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Cbl-b Is a Negative Regulator of Inflammatory Cytokines Produced by IgE-Activated Mast Cells

Sonja E. Gustin,2 Christine B. F. Thien, and Wallace Y. Langdon3

c-Cbl and Cbl-b E3 ubiquitin ligases are abundantly expressed in hemopoietic cells where they negatively regulate the activity and levels of many cell surface receptors and associated signaling molecules. By comparing bone marrow-derived mast cells from c-Cbl and Cbl-b-deficient mice it has recently been shown that Cbl-b is the dominant family member for negatively regulating signaling responses from high-affinity IgE receptors. In this study, we suggest that a possible reason for the greater enhancement of IgE receptor signaling in Cbl-b-deficient mice is the relatively higher levels of Cbl-b protein over c-Cbl in mast cells compared with other hemopoietic cells. We also directly compare mast cells from c-Cbl and Cbl-b-deficient mice and find that loss of Cbl-b, but not c-Cbl, increases cell growth, retards receptor internalization, and causes the sustained tyrosine phosphorylation of Syk and its substrates. However, loss of Cbl-b does not enhance the activation of ERK or Akt, nor does it promote a greater calcium response. Furthermore, loss of Cbl-b or c-Cbl does not increase levels of the Syk or Lyn protein tyrosine kinases. Most notable, however, is the extremely large increase in the production of proinflammatory cytokines TNF-α, IL-6, and MCP-1 by Cbl-b−/− mast cells compared with levels produced by c-Chl−/− or wild-type cells. This marked induction, which appears to be restricted to these three cytokines, is dependent on IgE receptor activation and correlates with enhanced Iba kinase phosphorylation. Thus, Cbl-b functions as a potent negative regulator of cytokinies that promote allergic and inflammatory reactions. The Journal of Immunology, 2006, 177: 5980–5989.

The activation of mast cells and basophils by IgE is regarded as a key effector mechanism for acute allergic reactions such as anaphylaxis and attacks of asthma. These reactions are triggered by Ag-induced cross-linking of IgE Abs that bind to high-affinity FcRs for IgE (FcεRII) on the surface of mast cells and basophils. FcεRII aggregation activates signaling cascades that promote many cellular effects including the release of preformed mediators of allergic reactions, the synthesis of proinflammatory lipid mediators, and the synthesis and secretion of cytokines, growth factors, and chemokines (reviewed in Refs. 1–3).

FcεRI does not possess intrinsic enzymatic activity but cross-linking with IgE and Ag does induce the activation of cytoplasmic protein tyrosine kinases (PTKs) of the Src, Syk, and Tec families. The initiating PTK is the Src family member Lyn, which is constitutively associated with the β subunit of FcεRI. Following activation, Lyn phosphorylates tyrosine residues in the ITAMs of the β and γ subunits of the receptor which can then recruit Syk and additional Lyn molecules through interactions with their Src homology 2 (SH2) domains. Syk is then activated by Lyn, or newly activated Syk, enabling it to phosphorylate many downstream substrates. These Syk substrates include linker for activation of T cells (LAT), SH2-containing leukocyte protein of 76 kDa (SLP-76), non-T cell activation linker (linker for activation of B cells (LAB)), and Vav which activate pathways involving phospholipase Cγ (PLCγ), Rac, Ras, JNK, and p38 (4–8). In addition, the adaptor protein Grb2-associated-binder-2 (Gab2) has also been identified as a key PTK substrate that functions as the principal activator of the Plk pathway in response to FcεRI engagement (9). Interestingly, Gab2 is phosphorylated by the Fyn kinase which is required for Akt activation and complete mast cell degranulation (10). Together, these signaling pathways contribute to inducing the release of preformed histamine and the subsequent synthesis of multiple cytokines with proinflammatory and immunoregulatory properties. Although many of these cytokines have protective roles in the clearance of bacteria and parasites, the overproduction of proinflammatory cytokines such as TNF-α, TGFβ1, IL-6, and MCP-1 can also mediate damaging effects by promoting allergic and inflammatory reactions (reviewed in Refs. 2 and 11).

The pivotal role of Syk in FcεRI signaling was first established by studies with the Syk-selective tyrosine kinase inhibitor piceatannol that effectively abolished Syk activation and IgE-mediated downstream responses without affecting Lyn (12). The role of Syk was further established by examining mast cells from fetal liver of Syk knockout mice and Syk-deficient RBL-2H2 cells, both of which show no evidence of FcεR-mediated signaling (13, 14). Thus the targeting of Syk provides a plausible strategy to suppress the pathogenic effects of allergic and inflammatory responses. A family of proteins that have emerged as candidates for suppressing the activity of Syk are the Cbl family of E3 ubiquitin ligases which have been found to direct the ubiquitylation of Syk (15–17). Cbl proteins possess E3 ubiquitin ligase activity by virtue of a RING
(really interesting new gene) finger domain that recruits ubiquitin-conjugating enzymes (E2s) thus allowing ubiquitin molecules to be transferred to substrates targeted by Cbl (18, 19). Cbl proteins also possess numerous protein-protein interaction domains that serve to identify Cbl-specific substrates for ubiquitylation and also recruit additional signaling molecules to activated receptor complexes (20-22).

The mammalian Cbl family consists of three homologs, c-Cbl, Cbl-b, and Cbl-3, and interestingly in mast cells it is Cbl-b that has emerged as the dominant Cbl protein that negatively regulates FcεRI signaling (23–25). This is an intriguing finding because Cbl-b and c-Cbl are very similar proteins and in vitro studies have shown that c-Cbl can function as an effective negative regulator of Syk in RBL-2H3 cells and B cells (16, 17, 26). However, the relative abundance of c-Cbl and Cbl-b in mast cells is not known and this may be a crucial factor in determining the more potent regulatory role of Cbl-b. Indeed, c-Cbl and Cbl-b show distinct roles in thymocytes and peripheral T cells, respectively, and this distinction appears to be largely due to their relative abundance in each cell type, i.e., in thymocytes, c-Cbl protein levels are markedly more abundant than Cbl-b and vice versa for peripheral T cells (27).

In this study, we directly compare the roles of these two proteins in regulating FcεRI signaling using bone marrow-derived cultured mast cells (BMMCs) from wild-type (wt), Cbl-b and c-Cbl-deficient mice. As recently shown by Zhang et al. (23), we find that FcεRI signaling is enhanced in BMMCs from the Cbl-b knockout mouse and that this enhancement appears to stem from a sustained activation of Syk. We also find that Cbl-b-deficient BMMCs have a block to FcεRI internalization following Ag cross-linking, and that the amount of Cbl-b protein relative to c-Cbl is markedly higher in BMMCs compared with other hematopoietic cells. Most strikingly, we show that Cbl-b is a potent negative regulator for the production of TNF-α, IL-6, and MCP-1 from BMMCs in response to FcεRI activation.

Materials and Methods

Cell culture and Abs

Tissue culture flasks were obtained from Nunc and tissue culture medium and supplements from ThermoTrace. Reconstituent mouse IL-3 was a gift from Dr. I. Young (John Curtin School of Medical Research, Canberra, Australia) and DNP-BSA was obtained from Molecular Probes. Affinity purified mouse anti-DNP IgE was a gift from Drs. J. Oliver and B. Wilson and has been previously described (28). Rabbit anti-LAT was a gift from Dr. L. Samelson (National Institutes of Health, Bethesda, MD) and rabbit anti-Syk (sc-1077), anti-Lyn (sc-15), anti-ERK 1 (sc-94), and mouse anti-c-Cbl (05–440) was purchased from Upstate Cell Signaling Solutions (Lake Placid, NY). Rabbit anti-c-Cbl (05–440) was purchased from Santa Cruz Biotechnology. Mouse anti-c-Kit was used from Stock No. 000617, a gift from Dr. A. W. F. lists (Rockefeller University, New York), and mouse anti-Syk (SYK-01) was obtained from Alexis Biochemicals. Mouse anti-phosphophoryosine (4G10) was a gift from Dr. B. Druker (Oregon Health Sciences University, Portland, OR) and Abs to Akt, phospho-Akt (Ser473), phospho-p42/44 MAPK (Thr202/Tyr204), phospho-LAT (Y191), IκB kinase (IKK) α, and phospho-IKK-κ (Ser181/183) were obtained from Cell Signaling Technology. Mouse monoclonal anti-actin (A 4700) was purchased from Sigma-Aldrich. FITC-conjugated rat anti-mouse IgE, FITC-conjugated rat anti-mouse c-Kit (CD117), biotinylated rat anti-mouse IL-3 receptor α-chain, and PE-conjugated streptavidin were purchased from BD Pharmingen.

Mice and bone marrow-derived mast cells

The generation of c-Cbl and Cbl-b deficient mice has previously been described (29, 30) and all experiments were performed in accordance with the Animal Ethics Committee at the University of Western Australia (approval 03/100/275). Normal control mice were obtained from our C57BL/6J × 129/Sv background. Bone marrow cells from 8- to 12-wk-old mice were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS (Invitrogen Life Technologies), 50 μM 2-ME, 2 mM l-glutamine, and rIL-3 at 37°C in 5% CO2. The cells were split weekly into fresh medium and tissue culture flasks at 5 × 105 cells/ml. By wk 4 of culture, >97% of the cells expressed uniformly high levels of FcεRI.

Mast cell stimulation and immunoblotting

BMMCs were stimulated by firstly priming with 1 μg/ml IgE anti-DNP for 4–6 h followed by washes and resuspension in Tyrode’s buffer (10 mM HEPES, pH 7.4, 130 mM NaCl, 1 mM MgCl2, 1 mM KCl, 5.6 mM glucose, and 0.1% BSA) at 105 cells/ml. The Ab-primed BMMCs were activated by the addition of 10 ng/ml DNP-BSA and incubation at 37°C over a range of times. The BMMCs were lysed in n-octyl-p-D-glucopyranoside/Nondenit P-40 lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1 mM sodium orthovanadate, 0.2% Nonidet P-40, 60 mM n-octyl-p-D-glucopyranoside (Sigma-Aldrich), 10 μg/ml aprotinin, 1 mM sodium orthovanadate, 10 mM NaF, and 1 μg/ml each of chymostatin, leupeptin, and pepstatin). After incubating for 15 min on ice, lysates were cleared by centrifugation at 3000 × g for 8 min. Cleared lysates were analyzed by immunoblotting as previously described (31). Quantification of protein bands was determined by densitometric analysis of scanned x-ray films (Canon CanoScan LiDE20) using Kodak Molecular Imaging Software version 4.0 (Eastman Kodak).

Cytokine assays

BMMCs were primed with IgE anti-DNP as described above, washed in complete medium then cultured with 10 ng/ml DNP-BSA for the indicated times. In some experiments, anti-FcεRI Ab (BD Pharmingen) was added at 10 μg/ml at the time of DNP-BSA stimulation. Supernatants were assayed for cytokine production using Cytometric Bead Array kits for Th1/Th2 and inflammatory cytokines according to the manufacturer’s recommendations (BD Biosciences). The beads were analyzed using a FACSCalibur flow cytometer and the data was analyzed using BD Biosciences CBA software.

Flow cytometry

Cell surface expression of FcεRI, c-Kit, and IL-3R α-chain were analyzed by FACScalibur or FACSCount flow cytometers (BD Biosciences). Cells were harvested 6 h after stimulation. Before fixation and permeabilization, FcεRI levels, the cells were primed with anti-DNP IgE as described above and IgE binding was detected with an FITC-conjugated rat anti-mouse IgE (BD Pharmingen). Cells were collected using CellQuest software (BD Biosciences) and further analyzed using FlowJo (Tree Star). To assay for FcεRI internalization, BMMCs were primed with IgE anti-DNP and stimulated with 10 ng/ml DNP-BSA at 37°C as described above. Receptor internalization was terminated over a range of times by adding 2 ml of ice-cold FACS buffer (PBS/1% FCS with 0.5 mg/ml NaNO3). Surface FcεRI levels at each time point were determined by staining with FITC-conjugated rat anti-mouse IgE and flow cytometry. To detect intracellular levels of Syk and Lyn, the cells were fixed and permeabilized in Cytofix/Cytoperm (BD Pharmingen) according to the manufacturer’s directions. Following incubation with Abs, the cells were washed in medium containing 0.1% saponin and the unbleached Abs detected with FITC-conjugated goat anti-rabbit IgG (BD Pharmingen). To detect intracellular levels of IL-6 and TNF-α, the cells were treated as above and stained with FITC-labeled anti-mouse IL-6 and TNF-α (eBioscience).

Intracellular calcium analysis

Intracellular calcium concentrations were analyzed by flow cytometry using BMMCs loaded with Fluo-4 (Molecular Probes) and primed with anti-DNP IgE Abs. Cells were analyzed for 55 s before addition of 10 ng/ml DNP-BSA to cross-link receptor-bound Abs. Data was collected from stimulated BMMCs for a further 6 min at room temperature and analyzed using Flow cytometry using BMMCs from wild-type (wt), Cbl-b and c-Cbl-deficient mice. As recently shown by Zhang et al. (23), we find that FcεRI signaling is enhanced in BMMCs from the Cbl-b knockout mouse and that this enhancement appears to stem from a sustained activation of Syk. We also find that Cbl-b-deficient BMMCs have a block to FcεRI internalization following Ag cross-linking, and that the amount of Cbl-b protein relative to c-Cbl is markedly higher in BMMCs compared with other hematopoietic cells. Most strikingly, we show that Cbl-b is a potent negative regulator for the production of TNF-α, IL-6, and MCP-1 from BMMCs in response to FcεRI activation.

Results

Growth advantage of Cbl-b−/− BMMCs

Fig. 1A shows the growth characteristics of cultures of BMMCs derived from wt, c-Cbl−/−, and Cbl-b−/− mice. The graphs represent the cell concentrations at the end of each week following reseeding of the cultures at a concentration of 5 × 104 cells/ml. In each of the four culture groups, the Cbl-b−/− BMMCs consistently showed a greater proliferative capacity than either the wt or c-Cbl−/−-derived cells. The BMMCs were also analyzed for the surface expression of FcεRI, c-Kit, and IL-3R α-chain (Fig. 1B)
and no enhancement was observed in either of the two Cbl mutants. The expression levels shown in Fig. 1A are from 29-day-old cultures and the levels of these receptors did not vary at later time points or with other cultures established during the study.

BMMCs from Cbl-b−/− mice show enhanced phosphotyrosine signaling following FcεRI stimulation
To investigate signaling responses following Ag stimulation of FcεRIs, we initially determined the concentration of DNP-BSA that would most effectively initiate a strong response from PTKs. This was determined by immunoblotting total lysates with anti-phosphotyrosine Abs from wt and c-Cbl−/− BMMCs stimulated with a range of concentrations of DNP-BSA for 2 min at 37°C. As shown in Fig. 2A, 10 ng/ml gave the strongest signal in both groups. It was also evident that the c-Cbl−/− BMMCs responded more strongly than wt cells. To further investigate this increased phosphotyrosine signal, we compared wt, c-Cbl−/−, and Cbl-b−/− BMMCs stimulated with 10 ng/ml DNP-BSA for times ranging from 2 to 20 min. As shown in Fig. 2B, at 2 and 5 min after stimulation BMMCs from c-Cbl and Cbl-b knockout mice showed equivalent enhancements in their phosphotyrosine signals compared with wt cells. The proteins that showed the largest increases in tyrosine phosphorylation are indicated and have molecular weights equivalent to SLP-76, Syk, Lyn, LAT, and LAB. At the later time points of 10 and 20 min, it was evident however that the anti-phosphotyrosine signal from Cbl-b−/− BMMCs was more sustained than those derived from c-Cbl-deficient mice (Fig. 2B).

Indeed, by 20 min the signal from c-Cbl−/− BMMCs had returned to a level equivalent to that of wt cells.

Syk and Lyn levels are not enhanced in Cbl-b−/− mast cells
Because Cbl proteins have been well-characterized as E3 ligases for both Syk and Src family kinases, we investigated whether the enhanced and persistent PTK signal in BMMCs from Cbl-b−/− mice was linked to increased levels of Syk and/or Lyn. Levels of both PTKs were initially analyzed by flow cytometry of fixed and permeabilized BMMCs and were found to be expressed at equivalent levels among the three groups (Fig. 2C). Thus, the enhanced phosphotyrosine signal in Cbl-b−/− cells is not due to higher levels of these PTKs. Further analysis by flow cytometry at 1, 2, 4, and 8 h after antigenic stimulation did not reveal changes in Syk or Lyn levels among the three groups (data not shown). The levels of Syk and Lyn were also examined by immunoblotting and in contrast to quantitation by flow cytometry the level of p72 Syk was found to be less in some unstimulated Cbl-b−/− BMMCs (Fig. 2D). Surprisingly, the level of p72 Syk in Cbl-b−/− cells increased following antigenic stimulation to a level similar to that expressed by wt and c-Cbl−/− cells. Although this was observed in three of five separate Cbl-b deficient cultures this phenomenon was not restricted to these cultures as it also occurred in one of the wt cultures (data not shown). This effect was detected with both rabbit polyclonal (Fig. 2D, upper panel) and mouse monoclonal anti-Syk Abs (lower panel), and when examined for lower molecular mass forms we found increased levels of a 30 kDa anti-Syk-reactive protein (Fig. 2D). Thus, the equivalent levels of Syk detected by flow cytometry are likely to represent the sum total of both forms. Interestingly, the effect was not evident if cells were lysed directly into SDS sample buffer suggesting that the time in Nonidet P-40 lysis buffer (usually 15–20 min), before the addition of sample buffer, may be sufficient to permit Syk proteolysis. At present, we are unable to explain the mechanism for the increase in the level of p72 Syk in some cells following antigenic stimulation, nor the significance of the 30-kDa protein, but it is clear that the degradation can be prevented by FcεRI activation.

It was also evident that the retarded electrophoretic mobility of p72 Syk was prolonged in Cbl-b−/− cells following antigenic stimulation (compare the three tracks at 20 min after stimulation in Fig. 2D) providing a further indication that the loss of Cbl-b results in sustained activity of Syk. The Syk immunoblots also revealed the
presence of higher molecular forms of Syk that were induced following antigenic stimulation, and that these were most prominent at the early time points of 2 and 5 min (Fig. 2D). This stimulation-induced modification is indicative of Syk ubiquitylation and it is notable that the effect was most prominent in wt cells and was markedly reduced in Cbl-b-deficient cells. This suggests that Cbl-b is necessary for the ubiquitylation of Syk in primary mast cell cultures.

The enhanced PTK signal in Cbl-b−/− BMMCs does not result in greater ERK or Akt activation or calcium mobilization

To determine whether the enhanced phosphotyrosine signal in Cbl-b−/− BMMCs has downstream consequences on the Ras and PI3K pathways, we examined the levels of activation of ERK and Akt in these cultures. A comparison of the extent of Akt and ERK phosphorylation over a 20 min period following receptor aggregation revealed no differences in ERK phosphorylation among the three genotypes and a less intense phospho-Akt signal was evident in both c-Cbl−/− and Cbl-b−/− cells compared with wt cells (Fig. 3A). Thus, the FceRI signal that enhances and prolongs the activation of Syk in Cbl-b-deficient mast cells is not transduced into higher intensity ERK or Akt signals. Indeed, the less intense phospho-Akt signal indicates that the Fyn/Gab2 pathway is not negatively regulated by Cbl proteins and provides further evidence that Cbl proteins play a positive role in the activation of the PI3K/Akt pathway (32–36). Furthermore, we found no change in Gab2 levels in either c-Cbl or Cbl-b deficient cultures (data not shown) even though overexpression of Cbl-b targeted to lipid rafts has been shown to markedly reduce Gab2 levels (24). In contrast, LAT phosphorylation, which is a measure of Syk activity, was enhanced and sustained in the Cbl-b−/− cultures. The extent of this enhancement was quantified by densitometry and the ratio of phospho-LAT to LAT for each sample is shown in Fig. 3A.

Because the Lyn/Syk/LAT pathway is also required for the induction of calcium flux following FceRI activation, we examined whether the sustained phosphorylation of LAT in Cbl-b−/− mast cells affected the extent to which intracellular calcium is mobilized. As with the ERK signal, we found no enhancement of an FceRI-induced calcium response in either Cbl-b or c-Cbl-deficient mast cells (Fig. 3B). This was not due to the calcium response
Cytometry were able to detect higher levels of calcium flux induced by the fluo-4 fluorescence was monitored by flow cytometry. The settings on the flow cytometer were able to detect higher levels of calcium flux induced by Fluo-4 concentration as indicated by Fluo-4 DNP and stimulation was induced by addition of DNP-BSA. The kinetics of changes in intracellular calcium concentration was 2.1-fold more receptor retained on the surface of Cbl-b and 30 min time points. Indeed, after 30 min of stimulation there was no significant differences were observed among the three groups among the three groups following IgE priming for 5 h (Fig. 4). Quantitation of the loss of surface receptor at this time was retared in the Cbl-b BMMCs (Fig. 4, compare 10, 20, 30 min time points). Following 2 min of exposure to DNP, a large proportion of the receptors were found to be internalized, however, no significant differences were observed among the three groups following IgE priming for 5 h (Fig. 4). Quantitation of the loss of surface receptor at this time was 2.1-fold more receptor retained on the surface of Cbl-b cells compared with wt cells, and 1.5-fold more than c-Cbl cells. The analysis was continued for 50 min however no further internalization was observed beyond 30 min for each of the three groups (data not shown).

**FcεRI internalization is retarded in Cbl-b<sup>−/−</sup> BMMCs**

The role of Cbl proteins in directing the internalization of activated receptor complexes, particularly receptor tyrosine kinases, is well-documented and involves synergy between Cbl’s E3 ligase activity and interactions with adaptors such as Grb2 and CIN85 (19, 37–40). Indeed, it has recently been shown that overexpression of CIN85 in RBL-2H3 cells accelerates FcεRI internalization and sorting into early endosomes (41). To determine whether rates of Ag-induced internalization of FcεRI are altered in Cbl knockout mice, BMMCs were primed with IgE anti-DNP and cross-linked with DNP-BSA for a range of times. Consistent with the results in Fig. 1B, no marked differences in receptor levels were evident among the three groups following IgE priming for 5 h (Fig. 4, unstimulated panel). Following 2 min of exposure to DNP, a large proportion of the receptors were found to be internalized, however, no significant differences were observed among the three groups (Fig. 4). Quantitation of the loss of surface receptor at this time point, as determined by geometric mean fluorescence, revealed a 64% decrease in receptor levels for wt, 69% for c-Cbl<sup>−/−</sup>, and 64% for Cbl-b<sup>−/−</sup> BMMCs. However, at later time points following antigenic stimulation it was evident that receptor internalization was retarded in the Cbl-b BMMCs (Fig. 4, compare 10, 20, and 30 min time points). Indeed, after 30 min of stimulation there was 2.1-fold more receptor retained on the surface of Cbl-b<sup>−/−</sup>

**Chl-b protein is more highly expressed in BMMCs than other hemopoietic cells**

Numerous cell culture systems studying the overexpression of c-Cbl and Cbl-b have shown that both proteins have equivalent capacities to function as E3 ubiquitin ligases (reviewed by Refs. 20 and 22). It was therefore unlikely that the differences in cell growth and signaling between the BMMCs derived from the two mutant mice were a consequence of functional variations caused by minor sequence divergences. To test whether variations in protein levels may explain the phenotypic differences, immunoblots were analyzed to compare the levels of Cbl-b and c-Cbl in BMMCs, thymocytes, spleen cells, and lymph node cells (Fig. 5). In this experiment, total cell lysates were prepared at 10<sup>7</sup>/ml for each of the BMMC samples and at 2 × 10<sup>7</sup>/ml for thymocytes, spleen, and lymph node cells. Immunoblotting with mouse monoclonal anti-Cbl-b and anti-c-Cbl Abs indicated comparatively higher levels of Cbl-b protein in BMMCs compared with the other cell types examined. On a cell-to-cell basis, the protein levels of Cbl-b were

**FIGURE 3.** A, Cbl-b<sup>−/−</sup> mast cells show enhanced and sustained phosphorylation of LAT but not ERK or Akt. IgE-primed wt (+/+), c-Cbl<sup>−/−</sup> (c<sup>−/−</sup>), and Cbl-b<sup>−/−</sup> (b<sup>−/−</sup>) mast cells were left unstimulated or activated with DNP-BSA for the times shown above. Cell lysates were analyzed by immunoblotting with the indicated phosphospecific Abs then reprobed with the corresponding protein-specific Abs. B, Calcium mobilization is not enhanced in Cbl knockout BMMCs. Fluo-4-loaded BMMCs from wt (+/+), c-Cbl<sup>−/−</sup> (c<sup>−/−</sup>), and Cbl-b<sup>−/−</sup> (b<sup>−/−</sup>) mice were primed with IgE anti-DNP and stimulation was induced by addition of DNP-BSA. The kinetics of changes in intracellular calcium concentration was indicated by Fluo-4 fluorescence was monitored by flow cytometry. The settings on the flow cytometer were able to detect higher levels of calcium flux induced by the addition of 1 μg/ml ionomycin to the cells.
FIGURE 5. Cbl-b protein is highly expressed BMMCs. Total cell lysates were prepared at 1 × 10^7/ml for each of the BMMC samples, and at 2 × 10^7/ml for thymocytes, spleen, and lymph node cells. The lysates were examined by immunoblotting with either anti-Cbl-b, anti-c-Cbl, or anti-actin mouse mAbs. The anti-Cbl-b and c-Cbl immunoblots were exposed on x-ray film for 20 s and the densitometry intensities are expressed relative to spleen within each blot after adjusting to equalize actin levels in each corresponding cell lysate. Note that lysates were prepared on the basis of cell concentrations rather than total protein levels therefore thymocytes, which have very little cytoplasm, have low actin levels whereas larger BMMCs have higher actin levels.

Cbl-b−/− BMMCs show increased production of inflammatory cytokines TNF-α, IL-6, and MCP-1

To determine whether the sustained FcεRI signaling observed in Cbl-b−/− BMMCs affects cytokine production, cells from each of the three groups were primed with IgE anti-DNP and subsequently cultured in the presence of DNP-BSA for 16 h. Analysis of the supernatants for the production of inflammatory cytokines by Cytometric Bead Array assay revealed that antigenic stimulation induced markedly elevated levels of TNF-α, MCP-1, and IL-6 from Cbl-b−/− BMMCs compared with wt and c-Cbl−/− cells (Fig. 6A). This effect was specific for these three cytokines because there were minimal effects on the production of the other inflammatory cytokines IFN-γ, IL-10, and IL-12 (data not shown). An analysis of Th1/Th2 cytokine production revealed low levels of induction of IL-2, IL-4 and IL-5 which were only moderately elevated in Cbl-b−/− cultures compared with wt and c-Cbl−/− (data not shown).

An examination of inflammatory cytokine production at the earlier time point of 4 h after DNP-BSA stimulation revealed the same pattern with very high levels of induction for TNF-α, MCP-1, and IL-6 from Cbl-b−/− BMMCs compared with wt and c-Cbl−/− cells (Fig. 6B). The release of high levels of cytokines at 4 h prompted us examine cytokine release at earlier times following antigenic stimulation. As shown in Fig. 6C, higher levels of TNF-α, MCP-1 and IL-6 were released into the supernatant from Cbl-b−/− BMMCs as early as 15 min after stimulation, but the most notable enhancements were observed after 60 and 120 min. The greater release of TNF-α and IL-6 from Cbl-b−/− cells was not due to higher levels of preformed cytokines as intracellular staining showed these to be at low and equivalent levels in unstimulated cells from the three culture groups (data not shown). This suggests that enhanced FcεRI signaling in Cbl-b−/− BMMCs is the stimulus responsible for directing higher levels of newly synthesized proinflammatory cytokines.

The induction of IL-6 and MCP-1 is not dependent on TNF-α

TNF-α production can promote its own induction and also the transcription of MCP-1 and IL-6, which primarily occurs at the level of initiation through NF-κB (43–45). Thus to test whether the high levels of MCP-1 and IL-6 produced by Cbl-b−/− mast cells are dependent on an autocrine loop driven by TNF-α we incubated DNP-stimulated mast cells in the presence or absence of neutralizing TNF Abs. As shown in Fig. 7A and B, incubation with anti-TNF Abs was effective in neutralizing TNF-α but had no detectable effect in reducing the levels of MCP-1 or IL-6. The flow cytometry profiles from the Cytokine Bead Array analysis for the Cbl-b−/− cultures are shown in Fig. 7A and these profiles illustrate that MCP-1 and IL-6 production are not affected by the neutralizing TNF Abs. The flow cytometry profiles for the Cbl-b−/− cultures also demonstrate that FcεRI cross-linking does not induce high levels of IL-10, IFN-γ, or IL-12p70 (Fig. 7A). We also measured the levels of TNF-α, MCP-1, and IL-6 produced by cultures of each genotype 4 h after DNP stimulation, with or without the addition of anti-TNF, and this clearly illustrated that MCP-1 and IL-6 do not require TNF-α for their induction (Fig. 7B).

Enhanced activation of IKK in Cbl-b-deficient mast cells

Many proinflammatory cytokine genes expressed in mast cells are regulated by NF-κB, and all three cytokines that are markedly elevated in Cbl-b−/− mast cells, i.e., TNF-α, IL-6, and MCP-1, contain NF-κB elements in their promoters (43, 46, 47). Indeed, the production of TNF-α and IL-6 in response to FcεRI cross-linking is strictly dependent on IKK and NF-κB activity (48–50).

To examine whether the greater production of these cytokines by Cbl-b−/− mast cells is linked to an enhancement of the NF-κB pathway, we analyzed the activation of the IKK complex in DNP-stimulated BMMCs by immunoblotting with a phospho-IKK Abs. As shown in Fig. 7C, the phosphorylation of IKK was more evident in Cbl-b−/− cells. This finding indicates that a high intensity signal that activates NF-κB may be responsible for directing the greater production of these cytokines in Cbl-b-deficient mast cells. To compare the levels of IKK activation with that of the Syk/LAT pathway, the immunoblots were also probed with anti-phospho-LAT Abs. This comparison revealed equivalent patterns of induction for both molecules; i.e., c-Cbl-deficient cells responded weakly to antigenic stimulation and produced low levels of both phospho-LAT and phospho-IKK following antigenic stimulation, whereas Cbl-b-deficient cells showed a very large induction of both. Thus it is possible that the enhanced and sustained activation of Syk and LAT in Cbl-b deficient mast cells is sufficient to enhance the NF-κB pathway to a level that is capable of inducing the very high levels of TNF-α, IL-6, and MCP-1.

Discussion

The analysis of BMMCs derived from Cbl-deficient mice has revealed that Cbl-b, but not c-Cbl, is a potent negative regulator of responses to FcεRI activation. This marked difference between the BMMCs from the two Cbl knockout mice may not however be due
to functional differences between the proteins but simply due to a higher expression level of Cbl-b over c-Cbl (Fig. 5). The most striking effects that we have observed from the loss of Cbl-b function are a retardation of Ag-induced FcεRI internalization, a prolonged phosphotyrosine signal, and a large induction of the proinflammatory cytokines TNF-α, MCP-1, and IL-6 in response to FcεRI engagement.

Whether the prolonged phosphotyrosine signal and enhanced cytokine production are consequences of the delay in FcεRI internalization is currently not known, however Syk and Lyn levels are clearly not increased in the absence of Cbl-b, nor is there an enhancement in the activities of the ERK, Akt and calcium pathways. The lack of an effect on Lyn levels in Cbl-b and c-Cbl knockout mast cells is surprising in view of the ability of Cbl proteins to target Src family kinases, including Lyn, for ubiquitylation and degradation (22, 51). Thus the targeting of Src kinases by Cbl proteins appears to differ depending on cell type because the levels of Fyn and Lck in thymocytes, and Lyn in B cells, are clearly elevated in c-Cbl−/− mice (31, 36, 51–53).

The effects on Syk in mast cells by the overexpression of c-Cbl and Cbl-b have been well-characterized and both proteins are found to suppress Syk kinase activity without reducing protein levels (24, 26). Furthermore, in RBL-2H3 basophils, c-Cbl overexpression directs the ubiquitylation of both Syk and FcεRI following receptor cross-linking (16). Therefore, in this current study, it is possible that the loss of Cbl-b causes a sustained Syk signal through dual mechanisms of retarding FcεRI internalization and enhancing Syk kinase activity. Although the mechanisms remain to be fully resolved it is likely that the loss of Cbl-b E3 ligase activity is central to these effects, a possibility that is implied by the absence of a high molecular mass protein smear for Syk following FcεRI cross-linking (Fig. 2D).

A recent report that also examined BMMCs from Cbl-b and c-Cbl deficient mice similarly found that some signaling responses
FL2 intensities. Cytokine in the culture supernatants are revealed by their intensities of the FL3-stained beads, and the levels of each cytokine is revealed by the "signature" in blotting with phosphospecific IKK shown above. Cell lysates were analyzed by immunostimulated or activated with DNP-BSA for the times of 10

A

B

C

FIGURE 7. Neutralizing anti-TNF Abs do not diminish Ag-induced induction of MCP-1 or IL-6. A, Flow cytometry profiles of a cytokine bead array assay showing the induction of IL-6, MCP-1, and TNF-α from a Cbl-b−/− BMMC culture stimulated with 10 ng/ml DNP-BSA for 4 h in the absence (upper panel) or presence of 10 μg/ml anti-TNF Abs (lower panel). The identity of each cytokine is revealed by the “signature” intensities of the FL3-stained beads, and the levels of each cytokine in the culture supernatants are revealed by their FL2 intensities. B, Concentrations of TNF-α, MCP-1, and IL-6 from wt (+/+), c-Cbl−/− (c−/−), and Cbl-b−/− (b−/−) BMMC cultures stimulated with DNP-BSA for 4 h with or without the addition of anti-TNF Abs at the time of Ag addition. In all cultures, the addition of anti-TNF Abs did not affect the production of MCP-1 or IL-6. C, Enhanced and sustained phosphorylation of IKK-αβ in Cbl-b-deficient mast cells. IgE-primed wt, c-Cbl−/− (c) and Cbl-b−/− (b) mast cells were left unstimulated or activated with DNP-BSA for the times shown above. Cell lysates were analyzed by immunoblotting with phosphospecific IKK-αβ or LAT Abs then reprobed with the corresponding protein-specific Abs.

through the FceRI were enhanced in Cbl-b−/− but not c-Cbl−/− mast cells (23). These included enhanced tyrosine phosphorylation of Syk and PLC-γ2, increased calcium mobilization, and a greater release of histamine following receptor cross-linking. In contrast to these enhanced effects on signaling Zhang et al. (23) found a decrease in ERK activation in Cbl-b−/− mast cells. Here, we similarly find an enhancement in Syk phosphorylation in Cbl-b−/− mast cells but we did not find a decrease in ERK activation or an increase in calcium mobilization. We do not currently have an explanation for these differences because increased signaling from Syk, and phosphorylation of substrates such as LAT, would be predicted to enhance both calcium mobilization and ERK activation.

An interesting finding from this current study is the greater proportion of Cbl-b to c-Cbl protein in mast cells compared with other hemopoietic cells (Fig. 5). This finding has raised the possibility that relative protein levels may provide the explanation for the level of enhancement in FceRI signaling in Cbl-b−/− mast cells compared with wt and c-Cbl−/− cells. The magnitude of induction is perhaps surprising in view of the level of enhancement in FceRI signaling in Cbl-b−/− mast cells that has been observed here and elsewhere (23) which, although clearly detectable, does not appear to approach the magnitude of proinflammatory cytokines released by these cells. Thus, although FceRI signaling in Cbl-b−/− BMCCs is elevated through an increased and sustained Syk signal, the levels of these cytokines appear disproportionately high. We proposed that the high level of induction of TNF-α may drive further production of itself, and IL-6 and MCP-1, through a well-characterized feedback mechanism. However, the addition of neutralizing anti-TNF Abs (Fig. 7, A and B) indicated that this is not the case and raised the possibility that a single, highly activated pathway may be responsible for inducing all three cytokines. A clue for identifying this pathway has come from the greater level of phosphorylation of the IKK complex in Cbl-b-deficient cells compared wt and c-Cbl−/− cells (Fig. 7C), thus implicating the involvement of NF-κB. Indeed, it is significant that NF-κB plays a crucial role in the induction of all three cytokines (47–49) and in thymocytes which correlates with the c-Cbl−/− mouse showing markedly enhanced TCR signaling in the thymus, whereas the Cbl-b−/− mouse shows no effects on thymocyte signaling (20).
Further analysis of this pathway by comparing Cbl-b-deficient mast cells with wt and c-Cbl−/− cells will be of considerable interest. From a physiological point of view, this study is significant because TNF-α and IL-6 have very similar effects in regulating local and systemic immune responses and both have been implicated in numerous pathological processes. Similarly, MCP-1 plays a key role in recruiting monocytes in allergic inflammation (54) and its expression during cutaneous allergic reactions is mast cell dependent and largely mediates the recruitment of monocytes to skin lesions (55). Thus, the induction of TNF-α, MCP-1 and IL-6 to levels equivalent to those seen in the Cbl-b-deficient cultures would in vivo provide a potent mix capable of causing a massive inflammatory reaction with harmful consequences. The tight regulation of Cbl-b levels and its E3 ligase activity therefore appears to levels equivalent to those seen in the Cbl-b-deficient cultures would in vivo provide a potent mix capable of causing a massive inflammatory reaction with harmful consequences. It will be important to determine in future studies if the negative regulatory role played by Cbl-b in mast cells also raises the potential negative role played by Cbl-b in mast cells also raises the potential negative role played by Cbl-b on cytokine production is totally dependent on the RING finger and if so whether expressing constitutively active forms of Cbl-b (56) can suppress proinflammatory responses.

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Disclosures

The authors have no financial conflict of interest.

References


