T-cell regulation by *casitas B-lineage lymphoma* (*Cblb*) is a critical failsafe against autoimmune disease due to *autoimmune regulator* (*Aire*) deficiency

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Autoimmune polyendocrinopathy syndrome type 1 (APS1) results from homozygous *Aire* mutations that cripple thymic deletion of organ-specific T cells. The clinical course in man and mouse is characterized by high variability both in the latent period before onset of autoimmune disease and in the specific organs affected, but the reasons for this are unknown. Here we test the hypothesis that the latent period reflects the failsafe action of discrete postthymic mechanisms for imposing self-tolerance in peripheral T cells. *Aire*-deficient mice were crossed with mice of a uniform major histocompatibility complex (MHC) haplotype and genetic background carrying specific genetic defects in one of four distinct peripheral tolerance mechanisms: activation-induced cell death (*Fasl*$^{gld/gld}$), anergy and requirement for CD28 costimulation (*Cblb*$^{-/-}$), inhibition of ICOS and T_{FH} cells (*Rc3h1*$^{san/san}$), or decreased numbers of Foxp3^{+} T regulatory cells (*Card11*$^{unm/unm}$). *Cblb*-deficiency was unique among these four in precipitating rapid clinical autoimmune disease when combined with *Aire*-deficiency, resulting in autoimmune exocrine pancreatitis with median age of survival of only 25 d. Massive lymphocytic infiltration selectively destroyed most of the exocrine acinar cells of the pancreas and submandibular salivary gland, and CD4^{+} and CD8^{+} subsets were necessary and sufficient to transfer the disease. Intrinsic regulation of peripheral T cells by CBL-B thus serves a uniquely critical role as a failsafe against clinical onset of autoimmune disease in AIRE deficiency, and multiple peripheral tolerance mechanisms may need to fail before onset of clinical autoimmunity to many organs.

autoimmunity | tolerance | anergy | pancreatitis | ubiquitin ligase

Autoimmune diseases affect 5% of people but are extraordinarily heterogeneous in their timing of onset, clinical presentation, and target organs. There is a strong inherited predisposition to most autoimmune diseases, with 30–70% concordance in identical twins (1), due in most cases to cumulative effects of variants in mostly unknown genes including the MHC (2–4). It is not known if these complex genetic factors disrupt a single tolerance mechanism, compromise several cooperating mechanisms, or act at other levels such as inflammatory responses to microbes. Many different cellular mechanisms of actively acquired self-tolerance have been described, including clonal deletion, clonal anergy, and regulatory T-cell differentiation, but it is not known if these mechanisms act in series as failsafes for one another, such that autoimmune disease would require multiple mechanisms to fail, (5) or if they act independently to protect different tissues from autoimmunity. It is currently unknown whether the compounding of inherited defects in discrete tolerance mechanisms will precipitate autoimmune phenotypes that are simply the sum of each individual defect or will exhibit highly cooperative behavior to produce emergent autoimmune phenotypes. Understanding how the individual mechanisms fit together for robust tolerance is critical for interpreting patterns of genetic and phenotypic variability in human autoimmune disease.

The first self-tolerance mechanism to be conceived (6) and experimentally confirmed in vivo (7) is clonal deletion of self-reactive T lymphocytes during their exposure to self-antigens at an immature stage of development within the thymus. Failure of this process explains the monogenic autoimmune polyendocrinopathy syndrome 1 (APS1) that develops as a result of mutations in the *Autoimmune Regulator* (*Aire*) gene (8–11). Despite complete loss of *Aire* function in most cases, clinical autoimmunity in AIRE-deficient humans and mice nevertheless unfolds only following a latent phase of years in man or months in mice and is highly variable in its onset, severity, and range of organs targeted (8, 12–16). Genetic differences in the region that includes the MHC and other genetic loci modify the pattern of autoimmunity in *Aire* deficiency (12–14, 17), but neither the genes nor the nature of the tolerance mechanisms altered by these genetic associations are known. To understand the clinical variability in APS1 and in more common autoimmune diseases, it is important to define how specific deficits in different tolerance mechanisms cooperate, but there has been very little experimental investigation of this issue to date.

One hypothesis to explain the long latent period and absence of autoimmunity against many organs in *Aire*-deficient individuals is that specific peripheral tolerance mechanisms exist that do not require AIRE and serve as failsafes to compensate for the failure of thymic deletion. A prediction of this hypothesis is that combination of AIRE deficiency with different defined molecular lesions in peripheral tolerance would dramatically accelerate autoimmunity and lead to emergent autoimmune disease not seen with either individual defect. By contrast, no acceleration of autoimmunity and an additive combination of the individual autoimmune phenotypes would result from compounding Aire deficiency with a mechanism that does not serve as a failsafe. Here we have directly tested the consequences of combining defective thymic deletion due to AIRE deficiency with four different molecularly defined defects that disrupt specific peripheral tolerance mechanisms. Consistent with the hypothesis that specific peripheral tolerance mechanisms serve as critical failsafes for defects in AIRE, absence of CBL-B cooperated with AIRE deficiency to produce an emergent, lethal pancreatic autoimmune disease within several weeks of life. By contrast, mutations in three other peripheral tolerance mechanisms showed no such cooperation, despite two having more severe autoimmune phenotypes on their own. These results provide cellular and molecular insights into the clinical variability of APS1 and other autoimmune diseases, establish that

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genetically distinct thymic and peripheral tolerance mechanisms do serve as failsafes for one another, and raise important questions about the need for additional tolerance defects before clinical autoimmunity can develop against many organs.

Results

***Aire*:*Cblb* Double-Deficient Mice Have a Gravely Shortened Lifespan and Emaciation.** To test the hypothesis that the delayed and variable onset of autoimmunity in *Aire* deficiency results from compensation by discrete peripheral tolerance mechanisms, *Aire* deficient mice bearing a truncating mutation similar to the major Finnish APS1 mutation (16) were intercrossed on a uniform genetic background that is not prone to autoimmunity (B10.BR/SgSnJ; $H2^k$) with mice carrying molecularly defined genetic defects disrupting one of four distinct peripheral T-cell tolerance mechanisms: (*i*) The $Fasl^{gld/gld}$ point mutation in the ligand for FAS/CD95 disrupts activation-induced cell death of mature T cells (18) to cause extensive T-cell lymphoproliferation and antinuclear autoantibodies on the B10.BR background; (*ii*) the $Cblb^{-/-}$ knockout mutation in the CBL-B ubiquitin ligase disrupts mature T-cell anergy and releases T-cell proliferation from the need for CD28 costimulation (19–27) but is insufficient to cause any detectable accumulation of activated T cells, autoimmunity, or autoantibodies on the B10.BR background; (*iii*) $Rc3h1^{san/san}$ point mutation in the ROQUIN ubiquitin ligase that causes accumulation of activated T cells and lupus-like autoimmunity in B10.BR mice due to elevated expression of the costimulatory ICOS receptor and excessive formation of T follicular helper cells (28, 29); and (*iv*) $Card11^{unm/unm}$ point mutation in the coiled-coiled domain of CARD11 (also called CARMA-1) (30) that decreases TCR-CD28 signaling and decreases $FoxP3^+$ regulatory T cells to one sixth of normal but allows T-cell activation and results in T_h2-biased inflammatory disease.

In each of the *Aire* by peripheral tolerance defect crosses, offspring with the different genotypic combinations were produced in the expected Mendelian ratios. Three of the four combinations, $Aire^{-/-}Fasl^{gld/gld}$, $Aire^{-/-}Rc3h1^{san/san}$, and $Aire^{-/-}Card11^{unm/unm}$ displayed normal survival rates, remained overtly healthy up to 140 d (Fig. 1 *A–C*), and showed no clinical signs of accelerated autoimmune disease compared with control mice with individual mutations. $Aire^{-/-}Fasl^{gld/gld}$ mice exhibited comparable or slightly reduced lymphoproliferation, accumulation of activated T cells, and antinuclear autoantibodies to $Aire^{+/+}Fasl^{gld/gld}$ counterparts, consistent with little or no interaction between the individual autoimmune defects. By contrast, most $Aire^{-/-}Cblb^{-/-}$ mice either died or needed to be euthanased due to emaciation with median survival of 25 d (Fig. 1*D*). Mice with single deficiencies in either *Aire* or *Cblb* remained healthy for at least 140 d. $Aire^{-/-}Cblb^{-/-}$

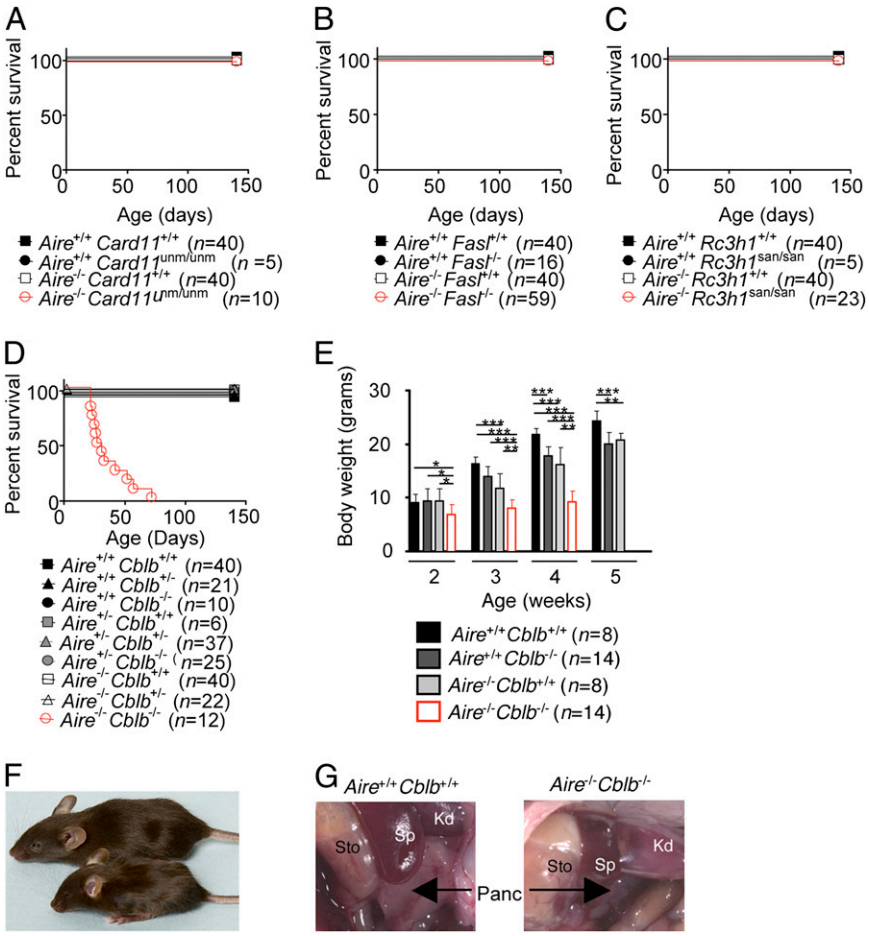

Fig. 1. Effects of combined defects in central and peripheral tolerance mechanisms. Survival measured from birth to 20 wk for mice with single and double mutations in: (*A*) *Aire* and *Card11*; (*B*) *Aire* and *Fasl*; (*C*) *Aire* and *Rc3h1*; (*D*) *Aire* and *Cblb*. All mice were on the B10.BR ($H2^k$) genetic background ($n \geq 6$ for each group). (*E*) Body weight at 2, 3, 4, and 5 wk after birth of male and female mice of the following genotypes: $Aire^{+/+}Cblb^{+/+}$ (n = 4 males, 4 females); $Aire^{+/+}Cblb^{-/-}$ (n = 7 males, 7 females) and $Aire^{-/-}Cblb^{+/+}$ (n = 4 males, 4 females) or $Aire^{-/-}Cblb^{-/-}$ (n = 7 males, 7 females). Statistical analysis by One-way ANOVA comparing all groups, followed by pairwise Bonferroni post-test. *, **, *** denote groups where the probability of no difference was $P < 0.05$, $P < 0.01$, or $P < 0.001$, respectively. Error bars represent s.d. (*F* and *G*) Runted appearance and pancreatic atrophy of a representative 24-d female $Aire^{-/-}Cblb^{-/-}$ mouse (*F, Upper; G, Left*) compared with a littermate control (*F, Lower; G, Right*). Sto, stomach; Sp, spleen; *Kd*, kidney; Panc, pancreas.

mice displayed normal appearance after birth but failed to gain weight after 2 wk (Fig. 1*E*) and rapidly developed an emaciated, runted appearance (Fig. 1*F*). Necropsy revealed a thin, translucent pancreas in all *Aire*$^{-/-}$*Cblb*$^{-/-}$ animals (Fig. 1*G*), evidence of emaciation including little or no body fat, involuted thymus, and peripheral lymphoid hypoplasia, but no other abnormalities were detected. The strong epistatic interaction between *Aire* and *Cblb* but not the other defects is surprising because the *Rc3h1*$^{san/san}$ and *Fasl*$^{gld/gld}$ mutations are sufficient on their own to cause autoantibodies and accumulation of abnormal T cells on the B10.BR background, whereas *Cblb*$^{-/-}$ is insufficient to cause these subclinical abnormalities on this background.

Aire:Cblb Double-Deficient Mice Have Lymphocytic Infiltration Destroying Pancreas and Salivary Gland Acinar Tissue. Histopathological survey of *Aire*$^{-/-}$*Cblb*$^{-/-}$ mice revealed extensive pathology restricted to the pancreas and submandibular salivary glands but not observed in mice with single deficiencies of *Aire* or *Cblb* (Fig. 2 *A* and *D* and Fig. S1). Autoimmune pancreatitis and sialoadenitis were observed in all double mutant mice tested, as young as 20 d. In the pancreas, diffuse lymphocytic infiltration of exocrine acinar tissue was observed, either as extensive patches centered on ductal areas or involving most or all of the acinar tissue of the pancreas. This was accompanied by loss of acinar cells, leaving fibrous and adipose tissue interspersed with ducts lined by simple cuboidal epithelium. The islets of Langerhans were usually not infiltrated by lymphocytes and appeared histologically normal, and the mice showed no evidence of hyperglycemia or diabetes. Mice that were *Aire*$^{-/-}$ and *Cblb*$^{+/-}$ developed mild pancreatitis but did not develop a lethal wasting syndrome, whereas the reciprocal *Aire*$^{+/-}$*Cblb*$^{-/-}$ combination had little or no pancreatitis (Fig. S2).

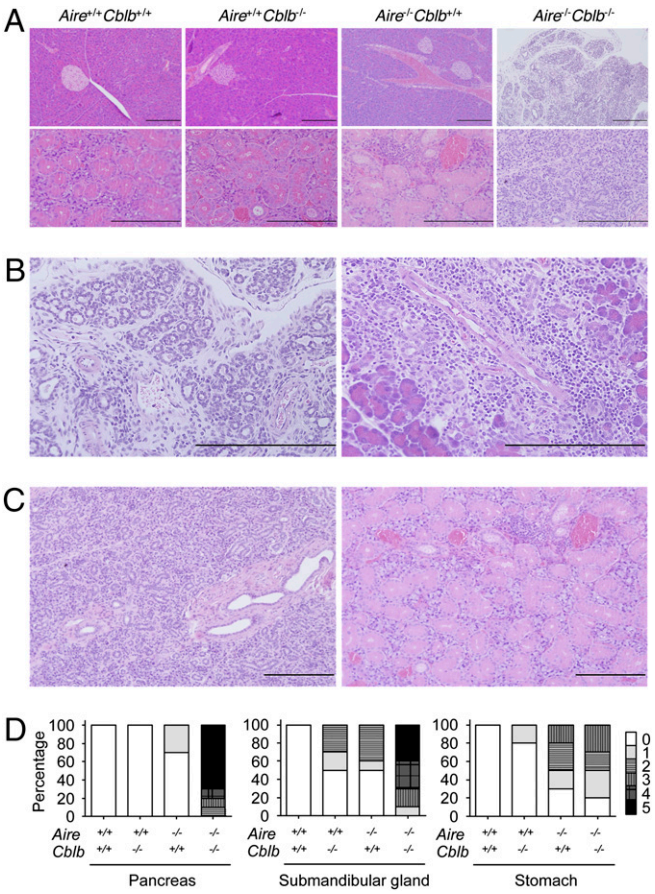

Fig. 2. Lymphocytic exocrine pancreatitis and sialoadenitis in *Aire*$^{-/-}$*Cblb*$^{-/-}$ mice. (*A*) Representative H&E stained sections of pancreas (*Upper*) and submandibular salivary gland (*Lower*) from 3- to 6-wk-old *Aire*$^{-/-}$*Cblb*$^{-/-}$ mice (taken at time of death or euthanasia) and 20-wk-old control counterparts (endpoint of study) of the indicated genotypes. (Scale bars: 200 μm.) (n = 10 for each group). (*B*) Higher power images of pancreas from *Aire*$^{-/-}$*Cblb*$^{-/-}$ mice. (*Left*) Grade 5 pancreatitis; (Right) grade 3 pancreatitis. (Scale bars: 200 μm.) (*C*) Submandibular salivary gland from *Aire*$^{-/-}$*Cblb*$^{-/-}$ mice. (*Left*) Grade 5 sialoadenitis; (*Right*) grade 1 sialoadenitis. (Scale bars: 200 μm.) (*D*) Percentage of mice of the indicated genotypes with different grades of pancreatitis, sialoadenitis, and gastritis (n = 10 for each group, all age 20 wk except *Aire*$^{-/-}$*Cblb*$^{-/-}$ mice 3–6 wk old). Pathology was scored blinded according to the scale shown in Fig. S1.

In the submandibular salivary gland, diffuse lymphocytic infiltration in *Aire*$^{-/-}$*Cblb*$^{-/-}$ double mutant mice was accompanied by partial or complete loss of acinar cells, leaving simple cuboidal epithelium-lined ducts (Fig. 2 *C* and *D* and Fig. S1). Other organs remained free of inflammatory infiltrates, including the parotid and sublingual salivary glands, eye, thyroid, lung, heart, liver, kidney, and testis or ovary. Only the stomach displayed moderate lymphocyte infiltration in *Aire*$^{-/-}$*Cblb*$^{-/-}$ animals, although it was no greater than in B10.BR mice with *Aire* deficiency alone (Fig. 2*D*). Malabsorption and exocrine pancreatic insufficiency occurs in a subset of AIRE-deficient patients (15), and this is likely to explain the emaciation of *Aire*$^{-/-}$*Cblb*$^{-/-}$ animals.

Autoantibodies reacting with pancreas and salivary gland were detected in the sera of *Aire*$^{-/-}$*Cblb*$^{-/-}$ mice and not in single mutant controls when tested against frozen cryosections of *Rag1*-deficient mice (Fig. S3*A*). The autoantibodies reacted with exocrine acinar cells in pancreas and not with the islets of Langerhans, thus correlating with the specificity of inflammatory destruction seen in histology. Autoantibody staining was most intense in the apical, zymogen granule-rich cytoplasm of the pancreatic acinar cells (Fig. S3*B*), resembling the pattern observed in *Aire*$^{-/-}$ NOD mice (12, 14).

Hematopoietic *Cblb* Deficiency Combines with Nonhematopoietic *Aire* Deficiency to Cause Rapid Onset Disease. The selective exocrine pancreas destruction precipitated by the combination of *Cblb* and *Aire* deficiency could have reflected a function of *Cblb* in the pancreas itself, as opposed to its established function in peripheral T cell regulation. To distinguish between these possibilities, bone marrow chimeras were generated by irradiating wild-type or *Aire*$^{-/-}$ mice and then reconstituting their hemopoietic system with bone marrow from either wild-type, *Cblb*$^{-/-}$, or a combination of wild-type and *Cblb*$^{-/-}$ donor cells (1:1 ratio). Thus the pancreas and other radioresistant tissues would always have a normal *Cblb* gene, whereas the T cells would either be normal, lack *Cblb*, or comprise a mixture of mutant and wild-type T cells (confirmed flow cytometrically). Within 6 wk after marrow transplantation, the majority of *Aire*$^{-/-}$ recipients of *Cblb*$^{-/-}$ marrow developed an emaciated appearance and either died or were euthanased (Table 1). Pancreas histology of *Aire*$^{-/-}$ recipients of *Cblb*$^{-/-}$ marrow captured shortly after death showed the same extensive inflammatory destruction of exocrine tissue seen in *Aire*$^{-/-}$*Cblb*$^{-/-}$ double mutant mice, whereas pancreatitis was absent or minimal in chimeric mice where one or both genes were wild-type. *Aire*$^{-/-}$ recipients receiving the mixed wild-type:*Cblb*$^{-/-}$ marrow also developed the wasting disease and exocrine pancreatitis, establishing that the *Aire*$^{-/-}$*Cblb*$^{-/-}$ tolerance defect was not corrected by the presence of wild-type T cells.

Both CD4 and CD8 T Cells Transfer Disease. Flow cytometric analysis of *Aire*$^{-/-}$*Cblb*$^{-/-}$ mice and control littermates showed an increased frequency of T cells from total lymphocytes measured in the blood and spleen compared with control littermates. The CD4:CD8 ratio remained comparable to wild-type counterparts (Fig. 3*A*). However, there was an increased proportion of CD44^{high} activated/memory T cells (Fig. 3*B*).

Table 1. Results of transplanting *Cblb*$^{-/-}$ or wild-type bone marrow, or an equal mixture of the two, into *Aire*-deficient or wild-type recipients

Recipients	*Aire*$^{-/-}$			*Aire*$^{+/+}$		
Donors	*Cblb*$^{-/-}$	*Cblb*$^{+/+}$	*Cblb*$^{-/-}$:*Cblb*$^{+/+}$	*Cblb*$^{-/-}$	*Cblb*$^{+/+}$	*Cblb*$^{-/-}$:*Cblb*$^{+/+}$
Experiment 1	2/4	n.d.	2/4	0/3	0/3	n.d.
Experiment 2	5/5	0/5	2/5	1/5	0/5	0/5
Experiment 3	6/10	0/5	n.d.	0/5	0/10	n.d.
Total	13/19	0/10	4/9	1/13	0/18	0/5

In the mixed chimeras the wild-type marrow carried a CD45.1 allelic marker, enabling flow cytometric analysis of thymi to confirm equal reconstitution of T lymphopoiesis, including Foxp3^{+} CD4 cells, by mutant and wild-type cells. Numerator, number of mice that died spontaneously or were euthanized when moribund; denominator, total number of mice tested. n.d., not done.

To test if the pancreatic disease was autoimmune, splenocytes from either wild-type or *Aire*$^{-/-}$*Cblb*$^{-/-}$ mice were adoptively transferred into *Rag1*-deficient mice. All mice receiving *Aire*$^{-/-}$*Cblb*$^{-/-}$ spleen cells became emaciated and moribund by 30 d following adoptive transfer and had to be euthanased, whereas all mice receiving wild-type splenocytes remained healthy (Fig. 3*C*). At necropsy, the only gross pathology detected was a small translucent pancreas in recipients of *Aire*$^{-/-}$*Cblb*$^{-/-}$ spleen cells, and histology demonstrated extensive lymphocytic infiltration of the exocrine pancreas and loss of most or all acinar cells (Fig. 3*D*). Flow cytometric analysis of peripheral blood lymphocytes in the recipients 12 d posttransfer revealed a marked overrepresentation of CD8 T cells in recipients of *Aire*$^{-/-}$*Cblb*$^{-/-}$ spleen cells (Fig. 3*E*).

To test the requirement for T cells in adoptively transferring pancreatitis, we MACS-depleted or -enriched T-cell subsets from the bone marrow chimeras that had developed pancreatitis (*Aire*-deficient recipients of *Cblb*-deficient marrow) and from control chimeras with wild-type *Aire* in the radioresistant tissues and wild-type *Cblb* in the hemopoietic system. The bone marrow chimeras were used as donors because insufficient lymphocytes could be isolated from small, emaciated *Aire*$^{-/-}$*Cblb*$^{-/-}$ 3-wk-old mice. Groups of *Rag1*-deficient mice received either whole splenocytes, splenocytes selectively depleted of either CD4 or CD8 T cells, or the corresponding number of CD4 or CD8 T cells selectively enriched from the starting splenocyte populations. To ensure removal of residual CD8 or CD4 cells in the T cell-depleted or

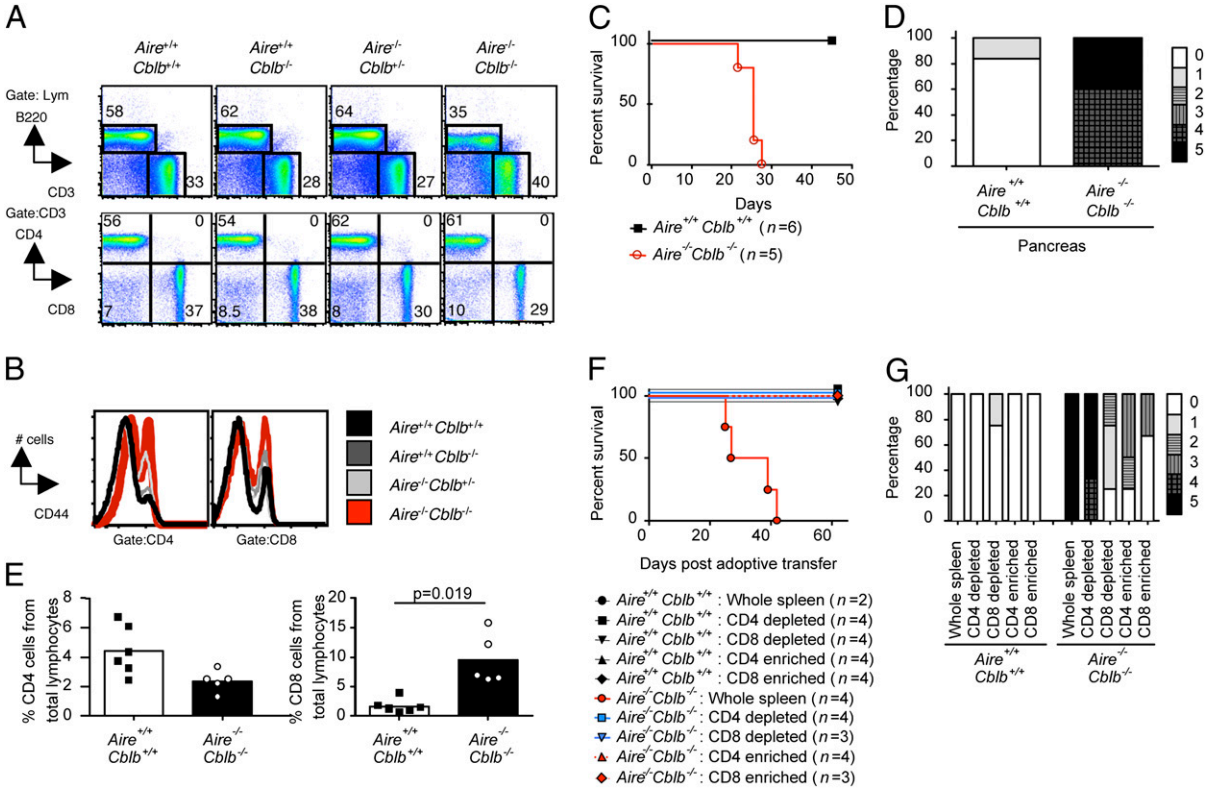

Fig. 3. Transfer of pancreatitis by T lymphocytes from *Aire*$^{-/-}$*Cblb*$^{-/-}$ mice. (*A* and *B*) Flow cytometric analysis of splenocytes from littermate 29 d mice of the indicated genotypes. Data are representative of three independent experiments analyzing whole litters. (*C*) Survival of *Rag1*-deficient B10.BR mice after adoptive transfer of 1–9 × 10^6 splenocytes from *Aire*$^{-/-}$*Cblb*$^{-/-}$ or wild-type mice. (*D*) Percentage of recipient mice in (*C*) with different grades of pancreatitis. (*E*) Percentage of CD4^{+} or CD8^{+} T cells in blood lymphocytes of mice in (*C*) 12 d posttransfer. Data in (*C*–*E*) are representative of two independent experiments ($n \geq 4$ for each group). (*F*) Survival of *Rag1*-deficient B10.BR mice after adoptive transfer of 2 × 10^6 unfractionated spleen cells (containing 2 × 10^5 CD4 or 1 × 10^5 CD8 cells), 2 × 10^6 spleen cells selectively depleted of CD4- or CD8-cells, or 2 × 10^5 CD4- or 1 × 10^5 CD8-enriched spleen cells, obtained from bone marrow chimeras of the indicated genotypes. Recipients of CD4-depleted or CD8-enriched cells, and recipients of CD8-depleted or CD4-enriched cells, were also treated with depleting antibody to CD4 or CD8, respectively, on days 0, 1, 7, and 14 to deplete any residual T cells of the relevant subset. (*G*) Percentage of recipient mice in *F* with different grades of exocrine pancreatitis.

-enriched inoculi, recipients were treated with depleting antibodies to CD8 or CD4 on days 0, 1, 7, and 14 following adoptive transfer. On day 17, the mice were bled and analyzed by flow cytometry, confirming that no residual CD4 T cells were detectable in recipients of CD4-depleted or CD8-enriched cells and that no residual CD8 T cells were detectable in recipients of CD8-depleted or CD4-enriched cells. All *Rag1*-deficient mice that received whole splenocytes from chimeric $Aire^{-/-}$ recipients of $Cblb^{-/-}$ marrow (abbreviated as $Aire^{-/-}Cblb^{-/-}$) died or were put down due to emaciation by 42 d posttransfer (Fig. 3*F*) and had extensive destruction of pancreatic acinar cells (Fig. 3*G*). By contrast, the wasting syndrome did not develop in recipients of CD4- or CD8-depleted splenocytes from $Aire^{-/-}Cblb^{-/-}$ donors nor in recipients of enriched CD4 or CD8 cells from these donors. Intermediate levels of pancreatic acinar destruction were nevertheless induced by the enriched T-cell subsets and by spleen cells depleted of one or other T-cell subset (Fig. 3*G*). Thus, both CD4 and CD8 T cells are needed for efficient transfer of autoimmune pancreatic destruction from $Aire^{-/-}Cblb^{-/-}$ mice.

Discussion

The findings above establish that variation in the latent period and organ specificity of autoimmune disease in *Aire* deficiency reflects the activity of a fail-safe tolerance mechanism mediated by the ubiquitin ligase CBL-B. Single deficiencies in *Aire* and *Cblb* resulted in no clinical autoimmune disease in the animals studied here on a genetic background not particularly prone to autoimmunity, and result in variable autoimmune disease with a long latent phase when studied on other genetic backgrounds (8, 16, 19, 20, 31). When both tolerance mechanisms were crippled, an emergent phenotype of complete autoimmune destruction of the exocrine pancreas occurred within weeks after T cells began emigrating from the thymus. The cooperation between *Aire* and *Cblb* was unique: although the $Fasl^{gld}$ and $Rc3h1^{san}$ mutations gave rise to more fully penetrant autoimmunity on their own, when compounded with $Aire^{-/-}$ they yielded autoimmune phenotypes that appear simply to be the sum of each individual defect. These findings reveal the higher level architecture assembling individual tolerance mechanisms together for robust resistance to autoimmunity.

CBL-B has a well-defined role in the regulation of mature T-cell activation. CBL-B is only expressed at low levels in immature T lymphocytes (23, 24) and consequently *Cblb* deficiency does not alter thymic selection or *Aire*-dependent thymic deletion. CBL-B is up-regulated in mature and anergic T cells, where it inhibits the PI3K and NFκB signaling pathways activated by TCR-CD28 costimulation, and is required for T-cell anergy to prevent proliferation of T cells that have recognized peripheral antigens with insufficient affinity or in the absence of adequate CD28 costimulation (19–27). Therefore, the rapid progression to pancreatic autoimmune disease observed here can be inferred to result from pancreas-specific T cells escaping thymic deletion due to *Aire* deficiency and then escaping anergy and the normal requirement for CD28 costimulation due to *Cblb* deficiency, allowing them to proliferate and differentiate into tissue-damaging effector T cells.

The role of CBL-B has a number of parallels with the inhibitory cell surface receptor, CTLA-4, suggesting they are both part of a single peripheral T-cell tolerance pathway for suppressing pancreatic and salivary gland autoimmunity. CTLA-4 is also induced by sustained antigen exposure on mature and anergic T cells and opposes CD28 costimulation, in part by binding more avidly to the CD28 ligands CD80 and CD86 (32, 33). Moreover, autoimmune exocrine pancreatic and salivary gland destruction are prominent features of the multiorgan inflammation that occurs in CTLA-4-deficient 129/Sv or BALB/c mice (34, 35). CTLA-4 opposes accumulation of pancreas-specific T cells by two routes: intrinsically by its expression on pancreas-specific effector T cells (35) and extrinsically by its expression on $Foxp3^+$ T regulatory cells where it down-regulates CD86 on antigen presenting cells to mediate their suppressive effects (35–37). Because *Cblb* deficiency relieves T cells of their dependence on CD86, it may deregulate pancreas-specific T cells both by preventing T-cell anergy and by making them independent from CD86 down-regulation by T regulatory cells. Experiments in vitro showed that *Cblb*-deficient T effector cells were refractory to growth inhibition by $CD4^+CD25^+$ T regulatory cells (38, 39). However *Cblb* deficiency does not result in the generalized T-cell activation that characterizes CTLA-4- or Foxp3 deficiency, indicating that it does not cause a general disruption of extrinsic regulation by T regulatory cells in vivo.

The cooperation between CBL-B and AIRE for suppressing pancreatic and salivary gland autoimmunity provides the basis for analyzing genetic variants within these pathways in human Sjogren syndrome, Mikulicz disease, and exocrine pancreatitis, which often occur together (40–42), and in other organ-specific autoimmune diseases. The early onset and rapid progression of autoimmune disease indicates that *Cblb*-mediated peripheral T-cell tolerance is the only effective failsafe preventing exocrine pancreatic autoimmune destruction when *Aire*-dependent thymic tolerance fails in B10.BR mice. It is striking that pancreatic endocrine tissues and other organs nevertheless remain unaffected by autoimmunity in the double-deficient animals, because *Aire* deficiency interferes with thymic deletion against hundreds of autoantigens that are highly expressed in different organs (8, 9, 43), and *Cblb* deficiency can predispose to pancreatic islet autoimmune destruction (21, 31, 44). This suggests that particular exocrine autoantigens possess unusual immunogenicity. One of the known targets of autoimmunity in CTLA-4-deficient mice and in *Aire*-deficient NOD mice is the abundant endoplasmic reticulum protein, pancreas-specific protein disulfide isomerase [PDIp (gene symbol *Pdia2*;14, 35)]. Interestingly, a similar pattern of autoantibody reactivity with apical exocrine pancreatic cells to that described in $Aire^{-/-}$ NOD mice (14) was observed in sera from $Aire^{-/-}Cblb^{-/-}$ mice (Fig. S3*B*). Hence, it will be interesting in future studies to determine the nature and immunogenicity of the "driver" autoantigens recognized by CD8 and CD4 T cells in $Aire^{-/-}Cblb^{-/-}$ mice.

The cooperation between the two self-tolerance defects has striking parallels with the cooperative onset of specific cancers without a latent phase in mice with combined activation of an oncogene and inactivation of a failsafe tumor suppressor gene. Like the multistep pathogenesis of different cancers, specific combinations of defects in compensating mechanisms of tolerance may be required to precipitate different autoimmune diseases. These findings provide a framework for understanding the latency and heterogeneity in clinical autoimmune disease and the complexity of its inheritance.

Materials and Methods

Mice. The mice were house in specific pathogen free conditions at the Australian National University Bioscience Facility. The original $Aire^{-/-}$ mice generated in 129/SV embryonic stem cells (16) were backcrossed to the B10.BR/SgSnJ ($H2^k$) genetic background for more than six generations. Heterozygous $Aire^{-/-}$ mice were crossed with heterozygous $Card11^{unm/unm}$, $Cblb^{-/-}$ $Fasl^{gld/gld}$, or $Rc3h1^{san/san}$ mice also on the B10.BR background. Mice of appropriate genotype were intercrossed for two generations to produce double-deficient mice. Experimental mice were age- and sex-matched and confirmed for genotyping by PCR. All animal procedures were approved by the Australian National University Animal Ethics and Experimentation Committee.

Histopathology. All tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Eight-micrometer sections were stained with H&E and scored for immune infiltrates. Slides were coded and scored blindly according to the criteria in Fig. S1.

Immunofluorescence. Frozen cryosections of pancreas, salivary gland, and stomach from $Rag1^{-/-}$ mice were stained with mouse sera diluted 1/30 in PBS. Alexa 488 goat anti-mouse IgG (Invitrogen) was used to detect the

primary tissue-specific antibodies in the mouse serum. Nuclear staining was detected by staining with 0.1 μg/mL DAPI (Invitrogen). Sections were imaged under a confocal microscope (TCS SP5; Leica) and scored by a reviewer blinded to the mouse identity and genotype.

Cell Isolation and Flow Cytometry. Spleens and thymi were harvested from mice. Single cells suspensions were prepared in FACS buffer (PBS supplemented with 10% FCS and 0.01% sodium aziade) by sieving and gentle pipetting through a 70-μm nylon mesh filters (BD Falcon). For surface staining, the single cell suspensions were washed twice in FACS buffer and incubated with each antibody and conjugate layer for 30 min with a single wash in between each layer. A BD LSRII Benchtop Flowcytometer (BD Biosciences) with Diva software (BD Biosciences) was used for acquisition of flow cytometric data, and Flowjo (Treestar, Inc.) was used for analysis.

Antibodies. Antibodies for flow cytometry were from BD Biosciences unless otherwise indicated: anti-mouse CD3 Alexa 700 (clone 145–2C11), anti-mouse CD4 PE Cy7 (clone RM-4.5), anti-mouse CD8 PerCP Cy5.5 (clone 53.6.7), anti-mouse B220-APC Cy7 (clone RA4-6B2), anti-mouse CD25-PE (clone PC61), and anti-mouse CD44 Pacific Blue (clone IM7; BioLegend).

Adoptive Transfer. Single cell spleen suspensions were prepared in RPMI medium 1640 (Gibco) supplemented with 5% FCS. Where $CD4^+$ or $CD8^+$ purification was necessary, cells were labeled using purified using anti-CD4 MACS beads (clone L3T4; Miltenyi Biotec) or anti-CD8 MACS beads (clone 53–6.7; Miltenyi Biotec) and separated using the autoMACS Separator (Miltenyi Biotec) according to manufacturer's instructions. The purity was more than 92% for both CD4 and CD8. Whole splenocytes, CD4-enriched, CD4-depleted, CD8-enriched, and CD8-depleted were transferred i.v. into B10.BR.*Rag-1*$^{-/-}$ recipients. On days 0, 1, 7, and 14 posttransfer, recipients of CD4- or CD8-enriched or CD4- or CD8-depleted cells were treated with 0.3 mg of anti-CD4 (clone GK1.5; Bio X Cell) or anti-CD8 (clone 2.43; Bio X Cell) to deplete any residual $CD4^+$ or $CD8^+$ cells.

Bone Marrow Chimeras. All recipients were sublethally irradiated with two doses of 450 rad and were reconstituted via i.v. injections with 2×10^6 donor bone marrow cells.

Statistical Analysis. Data were analyzed using the One-way ANOVA and Bonferroni posttest to compare pairs of columns using the GraphPad Prism software. Results were considered significant if $P < 0.05$.

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