulatory cell production. Our results are consistent with this hypothesis, because we show that agonistic stimulation through CD137 can prevent diabetes in NOD mice by the induction of a regulatory cell population. Although our results do not prove that *Cd137* is the *Idd9.3* gene, they are entirely consistent with this conclusion and supportive of prior studies defining the role of CD137 in type 1 diabetes.

Our results show that anti-CD137 mediates protection from diabetes via effects on $CD4^+CD25^+$ Treg cells. It has previously been shown that NOD $CD4^+CD25^+$ Treg cells numbers and functional capacity are decreased and that boosting NOD $CD4^+CD25^+$ Treg cell function can prevent diabetes (25,26). What we show for the first time is that NOD $CD4^+CD25^+$ Treg cells function may be linked to the *Idd9.3* candidate gene, *Cd137*. We show that $CD4^+CD25^+$ Treg cells upregulate CD137 compared with the $CD4^+CD25^-$ subset and that transfer of the $CD25^+$ subset completely prevented diabetes, whereas the $CD4^+CD25^-$ cell subset offered no significant protection. These findings are consistent with previous reports showing that $CD4^+CD25^+$ Treg cells upregulate CD137 (27,28) and with the increase of $CD4^+CD25^+$ cells in anti-CD137–treated mice in various models, although regulatory function was not proved in the latter reports (21,23). The effects of anti-CD137 antibody on $CD4^+CD25^+$ Treg cells are still controversial. 4-1BB ligand induces proliferation of $CD4^+CD25^+$ Treg cells and maintains their suppressor function (29), in contrast to no observable effect of anti-CD137 antibody on $CD4^+CD25^+$ Treg cells in vitro (30). This study also reported that regulatory cells from $CD137^{-/-}$ mice could control $CD45RB^{high}$ T-cell–induced colitis, implying that $CD4^+CD25^+$ Treg cells may exist independent of CD137 signaling (30). Moreover, in the setting of experimental autoimmune thyroiditis, the ad-

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ministration of anti-CD137 antibody breaks tolerance and worsens the disease (42). The double-edged effect of CD137 signaling likely depends on the balance of pathogenic and protective subsets at the time of treatment in any given model system, as we showed in Fig. 7. Nonetheless, our findings of specific binding of anti-CD137 antibody only to $CD4^+CD25^+$ cells in vivo plus our demonstration that these cells mediate the protective effect of the CD4 population in anti-CD137–treated mice strongly support the hypothesis that the $CD4^+CD25^+CD137^+$ Treg cell subset is a critical protective subset in type 1 diabetes.

We confirmed the observation recently published by Sakaguchi and colleagues (36,37) that CD25-depleted CD4 cells transfer several autoimmune diseases. In particular, we also confirmed their observation that the transfer of diabetes versus another organ-specific autoimmune syndrome, such as IBD or peripheral neuropathy, is mutually exclusive in this system (36). The mechanism for this striking organ specificity and exclusivity of autoimmune attack remains unknown. However, we have recently published a similar effect using genetic manipulation, in which we showed that modification of the NOD background by addition of *Idd* loci from B6/B10 on chromosomes 3 and 4 completely prevented diabetes while producing an autoimmune biliary disease (43,44). The remarkable "switching" of the tissue focus of autoimmune attack via environmental (CD25 depletion) or genetic (introgression of congenic regions) mechanisms is as yet unexplained but is clearly of great importance to understanding the pathogenesis of autoimmunity and type 1 diabetes.

In conclusion, we, for the first time, report that anti-CD137 antibody therapy suppresses type 1 diabetes in NOD mice and that this effect is dependent on induction of Treg cells. Treatment must be initiated before the development of a cadre of autoreactive T-cells, suggesting competing effects on protective and pathogenic cell subsets in type 1 diabetes. These results implicate a role for the *Idd9.3* candidate gene, *Cd137*, in type 1 diabetes pathogenesis and identify CD137 as a potential therapeutic target.

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