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Visualizing the Role of Cbl-b in Control of Islet-Reactive CD4 T Cells and Susceptibility to Type 1 Diabetes

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The E3 ubiquitin ligase Cbl-b regulates T cell activation thresholds and has been associated with protecting against type 1 diabetes, but its *in vivo* role in the process of self-tolerance has not been examined at the level of potentially autoaggressive CD4⁺ T cells. In this study, we visualize the consequences of Cbl-b deficiency on self-tolerance to lysozyme Ag expressed in transgenic mice under control of the insulin promoter (*insHEL*). By tracing the fate of pancreatic islet-reactive CD4⁺ T cells in prediabetic 3A9-TCR × *insHEL* double-transgenic mice, we find that Cbl-b deficiency contrasts with AIRE or IL-2 deficiency, because it does not affect thymic negative selection of islet-reactive CD4⁺ cells or the numbers of islet-specific CD4⁺ or CD4⁺Foxp3⁺ T cells in the periphery, although it decreased differentiation of inducible regulatory T cells from TGF-β–treated 3A9-TCR cells *in vitro*. When removed from regulatory T cells and placed in culture, *Cblb*-deficient islet-reactive CD4⁺ cells reveal a capacity to proliferate to HEL Ag that is repressed in wild-type cells. This latent failure of T cell anergy is, nevertheless, controlled *in vivo* in prediabetic mice so that islet-reactive CD4⁺ cells in the spleen and the pancreatic lymph node of *Cblb*-deficient mice show no evidence of increased activation or proliferation *in situ*. *Cblb* deficiency subsequently precipitated diabetes in most TCR:*insHEL* animals by 15 wk of age. These results reveal a role for peripheral T cell anergy in organ-specific self-tolerance and illuminate the interplay between *Cblb*-dependent anergy and other mechanisms for preventing organ-specific autoimmunity. *The Journal of Immunology*, 2011, 186: 000–000.

Organ-specific autoimmune diseases, such as type 1 diabetes, appear to result from inherited defects in different cellular checkpoints for acquiring self-tolerance within the T cell repertoire. Rare monogenic syndromes of organ-specific autoimmunity in humans—coupled with cellular analysis of mice bearing corresponding gene defects—have illuminated critical roles for the AIRE transcription factor in thymic deletion of organ-specific T cells (1–5) and for Foxp3, IL-2, and IL-2R α-chain in thymic formation of regulatory T cells (Tregs) (6–14). Analyses of polygenic susceptibility to autoimmunity also implicates defects in specific T cell tolerance checkpoints, notably subtle genetic variants of IL2RA and IL-2 associated with type 1 diabetes in humans and NOD mice, respectively, and polygenic defects in TCR signaling for thymic deletion in NOD mice (3, 15–19). Spontaneous type 1 diabetes in the Komada diabetes-prone (KDP) rat results from cooperation between a specific MHC haplotype—probably MHC class II—and a truncating mutation in the gene encoding Cbl-b (20–22). However, it is not known

whether Cbl-b deficiency disrupts thymic deletion of MHC class II-restricted diabetogenic CD4⁺ cells, formation of islet-specific Foxp3 T cells, or other peripheral self-tolerance checkpoints. Cbl-b has been linked to T cell anergy induction by the pharmacological agent ionomycin *in vitro* or upon artificial exposure to high concentrations of foreign peptides *in vivo* (23), but there is in fact little direct evidence for T cell anergy as a physiological mechanism for organ-specific self-tolerance and no information on how a defect in T cell anergy might be manifest in diabetogenic T cells.

Cbl-b and its close paralogue, c-Cbl, function as E3 ubiquitin ligases that regulate cell surface expression of activated receptors with receptor tyrosine kinase activity (e.g., the epidermal growth factor receptor) through endocytosis and targeting them for ubiquitin-mediated degradation (24, 25). Thymocytes predominantly express c-Cbl, whereas Cbl-b is more abundant in mature T cells (25, 26). C-Cbl is required for TCR sequestration and downregulation in thymocytes, whereas the two proteins appear to function redundantly for promoting TCR downregulation in peripheral T cells (23, 27, 28). Peripheral T cells from Cbl-b–deficient mice express relatively normal TCR levels on their surface but have a lower Ag concentration threshold for acute activation *in vitro* and can be activated to proliferate and synthesize cytokines in the absence of the normal requirement for CD28 costimulation (27, 29–33). Cbl-b–deficient T cells exhibit normal TCR-induced activation but show enhanced formation of three key TCR signaling events that normally depend on CD28 costimulation: NF-κB activation through the PI3K/Akt/protein kinase C-θ–Carma-1 pathway (34), FOXO-3a inactivation through the PI3K–Akt pathway (35, 36), and stable clustering of TCR with lipid rafts and signaling components in the T cell synapse (37). In response to viral infection, Cbl-b–deficient T cells undergo clonal expansion followed by a decline in numbers that is comparable to wild-type T cells, although during chronic infection, the deficient T cells resist the shutdown of IFN-γ secretion that occurs during “exhaustion” of wild-type CD8 cells (32). These data firmly

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Abbreviations used in this article: CD62L, L-selectin; HEL, hen egg lysozyme; iTreg, inducible regulatory T cell; KDP, Komada diabetes-prone; nTreg, natural regulatory T cell; SA, streptavidin; Treg, regulatory T cell.

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establish a unique role for Cbl-b in regulating naive T cell activation in response to foreign Ags.

The role of T cell anergy as a physiological self-tolerance mechanism is unclear. In tissue culture, T cell clones that receive a sustained TCR calcium signal in the absence of a CD28 costimulus are rendered unable to synthesize IL-2 and proliferate upon restimulation (38, 39). Because Cbl-b-deficient T cells are able to proliferate and make IL-2 without CD28 costimulation (27, 29), and Cbl-b together with other ubiquitin ligases is induced by chronic calcium-NFAT signaling mediated by the TCR or by the pharmacological agent ionomycin (37, 40), it has been suggested that Cbl-b is part of a program for physiological self-tolerance by T cell anergy. However, experimental studies of T cell anergy in vivo rely on acutely triggering naive T cells with high concentrations of foreign Ag in the absence of adjuvant (23). This does not trigger the outcome observed in T cells in culture, but instead, the T cells are triggered into several rounds of cell division in vivo followed by death of many of the T cell progeny. The residual cells are refractory to TCR-induced proliferation when placed in tissue culture, and this refractory state is diminished when the T cells lack Cbl-b (23). Although this high-zone desensitization regimen mirrors T cell exhaustion during chronic viral infection (32), it is not clear that the same process occurs in concert with clonal deletion and Treg formation during self-tolerance.

In this study, we analyze the effect of Cbl-b deficiency on the processes of self-tolerance occurring in CD4⁺ T cells that express a high-affinity transgenic TCR for a neo self-Ag (hen egg lysozyme; insHEL) that is expressed as an abundant membrane protein on pancreatic β cells (41). In these animals, most islet-reactive CD4⁺ T cells are deleted in the thymus through an Aire-dependent mechanism of insHEL expression on thymic medullary epithelial cells (3, 4). A subset of the islet-reactive CD4 cells escape deletion and are induced in the thymus to become Foxp3⁺CD25⁺ cells by a process that depends on IL-2 (12), whereas others escape both these fates but appear quiescent in the periphery for reasons that are unexplored. We find that Cbl-b deficiency leaves intact both Aire-dependent thymic deletion and IL-2-dependent Treg formation of islet-reactive CD4 cells and selectively cripples an intrinsic silencing of the proliferative response by islet-reactive cells that escape these other checkpoints. Cbl-b deficiency lead to a defect in islet-specific CD4 T cell anergy, as well as a defect in TGF- β -induced Foxp3 expression and suppressive activity of inducible Treg (iTreg) in vitro, and precipitated progression to diabetes over time.

Materials and Methods

Mice

3A9 TCR transgenic and ILK-3 insHEL transgenic mice produced in C57BL/6J mice were backcrossed more than 7 generations to B10.Br/SgSnJ (B10k JAX). *Cblb*^{-/-} mice (29), were backcrossed to C57BL/6 for 10 generations, and then backcrossed 3 generations to B10.Br 3A9 TCR:insHEL transgenic mice. Genotyping for the *Cblb* mutation was performed by multiplex PCR genotyping. Genomic DNA was prepared and amplified using a unique allele-specific forward primer that could distinguish the wild-type and mutant *Cblb* gene, whereas the reverse primer was common to both genotypes. All mice were bred and purchased from the Australian Phenomics Facility, Australian National University. All mice were housed in specific pathogen-free conditions (Canberra, Australian Capital Territory, Australia), and all animal procedures were approved by the Australian National University Animal Ethics and Experimentation Committee.

Abs and flow cytometry

Cell suspensions of spleen or thymus were prepared by sieving and gentle pipetting. For surface staining, cells were added at 2×10^6 cells/well and were maintained in the dark at 4°C throughout. Cells were washed twice in ice-cold

FACS buffer (2% FCS and 0.02% NaN₃ in PBS) and then incubated with each Ab and conjugate layer for 30 min and washed thoroughly with FACS buffer between each layer. The following Abs were used (all from BD Biosciences, except where otherwise indicated): CD3e-FITC, CD4-PerCP, CD4-allophycocyanin, CD4-FITC, CD5-PE, CD8-FITC, CD8-PE (Caltag Laboratories), CD8-PerCP, CD45.2-FITC, CD45.2-allophycocyanin, CD45.1-FITC, CD45.1-PE, L-selectin (CD62L)-biotin, B220-PerCP, B220-allophycocyanin, B220-PE, B220-biotin, CD19-PE, CD19-PE, CD25-allophycocyanin, CD69-FITC, CD69-biotin, and CD90-PE (BD Pharmingen). Secondary Abs and streptavidin (SA) conjugates were as follows: rat-anti-mouse IgG1-allophycocyanin, rabbit anti-rat-IgG-biotin (DakoCytomation), SA-PE, SA-allophycocyanin, and SA-Cychrome. Data were acquired on a FACSCalibur flow cytometer and analyzed using FlowJo software. Cells were sorted on a FACS Vantage (BD Biosciences). The rat-anti-mouse IgG1 mAb was a gift from E. Unanue and D. Peterson (Washington University School of Medicine, St. Louis, MO).

CFSE labeling

Cells were suspended at 5×10^7 /ml in warm RPMI 1640 medium and incubated for 10 min at 37°C with 5 μ M CFSE (Molecular Probes). Labeling was quenched with three washes of ice-cold RPMI 1640 medium/10% FCS. At the end of the culture period, cells were collected, washed, and stained with anti-CD4-allophycocyanin, anti-CD8-PE, 1G12/rat-anti-mouse IgG1 Abs (all from BD Pharmingen), and 7-aminoactinomycin D (Molecular Probes). Data were collected on a FACSCalibur (BD Biosciences) using CellQuest Pro and FlowJo (Tree Star) software analysis packages.

Diabetes incidence study

Diabetes incidence was measured using urine glucose testing with Diastix at weekly intervals or when a cage was wet. To be recorded as diabetic mice had to be diastix 4⁺ on at least two readings tested 1 wk apart. Nondiabetic mice were culled at 24 wk.

T cell culture

Single-cell suspensions of spleen cells from TCR or TCR:insHEL mice were prepared by passing a whole spleen through a 70- μ m nylon cell strainer in 5 ml R10 media (RPMI 1640 medium, 10% FCS, 3% penicillin/streptomycin/L-glutamine, 0.1 μ M/ml 2-ME, 1% MEM, 1% HEPES, and 1% sodium pyruvate), and the cells were centrifuged (7 min, 1500 rpm, 4°C) and re-suspended in 5 ml R10 culture medium. The cell density was adjusted to 2×10^6 cells/ml, and 200 μ l cells was plated on 96-well flat-bottom plates (Costar). HEL protein Ag was added at the indicated concentrations, and the cells were incubated for 72 h at 37°C. Cells were counted by preparing a 1:20 dilution of cells in trypan blue to stain dead cells. The cells were pulsed with [³H]thymidine for 6 h before the cells were harvested onto glass fiber filter papers and assayed using liquid scintillation chromatography (Topcount; Packard). To examine intracellular cytokine production, splenic CD4⁺ T cells were cultured in vitro in the presence of PMA plus ionomycin for 6 h in total, but over the last 2 h of culture, GolgiStop was added. Cells were stained with cell surface Abs and permeabilized and fixed prior to the addition of anti-IL-4, anti-IFN- γ , and anti-IL-17 secondary Abs. Cells were analyzed by flow cytometry.

iTreg development in vitro

Ninety-six-well microtiter plates (Nunc Maxisorp; eBioscience, San Diego, CA) were coated with 5 mg/ml anti-CD3 and 2 mg/ml anti-CD28. Spleen cells were obtained from wild-type, 3A9 wild-type, and 3A9 *Cblb*^{-/-} mice and were purified by FACS sorting to isolate CD4⁺CD62L⁺CD25⁻, CD44⁻ cells from wild-type, 3A9 wild-type, and 3A9 *Cblb*^{-/-}. The cells were cultured in vitro in the presence of 0.5 and 0.1 ng/ml recombinant human TGF- β for 3 d at 37°C, and 5 ng/ml recombinant human IL-2 was added for an additional 2 d. At the end of the culture period, the cells were stained and analyzed by flow cytometry to determine the frequency of CD4⁺CD25⁺Foxp3⁺ cells in the culture.

Suppression assay with iTregs in vitro

Naive responder T cells (CD4⁺CD62L⁺CD25⁻CD44⁻) were FACS sorted from the spleen of wild-type mice. The cells were washed and labeled with 5 mM CFSE for 10 min at 37°C in PBS/0.1% BSA. T-depleted splenocytes from wild-type mice were used as APCs and were obtained by FACS sorting CD3⁺CD8⁻CD4⁻ cells. Ninety-six-well microtiter plates (Nunc Maxisorp; eBioscience) were coated with 5 μ g/ml anti-CD3, and 10⁵ CFSE-labeled naive T cells plus 10⁵ iTregs plus 5×10^5 T-depleted splenocytes were cultured in a 96-well plate. The cells were cultured at

37°C for 3 d and then harvested, stained, and analyzed by flow cytometry for CFSE dilution.

Anti-Hel IgG ELISAs

Serum levels of anti-HEL Abs were detected using the ELISA system. Ninety-six-well microtiter plates (Nunc Maxisorp; eBioscience) were coated with 100 μ l of 100 μ g/ml HEL in carbonate buffer overnight at 4°C. Plates were washed twice with PBS 2% FCS and blocked with 100 μ l PBS and 50% FCS for 2 h at room temperature. Plates were washed twice with PBS 0.5% Tween 20, and serum samples were then added in a 1:50 dilution. Samples were incubated for 45 min at room temperature. Plates were washed three times with PBS 0.5% Tween 20, and alkaline phosphatase-conjugated secondary Abs were added (anti-mouse IgM, 1/500; anti-mouse IgG1, 1/1000; anti-mouse IgG, 1/1000; BD Pharmingen) for 45 min at room temperature. After washing twice with PBS 0.5% Tween 20, once with autoclaved H₂O and once with ELISA substrate buffer, plates were developed using Sigma Phosphatase substrate at 2 mg/ml in ELISA substrate buffer for 30 min. Plates were read using the Molecular Devices ThermoMax microplate reader (Sunnyvale, CA) plate reader and Softmax (San Diego, CA) software.

Bone marrow chimeras

Bone marrow chimeras were made by injecting 2×10^6 T cell-depleted bone marrow cells from *Cblb*^{+/+} or *Cblb*^{-/-} 3A9 TCR transgenic donors i.v. into B10.BR or B10.BR:*insHel* transgenic mice that had first been conditioned with two doses of 5 Gy gamma irradiation (Cs source; CSIRO, Canberra, Australian Capital Territory, Australia) given 3 h apart. The recipient animals were given water containing antibiotics for the first 3 wk, and mice were analyzed between 8 and 12 wk postinjection, at which time none were glucosuric.

Statistical analysis

Analysis was performed Student *t* test. The analysis of anti-Hel IgG levels was determined using ANOVA. The diabetes incidence was analyzed by log-rank test.

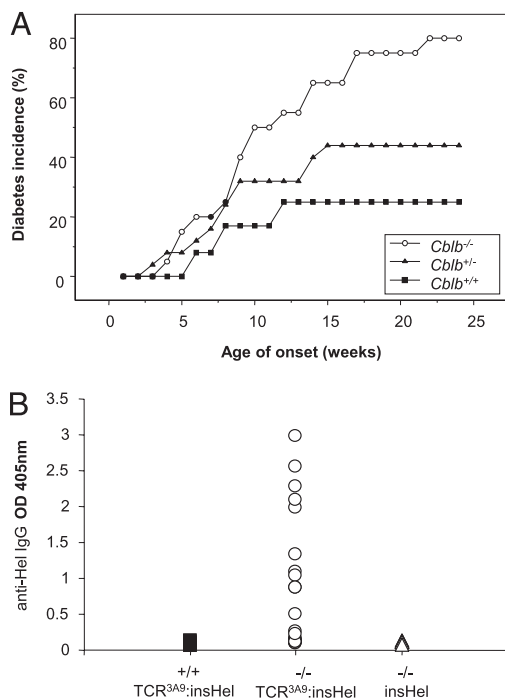


FIGURE 1. Loss of *Cbl-b* predisposes to development of type 1 diabetes. *A*, The incidence of type 1 diabetes was measured in wild-type, *Cblb*^{+/+}, and *Cblb*^{-/-} *TCR:insHel* mice ($n = 25$ /group). The incidence of diabetes was statistically higher in both *Cblb*^{-/-} and *Cblb*^{+/+} *TCR insHel* mice compared with *Cblb*^{+/+} *TCR insHel* mice ($p < 0.0001$; $p = 0.0007$) statistical comparison was made by log-rank test. *B*, Serum anti-HEL IgG levels were determined by ELISA. Data represent *Cblb*^{+/+} *TCR insHel* ($n = 6$), *Cblb*^{-/-} *TCR insHel* ($n = 13$), and *Cblb*^{-/-} *insHel* ($n = 6$) mice. Statistical analysis was made by Student *t* test between *Cblb*^{+/+} *TCR insHel* and *Cblb*^{-/-} *TCR insHel* mice and showed a significant difference between the groups ANOVA; $p = 0.0318$; 2 degrees of freedom.

Results

Cblb deficiency precipitates diabetes and anti-islet autoantibodies in *TCR:insHEL* transgenic mice

To study the role of *Cbl-b* in regulation of self-tolerance to an islet-specific Ag *Cblb*^{-/-}, mice were bred with 3A9 *TCR* \times *insHel* double-transgenic mice. The 3A9 TCR is I-A^k restricted, directs T cell development toward the CD4⁺ lineage, and recognizes the immunodominant 46–61 peptide of HEL bound to I-A^k (41, 42). The transgene carried by *insHEL* mice encodes a membrane-bound form of lysozyme under transcriptional control of the rat insulin promoter. The *insHEL* gene mirrors the pattern of proinsulin gene expression, with high expression in all pancreatic islet β cells (41) and lower expression in medullary thymic epithelial cells (3, 4). As a result of high numbers of islet-reactive CD4 T cells produced in the thymus, B10.BR *TCR:insHEL* mice develop insulinitis, but none develop anti-HEL IgG autoantibodies (Fig. 1*B*) and <25% develop type 1 diabetes (Fig. 1*A*). The low incidence of diabetes in the B10.Br double transgenic mice reflects Aire-dependent thymic deletion (3, 4) and IL-2-dependent thymic formation of islet-specific CD4⁺Foxp3⁺ cells (12), because >90% of B10.BR *TCR:insHEL* mice with *Aire* or *Il2* mutations lacking these mechanisms develop diabetes.

A similar increase in progression to diabetes occurred upon breeding the *Cblb* mutation into B10.BR *TCR:insHel* mice, with

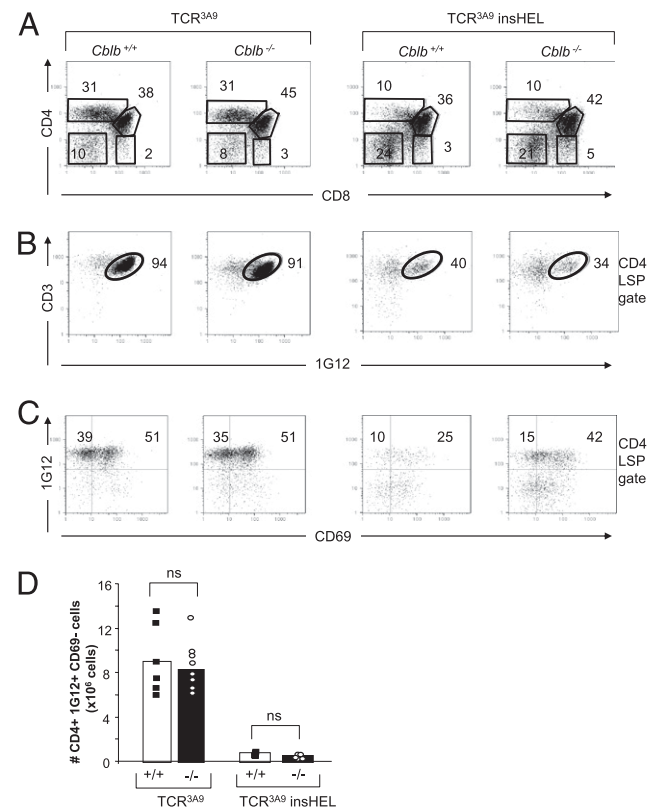


FIGURE 2. Normal positive and negative selection of HEL-specific T cells in thymus of mice lacking *Cbl-b*. *A–C*, Representative flow cytometric profiles of thymocytes showing: *A*, CD4 versus CD8 development and gates used to differentiate subsets; *B* and *C*, expression of HEL-specific 3A9 TCR detected with 1G12 anti-clonotypic mAb versus CD3 (*B*) or CD69 (*C*) on mature CD4 late single-positive (LSP) thymocytes. The numbers indicate the percentage of thymocytes (*A*) or CD4 LSP cells (*B*, *C*) within the gates shown. *D*, The absolute number of Hel-specific CD4⁺1G12⁺CD69⁻ cells in the thymus of *TCR* and *TCR:insHel* mice on a wild-type and *Cbl-b*-deficient background. Results of statistical comparison by Student *t* test are shown *TCR Cblb*^{+/+} versus *Cblb*^{-/-}, $p = 0.6538$; and *TCR:insHel Cblb*^{+/+} versus *Cblb*^{-/-}, $p = 0.1484$.

80% of the double-transgenic *Cblb*^{-/-} offspring developing diabetes (Fig. 1A). Many of the Cbl-b-deficient *TCR:insHEL* mice developed anti-HEL IgG autoantibodies in the serum, whereas none were present in Cbl-b-sufficient controls (Fig. 1B). Cbl-b deficiency alone was insufficient for spontaneous autoimmunity in the absence of the elevated T cell frequency conferred by the TCR transgene, because *Cblb*^{-/-} insHEL single-transgenic mice did not develop anti-HEL IgG autoantibodies (Fig. 1B) or diabetes (data not shown).

T cell selection in prediabetic mice is normal in the absence of Cbl-b

To define how Cbl-b deficiency predisposes to diabetes and islet autoantibodies, we analyzed 8- to 12-wk-old *TCR:insHEL* animals that had no evidence of glucosuria. We first asked whether the mutation had similar effects on thymic tolerance mechanisms to those previously defined for *Aire* or *Il2* mutations in the same transgenic model. The fate of HEL-specific CD4⁺ T cells was monitored by staining with a 3A9 clonotype TCR-specific mAb, 1G12. In *TCR* transgenic mice lacking the *insHEL* gene, positive selection of 1G12⁺ thymocytes into the CD4⁺ T cell lineage occurred equally in the presence or absence of Cbl-b, and there was no difference in the number of mature CD4⁺1G12⁺CD69⁻ T cells produced in thymus of wild-type or *Cblb*^{-/-} *TCR* mice (Fig. 2A, 2C). The c-Cbl and Cbl-b proteins regulate cell surface expression of the TCR-CD3 complex (28) but there was no difference ob-

served in the surface expression of TCR or CD3 on *Cblb*^{-/-} thymic T cells compared with wild-type TCR transgenic cells (Fig. 2B and data not shown).

Negative selection of islet-specific CD4⁺ T cells in *TCR:insHel* mice is under the control of the autoimmune regulator gene *Aire* (3, 4). Promiscuous expression of the HEL protein by medullary thymic epithelial cells causes deletion of the majority of CD4⁺ T cells during the single-positive stage of development (Fig. 2). Analysis of negative selection in the thymus of wild-type and *Cblb*^{-/-} *TCR:insHel* mice by flow cytometry revealed no difference between the deletion of Hel-specific CD4⁺1G12⁺ T cells, because there were equivalently low numbers of mature CD4⁺1G12⁺CD69⁻ cells in the thymus of both strains, and there was no change in the levels of CD3 and TCR (Fig. 2B–D). In the periphery, there was also no difference in the cell surface phenotype or frequency of the residual mature HEL-specific CD4⁺1G12⁺ T cells between wild-type and *Cblb*^{-/-} *TCR:insHel* mice (Fig. 3A, 3B).

We next examined the possibility that diabetes in *Cblb*^{-/-} *TCR:insHel* mice could reflect absence of thymic or peripheral 1G12⁺ CD4⁺CD25⁺Foxp3⁺ Tregs, as occurs in *TCR insHEL* mice lacking IL-2 (12). Flow cytometric staining nevertheless revealed no difference in the frequency or absolute number of Tregs in the thymus (data not shown) or the spleen of *Cblb*^{+/+} and *Cblb*^{-/-} *TCR:insHel* mice (Fig. 3C, 3D). Taken together, these results indicate that Cbl-b has little influence in regulating thymic selection of islet-specific CD4⁺ T cells, and furthermore, Cbl-b's role in regulating TCR/CD3 levels in thymocytes must be redundant presumably because of c-Cbl or other ubiquitin ligases.

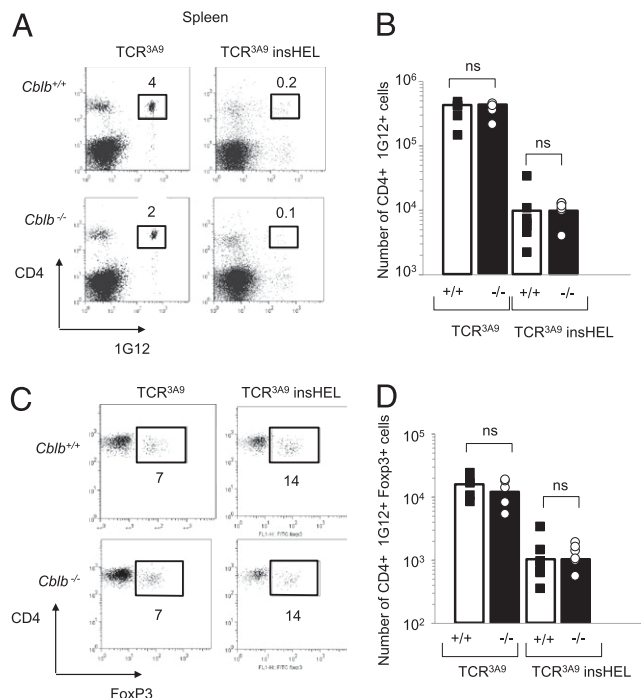


FIGURE 3. Normal control of peripheral islet-reactive CD4⁺ T cell frequency and subsets in mice lacking Cbl-b. *A*, Representative flow cytometric profiles showing the percentage of Hel-specific CD4⁺1G12⁺ cells in the spleen of *TCR* and *TCR:insHel* mice on a wild-type and Cbl-b-deficient background. *B*, The number of CD4⁺1G12⁺ cells in the spleen of wild-type and *Cblb*^{-/-} *TCR* ($n = 9$ /group) and *TCR:insHel* ($n = 9$ /group). There was no significant difference between *Cblb*^{-/-} and *Cblb*^{+/+} controls by Student *t* test. *C*, Representative flow cytometric profiles of spleen cells from mice of the indicated genotypes, gated on CD4⁺1G12⁺ and showing the percentage of gated cells expressing Foxp3. *D*, The number of spleen CD4⁺1G12⁺ cells expressing Foxp3 in individual mice of the indicated genotypes. There was no significant difference between *Cblb*^{-/-} and *Cblb*^{+/+} controls by Student *t* test. *TCR Cblb*^{+/+} versus *Cblb*^{-/-}, $p = 0.7363$; *TCR:insHel Cblb*^{+/+} versus *Cblb*^{-/-} mice, $p = 0.9496$.

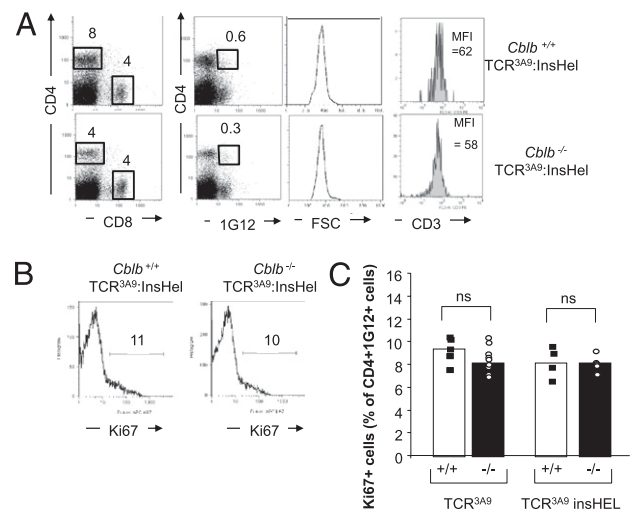


FIGURE 4. In vivo proliferation of islet-specific peripheral CD4⁺ T cells is prevented in *Cblb* deficient mice. *A*, Representative flow cytometric profiles of CD4⁺1G12⁺ T cells in the pancreatic lymph node of *Cblb*^{+/+} and *Cblb*^{-/-} *TCR:insHel* mice. The numbers indicate the frequency of CD4⁺ and CD8⁺ cells within the lymphocyte gate and the frequency of 1G12⁺ cells within the CD4 compartment. The mean fluorescence intensity (MFI) of forward light scatter and CD3 show that there is no enlargement of the islet-reactive CD4 cells and no increase in their surface TCR in Cbl-b-deficient animals. *B*, Intracellular flow cytometry staining for the cell cycle marker Ki67 in gated CD4⁺1G12⁺ T cells from *Cblb*^{+/+} and *Cblb*^{-/-} mice in the pancreatic lymph node. The percentage of Ki67⁺ cells is shown. *C*, Percentage Ki67⁺ cells among CD4⁺1G12⁺ cells in individual *TCR:insHel* mice on a *Cblb*^{+/+} and *Cblb*^{-/-} background. Each symbol represents one mouse. There was no significant difference between *Cblb*^{-/-} and *Cblb*^{+/+} controls by Student *t* test. *TCR Cblb*^{+/+} versus *Cblb*^{-/-}, $p = 0.6895$; *TCR:insHel Cblb*^{+/+} versus *Cblb*^{-/-}, $p = 0.6595$.

Defective anergy in islet-specific CD4⁺ T cells is masked in vivo in prediabetic mice

Because there was no effect of *Cbl-b* deficiency on thymic selection of islet-specific CD4 cells, we next explored the possibility that the cells that escaped thymic negative selection would fail to become anergic and proliferate in response to the high concentrations of insHEL produced in the pancreatic islets and presented in the draining pancreatic lymph node (3). Flow cytometric analysis, nevertheless, revealed no increase in the frequency or cell size of CD4⁺1G12⁺ cells within the draining pancreatic lymph node in the *Cblb*^{-/-} *TCR:insHel* mice (Fig. 4A). Similar to the thymus, there was no difference in the levels of TCR and CD3 expression on the Hel-specific CD4⁺ T cells in the lymph node of wild-type or *Cblb*^{-/-} *TCR:insHel* mice (Fig. 4A). Similar to the thymus, there was no difference in the levels of TCR and CD3 expression on the Hel-specific CD4⁺ T cells in the lymph node of wild-type or *Cblb*^{-/-} *TCR:insHel* mice (Fig. 4A). Moreover, flow cytometric staining for the nuclear cell Ag Ki67, which marks cells that have entered cell cycle revealed an equivalent low frequency of ~10% Ki67⁺ cells among CD4⁺1G12⁺ T cells of *Cblb*^{+/+} and *Cblb*^{-/-} *TCR:insHel* mice (Fig. 4B, 4C). Taken together, these results indicate that the majority of circulating islet-reactive CD4⁺ T cells remain tolerant and unresponsive to islet-derived autoantigen despite lacking *Cblb*.

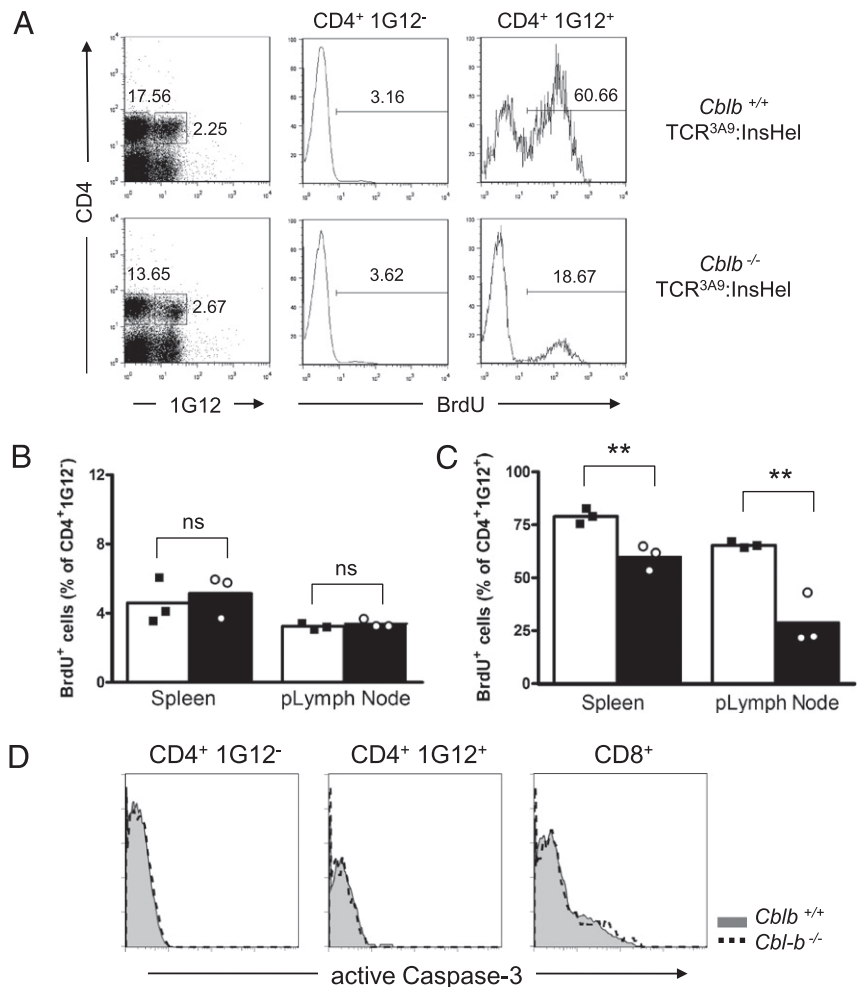
To explore this further, we set up bone marrow chimeras where wild-type or *Cblb*^{-/-} *TCR* transgenic bone marrow cells were transferred to irradiated *insHel* recipients. Twelve weeks after the transfer, none of the recipient mice had developed diabetes, and

they were treated with BrdU for 3 d and were analyzed on day 4 for the uptake of BrdU in both CD4⁺1G12⁺ and CD4⁺1G12⁻ cells using flow cytometry (Fig. 5A, 5C). This revealed that ~75 and 60% of wild-type CD4⁺1G12⁺ cells were BrdU⁺ in the spleen and pancreatic lymph node, respectively, compared with ~60 and ~25% of *Cblb*^{-/-} CD4⁺1G12⁺ T cells that were BrdU⁺ in the spleen and pancreatic lymph node, respectively (Fig. 5A, 5C). The CD4⁺1G12⁻ cells act as an internal control and displayed just 3–4% BrdU⁺ cells in secondary lymphoid tissues consistent with basal proliferation of naive T cells (Fig. 5A, 5B). These results indicate that in vivo the majority of circulating islet-reactive CD4⁺ T cells remain tolerant and unresponsive to islet-derived autoantigen in vivo despite lacking *Cbl-b*. We also examined the possibility that the loss of *cbl-b* may disrupt apoptosis that leads to the inappropriate survival of islet-reactive T cells. To explore this possibility, we analyzed expression of active caspase 3 by intracellular FACS staining. Analysis of spleen and lymph node cells for anti-caspase 3 staining revealed no difference between wild-type and *Cbl-b*^{-/-} CD4⁺ and CD8⁺ T cells (Fig. 5D). These results indicate that the *Cblb* mutation does not affect apoptosis in the absence of *Cblb*.

Although there was no evidence of a proliferative response to islet-derived HELs in vivo, we next asked whether there were intrinsic differences in the potential to proliferate that could be revealed by stimulating with different concentrations of HEL Ag in tissue culture.

Spleen cells from wild-type and *Cblb*^{-/-} *TCR* only or *TCR:insHel* mice were stimulated in vitro with HEL Ag, and proli-

FIGURE 5. In vivo proliferation of islet-specific peripheral CD4 T cells is prevented in *Cbl-b*-deficient mice. **A**, Representative flow cytometric profiles of CD4⁺1G12⁺ T cells in the pancreatic lymph node of *Cblb*^{+/+} and *Cblb*^{-/-} *TCR:insHel* mice. The numbers indicate the frequency of CD4⁺ and CD8⁺ cells within the lymphocyte gate and the frequency of 1G12⁺ cells within the CD4 compartment. The mean fluorescence intensity of forward light scatter and cell surface CD3 show that there is no enlargement of the islet-reactive CD4 cells and no increase in their surface TCR in *Cbl-b*-deficient animals. **B**, Intracellular flow cytometry staining for the BrdU⁺ in gated CD4⁺1G12⁺ T cells from *Cblb*^{+/+} and *Cblb*^{-/-} mice in the pancreatic lymph node. The percentage of BrdU⁺ cells is shown. **C**, Percentage BrdU⁺ cells among CD4⁺1G12⁻ cells in individual *TCR:insHel* mice on a *Cblb*^{+/+} and *Cblb*^{-/-} background. ***p* = 0.001. **D**, Percentage of BrdU⁺ cells among CD4⁺1G12⁺ cells in individual *TCR:insHel* mice on a *Cblb*^{+/+} and *Cblb*^{-/-} background. Each symbol represents one mouse. There was no significant difference between *Cblb*^{-/-} and *Cblb*^{+/+} controls by Student *t* test. *TCR Cblb*^{+/+} versus *Cblb*^{-/-}, *p* = 0.6895; *TCR:insHel Cblb*^{+/+} versus *Cblb*^{-/-}, *p* = 0.6595.

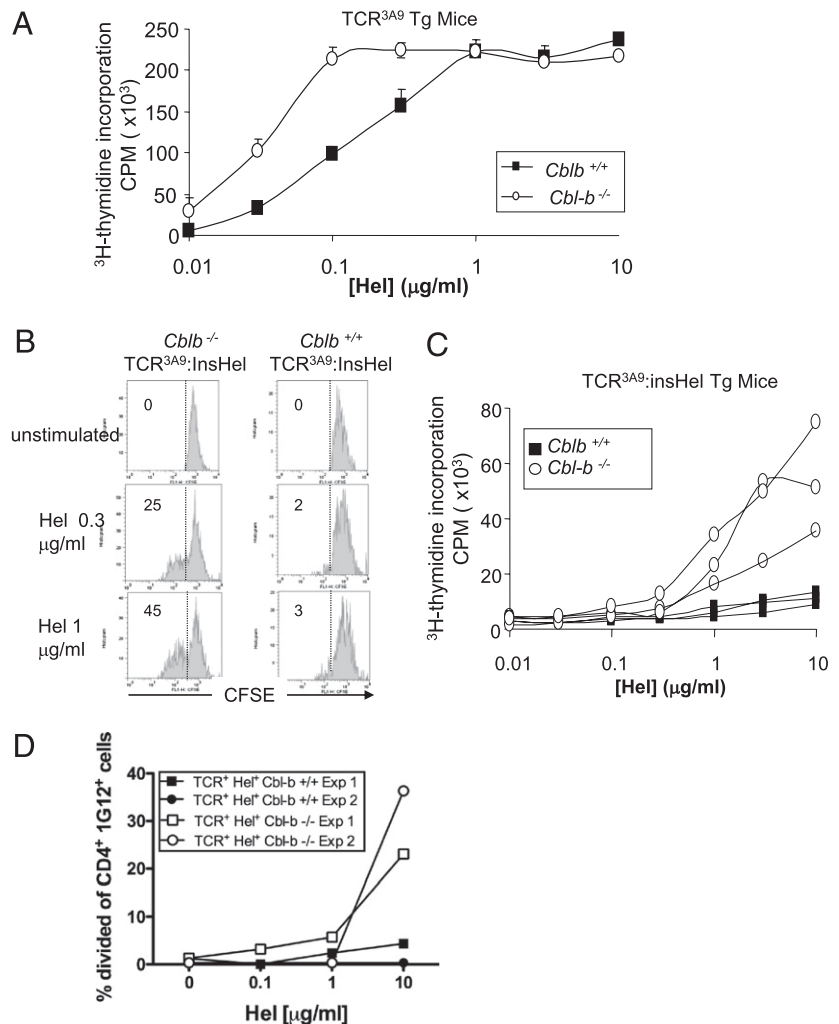


feration was measured after 72 h. Focusing first on the naive T cells from TCR-transgenic mice lacking insHEL, we confirmed previous observations that Cbl-b deficiency lowers the activation threshold such that the *Cblb*^{-/-} T cells proliferated at 5-fold lower Ag concentrations compared with wild-type T cells (Fig. 6A). Next, we examined HEL Ag-induced proliferation of T cells from wild-type and *Cblb*^{-/-} TCR:insHel mice by [³H]thymidine incorporation or by dilution of the cell membrane dye CFSE following Ag stimulation (Fig. 6B, 6C). By both assays, the HEL-specific 1G12⁺CD4⁺ T cells present in *Cblb*^{+/+} TCR:insHel mice made no T cell-proliferative response to exogenous HEL Ag in vitro (Fig. 6B, 6C), consistent with their being intrinsically anergic. By contrast, although a comparable frequency of 1G12⁺CD4⁺ T cells were present in *Cblb*^{-/-} TCR:insHel mice, these showed robust proliferation to exogenous HEL protein in vitro (Fig. 6B, 6C). Next, we examined the proliferative response on lymph node cells from wild-type and *Cblb*^{-/-} TCR:insHel mice following depletion of CD4⁺ Tregs by FACS sorting. The Treg-depleted lymph node cells were labeled with CFSE and cultured in the presence of Hel Ag, and proliferation was measured by CFSE dilution by FACS after 72 h. Wild-type T cells remained unresponsive to the Hel Ag in vitro following the removal of Tregs. In contrast, the *Cblb*^{-/-} T cells demonstrated a robust and dose-dependent proliferation to the Hel autoantigen (Fig. 6D). These results indicate that responsiveness of the *Cblb*^{-/-} T cells is independent of the presence of Tregs. We also examined the effector

response of T cells from wild-type and *Cblb*^{-/-} TCR:insHel mice and spleen cells from wild-type and *Cblb*^{-/-} TCR:insHel mice were activated directly ex vivo with PMA and ionomycin, and cytokine production was analyzed by intracellular flow cytometry. Analysis of the CD4⁺1G12⁺ cells from both wild-type and *Cblb*^{-/-} cells revealed no difference in the secretion of IFN- γ , IL-4, or IL-17, suggesting that the islet-reactive T cells in the spleen of *Cblb*^{-/-} TCR:insHel mice are not polarized toward any single Th subset (Supplemental Fig. 1).

Bone marrow chimeras were constructed to examine whether the defect in T cell anergy was a T cell-intrinsic effect. Bone marrow from wild-type or *Cblb*^{-/-} TCR mice was given to irradiated B10.Br insHEL or nontransgenic recipients. Flow cytometric analysis of the thymus and the spleen from nondiabetic chimeras 12 wk after reconstitution confirmed that in general there was no defect in positive or negative selection of CD4⁺1G12⁺ T cells in recipients of *Cblb*^{-/-} marrow (Fig. 7A). Although there was a statistically significant difference in the number of CD4⁺1G12⁺ cells in the thymus and the spleen of the mice receiving *Cblb*^{-/-} marrow in the insHel⁺ recipients compared with those that received *Cblb*^{+/+} marrow, negative selection still remained intact with a 10-fold reduction in CD4⁺1G12⁺ T cells in the insHel⁺ versus nontransgenic recipients (Fig. 7A; $p = 0.002$ and $p = 0.0029$, respectively). When the spleen T cells were assessed for their proliferative responses to exogenous HEL Ag in vitro, the *Cblb*^{-/-} T cells from insHEL⁺ recipients

FIGURE 6. Cbl-b-deficient islet-reactive CD4 T cells retain capacity to proliferate to HEL in vitro. **A**, Proliferation of nontolerant T cells from TCR transgenic mice lacking insHEL. Incorporation of [³H]thymidine into spleen cells from *Cblb*^{+/+} (■) and *Cblb*^{-/-} (○) TCR tg mice was measured after culture for 3 d with the indicated concentrations of HEL Ag. Data show the mean and SD of three mice per group. **B** and **C**, In vitro proliferation of islet-specific T cells from TCR:insHEL double-transgenic mice. **B**, Spleen cells from *Cblb*^{+/+} or *Cblb*^{-/-} TCR:insHel mice were labeled with CFSE, cultured for 3 d with the indicated concentrations of HEL protein, and analyzed flow cytometrically for CFSE. Histograms show the percentage of CD4⁺1G12⁺7AAD⁻ cells that had undergone cell division (i.e., CFSE^{low}) cells. The data shown are from one of three experiments performed. **C**, [³H]Thymidine incorporation of spleen cells from *Cblb*^{+/+} (■) and *Cblb*^{-/-} TCR:insHel mice (○) stimulated in vitro with different concentrations of Hel protein. Data show the response of cells from individual mice. Statistical analysis by Student *t* test showed a significant difference in the response at 1, 3, and 10 μ g/ml HEL. $p = 0.001$; $p < 0.0001$; $p < 0.0001$, respectively. **D**, Graphs shows the frequency of divided CD4⁺1G12⁺ cells in CD4⁺ Treg-depleted spleen cell cultures as measured by CFSE dilution following in vitro stimulation with different doses of Hel Ag. The data show results from two representative experiments for *Cblb*^{-/-} (open symbols) and *Cblb*^{+/+} (filled symbols) spleen cell cultures.



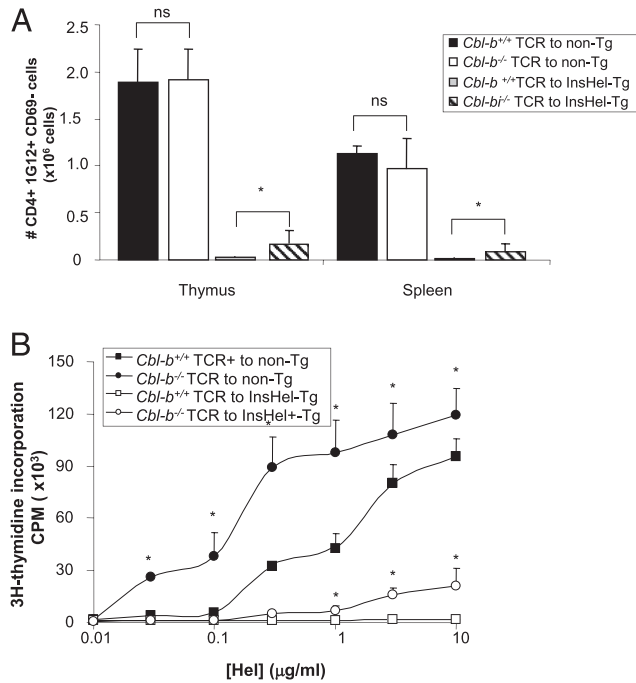


FIGURE 7. Hematopoietic cell-autonomous effect of *Cbl-b* deficiency on islet-reactive CD4⁺ T cells. *A*, Bone marrow chimeras were made by injecting bone marrow from *Cblb*^{+/+} or *Cblb*^{-/-} 3A9 TCR transgenic donors into irradiated B10.BR recipients that either expressed or lacked the *insHEL* transgene. *A*, Number of CD4⁺1G12⁺CD69⁻ T cells in thymus and spleen 12 wk after reconstitution. Data show the mean and SD for each group of *n* = 4 mice. Statistical analysis by Student *t* test showed that there was no difference in the numbers of CD4⁺1G12⁺ cells in the nontransgenic recipients, but there was a significant difference observed between *insHel*⁺ transgenic recipients receiving either *Cblb*^{+/+} bone marrow hematopoietic stem cells compared with *Cblb*^{-/-} bone marrow hematopoietic stem cells *p* = 0.002 and *p* = 0.0029 in thymus and spleen, respectively. *B*, In vitro proliferation of spleen cells from the mice shown in *A* stimulated with the indicated concentrations of Hel protein. Data show the mean and SD of four mice per group. **p* < 0.0001; analysis by Student *t* test showed a statistically significant increase in the proliferative response to 3 or 10 μg/ml HEL in cells from *insHEL*⁺ recipients of *Cblb*^{-/-} marrow compared with *insHEL*⁺ recipients of *Cblb*^{+/+} marrow. **p* < 0.0001; analysis on nontransgenic recipients showed a significant difference in proliferation of all HEL Ag concentrations for mice receiving *Cblb*^{-/-} marrow compared with *Cblb*^{+/+} marrow.

clearly retained responsiveness to Ag, whereas the *Cblb*^{+/+} T cells were completely unresponsive (Fig. 7*B*; *p* < 0.0001). These results confirm that the break in T cell energy in *Cblb*^{-/-} mice is due to a hematopoietic defect.

Induced Treg differentiation is impaired in Cbl-b-deficient CD4+ T cells

Recent studies have identified that *Cblb* mutation decreases Foxp3 induction by TGF-β in activated T cells, reducing the frequency and function of iTregs (36, 43, 44). To test whether the same defect occurs in HEL-specific 3A9 T cells, naive spleen CD4⁺ T cells from wild-type nontransgenic or *Cblb*^{+/+} and *Cblb*^{-/-} TCR mice were cultured in the presence of anti-CD3 and anti-CD28 in the absence or presence of 0.1 and 0.5 ng/ml TGF-β for 5 d to induce iTreg differentiation. In the presence of 0.5 ng/ml TGF-β, CD4⁺ T cells from wild-type nontransgenic and TCR transgenic mice strongly induced Foxp3 expression in 63 and 43% of cells, respectively (Fig. 8*A*). In contrast, the induction of Foxp3 was severely impaired in *Cblb*^{-/-} cells with 8% of cells expressing Foxp3 (Fig. 8*A*). When the suppressive activity of the TGF-β-induced iTregs was tested

against CFSE-labeled naive T cell responders in vitro, cells from both nontransgenic and TCR transgenic *Cbl-b* wild-type cultures decreased cell division by the responder cells, whereas *Cblb*^{-/-} cultures containing few iTregs were less suppressive (Fig. 8*B*). These findings confirm recently published data (36) and raise the possibility that iTreg formation may also be compromised under particular conditions in vivo but remain undetected because of the presence of normal numbers of thymic-derived natural Tregs (nTregs) in the *Cblb*^{-/-} TCR:*insHel* mice.

Discussion

The findings above extend previous evidence from the KDP rat strain that *Cbl-b* is important for regulating islet-specific autoimmunity (20–22) by showing that *Cblb* deficiency predisposes to diabetes and formation of islet autoantibodies in mice (Fig. 1). In the KDP rat strain, autoimmunity caused by *Cblb* deficiency requires additional susceptibility factors, notably a diabetes-susceptible MHC haplotype. Similarly, we find that autoimmunity is only caused by *Cblb* deficiency in mice when combined with a TCR transgene that makes high numbers of islet-reactive CD4 cells in their thymus. The current study provides a mechanistic insight into how *Cbl-b* deficiency predisposes to diabetes, showing that the mutation compromises a different self-tolerance

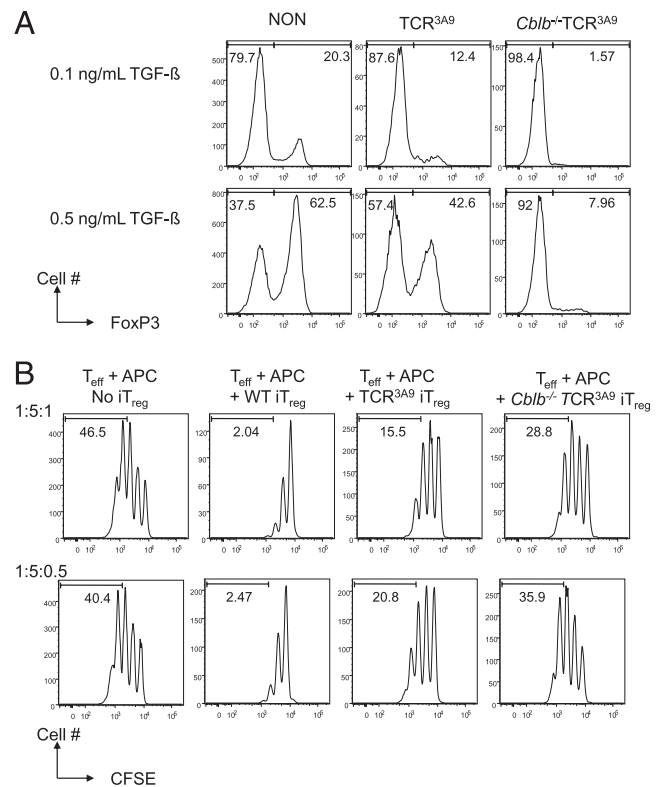


FIGURE 8. *Cblb*-deficient 3A9 T cells are resistant to TGF-β-induced iTreg formation. *A*, Naive CD4⁺CD62L⁺CD25⁻ T cells from nontransgenic or *Cblb*^{+/+} and *Cblb*^{-/-} TCR transgenic mice were stimulated with anti-CD3 and anti-CD28 in the presence of the indicated concentrations of TGF-β. Development of Foxp3⁺ iTregs was assessed by flow cytometry after 5 d. *B*, CFSE-labeled CD4⁺CD62L⁺CD25⁻ naive T cells from *Cblb*^{+/+} TCR mice were used as responder cells in suppression assays, mixed with irradiated T cell-depleted splenocytes as a source of APCs and with the cultured CD4 cells shown in *A*. The cells were mixed in the two indicated ratios (CFSE-labeled responder cells:APCs:TGF-β-treated CD4 cells). Anti-CD3 Ab was added to the cultures and CFSE dilution was assessed by flow cytometry 3 d later.

checkpoint from other diabetes susceptibility factors. Unlike diabetes susceptibility of NOD mice or Aire-deficient mice (3, 4, 17, 18), Cbl-b deficiency has no effect on the efficiency of thymic deletion of islet-specific CD4 T cells (Fig. 2). In contrast to mice lacking IL-2 (12), Cbl-b deficiency has no effect on thymic differentiation or peripheral numbers of islet-specific Foxp3⁺ CD4 cells (Fig. 3), although it does impair TGF- β -induced iTreg formation from mature naive T cells (Fig. 8). Instead, Cbl-b deficiency causes a selective lesion in the maintenance of proliferative unresponsiveness among islet-reactive T cells that have escaped thymic selection, providing direct evidence that T cell anergy occurs in vivo as one of the mechanisms for ensuring organ-specific self-tolerance. This selective defect in anergy is, nevertheless, masked in vivo through other *Cblb*-independent tolerance mechanisms for inhibiting the activation of islet-specific T cells.

The demonstration that Cbl-b is required for clonal anergy in islet-specific CD4 cells provides direct experimental support for the view that T cell clonal anergy occurs during physiological acquisition of self-tolerance. T cell anergy has been extensively characterized as a state that can be induced in previously activated T lymphoblasts or cloned T cell lines in tissue culture, where it is induced by TCR stimulation in the absence of CD28 costimulation or by pharmacological surrogates, such as prolonged exposure to ionomycin (37, 39). Under these conditions, *Cblb* mRNA and protein are strongly induced, and the cells become unresponsive to TCR restimulation as measured by proliferation in a manner that is abolished in *Cblb*-deficient T cells (37). Ionomycin-energized T lymphoblasts retain the ability to form TCR synapses on peptide-MHC/ICAM1-bearing lipid bilayers but compared with control T cells these synapses dissolve more rapidly over a period of 35–60 min in a process that is partly compromised in *Cblb*-deficient T cells (37). The role of Cbl-b in T cell clonal anergy has also been examined in vivo by treating TCR transgenic mice with repeated high doses of exogenous Ag (23). This treatment initially triggers proliferation of initially naive T cells, but after a wave of apoptosis, the residual cells remaining after 4–10 d have diminished capacity to proliferate or make cytokines upon restimulation in vitro or after adoptive transfer and challenge with exogenous Ag and adjuvant, unless the T cells are *Cblb* deficient (23). Similarly, during LCMV virus infection, naive T cells are initially activated to proliferate and produce cytokines, but during chronic infection following a high dose of virus, the remaining T cells become “exhausted” and no longer able to produce cytokines over the course of several weeks unless they are *Cblb* deficient (32). Collectively, these studies firmly establish a role for Cbl-b in the desensitization of previously activated T cells during the response to exogenous Ag but provide no information about the role of Cbl-b or T cell anergy in self-tolerance.

One of the two initial studies of *Cblb*-deficient mice concluded this mutation was sufficient on its own to cause a systemic autoantibody syndrome (29), but it is likely those findings were confounded by the mixed 129/Sv \times B6 strain background of these animals, which is sufficient on its own to cause the observed syndrome of systemic autoantibodies (45). We do not detect any significant increase in formation of antinuclear autoantibodies or lymphadenopathy in mice with the same *Cblb*-targeted mutation (29) backcrossed to the C57BL/6 background (data not shown), nor is there any spontaneous Ab formation to islet HELs despite the H2^k background being a high responder strain to HEL Ag (Fig. 1). In our studies, although Cbl-b deficiency caused a defect in islet-specific T cell anergy that could be measured upon stimulation with exogenous Ag in vitro, this defect is not manifest by the majority of islet-specific T cells in vivo because the *Cblb*-deficient T cells bearing the HEL-reactive TCRs were not en-

larged nor was there an increased percentage in cell cycle (Fig. 4). Also the *Cblb*-deficient islet-reactive CD4⁺ T cells are not polarized toward any Th subset but can secrete Th1, Th1, and Th17 cytokines.

A number of *Cblb*-independent tolerance processes could account for the maintenance of self-tolerance in the islet-reactive T cells in vivo. First, other ubiquitin ligases that are induced in islet-reactive CD4 cells could partially desensitize TCR signaling and synapse formation, as has been observed during ionomycin treatment of *Cblb*-deficient T cells (37). This residual anergy may prevent proliferation in response to the concentrations of HEL peptide presented in the pancreatic lymph node but be overwhelmed when higher concentrations of exogenous HEL peptide are presented in vitro. Second, *Cblb*-independent processes of anergy restraining islet-reactive T cells in vivo may be overcome in vitro because Ag is presented by activated dendritic cells in culture. Third, there may be active suppressive mechanisms at work in the pancreatic lymph node that do not function as efficiently in dispersed cultures of lymphocytes, such as Foxp3⁺ Tregs or local secretion of TGF- β . Given the poor differentiation of iTregs from activated *Cblb*^{-/-} TCR CD4 cells, but normal frequencies of Foxp3⁺CD4⁺ nTregs in vivo, the intrinsic defect in clonal anergy may be masked by the action of nTregs in vivo.

The findings in this study highlight that mutations in Cbl-b that compromise T cell anergy to islet-derived Ag are insufficient to trigger organ-specific autoimmune disease because of the presence of other self-tolerance checkpoints that prevent autoimmunity (46). The effects of *Cblb* deficiency are only manifest as autoimmunity when peripheral tolerance is stressed by increasing the frequency of autoreactive T cells through elevated thymic production in the TCR insHEL transgenic mice studied in this study. Similarly, the autoimmune effects of *Cblb* mutation in rats are only manifest as insulinitis, diabetes, and thyroiditis when combined with a diabetes-susceptible MHC haplotype (20–22) that, speculatively, may diminish thymic negative selection of islet-specific CD4 cells (47). Even with these combined defects, diabetes only develops after a variable latent phase. Rare islet-specific T cells may be activated to overcome other tolerance mechanisms in *Cblb*^{-/-} TCR insHEL in a manner analogous to that observed in this study in vitro, for example, triggered by intermittent increases in Ag release from pancreatic islets or transient activation of dendritic cells that could combine with a decreased functional capacity of iTregs. Thus, collectively, these factors could act to precipitate spontaneous organ specific autoimmunity.

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Disclosures

The authors have no financial conflicts of interest.

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