Colonic Bacterial Infection Abrogates Eosinophilic Pulmonary Disease

Andrew E. Williams, Lorna Edwards, and Tracy Hussell
Kennedy Institute of Rheumatology, Charing Cross Campus, Imperial College, London, United Kingdom

Induction of immunity to one pathogen in the lungs modifies the microenvironment and alters immunopathological changes that result from a second, unrelated pulmonary infection. However, it is unclear whether immunity generated at distant sites also affects lung immune responses. Here, we show that infection with the gut-restricted bacterium *Citrobacter rodentium* modifies immunopathological changes that result from pulmonary *Cryptococcus neoformans* infection. Th2 cytokine–driven pulmonary eosinophilia induced by *C. neoformans* infection was reduced, and the enhanced Th1 cytokine environment afforded more-rapid clearance of the fungus in *C. rodentium*–immune mice. The activated and intraepithelial (CD103+) T cell populations that expand after *C. neoformans* infection were diminished in *C. rodentium*–immune mice. T cell cross-reactivity was absent, but cross-reactive antibodies were detected. It is of importance to the “hygiene hypothesis” that these data indicate that an immune response induced by a gut-restricted pathogen can modify the immune outcome after pulmonary infection, suggesting that cell-phenotype modifications occur across mucosal sites.

The continued modulation of the immune system by sequential infections is increasingly acknowledged as a determining factor in the outcome of an immune response to unrelated pathogens [1–4]. This may contribute to variability in disease susceptibility after vaccination or infection in humans. Infection history may also provide the basis for differences in the development of atopic disorders, such as asthma and allergic rhinitis [5–7]. Such immune amendment early in life may reduce the predisposition toward an atopic state later in life—this hypothesis is known as the “hygiene hypothesis” [8, 9].

A reduction in Th2-driven atopic disorders can be achieved by the promotion of Th1 cytokines and/or the suppression of Th2 cytokines. Infection history in human and animal models suggests that both of these events occur. For example, pathogens able to induce a Th1-type immune response—such as *Mycobacteria* species [10, 11], measles virus [12], and influenza virus [13]—direct the immune system away from Th2-driven allergic responses [14]. In the developing world, on the other hand, a higher prevalence of Th2-inducing helminth infections is associated with reduced susceptibility to Th2-mediated allergies [15–17]. In this case, increased levels of parasite-induced interleukin (IL)–10 [18], a cytokine that down-regulates both Th1 and Th2 responses, ameliorate allergy. Indeed, a diminished frequency of atopy in children with elevated levels of IgE is associated with *Schistosoma haematobium* infection and IL-10 production [19].

We have previously shown that respiratory syncytial virus (RSV)–induced, Th2-driven pulmonary disease is abolished by prior exposure to the unrelated lung pathogen influenza virus [13]. Similarly, we have recently reported that exposure of the lungs to live bacille Calmette-Guérin (BCG) reduces the eosinophilia that develops after pulmonary *Cryptococcus neoformans* infection [11]. These findings are similar to those on the reduction in ovalbumin-induced pulmonary eosinophilia in mice previously immunized with heat-killed BCG or mycobacterial antigens [20]. The influence that
prior exposure to a pathogen has on the development of allergic or inflammatory disease has been well studied in isolated mucosal sites [2, 4, 18]. However, the extent of the immune interaction between anatomically distant mucosal sites has been the focus of much debate. Evidence for a gut-lung axis has been mostly circumstantial, with an association between experimental acute graft versus host disease and the development of idiopathic pneumonia syndrome [21].

In the present study, we investigate how previous exposure to a gut-restricted pathogen influences the immune response to an unrelated pathogen in the lungs. C57BL/6 mice were infected intragastrically with Citrobacter rodentium, the mouse model of enteropathogenic *Escherichia coli*, to induce a colon-restricted, Th1-biased immune response [22]. Mice were then infected with the lung pathogen *C. neoformans*, which induces Th2-mediated pulmonary eosinophilia [23, 24]. A significant reduction in eosinophilia was observed in *C. neoformans*-infected mice that had previously recovered from *C. rodentium* infection. This improved outcome was associated with a skewing of the cytokine response to a Th1-dominated phenotype and restricted pathogen dissemination. These results indicate that the immune response generated after gut infection is able to modulate the eosinophilic immune response to a lung pathogen, and they provide evidence for heterologous immunity across mucosal tissues.

**MATERIALS AND METHODS**

**Mice and pathogens.** Six–8-week-old female C57BL/6 mice (Harlan Olac) were housed in specific pathogen–free conditions, in accordance with institutional and Home Office (UK) guidelines. *C. rodentium* (formerly *Citrobacter freundii* biotype 4280) was grown and maintained in Luria broth (LB) containing 100 μg/mL nalidixic acid (Sigma), for 8–10 h at 37°C. Intimin-deficient *C. rodentium* (a gift from G. Frankl, Imperial College, UK) was grown in LB containing 100 μg/mL kanamycin. Intimin is necessary for bacterial attachment to gut epithelium, and a deficient strain is unable to colonize or cause immunopathological changes [25]. *C. neoformans* strain 52 was obtained from the American Type Culture Collection (24067). Yeast was grown to stationary phase (48–72 h) at room temperature on a shaker, in Sabouraud dextrose broth (1% neopeptone and 2% dextrose; Difco).

**Infection of mice.** Bacterial *C. rodentium* (biotype 4280) was centrifuged at 800 g for 15 min and washed in sterile PBS. Intimin-deficient *C. rodentium* was prepared the same way: 1 × 10⁹ bacteria in 200 μL of sterile PBS were drawn into a gavage needle, followed by ∼100 μL of air. The outside of the needle was then thoroughly washed with 70% ethanol and PBS to remove any bacteria. Mice were then intragastrically infected by gavage. Gavage infection reached a peak 14 days after infection, and the bacteria were cleared from the colon thereafter [26]. To establish that intragastric *C. rodentium* infection is restricted to the gut, mice were gavaged with *C. rodentium*, and lungs were removed after 2 h or at 1, 7, 10, or 14 days, for analysis of bacterial colony counts and lung histological properties, as described below. Unless otherwise stated, mice were intranasally infected with 2 × 10⁴ cfu of *C. neoformans* 14 days after *C. rodentium* infection and, in further experiments, 21 or 35 days after infection; the dose was determined by trypan blue hemocytometer counts of individual yeasts. Mice were killed 12 days after *C. neoformans* infection, by intraperitoneal injection of 3 mg of pentobarbitone and exsanguination of the femoral vessels.

**Cell recovery.** Bronchoalveolar lavage (BAL) fluid, lung tissue, and serum were obtained from each mouse, as described elsewhere [27]. In brief, BAL fluid was extracted by inflating the lungs 6 times with 1.5 mL of Eagle MEM (Sigma) containing 10 mmol/L EDTA and kept on ice. BAL fluid was centrifuged, the supernatant was removed, and the cell pellet was resuspended at 1 × 10⁶ cells/mL. Lungs were removed, one lobe was fixed in 2% formalin for histological analysis, and the remainder was placed in ice-cold RPMI 1640 (Sigma) containing 10% fetal calf serum, 2 mmol/L L-glutamine, 50 μg/mL penicillin, and 50 μg/mL streptomycin (R10F). The latter lung sample was disrupted using 0.8-μm filters (to obtain a single-cell suspension), red blood cells (RBCs) were lysed, and the cell pellet was resuspended at 1 × 10⁶ cells/mL in R10F.

**Enumeration of eosinophils and histological analysis.** Eosinophils were enumerated, by flow cytometry, by gating on the granulocyte population, using forward- and side-scatter properties. Counting cells from BAL fluid cyt centrifuge preparations stained with hematoxylin-eosin (HE) was performed to achieve identification and confirmation of the eosinophil population. Total cells were enumerated using a hemocytometer and trypan blue exclusion. Formaldehyde-fixed lung lobes were embedded in paraffin blocks, for histological analysis. Six-micrometer sections were cut and stained with HE. Lung tissue was examined under various objectives, and a representative field of view was photographed at ×200 magnification.

**Analysis of *C. neoformans* titer.** Twenty microliters of lung homogenate was diluted from 10⁻⁴ to 10⁻⁷ on Sabouraud dextrose agar plates for 72 h. The number of colonies was counted, and the total colony-forming units per lung were calculated as follows: number of colonies × dilution × 50 (to determine colony-forming units per milliliter).

**Flow-cytometric analysis of cell-surface and intracellular antigens.** Cells from BAL fluid and lungs were stained for anti-CD45RB–fluorescein isothiocyanate (FITC), anti-CD103–phycoerythrin (PE), allophycocyanin-conjugated anti-CD4, and peridinin chlorophyll protein complex–conjugated anti-CD8 (Pharmingen). Intracellular cytokines were detected by incubation of cells with 50 ng/mL PMA (Sigma), 500 ng of iono-
mycin (Calbiochem), and 10 μg/mL brefeldin A (Sigma) for 4 h at 37°C. Cells were then stained with anti-CD4 and anti-CD8 (as above) for 30 min on ice, washed, and fixed in 50 μL of 2% formaldehyde for 20 min at room temperature. Cells were permeabilized with 0.5% saponin in PBS containing 1% bovine serum albumin (BSA) and 0.1% azide (saponin buffer) for 10 min. Anti–tumor necrosis factor (TNF)–FITC and anti–IL-4–PE antibodies (Pharmingen) were diluted in saponin buffer and added to the cells. After 30 min, cells were washed once in saponin buffer and twice in PBS containing 0.1% azide and 1% BSA. All samples were analyzed on a 4-color FACScaliber (BD) flow cytometer, which collected data on at least 30,000 lymphocytes.

C. neoformans–specific ELISA for serum IgG. Ninety-six-well microtiter plates were coated with 1 × 10^8 cfu of UV-inactivated C. neoformans overnight at 4°C in 0.1 mol/L sodium carbonate buffer (8.4 g of NaHCO_3 and 3.56 g of Na_2CO_3, [pH 9.5]). Plates were washed with PBS containing 0.05% Tween 20 (Sigma) (wash buffer). Plates were blocked with PBS containing 6% BSA (PBA) for 2 h at room temperature and then were washed. Serum samples were diluted 1:50 in PBA, and 100 μL was added to the plate in triplicate. Normal mouse serum was used as a negative control. Samples applied to the ELISA plate were incubated for 2 h at room temperature. After washing, 100 μL of rat anti–mouse horseradish peroxidase–conjugated antibody (DAKO) was diluted 1:250 in PBA and incubated for 1 h at room temperature. After washing, 100 μL of o-phenylenediamine substrate (Sigma) was added to each well, and the reaction was stopped with 2 mol/L H_2SO_4. The absorbance was read at 492 nm.

Proliferation assay. Spleens were removed from individual mice under sterile conditions and passed through a 0.8-μm filter, to produce a single-cell suspension. After RBC lysis in 0.15 mol/L ammonium chloride (1 mol/L potassium hydrogen carbonate and 0.01 mmol/L EDTA [pH 7.2]), spleen cells were resuspended in R10F at 2 × 10^6 cells/mL; 4 × 10^3 cells, together with 1 × 10^4 inactivated C. neoformans yeast, were added to each well of a round-bottom 96-well plate. All samples were analyzed in triplicate. After 48 h at 37°C, 50 μCi/mL [H]^-thymidine (Amersham) was added for a further 24 h. Cells were harvested, the incorporated [H]^-thymidine was transferred to a filter, and the β emissions were read using a scintillation counter.

Statistical analysis. Data values were analyzed for statistical significance using a 2-tailed Student’s t test that assumed unequal variances. Statistical significance was determined by a Student’s t test that assumed unequal variances (P < .05).

**RESULTS**

Beneficial modification of the immune response to C. neoformans in the lungs, by gut infection with C. rodentium. To investigate whether immunity in the gut affects the outcome of pulmonary infection, mice were gavaged with C. rodentium 14 days before pulmonary infection with the fungus C. neoformans. In susceptible C57BL/6 mice, C. neoformans caused extensive Th2-driven pulmonary eosinophilia, lymphocyte infiltration, and pulmonary consolidation. Prior gut infection with C. rodentium reduced pulmonary lymphocytic infiltration.
after *C. neoformans* infection (figure 1A). More significant was the reduction in pulmonary eosinophils recovered by lavage (figure 1B) and the decreased *C. neoformans* burden in the lungs (figure 1C) and the airways (data not shown). *C. neoformans* causes extensive lung modifications. Alveoli and airways were consolidated with lymphocytic infiltrates. Lymphocytes and eosinophils were closely associated with clusters of *C. neoformans* yeast throughout the lungs (figure 1D). Prior infection with *C. rodentium* significantly moderated pulmonary immunopathological changes—lymphocytic infiltration was restricted to areas surrounding airways, and severe eosinophilia was absent (figure 1E and 1F).

**Dependence of modification of pulmonary immunopathological changes on bacterial replication.** Modification of the immunopathological changes that result from *C. neoformans* infection may reflect productive infection of the gut with *C. rodentium* or accidental contamination of the lungs during gavage. To test the first assumption, we gavaged mice with a *C. rodentium* strain lacking the intimin receptor. The intimin-deficient strain was unable to adhere to the gut epithelium and therefore did not cause immunopathological changes or elicit an immune response. To test the second assumption, we analyzed the lungs after gavage with *C. rodentium* alone. The intimin-deficient strain of *C. rodentium* did not abrogate lung cellularity (figure 2A) or eosinophilia (figure 2B), compared with wild-type (wt) *C. rodentium*, after *C. neoformans* infection of the lungs. Lung tissue removed from mice 2 or 24 h after gavage with wt *C. rodentium* did not contain inflammatory infiltrates or recoverable bacteria (data not shown). Furthermore, histological analysis of lungs at 7, 10, and 14 days after *C. rodentium* gavage revealed no immunological changes (figure 2C–2E) and no recoverable bacteria (data not shown).

**Modification of T cell recruitment after *C. neoformans* infection, by *C. rodentium* infection.** To further analyze the extent of the immune modulation in the lungs, T cell sub-

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**Figure 2.** No abrogation of pulmonary immunopathological changes, by intimin-deficient *Citrobacter rodentium*. Mice were gavaged with wild-type (wt) *C. rodentium* or a mutant strain lacking the critical adherence receptor intimin (int−/−). Fourteen days later, mice were intranasally infected with *Cryptococcus neoformans*. After another 12 days, pulmonary lymphocytes (A) and eosinophils (B) in bronchoalveolar lavage fluid were determined. Lung histological analysis was performed for immune-cell infiltrate after intragastric gavage with *C. rodentium* (wt) 7 days (C), 10 days (D), and 14 days (E) after infection. Statistical significance was determined by a Student’s *t* test that assumed unequal variances (*P* < .05). Citro, *C. rodentium*.

**Figure 3.** Decrease of activated T cells and intraepithelial T cells after gut infection. Activated CD4+ (A) and CD8+ T cells (B) were determined as CD45RBlo, and their total nos. were calculated from the total cell counts. CD4+ (C) and CD8+ (D) intraepithelial T cell populations were determined as CD103+. Five mice were used per group, and the data are representative of 3 separate experiments. Statistical significance was determined by a Student’s *t* test that assumed unequal variances (*P* < .05). Citro, *Citrobacter rodentium*; Crypto, *Cryptococcus neoformans*. 

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Enhancement of Th1-dominated immunity to *C. neoformans* in the lungs, by previous gut infection with *C. rodentium*. Since eosinophilic responses to *C. neoformans* infection were reduced by prior *C. rodentium* infection, we examined the cytokine profiles of CD4+ and CD8+ T cells by intracellular staining and flow cytometry. After infection with *C. neoformans*, the total number of both CD4+TNF+ (figure 4A) and CD8+TNF+ (figure 4B) T cells was enhanced in mice previously infected with the gut pathogen *C. rodentium*. Conversely, CD4+IL-4+ (figure 4C) and CD8+IL-4+ (figure 4D) T cell populations decreased in mice previously infected with *C. rodentium*, indicating a shift from a Th2 to a Th1 phenotype. No significant differences in levels of the regulatory cytokine IL-10 were observed (data not shown).

Enhancement of heterologous immunity and prevention of pathogen dissemination, by *C. rodentium* infection. Improvements in the immunopathological changes associated with *C. neoformans* infection, by prior *C. rodentium* infection, may be due to cross-reactivity in T and B cell responses. We...
Long life of C. rodentium–induced heterologous immunity.

To fully appreciate the extent of the protective immunity induced by C. rodentium infection on subsequent C. neoformans pulmonary infection, the time between each infection was increased. Gut infection with C. rodentium still modified the immune response to C. neoformans pulmonary infection, which was exemplified by a significant reduction in the proportion of pulmonary eosinophils, with 21 (figure 5E) and even 35 days (figure 5F) between the 2 infections.

DISCUSSION

Infection history represents an important sequence of events that influences the phenotype and severity of subsequent disease. It may also modify the immune response to vaccination. It has been clearly shown, in the context of pulmonary infection, that one viral infection can modify the immunological reaction to infection with a subsequent and unrelated virus [2, 13, 28, 29]. Furthermore, lung immunization with BCG reduces the pulmonary immunopathological changes associated with C. neoformans infection [11]. In the present study, we have shown that gut infection with the bacterium C. rodentium modifies the immune response to subsequent C. neoformans yeast infection in the lungs. This demonstrates that immune modification can act across 2 distinct mucosal sites and provides experimental evidence applicable to both the hygiene hypothesis and nonviral heterologous immunity.

The lack of a substantial immune reaction in the lungs after intestinal gavage with C. rodentium and the absence of recoverable bacteria suggest that there was no pulmonary colonization of the bacteria and confirm that the infection was restricted to the gut, with no subsequent bacteremia [30]. In addition, gavage with the intimin-deficient strain of C. rodentium did not modify the response to C. neoformans infection. Since intimin is necessary for adherence to and colonization of the intestine, these data demonstrate that the abrogation in eosinophilia was due to bacterial infection of the gut and subsequent immunity and, moreover, was not due to erroneous immunostimulatory products. For example, both the wt and intimin-deficient strains of C. rodentium contain endotoxin. Recent findings indicate that memory T cells traffic to nonlymphoid organs, irrespective of the site of initial antigen encounter [31]. Furthermore, it has previously been shown that T cells primed with reovirus in the intestine confer protection to reovirus infection of the respiratory tract [32]. This demonstrates that effective cross-protection against a homozygous virus challenge across mucosal sites can occur and supports the concept of a common mucosal immune system [33]. However, until now, the extent to which intestinal infection can modulate the subsequent immune response to an unrelated pathogen in the lungs was unclear. Only epidemiological evidence exists demonstrating the inverse relationship between intestinal infection...
and the development of immune abnormalities in the lungs [34]. It has been postulated that children are less likely to develop asthma if they live in a less-sterile environment with higher rates of intestinal bacterial infection [5]. In addition, gastric infection with Helicobacter pylori is associated with a decreased prevalence of asthma, eczema, and allergic rhinitis [35, 36]. Moreover, the acquisition of intestinal microflora during the postnatal period may be essential for proper T cell development [37], antigen tolerance [37], and maturation of Th1 immune responses [38–40]. A lack of appropriate immunoeeducation early in childhood may result in dysregulated immune responses later in life and the development of allergies [9, 18].

The mechanisms responsible for the reduced eosinophilic immunopathological changes that result from C. neoformans infection are likely numerous. C. rodentium–specific Th1 cells may enter the lungs during subsequent C. neoformans infection and influence anticyryptococcal immunity through the production of cytokines and other reactive mediators. The ability of immunity to one pathogen to suppress Th2 immunopathological changes that result from infection with another pathogen has previously been shown [13]. Influenza-specific CD8+ T cells reenter the lungs in response to RSV infection several weeks after the initial influenza virus infection [13] and prevent Th2-driven pulmonary eosinophilia. Similarly, lymphocytic choriomeningitis virus (LCMV) significantly reduces mortality by a subsequent vaccinia virus infection, although, unlike in the previous study, the effect most likely results from the generation of cross-reactive T cells [41]. Eosinophilic immunopathological changes that result from C. neoformans infection may equally be reduced by enhanced regulation in the lungs. The generation of regulatory T cells and the production of IL-10 are implicated in patients with high parasite burdens who, yet, have a low incidence of allergy [42, 43]. However, we did not observe an increase in IL-10 or T cells with a regulatory phenotype. A shift from a Th2 to a Th1 response may be more dominant.

An alternative explanation for reduced pathogen burden and subsequent eosinophilia may be the induction of cross-reactive heterologous immunity [2]. Given the complexity of both species, it is feasible that T cells share epitope specificity between the 2 organisms. Previously stimulated cross-reactive T cells could therefore enter the lungs in response to C. neoformans infection and clear the pathogen more quickly, as well as restrict its distribution to the airways, thus preventing excessive tissue damage. Moreover, these T cells would already have differentiated into Th1 T cells in response to C. rodentium infection, which would further augment protective immunity to C. neoformans infection. Cross-reactive T cells induced by LCMV infection influence the CD8+ T cell repertoire during subsequent viral infection with Pichinde virus [44]. The heterologous T cell response then controls the immunopathological changes and protective immunity to the second infection. However, despite the observation of enhanced pathogen clearance, we did not observe enhanced C. neoformans–specific T cell proliferative responses, irrespective of prior infection with C. rodentium. We did, however, observe cross-reactive antibody. Continued exposure to pathogens enhances the antibody repertoire [45], generates a polyclonal memory B cell pool [46], and provides the host with a broad range of antibody specificities that promote protective immunity [47]. Although cross-reactive antibody responses have been studied extensively in viral infections [48], the contribution of antibodies directed against other unrelated pathogens remains poorly described.

Our data showing expansion of intraepithelial T cells after C. neoformans infection and their reduction by prior infection with C. rodentium are novel. These intraepithelial T cell subsets express the integrin αEβ7 (CD103) and preferentially migrate to epithelia expressing E-cadherin [49]. CD8+CD103+ T cells have been implicated in immunopathological manifestations, such as renal allograft rejection, destruction of epithelia [50], and chronic obstructive pulmonary disease [51]. However, to our knowledge, we are the first to describe their expansion during infection-induced immunopathological changes and their reduction in less-pathogenic situations. We are currently attempting purification, but the low numbers of CD8+CD103+ T cells and the nature of the lung tissue make flow-cytometric sorting problematic.

In summary, we have described the capability of transferring immunity across different mucosal sites and the immunomodulatory influence of an intestinal bacterial infection on Th2-mediated pulmonary immunopathological changes. These findings support clinical observations in humans and extend the innate-imprinting hypothesis in the lungs [52] to include influences generated at distant sites.

References

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