Lipoxin Signaling in Murine Lung Host Responses to *Cryptococcus neoformans* Infection

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Abstract

Lipoxins (LX) are proresolving mediators that augment host defense against bacterial infection. Here, we investigated roles for LX in lung clearance of the fungal pathogen *Cryptococcus neoformans* (*Cne*). After intranasal inoculation of 5,000 CFU *Cne*, C57BL/6 and C.B-17 mice exhibited strain-dependent differences in *Cne* clearance, immunologic responses, and lipoxin A₄ (LXA₄) formation and receptor (ALX/FPR2) expression. Compared with C.B-17 mice, C57BL/6 lungs had increased and persistent *Cne* infection 14 days after inoculation, increased eosinophils, and distinct profiles of inflammatory cytokines. Relative to C.B-17 mice, bronchoalveolar lavage fluid levels of LXA₄ were increased before and after infection in C57BL/6. The kinetics for 15-epi-LXA₄ production were similar in both strains. Lung basal expression of the LX biosynthetic enzyme *Alox12/15* (12/15-lipoxygenase) was increased in C57BL/6 mice and further increased after *Cne* infection. In contrast, lung basal expression of the LXA₄ receptor *Alx/Fpr2* was higher in C.B-17 relative to C57BL/6 mice, and after *Cne* infection, *Alx/Fpr2* expression was significantly increased in only C.B-17 mice. Heat-killed *Cne* initiated lung cell generation of IFN- γ and IL-17 and was further increased in C.B-17 mice by 15-epi-LXA₄. A trend toward reduced *Cne* clearance and IFN- γ production was observed upon *in vivo* administration of an

ALX/FPR2 antagonist. Together, these findings provide the first evidence that alterations in cellular immunity against *Cne* are associated with differences in LXA₄ production and receptor expression, suggesting an important role for ALX/FPR2 signaling in the regulation of pathogen-mediated inflammation and antifungal lung host defense.

Keywords: lipoxin A₄; ALX/FPR2; *Cryptococcus neoformans*; pneumonia; resolution

Clinical Relevance

Most current antiinflammatory medicines are also immunosuppressive, so they can increase the risk for opportunistic infections, including fungal pneumonia. Lipoxin signaling via ALX/FPR2 augmented host defense mechanisms to *Cryptococcus neoformans* and contributed to mouse strain-dependent clearance of fungal pneumonia. Harnessing natural proresolving mediator signaling pathways, such as lipoxin-ALX/FPR2, may offer a novel therapeutic approach to inflammation that is host protective rather than immunosuppressive.

Acute respiratory tract infections remain a significant clinical problem worldwide, and genetic differences are known to influence host susceptibility and ability to

control pathogens (1). *Cryptococcus neoformans* (*Cne*) is an opportunistic fungal pathogen with the potential to cause severe respiratory disease (reviewed in

Ref. 2). Inhalation of *Cne* spores by immunocompetent hosts generally leads to asymptomatic infection; however, in susceptible populations, exposure can lead

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Table 1. Primers Used for Quantitative Polymerase Chain Reaction Analysis

Gene ID	TaqMan/Evagreen	Sequences or ID	Source
<i>Alox5</i>	Evagreen	ACTACATCTACCTCAGCCTCATT; GGTGACATCGTAGGAGTCCAC	MGH Primer Bank
<i>Alox12/15</i>	TaqMan	Mm00507789_m1	Applied Biosystems
<i>Alx/Fpr2</i>	Evagreen	CCGTCCTTTACGAGTCCTTACA; CAGGAGGTGAAGTAGAACTGGT	MGH Primer Bank
<i>Cdkn1b</i>	TaqMan	Mm00438168_m1	Applied Biosystems
<i>Ppia</i>	Evagreen	CAGTGCTCAGAGCTCGAA; GTGTTCTTCGACATCACGGG	MGH Primer Bank

Definition of abbreviation: MGH, Massachusetts General Hospital.

to *Cne* pneumonia, which can require extended periods of antifungal therapy (>6 mo) and, if left untreated, can disseminate to the nervous system, leading to significant morbidity and mortality (2, 3).

Differences in host responses to *Cne* have been documented in laboratory mouse and rat models (4–6). In a well-established mouse model of *Cne* pneumonia, C.B-17 mice are able to reduce their fungal burden by mounting a T helper cell type 1 (Th1) immune response, whereas a Type 2 (Th2) response predominates in C57BL/6 mice that are ineffective at limiting *Cne* infection (4). This has proven to be a useful experimental system to explore pulmonary host defense immunologic mechanisms.

Specialized proresolving mediators (SPMs) are generated in inflammatory exudates from essential fatty acids and serve as agonists at specific receptors to mediate cell type-specific actions (for review, see Ref. 7). SPM antiinflammatory actions include limiting granulocyte recruitment and activation, and SPM proresolving actions include enhancement of efferocytosis and bacterial killing by phagocytes (7). Together, SPM cellular responses augment host defense against bacterial pathogens and limit immunopathology associated with overly exuberant host responses (7), including in the lung (8).

Lipoxins are the lead family of SPMs. Lipoxin A₄ (LXA₄) is enzymatically derived from arachidonic acid via the actions of two lipoxygenases, often located in distinct cell populations. LXA₄ generation occurs during cell–cell interactions (i.e., transcellular biosynthesis) (9). There also exists a natural isomer of LXA₄, termed 15-epimer-LXA₄ (15-epi-LXA₄), that was first described as an aspirin-triggered epimer of LXA₄ generated by the collaborative actions of 5-lipoxygenase and

aspirin-acetylated COX-2 (10), but 15-epi-LXA₄ can also be formed by cytochrome P450 enzymes in an aspirin-independent mechanism (11). LXA₄ and 15-epi-LXA₄ are high-affinity ligands ($K_d \approx 0.7$ nM) for the G-protein–coupled receptor ALX/FPR2, through which they mediate many of their actions (7). ALX/FPR2 is highly conserved among humans, mice, and rats, with especially high degrees of similarity in key ligand binding residues (7). Expression of ALX/FPR2 has been documented in a variety of cell types, including leukocytes, epithelial cells, and

stromal cells, highlighting the broad impact of LX signaling (7).

In the present study, we show that distinctions in LXA₄ biosynthesis and ALX/FPR2 expression are associated with strain-dependent differences in the clearance of *Cne* pneumonia and pathogen-mediated inflammation, emphasizing homeostatic roles for resolution-signaling circuits in the successful host defense against this pathogen. Some of the results of these studies have been previously reported in the form of abstracts (12, 13).

Figure 1. *Cryptococcus neoformans* (*Cne*) lung infection was differentially cleared by two mouse strains. C.B-17 and C57BL/6 mice were inoculated intranasally with 5,000 CFU *Cne*. *Cne* infection was determined by CFU in tissue homogenates 7, 14, 18, 21, and 28 days after inoculation. Values represent the mean for $n \geq 5$ per time point. # $P \leq 0.01$ by two-way ANOVA for C.B-17 compared with C57BL/6. Data represent results of four experiments ($n \geq 4$ mice per group).

Figure 2. Bronchoalveolar lavage fluid (BALF) lipoxin A₄ (LXA₄) levels and strain-dependent kinetics after *Cne* infection. (A and B) Levels of LXA₄ (A) and 15-epi-LXA₄ (B) were measured by ELISA in BALF collected on Days 0, 7, 14, 18, and 21 after *Cne* infection. Values represent mean $\pm$ SEM for pg/mg protein. * $P \leq 0.05$, one-way ANOVA with Tukey's post test for differences compared with Day 7 ($n \geq 5$); # $P \leq 0.0002$, C57BL/6 versus C.B-17 as analyzed by Student's *t* test ($n \geq 5$ per group). Data reflect the results of four experiments. (C and D) The expression of the LX biosynthetic genes *Alox5* and *Alox12/15* and the LXA₄ receptor *Alx/Fpr2* was determined by quantitative PCR in lung tissue collected from C.B-17 (open bars) and C57BL/6 (solid bars) mice at Days 0 and 14. Values in C represent mean fold difference in naive C57BL/6 lungs relative to naive C.B-17. Values in D depict changes in expression levels in both strains at Day 14 relative to baseline levels. Student's *t* tests were performed on ΔC_t values. * $P \leq 0.002$ for comparisons between Days 0 and 14. # $P \leq 0.002$ for comparisons between strains ($n \geq 4$ per group) (Tables E1 and E2). ALX/FPR2 protein expression differed between strains at baseline. (E and F) Representative immunohistochemistry for ALX/FPR2 in naive C.B-17 (E) and C57BL/6 (F) mice. Panels on the left show incubation with primary antibody; panels on the right show incubation without primary antibody as negative controls. Arrows denote positively stained cells. Shown are representative images from $n = 3$ mice per group.

Materials and Methods

Detailed descriptions of the materials and methods are provided in the online supplement.

Mice

C.B-17 mice were bred at Lovelace Respiratory Research Institute, Albuquerque, NM. C57BL/6 mice were purchased from NCI (Frederick, MD). All studies conformed to National Institutes of Health guidelines and were approved by the Lovelace Respiratory Research Institute's Institutional Animal Care and Use Committee.

Organism and Inoculation

Cne strain 52D was prepared as previously described (14). *Cne* inoculum (50 μ l, 5,000 organisms) was deposited on the nares of each mouse. Inhalation of the inoculum was observed before mice were returned to their cages.

Harvest and Processing of Tissue

Mice were killed using approved protocols. After lavage with PBS containing 0.6 mM EDTA, lungs were perfused with PBS and transferred to ice-cold HBSS (Mediatech, Manassas, VA). Single-cell suspensions of liberase/DNase-digested lungs were prepared as described (14).

Quantitation of *Cne* in Lung

To quantify *Cne*, serial dilutions of lung homogenates were incubated on Sabaroud-Dextrose agar for 48 to 72 hours.

Leukocyte Differentials

Total cell enumeration and differential counts were performed using standard protocols.

Flow Cytometry

Immunophenotyping of enzyme-digested lung cells was performed using a panel of fluorescent antibodies. Cells were analyzed on a Becton Dickinson FACSCalibur or FACSCanto using FlowJo software (Tree Star Inc., Ashland, OR).

Cytokine Bead Array

Aliquots of bronchoalveolar lavage fluid (BALF) were submitted to Aushon for multiplex cytokine immunoassay (SearchLight Chemiluminescent Protein Array; Aushon, Billerica, MA).

Measurement of Lipoxins

BALF levels of LXA₄ and 15-epi-LXA₄ were quantitated by ELISA as described previously (Neogen, Lansing, MI) (15).

RNA Isolation and Quantitative PCR

mRNA was isolated using TRIzol, and cDNA was generated from DNase-treated mRNA using a reverse transcription kit (Life Technologies, Grand Island, NY). Quantitative PCR was performed using EvaGreen master mix (Biotium, Hayward, CA) or TaqMan primers and universal master mix (Life Technologies) on the Mx3005P system (Agilent Technologies, Santa Clara, CA). Primer sequences or ID numbers are listed in Table 1. Relative gene expression was determined using the $2^{-\Delta\Delta CT}$ method.

Immunohistochemistry

Lungs were fixed and embedded using standard protocols. Sections were blocked with 10% normal serum for 2 hours and incubated with primary antibody against ALX/FPR2 (M73) (1:50) overnight (4°C) (Santa Cruz Biotechnology, Inc., Dallas, TX). Staining was developed with 3,3'-diaminobenzidine; sections were counterstained with hematoxylin.

Cytokine Secretion Cultures/IL-17 Measurement

Nonadherent lung cells were exposed to 100 nM 15-epi-LXA₄ (EMD Millipore, Billerica, MA) or vehicle (<0.1% ethanol) before 5×10^5 heat-killed *Cne* (HK*Cne*) per ml or 5 µg/ml concanavalin A (Con A) (Sigma) or media alone and incubated at 37°C for 48 hours. Supernatants were analyzed for cytokine content by ELISA (14).

ALX/FPR2 Antagonist Intervention

C.B-17 Mice were inoculated with *Cne* as described. The ALX/FPR2 antagonist WRW4 (1 mg/kg/d in 0.1 ml 0.1% DMSO/PBS) or vehicle was injected intraperitoneally on Days 14 to 17 after infection. Tissues were collected on Day 18 for analysis of lung CFU and *in vitro* cytokine expression.

Statistical Analysis

Data were analyzed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). Data are expressed as mean $\pm$ SEM unless stated otherwise. Results were considered statistically significant if $P \leq 0.05$.

Results

C.B-17 Mice, but Not C57BL/6 Mice, Decreased Lung *Cne* within 4 Weeks

To determine the ability of C.B-17 and C57BL/6 mouse strains to limit the growth of *Cne*, log lung CFU were quantitated at several time intervals after inoculation with 5,000 CFU (details are provided in the online supplement). Significant differences in CFU between the strains were apparent from Day 14 onward (Figure 1). C.B-17 mice showed a 1.3 log reduction in lung CFU by Day 21 after inoculation (Day 7 versus Day 21; $P \leq 0.05$). In contrast, there was a significant increase in lung CFU in C57BL/6 mice (3-fold increase from Day 7 to Day 14; $P \leq 0.05$), after which the log CFUs remained high.

Strain-Specific Differences in LX Biosynthesis and Receptor Expression

Strain-specific LX levels were measured in BALF by ELISA (details are provided in the online supplement). After infection, levels of LXA₄ remained relatively steady in the C.B-17 mice and were not significantly increased by *Cne* inoculation. In contrast, in C57BL/6 mice, LXA₄ levels were often higher than those observed in C.B-17 mice both before and after infection with *Cne*. In comparison to C.B-17 mice, levels of LXA₄ were significantly higher at Days 14 and 18 in C57BL/6 mice compared with C.B-17 mice (213.7 ± 33.6 pg/mg protein [C.B-17] versus 817.0 ± 124.0 pg/mg protein [C57BL/6] on Day 14). The kinetics of 15-epi-LXA₄ generation were similar in the two strains, with peak levels detected at Day 14 in both. No significant differences were present either within strains after inoculation or between strains at any time point measured.

The strain-specific patterns for BALF LXA₄ levels suggested the possibility of differences in expression of 12/15-lipoxygenase (*Alox12/15*), which participates in LXA₄, but not 15-epi-LXA₄, biosynthesis. Analysis of *Alox12/15* expression at baseline demonstrated that C57BL/6 mice had higher levels in comparison to C.B-17 mice (Figure 2C; see Tables E1 and E2 in the online supplement). In addition, C57BL/6 mice showed increased expression of *Alox12/15* at Day 14 after inoculation (Figure 2D). For purposes of comparison, the expression of

5-lipoxygenase (*Alox5*), which is required for both LXA₄ and 15-epi-LXA₄, was determined. Comparison of lung *Alox5* expression revealed that, at baseline, C57BL/6 mice had higher levels relative to C.B-17 mice (Figure 2C; Tables E1 and E2). Despite the absence of increased BALF LXA₄ at the time points chosen for analysis, C.B-17 mice showed significant increases in both *Alox5* and *Alox12/15* in response to *Cne* infection (Figure 2D); however, their expression remained significantly lower than C57BL/6 mice (Tables E1 and E2).

The expression of the LXA₄ receptor *Alx/Fpr2* was next determined. Of interest, *Alx/Fpr2* expression was lower in the C57BL/6 mice relative to C.B-17 at baseline (Figure 2C; Tables E1 and E2). The differences in lung *Alx/Fpr2* expression were even more apparent by immunohistochemistry (Figures 2E and 2F). Although ALX/FPR2 is expressed in both strains at baseline, staining intensity was greater in C.B-17 mice (Figure 2E). ALX/FPR2 was primarily expressed at baseline in airway epithelial cells and alveolar macrophages (Figure 2E).

Leukocyte Trafficking to the Lung in Response to *Cne* Infection Differed between the Two Strains

In view of the differences in *Cne* clearance and the LXA₄ pathway, strain differences in pathogen-mediated inflammation were next assessed. Total and leukocyte differential cell counts for neutrophils, eosinophils, macrophages, and total lymphocytes were performed in lung digests (Figures 3A–3D) and BALFs (Figures E1 and E2). Dendritic cells, CD4⁺ T cells, and CD4⁺CD69⁺ T cells were identified and enumerated by flow cytometry (Figures 3E–3G). For confirmation of cytopins, tissue macrophages (large, autofluorescent, CD11c⁺, MHC II⁺ cells) were analyzed by flow cytometry (Figure E3). In C.B-17 mice, lung neutrophils were highest on Day 14 ($1.06 \times 10^7 \pm 2.11 \times 10^6$ cells/lung) (Figure 3A). C.B-17 eosinophils were also highest at Day 14 ($1.76 \times 10^6 \pm 4.26 \times 10^5$ cells/lung) (Figure 3B), but their numbers were approximately a log less than neutrophils (Figure 3A). Macrophages, total lymphocytes, and dendritic cells were highest on Day 18 ($1.10 \times 10^7 \pm 2.23 \times 10^6$ macrophages/lung; $2.63 \times 10^7 \pm 5.74 \times 10^6$ lymphocytes/lung; $3.264 \times 10^6 \pm 1.189 \times 10^6$ dendritic cells/lung) (Figures 3C–3E). CD4⁺ T cells and

Figure 3. *Cne* infection initiated different patterns of leukocyte trafficking in the lungs of C57BL/6 and C.B-17 mice. (A–D) Classes of leukocytes from liberase-digested whole lung were enumerated on cytospin slides by differential analysis from *Cne*-inoculated C.B-17 (open bars) and C57BL/6 (solid bars) mice at 7, 14, 18, 21, and 28 days after infection. Time course data are shown for neutrophils (A), eosinophils (B), macrophages (C), and lymphocytes (D). Lung single-cell suspensions were also subjected to flow cytometry to identify dendritic cells (E), CD4⁺ T cells (F), and CD4⁺CD69⁺ T cells (G) (details are provided in the online supplement). Values represent the mean $\pm$ SEM. * $P \leq 0.05$, one-way ANOVA with Tukey's post tests for differences from baseline to other time points within a strain. # $P \leq 0.03$. Data were analyzed by Student's *t* test for strain differences at specific time points ($n \geq 3$ per time point). Data represent results from four experiments.

activated effector T cells (CD4⁺CD69⁺) also peaked on Day 18 after inoculation (Figures 3F and 3G). Tissue macrophages identified by flow cytometry criteria peaked at Day 18 (Figure E3), confirming the cytospin results (Figure 3C). Neutrophil trafficking into BALF peaked at a later time point (Day 18) than in the lung digests (Day 14) (Figures 3A and E2). Similarly, macrophages peaked at Day 21 in BALFs (Figure E2) compared with Day 18 in lung digests (Figure 3C).

In C57BL/6 mice, the time course of neutrophil infiltration to the lungs mirrored C.B-17, but the other major leukocyte subclasses differed significantly in this regard (Figure 3), particularly in the BALF (Figure E2). Despite similar timing, peak neutrophil numbers in lung were significantly decreased in C57BL/6 relative to C.B-17 mice. The greatest numbers of neutrophils were detected on Day 14 in lung ($6.10 \times 10^6 \pm 0.95 \times 10^6$ cells/lung), which were approximately 50% of those seen in C.B-17 mice (Figure 3A). Although eosinophils were also highest at Day 14 ($1.14 \times 10^7 \pm 1.57 \times 10^6$ cells/lung), in contradistinction to the neutrophils, the eosinophil numbers were significantly greater in the C57BL/6 mice (Figure 3B). C57BL/6 macrophages and total lymphocytes were highest at Day 18 ($1.34 \times 10^7 \pm 3.23 \times 10^6$ macrophages/lung; $1.66 \times 10^7 \pm 4.48 \times 10^6$ lymphocytes/lung) (Figures 3C and 3D), both of which were less than the response in C.B-17 mice. Dendritic cell numbers were highest in C57BL/6 mice on Day 28 ($1.65 \times 10^6 \pm 0.43 \times 10^6$ cells/lung). The C57BL/6 total CD4⁺ and activated CD4⁺ cell numbers were also significantly decreased relative to the C.B-17 (Figures 3F and 3G).

BALF Cytokine Profiles of C.B-17 and C57BL/6 Mice in Response to *Cne*

To further characterize strain-specific differences in immune responses to *Cne* infection, the levels of BALF cytokines present in naive mice were compared with mice at Day 14 after inoculation (Figure 4). BALF IL-17 was significantly elevated in C.B-17 mice at Day 14 (27.7 ± 11.3 pg/mg protein) relative to baseline (3.8 ± 0.4 pg/mg protein), which was strain dependent (Figure 4A). IFN- γ and IL-4 showed trends toward elevation between Days 0 and 14 in C.B-17 mice (Figures 4B and 4C); however, only the change in IFN- γ appeared strain dependent (Figure 4B). On Day 14, IL-4 and TNF- α levels were significantly increased in C57BL/6

mice (Figures 4C and 4E). In addition, on Day 14, TNF- α levels were significantly increased in C.B-17 mice (Figure 4E). BALF levels for IL-13, IL-1 β , and IL-5 did not change with infection (Figures 4D, 4F, and 4G).

Regulation of Lung Cell IFN- γ and IL-17 Secretion by 15-epi-LXA₄

Given the selective regulation of cytokine levels, we measured IFN- γ and IL-17 in nonadherent lung cell suspensions obtained from both strains of mice at Days 0 (naive), 7, 14, 18, and 21 after inoculation (details are provided in the online supplement). IFN- γ levels were increased in cultures of unstimulated lung cell suspensions from C.B-17 mice at Days 14, 18, and 21 relative to uninfected (Day 0) controls (Figure 5A), indicating that *Cne* infection induces production of IFN- γ in this strain. In comparison to C57BL/6-derived cells, IFN- γ production was significantly higher at Days 7, 14, and 21 from C.B-17 cells. IL-17 levels from unstimulated C.B-17 lung cells were significantly greater on Days 7 and 21 after inoculation relative to control cultures (Day 0) (Figure 5B). A modest but statistically significant increase in IL-17 was also observed in the C57BL/6-derived cells on Day 7 relative to Day 0 controls (Figure 5B). Relative to C57BL/6-derived cells, IL-17 generation was markedly increased in cells from C.B-17 mice at all time points tested after *Cne* inoculation.

Lung cell suspensions were next exposed to HKC*Cne* and IFN- γ , and IL-17 generation was determined (Figures 5C and 5D). Concomitant exposure of HKC*Cne*-activated C.B-17 lung cells to 15-epi-LXA₄ (100 nM, 48 h) led to a significant increase in IFN- γ (Figure 5C). In contrast, with C57BL/6 cells, 15-epi-LXA₄ led to a modest increase in IFN- γ with HKC*Cne* (Figure 5C). IL-17 production was significantly increased by HKC*Cne* from only C.B-17 cells (Figure 5D). Exposure of HKC*Cne*-activated C.B-17 cells to 15-epi-LXA₄ markedly increased IL-17 production from $1,430 \pm 299.5$ pg IL-17/ml (vehicle) to $3,053 \pm 479.9$ pg IL-17/ml (15-epi-LXA₄), yet the LX had no significant effect on the C57BL/6 cells (Figure 5D). Of interest, when lung C.B-17 cells from mice infected with *Cne* 14 days earlier were stimulated with the lectin Con A, there was a dramatic increase in IFN- γ and IL-17 that was 40 to 50 times greater than that seen with HKC*Cne* (Figure 5E). In these incubations, the LX did not significantly change IFN- γ

levels, but it did significantly decrease IL-17 by approximately 40%, reaching significance only in C.B-17 mice. The C57BL/6 cell responses to Con A for both IFN- γ and IL-17 production were markedly less than C.B-17 (Figure 5E).

To determine the roles for endogenous signaling via ALX/FPR2, *Cne*-infected animals were given an ALX/FPR2 receptor antagonist WRW4 (1 mg/kg/d, as in Refs. 16 and 17) during protocol Days 14 to 17, when *Cne* clearance in C.B-17 mice increased relative to C57BL/6 mice (Figure 1). Upon harvest 18 days after inoculation, WRW4 led to a trend toward decreased lung *Cne* clearance (Figure 5E) and IFN- γ levels (Figure 5F) ($P = 0.07$).

Discussion

Here, we show strain-specific differences in the clearance of *Cne* that were associated with differences in immunologic responses and proresolving LXA₄ biosynthesis and receptor (ALX/FPR2) expression. Similar to previous reports (4, 5), strain-dependent differences in the reduction of *Cne* burden became apparent 14 days after lung infection. Unlike the C.B-17 mice, the C57BL/6 mice did not effectively lower lung *Cne*, which persisted and increased in the lungs of these mice over a 28-day interval. BALF LXA₄ was elevated in C57BL/6 compared with C.B-17 mice; however, 15-epi-LXA₄ levels were similar in both

Figure 4. Strain-specific cytokine production in response to lung *Cne* infection. Cytokine profiles of naive and *Cne*-infected (Day 14 after infection) C.B-17 (open bars) and C57BL/6 (solid bars) mice were measured in BALF by cytokine bead array (see MATERIALS AND METHODS). Cytokines analyzed included IL-17 (A), IFN- γ (B), IL-4 (C), IL-13 (D), TNF- α (E), and IL-1 β (F). Data are presented as mean $\pm$ SEM ($n = 5$ per group). * $P \leq 0.01$ for comparisons between Day 0 versus Day 14 within a strain. # $P \leq 0.01$ for comparisons between strains at a given time point. Data were analyzed by Student's t tests for differences within and between strains at specific time points.

strains. The differences in LXA₄, but not 15-epi-LXA₄, levels suggested strain-dependent differences in Alox12/15 activity, and expression of this enzyme was significantly increased in the C57BL/6 mice. In sharp contrast, expression of the LXA₄ and 15-epi-LXA₄ receptor ALX/FPR2 was decreased in the C57BL/6 mice relative to

C.B.17. Moreover, ALX/FPR2 expression was induced by *Cne* infection in only the C.B-17 mice. Concomitant with *Cne* reduction, C.B-17 mice mounted significant increases in lung neutrophils, macrophages, and lymphocytes, in particular activated CD4⁺ T cells. Although beyond the scope of the current study, immunophenotyping

results suggested the possibility of additional influences of LX signaling on the regulation of cellular immunity against *Cne*. The strains differed in proinflammatory cytokine generation, with C.B-17 mice producing more IFN- γ and IL-17 compared with C57BL/6, which trended to higher production of IL-4 and IL-13 and had a significant increase in lung eosinophils. IFN- γ and IL-17 production by HKCne-exposed C.B-17, but not C57BL/6, lung cells was significantly increased by 15-epi-LXA₄ exposure *in vitro*. In addition, blocking ALX/FPR2 signaling with the peptide antagonist WRW4 *in vivo* led to a trend toward reduced lung *Cne* clearance by C.B-17 mice. In parallel with the increase in *Cne* burden, there was a trend toward reduced secretion of the host-protective cytokine IFN- γ from nonadherent lung cells *in vitro*. Even these modest changes with disruption of LX-ALX/FPR2 signaling are notable because exposure to WRW4 did not enhance fungal host defense. This suggests that endogenous counterregulatory signaling mediated by LXs was not immunosuppressive for *Cne*; rather, there was a protective trend. Together, these results are the first to highlight distinctions in endogenous proresolving circuits between mouse strains and to link LX signaling to effective antifungal host defense.

Changes in ALX/FPR2 receptor expression regulate the kinetics and duration of acute inflammation (7, 18). ALX/FPR2-deficient mice have a resolution defect for acute peritoneal inflammation induced by the fungal glucan zymosan (19), and human ALX/FPR2 transgenic mice are protected from acute and allergic lung inflammation (20, 21). Acute inflammation and its resolution in humans are also related to ALX/FPR2 expression (22). The translational relevance of ALX/FPR2 in lung diseases is emphasized by its decreased expression in human severe asthma (13, 23) and the disruption of LX signaling at ALX/FPR2 in acute COPD exacerbations (24). Genetic variation in the human ALX/FPR2 gene has been identified with an association between decreased transcription and increased risk for atherosclerosis, a common disease of chronic vascular inflammation (25). Thus, the identification of decreased ALX/FPR2 expression in C57BL/6 mice and the lack of induction with lung *Cne* infection indirectly suggests a pivotal role for ALX/FPR2 in promoting

Figure 5. Lung lymphocytes showed strain-specific responses in IL-17 secretion. Nonadherent cells isolated from lung digests from the two strains of mice at various time points after inoculation with *Cne* were maintained in cell culture for 48 hours. IFN- γ and IL-17 levels in culture media were quantitated by ELISA; values are presented as mean $\pm$ SEM. (A and B) IFN- γ and IL-17 were measured in media from unstimulated cultures of lung cells of C.B-17 (open circles) and C57BL/6 (solid squares) isolated on Days 0, 7, 14, and 21 after inoculation. * $P \leq 0.05$, one-way ANOVA with Tukey's multiple comparisons for differences between time points within strain. # $P \leq 0.05$, Student's *t* test for differences between strains ($n \geq 3$ mice per group). (C and D) IFN- γ and IL-17 generation were measured in media from lung cell cultures collected on Day 14 after inoculation and stimulated with heat-killed *Cne* (HKCne) (5.0×10^5 /ml) $\pm$ 15-epi-LXA₄ (100 nM, 48 h) (two-way ANOVA with Sidak's multiple comparisons). * $P \leq 0.05$ ($n \geq 3$); # $P \leq 0.05$ ($n \geq 3$) for C.B-17 versus C57BL/6. Data reflect combined results from three experiments. (E) C.B-17 mice were inoculated with *Cne* as previously described and injected with the ALX/FPR2 antagonist WRW4 (1 mg/kg/d) or vehicle control on Days 14 to 17 after inoculation. Lung CFU were determined on Day 18 (Student's *t* test, $n \geq 13$ per group; data represent results from two experiments). (F) Nonadherent lung cells obtained from WRW4- or vehicle-treated C.B-17 mice were cultured as described. IFN- γ and IL-17 were measured in cell media (Student's *t* test, $n \geq 5$ per group).

antifungal host defense and provides a potential mechanistic explanation for the well-appreciated genetic susceptibility of this mouse strain for increased Type 2 lung inflammation relative to other strains (26, 27).

Strain-specific differences in LXA₄ levels were associated with differences in the expression of *Alox12/15*, which plays an important role in LXA₄ biosynthesis. Relative to C.B-17 mice, *Alox12/15* expression was significantly increased at baseline and in response to *Cne* in C57BL/6 mice. Eosinophils express high levels of *Alox12/15* (28), and, given the marked difference in lung eosinophilia, these cells were the most likely cause for the strain differences in lung *Alox12/15* expression and LXA₄ biosynthesis after infection. Both IL-4 and IL-13 levels were increased, and these Type 2 cytokines may have further increased *Alox12/15* expression because they can up-regulate the human homolog of *Alox12/15* in lung macrophages (29).

In addition to *Alox12/15*, *Alox5* is pivotal to LXA₄ biosynthesis (reviewed in Ref. 30) and was increased in C57BL/6 mice. 5-Lipoxygenase is also critical to proinflammatory leukotriene production (31) and antifungal host defense. In response to *Histoplasma capsulatum*, C57BL/6 mice had higher fungal burdens than 129/Sv mice and reduced *Alox5* expression (32). Moreover, *Alox5*-deficient 129/Sv mice display impaired control of *Histoplasma capsulatum* infection (33). In contrast, *Alox5*-deficient C57BL/6 mice display enhanced clearance of *Paracoccidioides brasiliensis* (16). These differences in host response suggest that, in addition to mouse strain specificity in lipid mediator signaling pathways, interactions between the microbial proteome and host

enzymes for transcellular biosynthesis of bioactive lipid mediators can influence pathogen clearance (34).

Similar to prior reports (14, 35), the immunologic responses of C.B-17 and C57BL/6 were markedly different, with Th1 and Th2 type responses, respectively. IFN- γ and IL-17 levels were increased in the C.B-17 mice to levels above those in similarly infected C57BL/6 mice. These cytokines are key contributors to *Cne* resistance and can suppress allergic responses to the fungus (36, 37). In contrast, C57BL/6 mice produced increased levels of IL-4 and the related Th2 cytokine IL-13, which can decrease IL-17 secretion and reduce polarization of T cells to a Th17 phenotype (38, 39). IL-17 deficiency can lead to impairments in host defense against *Cne*, related to increased leukocyte recruitment and IFN- γ production from CD4⁺ T cells (36). LX signaling regulates murine eosinophil trafficking, Th2 cytokines, and IFN- γ and IL-17 generation during allergic lung inflammation (20, 40). Here, 15-epi-LXA₄ increased IFN- γ production from C.B-17 mouse HKCne-activated lung cells. Stimulation with Con A led to dramatic increases in IFN- γ and IL-17 generation in C.B-17 cells relative to HKCne exposure. In contrast to HKCne, 15-epi-LXA₄ partially decreased this Con A-initiated IL-17 production, suggesting context-specific roles for LXs in restraining an overexuberant acute inflammatory response to Con A yet augmenting the host response to HKCne. To this end, an ALX/FPR2 receptor antagonist that can block LX actions *in vitro* (17) led to a trend toward decreased IFN- γ levels, suggesting a host protective role for the LXA₄ signaling pathway. LXA₄ can augment antimicrobial

host defense by several means, including induction of bactericidal permeability, increasing protein and NO synthase (41, 42). Together, the differences in ALX expression, cytokine production in response to HKCne in the presence of 15-epi-LXA₄, and endogenous modulation by WRW4 point to a regulatory role for the LX signaling pathway in immune responses to *Cne* and appear to suggest an important interdependence between cytokines and lipid mediators in host defense.

In summary, strain-specific differences in host defense during *Cne* pneumonia were associated with differences in LXA₄ biosynthesis and receptor expression, providing new evidence for the regulation of fungal infection by specialized proresolving mediators. Even with an increase in LXA₄ levels, a disruption of LX signaling from decreased ALX/FPR2 expression in C57BL/6 mice was associated with Th2 type lung inflammation and a decreased capacity to reduce fungal burden. In sharp contrast, C.B-17 mice decreased lung *Cne* infection with a more robust Th1 response and cytokine (IFN- γ and IL-17) production that was associated with inducible *Alx/Fpr2* expression and regulation of pathogen-mediated inflammation by 15-epi-LXA₄. Together, these findings suggest pivotal roles for SPM, like the LXs, in regulating fungal host defense in the lung. ■

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