Interleukin-17-Induced Protein Lipocalin 2 Is Dispensable for Immunity to Oral Candidiasis

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Oropharyngeal candidiasis (OPC; thrush) is an opportunistic fungal infection caused by the commensal microbe Candida albicans. Immunity to OPC is strongly dependent on CD4+ T cells, particularly those of the Th17 subset. Interleukin-17 (IL-17) deficiency in mice or humans leads to chronic mucocutaneous candidiasis, but the specific downstream mechanisms of IL-17-mediated host defense remain unclear. Lipocalin 2 (Lcn2; 24p3; neutrophil gelatinase-associated lipocalin [NGAL]) is an antimicrobial host defense factor produced in response to inflammatory cytokines, particularly IL-17. Lcn2 plays a key role in preventing iron acquisition by bacteria that use catecholate-type siderophores, and lipocalin 2−/− mice are highly susceptible to infection by Escherichia coli and Klebsiella pneumoniae. The role of Lcn2 in mediating immunity to fungi is poorly defined. Accordingly, in this study, we evaluated the role of Lcn2 in immunity to oral infection with C. albicans. Lcn2 is strongly upregulated following oral infection with C. albicans, and its expression is almost entirely abrogated in mice with defective IL-17 signaling (IL-17RA−/− or Act1−/− mice). However, Lcn2−/− mice were completely resistant to OPC, comparably to wild-type (WT) mice. Moreover, Lcn2 deficiency mediated protection from OPC induced by steroid immunosuppression. Therefore, despite its potent regulation during C. albicans infection, Lcn2 is not required for immunity to mucosal candidiasis.

Oropharyngeal candidiasis (OPC; thrush) is an opportunistic infection associated with CD4+ T cell loss and is caused by the commensal fungus Candida albicans. Individuals with HIV/AIDS are particularly prone to this disease, and OPC is considered an AIDS-defining illness (1, 2). In addition, OPC is common in patients taking immunodepleting chemotherapy, infants and the elderly, and patients with congenital immunodeficiencies, such as hyper-IgE syndrome (HIES) (3). OPC exhibits considerable morbidity and can cause impaired nutrition due to odynophagia (pain with swallowing) and failure to thrive in infants. To date, there are no clinically available vaccines for C. albicans, or indeed for any fungal pathogens (4, 5).

The cytokine interleukin 17 (IL-17; also known as IL-17A) is tightly associated with immunity to OPC (6). Mice lacking either IL-17 receptor subunit (IL-17RA and IL-17RC) are highly susceptible to OPC. Similarly, mice lacking IL-23 (either the IL-12p40 or the IL-23p19 subunit), a cytokine that induces IL-17-producing cells, are also susceptible (7–9). In agreement with the findings for mice, humans with mutations in IL-17RA or its ligand IL-17F suffer from chronic mucocutaneous candidiasis (CMC), characterized by both OPC and dermal candidiasis (10). In addition, neutralizing anti-IL-17 antibodies in autoimmune regulator (AIRE) deficiency or in some thymomas are linked to susceptibility to OPC (11, 12). IL-17 signals through an adaptor protein, Act1 (also known as CIKS) (13), and Act1-deficient humans were recently identified on the basis of their susceptibility to recurrent CMC (14). Antibodies against IL-17 and its receptor are currently in clinical trials for the treatment of autoimmune diseases such as psoriasis and rheumatoid arthritis (15–18), but the potential adverse effects of these therapies are still not well defined. On the basis of the evidence outlined above, OPC and CMC are obviously of particular concern for patients taking anti-IL-17 therapies. In fact, a recent report indicates that patients on anti-tumor necrosis factor (anti-TNF) drugs for inflammatory bowel disease show an increased risk of candidiasis, which had not been previously recognized (19).

Although IL-17 signaling is clearly essential for immunity to OPC, the downstream mechanisms by which IL-17 mediates immunity to this fungus remain largely unknown. IL-17 signaling induces a program of gene expression associated with innate immune responses, including cytokines (IL-6, granulocyte colony-stimulating factor [G-CSF]), chemokines (CXCL1, CXCL2, CCL20), and antimicrobial peptides (β-defensins and S100A8/9) (13). One of the most strongly induced IL-17 target genes encodes lipocalin 2 (Lcn2), which is regulated at the transcriptional level by IL-17 either alone or in conjunction with TNF-α (20–22). IL-17 signaling in a variety of cell types induces Lcn2 expression (20), and lcn2 mRNA is one of many IL-17 signature genes induced in the oral mucosa following C. albicans infection (7).

Lipocalin 2, also known as 24p3, urocin, or neutrophil gelatinase-associated lipocalin (NGAL), is an acute-phase protein produced by the liver and also by mesenchymal and epithelial cells in response to inflammatory cytokines. Lcn2 plays a key role in preventing iron acquisition by bacteria that use catecholate-type siderophores (23, 24). Accordingly, Lcn2−/− mice are highly susceptible to infection by Escherichia coli and Klebsiella pneumoniae (25–27), bacterial species that use this type of iron-scavenging system. However, the role of Lcn2 in mediating immunity to fungi...
has not been a focus of investigation. Accordingly, in this study, we evaluated the role of Lcn2 in immunity to mucosal \textit{C. albicans} infection. Surprisingly, Lcn2 was not required for the host defense against OPC, even though it was strongly upregulated in an IL-17-dependent manner following \textit{Candida} infection. Moreover, Lcn2 deficiency appeared to mediate protection from oral thrush induced by immunosuppression, suggesting an immunoregulatory role for this protein.

\textbf{MATERIALS AND METHODS}

\textbf{Mice used and oropharyngeal candidiasis model.} Mice were on the C57BL/6 background and were age and sex matched. IL-23p19\textsuperscript{−/−} mice were provided by Genentech (South San Francisco, CA), and IL-17RA\textsuperscript{−/−} mice were from Amgen (Seattle, WA). Lcn2\textsuperscript{−/−} mice were a kind gift from Tak Mak, University of Toronto, and have been described previously (25, 27). Act1\textsuperscript{−/−} mice have been described previously (28). All mice were housed under specific-pathogen-free (SPF) conditions. The genotypes of all mice were verified by PCR of ear biopsy specimens. Mice aged 7 to 10 weeks (\textit{n} = 3 to 10 per experiment) were inoculated sublingually with a 0.0025-g cotton ball saturated with a \textit{C. albicans} (strain CAF2-1) suspension of 2 × 10\textsuperscript{7} CFU/ml for 75 min under anesthesia, as described previously (29). If indicated, mice were administered cortisone acetate intraperitoneally (i.p.) at 225 mg/kg of body weight at days −1, +1, +3 relative to infection. The oral cavity was swabbed before each infection, and swabs were plated on YPD-AMP (yeast extract-peptone-dextrose agar plus ampicillin) agar plates to verify the absence of \textit{Candida}. Mice were weighed daily. After sacrifice, the tongue of each mouse was homogenized on a gentleMACS Dissociator (Miltenyi Biotec, Auburn, CA), and serial dilutions were plated on YPD-AMP agar in triplicate for colony enumeration. Data were analyzed by GraphPad Prism (version 5) by using \textit{t} tests with the Mann-Whitney correction (a \textit{P} value of <0.05 was considered significant). Protocols were approved by the University of Pittsburgh IACUC.

\textbf{Histology.} Tongue tissue was prepared for histology by the Research Histology Services core of the University of Pittsburgh. Samples were stained with periodic acid-Schiff stain (PAS) and were imaged at a magnification of ×10 to ×40.

\textbf{RNA preparation and real-time reverse transcriptase PCR.} RNA was extracted with RNeasy kits, and cDNA was synthesized with a SuperScript III first-strand synthesis system (Invitrogen). Relative quantification of gene expression was carried out by real-time PCR with SYBR green (Quanta BioSciences, Gaithersburg, MD) and normalization to the expression of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. All samples were analyzed in triplicate. Primers were from SABiosciences (Qiagen). Results were analyzed on an Applied Biosystems (Carlsbad, CA) 7300 real-time PCR system.

\textbf{RESULTS}

\textbf{Lcn2 is induced in an IL-17R-dependent manner during OPC.} To evaluate Lcn2 expression during OPC, we infected C57BL/6 mice orally with \textit{Candida albicans} (strain CAF2-1), using a standard OPC infection model with 75 min of sublingual exposure to \textit{C. albicans} strain CAF2-1 (29). As controls, we subjected wild-type (WT) mice administered high-dose cortisone, IL-17RA\textsuperscript{−/−} mice, and Act1\textsuperscript{−/−} mice to OPC (28), since the IL-23/IL-17 pathway is known to be essential for immunity to oral candidiasis (7). We then measured the \textit{C. albicans} load in the tongue at either day 2 or day 4 postinfection by plating serial dilutions of tongue homogenates onto YPD-AMP agar plates. At day 2, all animals, including WT mice, had similarly high fungal burdens, a result comparable to historical data with this model (Fig. 1A) (7). By day 4, WT mice had fully cleared the infection, whereas IL-17RA\textsuperscript{−/−} and Act1\textsuperscript{−/−} mice still had significant oral fungal loads. Weight loss measurements paralleled the fungal load data; WT mice fully recovered their original weight by day 4, correlating with fungal clearance, but IL-17RA\textsuperscript{−/−} mice, Act1\textsuperscript{−/−} mice, and cortisone-treated WT mice still showed reduced weight and persistent fungal
Lcn2 is dispensable for immunity to OPC. Since infection with OPC triggered upregulation of lcn2 in an IL-17- and Act1-dependent manner, we hypothesized that Lcn2 would be important for immunity to oral *Candida albicans* infection. Accordingly, WT or *lcn2*−/− mice (27) were subjected to OPC for 5 days, and fungal loads in the oral mucosa were assessed as described above. As controls, we also used IL-23−/− mice (which show the same susceptibility to OPC as IL-17RA−/− mice [8]). As an additional control, *lcn2*−/− mice were immunosuppressed with high-dose cortisone acetate. Both WT and *lcn2*−/− mice cleared the infection by day 5. However, cortisone-treated *lcn2*−/− mice were highly susceptible to disease, and so, to a lesser extent, were IL-23−/− mice (Fig. 2A). Weight loss tracked with susceptibility to disease; that is, mice that cleared *C. albicans* (WT and *lcn2*−/− mice) exhibited complete weight recovery, whereas IL-23−/− mice or cortisone-treated *lcn2*−/− mice showed progressive weight loss (Fig. 2B). In agreement with these findings, histological analysis of tongue tissue stained with periodic acid-Schiff stain (PAS) (to detect yeast) showed that WT and *lcn2*−/− mice had normal tissue architecture, with no detectable fungal organisms, at day 5. In contrast, IL-23−/− and cortisone-treated *lcn2*−/− mice showed destruction of the superficial epithelial layer by hyphal and pseudohyphal forms of *Candida albicans* (Fig. 2C), in a manner similar to that which we observed previously for cortisone-treated WT mice or IL-17RA−/− mice (7). These results indicate that Lcn2, though strongly induced by IL-17 during the course of oral *Candida* exposure, is not required to mediate fungal clearance.

Lipocalin 2-deficient mice show enhanced resistance to OPC induced by cortisone treatment. Since *lcn2*−/− mice were not susceptible to OPC, we questioned whether they might show enhanced resistance to infection. Accordingly, we compared the fungal susceptibilities of WT and *lcn2*−/− mice that were treated with cortisone acetate to induce immunosuppression. As shown, the fungal load in WT mice treated with cortisone was approximately 1 log unit greater than that in *lcn2*−/− mice treated with cortisone (4.8 × 105 compared with 3.72 × 104 CFU), a difference that was statistically significant (*P*, 0.0034) (Fig. 3A). In agreement with this observation, cortisone-treated *lcn2*−/− mice did not lose as much weight as cortisone-treated WT mice (Fig. 3B). Therefore, a deficiency in Lcn2 is associated with significant and reproducible, albeit modest, resistance to OPC.

**DISCUSSION**

In recent years, it has become clear that IL-17 is a central mediator of immunity to fungi, including *C. albicans* and other pathogens of fungal origin (30). The importance of IL-17 in mucocutaneous candidiasis is underscored by discoveries of patients with mutations in genes that impact the generation of Th17 cells (including *STAT3, STAT1*, and *CARD9*) or that block IL-17-mediated signaling (including *IL17F, IL17RA*, and *ACT1*) (6, 30, 31). Studies of humans also indicate that the vast majority of *Candida*-reactive Th cells express IL-17 (32). However, the downstream mecha-
Lipocalin 2 Is Not Required for OPC Immunity

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