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Differential Control of CD28-Regulated In Vivo Immunity by the E3 Ligase Cbl-b

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The E3 ubiquitin ligase Casitas B cell lymphoma-b (Cbl-b) plays a critical role in the development of autoimmunity and sets the threshold for T cell activation. In the absence of Cbl-b, T cells stimulated via the TCR respond similarly to those that have received a CD28-mediated costimulatory signal, suggesting that the absence of Cbl-b substitutes for CD28-mediated costimulation. In this study, we show that loss of Cbl-b restores Ig class switching and germinal center formation in Vav1 mutant mice in response to an in vivo viral challenge. Genetic inactivation of Cbl-b also rescues impaired antiviral IgG production in CD28-mutant mice. Moreover, loss of CD28 results in disorganization of follicular dendritic cell clusters, which is also rescued by the Cbl-b mutation. Intriguingly, despite restored antiviral in vivo immunity and follicular dendritic cell clusters, loss of Cbl-b did not rescue germinal center formation in CD28-deficient mice. Mechanistically, in vivo vesicular stomatitis virus-induced IL-4 and IFN-γ production and up-regulation of the inducible costimulatory molecule ICOS were dependent on CD28, and could not be rescued by the loss of Cbl-b. These data provide genetic evidence that CD28-dependent in vivo immune responses and Ig class switching can be genetically uncoupled from germinal center formation and ICOS induction by Cbl-b-Vav1-regulated signaling pathways. The Journal of Immunology, 2005, 174: 1472–1478.

Full T cell activation and a physiological response require an Ag-mediated signal mediated by the TCR (signal 1) and a second, costimulatory signal (signal 2) (1, 2). This two-step model for T cell activation is not static, but rather a dynamic process involving many factors. The classical costimulatory molecule, CD28, is constitutively expressed on T cells and facilitates initial T cell activation. CD28 stimulation has been linked to both PI3K and stress-activated protein kinase/JNK activation (3–6). Concurrent activation of CD28 along with TCR stimulation is required for sustained phosphotyrosine levels and cytoskeletal reorganization leading to supramolecular activation complex formation (6–9).

The cytoplasmic tail of CD28 contains a phosphotyrosine-based motif pYMNM, which, when phosphorylated, binds the Src homology 2 domain of the p85 subunit of PI3K (3, 4). Docking of PI3K to the CD28 cytoplasmic tail brings it into close proximity to the membrane, where it can phosphorylate lipids at the D3 position of phosphatidylinositol residues. CD28 costimulation leads to hyperphosphorylation of the GTPase exchange factor Vav1 and Vav1 phosphorylation is dependent on binding the products of PI3K (10, 11). In particular, the E3 ubiquitin ligase Casitas B cell lymphoma-b (Cbl-b) negatively regulates Vav1 phosphorylation (12, 13). Cbl-b can also inactivate p85-PI3K by targeting it for ubiquitination (14), and Cbl-b prevents the recruitment of p85-PI3K to both the TCR and CD28 (15).

Both Vav1- and CD28-mediated costimulation are required for initiating and maintaining T cell activation and effective immune responses in vivo (16–20). T cells deficient in either CD28 or Vav1 fail to proliferate and produce cytokines efficiently (16, 17, 20). Loss of Cbl-b results in autoimmunity and increased T cell proliferation and IL-2 production even in the absence of CD28 costimulation (12, 21). Moreover, Cbl-b has been identified as a major type I diabetes susceptibility gene in rats (22). We and others have shown that cbl-b−/−cd28−/− T cells, when stimulated with anti-CD3ε and Con A, proliferate to normal levels and produce IL-2 (12, 13). These results suggested that loss of Cbl-b compensates for CD28-mediated costimulation and uncouples T cell activation from the requirement for costimulation. In addition, these findings suggested that the loss of Cbl-b either restores the activity of a pathway downstream of CD28 or hyperactivates a pathway downstream of the TCR, which mimics CD28 costimulation. TCR stimulation in Cbl-b-deficient T cells leads to hyperactivation of the Vav1 pathway to levels comparable in wild-type (WT) cells given additional CD28-costimulatory signals (12). Therefore, the increased Vav1 activity observed in cbl-b−/− T cells most likely compensates for the loss of CD28.

However, CD28 activates signaling pathways other than the Vav1-regulated pathway. It is also important to distinguish whether the loss of Cbl-b can rescue expansion of T cells and/or differentiation to effector T cells in the CD28-deficient mice. Therefore, a more complete analysis of the role of Cbl-b in CD28-mediated T cell activation and differentiation was performed. CD28 is known to be required for effective and efficient Th cell

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2 Abbreviations used in this paper: Cbl, Casitas B cell lymphoma; FDC, follicular dendritic cell; GC, germinal center; huFc, human Fc; LT, lymphotoxin; PNA, peanut agglutinin; VSV, vesicular stomatitis virus; WT, wild type.
responses during vesicular stomatitis virus (VSV) infection. Therefore, we used the VSV infection model to determine whether Cbl-b can control all CD28-regulated functions.

In this study, we show that cbl-b−/−cd28−/− mice develop spontaneous autoimmunity similar to that seen in cbl-b−/− mice. Mutation of cbl-b restored Ig class switching in response to VSV immunization in both CD28- and Vav1-deficient mice. Intriguingly, whereas germinal center (GC) formation was completely restored in vav1+/−/cdl-b−/− mice, loss of Cbl-b did not rescue defective GC formation in cd28−/−/− mice. Mechanistically, CD28 is required for ICOS up-regulation in response to viral challenge, and the loss of Cbl-b cannot restore ICOS expression in the absence of CD28. Furthermore, IL-4 and IFN-γ production in response to challenge with VSV also requires CD28. We also demonstrate that CD28-deficient mice show a defect in the organization of splenic follicular dendritic cell (FDC) clusters. These data provide a molecular framework for Cbl-b/Vav1-dependent and Cbl-b/Vav1-independent CD28 activation pathways essential for the organization of secondary lymphoid tissues and in vivo immunity to viral infection.

Materials and Methods

Mice

The cbl-b−/−, vav1+/−, cd28−/−, cbl-b−/−vav1+/−, cbl-b−/−cd28−/− mice have been described (12, 16, 17, 21). Mice were genotyped by PCR and Southern blotting and absence of proteins confirmed via Western blotting and FACS. For histology, paraffin tissue sections were stained with H&E. If not otherwise stated, only 6- to 10-wk-old mice were used in all experiments. All mice were housed according to institutional guidelines.

Cell purification

In all assays, lymph node lymphocytes were isolated and T cells were purified by negative selection via magnetic separation of B cells with B220-coated Dynabeads (Dynal Biotech).

VSV infections and detection of VSV-neutralizing Abs

Mice were immunized with VSV-Indiana (2×10⁶ PFU, i.v.). Serum samples were collected on days 4 and 8, and neutralizing IgM and IgG Ab titers were determined, as described (23). In brief, 1/2 dilutions of 40-fold prediluted sera were incubated with VSV for 90 min. The presence of remaining infectious virus was determined by incubating the serum samples from VSV-infected mice with fibroblasts for another 24 h. Serum dilutions that reduced the number of viral plaques by 50% were taken as specific titers. IgG titers were determined after preincubation of sera with 2-ME, a procedure that eliminates IgM (23).

Analysis of costimulatory molecule expression

For VSV infections, splenic lymphocytes were harvested on day 8 postinfection. Harvested cells were analyzed by FACS for expression of OX40, CD40L, and ICOS. Human Fc (huFc) fusion proteins for B7RPH1 and OX40 ligand (kind gifts of Amgen) were used to detect ICOS and OX40, respectively. CD40L was detected using an anti-CD40L (biotin) Ab from BD Pharmingen. For cytokine analysis, cells were cultured with PMA (50 ng/ml) and ionomycin (100 ng/ml) for 4 h in the presence of Golgiplug (BD Biosciences). Cells were permeabilized and stained with Abs to IL-4 and IFN-γ (BD Pharmingen) using the Cytostain kit (BD Biosciences). Cytokine-producing cells were analyzed by FACS.

GC formation and immunohistochemistry

To determine formation of GCs, mice were immunized with VSV-Indiana, as described above. Splenectomies from VSV-infected animals were harvested 12 days after the initial infection, frozen in liquid nitrogen, and processed for cryosections. Cryostat sections (5 μm) were fixed in acetone (10 min). Sections were incubated with peanut agglutinin (PNA) (diluted 1/200), and bound PNA was detected by rabbit anti-PNA Abs (diluted 1/300; DakoCytomation). Macrophages were detected with anti-F4/80 and MOMA-1. FDCs were detected with FDC-M1 (clone 4C11), a FDC-specific marker. Binding of primary Abs was detected by alkaline phosphatase-labeled goat Abs to rabbit or rat Ig (1/80 dilution; Jackson ImmunoResearch Laboratories), followed by alkaline phosphatase-labeled donkey Abs against goat Ig (1/80 dilution; DakoCytomation). Alkaline phosphatase was visualized using Napthol AS-BI phosphatase and New Fuchsin as a substrate, which yields a red precipitate. Endogenous alkaline phosphatase activity was blocked by levamisole. VSV-specific B cells were detected, as described (24). In brief, dehydrated tissue sections were overlaid with a solution of UV-inactivated VSV (3×10⁶ PFU/ml) for 4 h. Specifically bound virus was detected by incubation with polyclonal rabbit anti-VSV-Indiana serum (diluted 1/1500), followed by alkaline phosphatase-labeled goat Abs to rabbit Ig and rabbit anti-goat Ig (diluted 1/80; Jackson ImmunoResearch Laboratories). Naphthol AS-BI phosphatase and New Fuchsin were used to develop the color reaction (24).

Results

The cbl-b−/−cd28−/− mice develop a spontaneous autoimmune phenotype

CD28 plays an important role in the development of autoimmune reactions (25). Blocking CD28 function in experimental autoimmune models often alleviates the severity of the disease (26). Furthermore, the severity of disease in mice predisposed to the development of autoimmune disease can be reduced when bred into a cd28 null background (27), and mice deficient in CD28 fail to develop experimental autoimmune encephalomyelitis when immunized with myelin basic protein (28). By contrast, mice deficient for Cbl-b are predisposed to the development of autoimmunity, and cbl-b−/− mice show increased susceptibility to experimental autoimmune encephalomyelitis (13). As they age, cbl-b−/− and cbl-b−/−vav1−/− mutant mice develop spontaneous autoimmunity. We have previously demonstrated that cbl-b−/−vav1−/− mice develop spontaneous autoimmunity and destructive lymphocyte infiltration into many organs similar to the cbl-b−/− mice (12). Considering the findings that the loss of Cbl-b can restore function in the vav1-deficient mice, but that CD28 signaling can be a requirement for autoimmune disease progression, we wanted to determine the impact of the absence of CD28 on the development of spontaneous autoimmunity in the cbl-b−/− mice.

To determine whether the loss of CD28 would prevent the development of autoimmunity in the cbl-b−/− mice, we aged cbl-b−/−cd28−/− and cd28−/− mice and examined them for signs of autoimmune disease. All old (>10 mo of age, n = 20) cbl-b−/−cd28−/− double-mutant mice display an autoimmune-like disorder very similar to that seen in the cbl-b−/− mice (12). We found lymphoid neo-organogenesis in the salivary glands (data not shown) and infiltration of mononuclear cells into the liver (Fig. 1A), lung (Fig. 1B), kidney (Fig. 1C), submandibular salivary gland (Fig. 1D), and intestine (data not shown). No significant infiltration could be detected in any organ of cd28−/− littermates of the same age (n = 13) (Fig. 1, E–H). Moreover, as seen in the serum of the cbl-b−/− mice, the cbl-b−/−cd28−/− mice produced autoantibodies to dsDNA (Fig. 2). Thus, loss of CD28 does not alleviate the spontaneous autoimmunity seen in cbl-b−/− mice.

Loss of Cbl-b restores viral specific IgG production and IgG class switching in vav1−/− and cd28−/− mice

Viral challenge with VSV was used to determine the effects of the loss of Cbl-b on CD28-mediated functions. VSV elicits a T cell-dependent immune response, resulting in the production of anti-VSV-neutralizing Abs. IgM production occurs in the absence of T cell help, while IgG production specifically requires CD4+–mediated T cell help (29, 30). CD28 function is dispensable for initial IgM responses to VSV, but is required for Ig class switch for the production of neutralizing IgG (16). Also, CD28 is required for GC formation following VSV infection (19).

To determine whether CD28- and Vav1-dependent Ig class switching could be rescued in vivo by the loss of Cbl-b during a VSV challenge, WT, cbl-b−/−, cd28−/−, vav1−/−, cbl-b−/−vav1−/−, and cbl-b−/−cd28−/− mice were infected with VSV, and
VSV-specific Ab production was monitored over time. The cbl-b<sup>+/−</sup> mice showed a moderate, but consistent, increase in levels of neutralizing anti-VSV IgG Abs on day 8 following VSV infection (Fig. 3A). As previously shown, both cd28<sup>+/−</sup> and vav1<sup>+/−</sup> mice produced less VSV-specific IgG than WT mice. Importantly, cbl-b<sup>−/−</sup>cd28<sup>−/−</sup> and cbl-b<sup>−/−</sup>vav1<sup>−/−</sup> double-mutant mice showed IgG production similar to that of WT mice, demonstrating that Ig class switch is also restored in response to virus challenge. These results demonstrate that the loss of Cbl-b restores IgG class switching in both cd28<sup>−/−</sup> and vav1<sup>−/−</sup> backgrounds.

PNA<sup>+</sup> GC formation is not rescued in cbl-b<sup>−/−</sup>cd28<sup>−/−</sup> mice in response to VSV infection

CD28 is also essential for the formation of GCs (19, 31). We hypothesized that if IgG class switching were restored in the cbl-b<sup>−/−</sup>cd28<sup>−/−</sup> and cbl-b<sup>−/−</sup>vav1<sup>−/−</sup> mice, then GC formation would also be restored. WT mice infected with VSV formed splenic GCs as determined by PNA staining (Figs. 3B and 4). PNA<sup>+</sup> cells typically localize to GCs and the marginal zone following immune challenge. Spleens from cbl-b<sup>−/−</sup> mice infected with VSV contained enhanced numbers of GCs as compared with WT spleens (Figs. 3B and 4). GC formation was severely impaired in both cd28<sup>−/−</sup> and vav1<sup>−/−</sup> mice following VSV infections. As expected, formation of PNA-positive GCs was restored to normal in cbl-b<sup>−/−</sup>vav1<sup>−/−</sup> mice. Surprisingly, even though IgG class switching was restored in cbl-b<sup>−/−</sup>cd28<sup>−/−</sup> mice infected with VSV, GC formation impaired in the spleens of the double-mutant mice and comparable to spleens from the cd28<sup>−/−</sup> single mutants (Figs. 3B and 4).

Because the cbl-b<sup>−/−</sup>cd28<sup>−/−</sup> mice produced large amounts of VSV-specific IgG, but failed to form GCs, the spleens were analyzed for VSV-specific plasma B cells. Whereas VSV-specific B cells can be readily detected in spleens from WT and cbl-b<sup>−/−</sup> mice using UV-inactivated VSV particles, VSV-specific plasma cells were markedly reduced in numbers in both cd28<sup>−/−</sup> and vav1<sup>−/−</sup> mice (Fig. 4). It should be noted that the spleens from cbl-b<sup>−/−</sup> mice contain increased numbers of VSV-specific B cells, which could explain the enhanced IgG production. Splenic VSV-specific B cell numbers were restored to normal in cbl-b<sup>−/−</sup>vav1<sup>−/−</sup> mice. Consistent with a rescue in IgG class switch seen in cbl-b<sup>−/−</sup>cd28<sup>−/−</sup> mice, spleens from cbl-b<sup>−/−</sup>cd28<sup>−/−</sup> mice contained VSV-specific B cells; however, they were not organized in focal GC-like areas as seen in WT spleens (Fig. 4). These data demonstrate that loss of Cbl-b restores CD28-dependent Ig class switching and the generation of VSV-specific plasma cells, but does not rescue CD28-dependent formation of PNA-positive GCs.

**CD28/Cbl-b regulate formation of FDC clusters**

Previously, it has been shown that lymphotixin α (LTα), TNF-α, and TNFR1 mutant mice can display Ig class switching in the absence of GC formation dependent on antigenic stimulation (32–36). In addition, FDC clusters fail to develop in the spleens of LTα, TNF-α, and TNFR1 knockout animals (32, 33, 36, 37). Splenic white pulp areas in LTα<sup>−/−</sup> mice lack the marginal zone of mAb MOMA-1-staining metallophilic macrophages, whereas TNFR1-deficient mice have preserved MOMA-1 staining (34, 36). We therefore analyzed the presence of MOMA-1-positive marginal zone macrophages and FDC clusters in cd28<sup>−/−</sup> and cbl-b<sup>−/−</sup>cd28<sup>−/−</sup> mice following VSV infections.

The macrophage markers F4/80 and MOMA-1 revealed no difference in macrophage distribution between all genotypes (Fig. 5). In WT and cbl-b<sup>−/−</sup> mice, FDC clusters were readily detectable when stained with FDC-M1 (clone 4C11), a FDC-specific marker (Fig. 5). Intriguingly, we observed a severe defect in FDC clusters in cd28<sup>−/−</sup> spleens. Loss of Cbl-b in cd28<sup>−/−</sup> mice restored the defective formation of FDC clusters to normal (Fig. 5). To our knowledge, this is the first description that CD28 is required for the establishment of FDC clusters.
Loss of Cbl-b cannot restore cytokine production by CD28-deficient CD4+ T cells

Our data indicate that not every aspect of CD28-mediated costimulation is restored by the loss of Cbl-b. Costimulation of CD4+ T cells is important for cytokine production. We therefore analyzed the ability of CD4 T cells to produce cytokines during VSV infection. Splenic T cells were harvested 8 days post-VSV infection and restimulated ex vivo with PMA and ionomycin. CD4+ T cells were analyzed by intracellular staining for IFN-γ and IL-4 production. There were fewer IFN-γ-producing CD4 T cells in the spleens from cd28−/− mice, as compared with WT controls (Fig. 6). Intriguingly, the number of IFN-γ-producing CD4+ T cells was not restored in the spleens from cbl-b−/− cd28−/− mice. Furthermore, there was a marked decrease in IL-4 production by CD4+ T cells in the cd28−/− mice, which was also not restored by the loss of Cbl-b (Fig. 6). These findings demonstrate that CD28 is important for cytokine production by CD4+ T cells in response to VSV infection, and that the loss of Cbl-b cannot restore IL-4 or IFN-γ production in the absence of CD28.

Defective ICOS up-regulation in both cd28−/− and cbl-b−/− cd28−/− T cells

Costimulation by molecules other than CD28 has been shown to be important for GC formation and cytokine production (38). For instance, CD40L, OX40, and ICOS, whose expression is induced on activated T cells, have been implicated in the regulation of GC formation (39–41), and CD28 signaling has been linked to the up-regulation of these costimulatory receptors (42–45). In addition, ICOS has been linked to the up-regulation of cytokines such as IL-4 (42, 46, 47). To provide a molecular explanation for Cbl-b-dependent and Cbl-b-independent CD28 activation pathways, we hypothesized that in the absence of both Cbl-b and CD28, one or more of these molecules were not up-regulated, leading to defects in GC formation and cytokine production.

We therefore examined the induction of CD40L, OX40, and ICOS cell surface expression on WT, cbl-b−/−, cd28−/−, and cbl-b−/− cd28−/− splenic CD4+ T cells following VSV infection. OX40 and ICOS were detected using huFc fusion proteins of OX40L and B7RP1, respectively. Splenic CD4+ T cells from mice were harvested following 8 days of VSV infection and analyzed for expression levels of costimulatory receptors (Fig. 7). OX40 up-regulation was similar across all genotypes. CD40L expression was undetectable on any of the splenic T cells over varying time points (data not shown). Approximately 20% of the WT and cbl-b−/− CD4+ T cells expressed high levels of ICOS following VSV infections. Importantly, both cd28−/− and cbl-b−/− cd28−/− T cells showed markedly reduced levels of ICOS expression (Fig. 7),
FDCs in zone metallophillic macropages, and 4C11-positive macrophages, MOMA-1-positive marginal demonstrating a rescue in CD4 contrast, loss of Cbl-b restored virus-specific GC formation in mice could not make splenic GCs, although VSV-specific B cells were clearly detectable in the spleens of the same animals. By

Discussion

Our findings demonstrate that IL-4 and IFN-γ production by CD4+ T cells in response to challenge with VSV requires a CD28-activated signaling pathway that is not regulated by Cbl-b. Furthermore, in the absence of CD28, ICOS up-regulation in response to viral challenge is impaired, which correlates with reduced cytokine production and GC formation.

Previously, it has been shown that T cells from cbl-b−/− mice no longer require costimulation for proliferation, IL-2 production, and lipid raft aggregation, suggesting that CD28-mediated costimulation is dispensable in the absence of Cbl-b (12, 13). Our genetic data using an in vivo viral challenge model show that the loss of Cbl-b cannot substitute for all aspects of CD28-mediated costimulation. CD28-dependent in vivo immune responses and Ig class switching can be genetically uncoupled from GC formation, IL-4 and IFN-γ production, as well as in vivo ICOS induction. These results provide novel insights into CD28-regulated signaling pathways required for antiviral immunity (Fig. 8).

In the absence of CD28 or Vav1, cbl-b−/−cd28−/− and cbl-b−/−vav1−/− mice were able to generate T-dependent Ag-specific IgG, demonstrating a rescue in CD4+ T cell help and Ig class switch in vivo. Intriguingly, despite rescued T cell help, cbl-b−/−cd28−/− mice could not make splenic GCs, although VSV-specific B cells were clearly detectable in the spleens of the same animals. By contrast, loss of Cbl-b restored virus-specific GC formation in vav1-deficient mice, suggesting that a signaling pathway regulated by Cbl-b can bypass the requirement for Vav1 in GC formation. Our data suggest that the CD28-regulated Cbl-b-Vav1 signaling cascade does not regulate GC formation, and that CD28 regulates GC formation via an unidentified pathway that may involve ICOS up-regulation.

CD28 stimulation induces the expression of costimulatory molecules, which are not constitutively expressed on T cells, such as OX40, CD40L, and ICOS. Because ICOS knockout mice do not make GCs in response to VSV, it is likely that the failure of the cbl-b−/−cd28−/− mice to form GCs is due to impaired ICOS expression. ICOS function is essential for antiviral immunity (48), and ICOS expression is specifically localized to GCs (49). Therefore, CD28 may act through ICOS to promote the organization of B cells into GCs following immune challenge. One study using icos−/− mice suggested that the defect in GC formation was due to impaired CD40L up-regulation (50). However, we observed normal CD40L expression in the cd28−/− and cbl-b−/−cd28−/− T cells in vitro (data not shown), suggesting that this may not be the same mechanism. However, because we failed to detect CD40L expression on T cells in our VSV infection experiments, we cannot completely exclude a role of CD40L. The observed defects in IL-4 production in the cd28−/− and cbl-b−/−cd28−/− T cells are in line with defects seen in ICOS up-regulation, as T cells deficient in ICOS fail to produce normal levels of IL-4 (42, 46, 47). IL-4 is a cytokine that is important for the growth and differentiation of Th2 cells. IL-4 is required for GC formation in Peyer’s patches, but is dispensable for GC formation in lymph nodes (51, 52). Whether

**FIGURE 5.** CD28-Cbl-b control formation of FDC clusters. A and B, Detection of splenic F4/80-positive macrophages, MOMA-1-positive marginal zone metallophillic macropages, and 4C11-positive FDCs in WT, cbl-b−/−, cd28−/−, and cbl-b−/−cd28−/− mice infected with 2 × 10⁶ PFU VSV-Indiana (i.v.). Serial spleen sections were processed for immunostaining 8 days after immunization. Note the absence of FDC clusters in cd28−/− mice and restoration of FDC clusters in cbl-b−/−cd28−/− double-mutant mice. B, Shows higher magnifications of FDC networks.
IL-4 contributes to the development of splenic GCs in response to VSV remains to be determined. Moreover, considering the defective production of both IL-4 and IFN-γ in VSV-infected cd28−/− and cbl-b−/− cd28−/− mice, it will be interesting to examine the Ig subclasses like IgG1 or IgG2a in the rescued in vivo anti-VSV responses.

Intriguingly, FDC clusters fail to form in the absence of CD28. FDCs are important for optimizing B cell responses and facilitating the GC reaction. CD28-deficient mice have a clear defect in FDC clusters, which may in turn cause the defect in GC formation. Our data show that the loss of Cbl-b restores FDC clusters in FDC-deficient mice. It is unknown whether the defect in FDC clusters in the CD28-deficient mice and the rescue in the cbl-b−/− cd28−/− mice are intrinsic to the FDCs or reflect a role for T cell help in FDC cluster formation. The rescue of IgG levels in the cbl-b−/− cd28−/− mice could simply be due to restored FDC clusters. Alternatively, Cbl-b is expressed in B cells; therefore, the rescue in IgG levels may be intrinsic to B cell function. Nonetheless, even though FDC clusters are restored in cbl-b−/− cd28−/− spleens, they are not sufficient to result in the accumulation of PNA+ B cells in GCs.

In the absence of Cbl-b, cd28−/− and vav1−/− mice mount a normal antiviral IgG Ig response, suggesting that the loss of Cbl-b negates the requirement for some aspects of CD28-mediated costimulation and Vav1 signaling. Spleens from VSV-infected WT and cbl-b−/− mice contained similar numbers of CD4+ ICOShigh splenic T cells, suggesting that the induction of ICOS expression in vivo is comparable in WT and cbl-b−/− T cells. Thus, cbl-b−/− T cells show no obvious deregulation of ICOS, CD40L, or OX40 in vivo. However, it is also possible that the loss of the requirement for CD28 could be due to enhanced expression of compensatory costimulatory molecules on cbl-b−/− T cells.

Our data show that cbl-b−/− cd28−/− mice develop spontaneous autoimmunity. Loss of Cbl-b restores Ig class switching and GC formation in Vav1 mutant mice in response to an in vivo viral challenge. Genetic inactivation of Cbl-b also rescues impaired Ig class switching and antiviral IgG production in CD28 mutant mice. Intriguingly, whereas GC formation was completely restored in vav1−/− cbl-b−/− mice, loss of Cbl-b did not rescue defective GC formation, but rescued FDC clusters in cd28−/− mice. Mechanistically, in vivo viral-induced IFN-γ and IL-4 production and up-regulation of the inducible costimulatory molecule ICOS were dependent on CD28, and could not be rescued by the loss of Cbl-b. These data provide a molecular framework for Cbl-b/Vav1-dependent and Cbl-b/Vav1-independent CD28 activation pathways essential for the organization of secondary lymphoid tissues and in vivo immunity to viral infections.
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