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Lipocalin 2-Dependent Inhibition of Mycobacterial Growth in Alveolar Epithelium

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_Mycobacterium tuberculosis_ invades alveolar epithelial cells as well as macrophages. However, the role of alveolar epithelial cells in the host defense against _M. tuberculosis_ remains unknown. In this study, we report that lipocalin 2 (Lcn2)-dependent inhibition of mycobacterial growth within epithelial cells is required for anti-mycobacterial innate immune responses. Lcn2 is secreted into the alveolar space by alveolar macrophages and epithelial cells during the early phase of respiratory mycobacterial infection. Lcn2 inhibits the in vitro growth of mycobacteria through sequestration of iron uptake. Lcn2-deficient mice are highly susceptible to intratracheal infection with _M. tuberculosis_. Histological analyses at the early phase of mycobacterial infection in Lcn2-deficient mice reveal increased numbers of mycobacteria in epithelial cell layers, but not in macrophages, in the lungs. Increased intracellular mycobacterial growth is observed in alveolar epithelial cells, but not in alveolar macrophages, from Lcn2-deficient mice. The inhibitory action of Lcn2 is blocked by the addition of endocytosis inhibitors, suggesting that internalization of Lcn2 into the epithelial cells is a prerequisite for the inhibition of intracellular mycobacterial growth. Taken together, these findings highlight a pivotal role for alveolar epithelial cells during mycobacterial infection, in which Lcn2 mediates anti-mycobacterial innate immune responses within the epithelial cells. _The Journal of Immunology_, 2008, 181: 8521–8527.
with mutations in the mtbB gene, which lack carboxy-mycobactin and mycobactin, exhibit impaired replication in low-iron medium and within macrophages (27). The mechanisms for the mycobactin-mediated iron acquisition within the phagosome of macrophages have recently been elucidated (29). Because pulmonary epithelial cells are also invaded by mycobacteria, host defense mechanisms that inhibit mycobacterial replication within these cells are expected to exist, however they currently remain unclear.

In the present study, we analyzed the role of Lcn2 in mycobacterial infection. Lcn2, which inhibits mycobacterial growth, was rapidly produced from alveolar macrophages and epithelial cells after mycobacterial infection. Furthermore, analyses using Lcn2-deficient mice revealed a pivotal role of alveolar epithelial cells in mycobacterial infection.

Materials and Methods

**Mice**

Lcn2−/− and H-2K-tsA58 transgenic mice have been generated (22, 30) and backcrossed to C57BL/6 for six generations. Lcn2−/− and wild-type littermates from intercrosses of Lcn2−/− mice were used for experiments at 6–8 wk of age. All animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Kyushu University and Osaka University.

**Mycobacteria**

*M. bovis* BCG (Tokyo strain) was purchased from Kyowa Pharmaceuticals. *M. tuberculosis* strains H37Ra (ATCC25177) and H37Rv (ATCC358121) were grown in Middlebrook 7H-9-ADC medium for 2 wk and stored at −80°C until use. GFP-expressing BCG, which was generated previously (5), was used for the experiment.

**Quantitative real-time RT-PCR**

Total RNA was isolated with the TRIzol reagent (Invitrogen), and reverse transcribed using M-MLV reverse transcriptase (Promega) and random primers (Toyobo) after treatment with RNase OUT (Promega). Quantitative real-time PCR was performed in ABI7300 (Applied Biosystems) using TaqMan Universal PCR Master Mix (Applied Biosystems). All data are shown as the relative mRNA levels normalized by the corresponding 18S rRNA level. The primers for 18S rRNA and Lcn2 were purchased from Assays on Demand (Applied Biosystems).

**Preparation of alveolar macrophages**

Bronchoalveolar lavage fluid (BALF) was collected from uninfected mice. To eliminate contamination by bacteria, the cells were cultured with 50 U/ml penicillin and 50 μg/ml streptomycin for 16 h, and then washed five times to remove nonadherent cells. The resultant adherent cells were used for experiments as alveolar macrophages, because >95% of the adherent cells were CD11b-positive.

**Preparation of recombinant Lcn2 (rLcn2) protein**

A mouse Lcn2 cDNA fragment was inserted into pGEX6P-2 (GE Healthcare) and transformed into E. coli BL21. The expressed GST-Lcn2 fusion proteins were purified using glutathione-Sepharose 4B (GE Healthcare) according to the manufacturer’s instructions. The purified proteins were incubated with PreScission Protease (GE Healthcare) to cleave the GST tag, and then purified with Glutathione-Sepharose 4B.

**Immunohistochemistry**

Lungs were fixed with 4% PFA and frozen in Tissue-Tec OCT compound (Sakura). The sections were incubated with anti-mouse Lcn2 Ab (R&D Systems), anti-pro-surfactant protein C (SP-C) Ab (Chemicon), anti-CD11b Ab (BD Biosciences), or anti-pan cytokeratin Ab (Sigma-Aldrich). The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes). Alveolar epithelial cells were infected with GFP-expressing BCG for 16 h, washed, and incubated with Dextran Conjugates (Cascade Blue; Molecular Probes) and Alexa Fluor 594-labeled rLcn2 for 6 h. rLcn2 was labeled using an Alexa Fluor 594 Protein Labeling Kit (Molecular Probes). The cells were fixed with 4% PFA and analyzed using a confocal microscopy (LSM 510; Carl Zeiss).

**Western blot assay**

BALF was collected from BCG-infected mice by catheterization techniques into 500 μl of PBS. To normalize BALF samples, we injected the same volume of PBS (500 μl) recovered equal volume, and used them for Western blot analysis. After removal of precipitates, the samples were separated on SDS-PAGE and transferred to PVDF membranes (Millipore). The membranes were incubated with anti-mouse Lcn2 Ab. Bound Ab was detected with Supersignal West Pico Chemiluminescent Substrate (Pierce).

**In vitro mycobacterial growth assay**

Mycobacteria were incubated in Middlebrook 7H9-ADC medium with the indicated concentrations of rLcn2 protein for 20 days at 37°C, and were plated on Middlebrook 7H10-OADC agar plates and incubated at 37°C for 30 days. In some experiments, BCG was incubated with the indicated concentrations of deferoxamine mesylate (DFO; Calbiochem), FeCl3, or mycobactin (Kyoritsu Seiyaku) on 7H10-OADC agar plates.

**In vivo infection of mycobacteria**

Mice were intratracheally infected with *M. tuberculosis* H37Rv (1 × 10⁵ CFU). At 6 wk after infection, homogenates of the lungs and livers were plated on 7H10-OADC agar plates. For histological analyses, the lungs were fixed with 4% PFA at 20 or 5 days after infection, embedded in paraffin, cut into sections, and stained with H&E or by the Ziehl-Neelsen method, respectively.

**Establishment of alveolar epithelial cell lines**

To establish alveolar epithelial cell lines (AECs) from wild-type and Lcn2−/− mice, the mice were crossed with H-2K-tsA58 transgenic mice, and used for experiments at 4 wk of age. Mouse pulmonary type II AECs were established as previously described (32). The cells were incubated at 33°C and passed over ten times. The cells were then stained with anti-SP-C Ab to confirm that they were type II alveolar epithelial cells.

**In vitro infection of mycobacteria**

Wild-type or Lcn2−/− derived AECs or alveolar macrophages were infected with BCG for the indicated periods. To eliminate extracellular BCG, the cells were cultured with 50 μg/ml streptomycin for 1 h, washed three times, and harvested. Lysates of the cells were plated on 7H10-OADC agar plates.

**Detection of intracellular growth of mycobacteria**

Wild-type and Lcn2−/− derived AECs were seeded onto 96-well plates, and infected with BCG for 6 h. To eliminate extracellular BCG, the AECs were cultured with 50 μg/ml streptomycin for 1 h, vigorously washed three times. The cells were pulsed with 37 kBq of [³H]uracil and cultured for 18 h. The cells were harvested on glass fiber filters and the incorporated [³H]uracil was measured using a liquid scintillation counter (Wallac). In some experiments, cytochalasin B (Sigma-Aldrich) or chlorpromazine (CPZ; Calbiochem) was added to the wells at 30 min before the [³H]uracil pulse or rLcn2 addition.

**Statistical analysis**

Differences between control and experimental groups were evaluated using Student’s t test or ANOVA plus posthoc testing. Values of *p* < 0.05 were considered to indicate statistical significance.

**Results**

Expression of lipocalin 2 in BCG-infected lungs

To assess the role of Lcn2 in mycobacterial infection, we first analyzed the expression of Lcn2 in the lungs of C57BL/6 mice intratracheally infected with BCG. Total RNA was extracted from the lungs at 2, 7, and 14 days after infection, and analyzed for Lcn2 mRNA expression by real-time quantitative PCR (Fig. 1A). Expression of Lcn2 mRNA was markedly increased at 2 days after infection and decreased thereafter. Because Lcn2 mRNA expression was shown to be induced in macrophages stimulated with TLR ligands (22), we analyzed whether alveolar macrophages expressed Lcn2 mRNA (Fig. 1B). Alveolar macrophages were isolated, infected with BCG, and analyzed for Lcn2 mRNA expression at 2 days.
after infection; BCG infection led to a marked increase in the expression of Lcn2 mRNA. We also analyzed the lungs by immunohistochemistry using an anti BCG-infected mice, several Lcn2-positive cells were observed. These cells mainly faced the alveolar surface and projected into the alveolar space, representing the typical morphology of type II alveolar epithelial cells. Costaining with an Ab to pro-SP-C, which is produced by type II alveolar epithelial cells, revealed that both Lcn2 and SP-C were produced by the same cells (Fig. 1D). These findings indicate that not only alveolar macrophages but also type II alveolar epithelial cells produce Lcn2 during respiratory mycobacterial infection. Type II alveolar epithelial cells are known to secrete several mediators into the alveolar space. Therefore, we analyzed whether Lcn2 is secreted into the alveolar space during intratracheal BCG infection. BALF was collected from uninfected and BCG-infected mice and analyzed for Lcn2 mRNA expression by real-time quantitative PCR. Data are shown as the relative mRNA levels normalized by the corresponding 18S rRNA level. Data are presented as means ± SD, and are representative of two independent experiments. B. Alveolar macrophages were isolated from uninfected wild-type mice, cultured with or without BCG for 48 h, and then analyzed for their Lcn2 mRNA expression by real-time quantitative PCR. C and D. At 2 days after intratracheal infection with BCG, lung tissue sections were stained with anti-Lcn2 Ab (red), DAPI (blue), and anti-SP-C Ab (green), and visualized by fluorescence microscopy. E. Wild-type mice were intratracheally infected with 2.5 × 10⁶ CFU of BCG. At the indicated time points after the infection, 500 μl of PBS was intratracheally injected and then recovered. The recovered BALF samples were subjected to Western blot analysis with anti-Lcn2 Ab.

Inhibition of in vitro mycobacterial growth by Lcn2.

We investigated whether Lcn2 inhibits mycobacterial growth by interfering with iron acquisition, similar to the case for inhibition of E. coli growth (16). First, we added DFO, an iron chelator, into in vitro BCG cultures (Fig. 2D). DFO reduced BCG growth in a dose-dependent manner, indicating that BCG requires iron for growth. Next, we added ferric iron into BCG cultures (Fig. 2E). Addition of ferric iron rescued Lcn2-mediated inhibition of BCG growth in a dose-dependent manner, indicating that Lcn2 inhibits use of iron from the culture medium. Addition of exogenous mbcacin (MB) also abolished Lcn2-mediated inhibition of BCG growth (Fig. 2F). These findings indicate that Lcn2 inhibits mycobacterial growth by sequestering iron.

In vivo anti-mycobacterial activity of lipocalin 2

Lcn2 has the ability to inhibit the growth of several mycobacterial strains.

A representative of two independent experiments. At the indicated time points after the infection, 500 μl of PBS was subjected to Western blot analysis with anti-Lcn2 Ab.
Increased numbers of mycobacteria in Lcn2-deficient alveolar epithelial cells

We next analyzed the localization of *M. tuberculosis* in the lungs at 5 days after intratracheal infection by staining acid-fast bacilli using the Ziehl-Neelsen method. In wild-type and Lcn2−/− mice, similar densities of *M. tuberculosis* were observed in granulomatous lesions, although the number and size of the granulomatous lesions were increased in Lcn2−/− mice (data not shown). In addition, *M. tuberculosis* exhibited similar staining of cells with a macrophage-like morphology in wild-type and Lcn2−/− mice (Fig. 4A). Strikingly, some of the alveolar epithelial cell layers in Lcn2−/− mice contained *M. tuberculosis* (Fig. 4B). In sharp contrast, *M. tuberculosis* was scarcely detected within the epithelial cell layers of wild-type mice. To corroborate these findings, we subjected the lungs of mice intratracheally infected with GFP-expressing BCG to immunohistochemical analyses. In both wild-type and Lcn2−/− mice, CD11b-positive cells contained GFP-expressing BCG. However, in the lungs of Lcn2−/− mice, GFP-expressing BCG was frequently observed in cells that did not express CD11b, in contrast to the low frequency observed in the lungs of wild-type mice (Fig. 4C). Visualization of epithelial cells using an anti-cytokeratin Ab indicated that increased numbers of alveolar epithelial cells in Lcn2−/− mice contained GFP-expressing BCG compared with those in wild-type mice (Fig. 4, D and E). Thus, in the absence of Lcn2, invasion and replication of mycobacteria in alveolar epithelial cells were increased.

Therefore, we assessed the sensitivities of alveolar macrophages and alveolar epithelial cells to in vitro infection with BCG. First, alveolar macrophages were isolated from wild-type and Lcn2−/− mice, and infected with BCG (Fig. 5A). The CFU titers of BCG in macrophages at 4 and 7 days after infection were comparable between wild-type and Lcn2−/− cells. Thus, the absence of Lcn2 did not affect the anti-mycobacterial activity in alveolar macrophages. Next, we established AECs from wild-type and Lcn2−/− mice. Because AECs are difficult to culture in vitro, we took advantage of transgenic mice harboring a temperature-sensitive mutation of the inducible H-2Kb promoter element (30–32). Using these mice, we were able to isolate AECs from wild-type mice expressing Lcn2 mRNA and secreting Lcn2 protein into the culture medium when infected with BCG (data not shown). Thus, these AECs showed the characteristics of type II alveolar epithelial cells. AECs were infected with BCG, and the CFU titers within the cells were counted at 1, 2, 3, and 4 days after infection (Fig. 5B). At 3 and 4 days after infection, the CFU titers in Lcn2−/− cells were increased compared with those in wild-type cells. Addition of exogenous rLcn2 reduced the CFU numbers in Lcn2−/− cells to wild-type levels, indicating that the high susceptibility of Lcn2−/− mice to *M. tuberculosis* infection is attributable to impaired clearance of mycobacteria from alveolar epithelial cells, rather than alveolar macrophages, in the absence of Lcn2.
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 [3H]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 [3H]uracil (Fig. 6A). Under these conditions, [3H]uracil incorporation was below 1 × 10^5 cpm in wells containing uninfected AECs or wells placed in contact with BCG and then extensively washed. After infection with each CFU, [3H]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 [3H]uracil by intracellular BCG (Fig. 6B). In alveolar macrophages, the [3H]uracil incorporation by intracellular BCG was comparable between wild-type and Lcn2−/− cells (Fig. 6C). Addition of rLcn2 did not effectively reduce the uptake of [3H]uracil by intracellular BCG in alveolar macrophages from both wild-type and Lcn2−/− mice (Fig. 6D). 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. 7A). 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. 7B), indicating that BCG and rLcn2 were localized in distinct cellular compartments within macrophages. We blocked...
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. 7C). We also analyzed the effects of the endocytosis inhibitor on rLcn2-mediated inhibition of BCG growth (Fig. 7D). Addition of CPZ abolished Lcn2-mediated inhibition of [3H]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, uuterocalin, 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 carboxy-mycobactin and bacilli-bactin (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 phagosomes 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 macrophosomes (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 and not macrophages. Therefore, Lcn2 might be the mechanism for inhibiting mycobacterial growth in alveolar epithelial cells.
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 Nrampl (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 sequestered 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
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