Visualizing the role of Cbl-b in control of islet-reactive CD4 T cells and susceptibility to Type 1 Diabetes

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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 autoggressive 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, Cbl-b-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 Cbl-b-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 implicate defects in specific T cell self-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 Komeda 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+ T 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 parologue, 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 degradation (24). 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...
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 Aire-dependent thymic deletion and IL-2–dependent 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 T 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 (Cunberra, 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 × 10⁶ 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): CD3ε–FITC, CD4–PerCP, CD4–allophycocyanin, CD4–FITC, CD5–PE, CD8–FITC, CD6–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–biotin, CD25–allophycocyanin, CD69–FITC, CD69–biotin, and CD90–PE (BD Pharmingen). Secondary Abs and streptavidin (SA) conjugates were used 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 FACSVantage (BD Biosciences).

**CFSE labeling**

Cells were suspended at 5 × 10⁶/ml in warm RPMI 1640 medium and incubated for 10 min at 37°C with 5 μM 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/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 shaking a whole spleen through a 70-μm nylon cell strainer in 5 ml R10 media (RPMI 1640 medium, 10% FCS, 3% penicillin/streptomycin/1-γ-glutamine, 0.1 μM/ml 2-ME, 1% MEM, 1% HEPES, and 1% sodium pyruvate), and the cells were centrifuged (7 min, 1500 rpm, 4°C) and resuspended in 5 ml R10 culture medium. The cell density was adjusted to 2 × 10⁶ cells/ml, and 200 μl 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. Splenocytes 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. Ninety-six-well microtiter plates (Nunc Maxisorp; eBioscience) were coated with 5 μg/ml anti-CD3, and 10⁶ CFSE-labeled naive T cells plus 10⁵ iTregs plus 5 × 10⁶ 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 µl of 100 µg/ml HEL in carbonate buffer overnight at 4°C. Plates were washed twice with PBS 2% FCS and blocked with 100 µl PBS and 50% FCS for 2 h at room temperature. Plates were washed twice 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 doses of 5 Gy gamma irradiation (Cs source; CSIRO, Canberra, Australian Capital Territory, Australia) given 3 h apart. The recipient animals were given doses of 5 Gy gamma irradiation (Cs source; CSIRO, Canberra, Australian Capital Territory, Australia) given 3 h apart.

**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.

**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 × 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 mouse 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 insulitis, but none develop anti-HEL IgG autoantibodies (Fig. 1B) and <25% develop type 1 diabetes (Fig. 1A). 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
80% of the double-transgenic Cblb<sup>−/−</sup> 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<sup>−/−</sup> 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 autoimmunity, 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<sup>+</sup> 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<sup>+</sup> thymocytes into the CD4<sup>+</sup> T cell lineage occurred equally in the presence or absence of Cbl-b, and there was no difference in the number of mature CD4<sup>+</sup>1G12<sup>+</sup>CD69<sup>+</sup> T cells produced in thymus of wild-type or Cblb<sup>+/−</sup> 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 observed in the surface expression of TCR or CD3 on Cblb<sup>−/−</sup> thymic T cells compared with wild-type TCR transgenic cells (Fig. 2B and data not shown).

Negative selection of islet-specific CD4<sup>+</sup> 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<sup>+</sup> T cells during the single-positive stage of development (Fig. 2). Analysis of negative selection in the thymus of wild-type and Cblb<sup>−/−</sup> TCR:insHEL mice by flow cytometry revealed no difference between the deletion of Hel-specific CD4<sup>+</sup>1G12<sup>+</sup> T cells, because there were equivalently low numbers of mature CD4<sup>+</sup>1G12<sup>+</sup>CD69<sup>+</sup> T 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<sup>+</sup>1G12<sup>+</sup> T cells between wild-type and Cblb<sup>−/−</sup> TCR:insHEL mice (Fig. 3A, 3B).

We next examined the possibility that diabetes in Cblb<sup>−/−</sup> TCR:insHEL mice could reflect absence of thymic or peripheral 1G12<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> 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<sup>−/−</sup> and Cblb<sup>−/−</sup> 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<sup>+</sup> 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<sup>+</sup> T cell frequency and subsets in mice lacking Cbl-b. A, Representative flow cytometric profiles showing the percentage of Hel-specific CD4<sup>+</sup>1G12<sup>+</sup> cells in the spleen of TCR and TCR:insHEL mice on a wild-type and Cblb<sup>−/−</sup> background. B, The number of CD4<sup>+</sup>1G12<sup>+</sup> cells in the spleen of wild-type and Cblb<sup>−/−</sup> TCR (n = 9/group) and TCR:insHEL (n = 9/group). There was no significant difference between Cblb<sup>−/−</sup> and Cblb<sup>+/+</sup> controls by Student t test. C, Representative flow cytometric profiles of spleen cells from mice of the indicated genotypes, gated on CD4<sup>+</sup>1G12<sup>+</sup> and showing the percentage of gated cells expressing Foxp3. D, The number of spleen CD4<sup>+</sup>1G12<sup>+</sup> cells expressing Foxp3 in individual mice of the indicated genotypes. There was no significant difference between Cblb<sup>−/−</sup> and Cblb<sup>+/+</sup> controls by Student t test. TCR Cblb<sup>−/−</sup> versus Cblb<sup>+/+</sup>, p = 0.7363; TCR:insHEL Cblb<sup>−/−</sup> versus Cblb<sup>+/+</sup> mice, p = 0.9496.
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). Furthermore, 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-
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 isolet-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...
occurs in HEL-specific 3A9 T cells, naive spleen CD4+ T cells from function of iTregs (36, 43, 44). To test whether the same defect four mice per group. * indicated concentrations of Hel protein. Data show the mean and SD of confirming that the break in T cell anergy in induction by TGF- of all HEL Ag concentrations for mice receiving cultured in the presence of 0.5 ng/ml TGF- or presence of 0.1 and 0.5 ng/ml TGF- from wild-type nontransgenic or CD4+ T 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 clearly retained responsiveness to Ag, whereas the Cblb+/− T cells were completely unresponsive (Fig. 7B; p < 0.0001). These results confirm that the break in T cell anergy 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 transgenic 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. 8A). In contrast, the induction of Foxp3 was severely impaired in Cblb−/− cells with 8% of cells expressing Foxp3 (Fig. 8A). 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. 8B). 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
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 Cblb 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 × 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 H2b 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 enlarged 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 insulitis, 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
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