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The B10 Idd9.3 Locus Mediates Accumulation of Functionally Superior CD137+ Regulatory T Cells in the Nonobese Diabetic Type 1 Diabetes Model

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CD137 is a T cell costimulatory molecule encoded by the prime candidate gene (designated Tnfrsf9) in NOD.B10 Idd9.3 congenic mice protected from type 1 diabetes (T1D). NOD T cells show decreased CD137-mediated T cell signaling compared with NOD.B10 Idd9.3 T cells, but it has been unclear how this decreased CD137 T cell signaling could mediate susceptibility to T1D. We and others have shown that a subset of regulatory T cells (Tregs) constitutively expresses CD137 (whereas effector T cells do not, and only express CD137 briefly after activation). In this study, we show that the B10 Idd9.3 region intrinsically contributes to accumulation of CD137+ Tregs with age. NOD.B10 Idd9.3 mice showed significantly increased percentages and numbers of CD137+ peripheral Tregs compared with NOD mice. Moreover, Tregs expressing the B10 Idd9.3 region preferentially accumulated in mixed bone marrow chimeric mice reconstituted with allotypically marked NOD and NOD.B10 Idd9.3 bone marrow. We demonstrate a possible significance of increased numbers of CD137+ Tregs by showing functional superiority of FACS-purified CD137+ Tregs in vitro compared with CD137− Tregs in T cell-suppression assays. Increased functional suppression was also associated with increased production of the alternatively spliced CD137 isoform, soluble CD137, which has been shown to suppress T cell proliferation. We show for the first time, to our knowledge, that CD137+ Tregs are the primary cellular source of soluble CD137. NOD.B10 Idd9.3 mice showed significantly increased serum soluble CD137 compared with NOD mice with age, consistent with their increased numbers of CD137+ Tregs with age. These studies demonstrate the importance of CD137+ Tregs in T1D and offer a new hypothesis for how the NOD Idd9.3 region could act to increase T1D susceptibility. The Journal of Immunology, 2012, 189: 5001–5015.

Type 1 diabetes (T1D) is a polygenic autoimmune disease, and several genetic elements implicated in T1D pathogenesis mediate their effects through disruption of immune tolerance (1). In the NOD mouse model of T1D, CD4+ CD25+Foxp3+ regulatory T cells (Tregs) are unable to control immune destruction of the β cells in the pancreatic islets during progression to diabetes. NOD.B10 Idd9.3 congenic mice (that have been shown by congenic mapping to have a 1.2-Mb B10 Idd9.3 region within a larger 5.5-Mb B10 region on chromosome four) have a 40% reduced incidence of diabetes compared with NOD mice (2, 3). The Idd9.3 region encodes 15 known genes including Tnfrsf9, which is the strongest candidate gene in the region. Tnfrsf9 encodes the CD137 protein, and there are three coding variants between the NOD and B10 Tnfrsf9 gene, two nonsynonymous single nucleotide polymorphisms (SNPs) and an alanine insertion in NOD (2). CD137 is an inducible T cell costimulatory molecule and a member of the TNFR superfamily (4). T cells with the B10 Tnfrsf9 allele have enhanced proliferation and IL-2 production when stimulated via CD137 compared with NOD T cells (2). We and others have shown that CD137 is constitutively expressed by a subset of CD4+CD25+ Tregs, but not by non-Treg CD4+ T cells (5–9). Marson et al. (10), in particular, showed that Tnfrsf9 is one of a small set of genes directly up-regulated by Foxp3. The Mathis group (11), moreover, showed that Tregs isolated specifically from NOD pancreatic islets up-regulated Tnfrsf9. It has also been recently shown that Idd9.2 and 9.3 protective alleles function in CD4+ T cells to prevent expansion of pathogenic islet-specific CD8+ T cells (12). CD137 signaling promotes proliferation and survival of natural Tregs in vitro, which is enhanced by IL-2 (6, 13). We have shown that agonist anti-CD137 Ab prevents diabetes in NOD mice and increases the number of CD4+CD25+ T cells in vivo (5). In this study, we show, for the first time to our knowledge, that the B10 Idd9.3 region mediates enhanced accumulation of peripheral CD137+ Tregs.

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The sequences presented in this article have been submitted to the European Molecular Biology Laboratory (http://www.ebi.ac.uk/embt) under accession numbers CU463327, CU210939, CU207373, CU424443, CU210934, CU210933, CU210932, CU207371, CU207342, and CU207366.

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in vivo. NOD.B10 Idd9.3 congenic mice accumulate significantly more CD137+ Tregs with age compared with NOD mice. We show that CD137+ Tregs are functionally superior to CD137- Tregs in suppressing T cells in vitro by both contact-dependent and -independent suppression. Treg-mediated contact-independent mechanisms include multiple short-range suppressive factors such as IL-10 (14), TGF-β (15), galectin (16), and IL-35 (17). Although contact-independent suppression is still not well understood, many papers have now demonstrated contact-independent suppression mediated in transwell plate assays (18–29). Alternate splicing produces two isoforms of CD137: full-length CD137 that is expressed on the cell membrane and soluble CD137 in which transmembrane exon 8 is spliced out (30). Soluble CD137 is increased in autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, and systemic lupus (31, 32). It has been shown that soluble CD137 can inhibit T cell proliferation and hypothesized that increased soluble CD137 functions as a negative-feedback mechanism to control overactivation of pathogenic cells in autoimmunity (32, 33). We present novel data showing that CD4+CD25+CD137+ Tregs are a major cellular source of soluble CD137. We also show that older NOD.B10 Idd9.3 congenic mice have significantly increased serum soluble CD137 compared with NOD mice. We suggest that the maintenance and long-term accumulation of functionally superior peripheral CD137+ Tregs (as we show in NOD.B10 Idd9.3 congenic mice protected from T1D) and their production of soluble CD137 may play a critical role in protection from autoimmune diseases such as T1D.

Materials and Methods

Mice and reagents

NOD/Mrktac mice were obtained from Taconic Farms. NOD.B10 Idd9.3 mice were developed as previously described (2, 3) and are available from The Jackson Laboratory (stock number 012311). The NOD.B6-Plpcre (hereafter referred to as NOD.CD45.2), which has a 1-Mb congenic interval, was developed as previously described (34) and is available from The Jackson Laboratory (stock number 011449). NOD.Foxp3-GFP knockin (Kl) mice (hereafter NOD.Foxp3-GFP mice) were a kind gift from Vijay Kuchroo and Ana Anderson of Harvard University. The mice were generated by crossing the C57BL/6 Foxp3-GFP Kl generated in Dr. Kuchroo’s laboratory (35) to NOD Mrktac for 12 generations and then intercrossed for hemi-homozygosity for Foxp3-GFP Kl mutation. NOD.B10 Idd9.3 Foxp3-GFP mice were produced by crossing the NOD.B10 Idd9.3 to NOD.Foxp3-GFP mice, intercrossing and selecting mice positive for both the Idd9.3 region and the Foxp3-GFP Kl mutation. NOD.Foxp3-GFP mice have normal incidence of T1D in our Cincinnati Laboratory Animal Medical Services facility, whereas the NOD.B10 Idd9.3 Foxp3-GFP mice show protection from T1D similar to NOD.B10 Idd9.3 mice in our colony. Mice 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 and the University of Cincinnati School of Medicine. Anti-CD137 mAb (clone 3H3) has been previously described (36). Abs against mouse 2.5G2-Fc, CD4-allophycocyanin, CD4-allophycocyanin-Cy7, CD25-PerCP-Cy5.5, CD25-FITC, streptavidin-PE, and streptavidin-allophycocyanin were purchased from BD Biosciences. CD3/CD28-coated beads and recombinant mouse IL-2 were purchased from Invitrogen. Primers for mouse Gapdh (435239E; 8001016) and β2-microglobulin (B2m; Mm00437762_m1) were purchased from Applied Biosystems. We used custom-designed primers for membrane-bound and soluble CD137 (Applied Biosystems). For membrane-bound CD137, the forward primer was 5‘-CTCCCCTGTTTGATGGACCTTTCC-3’ and the reverse primer was 5‘-AGGGAGGGACTCTTGACACGTTCC-3’; for soluble CD137, the forward primer was 5‘-GCCGCTGACTTCTGATGAGCTTTCC-3’ and the reverse primer was 5‘-GGGAGGACCAAGCATTAGGAAGA-3’. The probe for both the primers was 5‘-TCCCACTGATCACCATATTG-3’. Resequencing of the Idd9.3 interval in the NOD mouse strain and identifying polymorphisms

The resequencing of Idd9.3 in the NOD mouse strain involved aligning the bacterial artificial chromosome (BAC) clone end sequences of the NOD library against the B6 mouse genome sequence (37). From this, 10 NOD BAC clones that formed a minimal sequencing tile path spanning the 1.2-Mb Idd9.3 interval were selected and sequenced at the Wellcome Trust Sanger Institute and deposited at the European Molecular Biology Laboratory (http://www.ebi.ac.uk/embl; clone DN-135171; accession number CU463327; DN-79L21, CU210939; DN-382D20, CU207373; DN-27K19, CU424443; DN-12H14, CU210934; DN-17C24, CU210933; DN-8G22, CU210932; DN-12K97, CU207371; DN-266N3, CU207342; and DN-2A148, CU407306). As all of the B6 and B10 SNPs were found to be identical by descent throughout the Idd9.3 region (mouse phenome database: http://phenome.jax.org), we identified polymorphisms between NOD and B6 in the Idd9.3 interval. The NOD BAC clone sequences spanning the Idd9.3 interval were aligned to the B6 mouse genome sequence (National Center for Biotechnology Information) and aligned to the mouse genome assembly release 37.3 (38). The polymorphisms were entered into TIDBase (39, 40) and displayed graphically using GBrowse (41) (Supplemental Fig. 1). The SNP density plots were generated by counting the number of SNPs in 10-kb windows, sliding 2 kb at a time, and plotting the count at the midpoint of each window (Supplemental Fig. 1). The Welcome Trust Sanger Institute has next-generation sequenced 17 mouse strains, including NOD/ShiLtJ (http://www.sanger.ac.uk/resources/mouse/genes/). The SNP information was downloaded for the Idd9.3 region, entered into TIDBase, and can be viewed at www.tidbase.org.

Flow cytometry

For absolute cell counts, NOD and NOD.B10 Idd9.3 splenocytes or thymocytes were extracted and counted using a hemocytometer. For staining membrane-bound CD137, the cells were incubated with 2.4G2 Fc block. For FACS analysis, cells were stained with CD4-allophycocyanin and CD25-FITC and stained for CD137 using IgG2a anti-CD137 or IgG2a isotype control Ab, then stained with anti-IgG2a biotin and streptavidin-PE, and analyzed on an FACS Calibur (BD Biosciences). The cells were serially gated for total number of lymphocytes (by forward light and side scatter), CD4, CD4+CD25+, CD4+CD25+CD137+, and CD4+CD25+CD137+ T cells. The percentage staining in each gate was multiplied by the absolute number of cells to calculate the total number of lymphocytes, CD4+, CD4+CD25+, CD4+CD25+CD137+, and CD4+CD25+CD137+ T cells. For intracellular staining, the splenocytes were stained with CD4-allophycocyanin-Cy7, CD25-PerCP-Cy5.5, and CD137-PE, fixed with 2% formaldehyde (methanol-free), and permeabilized with 0.3% saponin. Intracellular staining was performed with Foxp3-PE or IgG2a isotype-PE (eBioscience), B2cxl-LEX-Alexa Fluor 488, or IgG isotype-Alexa Fluor 488 (Cell Signaling Technology), Bcl-2-Alexa Fluor 488 or IgG1 isotype-Alexa Fluor 488 (BioLegend), and Ki-67-Alexa Fluor 488 or IgG isotype-Alexa Fluor 488 (Novus Biologicals). Anti-mouse Ki-67 or IgG isotype control Ab was labeled with the APEX Alexa Fluor 488 Ab Labeling Kit (Invitrogen). Foxp3 staining was performed using the eBioscience Fixation/Permeabilization Kit. (NOD.CD4+CD25+×NOD.B10 Idd9.3) F1 bone marrow chimeras spleen and pancreatic lymph nodes were stained with CD4-allophycocyanin-Cy7, CD25-PerCP-Cy5.5, CD11b-FITC, CD25-allophycocyanin and CD137-PE or IgG2a isotype-PE and analyzed on an FACS Canto (BD Biosciences). All FACS data were analyzed using FlowJo (Tree Star).

Bone marrow chimera construction

Nine- to 13-wk-old (NOD.CD4+CD25+×NOD.B10 Idd9.3) F1 mice were irradiated with 800–1200 rad (the dose was varied as we gained experience in this procedure to optimize depletion of host cells). A total of 15–25 million bone marrow cells from 5–12-wk-old NOD B10 Idd9.3 and NOD.CD45.2 mice were extracted without RBC lysis. Mature CD4, CD8, and CD90 cells were removed using magnetic beads (Miltenyi Biotec), and the bone marrow was then mixed at a 1:1 ratio and injected into the irradiated F1 mice. Recipient mice were given water treated with antibiotic (neomycin trisulfate salt hydrate) for 2 wk after transfer. The recipient F1 mice were sacrificed 12–20 wk postinjection for analysis of peripheral T cell populations by FACS.

Quantitative real-time PCR

CD4 T cells were extracted from splenocytes using CD4 magnetic beads (Miltenyi Biotec). The CD4 T cells were blocked with 2.4G2 and stained with CD4-allophycocyanin-Cy7, CD25-FITC, and anti-CD3-allophycocyanin as above. The cells were sorted using a BD FACSAria machine (BD Biosciences) into CD4+CD25+CD137+, CD4+CD25+CD137-, and CD4+CD25+CD137- cell subsets; RNA was extracted from the sorted cells using an RNeasy mini kit (Qiagen) and converted into cDNA (Promega
Reverse Transcription System; Promega). Quantitative real-time PCR (RT-PCR) was performed on the cDNA using primers for B2m, soluble CD137, and membrane-bound CD137 using a StepOnePlus Real-Time PCR system (Applied Biosystems). The threshold cycle (CT) values of the gene of interest were subtracted from the CT of the housekeeping gene (Gadph or B2m) to produce the ΔCT (designated ΔΔCT) and the data graphed using GraphPad Prism 5 (version 5.02; GraphPad).

Proliferation assay

The CD4^+CD25^+CD137^+ T cells in the representative example shown. Because it harvest. On day 4, thymidine incorporation was assessed using a scintillation counter. The numbers of CD4^+CD25^+CD137^+T cells were cultured and sorted using BD FACSaria (BD Biosciences) with 90–95% purity. A total of 50,000 sorted cells were cultured at 37°C with 5% CO2: with 1) 25 U/ml IL-2; or 2) 25 U/ml IL-2 and 1.25 μg/ml anti-CD3 in triplicate wells. The cells were pulsed with 1 μCi [3H]-labeled thymidine on day 3 and harvested after 16 h using a β-scintillation counter. For the suppression assay, 50,000 CD4^+CD25^+ T cells were cultured in U-bottom 96-well plates with 1 μg/well soluble anti-CD3, 50,000 irradiated splenocytes (1500 rad), and varying numbers of CD4^+CD25^+CD137^+ or CD4^+CD25^+CD137^+Tregs. All cells were cultured and pulsed with 1 μCi [3H]-thymidine on day 3, 16 h before harvest. On day 4, thymidine incorporation was assessed using a β-scintillation counter.

Treg transwell suppression assay

A total of 100,000 sorted CD4^+CD25^+CD137^+ T cells were cultured with 100,000 irradiated splenocytes (1500 rad) and 1.25 μg/ml soluble anti-CD3 in the bottom wells of a 96-well transwell plate (Corning). A total of 25,000 or 50,000 CD4^+CD25^+CD137^+ or CD4^+CD25^+CD137^+Tregs were cultured in the top wells with 100,000 irradiated (1500 rad) splenocytes and 1.25 μg/ml soluble anti-CD3. In some assays, the sorted CD4^+CD25^+CD137^+ or CD4^+CD25^+CD137^+Tregs were cultured at the bottom of the transwell along with the CD4^+CD25^+CD137^+ effector T cells (with no Tregs in the top transwell) to directly compare contact-dependent- and independent suppression by the same sorted cells. In some cases, the cells were cultured with 50,000 CD3/CD28-coated beads (Invitrogen) in the absence of APCs. The cells were cultured at 37°C in 5% CO2 and pulsed with 1 μCi [3H]-thymidine on day 3. The cells in the bottom wells were harvested and counted using a β-scintillation counter.

ELISA

The mouse 4-1BB DuoSet ELISA system (R&D Systems) was used to detect soluble CD137 from serum and culture supernatants. A standard for each experiment. DeltaSoft software was used to calculate the amount of soluble CD137+ Tregs in NOD and NOD.B10 Idd9.3 congenic mice. The number of splenic CD4^+CD25^+ T cells increased significantly with age in both strains with no difference between the strains (Supplemental Fig. 2A). This explains the lack of decline of CD137^+ Treg number in NOD with age despite a significant drop in the percent of CD137^+ Tregs. Similarly, it also explains the significant increase in the total number of CD137^+ Tregs with age in NOD.B10 Idd9.3 mice (p = 0.03, Fig. 1C). However, the number of NOD CD137^+ Tregs did not change with age despite an age-related decline in the percent of NOD CD137^+ Tregs. (We also looked at the percentage of CD137^+ Tregs at 10–20 wk of age and observed no significant differences compared the other age groups of either strain, data not shown.) Consistent with previously published observations (44), the number of splenic CD4^+CD25^+ T cells increased significantly with age in both strains with no difference between the strains (Supplemental Fig. 2A). This explains the lack of decline of CD137^+ Treg number in NOD with age despite a significant drop in the percent of CD137^+ Tregs. Similarly, it also explains the significant increase in the total number of CD137^+ Tregs with age in NOD.B10 Idd9.3 mice (p = 0.03, Fig. 1C). The percentage of CD137^+ Tregs did not change with age (Fig. 1B). Thus, the increased number of NOD.B10 Idd9.3 CD4^+CD25^+CD137^+ T cells with age is due to a combination of an increased percentage of CD137^+ T cells and an increased absolute number of CD4^+CD25^+ T cells in older NOD.B10 Idd9.3 mice. There was also no difference in the percentage of CD4^+CD25^+ T cells with age in either strain (Supplemental Fig. 2B). Both NOD and NOD.B10 Idd9.3 congenic mice showed significant increases in CD4^+ T cells and total numbers of splenic lymphocytes with age (Supplemental Fig. 2C, 2D), but there was no difference between NOD.B10 Idd9.3 and NOD mice in the corresponding age groups.

We next studied the percentages and total number of thymic CD137^+ Tregs in NOD and NOD.B10 Idd9.3 congenic mice and found that they were consistent with the peripheral population results. The percentage and absolute number of thymic CD137^+ Tregs was significantly higher in 21–36-wk-old NOD.B10 Idd9.3 versus NOD mice (p = 0.002, Supplemental Fig. 3A; and p = 0.02, Supplemental Fig. 3B). The total number of thymic CD4^+CD25^+ T cells, however, remained approximately constant with age in both strains (Supplemental Fig. 3C). The percentage of NOD.B10 Idd9.3 CD4^+CD25^+ thymocytes rose significantly (p = 0.02, Supplemental Fig. 3D). The number of CD4^+ thymocytes significantly declined with age in both strains with no difference between the two strains (Supplemental Fig. 3E). The total number of thymocytes significantly decreased with age in NOD (p = 0.007, Supplemental Fig. 3F); it decreased, but not significantly, in older NOD.B10 Idd9.3 mice. In summary, it is clear that the decreased number of NOD thymic CD137^+ Tregs with age was due to a decreased percentage of these cells; in older NOD.B10 Idd9.3 mice, a significant increase in the percentage of CD137^+ Tregs caused a significant increase in the number of thymic CD137^+ Tregs.

To begin to understand possible reasons for increased NOD.B10 Idd9.3 CD137^+ Treg accumulation with age, we examined the per-cell surface expression of CD137 at the same time points. The mean fluorescent intensity (MFI) of CD137 on CD4^+CD25^+ CD137^+ T cells was significantly greater in young (3–9 wk old) NOD.B10 Idd9.3 versus age-matched NOD (p = 0.009, Fig. 1D). Although the percentage of CD4^+CD25^+CD137^+ T cells declined...
markedly in 21–36-wk-old NOD spleen (p = 0.006, Fig. 1B), the CD137\(^+\) Tregs found in old NOD spleen expressed a significantly higher level of CD137 per cell compared with young NOD splenic Tregs (p = 0.003, Fig. 1D), not significantly different from old NOD.B10 Idd9.3 cells (Fig. 1D). Similar CD137 MFI was found in thymic CD4\(^+\)CD25\(^+\)CD137\(^+\) T cells with age: old thymic NOD CD137\(^+\) Tregs expressed more CD137 per cell than young NOD cells (p = 0.0004, Supplemental Fig. 3G), and young thymic NOD.B10 Idd9.3 CD137\(^+\) Tregs were significantly higher than on young NOD cells (p = 0.0003, Supplemental Fig. 3G). These findings suggest that early, increased expression of CD137 on Tregs might enhance long-term accumulation of those cells, consistent with the previously published role of CD137 on CD8 and CD4 T cell survival in vivo and in vitro (42, 43, 45). The increased expression of CD137 on a per-cell basis in young NOD. B10 Idd9.3 congenic spleen is associated with the increase in the number of CD4\(^+\)CD25\(^+\)CD137\(^+\) T cells with age. Overall, these studies show that the B10 Idd9.3 region enhances accumulation of CD137\(^+\) Tregs in the NOD.B10 Idd9.3 congenic mice and supports the hypothesis that CD137 is important for the long-term accumulation of CD137\(^+\) Tregs.

The above results depended on identifying Tregs by CD25 expression. Although this is a recognized Treg marker, it can also be a marker of T cell activation, as can CD137. It was important to evaluate the peripheral Treg subsets by intracellular Foxp3 expression to ensure that the increased number of NOD.B10 Idd9.3 CD4\(^+\)CD25\(^+\)CD137\(^+\) T cells with age were truly Tregs. To evaluate this, we first performed intracellular Foxp3 staining at all age points. We showed that the overwhelming majority of CD4\(^+\)CD25\(^+\)CD137\(^+\) T cells are Foxp3 positive (Fig. 2A, 2B) and that there is no significant difference in percentage of Foxp3-positive cells between CD4\(^+\)CD25\(^+\)CD137\(^+\) and CD4\(^+\)CD25\(^+\)CD137\(^-\) T cells in either 5–9-wk-old or 20–30-wk-old NOD or NOD.B10 Idd9.3 congenic mice (Fig. 2A, 2B). Our results match a previous study that showed similar percentage Foxp3 expression in CD4\(^+\)CD25\(^+\) T cells in NOD with age (46) and shows that the increased number of NOD.B10 Idd9.3 CD4\(^+\)CD25\(^+\)CD137\(^+\) T cells is not due to an expansion of non-Tregs in this subset. We also found a slight increase in isotype staining with age (not shown), and because we subtracted the isotype staining (see Fig. 2C for a representative example for gating) to calculate true-positive Foxp3 cells, we may have underestimated the true Foxp3 expression [representative FACS plots for Foxp3 versus CD137 (Fig. 2D) and CD4 versus Foxp3 (Fig. 2E) for NOD and NOD.B10 Idd9.3 are also shown]. After all of the studies in this paper were performed, we obtained NOD.Foxp3-GFP mice and used these mice to create NOD.B10 Idd9.3 Foxp3-GFP mice. We aged these mice and used them to evaluate the percentage of Foxp3-GFP-positive cells in the CD4\(^+\)CD25\(^+\)CD137\(^+\) and CD4\(^+\)CD25\(^+\)CD137\(^-\) subsets in NOD and NOD.B10 Idd9.3 at the same time points as we examined above (Fig. 2A, 2B). The results (Fig. 3) support the conclusions of Fig. 2 above and show that well 90% of CD4\(^+\)CD25\(^+\)CD137\(^+\) and CD4\(^+\)CD25\(^+\)CD137\(^-\)
FIGURE 2. NOD and NOD.B10 Idd9.3 CD4+CD25+ Treg subsets show no difference in intracellular Foxp3 staining. (A and B) Splenocytes from 5–9-wk-old NOD (n = 3) and NOD.B10 Idd9.3 (n = 2) and 20–30-wk-old NOD (n = 4) and NOD.B10 Idd9.3 (n = 6) female mice were surface stained with CD4-APC.Cy7, CD25-PerCP-Cy5.5, and anti–CD137-APC, followed by intracellular staining for Foxp3-PE or IgG2a isotype control. (The CD137 gates are based on isotype staining for CD137, as shown in Fig. 1A.) (C) Representative dot plots of the FACS gating used to calculate the percent Foxp3 staining in (A) and (B) using 7-wk-old NOD (left panel) and 6-wk-old NOD.B10 Idd9.3 (right panel). Within the CD4+CD25+ gate, the CD4+CD25+CD137- and CD4+CD25+CD137+ Tregs were gated for Foxp3 or isotype histograms. The isotype histogram was used to establish gates for Foxp3-positive staining. The percent overlapping isotype staining in the gate was subtracted from the percent Foxp3+ in the gate (as noted at the top of each histogram) to calculate the true percent Foxp3 shown in (A) and (B). (D) The same NOD and NOD.B10 Idd9.3 mice used in (C) were gated for CD4+CD25+ T cells to show representative dot plots of Foxp3 and isotype (left panel) or Foxp3 and CD137 (right panels). (E) Representative dot plots of CD4 and Foxp3 within the lymphocyte gate of the same NOD and NOD.B10 Idd9.3 mice used in (C).
subsets in NOD and NOD.B10 Idd9.3, both young and old, were Foxp3-GFP positive, and that there were no significant differences between the strains (Fig. 3A, 3B show the pooled result, whereas Fig. 3C shows representative FACS plots in NOD. Foxp3-GFP and NOD.B10 Idd9.3 Foxp3-GFP mice). The combined results of Figs. 2 and 3 very strongly show that the increase of CD4+CD25+CD137+ cells in NOD.B10 Idd9.3 mice with age truly represents a significant increase in Foxp3-expressing CD137+ Tregs.

Increased accumulation of CD137+ Tregs with the B10 versus the NOD Idd9.3 region in mixed bone marrow chimeras in vivo

The finding of increased CD137+ Tregs in NOD.B10 Idd9.3 compared with NOD mice with age probably reflects multiple biological processes and complex intrinsic/extrinsic cellular effects in separate

![Graphs showing the percentage of Foxp3+ cells in NOD and NOD.B10 Idd9.3 Foxp3-GFP mice](image)

**FIGURE 3.** NOD.Foxp3-GFP and NOD.B10 Idd9.3 Foxp3-GFP mice demonstrate that the majority of CD4+CD25+CD137+ cells are Foxp3 Tregs and that there is no significant difference in the percent Foxp3+ cells between NOD and NOD.B10 Idd9.3. (A and B) Splenocytes from NOD.Foxp3-GFP (n = 3, 3–9 wk old and n = 2, 21–25 wk old) and NOD.B10 Idd9.3.Foxp3-GFP (n = 3, 3–9 wk old and n = 3, 21–25 wk old) were stained for CD4-APC-Cy7, CD25-PerCP-Cy5.5, and CD137-APC or IgG2a isotype control. The cells were analyzed for Foxp3-GFP expression in CD137+ and CD137− subsets from young NOD.Foxp3-GFP (top left panel), old NOD.Foxp3-GFP (bottom left panel), young NOD.B10 Idd9.3 Foxp3-GFP (top right panel), and old NOD.B10 Idd9.3.Foxp3-GFP (bottom right panel). (The CD137 gates are based on isotype staining for CD137, as shown in Fig. 1A.)
Enhanced proliferation of CD137+ Tregs ex vivo and in vitro versus CD45.2 proportions in CD4+CD25+CD137 Tregs from the same individual mice, in contrast, showed no significant difference in thymic CD45.1 versus CD45.2 population differences (Fig. 4A–C). Because the percentage of B10 and NOD allotype were from the CD137+ Treg population in the same bone marrow chimeric mouse, the total number of splenocytes or lymph node cells were the same for each cell population; hence the total numbers of B10 or NOD allotype CD137+ Tregs in the bone marrow chimeric mice were exactly proportional with these percentages. There was statistically no significant difference in the percentage of peripheral (splenic or pancreatic lymph node) CD4+CD25*CD137+ T cells expressing the B10 CD137 allele was significantly increased compared with the NOD allotype (Fig. 4B, 4C). CD4+CD25*CD137− Tregs from the same individual mice, in proportion, contrast, showed no significant difference in CD45.1 versus CD45.2 population proportions (Fig. 4A–C). Because the percentage of B10 and NOD allotype were from the CD137+ Treg population in the same bone marrow chimeric mouse, the total number of splenocytes or lymph node cells were the same for each cell population; hence the total numbers of B10 or NOD allotype CD137+ Tregs in the bone marrow chimeric mice were exactly proportional with these percentages. There was statistically no significant difference in thymic CD45.1 versus CD45.2 proportions in CD45.1 and CD45.2 population proportions (data not shown). These results, which are entirely consistent with the studies in Fig. 1, strongly support the hypothesis that the B10 CD137 region intrinsically and selectively mediates enhanced accumulation of CD137+ Tregs in the periphery.

Enhanced proliferation of CD137+ Tregs ex vivo and in vitro but no significant difference between NOD and NOD.B10 Idd9.3 congenic mice

CD137 costimulation causes proliferation of Tregs in vitro (13, 36) and in vivo (47). The increased frequency and accumulation of CD137+ Tregs in NOD.B10 Idd9.3 congenic mice could be due to intrinsic factors, such as greater proliferative capacity or enhanced cell survival mediated by the B10 CD137 allotype. To test proliferative capacity, we cultured NOD and NOD.B10 Idd9.3 CD137+ and CD137− Tregs in the presence of IL-2 (Fig. 5A), IL-2 and CD3 (Fig. 5B), or with no IL-2 (unstimulated) (Supplemental Fig. 4C). The proliferation of CD137+ Tregs was significantly greater than CD137− Tregs under both culture conditions and in both strains, but there was no difference in proliferation of CD137+ Tregs between NOD and NOD.B10 Idd9.3 mice (Fig. 5A, 5B). As expected, Tregs cultured with no IL-2 showed virtually no proliferation (Supplemental Fig. 4C). Next, we quantified expression of the nuclear protein Ki-67 as a marker for proliferation ex vivo. Consistent with the in vitro results, a significantly higher percentage of CD137+ Tregs were Ki-67 positive ex vivo compared with CD137− Tregs in both NOD and NOD.B10 Idd9.3 mice (Fig. 5C). Again, we found no difference in percentage of Ki-67−positive CD137− Tregs between NOD and NOD.B10 Idd9.3 mice. These studies suggest that the increased numbers and frequency of CD137+ Tregs in NOD.B10 Idd9.3 congenic mice with age could not be explained by enhanced proliferation mediated by the B10 allotype.

Viability studies in CD137+ Tregs expressing the B10 Idd9.3 region have shown that CD137 signaling prevents activation-induced cell death by repressing DNA fragmentation (48). Because CD137 signaling can upregulate the prosurvival molecule Bcl-xL (42), we tested the expression of Bcl-xL in NOD and NOD.B10 Idd9.3 Treg subsets. We found significantly increased Bcl-xL mRNA expression in NOD.B10 Idd9.3 versus NOD CD137+ Tregs (p = 0.04, Fig. 6A). We also found increased expression of Bcl-xL in NOD.B10 Idd9.3 versus NOD CD137− Tregs (p = 0.008), which suggests CD137 is not necessary for upregulation of Bcl-xL in these NOD.B10 Idd9.3 cells. We next tested Bcl-xL mRNA expression in the mixed bone marrow chimeric cell subsets,

FIGURE 4. CD137+ Tregs expressing the B10 Idd9.3 region demonstrate an intrinsic cell accumulation in mixed bone marrow chimeric mice. Total of 15–25 million bone marrow cells from 5–12 wk-old NOD.B10 Idd9.3 mice and NOD.B10 Idd9.3 mice were mixed at 1:1 ratio and injected into 9–13 wk-old irradiated (800–1200 rad in different experiments) (NOD.CD45.2 × NOD.B10 Idd9.3) F1 mice. Recipient nondiabetic mice were sacrificed 12–20 wk after reconstitution. (A) One representative experiment showing the expression of the CD45.1 (NOD. B10 Idd9.3) versus CD45.2 (NOD) allotype by CD137− (left panel) and CD137+ (right panel) Tregs 12 wk after reconstitution of the bone marrow chimeras. Pancreatic lymph nodes (n = 4) (B) and spleen (n = 5) (C) were harvested and stained with CD4-APC-Cy7, CD25-PerCP-Cy5.5, CD45.1-APC, CD45.2-FTTC, and anti-CD137-PE and analyzed on an FACS Canto cytometer (BD Biosciences). Percentage of each F1 (host) cells not shown. Statistical significance was calculated using the unpaired t test.
Similarly, CD137+ Tregs showed no difference in Bcl2 MFI in CD4+CD25+CD137 and anti–CD137-APC and FACS-sorted phycocyanin-Cy7, CD25-PerCP-Cy5.5, females were stained with CD4-allo-Idd9.3. cumulation of NOD.B10 Idd9.3 between NOD.B10 Idd9.3 and NOD (Fig. 6F). The greater accumulation of CD137+ Tregs in contrast to Bcl-xL (Fig. 6C). In the mixed bone marrow chimera mice, the CD137+ but not the CD137− Treg subset showed significantly increased Bcl2 mRNA in B10 allotype versus NOD allotype cells (p = 0.0003, Fig. 6D). To summarize, the CD137+ Treg subsets (in both normal NOD.B10 Idd9.3 mice and in the B10 allotype cells of bone marrow chimera mice) showed significant increases of Bcl-xL mRNA. However, in CD137− Tregs, NOD.B10 Idd9.3 Tregs also show increased Bcl-xL mRNA compared with NOD, and B10 allotype CD137+ Tregs in the bone marrow chimeras also showed upregulated Bcl2 message. To understand the significance of these findings, we examined protein levels of these survival molecules in both NOD and NOD.B10 Idd9.3 Treg subsets. Contrary to the PCR results, the Bcl-xL expression per cell did not vary between the NOD.B10 Idd9.3 versus NOD CD137+ Tregs (Fig. 6E). Similarly, CD137+ Tregs showed no difference in Bcl2 MFI in between NOD.B10 Idd9.3 and NOD (Fig. 6F). The greater accumulation of NOD.B10 Idd9.3 CD137+ Tregs compared with NOD could be due to enhanced survival of cells expressing the B10 CD137 allele. We tested this in vitro by culturing NOD and NOD.B10 Idd9.3 Tregs subsets in the presence of IL-2 or IL-2/CD3 as above. Under both conditions, the percentage of live, early apoptotic (Annexin positive), late apoptotic (Annexin/propidium iodide double positive), or dead (propidium iodide positive) cells did not differ between NOD and NOD.B10 Idd9.3 in both CD137− or CD137+ Tregs (data not shown). Therefore, although the increased expression of Bcl-xL mRNA in Tregs expressing the B10 CD137 allele is intriguing, it is not definitive. In addition, because we have no evidence of increased survival in vitro, we cannot confirm or exclude the possibility that expression of the B10 CD137 allele causes survival advantage of Tregs without further studies.

**Functionally superior contact-dependent and contact-independent suppression mediated by CD137+ versus CD137− Tregs**

Our results show enhanced accumulation of CD137+ Tregs in NOD.B10 Idd9.3 mice. To understand the possible significance of increased percentages and numbers of CD137+ Tregs with age, we investigated functional differences between CD137− and CD137+ Treg subsets. We performed an in vitro Treg contact-dependent suppression assay using NOD CD4+CD25−CD137− effector T cells and titrated numbers of syngeneic NOD CD137+ or CD137− Tregs (Fig. 7A). CD137+ Tregs were significantly functionally superior to CD137− Tregs at every ratio (through 1:32; p = 0.002) of Treg/effector T cells tested (Fig. 7A). Next, we tested NOD.B10 Idd9.3 Treg subsets in the same assay system and again found that NOD.B10 Idd9.3 CD137+ Tregs were functionally superior to CD137− Tregs when suppressing NOD.B10 Idd9.3 CD4+CD25− CD137− effector T cells (Fig. 7B); however, the amount of suppression by NOD.B10 Idd9.3 CD137+ Tregs was very comparable to NOD CD137+ Tregs (Fig. 7B versus 7A). We directly compared the suppressive capacity of NOD and NOD.B10 Idd9.3 CD137+ Tregs, in the same experiment, against NOD CD4+CD25− CD137− effector T cells and found no significant difference in the suppressive capacity of the CD137+ Tregs of these two strains (Fig. 7C). Next, we tested whether CD137− Tregs can mediate suppression in a contact-independent manner. Using transwell plates, we cul-

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**FIGURE 5.** No proliferative differences between NOD and NOD.B10 Idd9.3 CD137+ Tregs ex vivo or in vitro. (A and B) Splenocytes from 4–6-wk-old NOD and NOD.B10 Idd9.3 females were stained with CD4-allo-phycoerythrin-Cy7, CD25-PerCP-Cy5.5, and anti–CD137-APC and FACS-sorted for CD4+CD25−CD137+ and CD4+CD25−CD137− cells. A total of 50,000 sorted cells were cultured with 25 U/ml IL-2 (A) or 25 U/ml IL-2 and 1.25 μg/ml anti-CD3 (B) in triplicate wells. The cells were pulsed with 3H-labeled thymidine on day 3 and harvested after 16 h, and the data were pooled from n = 3 experiments. (C) Splenocytes from 5–7-wk-old NOD (n = 3) and NOD.B10 Idd9.3 (n = 3) females were surface stained with CD4-APC-Cy7, CD25-PerCP-Cy5.5, and anti–CD137-APC, followed by intracellular staining for Ki-67–Alexa 488. The stained cells were analyzed by flow cytometry. Statistical significance was calculated with the unpaired t test.
CD137+ Tregs express significantly higher levels of Bcl-xL mRNA than CD137− Tregs. The cultured CD4+CD25−CD137− T cells in the bottom well and Treg subsets in the upper well; both wells had irradiated splenocytes and anti-CD3 Abs. At a 1:2 ratio, both CD137+ and CD137− Tregs significantly suppressed the proliferation of T cells in a contact-dependent manner, but CD137+ Tregs were significantly more suppressive than CD137− Tregs (p = 0.008; Fig. 8A). Next, we directly compared Tregs from the same donor in both contact-dependent suppression (Tregs in the bottom well in contact with effector T cells) and contact-independent suppression (Tregs in the top well). Tregs were significantly more suppressive in the contact-dependent assay than in the contact-independent assay (Fig. 8A; p = 0.009 for CD137+ Tregs and p = 0.001 for CD137− Tregs). However, in both assays, the CD137− subset mediated significantly more suppression than the CD137+ subset.

Finally, we assessed contact-independent suppression via serial dilution assays. Ratios of Treg/effectector <1:2 did not show a significant difference in suppression between the CD137+ versus CD137− Tregs, consistent with a dilutional effect on a soluble factor (Fig. 8A). These results suggest that in a contact-independent system, both Treg subsets produce soluble suppressive factors when activated, but that CD137+ Tregs can produce either quantitatively higher or different soluble factors that contribute to their functional superiority to CD137− Tregs.

CD137+ Tregs are the major cellular source of alternately spliced soluble CD137 protein

Alternate splicing produces two isoforms of CD137: full-length CD137 expressed on the cell membrane and soluble CD137 in which transmembrane exon 8 is spliced out (30). Previously, however, production of soluble CD137 by Tregs has not been studied. We designed RT-PCR primers (see Materials and Methods) that discriminate soluble versus membrane-bound CD137 and used them to detect soluble CD137 versus membrane-bound CD137 from freshly sorted NOD and NOD.B10 Idd9.3 subsets. CD137+ Tregs expressed significantly higher levels of soluble and membrane CD137 mRNA compared with CD137− Tregs in NOD and NOD.B10 Idd9.3 mice (Fig. 9A, 9B). CD4+CD25+CD137− T cells produced negligible amounts of both isoforms of CD137 mRNA ex vivo (Fig. 9A, 9B). Next, we sorted the CD137+ and CD137− Tregs from NOD and NOD.B10 Idd9.3 mice and cultured them (with IL-2 alone, to promote survival in vitro under nonactivating conditions) to assess CD137 protein production. The cultured CD137+ Tregs (from either NOD and NOD.B10 Idd9.3 mice) produced significantly higher...
levels of soluble CD137 protein compared with CD137\(^+\) Tregs; CD4 non-Treg cells did not produce significant amounts of soluble CD137 protein (Fig. 9C). There was no significant difference in soluble CD137 protein production between NOD and NOD.B10 \(Idd9.3\) CD137\(^+\) Tregs and CD137\(^-\) and CD137\(^+\) Tregs (Fig. 9C). We did not detect significant soluble CD137 production from any cell subset in the absence of IL-2 (Supplemental Fig. 4C and data not shown). We have thus established, for the first time to our knowledge, that CD137\(^+\) Tregs are the primary T cell source of soluble CD137.

Given that activated T cells express CD137, it was possible that our CD137\(^-\) Tregs start producing soluble CD137 under activating conditions. To test this, we cultured CD137\(^-\) and CD137\(^+\) Tregs from NOD and NOD.B10 \(Idd9.3\) mice with IL-2 and anti-CD3 and tested culture supernatants on day 4 for soluble CD137. Under these activating conditions, CD137\(^-\) Tregs produced some soluble CD137, although still significantly less than CD137\(^+\) Tregs in both strains (Fig. 9D). Again, there was no significant difference in soluble CD137 production between the two strains. These...
Total of 100,000 irradiated (1500 rad) splenocytes were added to both the bottom and top transwells. The cells in the bottom well were pulsed with ³H-labeled thymidine on day 3 and harvested after 16 h ($n = 3$ experiments for all conditions). Control wells contained either CD4⁺ cells alone (first column), irradiated splenocytes alone (second column), or no Tregs (third column). **(B)** Mean percent proliferation in transwell suppression assays performed at 1:2 ($n = 10$) and 1:4 ($n = 3$) ratios of Treg/T cell. The percentage proliferation was calculated by dividing the cpm counts of the wells containing Tregs by the mean cpm count of the wells with only CD4⁺CD25⁺CD137⁺ T cells. Statistical analysis performed using the unpaired $t$-test.

Results suggest that Treg activation causes CD137⁺ Tregs to increase alternate splicing of *Tnfrsf9* with subsequent production of soluble CD137 protein, although still significantly less than CD137⁺ Tregs. Notably, non-Treg CD4⁺ cells still did not produce significant amounts of soluble CD137 after stimulation under these activating conditions (Fig. 9D). We also tested CD137 membrane expression in CD137⁺ and CD137⁻ Tregs from both strains upon IL-2 and CD3 stimulation as above. As expected, the cells sorted as CD137⁺ Tregs did not express any CD137 preculture (Supplemental Fig. 4A). However, after IL-2 and CD3 stimulation for 3 d, CD137⁻ Tregs from both strains showed increased CD137 expression on a per-cell basis, but the expression was still much lower than CD137⁺ Tregs (Supplemental Fig. 4B). Our data suggest that upon in vitro IL-2 and CD3 stimulation, CD137⁻ Tregs start expressing membrane and soluble CD137, but the expression level is still much lower than Tregs that originally express CD137 preculture. Next, we tested if soluble CD137 is produced by NOD Tregs during in vitro suppression in the presence of CD4 T cells and CD3/CD28 beads in the absence of APCs. Under these conditions, CD137⁻ Tregs produced soluble CD137 protein but still significantly less ($p = 0.0001$) than CD137⁺ Tregs (Fig. 9E). CD4 T cells cultured with CD3/CD28 beads (rather than irradiated APCs) also produced some soluble CD137 but significantly less than CD137⁻ Tregs ($p < 0.0001$). Our findings show that Tregs are the primary source of soluble CD137 in vitro. Our results also show that depending on in vitro culture conditions, CD137⁻ Tregs can produce some soluble CD137 but significantly less than CD137⁺ Tregs.

Increased serum-soluble CD137 in older NOD.B10 Idd9.3 mice

We have shown that NOD.B10 Idd9.3 mice accumulate significantly increased numbers of CD137⁺ Tregs with age (Fig. 1C). We have also shown that CD137⁺ Tregs are more suppressive (Fig. 7A, 7B) and express more soluble CD137 than CD137⁻ Tregs (Fig. 9A–D). However, NOD and NOD.B10 Idd9.3 CD137⁺ Tregs do not differ in either direct contact-mediated suppression (Fig. 7C) or soluble CD137 production on a per-cell basis (Fig. 9A–D). We hypothesized that the increased numbers of CD137⁺ Tregs with age in NOD.B10 Idd9.3 mice might be reflected in increased soluble CD137 serum levels in vivo. In fact, old NOD.B10 Idd9.3 mice had a significantly greater amount of serum-soluble CD137 levels than age-matched NOD mice (Fig. 9F). This result shows that the immunological effect of the B10 CD137 allotype could be quantitative, mediated by altering the accumulation of the affected cell subset (i.e., CD137⁺ Tregs). Increased numbers of Tregs expressing the B10 allele thereby produce increased total amounts of soluble CD137 and also quantitatively expand the overall available contact-mediated suppression.

In summary, we have established that CD137⁺ Tregs are a functionally superior Treg subset characterized by surface expression of CD137 and superior suppression and production of soluble CD137. Tregs expressing the B10 Idd9.3 region, compared with the NOD allotype, show increased peripheral accumulation of the functionally superior CD137⁺ Tregs with age. NOD.B10 Idd9.3 mice show a correlation between accumulation of CD137⁺ Tregs with age and increased serum-soluble CD137. The data presented in this study strongly support a critical role for CD137⁺ Tregs in the regulation of T1D.

**Discussion**

Our previous studies showed that anti-CD137 Ab treatment increased the number of CD4⁺CD25⁺ T cells in NOD mice and that anti-CD137 Abs bound specifically to CD4⁺CD25⁺CD137⁺ Tregs.
in vivo while preventing diabetes (5). The B10 Tnfrsf9 allele differs from the NOD allele by three coding variants (2, 3). These sequence polymorphisms differences are likely responsible for the previously described decreased T cell signaling by the NOD allele when stimulated through CD137 (2), and it is known that CD137 signaling enhances Bcl-xL production and mediates cell survival (42). It remained unclear how the decreased NOD allotype CD137 signaling could mediate increased T1D susceptibility (2). We hypothesized that decreased signaling through the NOD CD137 allele, or increased signaling through the B10 CD137 allele, could influence T1D incidence by affecting Treg accumulation and investigated this hypothesis using NOD and NOD.B10 Idd9.3 mice.

We found increased accumulation of Tregs expressing the B10 Idd9.3 locus in two separate systems (i.e., ex vivo in NOD versus

FIGURE 9. NOD and NOD.B10 Idd9.3 CD137+ Tregs are the major cellular source of soluble CD137 ex vivo. NOD.B10 Idd9.3 congenic mice have higher serum-soluble CD137 levels than NOD. (A and B) RT-PCR for soluble and membrane CD137: NOD and NOD.B10 Idd9.3 CD4+CD25+CD137+, CD4+CD25+CD137−, and CD4+CD25+CD137− T cells were sorted from 4–8-wk-old females as above. RNA was immediately extracted and converted to cDNA. RT-PCR was performed with a set of custom-designed primers used to detect either membrane (A) or soluble (B) CD137 [n = 3 experiments for both (A) and (B)]. B2m was used as an endogenous control. (C–F) ELISA for soluble CD137: CD4+CD25+CD137+, CD4+CD25+CD137−, and CD4+CD25+CD137− Tregs from 5–8-wk-old NOD and NOD.B10 Idd9.3 mice were sorted as above. Total of 50,000 cells were cultured in 96-well U-bottom plates with 25 U/ml IL-2 (NOD, n = 2; NOD.B10 Idd9.3, n = 5) (C) or 1.25 μg/ml of anti-CD3 and 25 U/ml IL-2 (NOD, n = 4; NOD.B10 Idd9.3, n = 4) (D) for 4 d. ELISA for soluble CD137 was performed on the supernatants. (E) NOD CD4+CD25+CD137+, CD4+CD25+CD137−, and CD4+CD25+CD137− T cells were sorted from 5–7-wk-old NOD mice as above. Total of 50,000 CD4+CD25+CD137− T cells were plated in U-bottom 96-well plates with 50,000 CD3/CD28 beads and 25,000 (1:2; n = 5 experiments) CD137− or CD137+ Tregs. The supernatant was collected on day 4, and ELISA was performed on the supernatants for soluble CD137. The statistical analysis was performed using the unpaired t test. (F) Twenty- to 37-wk-old nondiabetic NOD (n = 6) and NOD.B10 Idd9.3 (n = 7) mice were sacrificed and their serum tested for soluble CD137 by ELISA. Statistical analysis was performed using the unpaired t test.
NOD.B10 Idd9.3 mice and in a mixed bone marrow chimera system). The absolute number of splenic lymphocytes, CD4 cells, and CD4^+CD25^- T cells increased with age in both NOD and NOD.B10 Idd9.3 mice; however, the number of CD4^+CD25^+CD137^+ Tregs in mice was increased only in NOD.B10 Idd9.3 mice, reflecting a specific increase in their percentage in NOD.B10 Idd9.3 and a decreased percentage in NOD. Conversely, in the thymus, the number of CD4^+CD25^+CD137^- T cells decreased in NOD and increased in older NOD.B10 Idd9.3 mice, reflecting a significantly increased percentage of thymic CD137^+ Tregs in older NOD.B10 Idd9.3 mice. Our data suggest that increased thymic output of CD4^+CD25^-CD137^- T cells in older NOD.B10 Idd9.3 mice could contribute to the increased accumulation of these cells in the periphery. However, we have not conclusively proven increased thymic output, and there could be an additional mechanism for peripheral accumulation of CD4^+CD25^-CD137^- Tregs. Our data on cell numbers in spleen and thymus of NOD versus NOD.B10 Idd9.3 suggest a specific increase in CD137^+ Tregs in the NOD. B10 Idd9.3 mice, but these results could be influenced by many intrinsic and extrinsic causes. To address this point and to show in a separate system that the B10 Idd9.3 region supported increased accumulation of CD137^+ Tregs, we used a mixed bone marrow chimera approach, in which NOD and B10 Idd9.3-expressing Tregs could develop in the same mouse and hence have the same extrinsic cellular environment. The studies in bone marrow chimeras show that the increased accumulation of B10 Idd9.3-expressing Tregs is a cell-intrinsic feature in CD137^+ Tregs. This does not exclude the possibility that extrinsic factors, particularly IL-2 production from T cells, could also be contributing to expansion or survival of various cell types and protection from diabetes in NOD.B10 Idd9.3 mice. Given that the B10 allele mediates enhanced IL-2 production in NOD.B10 Idd9.3 CD4^+ T cells (2), this is a likely contributing factor that makes the system more complex and multifactorial.

We also explored possible mechanisms for accumulation of CD137^+ Tregs in old NOD.B10 Idd9.3 mice. First, we observed that the NOD.B10 Idd9.3 CD137^+ Tregs do not proliferate more in vitro or in vivo than the same cell subset in NOD mice, suggesting that proliferation does not account for increased accumulation of CD137^+ Tregs in NOD.B10 Idd9.3 mice. Next, we showed that increased CD137^+ Treg accumulation is correlated with increased CD137^+ Treg Bcl-xL mRNA expression in both NOD.B10 Idd9.3 mice and in the CD137^+ Tregs with the B10 allotype in the mixed bone marrow chimera mice. The lack of increased Bcl-xL protein expression and the increased Bcl-xL in CD137^+ Tregs in NOD.B10 Idd9.3 mice, however, makes this correlation hard to interpret. Furthermore, we did not see any difference in cell death between NOD and NOD.B10 Idd9.3 Treg subsets upon in vitro stimulation. Hence, further studies need to be performed to establish the mechanism of accumulation of CD137^+ Tregs in old NOD.B10 Idd9.3 mice.

It has been reported that Foxp3^+ TGF-β^+ T cells significantly decline with age in NOD mice and that the aged CD4^+CD25^- T cells are less suppressive against aged CD4^+CD25^- T cells (44, 49). In addition, the increased protection from diabetes in the NOD BDC2.5 model is also associated with increased Foxp3^+ T cells with age (50). Therefore, it is possible that the increased number of CD137^+ Tregs in NOD.B10 Idd9.3 mice with age could result in increased peripheral immune regulation that could regulate the onset of T1D. We observed that the amount of CD137 (MFI) on the cell surface of CD4^+CD25^+CD137^+ Tregs is not decreased on aged NOD CD137^- Tregs—the accumulating CD137^+ Tregs on aged NOD are all CD137^-high expressers. The CD137 MFI is also higher in young NOD.B10 Idd9.3 compared with young NOD CD137^+ Tregs. The decrease in cellular CD137 expression in young NOD versus NOD.B10 Idd9.3 CD137^+ Tregs might affect the accumulation of total number of surviving cells long term. Increased expression of an allele that in itself mediates increased signaling could combine to produce an intrinsically mediated signal that results in accumulation of these CD137^+ Tregs in NOD.B10 Idd9.3 mice. This is entirely consistent with studies of the function of CD137 in CD137 knockout mice, which have decreased long-term survival of Ag-specific CD8 T cells (4). Because we have shown that CD137^+ Tregs are functionally superior at regulation, an increase of this cell subset over time could result in increased peripheral regulation and increased protection from T1D. Our study has not been able to link increased accumulation with increased cell proliferation, cell survival, or cell death. Although further studies are needed to understand the link between CD137 and accumulation of Tregs, our results strongly suggest that CD137 costimulation is important for Treg-mediated diabetes prevention.

Mouse CD4^+CD25^+ Tregs have been differentiated into subsets based on their expression of cell-surface molecules such as CD134 (51), integrin αEβ7 (52), and CD62L (23, 53) that affect their suppressor activity or by molecules such as CD45RA^+ (54) and P-selectin (55) that delineate Treg differentiation in vitro or in vivo. Our results differentiate two subpopulations of CD4^+CD25^+ Tregs, CD137^+ and CD137^- Tregs. These subsets are not merely phenotypically differentiated by cell-surface expression of CD137, but by differences in functional cell-mediated suppression and by differences in the production of immunosuppressive soluble CD137.

Soluble CD137 has been reported in the sera of patients with rheumatoid arthritis and in the cerebrospinal fluid of patients with multiple sclerosis, and patients with multiple sclerosis have decreased expression of CD137 on their Tregs (31, 56–58). It has been shown that soluble CD137 acts to inhibit T cell proliferation (32, 33). Although the mechanism for soluble CD137-mediated suppression is not fully understood, it has been shown to bind with CD137L in vitro and likely mediates its effect through CD137L (32). In addition, soluble CD137 has been reported to arise later in the immune response to counteract overactivation of the immune system (33). These previous reports combined with the findings presented in this study suggest that production of soluble CD137 may act as a brake upon normal immune activation. In this scenario, activation of Ag-specific CD137^+ Tregs could produce soluble CD137 which, in combination with contact-dependent suppression, would downregulate the immune activation of both T cells and APCs expressing CD137L (59). Insufficient production of soluble CD137 (for example, in our system, mediated by a decrease in frequency of NOD Tregs producing soluble CD137 with age) could lead to exaggerated immune activation; conversely, increased numbers of such Tregs could act to decrease immune activation in NOD.B10 Idd9.3 mice. These considerations are strengthened by our observation of increased serum-soluble CD137 in the protected NOD.B10 Idd9.3 mice with age (Fig. 9F), correlating with the increased accumulation of CD137^+ Tregs in these mice, compared with NOD mice. We showed that there was no intrinsic cellular difference between NOD and NOD.B10 Idd9.3 cells in either contact-mediated suppression (Fig. 7A–C) or production of soluble CD137 (Fig. 9C, 9D). However, the accumulation of CD137^- Tregs in the NOD.B10 Idd9.3 mice was correlated with increased total amount of serum-soluble CD137 (Fig. 9F) and thus increased overall immunosuppression. Similarly, although NOD and NOD.B10 Idd9.3 CD137^- Tregs do not differ in contact-dependent suppression on a per-cell basis (Fig. 7), increased numbers of CD137^- Tregs increase the total available contact-mediated immunosuppression in NOD.B10 Idd9.3.
mice. Thus, the B10 Idd9.3 allele acts primarily to enhance accumulation of CD137+ cells, rather than by changing the immunosuppressive function on a per-cell basis, but the net overall effect is increased systemic immunosuppression. These conclusions are strongly supported by the evidence presented in this study but not directly proven, as will be done in future studies.

Our study explains how the hyporesponsive NOD CD137 allele can contribute to increased T1D susceptibility in NOD mice compared with the B10 allele or the NOD.B10 Idd9.3 mice. Thus, the NOD.B10 Idd9.3 allele could lead to increased accumulation of functionally superior CD137+ Tregs with age and thus downregulate autoimmunity, whereas NOD would have a quantitatively deficient allele of CD137+ Tregs compared with NOD.B10 Idd9.3, contributing to enhanced NOD autoimmunity with age. A decreased number of CD137+ Tregs results in decreased total cell-mediated suppression as well as decreased production of counterregulatory soluble CD137 (Fig. 9F), which might be even more important at the site of inflammation (e.g., the pancreatic islet). These considerations lead us to suggest that enhancing site-specific expression of soluble CD137 could downregulate autoimmunity, and we will explore this hypothesis in future studies.

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Disclosures

The authors have no financial conflicts of interest.

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