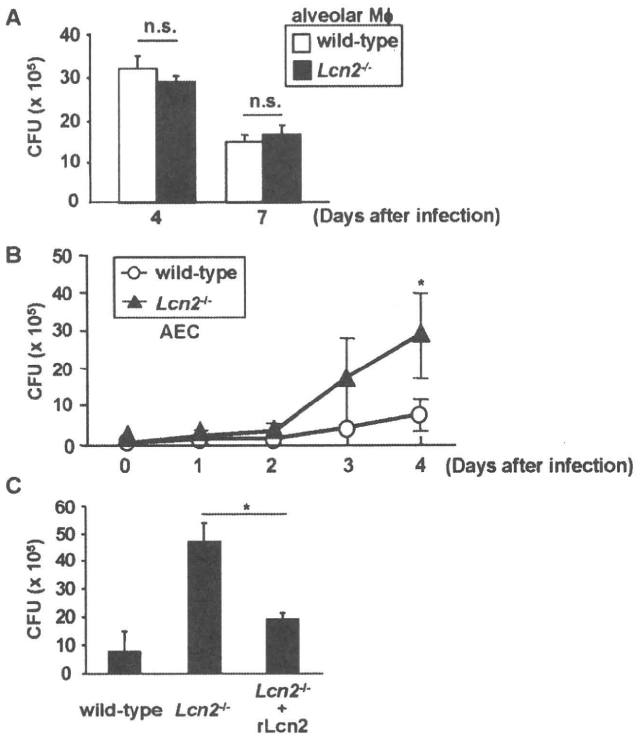

FIGURE 5. Increased BCG growth in *Lcn2*$^{-/-}$ alveolar epithelial cells. *A*, Alveolar macrophages were collected from uninfected wild-type and *Lcn2*$^{-/-}$ mice and cultured with BCG for the indicated periods. To eliminate external BCG, the cells were cultured with streptomycin for 1 h, washed three times, and harvested. Lysates of the cells were plated on 7H10-OADC agar, and the CFU numbers were counted. Representative data of two independent experiments are shown. n.s., not significant. *B*, Wild-type and *Lcn2*$^{-/-}$ AECs were cultured with BCG for the indicated periods. After removal of extracellular BCG, lysates the cells were plated on 7H10-OADC agar, and the CFU numbers were counted. Data are presented as means ± SD of triplicate determinations and are representative of three independent experiments. *, $p < 0.05$. Similar results were obtained when other AECs from wild-type and *Lcn2*$^{-/-}$ mice were used. *C*, Wild-type and *Lcn2*$^{-/-}$ AECs were cultured with BCG. At 2 days after infection, rLcn2 (final concentration 30 μg/ml) was added to the *Lcn2*$^{-/-}$ AEC. After an additional 2 days of culture, the cells were incubated with streptomycin for 1 h, washed three times, and harvested. Lysates of the cells were plated on 7H10-OADC agar, and the CFU numbers were counted. Representative data of three independent experiments are shown. Data are presented as means ± SD of triplicate determinations. *, $p < 0.05$.

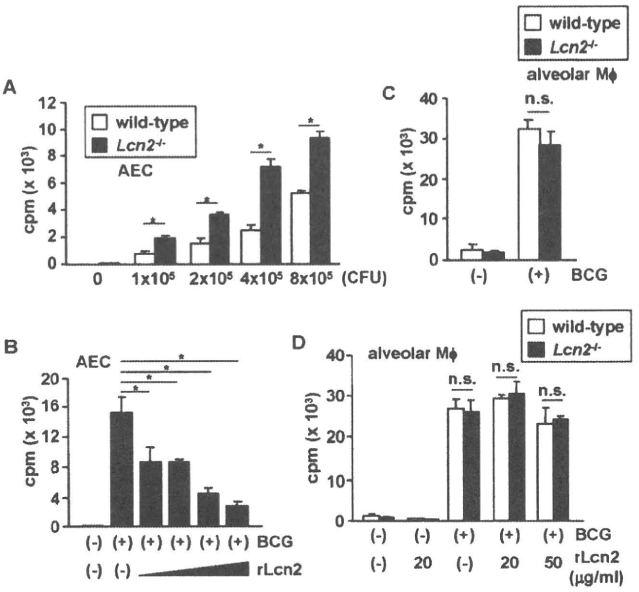

FIGURE 6. Lcn2-mediated inhibition of intracellular BCG growth. *A*, Wild-type and *Lcn2*$^{-/-}$ AECs were seeded onto 96-well plates and infected with the indicated CFUs of BCG for 6 h. The cells were then extensively washed to remove extracellular BCG and cultured in the presence of [^{3}H]uracil for 48 h. The incorporation of [^{3}H]uracil was measured. Data are presented as means ± SD of triplicate samples. Representative data of three independent experiments are shown. *, $p < 0.005$. *B*, *Lcn2*$^{-/-}$ AECs were seeded onto 96-well plates, and infected with BCG (2×10^5 CFU) for 6 h. After vigorous washing, the cells were cultured with increasing concentrations of rLcn2 (20, 30, 40, and 50 μg/ml) and [^{3}H]uracil for 48 h, before being measured for their [^{3}H]uracil incorporation. Data are presented as means ± SD of triplicate samples, and are representative of two independent experiments. * indicate a significant difference among groups, ANOVA, posthoc Scheffe, *, $p < 0.001$. *C*, Alveolar macrophages were collected from uninfected wild-type and *Lcn2*$^{-/-}$ mice, and cultured with BCG for 6 h. After vigorous washing, the cells were cultured in the presence of [^{3}H]uracil for 48 h, before being measured for their [^{3}H]uracil incorporation. Data are presented as the mean ± SD of triplicate samples. n.s., not significant. *D*, Alveolar macrophages from wild-type and *Lcn2*$^{-/-}$ mice were infected with BCG for 6 h. After vigorous washing, the cells were cultured in the presence of the indicated concentration of rLcn2 and [^{3}H]uracil for 48 h. Then, the [^{3}H]uracil incorporation was counted. Data are presented as means ± SD of triplicate samples. n.s., not significant.

Inhibition of intracellular mycobacterial growth by Lcn2

Mycobacteria are intracellular bacteria that replicate within cells. In the experiments performed so far, it is possible that extracellular growth was monitored as well as intracellular growth under the in vitro conditions. Therefore, to assess the intracellular growth of mycobacteria more precisely, we used [^{3}H]uracil, which is preferentially incorporated into mycobacterial nucleic acids (33). AECs derived from wild-type and *Lcn2*$^{-/-}$ mice were infected with several CFUs of BCG for 6 h, extensively washed with culture medium containing streptomycin to exclude extracellular BCG, and then cultured for 2 days in the presence of [^{3}H]uracil (Fig. 6*A*). Under these conditions, [^{3}H]uracil incorporation was below 1×10^2 cpm in wells containing uninfected AECs or wells placed in contact with BCG and then extensively washed. After infection with each CFU, [^{3}H]uracil incorporation was increased in *Lcn2*$^{-/-}$ cells compared with wild-type cells. In BCG-infected *Lcn2*$^{-/-}$ cells, addition of exogenous rLcn2 reduced the uptake of [^{3}H]uracil by intracellular BCG (Fig. 6*B*). In alveolar macrophages, the [^{3}H]uracil incorporation by intracellular BCG was comparable between wild-type and *Lcn2*$^{-/-}$ cells (Fig. 6*C*). Addition of rLcn2 did not effectively reduce the uptake of [^{3}H]uracil by intracellular BCG in alveolar macrophages from both wild-type and *Lcn2*$^{-/-}$ mice (Fig. 6*D*). These findings indicate that extracellular Lcn2 limits intracellular growth of BCG in AECs, but not in alveolar macrophages.

Because extracellular Lcn2 modulated intracellular mycobacterial growth in the AECs, we analyzed whether extracellular Lcn2 was incorporated into the AECs as described in several previous reports (18, 19). AECs were infected with GFP-expressing BCG and then treated with fluorescein-labeled rLcn2 (Fig. 7*A*). Lcn2 was detected within the AECs, and colocalized with dextran that was taken up into the cells by endocytosis. Furthermore, many BCG were colocalized with rLcn2, indicating that endocytosed Lcn2 was in close proximity to intracellular BCG. In contrast, although Lcn2 was incorporated into alveolar macrophages, the incorporated Lcn2 was not colocalized with BCG in alveolar macrophages (Fig. 7*B*), indicating that BCG and rLcn2 were localized in distinct cellular compartments within macrophages. We blocked

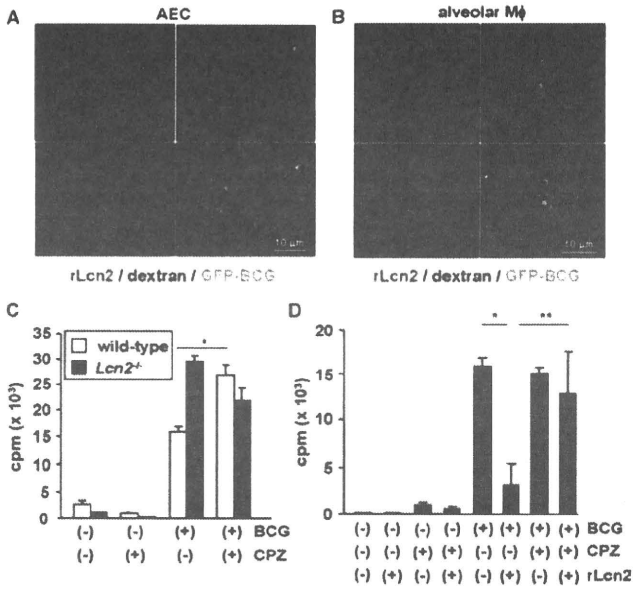

FIGURE 7. Requirement of Lcn2 incorporation for the inhibition of intracellular BCG growth. *A*, GFP-expressing BCG (green)-infected alveolar epithelial cells were cultured with dextran (25 μg/ml; blue) and fluorescein-labeled rLcn2 (15 μg/ml; red) for 6 h. The cells were then washed, fixed with 4% PFA, and analyzed by confocal microscopy. Data are representative of three independent experiments. *B*, GFP-expressing BCG (green)-infected alveolar macrophages were cultured with dextran (25 μg/ml; blue) and fluorescein-labeled rLcn2 (15 μg/ml; red) for 6 h. The cells were then washed, fixed with 4% PFA for 5 min, and analyzed by confocal microscopy. *C*, Wild-type and *Lcn2*$^{-/-}$ AECs were seeded onto 96-well plates and infected with BCG (2×10^5 CFU) for 6 h. After extensive washing, the cells were cultured with CPZ (10 μM) and [^{3}H]uracil for 48 h. The [^{3}H]uracil incorporation was then measured. Data are presented as means ± SD of triplicate samples, and are representative of two independent experiments. *, $p < 0.01$. *D*, *Lcn2*$^{-/-}$ AECs were seeded onto 96-well plates, and infected with BCG for 6 h. After washing, the cells were cultured with CPZ for 30 min and then cultured with rLcn2 (20 μg/ml) and [^{3}H]uracil for 48 h. The [^{3}H]uracil incorporation was measured. Data are presented as means ± SD of triplicate samples, and are representative of two independent experiments. * or ** indicate a significant difference among groups, ANOVA, posthoc Scheffe, *, $p < 0.005$; **, $p < 0.05$.

endocytosis of Lcn2 using CPZ after BCG infection. Addition of CPZ resulted in increased BCG growth in wild-type AECs, but not in *Lcn2*$^{-/-}$ cells (Fig. 7*C*). We also analyzed the effects of the endocytosis inhibitor on rLcn2-mediated inhibition of BCG growth (Fig. 7*D*). Addition of CPZ abolished Lcn2-mediated inhibition of [^{3}H]uracil incorporation in both wild-type and *Lcn2*$^{-/-}$ cells. Cytochalasin B, which also blocks endocytosis, had similar effects to those of CPZ on Lcn2-mediated inhibition of intracellular BCG growth (data not shown). These findings indicate that endocytosed Lcn2 inhibits the intracellular growth of BCG in AECs.

Discussion

Lcn2 has a variety of putative functions, as evident from its many different names such as neutrophil gelatinase-associated lipocalin, uterocalin, 24p3, and siderocalin (12, 13, 16, 19). In the context of its function in host defense, a structural study of the Lcn2 protein revealed that it associates with enterobactin-type bacterial siderophores (16). Subsequently, Lcn2 was shown to bind to several types of siderophores such as caroboxy-mycobactin and bacillibactin (20, 21). In addition, Lcn2 has been proposed to bind to an as-yet unknown mammalian siderophore (18, 34). Thus, Lcn2 has the ability to bind to a variety of types of siderophores. Furthermore, Lcn2 has been shown to inhibit the growth of *E. coli* through sequestration of iron uptake (22, 23). The present study has demonstrated that Lcn2 also participates in the inhibition of mycobacterial growth through similar mechanisms to those against *E. coli*. Indeed, Lcn2 has been shown to associate with the mycobacteria-derived hydrophilic siderophore carboxy-mycobactin (21). In accordance with our results, Lcn2 has been shown to be secreted from neutrophils during *M. tuberculosis* infection and inhibit their growth (35). Lcn2 was originally identified as a molecule that is secreted from neutrophils, which are rapidly recruited to *M. tuberculosis*-infected lungs. Therefore, neutrophils are presumably the source of Lcn2 as well as alveolar macrophages and epithelial cell during *M. tuberculosis* infection.

Regarding the high sensitivity of *Lcn2*$^{-/-}$ mice to *M. tuberculosis* infection, it is noteworthy that *Lcn2*$^{-/-}$ alveolar epithelial cells, but not macrophages, contained increased numbers of *M. tuberculosis* at the early phase of the infection, as evaluated by histopathological and immunohistochemical analyses. This finding was unexpected, because successful in vivo detection of mycobacteria in respiratory epithelial cells in wild-type mice has only been achieved through analyses of mycobacterial DNA or use of electron microscopy, even though mycobacteria have been shown to invade epithelial cells as well as macrophages in vitro (6–9, 36). In addition, *Lcn2*$^{-/-}$ alveolar epithelial cells, but not macrophages, exhibited defective inhibition of intracellular mycobacterial growth, suggesting that impaired inhibition of mycobacterial growth in alveolar epithelial cells due to the absence of Lcn2 may be a major cause of the high susceptibility *Lcn2*$^{-/-}$ mice to *M. tuberculosis* infection. Given that mycobacteria were easily detected in the alveolar epithelial cell layers by a typical histological approach in the absence of Lcn2 and the increased mycobacterial growth was observed in *Lcn2*$^{-/-}$ epithelial cells, but not in macrophages, epithelial cells may play an important role in the host immune responses against respiratory infection with *M. tuberculosis*.

Mycobacteria replicate within cells in vivo, and several lines of evidence indicate that mycobactin-mediated iron uptake is a prerequisite for intracellular mycobacterial growth (27, 29). Consistent with previous studies (18, 19), our findings indicated that Lcn2 is internalized into alveolar epithelial cells via endocytosis. Furthermore, addition of rLcn2 effectively inhibited intracellular mycobacterial growth in AECs, and this effect was abolished by endocytosis inhibitors. At present, it remains unclear how mycobacteria take up iron within epithelial cells using mycobactin. First, it is apparent that mycobacteria exist in the phagosome of macrophages. However, the subcellular localization of mycobacteria within epithelial cells has not been established, although mycobacteria have been shown to be localized in endosomes or macropinosomes (37, 38). Our results revealed colocalization of mycobacteria and dextran, indicating that mycobacteria exist in the endosome-like vacuole within epithelial cells. Second, it remains obscure whether mycobacteria secrete water-soluble carboxy-mycobactin into the cytoplasm to bind the cytosolic iron. It is also obscure how endocytosed Lcn2 approaches the carboxy-mycobactin/iron complexes within the cells. Given that Lcn2 and mycobacteria are colocalized within the endosome-like structure, it is possible that mycobacteria take up the iron entering the endosome using mycobactin, and endocytosed Lcn2, in turn, binds to the carboxy-mycobactin/iron complexes, thereby blocking iron acquisition by mycobacteria. Further studies are required to clarify the precise mechanisms for the interaction between Lcn2 and mycobacteria-derived carboxy-mycobactin.

In alveolar macrophages, the absence of Lcn2 did not affect the sensitivity to mycobacterial infection. This may be due to the differential localizations of mycobacteria in epithelial cells and macrophages. Lcn2 was colocalized with mycobacteria in epithelial

cells, indicating that mycobacteria exist within the endosome-like structure. In contrast, mycobacteria were localized within the phagosome in macrophages, leading to distinct localizations of Lcn2 and mycobacteria in macrophages. Alternatively, macrophages are professional cells that kill intracellular bacteria by producing several macrophage-specific anti-microbial mediators, including NO synthase and Nramp1 (39–41). These mediators may compensate the Lcn2 deficiency in macrophages. In contrast, they are not expressed in epithelial cells, resulting in the high sensitivity to mycobacterial infection in the absence of Lcn2. Thus, in alveolar epithelial cells, Lcn2 may be a major factor that mediates host resistance to mycobacterial infection.

Our results highlight a novel innate host defense system that inhibits mycobacterial infection at the respiratory mucosal surface. We would like to propose the following scenario with regard to the function of Lcn2. Lcn2 is secreted into the alveolar space by alveolar macrophages and epithelial cells during the early phase of respiratory mycobacterial infection. Lcn2 presumably inhibits mycobacterial growth within the alveolar space. In addition, Lcn2 is internalized into the alveolar epithelial cells, which are invaded by mycobacteria, and inhibits mycobacterial growth by sequestering iron uptake. This leads to a reduction in the number of infected mycobacteria at the early phase of infection, which may help to create sufficient time for effective activation of anti-mycobacterial innate and adaptive immune responses. Thus, respiratory epithelial cells play an active role in the resistance to mycobacterial infection, in addition to their functions as physical barriers and secretors of anti-bacterial mediators.

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Disclosures

The authors have no financial conflict of interest.

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