Correction

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**Mycobacterium tuberculosis** is protected from NADPH oxidase and LC3-associated phagocytosis by the LCP protein CpsA

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**Mycobacterium tuberculosis**' success as a pathogen comes from its ability to evade degradation by macrophages. Normally, macrophages clear microorganisms that activate pathogen-recognition receptors (PRRs) through a lysosomal-trafficking pathway called “LC3-associated phagocytosis” (LAP). Although **M. tuberculosis** activates numerous PRRs, for reasons that are poorly understood LAP does not substantially contribute to **M. tuberculosis** control. LAP depends upon reactive oxygen species (ROS) generated by NADPH oxidase, but **M. tuberculosis** fails to generate a robust oxidative response. Here, we show that CpsA, a LytR-CpsA-Psr (LCP) domain-containing protein, is required for **M. tuberculosis** to evade killing by NADPH oxidase and LAP. Unlike phagosomes containing wild-type bacilli, phagosomes containing the Δcspa mutant recruited NADPH oxidase, produced ROS, associated with LC3, and matured into antibacterial lysosomes. Moreover, CpsA was sufficient to impair NADPH oxidase recruitment to fungal particles that are normally cleared by LAP. Intracellular survival of the Δcspa mutant was largely restored in macrophages missing LAP components (Nox2, Rubicon, Beclin, Atg5, Atg7, or Atg16L1) but not in macrophages defective in a related, canonical autophagy pathway (Atg14, Ulk1, or eGAS). The Δcspa mutant was highly impaired in vivo, and its growth was partially restored in mice deficient in NADPH oxidase, Atg5, or Atg7, demonstrating that CpsA makes a significant contribution to the resistance of **M. tuberculosis** to NADPH oxidase and LC3 trafficking in vivo. Overall, our findings reveal an essential role of CpsA in innate immune evasion and suggest that LCP proteins have functions beyond their previously known role in cell-wall metabolism.

**M. tuberculosis** | autophagy | LC3-associated phagocytosis | NADPH oxidase | LytR-CpsA-Psr

The pathogen **Mycobacterium tuberculosis** (Mt) causes one of the world’s deadliest infections. Mtb survives within macrophages by preventing its own delivery to the degradative, phagolysosomal compartment (1). Recent work distinguished two related phagolysosomal pathways that are characterized by the association of LC3 with the phagosomal membrane, macroautophagy (hereafter autophagy) and LC3-associated phagocytosis (LAP) (2–5). Autophagy involves the capture of cytoplasmic components by a double-membrane compartment called the “autophagosome.” When this process sequesters microorganisms, it is called “xenophagy.” In both xenophagy and LAP, LC3-decorated organelles fuse with lysosomes, resulting in bacterial degradation. However, neither pathway is effective against Mt. Only a small fraction of Mt colocalizes with LC3, and autophagy-related (Atg) proteins that are required for both LAP and xenophagy make only a modest contribution toward Mt control (6–13), suggesting that Mt is able to circumvent both processes. A number of host and bacterial factors contribute to Mt’s ability to evade autophagy (14–17). How Mtb inhibits LAP is unclear.

LAP is initiated by bacterial binding to pathogen-recognition receptors (PRRs) (**Fig. S1**). LAP requires NADPH oxidase and the class III phosphatidylinositol 3-kinase complex, which generate reactive oxygen species (ROS) and phosphatidylinositol 3-phosphate [PI(3)P], respectively, at the incipient phagosome (2, 3, 5). ROS directly kill bacteria and, along with PI(3)P, recruit the Atg conjugation systems that deposit LC3 on the phagosomal membrane. Mtb activates numerous PRRs, so it is surprising that Mt does not robustly trigger LAP. This incongruity suggests that Mt has a LAP evasion strategy. Here, we show that an exported Mtb protein, CpsA (Rv3484), prevents clearance of Mt by NADPH oxidase and LAP. CpsA contains two domains: a LytR-CpsA-Psr (LCP) domain, which is found widely in Gram-positive bacteria, and a LytR domain, which has an unknown function. LCP domains can transfer cell-wall teichoic acids from their lipid-bound precursors to peptidoglycan (PGN) (18–20). Although mycobacteria do not have teichoic acid, arabinogalactan (AG) is linked to PGN in an analogous manner. Mtb has three proteins with LCP and LytR domains: Rv3267/Lcp1/CpsA1, Rv3484/CpsA/CpsA2, and Rv0822c.

**Significance**

**Mycobacterium tuberculosis** (Mt), the causative agent of the disease tuberculosis, grows in macrophages, cells that normally kill bacteria. Recent work has defined a macrophage pathway called “LC3-associated phagocytosis” (LAP) that can eliminate other microbes. LAP is characterized by the recruitment of NADPH oxidase to phagosomes, followed by phagosomal association with LC3 and delivery of the bacteria to a degradative lysosome. Here, we show that LAP does not effectively clear Mt. The ability of Mt to inhibit LAP and therefore cause disease depends upon CpsA, a member of the LytR-CpsA-Psr (LCP) protein family, which has previously been implicated in cell-wall metabolism. We demonstrate that Mt CpsA plays an unexpected role in antagonizing host innate immunity by inhibiting NADPH oxidase and LAP.


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Rv3267 is highly conserved in mycobacteria and is the main ligase responsible for catalyzing the transfer of AG to PGN (21, 22). CpsA shares 36% sequence identity with Rv3267, however, unlike Rv3267, CpsA is not conserved in rapidly growing, non-pathogenic mycobacteria (23), and the ΔcpsA mutant does not exhibit significant cell-wall defects (21). Here, we demonstrate that Mtb CpsA plays an unexpected role in antagonizing host innate immunity by inhibiting NADPH oxidase and LAP. Our findings provide an explanation for the limited oxidative burst seen in response to Mtb and establish a role for CpsA in inhibiting innate immune clearance of Mtb.

Results

CpsA Protects Mtb from Lysosomal Clearance. Previously, we screened secreted Mtb proteins for interactions with human proteins using a high-throughput yeast two-hybrid assay (24). CpsA was included in this screen because it had a predicted signal peptide, and it had been found in culture filtrate in mass spectrometry studies (25–27). We found that CpsA interacts with T-cell leukemia virus type 1 binding protein 1 (TAXIBPI) and nuclear dot protein 25 kDa (NDP52), two paralogs involved in xenophagy (13, 28, 29). This prompted us to delete cpsA from the WT H37Rv strain of Mtb to characterize its effect on autophagy (Fig. S2). When we infected LC3-GFP-expressing murine bone marrow-derived macrophages (BMDMs), we found that the ΔcpsA mutant was three times more likely to be in an LC3-GFP compartment than the WT strain at 4 h postinfection (hpi) (Fig. 1 A and B). We quantified the mean fluorescence intensity (MFI) around individual phagosomes using automated image analysis, as previously described in detail (24). We found a significant shift in ΔcpsA mutant phagosomes to brighter GFP populations compared with WT Mtb phagosomes (Fig. 1C), which closely approximated our visual scoring. The enhanced colocalization of the mutant with LC3-GFP was restored to WT levels in the complemented strain (ΔcpsAΔ::cpsA) (Fig. 1C). As shown previously, activating macrophages with IFN-γ before infection enhanced association of macrophages with WT Mtb (24, 32, 33). In contrast to the marked enhancement of LC3 association with the ΔcpsA mutant in unactivated macrophages, there was little difference between WT and ΔcpsA in IFN-γ-activated macrophages (Fig. 1D). Thus, CpsA inhibited LC3-associated trafficking specifically in unactivated macrophages.

To determine whether enhanced LC3 trafficking resulted in increased lysosomal delivery, we quantified the colocalization of the ΔcpsA mutant with lysosomal-associated membrane protein 1 (LAMP1), a late endosomal and lysosomal marker. We found that LAMP1 colocalized with the ΔcpsA mutant significantly more than it did with WT Mtb at 4 hpi, similar to what was seen when macrophages were pretreated with IFN-γ, which promotes lysosomal trafficking (Fig. 1E–G). LAMP1 colocalization was restored to WT levels in the complemented strain (Fig. 1F). There was no increased association of LAMP1 with the ΔcpsA mutant relative to WT Mtb in IFN-γ-activated macrophages, again suggesting that the altered trafficking was predominantly seen in unactivated macrophages (Fig. 1G). To determine whether the increased phagolysosomal trafficking depended upon autophagy proteins, we examined LAMP1 colocalization with Mtb in macrophages genetically lacking Atg5, which is required for both xenophagy and LAP (Fig. S1). In such macrophages, the enhanced association of LAMP1 with the ΔcpsA mutant was abrogated (Fig. 1H).

Next, we examined whether the failure of the ΔcpsA mutant to arrest lysosomal trafficking impaired its intracellular survival. We found that the ΔcpsA mutant, which grew normally in liquid medium (Fig. S3A), was killed by murine BMDMs, whereas WT Mtb and the complemented strain survived (Fig. 2A). IFN-γ–naive BMDMs cleared the ΔcpsA mutant to a similar degree as IFN-γ–activated macrophages controlled WT Mtb. We had similar findings in human THP-1 macrophages (Fig. 2B). To evaluate whether ΔcpsA was killed as a consequence of lysosomal trafficking, we used previously validated siRNA pools to deplete Ras-related protein Rab-7a (RAB7) or tumor-susceptibility gene 101 (TSG101), which are required for phagosome maturation (Fig. S4) (24, 32, 33). Silencing Rab7 or Tsg101 before infection partially restored the intracellular survival of ΔcpsA (Fig. 2 C and D and Fig. S4). Combined, these results suggested that CpsA protects Mtb from killing by an LC3–associated lysosomal-trafficking pathway.

CpsA Protects Mtb from LAP. Both xenophagy and LAP depend upon a common set of factors to deliver LC3 to the phagosomal membrane, including BECLIN1, ATG7, ATG5, and ATG16L1 (Fig. S1). To determine whether these shared factors were important in clearing the ΔcpsA mutant, we examined macrophages in which we silenced Atg7 or that were genetically lacking Atg5, Atg16L1, or Beclin1 for their ability to kill the ΔcpsA mutant. In all cases, macrophages defective in these core autophagy proteins were impaired in clearing the ΔcpsA mutant relative to WT macrophages (Fig. 3 A–D and Fig. S4). These results are consistent with the idea that CpsA protects Mtb from killing by an LC3–associated lysosomal-trafficking pathway, but they do not distinguish whether that pathway is LAP or xenophagy. Recent studies have defined distinct genetic requirements for xenophagy and LAP (4). Xenophagy requires the autophagy initiation machinery (ULK1, ATG14, and others), which is dispensable for LAP, whereas LAP requires the NADPH oxidase and RUBICON (Fig. S1). When we examined Ulk1–silenced and Atg14–deficient macrophages, we observed larger phagosomes containing Mtb and ΔcpsA survival (Fig. 3 E and F and Fig. S4). In addition, Mtb xenophagy depends upon the cGMP-AMP synthetase (cGAS) (12, 34–36). As we found with macrophages defective in the xenophagy-specific components ULK1 and ATG14, the intracellular growth defect of the ΔcpsA mutant was not rescued in macrophages lacking cGAS (Cgas−/−) (Fig. 3G). In addition, whereas Atg5 deficiency reversed the enhanced association of LAMP1 with the ΔcpsA mutant, the absence of Cgas did not (Figs. 1H and 3H). Moreover, when we examined the transcriptional response of macrophages infected with WT or ΔcpsA, there were few differences in gene expression (Fig. S6). We therefore examined macrophages lacking cGAS activated by cGAS (called the “cytosolic-surveillance pathway”) (Fig. S5). These data suggested that xenophagy and the cGAS–dependent pathway previously characterized during Mtb infection were not responsible for clearing the ΔcpsA mutant.

One distinction between xenophagy and LAP is the role of RUBICON, which inhibits autophagy and is required for LAP (4, 37, 38). In macrophages in which Rubicon was genetically absent or silenced, the attenuation of ΔcpsA was nearly completely rescued (Fig. 3 I and J and Fig. S4), consistent with the idea that ΔcpsA is cleared by LAP. Examination of all our data together revealed that the ΔcpsA mutant was restored to ∼80% of WT levels in macrophages selectively impaired in LAP (Rubicon) or jointly defective in xenophagy and LAP (Beclin1, Atg5, Atg7, or Atg16L1) (Fig. 3K). In contrast, there was little rescue of the survival of the ΔcpsA mutant in macrophages defective only in xenophagy (Atg14, Ulk1, and cGAS) (Fig. 3K and Fig. S1). We conclude that LAP, not xenophagy, plays the predominant role in clearing the ΔcpsA mutant.

CpsA Acts Upstream of NADPH Oxidase. In addition to a requirement for RUBICON, LAP also depends upon NADPH oxidase and ROS (4, 5), which are not required for xenophagy. To see if NADPH oxidase also played a role in clearing the ΔcpsA mutant, we examined its survival in Nos2-KO macrophages, which lack the NADPH oxidase catalytic core (gp91phox). We compared ΔcpsA to a mutant lacking katG, which encodes a catalase-peroxidase. As previously described, the ΔkatG mutant was attenuated in WT macrophages and survived in Nos2-KO macrophages (Fig. 4A) (39). The ΔcpsA mutant behaved similarly; it was attenuated in WT BMDMs, and its intracellular survival was restored in Nos2-KO macrophages (Figs. 3K and 4A). The ΔcpsA mutant with its being cleared by LAP. In contrast, there was no rescue of ΔcpsA in BMDMs lacking inducible nitric oxide synthase (Nos2−/−) (Fig. 3K). However, while both ΔkatG and ΔcpsA were similarly susceptible to NADPH oxidase inside macrophages, the ΔcpsA mutant was not nearly as susceptible to ROS in liquid medium.
Thus, we considered the possibility that ΔcpsA might be susceptible to NADPH oxidase because it induced more ROS during infection. To visualize ROS, we used 2′,7′-dichlorodihydrofluorescein diacetate (H$_2$DCFDA), a cell-permeant derivative of fluorescein that fluoresces upon oxidation. We found that ΔcpsA infection generated substantially more phagosomal ROS than WT Mtb in both murine BMDMs and human THP-1 cells (Fig. 4 B–D). The observed H$_2$DCFDA fluorescence was dependent upon ROS, as it could be blocked by diphenyleneiodonium (DPI), an inhibitor of superoxide production. Moreover, the ROS generated by ΔcpsA was attributable to NADPH oxidase, as it was largely absent in Nos2-KO macrophages (Fig. 4C). It has been shown previously that NADPH oxidase lies upstream of the Atg conjugation systems in LAP (Fig. S1) (4, 40). Consistent with

**Fig. 1.** CpsA inhibits LC3-associated lysosomal trafficking. (A) Fluorescence imaging of DsRed-expressing H37Rv or ΔcpsA (red) 4 hpi in murine BMDMs expressing GFP-LC3 (green). (B) The percentage of bacteria in an LC3-GFP$^+$ phagosome at 4 hpi was quantified by a blinded observer from over 500 bacteria in two independent experiments. **P = 0.0017, Student’s t test. (C) Automated image analysis was used to quantify the GFP MFI colocalized with over 250 bacilli, as shown in violin plots (described below). (D) Phagosomal LC3-GFP was quantified 4 hpi from BMDMs pretreated with IFN-γ or vehicle control before infection. (E) Immunofluorescence (IF) microscopy of LAMP1- (red) and GFP-expressing H37Rv or ΔcpsA (green) 4 hpi in BMDMs. (F and G) Phagosomal LAMP1 MFI in BMDMs pretreated with IFN-γ or vehicle control before infection was quantified at 4 hpi (F) and 24 hpi (G) from at least 100 bacilli. (H) Phagosomal LAMP1 MFI was quantified 4 hpi from Atg5-KO BMDMs (Atg5$^{lox/lox}$ LysM-Cre$^+$) and controls (Atg5$^{lox/lox}$ LysM-Cre$^-$) from at least 70 bacilli per sample. In A and E, arrows indicate bacilli that colocalize with the cellular marker. (Scale bars, 10 μm.) In C, D, and F–H, no contrast adjustment was performed before automated image analysis. The violin plots show the distribution of the data (the MFI of the indicated cellular marker associated with distinct intracellular bacteria) as its probability density. Within the violin plot, the box and whiskers plot indicates the median (horizontal line) and interquartile range (IQR) (boxes). The upper whisker extends to 1.5 × IQR from the top of the top box, and the lower whisker extends from the lower box by 1.5 × IQR IQR. Any data beyond the end of the whiskers are outlying points that are plotted individually. Data show one representative experiment from at least two independent experiments. *P ≤ 0.05; **P ≤ 0.0005; ****P ≤ 0.0001; ns, not significant; one-way ANOVA with Tukey’s multiple comparisons test.
Fig. 2. Mtb require CpsA to survive in macrophages. (A and B) Survival of H37Rv, ΔcpsA, and ΔcpsA::cpsA in BMDMs (A) and human THP-1 cells (B) that were pretreated with either IFN-γ or vehicle. Data show the mean ± SEM of one representative experiment from at least two independent experiments. In some cases the error bar is shorter than the height of the symbol. **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001, ns, not significant, one-way ANOVA with Tukey’s multiple comparisons test of H37Rv, ΔcpsA, and ΔcpsA::cpsA at the last time point. (C and D) Survival of H37Rv and ΔcpsA in BMDMs treated with siRNA control (con) or siRNA-targeting Rab7 (C) or Tsg101 (D) for 2 d before infection. The ratios of cfu 3 d after infection relative to day 0 were normalized to the H37Rv control samples. Data show the mean ± SEM from one representative experiment with at least three replicates from at least two independent experiments; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.0005; ns, not significant; one-way ANOVA with Tukey’s multiple comparisons test.

CpsA Blocks NADPH Oxidase Recruitment to Mtb Phagosomes.

NADPH oxidase is composed of two integral membrane subunits (p22phox and gp91phox) that form flavocytochrome b558 and a trimeric cytosolic complex (p47phox, p67phox, and p40phox). NADPH oxidase activity requires membrane trafficking of flavocytochrome b558 from recycling endosomes (41), assembly with the cytosolic subunits, and activation, which is contingent upon recruitment of GTP-bound Rac1/2 (42). Since the ΔcpsA mutant elicited more ROS, we assessed NADPH oxidase trafficking and assembly by examining recruitment of gp91phox, p47phox, and p40phox to mycobacterial phagosomes. We found that the ΔcpsA mutant colocalized with the membrane and cytosolic components of NADPH oxidase substantially more than WT Mtb (Fig. 5 A–G). As expected, the enhanced association was abrogated in Noc2-KO BMDMs (Fig. 5 C, E, and G). In contrast, enhanced colocalization of the NADPH oxidase with the ΔcpsA mutant did not depend upon Atg5 (Fig. 5 H–J), corroborating that ROS generation did not depend upon the Atg conjugation system (Fig. 4E). These findings demonstrate that CpsA blocks NADPH oxidase recruitment to the mycobacterial phagosome.

CpsA Is Sufficient to Inhibit Phagosomal NADPH Oxidase Recruitment.

CpsA belongs to the LCP family of proteins that are implicated in cell-wall biosynthesis in Gram-positive bacteria. Recent work demonstrated that in Actinobacteria LCP family members ligate AG to PGN (21, 22). In Mtb, there are two other full-length LCP members in addition to CpsA. Rv3267 plays the dominant role in cell-wall biogenesis, while CpsA is thought to have a minor role. Using GC/MS, we verified a slight decrease in AG attachment to PGN in the ΔcpsA mutant in our strain background, as had been seen in the CDC1551 strain background (Table S1). However, this did not result in substantial differences in growth under a variety of stress conditions (Fig. S3 B–F), as also is consistent with previous findings in the CDC1551 strain background (21).

Although the ΔcpsA mutant appeared to have only a mild cell-wall defect, we considered the possibility that even a mild perturbation in the cell wall might expose more pathogen-associated molecular patterns (PAMPs), rendering the ΔcpsA mutant hyperinflammatory and driving LAP. Since a number of Mtb Toll-like receptor 2 (TLR2) ligands are exported lipoproteins, we examined whether there was a difference in the proteins shed by the ΔcpsA mutant in culture filtrate compared with WT and the complemented strain. Mass spectrometry-based label-free quantitative analysis of the culture filtrate revealed no statistically significant differences except CpsA itself (Fig. S6). Since activation by other PAMPs was also possible, we next compared the transcriptional signature of uninfected macrophages with those infected with the ΔcpsA mutant and WT Mtb. At 4 hpi, thousands of genes were differentially regulated between uninfected and infected macrophages. However, using a false-discovery rate (FDR) cutoff of 0.05, only one gene was differentially expressed between WT and ΔcpsA–infected macrophages (TMA16, adjusted P = 0.04). Nonetheless, Kyto Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed a difference in the “Tuberculosis” response pathway between WT and ΔcpsA–infected macrophages, which largely reflected a difference in expression of NF-kB-regulated genes (Fig. S5B). However, contrary to our idea that the ΔcpsA mutant might drive LAP by virtue of being hyperinflammatory, the ΔcpsA mutant elicited diminished NFR response (Fig. S5 B–D).

Since enhanced PAMP signaling from an altered cell wall did not appear to explain ΔcpsA’s ability to increase LAP, we wondered whether CpsA’s role in virulence reflected a function independent of its activity on the cell wall. To determine whether CpsA was sufficient to alter intracellular trafficking in macrophages, we expressed a CpsA-GFP fusion protein in RAW264.7 cells, a murine macrophage cell line. CpsA has a putative signal peptide and is found in the culture filtrate as well as the cell envelope (Fig. S2) (25–27). We expressed CpsA without its putative signal peptide to mimic what would be exported from the bacterium and obtain access to the mammalian cell. As a control, we expressed a chloramphenicol acetyltransferase–fusion protein (CAT-GFP). We incubated macrophages expressing CpsA-GFP or CAT-GFP with zymosan, which has previously been shown to recruit the NADPH oxidase and traffic through the LAMP1 pathway (4). We found that CpsA dramatically impaired recruitment of p47phox and p40phox to zymosan-containing phagosomes as well as their delivery to a LAMPT1 compartment (Fig. 6 A–F). Thus, CpsA is sufficient to inhibit recruitment of NADPH oxidase to fungal cargo and to block lysosomal trafficking, demonstrating an activity beyond cell-wall biogenesis. Moreover, when the transfected macrophages were infected with the ΔcpsA mutant, CpsA expression inhibited recruitment of NADPH oxidase to ΔcpsA-mutant phagosomes and partially rescued the intracellular survival defect of the mutant, while WT Mtb infection was not altered (Fig. 6 G and H). Combined, these data support the idea that CpsA functions by acting on the host cell rather than the mycobacterial envelope to alter intracellular trafficking.

CpsA Protects Mtb in Vivo.

To assess whether cpsA is required in vivo, we infected C57BL/6 mice with WT or ΔcpsA via low-dose aerosol. After 17 d of infection, there was more than a two-log reduction in cfu of ΔcpsA compared with WT Mtb in the lungs, along with diminished consolidation in the lungs and defective dissemination to the spleen (Fig. 7 A–C). Virulence was restored to the ΔcpsA mutant by introduction of cpsA under control of its
native promoter (Fig. 7 A–C). Because the ΔcpsA mutant was attenuated early in infection, it suggested that Mtb requires CpsA to survive the innate immune response, consistent with our findings in macrophages. To further evaluate this, we infected SCID mice, which lack an adaptive immune response. Eight weeks postinfection, all mice infected with a strain producing CpsA died, whereas all ΔcpsA-infected mice survived (Fig. 7 D–F). Twenty weeks postinfection, we euthanized two of the ΔcpsA-infected mice to examine the bacterial burden. Remarkably, we could not recover any bacilli from the lungs. The remaining ΔcpsA-infected mice were thriving 32 wk postinfection. These results demonstrate that the innate immune response controls the ΔcpsA mutant in vivo, whereas WT Mtb resists the innate immune response, proliferates, and kills the host.

Previous work showed that catalase-deficient Mtb (ΔkatG) grows equivalently to WT during the first 2 wk of infection in mice, indicating that the bacilli are not experiencing oxidative stress during this time. This observation led McKinney and colleagues to speculate that delivery of NADPH oxidase to Mtb phagosomes is blocked during acute infection (39). Our in vitro data suggested that this might be mediated by CpsA. To determine whether CpsA protects Mtb from NADPH oxidase during acute infection in vivo,

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**Fig. 3.** CpsA-deficient Mtb are cleared by LC3-associated phagocytosis. (A, F, and I) Survival of H37Rv and ΔcpsA in BMDMs treated with siRNA control (con) or siRNA targeting Atg7 (A), Ulk1 (F), or Rubicon (I) for 2 d before infection. (B–E, G, and J) Survival of H37Rv and ΔcpsA in Atg5-KO (Atg5<sup>fl</sup>/<sup>fl</sup>-LysM-Cre<sup>+</sup>) and control (Atg5<sup>fl</sup>/<sup>fl</sup>-LysM-Cre<sup>−</sup>) (B), Atg16L1-KO (Atg16L1<sup>fl</sup>/<sup>fl</sup>-LysM-Cre<sup>+</sup>) and control (Atg16L1<sup>fl</sup>/<sup>fl</sup>-LysM-Cre<sup>−</sup>) (C), Beclin1-KO (Beclin1<sup>fl</sup>/<sup>fl</sup>-LysM-Cre<sup>+</sup>) and control (Beclin1<sup>fl</sup>/<sup>fl</sup>-LysM-Cre<sup>−</sup>) (D), Atg14-KO (Atg14<sup>fl</sup>/<sup>fl</sup>-LysM-Cre<sup>+</sup>) and control (Atg14<sup>fl</sup>/<sup>fl</sup>-LysM-Cre<sup>−</sup>) (E), WT (C57BL/6) and Cgas<sup>−/−</sup> (G), and WT and Rubicon<sup>−/−</sup> BMDMs (J). (H) Phagosomal LAMP1 quantified 4 hpi in WT or Cgas<sup>−/−</sup> BMDMs from at least 175 bacilli is shown in a violin plot (as described in the legend of Fig. 1). (K) The cfu ratios of ΔcpsA and H37Rv based on data from A–G, I, and J and Figs. 2 C and D and 4 A. Control BMDMs (WT, Cre<sup>+</sup>, or control-transfected (CON)) are shown in black. Loss-of-function experimental samples are colored according to their biological function as indicated. The dashed line indicates 80% restoration of intracellular growth. In A–G, I, and J the ratios of cfu 3 d after infection relative to day 0 were normalized to the H37Rv control samples. Data show the mean ± SEM from one representative experiment with at least three replicates from at least two independent experiments; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.0005; ****P ≤ 0.0001; ns, not significant; one-way ANOVA with Tukey's multiple comparisons test. Representative data before normalization are shown in Fig. S4.
we infected Nox2-KO mice with the ΔcpsA mutant and compared cfu in the lungs of these mice with that of C57BL/6 mice 17 d postinfection. We found that ΔcpsA grew 0.5 log more in the Nox2-KO mice than in WT mice (Fig. 7E), while Nox2 did not significantly affect WT Mtb. There was a trend toward increased growth of WT Mtb in mice lacking Atg5 in the myeloid compartment (Δatg5floxflox LysM-Cre+) compared with control animals, while no difference was observed in mice lacking Atg7 (Δatg7floxflox LysM-Cre+), consistent with findings by Kimmey et al. (8). In both Atg5 and Atg7 mutants, we observed partial rescue of the ΔcpsA mutant compared with Cre− littermate controls (Fig. 7F and G). Finally, in keeping with our in vitro data, Atg14 (Δatg14floxflox LysM-Cre+) and Atg14-deficient mice failed to rescue the ΔcpsA mutant (Fig. 7H and I). Overall, our data demonstrate that CpsA protects Mtb from NADPH oxidase and LAP in macrophages and mice.

Discussion

Mtb activates PRRs such as TLR2 and C-type lectin receptors (CLRs) but resists immune control from NADPH oxidase and LAP, which are normally activated by these PRRs. Our findings establish a role for CpsA in evading these antimicrobial pathways. Our results are consistent with previous work showing that NADPH oxidase fails to assemble on the mycobacterial phagosome and that the oxidative burst to Mtb is marginal (39, 43). Previous studies also suggested that Mtb inhibits NADPH oxidase in vivo during acute infection, since catalase-deficient Mtb grow normally during that time (39). Our data demonstrate that CpsA is a significant mediator of Mtb’s resistance to NADPH oxidase and LAP in vivo, as the growth of the mutant is improved in mice deficient in Nox2 or lacking Atg5 or Atg7 in hematopoietic cells, all of which are required for LAP. While recent work has called into question the role of autophagy in Mtb pathogenesis (8), our findings suggest that Atg proteins involved in LAP are poised to play a role but are subverted by the pathogen.

NADPH oxidase and LAP are crucial in the battle of host and pathogen, and our data demonstrate that CpsA plays an important role in Mtb’s ability to disarm the innate immune system. The ΔcpsA mutant is highly attenuated early during infection before the initiation of an adaptive immune response, and, remarkably, SCID mice do not succumb to infection. Our data highlight that even though Mtb is relatively resistant to ROS by virtue of catalase, NADPH oxidase plays an additional role in bacterial control by activating a potent lysosomal-trafficking pathway. The importance of blocking LAP is demonstrated by other pathogens as well. The growth of Salmonella typhimurium is restricted by LAP (40), and Aspergillus and Leishmania inhibit LAP (44, 45). In addition, a number of pathogens inhibit NADPH oxidase (46–49), although the link to LAP has not been clearly established. Even though Mtb can inhibit NADPH oxidase and LC3-trafficking systems, it must do so imperfectly, as these host mechanisms still play an important role in defense. For example, individuals with chronic granulomatous disease who have mutations in NADPH oxidase have increased susceptibility to mycobacterial diseases (50). We speculate that there may also be rare individuals who are relatively resistant to infection because Mtb cannot disarm their NADPH oxidase and LAP pathway.

Interestingly, the ΔcpsA mutant is less attenuated over time in mice (Fig. 7A and B), and it survives similarly to WT Mtb in activated macrophages (Fig. 2A and B). These findings suggest that once an adaptive immune response is initiated, different bacterial effectors compensate for the loss of cpsA or, alternatively, NADPH oxidase and LAP take on a less important role during chronic infection. We favor the idea that NADPH oxidase and LAP become less important as iNOS and canonical autophagy are...
stimulated under the influence of proinflammatory cytokines. At the same time, there would presumably be a corresponding shift in the virulence factors that Mtb needs to persist. Interestingly, two bacterial factors that impair Mtb autophagy in vitro, Eis and PE_PGRS47, do not play a role in acute infection in mice (16, 17). The PE_PGRS47-KO strain is attenuated during chronic infection, perhaps reflecting a switch from LAP to canonical autophagy as a dominant host response in the transition from acute to chronic infection.

CpsA is absent from nonpathogenic mycobacteria, so it appears that duplication of an LCP protein allowed CpsA to acquire an additional role in virulence. The evolution of critical virulence factors from proteins that play a basic role in bacterial physiology in environmental mycobacteria is a recurrent theme in Mtb pathogenesis, perhaps reflecting the lack of horizontal gene transfer. In Mycobacterium marinum, the close relative of Mtb, a cpsA transposon mutant is also defective in arresting phagosome maturation in macrophages and is attenuated in the CDC1551 strain background did not exhibit significant differences in growth, cording, antibiotic susceptibility, or total lipids, mycolic acids, or cell-wall polysaccharides (21). Thus, differences in growth, cording, antibiotic susceptibility, or total lipids, mycolic acids, or cell-wall polysaccharides (21). 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It remains to be determined how CpsA impairs NADPH oxidase recruitment and LAP. It is possible that it does so as a consequence of its cell-wall function. There is precedence for such a possibility: in Aspergillus melanin inhibits recruitment of the p22phox subunit of the NADPH oxidase to germinating conidia,
Fig. 6. CpsA is sufficient to inhibit LAP. (A, C, and E) RAW264.7 cells transfected with CpsA or control mRNA were examined by IF microscopy after the addition of zymosan (red) for the localization of p47<sup>phox</sup> (A), p40<sup>phox</sup> (C), and LAMP1 (E) (all pseudocolored green). Arrows indicate zymosan particles that colocalize with p47<sup>phox</sup><sup>max</sup>, p40<sup>phox</sup><sup>max</sup>, or LAMP1. (Scale bars, 10 μm.) (B, D, and F) Quantification of phagosomal p47<sup>phox</sup> (B), p40<sup>phox</sup> (D), and LAMP1 (F). (G) RAW264.7 cells transfected with CpsA or control mRNA were infected with H37Rv and ΔcpsA and were examined by IF microscopy. Phagosomal p40<sup>phox</sup> was quantified at 3 hpi. (H) Growth of H37Rv and ΔcpsA in RAW264.7 cells transfected with CpsA or control mRNA. The cfus are shown at the indicated time points. Data show mean ± SEM from one representative experiment from two independent experiments; *P ≤ 0.05, **P ≤ 0.01; ****P ≤ 0.0001; Student’s t test (B, D, F, and H) or ANOVA (G).
Macrophages. Murine hematopoietic stem cells were isolated from the tibia and femurs of 6- to 15-wk-old C57BL/6 mice (unless otherwise noted) as described in SI Materials and Methods. THP-1 cells (American Type Culture Collection) and RAW264.7 cells (American Type Culture Collection) were grown as described in SI Materials and Methods. RNA- mediated silencing was performed as described in SI Materials and Methods.

Microscopy. Macrophages were infected with GFP- or DsRed-expressing Mtb strains at a multiplicity of infection (MOI) of ~3. After 4 h, macrophages were washed and fixed with 1% paraformaldehyde/PBS overnight. Immunostaining, ROS visualization, and image acquisition were performed as described in SI Materials and Methods.

Intracellular Bacterial Survival Assays. To assess Mtb survival in vitro, BMDMs from C57BL/6 mice, unless otherwise specified, were seeded 1 d before infection and infected with a single-cell suspension of Mtb at a MOI of ~3, as previously described (24) and detailed in SI Materials and Methods. RAW264.7 cells were used when macrophages were transfected with CpsA as described in SI Materials and Methods.

Protein Extracts, Western Blotting, Mass Spectrometry, and RNA-sequencing. Preparation of protein extracts, Western blotting, mass spectrometry, and RNA-sequencing (RNA-seq) are described in SI Materials and Methods.
42. de Souza GA, Levenson NA, Quinlan MT (2009) Inhibition of the human neutrophil NADPH oxidase by Coxelia burnetti. 