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## CBLB constrains inactivated vaccine-induced CD8<sup>+</sup> T cell responses and immunity against lethal fungal pneumonia

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### Abstract

Fungal infections in CD4<sup>+</sup> T-cell immunocompromised patients have risen sharply in recent years. Although vaccines offer a rational avenue to prevent infections, there are no licensed fungal vaccines available. Inactivated vaccines are safer but less efficacious, and require adjuvants that may undesirably bias toward poor protective immune responses. We hypothesized that reducing the TCR signaling threshold could potentiate anti-fungal CD8<sup>+</sup> T cell responses and immunity to inactivated vaccine in the absence of CD4<sup>+</sup> T cells. Here, we show that Cblb, a negative regulator of TCR signaling, suppresses CD8<sup>+</sup> T cells in response to *inactivated* fungal vaccination in a mouse model of CD4<sup>+</sup> T cell lymphopenia. Conversely, Cblb deficiency enhanced both the type 1 (e.g., IFN $\gamma$ ) and type 17 (IL-17A) CD8<sup>+</sup> T cell responses to inactivated fungal vaccines, and augmented vaccine immunity to lethal fungal pneumonia. Furthermore, we show that immunization with live or inactivated vaccine yeast did not cause detectable pathology in Cblb<sup>-/-</sup> mice. Augmented CD8<sup>+</sup> T cell responses in the absence of CBLB also did not lead to terminal differentiation or adversely affect the expression of transcription factors, T-bet, Eomes and ROR $\gamma$ t. Additionally, our adoptive transfer experiments showed that CBLB impedes the effector CD8<sup>+</sup> T cell responses in a cell-intrinsic manner. Finally, we showed that ablation of Cblb overcomes the requirement of HIF-1 $\alpha$  for expansion of CD8<sup>+</sup> T cells upon vaccination. Thus, adjuvants that

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#### AUTHORS CONTRIBUTIONS

SGN conceived the project, designed the experiments, executed and analyzed the data, and wrote the manuscript. JSF executed and analyzed the HIF-1 $\alpha$  experiments and helped with editing the manuscript. MS and BSK provided conceptual and intellectual input for chronic viral infection and fungal vaccine experiments, respectively, and helped with editing the manuscript. SM executed experiments, analyzed the data, and helped with editing the manuscript.

#### DISCLOSURE

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target CBLB may augment inactivated vaccines and immunity against systemic fungal infections in vulnerable patients.

## INTRODUCTION

Fungal infections in immunocompromised patients, such as those with AIDS and CD4<sup>+</sup> T cell lymphopenia have sharply risen in recent years (1). Substantial evidence indicates that CD4<sup>+</sup> T cells are necessary to control the fungal infections, but these cells are lacking in CD4<sup>+</sup> lymphopenic individuals. We have previously shown that vaccine-induced antifungal cytokine-producing CD8<sup>+</sup> T cells, both type 1 (e.g., IFN $\gamma$ ) and type 17 (IL-17A) cells, can compensate to provide sterilizing, vaccine-immunity in the absence of CD4<sup>+</sup> T cells (2, 3). Although no licensed fungal vaccines are currently available, vaccines targeting CD8<sup>+</sup> T cells in vulnerable immune-compromised patients would require an additional layer of safety. Subunit vaccines are highly desirable, but protective fungal epitopes have not been determined for CD8<sup>+</sup> T cells. Inactivated vaccines are safe, but need adjuvants that may undesirably bias T-cell responses that support antibody production. Adjuvants for fungal vaccines tailored to CD4<sup>+</sup> lymphopenic individuals should rather induce type 1 (IFN $\gamma$ , TNF $\alpha$ , GM-CSF) and type 17 (IL-17A) CD8<sup>+</sup> T-cell cytokine responses that are important for vaccine immunity against pathogenic fungi.

CD8<sup>+</sup> T cells elicited by vaccination differentiate and clonally expand to become a large pool of effectors during the expansion phase, of which ~90% will die by apoptosis during the ensuing contraction phase. The remaining 5–10% of effectors, known as memory precursor effector cells, differentiate to become long-lasting memory cells. A threshold number of such memory cells is essential to rapidly clear infection upon re-exposure (4). Thus, the quantum of effector CD8<sup>+</sup> T cells following expansion dictates the threshold number of memory cells, whereas the inflammatory milieu affects the type and quality of the response. The inflammatory cytokines dictate counter-regulatory types of CD8<sup>+</sup> T cell responses; IL-12 promotes type 1 responses and a combination of IL-6, TGF $\beta$  and IL-1 promotes type 17 responses (5). Moreover, antigen load and inflammatory milieu regulate the quality of memory CD8<sup>+</sup> T cells. High antigen load causes the clonal deletion and functional exhaustion of effector CD8<sup>+</sup> T cells, whereas an inappropriate inflammatory milieu guides their terminal differentiation and dysfunction (6, 7). Thus, both TCR signaling and inflammatory milieu are key targets for developing rational fungal vaccines.

Productive TCR signaling requires at least two signals: signal 1 from the peptide-MHC-TCR complex, and signal 2 from co-stimulatory signals such as CD28. Upon induction of TCR signaling, a cascade of signaling pathways associated with cell proliferation, differentiation and survival are activated involving tyrosine kinases, MAPKs and cytoskeleton remodeling that culminate in NFAT-NF- $\kappa$ B-mediated activation of CD8<sup>+</sup> T cells (8). Casitas B-lymphoma-2 (CBLB), an E3 ubiquitin ligase, is important for maintenance of peripheral T-cell tolerance, and its absence leads to augmented TCR signaling resulting in autoimmunity (9). However, CBLB negatively regulates T cell responses during infection, and its deletion enhances CD8<sup>+</sup> T-cell mediated tumor immunity (10–12). CBLB controls TCR signaling pathways at various junctures by down-regulating the TCR complex, PI3K, PLC $\gamma$ 1 or Vav1,

and is associated with T-cell anergy and generation of poor quality effector T cells (11, 13–16). Thus, Cblb deficient T cells can overcome the requirement of CD28 signaling, signal 2, for T cell activation (17). Thus, CBLB targeting has been shown to be beneficial in controlling chronic viral infections and tumors (11, 18).

Our past work has shown that live attenuated fungal vaccination, in the absence of CD4<sup>+</sup> T cells, induces type1 and type17 CD8<sup>+</sup> T cells that are required for vaccine immunity, and are distinct from anti-viral and –bacterial effector CD8<sup>+</sup> T cells by portraying phenotypic attributes that portend stem-cell like behavior, and are maintained as long-lasting memory cells (19–21). In this study, we evaluated whether targeting CBLB would potentiate inactivated fungal vaccine immunity in a mouse model of CD4<sup>+</sup> T cell lymphopenia. We assayed the quantity and quality of CD8<sup>+</sup> T cell responses to inactivated *Blastomyces* and *Histoplasma* yeasts in comparison to Lymphocytic Choriomeningitis virus (LCMV). We also delineated the effect of CBLB on resistance following inactivated yeast vaccination. Finally, we established the T-cell intrinsic role of CBLB and present novel data that suggests Cblb deficiency can override the requirement of HIF-1 $\alpha$  for vaccine-induced CD8<sup>+</sup> T cell responses.

## METHODS

### Mice:

Cblb<sup>-/-</sup> mice were provided by P.S. Ohashi (University of Toronto, Ontario, Canada) with permission from Josef Penninger (IMBA, Austria). The C57BL/6, OT-I Tg (TCR $\alpha$ /TCR $\beta$  specific for OT-I epitope) and B6.PL-Thy1a/Cy/Thy1.1 (Thy1.1) were purchased from Jackson Laboratories. OT-I mice carrying Thy1.1 allele were generated by backcrossing OT-I Tg mice with Thy1.1 mice, which were then used to generate congenic Thy1.1<sup>+</sup> OT-I Tg-Cblb<sup>-/-</sup> mice by backcrossing with Cblb<sup>-/-</sup> mice. All mice were maintained under specific-pathogen free conditions at the University of Wisconsin-Madison and at the University of Illinois at Urbana-Champaign and were used in accordance with the guidelines of the institutional animal care committees.

### LCMV infection and DNA vaccination:

For acute viral infection, lymphocytic choriomeningitis virus (LCMV) Armstrong strain was given (10<sup>5</sup> pfu) by the intraperitoneal (i.p.) route. For chronic viral infection, LCMV Clone 13 strain was given (10<sup>6</sup> pfu) by the intravenous route (i.v.). To cause persistent infection (22), mice were infected with LCMV Clone 13 and were depleted of CD4<sup>+</sup> cells with GK1.5 antibody (200 $\mu$ g i.p. twice on days 0 and 4 post-infection). For DNA vaccination, 200  $\mu$ g of endotoxin-free pCMV-NP plasmid was given once via the intramuscular (anterior tibialis) route.

### Adoptive transfer experiments:

Naïve Cblb<sup>+/+</sup> and Cblb<sup>-/-</sup> OT-I cells (both Th1.1<sup>+</sup>) were purified from both draining lymph nodes (dLNs) and spleens using a CD8<sup>+</sup> T cell enrichment kit (BD Biosciences). A total of 10<sup>6</sup> OT-I cells was adoptively transferred i.v. into naïve recipient mice.

**Fungi, vaccinations and challenge infection:**

Wild-type virulent strains of *Blastomyces dermatitidis* (#26199) and *Histoplasma capsulatum* (G217B) were obtained from ATCC. The isogenic attenuated strain of *Blastomyces* lacking the virulence factor BAD1 (#55) (23) was used for vaccination ( $10^5$ - $10^6$  CFU). *Blastomyces* was maintained as yeast on Middlebrook 7H10 agar supplemented with Oleic acid-albumin complex (Sigma) at 39° C. To measure OT-I T cell responses, recombinant #55 strain engineered to carry the OT-I epitope, SIINFEKL, was used for vaccination ( $10^6$  CFUs) (19). *Histoplasma capsulatum* was maintained on *Histoplasma* Macrophage Medium (HMM) slants and used for vaccination ( $10^7$  CFU). To study vaccine resistance, mice were challenged intratracheally (i.t.) with  $\sim 10^4$  CFUs of virulent strain *B. dermatitidis* (#26199). Vaccinations were given subcutaneously (s.c.), divided at two sites, one dorsally and one at the base of tail (3, 24).

**CD4<sup>+</sup> T cell depletion:**

Except for the LCMV infection studies (unless indicated), CD4<sup>+</sup> T cells were depleted using 200 µg/mouse of GK1.5 mAb i.v., twice a week (BioXcell) for all experiments. The depletion efficiency was >99% as described previously (19).

**Flow cytometry:**

To assess vaccine-induced CD8<sup>+</sup> T cell responses, dLNs and spleens were harvested on indicated days. To measure recall responses, lung tissues were harvested at day 4 after pulmonary challenge. Tissues were homogenized on BD cell strainers, and RBCs were lysed using 4% ammonium chloride containing buffer. Cells were re-stimulated with anti-CD3 (clone 145-2C11; 0.1 µg/ml) and anti-CD28 (clone 37.51; 1 µg/ml) antibodies in the presence of Golgi Stop (BD Biosciences) for 5 hr at 37° C. Following incubation, cells were washed and stained with fluoro-chrome-conjugated antibodies for surface markers. Following fixation and permeabilization (BD CytoFix/CytoPerm buffer), cells were stained with antibodies against intracellular cytokine. All antibodies were purchased from BD Biosciences or eBioscience except anti-CD43 (Clone 1B11; Biolegend). Cells were analyzed by a BD LSRII or Cytek Aurora flow cytometer, and data was analyzed using FlowJo, LLC (Treestar). All flow cytometry data shown is after gating on lymphocyte gate followed by CD8α<sup>+</sup> T cell gate.

**CBLB and HIF-1α staining:**

Single cell suspensions were antibody stained for surface markers followed by fixation and permeabilization (BD Cytofix & Cytoperm). Cells were stained with anti-mouse CBLB antibodies (#SC-1435 or #SC-8006; Santa Cruz Biotech) followed by staining with fluoro-chrome conjugated rabbit anti-goat secondary antibody for SC-1435 clone (13) and rabbit anti-mouse HIF-1α (D1S7W, Cell Signaling). In some experiments, blocking peptide (SC-1435P, Santa Cruz) was used to block primary antibody binding sites before using for staining (13).

**Cytokine Bead Array:**

Blood was collected from mice when they were moribund and serum was separated. Serum cytokine concentrations were measured by flow cytometry using CBA kit (BD Biosciences) as per the manufacturer's protocol.

**Transcription factor staining:**

Antibodies against T-bet, ROR $\gamma$ t and Eomes were obtained from eBioscience, and cells were stained using a FoxP3 Staining Kit (eBioscience) along with cytokines staining.

**HIF-1 $\alpha$  inhibitor treatment:**

Echinomycin was purchased from Cayman chemical and stocks were made in methanol (1mg/ml). Stocks were diluted in sterile PBS and administered to mice i.p. every other day (~3  $\mu$ g/mouse) starting from day 4 post-vaccination.

**Statistical analysis:**

Statistical analysis of fungal load was measured by the non-parametric Mann-Whitney test, and all other analyses were performed using a two-tailed unpaired Student t-test. A two-tailed P value of 0.05 was considered statistically significant.

**RESULTS****Antigenic load dictates CBLB dependent CD8<sup>+</sup> T cell responses and immunopathology**

We previously reported that Cblb deficiency augments TCR signaling in CD8<sup>+</sup> T cells and enhances IFN $\gamma$  production during an acute infection with the Armstrong strain of LCMV (resolved in 8–10 days) (22) (13). In contrast to an acute infection, in immunocompetent mice the Clone 13 strain of LCMV replicates to very high levels, induces varying levels of CD8<sup>+</sup> T-cell dysfunction and results in a chronic viral infection lasting 3–6 months. Depletion of CD4<sup>+</sup> T cells during a chronic LCMV infection leads to severe functional exhaustion of CD8<sup>+</sup> T cells and life-long viral persistence (25). The expression levels of CBLB in CD8<sup>+</sup> T cells in this setting were directly proportional to the viral antigen load (**Fig. 1A**). Thus, it was of interest to determine the role of CBLB in regulating CD8<sup>+</sup> T cell responses under conditions of disparate antigenic loads that may be seen in immunocompromised hosts during systemic viral infection. To assess the effect of high viral load on immune regulation by CBLB, we evaluated CD8<sup>+</sup> T cell responses in Cblb<sup>+/+</sup> and Cblb<sup>-/-</sup> mice during chronic and persistent viral infections. Absence of CBLB significantly enhanced LCMV-specific (both NP396 & GP33 dominant epitopes) CD8<sup>+</sup> T cell responses during both chronic and persistent viral infections (**Fig. 1B**). Cblb deficiency enhanced CD8<sup>+</sup> T cell responses during chronic viral infection (18), but Cblb<sup>-/-</sup> mice succumbed within 8–9 days after LCMV infection (**Supp. Fig. 1A**). Remarkably, circulating levels of pro-inflammatory cytokines were sharply elevated following LCMV-Clone13 infection in Cblb<sup>-/-</sup> vs. Cblb<sup>+/+</sup> mice (**Supp. Fig. 1B**), which suggested that cytokine storm might underlie the lethality of Cblb<sup>-/-</sup> mice. Thus, under conditions of high viral load during a chronic LCMV infection, CBLB protects against immunopathology by downregulating anti-viral T cell responses.

We next assessed the role of CBLB in regulating T cell responses under conditions of relatively low antigenic stimulation induced by a DNA vaccine. As shown in **Fig 1C**, following DNA immunization, the percentage and IFN $\gamma$ -producing ability of antigen-specific CD8<sup>+</sup> T cells were significantly augmented in Cblb<sup>-/-</sup> mice compared to Cblb<sup>+/+</sup> mice. Together, these data suggest that CBLB augments the activation threshold of CD8<sup>+</sup> T cells. By doing so, CBLB limits CD8<sup>+</sup> T cell responses to weak antigenic stimuli, but protects against CD8<sup>+</sup> T-cell-mediated immunopathology induced by a high antigen load.

### **Ablation of Cblb enhances anti-fungal CD8<sup>+</sup> T cell responses following live yeast vaccination**

As illustrated above, sustained high antigenic load during a chronic LCMV infection led to fatal immunopathology in Cblb<sup>-/-</sup> mice, and we have shown previously that fungal antigen persists for an extended period of time (~7 weeks) following vaccination with live attenuated *Blastomyces* yeast (19). First, we asked if CBLB is induced following fungal vaccination. As shown in **Fig 2A**, fungal vaccination induced high levels of CBLB in activated CD8<sup>+</sup> T cells in both dLNs and spleens that waned in the ensuing weeks of rest (~8 wk post-vaccination) coinciding with the depletion of vaccine antigen (19). Next, we analyzed the response of Cblb<sup>-/-</sup> mice to vaccination with live attenuated yeast. We vaccinated Cblb<sup>+/+</sup>, Cblb<sup>+/-</sup> and Cblb<sup>-/-</sup> mice with live attenuated yeast and monitored their morbidity and survival. We did not observe overt signs of distress or mortality over 8-week post-vaccination in any of the groups. Lastly, we assessed the role of CBLB on anti-fungal CD8<sup>+</sup> T cell responses. We have previously shown that both type 1 (IFN $\gamma$ , GM-CSF, TNF $\alpha$ ) and type 17 (IL-17A) responses were important for fungal vaccine-immunity mediated by CD8<sup>+</sup> T-cells (2, 3). As shown in **Fig. 2B**, there was a significant increase in the frequency of both type 1 and type 17-cytokine producing CD8<sup>+</sup> T cells in Cblb deficient mice compared to Cblb<sup>+/+</sup> mice. Similarly, the number of type 1 and type 17 but not type 2 cytokine-producing CD8 T cells in dLNs and spleens were significantly higher in Cblb<sup>-/-</sup> mice compared to Cblb<sup>+/-</sup> and Cblb<sup>+/+</sup> controls (**Fig. 2C & Supp. Fig. 2A**). Notably, even a single copy of Cblb was enough to suppress the CD8<sup>+</sup> T cell responses. Collectively, these data suggest that Cblb deficiency enhances anti-fungal CD8<sup>+</sup> T cell responses to live fungal vaccination without causing lethality or obvious adverse effects.

### **CBLB constrains anti-fungal CD8<sup>+</sup> T cell responses to an inactivated vaccine**

Inactivated vaccines are safer for immune-compromised individuals, but they often fail to elicit protective T cell responses. After finding above that persistent fungal vaccine antigen does not cause lethality or aberrant gross pathology in Cblb<sup>-/-</sup> mice, we investigated whether: (i) CBLB constrains CD8<sup>+</sup> T cell responses to inactivated vaccine, and (ii) CBLB deficiency bolsters both type 1 and type 17 responses to inactivated vaccine. As shown in **Figure 3A**, vaccination with inactivated yeast, induced stronger Tc1 and Tc17 responses in Cblb<sup>-/-</sup> mice compared to Cblb<sup>+/+</sup> and unvaccinated controls, and responses were augmented with a higher vaccine dose. Similarly, total numbers of both IFN $\gamma$ <sup>+</sup> (Tc1) and IL-17A<sup>+</sup> (Tc17) CD8<sup>+</sup> T cells in Cblb<sup>-/-</sup> mice vaccinated with inactivated yeast were significantly higher than in Cblb<sup>+/+</sup> controls in both dLNs and spleens (**Fig. 3B & Supp. Fig. 2B**). CD43 expression (26), on cytokine producing cells in Cblb<sup>+/+</sup> and Cblb<sup>-/-</sup> mice were similar (**Supp. Fig. 2C**), suggesting that CBLB doesn't influence CD43 mediated



effector cell functions (26). Collectively, these data suggest that CBLB down-regulates anti-fungal Tc1 and Tc17 cell responses to inactivated fungal vaccine.

### **CBLB constrains inactivated vaccine-induced immunity against lethal fungal pneumonia**

We next investigated whether enhanced CD8<sup>+</sup> T cell responses to inactivated fungal vaccine in Cblb<sup>-/-</sup> mice translates to better resistance against lethal fungal pneumonia. Vaccination with heat-killed *Blastomyces* poorly protects Cblb<sup>+/+</sup> mice from lethal pulmonary challenge (27). In line with this finding, Cblb<sup>+/+</sup> mice vaccinated with inactivated yeast had only 5-fold less CFUs than unvaccinated controls after challenge. In sharp contrast, Cblb<sup>-/-</sup> mice that received inactivated vaccine had ~4 logs lower (1,295-fold) CFUs compared to their unvaccinated controls (**Fig. 4A**). Interestingly, unvaccinated Cblb<sup>-/-</sup> mice had ~2 logs lower (169-fold) CFUs compared to unvaccinated Cblb<sup>+/+</sup> mice, suggesting an innate, vaccine-independent role for CBLB. In concordance with CFU data, recall responses of cytokine-producing CD8<sup>+</sup> T-cells in infected lungs were significantly higher in Cblb<sup>-/-</sup> mice compared Cblb<sup>+/+</sup> mice (**Fig. 4B**). Thus, our data suggest that CBLB constrains antifungal immunity after vaccination with inactivated yeast and this finding correlates with poor induction and recall responses of CD8<sup>+</sup> T cells.

### **CBLB downregulates anti-fungal CD8<sup>+</sup> T cell responses to inactivated Histoplasma yeast**

To extend our observations to other pathogenic fungi, we evaluated the anti-fungal CD8<sup>+</sup> T cell responses in Cblb<sup>+/+</sup> and Cblb<sup>-/-</sup> mice following vaccination with inactivated *Histoplasma* yeast. Of note, *Histoplasma* induces strong type 1 (IFN $\gamma$ ) responses that are correlated with resistance (28–30). **Figure 5A** shows the frequency of cytokine-producing CD8<sup>+</sup> T cells in Cblb<sup>+/+</sup> and Cblb<sup>-/-</sup> mice vaccinated with either inactivated yeast or live yeast. As we observed with *Blastomyces* vaccination, the percentage of cytokine-producing CD8<sup>+</sup> T cells (especially type I cells) was significantly higher in Cblb<sup>-/-</sup> mice compared to Cblb<sup>+/+</sup> and unvaccinated mice (**Fig. 5 & Supp. Fig. 3**). Similar findings were present in live yeast vaccine groups except that Cblb<sup>-/-</sup> mice had significantly higher IL-17A<sup>+</sup> CD8<sup>+</sup> T cells compared to Cblb<sup>+/+</sup> controls (**Fig. 5B, Supp. Fig. 3B**). In sum, these findings together suggest that CBLB negatively regulates CD8<sup>+</sup> T cell responses to vaccination with multiple fungi.

### **Ablation of Cblb bolsters cytokine-producing ability of CD8<sup>+</sup> T cells without causing terminal differentiation**

We next investigated whether robust activation of Cblb deficient CD8<sup>+</sup> T cells causes their terminal differentiation or functional exhaustion, thereby potentially undermining long-term, vaccine-induced immunity. **Figure 6A** shows the expression of transcription factors in cytokine producing cells. Expression levels of prototypic transcription factors in Tc1 and Tc17 cells were similar in both Cblb<sup>+/+</sup> and Cblb<sup>-/-</sup> mice (**Fig. 6A**). The ratio of T-bet/Eomes, a prognostic feature of terminal differentiation and memory development (31, 32), was also similar in both Cblb<sup>+/+</sup> and Cblb<sup>-/-</sup> CD8<sup>+</sup> T cells (**Fig. 6B**). Also, the expression levels of CD127 (pro-survival) and KLRG-1 (terminal differentiation) (33) were similar in Cblb<sup>+/+</sup> and Cblb<sup>-/-</sup> mice (**Supp. Fig. 4A**). We also analyzed the quality of effector CD8<sup>+</sup> T cells by comparing their cytokine-producing ability on a per-cell basis. The MFIs of IFN $\gamma$

and IL-17A expression were significantly higher in Cblb<sup>-/-</sup> mice compared to Cblb<sup>+/+</sup> mice following vaccination with inactivated yeast (**Fig. 6C**), suggesting a better qualitative signature cytokine imprinted on these cells in the absence of CBLB. These data suggest that CBLB deficiency promotes CD8<sup>+</sup> T cell responses following vaccination with inactivated yeast without adversely causing their terminal differentiation.

### **CBLB downregulates anti-fungal CD8<sup>+</sup> T cell responses in a T-cell intrinsic mechanism**

CBLB is expressed not only in lymphoid cells but also in non-lymphoid cells, and affects their activation and functions (34). Although CBLB is a known negative regulator of TCR signaling, it is possible that CBLB expression in non-T cells affects the differentiation of anti-fungal CD8<sup>+</sup> T cells in a cell-extrinsic manner in our models. To address this possibility, we did “crisscross” adoptive transfer experiments involving vaccination. As shown in **Figure 7A**, irrespective of the background of recipient mice, OT-I cells lacking CBLB showed a significantly higher frequency of cytokine responses (1.5–2 fold) than OT-I cells expressing CBLB, suggesting a stronger intrinsic role of CBLB. Likewise, the total numbers of cytokine-producing CD8<sup>+</sup> T cells was significantly higher for Cblb<sup>-/-</sup>OT-I cells vs. Cblb<sup>+/+</sup> OT-I cells in either Cblb<sup>+/+</sup> and Cblb<sup>-/-</sup> recipients (**Fig. 7B**). Of note, although OT-I cells are known to resist becoming IL-17A<sup>+</sup> cells, the total number of IL-17A<sup>+</sup> Cblb<sup>-/-</sup> OT-I cells was significantly higher than Cblb<sup>+/+</sup>OT-I cells (**Fig. 7B**), even though the frequencies were similar between the groups. Thus, our findings indicate that CBLB has a prominent role in constraining anti-fungal CD8<sup>+</sup> T cell responses in a cell-intrinsic manner.

### **Cblb deficiency overcomes the requirement of hypoxic factor, HIF-1 $\alpha$ , for expansion of vaccine-induced CD8<sup>+</sup> T cell responses**

Hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) is critical for survival of cells under the hypoxic conditions that can prevail during inflammation or tumor growth (35). We have recently shown that HIF-1 $\alpha$  is necessary for the *expansion* of antifungal IFN $\gamma$ <sup>+</sup>, but not IL-17A<sup>+</sup>, CD8<sup>+</sup> T cell responses (21). Here, we investigated whether Cblb deficient CD8<sup>+</sup> T cells, resistant to apoptosis (36), require HIF-1 $\alpha$  for their response following fungal vaccination. We tested this hypothesis using the HIF-1 $\alpha$  specific inhibitor, Echinomycin. In concert with prior findings (21), Echinomycin treatment did not affect the expansion of IL-17A<sup>+</sup> (Tc17) CD8<sup>+</sup> T cells in Cblb<sup>+/+</sup> mice, but did significantly reduce IFN $\gamma$ <sup>+</sup> (Tc1) CD8<sup>+</sup> T cell responses in these mice in both dLNs and spleens (**Fig. 8 A & Supp. Fig. 4B**). In contrast, Echinomycin treatment did not affect Tc1 (or Tc17) cells in Cblb<sup>-/-</sup> mice, suggesting a dispensable role for HIF-1 $\alpha$  in the absence of CBLB. Next, we asked whether CBLB affects the protein levels of HIF-1 $\alpha$ . Our direct *ex vivo* staining showed increased levels of HIF-1 $\alpha$  in activated (CD44<sup>hi</sup>) CD8<sup>+</sup> T cells over naïve (CD44<sup>lo</sup>) T cells in Cblb<sup>+/+</sup> group (**Fig. 8B & C**), suggesting the induction of HIF-1 $\alpha$  following vaccination. Direct *ex vivo* staining showed the similar levels of HIF-1 $\alpha$  in both Cblb<sup>+/+</sup>- and Cblb<sup>-/-</sup>-activated (CD44<sup>hi</sup>) CD8<sup>+</sup> T cells, suggesting a negligible role of CBLB on HIF-1 $\alpha$  levels. Next, we evaluated the role of CBLB on the fate of HIF-1 $\alpha$  following restimulation (upon engagement of TCR signaling mimicking infection) in the presence of Cyclohexamide (protein synthesis blocker; new HIF-1 $\alpha$  synthesis) or MG132 (proteasome inhibitor; polyUb-mediated degradation of HIF-1 $\alpha$ ). We found higher levels of HIF-1 $\alpha$  with MG132 and relatively lower levels with Cycloheximide in both Cblb<sup>+/+</sup> and Cblb<sup>-/-</sup> CD8<sup>+</sup> T cells proportionately (**Fig. 8C**),



suggesting a minimal role of CBLB directly on HIF-1 $\alpha$  protein levels. Thus, HIF-1 $\alpha$  is redundant for *expansion* of CD8<sup>+</sup> T cells in the absence of CBLB following fungal vaccination.

## DISCUSSION

Despite the use of antifungals, individuals with deficiencies in CD4<sup>+</sup> T cells, such as AIDS patients, remain susceptible to life-threatening fungal infections. Inactivated vaccines would be desirable for such patients, if effective, but such vaccines have limited effectiveness due to weaker T cell responses. In this study, we show that modulating signaling downstream of the TCR is a strategy for developing efficacious fungal vaccines tailored to vulnerable individuals. Knocking out a negative regulator of TCR signaling, e.g. Cblb, augments type 1 and type 17 cytokine-producing CD8<sup>+</sup> T cell responses required for effective antifungal vaccine immunity. Our data supports the premise that adjuvants targeting such a pathway can bolster vaccine-immunity elicited by inactivated fungi. CD8<sup>+</sup> T-cell epitopes have not been identified for fungi; thus, the use of whole-cell based vaccines may be a rational method for inducing protective CD8<sup>+</sup> T cell immunity against such eukaryotic pathogens (37, 38).

Systemic fungal infections can be deadly in AIDS or CD4<sup>+</sup> lymphopenic individuals. Our study showed that chronic viral infections are lethal in Cblb<sup>-/-</sup> mice. However, inactivated fungal vaccination did not have this effect. Hence, perhaps one can vaccinate CD4<sup>+</sup> T cell lymphopenic patients by targeting CBLB without undesirable effects. Our idea is to use such adjuvants in a spatial (subcutaneous) and temporal (limited timeframe) manner. Perhaps, this method may potentiate a pool of virus-specific T-cell responses and boost their ability to clear virus (39, 40).

Vaccine-induced CD8<sup>+</sup> T cells compensate CD4<sup>+</sup> T cells for mediating immunity against lethal fungal pneumonia. Antifungal CD8<sup>+</sup> T cell immunity is correlated with the expression of both type 1 and type 17 cytokines (2, 3), and activation of neutrophils (3), an important leukocyte essential for killing yeast. Our study shows that CBLB deficiency fosters superior cytokine-producing CD8<sup>+</sup> T cells (**Fig. 3**) that correlate with vaccine-immunity (**Fig. 4**). Interestingly, unvaccinated Cblb<sup>-/-</sup> mice showed increased resistance compared to unvaccinated Cblb<sup>+/+</sup> mice, suggesting a role for innate (non-vaccine) immunity. Recent studies also showed that loss of CBLB promoted the activation of innate immune cells leading to superior *Candida* resistance (41–43). Nevertheless, our data indicate that vaccinated Cblb<sup>-/-</sup> mice are able to mediate much better resistance than vaccinated Cblb<sup>+/+</sup> mice. Thus, adjuvants targeting CBLB may bolster the efficacy of inactivated fungal vaccines to enhance CD8<sup>+</sup> T-cell immune correlates that govern vaccine-immunity. Our data also show that Cblb regulates vaccine-induced CD8<sup>+</sup> T cell responses in a cell-intrinsic manner, with little effect from non-T cells.

Qualitatively superior memory CD8<sup>+</sup> T cells are generated with optimal activation of TCR signaling (44). Overstimulation of TCR signaling, however, leads to CD8<sup>+</sup> T cell clonal deletion or functional exhaustion, marked by increased T-bet, decreased Eomes, and poor cytokine production (45). In the absence of Cblb, TCR down-regulation is inhibited, leading

to over-activation of TCR signaling and enhanced ERK phosphorylation (13). However, our data showed that vaccination with inactivated fungi did not cause terminal differentiation of Cblb deficient CD8<sup>+</sup> T cells, but enhanced their cytokine producing ability on a per-cell basis.

HIF-1 $\alpha$  is an important factor that facilitates survival, proliferation and tumor growth (35). Not surprisingly, tumor cells can thrive better in hypoxic niches by overexpressing HIF-1 $\alpha$ , and it serves as a target for treatment against certain cancers (46). T-cell specific ablation of HIF-1 $\alpha$  inhibits differentiation of Th17 cells by a ROR $\gamma$ t dependent mechanism (47); and Vhl (negative regulator of HIF-1 $\alpha$ ) deficiency in CD8<sup>+</sup> T cells enhances their effector functions, and causes immunopathology during persistent infection (48). We showed previously that antifungal Tc17 cells require HIF-1 $\alpha$  during their differentiation following fungal vaccination, but not for their expansion, while the converse was true for Tc1 cell responses (21). Here, our data showed that Cblb deficiency overcame the requirement of HIF-1 $\alpha$  for expansion of antifungal Tc1 cells. Perhaps, this may be one reason that Cblb<sup>-/-</sup> CD8<sup>+</sup> T cells function better at tumor sites, a niche known to have hypoxia. Regardless, in sum, we show here that CBLB can be a potential target for enhancing antifungal CD8<sup>+</sup> T cell responses following vaccination with inactivated yeast, thereby bolstering resistance against lethal fungal pneumonia.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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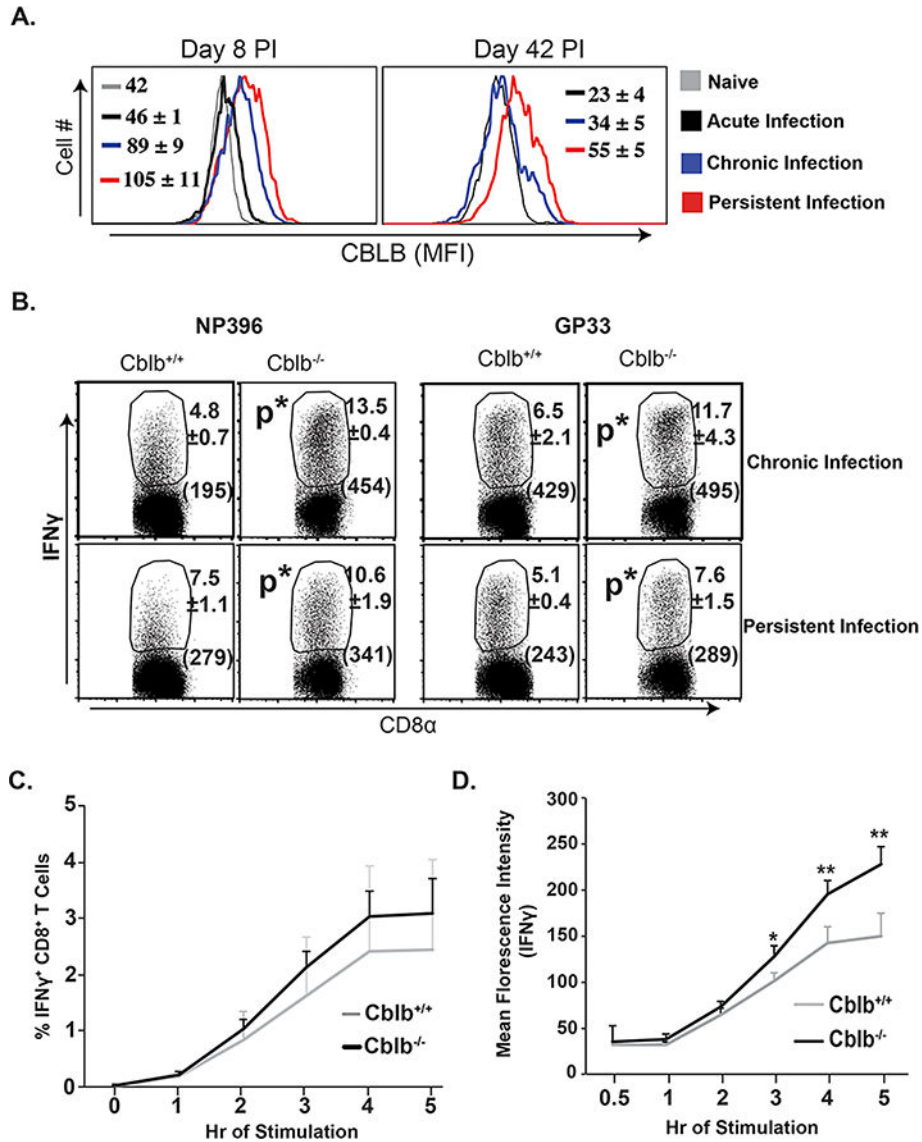
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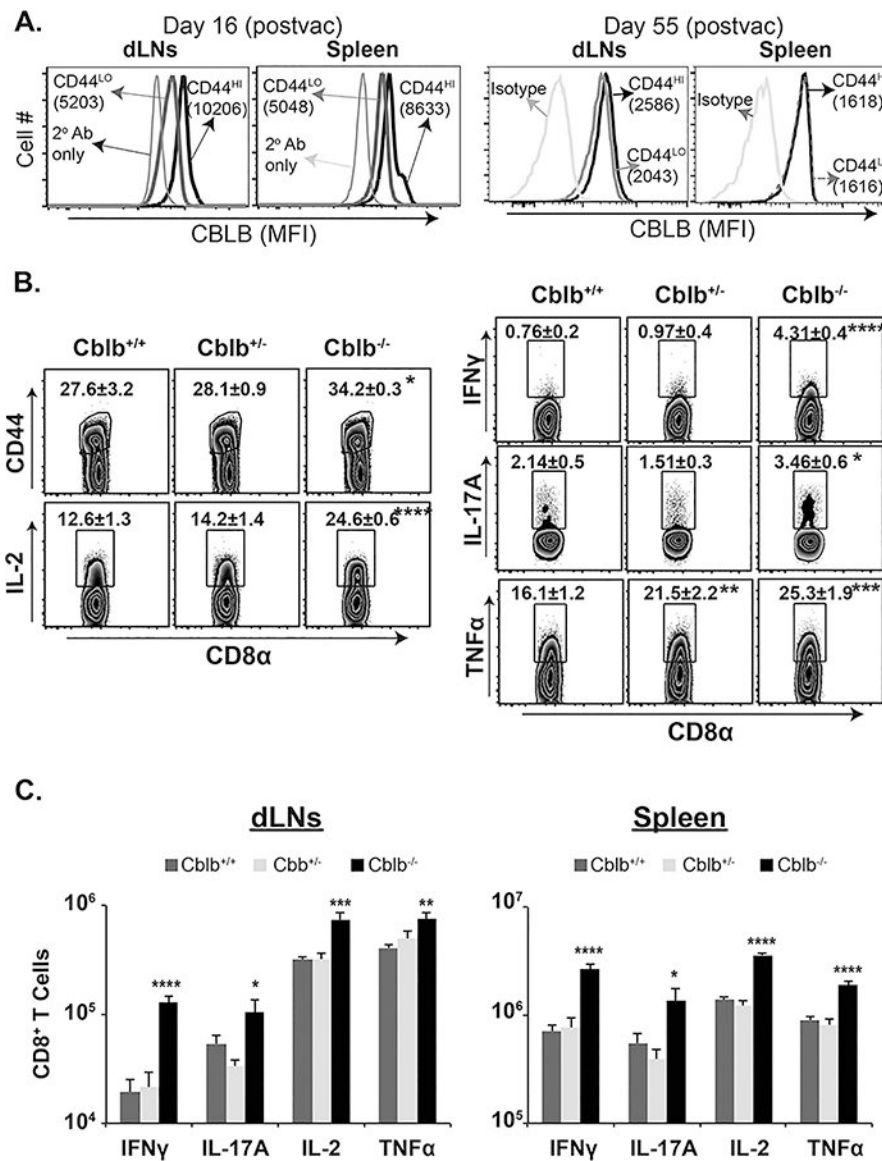
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**Figure 1. Role of CBLB during viral infections.**

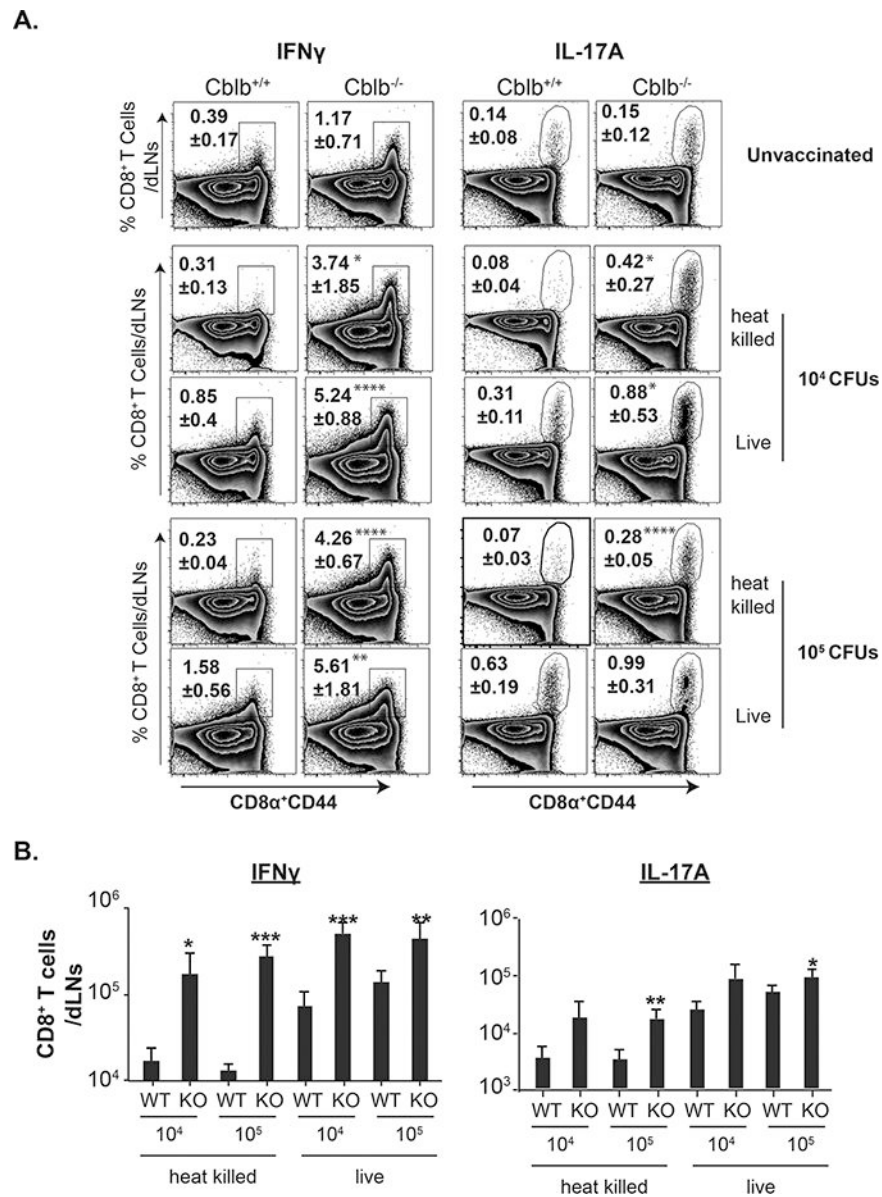
Naïve Cblb<sup>+/+</sup> and Cblb<sup>-/-</sup> mice were inoculated with LCMV strains to produce acute (Arm), chronic (Clone 13) or persistent (Clone 13, CD4-depleted) infections. (A) Levels of CBLB expression. Values are Mean Fluorescence Intensity (MFI) ±SD. (B) LCMV-specific CD8<sup>+</sup> T cell responses. Values are percent ± SD and MFI of IFNγ (parentheses). (C & D) Epitope-specific CD8<sup>+</sup> T cell responses following recombinant DNA (LCMV-NP) vaccination. Percent (C) and IFNγ expression in MFI (D) of CD8<sup>+</sup> T cell responses. N=4–5mice/group. \*p < 0.05.





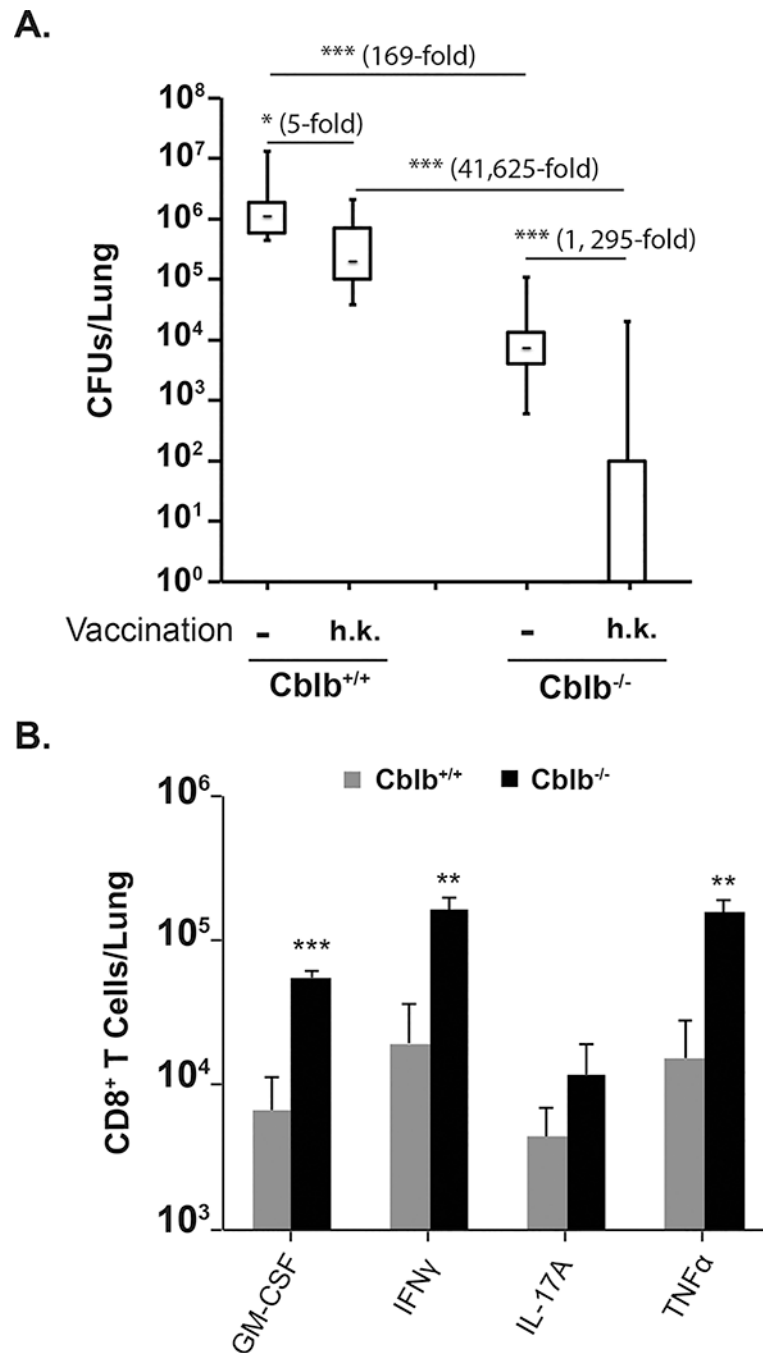
**Figure 2. CBLB constrains anti-fungal CD8<sup>+</sup> T cell responses.**

Naïve Cblb<sup>+/+</sup>, Cblb<sup>+/-</sup> and Cblb<sup>-/-</sup> mice were vaccinated s.c. with the live attenuated strain #55 of *B. dermatitidis* (10<sup>5</sup> cfu). On day 16, dLNs and spleens were harvested and cytokine-producing CD8<sup>+</sup> T cells were analyzed by flow cytometry. (A) CBLB expression (MFI) in naïve (CD44<sup>lo</sup>) and activated (CD44<sup>hi</sup>) CD8<sup>+</sup> T cells on indicated days post-vaccination. Frequency (B) and numbers (C) of cytokine-producing cells gated on activated (CD44<sup>hi</sup>) CD8<sup>+</sup> T cells. Values are mean  $\pm$  SD. N=4 mice/group. \*p 0.05. Data is representative of two independent experiments. CD4<sup>+</sup> T cells were depleted throughout the experiment.



**Figure 3. CBLB limits CD8<sup>+</sup> T cell responses to inactivated fungal vaccine.**

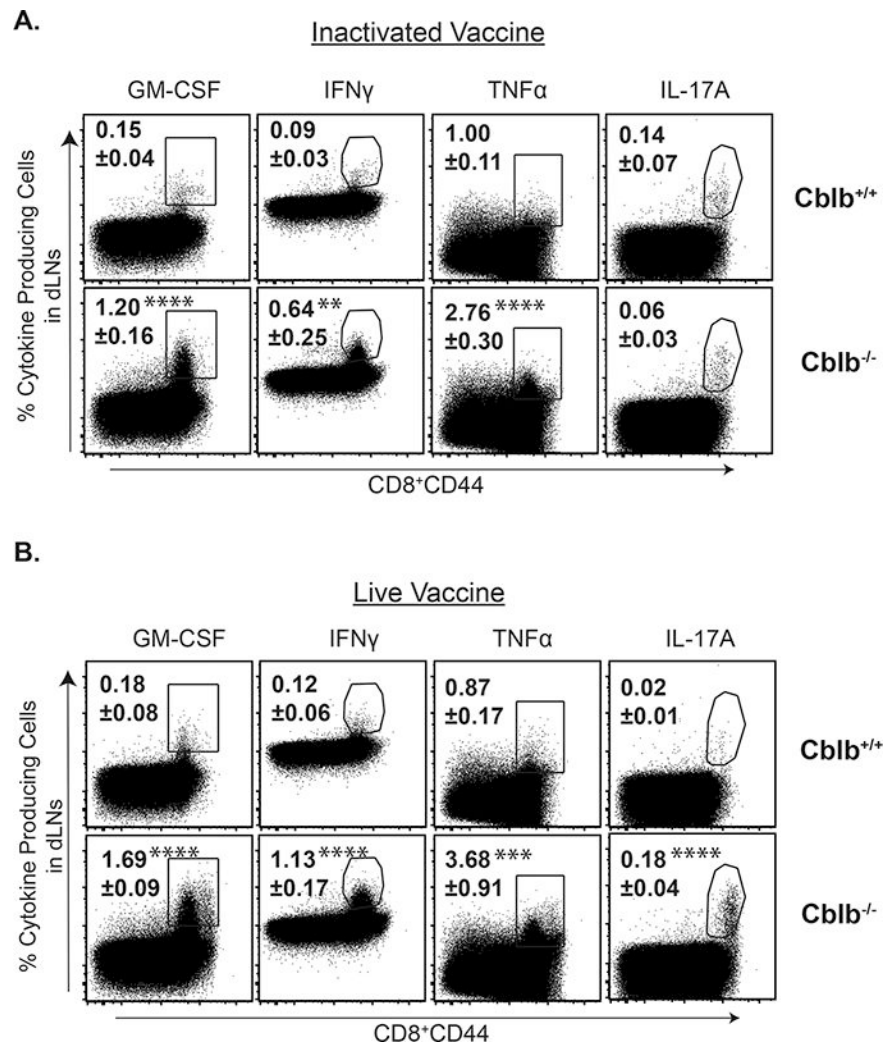
Naïve Cblb<sup>+/+</sup> and Cblb<sup>-/-</sup> were vaccinated with either live or heat-killed strain #55 yeast. On day 18, dLNs were harvested to analyze CD8<sup>+</sup> T cell responses by flow cytometry. Frequency (A) and numbers (B) of cytokine-producing CD8<sup>+</sup> T cells. Values are mean  $\pm$  SD. N=4–5 mice/group. \*p < 0.05. CD4<sup>+</sup> T cells were depleted throughout the experiment.



**Figure 4. CBLB regulates vaccine-immunity to inactivated fungal yeast.**

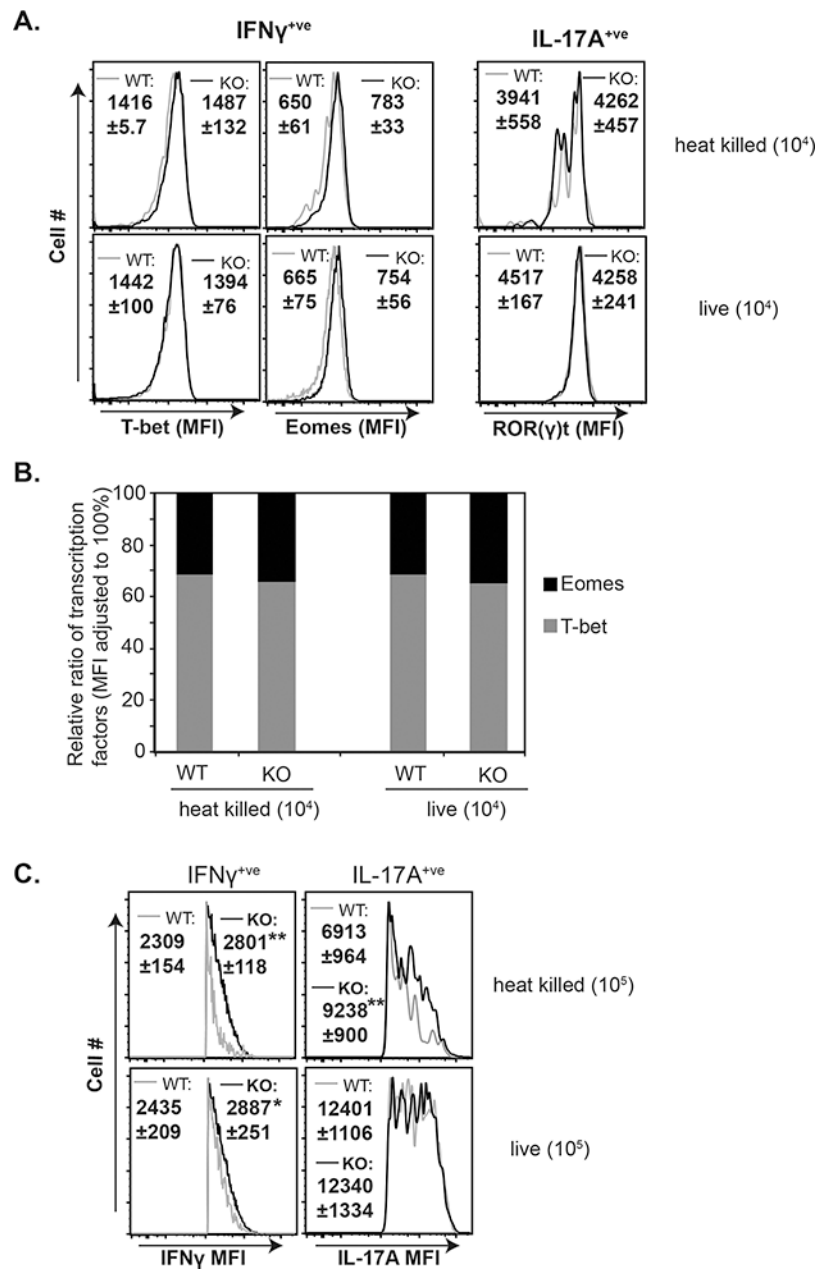
Naïve Cblb<sup>+/+</sup> and Cblb<sup>-/-</sup> were vaccinated with 10<sup>5</sup> cfu of heat-killed (h.k) strain #55 yeast.

(A) After a month rest, mice were challenged i.t. with a lethal dose (10<sup>4</sup> cfu) of virulent strain #26199. When unvaccinated mice were moribund (D16 post-infection; p.i.), lungs were harvested to quantify fungal burdens. CFUs are shown in whisker plots. N=4–14 mice/group. (B) Mice were vaccinated with killed yeast as above. Mice were challenged after 2 months rest and lungs were harvested (D4 p.i.) to enumerate cytokine-producing CD8<sup>+</sup> T cells. N=2–4 mice/group. Data is representative of two independent experiments. \*p 0.05.



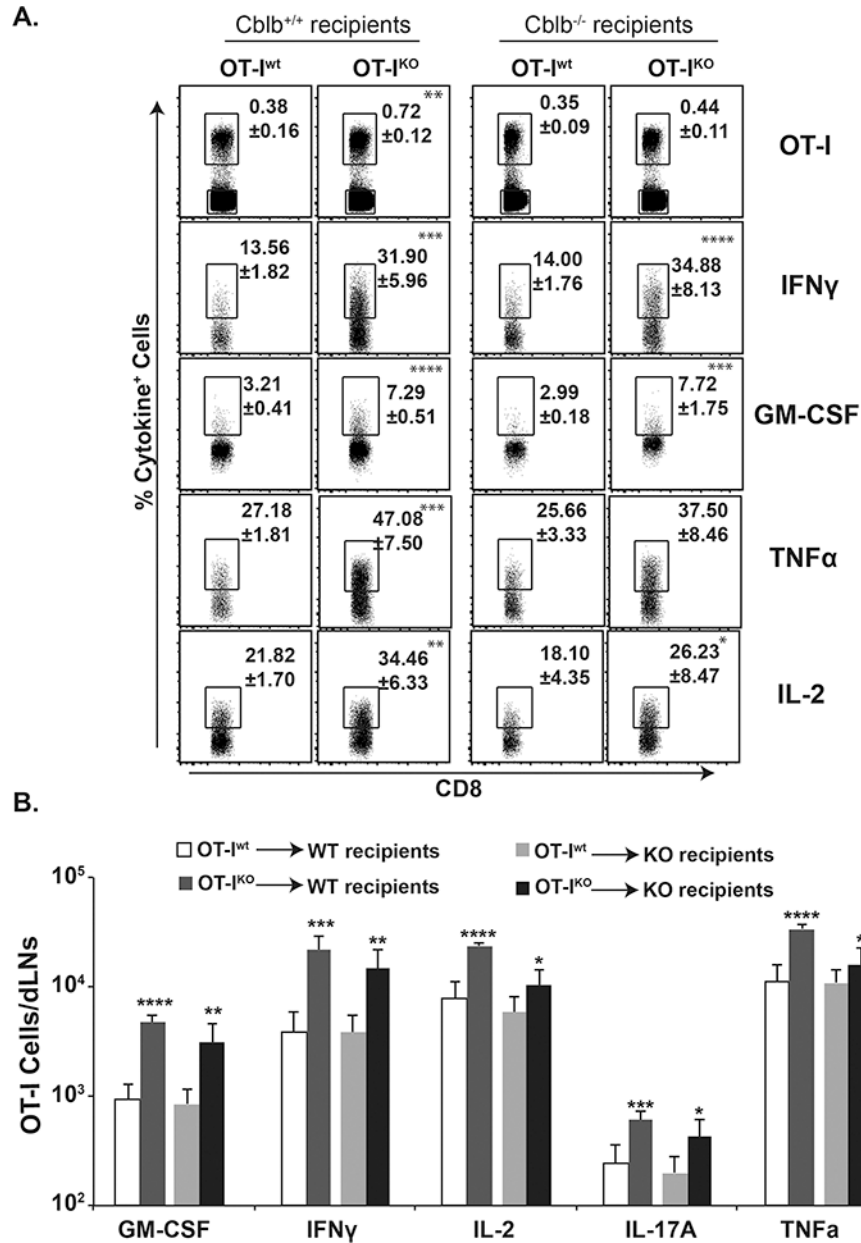
**Figure 5. CBLB limits CD8<sup>+</sup> T cell responses following vaccination with live or inactivated *Histoplasma* yeast.**

Naïve Cblb<sup>+/+</sup> and Cblb<sup>-/-</sup> were vaccinated with either live or heat inactivated (h.k.) *Histoplasma* yeast (10<sup>7</sup> cfu). On day 26, dLNs were harvested to analyze CD8<sup>+</sup> T cell responses by flow cytometry. Dot plots show the frequency of cytokine-producing CD8<sup>+</sup> T cells. Values are mean  $\pm$  SD. N=4–5 mice. \*p 0.05.



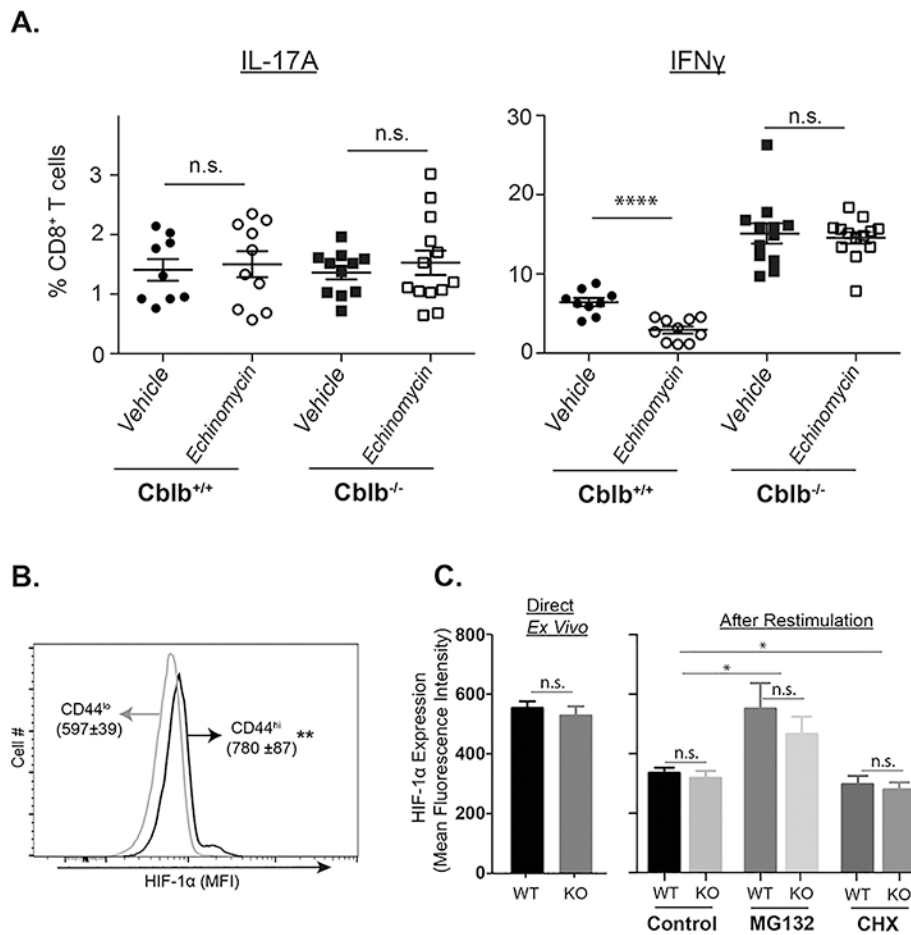
**Figure 6. Ablation of CBLB improves the quality of effector CD8<sup>+</sup> T-cells.**

Naïve Cblb<sup>+/+</sup> and Cblb<sup>-/-</sup> mice were vaccinated with either live or heat-killed strain #55 yeast. On day 18, dLNs were harvested to analyze CD8<sup>+</sup> T cell responses by flow cytometry. (A) Histograms show transcription factor profile of cytokine-producing CD8<sup>+</sup> T cells. (B) Relative proportions of expression of transcription factors in cytokine-producing CD8<sup>+</sup> T cells; relative expression was calculated by dividing MFI of each transcription factor by the sum of both MFIs. (C.) Histograms show MFI of cytokines gated on cytokine-positive CD8<sup>+</sup> T cells. Values are mean  $\pm$  SD. N=4–5 mice. \*p 0.05.



**Figure 7. Intrinsic role of CBLB for antifungal CD8<sup>+</sup> T-cell responses.** Enriched Thy1.1<sup>+</sup> OT-I T cells (~10<sup>6</sup>) from naïve Cblb<sup>+/+</sup> or Cblb<sup>-/-</sup> mice were adoptively transferred into naïve Thy1.2<sup>+</sup> Cblb<sup>+/+</sup> or Cblb<sup>-/-</sup> mice. On the following day, recipients were vaccinated with strain #55 yeast engineered to express the OT-I epitope (10<sup>6</sup> CFUs). On day 22, dLNs were harvested to enumerate cytokine-producing CD8<sup>+</sup> T cells. Frequency (A) and total numbers (B) of cytokine-producing OT-I cells. Values are mean ± SD. N=5–6 mice/group. Data is representative of two independent experiments. \*p 0.05, \*\*p 0.01, \*\*\*p 0.001, and \*\*\*\*p 0.0001, t-test comparing each cytokine-positive population between Cblb<sup>+/+</sup> and Cblb<sup>-/-</sup> cells in the same recipient genotype.





**Figure 8. HIF-1 $\alpha$  requirement for antifungal CD8<sup>+</sup> T cell responses.**

Naïve Cblb<sup>+/+</sup> and Cblb<sup>-/-</sup> mice were vaccinated with attenuated strain #55 yeast. Cohorts of mice received either vehicle or Echinomycin (3  $\mu$ g/mouse) every other day starting from day 4 post-vaccination (A). On day 14–16, dLNs were harvested to analyze cytokine-producing CD8<sup>+</sup> T cells by flow cytometry. Scatter diagrams show frequency of cytokine-producing cells among CD8<sup>+</sup> T cells. Each individual marker represents the value from a single mouse. Data is pooled from two independent experiments. (B & C) On day 16, dLNs were harvested to analyze HIF-1 $\alpha$  levels in CD8<sup>+</sup> T cells in the presence of Cyclohexamide (CHX; 10 $\mu$ M; EMD Millipore) or MG132 (50 $\mu$ M; EMD millipore). Data is representative of two independent experiments. Values are mean  $\pm$  SD. N=4–5 mice/group. \*p 0.05, \*\*p 0.01 and \*\*\*\*p 0.0001.