Casitas B-Lineage Lymphoma b Inhibits Antigen Recognition and Slows Cell Cycle Progression at Late Times during CD4+ T Cell Clonal Expansion

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Casitas B-Lineage Lymphoma b Inhibits Antigen Recognition and Slows Cell Cycle Progression at Late Times during CD4⁺ T Cell Clonal Expansion¹

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Optimal clonal expansion of CD4⁺ T cells during the primary response to Ag requires prolonged TCR recognition of peptide Ag/MHC complexes. In this study, we investigated the capacity of Casitas B-lineage lymphoma b (Cbl-b) to counter-regulate late TCR signals necessary for continued cell division in vivo. During the first 24 h of a primary response to Ag, Cblb−/− SC.C7 CD4⁺ T cells demonstrated no alteration in CD69, CD25, and CD71 up-regulation or cell growth as compared with wild-type cells. Nevertheless, beyond 24 h, both the expression of CD71 and the rate of cell division were increased in the genetic absence of Cbl-b, leading to an augmented clonal expansion. This deregulation of late T cell proliferation in the absence of Cbl-b resulted in part from an inability of Cblb−/− T cells to desensitize Akt, PLCγ1, and ERK phosphorylation events downstream of the TCR/CD3 complex, in addition to their failure to undergo a growth arrest in the absence of Ag. These observations now suggest a novel role for Cbl-b in triggering the exit from cell cycle at the end of a CD4⁺ T cell clonal expansion. The Journal of Immunology, 2008, 181: 5331–5339.

Effective clonal expansion by naive CD4⁺ T cells that recognize Ag for the first time in the peripheral immune system relies on a prolonged period of Ag/MHC complex presentation and TCR ligation. Such persistent Ag recognition is facilitated in part by the activation of dendritic cells via their TLRs or CD40, leading to a decrease in the rate of Ag/MHC turnover (1–3). Experimental Ags designed for only transient dendritic cell expression confirm that CD4⁺ T cell clonal expansion and differentiation to effector or memory cell phenotypes is suboptimal when TCR ligation is terminated prematurely (4, 5). A failure of CD4⁺ T cells to continue to detect Ag/MHC complexes 24 h into a primary response promotes dissolution of T cell/dendritic cell tight interactions and reduces their proliferative potential (6). The use of an anti-Ag/MHC mAb to antagonize late TCR signaling has further revealed that CD4⁺ T cells immediately undergo a growth arrest and slow their rate of cell division once TCR ligands become unavailable (7). This counter-regulation of cell cycle progression in the absence of ongoing Ag/MHC recognition appears to underlie the clonal competition that limits clonal expansions by responder T cells already present at high frequencies (8, 9). However, there are as yet no data to support the notion that this regulation of late clonal expansion protects against the development of immunopathology.

In contrast, Casitas B-lineage lymphoma b (Cbl-b)³ has previously been implicated in the control of T cell-mediated immunopathology. Mutation of Cblb in the Komeda rat strain leads to spontaneous diabetes development (10). Mice made genetically deficient in Cbl-b expression also demonstrate infiltrations of multiple organs with activated T and B cells and the spontaneous production of anti-dsDNA autoantibodies, in addition to heightened sensitivity to the development of experimental autoimmune encephalomyelitis (11, 12). The biochemical mechanisms by which Cbl-b molecules regulate autoreactivity development are not entirely clear; however, emerging data suggest that TCR-triggered NFκB-dependent gene expression is inhibited by Cbl-b in the absence of CD28 costimulatory signals (13). Consistent with this, Cbl-b limits the activation of protein kinase C-θ (PKC-θ) by vav-1, thus controlling the formation of signaling rafts containing PKC-θ, CARMA1, and Bcl10 that regulate the IκB kinase complex (11, 13). Cbl-b, a RING finger-containing E3 protein, has also been shown to bind to and catalyze the ubiquitination of the p85 regulatory subunit of PI3K, thereby inhibiting the activation of Akt in T cells (14, 15). Interestingly, the E3 ligase activity of Cbl-b is also thought to play an important role in the blockade of TCR signaling after T cell clonal anergy induction (16–18). Nevertheless, no previous experiments have investigated the role of Cbl-b in the regulation of late TCR signals.

This study now explores the capacity of Cbl-b to influence late events in CD4⁺ T cell clonal expansion in vivo. The results suggest that Cbl-b has little capacity to influence the entry of naive CD4⁺ T cells into cell cycle following immunization. However, the up-regulation of Cbl-b protein levels beyond 16–24 h of stimulation contributes to the premature growth arrest and slowing of the cell division rate that has been observed as Ag/MHC complex availability wanes. Therefore, these data support the hypothesis that Cbl-b opposes the development of immunopathology by facilitating CD4⁺ T cell exit from cell cycle.

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³Abbreviations used in this paper: Cbl-b, Casitas B-lineage lymphoma b; mTOR, mammalian target of rapamycin; PCCp, pigeon cytochrome c peptide 81-104; PKC, protein kinase C; Tg, transgenic; LN, lymph node.

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Materials and Methods

Mice and Ag

Chbl-b<sup>+</sup> as well as Chbl-b<sup>-/-</sup> Rag2<sup>-/-</sup> 5C.C7 mice were provided by R. H. Schwartz (National Institutes of Health, Bethesda, MD) and were produced by back-crossing Chbl-b<sup>-/-</sup> offspring (11) to Rag2<sup>-/-</sup> 5C.C7 B10.A mice (19). Lymph node (LN) T cells from these 5C.C7 animals uniformly express Vβ3<sup>+</sup> and CD4<sup>+</sup>, and recognize pigeon cytochrome c peptide 81–104 (PCCp)<sub>β3</sub>-E<sub>3</sub> complexed to B10.A/Cr mice (B10.A/Cr mice were purchased from Charles River Breeding Laboratories under a contract from the National Cancer Institute (Frederick, MD) and were used as syngeneic recipient mice for adoptive transfer studies. All mice were housed under specific pathogen-free conditions and used in accordance with guidelines put forth by the National Institutes of Health (Bethesda, MD) and the University of Minnesota Institutional Animal Care and Use Committee. Mice were sex and age matched for all experiments, ranging in age from 6 to 10 wk old.

T cell activation

For studies relying on the in vitro activation of CD4<sup>+</sup> T cells, 5C.C7 LN cells (>95% pure) were first labeled with 5 μM CFSE (Molecular Probes), and then were incubated in complete medium (RPMI 1640 (Mediatech) containing 10% FCS (Atlas Biologicals), 2 mM L-glutamine, penicillin, streptomycin, and 5 × 10<sup>-5</sup> M 2-ME) for 16–24 h at 37°C on plates, in some cases precoated with anti-CD3 mAb (145-2C11) (20) and in the presence or absence of soluble anti-CD28 mAb (37.51) (21), or with hamster IgG (BD Biosciences) as an irrelevant Ab control, as previously described (22). Alternatively, T cells were stimulated with ionomycin and PMA (Calbiochem), or with human rIL-2 (Chiron), as indicated. In some experiments, 10 μg/ml Brefeldin A (Calbiochem) was added to cultures to inhibit the secretion of newly synthesized IL-2, and before its detection by intracellular staining and flow cytometry. In other experiments, IL-2 secretion was monitored in the supernatant using an ELISA system (BD Pharmingen). Finally, some cells were analyzed by Western blot using Santa Cruz Biotechnology Ab to phospho-Cbl-b and actin, as well as Cell Signaling Technology Ab to phospho-PLCγ, -Akt, -ERK, and -STAT5, as previously described (22).

Adoptive transfer

For in vivo adoptive transfer experiments, 2 × 10<sup>6</sup> CFSE-labeled naive 5C.C7 CD4<sup>+</sup> T cells were injected i.v. into B10.A mice. The next day, mice were immunized with PCCp alone at the indicated dosage (or PBS as a control) to initiate a CD4<sup>+</sup> T cell clonal expansion at relatively high responder frequency and in the absence of an adjuvant. Alternatively, in vitro preactivated CFSE-labeled 5C.C7 CD4<sup>+</sup> T cells were adoptively transferred into B10.A mice and then immediately challenged with PCCp.

Flow cytometry

Lymphocytes were harvested from either tissue culture plates or from the spleen and LNs of adoptive transfer recipient animals, and then immediately fixed with 2% formaldehyde (Sigma-Aldrich). Cells were then mixed with 2 × 10<sup>5</sup> counting beads (Caltag Laboratories) and stained with an Ab mixture containing CD71- or CD69-PE, CD25-allophycocyanin, and CD4-PercP-Cy5.5 (BD Pharmingen). Alternatively, cells were permeabilized with 0.5% saponin (Sigma-Aldrich) and stained with IL-2-PE and CD4-allophycocyanin (BD Pharmingen) as previously described (23). The majority of 5C.C7 CD4<sup>+</sup> T cells were then distinguished from the endogenous polyclonal CD4<sup>+</sup> T cell population based on a persistence of CFSE fluorescence in the FL1 channel and CD4 staining, using either a BD FACScalibur or BD LSRII flow cytometer (BD Biosciences) and FlowJo analysis software (Tree Star).

Measurement of cell cycle progression in vivo

5C.C7 CD4<sup>+</sup> T cell G<sub>0</sub>-G<sub>1</sub> cycle entry was monitored by flow cytometry using the forward scatter determination. The average cell division rate for the CFSE-labeled 5C.C7 CD4<sup>+</sup> T cell population at various times after stimulation was calculated as previously described (7, 24). Briefly, based on the peaks of CFSE fluorescence intensity within the population, each T cell was assigned to a particular cell division group (with d = 0 to n cell divisions), and the number of T cell events (E) observed within each cell division group (E<sub>d</sub>) was determined. Average cell division (D) was then calculated using the following equation:

\[
D = \frac{\sum_{d=0}^{n} (2^{-d})E_d}{T_e}
\]

where \( T_e \) is the total number of cell events.

Note that this calculation of the average number of cell divisions is corrected for the 2-fold increase in cell number that is associated with each division. To
FIGURE 2. Cbl-b does not raise the threshold for initial Ag recognition. A total of $2 \times 10^6$ CFSE-labeled Rag2−/− 5C.C7 CD4+ T cells were adoptively transferred into B10.A recipients and exposed to varying doses of PCCp. Then, 5 h later, animals were sacrificed and CFSE-labeled LN CD4+ T cells were examined for expression of activation markers. A, CD25 expression by T cells from wild-type (top) and Cbl-b-deficient (bottom) donor mice exposed to either 0 (shaded histogram), 0.5 (solid tracing), 2.0 (dashed tracing), or 5 μg (dotted tracing) PCCp infusion. B, CD69 expression by wild-type (open square) and Cbl-b-deficient (closed circle) CD4+ T cells following immunization with the indicated dose of PCCp. Two to three animals were examined at each dose of Ag, and data are expressed as the mean fluorescence intensity ± SEM.

Results
Cbl-b counter-regulates the primary CD4+ T cell response to Ag stimulation in vivo
Mice made genetically deficient for Cbl-b have been shown to spontaneously produce autoantibodies and develop an immunopathology that is associated with multiorgan T and B cell infiltration (11, 12). Whether this immunopathology stems from a reduced threshold for the activation of the naïve autoreactive lymphocytes in the absence of Cbl-b or from an inability of the clonal anergy tolerance mechanism to control the clonal expansion and effector function of Cblb−/− self Ag-specific lymphocytes is uncertain. Furthermore, it remains unclear what effects a selection of autoreactive T cells and resulting immunopathology might have on the phenotype and function of CD4+ T cells taken from these mice.

To investigate the role of Cbl-b in the control of the CD4+ T cell primary response to Ag in the absence of confounding T cell autoreactivity or autoimmune, we examined the proliferation of Rag2−/− TCR-transgenic (Tg) 5C.C7 CD4+ T cells following their adoptive transfer into wild-type B10.A mice and stimulation with PCCp. Donor 5C.C7 mice lack the capacity to rearrange endogenous TCR gene loci and, therefore, avoid any self-reactivity. For these experiments, we also took advantage of an immunization regimen capable of only suboptimal CD4+ T cell priming and known to induce a partial peripheral tolerance to Ag (23); PCCp was administered in the absence of adjuvant as a single i.v. bolus to wild-type recipient mice that had been adoptively transferred with 5C.C7 CD4+ T cells at a relatively high precursor frequency ($2 \times 10^6$). Exposure of the naïve 5C.C7 CD4+ T cell population to 5 μg PCCp under these conditions elicited only a weak (∼4-fold) clonal expansion by 96 h that on average was associated with about two rounds of cell division (Fig. 1). Interestingly, Cbl-b acted in this system to moderate the response, as naïve Rag2−/− 5C.C7 CD4+ T cells made genetically deficient for Chlb demonstrated on average one additional round of cell division and accumulated to higher numbers following the immunization. Therefore, these data provided support for the hypothesis that Cbl-b acts within CD4+ T cells to counter-regulate Ag-induced proliferation in vivo even in the absence of systemic autoimmunity or lymphoproliferative disease.

Normal basal Cbl-b levels do not influence the recognition of rare peptide Ag/MHC complexes by naïve CD4+ T cells
To better understand the mechanism by which Cbl-b antagonizes Ag-dependent cell cycle progression in vivo, we examined the dose-response curve for Ag recognition by wild-type and Cblb−/− 5C.C7 CD4+ T cells. A titration of Ag in the immunization regimen revealed that, at higher doses of PCCp, cell divisions were inhibited in wild-type cells as compared with the Cbl-b-deficient CD4+ T cells (Fig. 1C). However, at limiting Ag concentration, Cbl-b-deficient T cells demonstrated no increased sensitivity to Ag, using CD69 and CD25 as markers of productive TCR engagement (Fig. 2). Consequently, our data offered no support for a model in which basal Cbl-b expression in naïve CD4+ T cells raises the threshold for initial Ag recognition. Rather, the data were more compatible with the hypothesis that Cbl-b expression or activity is increased only in response to intensified or prolonged TCR stimulation.

Cbl-b counter-regulates late cell division by inhibiting TCR-dependent CD71 expression and blastogenesis
Previously, we observed that toward the end of a primary CD4+ T cell response to Ag stimulation in vivo, the rate of cell division and mean cell size were highly correlated (7). Based on this, we have postulated that a T cell’s ability to achieve a blastogenesis after each successive round of cell division determines the subsequent cell division time. Given that in our experiments Cbl-b appeared to play no role in the regulation of the threshold for initial Ag recognition, we hypothesized that Cbl-b does not increase the time to first cell division, but rather slows subsequent cell divisions by inhibiting the rate of $G_1 \rightarrow S$ phase transition. A careful time course of in vivo CD4+ T cell activation demonstrated that wild-type 5C.C7 CD4+ T cells initiated and completed the majority of their cell divisions between 24 and 48 h (Fig. 3). Cbl-b-deficient T cells also initiated their proliferative response between 24 and 48 h, and demonstrated only a modestly increased cell division rate during this period as compared with the wild-type controls. Nevertheless, between 48 and 72 h of stimulation, the Cbl-b knockout T cells...
divided at least once more, suggesting that Cbl-b activity was most important to suppress cell divisions at the end of a response.

Consistent with these results, Cblb−/− T cells were on average bigger than wild-type at the 48-h time point, indicating an inhibition by Cbl-b of late G1 → S phase transitions (Fig. 3A). Cbl-b-deficient T cells also demonstrated a stronger and more durable expression of the transferrin receptor CD71. CD71 expression during cell cycle progression has previously been shown to be a useful marker of PI3K- and mammalian target of rapamycin (mTOR)-dependent G1 → S phase cell cycle progression (25). In our experiments, CD71 expression proved to be a better predictor of the rate of cell division than blastogenesis itself. The highest CD71 expression was always observed in T cells demonstrating the fastest cell division rate during the immunization (Fig. 3B). Interestingly, CD71 expression fell to near background level in wild-type CD4+ T cells after 72 h of stimulation, whereas Cblb−/− T cells exhibiting the fastest rate of cell division continued to express CD71 even at that late time point. Thus, these data suggested a new model where Cbl-b antagonizes the activity of PI3K and limits mTOR-dependent blastogenesis late during a primary response to Ag in the absence of infection or adjuvant.

The induction of Cbl-b both inhibits programming for autonomous cell division in the absence of ongoing TCR stimulation and desensitizes CD4+ T cells to low numbers of Ag/MHC complexes

Several reports have now demonstrated that CD4+ T cells remain fully aware of the continued presence of peptide Ag/MHC complexes throughout their primary clonal expansion. Intense competition for limiting Ag/MHC complexes at late times after initial Ag encounter leads to dissolution of CD4+ T cell/dendritic cell conjugates and, eventually, exit of T cells from the cell cycle (4–7). Given that Cbl-b does not appear to raise the threshold for initial Ag/MHC recognition in vivo, but instead promotes the cessation of cell division, we hypothesized that Cbl-b molecules are induced during the course of a primary Ag encounter to play a counter-regulatory role.

Consistent with this model, the activation of wild-type 5C.C7 CD4+ T cells in vitro led to a large increase in Cbl-b protein expression by 8 h of incubation that was sustained beyond 72 h (Fig. 4A). Ligation of the TCR/CD3 complex with CD3 mAb alone proved sufficient to induce this initial rise in Cbl-b protein levels.
It was previously suggested that CD28 signaling increases the rate of Cbl-b turnover in T cells and reduces protein expression (26). We, too, found some evidence for this CD28-Cbl-b antagonism at the 4-h time point; nevertheless, T cells stimulated with a combination of CD3 and CD28 mAbs ultimately demonstrated an increased Cbl-b protein level at the latest time points, perhaps as a consequence of sustained blastogenesis and improved in vitro T cell viability in the presence of CD28 costimulatory signaling (Fig. 4A and data not shown). Based on these data, it appeared that Cbl-b would have the greatest opportunity to exert its counter-regulatory effects at late times during T cell activation.

To further test the role of Cbl-b in the control of late cell cycle progression, we performed an overnight stimulation of the 5C.C7 CD4+ T cells in vitro with a combination of CD3 and CD28 mAbs, and then examined their capacity to undergo cell divisions in the absence of Ag following an adoptive transfer into normal B10.A hosts. As previously reported (7), wild-type CD4+ T cells showed decreased proliferative potential compared to Cbl-b−/− T cells (Fig. 4B and C).

**Figure 4.** Cbl-b up-regulation is associated with decreased proliferative potential. A, Cbl-b and actin protein levels were compared in normal 5C.C7 CD4+ T cells over time following stimulation in vitro with 1 µg/ml immobilized CD3 and/or 1 µg/ml soluble CD28 mAb as indicated. B, CFSE-labeled wild-type (gray tracing) and Cbl-b−/− (open tracing) 5C.C7 CD4+ T cells were stimulated in vitro for 24 h with a combination of 1 µg/ml immobilized CD3 mAb and 5 µg/ml soluble CD28 mAb (or with no mAb as a control), and then were adoptively transferred into normal B10.A mice without immunization. CFSE dye dilution 3 days later is shown for LN and spleen 5C.C7 CD4+ T cells.

**Figure 5.** Cbl-b limits the induction of IL-2, CD25, and CD71 expression during the first 22 h of T cell activation. A, Cbl-b−/− or Cbl-b+/− 5C.C7 CD4+ T cells were stimulated 22 h in vitro with either 2 µg/ml immobilized CD3 mAb alone or a combination of immobilized CD3 mAb plus 2 µg/ml soluble CD28 mAb. Brefeldin A (10 µg/ml) was added 9 h into the stimulation period. At the end of the experiment, 5C.C7 CD4+ T cells were examined by flow cytometry for intracellular IL-2 accumulation using IL-2-PE (open tracing) or isotype control PE-coupled mAb (gray tracing). B, Cumulative IL-2 production data for the experiment shown in A, expressed as the product % IL-2− × mean fluorescence intensity. Error bars indicate the SEM for four replicate samples per group. C, Stimulation of 5C.C7 CD4+ T cells (either Cbl-b+/−, top; or Cbl-b−/−, bottom) for 22 h with either medium alone (nil), or with 0.5 µg/ml immobilized CD3 mAb alone, or with immobilized CD3 mAb plus 0.5 µg/ml soluble CD28 mAb. At the end of the in vitro stimulation, viable CD4+ T cells were examined for CD25 and CD71 expression. All results shown were reproduced in at least one additional experiment.
failed to demonstrate a durable proliferative response after adoptive transfer into animals that lacked their Ag, with the T cells only infrequently dividing more than once and often not dividing at all (Fig. 4B). 5C.C7 CD4+ T cells made genetically deficient for Cbl-b, however, always divided at least once after adoptive transfer and frequently divided two, three, or four times. Thus, a 24-h period of CD3/CD28 stimulation was sufficient to up-regulate Cbl-b molecules that appeared to act as a brake on subsequent in vivo cell cycle progression in the absence of Ag. The expression of Cbl-b in the CD4+ T cells in effect prevented the development of a more durable proliferative “program.”

In addition to its effects on the programming of T cells for Ag-independent cell cycle progression, Cbl-b also reduced the sensitivity of primed CD4+ T cells to low doses of Ag at late times during their activation response. Following an overnight period of in vitro activation with CD3 mAb alone, the Cblb−/− 5C.C7 CD4+ T cells typically divided only once after adoptive transfer into unimmunized hosts (Fig. 4B). Transfer into a recipient animal that was also immunized with a low dose (1 μg) of PCCp, however, led to another round of cell division. In contrast, wild-type 5C.C7 CD4+ T cells demonstrated essentially no increased proliferation after transfer into PCCp-immunized mice. Taken together, the results indicated that Cbl-b molecules induced during the first 24 h of TCR/CD3 ligation both put a brake on subsequent cell division in the absence of Ag as well as desensitized the CD4+ T cells to further TCR signaling.

**Cbl-b dampens IL-2 production and CD25 expression during the first 24 h of stimulation**

To investigate further this capacity of Cbl-b to regulate programming for proliferation in the absence of ongoing Ag stimulation, we examined autocrine IL-2 production and response in vitro. Consistent with previous reports (11, 12), Cbl-b-deficient Rag2−/−/Cblb−/− CD4+ T cells demonstrated heightened IL-2 synthesis upon stimulation with either CD3 mAb alone or with the combination of CD3 and CD28 mAbs (Fig. 5, A and B). Furthermore, the pattern of in vitro CD25 and CD71 expression was altered by the Cblb gene deletion. Therefore, it appeared that newly synthesized Cbl-b molecules could antagonize the production of IL-2 and functional IL-2 receptors in vitro as well as inhibit the expression of CD71 during the first 24 h of Ag stimulation. Accordingly, a reduction in IL-2 receptor signaling and limiting iron stores following Cbl-b induction may lead to eventual growth arrest late during a clonal expansion.

**Cbl-b inhibits late TCR/CD3-dependent proximal signal transduction but cannot regulate growth factor receptor-dependent STAT5 phosphorylation**

The finding that Cbl-b is expressed and can inhibit TCR-triggered activation events within the first 24 h of stimulation complicated our initial interpretation of blocked late cell cycle progression. TCR antagonism by Cbl-b molecules during the first 24 h of activation may have simply blunted the blastogenesis response and ensured a premature exit from cell cycle by rapidly dividing T cells. Alternatively, up-regulated Cbl-b molecules (or their downstream target proteins) effectively antagonize the TCR throughout clonal expansion.

To differentiate these two possibilities, we took advantage of the calcium ionophore ionomycin and the PKC-activating phorbol ester PMA to initiate a strong G0/G1 phase progression that was independent of regulation by Cbl-b. As shown in Fig. 6, the combination of ionomycin and PMA strongly induced IL-2 production by CD4+ T cells, independent of the status of the Cblb gene. Similarly, 16 h of ionomycin plus PMA stimulation was sufficient to up-regulate an equivalently high level of CD71 and CD25 in both the wild-type and Cbl-b-deficient CD4+ T cells. Cbl-b protein expression was also strongly induced by 16 h of ionomycin and PMA stimulation (data not shown); therefore, the downstream signaling pathways triggered by these two agents and necessary for IL-2, CD71, and CD25 protein induction appeared to be insensitive to counter-regulation by Cbl-b. This failure of Cbl-b to act as an inhibitor in the absence of TCR/CD3 signals was revealing and further supported the model that Cbl-b targets TCR proximal signaling pathways for counter-regulation.

Using this ionomycin and PMA pharmacological system to activate equally the Cbl-b-sufficient and Cbl-b-deficient T cells, we then examined their late signaling characteristics and proliferative responsiveness. After a 16-h period of preactivation in the presence of ionomycin and PMA, and following a 2-h washout period, Cblb knockout 5C.C7 CD4+ T cells exhibited an enhanced level of anti-CD3 mAb-induced PLCγ-1, Akt, and ERK phosphorylation, as compared with wild-type T cells (Fig. 7A). Similarly, the adoptive transfer of these ionomycin plus PMA pretreated T cells into normal hosts revealed that the Cblb−/− CD4+ T cells could undergo more rounds of cell division following challenge with Ag
In contrast, these ionomycin plus PMA pretreated T cells demonstrated no capacity of Cbl-b to counter-regulate IL-2 receptor-triggered STAT5 phosphorylation and in vitro proliferation (Fig. 7, B and D). Therefore, the up-regulation of Cbl-b during CD4⁺/H11001 T cell activation leads to a selective antagonism of TCR-dependent proximal signaling events at late time points during the primary response to Ag but leaves T cells free to respond to cytokine stimulation.

Discussion

This work has explored the role of one counter-regulatory protein, Cbl-b, in the control of late Ag-driven cell cycle progression. During their primary response to Ag, CD4⁺ T cells rely on continuous TCR signaling to achieve the highest rate of cell division, maximal clonal expansion, and optimal differentiation to a helper phenotype (4, 5, 7). Prolonged clustering with Ag-bearing dendritic cells facilitates this durable TCR engagement but also forces CD4⁺ T cells with identical Ag specificities to compete for peptide Ag/MHC ligands if they are to remain in cell cycle (27). Despite this new appreciation of the necessity for continuous TCR signaling to prolong CD4⁺ T cell clonal expansion, the biochemical pathways involved in mediating this signal in vivo remain unknown.

Cbl-b has previously been implicated in the inhibition of p85 PI3K downstream signaling to NFκB (11, 13–15). We, therefore, chose to investigate this molecule in these studies based on our own in vitro data indicating a requirement for TCR- and CD28-dependent PI3K signaling to maintain an optimal rate of cell division at late time points after the initial Ag encounter (22). This study now establishes that, during a primary response to Ag stimulation in the absence of adjuvant or infection, the rate of cell division by CD4⁺ T cells that have already committed to a proliferative response becomes limited by their induction of Cbl-b protein and its subsequent inhibition of TCR-dependent proximal signaling events. Cbl-b molecules (and/or their target proteins) not only desensitize CD4⁺ T cell blasts to low numbers of Ag/MHC complexes at late times after the initiation of priming but also promote early exit from the cell cycle in the absence of ongoing TCR signaling.

These experiments were designed to address the concern that changes in T cell signaling observed in the absence of Cblb are a
consequence, rather than cause, of the spontaneous autoimmune disease that has sometimes been observed in these mice (11, 12). Use of the 5C.C7 TCR-Tg on the Rag2<sup>+/−</sup> background precluded the development of an autoreactive CD4<sup>+</sup> T cell repertoire in the Chlb<sup>−/−</sup> animals and prevented disease in our experiments. Adoptive transfer of these CD4<sup>+</sup> T cells into normal hosts also eliminated the effect of a loss of Chlb expression in other immune cells or tissues. Therefore, one can be confident that these results have not been confounded by some alteration in the specificity or differentiation state of the responder CD4<sup>+</sup> T cell population or by a secondary effect of systemic inflammation or non-T cell Chlb deficiency.

It is most important to note that this investigation did not reveal in vivo evidence for control of naive CD4<sup>+</sup> T cell initial recognition of Ag by Chlb. This result is remarkable given previous studies that have reported a selective capacity of Chlb molecules in naive T cells to interrupt signaling between p85 PI3K and NFkB even within the first 30 min of in vitro activation (11–15). We, too, have observed a capacity of Chlb to selectively antagonize NFkB translocation to the nucleus 4 h into an in vitro activation, particularly when CD28 costimulatory signals are lacking (R. Zhang, unpublished observation). Nevertheless, such alterations in PI3K and NFkB activation may be relatively unimportant to establishing the threshold for T cell activation in vivo, at least as measured by the early induction of CD69 and CD25 expression. Alternatively, early B7/CD28 interactions (even in the absence of infection) may be sufficient to overcome any inhibitory effect of a few Chlb molecules and allow for adequate early PI3K and NFkB signaling in vivo. Consistent with this latter theory, previously published in vitro activation studies have suggested that the costimulation of T cell cultures with anti-CD28 mAb alleviates much of the counter-regulatory effect of Chlb (26). The induction of CD69 and CD25 is thought to depend at least in part on the activation of NFkB (28, 29). Therefore, it is more likely that early during Ag priming of CD4<sup>+</sup> T cells in vivo there is insufficient Chlb activity to overcome the effects of CD28 costimulatory signaling and inhibit TCR signaling to PI3K, Akt, and NFkB.

In contrast to its inability to effectively antagonize initial Ag/MHC recognition in vivo, or prevent an early increase in cell size and CD71 surface expression by the Ag-specific CD4<sup>+</sup> T cells, Chlb did prove important in the exit of these T cells from cell cycle during the period between 48 and 72 h. Although the mechanism of this counter-regulation remains uncertain, Chlb expression was associated with a premature reduction in cell size and CD71 by 48 h. Our previous studies have implicated a CD28/PI3K/Akt/mTOR signaling pathway as necessary to maintain CD4<sup>+</sup> T cells in cell cycle following Ag stimulation in vitro (22, 30). Others have shown that the expression CD71 relies on signals mediated by PI3K and mTOR (25), and our own unpublished experiments confirm this (R. Zhang, unpublished observations). Therefore, we now speculate that during the in vivo primary response to Ag, PI3K, mTOR, and NFkB activities wane as Chlb protein levels rise and desensitize the CD4<sup>+</sup> T cells to further Ag stimulation, thus quenching their remaining proliferative program. It also follows that during a primary response to Ag in the presence of an adjuvant or infection, or in the clinical setting of systemic inflammation, increased PI3K activity associated with enhanced CD28 and OX40 costimulatory receptor signaling overcomes the negative effects of Chlb and facilitates a more durable clonal expansion response via the induction of mTOR regulators such as survivin and aurora B (31, 32).

Results obtained by others using P14 TCR-transgenic CD8<sup>+</sup> T cells primed during lymphocytic choriomeningitis virus infection support the conclusions that Chlb cannot interrupt early Ag recognition or entry into cell cycle by naive T cells, but instead puts a brake on late TCR signaling (33). Because CD8<sup>+</sup> T cells can be programmed for a durable clonal expansion, without need for persistent Ag recognition to maintain a high rate of cell division, the up-regulation of Chlb and subsequent TCR down-modulation are not accompanied by a slowing of the rate of proliferation (33, 34). In contrast, Chlb<sup>−/−</sup> P14 CD8<sup>+</sup> T cells do demonstrate augmented IFN-γ production on Ag rechallenge, consistent with a need for continuous TCR signaling to ensure optimal effector T cell generation and a capacity for Chlb to interfere with this TCR-dependent differentiation event (33, 35).

It is interesting that Chlb counter-regulates TCR/CD3-dependent activation signals but does not inhibit IL-2 receptor signaling in blasting CD4<sup>+</sup> T cells. Like the TCR, IL-2 receptors are thought to activate PI3K, with PI3K activity necessary for expression of cyclin D3 and degradation of p27kip1 before entry into cell cycle (36). Therefore, it might have been reasonable to expect that Chlb would also antagonize late IL-2-dependent PI3K signaling and cell cycle progression by 5C.C7 CD4<sup>+</sup> T cell blasts. However, experiments shown here using ionomycin plus PMA-generated CD4<sup>+</sup> T cell blasts (or T cells pretreated with CD3 and CD28 mAb; data not shown) did not demonstrate a significant increase in Akt phosphorylation during the first 30 min of exposure to the cytokine. In contrast, IL-2 was sufficient to stimulate equally the phosphorylation of STAT5 in both Chlb-expressing and Chlb-deficient CD4<sup>+</sup> T cell blasts. IL-2 treatment was also observed to protect both Chlb<sup>−/−</sup> and wild-type CD4<sup>+</sup> T cell blasts from growth factor withdrawal-induced cell death (R. Zhang, unpublished observation). Perhaps consistent with a dominant role for phospho-STAT5-dependent and Chlb/PI3K-independent signaling pathways in IL-2-triggered cell growth and survival in vitro, the PI3K inhibitor LY294002 blocks neither the expression of cyclin D3 and cyclin E nor the down-regulation of p27kip1 at late time points after treatment with IL-2 (37). Taken together, the data suggest there is little opportunity for Chlb to antagonize IL-2-dependent late cell signaling events. This capacity of IL-2 receptors to bypass the counter-regulatory effects of Chlb may help explain how IL-2 can promote a reversal of clonal anergy in CD4<sup>+</sup> T cells despite their high expression of Chlb (16–18, 38). Thus, the ability of Chlb to counter-regulate late cell cycle progression in our adoptive transfer system is most consistent with the model that IL-2 is neither necessary nor sufficient to mediate CD4<sup>+</sup> T cell proliferation in vivo (39).

In summary, this work details a new role for Chlb in the late regulation of CD4<sup>+</sup> T cell clonal expansion following a primary recognition of Ag. The accumulation of Chlb protein hours to days into an immune response antagonizes TCR-dependent proximal signal transduction and imposes a requirement for continued strong TCR signaling to maintain a high rate of cell division. When Ag/MHC complexes can no longer be detected during the course of a clonal expansion as a consequence of this Chlb-dependent desensitization, CD4<sup>+</sup> T cells then fail to maintain their expression of transferrin receptors (CD71) and undergo a premature growth arrest that prevents additional G<sub>1</sub>→S phase transitions. Such counter-regulation of TCR-dependent late cell growth by Chlb may underlie its ability to act as a barrier to the development of autoimmune disease following self Ag recognition.

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Disclosures
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References