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E3 ubiquitin ligase CBLB regulates innate immune responses and bacterial dissemination during nontuberculous mycobacteria infection

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Abstract

Nontuberculous mycobacteria (NTM) are emerging opportunistic pathogens causing pulmonary infection to fatal disseminated disease. NTM infections are steadily increasing in children and adults, and immune-compromised individuals are at a greater risk of fatal infections. The NTM disease's adverse pathology and resistance to antibiotics have further worsened the therapeutic measures. Innate immune regulators are potential targets for therapeutics to NTM, especially in a T cell–suppressed population, and many ubiquitin ligases modulate pathogenesis and innate immunity during infections, including mycobacterial infections. Here, we investigated the role of an E3 ubiquitin ligase, Casitas B-lineage lymphoma proto-oncogene B (CBLB), in immunocompromised mouse models of NTM infection. We found that CBLB is essential to prevent bacterial growth and dissemination. *Cblb* deficiency debilitated natural killer cells, inflammatory monocytes, and macrophages in vivo. However, *Cblb* deficiency in macrophages did not wane its ability to inhibit bacterial growth or production of reactive oxygen species or interferon γ production by natural killer cells in vitro. CBLB restricted NTM growth

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Author contributions

S.M. and J.S. designed and executed the experiments, analyzed the data, and edited the manuscript. H.F.M.A. designed, executed, and analyzed some in vitro and in vivo experiments. T.C.K. executed and analyzed some in vitro experiments. W.C. designed, executed, and analyzed protein expression. A.M.T. provided the *Mycobacterium* strains and helped in designing some experiments. M.D.V. analyzed the histopathological data and edited the manuscript. N.P. helped obtain the gene transcript data. S.G.N. conceived the project, designed and executed the experiments, analyzed the data, and wrote the manuscript.

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Supplementary material

Supplementary materials are available at *Journal of Leukocyte Biology* online.

and dissemination by promoting early granuloma formation in vivo. Our study shows that CBLB bolsters innate immune responses and helps prevent the dissemination of NTM during compromised T cell immunity.

Keywords

granuloma; macrophages; monocytes; NK cells; nontuberculous mycobacteria; NTM; T cell lymphopenia

1. Introduction

Nontuberculous mycobacteria (NTM) or atypical mycobacteria are widely distributed in the environment. Among more than 190 species of NTM, *Mycobacterium avium* complex (MAC) species have been predominantly isolated from infected patients. MAC-associated infections are globally prevalent, causing mortality of up to 42%.[1–3] NTM infections are on the rise in all individuals but pose a greater risk to immune-compromised individuals, including cystic fibrosis and emphysema patients.[4] The emergence of drug resistance and poor understanding of protective immune mechanisms during NTM infections further complicates disease prevention and treatment.[5] The understanding of regulators of innate immunity against NTM infections may help develop novel intervention strategies. The key cells involved in innate immunity to mycobacterial infections are macrophages, monocytes, neutrophils, and natural killer (NK) cells, regulating bacterial pathogenesis, persistence, and dissemination.

A prompt and effective innate immune response is necessary to clear NTM infection or contain the bacteria at the portal of entry. Macrophages are the first line of defense, and engulf and kill the mycobacteria. Activation of macrophages occurs through pattern recognition receptor signaling, which augments the effector functions of macrophages expressing tumor necrosis factor α (TNFα) and reactive oxygen species (ROS) and effectuates phagolysosome functions. However, mycobacterium has evolved to counter these immune mechanisms to establish infection within the macrophages. NTM can interfere with innate cell signaling pathways, impair activation, and inhibit the production of proinflammatory molecules, which can hinder the effective clearance of the infection.[6] Mycobacteria induce granulomatous inflammation but can disseminate in the immunocompromised host, often associated with their virulent factors.[7] For example, nonspecific glycopeptidolipids of NTM prevent TLR2 signaling and facilitate bacterial colonization leading to invasive infection.[8] Many virulence-related factors of NTM resist oxidative stress and phagosome acidification by interfering with the host proteins of macrophages.[9]

The role of monocytes and neutrophils during NTM infection is ambiguous. Cystic fibrosis patients with NTM infection harbor significantly higher classical monocyte/macrophages in the lung that correlate with tissue damage.[10] Monocytes from individuals with a history of NTM lung disease showed differential responses bearing hyperinflammatory signatures,[11] and tissue and circulating monocytes dynamically participate in tuberculosis infection. While their activation promotes immunity, monocytes are implicated in mycobacterial

dissemination, inflammation, and pathology.[12] Accordingly, the ratio of monocytes to lymphocytes on either side of the spectrum predicts active mycobacterial disease.[13] Like monocytes, the role of neutrophils during NTM infections is obscure. Neutrophils exert their antimycobacterial function by phagocytosis, degranulation, and NETosis,[14] and defective neutrophils or neutrophil deficiency enhances the susceptibility to NTM. In line with this, the adoptive transfer of functional neutrophils imparts increased host resistance to NTM infection, implying neutrophils' protective role.[15] However, contrary to protection, neutrophils have been shown to facilitate bacterial growth, dissemination, and tissue damage during mycobacterial infections.[16] While systemic infection showed some degree of NTM immunity by neutrophils, they are largely dispensable during pulmonary infection.[17]

In addition to myeloid cells, innate lymphocytes significantly contribute to immunity against NTM infection. Activated NK cells secrete cytokines, TNFα, interferon γ (IFNγ), and granulocyte-macrophage colony-stimulating factor to inhibit NTM growth.[18] IFNγ is a macrophage-activating factor, and deficiency of functional IFNγ exacerbates NTM infection and compromises the granuloma formation.[19] NK cell dysfunction increases the susceptibility to NTM infection in mice and humans.[6] Similarly, the depletion or absence of NK cells early during the pulmonary NTM infection results in higher bacterial load, severe pathology, and mortality associated with reduced macrophage phagocytosis, dendritic cells (DCs), and poor granuloma formation.[10,20] Thus, initial host-bacterial interactions are crucial to direct the outcomes of the mycobacterial infection.

E3 ubiquitin ligases are a class of molecules regulating the protein's turnover, function, trafficking, and cell signaling. Accumulating evidence shows that ubiquitin ligases are essential for mycobacterial host defense. E3 ubiquitin ligase TRIM27 reduced the mycobacterial replication by enhancing MAPK signaling and macrophage apoptosis.[21] Similarly, another ubiquitin ligase, LNX1, seems to target NEK6, which inhibits apoptosis by activating STAT3 in mycobacterial-infected macrophages and facilitates antimycobacterial activity.[22] Other ubiquitin ligases, Parkin, SMURF1, TRIM32, and NEDD4, mediated antimycobacterial immunity by promoting autophagy.[23] Contrasting these ubiquitin ligases, host E3 ligase ANAPC2 inhibited inflammation and favored bacterial replication in the macrophages.[24] Thus, E3 ubiquitin ligases are instrumental in regulating mycobacterial pathogenesis, and an understanding of their role during the in vivo pathogenesis, including opportunistic NTM, is needed for immune interventions.

Casitas B-lineage lymphoma proto-oncogene B (CBLB) is an E3 ubiquitin ligase that negatively regulates T cell responses.[25] While CBLB roles are well defined for adaptive immunity, its functions are poorly described for innate immunity, especially during infections. Accumulating data suggest that CBLB inhibits signaling by dectin-1/2/3 and lipopolysaccharide stimulation in innate immune cells.[25,26] However, the deficiency of *Cblb* minimally impacts the development of macrophages, DCs, and NK cells.[27] CBLB prevented LPS-induced septic shock by downregulating TLR4 in macrophages.[28] In a model of fungal infection, CBLB downregulates Syk kinase, and ablation/inhibition of CBLB enhances the production of proinflammatory cytokines, release of ROS, and killing of yeast by macrophages.[29–31] Further, Cbl family members have regulatory roles in DCs by modulating

the DC functions positively and negatively.[25,32] Nevertheless, the role of CBLB in innate immunity during mycobacterial disease is unknown.

In this study, we studied the function of CBLB in regulating bacterial growth, dissemination, innate cell responses, and granulomatous inflammation in a mouse model of NTM infection during T cell deficiency.

2. Methods

2.1 Mice

The OT-I Tg (TCRα/β specific for OT-I epitope; stock #003831) and B6.PL-Thy1a/Cy/Thy1.1 (Thy1.1; strain #000406) were purchased from the Jackson Laboratory. *Cblb*$^{-/-}$ mice were provided by P.S. Ohashi (University of Toronto, Canada) with permission from Josef Penninger (Institute of Molecular Biotechnology, Austria). OT-I Tg mice (strain #003831) were backcrossed with *Cblb*$^{-/-}$ to generate OT-I Tg-*Cblb*$^{-/-}$ mice. *TCRα*$^{-/-}$*Cblb*$^{-/-}$ mice were generated by backcrossing *TCRα*$^{-/-}$ (The Jackson Laboratory; strain #002116) with *Cblb*$^{-/-}$ mice. All mice were maintained under specific pathogen-free conditions at the University of Illinois at Urbana-Champaign (UIUC), and all animal experiments were conducted according to the strict guidelines of the Institutional Animal Care and Use Committee, who approved all the work.

2.2 Culture of bacteria and infections

Mycobacterium avium strain 104 (MAV104) and isogenic recombinant dsRED^{+} kanamycin-resistant MAV 104 were cultured in Middlebrook 7H9 (Difco) broth supplemented with albumin, dextrose, and catalase with or without kanamycin. At optical density (OD600) of ~0.8 to 1.0, the culture was centrifuged and resuspended in sterile phosphate-buffered saline (PBS) for infection. Six- to 8-wk-old mice were used for all the infections. Mice were infected either intravenously with 1×10^6 colony-forming units (CFUs) or intratracheally (by intubation under sedation) with 1×10^5 CFUs.

2.3 In vitro experiments

Bone marrow–derived macrophages (BMMs) (granulocyte-macrophage colony-stimulating factor 10 ng/mL for 6 days) were washed, rested overnight, and infected with either 5 multiplicity of infection (MOI) or 10 MOI (bacteria: cells) in RPMI supplemented with 10% fetal bovine serum (complete media). After 4 h incubation, BMMs were washed to remove any floating/nonadherent bacteria and were incubated further for indicated times in complete media. Bone marrow–derived neutrophils (BMNs) were isolated as described[33] and infected with 5 MOI of dsRED^{+} MAV104. Cells were washed, stained, and analyzed by flow cytometry.

2.4 Bacterial burden determination

Infected cells or tissues were harvested, homogenized, and plated on BD Middlebrook 7H10 agar plates supplemented with albumin, dextrose, and catalase and kanamycin. Infected culture cells were lysed using 1% Triton X-100 before plating.

2.5 Flow cytometry

Single-cell suspensions from the tissues were prepared using 40μm BD Cell Strainers. Red blood cells were lysed using 4% ammonium chloride-containing PBS buffer. Similarly, bone marrow–derived cells were harvested from the plates either by scraping (BMMs) or collecting (BMNs). Cells were surface stained with fluorochrome-conjugated antibodies (BD Biosciences, BioLegend, and Invitrogen) along with Live/Dead staining (Invitrogen) for 30 min at 4 °C in the dark. For measuring cytokine production by NK cells or macrophages, cells were incubated with GolgiStop (BD Biosciences) at the last 4 h of stimulation with NTM or during 4 h restimulation with PMA (5 ng/mL; Sigma-Aldrich)/ionomycin (500 ng/mL; Stemcell Technologies) before staining with fluorochrome-conjugated antibodies for surface markers and intracellular cytokines (Perm/Fix buffer; BD Biosciences). Anti-CBLB (Clone: G-1; Santa Cruz Biotechnology) and inducible nitric oxide synthase (iNOS) (Clone: CXNFT; Thermo Fisher) antibodies were added during intracellular staining following surface markers staining. Cells were analyzed by Cytek Aurora full spectrum flow cytometer (College of Veterinary Medicine, UIUC), and data were curated using FlowJo software (v10.9.1; BD Biosciences).

2.6 Confocal microscopy

BMM cells were plated on micro-slide glass bottom wells (ibidi) a day before infection. At 48 h postinfection (PI), wells were washed with PBS and stained with dyes (DAPI, LysoTracker, and CellROX, Molecular Probes/Thermo Fisher Scientific]) for 30 min at 37 °C. Wells were washed and resuspended in complete media before the microscopy. Images (at least 10 distinct fields) were taken on a 4-laser Nikon A1R confocal microscope (College of Veterinary Medicine, UIUC) at 60× plan apo λ/1.40 oil and analyzed using NIS-Elements C software (Nikon). For quantifying the ROS production in BMM cells from confocal images, ImageJ 1.52r software (National Institutes of Health; https://wsr.imagej.net/download/) was used for each cell of the representative field.

2.7 Histopathological studies

Tissues (lung, spleen, liver, and brain) were harvested and stored in 10% buffered formalin containers. Formalin-fixed tissues were paraffin embedded, and sections were mounted on slides for hematoxylin and eosin staining. Hematoxylin and eosin slides were read and interpreted by a board-certified veterinary anatomic pathologist in a double-blinded manner.

2.8 Statistical analysis

All statistical analyses were performed using a 2-tailed unpaired Student *t* test using GraphPad Prism 9.5.1 software (GraphPad Software). A 2-tailed *P*-value of ≤0.05 was considered statistically significant.

2.9. Ethics statement

This work was executed in accordance with the protocol approved by the IBC and IACUC committees at the University of Illinois at Urbana-Champaign.

3. Results

3.1 Ablation of *Cblb* promotes NTM growth and dissemination under T cell deficiency in mice

The role of CBLB in innate immune cells during infections, especially in the immunocompromised state, is not understood. Recent reports suggest that CBLB constrains innate immune responses during fungal infections, and the loss of CBLB or its functions enhances the antifungal activities of macrophages and DCs.[30,31,34] Because NTM infections primarily cause severe infections in individuals with compromised immunity,[8] we asked if CBLB impairs innate immunity during T cell deficiency. We used OT-I Tg mice (all $CD8^+$ T cells are specific for OT-I epitope of ovalbumin) and OT-I-Tg-*Cblb* KO (OT-I Tg lacking Cblb) in which OT-I Tg mice lack $CD8^+$ T cells recognizing NTM and have insignificant numbers of $CD4^+$ T cells. Because MAV among MAC species accounts for most NTM infections[1,5] and major routes of infections are oral or respiratory, resulting in pulmonary and disseminated infections,[35] we used MAV104 for our experiments to assess the Cblb-mediated innate immunity. Contrary to the established dogma of negative role of Cblb for innate immunity during fungal infections, we found significantly high bacterial loads in the lungs, spleens, and livers at 6, 12, and 22 wk PI in *OT-ICblb*$^{-/-}$ mice than in *OT-ICblb*$^{+/+}$ mice (Fig. 1A). Because OT-I Tg mice can have few functional $CD4^+$ T cells to NTM and may cause inadvertent readouts, we asked if our observations can be recapitulated in the absence of $\alpha\beta^+$ T cells using TCR$\alpha^{-/-}$ background mice. Analogous to the OT-I-Tg mouse infection data, we found significantly higher bacterial loads in the lung, spleen, and livers of TCR$\alpha^{-/-}$Cblb$^{-/-}$ mice than in tissues of WT control mice (Fig. 1B).

Because MAC bacteria are highly hydrophobic and aerosolized, inhalation is a common route of infection.[36] Next, we asked if *Cblb* deficiency promotes pulmonary NTM infection. Similar to systemic infection, we found higher bacterial loads in the lungs of *OT-ICblb*$^{-/-}$ mice than in the lungs of *OT-ICblb*$^{+/+}$ control mice following pulmonary infection (Supplementary Fig. 1). We also found increased bacterial dissemination into spleen and mediastinal lymph nodes in Cblb deficiency. Thus, CBLB inhibits NTM growth and bacterial dissemination during T cell deficiency.

3.2 *Cblb*-deficient macrophages are competent to inhibit NTM growth in vitro

Mycobacteria primarily target macrophages, and our preceding bacterial burden and dissemination data (Fig. 1) immediately piqued our interest in dissecting the cell-intrinsic role of CBLB for macrophage functions. BMMs were infected with MAV for 4 h and washed to remove any extracellular or nonadherent bacteria, and cells were further cultured for indicated days. At days 2 to 6 d PI, *Cblb*$^{-/-}$ BMM wells had significantly lower CFUs compared with *Cblb*$^{+/+}$ BMM wells (Fig. 2A, left). Although we did not notice significant differences in the later time points (days 8–13 PI) (Fig. 2A, right), *Cblb*$^{-/-}$ BMM wells consistently had lower bacterial burdens compared with *Cblb*$^{+/+}$ BMM wells. To dissect if the above phenotype is due to differences in bacterial uptake, we infected BMMs with fluorescent ($dsRED^+$) MAV104 for 4 h, washed to remove unbound bacteria, and analyzed by flow cytometry. We did not see significant differences in the frequency of $dsRED^+$ *Cblb*$^{+/+}$ and *Cblb*$^{-/-}$ BMMs (Fig. 2B), suggesting a minimal role of CBLB in

MAV binding or phagocytosis. Next, we assessed the role of CBLB in BMM responses to NTM. We infected BMMs with dsRED^{+} MAV104, and at 48 h, cells were stained with CellROX (Invitrogen; measuring cellular oxidative stress) to quantify ROS. The infected *Cblb*$^{-/-}$ BMMs had significantly higher CellROX fluorescence compared with *Cblb*$^{+/+}$ cells (Fig. 2C, Supplementary Fig. 2A). Lysotracker helped us to track the lysosomes, including phagolysosomes. To assess if CBLB is induced following MAV infection, we infected the BMMs and peritoneal macrophages to analyze its expression by flow cytometry. Infection of cells significantly induced the expression of CBLB compared with uninfected cells (Supplementary Fig. 2B). Thus, *Cblb* deficiency did not adversely affect the production of ROS or control of NTM growth by macrophages per se.

3.3 CBLB promotes inflammatory monocytes and alveolar macrophages during an NTM infection

Preceding in vitro data suggested a similar competitiveness of *Cblb*-deficient macrophages in controlling the bacteria compared with WT macrophages. Next, we set out to examine the fate of monocyte/macrophage lineage cells in vivo following the infection. Monocyte/macrophage lineage cells are instrumental in the pathogenesis of mycobacterial infection.[37] We employed the gating strategy of flow cytometry data (Supplementary Fig. 3) to identify the innate cells, including macrophages[38] and used CD90 (Thy1) monoclonal antibody to exclude any thymic-derived T cell population. We evaluated the inflammatory monocytes and macrophages during systemic and intratracheal infections. The frequencies of inflammatory monocytes were significantly lower in spleens of *OT-ICblb*$^{-/-}$ mice than in spleens of *OT-ICblb*$^{+/+}$ mice during the systemic infection ($P \leq 0.05$) (Fig. 3A, B). However, we did not see significant differences between the groups in inflammatory monocytes in the lung. Similar findings were recapitulated during systemic infection in TCR$\alpha^{-/-}$ background *Cblb*$^{-/-}$ and *Cblb*$^{+/+}$ mice (Fig. 3C). Next, we asked if *Cblb* alters the monocyte population in the lung following intratracheal infection. *Cblb* deficiency significantly reduced the inflammatory monocytes (Ly6C^{hi}) (Supplementary Fig. 4A, left). Additionally, we found reduced expression of major histocompatibility complex class II (MHC II) on inflammatory monocytes in the absence of *Cblb* (Supplementary Fig. 4A, right).

Because alveolar macrophages are lung resident cells distinct from the monocyte population, we next asked if CBLB influences their population. Like the monocyte population, we found a significant reduction of alveolar macrophages in *OT-ICblb*$^{-/-}$ mice compared with *OT-ICblb*$^{+/+}$ mice as early as 6 wk and up to 22 wk PI (Fig. 3D–E). The phenotype was recapitulated in infected TCR$\alpha^{-/-}$*Cblb*$^{+/+}$ and TCR$\alpha^{-/-}$*Cblb*$^{-/-}$ groups (Fig. 3F). Similarly, we found a significant reduction of alveolar macrophages in *Cblb*-deficient mice compared with *Cblb*-sufficient control mice following pulmonary infection (Supplementary Fig. 4B). Because NO and TNF influence host defense mechanisms during mycobacterial infections,[9,39,40] we measured their expression in alveolar macrophages. We found a reduced expression of iNOS in *Cblb*-deficient alveolar macrophages (Fig. 3G, H). However, the frequency of TNF producing alveolar macrophages was significantly high in *Cblb*-deficient mice compared with *Cblb*-sufficient mice (Fig. 3I). Thus, *Cblb* helps to sustain the monocytes and macrophage populations during NTM infection but not the TNF-producing ability of macrophages.

3.4 *Cblb* selectively rescues a subset of functional NK cells during NTM infection

NK cells are cytotoxic innate lymphocytes and one of the major IFNγ-producing cells mediating early immunity against mycobacterial infections, especially under T cell deficiency,[20,41,42] by augmenting the functions of macrophages. Importantly, CD11b expressing NK cells are more potent in their functions.[43–45] In line with the published reports on negative regulation of CBLB in NK cells,[46,47] we found significantly higher production of IFNγ by *Cblb*$^{-/-}$ NK cells, irrespective of their CD11b expression, after infection in vitro (Fig. 4A). Notably, the expression of CBLB was significantly induced in NK cells following the infection of splenocytes in vitro (Supplementary Fig. 4C). Next, we evaluated the fate and functions of NK cells in vivo after the infection. Infection enhanced the frequency of CD11b^{+} subset of NK cells at an earlier time (~40%–70%; week 6) (Fig. 4B) and remained proportionately similar until week 22 (Fig. 4C). Although *Cblb* deficiency did not affect CD11b^{-} NK cells, CD11b^{+} NK cells were significantly depleted during the infection. Next, we asked if CD11b-expressing NK cells are potent in IFNγ production. Single-cell suspension cells from infected mice were restimulated to determine the frequency of IFNγ- and TN—producing NK cells. The CD11b^{-} NK cell subset was poor in expression of IFNγ compared with CD11b^{+} NK cells (1% vs 15%), and *Cblb* deficiency significantly enhanced the expression of IFNγ (~30%) among CD11b^{+} NK cell subset (Fig. 4D). Similarly, we found significantly enhanced intrinsic ability of TNF expression by *Cblb*-deficient NK cells compared with *Cblb*-sufficient NK cells of either subset (Fig. 4E). Of note, the *Cblb*-deficient CD11b^{+} subset has the superior ability of TNF expression. However, *Cblb* deficiency severely depleted the CD11b^{+} subset (Fig. 4E). We found the similar phenotype following intratracheal infection (Supplementary Fig. 4D). Thus, *Cblb* selectively rescues functional CD11b^{+} NK cells during NTM infection.

3.5 Role of CBLB in neutrophils during an NTM infection

The neutrophil response during mycobacterial diseases is a double-edged sword, and their contribution to immunity is debated.[48,49] Here, we assessed neutrophil responses during NTM infection during T cell deficiency. During systemic infection, the neutrophils were unaffected in the absence of *Cblb*, and we found no significant differences at week 22 PI (Fig. 5A) and at earlier times (Supplementary Fig. 5A). The similar findings were recapitulated in TCRα knockout background mice except that the *Cblb*-deficient lungs endured more neutrophils (Fig. 5B). We also evaluated their activation phenotype using marker CD44 and found a consistent, significant reduction in *Cblb*$^{-/-}$ mice compared with *Cblb*$^{+/+}$ mice (Supplementary Fig. 5B). Similarly, intratracheal infection showed no differences between *Cblb*-sufficient and *Cblb*-deficient groups except in the lung at later period of infection. We asked if there was any difference in bacterial uptake due to *Cblb* deficiency. BMNs were incubated with 5 MOI of dsRED^{+}MAV104 and analyzed by flow cytometry. At 4 and 12 h PI, ~50% and ~90%of *Cblb*$^{+/+}$ and *Cblb*$^{-/-}$ BMNs, respectively, were dsRED^{+} (Fig. 5C), suggesting a minimal role of CBLB for phagocytosis. Thus, *Cblb* minimally affects the neutrophils during NTM infection but may help in their activation.

3.6 Role of CBLB in granulomatous inflammation during an NTM infection

The preceding data suggested that *Cblb* is protective in constraining mycobacterial growth, preserving monocyte/macrophage populations, and rescuing functional NK cell populations during T cell deficiency. However, we could not answer the enhanced dissemination without *Cblb*. Granulomatous inflammation during mycobacterial infections helps limit the dissemination and possibly contain bacterial growth. Next, we determined the effect of CBLB on NTM pathogenesis by histopathology. We harvested tissues following an NTM infection at weeks 3, 6, 9, and 13 and subjected to histopathological readings. The inflammation, characterized by small discrete granulomatous foci, was prominent in *Cblb*-sufficient mice as early as 3 wk PI in the spleen and liver (Fig. 6). Similarly, multiple small indiscrete granulomatous foci were found in the lungs in $Cblb^{+/+}$ mice. In contrast, granulomatous foci were not apparent in the *Cblb*-deficient mice in any tissues at 3 wk PI, and a few developed at weeks 6 and 9 PI. However, small discrete granulomatous inflammation foci were observed at 13 wk PI in all tissues of infected mice (Fig. 6), suggesting a delay in forming granulomas in the absence of *Cblb*. We noted higher numbers of acid-fast bacilli in $Cblb^{-/-}$ mice tissues compared with $Cblb^{+/+}$ mice starting from 3-wk PI (data not shown) that was reminiscent of CFU readings (Fig. 1). Collectively, our data suggest that CBLB facilitated the early formation of granulomas and thwarted the bacterial dissemination during an NTM infection under T cell deficiency.

4. Discussion

Given the increasing incidence of NTM infections in immunocompromised individuals, an understanding of host regulators of innate immunity is needed, especially during T cell deficiency. Our study showed that CBLB is necessary for inhibiting NTM growth and dissemination under T cell deficiency in vivo. We found that CBLB was significantly induced in primary macrophages after infection, but *Cblb* deficiency did not affect the competency in inhibiting NTM growth in vitro in contrast to fungal growth.[29,30] Interestingly, a recent study suggested that CBL sequesters LpqN, an *M. tuberculosis* virulence factor, for enhanced *M. tuberculosis* killing by macrophages,[50] and CBL-deficient macrophages were permissive for *M. tuberculosis* growth. The putative LpqN family protein is also present in *M. avium* (National Center for Biotechnology Information Basic Local Alignment Search Tool, sequence ID: EUA40567.1; and Broad Institute MAV4561 protein) species. Further studies are warranted to decipher the in vivo role of CBLB in macrophages during different mycobacterial diseases.

There is an immense interest in the role of newly recruited monocytes, including inflammatory monocytes, in the pathogenesis of mycobacterial diseases.[51,52] Recently recruited monocytes/macrophages expressing CCR2 seem highly permissive to *M. tuberculosis* due to their inability to get activation signals masked by phenolic glycolipids decoration on bacteria.[53,54] However, several studies have shown enhanced susceptibility under CCR2 deficiency, which may also depend on the virulence of mycobacteria.[55] Thus, newly recruited monocytes are critical regulators of the pathogenesis and outcome of mycobacterial diseases and can be targeted for therapeutic interventions.[56] Additionally, monocytes/macrophages can regulate defense against mycobacteria by producing reactive

oxygen/nitrogen species[57] and priming productive T cell responses.[58] In our study, we found a poor activation (low MHC-II) but higher production of ROS in *Cblb*-deficient inflammatory monocytes (data not shown). However, the numbers of inflammatory monocytes were relatively intact, except in the spleens of intravenous-infected groups, in which we found enhanced numbers of noninflammatory monocytes. In a recent study, noninflammatory monocytes, classified as myeloid-derived suppressor cells, subvert immune responses.[47] We reasoned if CBLB regulates the expression of CCL2, a chemokine involved in the recruitment of monocytes. We found either normal or less *CCL2* expression in infected $Cblb^{-/-}$ cells compared with $Cblb^{+/+}$, in vitro (data not shown), suggesting a minimal role of CCL2 for the observed phenotype. Nevertheless, in vivo dynamics of *CCL2* expression and its role in the activation of monocytes during NTM infection needs further investigation.[59] The low MHC-II expression levels on *Cblb*-deficient inflammatory monocytes may be due to their less differentiation status or commitment into macrophage lineage under altered micro milieu.[51,60] Nevertheless, we found an enhanced ROS production under *Cblb* deficiency, but not the iNOS, and maybe involved in dissemination in the lack of T cell help or enhanced cell death.[61]

NK cells are early regulators of mycobacterial pathogenesis, and their loss is associated with enhanced bacterial growth.[20,41] Our study showed a significant loss of NK cells in *Cblb*-deficient mice, especially the $CD11b^{+}$ subset that was a potent IFNγ and TNF producer in vivo. NTM infection significantly increased CBLB levels in NK cells, and as predicted,[62] $Cblb^{-/-}$ NK cells produced higher IFNγ and TNF compared with $Cblb^{+/+}$ cells, similar to antitumor NK cells.[63] Hence, we postulate that reduced NK cells in *Cblb*-deficient mice may be one of the causes of higher bacterial burden and dissemination. Our results align with the potential beneficial role of NK cells in controlling NTM infection,[20] and $CD11b^{+}$ NK cells may be superior in functions.[20,41,42,45] Although the role of *Cblb*-deficient NK cells during the early phases of infection and their loss during NTM infection is not known, we think that higher antigen levels may induce their depletion, akin to NK T cells or T cells.[64,65]

Neutrophils play a dichotomous role during mycobacterial infections, and their role in contributing to immunity or immunopathology is confounded.[49] Early recruitment and status of neutrophils seem to dictate their role in controlling mycobacterial infection, and many studies show the importance of neutrophil function in chronic granulomatous disease patients in defense against active tuberculosis.[66,67] However, exuberant neutrophil responses are associated with pathology.[48] Our data suggested that phagocytosis of bacteria by neutrophils was not affected by *Cblb* deficiency. However, CBLB seems to inhibit the activation of BMNs (Mean Fluorescence Intensity of CD44) in vitro. However, our in vivo studies suggested otherwise, in that neutrophils' activation status was significantly less in *Cblb*-deficient mice, but numbers were not affected. Similarly, we did not see significant differences in the numbers of neutrophils among the groups by histopathology, despite increased numbers of bacteria in the macrophages of *Cblb*-deficient groups. Although the role of CBLB in neutrophil functions is understudied, a recent study suggested a minimal effect of CBLB on neutrophil responses during candida infection.[29] Further functional studies are required to delineate CBLB's role in neutrophils during the early phases of NTM infection.[15]

Although the beneficial role of granuloma formation for the host defense and clearance of bacteria is debated,[68–70] the dogma, nevertheless, supports for prevention of dissemination.[71,72] In this study, CBLB promoted the formation of early granulomas in the lung, which correlated with reduced dissemination. Although granuloma formation reflects the failure of the initial protective immunity, the early formation of granulomas (mainly mononuclear phagocytes) walls off the bacteria.[70] In general, granulomas help restrict, segregate, and destroy the pathogen, evident during miliary tuberculosis, in which immune cells fail to form protective granulomas resulting in rapid lymphohematogenous spread of the bacteria.[73] Similarly, in an experimental model of schistosomiasis and cryptococcosis, mice that fail to elicit an effective granulomatous response have decreased survival accompanied by disseminated infection.[74,75]

We have shown that CBLB is a growing list of E3 ubiquitin ligases that regulates pathogenesis of mycobacterial diseases. CBLB was necessary for controlling bacterial growth and dissemination during an NTM infection under compromised T cell immunity. CBLB deficiency led to poor or altered innate cell subsets' status and the lack of early granulomatous inflammation. Future studies are warranted to uncover the relative roles and mechanisms of CBLB in innate immune cell subsets during NTM infection. Our studies demonstrate that CBLB can be a target for therapeutic measures in controlling NTM infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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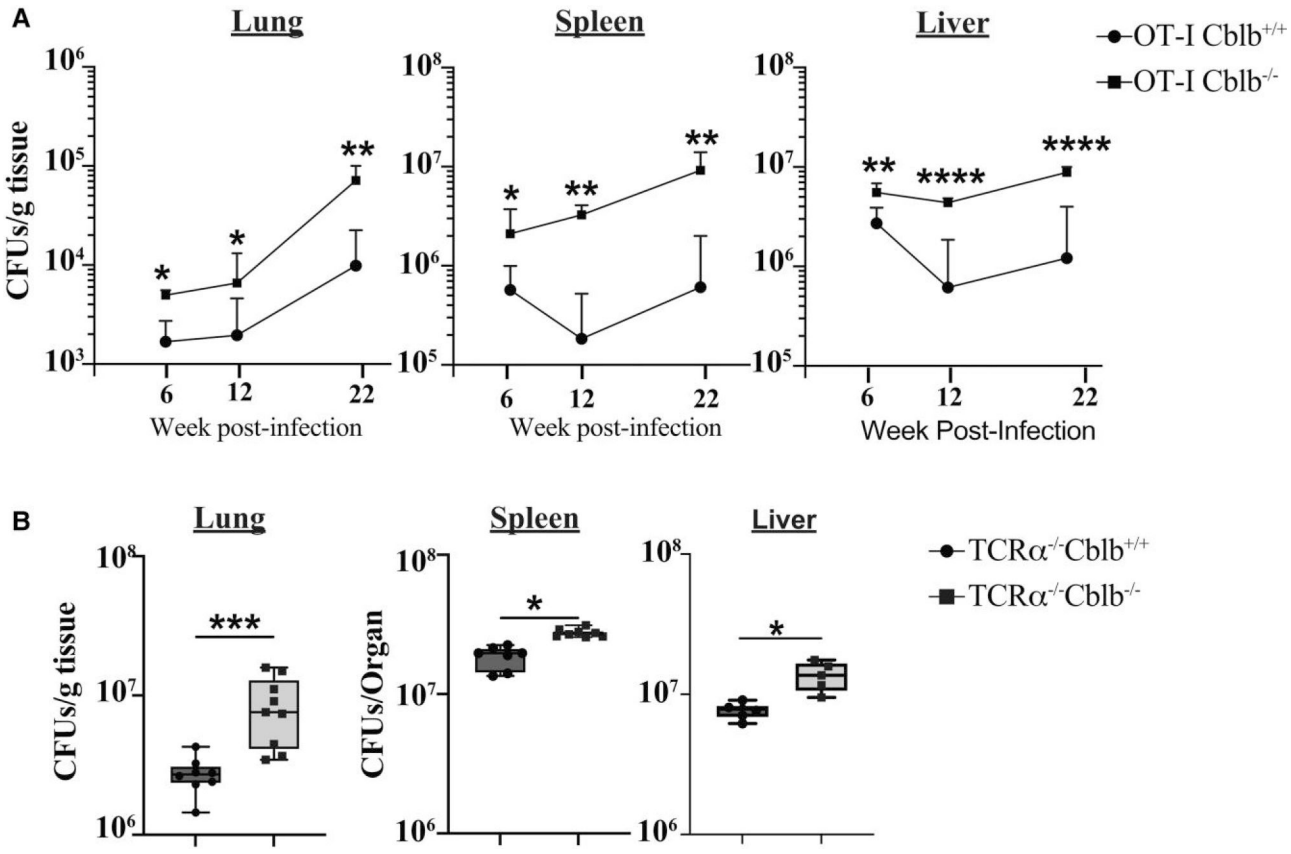

Fig. 1.

Cblb deficiency promotes NTM dissemination in mice. Six- to 8-wk-old *OT-I-Tg-Cblb*$^{+/+}$, *OT-I-Tg-Cblb*$^{-/-}$, *TCRα*$^{-/-}$*Cblb*$^{+/+}$, and *TCRα*$^{-/-}$*Cblb*$^{-/-}$ mice were infected with *M. avium* (MAV104; 10^6 CFUs) intravenously as described in the Methods. At indicated times PI (A) or at 8 wk PI (B), tissues were harvested and bacterial loads were enumerated. The data are representative of 2–3 experiments. Values are mean ± SD. n = 3–9 mice/group. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, and ****$P \leq 0.0001$.

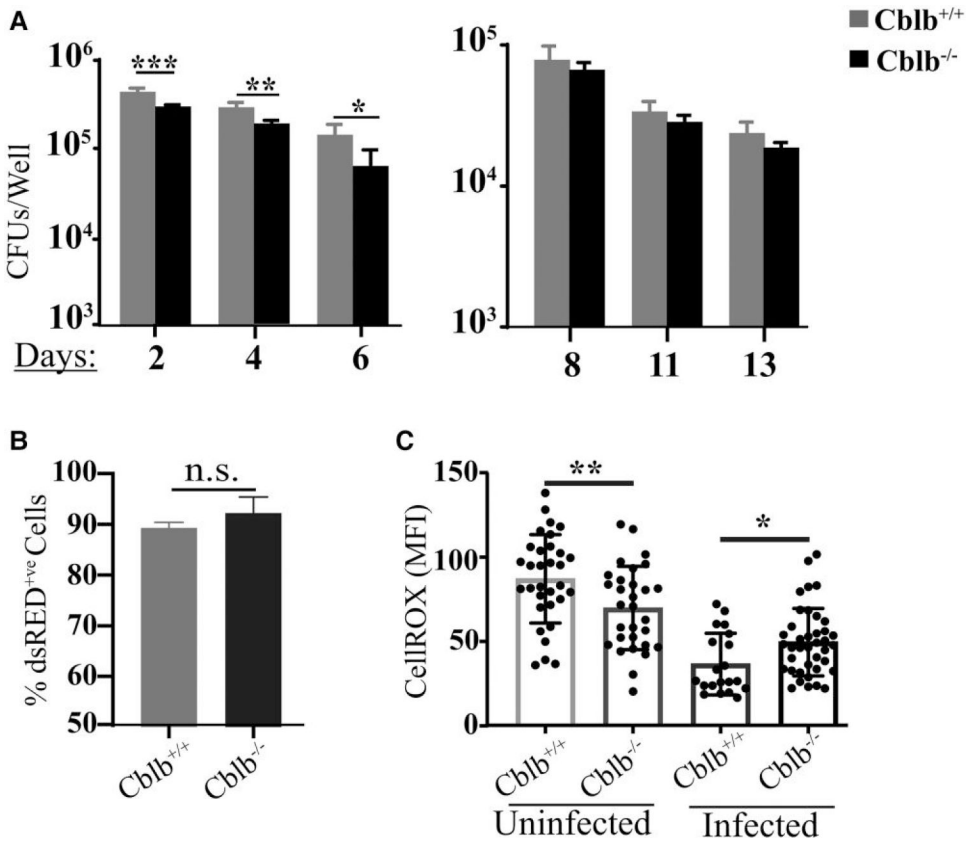

Fig. 2.

Effects of CBLB on NTM growth in vitro. Bone marrow cells from *OT-I-Tg-Cblb*$^{+/+}$ and *OT-I-Tg-Cblb*$^{-/-}$ mice were harvested to differentiate into macrophages (BMMs). On day 6, wells were washed and adherent BMMs were collected for seeding (1×10^6/well). Cells were infected with MAV104 (5 MOI), and at 4 h PI, wells were washed to remove nonadherent/extracellular bacteria. At indicated days of PI (A), BMMs were lysed with 1% Triton X-100 and CFUs were enumerated on 7H10 agar plates. At 4 h PI (B), the rate of phagocytosis (% dsRED$^+$ MAV104) by BMM cells was analyzed by flow cytometry. (A, B) Data is represented as mean ± SD of 4 replicates/experiment from at least 2 independent experiments. (C) At 48 h PI, BMMs were washed, stained with CellROX reagent, and analyzed by confocal microscopy. The ROS levels (Mean Fluorescence Intensity) were quantified using ImageJ software. Data are from 2–3 independent experiments. *$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$.

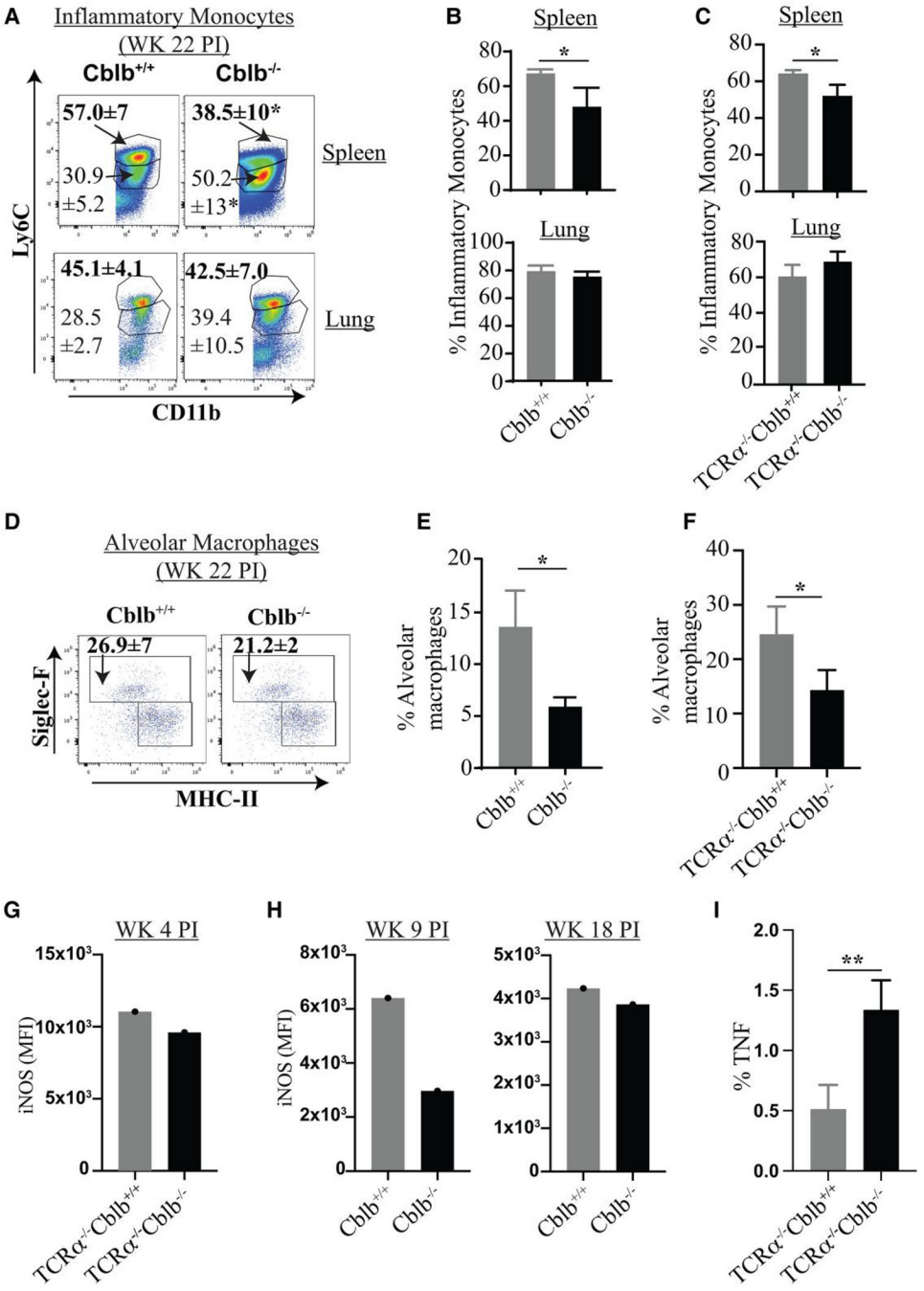

Fig. 3.

CBLB promotes inflammatory monocytes and macrophages during NTM infection. Cohorts of 6- to 8-wk-old mice were infected with MAV104 intravenously (~10^6 CFUs). At 22 wk (A), 6 wk (B, E), or 8 wk (C, F) PI, tissues were harvested, and single-cell suspensions were stained for inflammatory monocytes ($CD90^-$, $Ly6G^-$, $NK1.1^-$, $CD11c^-$, $CD11b^+$, $Siglec\text{-}F^-$, $Ly6C^{hi}$) and alveolar macrophages (AMs) ($CD90^-$, $Ly6G^-$, $NK1.1^-$, $CD11b^-$, $CD11c^+$, $Siglec\text{-}F^+/CD64^+$) using fluorochrome-conjugated antibodies. Stained cells were analyzed by flow cytometry. (A–C) Flow plots or bar diagrams show the percent inflammatory/

noninflammatory monocytes. (D–F, I) Flow plots or bar diagrams show the percent AMs and TNF producing AMs. (G, H) Bar diagrams show the mean fluorescence intensity of iNOS expression in AMs in pooled samples/group measured by flow cytometry. Values are mean ± SD. n = 3–6 mice/group. Data are representative of 2–3 independent experiments. *$P \leq 0.05$ and **$P \leq 0.01$. WK = week.

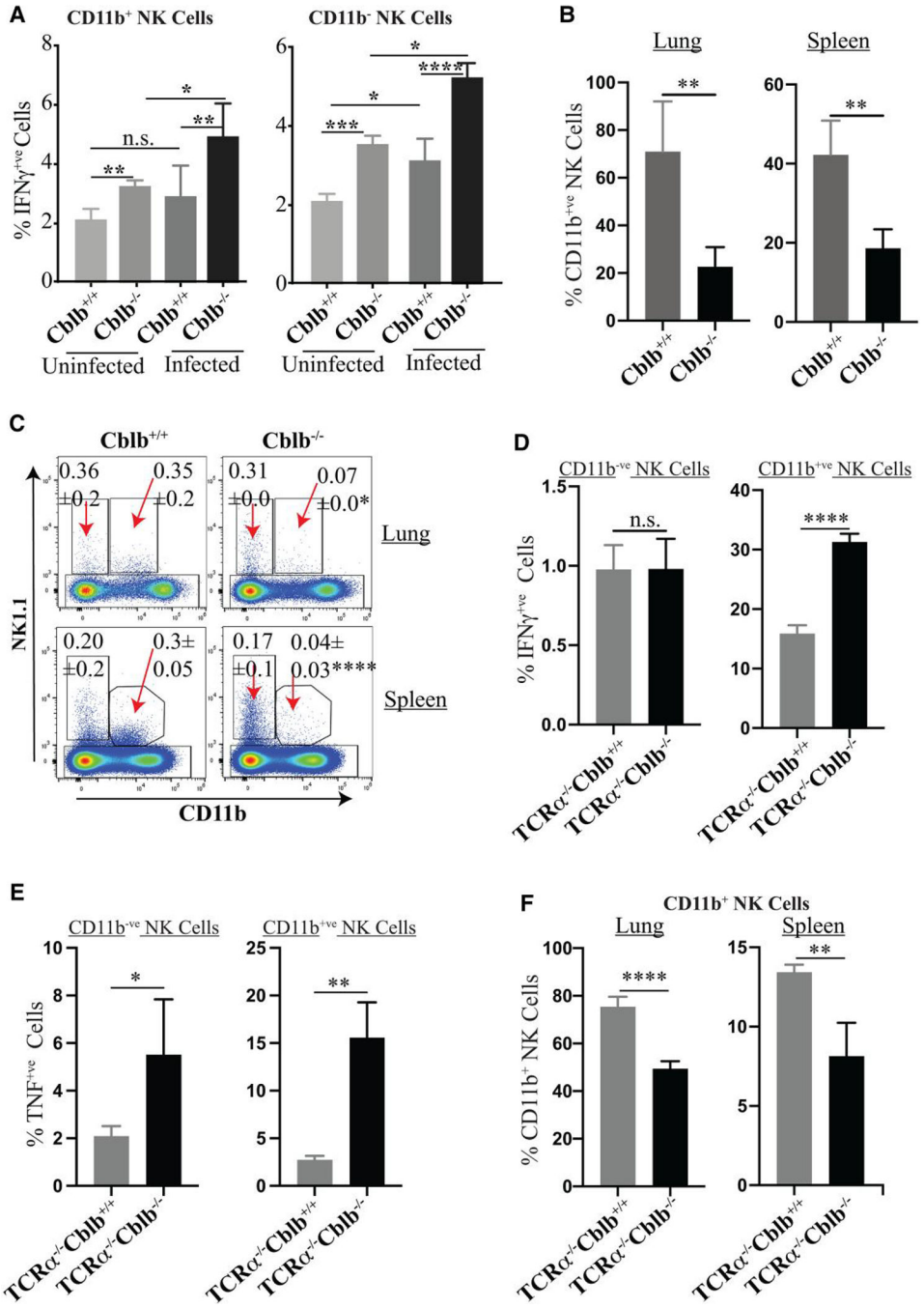

Fig. 4.

Selective depletion of CD11b$^+$ NK cells in *Cblb* deficiency. (A) Splenocytes (~5 × 10^6) from cohorts of naïve mice were incubated with MAV104 (5 MOI) for 48 h and GolgiStop (BD Biosciences) was added at last 4 h before surface and intracellular cytokine staining (4–6 replicates and 3 independent experiments) and cytokine producing cells were analyzed by flow cytometry. Cohorts of 6- to 8-week-old mice were infected with MAV104 intravenously (~10^6 CFUs). At 6 wk (B), 22 wk (C), or 4 wk (D–F) PI, tissues were harvested and single-cell suspensions were stained and analyzed by flow cytometry. (B, C, F) Flow plots

or bar diagrams show percent $CD11b^+/CD11b^-$ NK cells. (D, E) Single-cell suspensions from spleen and lungs were restimulated with PMA/ionomycin in the presence of GolgiStop for 4 h before staining, and cells were analyzed by flow cytometry. Bar diagrams show percent $IFN\gamma^+/TNF^+$ NK cell subsets. Values are mean ± SD. n = 3–6 mice/group. Data are representative of 2–3 independent experiments. $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, and $^{****}P \leq 0.0001$. n.s. = not significant.

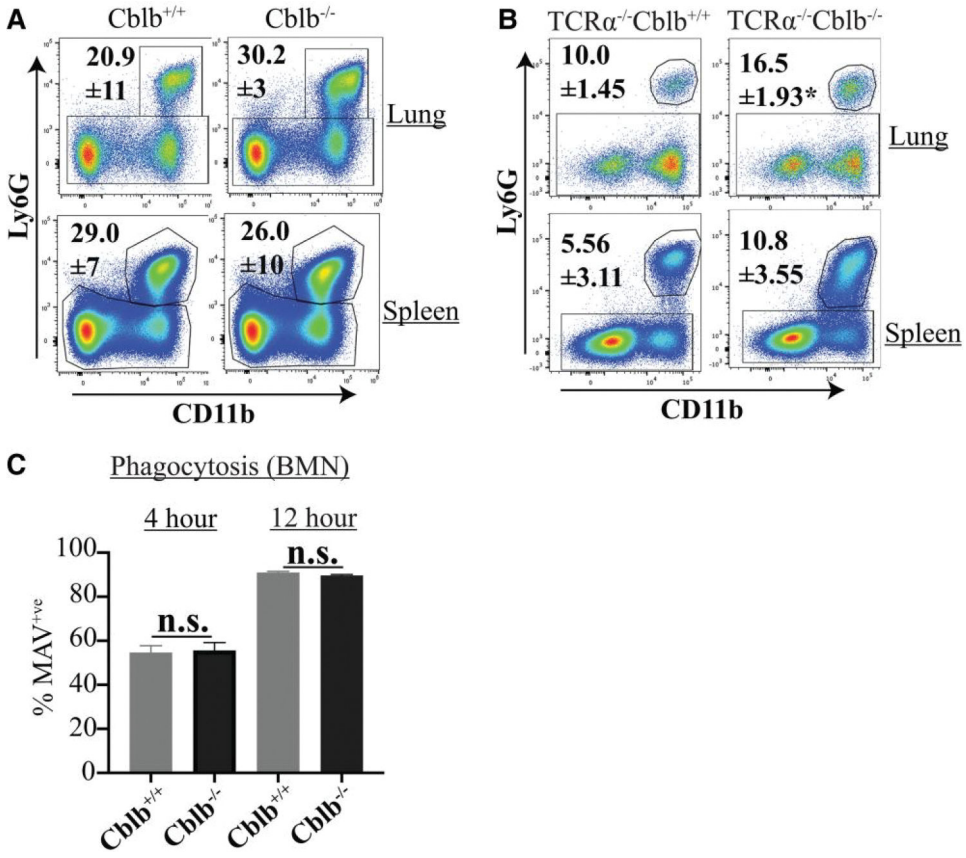

Fig. 5.

Minimal role of CBLB on neutrophils during NTM infection. Cohorts of 6- to 8-wk-old mice were infected with MAV104 intravenously (~10^6 CFUs). At 22 wk (A) or 8 wk (B) PI, tissues were harvested and single-cell suspensions were stained and analyzed by flow cytometry. n = 3–6 mice/group. (C) BMNs were infected with 5 MOI of $dsRED^+$ MAV104. At the indicated time points, the degree of phagocytosis (% $dsRED^+$) of neutrophils was assessed by flow cytometry. n = 6 replicates/group. Values are mean ± SD. $*P \leq 0.05$. n.s. = not significant.

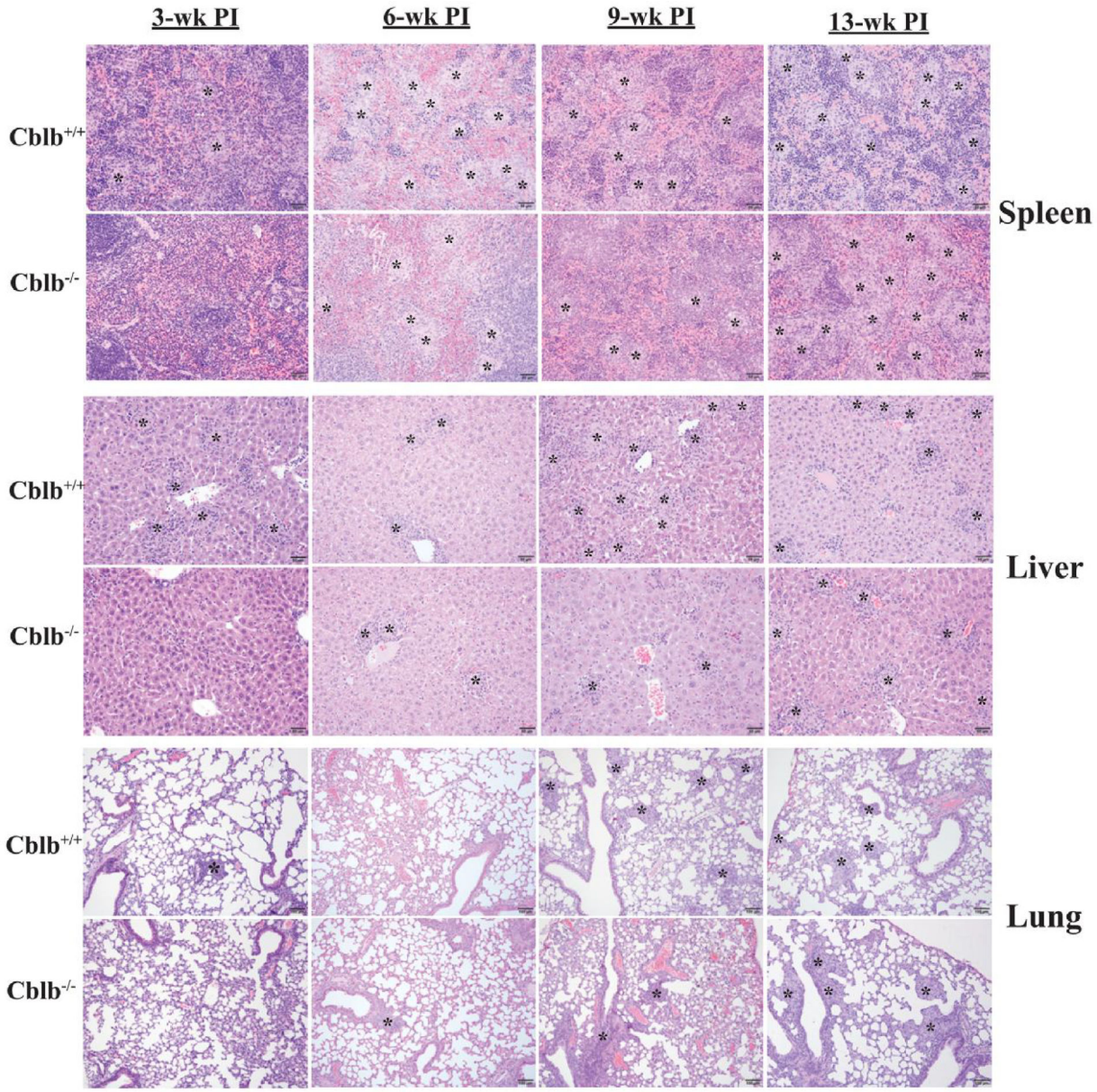

Fig. 6.

Histopathology readings of tissues. Cohorts of 6- to-8-wk-old mice were infected as described in Fig. 1 by intravenous route. At the indicated time PI, tissues were harvested and stored in 10% buffered neutral formalin. Tissues were paraffin embedded, sectioned, and mounted on the slides to stain with hematoxylin and eosin. Images are representative photomicrographs showing granulomatous inflammatory foci (asterisks). Bars = 50μm (liver and spleen) and 100μm (lung). n = 5–6 mice/group. Data are from 2–3 independent experiments.