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The E3 Ubiquitin Ligase Cbl-b Regulates Expansion but Not Functional Activity of Self-Reactive CD4 T Cells

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Cbl-b is an E3 ubiquitin ligase that limits Ag responsiveness in T cells by targeting TCR-inducible signaling molecules. Cbl-b deficiency thus renders T cells hyperresponsive to antigenic stimulation and predisposes individuals toward developing autoimmunity. In part because Cbl-b<sup>-/-</sup> T cells do not require CD28 costimulation to become activated, and insufficient costimulation is a critical parameter that confers anergy induction over effector differentiation, it has been hypothesized that Cbl-b<sup>-/-</sup> T cells are resistant to anergy. This possibility has been supported in models in which anergy is normally induced in vitro, or in vivo following exposure to soluble Ag boluses. In the current study, we characterized the response of Cbl-b<sup>-/-</sup> CD4 T cells in an in vivo system in which anergy is normally induced by a constitutively expressed peripheral self-Ag. Cbl-b expression increased in self-Ag-induced anergic wild-type CD4 T cells, and Cbl-b<sup>-/-</sup> CD4 T cells underwent more robust proliferation and expansion upon initially encountering cognate self-Ag compared with wild-type counterparts. Nevertheless, both wild-type and Cbl-b<sup>-/-</sup> CD4 T cells ultimately developed the same impaired ability to respond to antigenic restimulation. The more extensive expansion that occurred during the initial induction of anergy did, however, allow the anergic CD4 T cells to expand to greater numbers when they were functionally resuscitated following replacement of the initial source of tolerizing self-Ag with a viral form of the same Ag. The Journal of Immunology, 2009, 183: 4975–4983.

A critical aspect of immune function is the ability to respond to rare pathogen-derived Ags while remaining nonresponsive to more abundant self-Ags. For T cells, this is largely accomplished through negative selection in which thymic APC display ubiquitous and blood-borne self-epitopes to induce apoptosis of cognate developing T cells (1–3). The range of negative selection is extended by medullary epithelial cells that express nonlymphoid peripheral self-Ag (4, 5); however, this expression is low and sporadic (6), causing many self-reactive T cells to mature and migrate to the periphery (7, 8). In fact, negative selection is incomplete even for T cells that recognize self-Ags expressed at high levels on all APC subtypes (9). Peripheral T cell tolerance mechanisms are thus essential to prevent autoimmunity (10–12).

In contrast to negative selection that is mediated exclusively through apoptosis, several mechanisms contribute to peripheral tolerance. In some cases, naive self-reactive T cells remain unaware of their cognate self-epitopes and can be primed by pathogens expressing cross-reactive epitopes to mediate autoimmunity (13, 14). This ignorance may result from low-level self-Ag expression (15) or sequestration from lymphatic drainage due to expression in exocrine cells such as prostate epithelia (16).

Most often, peripheral self-Ags induce cognate T cell nonresponsiveness through a combination of dominant (extrinsic) or recessive (intrinsic/cell autonomous) mechanisms. The quintessential dominant mechanism involves several regulatory T cell populations that block autoreactive effector T cell function. The best characterized express CD4, CD25, CTLA-4, and the folate receptor (referred to as T regulatory cells (Tregs)) (17–19), and can either be selected during thymic development by self-epitopes with moderately high TCR affinity/avidity (20) or induced peripherally (21, 22).

Deletion/Apoptosis is the most definitive recessive/intrinsic peripheral tolerance mechanism and can be programmed by members of the TNF/nerve growth factor receptor superfamily such as CD95 (Fas) and other molecules like TNF and DR (23, 24), or through depletion of survival factors like IL-2 (25). Self-reactive T cells can also undergo functional inactivation/anergy in which they become hyporesponsive to antigenic stimulation due to blockages in the TCR signaling apparatus (26, 27). Although deletion and anergy are distinct events, the pathways leading to these outcomes might be linked because anergy and deletion are often observed in the same systems (28, 29). Self-reactive T cells may thus become anergic before undergoing apoptosis, although under certain conditions deletion is incomplete and anergic cells persist (30). Self-reactive T cells in the periphery undergo anergy and deletion because steady-state dendritic cells (DC) cross-presenting cognate parenchymally derived self-epitopes express suboptimal levels of critical costimulatory molecules and cytokines. In contrast, pathogen-reactive T cells expand and acquire effector functions during infection because pathogen-associated molecular patterns induce DC to express optimal levels of cytokines and costimulatory molecules (31–37).

Recent studies have identified several E3 ubiquitin ligases whose expression increases in anergic T cells, and that appear to

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Cbl-b−/− CD4 T CELLS UNDERGO SELF-Ag-INDUCED ANERGY

confer impaired antigenic responsiveness by ubiquitinating specific signaling molecules located downstream of the TCR (38, 39). In particular, Cbl-b can target molecules such as phospholipase C-yl (40) and the p85 regulatory subunit of PI3K (41). In addition, Cbl-b-deficient T cells are hyperresponsive to antigenic stimulation, do not require CD28 costimulation to produce IL-2 and proliferation (42, 43), and are less susceptible to negative regulation by TGF-β and Tregs (44, 45). Furthermore, in animal models, ablation or mutation of Cbl-b predisposes toward the development of autoimmunity (40, 42, 43, 46). Given that anergy normally results from TCR stimulation without sufficient costimulation, the ability of Cbl-b−/− T cells to become activated through TCR stimulation independently of costimulation might confer resistance to anergy and hence explain why Cbl-b−/− individuals develop autoimmunity. Consistently, anergic T cells express increased levels of Cbl-b (40, 47, 48), and Cbl-b−/− T cells have been reported to be resistant to anergy (40, 47). These studies, however, used either in vitro systems or in vivo systems in which anergy is induced by soluble Ag boluses.

To assess whether Cbl-b regulates CD4 T cell anergy induced by constitutively expressed peripheral self-Ags, in the current study we used our previously established model in which TCR transgenic (Tg) CD4 T cells specific for influenza hemagglutinin (HA) are rapidly and profoundly induced to become anergic following adoptive transfer into mice that express HA in a number of parenchymal organs in which steady-state DC present the cognate Ag in a number of parenchymal tissues at either low (self-HAlow (52)) or high (self-HAhigh (49)) amounts, as well as nontransgenic (NT) mice either in -

To begin to assess the role of Cbl-b in this tolerogenic response to self-Ag, expression of Cbl-b mRNA was measured by quantitative real-time RT-PCR in samples prepared from TCR Tg CD4 T cells recovered from self-HAlow, self-HAhigh, and viral-HA adoptive transfer recipients and purified by MACS or cell sorting (described in Fig. 5 of Ref. 54) were analyzed by quantitative SYBR Green-based real-time RT-PCR, as previously described (55). Briefly, after reverse transcribing cDNA, T-bet and Cbl-b mRNAs were quantified by first normalizing for input sample amounts by calculating the difference in threshold cycle (Ct) values relative to hypoxanthine phosphoribosyltransferase, and then using the ΔΔCt method to calculate the ratio between experimental samples and a representative naive CD4 T cell sample. Primers corresponding to T-bet were previously described (55). Primers corresponding to Cbl-b were forward, 5′-CGT CAC GCC CAA ACT CCA CTT TAA A and reverse, 5′- TCT GAA GEC CAA AT T CAG GGC TGT C. These sequences lack complementarity to other E3 ubiquitin ligases (data not shown).

Results

In vivo peripherally tolerated CD4 T cells express enhanced levels of Cbl-b mRNA

We previously found that when 6.5 TCR Tg CD4 T cells specific for influenza HA are adoptively transferred into C3-HA Tg recipients that express HA as a generic self-Ag in a variety of parenchymal tissues at either low or high levels (self-HAlow and self-HAhigh, respectively), they initially undergo moderate or robust proliferation and expansion, respectively, but subsequently develop an anergic phenotype marked by an impaired ability to proliferate and express cytokines in response to antigenic restimulation. In contrast, when the same HA-specific TCR Tg CD4 T cells are transferred into NT recipients infected with viral-HA, they expand and differentiate into Th1 effectors capable of expressing high levels of IFN-γ, TNF-α, and IL-2 (49, 54, 56).

To begin to assess the role of Cbl-b in this tolerogenic response to self-Ag, expression of Cbl-b mRNA was measured by quantitative real-time RT-PCR in samples prepared from TCR Tg CD4 T cells recovered from self-HAlow, self-HAhigh, and viral-HA recipients (Fig. 1). In comparison with naive TCR Tg CD4 T cells, counterparts recovered from self-HAhigh and self-HAhigh recipients expressed 2- and 3-fold increased amounts of Cbl-b mRNA, respectively, whereas expression in viral-HA-primed nontolerized Th1 effectors increased only 1.5-fold. As previously reported (54), expression of T-bet mRNA exhibited a reciprocal pattern in which it was robustly induced in viral-HA-primed Th1 effectors, but only partially induced in self-HA recipients. This trend in which Cbl-b mRNA expression was greater in self-Ag-tolerized compared with virally primed effector CD4 T cells was consistent with previous studies in which Cbl-b expression increased in T cells induced to undergo anergy in vitro or in vivo in response to soluble Ag boluses (40, 47, 48).

Cbl-b−/− CD4 T cells are not resistant to self-Ag-induced peripheral tolerization

To directly assess the role of Cbl-b in regulating tolerance induction to self-HA, a Cbl-b null mutation (43) was backcrossed into the 6.5 TCR Tg background. The parental Cbl-b−/− mice have an increased susceptibility toward developing autoimmunity (43), although young (4-mo-old) Cbl-b−/− TCR Tg mice contained similar numbers of peripheral CD4+ and CD8+ T cells compared with

Materials and Methods

Mice and adoptive transfer

Adoptive transfer recipients received 5 × 10^5 CFSE-labeled WT TCR Tg CD4 T cells specific for an I-E^d-restricted epitope deriving from influenza PR8 strain HA (H10SFERFEIPKE120) that were prepared from CD8-depleted lymph nodes of wild-type (WT) 6.5 TCR Tg mice (51) on the B10.D2 (H-2^b) Thy1.1 background. In the initial source of tolerizing self-Ag and exposed to a viral form of the same Ag. Thus, Cbl-b deficiency may in part predispose individuals toward developing autoimmunity by increasing the number of anergic self-reactive T cells that have the potential to be functionally resuscitated when they were removed from the initial source of tolerizing self-Ag and exposed to a viral form of the same Ag. Thus, Cbl-b deficiency may in part predispose individuals toward developing autoimmunity by increasing the number of anergic self-reactive T cells that have the potential to be functionally resuscitated, rather than preventing the initial induction of anergy.

Flow cytometry

The functional response of 6.5 TCR Tg CD4 T cells was analyzed by FACs following recovery from recipient spleens, as previously described (50, 55). Briefly, TCR Tg CD4 T cells (CD4+Thy1.1/1.2 or 1.1/1.2) were analyzed for frequency and CFSE dilution directly ex vivo. Intracellular cytokine staining was performed following restimulation with either the I-E^d-restricted HA peptide or PMA plus ionomycin (PMA plus I) in the presence of brefeldin A for 3 h, and subsequently staining with anti-Thy1.1, anti-Thy1.2, and the appropriate anti-cytokine mAbs following fixation and permeabilization. Cytokine expression plots were generated by gating on Thy1.1 CFSEhigh Tg cells, and total cytokine expression is expressed in arbitrary units (the percentage of cytokine+ cells multiplied by their mean fluorescence intensity), as previously described (50).
Cbl-b (WT) counterparts (Fig. 2A), and within the CD4+ compartment there was a similar proportion of naive (CD44 low) and memory (CD44 high) subsets that were able to express similar amounts of IL-2, TNF-α, and IFN-γ in response to either HA peptide or PMA plus I stimulation (Fig. 2B). Cbl-b−/− and WT TCR Tg mice also contained similar proportions of peripheral CD4+Foxp3+ cells (data not shown).

Cbl-b−/− and WT TCR Tg CD4 T cells were labeled with CFSE and cotransferred in equal numbers into self-HAlow, self-HAhigh, and viral-HA recipients (Fig. 3). Following recovery from recipient spleens, WT and Cbl-b−/− TCR Tg CD4 T cells were distinguished from each other, and from endogenous recipient-derived CD4 T cells, through their differential expression of congenic Thy1 markers. Thus, WT TCR Tg CD4 T cells were Thy11/1.1, Cbl-b−/− CD4 T cells Thy11/1.2 and endogenous CD4 T cells Thy11.2.

FIGURE 3. Cbl-b−/− peripheral T cells are phenotypically and functionally similar to Cbl-b+/+ (WT) counterparts. A, The total number of CD4+ and CD8+ cells in pooled spleen and peripheral lymph nodes from 4-mo-old Cbl-b−/− and WT TCR Tg mice (corresponding to the donors used in Figs. 3–5). B, Cbl-b−/− and WT CD4+ T cells were stimulated with HA peptide or PMA plus I and then stained for surface CD44 and intracellular IL-2, TNF-α, or IFN-γ. Data shown are representative of three independent experiments.

Despite the enhanced proliferation and expansion of Cbl-b−/− TCR Tg CD4 T cells to self-Ag (Fig. 3, A and B), the functional
activity that developed was limited (Fig. 3, C and D). Thus, whereas both WT and Cbl-b<sup>−/−</sup> TCR Tg CD4 T cells recovered from viral-HA recipients expressed high levels of IL-2, TNF-α, and IFN-γ in response to restimulation with HA peptide, Cbl-b<sup>−/−</sup> TCR Tg CD4 T cells recovered from both self-HA<sub>low</sub> and self-HA<sub>high</sub> recipients expressed very low amounts of all three cytokines that were no greater than in WT counterparts. Similar to what was observed in the spleen, Cbl-b<sup>−/−</sup> CD4 T cells recovered from lymph nodes and lung (an HA-expressing parenchymal organ (49)) of self-HA recipients were limited in their capacity to express cytokines (data not shown).

These results that Cbl-b<sup>−/−</sup> TCR Tg CD4 T cells exposed to cognate self-Ag possess similarly impaired function as WT counterparts suggested that Cbl-b<sup>−/−</sup> CD4 T cells are not resistant to peripheral tolerization/anergy. This contrasts with a previous study reporting that Cbl-b<sup>−/−</sup> T cells are resistant to anergy induced through administration of soluble boluses of cognate Ag (40). To assess whether Cbl-b<sup>−/−</sup> TCR Tg CD4 T cells in our system might be differentially resistant to anergy induced by endogenous self-Ag vs exogenous soluble Ag, we analyzed the response of WT and Cbl-b<sup>−/−</sup> TCR Tg CD4 T cells to soluble boluses of HA peptide administered either on 4 consecutive days or once on the day of adoptive transfer (Fig. 4). Not surprisingly, WT TCR Tg CD4 T cells underwent more complete CFSE dilution in response to daily peptide treatment than to a single peptide bolus, and Cbl-b<sup>−/−</sup> TCR Tg CD4 T cells diluted CFSE and expanded more robustly compared with WT counterparts in response to both peptide regimens (Fig. 4, A and B). The Cbl-b<sup>−/−</sup> TCR Tg CD4 T cells did develop a slightly enhanced capacity to express IFN-γ compared with WT counterparts following exposure to both single and daily peptide boluses, although IL-2 and TNF-α expression capacities were comparable in Cbl-b<sup>−/−</sup> and WT TCR Tg CD4 T cells treated with both peptide regimens (Fig. 4, C and D). Importantly, cytokine expression in soluble peptide-treated Cbl-b<sup>−/−</sup> TCR Tg CD4 T cells was weak compared with WT TCR Tg CD4 T cells exposed to an immunogenic form of HA Ag (i.e., viral-HA as in Fig. 3C). Thus, Cbl-b deficiency does not confer substantial resistance to soluble peptide-induced anergy.

**FIGURE 3.** Functional response of Cbl-b<sup>−/−</sup> CD4 T cells exposed to cognate self-Ag. CFSE-labeled Cbl-b<sup>−/−</sup> (Thy1.1/1.2) and WT (Thy1.1/1.1) TCR Tg CD4 T cells were adoptively cotransferred (5 × 10<sup>5</sup> each) into viral-HA, self-HA<sub>low</sub>, and self-HA<sub>high</sub> recipients and then recovered from spleens on day 4. A, Representative histograms of CFSE dilution. B, Expansion is plotted as the total number of TCR Tg CD4 T cells. C, Representative plots of intracellular IL-2, TNF-α, and IFN-γ expression following restimulation with HA peptide. D, Quantitation of cytokine expression corresponding to C. Total intracellular cytokine is expressed in arbitrary units, and was calculated by multiplying the percentage of cytokine expressing TCR Tg CD4 T cells by the corresponding mean fluorescence intensity values. n = 3 for each group, and all quantitative data are expressed as the mean ± SEM.
Cbl-b deficiency increases the number of anergic CD4 T cells that can be functionally resuscitated

Anergic T cells can regain responsiveness when no longer in the presence of the tolerizing Ag (58, 59). Thus, although Cbl-b deficiency does not appear to confer resistance to anergy in our system, the capacity of Cbl-b−/− CD4 T cells to expand more extensively during the induction of peripheral tolerance/anergy might allow a greater number to convert back to a functional state. To test this possibility, a strategy was used in which TCR Tg CD4 T cells were first tolerized following adoptive transfer into self-HA recipients, and then functionally resuscitated following retransfer into NT secondary recipients infected with viral-HA (54). Similar to the initial adoptive transfer experiment (Fig. 3), in self-HAhigh primary recipients Cbl-b−/− TCR Tg CD4 T cells underwent greater expansion compared with cotransferred WT counterparts (Fig. 5B), but exhibited comparably impaired abilities to express IL-2, TNF-α, and IFN-γ in response to HA peptide stimulation relative to viral-HA controls (Fig. 5, C and D). It was notable that in the current experiment the Cbl-b−/− TCR Tg CD4 T cells expanded to numbers that were ~10-fold greater than WT counterparts (Fig. 5B), whereas in the previous experiment this difference was only 2-fold (Fig. 3B), yet in both cases Cbl-b deficiency did not impact cytokine expression capacity. It should be pointed out that in the current experiment HA peptide stimulation induced weaker cytokine expression in all groups compared with stimulation with PMA plus I, which most likely resulted from limiting Ag presentation caused by the high ratio of specific CD4 T cells to APCs that resulted from the dramatically enhanced T cell expansion (as where PMA plus I stimulation is APC independent). Nevertheless, PMA plus I-induced expression of TNF-α and IFN-γ in self-HAhigh-exposed WT and Cbl-b−/−
TCR Tg CD4 T cells were both equivalently depressed in comparison with viral-HA counterparts. IL-2 expression in self-HA-high-exposed WT and Cbl-b-/- TCR Tg CD4 T cells were both rescued to control viral-HA levels by PMA plus I stimulation, consistent with our previous finding that IL-2 expression in tolerized CD4 T cells is limited by a blockage in TCR signaling that can be bypassed with PMA plus I (54). When the WT and Cbl-b-/- TCR Tg CD4 T cells that were initially tolerized in self-HA recipients were relabeled with CFSE and retransferred into viral-HA secondary recipients, they both diluted CFSE (Fig. 6A) and gained the capacity to express IL-2, TNF-α, and IFN-γ in amounts that were comparable to controls that had been twice exposed to viral-HA (Fig. 6C). This confirmed our previous finding that tolerized TCR Tg CD4 T cells can be functionally resuscitated when removed from the source of tolerizing self-HA Ag and then exposed to an immunogenic (viral) form of HA (54). Importantly, the number of functionally resuscitated Cbl-b-/- TCR Tg CD4 T cells was more than 10-fold greater than the number of WT counterparts (Fig. 6B). Thus, although Cbl-b deficiency might not confer resistance to anergy, it does appear to increase the number of self-reactive anergic CD4 T cells that have the potential to be functionally resuscitated.

Cbl-b regulation of cell cycle

Although our data analyzing the functional status of Cbl-b-/- TCR Tg CD4 T cells exposed to tolerogenic forms of cognate Ag prompt a redefinition of the role of Cbl-b in regulating anergy, our CFSE dilution and expansion data confirm a recent finding regarding how Cbl-b regulates cell cycle progression. Thus, Cbl-b deficiency increased the number of divided (CFSElow) TCR Tg CD4 T cells exposed to low-level self-HA and a single bolus of soluble HA peptide (Fig. 7A), yet the number of undivided (CFSEhigh) Cbl-b-/- and WT TCR Tg CD4 T cells was comparable (Fig. 7B). Note that this analysis was not performed for viral-HA, self-HA-high, or 4× HA peptide bolus groups because there were few undivided WT TCR Tg CD4 T cells to begin with. Nevertheless, the analysis of the self-HA-high and 1× soluble peptide groups confirms a recent finding by Zhang et al. (57) that Cbl-b regulates late cell cycle progression and/or survival, but not cell cycle entry.

Discussion

Cbl-b normally functions to limit Ag responsiveness of T cells by ubiquitinating TCR-inducible signaling molecules, and Cbl-b deficiency thus renders T cells hyperresponsive to antigenic stimulation and predisposes individuals toward developing autoimmunity (38–40, 42, 43, 46). In part because Cbl-b-/- T cells do not require CD28 costimulation to become activated (42, 43), and insufficient costimulation is a critical parameter that confers anergy induction over effector differentiation (31–37), it was hypothesized that Cbl-b-/- T cells would be resistant to anergy and thus help to explain why individuals lacking normal Cbl-b function develop autoimmunity. Consistent with this possibility, anergic T cells in various systems express increased Cbl-b (40, 47, 48), and Cbl-b-/- T cells have been reported to be resistant to anergy (40, 47). These studies, however, used either in vitro systems or in vivo systems in which anergy was induced with soluble Ag boluses.

In the current study, we tested the role of Cbl-b in regulating anergy using a TCR Tg adoptive transfer system in which CD4 T cell peripheral tolerization is induced by a constitutively expressed self-Ag. Consistent with previous studies (40, 47, 48), WT anergic CD4 T cells expressed greater levels of Cbl-b mRNA compared with nontolerized Th1 effectors. Additionally, Cbl-b-/- CD4 T cells that entered cell cycle upon encountering cognate Ag underwent greater expansion compared with WT counterparts, a result that is also consistent with previous studies documenting that Cbl-b-/- T cells are hyperproliferative to antigenic stimulation (42, 43) because they exit cell cycle more slowly (57). What was not consistent with previous reports, however, is that despite the greatly augmented expansion that Cbl-b-/- CD4 T cells undergo immediately following their initial encounter with self-Ag, on a per cell basis they become equally limited in their abilities to express key cytokines in response to antigenic restimulation in comparison with WT counterparts. In this regard, Cbl-b appears to play a functionally similar role to CTLA-4 because CTLA-4-/- CD4

FIGURE 5. Cbl-b deficiency allows self-reactive CD4 T cells to massively expand, but does not confer resistance to anergy. CFSE-labeled Cbl-b-/-/Thy1.1 and WT Thy1.1 TCR Tg CD4 T cells were adoptively cotransferred (5 × 10^6 each) into viral-HA and self-HA-high recipients and then recovered from spleens on day 4. A, Representative histograms of CFSE dilution. B, Expansion is plotted as the total number of TCR Tg CD4 T cells. C, Representative plots of intracellular IL-2, TNF-α, and IFN-γ expression following restimulation with HA peptide or PMA plus I. D, Total cytokine expression corresponding to C. n = 3 for each group.
T cells in another in vivo peripheral tolerance model undergo enhanced late expansion, yet still become anergic (60). In addition to indicating that Cbl-b<sup>−/−</sup> CD4 T cells are not resistant to peripheral self-Ag-induced anergy, these current results reinforce our previous findings that the magnitude of the proliferative response immediately following initial Ag encounter does not determine anergy induction vs priming (49), and that massive T cell expansion is not necessarily linked to the development of effector functions (61).

CD4 T cell anergy can be induced in vitro either by ligating the TCR (62) or directly activating the TCR-inducible Ca<sup>2+</sup>/NF-AT pathway pharmacologically (63) without providing costimulation. The cellular response leading to anergy in these in vitro models, however, is quite distinct from what occurs during the development of in vivo anergy induced by peripheral self-Ag. Thus, in vitro anergy develops in less than 1 day (62, 63) and results because the CD4 T cells do not enter cell cycle (64), whereas self-Ag-induced in vivo anergy is preceded by a vigorous proliferative phase of several days’ duration (49). Considering that proliferation per se is a critical determinant in regulating anergy in vitro (64), but not in vivo (49), and that Cbl-b clearly does regulate proliferation (42, 43, 57) (our current data), it is perhaps less surprising that Cbl-b plays a more critical role in regulating anergy in vitro than in vivo.

**FIGURE 6.** Cbl-b deficiency facilitates recovery of greater numbers of functionally resuscitated CD4 T cells. Pooled samples from Fig. 5 containing self-HA-tolerized or viral-HA-primed WT and Cbl-b<sup>−/−</sup> TCR Tg CD4 T cells were relabeled with CFSE and retransferred into viral-HA-infected NT secondary recipients and then recovered from spleens 4 days later. Each secondary recipient received 5 × 10<sup>5</sup> Cbl-b<sup>−/−</sup> TCR Tg CD4 T cells and the number of WT counterparts corresponding to the ratios described in Fig. 5B. A, Representative histograms of CFSE dilution. B, Expansion is plotted as the total number of TCR Tg CD4 T cells. C, Total intracellular IL-2, TNF-α, and IFN-γ expression following restimulation with HA peptide or PMA plus I. n = 3 per group.

**FIGURE 7.** Cbl-b regulates CD4 T cell expansion, but not entry into cell cycle. The total number of divided (CFSE<sup>light</sup>, A) and undivided (CFSE<sup>dark</sup>, B) TCR Tg CD4 T cells recovered from self-HA<sup>low</sup> (calculated from data corresponding to Fig. 3) and 1× peptide (calculated from data corresponding to Fig. 4) recipients is shown.
It has been reported that Cbl-b−/− T cells are resistant to soluble Ag-induced anergy in vivo (40), although in our current study we found that Cbl-b−/− CD4 T cells exposed to soluble peptide buffers failed to develop effector functions, suggesting that they too were susceptible to anergy. This possibility for anergy may relate to the increased resistance of Cbl-b−/− T cells to dominant/extrinsic peripheral tolerance factors such as TGF-β and Tregs (44, 45). Additionally, because self-reactive T cells can express effector functions transiently while they are undergoing peripheral tolerization (65–67), the augmented initial expansion of Cbl-b−/− T cells immediately following self-Ag recognition most likely results in a greater total amount of self-directed effector functions even though the effector activities of individual self-reactive T cells are not enhanced. Finally, oscillations in the expression levels of peripheral self-Ags could result in cycles of anergy induction, increased functional recovery, and ultimately autoimmunity when cognate self-Ag is re-expressed.

Disclosures

The authors have no financial conflict of interest.

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