Cutting Edge: Regulation of T Cell Activation Threshold by CD28 Costimulation Through Targeting Cbl-b for Ubiquitination

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Optimal T cell activation requires signaling through the TCR and CD28 costimulatory receptor. CD28 costimulation is believed to set the threshold for T cell activation. Recently, Cbl-b, a ubiquitin ligase, has been shown to negatively regulate CD28-dependent T cell activation. In this report, we show that CD28 costimulation selectively induces greater ubiquitination and degradation of Cbl-b in wild-type T cells than CD3 stimulation alone, and TCR-induced Cbl-b ubiquitination and degradation are significantly reduced in CD28-deficient T cells. Stimulation of CD28-deficient T cells with higher doses of anti-CD3 results in increased ubiquitination of Cbl-b, which correlates with enhanced T cell responses. Our results demonstrate that CD28 costimulation regulates the threshold for T cell activation, at least in part, by promoting Cbl-b ubiquitination and degradation. The Journal of Immunology, 2002, 169: 2236–2240.

C ooperative signals from the TCR and the costimulatory molecule CD28 are essential for T cell activation (1). TCR ligation in the absence of CD28 costimulation renders the T cell anergic (2), and this process represents one of the major mechanisms of the induction of peripheral T cell tolerance. In vitro studies demonstrated that in the absence of CD28 engagement, T cells require very high TCR occupancy and prolonged stimulation, whereas CD28 costimulation allows T cells to respond to lower degrees of TCR occupancy (3, 4). Consistent with this observation, CD28−/− T cells from myelin basic protein-immunized PL/J mice proliferate in vitro in response to Ag stimulation, albeit at a rate lower than wild-type (Wt) T cells (3). More importantly, immunization with high concentrations of myelin basic protein induces experimental autoimmune encephalomyelitis in CD28−/− PL/J mice at similar prevalence and severity as in Wt mice (3). These studies suggest that CD28 costimulation is needed for regulation of the threshold for T cell activation.

The ubiquitination process is regulated by a series of enzymes (termed E1, E2, and E3) which, in cyclic steps, transfer the ubiquitin (Ub) polypeptide to a host protein as a marker for degradation by the 26S proteasome (5–10). The Cbl family of proteins consists of an N-terminal (variant) Src homology 2 (SH2) domain, a RING finger domain, and a C-terminal proline-rich region with potential tyrosine phosphorylation sites. It is known that Cbl functions as an E3 Ub ligase with a RING finger domain that recruits an Ub-conjugating enzyme (E2), and an SH2 domain that recognizes target proteins for Ub conjugation (5, 11–14).

Peripheral T cells from Cbl-b−/− mice show enhanced proliferation and IL-2 production in response to TCR stimulation. Furthermore, the loss of Cbl-b results in an increased susceptibility to the development of autoimmunity (15, 16), supporting the exciting concept that Cbl-b is a key regulator of susceptibility to autoimmunity. Interestingly, loss of Cbl-b restores impaired T cell proliferation in CD28−/− mice (15, 16), suggesting that Cbl-b may be involved in CD28-dependent T cell activation. In support of this idea, Cbl-b has been shown to selectively regulate activity of Vav (15, 16), which we and others have shown can be synergistically activated by CD28 costimulation (17, 18).

The mechanism underlying the negative regulation of TCR signaling by Cbl proteins has recently been described (11, 19, 20). Cbl-b ubiquitinates the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3-K) (19, 21), resulting in its failure to associate with the 26S proteasome (5–10). The Cbl family of proteins consists of an N-terminal (variant) Src homology 2 (SH2) domain, a RING finger domain, and a C-terminal proline-rich region with potential tyrosine phosphorylation sites. It is known that Cbl functions as an E3 Ub ligase with a RING finger domain that recruits an Ub-conjugating enzyme (E2), and an SH2 domain that recognizes target proteins for Ub conjugation (5, 11–14).

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Materials and Methods

Mice and cell line
Female Wt and CD28−/− BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were used for experiments at 6–10 wk of age. Jurkat T cells were provided by Dr. T. L. Delovitch (The John P. Robarts Research Institute, London, Ontario, Canada).

Reagents

Purified and biotinylated anti-mouse CD3 (145-2C11), anti-mouse CD28 (37.51), and biotinylated anti-mouse LFA-1 (M174/7), anti-human CD3 (HT3a), and anti-human CD28 (CD28.2) mAbs were purchased from BD PharMingen (San Diego, CA). Abs against Cbl-b, Ub, c-Cbl, Lck, ZAP-70, Vav, phospholipase C (PLC)-y1, p38 mitogen-activated protein kinase (MAPK), SH2 domain-bearing protein tyrosine phosphatase-1 (SHP-1), PI3-K (p85), His, and hemagglutinin (HA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p44/p42 MAPK was purchased from New England Biolabs (Beverly, MA). T cell enrichment columns were obtained from R&D Systems (Minneapolis, MN). HRP-conjugated goat anti-rabbit IgG or rabbit anti-mouse IgG were purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD). Rabbit anti-hamster IgG, rabbit anti-mouse IgG, and streptavidin were purchased from Sigma-Aldrich (St. Louis, MO).

Plasmids

Cbl-b cDNA encoding full-length Cbl-b with an HA epitope in pcEFL was described previously (22). His6-tagged Ub plasmid was a gift from Dr. D. Bohmann (University of Rochester, Rochester, NY).

Cell culture and transfection

Jurkat T cells were cultured in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FBS and penicillin and streptomycin. To detect Cbl-b ubiquitination in Jurkat T cells, cells were transfected with the HA-tagged Cbl-b and His6-tagged Ub by electroporation (240 V, 960 μF). After 48 h, transfected cells were collected and subjected to stimulation.

T cell isolation and activation

Splenic T cells from naive Wt and CD28−/− mice were isolated (purity ≥95% as determined by FACS analysis of CD3+ cell surface expression) on T cell enrichment columns. For in vitro activation, T cells (1 × 10^6/ml) from naive Wt and CD28−/− mice were stimulated for the various time periods indicated by plate-bound anti-CD3 (2 μg/ml or as indicated) and/or soluble anti-CD28 (1 μg/ml or as indicated) mAbs in RPMI 1640 supplemented with 10 mM HEPES, 0.1 mg/ml streptomycin, 100 U/ml penicillin, 0.05 mM 2-ME, and 2 mM glutamine (all from Life Technologies, Grand Island, NY). For short-term T cell activation, Wt or CD28−/− T cells were incubated with anti-CD3 (2 μg/ml) and anti-CD28 (1 μg/ml) mAbs on ice, followed by cross-linking with rabbit anti-hamster IgG (10 μg/ml). Alternatively, Wt and CD28−/− T cells were incubated for 5 min at 37°C with biotinylated anti-CD3 or biotinylated anti-CD28 (2 μg/ml) and/or soluble anti-CD28 (1 μg/ml) mAbs, and followed by cross-linking for 5 and 15 min at 37°C with streptavidin (10 μg/ml). For stimulation of Jurkat T cells, 2 × 10^4/ml transiently transfected Jurkat T cells were incubated for 30 min on ice with anti-human CD3 (2 μg/ml) and anti-CD28 mAbs, followed by cross-linking for 5, 15, and 30 min at 37°C with rabbit anti-mouse IgG. The cells were lysed in 1% Nonidet P-40 lysis buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EGTA, 50 mM β-glycerophosphate, 2 mM Na3VO4, 10 mM NaF, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotonin) or in radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 0.5% SDS, 100 μM Na3VO4, 1 mM NaF, 1 mM PMSF, 10 μg/ml aprotonin, 10 μg/ml leupeptin) where indicated.

T cell proliferation assay

Splenic T cells (2 × 10^6/ml) from Wt and CD28−/− mice were cultured for 48 h at 37°C in round-bottom 96-well plates precoated with anti-CD3 mAb (0–40 μg/ml). The cells were pulsed with 1 μCi [3H]thymidine, and harvested 16 h later. The radioactivity was quantitated using a Wallac 1205 Betaplate beta liquid scintillation counter (PerkinElmer-Wallac, Gaithersburg, MD).

Immunoprecipitation and Western blot

Protein concentrations in the cell lysates were determined using a bicinechonic acid assay kit (Pierce, Rockford, IL). The conditions for immunoprecipitation and immunoblotting were described previously (18).

Quantitative real-time RT-PCR assays

Total RNA was extracted from cells with TRIzol reagents (Life Technologies, Gaithersburg, MD), and reverse-transcribed using a SuperScript RT kit (Invitrogen, Carlsbad, CA). Real-time RT-PCR was performed using Cepheid Smart Cycler (Cepheid, Sunnyvale, CA) applying SYBR Green I fluorescent dye to detect the PCR product. The following primers were used: forward primer 5′-TTCAGAGGCAAACTCATTG-3′ and reverse primer 5′-TACATCTCTCAGTCCTCTTTTA-3′ for Cbl-b, and the forward primer of 5′-CTGCCCCAGAACATCATCC-3′ and the reverse primer 5′-AGCGATTCTGTCATAC-3′ for GAPDH. Templates were initially denatured at 95°C for 5 min, which was followed by 45 cycles of amplification at 95°C for 40 s, 54°C for 40 s, and 72°C for 40 s for GAPDH. The relative differences among the samples at different time points were determined using the ΔΔCT threshold cycle method as described in the Applied Biosystems protocol for RT-PCR (Applied Biosystems, Foster City, CA).

Results and Discussion

In the absence of CD28 engagement, T cells require very high TCR occupancy and prolonged stimulation, whereas CD28 co-stimulation allows T cells to respond to lower degrees of TCR occupancy (3, 4). CD28 costimulation may not regulate T cell anergy in vivo, but rather it is needed for setting the threshold for T cell activation (3). To further test this hypothesis, Wt and CD28−/− T cells were stimulated for 72 h with increasing amounts of anti-CD3 mAb (0–40 μg/ml), and T cell proliferation was determined by [3H]thymidine incorporation. As shown in Fig. 1, anti-CD3 dose-dependent T cell proliferation was observed in CD28−/− T cells, albeit at lower levels than Wt T cells. This result supports the idea that in the absence of CD28 costimulatory molecules, the threshold for T cell activation is elevated.

Cbl-b is a negative regulator of T cell activation (19, 20). The loss of Cbl-b restores impaired T cell proliferation in CD28−/− mice, suggesting possible involvement of Cbl-b in CD28-dependent T cell activation (15, 16). Furthermore, it has been shown that Cbl-b not only targets other proteins for ubiquitination and degradation, but also itself can undergo ubiquitination and degradation in a proteasome-dependent manner (22). Based upon these findings, we hypothesized that CD28 costimulation may favor ubiquitination and degradation of Cbl-b which sets the threshold for T cell activation. To test this hypothesis, naive Wt T cells were stimulated for 0, 1, 2, 4, 8, and 16 h with anti-CD3 or anti-CD3 plus anti-CD28 mAbs, and lysed. The cell lysates were immunoprecipitated with anti-Cbl-b mAb, and blotted with anti-Ub mAb. Cbl-b ubiquitination occurred within 1–2 h after CD3 stimulation. Ubiquitination of Cbl-b was revealed by the appearance of a smear of anti-Ub immunoreactive species in the Cbl-b immunoprecipitates, indicating that multiple Ub molecules associated with Cbl-b.

FIGURE 1. Dose-dependent T cell proliferation in Wt and CD28−/− mice. T cells (2 × 10^6/ml) from naive Wt and CD28−/− BALB/c mice were stimulated for 72 h with different concentrations of plate-bound anti-CD3 mAb, and T cell proliferation was determined by [3H]thymidine incorporation. Data represent the mean ± SD.
SD costimulation significantly enhanced CD3-induced ubiquitination (Fig. 2A). Cbl-b degradation appeared at 1 h following stimulation, and was more prominent in CD3/CD28-costimulated T cells (Fig. 2A). To confirm this finding, we performed similar experiments using T cells from naive Wt and CD28−/− mice. In the absence of CD28, anti-CD3 stimulation

**FIGURE 2.** Promotion of Cbl-b ubiquitination and degradation by CD28 costimulation. **A,** Wt BALB/c splenic T cells were stimulated for 1, 2, 4, 8, and 16 h with plate-bound anti-CD3 (2 μg/ml) or anti-CD3 and anti-CD28 (1 μg/ml), and lysed. The cell lysates were immunoprecipitated with anti-Cbl-b mAb, and blotted with anti-Ub mAb. The membrane was then stripped and reprobed with anti-Cbl-b mAb. **B,** Splenic T cells from Wt and CD28−/− BALB/c mice were stimulated for 0, 1, 2, 4, and 16 h with plate-bound anti-CD3 mAb, and lysed. Cbl-b immunoprecipitates were blotted with anti-Ub mAb (**upper panel**). The amounts of Cbl-b were confirmed by stripping the membrane and reprobing it with anti-Cbl-b mAb (**middle panel**). The same supernatants were reimmunoprecipitated with anti-c-Cbl Ab, and blotted with anti-c-Cbl Ab (**lower panel**). **C,** Wt BALB/c T cells were stimulated for 0, 0.5, 1, 2, and 4 h with anti-CD3 and anti-CD28 mAbs. Total RNA was isolated from anti-CD3-stimulated T cells, and Cbl-b mRNA expression was determined by real-time RT-PCR. Real-time RT-PCR results were expressed as fold increase or decrease in the levels of Cbl-b mRNA compared with the 0-min control sample, after correction for loading differences. **D,** Wt BALB/c T cells were incubated for 30 min with anti-CD3 (2 μg/ml) and anti-CD28 (1 μg/ml) on ice, followed by cross-linking for 1, 5, 15, 30, and 60 min at 37°C with rabbit anti-hamster IgG (10 μg/ml), and lysed in RIPA buffer. The cell lysates were immunoprecipitated with anti-Cbl-b or control mouse IgG, and blotted with anti-Ub mAb. The membrane was stripped and reprobed with anti-Cbl-b mAb. **E,** Jurkat T cells, transiently transfected with HA-tagged Cbl-b and His6-tagged Ub, were incubated with anti-CD3 (2 μg/ml) and anti-CD28 (1 μg/ml), followed by cross-linking with rabbit anti-mouse IgG (10 μg/ml), and lysed. The cell lysates were immunoprecipitated with anti-Cbl-b, and blotted with anti-Ub mAb. **G,** Wt and CD28−/− T cells were incubated for 5 min with biotinylated anti-CD3 (2 μg/ml) or biotinylated anti-CD3 plus biotinylated anti-LFA-1 (2 μg/ml) followed by cross-linking for 5 and 15 min with streptavidin (10 μg/ml), and lysed. The cell lysates were immunoprecipitated with anti-Cbl-b, and blotted with anti-Ub mAb.
induced only minor ubiquitination and degradation of Cbl-b (Fig. 2B). The reduced amounts of Cbl-b in Wt T cells did not result from a suppression of its transcription, as the levels of Cbl-b mRNA were not down-regulated throughout the culture periods (Fig. 2C). These data suggest that Cbl-b may primarily be controlled at the posttranslational level. Note that TCR-induced degradation of Cbl-b is 26S proteasome-dependent as Cbl-b degradation was abrogated by lactacystin treatment (data not shown). Collectively, these results suggest that CD28 costimulation controls Cbl-b ubiquitination and degradation.

To examine whether ubiquitination and degradation of Cbl-b could occur at earlier time points following TCR ligation, Wt T cells were incubated for 30 min with anti-CD3 and anti-CD28 on ice, followed by cross-linking for 1, 5, 15, 30, and 60 min at 37°C with rabbit-anti-hamster IgG, and lysed. Ubiquitination and degradation of Cbl-b was detected as above. As shown in Fig. 2D, ubiquitination of Cbl-b occurred between 5 and 15 min following CD28 costimulation. Reprobing of the membrane with anti-Cbl-b mAb showed that Cbl-b degradation occurred at the same time kinetics to Cbl-b ubiquitination. Note that anti-Cbl-b immunoblotting also detected the same high molecular blot probe with anti-Ub mAb. This result suggests that Cbl-b ubiquitination observed did not represent ubiquitination of associated proteins. Similar results (data not shown) were also obtained using RIPA buffer which disrupts protein complexes (23), further excluding the possibility that Cbl-b ubiquitination may result from its binding proteins that are ubiquitinated in response to CD28 costimulation. CD28 costimulation-promoted Cbl-b ubiquitination was further confirmed by coexpression of Jurkat T cells with Cbl-b and Ub which resulted in a significant down-regulation of Cbl-b expression in CD28 costimulated Jurkat T cells (Fig. 2E).

It is possible that many proteins may undergo ubiquitination upon CD28 costimulation, and that Cbl-b ubiquitination and degradation may not be unique for CD28 costimulation. To exclude this possibility, several signaling molecules involved in TCR- and CD28-signaling pathways were detected for their ubiquitination and degradation. No ubiquitination of Lck, ZAP-70, SHP-1, p44/p42 MAPK, p38 MAPK, PLC-γ1, Vav, and c-Cbl was observed upon CD28 costimulation (data not shown). Consistent with this result, no decreases in the amounts of Lck, ZAP-70, SHP-1, p44/p42 MAPK, p38 MAPK, PLC-γ1, Vav, and c-Cbl were detected within 2 h following CD28 costimulation (Fig. 2F). It is also possible that Cbl-b ubiquitination can be induced by coligation of other coreceptors. To test this, Wt and CD28−/− T cells were incubated with biotinylated anti-CD3 and biotinylated anti-CD3 plus biotinylated anti-LFA-1 mAbs followed by cross-linking with streptavidin, and lysed in RIPA buffer. Cbl-b ubiquitination was determined. Costimulation of Wt T cells with LFA-1 did not enhance CD3-induced Cbl-b ubiquitination. Consistent with this observation, there was no Cbl-b ubiquitination observed in CD28−/− T cells upon LFA-1 costimulation (Fig. 2G). These data indicate that Cbl-b ubiquitination is not shared by other costimulatory receptor(s). Taken together, our results indicate that Cbl-b ubiquitination and degradation is a unique phenomenon for CD28 costimulation.

To confirm that CD28-mediated Cbl-b ubiquitination regulates the threshold for T cell activation, naive Wt T cells were first stimulated for 1 h with constant amounts of anti-CD3 in the presence of varying concentrations of anti-CD28 mAb. The cells were lysed, immunoprecipitated with anti-Cbl-b mAb, and then blotted with anti-Ub mAb. Stimulation by anti-CD3 together with increasing concentrations of anti-CD28 mAb induced a dose-dependent enhancement of ubiquitination of Cbl-b in Wt T cells (Fig. 3). In support of this result, stimulation with higher concentrations of anti-CD3 mAb enhanced ubiquitination of Cbl-b in CD28−/− T cells (Fig. 3), which correlated with an anti-CD3 dose-dependent increase in T cell proliferation (Fig. 1). This result confirmed that triggering TCR with very high amounts of anti-CD3 could, in part, overcome the requirement of CD28 costimulation for Cbl-b ubiquitination.

It has been shown that PI3-K may be an upstream regulator of Vav (24). Cbl-b constitutively associates with PI3-K (15, 21), targets the p85 regulatory subunit of PI3-K for ubiquitination (19, 21), and then negatively regulates the recruitment of p85 to CD28 and TCRζ in a proteolysis-independent manner (21). These observations suggest that inactivating Cbl-b by its ubiquitination induced by TCR stimulation may result in a decrease in PI3-K ubiquitination. To test this, Wt and CD28−/− T cells were incubated with anti-CD3, cross-linked with rabbit anti-hamster IgG for 1, 5, 15 and 30 min, and lysed in RIPA buffer. The cell lysates were immunoprecipitated with anti-PI3-K (p85), and blotted with anti-Ub. Interestingly, the highest level of PI3-K ubiquitination was observed in both Wt and CD28−/− T cells without stimulation (Fig. 4). After anti-CD3 stimulation, PI3-K ubiquitination gradually declined in Wt T cells, but this phenomenon was much less evident in CD28−/− T cells (Fig. 4). Taken together, these data indicate that Cbl-b may play an important role in the sequestration of PI3-K from CD28 and TCRζ.

In this study, we show that the threshold for T cell activation is regulated by CD28 costimulation, and this is in part achieved by targeting Cbl-b for ubiquitination and degradation. These findings provide an explanation of how CD28 costimulation eliminates the negative regulator of T cell activation, Cbl-b, thus leading to optimal T cell activation.

**FIGURE 3.** Dose-dependent ubiquitination of Cbl-b in Wt and CD28−/− T cells. Wt T cells were stimulated for 1 h with constant anti-CD3 (2 μg/ml) with various concentrations of anti-CD28 (0–16 μg/ml), and lysed. The cell lysates were immunoprecipitated with anti-Cbl-b, and blotted with anti-Ub mAb. CD28−/− T cells were stimulated for 1 h with increasing concentrations of anti-CD3 (0–40 μg/ml), and lysed. Cbl-b ubiquitination was detected as above.

**FIGURE 4.** PI3-K ubiquitination in Wt and CD28−/− T cells. Wt and CD28−/− T cells were incubated for 1, 5, 15, and 30 min with anti-CD3, and cross-linked with rabbit anti-hamster IgG. The cells were lysed, and cell lysates were immunoprecipitated with anti-p85, and blotted with anti-Ub mAb.
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