Lung Epithelial Cells Coordinate Innate Lymphocytes and Immunity against Pulmonary Fungal Infection

Graphical Abstract

Highlights

- Lung epithelial cells (LECs) mount a robust NF-κB-dependent response to inhaled fungi
- NF-κB signaling in LECs regulates IL-17A- and GM-CSF-producing innate lymphocytes
- Fungal killing by innate immune cells is dependent on LEC NF-κB, IL-17A, and GM-CSF
- IL-1α/IL-1R signaling in LECs promotes a CCL20-CCR6 axis to regulate innate lymphocytes

Authors

Nydiañez-Santos, Darin L. Wiesner, J. Scott Fites, Andrew J. McDermott, Thomas Warner, Marcel Wüthrich, Bruce S. Klein

Correspondence

bsklein@wisc.edu

In Brief

Hernández-Santos et al. show that the lung epithelium guards against inhaled fungi by providing early signals that orchestrate innate immunity. Signaling via the lung epithelial cell receptor IL-1R is required to release soluble signals that coordinate and recruit innate lymphocytes whose soluble products arm phagocytes to kill fungi.
Lung epithelial cells coordinate innate lymphocytes and immunity against pulmonary fungal infection

Nydiasir Hernandez-Santos, 1, Darin L. Wiesner, 1,2,6 J. Scott Fites, 1,5 Andrew J. McDermott, 1 Thomas Warner, 4 Marcel Wuthrich, 1 and Bruce S. Klein 1,2,3,6,*

1 Department of Pediatrics, School of Medicine and Public Health, University of Wisconsin-Madison, Madison, WI 53792, USA
2 Department of Internal Medicine, School of Medicine and Public Health, University of Wisconsin-Madison, Madison, WI 53792, USA
3 Department of Medical Microbiology and Immunology, School of Medicine and Public Health, University of Wisconsin-Madison, Madison, WI 53792, USA
4 Department of Pathology and Laboratory Medicine, School of Medicine and Public Health, University of Wisconsin-Madison, Madison, WI 53792, USA
5 These authors contributed equally
6 Lead Contact
*Correspondence: bsklein@wisc.edu
https://doi.org/10.1016/j.chom.2018.02.011

SUMMARY

Lung epithelial cells (LEC) are strategically positioned in the airway mucosa to provide barrier defense. LECs also express pattern recognition receptors and a myriad of immune genes, but their role in immunity is often concealed by the activities of “professional” immune cells, particularly in the context of fungal infection. Here, we demonstrate that NF-κB signaling in LECs is essential for immunity against the pulmonary fungal pathogen Blastomyces dermatitidis. LECs orchestrate innate antifungal immunity by augmenting the numbers of interleukin-17A (IL-17A)- and granulocyte-macrophage colony-stimulating factor (GM-CSF)-producing innate lymphocytes, specifically “natural” Th17 (nTh17) cells. Inhale lymphocyte-derived IL-17A and GM-CSF in turn enable phagocyte-driven fungal killing. LECs regulate the numbers of nTh17 cells via the production of chemokines such as CCL20, a process dependent on IL-1x-IL-1 receptor (IL-1R) signaling on LECs. Therefore, LECs orchestrate IL-17A- and GM-CSF-mediated immunity in an IL-1R-dependent manner and represent an essential component of innate immunity to pulmonary fungal pathogens.

INTRODUCTION

In the air we breathe, 4%–11% of the fine particle mass contains fungal spores (Fröhlich-Nowotny et al. 2009), some of which enter our airways. A fungal particles’ first point of contact in the airway is the epithelium lining the respiratory mucosa. This lining separates the environment from the lung parenchyma and enables lung epithelial cells (LEC) to guard the tissue against fungal invaders. However, the role of LECs as defenders against inhaled fungi has been overshadowed in favor of hematopoietic cells. LECs can participate in antimicrobial immunity by clearing particulates from the airway via the mucociliary escalator and by producing antimicrobial peptides and inflammatory mediators (Whitsett and Alosca, 2015). These mediators inform the immune system of microbial assault thereby directing early decisions (Swamy et al., 2010) about innate and adaptive immunity. Despite these LEC functions, little is known about how LECs promote antifungal immunity.

Interleukin-17 (IL-17) is a key component of immunity to fungi. CD4+ IL-17+ (Th17) cells were thought to be the main source of IL-17, but innate sources have now been described (Gaffen et al., 2014): T cell receptor αβ (TCRαβ) cells, TCRγδ cells, innate lymphoid cells type 3 (ILC3), invariant natural killer T cells, mucosal-associated invariant T (MAIT) cells (Rahimpour et al., 2015), neutrophils (Taylor et al., 2014), and eosinophils (Guerra et al., 2017). During ophthalmic candidiasis (OPO), Candida albicans induces natural T helper 17 (nTh17) cells, TCR γδ cells (Conti et al., 2014), and ILC3 (Giudici et al., 2013). In keratitis due to Aspergillus fumigatus, neutrophils auto-activate via para-crine IL-17A signaling and promote fungal clearance (Taylor et al., 2014). Lastly, in pulmonary aspergillosis, eosinophils produce IL-23 and IL-17A, and associate with and kill A. fumigatus (Guerra et al., 2017). Thus, both innate and adaptive sources of IL-17 promote immunity to fungi.

Cells that produce IL-17A may make other cytokines, alone or together with IL-17A. One such cytokine is granulocyte-macrophage colony-stimulating factor (GM-CSF), another key component of antifungal immunity. Humans with congenital or acquired defects in GM-CSF responses (pulmonary alveolar proteinosis) are susceptible to fungal infections due to impaired macrophage and neutrophil function (Punatar et al., 2012; Uchida et al., 2007). GM-CSF−/− mice are susceptible to Pneumocystis carinii infection (Paine et al., 2000) and mice lacking the β subunit of the GM-CSF receptor (GM-CSFFRβ) show impaired reactive oxygen species (ROS) production by neutrophils and inability to kill A. fumigatus (Kasahara et al., 2016). In Histoplasma capsulatum-infected macrophages (MO), GM-CSF causes zinc...
sequestration in the Golgi, prompting phagosomal zinc depriva-
tion, induction of ROS, and fungal death (Subramanian Vignesh
et al., 2013). A serine protease of Blastomyces yeast cleaves
GM-CSF, promoting escape from phagocyte killing (Sterkel
et al., 2016). Finally, in systemic candidiasis, IL-17 and Syk-
dependent IL-23 production by dendritic cells (DCs) enable
natural killer cells to produce GM-CSF, thereby promoting can-
didal activity of neutrophils (Bar et al., 2014). These findings
highlight the role of GM-CSF in potentiating phagocyte fungal
killing and the link between IL-17 and GM-CSF signaling path-
ways during fungal infection.

The relationship of the epithelium to GM-CSF-mediated anti-
fungal immunity is not well understood. LECs produce GM-CSF
early during lung development (Guilliams et al., 2013), and alve-
olar epithelial cell GM-CSF orchestrates DC responses to influ-
enza and antiviral CD8+ T cell responses (Unkel et al., 2012).
To our knowledge, LEC regulation of GM-CSF immunity to fungi
has not been investigated. Conversely, the epithelium and IL-17-
mediated immunity has received more attention. Epithelial cells
respond to IL-17. IL-17R on epithelial cells at different tissue
sites regulates antimicrobial peptides and chemokines that re-
cruit neutrophils. IL-17R on gut epithelium regulates γ-defen-
sins, which maintains levels of segmented filamentous bacteria
(Kumar et al., 2016). Likewise, IL-17R on oral epithelium regu-
lates β-defensin-3, which clears C. albicans from the oral cavity
(Conti et al., 2016). Similarly, IL-17R on lung club cells controls
CXCL5, neutrophil recruitment, and Klebsiella pneumonia
(Chen et al., 2016). Although LECs respond to IL-17, the mode
by which the epithelium regulates the function of IL-17-producing
cells remains ill defined.

Herein, we investigated how LECs regulate innate defense against
pathogenic fungi. We addressed three questions: (1) Are LECs es-
sential in host defense against inhaled fungi? (2) How do LECs
sense inhaled fungi, e.g., what are the signaling pathways and
upstream receptors? (3) How do LECs orchestrate innate anti-
fungal immunity, e.g., what are the effector cells, how do they
kill fungi, and how do LECs regulate these effector mechan-
isms? To address these questions, we exploited a murine model
involving the inhaled pathogenic fungus Blastomyces der-
matitis, the causative agent of fungal pneumonia and one of
the major endemic mycoses of North America. We show that the
fungus rapidly activates nuclear factor κB (NF-κB) signaling in
LEC, which is essential in orchestrating innate antifungal immu-
nity. LECs regulate antifungal activity by coordinating the function
of innate lymphocytes, including γδ T cells and innate
lymphocyte-derived IL-17A and GM-CSF arm phagocytes to kill
the fungus. This circuit is amplified by IL-1x-IL-1R signaling on
LEC. These findings provide fresh insight into the earliest stages
of lung host defense and highlight an unappreciated role for LECs
in orchestrating innate antifungal immunity.

RESULTS

LECs Mount a Robust, NF-κB-Dependent Antifungal
Response
To delineate the possible contribution of LECs to antifungal
immunity, we first ascertained whether the fungus interacts with
LECs and whether such interactions result in NF-κB activation;
this signaling event lies downstream of many pattern recognition
pathways and mediates transcription of various immune media-
tors (Lawrence, 2009). Following infection, histological examina-
tion of infected lung tissue revealed yeast in close proximity
to upper LECs (Figure 1A, arrowhead 1). Yeasts also were
observed in the lower airway, but inflammation obstructing the
alveoli obscured the location of yeasts relative to alveolar epithelial
cells (Figure 1A, arrowhead 2). LEC response to yeast resulted in
NF-κB activation, as shown by GFP+ LECs in histological
sections of infected NF-κB reporter mice (Figure 1B), where
GFP expression is under the transcriptional control of NF-κB dis-
acting elements (Magness et al., 2004). Flow cytometric quanti-
fication revealed an increased proportion of GFP+ LECs upon
fungal infection in these mice (Figure 1C). We also observed
translocation of p65 (ReA) into the nucleus as early as 1 h
post-infection (p.i.) and peaking at 4 h p.i. (Figures S1A and S1B).

If LECs rapidly sense inhaled fungi, NF-κB signaling in LECs
may help restrain fungal invasion of the lungs. To test this hypo-
thesis, we employed two murine models of infection in which NF-κB
is deleted either in a select subset of LECs or throughout the
airway epithelium. In DNTA mice, the club cell-specific promoter
CC10 drives expression of a dominant-negative form of IκB upon
doxycycline administration, preventing NF-κB translocation to the
nucleus (Cheng et al., 2007). DNTA mice treated with doxycycline
exhibited a 2- to 3-log higher fungal burden than DNTA mice not
treated with doxycycline or corresponding littermate controls
(rtTA) (Figure 1D). Thus, club cells may regulate early antifungal
immunity. However, perturbations in the intestinal microbiota as
a result of antibiotic treatment may confound interpretation of
these results. Therefore, we also used IKK2ALOE mice (Perez-
Navarro et al., 2013), in which the IKK2 (IKKβ) component of IκB ki-
nase is deleted in most of the airway epithelium, sequestering
NF-κB in the cytoplasm. In this model, lung-specific surfactant
protein C drives expression of cre recombinase, which recognizes
loxP sites flanking exons 5 and 6 of IKK2 with a predicted effi-
ciency of 60%–100% (Figures S1C and S1D). IKK2FL mice
phenotypically resemble wild-type (WT) and are used as littermate
controls. Infection with fungal spores or yeast yielded a respective
1 and 3 log increase in fungal burden in IKK2ALOE versus IKK2FL
mice (Figures 1D and 1F). Survival post-infection was also signifi-
cantly shortened in IKK2ALOE versus IKK2FL mice (Figure 1G).
Thus, NF-κB signaling in LECs promotes antifungal immunity and
prolongs survival during fungal infection.

Due to early activation of NF-κB signaling upon infection (Fig-
ures 1 and S1), we postulated that this pathway in LECs may
restrain fungal invasion early during the innate phase of the
host immune response. Upon kinetic analysis, the fungal burden
rose significantly in IKK2ALOE versus IKK2FL mice by as early as
48 h p.i. (Figure 1H), indicating that LECs orchestrate early resis-
tance in this model.

LECs-Mediated Antifungal Immunity Is IL-17A
and GM-CSF Dependent
Immunity to several respiratory fungal infections requires IL-17A
and GM-CSF. IL-17A−/− or IL-17A−/− mice show impaired
secondary immunity (Wultrich et al., 2011) to Blastomyces der-
matitis, as well as Histoplasma capsulatum and Coccidioides
posadasii. Moreover, inactivation of GM-CSF greatly enhances
virulence of B. dermatitis yeast (Sterkel et al., 2016). Here,
we analyzed the requirement for IL-17A and GM-CSF within 48 hr p.i. (day 2) to see if these products are required during the same time frame in which LECs orchestrate innate immunity. We neutralized IL-17A and GM-CSF in WT mice during 48 hr p.i., and assayed fungal burden at the end of this interval. Neutralization of IL-17A and GM-CSF significantly increased the burden at 2 days p.i. (Figure 2A), suggesting a temporal association between the requirements for LEC NF-κB signaling and IL-17A and GM-CSF. To assess a link, we analyzed these products in IKK2ΔLEC and IKK2ΔLIE mice. IKK2ΔLEC mice had reduced IL-17A in the bronchoalveolar lavage fluid (BALF) (Figure 2B) and also reduced numbers of IL-17A- and GM-CSF-producing innate lymphocytes compared with IKK2ΔLIE mice (Figure 2C). Thus, LECs may orchestrate innate antifungal immunity via the actions of IL-17A and GM-CSF, perhaps through regulating the function or number of innate lymphocyte populations.

NF-κB Signaling in LECs Regulates IL-17A- and GM-CSF-Producing Innate Lymphocytes

We sought to delineate the cell sources of IL-17A and GM-CSF. Several innate sources of IL-17A have been described in the oral cavity; for example, nTh17, TCRγδ, and ILC3 produce IL-17A during C. albicans infection in OPC (Conti et al., 2014; Gladiator et al., 2013). We used IL-17AΔRosa 26R-YFP, fete reporter mice (IL-17A producers are permanently labeled eYFP+) to identify cell sources in the lung (Hirot a et al., 2011). In naive lung tissue, TCRβ⁺CD4⁺ cells constituted the largest proportion of IL-17A-producing cells (46%), followed by TCRβ⁺CD4⁺ (nTh17) (26%) and TCRγδ (26%), and a minor population of ILC3 (2%). Following infection, TCRβ⁺CD4⁺ cells (nTh17) increased in proportion to 41%, TCRγδ remained unchanged (25%), TCRβ⁺CD4⁻ decreased to 30%, and ILC3 underwent a minor increase to 4% (Figures S3A and 3A). We detected a minor population of IL-17A⁺CD8⁺ T cells, but no IL-17A⁺ neutrophils (Figure S3B). Quantification of IL-17A-producing cells revealed an infection-dependent increase in the absolute number of all these cell populations (Figure 3B). Upon further characterization, we found that TCRβ⁺CD4⁺ cells were CD49a⁺, CD49d⁺ (bimodal distribution in infected animals), and CD29⁺ (Figure S3C), resembling the phenotype of nTh17 cells (Conti et al., 2014) and henceforth referred to as such. TCRγδ cells were CD27⁺, Vγ1∥Vγ4⁺, or Vγ1∥Vγ4⁺ (Heilig and Tonegawa, 1986) (Figure S3D), similar to reports that identified IL-17A⁺TCRγδ cells as CD27⁺ (Ribot et al., 2009). The TCRβ⁺CD4⁺ cells are a heterogeneous population.
Figure 2. LEC-Mediated Antifungal Immunity Is IL-17A and GM-CSF Dependent
(A) WT mice were infected i.t. with yeast. IL-17A and GM-CSF were neutralized as noted in the STAR Methods. At 48 hr p.i., lung CFU was enumerated. Each panel represents a separate experiment (two pooled experiments depicted in left panel); each symbol denotes one mouse. Mann-Whitney test (48 hr p.i.).
(B) IKK2\textsuperscript{WT} and IKK2\textsuperscript{ALEC} mice were infected i.t. with yeast, and IL-17A content was analyzed by ELISA in BALF at 48 hr p.i. A representative of four experiments is depicted. Mann-Whitney test.
(C) IKK2\textsuperscript{WT} and IKK2\textsuperscript{ALEC} mice were infected i.t. with yeast. At 48 hr p.i., lung cell suspensions were analyzed by flow cytometry for intracellular IL-17A and GM-CSF in lymphocytes (CD90.2\textsuperscript{+} CD44\textsuperscript{+}). Two pooled experiments depicted. Mann-Whitney test.
(D) WT, IL-17A\textsuperscript{−/−} and GM-CSF\textsuperscript{−/−} were infected with 5 \times 10\textsuperscript{6} yeasts; at 48 hr p.i., lungs were analyzed by confocal imaging. Club cells depicted in green (CC10), nuclei in blue (DAPI) and GM-CSF in red.
(E) Procedure was done as in (A) with one further injection of antibody at 48 hr p.i. At 96 hr p.i., lung CFU were quantified. Data representative of three experiments. Kruskal-Wallis with Dunn\'s multiple comparison.

See also Figure S2.

CSF and club cells. In WT mice, GM-CSF production was detected in cells infiltrating the airway (likely leukocytes), but not in club cells (Figure 2D). In IL-17A\textsuperscript{−/−} mice, GM-CSF staining was absent, indicating that IL-17A regulates GM-CSF production in these infiltrating cells. The GM-CSF defect was not due to defective cell recruitment since the absolute numbers of lymphoid and myeloid cells were similar in IL-17A\textsuperscript{−/−} and WT mice (Figure S2). While in vivo neutralization of either IL-17A or GM-CSF impaired resistance to infection, neutralization of both together was not additive, suggesting that the two products act in the same pathway, e.g., GM-CSF is a target of IL-17 signaling (Figure 2E).

nTh17 Cells Are Indispensable for Innate Antifungal Immunity
We investigated the roles of IL-17A- and GM-CSF-producing innate lymphocytes in innate antifungal defense. Given the reduced proportion and number of IL-17A\textsuperscript{+} and GM-CSF\textsuperscript{+} TCR\textgamma\textsuperscript{+} cells upon ablation of NF-\kappaB in LECs, we first tested if TCR\textgamma\textsuperscript{+} cells require anti fungal immunity at 48 hr p.i. Surprisingly, the fungal burden was similar in TCR\textgamma\textsuperscript{−/−} versus WT mice (Figure 4A). On the other hand, the fungal load was significantly higher in TCR\textalpha\textsuperscript{−/−} versus WT mice, and similar to that in Rag\textgamma\textsuperscript{−/−} mice, which lack all lymphocyte populations (Figure 4A). Thus, TCR\textalpha\textsuperscript{+} lymphocytes are dispensable for innate antifungal defense, whereas TCR\textgamma\textsuperscript{+} cells participate but are

composed of MAIT cells and an as-yet undefined subset (Figure S3E). Intracellular cytokine staining in WT animals revealed that each of these lymphocyte subsets also produce GM-CSF (Figures 3C and 3D).

Importantly, further analysis of innate lymphocyte populations revealed that the number of cytokine-producing cells during infection was severely impaired in IKK2\textsuperscript{ALEC} versus IKK2\textsuperscript{WT} mice (Figures 3C and 3D), although the total number of innate lymphocytes in the lung at baseline was unaffected by ablation of NF-\kappaB in LECs (Figure S3F).

Specifically, nTh17 and TCR\textgamma\textsuperscript{+} cells exhibited decreased numbers of IL-17A\textsuperscript{+} cells, and TCR\textgamma\textsuperscript{+} CD4\textsuperscript{+} and TCR\textgamma\textsuperscript{+} cells showed decreased numbers of GM-CSF\textsuperscript{+} cells (Figure 3D). Thus, NF-\kappaB signaling in LECs regulates the numbers of IL-17A\textsuperscript{+} and GM-CSF-producing innate lymphocytes during fungal infection.

LEC Are Not a Source of GM-CSF
LEC can produce GM-CSF (Unkel et al., 2012). To determine if LECs are a source of GM-CSF during fungal infection, we co-stained infected lung tissue with antibodies against GM-CSF and club cells. In WT mice, GM-CSF production was detected in cells infiltrating the airway (likely leukocytes), but not in club cells (Figure 2D). In IL-17A\textsuperscript{−/−} mice, GM-CSF staining was absent, indicating that IL-17A regulates GM-CSF production in these infiltrating cells. The GM-CSF defect was not due to defective cell recruitment since the absolute numbers of lymphoid and

myeloid cells were similar in IL-17A\textsuperscript{−/−} and WT mice (Figure S2). While in vivo neutralization of either IL-17A or GM-CSF impaired resistance to infection, neutralization of both together was not additive, suggesting that the two products act in the same pathway, e.g., GM-CSF is a target of IL-17 signaling (Figure 2E).

nTh17 Cells Are Indispensable for Innate Antifungal Immunity
We investigated the roles of IL-17A- and GM-CSF-producing innate lymphocytes in innate antifungal defense. Given the reduced proportion and number of IL-17A\textsuperscript{+} and GM-CSF\textsuperscript{+} TCR\textgamma\textsuperscript{+} cells upon ablation of NF-\kappaB in LECs, we first tested if TCR\textgamma\textsuperscript{+} cells require anti fungal immunity at 48 hr p.i. Surprisingly, the fungal burden was similar in TCR\textgamma\textsuperscript{−/−} versus WT mice (Figure 4A). On the other hand, the fungal load was significantly higher in TCR\textalpha\textsuperscript{−/−} versus WT mice, and similar to that in Rag\textgamma\textsuperscript{−/−} mice, which lack all lymphocyte populations (Figure 4A). Thus, TCR\textalpha\textsuperscript{+} lymphocytes are dispensable for innate antifungal defense, whereas TCR\textgamma\textsuperscript{+} cells participate but are
dispensable. TCRαβ lymphocytes are comprised of two subsets: CD4+ and CD4-. After antibody treatment in vivo, fungal load rose significantly in CD4+ cell-depleted mice versus isotype control-treated mice and was similar to levels in TCRαβ−/− (Figure 4B). Thus, TCRβ−CD4+ cells are required for antifungal immunity.

Although LECs regulate innate antifungal immunity through the requisite action of nTh17 cells, we asked if the dispensability of TCRγδ cells in this model is due to compensation (Figure 4C). Indeed, the number of C-C chemokine receptor 6 (CCR6)+nTh17 cells increased greatly in TCRγδ−/− versus WT mice, whereas the number of CCR6+TCRγδ cells in TCRαβ−/− mice was similar to that in WT mice (Figure 4C). Thus, nTh17 cells appear to compensate for the loss of TCRγδ cells.

We explored the role of these innate lymphocytes in human patients. nTh17 cells cannot be reliably detected at the time patients present with illness, but TCRγδ cells could be analyzed. We considered that, whereas TCRγδ cells are dispensable for antifungal immunity in mice, this might not be the case in humans. In healthy individuals, 1%–5% of the circulating CD3+ cells are TCRγδ+ (Carding and Egan, 2002), and the majority of this subset is Vγ9Vδ2+. In psoriasis, Vγ9Vδ2+ cells infiltrate skin lesions, and the increase of these cells in the skin corresponds to a decreased proportion of circulating Vγ9Vδ2+ cells, which is partly restored after treatment (Laggener et al., 2011). Likewise, Vγ9Vδ2+ exit the circulation and enter the pleural fluid of tuberculosis patients (Balbi et al., 1993). We hypothesized that, in pulmonary fungal infection, Vγ9Vδ2+ cells leave the circulation and infiltrate the tissue in response to inflammatory signals. Thus, we analyzed the blood of patients with active or resolved blastomycosis. At the time of presenta-
myeloid cells in IKK\textsuperscript{\textalpha\textbeta\textgamma\textdelta} and control mice. Although the levels of CXCL1 were decreased in the lungs of IKK\textsuperscript{\textalpha\textbeta\textgamma\textdelta} relative to IKK\textsuperscript{\textalpha\textbeta\textgamma\textdelta} at 24 hr p.i. (Figure S4A), the numbers of alveolar MØs, DCs, monocytes, and neutrophils at 12, 24, and 48 hr p.i. were unaltered in the absence of NF-κB signaling in LECs (Figures S4B and S4C). To assess phagocyte function in IKK\textsuperscript{\textalpha\textbeta\textgamma\textdelta} mice, we tracked leukocyte-yeast interactions and killing in vivo using dsRed reporter yeast (Sterkel et al., 2016). Loss of dsRed fluorescence in Uvitex-stained yeast denotes loss of viability, e.g., Uvitex*dsRed* and Uvitex*dsRed* represent live and dead yeasts, respectively (Figure S5A). Similar to the elevated fungal load in IKK\textsuperscript{\textalpha\textbeta\textgamma\textdelta} mice at 48 hr p.i. (Figure 1H), the number of dsRed* (live) yeasts was significantly higher in IKK\textsuperscript{\textalpha\textbeta\textgamma\textdelta} versus IKK\textsuperscript{\textalpha\textbeta\textgamma\textdelta} mice (Figure S5B, left) and paralleled the decreased proportion of dead yeasts in IKK\textsuperscript{\textalpha\textbeta\textgamma\textdelta} versus IKK\textsuperscript{\textalpha\textbeta\textgamma\textdelta} mice (Figure S5B, right). The proportion of yeasts killed by alveolar MØs, DCs, and neutrophils was significantly reduced in IKK\textsuperscript{\textalpha\textbeta\textgamma\textdelta} versus IKK\textsuperscript{\textalpha\textbeta\textgamma\textdelta} mice (Figure S5C), indicating these findings support a model in which NF-κB activity in LECs regulates the number of IL-17A- and GM-CSF-producing innate lymphocytes, which in turn arm phagocytes to kill the yeast.

The IL-1α/IL-1R Signaling Pathway in LECs Is Essential for Antifungal Immunity

To uncover the LEC-intrinsic signaling pathways of antifungal immunity, we analyzed pattern recognition receptors and adaptor molecules that promote antifungal immunity. Here, CARD9<sup>−/−</sup> and MyD88<sup>−/−</sup> mice had significantly higher fungal burdens than WT mice at 48 hr p.i. (Figure S5A). We next analyzed receptors upstream of CARD9 and MyD88, Dectin-1<sup>−/−</sup>, Dectin-2<sup>−/−</sup>, Dectin-3<sup>−/−</sup>, Mince<sup>−/−</sup>, and TLR23479<sup>−/−</sup> mice had fungal loads similar to WT mice. However, the burden in IL-1R<sup>−/−</sup> mice was significantly higher than that in WT mice, and similar to MyD88<sup>−/−</sup> mice (Figure S5A). Moreover, fungal burden was inversely correlated with IL-17A level in lung
homogenate (Figure S5B). Thus, IL-1R signaling via MyD88 and CARD9 are each required for innate antifungal immunity.

We next analyzed the tissue-specific roles of adaptors and receptors implicated above. Bone marrow chimeric mice showed that IL-1R was required for antifungal immunity in nonhematopoietic cells. The role of CARD9, on the other hand, was restricted to the hematopoietic compartment (Figure 6A). Furthermore, using in vivo neutralization of the ligands for IL-1R, we found that IL-1x, but not IL-1β, is required for IL-1R signaling in LECs, as demonstrated by the significantly increased fungal load in IL-1x-neutralized mice (Figure 6B). We also observed that IL-1x is induced in the lung by 24 hr p.i. (Figure S6A), and that LECs themselves are a source of this cytokine (Figure S6B).

Our results imply that the IL-1x-IL-1R signaling axis may regulate expression of LEC products that promote increased innate lymphocyte numbers, thereby fostering antifungal immunity. If this hypothesis is correct, in the absence of IL-1R, innate lymphocytes should be curtailed. Indeed, we found that both the proportion and number of nTh17 cells are reduced in IL-1R−/− versus WT mice (Figures 6C and 6D), further supporting the requirement we observed for nTh17 cells in antifungal immunity (Figures 4A and 4B). IL-17A-producing TCRyδ cells were also impaired in IL-1R−/− mice (Figures 6C and 6D), in accordance with prior studies that demonstrated the requisite role of IL-1R for proper TCRyδ function (Duan et al., 2010).

To specifically target the IL-1R in LECs, we generated mice in which the IL-1R is deleted only in LECs (IL-1R1EC). Targeted deletion of IL-1R in LECs resulted in a sharp increase in lung fungal burden compared with littermate controls (IL-1R0/0) (Figure 6E). Collectively, our data establish the indispensable role for IL-1x-IL-1R signaling in LECs for innate antifungal immunity and suggest that such signaling ultimately regulates innate lymphocyte number and function.

**IL-1R Regulates CCL20 Production by LECs, and CCL20 Partners with Other Chemokines to Regulate nTh17 Numbers**

Given the requisite role of IL-1x-IL-1R signaling in LECs, and the importance of nTh17 cells in antifungal immunity, we investigated IL-1R-dependent, LEC-derived signals regulating nTh17 function during fungal infection. C-C motif chemokine ligand 20 (CCL20) is produced by epithelial cells (Reisman et al., 2003), and recruits IL-17-producing cells expressing CCR6 to inflammatory sites (Hirota et al., 2007). Moreover, CCL20 production is induced by IL-1 in LECs (Stamper et al., 2003). We
hypothesized that *B. dermatitidis* induces IL-1α-IL-1R-dependent production of CCL20 in LECs, which in turn regulates nTh17 numbers. We found that CCL20 is induced in infected lung tissue at 24 hr p.i. (Figure 7A). Likewise, the proportion and number of nTh17 (CCR6⁺IL-17A⁺) cells increased by 48 hr p.i. (Figure 7B). Lung CCL20 levels were also lower in the BAF of infected IKK2ΔLEC (or IL-1RΔIκB mice) versus IKK2ΔIκB mice, indicating that CCL20 production is contingent on LEC NF-κB activity and IL-1R signaling (Figure 7C).

Since cell sources other than LECs may account for CCL20 in the BAF, we investigated LECs as a source. We purified LECs (CD31⁺ CD45⁻ CD326⁺) (Figure S7) from infected IKK2ΔIκB, IKK2ΔLEC, and IL-1RΔIκB mice, and measured CCL20 in LECs. Consistent with our findings in BAF, CCL20 levels in IKK2ΔLEC and IL-1RΔIκB LECs were significantly decreased relative to IKK2ΔIκB (Figure 7D). *In vivo* administration of recombinant CCL20 to IKK2ΔLEC mice only partially restored the fungal burden to levels in IKK2ΔIκB mice (Figure 7E), suggesting that CCL20 may collaborate with other factors to recruit or expand the numbers of CCR6⁺nTh17 cells in the lung upon infection.

To test the role of CCR6 signaling, we blocked this G-protein-coupled receptor with pertussis toxin (PTx). The absolute number of nTh17 cells decreased sharply in mice treated with PTx, whereas the number of IL-17A⁺TCRγδ⁺ cells remained unchanged (Figure 7F), suggesting that chemokine receptor signaling regulates the numbers of nTh17 cells in the lung. Both PTx-treated and CCR6-deficient mice exhibited significantly higher lung fungal burden (Figures 7G and 7H), supporting a role for chemokine signaling, particularly the CCL20-CCR6 axis, in antifungal immunity.

We considered that differences in innate lymphocyte numbers may arise from proliferation in situ. We quantified the number of proliferating, IL-17-producing innate lymphocytes during infection. We found that the frequency and absolute number of CCR6⁺Ki67⁺ and CCR6⁺IL-17A⁺ nTh17 and TCRγδ⁺ cells increased in infected mice (Figures 7I and 7J). These results, together with the decreased numbers of nTh17 cells upon PTx treatment, suggest that, while TCRγδ⁺ proliferate in situ, nTh17 are both recruited to the lung and proliferate during fungal infection. Thus, IL-1 induces CCL20 in LECs, which synergizes with other soluble factors to augment nTh17 numbers and foster fungal killing.

**DISCUSSION**

We report a requisite role for NF-κB signaling in the lung epithelium in regulating innate immunity to an inhaled fungal pathogen. A primary and indispensable role for the lung epithelium has been neglected in favor of the general perception that hematopoietic cells, such as alveolar MØs, serve as the initial reservoir and sentinel of pulmonary fungal infection. For example, inhaled *A. fumigatus* spores are thought to initially enter resident alveolar
MØ where either they are recognized and killed or they germinate and proliferate in the setting of impaired immunity (Amananda et al., 2009). Likewise, H. capsulatum is generally thought to parasitize and multiply in alveolar MØ. Finally, we recently reported that entry of alveolar MØ by B. dermatitidis spores early in infection accelerates conversion to yeast and escape from host immunity (Sterkel et al., 2015). These perceptions notwithstanding, MyD88 signaling in epithelium is known to regulate early recruitment of neutrophils into the lung in murine aspergillosis (Jhingran et al., 2015), and NF-κB signaling in LECs promotes adaptive immunity to P. carinii infection (Perez-Nazario et al., 2013).

In our study, NF-κB signaling in LECs was required to contain early fungal growth, but the critical epithelial cell subset(s) remains to be determined. Although IKKε−/− mice have impaired NF-κB signaling throughout most of the lung epithelium (Figure S1), in DNTA mice the defect is restricted to club cells (Cheng et al., 2007), indicating that club cells may regulate innate antifungal responses. The lung has seven subsets of epithelial cells, conferring distinct functions. In mouse lung, club cells comprise much of the lining of the bronchi and bronchioles (Wasaki et al., 2016). Although our data pointing to a central role for club cells may be confounded by antibiotic (doxycycline) treatment effects on the microbiota, club cells have been implicated in host defense against Klebsiella pneumonia (Chen et al., 2016).

Our data show that NF-κB signaling in airway epithelial cells orchestrates innate antifungal immunity via innate lymphocytes, particularly nTh17 cells. Innate lymphocyte-derived IL-17 and GM-CSF enabled fungal killing by alveolar MØ, DCS, and neutrophils. Airway epithelial cell and innate lymphocyte function were connected by chemokines such as CCL20, which was induced in epithelial cells by IL-1. Thus our results uncovered an unappreciated network in which LECs foster antifungal immunity, not only by responding to IL-17, but also by directing the function of IL-17- and GM-CSF-producing innate lymphocytes, in part via CCL20.

The early infection-dependent translocation of NF-κB to the nucleus of LECs raises the question of whether yeasts induce such signaling directly through physical interactions with LECs, as in P. carinii infection (Millard et al., 1990), or indirectly via

Figure 7. IL-1R Signaling Regulates CCL20 Production by LECs and CCL20 Partners with Other Chemokines to Regulate nTh17 Numbers

(A and B) CCL20 levels in lung homogenate were quantified by ELISA at various times after infection with yeast. One-way ANOVA with Bonferroni correction was used. Dotted line is the upper limit of detection (9). Proportion (left) and number (right) of CCR6+IL-17A+ nTh17 cells in IL-17TRosa26RFP mice after infection, Mann-Whitney test was used.

(C) CCL20 levels in BAL of IKKε−/−, IKKε−/−, and IL-1R−/− quantified by ELISA. 48 hr p.i. Four pooled experiments are depicted. One-way ANOVA with Bonferroni correction was used, n.s., not significant.

(D) LECs (CD31+CD45−/CD31−) were purified from infected mice (48 hr p.i.) and levels of CCL20 in cell lysates quantified by ELISA. Two pooled experiments are depicted. One-way ANOVA was used.

(E) Yeasts were given i.t. alone or together with rCCL20. At 48 hr p.i., lung CFU was quantified by one-way ANOVA.

(F) IL-17TRosa26RFP mice were infected with yeast i.t. alone or together with PTX. At 48 hr p.i., lungs were harvested and the number of IL-17A+nTh17 and TCRγδ+ cells quantified by flow cytometry, Mann-Whitney test was used.

(G) Yeasts were given i.t. alone or together with PTX. At 48 hr p.i., lung CFU was quantified. Mann-Whitney test was used.

(H) Mice were infected with yeast i.t. and at 48 hr p.i., lung CFU was quantified. One-tailed, unpaired t test was used.

(I and J) WT mice were infected with yeast and, after 48 hr, the proportion (%) and number (×) of CCR6+IL-17A+ and IL-17A+K67−CCR6+ cells were quantified by flow cytometry. K67−IL-17A+ and K67+CCR6+ cells in the TCRγδ+CD4− gate (%) denote proliferating nTh17 cells; enumerated in Ref. Mann-Whitney test was used.

See also Figure S7.
pattern recognition of fungal molecules by hematopoietic cells. Our finding of NF-\(\kappa\)B signaling in lung epithelium as early as 1 hr p.i. may be consistent with initial interaction of yeast with LECs. However, it remains unclear how LECs would directly sense the yeast. We excluded C-type lectin receptors (CLR)s on LECs, since known CLRs signal via CARD9, which we showed in bone marrow-chimeric mice exerts its effect exclusively through hematopoietic cells. It is possible that LEC signaling via MyD88, through an as yet unidentified non-CR receptor, promotes initial recognition of yeast. A third possibility is that hematopoietic cells such as CD103+DCs in apposition with airway epithelium initially sense yeast and respond by producing IL-1 that rapidly triggers engagement of LECs via the IL-1R-MyD88 signaling axis, which is amplified by autocrine production of IL-1\(\alpha\) in a feedback loop.

We found that NF-\(\kappa\)B signaling in LECs is crucial in establishing adequate IL-17A and GM-CSF production, which in turn regulates phagocyte killing. Traditionally, IL-17 is functionally linked to the recruitment of neutrophils to peripheral inflammatory sites via induction of C-X-C chemokines at epithelial surfaces. This is evident in K. pneumoniae infection, where IL-17R deficiency in club cells results in decreased expression of CXCL5 and impaired bacterial clearance from the lung (Chen et al., 2016). However, in OPC, the number of neutrophils recruited to the tongue in mice with IL-17R-deficient oral epithelium (IL-17R\(\Delta\kappa\)13) was indistinguishable from that observed in littermate controls (Conti et al., 2016). The increase in the fungal burden of IL-17R\(\Delta\kappa\)13 mice was attributed to decreased production of \(\beta\)-defensin-3 by the oral epithelium (Conti et al., 2016). Our data showed that NF-\(\kappa\)B deficiency in LECs had no effect on the recruitment of neutrophils, but it did severely affect their function. These disparities in the mechanisms of IL-17-mediated immunity suggest that the effects of IL-17 signaling in epithelial cells depend on the microbe and anatomical location of infection.

We identified several sources of IL-17 and GM-CSF upon pulmonary fungal infection, including nTh17 and TCR\(\gamma\)\(\delta\) cells, which were previously documented as IL-17 sources in the gut (Conti et al., 2014). In addition, we identified MAIT cells as a source of IL-17A during fungal infection. Our experiments in TCR\(\kappa\)\(\alpha\)- and TCR\(\kappa\)\(\delta\)- mice showed that TCR\(\gamma\)\(\delta\) cells are dispensable, whereas TCR\(\kappa\)\(\beta\), specifically CD4+ cells, are indispensable for innate antifungal immunity. This result was surprising given the preferential location of TCR\(\gamma\)\(\delta\) cells at epithelial surfaces (Chen et al., 2014), and the fact that NF-\(\kappa\)B deficiency in LECs led to severely impaired TCR\(\gamma\)\(\delta\) cell responses. Our result may be explained by compensatory TCR\(\kappa\)\(\beta\) cell responses in TCR\(\kappa\)\(\alpha\)- mice. Intestinal TCR\(\kappa\)\(\beta\) cells increase in TCR\(\kappa\)\(\alpha\)- mice (Koman et al., 1995), and dendritic epithelial T cells, which are TCR\(\gamma\)\(\delta\)- in the skin of WT mice, are temporarily replaced by TCR\(\kappa\)\(\beta\) cells in TCR\(\kappa\)\(\delta\)- mice (Jameson et al., 2004). Furthermore, in a model of hypersensitivity pneumonitis, in which the main source of IL-17 is TCR\(\gamma\)\(\delta\) cells, CD4+ TCR\(\kappa\)\(\beta\) cells compensate and produce IL-17 in TCR\(\kappa\)\(\delta\)- mice (Simonian et al., 2009). We found that the number of CCR6+ nTh17 cells increased in TCR\(\kappa\)\(\delta\)- versus WT mice, whereas the number of CCR6+ TCR\(\gamma\)\(\delta\) cells was similar in TCR\(\kappa\)\(\alpha\)- and WT mice. Thus, TCR\(\kappa\)\(\beta\) cells may compensate for loss of TCR\(\gamma\)\(\delta\) cells to conceal their function in mice. However, in humans presenting with active blastomycosis, we found that the frequency of circulating V\(\gamma\)9\(\delta\)\(\delta\)2+ cells was decreased compared with healthy donors, suggesting that V\(\gamma\)9\(\delta\)\(\delta\)2+ cells leave the bloodstream to infiltrate inflamed tissue, as in psoriasis (Lagney et al., 2011). Remarkably, effective antifungal treatment restored the frequency of circulating V\(\gamma\)9\(\delta\)\(\delta\)2+ cells to levels found in healthy donors. TCR\(\gamma\)\(\delta\) cells infiltrate infected tissue in other granulomatous diseases such as tuberculosis (Balbi et al., 1993), but their role in such disorders remains ill defined.

The LEC cytokine and chemokine network that instructs type 17 responses is poorly understood. We show that CCL20, a product of epithelial cells at other anatomical locations (Reibman et al., 2003; Sierro et al., 2001), and a signal recruiting CCR6+ type 17 cells to sites of inflammation (Hirota et al., 2007), is made by LECs and, in conjunction with other factors, regulates the number of nTh17 cells upon fungal infection. CCL20 production by LECs required IL-1x/IL-1R signaling, itself indispensable in restraining infection. The chemokine response of alveolar epithelial cells infected with P. carinii in vitro depends on IL-1R signaling (Bello-Irizarry et al., 2012), and the first wave of neutrophils recruited following A. fumigatus infection also requires IL-1R signaling in LECs (Jingnan et al., 2015). The key role of the IL-1x/IL-1R axis in LEC-mediated antifungal immunity prompts the question: What IL-1R-dependent LEC products, in addition to CCL20, comprise the network of early signals that restrain fungal infection? Although the answer to this question remains to be investigated, our findings highlight the role of LECs as key orchestrators of antifungal immunity and potential targets of therapeutic approaches to enhance resistance against inhaled fungal pathogens, which represent a growing public health problem worldwide.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **CONTACT FOR REAGENT AND RESOURCE SHARING**
  - **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
    - Peripheral Blood Mononuclear Cells (PBMC)
    - Mice
    - Growth of Fungi
    - Experimental Model of Infection
- **METHOD DETAILS**
  - Flow Cytometry
  - In Vivo Antibody Neutralization and Depletion
  - In Vivo Administration of CCL20 and Pertussis Toxin (PTx)
  - IL-17A and CCL20 ELISA
  - In Vivo Killing Assay
  - Epithelial Cell Purification
  - Bone Marrow Chimeras
  - NF-\(\kappa\)B Immunoﬂuorescence
  - Immunofluorescence to Detect GM-CSF in the Lung
- **QUANTIFICATION AND STATISTICAL ANALYSIS**

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes seven figures and can be found with this online article at https://doi.org/10.1016/j.chom.2018.02.011.
ACKNOWLEDGMENTS

We thank the University of Wisconsin Carbone Cancer Center and Joe Hardin for preparation of immunofluorescent lung sections and Robert Gordon, Department of Pediatrics, for assistance with graphic illustrations. The work was supported by Burroughs Wellcome Fund grant 133-AAB41476 (to N.H.-S.), NIAID T32AI007635 (to A.J.M.), NHLBI T32HL007899 (to D.L.W.), American Heart Association 17POST32790004 (to J.S.F.), USPHS grant AI035681 (to B.S.K.), and the Carbone Cancer Center Grant P30 CA014520.

AUTHOR CONTRIBUTIONS

N.H.-S., D.L.W., J.S.F., A.J.M., T.W., and M.W. designed and performed experiments. N.H.-S. drafted the paper. B.S.K. helped design and supervise the study and write the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: July 24, 2017
Revised: December 23, 2017
Accepted: February 9, 2018
Published: March 22, 2018

REFERENCES


**STAR METHODS**

**KEY RESOURCES TABLE**

<table>
<thead>
<tr>
<th>REAGENT or RESOURCE</th>
<th>SOURCE</th>
<th>IDENTIFIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibodies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-mouse B220 (RA3-6B2)</td>
<td>BD Biosciences</td>
<td>Cat# 561086; RRID:AB_2034009</td>
</tr>
<tr>
<td>Anti-mouse CC10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-mouse CCR6 (29-2L17) or CCR6 (140706)</td>
<td>BioLegend or BD Biosciences</td>
<td>Cat# 129819; RRID:AB_2562513 Cat# 557976, RRID:AB_2228783</td>
</tr>
<tr>
<td>Anti-mouse CD103 (2E7)</td>
<td>BioLegend</td>
<td>Cat# 121426; RRID:AB_2563891</td>
</tr>
<tr>
<td>Anti-mouse CD11b (M1/70)</td>
<td>BD Biosciences or BioLegend</td>
<td>Cat# 564443 Cat# 101212; RRID:AB_3127895</td>
</tr>
<tr>
<td>Anti-mouse CD11c (N418)</td>
<td>BioLegend or eBiosciences</td>
<td>Cat# 117339; RRID:AB_2562414 Cat# 17-0114-82</td>
</tr>
<tr>
<td>Anti-mouse CD11c-biotin (N418) LEC enrichment</td>
<td>eBiosciences</td>
<td>Cat# 13-0114-82</td>
</tr>
<tr>
<td>Anti-mouse CD27 (LG.3A10)</td>
<td>BioLegend</td>
<td>Cat# 124215; RRID:AB_10645330</td>
</tr>
<tr>
<td>Anti-mouse CD29 (HMp1-1)</td>
<td>BioLegend</td>
<td>Cat# 102221</td>
</tr>
<tr>
<td>Anti-mouse CD31 (930)</td>
<td>BioLegend</td>
<td>Cat# 102420; RRID:AB_10613644</td>
</tr>
<tr>
<td>Anti-mouse CD31-biotin (930) LEC enrichment</td>
<td>BioLegend</td>
<td>Cat# 102404; RRID:AB_312899</td>
</tr>
<tr>
<td>Anti-mouse CD32e (G8.8) LEC enrichment</td>
<td>BioLegend or eBiosciences</td>
<td>Cat# 12-5791-83</td>
</tr>
<tr>
<td>Anti-mouse CD4 (GK1.5) in vivo neutralization</td>
<td>BioXcell</td>
<td>Cat# BE0003-1; RRID:AB_1107636</td>
</tr>
<tr>
<td>Anti-mouse CD4 (RMA4-5)</td>
<td>BD Biosciences</td>
<td>Cat# 564933 Cat# 553051; RRID:AB_398528</td>
</tr>
<tr>
<td>Anti-mouse CD44 (4/17)</td>
<td>BD Biosciences</td>
<td>Cat# 553133; RRID:AB_2076224</td>
</tr>
<tr>
<td>Anti-mouse CD45-biotin (80-F11) LEC enrichment</td>
<td>eBiosciences</td>
<td>Cat# 13-0451-85</td>
</tr>
<tr>
<td>Anti-mouse CD45.2 (104)</td>
<td>BD Biosciences</td>
<td>Cat# 560697; RRID:AB_1727495</td>
</tr>
<tr>
<td>Anti-mouse CD49a (Ha31/8)</td>
<td>BD Biosciences</td>
<td>Cat# 740375</td>
</tr>
<tr>
<td>Anti-mouse CD49d (R1-2)</td>
<td>BioLegend</td>
<td>Cat# 103607; RRID:AB_313038</td>
</tr>
<tr>
<td>Anti-mouse CD64 (K54-57.1)</td>
<td>BioLegend</td>
<td>Cat# 139304; RRID:AB_10612740</td>
</tr>
<tr>
<td>Anti-mouse CD8a (53.6-7)</td>
<td>BioLegend</td>
<td>Cat# 100712</td>
</tr>
<tr>
<td>Anti-mouse CD90.2 (30-H12) or CD90.2 (53.2)</td>
<td>BioLegend or BD Biosciences</td>
<td>Cat# 105316; RRID:AB_4928861; Cat# 565257</td>
</tr>
<tr>
<td>Anti-mouse Donkey anti-chicken</td>
<td>Jackson Immuno Research</td>
<td>Cat# 703-545-155; RRID:AB_2340375</td>
</tr>
<tr>
<td>Anti-mouse F4/80-biotin (BM8) LEC enrichment</td>
<td>eBiosciences</td>
<td>Cat# 13-4801-85</td>
</tr>
<tr>
<td>Anti-mouse GFP</td>
<td>Abcam</td>
<td>Cat# ab 13970; RRID:AB_300798</td>
</tr>
<tr>
<td>Anti-mouse GM-CSF (MP1-22E9)</td>
<td>BioLegend</td>
<td>Cat# 505404; RRID:AB_315380</td>
</tr>
<tr>
<td>Anti-mouse GM-CSF in vivo neutralization</td>
<td>Sterkell et al., 2016</td>
<td>N/A</td>
</tr>
<tr>
<td>Anti-mouse Goat anti-rat</td>
<td>Invitrogen</td>
<td>Cat# A21434; RRID:AB_141733</td>
</tr>
<tr>
<td>Anti-mouse IL-17A (17F3) in vivo neutralization</td>
<td>BioXcell</td>
<td>Cat# BE0173; RRID:AB_10950102</td>
</tr>
<tr>
<td>Anti-mouse IL-17A (TC11-18H10)</td>
<td>BD Biosciences</td>
<td>Cat# 559502; RRID:AB_397256</td>
</tr>
<tr>
<td>Anti-mouse IL-1x (ALF 161)</td>
<td>BioXcell</td>
<td>Cat# BE0243; RRID:AB_2687724</td>
</tr>
<tr>
<td>Anti-mouse IL-1r (B122)</td>
<td>BioXcell</td>
<td>Cat# BE0246; RRID:AB_2687727</td>
</tr>
<tr>
<td>Anti-mouse Ly6C (HIK1.4)</td>
<td>BioLegend</td>
<td>Cat# 128014</td>
</tr>
<tr>
<td>Anti-mouse Ly6G (1A8)</td>
<td>BD Biosciences</td>
<td>Cat# 563978; RRID:AB_2716852</td>
</tr>
<tr>
<td>Anti-mouse MHC Class II (M5/114.15.2)</td>
<td>BioLegend</td>
<td>Cat# 127604</td>
</tr>
<tr>
<td>Anti-mouse MR1 tetramer (5-OP)</td>
<td>Rahimpour et al., 2015; NIH Tetramer Facility</td>
<td>N/A</td>
</tr>
<tr>
<td>Anti-mouse MR1 tetramer (6-FP) - control</td>
<td>Rahimpour et al., 2015; NIH Tetramer Facility</td>
<td>N/A</td>
</tr>
<tr>
<td>Anti-mouse NK1.1 (PK136)</td>
<td>BioLegend</td>
<td>Cat# 108710</td>
</tr>
<tr>
<td>Anti-mouse p65 (D14E12)</td>
<td>Cell Signaling</td>
<td>Cat# 8242; RRID:AB_10859369</td>
</tr>
<tr>
<td>Anti-mouse Siglec F (E50-2440)</td>
<td>BD Biosciences</td>
<td>Cat# 565526</td>
</tr>
</tbody>
</table>

(Continued on next page)
**Continued**

<table>
<thead>
<tr>
<th>REAGENT or RESOURCE</th>
<th>SOURCE</th>
<th>IDENTIFIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-mouse TCRβ (H57-597)</td>
<td>BioLegend or BD Biosciences</td>
<td>Cat# 109222 Cat# 560706</td>
</tr>
<tr>
<td>Anti-mouse TCRγδ (GL3)</td>
<td>BioLegend or BD Biosciences</td>
<td>Cat# 118120 Cat# 563993</td>
</tr>
<tr>
<td>Anti-mouse Vα1 (2.11)</td>
<td>BioLegend</td>
<td>Cat# 141105</td>
</tr>
<tr>
<td>Anti-mouse Vγ4 (UC3-10A6)</td>
<td>BioLegend</td>
<td>Cat# 137707</td>
</tr>
<tr>
<td>Anti-human CD3 (SK7)</td>
<td>BD Biosciences</td>
<td>Cat# 565466</td>
</tr>
<tr>
<td>Anti-human Ki67 (B56)</td>
<td>BD Biosciences</td>
<td>Cat# 565929</td>
</tr>
<tr>
<td>Anti-human TCRγδ (B1)</td>
<td>BD Biosciences</td>
<td>Cat# 564156</td>
</tr>
<tr>
<td>Anti-human Vα9 (B3)</td>
<td>BioLegend</td>
<td>Cat# 331305</td>
</tr>
<tr>
<td>Anti-human Vα2 (B6)</td>
<td>BioLegend</td>
<td>Cat# 331407</td>
</tr>
</tbody>
</table>

**Bacterial Samples**

| **Blastomyces dermatitidis** | ATCC | 26199 |
| **Blastomyces dermatitidis (spore-forming)** | Clinical isolate Wisconsin State Laboratory of Hygiene | 14081 |
| **Blastomyces dermatitidis DsRed** | Sterkel et al., 2016 | N/A |
| **Peripheral Blood Mononuclear Cells (PBMC)** | Infected patients (University of Wisconsin Hospital and Clinic) and healthy controls | N/A |

**Chemicals, Peptides, and Recombinant Proteins**

<table>
<thead>
<tr>
<th>REAGENT or RESOURCE</th>
<th>SOURCE</th>
<th>IDENTIFIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinant IL-17</td>
<td>BioLegend</td>
<td>Cat# 576004</td>
</tr>
<tr>
<td>Recombinant GM-CSF</td>
<td>PeproTech</td>
<td>Cat# 31503</td>
</tr>
<tr>
<td>Recombinant CCL20</td>
<td>BioLegend</td>
<td>Cat# 582302</td>
</tr>
<tr>
<td>Pertussis Toxin (PTx)</td>
<td>Calbiochem</td>
<td>Cat# 516560</td>
</tr>
</tbody>
</table>

**Critical Commercial Assays**

<table>
<thead>
<tr>
<th>REAGENT or RESOURCE</th>
<th>SOURCE</th>
<th>IDENTIFIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-17 ELISA</td>
<td>R&amp;D Systems</td>
<td>Cat# DY421</td>
</tr>
<tr>
<td>CCL20 ELISA</td>
<td>R&amp;D Systems</td>
<td>Cat# MCC200</td>
</tr>
</tbody>
</table>

**Experimental Models: Organisms/Strains**

<table>
<thead>
<tr>
<th>REAGENT or RESOURCE</th>
<th>SOURCE</th>
<th>IDENTIFIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-κB-eGFP</td>
<td>Dr. Christian Jobin; Magness et al., 2004</td>
<td>N/A</td>
</tr>
<tr>
<td>IL-17/CreRosa26RtTA</td>
<td>Hirota et al., 2011</td>
<td>N/A</td>
</tr>
<tr>
<td>Decin-1/Cre</td>
<td>Dr. Gordon Brown; Taylor et al., 2007</td>
<td>N/A</td>
</tr>
<tr>
<td>Decin-2/Cre</td>
<td>Dr. Yoichiro Iwakura; Saio et al., 2010</td>
<td>N/A</td>
</tr>
<tr>
<td>Decin-3/Cre</td>
<td>Consortium for Functional Glycomics and Mutant Mouse Resource and Research Centers (MMRCC)</td>
<td>Cat# 031935-UCD</td>
</tr>
<tr>
<td>CARD9−/−</td>
<td>Dr. Xin Lin; Hsu et al., 2007</td>
<td>N/A</td>
</tr>
<tr>
<td>Mincle−/−</td>
<td>Consortium for Functional Glycomics and Mutant Mouse Resource and Research Centers (MMRCC)</td>
<td>Cat# 031936-UCD</td>
</tr>
<tr>
<td>TLR 23479−/−</td>
<td>Dr. Carsten Kirschning; Conrad et al., 2009</td>
<td>N/A</td>
</tr>
<tr>
<td>DNMTA and rtTA</td>
<td>Dr. Timothy S. Blackwell; Cheng et al., 2007</td>
<td>N/A</td>
</tr>
<tr>
<td>IL-1R−/−</td>
<td>The Jackson Laboratory</td>
<td>Cat# 003245</td>
</tr>
<tr>
<td>IL-1RαX-RE/RE (IL-1Rα−/−)</td>
<td>The Jackson Laboratory</td>
<td>Cat# 002839</td>
</tr>
<tr>
<td>MyD88−/−</td>
<td>The Jackson Laboratory</td>
<td>Cat# 009088</td>
</tr>
<tr>
<td>TCRβ−/−</td>
<td>The Jackson Laboratory</td>
<td>Cat# 002120</td>
</tr>
<tr>
<td>RAG−/−γc−/−</td>
<td>Taconic</td>
<td>Cat# 4111</td>
</tr>
<tr>
<td>IKKα/β/γ</td>
<td>Dr. Terry Wright; Bello-irizarry et al., 2012</td>
<td>N/A</td>
</tr>
</tbody>
</table>

(Continued on next page)
## CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Bruce S. Klein (bsklein@wisc.edu).

## EXPERIMENTAL MODEL AND SUBJECT DETAILS

### Peripheral Blood Mononuclear Cells (PBMC)

Peripheral blood from healthy donors and blastomycesis patients was overlaid on Ficoll and centrifuged at 400 rcf for 20 min at RT without acceleration and without break. The PBMC layer was subsequently collected and the residual ficoll washed. Approximately 1-2 million PBMC were stained and analyzed by flow cytometry. Work with human donors was performed with informed consent from the participants and approved by the appropriate institutional IRB at University of Wisconsin-Madison.

### Mice

All strains were in the C57BL/6 background, except Dectin 3−/− which are a combination of multiple genetic backgrounds. Experiments conducted with Dectin 3−/− mice included the appropriate isogenic controls. Mice were bred in-house under specific pathogen free (SPF) conditions. WT animals were purchased as needed from the NCI repository, NFXB-eGFP (Magness et al., 2004), IL-17R−/−Rosa26RiPP (Hirot a et al., 2011), Dectin-1−/− (Taylor et al., 2007), Dectin-2−/− (Saito et al., 2010), Dectin-3−/− (Wang et al., 2015), Mincle−/− (Wells et al., 2008), CARD8−/− (Hsu et al., 2007), TLR 23479−/− (Conrad et al., 2009), IL-17A−/− (Nakae et al., 2002), DNTA and rTα mice (Cheng et al., 2007) (the latter two strains were a kind gift from Drs. Tim Blackwell and Fiona Yull at Vanderbilt University, TN) have been previously described. IL-1R−/−, IL-1RI−/−, MyD88−/−, TCRδ−/− and RAG−/−−−−− were purchased from the Jackson Laboratories. SPC-CRE mice were obtained by backcrossing IKK2ΔLec mice with WT to eliminate the IKK2δ allele. IKK2ΔLec mice were a kind gift from Dr. Terry Wright at the University of Rochester Medical Center (Bello-irizary et al., 2012). In these animals, the IKK2 component of the NF-κB signaling pathway is floxed (fl) by loxP sites, and cre recombinase expression is driven by the lung-specific surfactant protein C, resulting in deletion of IKK2 exclusively in airway epithelial cells. In all experiments, the CRE-IKK2fl mice were used as controls. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC).

### Growth of Fungi

*B. dermatitidis* strain ATCC 26199 was grown in 7H10 media slants, in a humidified incubator at 37°C - 39°C, at which temperature the organism maintains its yeast morphology. In order to generate spores, *B. dermatitidis* strain 14081 was grown to log phase at 37°C and plated onto potato dextrose agar (PDA) to promote spore formation. Plates were incubated at 25°C for 2 weeks in BSL3 conditions and spores were subsequently harvested in PBS. For enumeration of CFU, lungs were plated onto Brain Heart Infusion (BHI) (strain 26199) or Histoplasma Macrophage Medium (HMM) (strain 14081) agar and incubated for 7 days at 37°C.

### Experimental Model of Infection

Seven to twelve week old mice were anesthetized with isoflurane, intubated using the BioLTE system (Brantree Scientific, Inc.), and inoculated i.t. with 2 x 10⁶ *B. dermatitidis* yeasts or 1 x 10⁶ spores, unless stated otherwise. At various timepoints post-inoculation (indicated in each Fig.), the bronchoalveolar lavage fluid (BALF) and the lungs were collected and subsequently processed for colony forming unit (CFU) analysis, flow cytometry, or enzyme-linked immunoabsorbent assay (ELISA).

### METHOD DETAILS

#### Flow Cytometry

The lungs were perfused by injecting 10ml of PBS into the heart’s left ventricle, mechanically disrupted with a syringe plunger, and incubated in 2 mg/ml Collagenase D and 10 μg/ml DNase at 37°C for 20 minutes. Collagenase was inhibited with 50mM...
EDTA in PBS. The resulting single-cell suspension was filtered through a 40 μm nylon mesh and the remaining red blood cells were lysed in ACK lysis buffer. In some experiments circulating leukocytes were excluded from analysis by injecting 2 μg/mouse i.v. of fluorescently labeled anti-CD45 and allowing it to circulate for 3 minutes prior to harvesting the lungs. Cell suspensions were incubated with 10 μg/ml of Brefeldin A for 4 hours at 37 °C and surface and intracellular antigens were subsequently stained with fluorochrome-conjugated antibodies. Dead cells were excluded from data analysis with Live/Dead fixable dead cell stains (Molecular Probes, Life Technologies). Non-specific binding of antibodies was blocked with anti CD16/32 (Fc Block) and surface antigens were stained on ice for 20-60 minutes. Cells were then permeabilized with BD Cytofix/CytoPerm at RT for 20 minutes or at 4°C overnight and intracellular cytokines were stained on ice for 20-60 minutes. For Ki67 staining the cells were fixed and permeabilized with Foxp3 transcription factor staining buffer set (eBiosciences). Fluorochrome-conjugated antibodies were purchased from BioLegend, BD Biosciences or e-Biosciences and are listed in the Key Resources Table. Mouse MR1 tetramer (5-OP), and the corresponding control (6-OP), for detection of MAIT cells was developed by Dr. Dale I. Godfrey (Rahimpour et al., 2015) and purchased from the NIH tetramer facility. Data were acquired in five-laser BD Fortessa or BD LSRII and analyzed with FlowJo Software v10.

In Vivo Antibody Neutralization and Depletion
Two hundred and fifty micrograms of anti-IL-1x (BioXcell ALF 161), anti-IL-1β (BioXcell B122), anti-IL-17A (BioXcell 17F3), anti-GM-CSF (BioXcell MP1-22E9) and anti-CD4 (BioXcell GK1.5) were injected i.v retroorbitally at the time of inoculation with B. dermatitidis.

In Vivo Administration of CCL20 and Pertussis Toxin (PTx)
100 ng of CCL20 (BioLegend) and 400 ng of PTx were given i.t at the time of infection together with the inoculum (2 x 10^5 yeasts) in 20μl.

IL-17A and CCL20 ELISA
Mice were bled retroorbitally and lungs were perfused with 10ml of PBS. BALF was collected by instilling 1ml of ice-cold PBS containing protease inhibitors (Complete Mini EDTA-free protease inhibitor tablets, Roche) via the trachea and aspirating it back with a syringe. BALF was obtained by centrifuging at maximum speed at 4°C to pellet the cells. The supernatant was collected and stored at -70°C. The lungs were collected in PBS containing protease inhibitors and homogenized. 1% Triton-X was added to the homogenate and debris was eliminated by centrifuging at maximum speed at 4°C and the supernatant was stored at -70°C. IL-17A and CCL20 content in BALF and lung homogenate was analyzed by ELISA according to the manufacturer’s specifications (R&D Systems).

In Vivo Killing Assay
Mice were inoculated with DsRed yeast stained with 20 μg/ml Uvitex and at 48 h.p.i. the lungs were harvested and processed for analysis by flow cytometry as described (Sterkel et al., 2018). When cytokine neutralization was required, antibodies were given i.v. at the time of inoculation.

Epithelial Cell Purification
The protocol for purification of airway epithelial cells was adapted from Messier, E.M. et al. (Messier et al., 2012). Mice were bled retroorbitally and lungs were perfused with 10ml of PBS. The lungs were then instilled with 2-3ml of undiluted 37°C Dispase via the trachea. The backflow of enzyme was prevented by injecting 0.5ml of 1% low-melt agarose subsequently solidified by icing the trachea. The lungs were then incubated in 2ml of Dispase at 37°C for 6 minutes and homogenized in pre-warmed (37°C) complete High glucose DMEM containing DNase (10 μg/ml) using a Gentle MACS dissociator (Miltenyi Biotec). The resulting single-cell suspensions were filtered sequentially through 70μm and 40μm nylon meshes. Non-specific binding of antibodies was blocked with anti-CD16/32, leukocytes (anti-CD45, anti-CD11c, anti-Ly6G, anti-F4/80) and endothelial cells (anti-CD31) were labeled with biotinylated antibodies and subsequently depleted by positive selection with Streptavidin Microbeads (Miltenyi Biotec) in LS columns (Miltenyi Biotec). The resulting leukocyte- and endothelial cell-depleted fraction was labeled with anti-CD326 PE and epithelial cells were positively selected with anti-PE magnetic Microbeads and LS Columns. The purity of the epithelial cell fraction was verified by flow cytometry (Figure S7). Purified epithelial cells were lysed in NP40 lysis buffer (1% NP40, 50mM NaCl, 50mM Tris, 5mM EDTA) and lysates were analyzed for cytokine content by ELISA.

Bone Marrow Chimeras
Recipient mice received lethal irradiation in two sequential doses of 550Gy each (1,100Gy total), with a 3-hour resting period in between doses. Twenty-four hours post-irradiation, mice received 1 x 10^6 donor bone marrow cells i.v. Eight weeks after transplant, chimeric animals were infected with B. dermatitidis yeasts and 48-hours later the lungs were plated onto BHI agar for quantification of fungal burden.

NFκB Immunofluorescence
Mice were infected with 5 x 10^6 B. dermatitidis yeasts. In some experiments, yeast were labeled with 100 μg/ml calcofluor white. Lung tissue was fixed in 10% buffered formalin for 48 hours, embedded in paraffin and sectioned. In order to assess
nuclear localization of NF-κB by immunofluorescence, sections were deparaffinized and antigen retrieval was performed in citrate buffer pH 6.0 (10mM citric acid, 0.05% tween 20) for 3 minutes in a Biocare decloaker (Biocare Medical, Concord, CA). After cooling and serum blocking, sections were incubated with 1:800 rabbit anti-p65 antibody (Cell Signaling, 8242S) in PBS with 1% goat serum overnight at 4C. After washing, sections were subsequently treated with 1:500 Alexa Fluor 488 goat anti-rabbit immunoglobulin (Invitrogen) for half an hour at room temperature, at which time point sections were washed and counterstained with Prolong Gold antifade with DAPI (Invitrogen). All images displayed were taken at a total magnification of 400X, with identical microscope settings and image processing for all pictures. Individual airways from lung sections from each time point were examined, and the percent of lung epithelial cells with nuclear-localized NF-κB was determined. When cryosections from NF-κB-GFP reporter mice were used, the tissue was processed as described in the section Immunofluorescence to detect GM-CSF in the lung. Samples were stained with primary antibodies to GFP (1:1000) and CD326 (1:100) followed by secondary antibodies donkey anti-chicken Alexa Fluor 488 and goat anti-rat Alexa Fluor 555.

**Immunofluorescence to Detect GM-CSF in the Lung**

WT and GM-CSF−/− were infected with 5 x 10⁶ B. dermatitidis yeasts and the lungs were processed for tissue sectioning 48 h.p.i. Six hours prior to harvest, mice were injected with 500μg i.p. and 42μg i.n. of Brefeldin A in order to retain cytokines in the cytoplasm. The lungs were fixed in 10% buffered formalin for 1 hour and subsequently cryoprotected by infusion with 30% sucrose for approximately 3 days. The left lobes were embedded in OCT and sectioned. Non-specific binding of antibodies was subsequently blocked for 1 hr at room temperature with vector lab animal-free blocking solution with 1% Tween. Samples were stained with 1:20,000 anti-CC10 (Seven Hills) and 1:100 anti-GM-CSF diluted in blocking solution overnight at 4°C. Antigens were visualized upon secondary staining with 1:1000 anti-rat Alexa Fluor 564 (Invitrogen) and 1:1000 anti-rabbit Alexa Fluor 488 (Jackson Immune Res) for 2 hours at room temperature in blocking solution. Confocal images were collected on Nikon A1R+ 20X optical with 2X digital zoom.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical analysis was performed using GraphPad Prism and is specified in each figure legend.