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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: 000–000.

Type 1 diabetes (T1D) is a polygenic autoimmune disease, and several genetic elements implicated in T1D pathogenesis mediate their effects through disruption of im-

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Abbreviations used in this article: BAC, bacterial artificial chromosome; B2m, β2-microglobulin; CT, threshold cycle; Ki, knockin; MFI, mean fluorescence intensity; RT-PCR, quantitative real-time PCR; SNP, single nucleotide polymorphism; TID, type 1 diabetes; Treg, regulatory T cell.
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/MtkTac 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-Ptprc (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 014149). NOD.Foxp3-GFP knockout (KO) 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 KO driven in Dr. Kuchroo’s laboratory (35) to NOD MtkTac for 12 generations and then intercrossed for hemi–homozygosity for Foxp3-GFP KO mutation. NOD.B10 Idd9.3 Foxp3-GFP mice were produced by crossbreeding 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 KO 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 as above. The cells were sorted using a BD FACSAria (BD Biosciences). The cells 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), Bcl-xL–Alexa Fluor 488, or IgG isotype-Alexa-Fluor488 (Cell Signaling Technology), Bcl-2-Alexa Fluor 488 or IgG1 isotype-Alexa-Fluor488 (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.CD45.2 × NOD.B10 Idd9.3)F1 bone marrow chimera spleen and pancreatic lymph nodes were stained with CD4-allophycocyanin-Cy7, CD25-PerCP-Cy5.5, CD137-FITC, CD45-allophycocyanin, and CD137-PE or IgG2a isotype-PE, and analyzed on an FACS caliber (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.

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.

Bone marrow chimera construction

Nine- to 13-wk-old (NOD.CD45.2 × 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 purchased using magnetic beads (Miltenyi Biotech), 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 Biotech). The CD4 T cells were blocked with 2.4G2 and stained with CD4-allophycocyanin-Cy7, CD25-FITC, and anti-CD137-allophycocyanin as above. The cells were sorted using a BD FACSAria (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 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-1351J8, accession number CU463327; DN-79L21, CU210939; DN-382D20, CU207373; DN-27K19, CU424443; DN-192H14, CU210934; DN-117C24, CU210933; DN-382G2, CU210932; DN-129J7, CU207371; DN-266N3, CU207342; and DN-14M18, CU407306). As all of the B6 and B10 SNPs were found to be identical by descent throughout the Idd9.3 region (mouse genome 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 m37 mouse assembly) using the sequencing program and alignment tools of the BLAST program (38). The polymorphisms were entered into T1DBase (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 Wellcome 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 T1DBase, and can be viewed at www.t1dbase.org.
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 ΔCT) and the data graphed using GraphPad Prism 5 (version 5.02; GraphPad).

**Proliferation assay**

The CD44CD25CD137−, CD44CD25CD137−, and CD44CD25CD137− splenocytes were stained 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 μCi3H-labeled thymidine on day 3 and harvested after 16 h using a β-scintillation counter. For the suppression assay, 50,000 CD44CD25CD137− T cells were cultured in U-bottom 96-well plates with 1 μg/ml soluble anti-CD3, 50,000 irradiated splenocytes (1500 rad), and varying numbers of CD44CD25CD137− or CD44CD25CD137− Tregs. All cells were cultured and pulsed with 1 μCi3H-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 CD44CD25CD137− 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 CD44CD25CD137− or CD44CD25CD137− 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 CD44CD25CD137− or CD44CD25CD137− Tregs were also cultured in the bottom of the transwell along with the CD44CD25CD137− 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 μCi3H-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. The kit uses rat anti-mouse 4-1BB capture Ab and biotinylated goat anti-mouse 4-1BB detection Ab. Recombinant mouse 4-1BB, provided in the kit, was used as a standard. DeltaSoft software was used to calculate the amount of soluble CD137 in each well based on the standard for each experiment.

**Treg culture**

A total of 50,000 CD44CD25CD137− or CD44CD25CD137− Tregs were cultured in 96-well U-bottom plates with no IL-2 (unstimulated) or 25 U/ml mouse rIL-2 alone with or without 1.25 μg/ml anti-CD3 Ab for 4 d, and the supernatants were tested for soluble CD137 as above by ELISA.

**Data analysis**

All statistical analysis was performed using either the unpaired t test or the Mann–Whitney U test in GraphPad Prism 5 (version 5.02; GraphPad).

**Results**

**Increased accumulation of CD137+ Tregs with the B10 versus the NOD Idd9.3 region in vivo**

We previously demonstrated that agonist anti-CD137 treatment prevents diabetes in NOD mice, that a subset of Tregs constitutively expresses CD137, and that anti-CD137 binds to CD44CD25CD137− Tregs in vitro and in vivo (5). Our results suggested that CD137+ Tregs may be important in T1D pathogenesis. We quantified CD137+ Tregs at 10–20 wk of age and observed no significant differences compared the other age groups, data not shown.) Consistent with previously published observations (44), the number of splenic CD44CD25− 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), despite the finding that the percentage of CD137+ Tregs did not increase with age (Fig. 1B). Thus, the increased number of NOD.B10 Idd9.3 CD44CD25CD137+ T cells with age is due to a combination of an increased number of CD44CD25− T cells and an increased number of CD44CD25− T cells in older NOD.B10 Idd9.3 mice. There was also no difference in the percentage of CD44CD25− T cells with age in either strain (Supplemental Fig. 2B). Both NOD and NOD.B10 Idd9.3 congenic mice showed significant increases in CD44+ 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 CD44CD25− T cells, however, remained approximately constant with age in both strains (Supplemental Fig. 3C). The percentage of NOD.B10 Idd9.3 CD44CD25− thymocytes rose significantly (p = 0.02, Supplemental Fig. 3D). The number of CD44+ 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.

We began 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 fluorescence intensity (MFI) of CD137 on CD44CD25− 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 CD44CD25CD137+ T cells declined

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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 1.** Percentages and absolute numbers of CD137+CD4+CD25+ T cells are significantly lower in aged NOD versus NOD.B10 Idd9.3 mice. (A) Splenocytes from 6-wk-old NOD mice were stained with CD4-allophycocyanin, CD25-APC, and anti–CD137-PE or IgG2a isotype control and analyzed by flow cytometry. The IgG2a isotype control staining was used to establish true CD137 staining. Percentages below the figure show the percent CD137+ versus CD137− in the CD4+CD25+ gate. One representative of multiple experiments. (B–D) NOD and NOD.B10 Idd9.3 splenocytes were isolated from younger (3–9 wk) and older (21–36 wk) nondiabetic females, stained with CD4-APC, CD25-FITC, and anti–CD137-PE, and analyzed by flow cytometry. (B) Isotype staining was used to gate for percentage of CD137+ T cells in the CD4+CD25+ T cell subset in NOD (n = 18, 3–9-wk-old and n = 12, 21–36-wk-old mice) and NOD.B10 Idd9.3 (n = 12, 3–9 wk old and n = 16, 21–36 wk old) spleen. (C) The number of CD4+CD25+CD137+ T cells was counted in NOD (n = 8, 3–9 wk old and n = 7, 21–36 wk old) and NOD.B10 Idd9.3 (n = 5, 3–9 wk old; and n = 7, 21–36 wk old) spleen. (D) The MFI of CD137 on CD137+CD4+CD25+ T cells was analyzed on NOD (n = 10, 3–9 wk old and n = 5, 21–36 wk) and NOD.B10 Idd9.3 (n = 7, 3–9-wk-old and n = 9, 21–36-wk-old mice) spleen.
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 chimera 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 CD4+CD25+CD137+ cells](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 marrow chimera. Pancreatic lymph nodes (Tregs 12 wk after reconstitution of the bone marrow chimeric mouse, the total number of splenocytes or lymph node cells were the same for each cell population; hence the total number of B10 and NOD allotype were from the CD137 + Treg population in the same bone marrow chimeric mouse. 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 alleotype. 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 alleotype.

Viability studies in CD137 stimulated and unstimulated T cells 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 chimera cell subsets.

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 alleotype. 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 alleotype.

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**FIGURE 4.** CD137+ Tregs expressing the B10 Idd9.3 region demonstrate an intrinsic cell accumulation in mixed bone marrow chimera mice. Total of 15–25 million bone marrow cells from 5–12-wk-old NOD.B10 Idd9.3 mice and NOD.CD45.2 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) alleotype by CD137− (left panel) and CD137+ (right panel) Tregs 12 wk after reconstitution of the bone marrow chimera. 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 FACSCanto cytometer (BD Biosciences). Percentage of each F1 (host) cells not shown. Statistical significance was calculated using the unpaired t test.
stratified by allotype. In the mixed bone marrow chimera experiments, CD137+, but not CD137− Tregs, with the B10 allotype expressed significantly increased Bcl-xL (p = 0.008, Fig. 6B). As a control, we tested levels of Bcl-2, a prosurvival molecule not associated with CD137 signaling. There were no significant differences in expression of Bcl2 in NOD versus NOD.B10 Idd9.3 CD137+ or 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 Treg 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 was 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-

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-allophycocyanin-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 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 higher levels of Bcl-xL mRNA than NOD CD137− Tregs. (A) Splenocytes from 4–7-wk-old NOD (n = 9) and NOD.B10 Idd9.3 (n = 6) females were used for sorting up to 50,000 CD4+CD25− CD137−, CD4+CD25+CD137−, and CD4+CD25+CD137+ cells as described above. RNA was extracted from the sorted cells and converted to cDNA as described in the Materials and Methods. RT-PCR was performed on the cDNA using either B2m or Gapdh and Bcl-xL primers. (B) NOD.B10 Idd9.3 and NOD.CD45.2 mixed bone marrow chimeric mice were made as above. Recipient nondiabetic mice were sacrificed 9 to 10 wk postinjection. CD137+ and CD137− Tregs were sorted according to their CD45.1 (NOD.B10 Idd9.3) and CD45.2 (NOD) allotype and used for RT-PCR with B2m and Bcl-xL primers. The mean of n = 3 separate experiments is shown. (C) Four- to 7-wk-old NOD (n = 3) and NOD.B10 Idd9.3 (n = 3) female splenocytes were used for sorting 50,000 CD4+CD25− CD137−, CD4+CD25+CD137−, and CD4+CD25+CD137+ cells and used for RT-PCR with B2m and Bcl2 primers. (D) CD45.1- or CD45.2-expressing CD137− and CD137+ Tregs were sorted from mixed bone marrow chimera as in (B) and used for RT-PCR with B2m and Bcl2 (n = 3). Splenocytes from 4–9-wk-old NOD (n = 4) 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 Bcl-xL Alexa Fluor 488 (E) or Bcl2 Alexa Fluor 488 (F) and their matched isotype control. The stained cells were analyzed by flow cytometry for MFI for Bcl-xL or Bcl2 within CD4+CD25+CD137− and CD4+CD25+CD137+ cells. All statistical calculations were performed using the unpaired t test.

**FIGURE 6.** Peripheral NOD.B10 Idd9.3 CD137− Tregs express higher levels of Bcl-xL mRNA than NOD CD137+ Tregs. (A) Splenocytes from 4–7-wk-old NOD (n = 9) and NOD.B10 Idd9.3 (n = 6) females were used for sorting up to 50,000 CD4+CD25− CD137−, CD4+CD25+CD137−, and CD4+CD25+CD137+ cells as described above. RNA was extracted from the sorted cells and converted to cDNA as described in the Materials and Methods. RT-PCR was performed on the cDNA using either B2m or Gapdh and Bcl-xL primers. (B) NOD.B10 Idd9.3 and NOD.CD45.2 mixed bone marrow chimeric mice were made as above. Recipient nondiabetic mice were sacrificed 9 to 10 wk postinjection. CD137+ and CD137− Tregs were sorted according to their CD45.1 (NOD.B10 Idd9.3) and CD45.2 (NOD) allotype and used for RT-PCR with B2m and Bcl-xL primers. The mean of n = 3 separate experiments is shown. (C) Four- to 7-wk-old NOD (n = 3) and NOD.B10 Idd9.3 (n = 3) female splenocytes were used for sorting 50,000 CD4+CD25− CD137−, CD4+CD25+CD137−, and CD4+CD25+CD137+ cells and used for RT-PCR with B2m and Bcl2 primers. (D) CD45.1- or CD45.2-expressing CD137− and CD137+ Tregs were sorted from mixed bone marrow chimera as in (B) and used for RT-PCR with B2m and Bcl2 (n = 3). Splenocytes from 4–9-wk-old NOD (n = 4) 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 Bcl-xL Alexa Fluor 488 (E) or Bcl2 Alexa Fluor 488 (F) and their matched isotype control. The stained cells were analyzed by flow cytometry for MFI for Bcl-xL or Bcl2 within CD4+CD25+CD137− and CD4+CD25+CD137+ cells. All statistical calculations were performed using the unpaired t test.

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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 (Fig. 9C, 9D). 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
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 increased only in NOD.B10 Idd9.3 mice, reflecting a specific increase in their proportion 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 additional mechanisms 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 allele type 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-\(\beta \) 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 cosimulation 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 \(\alpha E \beta 7 \) (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\(^-\). 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 increase 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 Id9.9 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 al- etype can contribute to increased T1D susceptibility in NOD mice compared with the B10 alertype in the NOB.B10 Id9.9 mice. Thus, the NOB.B10 Id9.9 allele could lead to increased accumulation of functionally superior CD137+ Treggs with age and thus downregulate autoimmunity, whereas NOD would have a quanti- tative deficiency of CD137+ Treggs compared with NOB.B10 Id9.9, contributing to enhanced NOD autoimmunity with age. A decreased number of CD137+ Treggs 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 consider- ations 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.

References


**Supplemental Figure one. ldd9.3 gene content and sequence polymorphisms**

(a) The gene content of the ldd9.3 interval is displayed in the T1DBase Gene Span track. The NOD tile path track shows the ten NOD/Tac BAC clones that formed a minimal tilepath spanning the ldd9.3 region. The NOD BAC SNP graph displays the density of SNPs identified by comparing the NOD/Tac BAC clone sequence to the B6 reference (SNPs per 10 kb). The NOD/ShiLtJ strain has been next generation sequenced (NGS) by the Wellcome Trust Sanger Institute and the NOD NGS SNP graph displays the density of SNPs identified by comparing the NOD/ShiLtJ NGS sequence to the B6 reference. (b) Zoomed in view of Tnfrsf9. The NOD variation track represents the location of polymorphic NOD/B6 SNPs across Tnfrsf9, identified by comparing the NOD/Tac BAC clones to B6; black, red, blue, and green lines represent G, T, C, and A NOD alleles, respectively. The NGS NOD track shows the NOD/B6 SNPs identified by comparing the NOD/ShiLtJ NGS sequence to the B6. Note that where multiple SNPs are located close together the lines in the B6/NOD SNPs tracks may represent more than one SNP.

**Supplemental Figure two. Absolute number of NOD and NOD.B10 ldd9.3 CD4pos, CD4posCD25pos, and splenic lymphocytes increase with age**

NOD and NOD.B10 ldd9.3 splenocytes were counted with a hemocytometer and stained with CD4-APC, CD25-FITC and anti-CD137-PE. The number of CD4posCD25pos T cells (a), the number of CD4 cells (c), and the number of splenic lymphocytes (d) were calculated by multiplying the total number of splenocytes by the percentage of cells in each gate. (b) shows the percentage of CD4posCD25pos T cells. (a-d) performed on NOD (n=8, 3-9 wk old and n=7, 21-36 wk old) and NOD.B10 ldd9.3 (n=5, 3-9 wk old and n=7, 21-36 wk old) spleen using the staining protocol from Fig 1d.
Supplemental Figure three. Percent and absolute number of thymic CD4<sup>pos</sup>CD25<sup>pos</sup>CD137<sup>pos</sup> T cells decline with age in NOD but not NOD.B10 idd9.3 mice

NOD and NOD.B10 idd9.3 thymocytes were stained with CD4-APC, CD25-FITC and anti-CD137-PE. (a) The percentage CD4<sup>pos</sup>CD25<sup>pos</sup>CD137<sup>pos</sup> T cells within the CD4<sup>pos</sup>CD25<sup>pos</sup> T cells stained in NOD (n=10, 3-9 wk old and n=11, 21-36 wk old mice) and NOD.B10 idd9.3 (n=13, 3-9 wk old and n=14, 21-36 wk old) thymus. (b - f) The number of CD4<sup>pos</sup>CD25<sup>pos</sup>CD137<sup>pos</sup> T cells (b), the total number of CD4<sup>pos</sup>CD25<sup>pos</sup>CD137<sup>pos</sup> T cells within CD4 T cells (d), the total number of CD4<sup>pos</sup> T cells (e), and the total number of thymic lymphocytes (f), were calculated from counting the absolute number of thymocytes in NOD (n=5, 3-9 wk old and n=7, 21-36 wk old) and NOD.B10 idd9.3 (n=4, 3-9 wk old and n=6, 21-36 wk old) and applying the indicated gates. (g) The mean florescence intensity of CD137 on CD4<sup>pos</sup>CD25<sup>pos</sup>CD137<sup>pos</sup> thymocytes was analyzed in NOD (n=5, 3-9 wk old and n=4, 21-36 wk) and NOD.B10 idd9.3 (n=9, 3-9 wk old and n=8, 21-36 wk old mice) mice. Statistical analysis was performed using the unpaired t test.

Supplemental Figure four. CD137<sup>neg</sup> Tregs upregulate CD137 after in vitro stimulation, but to a lower MFI level than CD137<sup>pos</sup> Tregs

Splenocytes from 4-7 week old NOD and NOD.B10 idd9.3 females were sorted for CD4<sup>pos</sup>CD25<sup>neg</sup>CD137<sup>neg</sup> T cells and CD137<sup>neg</sup> and CD137<sup>pos</sup> Tregs as described above. (a) Samples of the sorted cells were stained for CD4, CD25 and CD137 and analyzed by flow cytometry; CD137 MFI for each subset is shown beneath each FACS plot (b) 25,000 sorted cells were cultured with 25U/ml IL-2 and 1.25ug/ml anti-CD3 for three days. The cultured cells were stained for CD4, CD25 and CD137 as above and analyzed by flow cytometry; CD137 MFI for each
subset is shown beneath each FACS plot. One representative of three experiments shown. (c) Splenocytes from 4-6 week old NOD and NOD.B10 Idd9.3 females were stained with CD4-APC.Cy7, CD25-Percep-Cy5.5, and anti-CD137-APC, and FACS-sorted for CD4^{pos}CD25^{pos}CD137^{neg} and CD4^{pos}CD25^{pos}CD137^{pos} cells. 50,000 sorted cells in each group were left unstimulated in a 96 well plate. The cells were pulsed with ^3H labeled thymidine on day 3 and harvested after 16 hours. The data was pooled from n=3 experiments.
Supplemental Figure Two

a. 

\[ \# \text{CD}4^{+}\text{CD}25^{+} \text{T cells} \times 10^6 \]

3-9 wks NOD 21-36 wks NOD 3-9 wks NOD.B10 Idd9.3 21-36 wks NOD.B10 Idd9.3

\*P=0.0004  *P=0.02

b. 

\%CD4^{+}\text{CD}25^{+} \text{of CD4} \]

3-9 wks NOD 21-36 wks NOD 3-9 wks NOD.B10 Idd9.3 21-36 wks NOD.B10 Idd9.3

\*P= 0.0004 *P= 0.02

c. 

\# \text{CD}4 \text{ cells} \times 10^7 \]

3-9 wks NOD 21-36 wks NOD 3-9 wks NOD.B10 Idd9.3 21-36 wks NOD.B10 Idd9.3

\*P=0.01  *P=0.0002

d. 

\# \text{spleenic lymphocytes} \times 10^8 \]

3-9 wks NOD 21-36 wks NOD 3-9 wks NOD.B10 Idd9.3 21-36 wks NOD.B10 Idd9.3

\*P= 0.01 *P<0.0001
Supplemental Figure Four

a.

NOD

NOD.B10 Idd9.3

Counts

0 10^2 10^3 10^4 10^5

0 10^2 10^3 10^4 10^5

CD137neg Tregs (red): 6
CD137pos Tregs (blue): 446

CD137neg Tregs (red): 13
CD137pos Tregs (blue): 560

b.

NOD

NOD.B10 Idd9.3

Counts

0 10^2 10^3 10^4 10^5

0 10^2 10^3 10^4 10^5

CD137neg Tregs (red): 757
CD137pos Tregs (blue): 1438

CD137neg Tregs (red): 727
CD137pos Tregs (blue): 1600

c.

CPM counts

0 20000 40000 60000 80000 100000

CD137neg/pos
CD137neg/pos
CD137neg/pos
CD137neg/pos